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ORIGINAL ARTICLE

Dehydroascorbate Reductase and Glutathione Reductase Play an Important Role in Scavenging Hydrogen Peroxide during Natural and Artificial Dehydration of *Jatropha curcas* Seeds

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Abstract Changes in H_2O_2 and the main antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), in endospermic and embryonic tissues were studied in developing and artificially dried *Jatropha curcas* seeds. Immature seeds were desiccation-tolerant at 80 days after flowering, as they were able to germinate fully after artificial drying on silica gel had reduced their water content to 10-12% of fresh weight. In both endospermic and embryonic tissues, H_2O_2 level and, consequently, lipid peroxide content, decreased during seed development as well as after artificial dehydration of developing seeds. All examined antioxidant enzymes except DHAR showed a decrease in total activity in mature stages as compared with early stages. Expression analysis of SOD genes revealed that the decrease in total SOD activities was related to the decrease in Cu/Zn-SOD expression, while the continuous activity of SOD during maturation was related to an increase in Mn-SOD expression. Artificial drying resulted in increased SOD and DHAR activity, irrespective of the developmental stage. Our results revealed weak participation of CAT and APX in H_2O_2 scavenging, as well as no significant alterations in GR activities either during maturation or after artificial drying. Changes in SOD and GR isoenzyme patterns occurred during maturation-related drying, but not after artificial drying. These results highlight the role of ascorbate-glutathione cycle enzymes (DHAR and GR) in H_2O_2 scavenging during maturation or after artificial drying of developing *J. curcas* seeds.

Key words: dehydroascorbate reductase, glutathione reductase, hydrogen peroxide, *Jatropha curcas*, scavenging

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Introduction

Plant tissue dehydration is associated with an increased production of reactive oxygen species (ROS), which can react and trigger numerous deleterious oxidative processes (Leprince et al. 1993; Smirnoff 1993; Allakhverdiev and Murata 2004, 2008; Nishiyama et al. 2006, 2011; Murata et al. 2007, 2012; Allakhverdiev et al. 2008; Posch and Bennett 2009; Gruissem et al. 2012). In order to protect the cellular membrane and organelles from the damaging effects of ROS, plants possess an antioxidant system that consists of low-molecular-weight antioxidants, such as ascorbate, glutathione, α-tocopherol, and carotenoids, (Alscher and Hess 1993; Bukhov et al. 2004; Nishiyama et al. 2006, 2011; Haberer et al. 2007; Murata et al. 2007, 2012) as well as enzymes such as superoxide dismutase (SOD), catalase

(CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Bewley and Black 1994; Bowler et al. 1994; Creissen et al. 1994; Foyer et al. 1994; Kaniuga 2008).

Dehydration of recalcitrant (desiccation-intolerant) seeds has often been associated with impaired antioxidative mechanisms, leading to oxidative damage and numerous lethal lesions (Hendry et al. 1992; Finch-Savage et al. 1993; Vertucci and Farrant 1995; Li and Sun 1999; Pammenter and Berjak 1999). In contrast, the ROS production and scavenging associated with dehydration of orthodox (desiccation-tolerant) seeds has only been documented in sunflower (Bailly et al. 2004), Norway maple (Pukacka and Ratajczak 2007) and maize (Huang et al. 2008). Moreover, the responses of antioxidant enzymes to artificial dehydration in orthodox seeds at different developmental stages have been poorly documented - with the exception of CAT in sunflower (Bailly et al. 2004), and ascorbate-glutathione cycle enzymes in Norway maple (Pukacka and Ratajczak 2007). Furthermore, there have also been few studies at the level of RNA that deal with the participation of antioxidant enzymes in seed development or seed dehydration. For example, studies on CAT in developing sunflower seeds (Bailly et al. 2004) and Mn-SOD in developing embryos of the sacred lotus (Li et al. 2009).

H_2O_2 is moderately reactive and is therefore a relatively long-lived molecule (half-life of 1 ms) that can diffuse some distance from its production site (Bhattacharjee 2005). H_2O_2 plays a role in biosynthetic processes and acts as a secondary messenger, regulating plant growth and development as well as stress responses (Foyer et al. 1997; Allakhverdiev and Murata 2004, 2008; Nishiyama et al. 2006, 2011; Murata et al. 2007, 2012; Allakhverdiev et al. 2008). The cellular balance between SOD and the different H_2O_2-scavenging enzymes is crucial in determining the steady-state levels of O_2^- and H_2O_2 (Asada and Takahashi 1987; Bowler et al. 1991). The effective concentration of ROS in plant tissues is determined by a dynamic equilibrium between the rates of production and scavenging (Allakhverdiev and Murata 2004, 2008; Nishiyama et al. 2006, 2011; Murata et al. 2007, 2012; Allakhverdiev et al. 2008; Garnczarska and Wojtyla 2008; Dizengremel et al. 2009).

Seeds of Jatropha plant are an important source of oil for biodiesel production (Openshaw 2000; Henning 2003; Srikanta Dani et al. 2011). Many studies were provided on Jatropha seeds however, the factors affecting its germination mechanism are still unknown (Mattana 2012). The objective of the present study is to verify the existence of a causal relationship between ROS level and ROS scavenging enzymes in orthodox seeds. The study focuses on the direct determination of H_2O_2 production status, gene expression and the activity of antioxidant enzymes during the acquisition of desiccation tolerance and after the artificial drying of seeds in different developmental stages of the true orthodox *J. curcas* seeds.

