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Full Length Article

Effects of Zinc on Growth and Antioxidant Responses in *Jatropha curcas* Seedlings

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ABSTRACT

Jatropha (*Jatropha curcas*) embryos were geminated and grown *in vitro* under different zinc concentrations (0, 0.25, 0.5, 1, 2 & 3 mM) to investigate its influence on the growth and the changes of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and phenylalanine ammonia-lyase (PAL) activities in Jatropha seedlings. The biomass of the cotyledons, hypocotyls and radicles was increased with increasing zinc concentration and the largest increments were observed at 0.25, 0.5 and 0.5 mM, respectively. SOD activity in the cotyledons, hypocotyls and radicles was increased gradually with the increase in zinc concentrations. While POD activity in the cotyledons, hypocotyls and radicles was enhanced with increase in zinc concentrations. CAT activity in the cotyledons, hypocotyls and radicles reached the largest increments at the zinc concentrations of 0.5, 2 and 0.5 mM, respectively. PAL activity showed a similar trend compared to the changes of CAT activity. Electrophoresis anaylsis suggested a significant correlation between zinc concentrations and isoenzyme patterns of SOD, POD and CAT and these results were consistent with the changes of the activities assayed in solutions. These results suggest that SOD, POD, CAT and PAL, may play an important role in the defensive mechanisms of *Jatropha curcas* seedlings exposed to excessive zinc.

Key Words: Jatropha; Zinc; Antioxidant enzyme; Isoenzyme patterns

INTRODUCTION

Zinc (Zn), as one of the essential micronutrients in plants is necessary for plant growth and development. However, excessive Zn in plants can profoundly affect normal ionic homeostatic systems by interfering with the uptake, transport, osmotic and regulation of essential ions and results in the disruption of metabolic processes such as transpiration, photosynthesis and enzyme activities related to metabolism (Rout & Das, 2003; Broadley *et al*., 2007; Abbas *et al*., 2009). Zn phytotoxicity also induces oxidative stress by generating free radicals and reactive oxygen species (ROS) (Weckx & Clijsters, 1997). These ROS are highly reactive and cause the death of plants by damaging membrane lipids, proteins, pigments and nucleic acids. To cope up with the damages caused by the ROS, cells possess their own comprehensive and integrated endogenous antioxidant defense system composed of both enzymatic as well as non-enzymatic components (Miller *et al*., 2008). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) represent the endogenous defense of plant cells. These enzymes are present in different isoforms in several cell compartments and their expression is genetically controlled and regulated both by developmental environmental stimuli, according to the necessity to remove ROS produced in cells (Mittler *et al*., 2004).

Jatropha *(Jatropha curcas* L.) or physic nut is a multipurpose and drought-resistant, large shrub. Various parts of the plant hold potential for use as animal feed, inclusion in medicinal preparations and as a source of honey. Recently, Jatropha has been investigated mainly as a potential source of oil that has been recognized as an adequate sub-stitute motor fuel (Debnath & Bisen, 2008). Our previous researches suggested that Jatropha seedlings could adjust themselves to adapt to higher concentration copper under sand culture condition as well as lead toxicity *in vitro* embryo culture (Gao *et al*., 2008 & 2009). A key role of ROS-scavenging enzymes in the protection against harmful oxidative reaction resulting from zinc stress has also been reported (Prasad *et al*., 1999; Bonnet *et al*., 2000; Khudsar *et al*., 2004; McGeer *et al*., 2005). These findings suggest that these plants try to counteract high concentrations of oxygen species produced under zinc toxicity through a co-ordinated increase in the activities of enzymes involved in their detoxification. However, the effects of zinc on growth and the possible induction of a defense mechanism in Jatropha have not been studied. Therefore, the aim of the present study was to investigate the effects of zinc on the growth, SOD, POD, CAT and PAL in Jatropha seedlings.

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MATERIAL AND METHODS

Mature Jatropha (*Jatropha curcas* L.) seeds were collected in September, 2008 from more than 10 individual wild trees in Panzhihua, Sichuan provice, China. Seeds were selected and stored in a plastic box with labeled (No. 20080926) were deposited at 4°C until processing. Nitro blue tetrazolium (NBT), l-phenylalanine and methionine were purchased from Sigma (St. Louis, MO, USA). Other reagents were of grade or higher.

