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Full Length Research Paper

Stimulative effect induced by low-concentration Cadmium in *Lonicera japonica* Thunb.

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A hydroponics experiment was carried out to study the stimulative effect in *Lonicera japonica* Thunb. induced by cadmium (Cd). The results showed that the plants did not show any visual symptoms at low concentrations of Cd after 2 weeks exposure. Furthermore, roots elongation and contents of photosynthetic pigments displayed a biphasic curve with the increase of added Cd in nutrient medium, indicating plant growth was stimulated at low doses of Cd and inhibited at high doses of Cd. In roots, low-concentration Cd (5 to 25 µmol/L) reduced the content of malondialdehyde (MDA), superoxide anion radical ($O_2^{\cdot-}$) production decreased to 13.07 and 21.39% compare with the control, respectively. The activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) was slightly declined at 5 and 25 µmol/L of Cd, respectively and then significantly increased at higher Cd (> 100 µmol/L) treatments in roots. Low Cd led to a slight increase in the activity of catalase (CAT) after 2 week in roots, but lipid peroxidation was not higher than in the control. According to these results, low-concentration Cd (< 100 µmol/L) did not induce oxidative stress and even alleviated the oxidative stress in roots of *L. japonica*., which might be a mechanism of the stimulative effect in plants.

Key words: Cadmium, stimulative effect, oxidative stress, lipid peroxidation and *Lonicera japonica* Thunb.

INTRODUCTION

In the last half century, heavy metals contamination has increased drastically, causing hazardous effects on human, animal and plant organisms (Sawidis, 2008). Among them, Cd has attracted the most attention due to its potential toxicity to human and relatively high mobility into the plant system (McLaughlin and Singh, 1999). Although Cd is not essential for plant growth, its ions are taken up readily by the plant roots and translocated to the above-ground vegetative parts (Shamsi et al., 2008a). At high concentrations, it causes a number of harmful, widely investigated effects in plants. Some of the most representative symptoms are the inhibition of root elongation (Kahle, 1993; Arduini et al., 1997; Wójcik and Tukendorf, 1999), membrane degradation in chloroplasts (Vassilev et al., 2004), suppression of the photosynthetic activity (Krupa and Baszyński, 1995; Vassilev et al, 1998; Prasad et al., 2001), induction of oxidative stress, enzyme inactivation and even cells death (Dominguez, 2007; Padmaja et al., 1990; Toppi and Gabbrielli, 1999; Devi et al., 2007; Liu et al., 2009). The effect may depend on the species and age of plants, the treated organ, and the concentration of the stress agent.

While high concentrations of chemical stressors such as heavy metals and herbicides are toxic, stressors in low concentrations may have a beneficial effect (Tang et al., 2009; Simard et al., 1990; Karavaev et al., 2001; Cedergreen, 2008). Low-concentration stressors induced stimulation effects have been reported in many agricultural and ornamental plant species: rice, bean, barley and maize (Nyitrai et al., 2003, 2004, 2007; Mishra and Kar, 1973; Kovács et al., 2009), *B. pilosa* (Sun et al.,

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2009), *S. alfredii* (Zhou and Qiu, 2005), *B. juncea* (Seth et al., 2008). These stimulative effects include Chl-accumulation, an increase in the amount of cytokinins (Kovács et al., 2009), enhancement of plant biomass, promotion of roots elongation. Several studies have shown that the stimulative effect may be linked to levels of oxidative stress (Yang et al., 2007; Qiu et al., 2008; Zhang et al., 2009; Wang et al., 2010). Cd is known to induce a burst of reactive oxygen species (ROS) in plant tissues at high concentrations, leading to oxidative stress (Gouia et al., 2003; Dominguez et al., 2007; Meng et al., 2009). To alleviate oxidative stress due to ROS, plants have developed a series of antioxidant defense systems (Allen, 1995; Stroinski, 1999). Among these defense systems, anti-oxidative enzymes such as SOD, POD and CAT play an important role in scavenging ROS through a series of complex reactions (Salin, 1988; Asada, 1992; Mishra et al., 2006). Generally, these enzymes, which may be expressed by distinct regulatory mechanisms in response to various environmental stresses and play the cooperative role in protecting each organelle and minimizing tissue injury (Mittler, 2002). However, little information is available on the physiological response and antioxidant enzyme changes in plants at low concentrations of Cd. Therefore, the activities of antioxidant enzymes (SOD, CAT, POD), the amount of MDA and $O_2^{\cdot-}$ generation rate in roots were measured in the present study to determine whether low-concentration Cd could cause oxidative stress.

