Plant Physiol. (1991) 96, 1093–1098
0032-0889/91/96/1093/06/$01.00/0

Received for publication November 6, 1990
Accepted March 9, 1991

Homeohydrous (Recalcitrant) Seeds: Dehydration, the State of Water and Viability Characteristics in *Landolphia kirkii*

N. W. Pammenter, Christina W. Vertucci*, and Patricia Berjak

Plant Cell Biology Research Group, Department of Biology, University of Natal, Durban, 4001 South Africa (N.W.P., P.B.); and U.S. Department of Agriculture, Agricultural Research Service, National Seed Storage Laboratory, Fort Collins, Colorado 80523 (C.W.V.)

ABSTRACT

Differential scanning calorimetry was used to study the relationships among drying rate, desiccation sensitivity, and the properties of water in homeohydrous (recalcitrant) seeds of *Landolphia kirkii*. Slow drying of intact seeds to axis moisture contents of approximately 0.9 to 0.7 gram/gram caused lethal damage, whereas very rapid (flash) drying of excised embryonic axes permitted removal of water to approximately 0.3 gram/gram. The amount of nonfreezable water in embryonic axes (0.28 gram H_2O/gram dry mass) did not change with drying rate and was similar to that of desiccation-tolerant seeds. These results suggest that the amount of nonfreezable water *per se* is not an important factor in desiccation sensitivity. However, flash drying that removed all freezable water damaged embryonic axes. Differences between desiccation-sensitive and -tolerant seeds occur at two levels: (a) tolerant seeds naturally lose freezable water, and sensitive seeds can lose this water without obvious damage only if it is removed very rapidly; (b) tolerant seeds can withstand the loss of a substantial proportion of nonfreezable water, whereas sensitive seeds are damaged if nonfreezable water is removed.

Desiccation tolerance of organisms involves, *inter alia,* the ability to withstand loss of water sorbed to macromolecular structures, particularly the surfaces of membranes, without irreversible denaturation (3, 4). A current theory, the "water replacement hypothesis," suggests that, in desiccation-tolerant tissues, water closely associated with macromolecular surfaces can be replaced by polyhydroxyl compounds that stabilize the macromolecules as water is withdrawn (3, 4). This hypothesis implies that such water replacement does not occur in desiccation-sensitive tissue.

Recalcitrant, or "homeohydric" (1), seeds are shed when the water content is high and when they are desiccation intolerant. A model describing homeohydric seed behavior suggests that the more rapid the rate of dehydration, the lower the water content the seeds can tolerate (6). Both experimental (5) and observational (8) evidence support this. Most homeohydric seeds are too large to be dried quickly, but if embryonic axes are excised, they can be dried rapidly. Isolated axes subjected to this "flash" drying retain viability to a much lower water content than axes in intact seeds dried more slowly over silica gels (1).

In this paper, we address (a) to what extent drying rate can affect the minimum moisture content that homeohydric tissues can survive, (b) whether the rate of water removal affects the thermal properties of water remaining in the tissues, and (c) whether there is a correlation between the thermal properties of water and expression of desiccation sensitivity. If desiccation tolerance is related to the ability of tissue to lose "bound" water without the denaturation of macromolecules, there may be differences in the thermal characteristics of water in axes of homeohydrous seeds flash dried and those dried more slowly. The relationships among dehydration rate, desiccation tolerance, and the state of water were studied using embryonic axes of the homeohydric seeds of *Landolphia kirkii* Dyer. This species is a viny shrub native to Mozambique and the inland region of northeastern South Africa. Its large (approximately 1.5 g) endospermic seeds are typical of many other seeds from tropical species in that they are intolerant to chilling and desiccation, the embryonic axis is fully developed when it sheds, and there are no tendencies toward dormancy. If maintained at their original water content, the seed will lose viability in approximately 1 month.

