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Full Length Research Paper

Desiccation-induced changes in viability, lipid peroxidation and antioxidant enzyme activity in *Mimusops elengi* seeds

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Intermediate seeds of *Mimusopsis elengi* showed obvious membrane lipid peroxidation during desiccation. When the moisture content (MC) decreased from initial 41.8 to 6.1%, seed viability significantly decreased from 100 to 23%, consorted with activity changes of a few anti-oxidative enzymes. The activities of superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) gradually showed a similar change rend in the process of desiccation, namely their activities firstly increase to the maxima, and then decreased. The exception was catalase (CAT) which showed a general decrease in activity during the whole experimental period. It indicated that seed deterioration of *M. elengi* was related with uncontrolled production of reactive oxygen species (ROS) and the ability of enzymes mentioned to eliminate such toxic products during drying seeds. Thus, the fact that oxidative stress induced unmendable damage was considered as one of the critical factors which resulted in viability loss of *M. elengi* seeds.

Key words: Intermediate seeds, desiccation, reactive oxygen species, antioxidant enzymes, lipid peroxidation, *Mimusops elengi*.

INTRODUCTION

Orthodox seeds that can withstand dehydration to about 5% are generally regarded as desiccation tolerant (Roberts, 1973). Unless debilitated by zero-tolerant storage fungi, orthodox seeds should maintain high vigor and viability at least from harvest until the next growing season or for many decades at -18°C (Ellis and Roberts, 1980). However, some seeds, often of tropical origin, cannot withstand dehydration and are also often chilling sensitive, and were described as recalcitrant (Corbineau and Côme, 1988; Hong and Ellis, 1996). A further group of seeds that demonstrated intermediate behavior has been described (Ellis et al., 1990; 1991). When they originate from tropical climates, intermediate seeds are often chilling sensitive. One of main features is that such seeds can tolerate desiccation to moisture contents (MCs) in equilibrium with about 40 to 50% relative humidity (RH), that is, about 7 to 12% MC depending on the species, but further drying leads more rapid loss in viability and sometimes immediate damage occurs on further desiccation (Ellis et al., 1990; Hong and Ellis, 1996).

During desiccation, intracellular structures are highly vulnerable, as conditions for radical generation are enhanced (Vertucci and Farrant, 1995; Walters et al., 2005). Different stressors, biotic and abiotic, often result in producing reactive oxygen species (ROS), including the superoxide anions ($\cdot O^{2-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$), which cause damage to proteins, lipids and DNA (Møller and Kristensen, 2004), especially an extensive peroxidation and de-esterification of membrane lipids (Hoekstra et al., 2001; França et al., 2007), leading to irreversible formation of gel phase domains and loss of membrane function (Mckersie et al., 1990). The balance between ROS production and cell defenses determines

Abbreviations: MC, Moisture content; **SOD,** superoxide dismutase; **POD,** peroxidase; **APX,** ascorbate peroxidase; **GR,** glutathione reductase; **CAT,** catalase; **ROS,** reactive oxygen species.

the degree of oxidative stress. To protect against oxidative damage, cell possess defense mechanisms that include enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and antioxidants, such as glutathione, vitamins C and E (Leprince et al., 1990; Bailly, 2004; Berjak, 2006). Generally, a primary defense against oxygen toxicity involves at least one form of the enzyme SOD. This enzyme is involved in the conversion of super-oxide anion to oxygen and hydrogen peroxide, which is further degraded by CATs or POD. Among antioxidants, the ascorbate-glutathione pathway is considered to play a major role in protection from ROS (Noctor and Foyer, 1998). In this pathway, reduced glutathione (GSH) is needed for reduction of dehydroascorbate, which is formed via monodehydroascorbate by the action of APX or by non-enzymatic reactions of ascorbate (ASC) with oxidants. As a cellular antioxidant, GSH can also protect thiol-containing enzymes and can directly scavenge activated oxygen species (Asada, 1992, 1999; França et al., 2007). Also, the maintenance of GSH, which is oxidized to glutathione disulfide (GSSG) is mediated by GR at the expense of nicotinamide adenine dinucleotide phosphate (NADPH) (Hausladen and Alscher, 2004).

