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Growth and antioxidant responses in *Jatropha curcas* seedling exposed to mercury toxicity

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ABSTRACT

Jatropha curcas seedlings were exposed to varying concentrations of mercury in order to investigate mercury accumulation, and the changes in growth and antioxidant enzyme activities using *in vitro* embryo germination and culture. Our results showed that mercury is readily accumulated by germinating embryos and growing seedlings, and its content was greater in the radicles than those of in the cotyledons and hypocotyls. This accumulation was directly correlated with an increase in tested mercury concentrations in the medium. Biomass in the cotyledons, hypocotyls and radicles increased gradually with increasing mercury concentrations, peaking in seedlings exposed to mercury concentration of 50 μM, and then decreased. Superoxide dismutase activities in the cotyledons, hypocotyls and radicles showed largest increment at mercury concentration of 100 μM. Peroxidase activities in the cotyledons and hypocotyls reached peaks at mercury concentration of 200 μM, and the highest activity in the radicles was observed at 100 μM. Catalase activities in the cotyledons and hypocotyls were significantly induced, and the highest activity in the radicles was observed at mercury concentration of 200 μM. Phenylalanine ammonia-lyase activities in the hypocotyls had a positive correlation to mercury concentrations, and the highest activities in the cotyledons and radicles were found at mercury concentrations of 200 and 100 μM, respectively. Analysis of superoxide dismutase, peroxidase and catalase isoenzymes suggested that different patterns depend on mercury concentrations and tissue types, and the staining intensities of these isoenzymes are consistent with the changes of these enzyme activities assayed in solutions.

1. Introduction

Heavy metals pollution is one of the most important ecological problems on the world scale. Excessive heavy metals in contaminated soil can result in decreased soil microbial activity and soil fertility, and losses in agricultural yield. Their presence in the environment can be highly dangerous [1]. Amongst heavy metals, mercury (Hg) is one of the most toxic heavy metals commonly found in the global environment. The interaction between Hg and plant systems is of particular importance because this metal has been widely used in seed disinfectants, fertilizers and herbicides [2,3]. In plants, the toxic effects of Hg can be characterized by the following: (a) blocking of essential functional groups in biomolecules [4,5], (b) displacement of essential metal ions from biomolecules in photosynthetic pigments, causing a decrease in photosynthesis rates [6], and (c) inhibiting root and shoot growth and yield production, affecting nutrient uptake and homeostasis [7]. The effects of Hg toxicity on cellular systems in many plant species have received a great deal of attention, but researches in this area will be continued.

Being a transition metal, Hg can induce oxidative stress in plant cells and has been linked to the excess production of reactive oxygen species (ROS), which may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death [5,7]. In order to limit oxidative damage, plants have therefore developed mechanisms enabling them to counteract these events. These protective mechanisms include change in lipid composition, changes in antioxidant enzyme activity, increased sugar or amino acid contents, and changes in the level of soluble proteins and gene expressions [8]. In several plants, it has been reported that the activities of ROS-scavenging enzymes, including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), is an important protective mechanism to minimize oxidative damage exposed to Hg toxicity [9]. Therefore, increased activities of these enzymes may be considered as typical defense components against Hg toxicity.

Some plant species are sensitive to heavy metal stress, whereas others are tolerant. The latter shows little inhibition or damage, even they may cope with higher contents of heavy metals in growth medium as well as accumulate high concentrations of heavy metals in their tissues [10,11]. Much research has been done on Hg

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accumulation in several plant species, such as *Oryza sativa* [1], willow (Salix) [4], tomato [9], *Medicago sativa* [12], *Solanum melongena* [13], Indian mustard [14], *Pteris vittata* and *Nephrolepis exaltata* [15]. There is also some evidence that certain plant species have the ability to extract and accumulate Hg both the atmospheric and soil [13,16]. Currently, however, no plant species with Hg hyperaccumulating properties has been identified. Recently, industrial and energy crops have attracted the interest of researchers for meeting the increasing requirements of petroleum and its products. However, cultivation of oil crops on large scale has to be initiated without scarifying the land under agriculture. Therefore, other types of land, which we consider as wastelands, may be put to potential beneficial re-use by producing biomass for energy purposes.

