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Growth Performance and Tolerance Responses of Jatropha (*Jatropha curcas*) Seedling Subjected to Isolated or Combined Cadmium and Lead Stresses

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ABSTRACT

A pot-culture experiment was conducted to investigate growth performance, physiological and biochemical responses, and cellular ultrastructure of the Jatropha (*Jatropha curcas* L.) seedling exposed to cadmium (Cd), lead (Pb) and their combined stress. In response to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, plant biomass, gas exchange rate and photosynthetic pigment contents decreased, while leaf conductivity, MDA, the soluble proteins and free proline content increased significantly. Antioxidant enzyme activities significantly increased when the plant seedlings were exposed to Cd50, Cd100 and Pb500 treatments but drastically decreased under Pb1000 and Cd50-Pb500 treatments. This indicated that low Cd and Pb levels activated the antioxidant defense system, but higher Cd and Pb levels transcended the defensive capability of Jatropha. The cellular ultrastructure of seedling fronds under Cd100 and Pb1000 treatments were evaluated by transmission electron microscopy. The deleterious effects on mitochondria and chloroplast were most obvious. The cell walls and plasma membranes also showed distortion under intracellular observation. Overall, the Jatropha exhibited inherent heavy metal-tolerance and possibly becomes a suitable candidate to phytoremediation of heavy metal-contaminated soils or develop multipurpose biomedical applications.

Key Words: Antioxidant; Biomass; Cd; Growth parameter; Jatropha; Pb

INTRODUCTION

Cadmium (Cd) and Lead (Pb) are two components of the most common heavy metal pollutants in the environment and they have often been reported to enter the environment together through industrial processes in the mineral areas because of the similar chemical properties (Pilon-Smits, 2005; Rai, 2008; Yadav *et al.,* 2009). The phytotoxicities of Cd and Pb in higher vascular plants were observed at multiple levels, ranging from plant growth and development to various metabolic processes (Pilon-Smits, 2005; Liu *et al.,* 2007; Zhou *et al.,* 2008; Piotrowska *et al.,* 2010). Currently, the economic crop species, such as *Triticum aestivum*, *Oryza sativa Cucumis sativus* and *Zea mays* show absorption, accumulation and translocation capacities regarding Cd or Pb heavy metals and the relevant tolerance mechanism (Song *et al.,* 2002; Wang *et al.,* 2003; Huang *et al.,* 2009 Karak & Bhattacharyya, 2010; Perveen *et al.*, 2011). However, no research paper has reported the growth performance and tolerance-related cellular mechanisms of the Jatropha (*Jatropha curcas* L.) in response to Cd and Pb heavy metal stresses.

Jatropha, commonly known as physic nut, is a large shrub belonging to the family Euphorbiaceae. This plant species has currently become a new interest for researchers. It is regarded as a potential biofuel crop with high economic value to biodiesel and simultaneously produce anti-cancer medicine, pesticide, cosmetics, and feedstuff et al and its leaves and other parts are used for the treatment of various diseases (Jamil *et al.,* 2009; Li *et al.,* 2010a; Yadav *et al.* 2010). For example, Curcin, a kind of type I ribosome-inactivating protein with anti-tumor and anti-virus activity, is first found from the seeds of Jatropha (Stirpe *et al.,* 1976; Luo *et al.,* 2006). This plant species had developed highly efficient protective mechanisms against various abiotic stresses and grows in wide geographic regions, ranging from tropical to subtropical zones in China, India, Brazil and other continents (Gao *et al.,* 2009; Gao *et al.,* 2010; Silva *et al.,* 2010). In the Panzhihua mineral region in southwest China, Jatropha is a facultative halophyte that has a high seed production and a short gestation period and is suitable for versatile development. Except that it has pharmacological features, this plant also serves as a pioneer in phytoremediation, capable of scavenging heavy metals from the wasteland, a cost-effective, environment-friendly, and sustainable green technology (Li *et al*., 2010a; Yadav *et*

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al., 2010; Abhilash *et al.,* 2011; Abdulla *et al.,* 2011). For these reasons, the growth and physiological responses of Jatropha to Cd and Pb heavy metals and relevant cellular or molecular mechanisms are of considerable practical and ecological importance.

The tolerant capacity of plants to heavy metals depended on an interrelated network of physiological and molecular mechanisms. Numerous plants have evolved enzymatic (catalase, ascorbate, peroxidase) and nonenzymatic (ascorbate, glutathione) antioxidant mechanisms to prevent oxidative damages (Piotrowska *et al*., 2010), but the cellular mechanisms of oxidative metabolism remain poorly understood. In our previous works, the Jatropha plant was a good candidate for eco-toxicological research. We noted that this plant species developed efficient antioxidant systems against heavy metal damage, including superoxide dismutase (SOD) and peroxidase (POD) isoenzymes' pattern of cotyledon, hypocotyls and radicle exposed to heavy metals (Juwarkar *et al.,* 2008; Gao *et al.*, 2009, 2010); however, the oxidative metabolism induced by heavy metal phytotoxicity remain elusive. To address this particular concern, our study seeks to assess the growth performance of Jatropha seedling, ascertain the phytotoxic extent of Cd, Pb and their combined treatments on the gas-exchange parameters and photosysthetic pigments, and identify the cellular ultrastructure of the plant fronds.