Water stress increases the formation of ROS resulting in lipid peroxidation, denaturation of proteins and nucleic acid damage with severe consequences on overall metabolism (França et al., 2007). As was reported, regulation of the antioxidant defense system is complex and its role in desiccation tolerance is obvious. Rapid decline in viability in response to desiccation below intermediate moisture (6.2 to 10.9%) in neem seeds (Sacandé et al., 2000, 2001) was caused by oxidative stress like *Quercus robur* (Hendry et al., 1992), *Acer platanoides* (Pukacka, 1991) and *Acer saccharinum* (Pukacka and Ratajczak, 2006).

However, oxidative effects on viability of intermediate seeds of *Mimusopsis elengi* (Truong et al., 2006), an all-purpose tree has been yet unknown. To better understand the relationship between the dynamic change of seed viability and various antioxidative enzymes during desiccation to effectively facilitate *ex situ* conserving seeds of this species, changes of seed viability, lipid peroxidation and activities of a few enzymes were investigated.

MATERIALS AND METHODS

Seed collection, MC and viability test

Mature drupe fruits of *M. elengi* were collected from trees growing in the Xishuangbanna Tropical Botanical Garden (XTBG) (21°41´N, 101º25´E, the altitude of 573 m) of the Chinese Academy of Sciences in China on 8 March, 2009. Seeds were immediately extracted by hand, cleaned and surface dried in the laboratory. A sample of 100 seeds was tested for ability to germinate and other seeds were used for other treatments. To establish seed population responses to different levels of desiccation, *M. elengi* seeds were dried over newly-regenerated silica gel to various MCs, and then seed viability was tested by direct germination. The seed MCs were determined according to the method given by International Seed Testing Association (ISTA, 1999). Twenty (20) seeds were used for determining both the initial and other MCs. Seeds were weighed before and after drying at 103°C for 17±1 h and MC was expressed as percentage (%) based on fresh weight (FW). Both freshly harvested and dried seeds set in 12 cm glass Petri dishes with a sheet of filter paper moistened by distilled water were incubated at 30°C with a photoperiod of 12 h/d (HPG-280B Illuminating Incubator; Har'erbin Electronic Apparatus Manufactory, Har'erbin, China). Photosynthetic photo flux density (PPFD) inside growth chambers was 60 μmol s^{-1} m^{-2} (LI-COR, Inc., Nebraska, USA). The germination tests were observed for 40 days. The seeds with 2 mm visual radicle were considered as germinated.

Lipid peroxidation

The level of lipid peroxidation in seed samples was determined in terms of malondialdehyde (MDA) content according to the method of Lima et al. (2002).

Enzyme extraction and assays

All operations were performed at 4°C. For protein and enzyme extractions, 0.5 g of seed samples were homogenized with an ice-cold 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% (w/v) polyvinylpoly-pyrrolidone (PVPP). Homogenates were centrifuged at 13, 000 ×g for 30 min, and supernatants were used for measuring protein content and enzyme activity. Total soluble protein contents were determined according to Bradford (1976) by using bovine serum albumin as standard.

Total SOD (EC 1.15.1.1) activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetra-zolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971). One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT photoreduction.

CAT (EC 1.11.1.6) activity was estimated according to Del Rio et al. (1977) which measures the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture contained 0.05 M Na-phosphate buffer (pH 7.0) with 0.1 mM EDTA and 3% H_2O_2. The decrease in the absorption was followed for 3 min and µmol H_2O_2 destroyed per minute was defined as one unit of CAT activity. POD (EC 1.11.1.7) activity was based on the method described by Chance and Maehly (1975). A unit of POD activity was defined as µmol mL^{-1} H_2O_2 decomposed per minute.

