

Changes of polyphenol oxidase activity during *Jatropha curcas* seed germination

Key words: Catechol, polyphenol oxidase, *Jatropha curcas*, seed germination.

1 SUMMARY

Seed germination experiments were performed to investigate the changes of polyphenol oxidase (PPO) activities in different tissues of *Jatropha curcas* during the germination process. PPO activities with catechol as substrates in the degrading endosperms decreased up to the 4th day, and they increased up to the 8th day. The activities using catechol as substrate dropped at the first day and then gradually increased to 220 % at the 2th day and decreased gradually up to the 5th day. The activity in the developing cotyledons initially decreased slightly but increased during further development. PPO activities in the developing shoots/roots increased up to the 5th day, decreased until the 8th day and remained constant after this period. The activities in the developing stems detected using catechol as substrate enhanced in developing process. Electrophoresis analysis showed various isoenzymes were differentially expressed between germination process and tissue types. These results were also consistent with the changes of the enzyme activities assayed in extract solutions.

2 INTRODUCTION

Polyphenol oxidases (PPOs) are enzymes with a dinuclear copper centre, which catalyze the hydroxylation of monophenols to o-diphenols and the oxidation of o-dihydroxyphenols to o-quinones, utilizing molecular oxygen (Thipyapong *et al.*, 2007). They are widely distributed in higher plants, animals, and microorganisms and located in the chloroplast bound to thylakoid membranes (Ono *et al.*, 2006). PPO has been studied in many plant tissues, but its biological function remains hard to define. Earlier reports suggest that they may be involved in a number of cellular processes, such as defense against pests and pathogens, control of oxygen levels in the chloroplasts, wound-induced rooting, synthesis of phenolic compounds and wound healing (Constabel *et al.*, 1995; Tyagi *et al.*, 2001; Strack and Schliemann 2001; Thipyapong *et al.*, 2007).

Plant PPOs are encoded by a multigene family. The PPO multigene family was also reported from tomato, faba bean leaves, pokeweed suspension culture, tobacco flower tissues, red clover and apricot fruits. PPO appears to exist in multiple forms which differed by one or more characteristics, such as latency, catalytic behavior, molecular mass, isoelectric point, immunological specificity and hydrophobicity (Thygesen *et al.*, 1995, Thipyapong *et al.*, 2007). Recent molecular studies have revealed that PPO gene family is differentially expressed in different organs, and at distinct developmental and physiological conditions (Chang *et al.*, 2007, Massa *et al.*, 2007).

Jatropha curcas, is a multipurpose and drought-resistant, large shrub or small tree in many tropical and subtropical countries. It is also suitable for preventing soil erosion and



shifting of sand dunes. Various parts of the plant hold potential for use as a source of oil, animal feed and medicinal preparations (Openshaw 2000). In previous reports, changes of PPO activity in different organs and development stages have been studied (Demeke *et al.*, 2001, Kim *et al.*, 2001, Doğan *et al.*, 2005, Maki and Morohashi 2006). Seed germination and post-germination seedling growth are well-regulated processes that involve high

3 MATERIALS AND METHODS

3.1 Plant material and chemical: *J. curcas* (physic nut) 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 with labeled and deposited at 4 °C until processing. Sands was gently crumbled by hand, air-dried at room temperature and sieved through a 3 mm mesh to remove root residues and small rocks. Catechol was purchased from Sigma (St. Louis, MO, USA). Others reagents were of the grade or higher purity available.

