Effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedlings

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ABSTRACT

The effects of increasing NaCl concentrations on biomass, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) in *Jatropha curcas* L. seedlings were investigated. The fresh weights of cotyledons and radicles with increasing NaCl concentrations decreased progressively, and the fresh weight of hypocotyls reached the lowest level at NaCl concentration of 150 mmol and then increased. SOD activity in the cotyledons, hypocotyls and radicles increased gradually up to NaCl concentrations of 150, 200 and 150 mmol, respectively. The highest POD activities in the cotyledons, hypocotyls and radicles were observed at NaCl concentrations of 150, 200 and 150 mmol, respectively. CAT activity in the cotyledons, hypocotyls and radicles enhanced gradually up to 100, 200 and 150 mmol NaCl concentrations, respectively. Increased PAL activity in the hypocotyls and radicles was linearly and positively correlated with increasing NaCl concentrations, but the peak activity in the cotyledons was observed at NaCl concentration of 150 mmol. Electrophoresis analysis suggested that different patterns in SOD and POD isoenzymes depend on NaCl concentrations and organ type, and the staining intensities of these isoforms are consistent with the changes of enzyme activity assayed in solutions.

Keywords: ROS-scavenging enzymes; *in vitro* embryo culture; salt tolerance; isoenzyme pattern

Salt stress in soil or water is one of the major abiotic stresses especially in arid and semi-arid regions and can severely limit plant growth and yield. Salt stress can lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species (ROSs) and induce oxidative stress (Parida and Das 2005, Parvaiz and Satyawati 2008).

ROSs have potential to interact with many cellular components, causing significant damage to membranes and other cellular structures. However, an elaborate and highly redundant plant ROS network, composed of antioxidant enzymes and antioxidants, is responsible for maintaining the levels of ROS under tight control. In plant cell, antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) have been considered as a defensive team, whose combined purpose is to protect cells from oxidative damage (Mittler 2002). Increased SOD, POD and CAT activities are closely related to salt tolerance of many plants as reported in various researches (Rahnama and Ebrahimzadeh 2005, Azevedo Neto et al. 2006, Koca et al. 2007). These findings suggest that the induction of ROS-scavenging enzymes, such as SOD, POD and CAT, is the most common mechanism of salt tolerance for detoxifying ROS synthesized.

Jatropha curcas L., commonly known as physic nut or purging nut, is a drought resistant shrub or tree belonging to the family *Euphorbiaceae*, which is cultivated in many tropical and subtropical coun-

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tries. Different parts of this plant could be used for various purposes, such as energy source, therapeutic uses, fertilizer and animal feed. *Jatropha curcas* is also well adapted to gravelly, sandy and saline soils where salinity might be the major problem due to limited water supply (Openshaw 2000). Prior to this study little data known on the changes of antioxidant enzymes and isoenzyme of these enzymes with respect to salt stress or to possible roles in *Jatropha curcas* seedlings. In the present study, the effects of increasing NaCl concentrations on growth, SOD, POD, CAT and phenylalanine ammonia-lyase (PAL) in *Jatropha curcas* L. seedlings were investigated. Moreover, patterns of SOD and POD isoenzymes were also detected, to localize or demonstrate specific SOD and POD isoenzymes to specific organs and different NaCl concentrations.

MATERIAL AND METHODS

Mature *Jatropha curcas* L. seeds were harvested 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 (labelled, No. 20070822) and were deposited at 4°C until use.

The seeds were subjected to 70% ethanol for 30 s, and then to 0.1% mercuric chloride for 8 min. The seeds were rinsed several time with sterile distilled 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. Three embryos were sown in wide-neck bottles (100 ml) containing Murashige and Skoog (MS) medium (25 ml) for germination and growth. Embryos were separated into five lots. One lot was allowed to grow on MS medium to serve as control. The remaining four lots were exposed to four concentrations of NaCl: 50, 100, 150 and 200 mmol. MS medium pH was adjusted to 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. Germination experiment was carried out at 30°C in a greenhouse. Rotten and contaminated embryos were removed promptly. When the cotyledons of seedlings had developed, cotyledons, hypocotyls and radicles were washed with distilled water, blotted and immediately frozen in liquid nitrogen or stored at –80°C for analysis. Three sets of seedlings were analyzed for each NaCl concentration, with 15 embryos per set.