MATERIALS AND METHODS

Seed Collection and Transport

The details of collection and transport of recalcitrant seeds for experimental use may materially affect results (2). For these experiments, intact mature fruits of *Landolphia kirkii* were hand harvested in the field and transported, within 48 h of collection, in plastic bags by road to Durban, South Africa. The fruits are hard coated and contain 15 to 30 seeds to which the sticky fruit pulp adheres. The seeds lose virtually no water in the intact fruit. On receipt, seeds were removed from the fruit, cleaned by rubbing with paper towels, briefly surface sterilized with 10% bleach, dusted with a fungicidal powder, and sealed in plastic bags. They were then immediately air freighted in the temperature/pressure-controlled hold to the laboratory in Fort Collins, CO, where they were stored in the plastic bags at 25°C. The time lag between removal of the seeds from the fruits and receipt in Fort Collins was 5 to 7 d and between receipt and initiation of experiments never more than 7 d.

Drying Treatments

Rapid (flash) drying was achieved by passing air at room temperature (approximately 22°C) at 9 L/min through four fish-tank air diffusers in parallel evenly spaced on the bottom of a plastic box 10 cm long, 10 cm wide, and 4 cm deep. Excised embryonic axes were placed on a fine-mesh nylon supported 2 cm above the air stones and removed after the required drying time. Axes that were used in subsequent tissue culture were briefly surface sterilized before flash drying. For slow drying, intact seeds were stored in a monolayer 7 cm above a 2-cm deep layer of activated silica gel in a sealed container. The silica gel was replaced when it began to lose the deep blue color of the indicator. Water contents were determined gravimetrically on isolated axes (both flash and slow dried) by enclosing them in aluminum DSC[1] volatile sample pans, puncturing the lids and heating at 90°C for 24 h. Water contents are expressed as g H_2O/g dry mass.

Viability Assessment

Viability of flash-dried axes was assessed by embryo culture. The culture medium was that formulated by Normah *et al.* (10) for *Hevea brasiliensis*. A surviving axis expanded, greened, and developed roots in 2 to 4 weeks. Axes that were necrotic or showed no development after 2 to 3 months were scored as nonsurviving. Whole seeds that had been dried over silica gel were set out to germinate in damp vermiculite. In most cases, the germination assay was complete within 2 weeks, by which time radicles of surviving seeds had extended at least 2 cm, and seeds that did not germinate had severe microbial infestation and were mushy.

The degree of dehydration damage was also assessed by measuring the rate of leakage of electrolytes from individual axes (11) using a conductivity meter (ASAC-1000 seed analyzer, Neogen Corp.). Conductivity of the soaking water was measured at 5-min intervals for the initial 40 min of soaking. Leakage rate was determined from a linear regression of the leakage on time data.

Calorimetry

Thermal transitions of water freezing and melting were measured using a Perkin Elmer DSC-4 or DSC-7 with temperatures calibrated between −95 and 156°C using methylene chloride and indium standards. Axes dried for different times were sealed in aluminum sample pans and were cooled at 10°C/min to −70°C and then heated at 10°C/min to 20°C. The energy of a thermal transition was determined from the area of the peak above or below the baseline. After the thermal transitions were recorded, the pans were punctured and dry weights and water contents determined as described above. Transition enthalpies expressed per unit dry weight of the sample were plotted against sample water content, the intercept on the abscissa giving the water content at which water is nonfreezable under the conditions used here. The temperature of the transitions was determined as the intersection between a line drawn tangential to the steepest part of the peak and the baseline (the onset temperature).

RESULTS

Flash drying and drying over silica gel resulted in considerably different drying rates (Fig. 1). Within 30 min, flash drying reduced embryonic axis water content from approximately 1.5 to approximately 0.32 g/g, after which further water loss was slow. It took 3 d of drying intact seeds over silica gel to reduce axis water content to a similar level.

