Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds

Changrun Li and Wendell Q. Sun*

Department of Biological Sciences, National University of Singapore, Kent Ridge Crescent, Singapore 119260

Abstract

Mature and immature axes of *Theobroma cacao* (cocoa) seeds tolerated desiccation under a rapid-drying regime to critical water contents of 1.0 and 1.7 g g^{-1} dw, respectively. These critical water contents corresponded to water contents below which activities of free radical-scavenging enzymes (ascorbate peroxidase, peroxidase and superoxide dismutase) decreased rapidly during desiccation. The decline in axis viability below the critical water content was correlated with sharp increases in lipid peroxidation and cellular leakage. Cotyledon tissues were more desiccation-tolerant than axes, with a low critical water content of 0.24 g g^{-1} dw. Desiccation sensitivity in cotyledon tissues was also correlated with the decrease in superoxide dismutase activity and increased lipid peroxidation products. However, in the cotyledons, no ascorbate peroxidase activity was detected at any water content, and peroxidase activity was gradually reduced as desiccation proceeded. Cocoa embryonic axes contained large amounts of sucrose, raffinose and stachyose but only traces of reducing monosaccharides. Desiccation sensitivity of recalcitrant cocoa axes did not appear to be due to the lack of sugar-related protective mechanisms during desiccation, and it was more likely related to the decrease of enzymic protection against desiccation-induced oxidative stresses.

Keywords: ascorbate peroxidase, carbohydrate, desiccation tolerance, lipid peroxidation, recalcitrant seed, superoxide dismutase, Theobroma cacao.

Introduction

A large number of tropical species produce recalcitrant seeds. Desiccation sensitivity of recalcitrant seeds is a major problem for long-term conservation of genetic resources. Relatively high water content and temperature must be maintained to avoid desiccation injury and chilling injury, but at a high water content, stored seeds lose their viability rapidly (Chin and Roberts, 1980). Specific mechanisms associated with desiccation sensitivity of recalcitrant seeds are still not resolved. Desiccation sensitivity is a complex physiological phenomenon and is likely to be mediated by a series of deleterious and/or protective mechanisms.

Several deleterious processes may occur in desiccation-sensitive tissues during desiccation. Mechanical stresses upon the removal of water can cause structural changes at subcellular, cellular and tissue levels. Alterations in membrane structural integrity and function are well documented and reflected by the increased leakage of ions, sugars and proteins (Crévecoeur *et al*., 1976; Sargent *et al*., 1981; Senaratna *et al*., 1984). Metabolic stresses at a given water content can impair specific metabolic activities. The breakdown of metabolic co-ordination in cells may initiate uncontrolled free radical attack and decrease enzymic and non-enzymic protection against such oxidative damage (Leprince *et al*., 1990; Hendry *et al*., 1992; Pammenter *et al*., 1994). It has been shown that the loss of viability during drying in several desiccation-sensitive seeds was accompanied by increased lipid peroxidation and the accumulation of stable free radicals (Leprince *et al*., 1990; Hendry *et al*., 1992; Chaitanya and Naithani, 1994; Finch-Savage *et al*., 1994, 1996).

A number of protective mechanisms associated with seed desiccation tolerance have been identified through studies on acquisition and loss of desiccation tolerance in orthodox seeds. These mechanisms include the change in relative composition of membrane phospholipids, the accumulation of soluble carbohydrates such as sucrose and oligosaccharides, the synthesis of specific LEA proteins, and the increased ability to prevent, tolerate or repair free radical attack (Leprince *et al*., 1993; Sun and Leopold, 1997). The accumulation of sucrose and oligosaccharides is associated with membrane and protein stabilization as well as cytoplasmic glass

*Correspondence
Tel: 65-874-7932
Fax: 65-779-2486
Email: dbssunwq@nus.edu.sg

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formation during desiccation (Crowe *et al*., 1984; Carpenter *et al*., 1987; Williams and Leopold, 1989; Hoekstra *et al*., 1991; Sun *et al*., 1994, 1996). However, desiccation tolerance is not an "all-or-nothing" event, and the extent to which desiccation can be tolerated varies among recalcitrant species (Chin and Roberts, 1980; Berjak and Pammenter, 1994). Mechanisms of desiccation sensitivity may well differ among seeds of recalcitrant species.

Theobroma cacao (cocoa) seeds are desiccation-sensitive, and the biochemical basis remains to be studied. In the present study, we have focused on the relationship between desiccation sensitivity and activities of free radical-scavenging enzymes (ascorbate peroxidase, peroxidase and superoxide dismutase) in seeds. Desiccation sensitivity and the loss of viability in cocoa seeds during desiccation were related to the rapid decrease in activities of free radical-scavenging enzymes and did not appear to be due to the lack of sugar-related protective mechanisms.