Fresh tissues were homogenized with 50 mmol sodium phosphate buffer (pH 7.0, 1/10, w/v) including 150 mmol NaCl and 0.5 mmol EDTA. The homogenized suspension was obtained by centrifuging at 12 000 rpm for 10 min at 4°C. The supernatant was used as assaying antioxidant enzyme and PAL. Protein content was quantified by the Lowry method and bovine serum albumin as the standard.

SOD activity was determined by the Chen and Pan method (Chen and Pan 1996). One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium to blue formazan by 50%. POD activity was performed according to the Sakharov and Aridilla method (Sakharov and Aridilla 1999) with slight modification. One unit of POD activity was expressed as the amount of enzyme that produced a change of 1.0 absorbance per min at 470 nm. CAT activity was measured following the change of absorbance at 240 nm for 1 min due to H_2O_2. One unit of CAT activity was expressed as the amount of enzyme needed to reduce 1 μmol of H_2O_2 per min (Montavon et al. 2007). SOD, POD, and CAT activities were expressed as unit per gram fresh weight (U/g fw).

Fresh tissues were homogenized with chilled 50 mmol Tris-HCl (pH 8.8, 1/10, w/v), supplemented with 0.5 mmol EDTA and 1% polyvinyl pyrrolidone. Other steps are the same as above. PAL activity was measured by monitoring the reaction product, *trans*-cinnamate (Hahlbrock and Ragg 1975). One unit of PAL activity was defined as the amount causing an increase of 0.01 in A_{290} per min. PAL activity was expressed as unit per gram fresh weight (U/g fw), as well.

SOD gel activity was carried out by the Beauchamp and Fridovich method with some modifications (Beauchamp and Fridovich 1971). After completion of electrophoresis, gels were incubated in 50 mmol phosphate buffer (pH 7.5) containing 28 μmol riboflavin, 28 mmol *N,N,N,N*-tetramethyl ethylenediamine (TEMED) for 30 min under dark conditions, followed by washing in distilled water for 1 min and incubation in the same buffer containing 2.45 mmol nitroblue tetrazolium (NBT) for 20 to 30 min exposed to light at room temperature. Isoenzymes appeared as colorless bands on a purple background. POD isoenzyme activity was determined by the procedure described by Ros Barcelo (1987). Gels, rinsed in water, were incubated in a solution composed of 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till brown bands appear, and the reaction was stopped by rinsing the gels with deionized water.

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

RESULTS AND DISCUSSION

Soil salinity is a prevalent abiotic stress for plants. Growth inhibition is a common response to salinity and plant growth is one of the most important agricultural indices of salt stress tolerance as indicated by different studies (Parida and Das 2005). In order to define salt stress tolerance or sensitivity of seedlings, the effects of NaCl treatment on fresh weights of the cotyledons, hypocotyls and radicles were tested (Figure 1). The fresh weight of cotyledons and radicles showed no significant changes at NaCl concentration of 50 mmol, but they decreased progressively with increasing NaCl concentration. The fresh weights of hypocotyls decreased gradually up to 150 mmol NaCl concentration then increased at NaCl concentration of 200 mmol. The cotyledon area and height of seedlings were the most sensitive to salt stress, especially under higher NaCl concentrations, which led to below medium of seedlings more sensitive to salt stress than above medium. It is worth noting that at NaCl concentration of 200 mmol the radicles of seedlings did not develop, and the hypocotyls became rough (data not shown). These growth parameters were consistent with the changes in the fresh weight of cotyledons, hypocotyls and radicles at all tested NaCl concentrations. Our findings suggested that different changes are related to the effects of different NaCl concentrations on different organs of seedlings.