The moisture contents at which at least 80% survival was maintained differed for the two drying regimens (Fig. 2). Flash-dried axes showed a decline in survival in culture only after water content had been reduced to <0.32 g/g. Seeds dried over silica gel showed only 30% germination at a water content of 0.75 g/g. Leakage of electrolytes from isolated axes substantiated these observations (Fig. 3). There was a marked increase in leakage rate, indicative of substantial subcellular damage, from flash-dried axes only at water contents of approximately 0.4 to 0.3 g/g, and a similar increase in leakage rate was observed from axes of seeds dried over silica gel at 0.9 to 0.7 g/g water content.

DSC thermograms were used to study some thermal characteristics of water in tissues at different hydration levels (Fig. 4). Although both cooling behavior and heating behavior were measured, only heating thermograms are presented in Figure 4 because the freezing exotherms were usually single sharp peaks that could be described by onset temperature and energy of melt. Tissues at high water contents showed a broad endothermic peak, presumably from melting of ice. Often, a sharp peak centered around 0°C was superimposed on the broad peak. The sharp peak was always present at water contents >0.7 g/g but was not observed at water contents

[1] Abbreviations: DSC, differential scanning calorimetry; g/g, g H_2O/g dry mass.

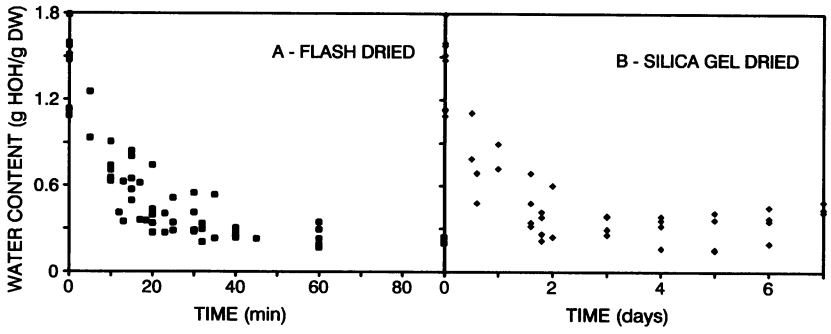

Figure 1. Drying time course for embryonic axes of *L. kirkii*. A, Isolated axes flash dried; B, axes dried in intact seeds stored over silica gels. Note the different time scales for A and B.

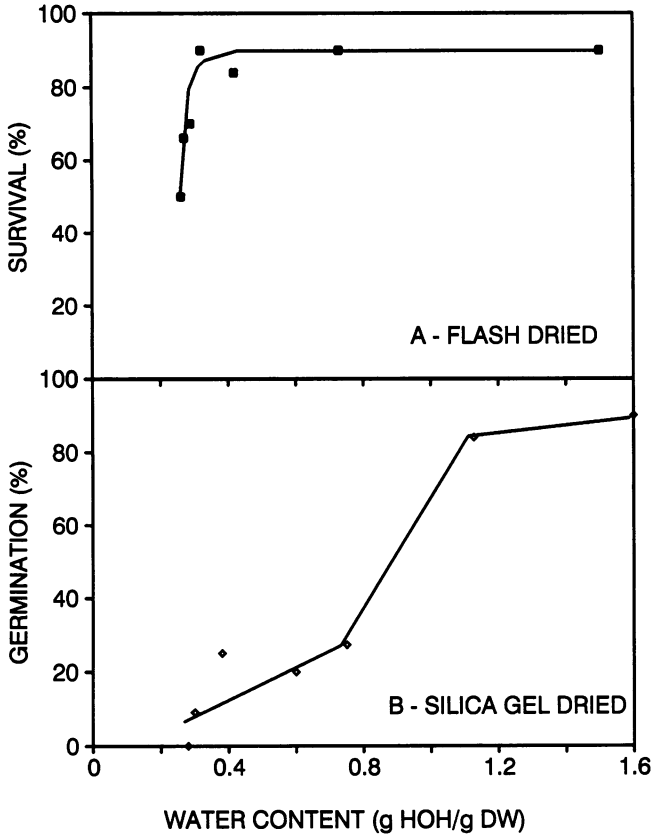

Figure 2. Response of desiccation sensitivity of embryonic axes of *L. kirkii* to drying rate. A, Flash-dried isolated axes; B, axes slowly dried in intact seeds stored over silica gels.