MATERIALS AND METHODS

Seed germination and preparation of heavy metal-spiked soil: In August 2009, mature Jatropha seeds were collected from several native habitats in Panzhihua region. Seeds were kept dry, stored in a plastic box, and labeled for identification. The selected seeds were sterilized in 0.1% mercuric chloride for 15 min, rinsed 5 times with sterile double distilled water (ddH_2O), and soaked for 2 h in ddH_2O and were aired in a culture room. The prepared seeds were spread on double-layer filter paper to germinate naturally on a plate for 4 days. Each plate held 10 granules of seeds. The germinated seeds were grown in the field and received normal exposure to daylight, temperature and humidity. The plant seedlings having approximately the same height and weight were carefully replanted in experimental pots. According to Chinese Environmental Quality Standard for soils (GB15618-1995, Grade II for soil pH < 6.5: Cd ≤ 0.3 mg/kg & Pb ≤ 250 mg/kg, indicating a pollution warning threshold), phytotoxicities of the soil were associated with 70 ~ 150 mg/kg total Cd or 500 ~ 1000 mg/kg total Pb (Li *et al.*, 2007). However, as far as the topsoil (0 ~ 20 cm) was concerned, the average Cd and Pb concentrations were generally 0.45 and 24.2 mg/kg, respectively, and inferred to induce no toxic effect on the colonized plants (Li *et al.*, 2007). The experimental soil for seedling growth was collected with a topsoil depth of 0 to 20 cm from the wild field with semi-dry continental climate with 20 ~ 35°C average annual temperature. The soil samples were placed into plastic bags, transported to the laboratory, and air-dried. The samples were subsequently sieved through a 2 mm mesh sieve and mixed with concentrations of Cd at 0, 50, and 100 mg/kg (termed CK, Cd50 & Cd100) and Pb at 0, 500, and 1000 mg/kg (termed CK, Pb500 & Pb1000) by $CdCl_2 \cdot 2H_2O$ or Pb $(CH_3COO)_2 \cdot 3H_2O$ salt formation evenly, respectively. Soils without the addition of Cd or Pb served as the control. The combined Cd50-Pb500 treatment was prepared by mixing both Cd at 50 mg/kg and Pb at 500 mg/kg evenly with the soil. The prepared soil samples were incubated in pots for a minimum of 30 days.

Pot-culture experiment: Pot-culture experiments were conducted to assess the growth parameters, various physiological and biochemical responses of the Jatropha seedlings, and to evaluate the cellular ultrastructure of the fronds. The plant seedlings were planted on experimental pots on April 20, 2010, and samples were harvested during the summer season. The uniform seedlings (3 cm in root length, 15 cm in seedling height & 4 ~ 5 fronds) were selected and transplanted to the pots, which were arranged in a completely randomized design with two levels of Cd and Pb and one combination. Each treatment contained 5 replicates. Each replicate consisted of 5 seedlings and all treatments were grown under open field conditions. The distilled water was added daily to maintain the proper soil moisture. After 30 days in the pot culture, the plants were harvested, washed thoroughly with distilled water and separated into root, leaves and shoot. The following parameters were determined: total plant biomass, plant height, fresh leaf weight, fresh stem weight, fresh root weight, and root length. Four seedlings in each replicate of one treatment were selected randomly in order to obtain the aforementioned parameters. The tolerance index (TI) was calculated as a percentage according to plant biomass, which is the ratio of the variable measured in treated plants to that of control plants (Kumar *et al.,* 2008; Yadav *et al.,* 2009). The root/shoot ratio was determined as a percentage differential between the total belowground and the total aboveground biomass.

Gas exchange and chlorophyll fluorescence: Photosynthesis and transpiration rates were performed in fully expanded leaves with a portable LI-6400 photosynthesis system (LI-COR, Inc., Lincoln, NE, USA), which was operated in an open environment at a constant air flow of 200 mL/min and at saturation irradiance with an incident photosynthetic photon flux density of 1200 $\mu moL/m^2s$. All measurements were conducted in quadruplicates for intact leaves from five randomly chosen cuttings. Full photosynthetic induction was obtained with LED light source equipment to provide saturated photosynthetic photon flux density (PPFD) and illuminate samples for 10 ~ 30 min. The saturated PPFD was determined by preliminary experiments. After the apparent steady state gas exchange was achieved, the data were recorded. Leaf CO_2 assimilation rate (Pn), transpiration rate