3.2 Seed germination and culture conditions: *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 water and soaked for 24–36 h at room temperature, before being sown in trays filled with sand for germination and growth. Germination experiment was carried out at 30 °C temperature greenhouse. Trays were labeled with sampling dates and arranged to a complete randomized design on a table kept with an even light supply. These trays containing seeds were supplied with ample water to maintain approximately 100% relative humidity within each tray before germination. Distilled water was used to maintain the 70% relative humidity during germination and early seedlings growth. Seeds were considered as having germinated when seed coat dehiscence, occurred usually after 44–48 h of incubation. The moment two cotyledons of seedlings had developed (10 days) is referred to as “developed seedling”. Ten germinated seeds or

metabolic activity (Bailly, 2004). Prior to these studies little were known of the changes of PPO activities and patterns of PPO isoenzymes with respect either to germination process or to a possible role during *J. curcas* seed germination and early seedlings growth. The main objective of this investigation took a systematic approach to determine the changes of PPO activity and isoenzyme patterns during *J. curcas* seed germination and early seedlings growth.

developed seedlings were selected from the trays every two days, and endosperms, cotyledons, hypocotyls and radicles of different stages were separated. These tissues were recorded and stored immediately at -80 °C for further analysis. Each germination experiment was performed three repetitions with 500 seeds.

3.3 Protein extraction and estimation: Fresh endosperms, cotyledons, hypocotyls and radicles were homogenized with 50 mM sodium phosphate buffer (pH 7.0, m/v, 1/10) including 0.5 mM EDTA and 150 mM NaCl. The homogenate was centrifuged at 12 000 rpm for 10 min at 4 °C, and the supernatant was used for protein content and enzyme assays. Protein concentrations were measured by the Lowry method.

3.4 Assay of polyphenol oxidase (PPO) activity: Assays of enzyme activity was performed using a UV–Vis spectrophotometer (TU-1901, Purkinje General, Beijing, China). PPO activity was expressed as enzyme units per gram fresh weight (U g⁻¹ FW). PPO activity was measured using catechol as substrate by incubating 100 µl of enzyme extract to 2.9 ml of buffered substrate (50 mM sodium phosphate, pH 6.4 and 10 mM catechol), and then monitoring the change of absorbance at 398 nm. One unit of activity of PPO was defined as the amount of enzyme causing 0.01 absorbance increase per minute.

3.5 Activity gel electrophoresis analysis: Native PAGE was performed with 7% acrylamide gels under non-denaturing conditions. PPO isoenzyme was measured by

the Dicko methods (2006). The gel was incubated for 30 min in 50 mM sodium phosphate (pH 6.4), containing 0.1% p-phenylenediamine and 10 mM catechol. Brown bands that appeared were

photographed.

3.6 Statistical analysis: All values shown in present study were the mean of three assays. Data were tested at significant levels of *P* value were less than 0.05 using one way ANOVA.

4 RESULTS

Changes of PPO activity in the degrading endosperms were shown in Figure 1. PPO activity increased at germination (day 2), then the activity decreased up to day 10, representing 0.89 times that of the control. Patterns of PPO isoenzymes in the degrading endosperms are shown in Figure 2. These results suggested that at least three PPO isoenzymes are detected, and different patterns are found. The staining

intensities of isoform II reduced gradually up to day 8, and disappeared at day 10. However, isoform III appeared at day 8 and then the staining intensity enhanced. Based on the above results, our results suggested that the changes of PPO activity in the degrading endosperms observed (Figure 1) are well correlated with the staining intensities of isoforms (Figure 2).

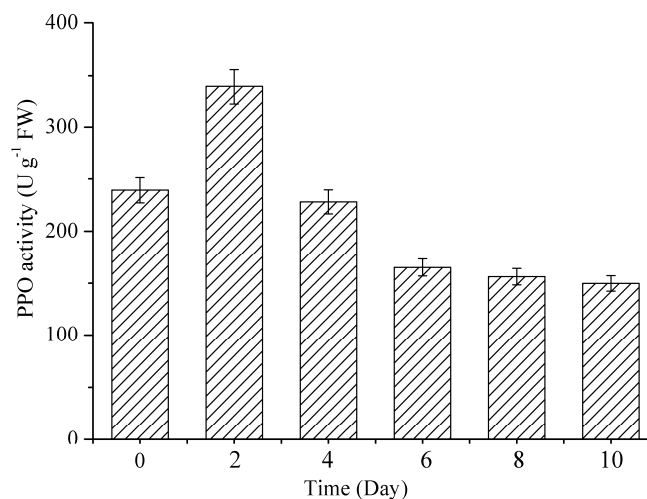


Figure 1: Changes of polyphenol oxidase activities in the degrading endosperms of *J. curcas* seeds germinated and grown in sands for 10 days. The values and standard errors (vertical bars) of three replicates are shown. Control (0): ungerminated endosperms.

