Desiccation tolerance of recalcitrant *Theobroma cacao* embryonic axes: the optimal drying rate and its physiological basis

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**Abstract**

Recalcitrant seed axes were reported to survive to lower water contents under fast-drying conditions. The present study was to examine the hypothesis that drying rate and dehydration duration could interact to determine desiccation tolerance through different physico-chemical mechanisms. The effect of drying rate on desiccation tolerance of *Theobroma cacao* seed axes at 16 °C was examined. Rapid-drying at low relative humidity (RH) and slow-drying at high RH were more harmful to cocoa axes, because electrolyte leakage began to increase and axis viability began to decrease at high water contents. Maximum desiccation tolerance was observed with intermediate drying rates at RH between 88% and 91%, indicating the existence of an optimal drying rate or optimal desiccation duration. This maximum level of desiccation tolerance for cocoa axes (corresponding to a critical water potential of −9 MPa) was also detected using the equilibration method, in which axes were dehydrated over a series of salt solutions or glycerol solutions until the equilibrium. These data confirmed that the physiological basis of the optimal drying rate is related to both mechanical stress during desiccation and the length of desiccation duration during which deleterious reactions may occur. The optimal drying rate represents a situation where combined damages from mechanical and metabolic stresses become minimal.

**Key words:** Critical water potential, desiccation sensitivity, drying rate, recalcitrant seed, *Theobroma cacao*, water stress.

**Introduction**

Recalcitrant seeds lose their viability at relatively high water content during dehydration. Their desiccation sensitivity imposes a serious problem for the long-term conservation of many tropical plant genetic resources. The effect of drying rate on desiccation sensitivity of recalcitrant *Avicennia marina* seeds was observed some time ago (Farrant *et al.*, 1985). Since then, many studies have examined the effect of drying methods on desiccation tolerance and cryopreservation of recalcitrant seeds or excised axes (Normah *et al.*, 1986; Berjak *et al.*, 1990, 1993; Pammeter *et al.*, 1991, 1998, 1999; Pritchard, 1991; Kioko *et al.*, 1998; Pammeter and Berjak, 1999; Sun, 1999). Fast-drying was generally found to permit recalcitrant seeds or excised axes to survive to lower water contents, and to improve the survival after cryopreservation (Potts and Lumpkin, 1997; Kioko *et al.*, 1998; Pritchard and Manger, 1998). The improvement in desiccation tolerance under fast-drying conditions has been attributed to the fact that recalcitrant seeds or excised axes spend less time at a partially dried state (Pammeter *et al.*, 1998). In addition, uneven water distribution in seed tissues may be related to the improved desiccation tolerance during the fast-drying of axes (Pammeter *et al.*, 1998; Tompsett and Pritchard, 1998). Meristemic tissues have higher water contents than other parts of axes (Pammeter *et al.*, 1998). Drying rates also affect the desiccation tolerance of somatic embryos (Senaratna *et al.*, 1989, 1991; Tetteroo *et al.*, 1995; Timbert *et al.*, 1996; Li *et al.*, 1999) and immature zygotic embryos or seeds (Blackman *et al.*, 1992; Hong and Ellis, 1997; Lima *et al.*, 1998). Unlike mature recalcitrant seed tissues, however, the slow-drying method was beneficial.

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to somatic embryos and immature seeds. In the latter case, it was believed that seed tissues lost water slowly and could acquire desiccation tolerance during the period of prolonged dehydration.

A common problem in many earlier studies dealing with dehydration of recalcitrant seed tissues was that drying conditions were poorly defined and that drying rates were not quantified or specified. In those studies, the fast-drying condition was normally referred to as the dehydration of seed tissues under a laminar flow cabinet or with silica gel, while slow-drying was the drying of seed tissues with testa or in closed containers. The terms fast-drying, intermediate-drying and slow-drying were used without measuring the rate of water loss. Moreover, the response of recalcitrant seeds to dehydration was mostly studied within a narrow range of drying rates. In order to understand the relationship between drying rate and desiccation tolerance, one has to study the response of recalcitrant seeds to a wide range of drying rates or desiccation conditions. The mechanism, by which drying rate affects desiccation tolerance, needs to be investigated within a theoretical framework of physico-chemical stresses during the period of dehydration.

In the present study, a new measure has been devised to quantify the drying rate and to examine the expression of desiccation tolerance of *Theobroma cacao* axes under a wide range of drying rates. These data show the existence of an optimal drying rate for maximum desiccation tolerance in *Theobroma cacao* axes. The objective of the present study is to examine the hypothesis that the interaction between drying rate and dehydration duration affects mechanical and physico-chemical parameters that are associated with the degree of desiccation damage and desiccation tolerance of recalcitrant seeds.