Materials and methods

Source of plant materials

Immature and mature cocoa (*Theobroma cacao* L.) fruits were purchased from a cocoa plantation in Malaysia. A fruit was considered mature when its color had turned to yellow completely, and the seeds had attained maximum dry weight. Immature fruits were green with seed cotyledons just fully expanded. Cocoa fruits were stored temporarily at 16°C before use. Under the storage conditions used, recalcitrant cocoa seeds (in the fruit) can be easily stored for more than two months without any significant loss of seed viability and vigor. For our experiments, cocoa fruits were normally stored for less than 15 days after harvest. Extracted seeds were used within the same day.

Determination of desiccation sensitivity

Embryonic axes were surface-sterilized in 0.5% sodium hypochlorite for 5 min after excision from seeds. After rinsing with sterilized water, the axes were blotted dry and air-dried on filter paper in a laminar air-flow cabinet for up to 6 h at approx. 25°C and 60% RH. The initial water content of cocoa axes isolated from immature and mature seeds was about 4.8 and 2.8 g g^{-1} dw, respectively. At different time intervals, samples were taken for measurements of water content and electrolyte leakage. Fifteen embryonic axes were soaked in 30-ml water for 1 h, after which electrolyte leakage was measured and expressed as the percentage of total electrolyte content (Sun and Leopold, 1993). Water content of axes was determined after drying for 16 h at 103°C and expressed in g water g^{-1} dry weight (g g^{-1} dw). The viability of embryonic axes after desiccation was determined for 30 axes (in two replicates) cultured on modified Murashige and Skoog medium. The medium was supplemented with 1 mg L^{-1} naphthalene acetic acid, 1 mg L^{-1} benzylaminopurine, 0.17 g L^{-1} NaH_2PO_4 and 2 g L^{-1} fine charcoal (Sigma Co.). Axis growth was assessed after 30 days at 25°C under white fluorescent light (40 W, 12-h photoperiod). Shoots were considered to have survived if they had a green and swollen plumule. Roots were considered alive when the radicle remained whitish and was longer than 1.0 cm.

Desiccation tolerance of seed cotyledons was measured with the method of electrolyte leakage only. Cotyledon tissues of cocoa seeds were cut into small pieces (~ 3 × 3 × 1 mm), and the tissues were thoroughly washed with distilled water for 1 h. After blot-drying, cotyledon tissues were dried on filter paper in a laminar air-flow cabinet at about 25°C and 60% RH. Water content and electrolyte leakage were monitored during drying.

The water content below which electrolyte leakage increased rapidly or axis survival decreased rapidly was considered as the critical water content. The data were fitted with two separate linear regression lines, and the water content at the intercept between the two lines was used as the critical water content according to Sun and Leopold (1993).

Measurement of activities of free radical-scavenging enzymes

Seed tissues (about 250 mg fresh weight) were homogenized with 1.0 ml of 100 mM phosphate buffer (pH 7.8) containing 2% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 18,000 × g (4°C) for 15 min, and the supernatant was used for enzyme activity assays. Activities of superoxide dismutase and peroxidase were determined according to Giannopolitis and Ries (1977) and Kato and Shimizu (1987), respectively. Ascorbate peroxidase activity was measured using a modified procedure of Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 1 mM hydrogen peroxide and 0.1 mM EDTA in a total volume of 1.0 ml. Enzyme activity was expressed on the basis of protein content. Protein content of the extract was determined with the Bradford ready-to-use Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of lipid peroxidation product

The content of lipid peroxidation products was measured using the TBA-TCA reagent (0.25%

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thiobarbituric acid in 10% trichloroacetic acid) according to Heath and Packer (1968). Seed tissues (ca. 250 mg fresh weight) were homogenized at room temperature in 100 mM phosphate buffer (pH 7.5) and centrifuged at 5,000 × g for 1 min. An aliquot of 0.2 ml supernatant was added to 2.0 ml TBA-TCA reagent and incubated at 95°C for 30 min. The sample was cooled and centrifuged at 18,000 × g for 10 min. The absorbance of the supernatant was measured at 532 nm corrected by subtracting the absorbance at 600 nm. Samples incubated with 10% TCA without TBA were used as control.

Measurement of soluble carbohydrates

Carbohydrate content was determined according to Sun *et al*. (1994). Axis tissues (about 80 mg dry weight) were extracted at 75°C with 50% ethanol containing phenyl-α-D-glucoside as the internal standard. Extraction for each sample was repeated three times with 50% ethanol, and all supernatants were pooled for each sample. Pooled supernatant was dried under vacuum, re-dissolved in 1 ml water, and loaded on a column containing equal volumes (0.8 ml) of Dowex 50w (top layer), IRA-94 (middle layer) and polyvinylpolypyrrolidone (bottom layer) (all resins from Sigma, St. Louis, MO, USA). The column was washed with 5 ml water, and the eluant was collected and filtered through a 0.2 µm pore nylon membrane. Extracted carbohydrates were derivatized with trimethylsilylimidazole (in pyrrodine, 1:4, v/v) at 90°C for 1 h and separated by gas chromatography (GC) using a HP-1 capillary column (0.32-mm ID, 0.25-µm thickness) (Hewlett Packard, Avondale, PA, USA).

