Journal of Integrative Plant Biology 2009, **51** (7): 646–653

The Response Difference of Mitochondria in Recalcitrant *Antiaris toxicaria* Axes and Orthodox *Zea mays* Embryos to Dehydration Injury

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Abstract

Long-term preservation of recalcitrant seeds is very difficult because the physiological basis on their desiccation sensitivity is poorly understood. Survival of *Antiaris toxicaria* axes rapidly decreased and that of immature maize embryos very slowly decreased with dehydration. To understand their different responses to dehydration, we examined the changes in mitochondria activity during dehydration. Although activities of cytochrome (Cyt) *c* oxidase and malate dehydrogenase of the *A. toxicaria* axis and maize embryo mitochondria decreased with dehydration, the parameters of maize embryo mitochondria were much higher than those of *A. toxicaria*, showing that the damage was more severe for the *A. toxicaria* axis mitochondria than for those of maize embryo. The state I and III respiration of the *A. toxicaria* axis mitochondria were higher than those of maize embryo, the former rapidly decreased, and the latter slowly decreased with dehydration. The proportion of Cyt *c* pathway to state III respiration for the *A. toxicaria* axis mitochondria was low and rapidly decreased with dehydration, and the proportion of alternative oxidase pathway was high and slightly increased with dehydration. In contrast, the proportion of Cyt *c* pathway for maize embryo mitochondria was high, and that of alternative oxidase pathway was low. Both pathways decreased slowly with dehydration.

Key words: alternative oxidase pathway; cytochrome *c* oxidase pathway; dehydration; mitochondria; orthodox; recalcitrance; respiration rate; seed.

Song SQ, Tian MH, Kan J, Cheng HY (2009). The response difference of mitochondria in recalcitrant *Antiaris toxicaria* axes and orthodox *Zea mays* embryos to dehydration injury. *J. Integr. Plant Biol.* **51**(7), 646–653.

Available online at www.jipb.net

Orthodox seeds are shed from parent plants when water content is low, having undergone maturation drying prior to this event, and can generally be further dried until the water content is in the range of 1%–5% (wet mass basis) without damage. Recalcitrant seeds, however, do not undergo maturation drying, and are shed at relatively high water content when still metabolically active. Such seeds are highly susceptible to desiccation injury, and thus are not storable under conditions suitable for orthodox seeds. Furthermore, many recalcitrant seeds are sensitive to chilling injury (Pammenter and Berjak 1999). It has been suggested that desiccation sensitivity of seeds was associated with some processes involving late embryogenic abundant (LEA) or LEA-like proteins, accumulation of sugars, aspects of reactive oxygen species (ROS) and non-enzymic and enzymic antioxidants (Berjak 2006; Berjak and Pammenter 2008). Different processes may confer protection against the consequences of water loss at different hydration levels (Vertucci and Farrant 1995), and the absence or ineffective expression of one or more of these could determine the relative degree of desiccation sensitivity of seeds of individual species (Song et al. 2003; Berjak and Pammenter 2004). However, to date the various deficiencies underlying desiccation sensitivity of recalcitrant seeds are generally conjectural.

Mitochondrial activity in seeds, as in other aerobic organisms, provides energy and carbon skeletons for other cellular processes, and are also involved in the production of ROS through one-electron carriers in the respiratory chain (Møller 2001). It has been shown that structures and functions of

Received 8 Oct. 2008 Accepted 9 Apr. 2009
Supported by the National Natural Science Foundation of China (30870223).
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doi: 10.1111/j.1744-7909.2009.00836.x

mitochondria are also very susceptible to oxidative stress (Lenaz 1998). Furthermore, plant mitochondria contain two terminal oxidases, cytochrome *c* oxidase (CCO) and a cyanide-resistant, alternative oxidase. Electron flux through these two respiratory pathways is controlled by environmental conditions and stimuli received by mitochondria (McIntosh et al. 1998). These stimuli include low temperature, wounding, pathogen attack, elevated carbohydrate status, cell culture stage, addition of ethylene, fruit ripening and elevation of salicylic acid levels (McIntosh 1994; Vanlerberghe and McIntosh 1997).

