Cryopreservation of Desiccation-Sensitive Axes of *Camellia sinensis* in Relation to Dehydration, Freezing Rate and the Thermal Properties of Tissue Water

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Summary

The inter-relationships between water content, rate of freezing to -196 °C, thermal properties of water, and survival were studied in excised embryonic axes of tea seeds. Three freezing rates were used: 10 °C min$^{-1}$, about 200 °C min$^{-1}$ and rapid freezing (attained by plunging samples into nitrogen slush). Differential scanning calorimetry yielded three categories of melting endotherms: those without endothermic peaks (no freezable water), those with a broad endothermic peak with onset temperature varying with water content, and those with an additional sharp peak at about -2 °C. When axes were cooled slowly, the sharp peak was present at high water contents (> 1.8 g H$_2$O g$^{-1}$ dw) and was diminished as axes were dried. The sharp peak was not apparent when axes were subjected to rapid freezing. Axes at moisture contents and subjected to freezing rates such that the sharp peak was present in the melting endotherms did not survive in tissue culture. Axes rapidly dried to water contents between 1.1 and 1.6 g H$_2$O g$^{-1}$ dry mass prior to rapid freezing showed 100% survival in tissue culture. Ultrastructural studies of freeze fractured replicas showed considerable freezing damage at intermediate freezing rates, but good preservation of subcellular detail under rapid freezing conditions. It is suggested that the sharp peak observed on melting endotherms represents the melting of pure water arising from ice crystals formed during freezing. If axes with a sufficiently high water content are cooled at a freezing rate that prevents the formation of large ice crystals, damage is minimized and survival enhanced. If axes are dried to water content close to the level of non-freezable water, the additional stress of freezing is deleterious.

**Key words:** Calorimetry, cryopreservation, flash drying, freezing rate, recalcitrant seed.

**Abbreviations:** DSC = differential scanning calorimetry; dw = dry weight; g g$^{-1}$ = g H$_2$O g$^{-1}$ dw.
Introduction

The long term storage of desiccation-sensitive (recalcitrant) seeds is restricted not only by their desiccation sensitivity, but also by their inability to survive sub-zero temperatures when fully hydrated (Normah et al., 1986; Pritchard and Prendergast, 1986; Pence, 1990, 1991, 1992; Vertucci et al., 1991). Desiccation tolerance of recalcitrant seeds varies among species, and even between different developmental stages within the same species. Previous studies (Normah et al., 1986; Pritchard and Prendergast, 1986; Berjak et al., 1989a, 1992; Pammenter et al., 1991) have shown that, for mature seeds of some recalcitrant species, dehydration to relatively low moisture levels (between 0.2 and 0.4 g H2O g−1 dw) may be attained without adversely affecting viability. However, to achieve this level of tolerance, water must be re-
moved rapidly. This is accomplished by drying excised embryonic axes in an air-stream (flash drying [Berjak et al., 1990]). The resulting reduction in moisture content enhances the potential for cryopreservation (Normah et al., 1986; Pritchard and Prendergast, 1986; Chaudhury et al., 1991; Pence, 1990, 1992; Vertucci et al., 1991).

The limited success in cryopreservation of recalcitrant species may be attributable to desiccation damage. Freezing injury or a combination of both. Differential scanning calorimetry (DSC) was used to determine the types of thermal transitions associated with these two types of injury (Vertucci, 1989 a, b; Pammenter et al., 1991; Vertucci et al., 1991; Berjak et al., 1992). Removal of unfreezable water was linked to desiccation damage (Pammenter et al., 1991; Berjak et al., 1992). The presence of freezable water in seeds was not necessarily linked to freezing injury if the water content of the axes was less than a critical level (Vertucci 1989 a, b, 1990; Vertucci et al., 1991). Necrosis and decreased survival of embryonic axes of *Landolpha kirkii* in culture was associated with the presence of a sharp peak at about 0°C in melting thermograms of axes cooled to −70°C (Vertucci et al., 1991). This peak was most evident in axes with moisture contents greater than 0.58 g.g⁻¹. The correlation between the presence of the sharp peak and loss of viability led those authors to speculate that such a peak was indicative of the existence of a particular type of water which was physiologically relevant to freezing damage.