Jatropha curcas L., commonly known as physic nut, belongs to the family *Euphorbiaceae* and is today recognized as a petro-substitute. Its cultivation can also help in the reclamation of wastelands, degraded lands and mine contaminated lands [17]. Panzhihua, an important industrial and mining base with abundant mineral resources, is located in the southwest of China. In particular, the area around mine facilities in Panzhihua has the higher levels of environmental Hg, due to the extraction and processing of Hg-mineral ore for centuries [3]. In Panzhihua, *J. curcas* is often grown on riverside, and in mining areas where soils are usually contaminated by heavy metals. Our previous results suggested that *J. curcas* could adjust themselves to tolerate copper and lead stresses by an effective antioxidant defensive mechanism [18,19]. Although no reports of eco-toxicological research on *J. curcas* plant have been published, these results indicate a promising capacity for bioaccumulation, phyto-translocation and phytoremediation of heavy metals in *J. curcas* [20,21]. Thus, *J. curcas* might be a good candidate for eco-toxicological research. Thus, the aim of this study is to achieve a better understanding of adaptive and tolerance mechanism of *J. curcas* plants exposed to Hg toxicity. Though results obtained with plants grown in pots experiments cannot be directly compared to those in field conditions, they are crucial to highlight the growth and antioxidant responses of plants in control conditions.

2. Materials and methods

2.1. Plant materials and culture

Mature *J. curcas* seeds were collected in August, 2007 and August, 2009 from more than 10 individual wild trees in Panzhihua, Sichuan, China. Seeds were oven-dried at 30 °C, selected and stored in a plastic box (Labeled, No. 20070822) were deposited at 4 °C. *J. curcas* seeds were surface sterilized with 70% ethanol for 30 s, and then in 0.1% mercuric chloride for 8 min. Seeds were rinsed several time with distilled sterile water, and soaked in water at room temperature for 24–36 h. Embryos were dissected from the seeds on a clean bench. These embryos were placed in Murashige and Skoog (MS) medium in wide-neck bottles (100 ml) for germination and growth in *in vitro* culture for 7 days [22]. The bottles containing culture medium and three embryos were separated into five lots. One lot was allowed to grow on MS medium with 30 g l^{-1} sucrose and 0.6% agar powder to serve as control. The remaining four lots were cultured on basic MS medium supplemented with Hg added as $HgCl_2$ at concentrations of 50, 100, 200 and 400 μM, respectively. The pH value of these medium was adjusted to 5.8 ± 0.1 prior to autoclaving at 121 ± 2 °C for 15 min. The cultures were incubated at 30 ± 2 °C under a 12-h photoperiod in cool, white fluorescent light. Rotten and contaminated embryos were removed promptly. When cotyledons of these seedlings had developed, cotyledons, hypocotyls and radicles were washed with double distilled water, blotted and immediately frozen in liquid nitrogen or stored at −80 °C for analysis. Plant tissues were oven-dried at 80 °C followed by the estimation of dry weights. The relative water content (RWC) was calculated using RWC (%) = [(FW − DW)/FW] × 100. The experiments were arranged in a completely randomized design with three replicates per treatment and each replicate contained 75 embryos (25 bottles).

2.2. Estimation of mercury content

Radicles were washed with 1% (v/v) HCl for several seconds, followed by washing three times with distilled water in order to remove the mercury adhering to the surface of the radicles. Then, seedlings were washed thrice with distilled water and finally with de-ionized water, and oven-dried at 70 °C for approximately 72 h. Dried cotyledons, hypocotyls and radicles samples of *J. curcas* were digested with 5 ml concentrated HNO_3 and 1 ml H_2O_2 for 20 min using microwave-digestion method, and then diluted to 25 ml with de-ionized water. Hg contents in the cotyledons, hypocotyls and radicles were determined using flame atomic absorption spectrophotometer (SpectrAA-220Fs, VARIAN, USA) and Integrated Couple Plasma Mass Spectrophotometer (ICP-MS: PQExCell, VG Elemental). Hg content was expressed in μg/g dry weight of tissue.

2.3. Protein extraction and estimation

Fresh cotyledons, hypocotyls and radicles were homogenized and extracted with 50 mM sodium phosphate buffer (pH 7.0, m/v, 1/10) including 0.1 mM EDTA and 150 mM NaCl. Crude extract was centrifuged at $15{,}294 \times g$ for 5 min at 4 °C and the supernatant was used for assaying of protein contents, antioxidant enzyme and PAL as well as gel electrophoresis. Protein content was determined according to Lowry's method using bovine serum albumin as standard [23].

2.4. Assay of antioxidant enzymes

SOD assay was performed according to McCord and Fridovich method with some slight modifications [24]. The 3 ml reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin and 50 μl enzyme extract. Absorbance was read at 560 nm using a UV/vis spectrophotometer (TU-1901, Purkinje General, Beijing, China). The enzyme volume corresponding to 50% inhibition of the reaction (one unit) was calculated. The activity was expressed as enzyme units per mg protein (U g^{-1} mg protein).