Antiaris toxicaria is a tree species that is endemic to tropical forests of Xishuangbanna, and is protected by law in China (State Environmental Protection Administration of China and Institute of Botany of the Chinese Academy of Sciences 1987). It has been reported that *A. toxicaria* seeds are sensitive to dehydration and are typical recalcitrant seeds (Cheng and Song 2008). To our knowledge, little is known about the relationship between dehydration sensitivity of seeds and mitochondria activity. In the present paper, we used a system of recalcitrant *A. toxicaria* axes and orthodox maize embryos, and isolated and purified their mitochondria to investigate the response difference of mitochondria on dehydration injury.

Results

Changes in water content and survival during dehydration

Mean water contents of *A. toxicaria* axes and maize embryos were 1.111 and 1.666 g/g, respectively. When they were dehydrated at 45% relative humidity (RH) and at 15 $\pm$ 1 °C, the water content of *A. toxicaria* axes and maize embryos rapidly decreased ($P \leq 0.001$); the time at which their water content decreased by 50% were 0.81 and 0.51 d, respectively (Figure 1A).

Survival of *A. toxicaria* axes rapidly decreased with dehydration ($P \leq 0.001$), and the W_{50} were 0.374 g/g. The maize embryos slowly decreased during the early phase of dehydration, and then markedly decreased when water content of embryos was lower than 0.235 g/g ($P \leq 0.001$), and the W_{50} was 0.11 g/g (Figure 1B). The W_{50} of maize embryos was much lower than that of *A. toxicaria*. It was noted that survival of *A. toxicaria* axes was zero when they were dehydrated to a water content of 0.34 g/g. Maize embryos, when dehydrated to a water content of 0.10 g/g, were still 23%, showing that dehydration tolerance of maize embryos was much larger than that of *A. toxicaria* axes (Figure 1B).

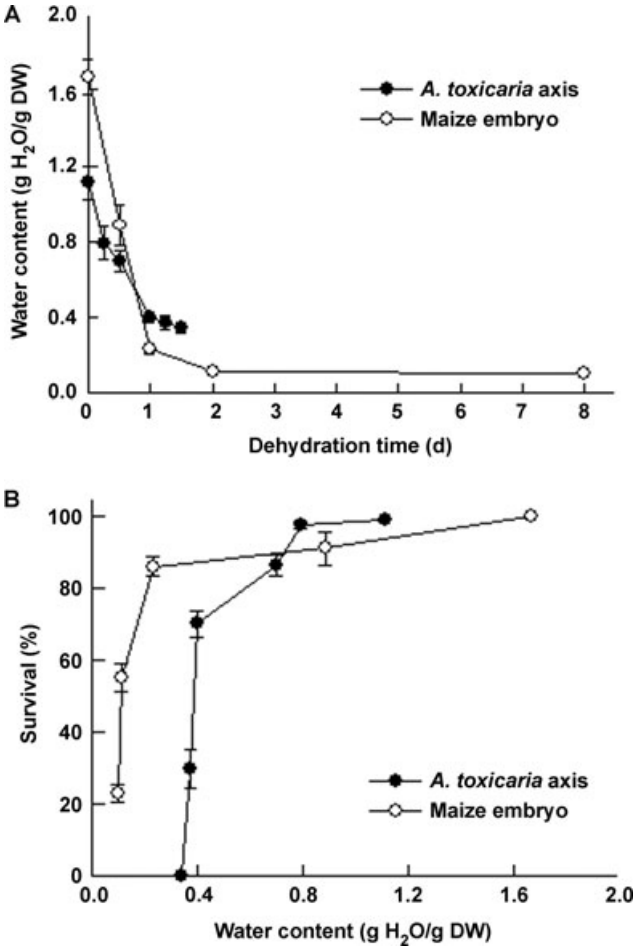

Figure 1. The changes in water content (**A**) and survival (**B**) during dehydration of *Antiaris toxicaria* axes and maize embryos.

A. toxicaria axes and maize embryos were dehydrated for different times at 15 °C and 45% relative humidity (RH), and then germinated. All values are means $\pm$ *SD* of four replicates of 25 axes or embryos each.

Changes in CCO activities and CCO activity latencies of mitochondria

After dehydration to different water contents, mitochondria of *A. toxicaria* axes and maize embryos were prepared, and CCO activities and CCO activity latencies of their mitochondria were assayed. CCO activity of the *A. toxicaria* axis and maize embryo mitochondria gradually decreased with dehydration ($P \leq 0.001$) (Figure 2A). The CCO activities of maize embryo mitochondria were higher than those of the *A. toxicaria* axis after dehydration. For example, when water content of *A. toxicaria* axes and maize embryos decreased by 50%, the CCO activities of their mitochondria decreased by 47% and 33%, respectively (Figure 2A).