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). One unit of APX was defined as 1 µmol mL^{-1} ASC oxidized per minute. GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). The assay medium contained 0.025 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH Na4 and 0.1 mL enzyme extract in a final assay volume of 1 mL. NADPH oxidation was fallowed at 340 nm. Activity was calculated using the extinction coefficient (6.2 $mM^{-1}cm^{-1}$) for GSSG. One unit of GR was defined as 1 mmol mL^{-1} GSSG reduced per minute. The specific enzyme activities were expressed as Umg^{-1} protein g^{-1} FW for all enzymes assayed.

Statistical analysis

The mean of both a certain seed moisture and MDA

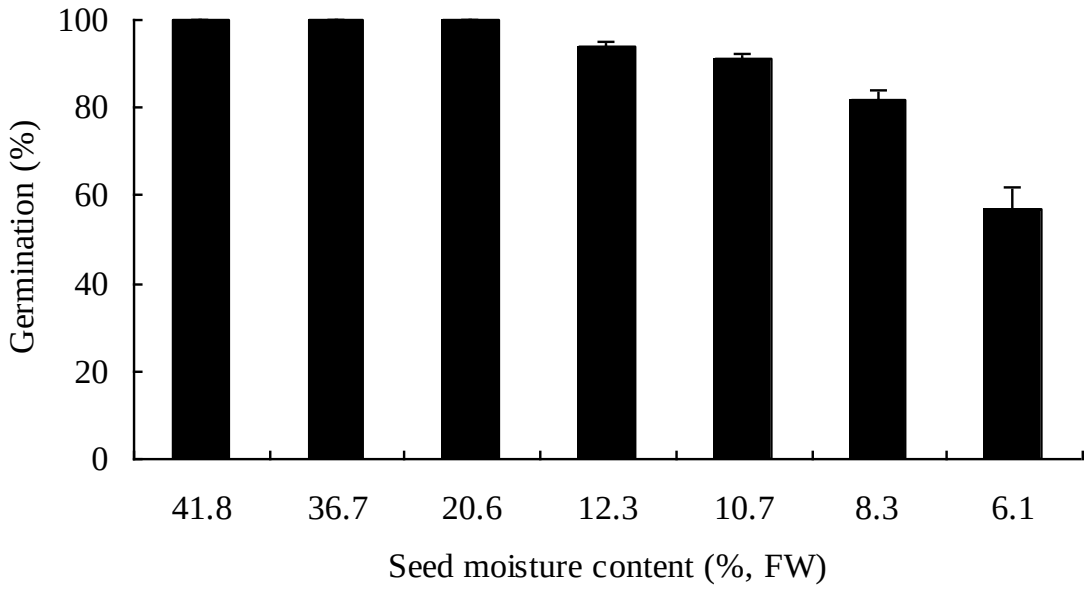

Figure 1. Changes of the germination of *M. elengi* seeds with different MC after desiccation. Bars indicate ±1 SD.

content was calculated on four replicates. Other statistical data were expressed as the mean values of four replicates with standard deviations.

RESULTS

MC and germination

Seeds of *M. elengi* with an initial MC of 41.8%, germinated completely without drying (Figure 1). These seeds exhibited a rapid decline in MC during drying (data not shown). The final germination percentages were 91 and 82% at 10.7% MC and 8.3% MC, respectively. Further drying significantly reduced seed viability. Consequently, the germination percentage declined to 23% at 6.1% of seed MC (Figure 1).

Lipid peroxidation

Lipid peroxidation levels in seeds assessed as the content of MDA are shown in Figure 2. MC-dependent increase in MDA content in *M. elengi* seeds was indicated when compared to fresh seeds. With decrease in seed MC, the MDA content increased throughout the experimental desiccation time. In particular, extreme water loss (for example, 6.1% MC) induced significant lipid peroxidation.

Antioxidant enzyme activities

SOD activities in seeds of *M. elengi* seeds desiccated is shown in Figure 3. With decrease in water content, SOD activity gradually reached 11.8U and subsequently decreased.

