Effects of copper on growth, antioxidant enzymes and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedling

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ABSTRACT

The effects of different concentrations of copper (0–800 μmol) on growth, protein contents, peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and phenylalanine ammonia-lyase (PAL) in *Jatropha curcas* L. seedlings were assessed by means of pot experiments. Results suggested that increased copper concentrations lead to decreased shoot elongation and seedling biomass. Protein content in the leaves and roots reached their highest levels at the copper concentrations of 400 μmol, while the highest protein content in the stem was observed at 800 μmol copper. POD activity in leaves and stems was unaffected at low copper concentrations, but showed a considerable variation at high copper concentrations. In roots, the highest POD activity was observed at 200 μmol copper. Under copper stress, SOD activity in leaves increased concomitantly with increasing copper up to 400 μmol, and SOD activity in stems and roots showed a slight increase. Catalase activity significantly elevated in leaves and roots but showed no significant changes in stems of the seedlings exposed to copper. A gradual increase of PAL activity in leaves and roots at the copper concentration of 400 and 200 μmol was observed, while PAL activity remained unchanged in stems.

Keywords: toxic element; ROS-scavenging enzymes; defensive mechanism of plant; abiotic stress

Copper (Cu) is an essential element for plant growth and plays a significant role in many physiological processes, including photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, antioxidant activity, cell wall metabolism and hormone perception. In general, copper concentrations in cells need to be maintained at low levels. However, plants usually find an ample supply of copper in soils, and copper at high concentrations can be a stress factor triggering physiological responses (Yruela 2005). At the cellular level, copper is a structural and catalytic component of many proteins and enzymes involved in a variety of metabolic pathways (Pilon et al. 2006). It has been previously reported that an excess of Cu can result in production of reactive oxygen species (ROS) and free radicals. These substances can in turn damage cell membranes by binding to the sulfhydryl groups of membrane proteins or by increasing rates of lipid peroxidation (Liu et al. 2004).

It is generally accepted that heavy metals can be a major toxicant in plant cells due to their potential inhibitory effects on many physiological and biochemical processes. The toxicity of heavy metals may arise as a result of the generation of ROS that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Mittler 2002). To cope with the damages caused by ROS, plant cells have evolved a number of defence mechanisms. Primary defence mechanisms prevent metal to enter into the cell via exclusion, or binding of metal to cell wall and other ligands, organic acids, amino acids, glutathione (GSH) or phytochelatins (PCs) to render them harmless (Antosiewicz and Wierzbicka 1999). Allosteric regulation of glutamate kinase activity by free proline creates a possibility for an increase in glutamic

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acid content due to the synthesis of glutathione and phytochelatines in plant cells (Pavlíková et al. 2007). Secondary defence system includes various antioxidants and enzymatic mechanisms to combat an increased production of ROS caused by the metal (Mittler 2002).

Jatropha curcas L. belongs to the family *Euphorbiaceae*, and is cultivated as a medicinal plant in many tropical and subtropical countries (Openshaw 2000). Early studies showed that copper can interact with a wide range of physiological and biochemical processes in plant species (Jouili and Ezzedine 2003, Draobzkiewicz et al. 2004). However, the effects of elevated copper levels on growth, antioxidant and defence responses of *Jatropha curcas* are still unclear. The aim of this study was to investigate the effects of copper on plant growth, and changes in POD, SOD, CAT and PAL in *Jatropha curcas* L. seedlings.

MATERIAL AND METHODS

Mature *Jatropha curcas* L. (physic nut or purging nut) seeds were collected in 2006 from more than 10 individual wild trees in Panzhihua, Sichuan province, China. The sands were gently crumbled by manual means, air-dried at room temperature and sieved through a 3 mm mesh to remove root residues and small rocks. Guaiacol, L-phenylalanine, methionine, nitro blue tetrazolium (NBT), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Other chemicals used were analytical grade reagent.

For the seed germination experiment, healthy seeds were surface sterilized for 15 min by 1% sodium hypochlorite, then washed several times with distilled water and divided into five portions. Each group of 25 seeds was soaked in beakers containing 100 ml of the testing solutions at 30°C in the light as follows: 48 h in $CuSO_4$ at 0, 100, 200, 400 and 800 μmol. During soaking, testing solutions were changed thrice. After soaking, all seed samples were placed in 20 cm diameter glass petri dishes, one sample per dish, and allowed to germinate and grow in a greenhouse at the constant temperature of 30°C and 24 h light. Each petri dish was filled with 500 g of sand (dry weight) and then $CuSO_4$ solution was added to the pods at five concentrations: 0, 100, 200, 400 and 800 μmol/kg sand. Germinated seeds and rotten seeds were counted every other day and the rotten seeds were removed. When the seedlings had developed 2 leaves, healthy seedlings that had germinated at the same time were selected, and washed with distilled water. Leaves, stems and roots of 5 seedlings of each group were weighed (fresh weight), homogenized and assayed for protein contents and enzyme activity. All assays and measurements were carried out in three repetitions.