Although CCO activity latencies of the *A. toxicaria* axis ($P = 0.004$) and maize embryo ($P \leq 0.001$) mitochondria decreased with dehydration, those of maize embryo mitochondria were consistently higher than those of the *A. toxicaria* axis during dehydration, and those of the maize embryo mitochondria obviously decreased until their water content was lower than 0.11 g/g (Figure 2B).

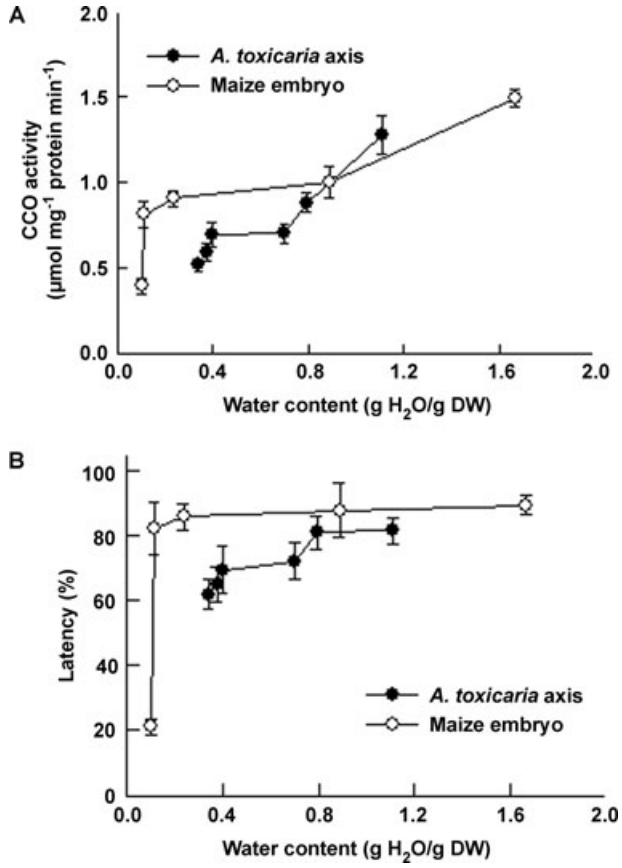

Figure 2. Changes in activities **(A)** and latencies **(B)** of cytochrome *c* oxidase (CCO) of isolated mitochondria during dehydration of *Antiaris toxicari* axes and maize embryos.

After *A. toxicaria* axes and maize embryos were dehydrated to different water contents at 15 °C and 45% relative humidity (RH), their mitochondria were immediately isolated. All values are means ± *SD* of four replicates.

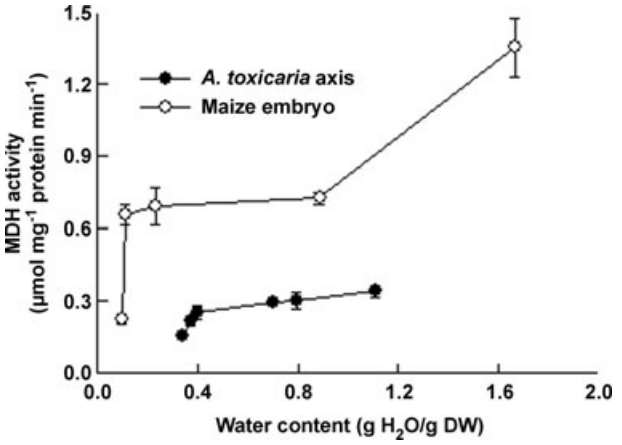

Figure 3. Changes in NAD$^+$-malate dehydrogenase (MDH) activities of isolated mitochondria during dehydration of *Antiaris toxicaria* axes and maize embryos.

After *A. toxicaria* axes and maize embryos were dehydrated to different water contents at 15 °C and 45% relative humidity (RH), their mitochondria were immediately isolated. All values are means ± *SD* of four replicates.

Changes in NAD$^+$-malate dehydrogenase activities of mitochondria

NAD$^+$-malate dehydrogenase (MDH) activities of the *A. toxicaria* axis and maize embryo mitochondria decreased continuously with dehydration ($P \leq 0.001$), and those of maize embryo mitochondria were much higher than those of the *A. toxicaria* axis (Figure 3).