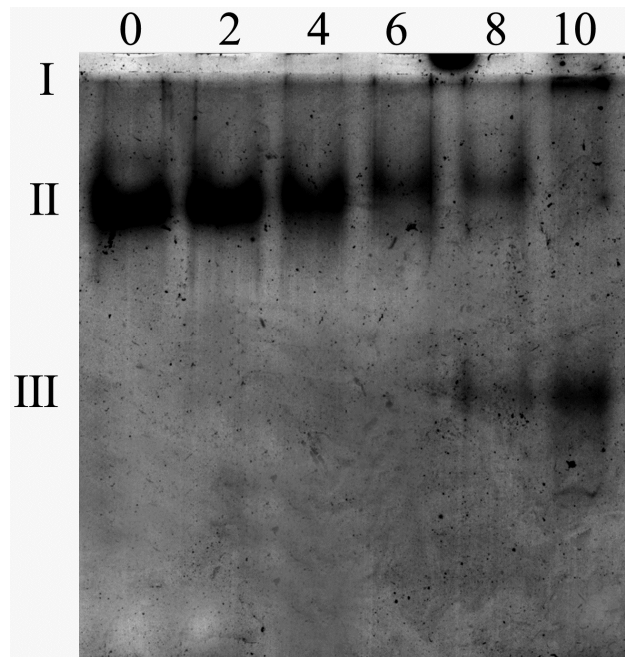


Figure 2: Patterns of PPO isoenzymes in the degrading endosperms of *J. curcas* seeds germinated and grown in sands for 10 days. Control (0): ungerminated endosperms. About 30 μ l extract from each sample was loaded.

Changes of PPO activity in the developing cotyledons were shown in Figure 3. As shown in Figure 3, PPO activity in the developing cotyledons increased gradually up to day 10. Electrophoresis analysis suggested that there are four PPO isoenzyme bands visualized when using L-Dopa as substrate, but three isoenzyme bands using catechol as substrate are detected

(Figure 4). In the developing cotyledons, different isoenzyme patterns were found when using different substrate. Results showed that the staining intensity of isoenzyme II in the developing cotyledons reduced gradually up to disappearing, and two new isoenzyme bands (III and IV) emerged.

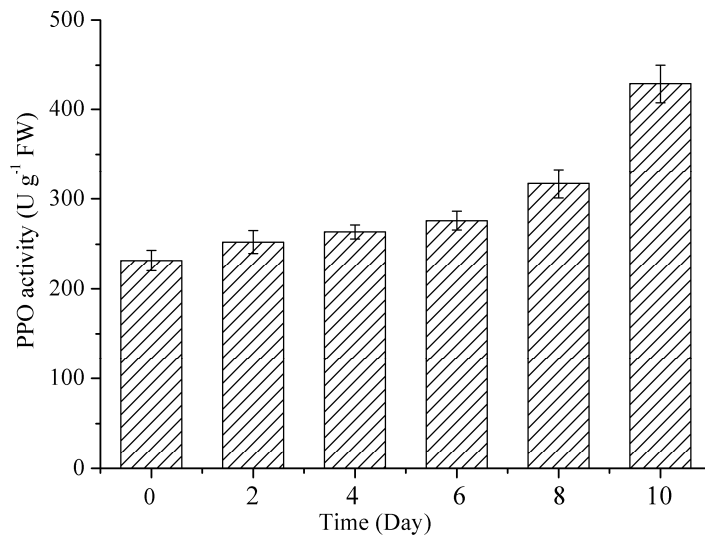


Figure 3: Changes of polyphenol oxidase activities in the developing cotyledons. *J. curcas* seeds were germinated and grown in sands for 10 days. The values and standard errors (vertical bars) of three replicates are shown. Control (0): ungerminated cotyledons.