In order to assay protein content and enzyme activity, fresh leaves, stem and roots (0.5 g) were ground with liquid nitrogen and homogenized in 5 ml of 50 mmol sodium phosphate buffer (pH 7.0) including 0.5 mmol EDTA and 0.15 mol NaCl. The homogenate was centrifuged at 12 000 g for 10 min at 4°C and the supernatant was used for protein determination and enzyme assays. Protein concentration was assayed by the Lowry spectrophotometric method, using bovine serum albumin as a standard.

POD activity was determined by measuring the increase in absorbance at 470 nm with a recording spectrophotometer (TU-1901 UV-Vis Spectrophotometer, Purkinje General, Beijing, China) (Sakharov and Aridilla 1999). The mixture consisted of 2.8 ml of guaiacol (3%), 0.1 ml H_2O_2 (2%) and 0.1 ml enzyme extract. One unit of POD activity was defined as an increase in absorbance of 1.0 per min. POD activity was expressed as enzyme units per gram fresh weight (U/g fw).

SOD activity was assayed in a 3-ml reaction mixture containing 50 mmol sodium phosphate buffer (pH 7.0), 10 mmol methionine, 1.17 mmol riboflavin, 56 mmol NBT and 100 μl enzyme extract. The absorbance of solution was tested by measurement of its capacity of inhibiting the photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the enzyme activity that reduced the photoreduction of nitroblue tetrazolium to blue formazan by 50% (Chen and Pan 1996). SOD activity was expressed as enzyme units per gram fresh weight (U/g fw).

CAT activity was determined by a decrease in absorbance of the reaction mixture at 240 nm. The activity was assayed for 1 min in a reaction solution (3 ml final volume) composed of 2.85 ml phosphate buffer (50 mmol, pH 7.0), 100 μl H_2O_2 (1%) and 50 μl of crude extract. One unit of CAT activity was defined as the amount of enzyme, which caused 1 μl H_2O_2 decomposition in one minute (Montavon et al. 2007). CAT activity was expressed as enzyme units per gram fresh weight (U/g fw).

Half g samples of fresh leaves, stem and roots were ground with liquid nitrogen and homogenized in 5 ml of 50 mmol Tris-HCl (pH 8.8) including 0.5 mmol EDTA. The homogenate was centrifuged

at 12 000 g for 10 min at 4°C and the supernatant was used for enzyme assays. PAL activity was determined by monitoring the production of *t*-cinnamic acid at 290 nm (Hahlbrock and Ragg 1975). The reaction mixture contained 50 mmol Tris-HCl buffer (pH 8.8), 20 mmol L-phenylalanine and enzyme extract. Incubation was at 30°C, and the reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid. Absorbance at A_{290} nm was measured after 30 min. One unit of enzyme activity was defined as the amount of enzyme causing the decrease in absorbance of 0.01 per min. PAL activity was expressed as enzyme units per gram fresh weight (U/g fw).

All values shown in this paper were the means of three assays carried out for each value. Data were tested at significant levels of P value < 0.05 using one-way ANOVA.

RESULTS AND DISCUSSION

Figure 1 shows the changes of the biomass in leaves, stems and roots of seedlings under Cu stress. Overall, biomass in leaves increased slightly at lower Cu concentration, while showing a slight decrease at higher Cu concentration. There was a correlation between increasing Cu concentrations and reduced seedling stems and roots mass. These results are consistent with observed morphological responses of germinated seedlings exposed to different copper concentration (data not shown). Copper is an essential micronutrient for normal plant growth and metabolism, but it can be toxic to the plants at higher concentrations (Yruela 2005). It has been demonstrated that an excess of copper can inhibit the growth of young seedling, root elongation and cause damage to root epidermal cells and root cell membranes (Xiong and Wang 2005, Tanyolac et al. 2007). On the basis of these results, our findings suggested that elevated copper concentration can inhibit the normal growth and development of *Jatropha curcas* seedling.