CAT activity was greatly affected by drying throughout the experimental period (Figure 3). When decrease in seed MC from 41.8 to 6.1%, the activities of CAT gradually declined from 25.38 to 15.03 U. The activity of POD firstly increased to the maximum of 225.6 U, and then declined to 71.6 U under deeply desiccation stress. APX detoxifies H_2O_2 in favor of ASC oxidation in Asada Halliwell pathway in different cell compartments.

In the process of drying, APX activity showed a first increase and subsequent decrease with decrease in seed MC (Figure 4). However, constitutive GR activity in seeds tested gradually increased to the maximum of 20.1 U at 10.7% of seed MC, and subsequently decreased to 12.6 U (Figure 4).

DISCUSSION

At different hydration levels, because the thermodynamic properties of water change, different metabolic processes can take place (Vertucci and Farrant, 1995). A decrease in enzymic protection against oxidative attack in non-orthodox seeds when their water is lost was directly linked with lipid peroxidation and free radical formation, especially ROS, contributing to viability loss in these non-orthodox seeds (Hendry et al., 1992; Leprince et al., 1999; Varghese and Naithani, 2002; Pukacka and Ratajczak, 2006; Dussert et al., 2006).

One of the two main characteristics is that intermediate seeds are able to withstand considerable drying [down to a relative humidity (RH) of 30 to 40%] in comparison with

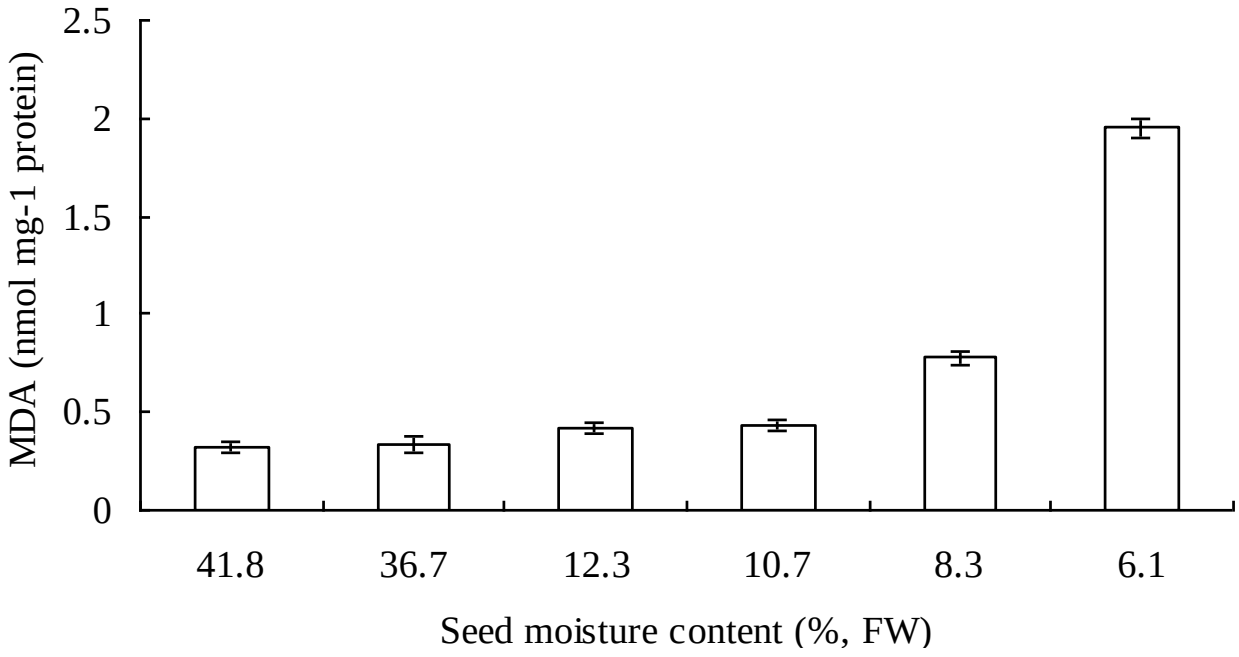

Figure 2. MDA content (nmol g^{-1} FW) in *M. elengi* seeds with certain MCs. Bars indicate ±1 SD.