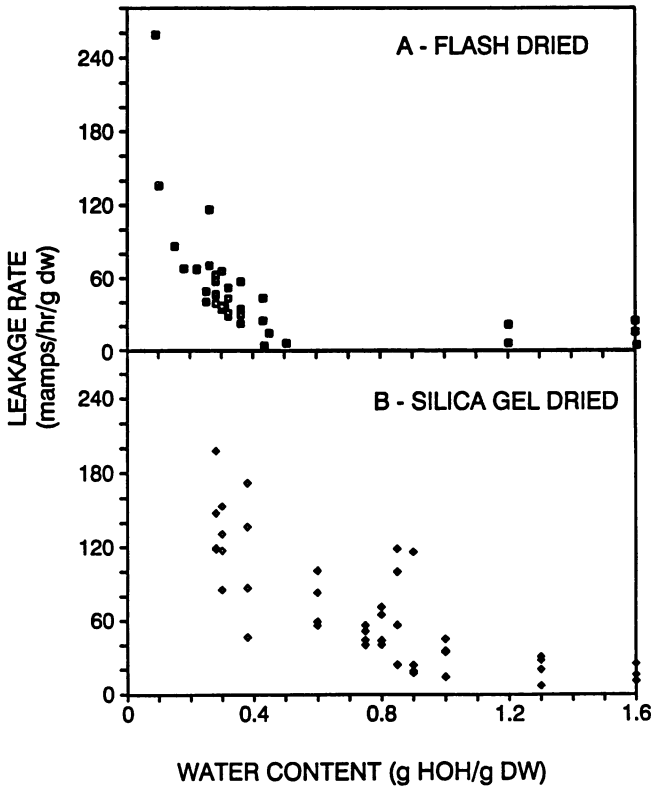

Figure 3. Rate of electrolyte leakage from embryonic axes of *L. kirkii* dried at different rates. A, Flash-dried isolated axes; B, axes slowly dried in intact seeds stored over silica gels.

<0.5 g/g. Neither freezing nor melting transitions were apparent at water contents below approximately 0.37 and 0.28 g/g, respectively. Essentially the same patterns were observed for flash-dried axes and axes from seeds dried over silica gel.

The onset temperature of the freeze (Fig. 5A) occurred between −15 and −55°C. At moisture contents between 1.6 and 0.8 g/g, the onset temperature declined slightly with decreasing water content, as would be expected for a dilute solution of increasing concentration. At water contents <0.8 g/g, the onset temperature of the freeze declined sharply with decreasing water content. A similar trend was observed for the onset temperature of the broad endothermic peak observed during heating (Fig. 5B). However, the onset temperature of the sharp peak did not change with water content.

At water contents <1.2 g/g, the enthalpies of the melting and freezing transitions were linearly related to axis water content (Fig. 6). For both transitions, the results from flash-dried axes and axes from seeds dried over silica gel were similar (the enthalpy of the melt in axes from seeds dried over silica gel was slightly lower; Table I). The intercepts of the plots on the abscissae give the water contents at which freezable water is no longer present. These values for the melting and freezing transitions were 0.28 and 0.37, respectively, and there was no significant difference between the drying treatments.