Jatropha seeds were surface sterilized with 70% enthol for 30 sec 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. Culture medium was separated into five lots. One lot was allowed to grow MS medium with 30 g/L sucrose and 0.6% agar powder to serve as control. The remaining four lots were cultured on basic MS medium supplemented with zinc added as $ZnCl_2$ at concententions of 0.25, 0.5, 1, 2 and 3 mM, 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. The experiments were arranged in a completely randomized design with three replicates per treatment and each replicate contained 15 embryos.

Fresh cotyledons, hypocotyls and radicles were homogenized in 50 mM sodium phosphate buffer (pH 7.0, 1/10, w/v) containing 0.1 mM EDTA. The homogenate was centrifuged at 12000 rpm for 10 min at 4°C and the resulting supernatant was used for enzyme assays. The experiment was randomly arranged three replicates with each treatment.

SOD, POD and CAT isozymes were separated by non-denaturing PAGE. PAGE for isoenzymes assay was performed with 10% acrylamide gel at room temperature. A vertical electrophoresis apparatus (model DYCZ-24DN, Beijing Liuyi Instrument Factory, China) was used. The electrophoretic run was carried out with 150 mV per plate towards the cathode. SOD activity was detected as described by the Beauchamp and Fridovich method (1971). Briefly, gels were incubated at ambient temperature in 2.45 mM NBT solution for 20 min. This solution was replaced with riboflavin buffer (50 mM sodium phosphate, pH 7.5, containing 28 mM tetramethylethylenediamine & 28 µM riboflavin) for 15 min. Gels were then transferred into distilled water and exposed to light for 15-30 min. For the detection of POD isoenzyme activity and the gel was stained in a solution containing 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid. The drawing of the gel was made immediately after staining (Ros Barcelo, 1987). CAT isoenzymes were measured by the Woodbury method (1971). Gels were incubated in 0.01% H_2O_2 for 10 min and developed in a 2% (m/v) $FeCl_3$ and 2% $K_3Fe(CN)_6$ (m/v) solution for 10 min until the colourless bands appear.

SOD activity was determined by rate inhibition of nitro blue tetrazolium at 560 nm. One unit of SOD was defined as the amount of enzyme that inhibited the reduction of nitro blue tetrazolium by 50% (Chen & Pan, 1996; Gao *et al*., 2008). The activity was expressed in U/g fresh weight.

POD activity was assayed using guaiacol and H_2O_2 as sub-strates. The reaction medium (3 mL final volume) consisted of 2.8 mL 50 mM Tris-HCl buffer (pH 7.0), 0.1 mL H_2O_2 (2%) and 0.1 mL enzyme extract (Sakharov & Bautista, 1999). One unit of enzyme activity was reported as the amount, which increased the absorbance by 1 per min. The activity was expressed in U g^{-1} fresh weight.

CAT activity was determined in a 3-mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 100 µL H_2O_2 (1%) and 50 µL of enzyme extract. The rate of decrease in absorbance was measured at 240 nm. One unit of catalase is defined as the amount that decomposes 1 µmol H_2O_2 in 1 min (Montavon *et al*., 2007). The activity was expressed in U g^{-1} fresh weight.

PAL activity was determined in crude extracts obtained by grinding these tissues (0.2 g) with 2 mL of 50 mM Tris-HCl buffer containing 10% polyvinylpyrrolidone. The protein extract obtained after centrifugation (15294 x *g* for 10 min) was used to assay the PAL activity. PAL assay was performed in 3 mL of reaction mixture containing 50 mM Tris-HCl buffer pH 8.8, 20 mM L-Phe and 50 µL enzyme extract. The increase in absorbance at 290 nm was recorded after 30 min (Hahlbrock & Ragg, 1975). One unit of activity was defined as the amount of enzyme required for the formation of 1 mmol of product in 1 min under the assay condition. The results were expressed as PAL units per gram of tissues (fresh weight).

Values reported in this paper were the mean of three replicated treatments. Data were tested at significant levels of $P < 0.05$ using one way analysis of variance (Kleinbaum *et al*., 1998).

RESULTS

The effects of different zinc concentrations on the growth and biomass of Jatropha seedlings are given in Fig. 1. The biomass of cotyledons, hypocotyls and radicles increased gradually up to 0.25, 0.5 and 0.5 mM zinc concentration, respectively and then decreased. The development of cotyledons was inhibited, when zinc concentration is higher than 0.5 mM and toxic symptoms were observed on the cotyledons, hypocotyl and radicles.