Changes in respiratory rate of mitochondria

The basic respiratory rate (state I) of mitochondria from newly collected *A. toxicaria* axes was much higher than that of sample from newly collected maize embryos. The basic respiratory rate of the *A. toxicaria* axis mitochondria rapidly decreased during the early phase of dehydration ($P \leq 0.001$), while that of the maize embryo mitochondria decreased relatively slowly with dehydration ($P \leq 0.001$) (Figure 4A). When the water contents of *A. toxicaria* axes and maize embryos decreased by 50%, the basic respiratory rate of their mitochondria decreased by 70% and 17%, respectively (Figure 4A).

The changes in state III respiratory rate of the *A. toxicaria* axis and maize embryo mitochondria were similar to those of their basic respiratory rate; that is, with dehydration, the respiratory rate of the *A. toxicaria* axis mitochondria dramatically decreased ($P \leq 0.001$), and that of the maize embryo mitochondria decreased relatively slowly ($P \leq 0.001$). It is noted that the respiratory rate of the *A. toxicaria* axis mitochondria were consistently higher than that of the maize embryo during dehydration (Figure 4B).

Proportional changes in the respiratory pathway

To assess the proportional changes in the cytochrome (Cyt) *c* and alternative oxidase (AOX) pathways in respiratory state III (respiratory rate) during dehydration, potassium cyanide (KCN) (a CCO inhibitor) and salicylhydroxamic acid (SHAM, an AOX inhibitor) were used in the present study. The respiratory rate is composed of activities of the Cyt *c* pathway, the AOX pathway and other oxygen consumption pathways. For the *A. toxicaria* axis mitochondria, the proportion of the respiratory rate inhibited by KCN to the state III respiratory rate was 32.5%, and rapidly decreased with dehydration ($P \leq 0.001$), and decreased to zero when the water contents of *A. toxicaria* axes were decreased to 0.400 g/g (Figure 5A). However, for the maize embryo

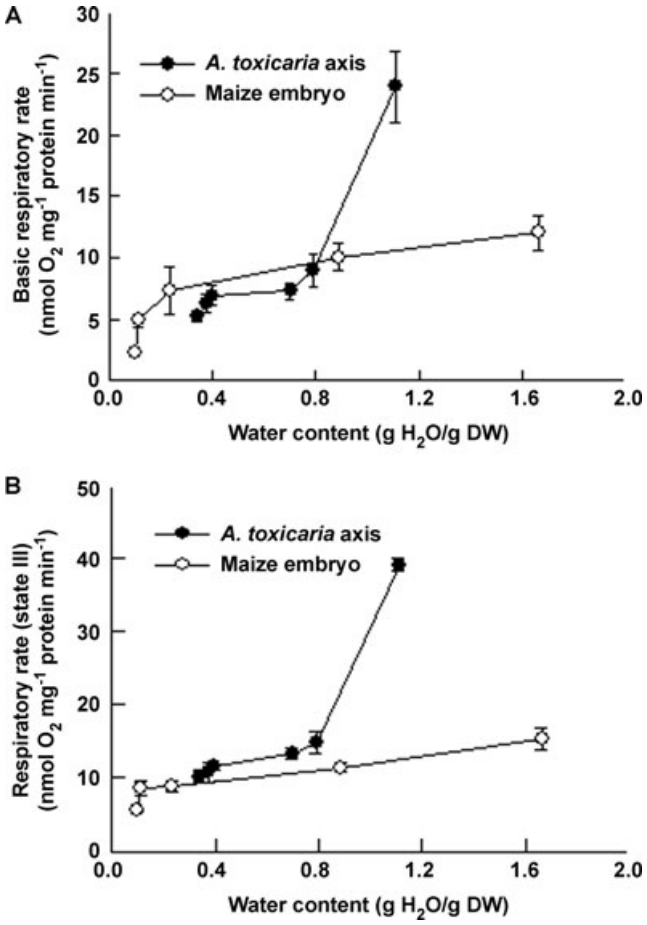

Figure 4. Changes in basic respiratory rate **(A)** and respiratory rate (state III) **(B)** of isolated mitochondria during dehydration of *Antiaris toxicaria* axes and maize embryos.

