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Effects of aluminum toxicity on the growth and antioxidant status in *Jatropha curcas* seedlings

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In the present study, the effects of aluminium (Al) concentrations on growth, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and phenylalanine ammonia-lyase (phenylalanine ammonia-lyase (PAL), EC 4.3.1.5) activities in *Jatropha curcas* L. seedlings were investigated. To carry out such investigations, *J. curcas* embryos were germinated and grown *in vitro* under Al concentrations of 0, 0.5, 1, 2 and 3 mM over a 7-day period. Biomass and the activities of antioxidant defense enzymes, such as SOD, POD, CAT and PAL in Jatropha curcas seedlings were observed. Results indicated that with the increasing Al concentrations, the biomass of cotyledons increased initially and then decreased but the biomass of hypocotyls and radicles decreased gradually. The SOD, POD, CAT and PAL activities in the cotyledons, hypocotyls and radicles were mainly increased, but the change trends were different.

Key words: Aluminium (Al) toxicity, *Jatropha curcas*, antioxidative, plant defense system.

INTRODUCTION

Aluminum (Al) is a light metal that makes up 7% of the earth's crust and is the third element. Al is a major component of the soil and most exists in a fixed status and has no hazard to plants. However, when soils become acidic as a result of natural processes or human activities, the fixed Al turns to soluble forms and the soluble Al could do harm to plants. About 40% of the world's arable soils are acidic and therefore present Al toxicity hazards (Uexküll and Mutert, 1995). Al toxicity has been considered to be a main limiting factor of crop productivity on acid soil (Foy et al., 1978; Uexküll and Mutert, 1995). The most distinct and earliest symptoms of Al toxicity in plants is the inhibition of root growth, which occurs within hours or even minutes of exposure to Al (Blamey et al., 2004; Dipierro et al., 2005; Kochian et al., 2005; Llugany et al., 1995; Ma, 2007; Ryan et al., 1993). The root meristem has been considered to be the primary site of Al accumulation and toxicity, suggesting that Al interacts with actively dividing and elongating cells (Delhaize and Ryan, 1995), but the mechanism of inhibition of root elongation is not yet well understood (Kopittke et al., 2008; Ryan et al., 1993). In fact, Al can interact with the root cell walls, apoplastic and/or symplastic constituents, disrupt the normal function of plasma membrane and plasma membrane transport system (Ahn et al., 2001; Blamey et al., 2004; Horst et al., 2010; Ishikawa and Wagatsuma, 1998; Jones and Kochian, 1997; Kopittke et al., 2008).

Some reports have shown that the common feature of several metal toxicity symptoms is the enhanced production of reactive oxygen species (ROS) and this results in oxidative stress (Cheng, 2003; Mithöfer et al., 2004; Valko et al., 2005). In order to alleviate oxidative damage, plants have developed comprehensive and integrated antioxidant enzyme and non-enzyme systems. The antioxidant enzyme systems include a series of

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enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), which, together with other enzymes, promote the scavenging of ROS (Alscher et al., 2002; Mittler et al., 2004; Veljovic-Jovanovic et al., 2006). If ROS is in excessive accumulation, it makes the antioxidant enzyme systems and non-enzyme systems of plant disorderly and leads to the oxidation of biomolecules (Boscolo et al., 2003) or even cell death (Cargnelutti et al., 2006).

Al stress, like other metal stress in plants, could lead to oxidative stress (Giannakoula et al., 2010; Schuch et al., 2010; Xu et al., 2011). In most cases, Al was considered to be toxic and had negative effects on plant development (Horst et al., 2010; Sun et al., 2007), but under some conditions, low concentrations can increase growth or produce other desirable effects. Plants that have shown positive growth to Al in nutrient cultures include sugar beet (Keser et al., 1975), tea shrub (Matsumoto et al., 1976), *Pinus radiate* D. Don (Huang and Bachelard, 1993), *Melastoma malabathricum* (Watanabe et al., 2005).

Jatropha curcas L. is a drought resistant shrub or tree belonging to the family Euphorbiaceae. Because of the high content of oil in the seeds, *Jatropha* has been investigated mainly as a potential source of oil and has been recognized as an adequate substitute for fossil oil (Debnath and Bisen, 2008). Before this study, there is little data about Al stress in *J. curcas* L. (de Macedo et al., 2011). Keeping in view the effects of aluminum toxicity on the growth and antioxidant status in *J. curcas* seedlings, the present study aimed to investigate the relationship between the concentrations of aluminum (Al) in *J. curcas* seedlings and the growth, as well as antioxidant enzymes.