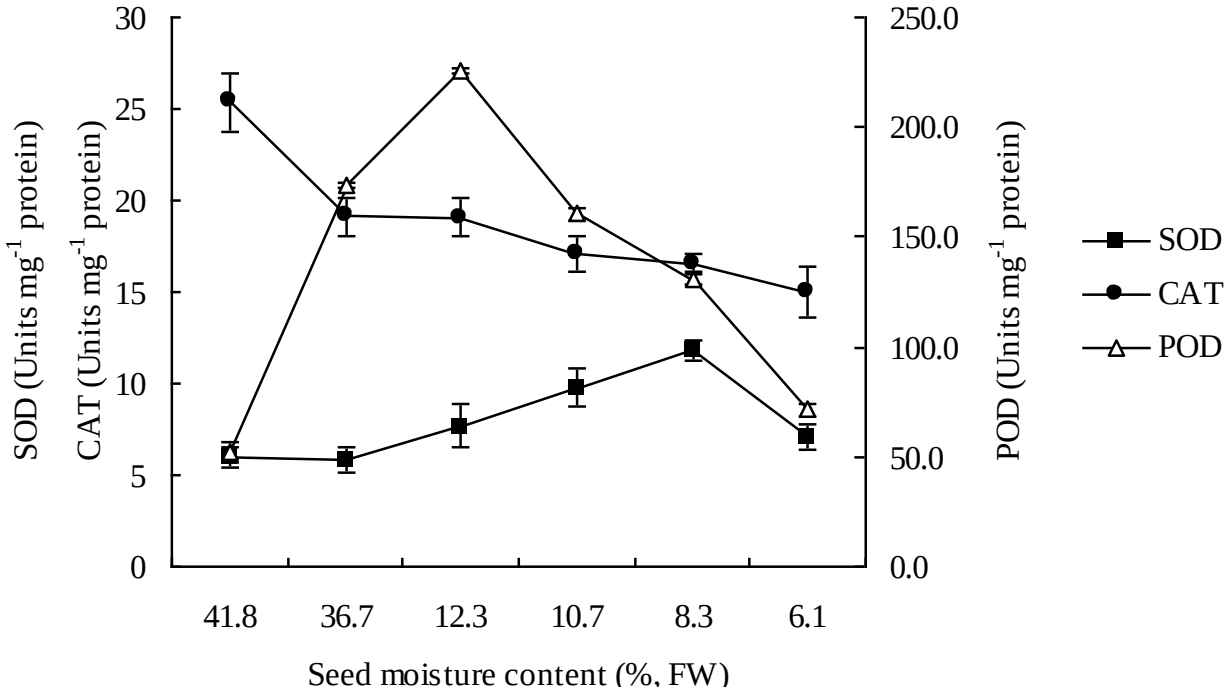

Figure 3. Activities of SOD, CAT and POD enzymes (Units mg^{-1} protein) in *M. elengi* seeds during desiccation. Bars indicate ±1 SD.

recalcitrant seeds, but cannot tolerate extreme water loss as is the case in orthodox seeds (Ellis et al., 1990). In this study, *M. elengi* seeds were severely deteriorated when they were dehydrated to 6.1%. Actually, the ability of *M. elengi* seeds to germinate was badly impaired at 6.1% MC (Figure 1). These results supported the findings of Truong et al. (2006) who confirmed that *M. elengi* seeds lost viability below 8% MC considered as intermediate.

