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Full Length Research Paper

Cadmium-induced changes in growth and antioxidative mechanisms of a medicine plant (*Lonicera japonica* Thunb.)

Zhouli Liu, Wei Chen* and Xingyuan He

Institute of Applied Ecology, Chinese Academy of Sciences, 72 Wenhua Road Shenhe District, Shenyang 110016, People's Republic of China.

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The responses of a medicine plant (*Lonicera japonica* Thunb.) to different cadmium (Cd) levels in the nutrient medium were studied. The results showed that the high Cd concentrations accumulated in roots and shoots of *L. japonica*, which increased edible security risk of the plant. The chlorophyll content showed slightly increase in plants exposed to 5 mg L^{-1} Cd, but decreased exposed to higher Cd concentrations. The variation in chlorophyll content in plants in relation to Cd concentrations in medium is considered as a good biomarker for Cd stress. Elevated malondialdehyde (MDA) content in leaves and roots indicated the plants were subjected to Cd-induced oxidative stress. As a defensive mechanism, the cooperation of superoxide dismutases (SOD, EC 1.15.1.1), peroxidases (POD, EC 1.11.1.7) and catalases (CAT, EC 1.11.1.6) may play an important role in plant tolerance to Cd. These enzymes activity showed significant increase by exposure to low Cd concentrations, indicating some acclimation effect, but decrease by exposure to high Cd concentrations implicating a potential injury to the plant.

Key words: Antioxidant enzyme, growth, cadmium, *Lonicera japonica* Thunb., tolerance.

INTRODUCTION

Nowadays, the contamination of soil by high level of heavy metals has become a serious problem. Cd is one of the main metals in polluted soil (Moller et al., 2005), and commonly found in soils affected by intensive human activity, such as automobile and industry (Solis-Dominguez et al., 2007). Over the past five decades, the worldwide release of Cd has reached 22,000 t (metric ton) (Singh et al., 2003). It makes the people pay more attention to the safety problem of food polluted by Cd.Cd is not essential to plant growth, and it can cause various phytotoxic symptoms including leaf chlorosis, root putrescence, growth inhibition (Skórzyńska-Polit et al., 2010; Valentovičová et al., 2010). Cd is also known to induce a burst of reactive oxygen species (ROS) in plant tissues, leading to oxidative stress (Gouia et al., 2003; Solis-Dominguez et al., 2007; Meng et al., 2009). To control the level of ROS, plants have evolved enzymatic and non-enzymatic defense systems (Allen, 1995; Stroinski, 1999). Among these defense systems, antioxidative enzymes play an important role in scavenging reactive oxygen species (ROS) through a series of complex reactions, which include the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 by superoxide dismutases (SOD, EC 1.15.1.1) (Bowler et al., 1992), and the detoxification of H_2O_2 by peroxidases (POD, EC 1.11.1.7), catalases (CAT, EC 1.11.1.6) and ascorbate

*Corresponding author. E-mail: forestry83@gmail.com; chenwei5711@163.com. Tel: +86 24 83970349. Fax: +86 24 83970300.

Abbreviations: CAT, catalase; **Cd,** cadmium; **H_2O_2,** hydrogen peroxide; **MDA,** malondialdehyde; **POD,** peroxidases; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase.

peroxidase (APX, EC 1.11.1.11) (Salin, 1988; Asada, 1992; Mishra et al., 2006). Cd-induced changes in the activities of these enzymes have been reported in many agriculture and forestry plant species: pea (Chaoui and Ferjani, 2005), soybean (Cataldo et al., 1981), bean (Chaoui, 1997), willow (Cosio et al., 2006), wheat (Luo et al., 1998), rice (Chien et al., 2001) and sunflower (Di Cagno et al., 1999). However, little information is available on medicinal plants. Presently, the involvement of antioxidative enzymes in plant responses against Cd toxicity is unclear because Cd is not contained in the group of transition metals like copper and zinc, and it is not involved directly on the production of ROS via Fenton-type reactions or Haber-Weiss reactions (Schutzendubel and Polle, 2002).