Materials and Methods

Plant Materials

J. curcas fruits were collected from plants grown in the Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences, Yunnan, southwest China. The plants were propagated under field conditions. The plants checked every day throughout the flowering period and, if a floral bud appeared, the flower was tagged. Seeds were staged with regard to the time of flowering, fresh weight and morphological changes. Seeds were utilized at different developmental stages, starting from 55 days after flowering (DAF) (31.21% of the maximum dry weight (DW) with water content (WC) of 1 g $H_2O \cdot g^{-1}$ DW) until 100 DAF (100% of the maximum DW with WC of 0.12 g $H_2O \cdot g^{-1}$ DW). The initial time point (55 DAF) was chosen because at this stage in development the embryos are large enough to be isolated from the endosperm tissues for separate analysis.

Fresh Weight, Dry Weight and Water Content of *Jatropha* Seeds

Seeds at different developmental stages were weighed to determine fresh weight (FW). The DW and WC of whole seeds were determined by reweighing after oven drying at 103°C for 17 h. WC was expressed in terms of fresh weight (% FW).

Germination Test

Batches of 20 seeds were germinated in a 1% agar medium in 15-cm Petri dishes at 30±1°C for 30 days. Seeds were recorded as having germinated if the axes had elongated and broken through the seed coat.

Desiccation Treatments

Fresh seeds at different developmental stages were dried in desiccators containing silica gel at 25-28°C in an air-conditioned room. The seeds were buried in silica gel with alternating layers of seeds and gels. The silica gels were renewed when their color changed from blue to red. Seeds were sampled for WC determination after different incubation periods and were analyzed when WC was about 10-12% of

that recorded for FW.

Conductivity Test

Electrolyte leakages (EL) of individual seeds were measured using a conductivity meter (DDS-307, Leici, Shanghai, China). Ten replicates were used for each seed which was placed in a vial containing 20 mL of de-ionized water. After the vials were shaken slightly, the conductivity of the solution was measured immediately. The conductivity of the solution was measured again after 1 hour. Finally, each vial was placed in boiling water for 1 h, cooled to room temperature (about 20±2°C) and then shaken, after which total conductivity was measured. Leakage rate of electrolytes (expressed in $\mu S \cdot cm^{-1} FW \cdot h^{-1}$) was calculated as the net conductivity of the solution with seeds immersed for 1 h divided by the total conductivity after boiling.

Evaluation of Hydrogen Peroxide Content

Hydrogen peroxide was extracted with cold acetone according to the procedure described in Patterson et al. (1984). The resulting extract was reacted quantitatively with titanium tetrachloride and ammonia to produce a peroxide-Ti complex. The complex was collected through centrifugation and then dissolved in 2 M sulfuric acid. The absorbance of the solution was measured at 415 nm and H_2O_2 content was calculated according to a standard curve.

Evaluation of Lipid Peroxidation Product

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content from 0.5 g of plant tissue as originally described by Heath and Packer (1986), with slight modifications by Hendry et al. (1993).

Antioxidant Enzyme Assays

All procedures were carried out at 0-4°C. Embryos and endosperms were ground in a cold mortar using specific buffers for each enzyme extraction. The homogenates were centrifuged at 12,000 rpm for 15 min, and the obtained supernatants were stored at -20°C for later determinations of enzyme activity. The supernatants were used for both spectrophotometric and native-PAGE analyses. Conditions for all assays were selected such that the rate of reaction was constant for the entire experimental period and proportional to the amount of enzyme added. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard. The specific activity of the enzymes was expressed in units as function of protein content; 1 unit = 1 μmol substrate metabolized mg^{-1} protein min^{-1}.

SOD Assay

SOD (EC 1.15.1.1) was extracted with 50 mM phosphate buffer (pH 7.0), containing 1 mM EDTA, 0.05% Triton X-100, 2% (w/v PVP and 1 mM ascorbic acid (AsA). SOD activity was assayed in terms of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Beauchamp and Fridovidsh (1971) as described in Donahue et al. (1997).

CAT, APX and GR Activity Assays

CAT, APX and GR were extracted with 50 mM Tris-HCl (pH 7.5), containing 20% (v/v) glycerol, 1 mM AsA, 1 mM DTT, 1 mM EDTA, 1 mM GSH, 5 mM $MgCl_2$ and 1% (w/v) PVP.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm (0.04 $mM^{-1} \cdot cm^{-1}$) as described by Aebi (1983).

APX (EC 1.11.1.11) activity was assayed as the decrease in absorbance due to ascorbate oxidation observed at 290 nm (2.8 $mM^{-1} \cdot cm^{-1}$) and 25°C, according to the method of Nakano and Asada (1981).

GR (EC 1.6.4.2) activity was determined by monitoring NADPH oxidation by glutathione disulfide (GSSG) as a substrate for GR to regenerate glutathione (GSH) at 25°C through the decreased absorbance at 340 nm (6.2 mM^{-1} cm^{-1}) (Halliwell and Foyer 1978). The assay mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH, and 20 μL enzyme sources in 1 mL final volume.

DHAR Assay

DHAR (EC 1.8.5.1) was extracted with 50 mM Tris-HCl (pH 7.2), containing 0.3 mM mannitol, 1 mM EDTA, 0.1% (w/v) BSA and 0.05% cysteine. DHAR was assayed directly by following the increase in the absorbance at 265 nm due to the GSH-dependent production of ascorbate (Hossain and Asada 1984). The assay mixture consisted of 1 mM DHA, 1 mM GSH and 100 mM potassium phosphate buffer (pH 6.3).

