Effects of cadmium on growth and antioxidant responses in *Glycyrrhiza uralensis* seedlings

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ABSTRACT

In the present study, *Glycyrrhiza uralensis* (Leguminosae) seeds were germinated and grown with different concentrations (0, 0.05, 0.1, 0.2 and 0.4 mmol/l) of cadmium acetate, in order to investigate the effects of cadmium on the growth, uptake, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities in *Glycyrrhiza uralensis* seedlings. Uptake of Cd in different tissues of seedlings increased with increasing Cd concentrations in the tested medium, with most accumulation in the radicles. Results suggested that increased cadmium concentrations lead to decreased shoot elongation and seedling biomass. SOD activity in the cotyledons, hypocotyls and radicles increased gradually up to 0.2, 0.1 and 0.4 mmol/l, respectively. POD activity in the cotyledons, hypocotyls and radicles concentrations increased continuously with rising cadmium concentrations up to 0.2, 0.1 and 0.1 mmol/l, respectively. CAT activity in the cotyledons, hypocotyls and radicles increased gradually with increasing cadmium concentrations up to 0.2, 0.2 and 0.1 mmol/l, respectively. PPO activity showed significant increases in the cotyledons, hypocotyls and radicles at 0.4, 0.1 and 0.2 mmol/l cadmium, respectively. A significant change of PAL activity in the cotyledons, hypocotyls and radicles was observed with increasing cadmium concentrations up to 0.2, 0.4 and 0.2 mmol/l, respectively. Results of POD isoenzymes suggested that the staining intensities of isoform patterns were consistent with the changes of the activities assayed in solutions. These results suggested that *Glycyrrhiza uralensis* seedlings may have a better protection against oxidative stress by increasing antioxidant enzymes and PAL activity exposed to cadmium toxicity.

Keywords: licorice; reactive oxygen species (ROS); plant oxidative damage; antioxidant phenolic substances; heavy metal; toxic element

Generally, heavy metals cause oxidative damage to plants, either directly or indirectly through reactive oxygen species (ROS) formation. However, cadmium is not a redox active metal, unlike copper or iron that appear to act directly on the production of ROS through the Fenton and Haber Weiss reactions (Shah et al. 2001, Cho and Seo 2005). Chloroplasts, the major component of photosynthetic organ, are highly sensitive to damage-exposed Cd toxicity (Sandalio et al. 2001). ROS are also produced by the reaction of chloroplast O_2 and electrons that escape from the photosynthetic electron transfer system under normal circumstances. Cd inhibits the photoactivation of photosystem II (PSII) by inhibiting electron transfer (Sigfridsson et al. 2004). Thus, Cd could lead to the generation of ROS indirectly by production of a disturbance in the chloroplasts. In addition, other reports suggested that cadmium may stimulate the production of ROS in the mitochondrial electron transfer chain (Heyno et al. 2008). Based on the above results, Cd toxicity also brings about oxidative stress since the balance between ROS generation and removal was broken. Heavy metals, including Cd, are associated with oxidative stress, plant damage and changes of metabolism (nutrient uptake, contents of pigment, protein, chlorophyll synthesis, the profiles concentration or activity of isozymes) and enzyme concentration to stress metabolism (Clemens 2006, Ghani and Wahid 2007, Monteiro et al. 2009). Metabolic reactions are all possible strategies for adaptation of plants to metal stress.

Glycyrrhiza uralensis Fisch. (Licorice), belongs to the family Leguminosae, and is known as 'Gancao' in China. It is a well-known Chinese herbal medicine used for the treatment of various diseases as well as a tonic medicine for thousands of years. Besides its pharmaceutical uses, licorice is also a drought-tolerant and deep-rooted plant and very important for wind breaking, sand fixing, and soil formation in semiarid ecosystems (Zhang and Ye 2009). Earlier reports showed that Cd can either inhibit or stimulate the activity of several antioxidant enzymes, like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and phenylalanine ammonia-lyase (PAL), before any visible symptoms of toxicity appear. In addition, the response of antioxidant enzymes to Cd, and in general to metals, can also vary among plant species and different tissues (Dai et al. 2006, Mobin and Khan 2007, Zhang et al. 2009). These results would be meaningful to evaluate the role of the antioxidant system in Cd tolerance and detoxification. In the present study, we aimed to provide an insight view to cadmium stress and antioxidant enzyme system in licorice plant, and establish appropriate methods of *in vitro* culture for selection of useful plant biomarkers for further studies in real-scenario conditions.