Therefore, it is necessary to examine the Cd-induced stress in a medicinal plant in order to test the hypothesis that the antioxidative enzymatic system may be a sensitive index in Cd toxicity in plants. *Lonicera japonica* Thunb. (Japanese honeysuckle) has become established in temperate and tropical regions worldwide in the past 150 years (Larson et al., 2007). The shoots and roots of *L. japonica*. are widely used in Asian medicine, and the flowers of the plant are used in tea. Presently, it is also widely used all over China, as an ornamental plant for vertical gardening. It was well known for its various characteristics, such as high biomass, easy cultivation, extensive competitive ability, wide geographic distribution, strong resistance to environmental stress like bacterial, viral and oxidative stress (Thanabhorn et al., 2006). In our previous study, it was shown that *L. japonica* had strong tolerance to Cd in the nutrient medium and strong accumulation capability of Cd in its stem (Liu et al., 2009). The aims of the present study were to assess the role of antioxidative metabolism of plant tolerance to Cd stress, identify valuable biomarkers for the pollutant and evaluate the edible security of *L. japonica*. This study should be also useful in providing a reference for selecting phytoremediators of soils contaminated by Cd.

MATERIALS AND METHODS

Plant culture and treatment

Cuttings of *Lonicera japonica* Thunb. were collected from a non-contaminated field in the Shenyang Arboretum, Chinese Academy of Sciences and propagated in sterilized sand. After two months, plants were transferred to a 500 ml adumbral containers for hydroponics culture in a greenhouse, 4 plants for each. The nutrient medium was a modified Hoagland solution (Hoagland and Arnon, 1950) containing the following ingredients (mM): $Ca(NO_3)_2 \cdot 4H_2O$ 5.00, $MgSO_4 \cdot 7H_2O$ 2.00, KNO_3 5.00, KH_2PO_4 1.00, H_3BO_3 0.05, $ZnSO_4 \cdot 7H_2O$ 0.80×10^{-3}, $MnCl_2 \cdot 4H_2O$ 9.00×10^{-3}, $CuSO_4 \cdot 5H_2O$ 0.30×10^{-3}, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.02×10^{-3}, Fe-EDTA 0.10. The nutrient medium was continuously aerated with an aquarium air pump, renewed once every 3 days, and the pH was daily adjusted to 5.8 ± 0.1 with 0.1 M HCl or 0.1 M NaOH. After the plants were cultivated for 1 week in 50% Hoagland solution, the nutrient medium was changed into 100% Hoagland solution for next 2 weeks. Then $CdCl_2 \cdot 2.5H_2O$ was added into the solution to get: 0 (CK), 5, 10, 25 and 100 (mg L^{-1}), respectively. The experiment was repeated 3 times. The plants were grown in a greenhouse at a temperature of 22 ± 2℃ in 2008 and harvested 2 and 4 weeks later for analysis.

Sampling and enzyme assays

Fresh tissue (0.1 g) was 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 12,000 × g for 30 min at 4℃ the supernatant was used for further analyses. The chlorophyll content was measured in 80% acetone extract of 0.1 g leaf tissue (Hegedüs et al., 2001). Lipid peroxidation was estimated by the concentration of malondialdehyde (MDA), the major trichloroacetic acid (TCA) reactive material, as described by Heath and Packer (1968). All enzyme activities were calculated on the basis of fresh weight (FW). Superoxide dismutase (SOD) activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction (Krivosheeva et al., 1996), taking the enzyme extract that inhibites 50% of the reduction as one unit. Catalase (CAT) activity was measured by the decrease in absorbance at 240 nm (extinction coefficient, 39.4 $mM^{-1}cm^{-1}$) (Pinhero et al., 1997).

Peroxidase (POD) activity was measured using guaiacol and H_2O_2 as substrates and the increase in the absorbance at 470 nm due to oxidation of guaiacol was recorded (Wu and von Tiedemann, 2002). All enzyme activities were calculated on the basis of fresh weight (FW). Superoxide dismutase (SOD) activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction (Krivosheeva et al., 1996), taking the enzyme extract that inhibites 50% of the reduction as one unit. Catalase (CAT) activity was measured by the decrease in absorbance at 240 nm (extinction coefficient, 39.4 $mM^{-1}cm^{-1}$) (Pinhero et al., 1997). Peroxidase (POD) activity was measured using guaiacol and H_2O_2 as substrates and the increase in the absorbance at 470 nm due to oxidation of guaiacol was recorded (Wu and von Tiedemann, 2002).