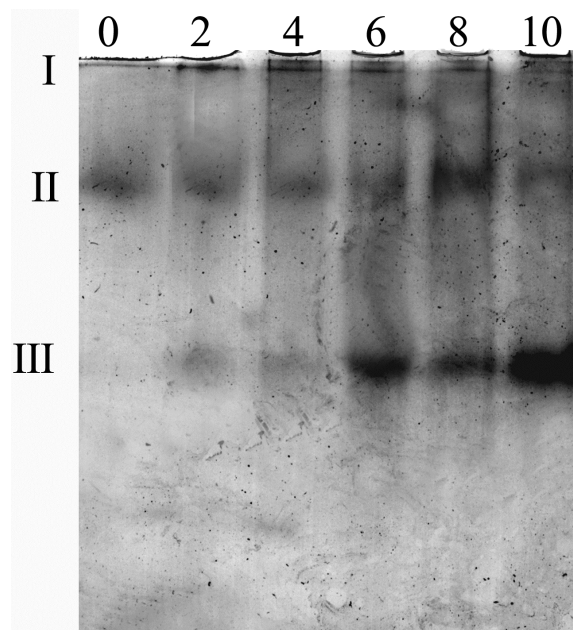


Figure 4: Patterns of PPO isoenzymes in the developing cotyledons of *J. curcas* seeds germinated and grown in the sands for 10 days. Control (0): ungerminated cotyledons. About 30- μ l extract from each sample was loaded.

Changes of PPO activity in the developing hypocotyls were shown in Figure 5. According to Figure 5, PPO activity in the developing hypocotyls with developing process increased gradually up to day 10, and the peak activities reached 100.5%. Electrophoresis analysis

suggested that there are at least three isoenzyme bands in the developing hypocotyls (Figure 6). The staining intensity of isoenzyme bands were consistent with the changes of the activity assayed in solutions.

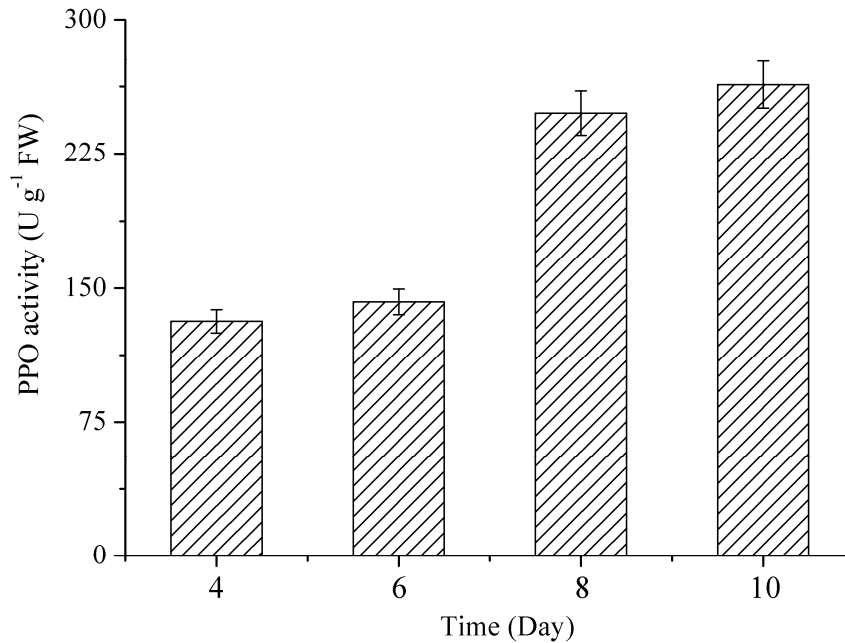


Figure 5: Changes of PPO activities in the developing hypocotyls of *J. curcas* seeds germinated and grown in sands for 10 days. The values and standard errors (vertical bars) of three replicates are shown.