Native PAGE Analysis of Antioxidant Enzymes

Native PAGE was performed on protein extracts from embryonic and endosperm tissues isolated from various developmental stages. Native PAGE was performed using the Mini Protean 3 Cell (Bio-Rad, Hercules, CA, USA) apparatus and 10×12-cm slab gels. A stacking gel containing 5.0% acrylamide and a separating gel containing 7.5% (for CAT), 10% (for APX and GR) or 12% (for SOD) acrylamide was used with a running buffer of 4 mM Tris-HCl (pH 8.3)

and 38 mM glycine. Electrophoretic separation was performed at 4°C and 200 V (Laemmli 1970). An equal amount of protein (enzyme extract), as determined by Bradford assay (Bradford 1976), was loaded on to each lane (100 μg protein for SOD and APX, 15 μg protein for CAT, 50 μg protein for GR).

SOD Activity

SOD isoforms on the gels were detected by nitroblue tetrazolium (NBT) reduction by superoxide radicals that were generated photochemically (Beauchamp and Fridovich 1971). After electrophoresis, the gels were covered with a solution that contained 0.25 $mg{\cdot}mL^{-1}$ NBT and 0.1 $mg{\cdot}mL^{1}$ riboflavin, and then exposed to light. The two types of SOD (Mn-SOD and Cu/Zn-SOD) were identified using inhibitors. Mn-SOD was diagnosed by its insensitivity to 5 mM H_2O_2 and 1 mM KCN, while Cu/Zn-SOD was identified by its sensitivity to 1 mM KCN (Navari-Izzo et al. 1998).

CAT Activity

CAT activity in native PAGE gels was determined using the methodology of Woodbury et al. (1971). Gels were incubated in 0.003% H_2O_2 for 20 min and developed in a 1% $FeCl_3$ and 1% K_3Fe (CN_6) solution for 10 min.

APX Activity

APX isoform activity was evaluated according to Lee and Lee (2000), with some modification. A running buffer with 2 mM AsA added was used for pre-running (30 min). After electrophoretic separation, the gels were equilibrated with 50 mM Na-phosphate buffer (pH 7.0) containing 2 mM AsA for 30 min, and then incubated in the same buffer + 4 mM AsA + 2 mM H_2O_2 for 20 min. H_2O_2 was added to the solution just before gel incubation. Gels were then incubated with 50 mM Na-phosphate buffer (pH 7.8), 28 mM TEMED and 2.45 mM NBT for 10-20 min with gentle agitation. The reaction was terminated by a brief wash in distilled water.

GR Activity

To detect GR activity, gels were soaked in a reaction medium containing 0.25 M Tris-HCl buffer (pH 8.4), 4 mM GSSG, 1.5 mM NADPH and 2 mM 5',5'-dithiobis (2-nitrobenzoic) acid in the dark for 1 h at room temperature (Foyer *et al.,* 1991). The reaction was stopped by adding 7.5% glacial acetic acid to the staining buffer.

Total RNA Extraction and DNAse Treatment

Total RNA from *J. curcas* seeds at different developmental stages was extracted using silica particles protocol (Ding et al. 2008). Contaminating DNA was removed during 30 min incubation at 37°C by adding 5 μL 10x DNase I Buffer (TaKaRa), 2 μL DNase I (RNase-free, 5 U/μL, TaKaRa) and 0.5 μL RNase inhibitor (RNasin plus Rnase Inhibitor 40 U/μL, Promega) to 30 g RNA in 50 μL sterile de-ionized distilled water (sdd H_2O). The DNase treatment was terminated by phenol/chloroform extraction.

cDNAs Synthesis and Semi Quantitative Analysis

For semi quantitative RT-PCR the concentration of RNA was quantified by spectrophotometeric measurements. cDNAs were synthesized from 2 μg DNase-treated total RNA with M-MLV reverse transcriptase (Promega) and Oligo d(T)18N according to manufacturer's recommendations. The gene specific primers were based on sequencing data from a database of expressed sequence tags (ESTs) for developing *J. curcas* seeds (unpublished data). The cDNA samples were standardized on actin transcript amount (FM894455) using gene specific primers in a polymerase chain reaction (PCR) (Table 1). PCR was used to amplify the number of copies of specific DNA sequences *in vitro.* The number of cycles was optimized for each gene to yield optimal contrast between

Table 1. Selected genes and their primer sequences used for semi-quantitative RT-PCR analysis to verify the expression patterns of the selected genes

Gene	GenBank Accession no.	Primer pairs	Primer sequence (5'–3')
Actin	FM894455	XT24/XT25	TGCAGACCGTATGAGCAAGGAGATC/ CCAGAGGGACCATTACAGTTGAGCC
Cu/Zn-SOD	FM893551	XT46/XT47	AGCAGTGAGGGTGTTAAAGGGAC/ TGGAATGTGGACCAGTAAGAGGA
Mn-SOD	FM893578	XT48/XT49	TCAAGTTCAATGGTGGAGGTCAT/ GGTCCTGGTTTGCAGTTGTCTCA
CAT	FM893289	XT42/XT43	ACCACAGTCACGCCACTCAGGAT/ AAGACGGTGCCTTTGGGTATCAG
APX	FM896492	XT44/XT45	TTGTCTTTGGCGAACAGATGGGTC/ GCAAGCAGGCCATTAGGCAACAT
DHAR	FM892733	XT40/XT41	TGATGTTATTGTTGGGATTTAGAGG/ ATCAAGGTGGTACAACTTTGGTG

samples in the performed EtBr-agarose gel electrophoresis. For a typical PCR reaction the cDNAs of different developmental satges were used as a template in a 50 μL sample containing 1x Taq-buffer, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.2 μM of each primer (reverse and forward) and 2 units Taq-polymerase.