**Materials and methods**

**Plant materials**

Mature fruits of *Theobroma cacao* (cocoa) were harvested from a Malaysian plantation. Fruits were temporarily stored at 16 °C and normally used within one week. Cylindrical axes were large with a diameter of ~2–3 mm and a length of 8–10 mm. Fresh axes weighed about 35–40 mg each, with a dry weight about 8–10 mg. Isolated axes were washed and imbibed in distilled water for 2 h before dehydration treatments.

**Control of drying conditions**

In the first set of experiments, saturated salt solutions (with excessive salt present) were used to control varying relative humidities (RH). Various salts (with the equilibrium RH of their saturated solutions at 16 °C in brackets) included NaOH (6%), K acetate (23.5%), MgCl₂ (33%), K₂CO₃ (44%), Ca(NO₃)₂ (58%), NH₄NO₃ (70%), NaCl (75.5%), NH₄Cl (79.5%), KCl (86%), K₂CrO₄ (88%), ZnSO₄ (92%), KNO₃ (95.5%), and K₂SO₄ (97%). Excised axes were dried over saturated solutions in sealed, pre-equilibrated GA-7 culture vessels, which contained 10 ml of saturated solutions plus 20 g of additional salt. In another set of experiments, drying of axes was achieved through the equilibration with glycerol solutions and unsaturated NaCl solutions. Sixteen solutions were prepared with water potential ranging from ~25 to ~2 MPa. Each GA-7 culture vessel contained 200 g of solution. The final water potential of solutions after the dehydration experiment was determined according to the weight change of solutions (as described by Sun and Gouk, 1999). Since the mass ratio of solution to seed axes was at least 200, the difference between the initial water potential and the final water potential was negligible.

**Dehydration of embryonic axes**

Various drying rates were obtained under different RH. About 20 axes were dried over saturated salt solutions in a GA-7 culture vessel at 16 °C. Axes were spread into a single layer onto a nylon mesh basket supported by a plastic stand. For each drying rate experiment, 15 samples (vessels) were prepared. Samples were regularly taken for the measurement of water content, electrolyte leakage and axis viability. Experiments were repeated 2–4 times for each RH. To investigate the influence of temperature, the effect of drying rates on desiccation tolerance was also studied at 25 °C. In the other experiment, axes were allowed to reach equilibrium with the dehydrating solutions. Samples were taken after 6 d and 12 d for the determination of water content, electrolyte leakage and axis viability. Water content of axes was determined gravimetrically after drying at 103 °C for 24 h, and was expressed in g water g⁻¹ dry weight (g g⁻¹ dw).

**Measurement of desiccation damage**

To determine electrolyte leakage, 8–10 axes were soaked in a vial containing 30 ml distilled water. The vial was gently shaken for 1 h, and the conductivity of imbibition water was then measured. Total conductivity was measured after axes were killed in boiling water for 20 min and cooled to room temperature (overnight). Electrolyte leakage due to desiccation damage was expressed as the percentage of the total conductivity. In order to measure the loss of axis viability during dehydration, axes were first disinfected before desiccation with 0.1% HgCl₂ for 3 min and rinsed with sterilized water 4–5 times. The axes were then dried over aseptic saturated salt solutions or under a laminar flow cabinet. Samples of 20–25 axes were taken after different periods of drying and were cultured on hormone-free Murashige and Skoog medium. Axes dehydrated over unsaturated NaCl and glycerol water potential solutions were disinfected after drying. Root survival, shoot survival and axis length were recorded after 10 d of culture. Shoots were considered viable if they had new growth or remained healthy (white), while roots were considered viable if radicals were healthy (white) and showed elongation and no crack. (Cocoa axes contain high concentrations of lipids and phenolic compounds. Once damaged, axes would turn brownish immediately and then die. Axes that are white can recover.)

**Results**

**Drying rates of cocoa axes under various RH conditions**

Water content curves during desiccation were biphasic for all RH (Fig. 1). During the first phase of drying, the loss of water followed a simple exponential function. During
the second phase, axes had reached equilibrium with the saturated solutions, and thus water content did not decrease further. Because water loss during the first phase could be described by an exponential function, the rate constants of drying \((k)\) were calculated according to the first-order kinetics (Fig. 1), and were used as an expression of drying rate in the present study. Figure 2 shows the rate constants of drying and the time to reach apparent equilibrium under various RH. The equilibration between axes and salt solutions was achieved rapidly at RH < 80%, whereas at 85% < RH < 96%, the equilibration time increased exponentially. For example, it took more than 10 d to reach the apparent equilibrium at 94% RH. At RH > 96%, the equilibration time decreased again.