POD activity was determined by measuring the increase in absorbance at 470 nm due to the formation of tetraguaiacol [25]. Reaction mixture (3 ml) consisted of 2.8 ml 3% guaiacol in 50 mM Tris–HCl (pH 7.0) and 100 μl 2% H_2O_2. The reaction was started by adding 100 μl enzyme extract and the increase in absorbance at 470 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which produces 1.0 absorbance change at 470 nm per min in assay conditions. The activity was expressed as enzyme units per mg protein (U g^{-1} mg protein).

CAT activity was measured by the Montavon method [26]. The activity was assayed for 1 min in a reaction solution (3 ml total volume) composed of 2.8 ml phosphate buffer (50 mM, pH 7.0), 100 μl H_2O_2 (2%) and 100 μl of crude extract. One unit of CAT is defined as the amount causing the decomposition of 1 μM of H_2O_2 per min. The activity was expressed as enzyme units per mg protein (U g^{-1} mg protein).

2.5. Enzyme extraction and PAL activity assay

Fresh cotyledons, hypocotyls and radicles were ground in ice-cold 50 mM Tris–HCl buffer pH 8.8 containing 1% polyvinylpolypyrrolidone and 0.1 mM EDTA. The homogenate was centrifuged at 15,294 × *g* for 5 min at 4 °C and the supernatant was assayed for enzyme activity. PAL activity was determined by monitoring the reaction product *trans*-cinnamate at 290 nm [27]. The reaction mixture contained 50 mM Tris–HCl, pH 8.8, 20 mM l-phenylalanine, and 100 μl enzyme extract in a 5 ml volume. The reaction was allowed to proceed for 30 min at 30 °C and was stopped by the addition of 0.5 ml 10% trichloroacetic acid. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01 per min under assay conditions. The activity was expressed as enzyme units per mg protein ($U\,g^{-1}$ mg protein).

2.6. Polyacrylamide gel electrophoresis (PAGE)

Samples of crude protein extracts were electrophoresed in 8% (w/v) polyacrylamide slab gel under non-denaturing conditions. For SOD, POD and CAT isoenzymes staining, protocols developed by Beauchamp and Fridovich [28], Ros Barcelo [29] and Woodburry et al. [30] were followed, respectively.

2.7. Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. Data were expressed as means ± S.D. Statistical significance was evaluated with a Student's *t*-test, and differences were considered significant if *P* values were less than 0.05.

3. Results and discussion

3.1. Effects of different Hg concentrations on the seedling growth

Most plants show inhibited growth when exposed to heavy metals, though the actual tolerance varies among plant species. Thus, knowing the cascade of events produced by mercury exposure and relating it with growth effects would be an important consideration for risk assessment in plants [1,16]. Amongst the heavy metals, Hg has been shown to affect growth and metabolism of plants to varying degrees depending on the concentration and status of Hg in the plant tissues [7,15]. Effects of Hg on the biomass of cotyledons, hypocotyls and radicles in seedling are shown in Fig. 1. The fresh weight of cotyledons increased in all Hg concentrations though the increase was smaller at higher Hg concentrations. The fresh weight increment of hypocotyls showed a similar trend, but was slightly decreased at Hg concentration of 400 μM compared to the control. The fresh weight of radicles increased by 51.6% at Hg concentration of 50 μM, whereas those at Hg concentration of 100 and 200 μM decreased compared to the control (Fig. 1). Earlier reports suggested that Hg at low levels may not significantly affect plant growth, and only at higher concentrations does Hg become strongly phytotoxic to cells, inducing visible injuries and physiological disorder [12,31]. In the present study, *J. curcas* seedlings grew well at all tested Hg levels except for 400 μM, and no significant toxic symptoms were observed. However, growth in a medium with 400 μM Hg concentration caused significantly toxicity effects on seedlings growth and development. Significant morphological aberrations included impaired radicles development, coarser hypocotyls and cotyledons chlorosis (data not shown). Various authors have also reported inhibition of seedling growth when exposed to Hg toxicity [1,7,14], but results of the present study indicated that lower tested Hg concentrations increased the production