Statistical Analysis

Data were statistically analyzed and are presented as the mean±standard error of the mean (±S.E.M.) of the 20 independent replicates for FW, DW and WC, and the four independent replicates for H_2O_2 and MDA contents, as well as all enzymes activity.

Results

Seed Development and Acquisition of Desiccation Tolerance

Seed development was observed until 100 DAF when the seeds were fully dry (Fig. 1A). The changes in FW, DW and WC expressed as a percentage of FW of the seeds during their development on the mother plant are shown in Fig. 1B. DW gradually increased along with the decrease in WC during reserve accumulation (Fig. 1B, from 55 to 90 DAF). No changes were observed in DW during the natural desiccation process (from 90 to 100 DAF). There was a drop in WC from 42% in mature seeds (90 DAF) to about 12% in dry seeds (100 DAF) as a result of the natural process of desiccation. General increases were observed in germination percentage for both of fresh and artificially dried seeds in relation to the degree of seed development. Germination and conductivity tests confirmed that both fresh and artificially dried seeds became fully tolerant to desiccation at 80 DAF (Fig. 2).

H_2O_2 and MDA Contents

There was a decrease in H_2O_2 and MDA throughout seed development in both endosperm and embryo tissues (Fig. 3). H_2O_2 content was higher in endosperm than in embryo tissues, regardless of the developmental stage. Artificially

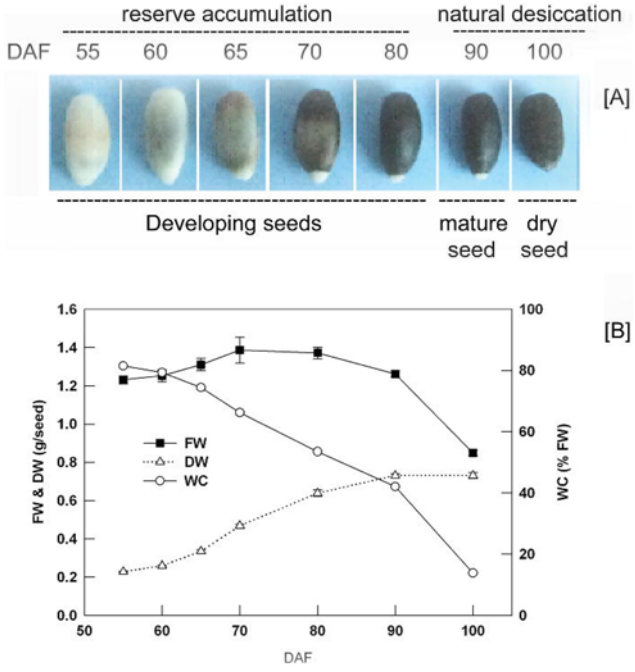

Fig. 1. *J. curcas* seeds at different developmental stages [A] and changes in seed fresh weight (FW), dry weight (DW) and water content (WC) during seed development [B].

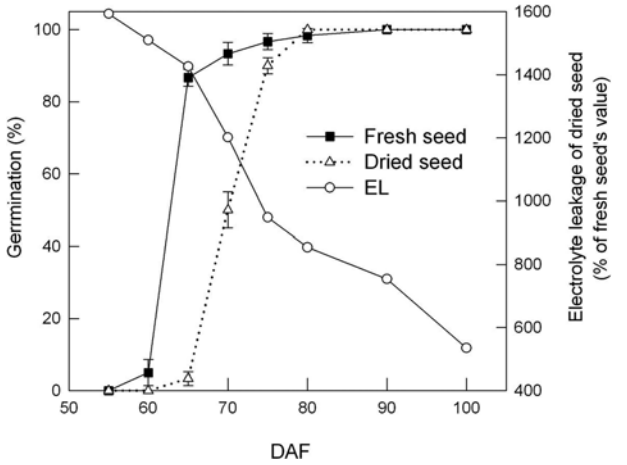

Fig. 2. Changes in germination percentage of fresh and artificially dried seeds at different developmental stages and electrolyte leakage (EL) of dried seeds, expressed as a percentage of initial value of fresh seeds.

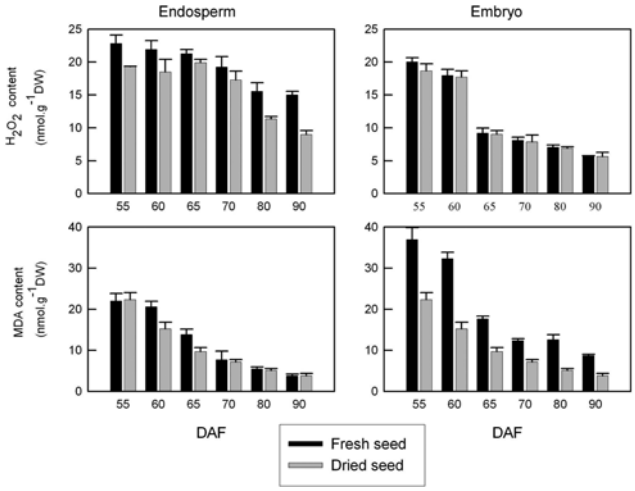

Fig. 3. Changes in H_2O_2 and MDA contents in endosperm and embryonic tissues of fresh (black bars) and artificially dried (gray bars) seeds at different developmental stages.