MATERIALS AND METHODS

Plant materials and seedling culture. Mature licorice seeds were harvested in September 2008 from one plant, growing on the Wu-wei Desert Garden, Gansu, China. These seeds were inspected and deposited in a plastic box with labels (No. 20080925) at 4°C until using. Licorice seeds were surface sterilized in 70% ethanol for 30 s, and then in 0.1% mercuric chloride for 8 min. The seeds were rinsed several times with distilled water, and soaked in water at room temperature for 12 h. The soaked seeds were sown in wide-neck bottles (100 ml) for germination and growth, containing the Murashige and Skoog (MS) medium (about 25 ml). MS medium pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 121 ± 2°C for 15 min, with 30 g/l sucrose and 6 g/l agar powder, containing 0, 0.05, 0.1, 0.2 and 0.4 mmol/l cadmium acetate. Germination experiment was carried out at the greenhouse temperature of 25°C under a 12 h photoperiod in cool, white fluorescent light. The seeds were placed for germination and growth in *in vitro* culture for 7 days when the cotyledons of seedlings had developed. Rotten and contaminated embryos were removed promptly. After culturing, the cotyledons, hypocotyls and radicles of seedlings of each group were weighed (fresh weight), homogenized and immediately stored at –80°C for protein contents and enzyme activity. Five sets of seedlings were analyzed for each cadmium concentrations, with 25 embryos per set.

Estimation of cadmium content. After 7 days, seedlings were selected from each pot for the determination of Cd concentrations. Cotyledons, hypocotyls and radicles were oven dried at 60°C for 48 h, weighed and digested with 5 ml concentrated HNO_3 and 30% w/v H_2O_2 (1 ml) for 30 min in a microwave digester system, and then diluted to 25 ml with deionized water. Cd concentrations of all samples were analyzed with the Integrated Couple Plasma Mass Spectrophotometer (ICP-MS: PQExCell, VG Elemental). Cd contrations were expressed in μg/g dry weight of tissue.

Protein extraction and estimation. Fresh tissues (0.2 g) were homogenized with 2 ml ice-cold 50 mmol/l sodium phosphate buffer (pH 7.0, 1/10, w/v) including 150 mmol/l NaCl and 1 mmol/l EDTA. The homogenized suspension was obtained by centrifuging at 12 000 rpm for 10 min at 4°C. The supernatant was used as the crude extract for assaying POD isozymes, following for the assay of three antioxidant enzymes. Protein content was quantified by the Bradford method and bovine serum albumin as the standard.

Assay of SOD activity. SOD activity was determined by the method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium to blue formazan by 50%. The activity was expressed as the enzyme units per gram fresh weight (U/g FW).

Assay of POD activity. POD activity was performed according to the method of Sakharov and Bautista (1999) with slight modifications. A unit of POD activity (OD_{470}) was expressed as the change in absorbance per minute. The activity was expressed as enzyme units per gram fresh weight (U/g FW).

Assay of CAT activity. CAT activity was measured following the change of absorbance at 240 nm for 1 min due to H_2O_2 (Aebi 1984). Catalase activity was expressed as enzyme units per gram fresh weight (U/g FW).

Assay of PAL activity. Fresh tissues were homogenized with chilled Tris-HCl (50 mmol/l, pH 8.5, 2 ml/g FW), supplemented with 0.5 mmol/l EDTA and 1% polyvinyl pyrrolidone. Other steps are the same as previously. PAL activity was measured by

monitoring the reaction product *trans*-cinnamate at 290 nm (Dai et al. 2006). PAL activity was calculated as the change in optical density during 30 min and one enzyme unit was defined as the amount causing an increase of 0.01 in A_{290} per min. The activity was expressed as enzyme units per gram fresh weight (U/g FW).

Assay of PPO activity. PPO activity was determined with L-Dopa as substrate by measuring the initial rate of dopachrome formation, as indicated by an increase in absorbance at 475 nm (Duckworth and Coleman 1970). The activity was expressed as enzyme units per gram fresh weight (U/g FW).