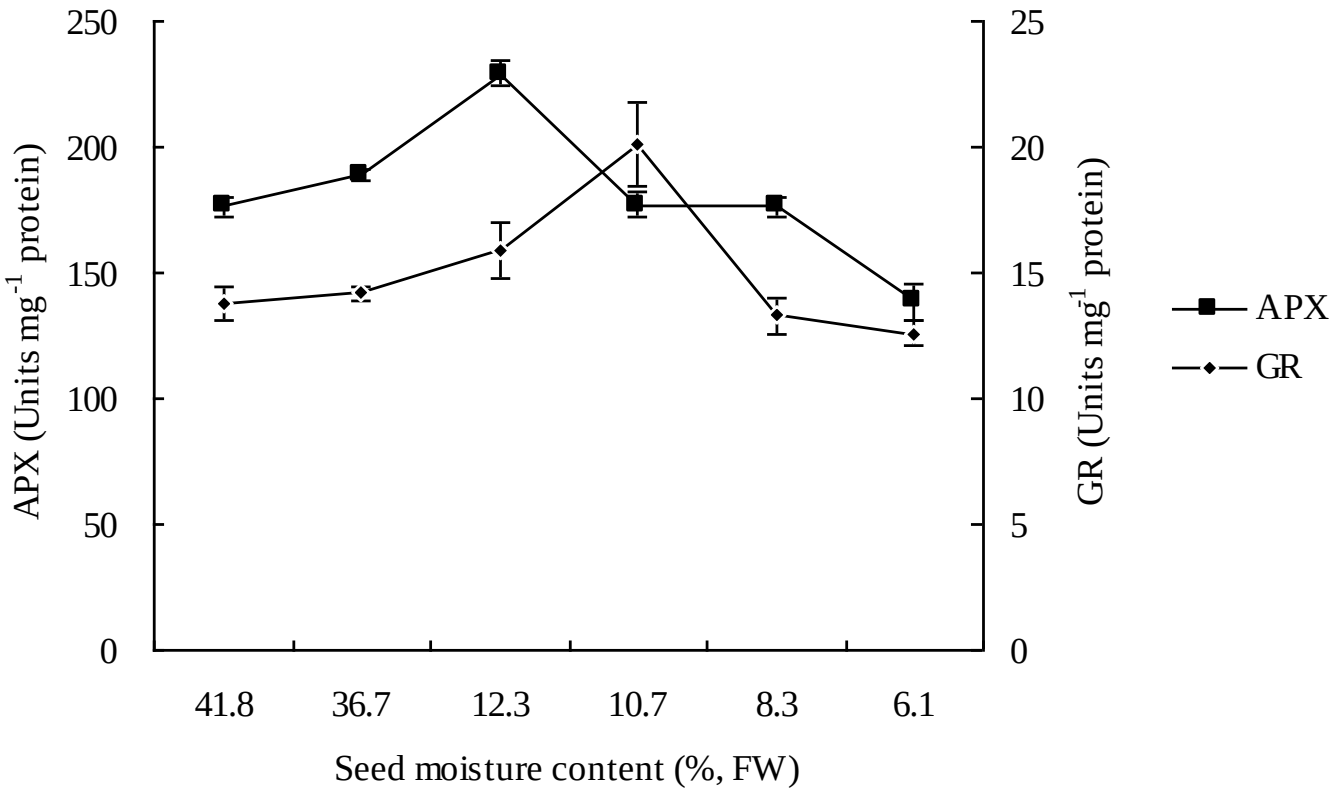

Figure 4. Activities of APX and GR enzymes (Units mg^{-1} protein) in *M. elengi* seeds with different MCs. Bars indicate ± 1 SD.

Similar to previous studies on axes and/or cotyledons of recalcitrant species, such as *Castanea sativa* (Leprince et al., 1994), *Q. robur* (Hendry et al., 1992) and *A. saccharinum* (Pukacka and Ratajczak, 2006) and intermediate seeds like *Azadirachta indica* (Varghese and Naithani, 2002), *Coffea Arabica* (Dussert et al., 2006), indicating that loss of seed viability is associated with increased free radical attack and peroxidative damage; the present study confirmed that damage to cell membrane was the main cause of viability loss of *M. elengi* seeds during desiccation (Figures 1 and 2). The increase in lipid peroxidation of whole *M. elengi* seeds coincided with the decrease in seed MC (Figures 1 and 2). With water loss, there was a dramatic increase in MDA content in seeds tested. Similarly, Greggains et al. (2001) found that increased in advance of viability loss and decreased at 54 to 57% MC, where most seeds of *Avicennia marina* lost viability. Thus, these results suggested that oxidative processes increasingly induce baneful effects as the seeds were dried.