Cd determination

The plants were harvested after they were exposed to Cd for 28 days. The harvested plants were rinsed with tap water, and the roots were immersed in 20 mM Na_2-EDTA for 15 min to remove Cd adhered to the root surface (Yang et al., 2004). Then the plants were separated into leaves, stems and roots. They were then separately rinsed with running tap water and distilled water, wiped with tissues and weighed. They were then dried at 105℃ for 30 min, then at 70℃ until weight was constant. Dried plant materials were weighed and ground. The powders were digested with a concentrated acid mixture of HNO_3/ $HClO_4$ (3:1, v/v). The Cd concentration in plant tissues was determined with an Optima 3000 ICP-AES instrument (Perkin-Elmer, USA). Statistical analyses Average values and standard deviations (SD) were calculated for all the data in this paper. One-way analysis of variance was carried out with SPSS 11.0. The significant difference was set between treatments at $p < 0.05$ or $p < 0.01$. Multiple comparisons were also made by the LSD (least significant difference) test.

RESULTS

Cd accumulation in plant tissues

After 28 days Cd-exposure, Cd concentrations in shoots

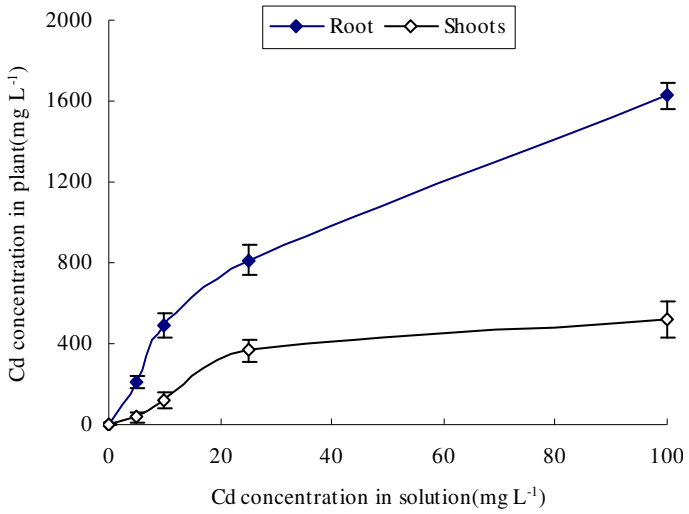

Figure 1. Effects of Cd stress on root and shoots Cd concentrations in L. japonica after 28 days exposure. Values represent mean ± SD (n=3).

and roots of *L. japonica* increased markedly with increasing Cd concentrations in the medium (Figure 1). It was shown that Cd accumulated mainly in roots. At the same level of Cd concentration, there was a significant gradient of Cd concentrations from roots to shoots. When plants were exposed to 25 mg L^{-1} Cd, the concentration of Cd accumulated in shoots was 366.35 μg g^{-1} DW. Furthermore, in plants exposed to 100 mg L^{-1} Cd, the Cd concentrations accumulated in roots and shoots reached the highest level—1625.93 and 518.67 μg g^{-1} DW, respectively.

Effects of Cd on plant growth

The toxic effects of Cd on plant growth measured in terms of dry biomass and height are shown in Figures 2 and 3. Leaf, root and total biomass showed similar trends with increasing Cd concentrations in the medium (Figure 2). The biomass of leaves, root and total exposed to 5 mg L^{-1} Cd showed increases above the control. When Cd concentrations in the medium were up to higher concentrations (25 mg L^{-1}), leaf, root and total biomass began to decrease with increasing Cd concentrations in the medium. The change in height corresponds well with the change in biomass. The height of the plant increased when plants were exposed to 5 mg L^{-1} Cd (Figure 3). When Cd concentration reached as high as 100 mg L^{-1} in the medium, the height only decreased by 6.79% and had no significant differences compared to the control ($p > 0.05$). As shown as Figures 2 and 3, the increase of leaf, root and total biomass and height indicated that low-concentration-Cd had significant stimulative effects on plant growth.