Assay of POD isoenzymes activity. PAGE for isoenzymes assay was performed with 10% acrylamide gel. POD activity was determined on the gel as described by Ros Barcelo (1987). The gels were rinsed in water and the gel was stained in a solution containing 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till the brown color.

Statistical analysis. Data shown in this paper are reported as the mean ± SD. We performed three independent experiments in duplicate for each condition. Statistical significance was considered to be significant when the *P* value was less than 0.05 using one way analysis of variance.

RESULTS AND DISCUSSION

Plant growth inhibition is a non-specific manifestation of alterations at a biochemical level that are produced as a classical parameter commonly used in the assessment of heavy metal toxicity to plant (Clemens 2006). In order to investigate cadmium stress tolerance or sensitivity of *Glycyrrhiza uralensis*, growth parameters like leaf area, length and fresh weight of hypocotyls and radicles were observed under Cd stress. Under cadmium stress, cotyledon areas as well as hypocotyls and radicles lengths reduced significantly by cadmium toxicity treatments (Figure 1A). However, the rate of decline in radicle lengths was more pronounced under cadmium stress compared to hypocotyls length. The fresh weight of cotyledons decreased gradually up to 0.4 mmol/l cadmium, and the fresh weight of hypocotyls and radicles were also affected by cadmium treatment and the similar trend (decline) can be seen on day 7 (Figure 1B). In higher plants, roots are the first organs in contact with the toxic metal ions, and significantly inhibit root growth and cell division at higher metal concentrations (Clemens 2006). The present results suggested that the length of radicles gradually shortened with increasing cadmium concentrations compared to the control (Figure 1). Growth reduction under Cd toxicity conditions was observed for several species tested, including *Miscanthus sinensis* (Scebba et al. 2006), *Cucumus sativus* (Abu-Muriefah 2008) and *Lemna polyrrhiza* (John et al. 2008). In this work, a significant inhibition was showed in the cotyledons, hypocotyls and radicles, especially in the radicles (Figure 1). Thus, our findings lend further support to the previous results.

The uptakes of Cd in the different tissues of seedlings are presented in Table 1. The Cd concentrations in seedlings increased with increasing tested Cd levels in the medium. The highest Cd concentrations in the cotyledons, hypocotyls and radicles were 1620.9, 3161.3 and 5843.7 μg/g dry weight, when exposure to the Cd concentration of 400 μmol/l, respectively. Partitioning of metals in different plant tissues is a common strategy to

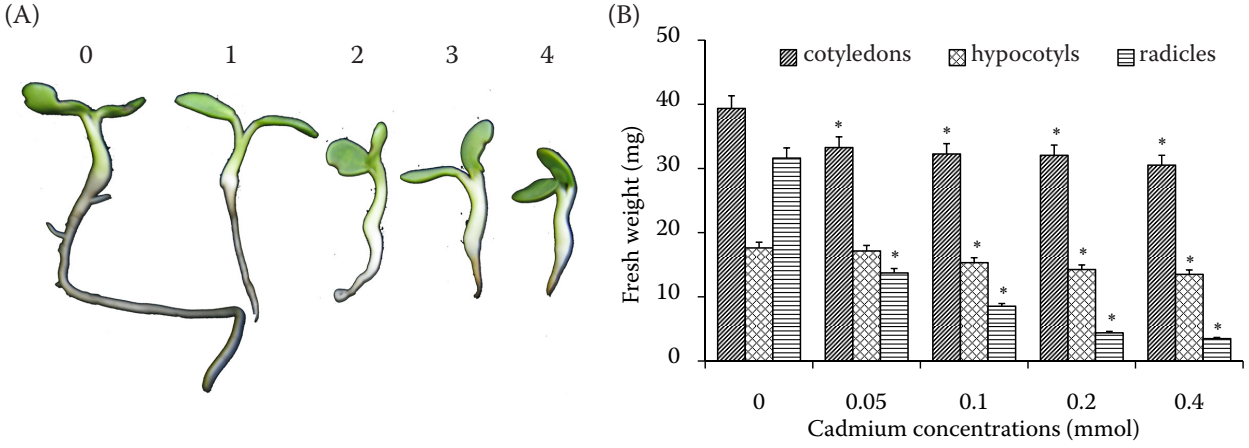

Figure 1. Effects of different cadmium concentrations on growth (A) and (B) fresh weights of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown. (A) lanes from 0 to 4 were 0, 0.05, 0.1, 0.2 and 0.4 mmol/l, respectively