L. japonica Thunb., a popular ornamental, has become established in temperate and tropical regions worldwide in the past 150 years, and it is also widely used in Asian medicine (Larson et al., 2007). In our previous work, it was shown *L. japonica* had strong tolerance to Cd in the nutrient medium and strong accumulation capability of Cd in its stem. When exposed to low concentrations Cd, the plants did not show any visual symptoms, even the plant growth seemed to be improved (Liu et al., 2009). In the present study, we investigated the stimulative effect induced by low-concentration Cd in *L. japonica*. Our primary aim was to explore the possible mechanism of stimulation. Furthermore, it may provide a new model for further investigating the stimulative effect in plants at low-concentration chemical stressors.

MATERIALS AND METHODS

Plant culture and Cd exposure

Cuttings of *L. japonica* were collected from Linyi, Shandong Province, China and propagated in perlite in the laboratory of Institute of Applied Ecology, Chinese Academy of Sciences. After two months, plants were transformed to 500 ml adumbral containers with modified Hoagland solution (Hoagland and Arnon, 1950) and continual aeration, six plants for each. The nutrient medium contain the following ingredients (mmol/L): 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 experiment was performed in a growth chamber under controlled conditions: 8 h illumination per day (9.00 am to 5.00 pm), 1200 $\mu molm^{-2}$ s^{-1} PPFD, with a 25/18°C day/night temperature and a relative humidity of 60%.

Solutions were renewed once every 4 days to prevent nutrient depletion, 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, 25, 50, 100, 200, 400 (μmol/L), equal as 0 (CK), 0.56, 2.81, 5.62, 11.24, 22.48, 44.96 (mgL^{-1}), respectively. Samples were taken after 2 weeks of treatment from roots and leaves. The experiment was repeated three times.

Root elongation

The maximum root length of each plant was measured before and after Cd treatments. The root elongation rate was calculated as the difference between the final and initial length.

Leaf chlorophyll content

The extraction procedure was similar to that of Booker and Fiscus (2005) with small modification. Tissue samples (0.2 g) were soaked in 25 ml 95% (v/v) ethanol at 4°C in darkness until the tissues became white. Extract was used to measure the absorbance at 649 and 665 nm. Chlorophyll content was calculated according to Lichtenthaler and Wellburn (1983).

Determination of lipid peroxidation and enzymes´ activities in roots of *L. japonica*

Fresh roots (0.2 g) was ground under liquid nitrogen and homogenized in 5.0 ml 50 mmol/L cold Na-phosphate buffer (pH 7.8), with 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). After centrifuging at 13,000 × g for 30 min at 4°C , the supernatant was used for further analyses. Lipid peroxidation was estimated by the concentration of MDA, the major thiobarbituric acid (TCA) reactive material, as described by Bowler (1992).

All enzyme activities were calculated on the base of fresh weight (FW). SOD activity was assayed by the inhibition of nitroblue tetrazolium (NBT) reduction (Krivosheeva et al., 1996), taking the enzyme extract that inhibits 50% of the reduction as one unit. CAT activity was measured by the decrease in absorbance at 240 nm (Pinhero, 1997). 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 Tiedemann, 2002).

Assay of $O_2^{\cdot-}$ generated in roots of *L. japonica*

Fresh roots (0.2 g) were quickly ground in liquid nitrogen and homogenized in 5.0 ml 50 mmol/L cold Na-phosphate buffer (pH 7.8), with 1 mmol/L EDTA and 2% (w/v) PVP. After centrifuging at 13,000 × g for 10 min at 4°C, the supernatant was used for further analyses. $O_2^{\cdot-}$ generation rate was determined by the reaction of hydroxylamine and $O_2^{\cdot-}$. The specific absorption at 530 nm was assayed. The generation rate was calculated according to the standard curve, which was prepared with sodium nitrite (Wang et al., 2010).