The Cd-induced growth changes were associated with the appearance of visible toxicity symptoms such as chlorosis. When plants were exposed to 5 and 10 mg L^{-1} Cd, the leaves of *L. japonica* did not show any visual symptoms and the color of leaves became darker. However, along with the increase of Cd concentration in the medium, leaf chlorosis began to develop acropetally along the leaf veins. Such symptoms were observed mainly at higher Cd concentrations (100 mg L^{-1}).

Effects of Cd on chlorophyll content

The correlation between chlorophyll and carotenoid contents in leaves and Cd concentration in medium was quite clear (Table 1). The chlorophyll a, chlorophyll and carotenoid contents were increased by 5 and 10 mg L^{-1} Cd, but lowered by higher concentrations Cd exposure, which indicated that low dosage of Cd may be beneficial to plant. The results are consistent with the visual observation on the leaf color changes. When plants were exposed to 100 mg L^{-1} Cd, chlorophyll a, chlorophyll b, chlorophyll and carotenoid contents all decreased.

Effects of Cd on lipid peroxidation and enzyme activity

The level of lipid peroxidation products, measured as malondialdehyde (MDA) contents, is presented in Table 2. Although no significant changes in MDA contents exposed to 5 mg L^{-1} Cd during most of treatment time, MDA contents in leaves and roots of *L. japonica* increased with the increased Cd concentration in the medium. MDA contents in leaves reached the maximum by 14 and 28 days exposure to 25 mg L^{-1} Cd, but not as same as that in roots. However, decrease was observed in leaves and roots when plants exposed to 100 mg L^{-1} Cd.

Similar trend such as the inducing effect of Cd, the increased superoxide dismutase (SOD) activity showed after only 14 days Cd-exposure and then turned to decline, all can be seen in leaves, but not as clear as in roots (Table 2). Twenty five mg L^{-1} was the best Cd concentration in inducing the activity in leaves. When the exposure extended to 28 days, SOD activity had significant increase in leaves exposed to 10 mg L^{-1} Cd, and the activity reached the highest level in roots exposed to 25 mg L^{-1} Cd and then decreased, but still higher than that after 14 days. The SOD activity, whether in leaves or roots, showed the maximum after 28 days Cd-exposure. It was much higher in roots than in leaves, and the highest level of the activity in roots was 972.02 U g^{-1} FW, which was related to the accumulation of Cd in plant tissues.

After 14 days Cd-exposure, catalase (CAT) activity was significantly increased, reached the highest level in