After *A. toxicaria* axes and maize embryos were dehydrated to different water contents at 15 °C and 45% relative humidity (RH), their mitochondria were immediately isolated. Respiratory rate was expressed as nmol O_2/mgprotein/min. All values are means ± *SD* of four replicates.

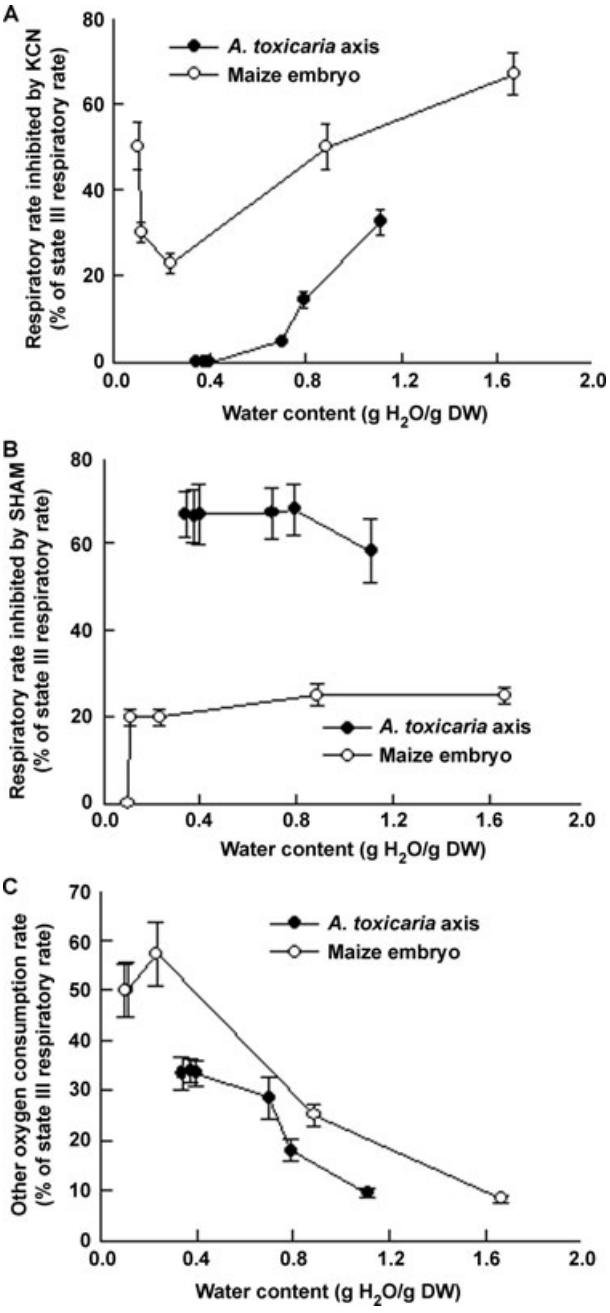

Figure 5. Changes in respiratory rate inhibited by potassium cyanide (KCN) **(A)**, and by salicylhydroxamic acid (SHAM) **(B)** and other oxygen consumption pathways **(C)** of isolated mitochondria during dehydration of *Antiaris toxicaria* axes and maize embryos.

Respiratory rate was expressed as a percentage of the state III respiratory rate. All values are means ± *SD* of four replicates.

mitochondria, the proportion of respiratory rate inhibited by KCN to the state III respiratory rate was 66.7%, and decreased during the early phase of dehydration, but then increased ($P \leq 0.001$) (Figure 5A). The respiratory rate inhibited by KCN of the maize embryo mitochondria was much higher than that of *A. toxicaria* mitochondria (Figure 5A).

In addition, for the *A. toxicaria* axis mitochondria, the proportion of respiratory rate inhibited by SHAM to the state III respiratory rate was 58%, and increased during the early phase of dehydration, and then maintained a constant level ($P \leq 0.472$) (Figure 5B). In contrast to *A. toxicaria* axis mitochondria, the proportion of respiratory rate inhibited by SHAM of maize embryo mitochondria to state III respiratory rate was 25%, and decreased slightly with dehydration up to a water content of 0.113 ± 0.007 g/g, and then decreased rapidly to zero when the water content of embryos was 0.101 g/g ($P \leq 0.001$) (Figure 5B).

The respiratory rate of maize embryo mitochondria inhibited by SHAM was much less than that of *A. toxicaria* mitochondria (Figure 5B).