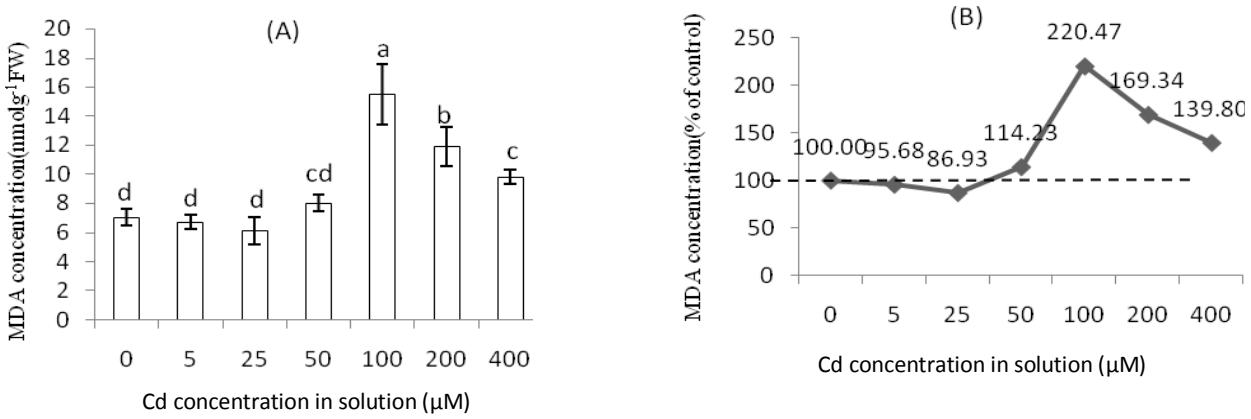

Figure 1. (A) Effect of Cd concentrations in medium on MDA content in roots of *L. japonica* after 14 days exposure. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the LSD test. (B) Effect of Cd on MDA content in roots of *L. japonica* expressed as percentages of the value for the control, which is set at 100%.

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

Cd (μmol/L)	Chlorophyll a	Chlorophyll b	Carotenoid
0	2.22 ± 0.20ab	1.37 ± 0.05a	0.20 ± 0.09ab
5	2.22 ± 0.13ab	1.45 ± 0.06a	0.17 ± 0.04ab
25	2.34 ± 0.02ab	1.46 ± 0.10abc	0.25 ± 0.02ab
50	2.60 ± 0.12a	1.37 ± 0.04ab	0.33 ± 0.04a
100	2.04 ± 0.18b	1.08 ± 0.04bc	0.20 ± 0.07ab
200	1.75 ± 0.29bc	1.11 ± 0.19abc	0.09 ± 0.02b
400	0.92 ± 0.01c	1.00 ± 0.01c	0.09 ± 0.01b

Statistical analyses

All measurements were replicated three times. Average values and standard deviations (S.D.) were calculated by the Microsoft Office Excel 2007 for all the data in this paper. One-way analysis of variance was carried out with SPSS13.0. The significant difference was set among Cd concentrations at $p < 0.05$ or $p < 0.01$. Multiple comparisons were made by the least significant difference (LSD) test.

RESULTS

Roots elongation

After 14 days exposure to 5 to 100 μmol/L Cd, the roots of *L. japonica* did not show any visual symptoms. When the concentration up to 200 μmol/L, roots turned to dark brown and elongation was inhibited. At low Cd concentrations (5 to 50 μmol/L), the roots elongation was higher than the control, and then turned to decrease when the concentration was higher than 100 μmol/L (Figure 1A). Roots elongation rate was elevated by 18.92, 20.38 and 2.93% compared with the control, respectively, in treated with 5, 25 and 50 μmol/L Cd. In contrast, it was marked decreased by 39.75 and 44.65% compared with the control at the doses of 200 and 400 μmol/L (Figure 1B). Data indicated that Cd improved the roots growth at low concentrations, but inhibited roots elongation at high concentrations, which is reflected in an inverted U-shaped dose-response curve (Figure 1).

