Growth and Antioxidant Responses in *Jatropha curcas* Cotyledons under Lead Stress

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Jatropha curcas embryos were grown *in vitro* to observe the effects of lead on cotyledon responses. The cotyledon biomass increased initially and then decreased with increasing lead concentration. The SOD activity increased gradually up to 200 μM and then decreased. The POD activity showed a similar trend. The CAT activity was increased at all lead concentrations, the highest activity being observed at 200 μM. However, the PAL activity was inhibited significantly except for 100 μM. Anaylsis by electrophoresis suggested a significant correlation between lead concentration and patterns of SOD, POD and CAT isoenzymes, and these results were consistent with changes of the antioxidant enzyme activities as assayed in solution.

Key words: Heavy Metal Stress, ROS-Scavenging Enzymes, *in vitro* Embryo Culture, Defensive Mechanism of Plant

Introduction

Lead (Pb) is a non-essential element in metabolic processes and may be toxic or lethal to organisms even when absorbed in small amounts (Sharma and Dubey, 2005). The toxicity of heavy metals can result in the generation of reactive oxygen species (ROS) that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death. Correspondingly plant cells develop a broad range of defense responses to control the cellular ROS level. SOD plays a role in the dismutation of superoxide radicals, while POD and CAT contribute to the elimination of hydrogen peroxide (Cobbett, 2003; Mittler *et al*., 2004). It is imperative to compare the activity of ROS-scavenging enzymes among plant species with their ability to tolerate different lead concentrations, as it will tell us the intrinsic role of antioxidant enzymes in lead tolerance (Sharma and Dubey, 2005). Previous studies have shown that toxic concentrations of lead in plants cause oxidative stress, resulting in enzymatic and non-enzymatic antioxidant responses of plants (Verma and Dubey, 2003; Thomas *et al*., 2004; Mishra *et al*., 2006; Qureshi *et al*., 2007). Thus it is important to understand the behaviour of those enzymes in the presence of highly toxic metals.

Jatropha curcas L., commonly known as physic nut, belongs to the family Euphorbiaceae and has potent medicinal qualities and considerable commercial value. Recently, its seed has been investigated as a potential source of oil (Openshaw, 2000). The present work was conducted in order to study the effects of different levels of lead stress on biomass, antioxidant enzymes and PAL activities in *Jatropha curcas* cotyledons.

Material and Methods

Plant materials and chemicals

Mature *Jatropha curcas* seeds were collected in August, 2007 from more than 10 individual wild trees in Panzhihua, Sichuan province, China. Seeds were oven-dried, selected and stored in a plastic box (labeled No. 20070822) deposited at 4 °C until processing. Nitro blue tetrazolium (NBT) and *N*,*N*,*N*,*N*-tetramethyl ethylenediamine (TEMED) were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of reagent grade or higher.

Embryo germination and seedlings growth

Jatropha curcas seeds were surface-sterilized in 70% ethanol for 30 s, and then in 0.1% mercuric chloride for 8 min. Seeds were rinsed with

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distilled sterile water and soaked in sterile water for 24–36 h in a culture room. Each embryo was dissected from the seeds on a clean bench. Murashige and Skoog (MS) medium was adjusted to pH 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. Culture media (25 ml) were dispensed into wide-neck bottles (100 ml), containing 0, 100, 200, 400 and 800 μM $Pb(NO_3)_2$. The embryos were placed for germination and growth in *in vitro* cultures for 7 d. The cultures were incubated at (30 ± 2) °C and a 12-h photoperiod in coldness, white fluorescent light. Rotten and contaminated embryos were removed promptly. When the cotyledons of seedlings had developed, cotyledons were washed with double distilled water, blotted and immediately frozen in liquid nitrogen or stored at –80 °C for analysis. Three sets of seedlings were analyzed for each lead concentration, with 15 embryos per set.

Protein extraction and estimation

Protein extraction of fresh cotyledons was performed as previously described (Gao *et al.*, 2008). Fresh cotyledons were homogenized in 50 mM sodium phosphate buffer (pH 7.0, 1/10, w/v) containing 1 mM EDTA and 150 mM NaCl. The homogenate was centrifuged at 12000 rpm for 10 min. The supernatant was used immediately or frozen and stored at –80 °C for assaying of enzyme activity at a later date. Protein was quantified by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Assay of antioxidant enzymes

The POD activity was determined by the Sakharov and Aridilla method (1999). One enzyme unit was defined as the amount of enzyme that produced a change of 1 absorbance unit per min at 470 nm. The SOD activity was determined by measuring its ability to inhibit the photochemical reduction of NBT (Chen and Pan, 1996). One unit of SOD was defined as the amount of enzyme that caused 50% inhibition of the photoreduction of NBT under the assay conditions. The CAT activity was determined by the Montavon method (Montavon *et al.*, 2007). One unit of CAT activity was defined as the amount of enzyme needed to reduce 1 μM of H_2O_2 per min. The activities were expressed as unit per gram fresh weight (U g^{-1} FW).

Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis for isoenzymes was carried out with 7% acrylamide gel. The SOD isoenzyme activity was determined by the Beauchamp and Fridovich method (1971). Gels were equilibrated with 50 mM phosphate buffer (pH 7.5) containing 28 μM riboflavin and 28 mM TEMED for 30 min, then washed in distilled water for 1 min, and resubmerged in the same buffer containing 2.45 mM NBT for 10–20 min with gentle agitation in the presence of light. Enzyme bands appeared as colourless bands on a purple background. For the POD isoenzymes activity assay, the gel was soaked in deionized water for 5 min, and then incubated in 0.03% H_2O_2, 0.2% (w/v) benzidine and 0.1% (v/v) acetic acid for 3–5 min. When maximum contrast was achieved, the reaction was stopped by rinsing the gel with deionized water (Ros Barcelo, 1987). The CAT isoenzyme activity was assayed by the Woodburry method (Woodburry *et al.*, 1971). Gel was firstly incubated in 0.03% H_2O_2 for 5–10 min, and then transferred into 4% soluble starch for 1 h. The gel was stained in a 2% (m/v) $FeCl_3$ and 2% $K_3[Fe(CN)_6]$ (m/v) solution for 5–10 min until the colourless bands were clearly visible on the deep blue gel.

Enzyme extraction and PAL activity assay

Enzyme extraction for the PAL activity assay was carried out as previously described (Gao *et al.*, 2008). Fresh cotyledons were ground and homogenized in 50 mM tris (hydroxymethyl) aminomethane (Tris)-HCl (pH 8.8, 1/10, w/v) including 0.5 mM EDTA. The homogenate was centrifuged at 15294 × *g* for 10 min at 4 °C, and the supernatant was used for enzyme assays. The PAL activity was determined by assaying the L-Phe decomposition product *trans*-cinnamate, as measured by the increase of absorbance at 290 nm (Hahlbrock and Ragg, 1975). One unit of enzyme activity was defined as the amount of enzyme needed to decrease the absorbance by 0.01 unit per min. The PAL activity was expressed as unit per gram fresh weight (U g^{-1} FW).

Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. All data were expressed as means ± SD. Statistical significance was evaluated with a Student's *t*-test, and

differences were considered significant if P values were 0.05.

Results and Discussion

Effects of lead on the biomass, SOD, POD, PAL and CAT activities in cotyledons are displayed in Table I. The biomass of *Jatropha curcas* cotyledons increased with increasing Pb concentration up to 200 μM and then decreased. The largest increase over the control was 128.3%. Although Pb is generally considered to be a highly toxic element and its negative effects on plant development and growth have been well documented, positive effects on plant growth at lower concentration have also been reported (Sharma and Dubey, 2005; Mishra *et al*., 2006). This study lends further support to those findings. Although results obtained with plants grown *in vitro* can not be directly compared to those grown under field conditions, they are important in detailing plant growth response under controlled conditions.

Treatments with 100 and 200 μM Pb induced a progressive increase in SOD activities; a 1.74-fold increase in the activity was observed at the 200 μM Pb concentration compared with the control. However, the activities were relatively lower at 400 and 800 μM Pb. Earlier reports indicated that Pb induced changes of the SOD activity in *Oryza sativa* (Verma and Dubey, 2003), *Sesbania drummondii* (Thomas *et al*., 2004), and *Cassia angustifolia* Vahl. (Qureshi *et al*., 2007). Increased SOD activity might be either due to increased production of ROS or be a protective measure adopted by *Jatropha curcas* plants against oxidative damage. At least three bands of SOD isoenzymes were detected by gel electrophoresis (I–III), but the isoenzyme intensities differed between different Pb concentrations (Fig. 1). The diverse responses of SOD isoenzymes suggested that each condition produces its own unique effects, and further study of these should help to clarify the overall importance of oxidative stress phenomena in plants.

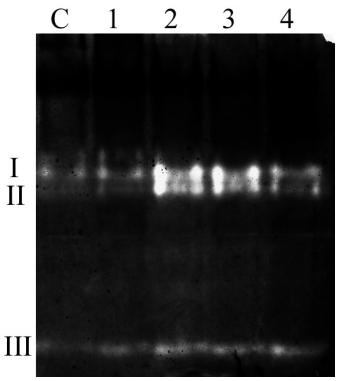

Fig. 1. Patterns of SOD isoenzymes from cotyledons of *Jatropha curcas*. Lanes from left to right (C, 1–4) are 0, 100, 200, 400 and 800 μM, respectively. About 25 μl of extract from each sample were loaded. I, II and III represent different SOD isoenzyms.

In the present study, the protective enzymes POD in the Pb-treated seedlings were generally activated. A marked elevation of POD activity was recorded overall compared to the control, and the largest increase was observed at the 200 μM Pb concentration, representing a 616.2 % increase. The activation of this enzyme might present circumstantial evidence for the occurrence of oxidative stress in *Jatropha curcas* seedlings. Increased POD activity has been reported in *Oryza sativa* (Verma and Dubey, 2003), *Ceratophyllum demersum* L. (Mishra *et al*., 2006), and *Sesbania drummondii* (Thomas *et al*., 2004) plants subjected to growth under elevated Pb contents.