Effect of drying rates on desiccation tolerance of excised axes

Under the condition used in the present study, cocoa seeds can be easily stored for more than 2 months without significant loss of seed viability and vigour. Figure 3 shows changes in water content and electrolyte leakage of axes during drying at 95.5% and 97% RH for up to 28 d. At 97% RH, no increase in electrolyte leakage was observed after dehydration to \(\sim 2.0 \text{ g g}^{-1} \text{ dw}\) and during subsequent storage. At 95.5% RH, electrolyte leakage increased significantly after water content of seed axes decreased to below a critical level. The increase of electrolyte leakage was apparently not due to their short storage life but desiccation damage during drying. Figure 4 shows the changes of electrolyte leakage of cocoa axes after dehydration to different water contents under various RH. In all cases, electrolyte leakage increased rapidly after water content decreased to below a critical level. The relationship between critical water content and dehydrating RH was shown in Fig. 5.
Critical water content was higher when axes were dried under low RH or very high RH. Maximum desiccation tolerance for cocoa axes was observed between 88% and 91% RH.

Figure 6 shows the effect of drying rates on critical water contents of cocoa axes at 16 °C. At rate constants of drying > 0.12 h⁻¹, critical water content maintained at ~1.1–1.2 g g⁻¹ dw. Critical water content began to decrease rapidly when rate constant of drying decreased from 0.12 h⁻¹ to 0.02 h⁻¹. However, a further decrease in drying rate was accompanied with a sharp increase in critical water content. There was an optimal drying rate around 0.02 h⁻¹ for cocoa axes to achieve maximum desiccation tolerance. The effect of temperature was also studied. In these experiments, axes were dried at 25 °C under a laminar flow cabinet or different RH. A similar relationship between drying rate and the critical water content was observed at both 16 °C and 25 °C (Fig. 6). The temperature effect appears to be related to the drying rate.

The loss of axis viability was also used as an indicator of desiccation damage. Figure 7 shows the survival of
roots and shoots after axes were dried to various water contents. Critical water content was calculated to be 0.87, 0.92 and 0.90 g g⁻¹ dw according to root survival, shoot survival and axis growth, respectively. Critical water contents obtained from viability tests at different drying rates were superimposed in Fig. 6, and show an excellent agreement with results from the electrolyte leakage measurements.

In an attempt to understand the physiological basis of the optimal drying rate, the critical water content and equilibrium water content of axes that were dried under different atmospheric water potentials were compared (Fig. 8). The maximum desiccation tolerance of axes (0.63 g g⁻¹ dw, −9 MPa) was achieved at dehydrating atmospheric water potentials between −12 and −17 MPa (88–91% RH).

**Determination of desiccation tolerance by the equilibrium dehydration method**

Figure 9 shows changes in axis viability and electrolyte leakage after equilibration for 6 d and 12 d over a series of solutions with different water potentials. Preliminary results showed that water content of axes remained constant after 9–10 d of equilibration. Axes had not reached equilibrium with solutions in 6 d, and therefore axis viability (shoot and root survival) remained very high except for samples equilibrated at water potential below −16 MPa. When axes reached the equilibrium with the solutions after 12 d, a critical water potential was found at −8.5 MPa, below which axis viability decreased sharply. However, axis growth decreased at higher water potential (−6 MPa). No axis survived dehydration to −12 MPa (Fig. 9). Electrolyte leakage of axes increased rapidly when water potential was below −8.5 MPa. Figure 10 shows the relationship between the axis survival and tissue water content after equilibration. When data were plotted against water content, identical curves were observed for samples equilibrated for 6 d and 12 d, and a critical water content of ∼0.7 g g⁻¹ dw was observed. Axis length was affected at slightly higher water content (Fig. 10C). The critical water content of axes that were dried under different atmospheric water potentials were compared (Fig. 8). The maximum desiccation tolerance of axes (0.63 g g⁻¹ dw, −9 MPa) was achieved at dehydrating atmospheric water potentials between −12 and −17 MPa (88–91% RH).