Chlorophyll contents

The contents of chlorophyll a, chlorophyll b and carotenoid in leaves after 14-day Cd treatments were presented in Table 1. Interestingly, the low concentrations of Cd in the nutrient solution resulted in the increase of chlorophyll a, chlorophyll b and carotenoid contents. They were increased at low-concentration Cd (5 to 50 μmol/L), the maximum increase rate was 17.18, 8.76 and 65.00% compared with the control, respectively. When at the high concentration Cd (> 100 μmol/L), the content of photosynthetic pigments tended to decline with the increase of Cd concentrations in the solution. They marked decreased by 58.56, 27.01 and 55.00% of the control at 400 μmol/L of Cd. The concentrations of chlorophyll and carotenoid generally showed an inverted

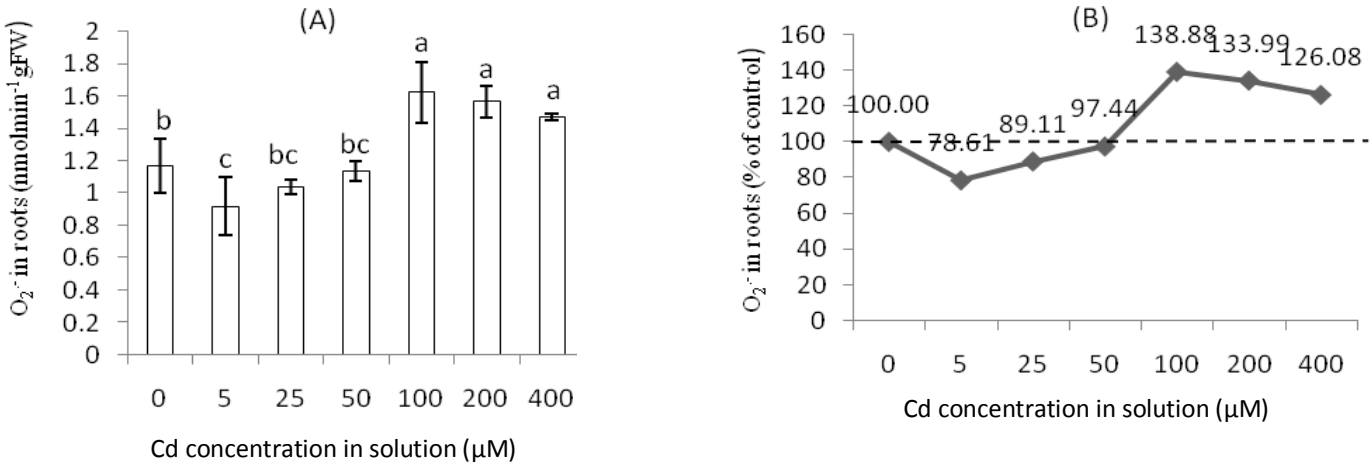

Figure 2. (A) Effect of Cd concentrations in medium on generation in roots of *L. japonica* after 14 days exposure. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the LSD test. (B) Effect of Cd on generation rate in roots of *L. japonica* expressed as percentages of the value for the control, which is set at 100%.

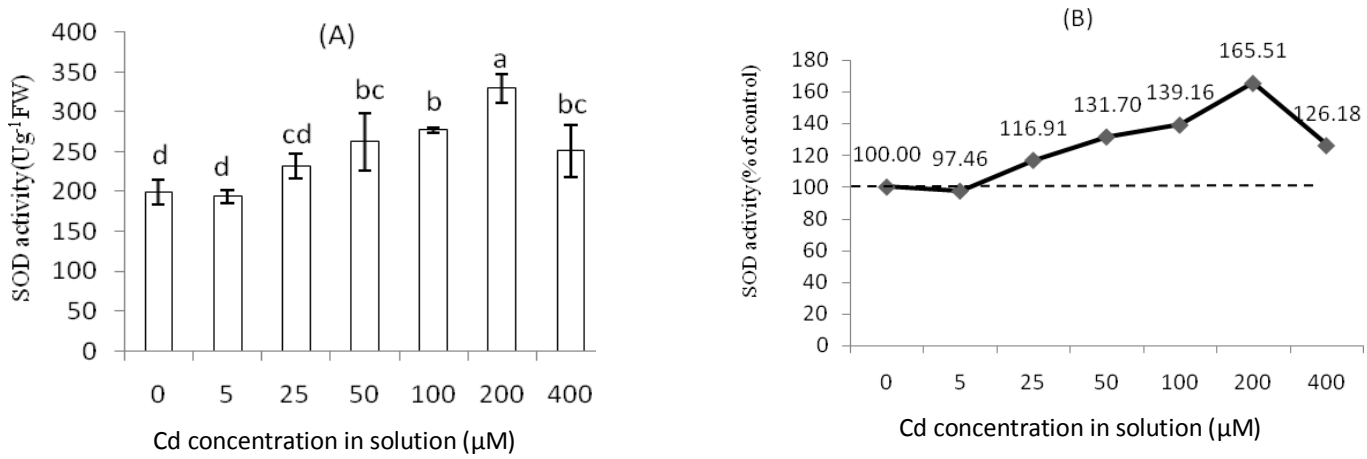

Figure 3. (A) Effect of Cd concentrations in medium on SOD in roots of *L. japonica* after 14 days exposure. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the LSD test. (B) Effect of Cd on SOD activity in roots of *L. japonica* expressed as percentages of the value for the control, which is set at 100%.