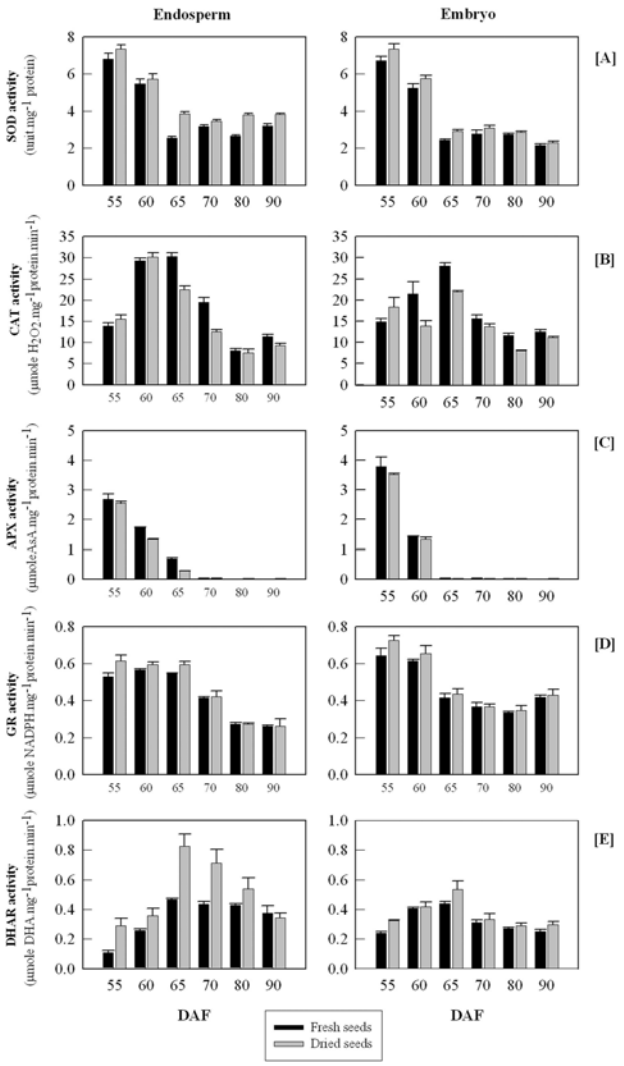

Fig. 4. Changes in SOD [A], CAT [B], APX [C], DHAR [D] and GR [E] activities in endosperm and embryonic tissues of fresh seeds (black bars) and artificially dried (gray bars) seeds at different developmental stages. The activities are expressed as a function of tissue protein content.

drying the seeds caused a noticeable decrease in H_2O_2 levels in endosperm tissues of dried seeds as compared to fresh ones for all developmental stages, whereas the decrease was barely observable in the dried embryo tissues. In contrast to H_2O_2, MDA content remained at a higher level in the embryo than in the endosperm across all developmental stages. Artificially drying seeds resulted in a significant decrease of the MDA content in embryo tissues for all developmental stages, whereas similar MDA amounts were found in endosperm tissues of both fresh and dried seeds at 70, 80 and 90 DAF. A decrease in MDA content was observed in the endosperm tissues at 60 and 70 DAF as a result of artificial drying.

Antioxidant Enzyme Activities

Changes in the activity of antioxidant enzymes including SOD, CAT, APX, DHAR and GR in endosperm and embryo of fresh and artificially dried seeds at various developmental stages are illustrated in Fig. 4.

SOD

SOD shows a similar activity pattern in both endosperm and embryo tissues during seed development (Fig. 4A). SOD activity decreased during the early stages of development (55, 60, and 65 DAF) and remained low during maturation (70, 80 and 90 DAF). Cu/Zn-SOD activity increased slightly at all stages in both endosperm and embryo tissues in dried seeds as compared to fresh seeds. Staining for SOD activity on non-denaturing PAGE revealed the changes in the pattern of isoenzyme activity in the endosperm and embryo during development (Fig. 5A). Three SOD isoenzymes, Mn-SOD isoenzyme, Cu/Zn-SOD isoenzyme and Fe-SOD isoenzyme, were identified by SOD activity staining in the endosperm (Fig. 5A, left panel). However, the Fe-SOD isoenzyme was not detectable in the embryonic tissues at any developmental stage (Fig. 5A, right panel). In both endosperm and embryo, the Mn-SOD isoenzyme exhibited a general increase in activity during development. In contrast, the Cu/Zn-SOD isoenzyme exhibited high levels of activity in immature stages but was absent during maturation. Fe-SOD isoenzyme activity was detected in the endosperm after 65 DAF, after which there was a slight increase during maturation. According to the non-denaturing PAGE analysis of SOD activity (Fig. 5A), artificial dehydration did not cause any detectable changes in the isoenzyme patterns of either endosperm or embryo tissues for any developmental stage.

CAT

CAT activity increased greatly during the early stages of development; almost doubling from 55 to 60 DAF (Fig. 4B). After 65 DAF, CAT activity rapidly decreased and remained relatively constant at 10 μmol H_2O_2 mg^{-1} protein min^{-1} during the late developmental stages (80 and 90 DAF). Artificial dehydration caused a slight increase in CAT activity, compared with values of fresh tissues, in the endosperm tissues of seeds at 55 and 60 DAF and embryonic tissues of seeds at 55 DAF. In contrast, artificial dehydration caused a general decrease in CAT activity in the later stages of both endosperm and embryonic tissues.