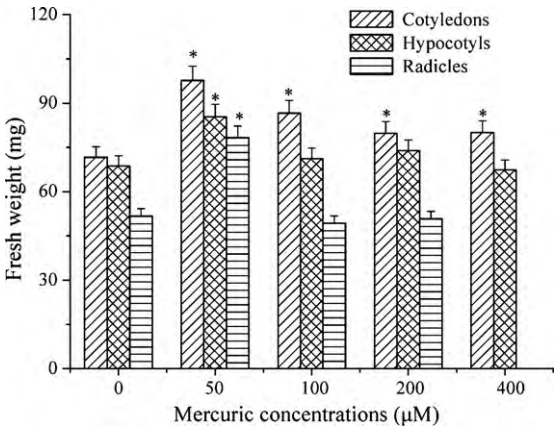

Fig. 1. Effects of mercury on the biomass of cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg. Data points and error bars represent means ± S.D. of three replicates ($n = 3$). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

of fresh weight in the seedlings (Fig. 1). This may be due to an increase in relative moisture content in *J. curcas* seedling (Table 1). This might also be due to long-term adaptation of *J. curcas* to polluted environments, or even the development of a mechanism of heavy metal tolerance to adjust to change in the Panzhihua region. Plants can hyperaccumulate heavy metals according to their unique physiology. Hyperaccumulators are highly tolerant of extreme environmental conditions and exhibit a variety of responses to heavy metal stresses. These adaptations enable them to tolerate and evolve resistance to adverse conditions that are toxic to most other plants species [10,11,16]. The changes observed in the growth responses of *J. curcas* seedling are in agreement with the results obtained at lower tested Hg concentrations in some plant species [9,12,32]. However, *J. curcas* seedlings have higher tolerance to the excessive levels of Hg in the medium during embryo germination and seedlings growth.

3.2. Effects of different Hg concentrations on the relative moisture content (RWC)

Relative moisture content has been reconsidered as an indicator of phytotoxicity after heavy metal stress in plant species. Earlier studies suggested that RWC is correlated to concentrations and phytotoxicity of heavy metals in plants [14,15]. Effects of Hg on the relative moisture content of cotyledons, hypocotyls and radicles in seedling are shown in Table 1. RWC in the cotyledons and radicles increased significantly at Hg concentration of 50 μM, and the greatest increase being 8.28% and 5.64%, respectively. However, at 400 μM RWC decreased significantly by 18.9% in cotyledons when compared to the control. RWC in the hypocotyls showed no sig-

Table 1
Effects of Hg on the relative moisture content of cotyledons, hypocotyls and radicles in *Jatropha curcas* seedling germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg.

Hg treatment (μM)	Relative moisture content (RWC, %)		
	Cotyledons	Hypocotyls	Radicles
0	71.8 ± 2.89	90.9 ± 3.75	85.5 ± 3.67
50	77.7 ± 3.36*	91.4 ± 3.87	90.3 ± 3.71*
100	73.2 ± 3.12	90.9 ± 4.05	87.6 ± 3.88
200	68. 8 ± 2.74	90.5 ± 3.32	86.6 ± 3.65
400	58.3 ± 2.31*	71.1 ± 3.16*	–

Data represent the mean ± S.E. of three replicates. Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

Table 2
Mercury concentrations in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg.

Hg treatment (μM)	Hg content (μg g^{-1} dry weight)		
	Cotyledons	Hypocotyls	Radicles
0	ND	ND	ND
50	175.2 ± 7.43	384.4 ± 16.7	1121.1 ± 48.1
100	364 ± 15.5	685.3 ± 29.4	2037.6 ± 89.8
200	672.4 ± 28.6	1363.2 ± 59.2	3392.4 ± 144.3
400	1930.5 ± 81.2	6881.9 ± 294.9	-

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

nificant increase at lower tested Hg concentrations, but a decrease by 21.8% was observed at 400 μM Hg when compared to the control. Since RWC indicated the water status in plant tissues, reduced RWC seems to have disturbed the homeostasis of plant due to heavy metal stresses [15]. However, RWC in the cotyledons, hypocotyls and radicles was not suppressed by Hg exposure except for Hg concentration of 400 μM. This may be explained by the fact that the changes in the fresh weight of seedlings in response to mercury concentration correlate with the observed changes in RWC (Fig. 1 and Table 1).