Other oxygen consumption pathways, besides those inhibited by KCN and SHAM, occurred in mitochondria of *A. toxicaria* axes and maize embryos. The proportion of other oxygen consumption pathways to the state III respiratory rate increased with dehydration ($P \leq 0.001$) (Figure 5C).

Discussion

Survival of *A. toxicaria* axes decreased significantly with dehydration, and their W_{50} was 0.374 g/g (Figure 1B), showing that *A. toxicaria* axes are highly sensitive to dehydration, and are typically recalcitrant. The response of *A. toxicaria* axes to dehydration was similar to those obtained by Cheng and Song (2008), who reported that seeds and axes of *A. toxicaria* are sensitive to desiccation, and were correlated with the increase in $\cdot O_2^-$ production rate, content of hydrogen peroxide and thiobarbituric acid (TBA)-reactive products, and the decline in the activities of antioxidant enzymes of seeds and axes. In the present study, the water content of maize embryos was 1.67 g/g (Figure 1), indicating that they had not completed maturation drying. Although survival of maize embryos also decreased with dehydration, their desiccation tolerance (W_{50}= 0.11 g/g) was much higher than that of *A. toxicaria* axes (W_{50}= 0.374 g/g); for example, when maize embryos were dehydrated to a water content of 0.113 g/g, their survival was still 55% (Figure 1B).

Cytochrome *c* oxidase is the terminal complex of the electron transport chain (ETC), and a marker enzyme of mitochondria (Douce 1985). The CCO activity of the *A. toxicaria* axis and the maize embryo mitochondria gradually decreased with dehydration (Figure 2A), suggesting that the CCO was subjected to damage by dehydration. Alternatively, enzyme activity may have declined as water content became limited. However, as the CCO activity latencies of the *A. toxicaria* axis mitochondria decreased with dehydration (Figure 2B), it may be assumed that the mitochondria membranes gradually deteriorated with dehydration, and that the damage was more severe for the *A. toxicaria* axis mitochondria than for the maize embryo.

NAD^+-malate dehydrogenase occurs in the mitochondrial matrix, where it converts malate into oxaloacetate. Mitochondria of the *A. toxicaria* axes and maize embryos showed gradually decreasing MDH activity with dehydration (Figure 3), suggesting either damage to the enzyme or inactivation as a consequence of declining water content. MDH activities of maize embryo mitochondria were much higher than those of the *A. toxicaria* axis mitochondria (Figure 3), which also showed that the injury of the *A. toxicaria* axis mitochondria was greater than that of the maize embryos.

The basic respiratory rate and the respiratory rate (state III) of the *A. toxicaria* axis mitochondria were much higher than those of the maize embryos (Figure 4). These results are in agreement with Berjak and Pammenter (1997), who proposed that recalcitrant seeds are metabolically active at shedding, and may be equated in many cases to 'developing seedlings', not mature orthodox seeds. With dehydration, the basic respiratory rate and the respiratory rate of the *A. toxicaria* axis mitochondria rapidly decreased, while those of the maize embryo mitochondria slowly decreased (Figure 4). This suggests that the decrease in the basic respiratory rate and the respiratory rate of mitochondria in recalcitrant *A. toxicaria* axes and in immature orthodox maize embryos was related to desiccation sensitivity, and that desiccation sensitivity of the *A. toxicaria* axis mitochondria was much higher than that of the maize embryo.