Table 1. Accumulation of cadmium in the cotyledons, hypocotyls and radicles of *Glycyrrhiza uralensis* seedlings after 7 days of exposure to different Cd concentrations

Cd treatment (μmol)	Cd concentratent (μg/g dry weight)		
	cotyledons	hypocotyls	radicles
0	ND	ND	ND
50	195.3 ± 8.75	347.8 ±15.3	738.8 ± 31.9
100	339.6 ± 12.8	718.1 ± 29.5	1350.1 ± 61.2
200	752.4 ± 31.6	1344.6 ± 57.3	2726.5 ±106.2
400	1620.9 ± 68.4	3161.3 ± 128.1	5843.7 ± 252.8

Data represent the mean ± S.E. of three replicates. ND – not determined

avoid toxicity in above-ground parts. The first barrier against Cd toxicity occurs in the roots where Cd may be immobilized by ligands on cell walls and extracellular carbohydrates (Sanitá di Toppi and Gabbrielli 1999, Zhang et al. 2009). In the present study, different concentrations of Cd in the medium may result in an accumulation of Cd at higher levels in the radicles than in the cotyledons and hypocotyls. The present findings support the fact that Cd ions are mainly retained in the roots and that only small amounts are transported to the shoots and leaves.

In plants, earlier results have suggested that Cd can cause oxidative damage to plant cells either directly or indirectly through the burst of ROS in many species, like rice and *A. thaliana*. Although Cd is not a redox metal, it is readily taken up and both directly and indirectly affects several metabolic activities in different tissues and species (Shah et al. 2001, Cho and Seo 2005, Zhang et al. 2005). SOD is considered a key enzyme in the regulation of intracellular concentrations of ROS. Thus, increased SOD activity in plant cells showed that it plays a positive role in controlling the cellular level of these ROS and/or repairing oxidative damage (Miller et al. 2008). As shown in Figure 2, SOD activity was strongly affected by cadmium toxicity. SOD activity in the cotyledons increased up to 0.2 mmol cadmium, and the highest activities increased by 110.8%. Similarly, the maximal activities in the hypocotyls and radicles were found at 0.1 and 0.4 mmol/l cadmium concentrations, and increased by 55.1% and 68.3%, respectively. The tolerance of some plants to cadmium stress was associated with the higher abundance of SOD (Shah et al. 2001, Scebba et al. 2006, Mobin and Khan 2007). In the present study, SOD activity was largely induced by different cadmium concentrations, and a positive correlation between cadmium stress and the abundance of SOD in different tissues of licorice plants is also observed (Figure 3). Thus, our findings indicated that SOD, in some extent, is involved in the defense mechanism of licorice plants cadmium-induced oxidative damages.

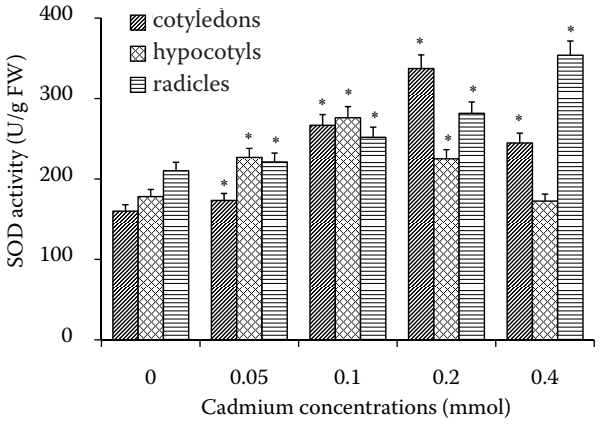

Figure 2. Effects of different cadmium concentrations on superoxide dismutase (SOD) of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown

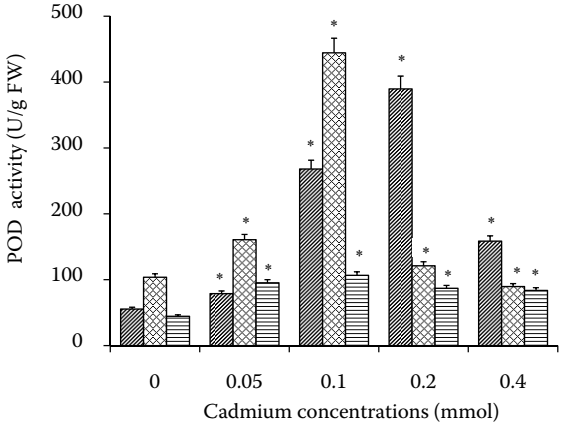

Figure 3. Effects of different cadmium concentrations on peroxidase (POD) of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown

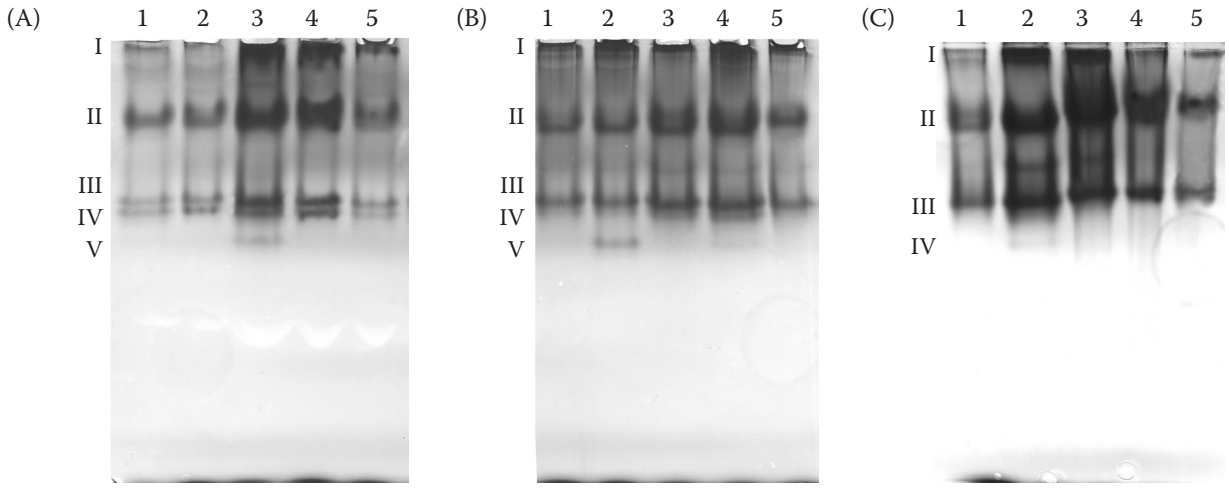

Figure 4. Patterns of peroxidase (POD) isoforms in cotyledons, hypocotyls and radicles of *Glycyrrhiza uralensis* seedlings. (A) patterns of POD isoenzymes in the cotyledons; (B) patterns of POD isoenzymes in the hypocotyls; (C) patterns of POD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 0.05, 0.1, 0.2 and 0.4 mmol/l, respectively. About 25 µl of each sample was loaded

Pioneer studies suggested that POD is not only one of the defense proteins, but it as well an important antioxidant enzyme involved in the response to environmental stresses (Flohé and Ursini 2008). POD activity in the cotyledons increased significantly exposed to 7 days of cadmium treatment, and reached 602.6% at 0.2 mmol/l cadmium compared to the controls. In the hypocotyls and radicles, POD activity rose further up to 0.1 and 0.1 mmol/l cadmium concentrations, increasing by 328.1% and 140%, respectively (Figure 3). POD activity was shown to be induced in several plants when subjected to cadmium stress, such as barley (Huttová et al. 2006), *Miscanthus sinensis* (Scebba et al. 2006) and *Vicia faba* (Zhang et al. 2009). In the present study, increasing Cd concentrations sequentially induced POD activity in the cotyledons, hypocotyls and radicles of *Glycyrrhiza uralensis*, especially in the radicles (Figure 3). Considering this in relation to the results of growth parameters and SOD activity, POD may play an important role in resistance to cadmium stress in licorice plants. To get an insight into the effect of cadmium on isoenzymes pattern, a non-denaturing PAGE for POD activities was performed (Figure 4). In the cotyledons, hypocotyls and radicles, there were five, five and three isoenzyme bands of POD visualized, respectively, but different staining densities were found. The staining densities of POD isoenzymes (I, II, III and IV) in the cotyledons increased with increasing cadmium at 0.05–0.2 mmol/l, and a new isoenzyme was observed when subjected to 0.1 mmol/l cadmium concentration. POD isoforms are known to occur in a variety of plant species, and the expression pattern of isoforms varies in different tissues of healthy plants and is developmentally regulated and influenced by environmental factors (Huttová et al. 2006, Flohé and Ursini 2008, Paynel et al. 2009). In the hypocotyls and radicles, the activity of isoenzymes (II and III) was significantly induced by cadmium stress compared to the control. In addition, a new band in the hypocotyls (IV) and radicles (V) was detected. In the present study, the relationship between cadmium concentrations and isoform pattern in different tissues of licorice is detected, and different staining densities of specific isoform also show different changes with the increasing cadmium concentrations (Figure 4). Although the activity of POD isoform in different tissues of licorice seedlings shows different patterns exposed to cadmium stress, the total POD activity was significantly enhanced, suggesting that POD could reflect an increased degree of oxidative stress. The roles of these isoform might be repair of oxidative damage provoked by cadmium stress.