3.3. Hg uptake

Numerous studies have demonstrated that heavy metal toxicity to plants is positively correlated to heavy metals concentration in plant tissues. It has been shown to affect growth and metabolism of plants to varying degrees depending on the concentration of Hg accumulation in plant tissues [9,12,15], but physiological changes in *J. curcas* plants exposed to Hg toxicity has not been identified. Hg concentrations in the cotyledons, hypocotyls and radicles of *J. curcas* seedling are shown in Table 2. The Hg concentrations in the cotyledons, hypocotyls and radicles of *J. curcas* seedling showed positive and linear relationships with tested Hg concentrations, and maximum Hg concentrations were 1930.5, 6881.9 and 3392.4 μg g^{-1} DW, respectively (Table 2). Partitioning of metals in different plant tissues is a common strategy to avoid metal toxicity in above-ground parts. The first barrier against heavy metal toxicity occurs in the roots where metal ions may be immobilized by ligands on cell walls and extracellular carbohydrates [16,33]. In the present study, *J. curcas* showed Hg accumulation without any adverse effect on growth, and the increase in the fresh weight might be due to an increase in RWC of *J. curcas* seedlings at the tested Hg concentration of 50 μM (Fig. 1 and Table 1). In addition, the amounts of Hg uptake in the radicles were greater than those of in the cotyledons and hypocotyls of *J. curcas* seedlings except for tested Hg concentration of 400 μM. The present results are consistent with the findings of several studies that demonstrated that Hg ions are mainly retained in the roots and that only small amounts are transported to the leaves and shoots, and Hg uptake into roots is relatively fast, whereas trans location to leaves and shoots is slower [16,34]. On the other hand, a high mercury accumulation (about 2500 μg g^{-1} DW) has been reported in the *Sesbania drummondii* roots, which was considered for phytoremediation of a mercury-contaminated environment [5]. On the basis of these results, the present findings have provided information on how Hg concentration in different tissues varies in response to elevated Hg concentrations in MS medium. Such a high increase in Hg concentrations also suggested that *J. curcas* plants possess significant potential to tolerate Hg and that plants presumably rely on more than a single mechanism to achieve this.

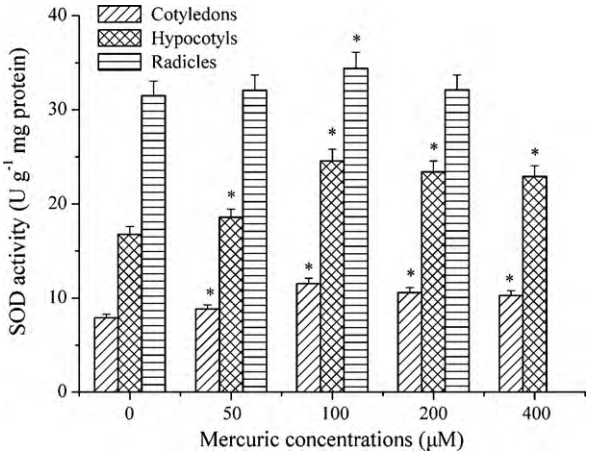

Fig. 2. Effects of mercury on superoxide dismutase (SOD) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg. Data represent the mean ± S.E. of three replicates. Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

3.4. Effects of different Hg concentrations on superoxide dismutase activity

SODs constitute the first line of defense to scavenge O_2^- radicles generated in various physiological conditions [8,12,35]. Effects of Hg on SOD activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings were shown in Figs. 2 and 3. SOD activity in the cotyledons and radicles showed maximum increases of 46% and 9.24% at 100 μM Hg concentrations, and the greatest increase in the hypocotyls was observed at 200 μM Hg concentrations. Being a transition metal, Hg ion can induce oxidative stress in plants, resulting in lipid peroxidation, K^+ leakage, and alteration of antioxidant enzyme activities and induction of thiol-containing compounds [9,34,36]. SOD activity in the cotyledons and hypocotyls increased significantly compared to the control. Previous studies have suggested that increased SOD and CAT activities present a positive protection effect under varying degrees of Hg conditions (1–10 mg l^{-1}). However, the protection effect disappeared at higher levels (50 mg l^{-1}) of Hg [34]. Enhanced SOD and POD activities in *J. curcas* seedlings under Hg stress are circumstantial evidence for tolerance mechanisms developed by this plant. Plant varieties that over-expressing of SODs and other scavenging enzymes have been engineered with the goal of increasing stress tolerance. However, the ROS-scavenging pathway is quite complex and attempts to create resistant plants have been met with varying success. Plants have multiple genes coding SOD and different SOD isoenzymes are specifically targeted to chloroplasts, mitochondria, peroxisomes, cytosol and apoplasts. Specialization of function among SODs may be due to a combination of the influence of subcellular location of the enzyme and upstream sequences in the genomic sequence [8,35]. Patterns of SOD isoenzyme expression obtained from different organs of *J. curcas* seedlings are shown in Fig. 3. In the cotyledons, hypocotyls and radicles, at least three isoforms of SOD visualized, respectively, but the staining intensities of these isoenzymes differ depending on Hg concentrations and plant tissues. The expression of SOD genes are involved in many life aspects including developmental course and in response to environmental stress [35]. Although we did not identify the specific activity of each isoenzymes, the total SOD activity shows the significant increases with increasing Hg concentrations (Fig. 2). These results suggest that increased activity of SOD isozymes in different tissues might be connected with increased requirement of each tissue to combat Hg toxicity. Moreover, the changes in the staining