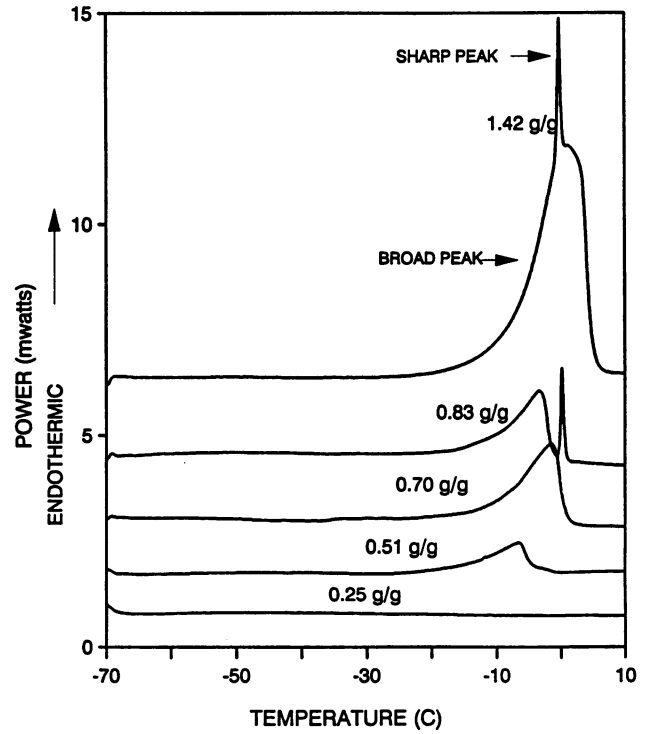

Figure 4. Heating DSC thermograms of isolated axes of *L. kirkii* flash dried for varying times. The figures above each curve give the axis water content. Similar thermograms were obtained from axes that had been slowly dried in intact seeds stored over silica gels.

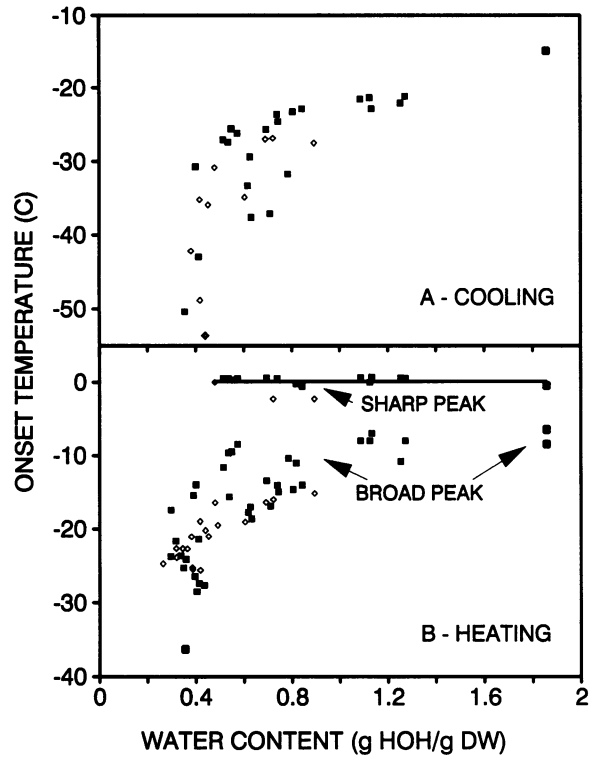

Figure 5. Temperatures of the onset of the (A) freezing and (B) melting transitions in thermograms of axes of *L. kirkii* of different water contents. ■, isolated axes that were flash dried; ◇, axes slowly dried in intact seeds stored over silica gels.

DISCUSSION

We investigated the relationships among rate of drying, dehydration tolerance, and the properties of water in desiccation-sensitive seeds of *L. kirkii*. The seed material dried at the two rates used in this study showed considerable differences in desiccation sensitivity. Isolated axes were flash dried to water contents of approximately 0.3 g/g before viability, as assessed by growth on culture medium, declined (Fig. 2) or electrolyte leakage increased markedly (Fig. 3). Loss in viability and increase in electrolyte leakage was observed in seeds dried over silica gel to an axes water content of approximately 0.75 g/g.