SOD is one of several important antioxidant enzymes with the ability to repair oxidation damage caused by ROS. Thus, SOD is considered a key enzyme for maintaining normal physiological conditions and coping with oxidantive stress in the regulation of intracellular levels of ROS (Mittler 2002). Effects of NaCl on SOD activity in the cotyledons, hypocotyls and radicles were shown in Figure 2. SOD activity in the cotyledons increased significantly with increasing NaCl concentrations compared to the control, and the maximal levels increased by 70.8% at NaCl concentration of 150 mmol. Similarly, the highest SOD activities in the hypocotyls and radicles increased by 86.8% and 72.8% at NaCl concentrations of 200 and 150 mmol compared to the control, respectively. Many studies found a positive correlation between salt stress and the abundance of SOD in plants cells (Badawi et al. 2004, Cavalcanti et al. 2007). Results in published reports also indicate that SOD overexpression may be involved in the increase of stress protection observed in some transgenic plants (Yiu and Tseng 2005, Tseng et al. 2007). A significant increase of SOD activity in our study may be either necessary for the increased production of ROS or be a defensive mechanism developed by *Jatropha curcas* seedlings against

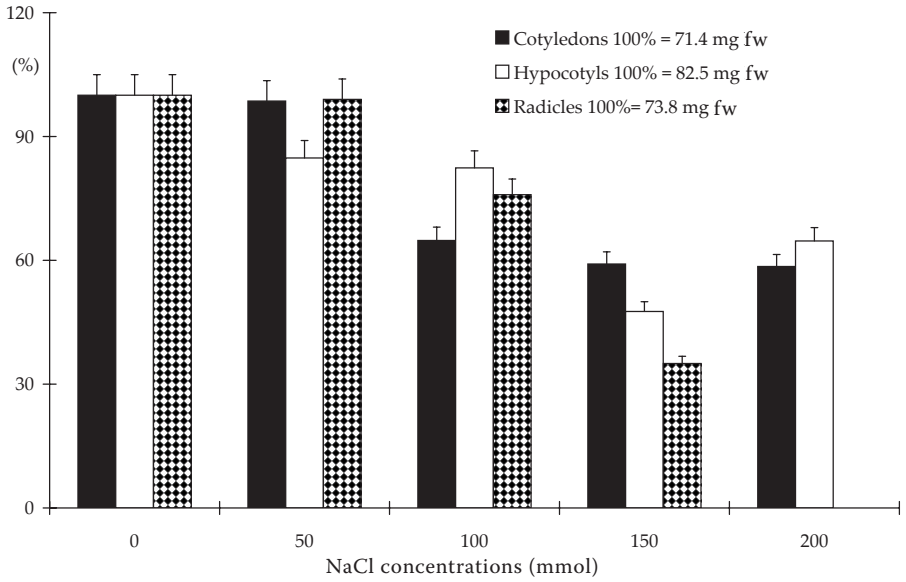

Figure 1. Effects of NaCl stress on biomass of the cotyledons, hypocotyls and radicles in *Jatropha curcas* L. seedlings. The values and standard errors (vertical bars) of three replicates are shown

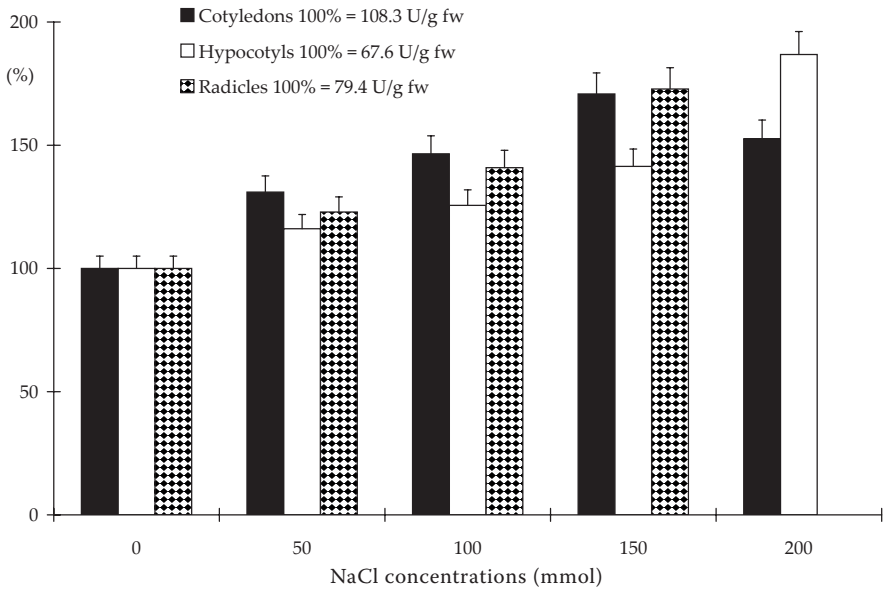

Figure 2. Effects of NaCl stress on superoxide dismutase (SOD) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings. The values and standard errors (vertical bars) of three replicates are shown