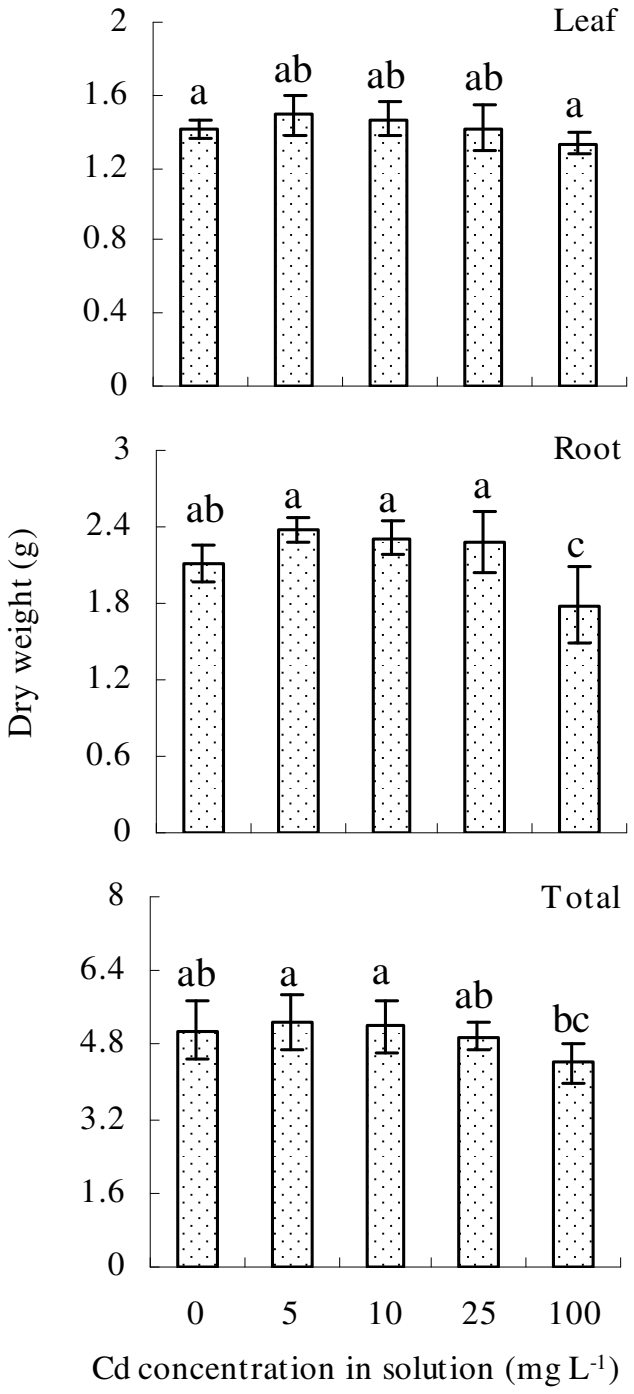

Figure 2. Effects of Cd on dry weight of leaf, root and total biomass in L. japonica. Values represent mean ± SD. Different letters indicate significant differences at p<0.05.

leaves exposed to 25 mg L^{-1} Cd, and in roots exposed to 10 mg L^{-1} Cd (Table 2). This might be related to the higher Cd concentration in roots. Two weeks later, CAT activity decreased both in leaves and roots, and the higher the Cd concentration, the lower the activity.

Peroxidase (POD) activity in leaves and roots was higher than the control by 14 days Cd-exposure, especially significantly exposed to 100 mg L^{-1} Cd ($p < 0.05$). After 28 days Cd-exposure, the increase in POD activity in roots began much faster than that in leaves. The best concentration in inducing the activity in roots was 5 rather than 10 mg L^{-1} Cd. Considering the much lower Cd concentration in roots, the results indicate that Cd is much more efficient in enhancing POD activity in roots than that in leaves.

DISCUSSION

The metals bioaccumulation in plants often induces a series of cellular changes, some of which are directly attributed to metal tolerance capacity of the plants. In our study, Cd accumulation in *L. japonica* is accompanied by an induction of many physiological changes. The dose-responses of Cd accumulation in plant tissues were studied (Figure 1). The results showed Cd concentrations in roots and shoots of *L. japonica* increased obviously along with increasing Cd concentrations in the medium, and Cd accumulated in roots at a faster rate than in shoots. Our present results are compatible to those reported in other studies (Hegedüs et al., 2001; de la Rosa et al., 2004; Chen et al., 2007). Cd^{2+} can be easily absorbed as free ion, and roots of most plants have been found to be a major site of Cd accumulation (Di Cagno et al., 1999). More important in the study is that exposed to 25 mg L^{-1} Cd, the plant did not induce significant damage, indicated by the biomass and height had no significant differences compared with the control, and Cd concentration in shoots got up to 366.35 μg g^{-1} DW, more than 100 μg g^{-1} dry tissue, which is the threshold value of Cd-hyperaccumulator (Baker and Brooks, 1989; Sun et al., 2008), indicated that *L. japonica* had a potential as phytoremediator of Cd-contaminated soil.

With Cd accumulation in the tissues, the acropetal development of the chlorosis is a strong evidence that the symptom is really caused by the absorbed and acropetally translocated Cd. In the present study, the increase in chlorophyll content by exposure to 5 mg L^{-1} Cd may indicate an improved growth. The same phenomenon has been found for studying the enhancement of Aluminum to plant growth as Kinraide (1993), the explanation of which contained increased Fe solubility, promotion of P uptake, prevention of Ca depletion, and protection against Cu/Mn toxicity. However, the decreased chlorophyll content related to higher Cd concentration could be used to monitor Cd-induced damage (Hegedüs et al., 2001). The phenomenon is also proposed as hormesis by de la Rosa et al. (2004).