Results

Drying rate and dehydration response of cocoa axes

The drying rate can affect the response of recalcitrant seeds to desiccation. Drying curves of immature and mature cocoa axes were compared (Fig. 1). Under these rapid-drying conditions, water contents of isolated immature and mature axes decreased to 0.4 to 0.5 g g^{-1} dw within six hours. Due to high initial water content of immature axes before desiccation, the water content of immature axes remained higher than in mature axes during drying (Fig. 1A). For both immature and mature axes, the rate of water loss (drying rate) could be described by a simple exponential function (Fig. 1B), demonstrating that water loss in axes followed first-order-kinetics during desiccation under our experimental conditions. Drying rate, expressed as the rate constant of water loss, was similar for immature (0.312 ± 0.010 h^{-1}) and mature axes (0.297 ± 0.016 h^{-1}).

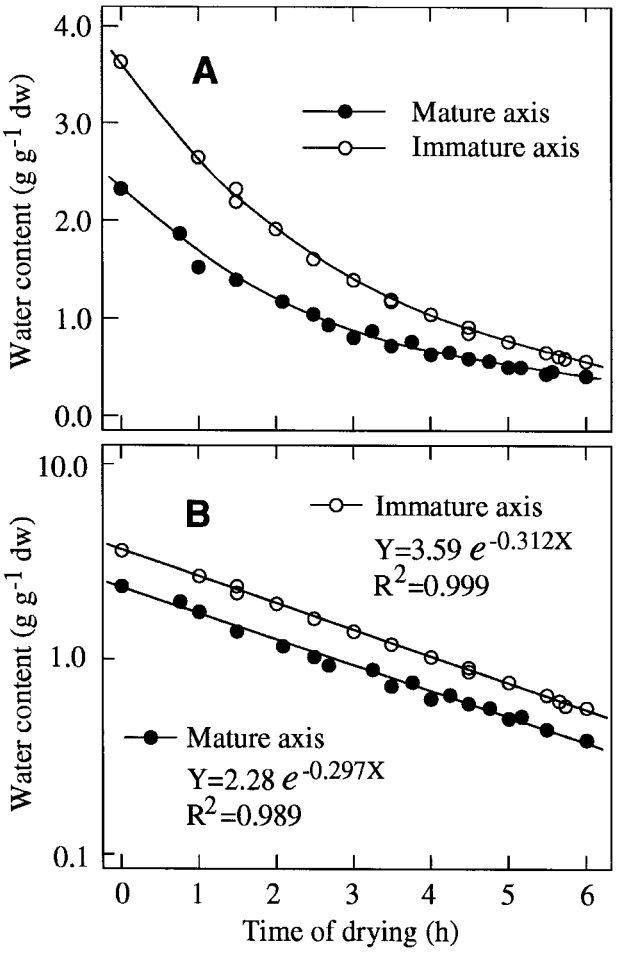

Figure 1. Drying curves of isolated immature and mature *Theobroma cacao* axes under a laminar airflow cabinet at 25°C. Water content of axes was plotted on a linear scale (A) and logarithmic scale (B). The slopes of the logarithmic plots are proportional to the rate constants of water loss (see text).

Desiccation sensitivity of cocoa seed tissues

Electrolyte leakage in mature axes increased only slightly after desiccation to a water content of about 1.0 g g^{-1} dw (Fig. 2A). However, electrolyte leakage increased remarkably when axes were desiccated to a water content of < 1.0 g g^{-1} dw. For immature axes, electrolyte leakage increased when water content was reduced to < 1.7 g g^{-1} dw. Results from leakage measurements corresponded very well to axis root survival after desiccation (Fig. 2B). Desiccation of mature axes from the initial water content to 1.0 g g^{-1} dw did not affect root survival. However, desiccation to water content < 1.0 g g^{-1} dw led to a rapid decrease in root survival. For immature axes, root survival began to decline at a much higher water content (1.7 g g^{-1} dw). Therefore, the critical water contents were 1.7