U-shaped curve with the increasing addition of Cd in the solution, which similar to the variation pattern of roots elongation. The result indicated that low dosage of Cd may be beneficial to plant.

Lipid peroxidation induced by Cd in roots

It is known that the most widely accepted indicator of oxidative damage is the accumulation of MDA, which is a breakdown product of lipid peroxidation (Smirnoff, 1993). In our study, the contents of MDA in roots declined by 4.32 and 13.07% compared with the control at 5 and 25 μmol/L Cd and then enhanced with the increase of Cd concentrations in the solution, indicating a U-shaped curve of MDA levels (Figure 2). This result showed that low concentrations of Cd did not cause peroxidation but induced beneficial effect to some extent.

Generation of $O_2^{\cdot-}$ induced by Cd in roots

After exposure of 14 days, $O_2^{\cdot-}$ generation in the roots significantly declined at low concentrations of Cd, and then enhanced along with the increase of Cd (Figure 3A). According to Figure 3B, $O_2^{\cdot-}$ generation rate was decreased by 21.39, 10.89 and 2.56% compared with the control, respectively, in treated with 5, 25 and 50 μmol/L

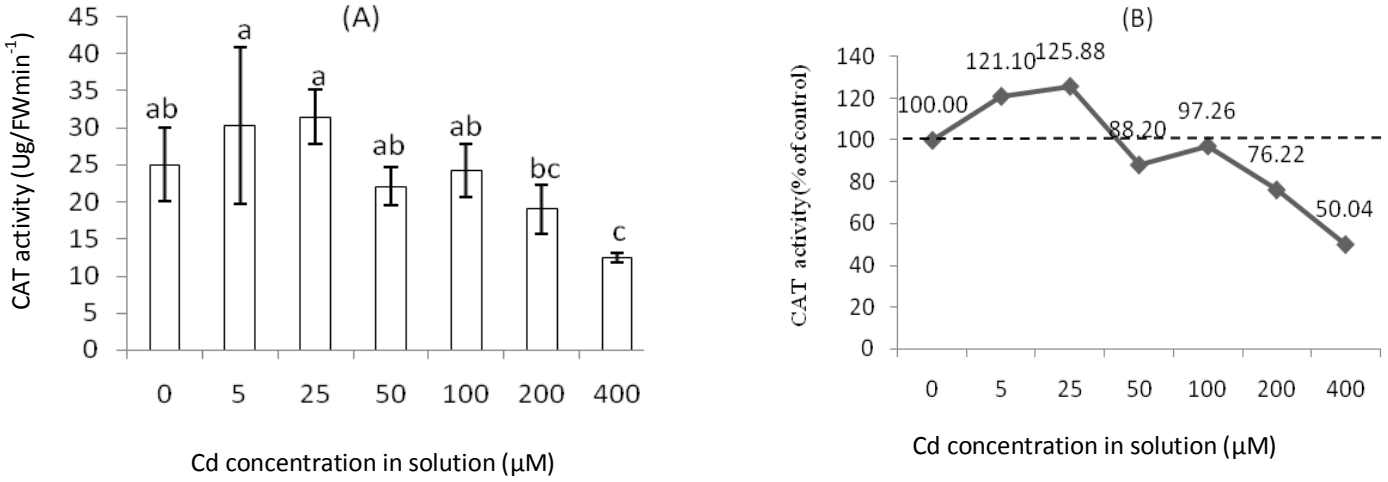

Figure 4. (A) Effect of Cd concentrations in medium on CAT in roots of *L. japonica* after 14 days exposure. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the LSD test. (B) Effect of Cd on CAT activity in roots of *L. japonica* expressed as percentages of the value for the control, which is set at 100%.