MATERIALS AND METHODS

Plant materials and chemicals

Mature *J. curcas* seeds were collected in August, 2010 from more than 10 individual wild trees in Panzhihua, Sichuan province, China. Seeds were oven dried, selected and stored in a plastic box (Labeled, No. 20100822) and were deposited at 4°C until processing. Other reagents used were of reagent grade or higher.

Embryo germination and seedlings growth

J. curcas seeds were surface sterilized in 70% ethanol for 30 s, and then in 0.1% mercuric chloride for 8 min. Seeds were rinsed with distilled sterile water several times and soaked in sterile water for 24 to 36 h in a culture room. Each embryo was dissected from the seeds on a clean bench. Murashige and Skoog (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. Culture mediums (25 ml) were dispensed into Wide-neck bottles (100 ml), containing 0, 0.5, 1, 2 and 3 mM Al concentrations. Al was supplemented as $AlCl_3$. The embryos were placed for germination and growth in *in vitro* culture for 7 days. The cultures were incubated at 30 ± 2°C under a 12-h photoperiod in cool, white fluorescent light. When the cotyledons of seedlings had developed, cotyledons were washed with double distilled water, blotted and immediately frozen in liquid nitrogen or stored at -80°C for analysis. Three sets of seedlings were analyzed for each Al concentration, with 15 embryos per set.

Protein extraction

Protein extraction of fresh tissues was performed as previously described (Gao et al., 2010). The supernatant was used immediately or frozen and stored at -80°C for assaying of enzyme activity at a later date. Protein was quantified by the Lowry method using bovine serum albumin as standard.

Assay of antioxidant enzymes

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

Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis for isoenzymes was carried out with 10% acrylamide gel. SOD isoenzyme activity was determined by the Beauchamp and Fridovich method (Beauchamp and Fridovich, 1971). Gels were equilibrated with 50 mM phosphate buffer (pH 7.5) containing 28 µM riboflavin, 28 mM *N*,*N*,*N*,*N*-tetramethyl ethylenediamine (TEMED) for 30 min, then washed in distilled water for 1 min and resubmerged in the same buffer containing 2.45 mM NBT for 10 to 20 min with gentle agitation in the presence of light. Enzyme bands appeared as colorless bands on a purple background. For the POD isoenzymes activity assay, the gel was soaked in deionized water for 5 min, and then incubated in 0.03% H_2O_2, 0.2% (w/v) benzidine and 0.1% (v/v) acetic acid for 3 to 5 min. When maximum contrast was achieved, the reaction was stopped by rinsing the gel with deionized water (Gao et al., 2009).

Assay of phenylalanine ammonia-lyase (PAL) activity

Enzyme extraction for the PAL activity assay was carried out as previously described (Gao et al., 2010). PAL activity was determined by assaying the reaction L-Phe decomposition product *trans*-cinnamate, as measured by the increase of absorbance at 290 nm (Hahlbrock and Ragg, 1975). One unit of enzyme activity was defined as the amount of enzyme needed to decrease in absorbance of 0.01 per min. PAL activity were expressed as unit per gram fresh weight (U/g fw).

Statistical analysis

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

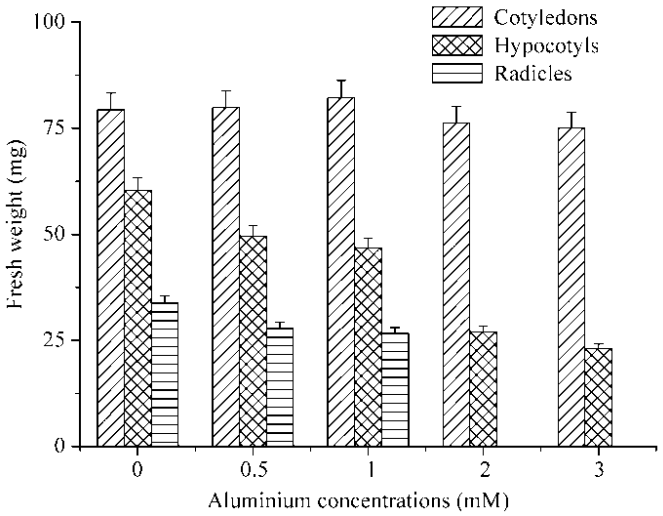

Figure 1. Effects of Al on the fresh weights in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (n = 3).