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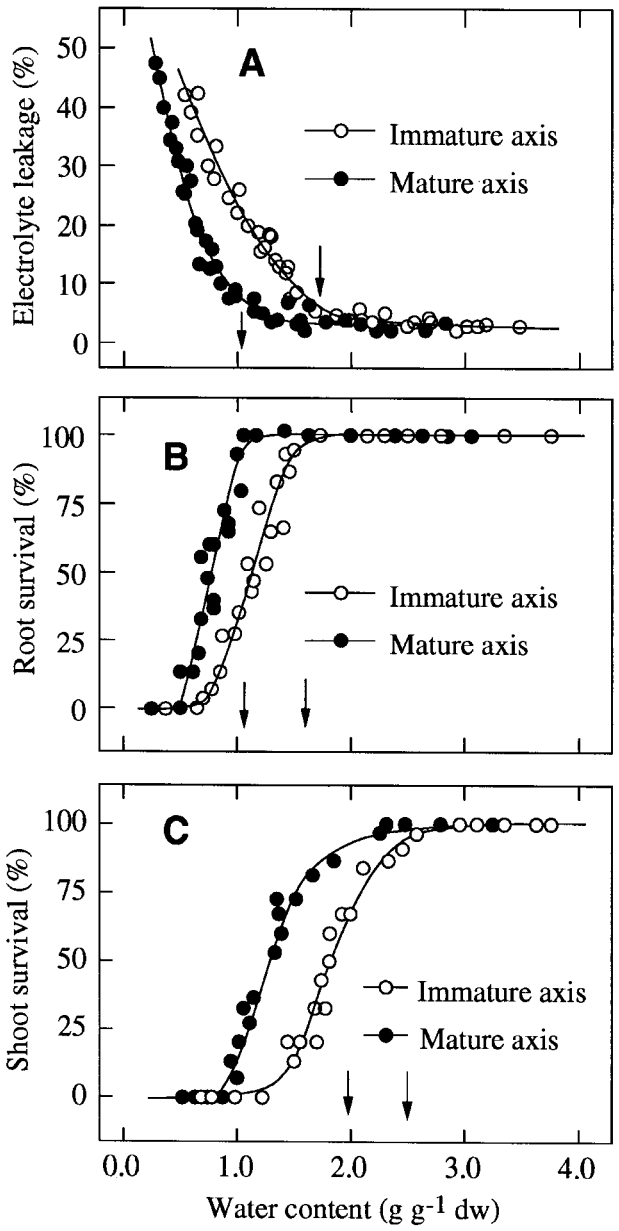

Figure 2. Desiccation sensitivity of immature and mature *Theobroma cacao* axes. Electrolyte leakage (**A**), root survival (**B**) and shoot survival (**C**) after desiccation to different water contents. Arrows indicate the start-point for the increase in electrolyte leakage or the decrease in shoot and root survival. The critical water content was determined by fitting the data with two linear regression lines and calculating water content at the intercept between the two lines.

and 1.0 g g^{-1} dw for immature and mature cocoa axes, respectively under the dehydration conditions used. Cotyledon tissues were able to tolerate drying to a much lower water content than embryonic axes

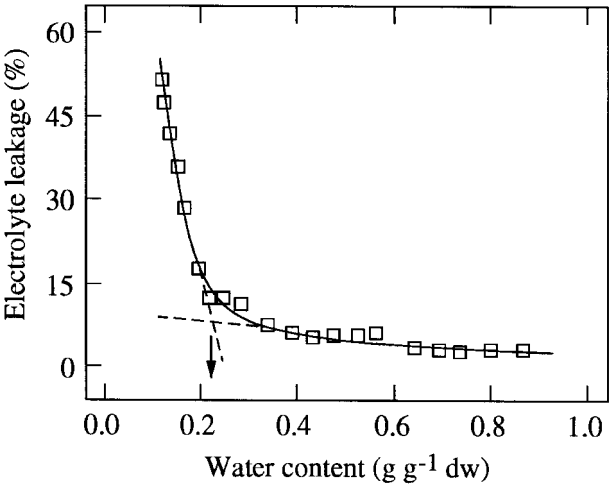

Figure 3. Electrolyte leakage of cocoa cotyledon tissues after desiccation to different water contents. The arrow indicates the critical water content for desiccation tolerance.

(Fig. 3). The critical water content for mature cotyledon tissues was 0.24 g g^{-1} dw.

Shoot survival was more sensitive to desiccation than root survival for both immature and mature axes (Fig. 2C). Shoot survival decreased after mature axes were desiccated to a water content < 2.0 g g^{-1} dw. Water contents for 50% and 0% shoot survival after desiccation were 1.3 and 1.0 g g^{-1} dw, respectively. Shoot survival for mature axes was much higher than for immature axes. Shoot survival of immature axes began to decline after desiccation to a water content < 2.5 g g^{-1} dw and was reduced to 50% and 0% after desiccation to 2.0 and 1.5 g g^{-1} dw, respectively. Lower shoot survival, relative to root survival, was probably caused by the difference in tissue size which affected the drying rate between the plumule and the root.