CAT activity staining revealed one CAT band that was present in both endosperm and embryo during all developmental stages (Fig. 5B). CAT activity staining exhibited a pattern of

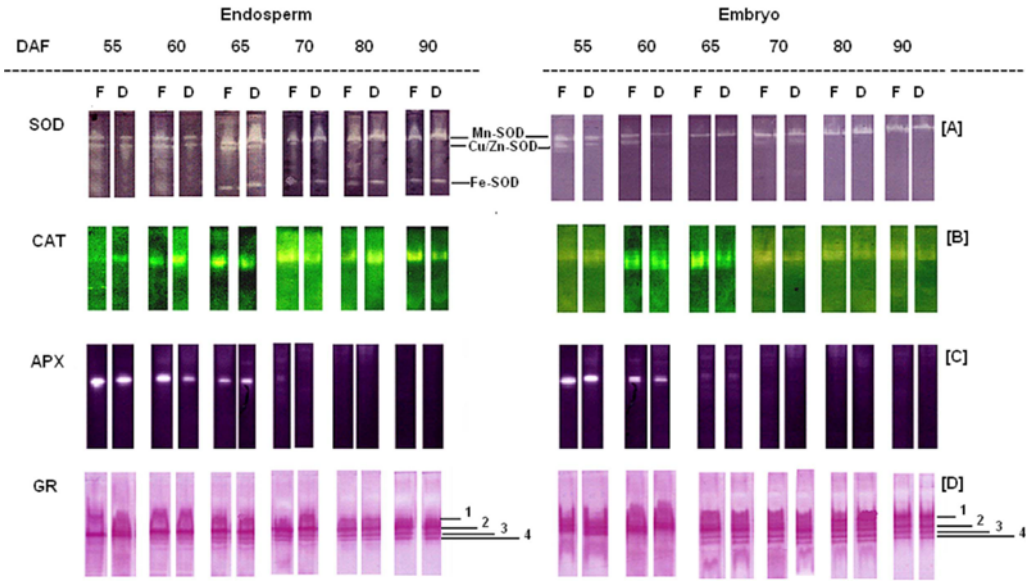

Fig. 5. Native gel staining for SOD [A], CAT [B], APX [C] and GR [D] activities in endosperm and embryonic tissues of fresh (F) and artificially dried (D) seeds at different developmental stages. Numbers from 1 to 4 in [D] indicate different GR isoforms. Equal amounts of total proteins (100 μg for SOD and APX, 15 μg for CAT and 50 μg for GR) were loaded in each lane of staining gels.

activity (Fig. 5B) similar to that determined by the spectrophotometric assay for total CAT activity in both fresh and artificially dried seeds (Fig. 4B).

APX

APX activity showed a similar pattern to SOD activity with higher levels at early stages of development of the endosperm and embryo (Fig. 4C). APX activity decreased from 2.6 and 3.7 μmol AsA mg^{-1} protein min^{-1} in endosperm and embryo at 55 DAF respectively, until it became undetectable at 70 DAF in the endosperm and at 65 DAF in the embryo. Artificial dehydration caused a decrease in APX activity in both endosperm and embryo tissues. APX activity staining revealed that one major APX bands was present in both endosperm and embryo during early developmental stages (Fig. 5C). APX activity staining also generated a pattern of activity very similar to that revealed by the spectrophotometric assay in both fresh and artificially dried seeds (Fig. 4C).

DHAR

DHAR activity increased transiently during the early stages of development (Fig. 4D). Enzyme activity increased by about 4-fold in endosperm and about 2-fold in embryonic tissues over the period from 55 to 65 DAF, and then decreased during the late developmental stages (Fig. 4D). Artificial dehydration resulted in a noticeable increase in DHAR activity in both of endosperm and embryonic tissues of the seeds at 55, 60 and 65 DAF and no significant changes in either tissue at 70, 80 and 90 DAF.

GR

GR activity showed slight changes during the early developmental stages (from 55 to 65 DAF), after which levels declined, reaching values approximately 50% lower than those at 55 DAF (Fig. 4E). The GR activity levels remained low during the late developmental stages. The effect of dehydration on GR activity was negligible in both endosperm and embryonic tissues. GR activity staining showed changes in the activity and appearance of four GR isoenzymes in both endosperm and embryo (Fig. 5D). Isoenzyme 2 showed high activity during the early development stages, whereas the isoenzymes 1, 3 and 4 were more prominent during the late development stages. Artificial drying had no obvious effect on the pattern of GR isoenzyme activity in either endosperm or embryonic tissues (Fig. 5D).

Expression Profiling of Antioxidant Enzyme Genes During Seed Development

Expression patterns of the antioxidant enzyme genes were further investigated to confirm their participation in H_2O_2 scavenging during seed development. The transcript amounts of five antioxidant enzymes (Mn-SOD, Cu/Zn-SOD, CAT, APX and DHAR) in seeds at different developmental stages were analyzed by semi-quantitative RT-PCR. The transcript levels of Mn-SOD increased gradually during seed development (Fig. 6A). In contrast, the transcript levels of Cu/Zn-SOD