CAT is one of the major antioxidant enzymes that eliminates hydrogen peroxide by converting it into oxygen and water (Miller et al. 2008). CAT activity in the cotyledons was significantly induced at 0.2 mmol/l cadmium compared to the control, and the highest activity increased by 101.3%. However, the activity in the hypocotyls and radicles increased significantly with the increasing cadmium concentrations up to 0.2 and 0.1 mmol/l, representing 35.1% and 89.9% increments, respectively (Figure 5). Earlier reports showed that CAT activity was significantly induced

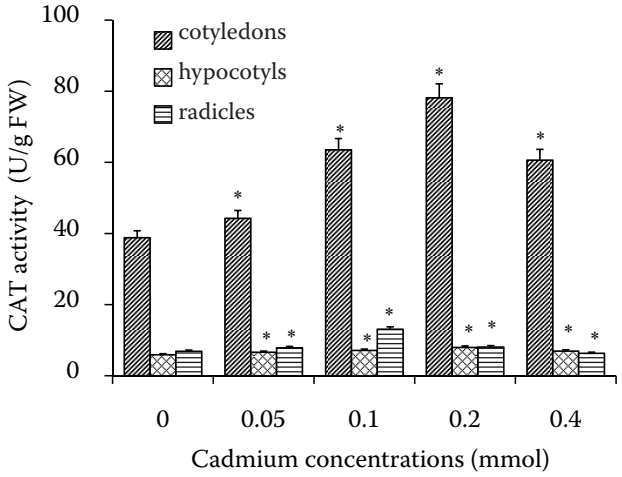

Figure 5. Effects of different cadmium concentrations on catalase (CAT) of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown

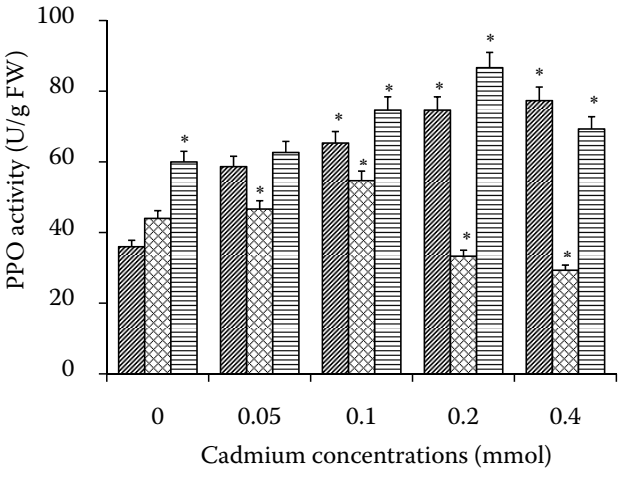

Figure 6. Effects of different cadmium concentrations on polyphenol oxidase (PPO) of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown

by cadmium stress. This suggested that it plays a very important role in the protection against oxidative damage caused by cadmium (Scebba et al. 2006, Mobin and Khan 2007, Zhang et al. 2009). In the present study, CAT activity in the cotyledons was significantly induced exposed to cadmium toxicity, but the activity in the hypocotyls and radicles showed no significant changes compared to those of in the cotyledons (Figure 5). Our results suggested that CAT, at least in the hypocotyls, appears not to be an effective scavenger of ROS compared to those of in the cotyledons and radicles.