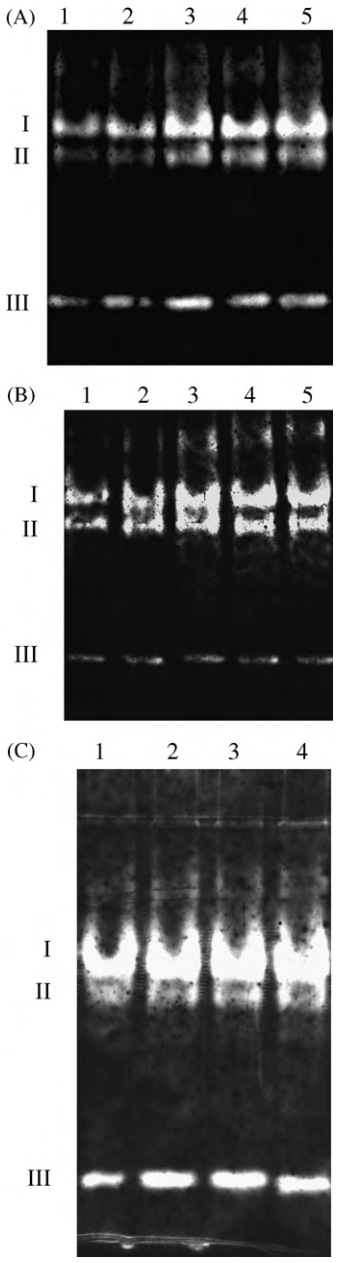

Fig. 3. Patterns of SOD isoenzymes present in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings. (A) Patterns of SOD isoenzymes in the cotyledons; (B) patterns of SOD isoenzymes in the hypocotyls. (C) Patterns of SOD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 50, 100, 200, and 400 μM, respectively. About 50 μg proteins from each sample were loaded into the native PAGE.

intensities of these isoenzymes were correlated with the quantitative changes of SOD activity assayed in solutions (Figs. 2 and 3).

3.5. Effects of different Hg concentrations on peroxidase activity

POD, which is another indicator of oxidative stress in plant cells, is involved in several important physiological and developmental processes. Therefore, POD activity has often served as an indicator of metabolism in response to changes in growth conditions and/or environmental stress conditions [37]. Effects of Hg on POD activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings were shown in Figs. 4 and 5. POD activity in the cotyledons and hypocotyls increased significantly up to 200 μM, with maximum increases of 86% and 158.6% compared to the control, respectively. POD activity in the radicles increased progressively up to 100 μM,

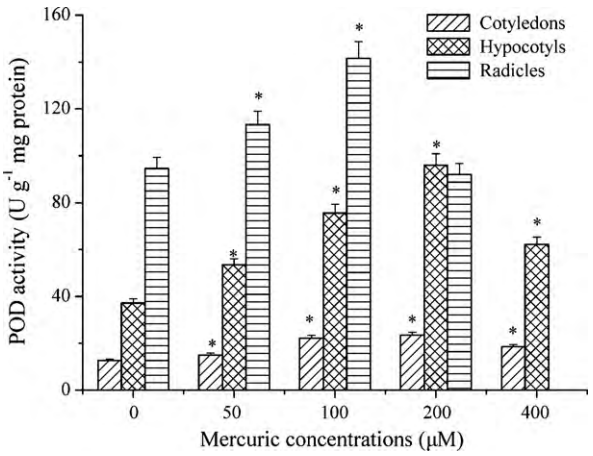

Fig. 4. Effects of mercury on peroxidase (POD) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg. Data represent the mean ± S.E. of three replicates. Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