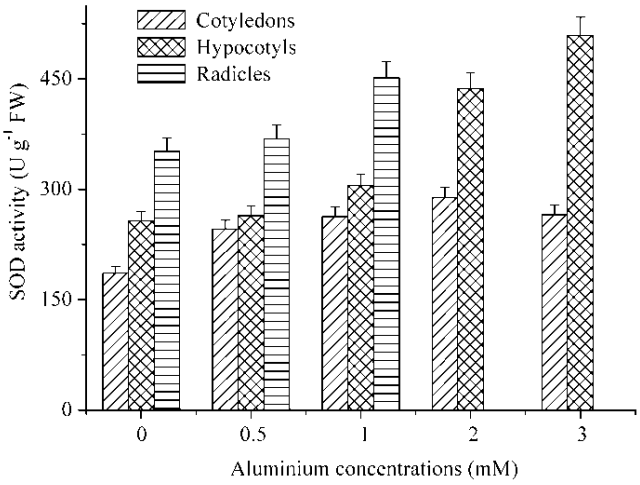

Figure 2. Effects of Al on superoxide dismutase (SOD) activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (n = 3).

RESULTS AND DISCUSSION

Effects of Al on plant growth

Figure 1 showed the changes of the fresh weights of cotyledons, hypocotyls and radicles in *J. curcas* seedlings. With the increasing of $AlCl_3$ concentration up to 1 mM, the fresh weight of cotyledons had a little increase. When $AlCl_3$ concentration was up to 2 and 3mM, the fresh weight of cotyledons, compared to the control, was slightly decreased and the changes were not significant. The fresh weight of hypocotyls decreased gradually with increasing Al concentration up to 3 mM and the fresh weight of radicles showed a similar trend, but when Al concentration was higher than 1 mM, the development of radicles was completely suppressed and could be observed, significant morphological aberrations included impaired radicles development, coarser hypocotyls and cotyledons chlorosis (data not shown). Al, at low concentrations, increased the fresh weight of cotyledons. This could not exclude the reason that low Al concentrations might have positive effect to cotyledons, as reported by other researchers (Ma, 2007; Watanabe et al., 2005). With the inhibition of Al toxicity on radicles development, it inhibited the absorption of nutrients and affected the photosynthesis, thereby suppressed the growth of hypocotyls and cotyledons. At the same time, high Al concentrations possibly enhance the ROS production, which led to the oxidative damage to plant cells and blocked the growth. On the base of these results, our findings suggested that high Al concentrations (> 1 mM) can inhibit the normal growth and development of *J. curcas* seedling.

Effects of Al on SOD activities

Al stress, like other abiotic and biotic stress, can induce oxidative stress reactions (Giannakoula et al., 2010). Effects of Al on SOD activity in *J. curcas* seedlings were shown in Figures 2 and 3. Compared to the control, SOD activity in the cotyledons, hypocotyls and radicles was all enhanced by Al stress. SOD activity in the hypocotyls increased significantly with increasing Al concentrations, and the maximal levels increased by 98.1% when Al concentration was 3 mM. In the cotyledons and radicles, the SOD activity increased by 55.1 % and 28.2 % at Al concentrations of 2 and 1 mM compared to the control, respectively. Many research have also indicated that Al could increase SOD activity in plants (Schuch et al., 2010; Du et al., 2010; Li et al., 2011) and this may be due to Al inducing the cell to initiate SOD synthesis to remove the superoxide radicals (Giannakoula et al., 2010). However, when the amount of free radicals exceeds cell's capacity, enzymatic activities start decreasing and if unchecked could ultimately lead to DNA damage (Meriga et al., 2004). The significant increase of SOD activity in our study may be induced by the increased production of ROS and can be a defensive mechanism developed by *J. curcas* seedling against stress. The pattern of SOD isoforms was analyzed by native PAGE, and activity staining revealed that at least four SOD isoenzyme bands in the cotyledons, hypocotyls and radicles were detected, respectively (Figure 3A to C).