salt stress. Patterns of gel electrophoresis analysis suggested that at least three SOD isoenzyme bands in the cotyledons, hypocotyls and radicles are detected, respectively (Figure 3). The staining intensities of isoenzyme I and II in the cotyledons, hypocotyls and radicles were induced significantly with increasing NaCl concentrations, but that of isoenzyme III was minimally changed in response to NaCl concentrations. Thus, the changes of staining intensities of these isoenzymes strongly depended on NaCl concentrations and plant organs. Increased activity of SOD isozymes in different organs could contribute to the tolerance mechanism of *Jatropha curcas* seedlings against salt stress. Moreover, the staining intensities of these isoenzymes showed a similar change tread compared to the quantitative changes assaying in solutions (Figures 2 and 3). These findings suggest that different SOD isoenzymes might be regulated by salt stress and be involved in distinct physiological processes. The present findings could also be used as a basis for elucidating the mechanisms how the levels of SOD transcripts are induced in response to salt stress.

POD is widely distributed in higher plants where it is involved in various processes, including lig-

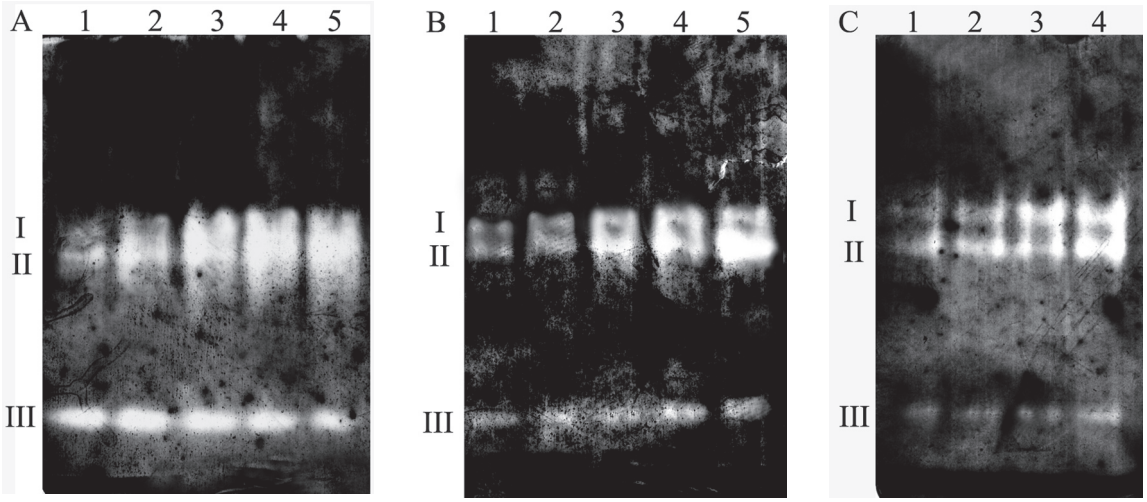

Figure 3. Patterns of SOD isoenzymes in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings: (A) patterns of SOD isoenzymes in the cotyledons; (B) patterns of SOD isoenzymes in the hypocotyls; (C) patterns of SOD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 50, 100, 150 and 200 mmol, respectively. About 25 µl extract from each sample was loaded