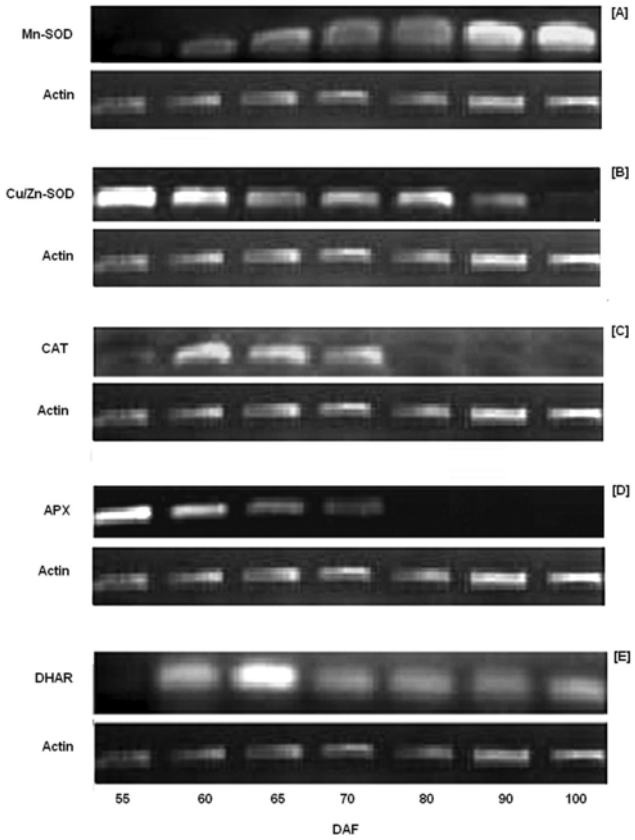

Fig. 6. Transcript levels of antioxidant enzymes in seeds at different developmental stages. The number of amplification cycles was optimized for each gene (26 cycles for Mn-SOD and CAT, 25 cycles for Cu/Zn-SOD, 24 cycles for APX and DHAR and 23 cycles for actin) to yield optimal contrast between samples in EtBr-agarose gel electrophoresis. Actin gene was included as an internal control.

decreased gradually with seed development (Fig. 6B). This differential expression pattern of the two SOD isoenzymes, Mn-SOD and Cu/Zn-SOD, is consistent with their activity pattern (as shown in Fig. 5A). The transcript amount of CAT showed a sharp increase from 55 to 60 DAF then continued with a slight decrease until 70 DAF after which it was characterized by a very low level of expression from 80 to 100 DAF (Fig. 6C). In contrast to CAT (Fig. 6C), APX expression level decreased gradually during early development stages (50-70 DAF, Fig. 6D). Both CAT and APX transcripts were undetectable during the late maturation and desiccation phases (80-100 DAF, Fig. 6C and D). DHAR transcript amount increased during 55-65 DAF, reaching the maximum level at 65 DAF and then remained low from 70 DAF until the end of seed development (Fig. 6E). When considered collectively, the expression analysis of the antioxidant enzyme genes (Fig. 6) demonstrated the dominance of Mn-SOD and DHAR transcripts during the desiccation phase of seed development (from 90-100 DAF).

Discussion

J. curcas seed development displays the classic developmental pattern of orthodox seeds (Bewley and Black, 1994). However, in these seeds the natural desiccation process that occurs during the final stage of seed development is not a prerequisite to switch from development to germination. The seed reaches its full germinability (100%) at 80 DAF before the onset of the dehydration process. Increasing rates of germination throughout seed development among whole seeds (Fig. 2) indicate the importance of reserve compounds and the maturation process itself in supporting post-germination growth (Bewley and Black 1983). Minimizing the increase in electrolyte leakage after desiccation treatment in late-stage seeds (Fig. 2) revealed a potential method for mitigating the deleterious effect of desiccation on membrane properties during the late stages of seed development. Therefore, changes in membrane properties during seed development are very important for the acquisition of desiccation tolerance (Parrish and Leopold 1978; Yang et al. 2004).

The changes observed in H_2O_2 and MDA content during *J. curcas* seed development provide insights on the relationship between H_2O_2 level and MDA content (Fig. 3). Although increased ROS production appears to be a normal result of plant tissue dehydration (Leprince et al. 1993; Smirnoff 1993), our findings demonstrate a decrease in H_2O_2 production and, consequently, MDA content (Fig. 3) through the natural desiccation of the seeds during development and maturation. Moreover, artificial drying of *J. curcas* seeds at different developmental stages resulted in reduced H_2O_2 and MDA content in dried seeds as compared with fresh ones. Consistent with this observation, Bailly et al. (2004) found that the reduced levels of H_2O_2 and MDA were the result of the artificial dehydration of orthodox sunflower seeds. Decreased metabolic activity is probably the reason for reduced H_2O_2 production during maturation and dehydration of orthodox seeds (Leprince et al. 2000; Vertucci and Farrant 1995).

The effective concentration of free radicals in plant tissues is a consequence of a dynamic equilibrium between the rates of production and scavenging (Garnczarska and Wojtyla 2008; Goh et al. 2012). Thus, a decrease of H_2O_2 level can be due either to a low production rate or to efficient scavenging by antioxidant enzymes. A possible explanation for the observed increase in SOD activity after artificial drying of all developmental stages (Fig. 4A) is that SOD more effectively catalyzes the reduction of superoxide anions to H_2O_2 (Alscher et al. 2002). Interestingly, our results show an inverse relationship between increasing SOD activity (Fig. 4) and decreasing H_2O_2 content (Fig. 3) after drying, highlighting the importance of protection mechanisms.

The involvement of antioxidant enzymes in detoxification and maintenance of a cellular metabolic balance in orthodox seeds has been reported in multiple species, including Norway maple and European beech (Pukacka et al. 2003), wheat (De Gara et al. 2003) and yellow lupine (Garnczarska et al. 2009). In the present study, ROS scavenging enzymes (SOD and H_2O_2 scavenging enzymes) showed striking differences in their activity patterns during seed development (Fig. 4, 5 and 6) as well as after artificial drying (Fig. 4 and 5). High levels of SOD activity during the early stages of development are related to the abundance of Cu/Zn-SOD transcript (Fig. 6B), while the continuous SOD activity during maturation and natural desiccation was accompanied with increases of Mn-SOD transcript (Fig. 6A). Abundance of Mn-SOD transcript in mature and dry embryos has also been found in lotus seeds (Li et al. 2008).