As shown in Fig. 2, SOD activity in the cotyledons, hypocotyls and radicles increased gradually with the increasing zinc concentrations up to 3 mM, increased by 40.4%, 40.1% and 21.4%, respectively. Results of

isoenzyme patterns suggested that at least four isoenzyme bands are detected and a novel isoenzyme is found in the cotyledons (III), hypocotyls (III) and radicles (II) at the higher zinc concentrations (Fig. 3). These isoenzymes show different staining intensity with the increased zinc concentrations and the staining densities of SOD isoenzyme were consistent with the changes of the activities assayed in solutions.

POD activity in the cotyledons and hypocotyls with the increasing zinc concentrations gradually increased and the highest activities reaching 6.63 and 7.84 times compared to the controls. However, POD activity in the radicles reached the highest value at the zinc concentrations of 0.5 mM and then decreased (Fig. 4). Isozyme pattern analysis suggested that at least seven, six and five isoenzyme bands in the cotyledons, hypocotyls and radicles are detected, respectively and the staining densities of these isoenzymes show different characteristics with the increasing zinc concentrations (Fig. 5).

CAT activity in the cotyledons and hypocotyls increased with the increasing zinc concentrations up to 0.5 mM and 2 mM, representing 36.9% and 38.5% increment, respectively (Fig. 6). However, the activity in the radicles showed no significant changes and the activity remained low, representing about 9 U per gram fresh weight. Only one CAT isoenzyme band in the cotyledons, hypocotyls and radicles was observed using electrophoresis (Pattern no shown). Its intensity varies with zinc concentrations and plant tissues and is consistent with the changes of the activities assayed in solutions.

PAL activity in the cotyledons, hypocotyls and radicles was significantly induced with the increasing zinc concentrations up to 0.5, 2 and 2 mM, respectively and then decreased. The highest activities increased by 87.8%, 107.1% and 140% compared to the control, respectively (Fig. 7).

DISCUSSION

Growth inhibition is a general phenomenon associated with most of heavy metals, while the tolerance limits for heavy metal toxicity are specific for each species and even for each variety of cultural plants (Broadley *et al*., 2007). Being an essential micronutrient, zinc may promote the growth of Jatropha seedlings, when present at lower concentrations, but if present at high levels, zinc inhibited growth by interfering with normal cellular metabolic events and inducing visible injuries and physiological disorder, as are reported by us and other workers (Ali *et al*., 1999; Kaya *et al*., 2000). The first visible damage due to excessive zinc was on root growth due to reduction in cell division (Prasad *et al*., 1999). In the present study, the decreases of area, length and biomass in cotyledons and radicles were observed in zinc concentrations higher than 1 mM. Thus, our results suggested that Jatropha seedlings showed a negative response to higher zinc toxicity, possibly through

Fig. 1. Biomass in cotyledons, hypocotyls and radicles of *Jatropha curcas* exposed to different zinc concentrations. Data are displayed as mean ± standard deviation (bars) for three replications. Significant difference (*P*< 0.05) is denoted as asterisk (*) between the control and treatments

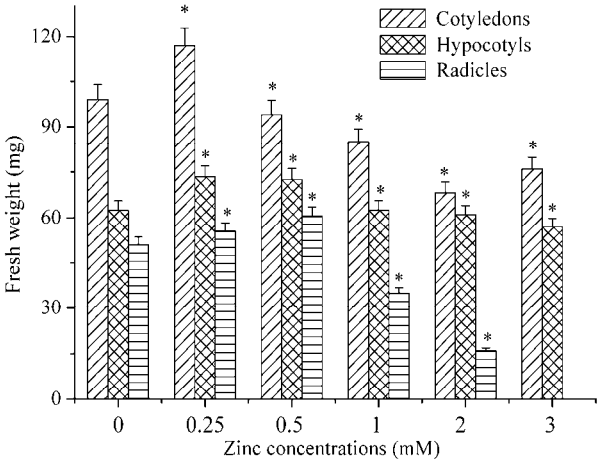
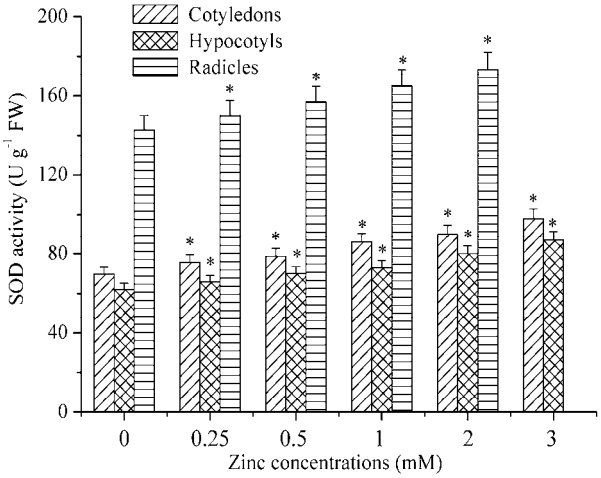