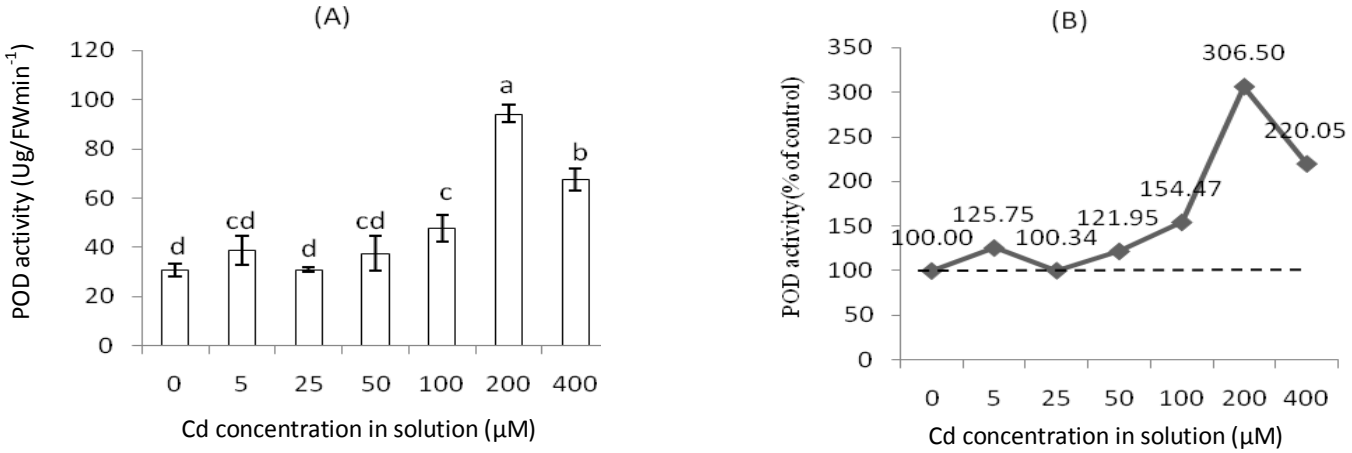

Figure 5. (A) Effect of Cd concentrations in medium on POD in roots of *L. japonica* after 14 days exposure. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the LSD test. (B) Effect of Cd on POD activity in roots of *L. japonica* expressed as percentages of the value for the control, which is set at 100%.

Cd. Significant enhancement was showed at concentrations from 100 to 400 μmol/L. A U-shaped dose response curve of $O_2^{\cdot-}$ generation rate was observed in the experiment (Figure 3).

Antioxidant enzymes´ activities induced by Cd in roots

In our study, SOD activities slightly decreased at 5 μmol/L of Cd, increased with the increasing concentrations of Cd from 25 to 200 μmol/L, and then tended to decrease thereafter, displaying a biphasic curve in all the treatments (Figure 4A). As shown in Figure 4B, compared with the control, SOD activity declined by 2.54% at the lowest concentration of Cd. Significant enhancement of SOD activities was observed at 200 μmol/L of Cd, the highest increase was 65.51% to the control. CAT activities increased at the low Cd concentrations (5 to 100 μmol/L), and then declined (Figure 5A). According to Figure 5B, CAT activities increased by 21.10 and 25.88% compared with the control, in treated with 5 and 25 μmol/L Cd. In contrast, it was significant reduction to 76.22 and 50.04% of the control at the doses of 200 and 400 μmol/L Cd. The results also showed that the total activity of POD enzymes slightly decreased at 25 μmol/L, then increased with the increased Cd concentration in the medium from

50 to 400 µmol/L. POD activities had no significant changes compared with the control at low concentrations of Cd (5 to 50 µmol/L).

DISCUSSION

An increase in elongation of roots and contents of photosynthetic pigments of *L. japonica* at lower concentrations of Cd (< 100 µmol/L) exposure followed by a marginal decrease with increasing Cd concentrations was observed after 14 days of exposure, which suggested that a certain concentrations of Cd could facilitate the plant growth. On the contrary, the elongation of roots and contents of photosynthetic pigments was significantly decreased at higher concentrations of Cd (> 100 µmol/L), which could be used to monitor Cd induced damage (Hegedüs et al., 2001). Our results indicated that Cd improved the plant growth at low concentrations, but inhibited at high concentrations, which is also proposed as hormesis by de la Rosa et al. (2004). The phenomenon of stimulation in plant growth has also been found for studying the enhancement of aluminum to plant growth according to Kinraide (1993), the explanation of which contained increased Fe solubility, promotion of P uptake, prevention of Ca depletion, and protection against Cu/Mn toxicity. Videa (2002) also found that the reason for the increase in plant growth could be due to an increase in the uptake of essential elements from the growth medium leading to intrinsic synthesis of biomolecules as a result of stress due to Cd. However, it is more likely that stimulative effects should be found in some physiological mechanisms that are triggered by some stress.