(Tr), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were measured, and the instantaneous carboxylation efficiency (Pn/Ci) was calculated. The chlorophyll content was determined according to the method of Knudson (Knudson *et al*., 1977). Four fully-expanded and exposed fresh seedling leaves were extracted in 2 mL of 95% ethanol in the dark, and extracted solution was analyzed on a spectrophotometer at wavelengths of 665, 649, and 470 nm using the following equations to calculate the pigment content: Chl a = 13.95 (A665) – 6.88 (A649); Chl b = 24.96 (A649) – 7.32 (A665); Car = [1000 (A470) – 2.06 (Chl a) – 114.8 (Chl b)]/245 (Chl a: chlorophyll a; Chl b: chlorophyll b; Car: carotenoid; unit: μg/g). A digital electrical conductivity meter (DDS-11A, Thermo Scientific, USA) was used to assess electrolyte leakage percentage based on the method of (Lutts *et al.,* 1996). Jatropha seedling leaf samples were harvested and cut into 1 cm segments in individual stoppered vials containing 10 mL ddH_2O after three washes with deionized H_2O to remove surface-adhered electrolytes. Leaf segments were incubated at room temperature (25°C) on a shaker (100 rpm) for 24 h. Electrical conductivity of bathing solution (EC_1) was measured after incubation. Samples were then placed in thermostatic water bath at 95°C for 15 min and a last conductivity reading (EC_2) was obtained upon equilibration at 25°C. The electrolyte leakage was calculated as EC_1/EC_2 and expressed as percent.

Lipid peroxidation, soluble proteins and free proline measurements: Oxidative damage of lipids in seedling tissue was measured in terms of the total content of two thiobarbituric acid (TBA) reactive substances, one of which is malondialdehyde (MDA) (DeLong *et al*., 2002). MDA content was analyzed by a colorimetric method at 532 nm, and nonspecific absorption at 600 nm was subsequently subtracted. The concentration of MDA was calculated at its extinction coefficient (155 mM^{-1} cm^{-1}) (Heath & Packer, 1968). Total water-soluble proteins in seedling fronds' homogenate were measured by Bradford assay with unit of mg/g FW (Bradford, 1976). Free proline was extracted, and its concentration was determined by the method described by Bates *et al*. (1973). Seedling leaves were homogenized with 3% sulfosalicylic acid. The supernatant was treated with acetic acid and acid ninhydrin and boiled for 1 h. The optical density at 520 nm was subsequently determined. The proline content was recorded as μg/g FW.

Antioxidant enzyme assay: Fresh seedling leaves (1.0 g) were homogenized with a mortar pestle using liquid nitrogen in 10 mL of an extraction buffer (20 mM Tris-HCl in 1% polyvinylpyrrolidone, pH 7.4). The detailed methods were described by Polle *et al.* (1997). After filtration through two layers of gauze to remove any debris, the homogenate was centrifuged at 10,000 g for 20 min. The supernatant was used for both the enzyme activity and some other assay. The Coomassie Brilliant Blue G-250 was used to quantify the enzyme content (Bradford, 1976). SOD was determined on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Beauchamp & Fridovich, 1971). The reaction solution contained 50 mM phosphate buffer (pH7.8), 13 mM methionine, 75 μM nitro blue tetrazolium, 2 μM riboflavin, 100 nM EDTA and dd H_2O. The riboflavin was added last. The reaction mixture was read at 560 nm. One unit of SOD activity (U) was designated as the amount of enzyme that caused 50% inhibition of initial reaction rate. Total SOD activity used U/g fresh weight (FW). Catalase (CAT) was assayed by Aebi method (Aebi, 1984). Based on H_2O_2 hydrolysis, the decreasing absorbance was measured at 240 nm (A240). Reduction of 0.1 at A240 in 1 min was designated as one unit of enzyme activity (U/g. min FW). POD activity was measured by guaiacol spectrophotometry (Lagrimini, 1991). When exposed to H_2O_2, POD catalyzed the guaiacol to tetraguaiacol, which had optical density (OD) of 470 nm. The reaction solution contained 100 mM phosphate buffer (pH 6.0), 33 mM guaiacol and 0.3 mM H_2O_2. Specific activity of POD was calculated from the increase in OD470 for 30 s and expressed as U/g.min FW.

Cellular ultrastructure observation: Transmission electron microscopy (TEM) was performed on small sections (1–2 mm in length) of four fully expanded seedling fronds. Leaflets were fixed with 3% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 6–8 h under 4°C condition, post-fixed in 1% osimium tetraoxide for 1 h and immersed in 0.1 M phosphate buffer (pH 7.2) for 1–2 h. The leaflets were dehydrated in a graded ethanol series of 50, 60, 70, 80, 90 and 100% concentration for 15–20 min. The tissues were embedded in epon-araldite. Ultra-thin sections (80 nm) were sliced, stained with uranyl acetate and lead citrate and mounted on copper grids for viewing in the H-600IV TEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 60 kV at various magnifications.

Statistical analysis: All data presented here are the mean value ± standard deviation (SD) calculated from the replicates. All statistical analysis was performed using a data processing system (DPS) statistical software package, such as Origin 8.0, SPSS11.5 and used two-way ANOVA followed by the least significant difference (LSD) test to evaluate significant differences among the treatments at the defined level of $P < 0.05$.