the highest value increasing by 49.7% compared to the control. Accordingly, our findings showed that Hg concentrations in the medium really influenced the changes in POD activity in different degree, which may be the results of commonly response to Hg toxicity. Induction of POD activity has previously been reported in tomato, cucumber, *Medicago sativa* plants exposed to Hg stress [9,12,34]. Compared to previous studies, our findings showed that POD activity in *J. curcas* seedlings was induced gradually by increasing Hg concentrations, especially in the cotyledons and hypocotyls. The changes in POD activity may result from a complex interaction between Hg concentrations and different organs, and warrants further study. Many plants encode POD as small or large multigenic families, which may reflect the different roles of this enzyme. Multiple POD isoforms have been found in many plant species including maize, *Nicotiana tabacum*, rice, and *Arabidopsis thaliana*. Expression of POD genes is complicated since they are regulated at different times and places in response to various kinds of biotic and abiotic stresses [37,38]. Results of isoenzyme patterns suggested that at least six, five and six POD isoenzyme bands in the cotyledons, hypocotyls and radicles are visualized, respectively (Fig. 6). POD isoenzymes (I, II, IV and V) in the cotyledons showed an increase in the staining intensities with increasing Hg concentrations up to 200 μM. In the hypocotyls, the change in the staining intensities of POD isoenzyme (II, III and VI) was characteristic of a response to increasing Hg stress. The staining intensities of POD isoenzymes (III, IV and V) in the radicles enhanced significantly at Hg concentrations of 50 and 100 μM, and the maximum change was found at 100 μM. Although these POD isoenzymes show different patterns of activities under the Hg exposure, the total activity of POD in *J. curcas* seedlings was significantly enhanced, suggesting that POD activity could reflect an increased degree of oxidative stress. Moreover, the changes in the staining intensities of these isoenzymes are similar to the changes of activity assayed in solutions (Figs. 4 and 5). Our findings suggest that the differential expression of these isozymes occurs in a tissue specific manner and differential regulation in response to Hg concentrations. Thus, POD activity may be useful as a heavy metal biomarker for *J. curcas* plant, indicating that it is the most important ROS-scavenging enzyme.

3.6. Effects of different Hg concentrations on catalase activity

CAT, known to be one of the most efficient antioxidant enzymes, plays a very important role in maintaining the redox homeosta-

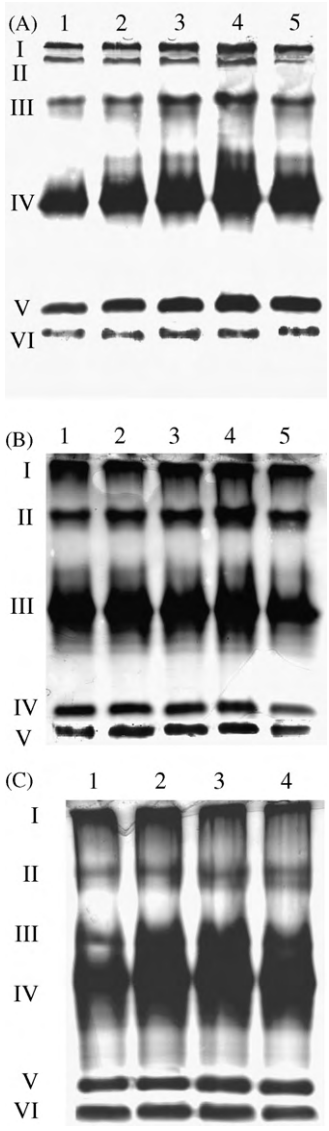

Fig. 5. Patterns of POD isoenzymes present in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings. (A) Patterns of POD isoenzymes in the cotyledons; (B) patterns of POD isoenzymes in the hypocotyls. (C) Patterns of POD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 50, 100, 200, and 400 μM, respectively. About 30 μg proteins from each sample were loaded into the native PAGE.

sis of the cell [39]. Effects of Hg on CAT activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings are shown in Fig. 6. CAT activity in the cotyledons and hypocotyls increased gradually with increasing Hg concentrations up to 400 and 200 μM, and the peak activity increased by 97.9% and 156.1% compared to the control, respectively. Similarly, CAT activity in the radicles showed exposure of tested Hg concentrations dependent, the greatest increase in activity being only 23.3% at Hg concentration of 200 μM. CAT, SOD and POD in plant cells are generally considered as typical defense components against heavy metals stress. Our findings suggested that the changes in CAT activity show a trend similar to that of POD activity (Figs. 4 and 6). This perhaps indicates that a complete set of antioxidant defense system, rather than a single antioxidant is responsible for protection in *J. curcas* plant exposed to Hg toxicity. Similar to the present study, an increase in CAT activity has been reported in *S. drummondii*, tomato, alfalfa exposed to Hg [5,9,36]. In addition, there was a significant positive correlation between Hg concentrations and CAT activity, which was also found to differ in different organs. CAT is presented as multiple isoforms encoded