The staining intensities of isoenzyme I and II in the cotyledons, hypocotyls and radicles were induced with increasing Al concentrations, but isoenzyme III and IV had virtually no change. The different expression of SOD genes may due to the subcellular location of the enzyme, the upstream sequences in the genomic sequences and

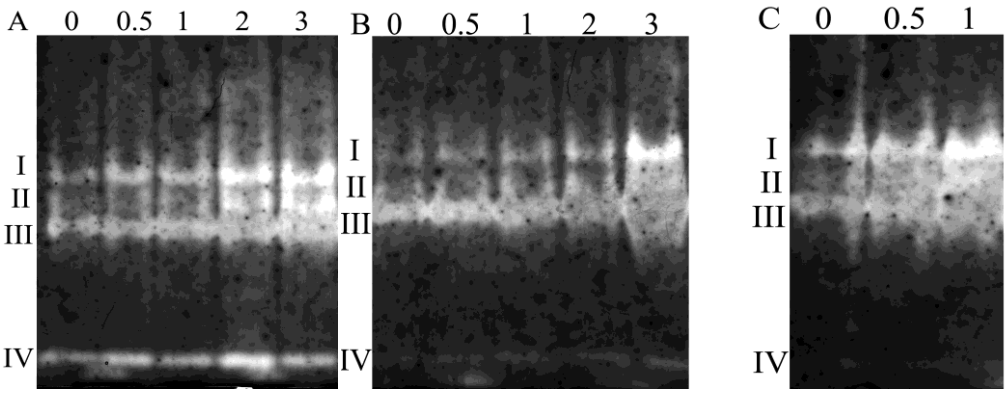

Figure 3. Patterns of SOD isoenzymes of cotyledons, hypocotyls and radicles in *J. curcas* seedlings. A: patterns of SOD isoenzymes in the cotyledons; B: patterns of SOD isoenzymes in the hypocotyls. C: patterns of SOD isoenzymes in the radicles. Lanes from left to right were 0, 0.5, 1, 2 and 3 mM, respectively.

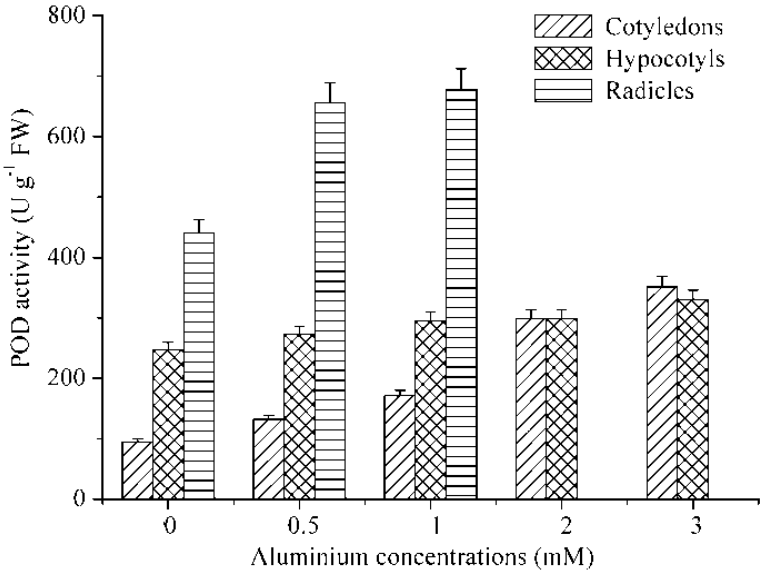

Figure 4. Effects of Al on peroxidase (POD) activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (n = 3).

the environmental stress (Mittler et al., 2004). The staining intensities of these isoenzymes showed a similar change compared to the changes of SOD activity assayed in solutions (Figures 2 and 3). With the increase of Al concentrations, the SOD activities were induced and this may promote the tolerance of *J. curcas* seedlings against Al stress.

Effects of Al on POD activities

Effects of Al on POD activity in the cotyledons, hypocotyls and radicles were shown in Figure 4. Al stress significantly affected the POD activity in the cotyledons with an increase of 270% when Al concentration was up to 3 mM. At the same time, POD activity in the hypocotyls and radicles was also induced, with the maximum increases of 33.4 and 37% compared to the control when Al concentration was 3 and 1 mM, respectively. As an important enzyme in plant defense system, POD can play an important role when plant is in adverse condition, and multiple POD isoforms have been found in many plant species (Passardi et al., 2005). The expression pattern of *J. curcas* seedling was shown in Figure 5. On the activity gels, at least six bands in the cotyledons, hypocotyls and radicles were observed. POD isoenzyme (II and III) in the