RESULTS

Growth parameters: Our studies showed that the Jatropha plant had capability to grow in the designed heavy metal-spiked soils, as the seedlings survived at least 50 days in Cd100, Pb1000 and Cd50-Pb500 treatments, respectively. In respect to fresh leaf weight, fresh root weight, and biomass, there were slight decreases in growth parameters when exposed to Cd50 treatment and moderate decreases when exposed to Cd100 and Pb500 treatments and drastic decreases when exposed to Pb1000 and Cd50-Pb500 treatments when compared to the control (CK) (Fig. 1a, c & d). Pb1000 treatment appeared to have the strongest

Fig. 1: Biomass measurement of Jatropha seedling when exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments for 30 days. (a) fresh leaf weight; (b) fresh stem weight; (c) fresh root weight; (d) biomass. Values were the mean ± SD of four replicate measurements. Different letters indicate the significant difference between data points (P<0.05)

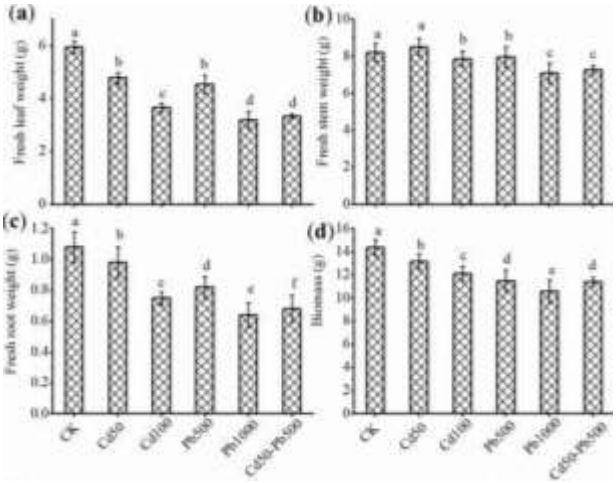
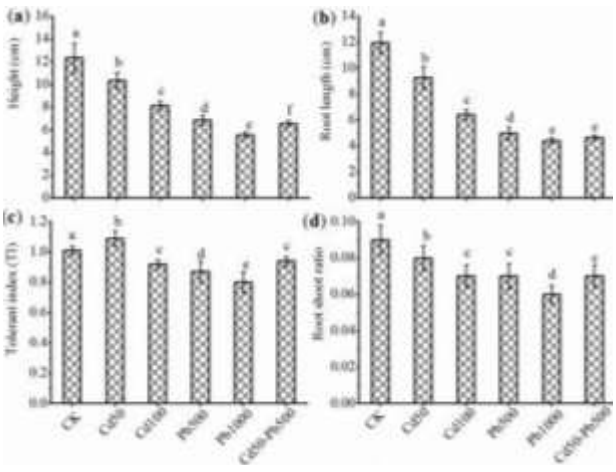


Fig. 2: The plant height, root length, and other parameters of Jatropha seedling when exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments for 30 days. (a) plant height; (b) root length; (c) tolerance index (TI); (d) root/shoot ratio. Values were the mean ± SD of four replicate measurements. Different letters indicate the significant difference between data points (P<0.05)



phytotoxic effect on the seedling growth compared to other treatments. Fresh stem weight was not significantly affected by Cd50 treatments, but Cd100 and Pb500 treatment showed more significant phytotoxicity to stem, and Pb1000 and Cd50-Pb500 treatments showed most strong phytotoxicity in stem biomass (Fig. 1b). The plant height and root length both decreased proportionally when accompanied with Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments. Obviously, Cd and Pb heavy metals both had phytotoxic effects in a dose-dependent manner, since Pb500 and Pb1000 treatments showed stronger phytotoxic effects than Cd50 and Cd100 treatments, respectively (Fig. 1). Cd50-Pb500 treatment showed moderate phytotoxicity between Pb500 and Pb1000 treatments (Fig. 2a & b). Pb1000 treatment showed the strongest phytotoxic effect on the belowground and aboveground biomass. In respect to tolerant indexes (TI), Cd, Pb and the combined treatments all caused significant reduction of TI compared to the control, except that Cd50 treatment had slightly higher TI than that of the control (Fig. 2c). Alternatively, Cd100, Pb500 and Cd50-Pb500 treatments had similar root/shoot ratios, and had significantly decreased root/shoot ratios compared to Cd50 treatment and control, respectively (Fig. 2d). Most phytotoxic effect was observed under Pb1000 treatment, which drastically decreased all seedling growth parameters (Fig. 1 & 2).