Plant mitochondria possess a branched ETC comprising two main pathways: the Cyt *c* pathway (terminating at CCO) and the AOX pathway (terminating at AOX). Both pathways obtain their electrons from reduced ubiquinone (Atkin et al. 2002). Clearly, the Cyt *c* pathway (inhibited by KCN), the AOX pathway (inhibited by SHAM) and other oxygen consumption pathways (not inhibited by KCN and SHAM) were all operative in the *A. toxicaria* axis and maize embryo mitochondria, and the proportion of oxygen consumption by these pathways during dehydration was different (Figure 5). For the *A. toxicaria* axis mitochondria, the proportion of the Cyt *c* pathway to state III respiratory rate was 32.5%, and rapidly decreased with dehydration (Figure 5A). For example, the Cyt *c* pathway decreased by 91% when water contents of the *A. toxicaria* axes decreased by 50% (Figure 5A). The proportion of the AOX pathway to the state III respiratory rate was 58%, and increased during the early phase of dehydration, and then maintained a constant level (Figure 5B). The reason why the proportion of the AOX pathway slightly increased might be a decrease in the proportion of the Cyt *c* pathway during dehydration. However, for the maize embryo mitochondria, the proportion of the Cyt *c* pathway to the state III respiratory rate was 66.7%, and decreased relative slowly during the early phase of dehydration. For example, the Cyt *c* pathway decreased by 27% when water contents of the maize embryos decreased by 50% (Figure 5A). The proportion of AOX to the state III respiratory rate of the maize embryo mitochondria was 25%, and decreased slightly with dehydration, and finally decreased rapidly to zero (Figure 5A). These results showed that for the recalcitrant *A. toxicaria* axis mitochondria, the Cyt *c* pathway was very sensitive to dehydration as indicated by KCN inhibition; but for immature orthodox maize embryo mitochondria, sensitivity of the Cyt *c* pathway to dehydration was much less than that of the *A. toxicaria* axis mitochondria. The AOX pathway of the *A. toxicaria* axis mitochondria was not desiccation sensitive, but that of the maize embryo mitochondria was slightly sensitive during the early phase of dehydration, and was very sensitive during the late phase of dehydration.

The proportion of other oxygen consumption pathways of the *A. toxicaria* axis and maize embryo mitochondria increased with dehydration (Figure 5C); however, it is not known whether these oxygen consumption pathways were implicated in rotenone-resistant "external" and "internal" NAD(P)H dehydrogenases of mitochondria.

To our knowledge, this is the first report on the response differences of mitochondria in recalcitrant and orthodox embryos (or axis) to dehydration injury. Whether these results are in keeping with the mitochondria of other seeds requires more research. It has been proposed that the AOX pathway increases in response to several stress situations, such as low temperature (Vanlerberghe and McIntosh 1992), inhibition of the Cyt *c* pathway (Wagner and Krab 1995), the application of inhibitors of mitochondrial protein synthesis (Day et al. 1996), and the production of ROS (Wagner and Krab 1995). A possible role of the AOX pathway may be in protecting plants from ROS, or in sustaining respiration under conditions where the cytochrome *c* pathway is restricted (Lennon et al. 1997; Lambers et al. 2005).

Materials and Methods

Plant material

Fruits of *Antiaris toxicaria* (Pers.) Lesh were manually collected at maturity in May, 2005 from trees growing in Xishuangbanna Tropical Botanical Garden (21°41′N, 101°25′E; altitude, 570 m), Menglun, Mengla, Yunnan, China. The annual mean temperature of this location is 21.4 °C with a mean winter minimum of 15.6 °C and a mean summer maximum of 25.3 °C; rainfall is 1557 mm per year, of which 264 mm is in the dry season (November–April), and the remainder in the rainy season (May–October). After extraction from the fruits, seeds were cleaned in water, and then surface-sterilized in a solution of 1% sodium hypochlorite, rinsed three times in sterilized water, and kept at 15 °C until use after the water content of seed surface was dried.

Maize (*Zea mays* L. *cv*. Nongda 108) ears were collected at 28 d after pollination from plants growing in Xishuangbanna Tropical Botanical Garden in July 2005. After extraction from the cobs, seeds were kept at 15 °C until use.

Desiccation treatment

After extraction from the seeds, the *A. toxicaria* axes and maize embryos were dehydrated for different times at 45% RH and at 15 ± 1 °C.

Water content determinations

Water contents of the *A. toxicaria* axes and maize embryos were determined gravimetrically (after drying at 80 °C for 48 h). Five axes or embryos were sampled each time for these determinations. Water contents of the *A. toxicaria* axes and maize embryos are expressed on a dry mass basis (g H_2O/g dry weight (DW), g/g).

The term, W_{50}, was used for the water content at which 50% of axes, or embryos were killed by dehydration.

Survival assessment

Four replicates of 25 axes or 25 embryos each were germinated on moist filter paper in closed Petri dishes in the dark at 30 ± 1 °C for 7 d. The axes or embryos showing radicle extension of 2 mm were scored as having survived.