Seeds have evolved a complex antioxidant system to protect cellular membranes and organelles against damaging effects of ROS (Walters et al., 2005; Dussert et al., 2006), such as antioxidative enzymes and small molecular antioxidants. The oxidative stress in dehydrated organisms is likely to be due to an uncontrolled formation of ROS (Oliver et al., 2001) and is expected to occur within an interval of intermediary hydration levels where the down-regulation of metabolism becomes uncoordinated (Leprince et al., 1999, 2000). According to Vertucci and Farrant (1995), at lower water contents of 0.08 to 0.25 g H_2O/g dry mass, low level catabolic events still occur slowly in seeds. This assumption is in accordance with the fact that oxidation stress occurred when *M. elengi* seeds crossed the interval of hydration levels where ROS were produced during drying. SOD catalyses the disproportion of superoxide radical to molecular oxygen and H_2O_2 and hence, decreases the risk of hydroxyl radical formation from superoxide via the Haber-Weiss type reaction (Pereira et al., 2003). SOD activity in seeds of *M. elengi* showed a gradual increase and subsequent decrease when exposed to substantive water loss (Figure 3). Also, POD activity showed a similar change track (Figure 3). In addition, activities of APX and GR showed similar change tracks when compared to SOD (Figure 4). However, CAT activity presented an adverse change when compared to SOD and POD (Figure 3). POD is involved in many different physiological functions, including the oxidation of toxic compounds, the biosynthesis of cell walls (lignin and suberin), growth and developmental processes, etc (Dicko et al., 2006). Activity of POD has been reported to firstly increase and then decrease during drying seeds of *Clausena lansium* (Rutaceae) (Song and Fu, 1997).

Therefore, it was inferred that *M. elengi* seeds were experiencing intense oxidative stress.

APX and GR, the key enzymes in the Halliwell-Asada pathway, are involved in the reduction of H_2O_2 by using ASC as an antioxidant and NADPH as a reductant, to regenerate ASC (Asada, 1992,1999) and GSH (Foyer and Halliwell, 1976) and thereby protection of plants against oxidative stress. At present, ASC-GSH cycle enzymes are well characterized in some organs of plants (Vanacker et al., 1998; Mittova et al., 2000; Hernández et al., 2006). Also, ASC and GSH are reported to be major redox buffers in plant cells (Shigeoka et al., 2002). A decrease in their redox status leads to a loss of cell redox homeostasis (Foyer and Noctor, 2003). Thus, the inactivation of APX in the present study might be attributed to the loss of ascorbic acid because ascorbic is believed to be the first line of defense against oxidative stress (Barnes et al., 2002). This was also reflected by the increase in lipid peroxidation after desiccation.

Under physiological conditions, the normal ascorbic acid regeneration cycle is expected to function and able to maintain the ascorbic acid concentration (Shigeoka et al., 2002). To compensate the loss of ascorbic acid, the regeneration system was timely initiated. This present study showed that the activities of GR responded positively to desiccation. Upon suffering the desiccation stress, activities of GR was instantly up-regulated up to 10.7% MC, but fell to low levels with further drying down to 6.1% MC (Figure 4), probably due to a decreasing GSH level (data not determined). The similar changes of GR occurred in the axes of *Q. robur* seeds (Hendry et al., 1992). With further desiccation, the activities of all the enzymes tested were impaired, which implied that activities of these enzymes were not enough to prevent peroxidation of lipid membranes caused by extreme water loss. It was inferred that built-up ROS in dried seeds accelerated loss of membrane integrity and loss of viability. According to the present results and similar findings in literatures, this study argued that the main difference among three categories of seeds should be their levels of desiccation tolerance but not the mechanisms underlying their storage behavior.

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