Physiological responses and photosynthetic function: The growth reduction of Jatropha seedling was accompanied by some significant changes in the gas exchange parameter and photosynthetic pigment. When exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, the physiological responses of plant seedling were indicated in Fig. 3. Pn, Tr , gs and Pn/Ci values were all drastically reduced by the phytotoxicity of heavy metals and showed similar alteration trends in all treatments, and Cd50-Pb500 treatment showed the strongest phytotoxic effect on the leaf gas-exchange parameter and Pn/Ci ratio (Fig. 3). In case of photosynthetic pigments (Fig. 4). Chl a and Chl b contents decreased significantly in all treatments compared with that of the control (Fig. 4a & b). In contrast, Car was less sensitive than Chl a or Chl b to heavy metal stresses (Fig. 4c). Cd50-Pb500 treatment showed a strong photosynthetic inhibition compared with the other treatments. Additionally, Cd100, Pb500, P1000 and Cd50-Pb500 treatments had the highest leaf electrolyte leakage compared with control and Cd50 treatment (Fig. 4d). Therefore, one could hypothesize that the Cd and Pb heavy metals had an interactive phytotoxic effects on the gas exchange and photosynthetic function of plant seedling.

Soluble proteins, free proline and peroxidation products: Soluble proteins' contents gradually increased proportionally with Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments after 30 days of the exposure periods, respectively, except that the soluble protein content of Cd50-Pb500 treatment was lower than that of Pb1000 treatment (Fig. 5a). The proline content and MDA content of Jatropha seedling significantly increased proportionally with Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, except for MDA content of the combined Cd50-Pb500 treatment during 30 days of the experiment (Fig. 5b & c). Specifically, the proline contents had about 1.3, 2.1, 1.8, 2.3 and 2.9 time increase over control in Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, respectively, and MDA

Fig. 3: Leaf gas exchange of Jatropha seedling when exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments for 30 days. (a) Pn; (b) Tr; (c) Gs; (d) Pn/Ci. Values were the mean ± SD of four independent experiments. Different letters indicate the significant difference between data points (P<0.05)

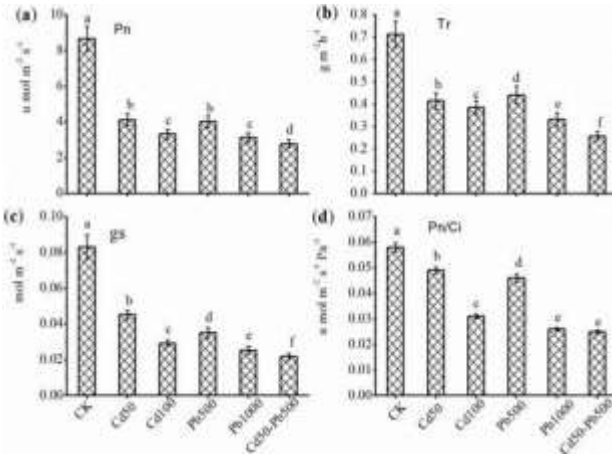
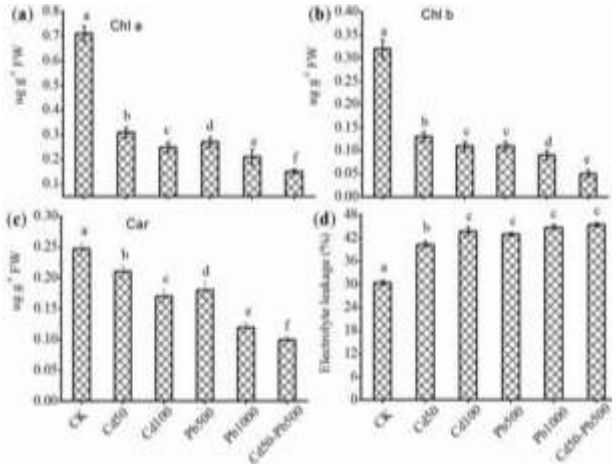


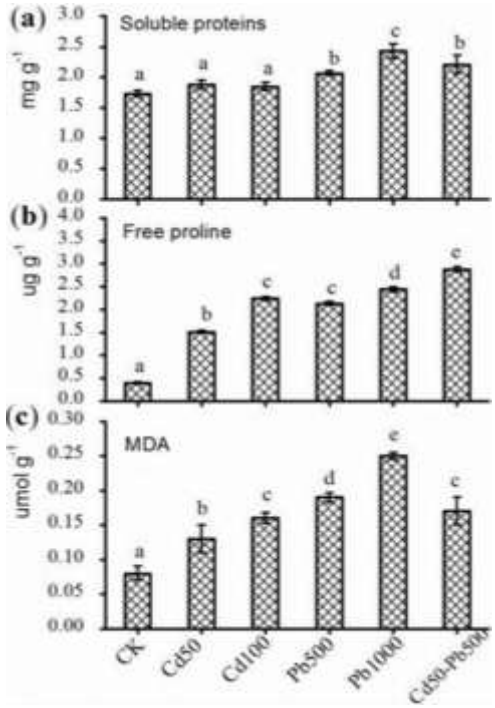
Fig. 4: Leaf pigment contents and electrolyte leakage percentage of Jatropha seedling when exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments for 30 days. (a) Chla; (b) Chlb; (c) Car; (d) Electrolyte leakage. Values were the mean ± SD of four independent experiments. Different letters indicate the significant difference between data points (P<0.05)



contents increased by 2.1, 3.1, 3.9, 4.8 and 3.2 time over control in Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, respectively. These nonenzymatic antioxidant components indicated that the Jatropha seedlings had strong response patterns to Cd, Pb, and the combined heavy metal stresses.