Many Cd-induced potential damages are difficult to measure, but the most widely accepted indicator of oxidative damage is the accumulation of malondialdehyde (MDA), which is a breakdown product

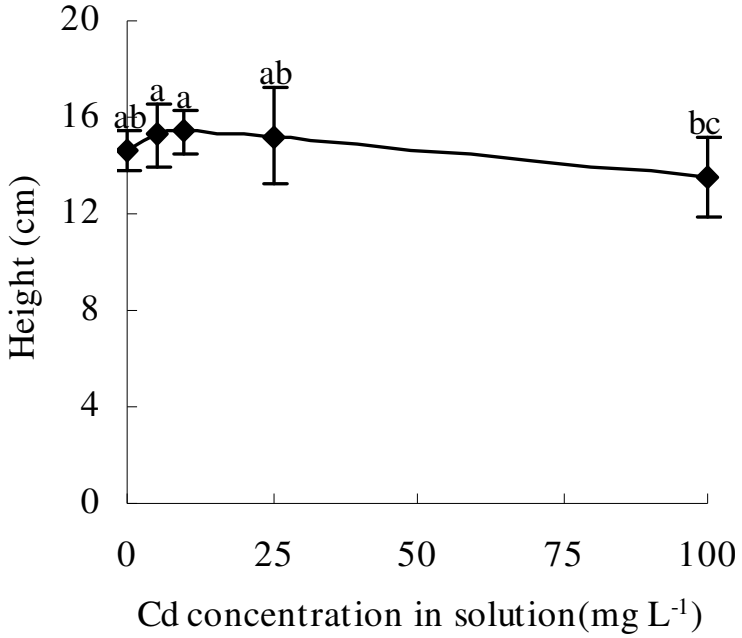

Figure 3. Effects of Cd on height of L. japonica. Values represent mean ± SD. Different letters indicate significant differences at p<0.05.

Table 1. Effects of Cd on chlorophyll and carotenoid contents (mg g^{-1}FW) in leaves of *L. japonica.*

Cd stress levels (mg L^{-1})	Chlorophyll a	Chlorophyll b	Chlorophyll	Carotenoid
0	1.98±0.01ab	2.14±0.01a	4.12±0.02a	2.07±0.01a
5	2.27±0.03a	2.09±0.02a	4.37±0.01ab	2.13±0.01ab
10	2.29±0.02a	2.08±0.01a	4.37±0.04ab	2.15±0.03ab
25	2.05±0.02ab	1.91±0.03ab	4.96±0.03ab	2.09±0.01a
100	1.51±0.01bc	1.67±0.02b	3.19±0.01c	1.74±0.02bc

Data are means ± SD (n=3). Different letters indicate significant differences at p<0.05.

of lipid peroxidation (Smirnoff, 1993). In the present study, the elevation in MDA content in leaves and roots indicated the plants were subjected to Cd-induced oxidative stress (Table 2). This is in accordance with other study (Chaoui et al., 1997). However, a slight decrease was observed in plant exposed to higher Cd concentrations. These results coincide with earlier study by Hegedus et al. (2001). MDA content was not greatly affected by exposure to 5 mg L^{-1} Cd during most of the treatment time. This indicated that the capabilities of the plant to adapt to lower concentration of Cd might be related to a low degree of lipid peroxidation and thus an improved growth was maintained.

In the present study, it has been shown that Cd induced lipid peroxidation, resulting in ROS formation. As a defensive mechanism, antioxidative enzymes, SOD, POD and CAT are correspondingly induced for removing free radicals and scavenging ROS. The significant responses of SOD, POD and CAT activities in *L. japonica* suggested antioxidative enzymatic system might play an important role in the resistance of plant to Cd stress (Table 2). In our study, when the exposure extended to 28 days, the activity of SOD was much higher in roots than in leaves, and this may be related to the much higher Cd concentration in roots than that in leaves. After 28 days Cd-exposure, SOD activities still higher, which might be an indicator of reduced tolerance. The changes of CAT activity indicated low Cd concentrations in the medium were more efficient in inducing CAT activity in roots. On the contrary, higher Cd concentration was needed in the induction in leaves. After 28 days Cd-exposure, it was shown CAT activity in roots decreased with the increase of Cd concentration in the medium, but the changes in leaves were not as significant as in roots. The decrease in CAT activities seems to be an indicator of potential damage. Our result is compatible to the study by Sun et al. (2007). In plant cells, H_2O_2 is removed by CAT, but CAT is not a robust enzyme and its