Changes in protein contents of leaves, stems and roots of seedlings at different copper concentrations are shown in Figure 2. Protein content in leaves and roots increased significantly compared to the control, and the highest increments in protein content (about 50% and 32.7%) occurred at the copper concentration of 400 µmol. However, protein concentration in stems increased concomitantly with rising copper concentrations. Soluble protein content in organisms, an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressors such as natural and xenobiotic (Singh and Tewari 2003). In this study, soluble protein content of *Jatropha curcas* seedlings was increased by copper stress. However, early evidences suggest that high levels of Cu induced the reduction in leaf total soluble protein in barley plants (Guo et al. 2007). The mechanism by which copper affects protein content is complex and needs a further study.

The observed changes of POD activity in leaves, stems and roots of seedlings under copper stress were shown in Figure 3. No significant changes in POD activity were observed in the leaves of seedlings exposed to 100 and 200 µmol Cu stress

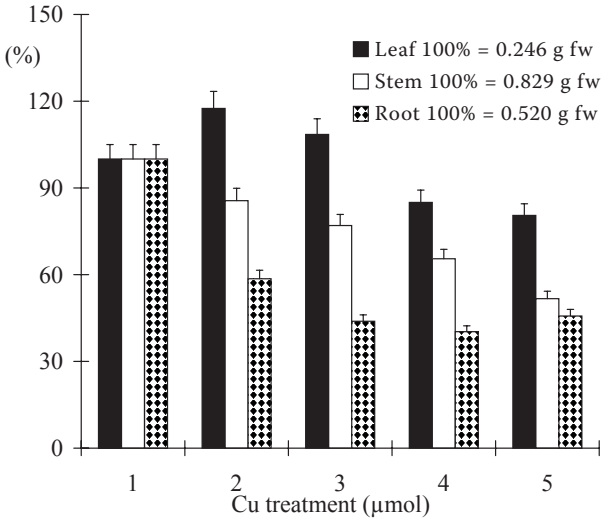

Figure 1. Effects of copper on the biomass in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

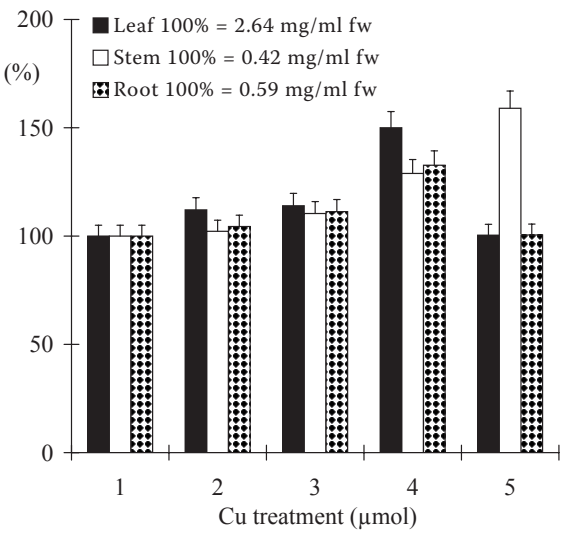

Figure 2. Effects of copper on protein content in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

environments, while the activities showed increases of about 60.3% and 29.7% under 400 and 800 µmol Cu stress, respectively. POD activity in stems increased by about 13.2%, 45.9%, 48.1% and 150.3% at copper concentrations of 100, 200, 400 and 800 µmol, respectively. There was a significant increase in POD activities in roots under copper stress. POD activity showed the largest increase (about 130.1%) at the copper concentration of 200 µmol; with increasing copper concentration it declined. These results are in agreement with the results of some other studies with several plant species that suggest increased peroxidase activity in response to elevated copper concentrations (Jouili and Ezzedine 2003, Tanyolac et al. 2007). POD activity correlates with copper concentration in plant shoots (Xiong and Wang 2005). Peroxidases are widely distributed in plants tissues involved in growth, development and senescence processes of plants. POD activity is also considered a useful biomarker for sublethal metal toxicity in examined plant species. In metal-tolerant plant species, POD activity was found to be sufficiently high to enable the plants to protect themselves against oxidative stress (Passardi et al. 2005). Copper, a redox active metal, can catalyze the formation of harmful free radicals such as hydroxyl, peroxyl and alkoxyl radicals, resulting in oxidative stress. Activity of one or more antioxidant enzymes generally increases in plants exposed to stress conditions, and this elevated activity correlates with increased stress tolerance (Pilon et al. 2006). Studies have suggested that POD activities in roots were higher than those in leaves and stems. Numerous studies reported that POD activity response to excess copper can vary among plant species and among different tissue (Passardi et al. 2005); their findings suggested that increased POD activity in *Jatropha curcas* might be sufficient to protect proteins, chlorophyll and lipids of some parts of plants against ROS attack.