Antioxidant enzymes: There were approximately 65, 32, 67, 85 and 18% increases in CAT activity at Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments, respectively (Fig. 6a). POD activity increased approximately 10, 11 and 22% in the Cd50, Cd100, and Pb500 treatments,

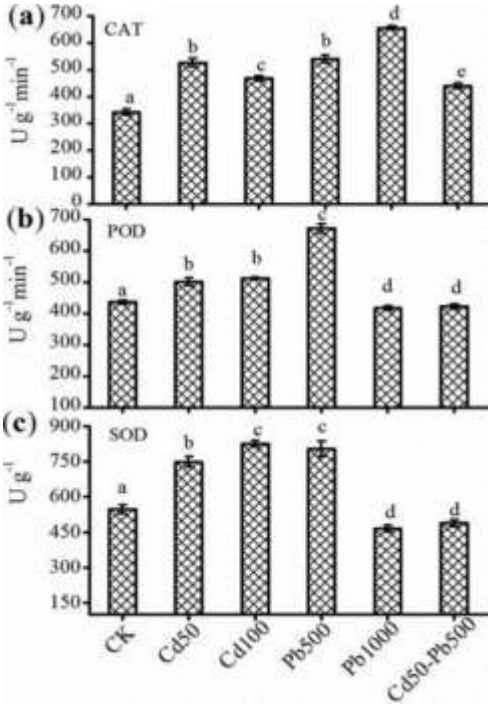
Fig. 5: The soluble proteins (a), free proline (b) and MDA (c) contents of seedling exposed to Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 treatments for 30 days. Values recorded here represent the mean ± SD from three independent replicates. Different letters indicate the significant difference between data points (P<0.05)



respectively. When subjected to Pb1000 and Cd50-Pb500 treatments, POD activity significantly decreased by 8 and 7%, respectively, as compared to control (Fig. 6b). SOD activity increased by approximately 50, 68 and 62% in Cd50, Cd100 and Pb500 treatments, respectively, but in Pb1000 and Cd50-Pb500 treatments, SOD activity decreased by approximately 10 and 12% than that of control (Fig. 6c). Pb1000 and Cd50-Pb500 spiked soils had strongly phytotoxic effects on the seedling growth and maybe have destroyed the inherent SOD-APX-CAT system in intracellular compartments.

Cellular ultrastructure: TEM observations are indicated in Fig. 7. The subcellular arrangement of the leaf cell in the control plant seedling was regular, integrated, intact and clear-cut in appearance (Fig. 7a), for example, it contained compact and smooth cell wall layers, typical elliptical chloroplasts containing 8-12 grana with large and plump osmiophilic granules, and the mitochondria showed an elliptical shape in intracellular compartment (Fig. 7b). Stroma-thylakoid layers had a distinct structure and were arranged in order. The grana lamella membrane and chloroplast membrane were distinctly legible (Fig. 7c). When plant seedling was grown in Cd100 spiked soils for 30 days, the leaf cell showed distinctly larger volume,

Fig. 6: Activities of antioxidant enzyme CAT (a), POD (b), SOD (c) in Jatropha seedling leaves treated with Cd50, Cd100, Pb500, Pb1000 and Cd50-Pb500 for 30 days. Values recorded here represent the mean ± SD from three independent replicates. Bars with the same letters are not significantly different at P < 0.05