Reduction of activities of free radical scavenging enzymes

Activities of three free radical-scavenging enzymes — ascorbate peroxidase, peroxidase and superoxide dismutase — were monitored during desiccation of embryonic axes (Fig. 4). Activities of all three enzymes decreased as water content in axes decreased during desiccation; however, mature axes always maintained much higher enzyme activities than did immature axes. Water contents, below which activities of the three enzymes decreased rapidly, corresponded to the critical water contents for desiccation sensitivity of immature axes and mature axes. Activities of all enzymes were expressed on the basis of protein content in samples. There was no change of protein content on dry weight basis in embryonic axes during desiccation.

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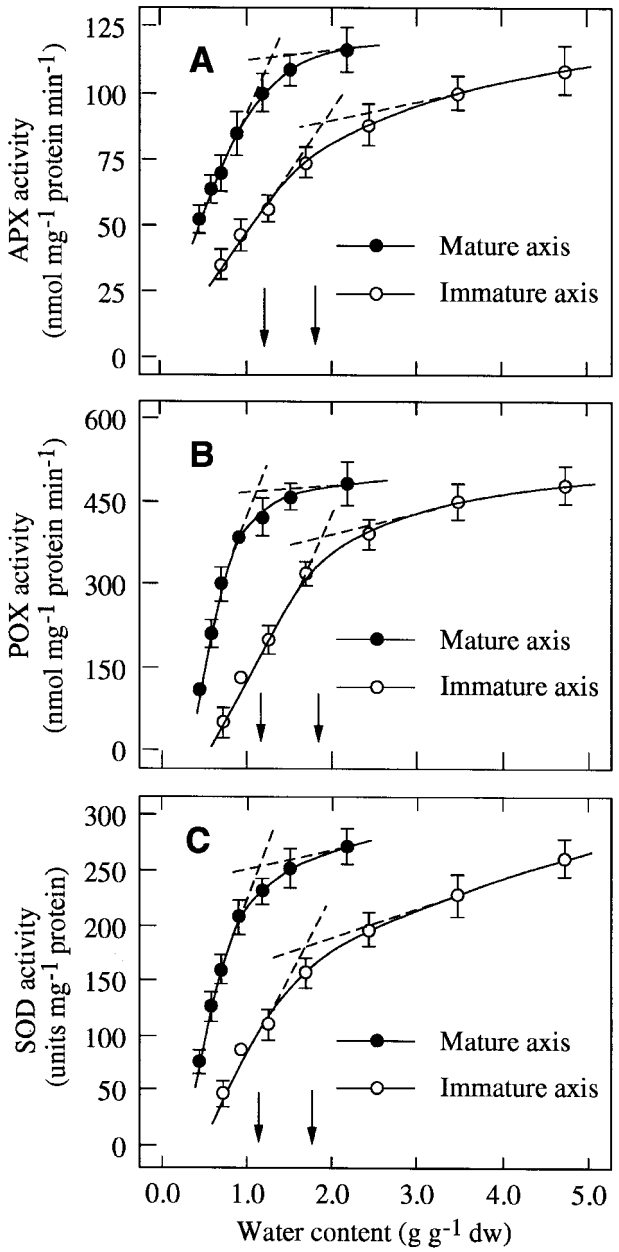

Figure 4. Effects of desiccation on activities of ascorbate peroxidase (APX, **A**), peroxidase (POX, **B**) and superoxide dismutase (SOD, **C**) in immature and mature *Theobroma cacao* axes. Data are means ± SE of three replicates. Arrows indicate the critical water content for desiccation tolerance of cocoa axes.

Accumulation of lipid peroxidation products in desiccated axes

During desiccation, the content of TBA-reactive products in embryonic axes increased (Fig. 5). TBA-reactive product content was much higher in immature axes than in fully mature axes. Water contents below which TBA-reactive product content increased sharply also corresponded to the critical water content for desiccation sensitivity. The increase in TBA-reactive products in axes was strongly correlated with the decline in the activities of free radical processing enzymes (Fig. 6).

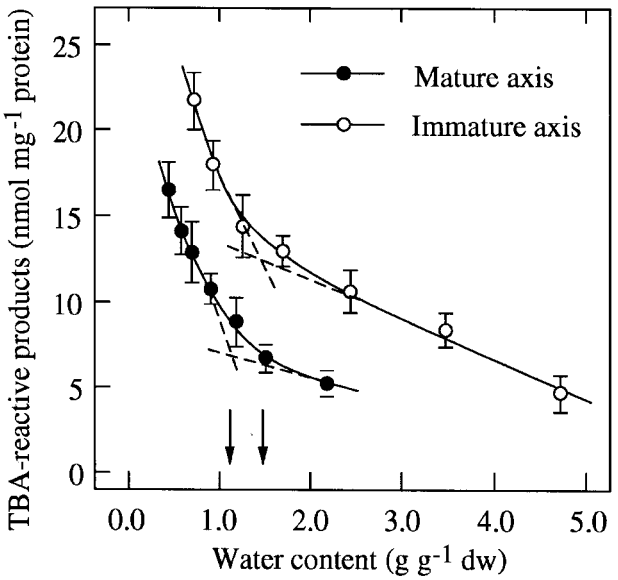

Figure 5. The content of lipid peroxidation products (TBA-reactive products) in *Theobroma cacao* axes after desiccation to the indicated water contents. The data are means ± SE of three replicates. Arrows indicate the critical water content of cocoa axes.