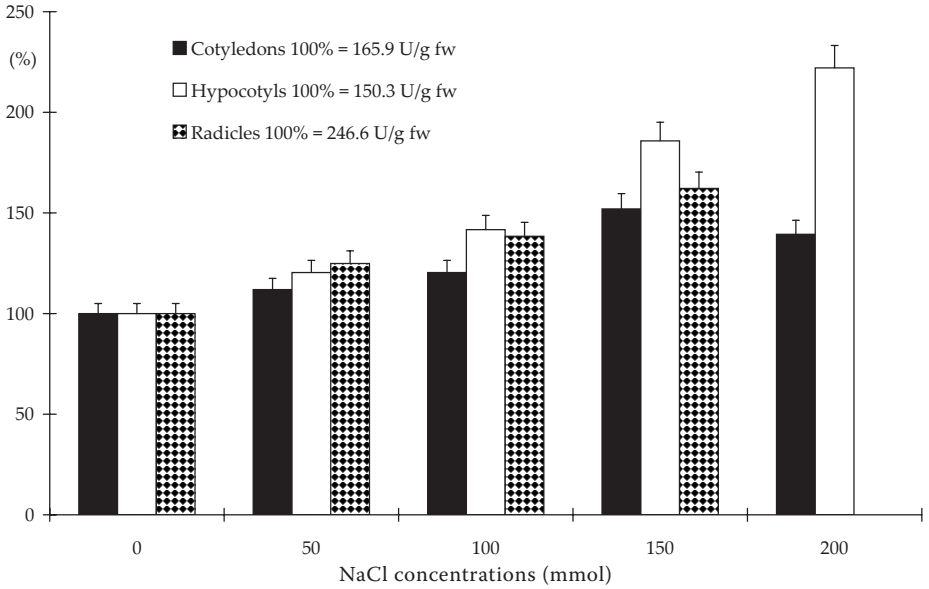

Figure 4. Effects of NaCl stress on peroxidase (POD) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings. The values and standard errors (vertical bars) of three replicates are shown

nification, auxin metabolism, salt tolerance and heavy metal stress (Passardi et al. 2005). Therefore, POD has often served as a parameter of metabolism activity during growth alterations and environmental stress conditions. Effects of NaCl on POD activity in the cotyledons, hypocotyls and radicles were shown in Figure 4. In general, POD activity in the cotyledons, hypocotyls and radicles increased significantly with rising NaCl concentrations up to 150, 200 and 150 mmol, respectively. Thus, the activities increased most by 52%, 122.2% and 62.2% compared to the control. Increased POD activity in the hypocotyls was more pronounced as compared to those in the cotyledons and radicles, especially under higher salt concentrations (Figure 4). Considering those in relation to growth parameters and the changes in SOD activity (Figures 1 and 2), these results suggest a possibility that increased POD and SOD activities might enable plants to protect themselves against salt stress. POD isoenzymes are known to occur in a variety of plant tissues. The expression pattern of these isoenzymes varies in different tissues of healthy plants and is developmentally regulated or influenced by environmental factors (Passardi et al. 2005). To determine whether there were developmental or salt stress-mediated differences among individual POD isoenzymes, POD activity assays were also performed by activity staining (Figure 5). On the activity gels, at least

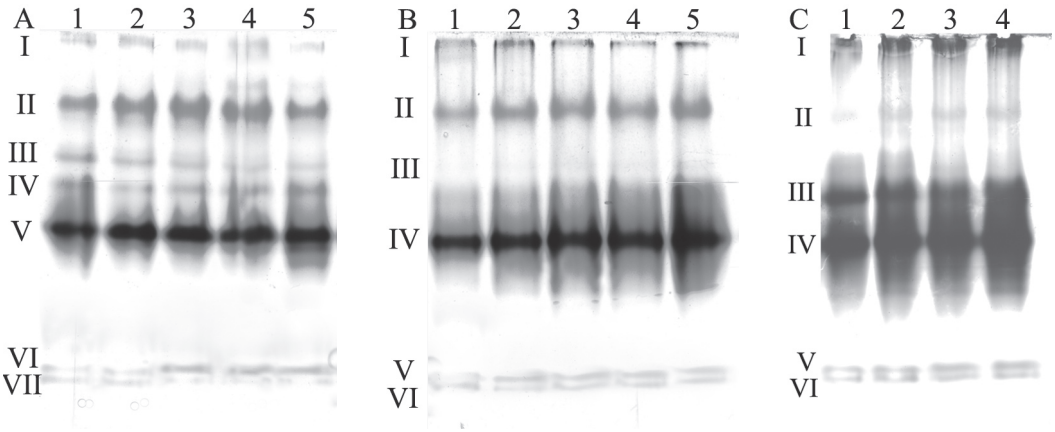

Figure 5. Patterns of peroxidase (POD) isoenzymes in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings: (A) patterns of POD isoenzymes in the cotyledons; (B) patterns of POD isoenzymes in the hypocotyls; (C) patterns of POD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 50, 100, 150 and 200 mmol, respectively. About 10 µl extract from each sample was loaded