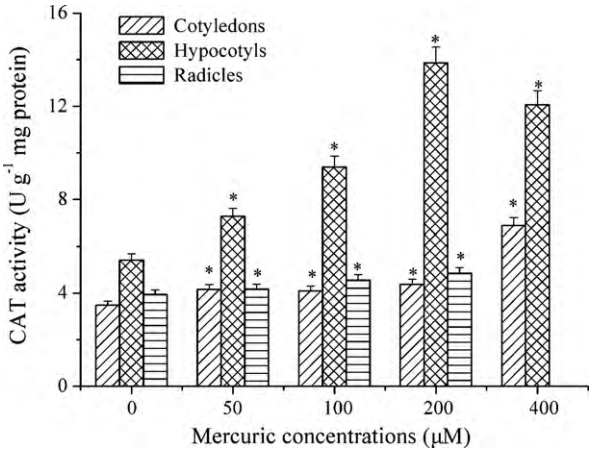

Fig. 6. Effects of mercury on catalase (CAT) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg. Data represent the mean ± S.E. of three replicates. Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

by a small gene family in many plants [8]. However, our results suggest the presence of at least one CAT isoenzyme in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings is detected. Its staining intensity varies with Hg concentrations and plant tissues and is consistent with the changes of the activities assayed in solutions (pattern no shown). Our findings provide evidence that CAT may provide an additional protection against the oxidative damage induced by Hg toxicity.

3.7. Effects of different Hg concentrations on phenylalanine ammonia-lyase (PAL) activity

PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism, and could perform defense-related functions. PAL could be induced by various biotic and abiotic stresses, resulting in the accumulation of defense-related products, such as phenolic and flavonoids [40]. Thus, PAL activity and activation are generally recognized as a marker of environmental stress in different plant species. Effects of Hg on PAL activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings are shown in Fig. 7. PAL activity in the cotyledons enhanced con-

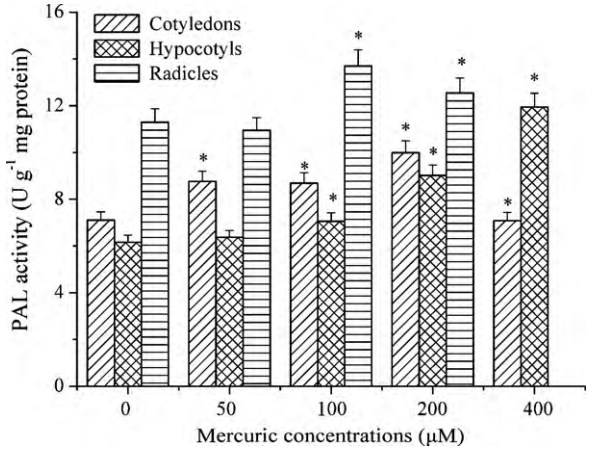

Fig. 7. Effects of mercury on phenylalanine ammonia-lyase (PAL) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings germinated and grown in MS medium containing 50, 100, 200, and 400 μM Hg. Data represent the mean ± S.E. of three replicates. Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

comitantly with increasing Hg up to 200 μM, and the peak activity increased by 40.7% compared to the control. Similarly, PAL activity in the hypocotyls and radicles increased with increasing Hg concentrations up to 400 and 100 μM, to maximum of 93.7% and 21.2%, respectively. Induction of PAL activity has also been observed in some plant species under heavy metals stress and depends on the stress and species of plant, although the significance of such induction was not clear. In general, the expression of PAL in many plant species is made more complex by the existence of multiple PAL-encoding genes, some of which are expressed only in specific organs or only under certain stress conditions [40]. A few reports on the induction of PAL activity under heavy metals stress is reconsidered for H_2O_2 generation, which occurs as primary reaction in response to stress [41]. Our results indicated that PAL activity appear to be an effective scavenger of ROS exposed to Hg toxicity. These findings suggest that PAL may also be involved in modulating the resistance of *J. curcas* plants exposed to Hg toxicity and their biological roles are more complex than expected.

4. Conclusion

Based on *in vitro* embryo germination and culture, the present study could be useful for selecting tolerant plants to heavy metals stresses. Findings allow us to conclude that growth and antioxidant responses in *J. curcas* seedlings exposed to Hg toxicity are significantly different among different tissues and Hg concentrations. Higher SOD, POD, CAT, PAL activities suggest an increased tolerance and capacity of *J. curcas* seedlings to protect the plant from oxidative damage caused by exposure to Hg. In fact, *J. curcas* plants are growing in a complicated environment including multiple heavy metals. Thus, further in-depth studies on *J. curcas* plants might not only help to understand plant defense mechanisms or some toxicity/tolerance issues, but also possibly identify marker proteins under multiple heavy metal stress.

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