Table 2. Effects of Cd on malondialdehyde (MDA, nmol g^{-1}FW), superoxide dismutase (SOD, U g^{-1}FW), peroxidase (POD, U g^{-1}FW min^{-1}) and catalase (CAT, μmol g^{-1}FW min^{-1}) activities in *L. japonica.*

Parameters	Cd treatments (mg L^{-1})	Leaf		Root	
		14 days	28 days	14 days	28 days
MDA	0	16.03±1.13ab	15.21±0.89a	15.47±1.23a	12.09±1.47b
	5	18.27±1.36ab	16.19±1.33a	19.82±1.55a	15.24±0.57b
	10	19.18±1.51a	17.82±1.01a	21.05±1.70b	19.70±3.47ab
	25	21.59±0.78a	19.35±1.53ab	20.79±2.23ab	17.93±1.70ab
	100	17.85±2.03ab	16.44±1.18a	17.36±0.91a	13.21±2.04b
SOD	0	212.93±38.04a	345.30±57.13a	291.32±30.62ab	427.79±76.53a
	5	331.25±39.62ab	377.94±68.33a	312.48±28.81ab	480.52±78.21a
	10	340.62±38.10ab	462.33±92.58b	325.65±50.39ab	631.93±35.84ab
	25	387.83±45.73b	407.12±37.67ab	383.27±34.97b	972.02±101.36bc
	100	303.14±40.35ab	381.65±71.92ab	380.58±77.25b	719.87±87.59abc
CAT	0	40.39±5.23a	37.17±2.62a	23.93±2.68ab	39.62±3.87a
	5	52.77±8.01a	37.69±2.09a	58.62±5.05b	38.15±4.24a
	10	71.84±10.12ab	35.84±7.58a	77.35±6.22bc	36.42±2.39a
	25	102.06±14.38bc	33.25±1.67a	39.79±5.31ab	35.21±7.66a
	100	62.52±9.11ab	30.71±3.18ab	20.55±3.94ab	27.39±2.28ab
POD	0	27.05±3.65a	33.23±2.41a	15.91±0.82a	32.81±4.53a
	5	29.93±1.42a	41.71±3.03ab	16.37±1.52a	53.22±5.09ab
	10	36.71±3.38ab	45.92±5.16ab	21.95±3.72ab	49.25±8.37ab
	25	34.45±5.90ab	43.10±2.92ab	27.32±5.23ab	41.69±2.66a
	100	40.38±1.72bc	38.27±5.73a	32.18±3.01b	39.07±3.82a

Data are means ± SD (n=3). Different letters indicate significant differences at $p<0.05$.

effectiveness is also limited by relatively poor affinity for H_2O_2 and its subcellular localization in peroxisomes (Feierabend et al., 1992). The changes in POD activity may be related to the complexity of its physiological functions. POD not only scavenges H_2O_2, but also catalyzes the synthesis of H_2O_2 needed for cell wall formation (Baker et al., 2000). In our study, increased POD activity was shown, suggested that it could be the reason of either ionic microenvironment or tissue specific gene expression (Hegedüs et al., 2001). Other studies have reported increase (Shah et al., 2001), decrease (Hassan et al., 2005) and no changes (Schutzendubel et al., 2001) of POD activity in response to Cd exposure. In the present study, variations in SOD, POD and CAT activity indicated low concentration Cd induced acclimation, resulting in the increase of enzyme activity and tolerance to Cd stress, but when the stress intensity was too strong, these enzymes would be inhibited causing decrease in tolerance and eventually lead to damage to the plant. The differences of SOD, POD and CAT activity in leaves and roots were significant and it may have two reasons: one is Cd concentration in roots is higher than that in leaves under the same concentration Cd stress; the other is that different isozymes may exist in leaves and roots and they respond differently.

Conclusion

In the present study, the accumulation of Cd in *L. japonica* contributed to the formation of AOS, confirmed by elevated MDA content. The antioxidative enzymatic defense systems may play an important role in plant tolerance to Cd stress, especially the cooperation of SOD, POD and CAT activities for the detoxification of ROS, which is directly attributed to metal tolerant ability of the plant. In addition, the high Cd concentrations accumulated in the plant tissues were found, indicated that *L. japonica* had a potential as a phytoremediator of Cd-contaminated soil and the plant used for medicine would be not safe for human health if grown under Cd-contaminated conditions.

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