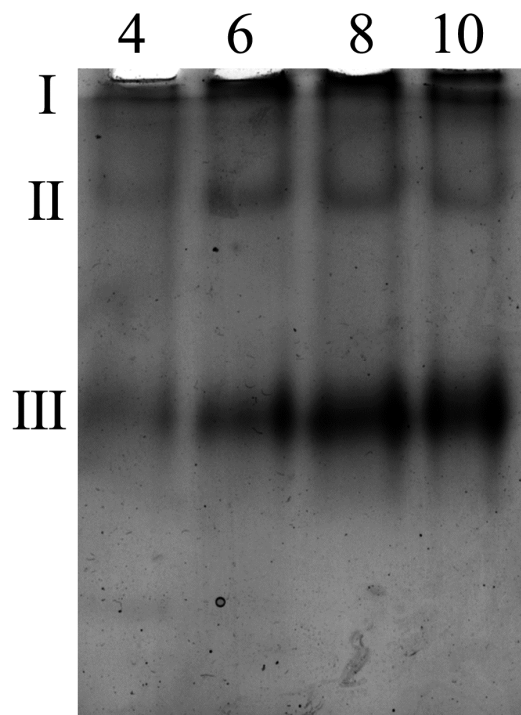


Figure 6: Patterns of PPO isoenzymes in the developing hypocotyls of *J. curcas* seeds germinated and grown in sands for 10 days. About 30- μ l extract from each sample was loaded.

Changes of PPO activity in the developing radicles were shown in Figure 7. PPO activity in the developing radicles is positively correlated with developing process, and the highest activity increased 346.2% compared to that of day 3. Electrophoresis analysis suggested that four isoenzyme bands are detected in the developing radicles (Figure 8).

The staining intensity of these bands showed different patterns of PPO activities depending on the developing process. Thus, there is a similar relationship between the quantitative changes in PPO activity and the staining intensity changes in POD isoenzyme pattern during the germination.

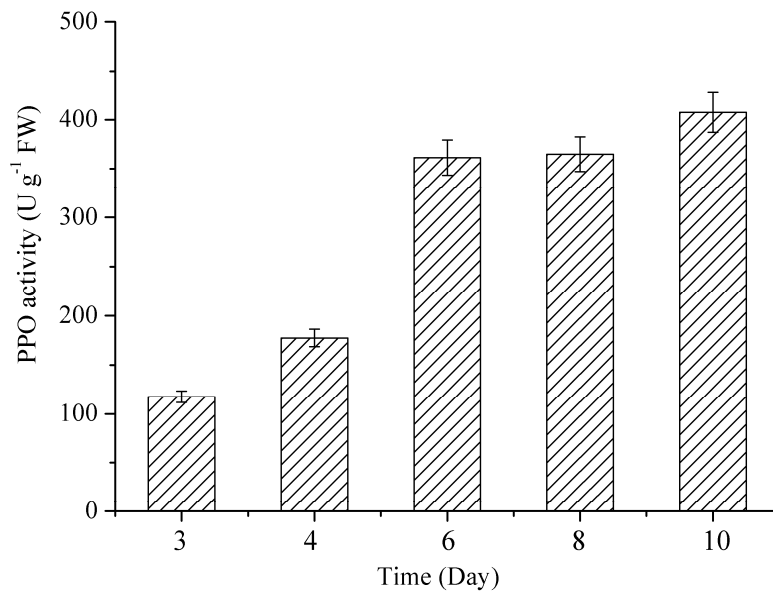


Figure 7: Changes of polyphenol oxidase (PPO) activity in the developing radicles. *J. curcas* seeds were germinated in sands for 10 days. The values and standard errors (vertical bars) of three replicates are shown.