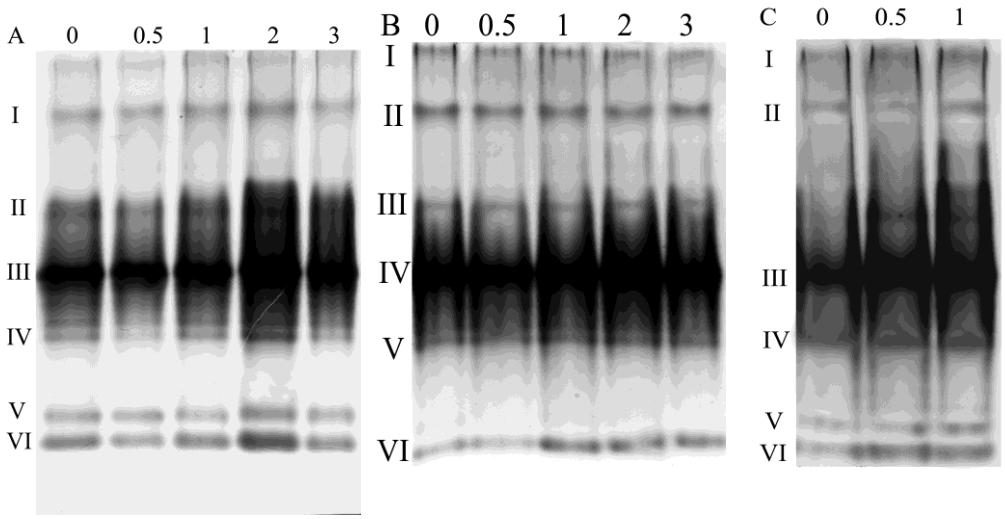

Figure 5. Patterns of POD isoenzymes of cotyledons, hypocotyls and radicles in *J. curcas* seedlings under Al stress condition. A: patterns of POD isoenzymes in the cotyledons; B: patterns of POD isoenzymes in the hypocotyls. C: patterns of POD isoenzymes in the radicles. Lanes from left to right were 0, 0.5, 1, 2 and 3 mM, respectively.

cotyledons showed an increase in the staining intensities with the increasing of Al concentration. In the hypocotyls and radicles, the main increase in the staining intensities was isoenzyme IV and III, respectively.

According to Figure 4 and 5, the changes of total staining intensities on activity gels and POD activity assayed in solutions were similar. In Al stress response, POD isozymes might consist in scavenging the toxic lipid hydroperoxides generated by the peroxidation of membrane lipids and they could participate in lignin biosynthesis to build up the physical barrier against toxic metals entering the cell (Ezaki et al., 1996; Hegedüs et al., 2001). Our findings suggested that POD, together with SOD and CAT, can increase their activities when *J. curcas* seedlings were exposed to Al stress, and they could scavenge ROS and reduce the damage caused by Al stress.

Effects of Al on CAT activities

CATs and PODs are the two major systems for the enzymic removal of hydrogen peroxide (H_2O_2) in plants, and CATs have mainly been associated with the removal of H_2O_2 in peroxisomes (Willekens et al., 1995). The changes of CAT activities in *J. curcas* seedlings exposed to Al stress were shown in Figure 6. Compared to control, CAT activities in hypocotyls and radicles were all increased, with the maximum increases of 46.4 and 10.7% when Al concentration was 2 and 1 mM, respectively. In cotyledons, CAT activities were increased first and then decreased with the increasing Al concentration. When the Al concentration was 0.5 mM, the CAT activity in cotyledons was the highest with increase by 58% but when concentration increased to 3 mM, the activity was lower than control. The CAT activity in the hypocotyls and radicles was very low; this might indicate that H_2O_2 degradation occurred due to POD rather than CAT. Similar results have already been observed in maize exposed to Al stress (Boscolo et al., 2003).

According to Figures 4 and 6, at low Al concentration, the removal of H_2O_2 in the cotyledons was mainly due to CAT rather than POD and at high Al concentration, this situation was just opposite. The decrease in CAT activity at highly Al stressed seedlings might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under such conditions (Sharma and Dubey, 2007). Our findings suggested, at least here, that CAT appeared not to be an effective ROS-scavenger exposed to Al toxicity.