Figure 4 showed changes of SOD activity in leaves, stems and roots of seedlings under copper stress. SOD activity in leaves showed the largest increase at 400 µmol Cu (about 100.3%) and an increase about 40.4% at 800 µmol copper. SOD activity in stems increased by about 14.8%, 30.3% and 5.2% at copper concentrations of 100, 200 and 400 µmol, respectively. SOD activity in roots increased by about 9.8%, 25.3% and 35.2% at copper concentrations of 100, 200 and 400 µmol, respectively. However, the SOD activities in stems and roots were inhibited under 800 µmol Cu stress. SOD is an essential component of the antioxidative stress defence system in plants, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (Mittler 2002). The results of this study regarding an increased SOD activity in response to excess of copper are in agreement with those obtained in oat, wheat and *Arabidopsis thaliana* (Alscher et al. 2002, Draobzkiewicz et al. 2004). Moreover, previous studies suggested that exposure to higher Cu concentrations results in a significantly decreased SOD activity (Tanyolac et al. 2007); their study suggested that SOD activity is decreased in stems and roots at copper concentration of 800 µmol. Our findings suggested that SOD is involved in the oxidative stress defence

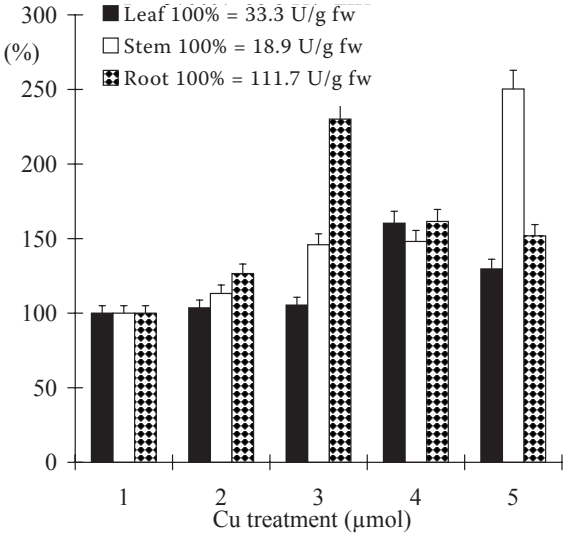

Figure 3. Effects of copper on peroxidase (POD) activity in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

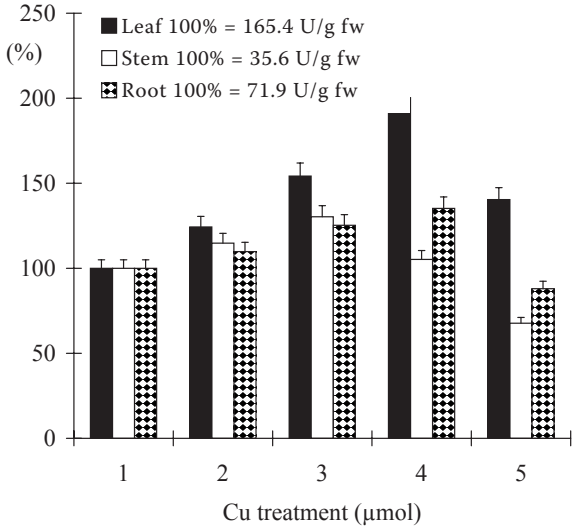

Figure 4. Effects of copper on superoxide dismutase (SOD) activity in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

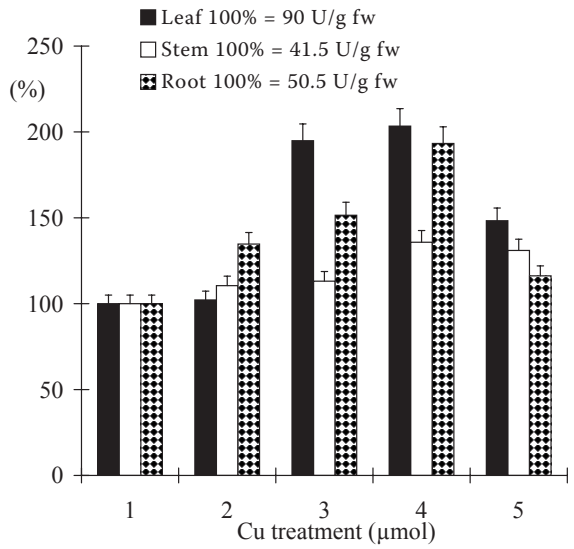

Figure 5. Effects of copper on catalase (CAT) activity in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