Preparation of mitochondria

All procedures were carried out between 0 and 4 °C. Mitochondria were prepared according to the method of Struglics et al. (1993) modified as follows: 200 *A. toxicaria* axes, or 400 maize embryos were homogenized in a pre-cooled homogenizer in 250 mL of ice-cold extraction medium containing 0.3 mol/L sucrose, 20 mmol/L 3-N-morpholinopropanesulphonic acid (MOPS) (pH 7.2) and 1 mmol/L ethylenediaminetetraacetic acid (EDTA). The brei was squeezed through a 300 μm mesh nylon cloth. The filtrate was centrifuged at 1 000 *g* for 10 min. The supernatant was then centrifuged at 41 400 *g* for 30 min. The pellet was resuspended in washing medium (0.3 mol/L sucrose, 10 mmol/L MOPS, 1 mmol/L EDTA, pH 7.2), centrifuged at 1 000 *g* for 10 min, and then the supernatant was centrifuged at 41 400 *g* for 30 min. The resultant pellet (crude fraction) was resuspended in a small volume of washing medium.

The crude fraction (containing mitochondria, peroxisomes and amyloplasts) was separated on a self-generating Percoll gradient. In the bottom of a centrifuge tube 15 mL of 28% Percoll in 0.3 mol/L sucrose and 10 mmol/L MOPS (pH 7.2) was introduced, on top of which, 21 mL of the Percoll/MOPS solution in 0.3 mol/L mannitol was layered. Three milliliters of the crude fraction were layered on the top, and a Percoll gradient generated by centrifugation at 41 400 *g* for 35 min.

The mitochondrial bands were removed using a pump, diluted 10 times in washing medium and pelleted at 15 000 *g* for 30 min to remove the Percoll. This procedure was repeated once. The pellets were finally resuspended in washing medium. The volume of the resultant samples was measured, and dimethylsulphoxide (DMSO, an ultra-low-temperature protector) then added to the sample to make the final DMSO concentration of 5%. The samples were frozen in liquid nitrogen and stored at –80 °C.

Assay of Cytochrome *c* oxidase

Cytochrome *c* oxidase (EC 1.3.9.1) activity was measured using a DU 800 spectrophotometer (Beckman Coulter Co., Fullerton, CA, USA) in the presence or absence of 0.025% (w/v) Triton

X-100 (TX). The percentage latency of CCO was calculated as 100 × ((rate + TX) – (rate – TX)) / (rate + TX) (Møller et al. 1987; Rasmusson and Møller 1991).

Assay of malate dehydrogenase

NAD^+-malate dehydrogenase (EC 1.1.1.37) was assayed using a DU 800 spectrophotometer in the reverse direction (oxidation of NADH) according to Møller et al. (1987).

Measurement of oxygen consumption

Respiratory rate was measured according to the method of Attucci et al. (1991) modified as follows. Oxygen consumption by mitochondria was measured at 25 °C using a Clark electrode system (Hansatech Ltd, Norfolk, UK). The reaction medium contained 0.3 mol/L sucrose, 5 mmol/L MOPS (pH 7.2), 5 mmol/L KH_2PO_4, 2.5 mmol/L $MgCl_2$, 0.1% bovine serum albumin (BSA), 20 μL 1.0 mol/L succinate, 4 μL 25 mmol/L adenosine diphosphate (ADP), and 20 μL of mitochondria, in a total volume of 1 mL. The O_2 concentration in the air-saturated medium was taken as 250 nmol/L. Respiratory rate was corrected for the oxygen consumption by the electrode, and was expressed as nmol O_2/mg protein/min.

Inhibitor experiments

Respiratory rate of mitochondria inhibited by KCN and SHAM was measured according to the method of Attucci et al. (1991) modified as follows. Oxygen consumption by mitochondria was measured at 25 °C using a Clark electrode system. Four microliters of the inhibitors, 500 mmol/L KCN or 500 mmol/L SHAM, were added to a total volume of 1 mL of the mitochondria, containing the reaction medium described above. Respiratory rate was expressed as a percentage of the state III respiratory rate. The other oxygen consumption rate = 100 – (respiratory rate inhibited by KCN and SHAM).

Protein assay

Protein was measured following the procedure of Bradford (1976), using BSA as a standard.

Statistical analysis

All data were analyzed using a one-way ANOVA model from the SPSS 11.0 package for Windows (SPSS Inc., 2006).

Acknowledgements

We are grateful to Professor Patricia Berjak and Professor Norman Pammenter (School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa) for their comments on this paper.

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(Handling editor: Pieter B. F. Ouwerkerk)