Fig. 2. SOD activities in cotyledons, hypocotyls and radicles of *Jatropha curcas* exposed to different zinc concentrations. Data are displayed as mean ± standard deviation (bars) for three replications. Significant difference (*P*< 0.05) is denoted as asterisk (*) between the control and treatments



the enhancement of ROS production, which in turn led to the oxidative damage to plant cells and blocked the growth.

SOD play an important role in detoxification processes by catalyzing the conversion of free O_2^- to O_2 and H_2O_2 and is associated with stress situations including zinc stress (Bonnet *et al*., 2000). In plants, environmental adversity often leads to the increased generation of reduced oxygen species and consequently, SOD has been proposed to be important in plant stress tolerance (Mittler *et al*., 2004). In the present study, SOD activity in the cotyledons are

Fig. 3. Patterns of SOD isoenzymes in cotyledons, hypocotyls and radicles of Jatropha 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 left to right were 0, 0.25, 0.5, 1, 2 and 3 mM, respectively. About 25 µl extract from each sample was loaded

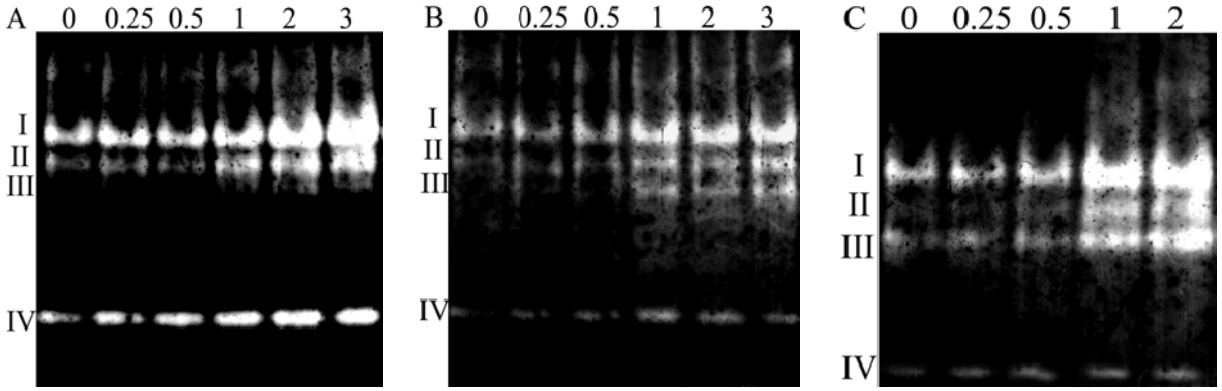
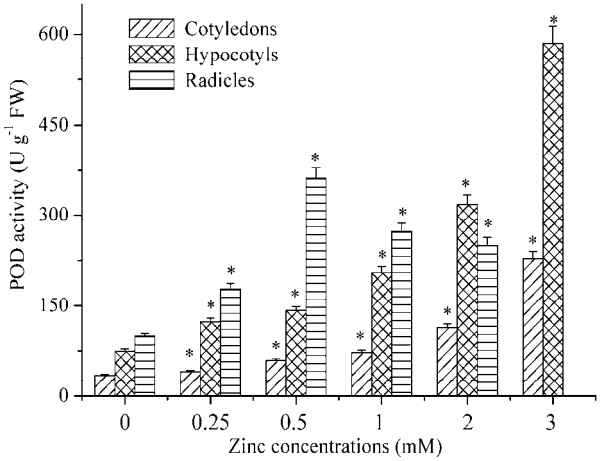