Table I. Biomass, SOD, POD, PAL and CAT activities in cotyledons of *Jatropha curcas* exposed to different lead concentrations for 7 d.

Parameter	Pb concentration [μM]				
	0	100	200	400	800
Fresh weight [mg]	69 ± 2.85	76 ± 3.11	88.5 ± 3.63	63.7 ± 2.19	50 ± 2.13
SOD activity [U/g FW]	103.1 ± 4.16	121.8 ± 5.59	179.5 ± 7.38	148.9 ± 5.95	127.8 ± 4.79
POD activity [U/g FW]	168.1 ± 6.41	514.4 ± 22.7	1035.9 ± 43.7	667.6 ± 30.1	560 ± 24.3
PAL activity [U/g FW)	44 ± 1.95	78.3 ± 2.62	66.1 ± 2.51	53.2 ± 2.16	51.6 ± 1.78
CAT activity [U/g FW]	95.2 ± 3.16	100.9 ± 3.95	66.1 ± 2.51	67.5 ± 2.78	57.5 ± 2.18

Data represent mean values ± SD (n = 3).

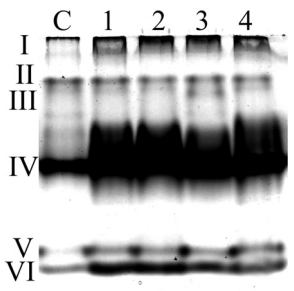

Fig. 2. Patterns of POD isoenzymes from cotyledons of *Jatropha curcas*. Lanes from left to right (C, 1–4) are 0, 100, 200, 400 and 800 μM, respectively. About 15 μl of extract from each sample were loaded. I, II, III, IV, V and VI represent different acidic POD isoenzymes..

Patterns of POD isoenzymes indicate that at least six isoforms are observed (I–VI), and three isoforms, IV, V and VI, are significantly stimulated in all high Pb concentrations, especially at 200 μM. Moreover, one isoform, III, is induced at 400 and 800 μM Pb (Fig. 2). Although the majority of POD isoenzymes exposed to toxic Pb shows different patterns of activity, the total POD activity was significantly enhanced (Table I and Fig. 2), suggesting that an elevated POD activity could be an indicator of increased oxidative stress. These results are in accordance with those observed in plants such as *Lemna minor*, in which enhanced antioxidant enzyme activities improved Pb tolerance (Rataczak and Garnczarska, 2000). Moreover, the spatial pattern of POD activity is consistent with the changes of solution assays. Our findings suggest that the increase of SOD and POD activities in *Jatropha curcas* seedlings under Pb stress (Table I) may be a circumstantial evidence for tolerance mechanisms developed by this plant.

CAT activities increased significantly at all Pb concentrations compared with the control values, and the highest activity was observed at 100 μM Pb, representing a 78 % increase. Our results suggest that effective induction of these enzymes may help to clear the peroxides accumulated under stress. Increased activation of antioxidant enzymes has previously been reported under toxic Pb conditions (Verma and Dubey, 2003; Thomas *et al.*, 2004, Mishra *et al.*, 2006). Our findings are in agreement with those for CAT activities in plants exposed to lead stress. Parallel changes in SOD, POD and CAT activities seen in cotyledons suggest that these three enzymes act interdependently to scavenge ROS (Table I). Thus, we observed a coordinated response among the antioxidant enzymes to enable the *Jatropha curcas* plants to tolerate oxidative stress. In plants, CAT is present as multiple isoforms encoded by a small gene family (Willekens *et al.*, 1995). However, only one CAT isoform is found in the control and treated group, and the intensities of CAT isoenzymes are consistent with the changes in the activities assayed in solutions (patterns no shown). This difference in isoenzyme patterns should be further investigated.

PAL activities only increased by 6 % at 100 μM Pb compared with the control. However, a further increase in Pb concentrations led to a decrease in the PAL activity to the basal level. Several lines of evidence suggest that the activation of PAL under stress conditions is part of a defense mechanism (MacDonald and D'Cunha, 2007). So, it seems that, in *Jatropha curcas* cotyledons, PAL might not play an essential role in modulating the resistance of plant tissues to Pb stress. Therefore, our findings indicate that the synthesis of PAL might be inhibited or blocked when exposed to toxic Pb.

In conclusion, our findings suggest that increased SOD, POD and CAT activities as well as the changes of isoenzymes patterns in cotyledons may be important in determining the ability of plants to survive under heavy metal stress and help to reduce the build up of ROS. Such responses may be of considerable value in understanding the response mechanisms of *Jatropha curcas* to lead toxicity and gaining insights into metal-microbe interactions in natural environments. Therefore, *Jatropha curcas*, based on the present results, can be successfully grown in Pb-rich areas.

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