Activities of free radical scavenging enzymes and lipid peroxidation in cotyledon tissues

Activities of ascorbate peroxidase, peroxidase and superoxide dismutase were also measured in cocoa cotyledon tissues (Fig. 7). Superoxide dismutase activity decreased during desiccation, and the critical water content for desiccation sensitivity corresponded to the water content below which superoxide dismutase activity decreased sharply. However, ascorbate peroxidase activity was very low and did not change, whereas peroxidase activity decreased steadily during desiccation. These results showed that axis and cotyledon tissues of cocoa seeds exhibited different patterns of enzymic antioxidative responses. In correlation with the decreased superoxide dismutase activity, lipid peroxidation products in cotyledon tissues increased sharply when water content was reduced to below the critical water content of desiccation sensitivity.

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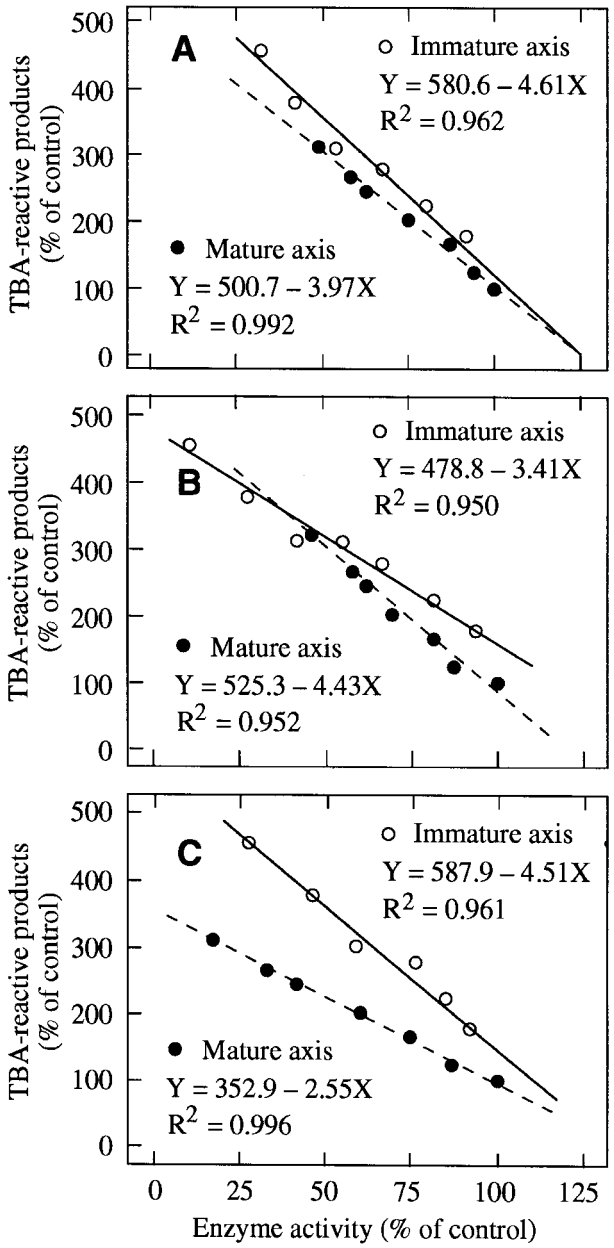

Figure 6. The correlation between lipid peroxidation products and the activities of ascorbate peroxidase (**A**), peroxidase (**B**) and superoxide dismutase (**C**) in *Theobroma cacao* axes after desiccation. The activities of enzymes and the content of lipid peroxidation products are presented as the percentage of those in undried control axes.

Soluble carbohydrates in embryonic axes

The axes of mature cocoa seeds contained large amounts of sucrose, raffinose and stachyose but only traces of reducing monosaccharides (Table 1). The total soluble carbohydrate content in fresh axes attained 219.0 mg g^{-1} dw. The axes had a sucrose-to-oligosaccharide mass ratio of 4.5:1, which is similar to many desiccation-tolerant orthodox seeds. No significant decrease in carbohydrate content was found after embryonic axes were desiccated to an average water content of 0.6 g g^{-1} dw (Table 1).