From the DSC analyses we can distinguish at least three types of water in the axes of these seeds (Fig. 4): (a) water associated with the sharp peak in melting transitions in axes with moisture contents >0.5 g/g, (b) water associated with the broad peak in melting transitions in axes with moisture contents >0.3 g/g), and (c) water giving no observable transition in axes with moisture contents <0.28 g/g. This last type of water is considered nonfreezable, given the cooling and heating protocols of this experiment. As can be seen from the drying kinetics (Fig. 1), nonfreezable water is not readily lost during either flash drying or dehydration over silica gels. In addition to the three types of water mentioned, there is a discrepancy between the amount of nonfreezable water calculated from freezing and melting transitions. Although this difference may be a result of energy dissipation during an exothermic event (and not measured in a heat-compensated DSC), it also may be relevant to the expression of freezing injury, as has been suggested for orthodox seeds (12).

Based on measurements of heat capacity and characteristics of water transitions, Vertucci (12) identified five types of water in cotyledons of soybean and pea seeds. There are similarities between the types of water identified in those desiccation-tolerant seeds and in the desiccation-sensitive seeds described here. The water content at which water is nonfreezable and the temperatures of the freeze and melt are comparable in the two tissue types. However, the energies of the freeze and melt (calculated as the slope of the lines in Fig. 6) are different between the two tissue types. In addition, although the sharp peak was sometimes apparent in the hydrated pea cotyledon, it was a far more prominent feature in the warming thermograms of *L. kirkii*.

Current hypotheses suggest that the level of desiccation tolerance of organisms is related to the amount of bound water present. Three hypotheses have been presented: (a) Desiccation-tolerant organisms have the capacity to substitute sugars or other low mol wt solutes for water on membranes and other structural elements, thereby providing the hydrophilic interactions required for structural stability (3, 4). Because water would be replaced, desiccation-tolerant organisms would have less measurable bound water when in the desiccated state. (b) Desiccation-sensitive organisms have a high

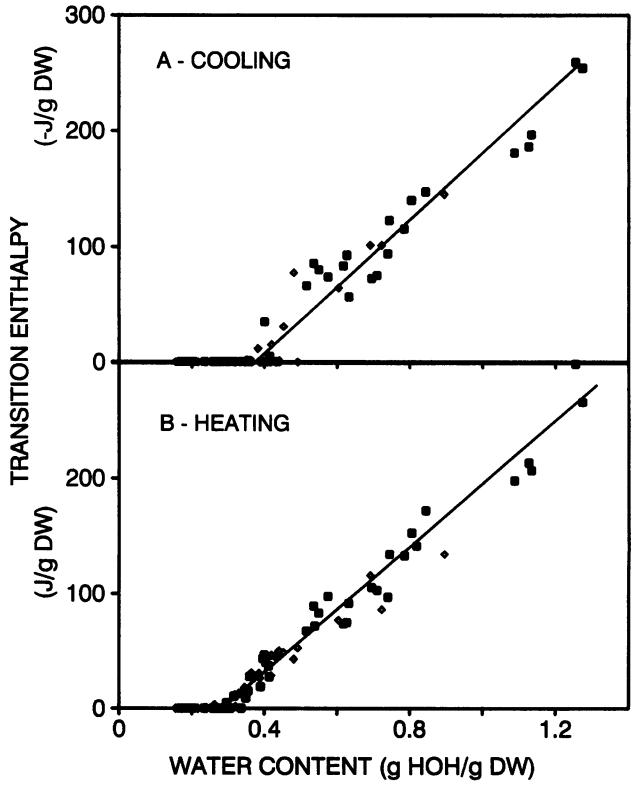

Figure 6. Enthalpies of the (A) freezing and (B) melting transitions as a function of water content in axes of *L. kirkii*. ■, isolated axes that were flash dried; ◇, axes slowly dried in intact seeds stored over silica gels. Lines are the least squares fit of data from flash-dried axes.