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potential (−8.5 MPa) and the critical water content (≈ 0.7 g g⁻¹ dw), detected with the equilibrium dehydration method, were in good agreement with the maximum desiccation tolerance (0.63 g g⁻¹ dw, −9 MPa) observed in non-equilibrium drying studies (Figs 4, 5, 6).

Discussion

Water loss kinetics and the rate constant of drying under controlled dehydration

Although drying rate has been known to affect desiccation sensitivity of recalcitrant seed tissues for more than a decade, a good measure for the rate of water loss during drying has not been developed. The lack of quantitative data about the effect of a wide range of drying rates on desiccation tolerance was, in part, attributed to the lack of a quantitative measure of drying rate. In most cases, drying curves cannot be described by a linear function of time. During drying, the initial rate of water loss from seed tissues is more rapid, and the rate of water loss decreases thereafter. Recently, it was found that, under a constant drying condition, water loss from cocoa axes follows a negative exponential function, which can be described by using the first-order-kinetics. The first-order relationship between water loss and drying time was used to compare drying curves for axes at different maturity with varying initial water contents (Li and Sun, 1999). In the present study, water loss of mature cocoa axes, dried under constant RH between 6% and 97% or in a laminar air flow cabinet, was found to conform to the first-order-kinetics before the tissue reaches an equilibrium with the surrounding atmosphere (Fig. 1). Therefore, drying rate of axes can be expressed by the rate constant of water loss (i.e. the slope k) during drying. The data of drying curves for seeds of *Aesculus hippocastanum* (Tompsett and Prichard, 1998), *Ekeberia capensis* (Pammenter et al., 1998) and *Trichilia dregeana* (Pammenter et al., 1999) have been analysed and it was found that the first-order-kinetics is also applicable to other seed tissues.

The rate constant (k) of water loss is not just a convenient parameter for the expression of drying rate. The biological meaning of drying rate constant becomes apparent when one considers the hydraulic conductivity of seed tissues. The volume flow of water from seed tissue to dehydrating atmosphere is proportional to the difference in water potential (∆Ψ) between seed tissue and surrounding atmosphere, and can be written as

\[ V_w = A L_p (Ψ_o - Ψ_i) \]  

where \( V_w \) is the volume flow of water per unit time (m³ s⁻¹), \( A \) is the surface area of seed tissue (m²), and \( L_p \) is the hydraulic conductivity coefficient of seed tissue (m s⁻¹ Pa⁻¹). The \( Ψ_o \) and \( Ψ_i \) are external (air) and internal (tissue) water potential, respectively. The rate constant \( k \) is related to the hydraulic conductivity coefficient \( L_p \) and to external (air) water potential (Ψo). The difference in water potential (∆Ψ or Ψo−Ψi) is the hydrostatic pressure, a measure of the mechanical force for dehydration stress. Under constant RH (i.e. constant Ψo), Ψi is a function of drying time (t) that describes the change of tissue water potential during drying, whereas \( k \) is a property of seed tissue and its value is proportional to \( L_p \). When \( L_p \) is small and limits the volume flow of water within the tissue, uneven dehydration occurs. A careful study of water loss dynamics would allow us to better understand the complex stress-time-viability relationship for recalcitrant seeds during drying. The relationship between \( k \) and \( L_p \), as well as the contribution of tissue or cellular parameters to \( k \) and \( L_p \) are still under investigation.

The optimal drying rate and its physiological basis

The response of recalcitrant seeds or excised axes to desiccation is affected by seed developmental status (Hong and Ellis, 1990; Farrant et al., 1992; Finch-Savage, 1992; Sun and Leopold, 1993; Sun et al., 1994; Farrant and Walters, 1998) and dehydration conditions, such as drying rate (Normah et al., 1986; Berjak et al., 1990, 1993; Pammenter et al., 1991, 1998; Pritchard, 1991) and temperature (Leprince et al., 1995). Drying rate affected desiccation tolerance significantly, and fast-drying has been reported to improve desiccation tolerance in a number of recalcitrant seeds (reviewed by Pammenter and Berjak, 1999). Under slow-drying condition, the seed tissue has to stay longer at intermediate water contents, at
which aqueous-based deleterious processes would occur due to the loss of co-ordinated regulation of metabolism or failure of antioxidant systems (Pammenter et al., 1991; Berjak and Pammenter, 1997; Pritchard and Manger, 1998). It was observed in Ekebergia capensis that, at similar water contents, slowly-dried axes showed more extensive deterioration of cellular membranes than the rapidly-dried seed tissues (Pammenter et al., 1998). In some recalcitrant seeds, slow-drying might permit the initiation of germination so that seeds become increasingly more sensitive to desiccation (Farrant et al., 1985). These interpretations imply that greater desiccation tolerance may be achieved with a faster drying rate. In the present study, it was shown that an optimal drying rate existed for recalcitrant cocoa axes to achieve maximum desiccation tolerance (Figs 4, 5, 6). Data points for Ekebergia capensis axes (Pammenter et al., 1998) were presented for comparison in Fig. 6. The optimal drying rate could not be determined because drying rate >0.10 h⁻¹ was not studied.