seven POD isoenzyme bands in the cotyledons were visualized (Figure 5A), whereas six POD isoenzyme bands in the hypocotyls and radicles were observed (Figure 5B and C). These isoenzymes showed different staining intensities with individual NaCl concentrations and organs of seedlings. The regulatory mechanism of POD isoenzymes against salt stress might be complicated, and the relationship of the genes to these enzymes requires further analysis. In addition, the changes in the staining intensities of these isoenzymes showed a similar tread compared to the quantitative changes of POD activity assaying in solutions (Figures 4 and 5). These results suggest that the increased POD activity could contribute to the antioxidant mechanism of *Jatropha curcas* seedlings against higher NaCl concentrations stress.

CAT, which is involved in the degradation of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Willekens et al. 1995, Mittler 2002). Effects of NaCl on CAT activity in the cotyledons, hypocotyls and radicles were shown in Figure 6. CAT activity in the cotyledons increased gradually with increasing NaCl concentrations up to 100 mmol, and the highest activity increased by 75.4% compared to the control. CAT activity in the hypocotyls enhanced significantly and reached the maximal level at NaCl concentration of 200 mmol, increasing by 246.9%. Similarly, the peak CAT activity in the radicles was observed at NaCl concentration of 150 mmol, increasing by 588.1%. Similar to our findings, increased CAT activity in *Cassia angustifolia* L. (Agarwal and Pandey 2004), maize (Azevedo Neto et al. 2006) and *Sesamum indicum* (Koca et al. 2007) differing in salt tolerance were found. The changes in CAT activity may depend on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Chaparzadeh et al. 2004). This study lends further support to those findings. Increased CAT activity in *Jatropha curcas* seedlings was observed, especially in the radicles, which suggested the existence of an effective ROS-scavenging mechanism because radicles are the first organs which come in contact with salt and are thus thought to play a critical role in plant salt tolerance. Therefore, our findings showed that increased CAT activity coordinated with the changes of SOD and POD activities plays an important protective role in the ROS-scavenging process and the active involvement of these enzymes are related, at least in part, to salt-induced oxidative stress tolerance in *Jatropha curcas* seedlings.

PAL, a key enzyme in the phenylpropanoid pathway, is involved in the defence response of plant cells. Thus, PAL has been generally recognized as a marker of environmental stress in different plant species (MacDonald and D'Cunha 2007). Effects of NaCl on PAL activity in the cotyledons, hypocotyls and radicles were shown in Figure 7. PAL activity in the cotyledons increased gradually up to NaCl concentration of 150 mmol, and the highest activity increased by 117.2% compared to the control. A significantly increasing PAL activity in the hypocotyls

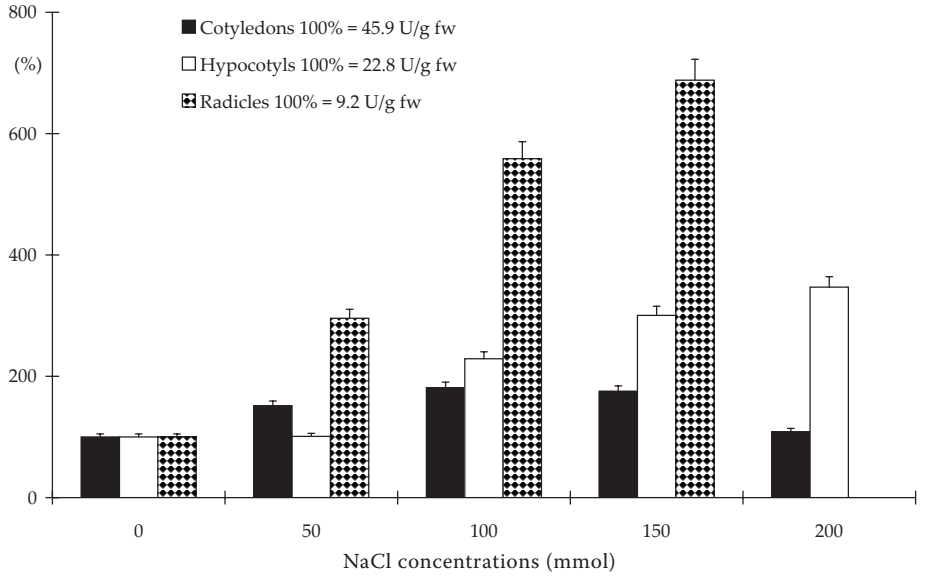

Figure 6. Effects of NaCl stress on catalase (CAT) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings. The values and standard errors (vertical bars) of three replicates are shown