Effects of Al on PAL activities

PAL, a key enzyme involved in the metabolism of phenolics and lignification of cell walls, was mainly involved in defense mechanisms (Kovacik and Backor, 2007). Effect of Al on PAL activity in the cotyledons, hypocotyls and radicles are shown in Figure 7. Compared to the control, the PAL activities were all increased but the change trends were different. In the cotyledons and radicles, PAL activities were increased first and then decreased with the increasing Al concentration, and when exposed to 0.5 mM Al, the activity was the highest which increased by 37.2 and 23.4%, respectively. In the

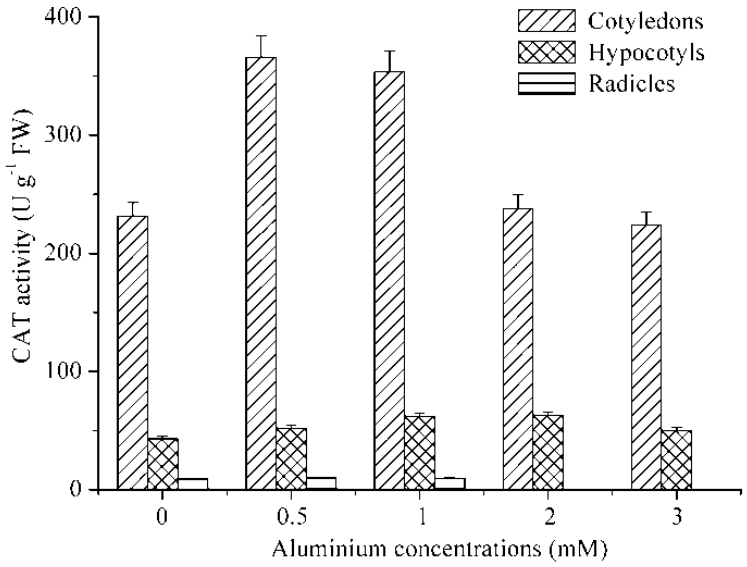

Figure 6. Effects of Al on catalase (CAT) activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (*n* = 3).

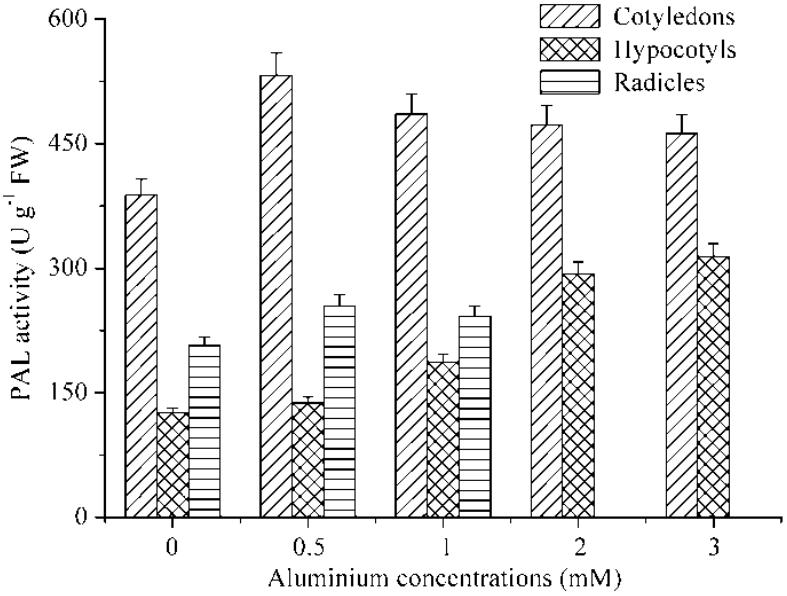

Figure 7. Effects of Al on phenylalanine ammonia-lyase (PAL) activity in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (*n* = 3).

hypocotyls, the maximum activity was observed at 3 mM Al and the increase was 149.9%. Similar changes were also observed in the previous studies (Kovacik and Backor, 2007; Dai et al., 2006). Some researchers indicated that PAL enhancement in the environmental stressed conditions is due to H_2O_2 generation which occurs as primary reaction in response to stress (Dorey et al., 1999). So, our results suggested that increased PAL activities may be related to *J. curcas* seedlings response to Al stress.

Conclusion

The changes of SOD, POD, CAT and PAL activity were studied when *J. curcas* seedlings were exposed to different Al concentration and the expression patterns of SOD and POD were also shown based on *in vitro* embryo germination and culture. The results in this study showed that the increases of SOD, POD, CAT and PAL activity might be an important part of *J. curcas* seedlings resistance mechanisms to Al stress and the synergistic effects might help to reduce the accumulation of ROS and the oxidative damage. This research might provide some evidences for further study into the response mechanisms of *J. curcas* to Al stress.

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