The finding of an optimal drying rate is of interest to further studies on the mechanism of desiccation sensitivity of recalcitrant seeds. The effect of drying rate on desiccation tolerance is associated not only with the regulation of metabolisms (the physico-chemical aspects), but also with the physical process of dehydration itself (the mechanical aspects). It is conceivable that under low RH the water potential of seed axes will change very rapidly (i.e. extremely high ∂Ψ/∂T). As a result, the uneven, rapid volumetric change would inevitably induce great damage within the well-organized seed tissues (and also cells), unless the seed tissues are able to withstand such enormous mechanical stresses. Even for desiccation-tolerant orthodox seed tissues, few are able to survive very rapid-drying without showing desiccation damages. As the drying rate reduces, the mechanical force of dehydration stress (rapid change of tissue water potential) will decrease significantly, whereas the dehydration time will increase exponentially (Fig. 2). Therefore, under very slow-drying conditions, seed axes may be damaged by various deleterious processes that take place during the period of prolonged dehydration, ranging from the disruption of metabolic regulation to the failure of antioxidant systems. Figure 11 shows the relationship between critical water content, drying rate constant (k) and time to reach critical water content. The rate constant of drying is used here as a convenient indicator for the mechanical force of dehydration stress. It is clearly shown that the optimal drying rate represents a situation, where combined damages from mechanical stress and metabolic (physico-chemical) stress would probably reach minimal.

A simple method to determine the optimal drying rate

The determination of the optimal drying rate has practical significance to the successful cryopreservation of recalcitrant seed materials. The optimal drying rate is expected to vary with recalcitrant species. The method used to examine the dehydration responses of cocoa axes in the present study is not easy and is time-consuming, which involves the careful monitoring of water loss and axis viability under various drying conditions, and the calculation of drying rate and critical water content for every dehydration regime. In order to develop a simple protocol, an equilibrium dehydration method was tested, in which seed axes were allowed to equilibrate with a series of salt solutions or glycerol solutions of known water potentials or RH (Figs 9, 10). Upon equilibrium, desiccation damage can be assessed for seed axes dehydrated at different water potentials or RH, and the critical water potential or RH of desiccation damage (i.e. the onset level) could be determined. This critical water potential or RH should correspond to the dehydration condition, under which the optimal drying rate is achieved. For cocoa axes, this test shows that the critical water potential for desiccation damage (~8.5 MPa), determined by the equilibrium dehydration method (Fig. 9), indeed, corresponded very well to the water potential of maximum desiccation tolerance (Ψₘ, ~9 MPa) (Fig. 8).

The theoretical basis for the equilibrium dehydration method is that the optimal drying rate is related to a minimal level of combined damages from mechanical and metabolic stresses during dehydration (Fig. 11). Mechanical stress within seed tissues during drying is minimized when Ψₒ increases (less negative), whereas metabolic stress (i.e. the stress-time phenomenon) is minimized as Ψₒ decreases and k increases. Under the equilibrium condition, the minimal level of combined stress damage will be found when Ψₒ approaches Ψₘ, the water potential of the maximum desiccation tolerance. At Ψₒ < Ψₘ, seed tissues will be damaged due to excessive dehydration upon equilibrium. Therefore, the onset water
potential of desiccation damage equals $\Psi_m$ and the optimal $\Psi_o$. When a series of $\Psi_o$ or RH are properly selected, it is possible to determine the optimal $\Psi_o$ or RH for a recalcitrant seed. It should be pointed out, however, that desiccation damage has to be assessed immediately upon equilibrium. Electrolyte leakage and axis viability at equilibrium with specific water potential would change with increasing storage time, and therefore would affect experimental results.

In conclusion, there exists an optimal drying rate for cocoa axes to achieve the maximum desiccation tolerance. The optimal drying rate of cocoa axes is $\sim 0.02$ h$^{-1}$, corresponding to RH between $88\%$ and $91\%$. The optimal drying condition for recalcitrant seeds can be determined using the equilibrium dehydration method.

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