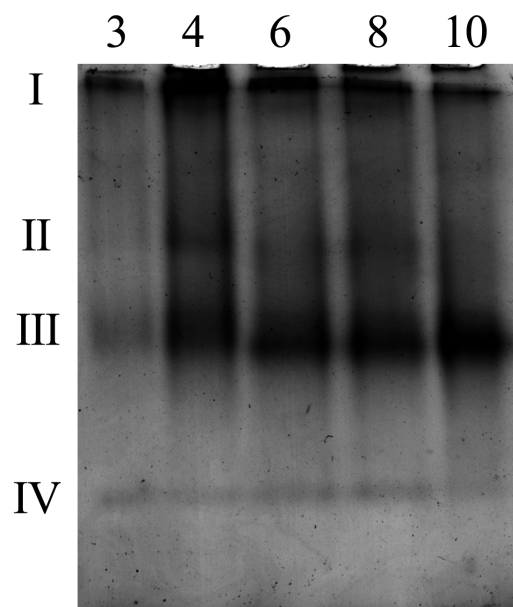


Figure 8: Native PAGE for polyphenol oxidase activities in the developing radicles. About 30 μ l extract from each sample was loaded.

5 DISCUSSION

In the present study, these results approach enabled us show a correlation between germination period and changes of PPO activities. Our findings here show a complex regulation of steady-state PPO activities at a temporal and spatial level, as well as provide evidence for the developmentally programmed turnover of PPO. PPOs are generally organized into multi-gene families. Multiple PPO isoforms reported for some plants were detected in the same tissue at the same developmental stage (Thipyapong *et al.*, 1997; Kim *et al.*, 2001, Escribano *et al.*, 2002). Electrophoresis analysis revealed the presence of different forms of PPO with distinct activities in the different tissues of *J. curcas*. For instance, there were three isoforms in endosperms, three isoforms in the cotyledons, three isoforms in hypocotyls and four isoforms in radicles of *J. curcas* (Figure 2, Figure 4, Figure 6 and Figure 8), respectively. These isoenzymes show different banding pattern and intensity in different development stages of same tissues. The isoforms of PPO activities detected at different developmental stages or between different tissues may suggest that they carry specific functions and contribute to its unique properties during the germination and early seedlings development. Actions of PPO often occur after wounding by insect attack and plant pathogen or treatment with methyl jasmonate. Thus, rapid changes in PPO activity have been proposed to may be involved in necrosis

development around damaged leaf surfaces and in defense mechanisms against insects and plant pathogen attack (Tyagi *et al.*, 2001, Thipyapong *et al.*, 2007). The quantitative analysis shows that PPO activities decrease initially and increase gradually during the germination (Figure 1 and Figure 3). The presence of PPO activities primarily at early developmental stages has been observed previously in some plant species (Thygesen *et al.*, 1995, Demeke *et al.*, 2001, Kim *et al.*, 2001, Doğan *et al.*, 2005, Maki and Morohashi, 2006). Based on the above data, changes of PPO activities indicate their possible participation in the defense mechanism of *J. curcas* development process. PPO activities during germination were also described from several other sources, such as maize, tomato, wheat. The present study showed that a novel PPO with peroxidase activities was expressed in cotyledons of germinating seed. This study results support the fact that the presence of PPO activity in the germinating *J. curcas* seed strongly depended on the variety of the tissue and germination phases. During the germination and early seedling development, the changes of PPO activities correlated closely with the changes of PPO isoenzymes. Studies involving purification and molecular characterization of PPO isoenzyme during the germination may provide further insight into the significance of individual isoenzymes to the defense response in the *J. curcas* plant.

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