Cd can lead to the generation of ROS (Gallego et al., 1996), such as $O_2^{\cdot-}$, $^1O_2$, ·OH, which can initiate lipid peroxidation (Halliwell and Gutteridge, 1989). The peroxidation of cell membranes severely affects its functionality and integrity and can produce irreversible damage to the cell function (Halliwell and Gutteridge, 1989). Oxidative stress is due to a disturbance in the balance between the production of ROS and the efficiency of the antioxidant defense (Wiegand and Pflugmacher, 2005). In other words, oxidative stress results if excessive production of ROS overwhelms the antioxidant defense system or when there is a significant decrease or lack of antioxidant defense. MDA is an index of lipid peroxidation, and high accumulation of MDA often indicates severe lipid peroxidation. Various studies showed that the application of heavy metals will increase the MDA content in plant tissues.

However, regarding MDA content (Figure 2), $O_2^{\cdot-}$ generation rate (Figure 3), the biphasic dose-response curves were observed in this experiment. The MDA content in roots of *L. japonica* decreased compared to the control when treated with low concentrations of Cd (5 to 25 µmol/L). A similar phenomenon has been observed in $O_2^{\cdot-}$ production in roots of *L. japonica* at (5 to 50 µmol/L) Cd treatment. It is possibility that low levels of Cd did not cause lipid peroxidation in roots, but actually alleviated oxidative stress. The fact that the concentrations of $O_2^{\cdot-}$ and MDA as well as the activities of SOD and POD all slightly decreased at low Cd levels suggested that the scavenging of ROS was probably not entirely the result of antioxidative enzymes, but probably caused by Cd application. Similar results were report in horsebean seedlings under Pb stress (Wang et al., 2010). The elevation in MDA content and $O_2^{\cdot-}$ production in roots at high concentration of Cd (> 100 µmol/L), indicating the plants were subjected to Cd-induced oxidative stress, which is in accordance with our earlier study (Liu et al., 2011). Our results coincided with the phenomenon of hormesis in toxicology (Calabrese and Baldwin, 2005), which implies a stimulation effect at low-dose and reduction effect at high dose.

As a defensive mechanism, antioxidative enzymes, especially SOD, POD and CAT play an important role in scavenging ROS (Liu et al., 2009). In our study, the activities of SOD and POD was slightly declined at 5 and 25 µmol/L of Cd, respectively (Figure 4, 6) and then significantly increased at higher Cd (> 100 µmol/L) treatments in roots. Low Cd led to a slight increase in the activity of CAT after 2 week in roots (Figure 5), but lipid peroxidation was not higher than in the control, indicating the low-concentration Cd did not induce oxidative stress in roots of *L. japonica*. The activities of SOD, POD and CAT may be stimulated by the generation of ROS (Somashekaraiah et al., 1992). The activities of SOD, POD and CAT were not significant increase at low Cd treatments might be attributed to the low level of ROS (Mishra et al., 2006). Our results supported that low production of MDA and $O_2^{\cdot-}$ might be a mechanism of the stimulative effect induced by low-concentration Cd in plants. This hypothesis was confirmed by Allender et al. (1997), who presented a clear evidence of low production of reactive oxygen species ROS as a mechanism to enhance plant growth, possibly through associated effect on Ca^+ membrane transport.

In conclusion, *L. japonica* did not show any visual symptoms at low concentrations (5 to 50 µM) Cd after 14 days exposure. Low-concentration Cd could stimulate the roots elongation, increase contents of photosynthetic pigments and alleviate the oxidative stress in roots. Therefore there is a possibility that Cd could be nutrimental for the growth of *L. japonica*, or there exist other Cd enzymes or Cd-binding proteins in *L. japonica* that are beneficial for ROS elimination. However, the reason for the stimulation phenomenon is not completely understood, which might be regulated by multiple mechanisms. The underlying mechanisms needs for further investigated. *L. japonica* could be considered as a new model to investigate the underlying mechanisms of the stimulative effects in plants.

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