High level of SOD and APX enzymes during early stages and increased CAT and DHAR activity during the reverse accumulation phase suggest that the antioxidant system was sufficiently effective during these stages to minimize further dehydration damage and to control H_2O_2 content (Lehner et al. 2006; Zulfugarov et al. 2011). The absence or very low activity of ROS-scavenging enzymes is typical of all mature and viable orthodox seeds (Cakmak et al. 1993; De Gara et al. 1997; Tommasi et al. 1999). Bailly et al. (2004) suggested that CAT activity plays a primary role in H_2O_2 scavenging during late stages of development or after artificial drying of sunflower seeds, where higher CAT activity associated with reduced levels of H_2O_2. However, this general activity pattern was not observed in *J. curcas* seeds. Our results demonstrate that this relationship is apparent only during the early stages of development and is reversed completely during the later stages as well as after artificial drying in both endosperm and embryonic tissues (Fig. 4B). Even at low levels, the continuous CAT activity during late developmental stages and after artificial drying suggests a role for CAT in fine-tuning the cellular H_2O_2 balance and modulating the related signaling pathways (Mittler 2002; Neill et al. 2002). Reduced CAT and APX activities at late stages and after artificial drying indicate the weak participation of CAT and APX in H_2O_2 removal. The reduction of APX and CAT activities in mature and dry seeds were related to the decrease in the amount of APX and CAT gene transcript (Fig. 6C and 6D). The observed APX activity pattern (Fig. 4C, Fig. 5C and Fig. 6D) is consistent with previous observations that APX is always very active in dividing cells and tissues undergoing differentiation (De Gara et al. 1996; De Pinto et al. 2000), whereas the APX activity decreases in senescent tissues (Borraccino et al. 1994). The pool of ascorbic acid as an electron donor in ascorbate–glutathione cycle (A-G cycle) is regenerated by monodehydroascorbate reductase (MR) and DHAR, with the participation of GSH (reduced glutathione) and NADPH. GSH is oxidized to GSSG and reduced back to GSH by GR (Pukacka and Ratajczak, 2007). Other studies on AsA content, DHA content, and AsA:DHA ratio, revealed that it is difficult to determine what role the ascorbate system plays in preparing seeds for desiccation. There were no significant differences in ascorbic acid content and APX activity between the orthodox and the recalcitrant seeds (Tommasi et al. 1999; Pukacka and Ratajczak, 2007). In seeds of Norway maple (orthodox seed), an undoubtedly important element of cell protection against damage caused by dehydration is the A-G system and glutathione metabolism, which scavenge ROS and protect proteins against denaturation.

Increasing or semi-stable DHAR and GR activities were observed, respectively, in *J. curcas* seeds during the late stages of development and after artificial dehydration in both of endosperm and embryonic tissues (Fig. 4D and 4E). This is in general concordance with other studies: GR activity in beans (Bailly et al. 2004), GR and DHAR activities in Norway maple (Pukacka and Ratajczak 2007), wheat (De Gara et al. 2003) and yellow lupine (Garnczarska et al. 2009). The key role of the GR reaction to produce GSSG using the reducing power of NADP and the involvement of GSSG in protein glutathionylation, which increases protein stability (Buchanan and Balmer 2005), may explain the semi-stability of GR activity during late stages of orthodox seed development. In seed development, disulphide isomerase plays a key role in the maturation of storage proteins (De Gara et al. 2003). Therefore, the nature of the endosperm as a storage tissue explains the increasing DHAR activity in the endosperm relative to the embryo during development. Considering the metabolic and regulatory properties of DHAR and the activity pattern of this enzyme (Fig. 4D) as well as the expression profile (Fig. 6E) during the early stages of development, we can speculate that DHAR plays an important role in the acquisition of germinability and in increasing the germination percentage of immature seeds.

Decreasing GR activity during early developmental stages is related to decreasing activity of GR isoenzyme 3 (Fig. 5D). However, the synthesis of GR isoenzyme 2 and 4 was primarily responsible for sustaining moderate activity during maturation and desiccation phases. The clear transition between presence and absence of different GR isoenzymes during seed development suggests that they have different properties and roles. Although CAT activity has been shown to be essential for removing potentially toxic H_2O_2 produced under various stress conditions and for avoiding oxidative–stress related damage (Willekens et al. 1995), the present data demonstrate that GR and DHAR activity may play a primary role in H_2O_2 management during late stages of development and after artificial drying of *J. curcas* seeds.

Our results suggest that ascorbate–glutathione cycle

enzymes may play a key role in H_2O_2 scavenging during the natural and artificial drying of *J. curcas* seeds. The activity patterns of antioxidant enzymes documented in this study may allow for the replacement of different protective mechanisms in orthodox seeds. ROS-scavenging enzymes are well organized during the early stages of seed development and the level of H_2O_2 is very high. On the other hand, during late stages and after artificial drying, GR and DHAR activity represent more convenient parameters with which to measure the stability of protective proteins known to be expressed during the late stages of orthodox seed development. Moreover, the association of reduced H_2O_2 levels with the weak responses of antioxidant enzymes after artificial drying elevates the role of non-enzymatic antioxidant components, which remain a subject for future investigations.

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