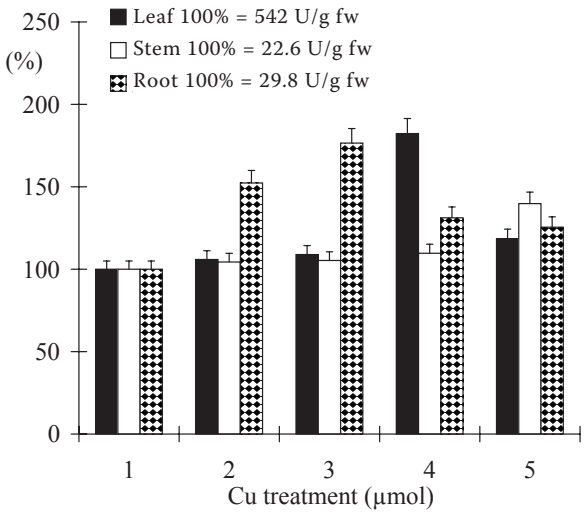

Figure 6. Effects of copper on phenylalanine ammonia-lyase (PAL) activity in leaves, stems and roots of seedlings. Values are the means ± SD (n = 3)

system of *Jatropha curcas* and up-regulation of SOD activity would help to reduce ROS.

Changes in CAT activity in leaves, stems and roots of seedlings under copper stress are shown in Figure 5. CAT activity in leaves increased by about 2.2%, 94.9%, 103.4% and 48.3% at Cu concentrations of 100, 200, 400 and 800 µmol, respectively. The activities in stems increased by 10.5%, 13.1%, 35.8% and 31% at copper concentrations of 100, 200, 400 and 800 µmol, respectively. Similarly in roots, the activities were increased by about 34.7%, 51.5%, 93.3% and 16.2%, respectively. These results demonstrate that any H_2O_2 formed as a result of SOD activity was consumed by catalase and/or peroxidase. Catalase is one of the most important plant enzymes catalyzing the dismutation of hydrogen peroxide, and is known as a mediator of oxidative damage, into oxygen and water (Mittler 2002). Accumulating evidence indicated that catalase activity varies during plant development, such as seed maturation and germination (Kunce and Trelease 1986), seedling development and leaf maturation (Havir and McHale 1987). However, CAT activities are always found suppressed under high copper stress (Draobzkiewicz et al. 2004). In this respect, our study seems to contradict previously reported findings, and further research is thus required.

Changes of phenylalanine ammonia lyase in leaves, stems and roots of seedlings under copper stress were also analysed (Figure 6). PAL activities in leaves at Cu concentrations of 100, 200, 400 and 800 µmol increased by 5.9%, 8.9%, 82.3% and 18.4%, respectively. The activities in roots were increased by 52.3%, 76.5%, 31.2% and 25.5% at copper concentrations of 100, 200, 400 and 800 µmol, respectively. No significant changes in the stems under Cu stress were observed except for PAL activity increased about 40% at 800 µmol Cu stress. The results suggest that PAL activity in *Jatropha curcas* seedlings strongly depends on tissue type and copper concentration. PAL, a key component of plant phenylpropanoid metabolism, is also involved in plant defence against oxidative stress; it increases during biotic and abiotic stress caused by heavy metal, light (through its effect on phytochrome), and fungal infection (Jouili and Ezzedine 2003, MacDonald and D'Cunha 2007). Regulation of PAL activity in plants is complex, as there are multiple PAL encoding genes, some of which are expressed only in specific tissues or only under certain environmental conditions (MacDonald and D'Cunha 2007). This study has provided information on PAL activity changes in response to elevated copper concentrations in different tissues.

The findings in this study lend support to the hypothesis that *Jatropha curcas* ability to cope with metal stress depends on oxidative stress defence mechanisms. Changes in protein content, SOD, CAT, POD and PAL in *Jatropha curcas* L. seedlings showed a clear correlation with copper concentrations. Changes also differed between plant tissues. Findings suggested that leaf response to high copper concentrations involves mainly the SOD, CAT and PAL enzymes, while root response involves POD. These variable responses may be attributed to differences in gene expression and protein function in different plant tissues. It is presumed that these findings might contribute to a better understanding

of the response mechanisms of *Jatropha curcas* to metal stress and to further insights into metal-microbe interaction in natural environments. Studies involving molecular cloning and localizing specific POD, SOD and CAT isoenzymes are in progress in order to elucidate the gene regulation mechanisms of antioxidant enzymes.

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