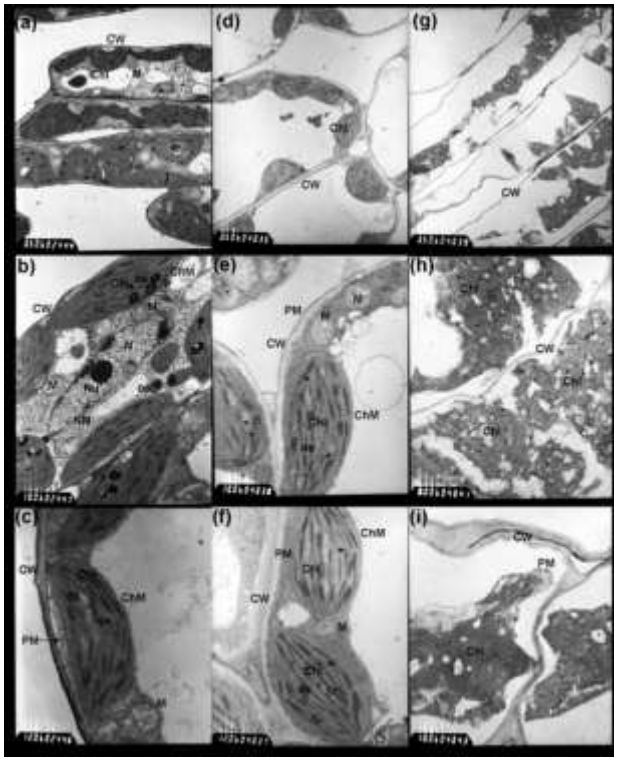


chloroplasts and mitochondria were slightly distorted, became smaller in size, and attached to internal cell walls with several vacuoles. In addition, an indistinct cell wall layer was observed (Fig. 7d). The mitochondria appeared round in shape and overflowed a number of matrices, and the mesophyll cells increased the thickness of cell walls and had a disorderly thylakoid structure (Fig. 7e). Mitochondria appeared to be highly vacuolated in response to Cd heavy metal. The dense lysosomes, similar to organelles, were also present in the intracellular compartment (Fig. 7f). When the plant seedling was grown in Pb1000 spiked soil for 30 days, the subcellular structure was distorted, and membrane structure was illegible and exhibited distinct damnification (Fig. 7g). The chloroplast membranes showed degradation and overflowed a large number of matrices, and the cell wall was detached from the plasma membrane (Fig. 7h). The plasma membrane was distorted. A large number of vacuoles emerged. The electronic opaque particles were observed in the intracellular compartment. The plasmolysis and the damage to the cell membrane were obvious, too. Furthermore, the chloroplast membrane was completely broken and the matrix flowed out of the chloroplast (Fig. 7i).

DISCUSSION

In response to Cd and Pb treatments, the Jatropha seedling exhibited a retardation of growth, a depression of gas exchange rate and a photosynthetic efficiency, as well as the inhibition of several antioxidant enzymes, the production of lipid peroxidation components, and the extensive impairments in cellular ultrastructure. These results indicated that the phytotoxicities of Cd, Pb and their combination were directly associated with the heavy metal-tolerance capability of the plant and accompanied by an occurrence of a variety of morphological, molecular or cellular events. Firstly, the most notable symptom of Cd and Pb phytotoxicity was the retardation of plant growth. The growth performance of plant seedling in heavy metal-spiked soils provided explicit insights into the tolerant potential against heavy metal stresses. In general, the growth parameters, such as root length, fresh weight, and biomass production, are obvious responses to heavy metal toxicity in tree species (Dominguez *et al.,* 2009). This study revealed that at high heavy-metal concentrations, the plant height was significantly reduced, and the biomass was decreased. The root growth was more sensitive than other parameters, as

Fig. 7: Transmission electron micrographs of Jatropha seedling leaf ultrastructure (without the addition of Cd (a, b & c) and addition of 100 mg Cd kg^{-1} (d, e & f) or addition of 1000 mg Pb kg^{-1} (g, h & i)). Chl: chloroplast, chM: chloroplast membrane, M: mitochondria, CW: Cell Wall, os: osmiophilic granules, St: Stroma-thylakoid, PM: plasma membrane. The magnification of panel (a), (d) and (g) is 3500×; The magnification of panel (b), (e) and (h) is 10000×; The magnification of panel (c), (f) and (i) is 12000×



roots rapidly absorbed water and had higher accumulations of heavy metal elements. The results presented by this study were in agreement with earlier reports on other plants, such as aquatic plant *wolffia arrhiza* (Piotrowska *et al.*, 2010), barley *Hordeum vulgare* (Tiryakioglu *et al*., 2006) and *typha angustifolia* (Bah *et al.*, 2011). Other studies with woody plant reported a higher inhibition of root elongation (Dominguez *et al*., 2009). In particular, Jatropha plants could bioaccumulate and bioconcentrate toxic heavy metals from an aqueous solution (Mohammad *et al*., 2010) and could be used as phytoremediation candidates in some countries (Juwarkar *et al.*, 2008; Kumar *et al.*, 2008; Jamil *et al*., 2009). Additionally, the plant seedling exhibited a high root/shoot ratio throughout the experiment. An alternative explanation might relate to a strong root system with many roots spread out over the entire soil for survival because root/shoot ratio could reflect plant's response to various environment factors (Otieno *et al.,* 2005; Lukacova Kulikova & Lux, 2010; Li *et al.,* 2010b).

The physiological responses, such as the gas exchange rate and photosynthetic function, can be ascribed to the different effects of physico-chemical properties of heavy metals on the integrity and function of the photochemical apparatus of plant seedling fronds, as well as the impact on the chlorophyll concentrations in the leaves. The photosynthesis rate, CO_2 assimilation rate, and stomatal conductance in response to Cd and Pb heavy metals have been well documented (Chen *et al*., 2012). Low leaf Pn values were partially associated with low gs and metabolic limitations (low Pn/Ci) (Wahid *et al.* 2007; Silva *et al.*, 2010). The maintenance of an intercellular CO_2 concentration is concomitant with the leaf CO_2 assimilation rate and reflected photosynthesis function of seedling in the different heavy metal-spiked soils. The chlorophyll and carotenoid contents played a central role in the energy manifestation of green plant. Any significant alteration of their contents possibly resulted in a marked effect on the entire metabolism of the plant (Piotrowska *et al*., 2010). In this study, either Cd or Pb alone, or their combination all resulted in a significant reduction in the chlorophyll contents, possibly due to the inhibition of chlorophyll biosynthesis or a breakdown of pigments and their precursors (Agrawal & Mishra, 2009). Cd or Pb might replace the central Mg from chlorophyll molecules and thereby reduce the photosynthetic light-harvesting ability of plant (Agrawal & Mishra, 2009). In contrast, Car were less sensitive than Chl a and Chl b in response to both Cd and Pb heavy metals, which probably facilitated the maintenance of photosynthetic apparatus against heavy metal stress (Piotrowska *et al.,* 2010). Car stabilized and protected the lipid phase of the thylakoid membrane by serving as the antioxidant to scavenge the free radicals (Polle *et al.,* 1992; Piotrowska *et al.*, 2010). So, the Jatropha plant displayed efficient protective mechanisms against oxidative damages induced by Cd or Pb heavy metals.