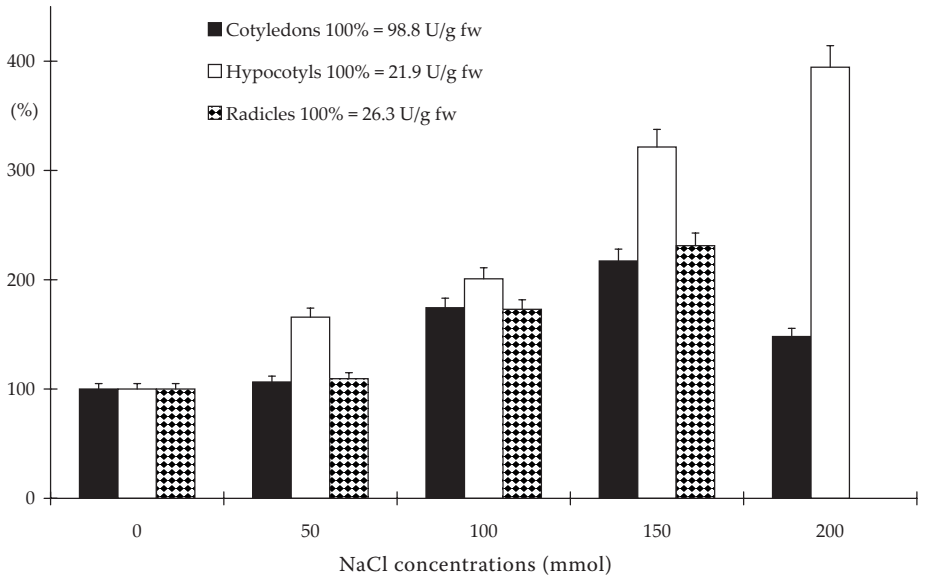

Figure 7. Effects of NaCl stress on phenylalanine ammonia-lyase (PAL) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* L. seedlings. The values and standard errors (vertical bars) of three replicates are shown

was observed at all tested NaCl concentrations, and the highest activity increased by 294.5% at NaCl concentration of 200 mmol. Similarly, the highest PAL activity in the radicles was observed at NaCl concentration of 150 mmol. In a few investigations, PAL activity was induced by various biotic and abiotic stresses, such as wounding, chilling, heavy metal and infection by viruses, bacteria or fungi (MacDonald and D'Cunha 2007). Now the responses of PAL activity of *Jatropha curcas* plants grown under higher NaCl concentrations are also known. Increased PAL activity could be a response to the cellular damage provoked by higher NaCl concentrations. So it seems that in *Jatropha curcas* L. seedlings the enhancement of PAL activity could be related to the implication of this enzyme in the plant response to salt stress. Moreover, our results suggest that PAL activity and the changes in PAL activity strongly depend on the organ of seedlings and NaCl concentrations.

Our findings show significant differences between responses of antioxidant defence enzymes and different plant organs when exposed to increasing NaCl concentrations. The presented results also clearly indicate that *Jatropha curcas* L. seedlings tolerate NaCl up to a concentration of 150 mmol, and higher concentrations cause eco-toxicity to the plant. Increased SOD, POD, CAT and PAL in *Jatropha curcas* seedlings suggest the tolerance capacity of the plant to protect themselves from oxidative damage. Such a test may be useful for elucidating the salt tolerance mechanisms of *Jatropha curcas* plant, and may provide useful bioassays that help to devise strategies for reducing the risks associated with salinity toxicity, especially when they are combined with field experiments. Further experiments are necessary to understand the induction and regulation of these enzymes as well as the regulation mechanism of metabolism in *Jatropha curcas* plants against salt stress; this experiment is going on in our laboratory.

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