Table 1. Carbohydrate contents in hydrated and desiccated axes of mature *Theobroma cacao* seeds. Reducing sugars, such as fructose, glucose and galactose, were present in trace amount and were not quantified. Data are means ± SE of 8 to 10 samples.

Saccharide	Content (mg g^{-1} dw)	
	Hydrated axes (1.8 g water g^{-1} dw)	Dehydrated axes (0.6 g water g^{-1} dw)
Myo-inositol	3.9 ± 0.4	3.8 ± 0.3
Sucrose	176.5 ± 10.5	163.7 ± 7.5
Raffinose	22.4 ± 2.2	20.9 ± 1.9
Stachyose	16.2 ± 3.0	15.2 ± 1.8

Discussion

The relationship between the viability loss of recalcitrant seeds and oxidative stress during desiccation has been investigated by several workers (Hendry *et al*., 1992; Chaitanya and Naithani, 1994, 1998; Finch-Savage *et al*., 1994, 1996). Recalcitrant seed tissues possess both enzymic and non-enzymic protective mechanisms against free radical attack. Enzymic protective processes involve a number of enzymes including superoxide dismutase, catalase, glutathione reductase and peroxidases. The roles of enzymic and non-enzymic protective mechanisms in desiccation sensitivity of recalcitrant seeds are not generally resolved. In *Quercus robur* seeds, activities of superoxide dismutase, glutathione reductase and ascorbate peroxidase are low in axes, and the decrease in activities of these enzymes during desiccation does not correspond to desiccation sensitivity specifically. The defence mechanism in *Q. robur* axes is largely dependent on antioxidants (ascorbic acid, α-tocopherol and its precursor γ-tocopherol), whereas in cotyledons the defence mechanism is predominantly enzymic (Hendry *et al*., 1992). In *Shorea robusta* seeds, however, superoxide dismutase appears to play an active role in desiccation tolerance against free radical attack. The initial activity of superoxide dismutase is low in fresh embryonic axes, but increases significantly upon desiccation in viable seeds (Chaitanya and Naithani, 1994). The treatment that prolongs the seed storage life increases superoxide dismutase activity and decreases the O_2^- level (Chaitanya and Naithani, 1998). In cocoa axes, the

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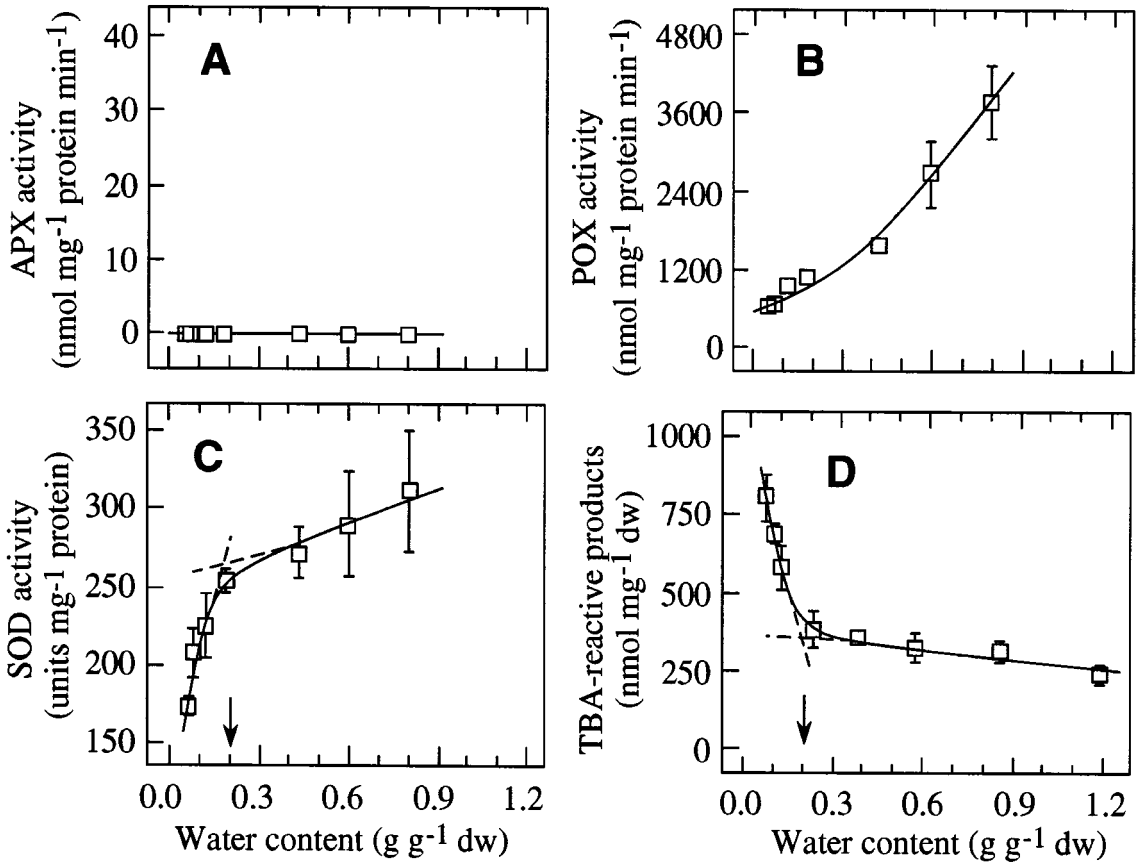