Fig. 4. POD activities in cotyledons, hypocotyls and radicles of Jatropha exposed to different zinc concentrations. Data are displayed as mean ± standard deviation (bars) for three replications. Significant difference (*P*< 0.05) is denoted as asterisk (*) between the control and treatments



significant higher than those of the hypocotyls and radicles at the same zinc level, suggesting that the cotyledons are most sensitive, when exposed to zinc toxicity. Isoform enzymes of PAGE analysis showed that the levels of SOD transcripts are induced in response to zinc stress; however, they differ in different tissues and zinc concentrations. These results might suggest that a hierarchy of regulatory events act at the transcription of SOD genes. Pioneer studies had shown a general stimulation of constitutive SODs and the induction of specific SOD isoenzymes in different plant species (Prasad *et al*., 1999). Enhanced SOD activity could potentially increase oxidative stress due to increased production of H_2O_2. In this study, there was an increase in SOD activity over time for the control and the treatment seedlings, with the treatments having markedly higher activity overall compared to the control (Fig. 2 & 3). Based on the above results, the increased SOD activities and their isoenzymes may play an important role in the defensive mechanisms of plant seedling against zinc toxicity.

POD, along with SOD and CAT, are redox metalloenzymes involved in cell defense against oxidative stress. Plant PODs, which are encoded by small or large multigenic families, are involved in several important physiological and developmental processes (Passardi *et al*., 2005). POD can also be considered useful markers for environmental stresses since their activity is affected by heavy metal, salt and other environment conditions (Prasad *et al*., 1999; Bonnet *et al*., 2000; Gao *et al*., 2008 & 2009). Our results suggested that POD activity in Jatropha seedlings was induced gradually by increasing zinc concentrations. Previous studies have also shown that induction of POD activity has been reported in many plant species, when exposed to zinc stress (Prasad *et al*., 1999; McGeer *et al*., 2000). Electrophoresis analysis revealed the presence of different forms of POD with distinct activities in the different tissues of Jatropha (Fig. 5). In addition, a new POD isoenzyme band in the cotyledons (VI), hypocotyls (V) and radicles (IV) is detected at the highest zinc concentration, indicating that this POD isoenzyme probably plays a specific physiological role at specific plant organs. Although these POD isoenzymes show different patterns of activities exposed to zinc toxicity, the total activity of POD in Jatropha seedlings was significantly enhanced, suggesting that POD activity could reflect an increased degree of oxidative stress. Our findings suggested that the defensive system of plant regulated the changes of enzyme activities or the types of isoenzyme in order to enhance the defensive function against excessive zinc.

CAT is one of the major antioxidant enzymes that plays a very important role in the protection against oxidative damage by breaking down hydrogen peroxide. Accumulating evidence indicates that catalase plays an important role in plant defense, aging and senescence

Fig. 5. Patterns of POD isoenzymes in 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 left to right were 0, 0.25, 0.5, 1, 2 and 3 mM, respectively. About 15 µL extract from each sample was loaded

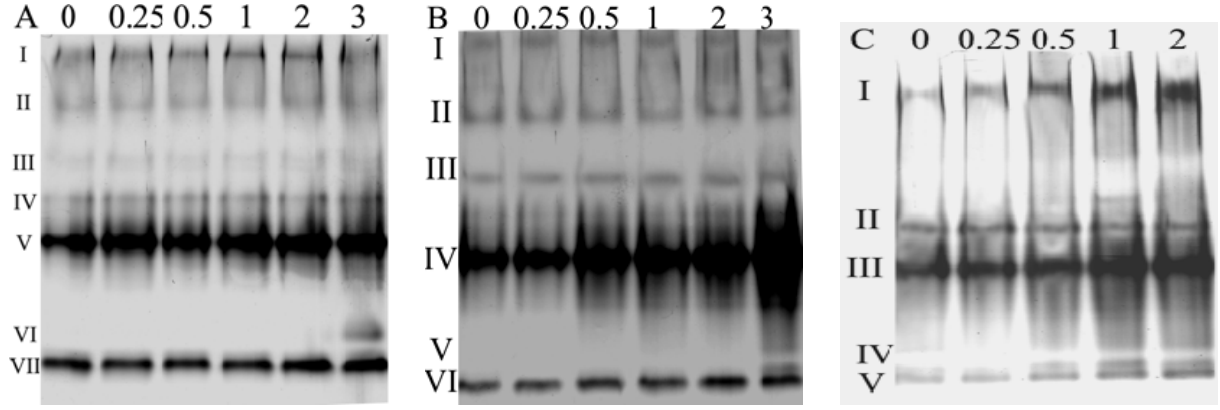


Fig. 6. CAT activities in cotyledons, hypocotyls and radicles of *Jatropha curcas* exposed to different zinc concentrations. Data are displayed as mean ± standard deviation (bars) for three replications. Significant difference (*P*< 0.05) is denoted as asterisk (*) between the control and treatments