PPO is a terminal oxidase which can directly pass electrons to O_2 when the intermediate products of plant respiration are oxidized. It could catalyze the oxidization of such a group of compounds as phenol to quinone. PPO and POD relate closely with 'the callus reaction', meanwhile, they also have some relationships with the synthesis of cell compounds containing phenol groups such as lignin (Thipyapong et al. 2007). As shown in Figure 6, the total PPO activity in licorice plants increased significantly exposed to cadmium stress. In the cotyledons and radicles, PPO activity was also affected by different cadmium concentrations treatment, and the highest level increased by 114.8% and 44.4% compared to the control, respectively. The activity in the hypocotyls increased by 6.1% and 24.2% at 0.05 and 0.1 mmol/l cadmium concentrations, respectively. However, the activity decreased significantly at 0.2 and 0.4 mmol/l cadmium concentrations, representing 24.2% and 33.3% decrease, respectively. Previous reports showed that rapid changes in PPO activity were proposed that may be involved in necrosis development around damaged leaf surfaces and in defense mechanisms against insects and plant pathogen attack (Thipyapong et al. 2007). PPO activity in some plant species was observed under heavy metal stress, and showed a significant increase compared to the control (Kováčik and Klejdus 2008, Saffar et al. 2009). The present results suggested that PPO activity in the cotyledons, hypocotyls and radicles increased initially up to 0.4, 0.1 and 0.2 mmol/l cadmium and then decreased, respectively (Figure 6). The induction of PPO activity might be due to its role in phenolic compound synthesis, which plays an important

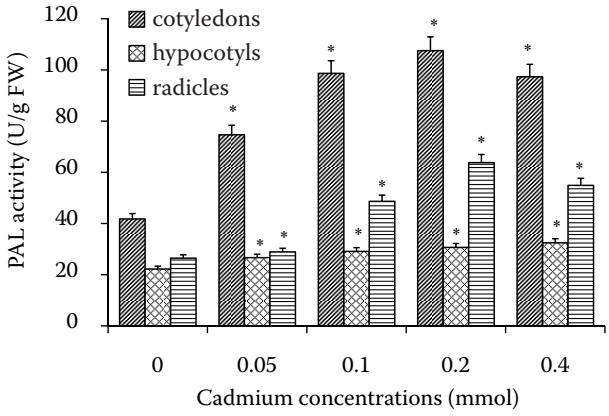

Figure 7. Effects of different cadmium concentrations on phenylalanine ammonia-lyase (PAL) of *Glycyrrhiza uralensis* seedlings. The values and standard errors (vertical bars) of three replicates are shown

role in detoxification of heavy metals in plants (Ruiz et al. 1999). On the basis of these results, our results indicated that changes of PPO activity might participate in the defense mechanism of licorice plants against cadmium toxicity.

PAL is the initial rate-controlling enzyme in phenolic synthesis and many phenolic compounds in plants possess antioxidant activity. In plants, PAL activity may differ widely according to developmental stage, genotype and environmental stresses (Dai et al. 2006). PAL activity in the cotyledons was significantly increased at 0.05, 0.1, 0.2 and 0.4 mmol/l cadmium by 78.7%, 136.2%, 157.5% and 133% compared to the control, respectively. In the hypocotyls and radicles, the activities increased gradually up to 0.4 and 0.2 mmol/l cadmium, and the highest activities enhanced by 46% and 141.2%, respectively (Figure 7). Stimulation of PAL activity was previously reported, and can be considered as an important factor contributing to higher antioxidant protection under cadmium stress. This might suggest that increased PAL activity may enhance phenolic metabolism to produce antioxidant phenolics and lignin precursors aimed at reducing the toxic effects of cadmium (Dai et al. 2006, Kováčik and Bačkor 2007). In the present study, the responses of PAL activity in different tissues of licorice seedlings are reported. Results showed that PAL activity significantly increased in licorice plants. Thus, the defense mechanism of plants by which cadmium affects phenolic metabolism and PAL activity is complex and needs further study.

In conclusion, our findings suggest that Cd toxicity induce growth inhibition in licorice seedlings, and an increase in the overall antioxidant capacities of licorice plants. Our results support the fact that the growth and antioxidant responses in licorice seedlings strongly depend on the variety of the tissue and Cd concentrations. These biomarkers, and in particular antioxidant enzymes, could be used in integrative approaches with classical endpoints in ecotoxicological tests with Cd and after further studies in real field conditions.

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