Figure 7. Changes in activities of ascorbate peroxidase (APX, **A**), peroxidase (POX, **B**) and superoxide dismutase (SOD, **C**) as well as the content of lipid peroxidation products (TBA-reactive products, **D**) in *Theobroma cacao* cotyledon tissues during desiccation. Data are means ± SE of three replicates. Arrows indicate the critical water content for cocoa cotyledons.

changes in activities of superoxide dismutase and peroxidases in response to desiccation were different from *Q. robur* and *S. robusta* (Fig. 4). The rapid decreases in activities of superoxide dismutase and peroxidases during desiccation in mature and immature cocoa axes were found to correspond to desiccation sensitivity (critical water content), as determined by viability tests and electrolyte leakage measurement (Figs 2 and 4). For cotyledon tissues, the decrease in superoxide dismutase activity was also correlated to desiccation sensitivity (Fig. 7). The results support the hypothesis that the enzymic protection against free radical attack plays an important role in desiccation sensitivity of recalcitrant seeds during desiccation. Differences between the present study and two earlier studies (Hendry *et al*., 1992; Chaitanya and Naithani, 1994) may indicate different patterns of biochemical and physiological responses to the oxidative stress among recalcitrant species. Non-enzymic antioxidants may also play an important role in desiccation sensitivity of cocoa embryonic axes and cotyledon tissues. The changes of non-enzymic antioxidant components in axes and cotyledons during desiccation are under investigation.

An increase in lipid peroxidation was reported in embryonic axes of several recalcitrant species during desiccation (Hendry *et al*., 1992; Finch-Savage *et al*., 1994, 1996; Chandel *et al*., 1995). In the present study, we observed a progressive increase in lipid peroxidation during desiccation in cocoa seed tissues (Figs 5 and 7). A decrease in enzymic protection was probably associated with lipid peroxidation in cocoa seed tissues (Figs 6 and 7). Lipid peroxidation produces highly reactive free radical intermediates that can damage membranes, proteins and nucleic acids, and was observed to precede the loss of viability in *Q. robur* axes (Finch-Savage *et al*., 1996). Although we did not measure the free radical levels in axis and cotyledon tissues, the data on activities of free radical-scavenging enzymes and lipid peroxidation suggested the presence of increased free radical oxidative pressure during desiccation. The increase in electrolyte

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leakage and the loss of seed viability probably resulted from the increased oxidative damage.

The accumulation of soluble carbohydrates such as sucrose and oligosaccharides is commonly associated with the desiccation-tolerant state in orthodox seeds. The opposite trend was found in many desiccation-sensitive tissues and germinating seeds which contain high levels of reducing monosaccharides (Koster and Leopold, 1988; Leprince *et al*., 1990, 1993; Ooms *et al*., 1993; Lin and Huang, 1994; Sun *et al*., 1994; van der Toorn and McKersie, 1995). Sucrose and oligosaccharides can stabilize membranes and macromolecules during desiccation possibly through several mechanisms such as direct hydrogen-bonding interaction and glass formation (Crowe *et al*., 1984; Carpenter *et al*., 1987; Hoesktra *et al*., 1991). Reducing monosaccharides would initiate a series of oxidative Amadori/Maillard reactions, leading to the inactivation of proteins and enzymes as well as damage to membranes and DNA (Sun and Leopold, 1995; van der Toorn and McKersie, 1995). The present study showed that mature cocoa axes contained more than 20% soluble sugars on dry weight basis and only traces of reducing monosaccharides (Table 1). The mass ratio between sucrose and oligosaccharide was 4.5 to 1, which was similar to that of some orthodox seeds (see Lin and Huang, 1994).

In conclusion, under our rapid-drying conditions cocoa embryonic axes of mature and immature seeds were unable to tolerate desiccation to a water content < 1.0 and < 1.7 g g^{-1} dw, respectively. Cotyledon tissues were more desiccation-tolerant than embryonic axes. Desiccation sensitivity of cocoa axes and cotyledons was correlated with a rapid decrease in enzymic protection against oxidative attack. Recalcitrance of cocoa axes was not caused by a lack of sugar-related protective mechanisms.

Acknowledgments

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