Lipid peroxidation, soluble proteins and free proline contents in plant cells were some important indicators of reversible and irreversible changes in metabolism response to a wide variety of stressors (Singh & Tewari, 2003). Oxidative stress subjected to toxic heavy metals could be demonstrated by MDA content, which often reflects the extent of injuries incurred when plants were exposed to any adverse environments (Xu *et al*., 2009). In this study, MDA content significantly increased concomitantly with various treatments described above. It is evident that the membrane lipid has been peroxidated under these heavy metal stresses and the plant species had a potent membrane lipid peroxidation capability against heavy metal phytotoxicity. Due to acute oxidative stress caused by heavy metals in plant cells, the soluble protein contents slightly increased. Free proline is closely related to the antioxidation of plant and could eliminate active oxygen in plant (Xu *et al.*, 2009). In this study, the combined Cd50-Pb500 treatment had the maximum proline increase in contrast to other treatments due to the synergistic effect of Cd and/or Pb heavy metals.

It has been previously documented extensively that SOD, POD and CAT were major protective enzymes that could maintain redox homeostasis of plant cells and scavenge active oxygen radicals when oxidative damage occurred (Gao *et al*., 2010; Bah *et al.*, 2011). Jatropha seedling suffered from serious damage and manifested a defensive response capacity when subjected to too high concentrations of Cd and Pb heavy metals. The activities of SOD, POD and CAT enzymes were inhibited, and organelles, including mitochondria and chloroplasts, were destroyed extensively. Some investigations demonstrated that the coordinated SOD and CAT transferred hazardous O_2^- and H_2O_2 to intracellular O_2 and H_2O, and reduced the formation of actively hazardous ^-OH (Liu *et al.*, 2007). Specifically, SOD was the first component of the antioxidant defense molecules to scavenge O_2^- and transform O_2^- to H_2O_2 and H_2O. It directly reduced the level of intracellular free radicals (Gao *et al.*, 2010; Piotrowska *et al.*, 2010; Visioli *et al.,* 2010). So, the antioxidant defense of enzymes was also directly associated with the heavy metal-tolerance of plants. The Jatropha plant has a strong antioxidant enzyme system against heavy metal phytotoxicity. The results in this study are in accordance with other reports of the Jatropha, including the acclamatory responses to heat and drought stresses and mercury phytotoxicity (Gao *et al*., 2010; Silva *et al.*, 2010). The Jatropha may have some inherent adaptive tolerance mechanism in a multiple heavy metal elements-contaminated environment (Jamil *et al*., 2009; Gao *et al*., 2010). Therefore, Jatropha plant species had strong response patterns to phytotoxic effects of heavy metals, including nonenzymatic components, such as soluble proteins, free proline, MDA, et al., and various enzymatic antioxidants, such as POD, SOD, CAT etc.

Cells are the structural and functional unit to control all types of the physiological and biochemical processes. Heavy-metal phytotoxicity mainly impairs the seedling development, transpiration, chlorophyll production,

oxidative metabolism, and lamellar organization in the chloroplasts (Pourrut *et al.*, 2011). So, the cellular ultrastructure was evaluated by transmission electron microscopy and directly illustrated the cellular mechanisms in intracellular compartments. Apart from the influence on cell wall, plasma membrane, and chloroplast membrane, the ultrastructures of the chloroplast and mitochondria were the most striking effect. The cellular ultrastructural analysis directly indicated that the plant seedlings exhibited a degree of resistance against heavy-metal phytotoxicity and had efficient heavy metal-tolerant mechanisms. So, the current study showed a better understanding of the heavy-metal tolerance of the Jatropha plant. It abundantly grows in multiple heavy metal-contaminated Panzhihua region and evolves gradually to be a native plant species suitable for the phytoremediation of heavy metal-contaminated soils. Our study also has a high importance that we cultivate the Jatropha plant to find the natural compounds for medicine applicability. Thus, further in-depth studies on the Jatropha plants might not only help to understand plant defense mechanisms or some toxicity/tolerance issues, but also possibly identify some new marker proteins under multiple heavy metal stresses.

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