Table I. *Parameters Derived from the Plots of Transition Enthalpies versus Water Contents (Fig. 6)*

Drying Treatment	Melt Slope	X Intercept	Freeze Slope	X Intercept
	J/g H_2O	*g/g*	*J/g H_2O*	*g/g*
Flash dried	270	0.29	272	0.37
Silica gel dried	257	0.28	270	0.37

degree of subcellular and metabolic structure that can be maintained only at relatively high water contents, implying a high proportion of bound water (7). (c) Desiccation-intolerant organisms lack the capacity to tightly bind water (13).

If the level of nonfreezable water measured here is related to the amount of water associated with macromolecular surfaces, the three hypotheses predict the levels of nonfreezable water that might be expected in desiccation-tolerant and -sensitive organisms. The sensitivity of *L. kirkii* axes to desiccation differed with drying regimen, and yet the drying treatments had no effect on either the types of water present or the water contents at which the different types could be detected. The water contents at which the melting and freezing transitions become apparent in soybean and pea seed tissue (0.22 and 0.33; 0.24 and 0.35 g/g, respectively [12]) are similar to the water contents at which these transitions become apparent in *L. kirkii* (0.28 and 0.37 g/g). These data suggest that the desiccation tolerance of seed tissue may not be related to the amount of nonfreezable water.

We propose the following relationships among water content, types of water, desiccation sensitivity, and dehydration rate in homeohydric seeds: (a) Homeohydric seeds are normally shed at water contents in excess of 0.8 g/g and can withstand the loss of water to approximately this level. Water in tissues at this moisture content or greater has three components to the melting endotherm: a sharp peak with onset temperature of 0°C, a broad peak with onset temperature of approximately −8°C, and a portion that does not exhibit a peak. (b) When dried slowly, homeohydric seeds do not withstand the loss of water between 0.8 and 0.28 g/g without some damage. However, when dried rapidly (flash drying of excised axes), loss of this water can be tolerated, at least in the short term. This is unlikely to be a result of an inherent desiccation tolerance but because water content is reduced to levels inhibiting both metabolic and degenerative processes before these can occur (2). Desiccation-tolerant seeds normally lose this water in an organized manner during maturation drying. Some water in tissues at this moisture level is observed to melt, although the temperature of the transition decreases from −8 to −38 with decreasing moisture content. (c) Loss of nonfreezable water from axes of homeohydric seeds, *i.e.* that present below 0.28 g/g, cannot be tolerated, even if it is removed very rapidly. Vertucci (12) established that the moisture content at which water is not observed to freeze under the given protocols corresponds to a relative humidity of approximately 92% (equivalent to water potential of −11.3 MPa). This is similar to the water potential at which mitochondrial electron transport is facilitated (14) and at which membranes are believed to undergo conformational changes (9, 15). We suggest that the removal of nonfreezable water from homeohydrous tissue results in the loss of structural integrity of cell components. However, a substantial proportion of this water is normally lost during maturation drying of desiccation-tolerant seeds without the concomitant loss of integrity.

In conclusion, there are several types of water associated with desiccation-sensitive seeds that bear some correspondence to the types of water found in desiccation-tolerant seeds. The proportion of nonfreezable water does not appear to be critical in terms of desiccation tolerance; rather, it is the differing responses to removal of this type of water that distinguishes between desiccation-tolerant and -intolerant seeds.

ACKNOWLEDGMENTS

The authors would like to thank George White from the Germplasm Services Laboratory (Agriculture Research Service, Beltsville, MD) for helping us import the seed materials. We would also like to express our gratitude to those at the University of Natal (Durban, South Africa) who collected, prepared, and shipped the materials and who also offered expertise via phone and fax on how to handle materials after they arrived in Fort Collins. Thanks are also due to those at the National Seed Storage Laboratory (Fort Collins, CO) for assistance, particularly with the tissue culture.

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