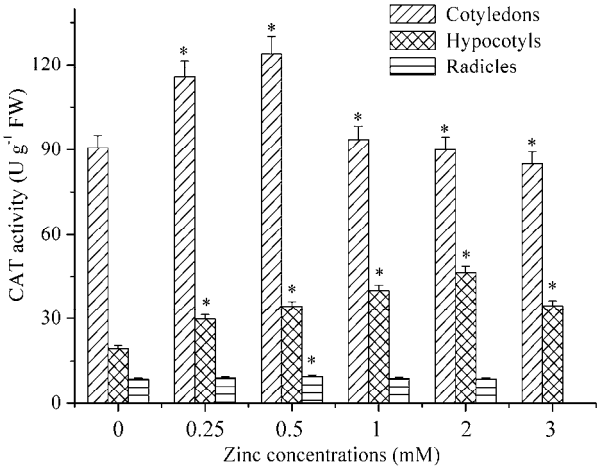
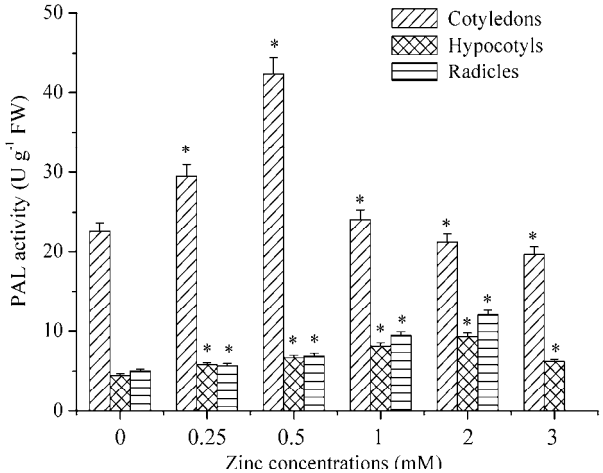


Fig. 7. PAL activities in cotyledons, hypocotyls and radicles of *Jatropha curcas* exposed to different zinc concentrations. Data are displayed as mean ± standard deviation (bars) for three replications. Significant difference (*P*< 0.05) is denoted as asterisk (*) between the control and treatments



(Mittler *et al*., 2004). The CAT activities in the cotyledons and radicles significantly increased with the zinc concentrations at 0.5 and 1 mM, but the activities in the hypocotyls increased gradually up to 2 mM. The induction of this enzyme under zinc stress indicated that it helps in inhibiting the oxygen radical accumulation. Similar to the present study, an increase in CAT activity has been reported in other plant species exposed to zinc stress (Prasad *et al*., 1999; McGeer *et al*., 2000). Based on the above results, our findings provide evidence that CAT may provide an additional protection against the oxidative damage induced by zinc stress.

PAL has been widely studied in plant tissues, especially with regard to its induction by various environmental factors such as light, wounding excision and infection. In plants, PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism and could perform defense-related functions (MacDonald & D'Cunha, 2007). In the present work, PAL activities in the cotyledons, hypocotyls and radicles showed an enhanced tread. Earlier results suggested that a reduced PAL activity is directly correlated with lower levels of phenylpropanoids in transgenic tobacco plants, whereas gene expression and enzyme induction are correlated with phenylpropanoid accumulation (Boudet *et al.*, 2003). Induction of PAL activity and total phenolics has been also observed in some plant species under heavy metals stress and depends on the stress and species of plant (Kováčik & Bačkor, 2007). These

results suggest that PAL may modulate the resistance to stresses by regulating the biosynthesis of phenolic compounds. Thus, our findings suggest that induced PAL activities may also be involved in modulating the resistance of Jatropha plants exposed to excessive zinc.

In conclusion, the presence of high levels of ROS-scavenging enzymes and PAL activities in *Jatropha curcas* seedlings reflects a positive adaptive response aimed at enhancing protection not only against zinc toxicity, maintains the balance of ROS, but also towards further oxidative damage. These biochemical responses to excessive zinc in *in vitro* embryo culture were sensitive. Thus, they are useful for elucidating the mechanisms by which plants take up heavy metal and can provide useful bioassays that help to assess heavy metals contamination in agricultural environments, especially, when they are combined with field experiments.

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