Recent work (Berjak et al., unpublished) has shown that the melting endotherms of embryonic axes of *Camellia sinensis* (tea), flash dried to a range of moisture contents and cooled at 10°C min⁻¹ to −70°C, lacked a sharp peak only at moisture levels less than 0.44 g.g⁻¹. Axes flash dried to moisture levels less than about 0.40 g.g⁻¹ showed decreased survival in culture, even in the absence of a freezing treatment. Thus, the optimal moisture contents for cryopreservation of tea axes, frozen under these conditions, represent an extremely narrow range which is flanked by desiccation damage on one side, and freezing injury (as indicated by a sharp peak) on the other. Consequently, efforts to cryopreserve tea might be hampered by the unusually small window of permissible moisture contents.

Traditional views on the freezing process have favoured slow cooling rates (because these encourage the formation of a few, large, extracellular ice crystals) over faster rates which result in a larger number of intra- and extracellular nucleation events (Meryman, 1966; Liebo and Mazur, 1971; Levitt, 1980; Fahy et al., 1984; Franks, 1985; Kartha, 1985). However, rapid cooling rates have also been used for biological samples because they limit the growth of the ice crystals and encourage vitrification of the cellular matrix (Liebo and Mazur, 1971; Fahy et al., 1984; Franks, 1985). Cryo-electron microscopy (EM) of hydrated material demands that the native ultrastructure of the specimen be maintained free from artefacts which arise from the freezing process. This requires the attainment of ultra-rapid cooling rates. While the vitrification approach emphasizes the retention of viability and the cryo-EM approach emphasizes the preservation of ultrastructure, both disciplines have the avoidance of cell damage as their common goal. Thus, it was decided to adopt the methodology of cryomicroscopical specimen preparation and apply it to survival studies of cryopreserved germplasm.

At sufficiently high cooling rates, water can be vitrified into an amorphous state, while lower rates result in the formation of ice in its cubic or hexagonal configuration (Dubochet et al., 1982). Vitreous water will undergo, upon warming, a transition into cubic ice, and then to its larger hexagonal form (Franks, 1985). These transitions occur between −138°C and −40°C, which is a critical temperature range that a specimen must traverse rapidly during freezing and warming to avoid the formation of disruptive hexagonal ice crystals (Robards and Sleytr, 1985). Freezing rates in excess of 10,000°C s⁻¹ (Costello et al., 1982) must be achieved in order to prevent crystallization of the water present in the sample or, at least to keep it within its small (0.1 nm) cubic form (Robards and Sleytr, 1985). One of the prerequisites for achieving such rates is a small specimen not exceeding 0.1 mm³. Sample size must be kept to such dimensions to ensure an adequate freezing rate throughout the specimen. Good thermal contact between the sample and the cryogen is also essential to achieve ultra-rapid cooling rates. Unfortunately working with such minute specimens is impractical in the context of cryopreservation of recalcitrant embryonic axes. Cryoprotectants, which lower the nucleation temperature and raise the recrystallization temperature, may be added to narrow the critical temperature range where damage can occur (Robards and Sleytr, 1985). Partially drying the tissue may have similar effects: low moisture contents reduce the heat capacity of the tissue and encourage the formation of glasses in pea and soybean seeds (Vertucci, 1989 a, b, 1990) and corn embryos (Williams and Leopold, 1989). Thus, the conditions for optimal freezing may be maintained in larger samples by adding a cryoprotectant or by partially drying the sample.

In the present study, whether or not the rate of cooling to liquid nitrogen temperatures affects the properties of water in embryonic axes of tea, and whether this is relevant to their survival, is tested. Embryonic axes were flash dried to various moisture contents and subsequently frozen at three different rates, viz. slow (10°C min⁻¹), intermediate (200°C min⁻¹), and rapid freezing by plunging the samples into subcooled nitrogen. Calorimetric properties of the water present in the axes were recorded during warming using DSC; ultrastructural preservation was evaluated by freeze-fracture EM; and survival after freezing of axes was monitored by in vitro culture.

**Materials and Methods**

**Plant Material and Drying Treatment**

Seeds of *Camellia sinensis* were harvested in the northeastern Transvaal region of South Africa and transported by air under temperature- and pressure-controlled conditions to Fort Collins, CO. Time from harvest to receipt of material in Fort Collins, USA, did not exceed 7 days. Seeds were used within 2 weeks of arrival.

Embryonic axes were excised and placed on moist filter paper prior to transfer to the flash drying chamber (Pammenter et al., 1991). The axes were dried for different intervals from 0 to 100 minutes in an air-stream of compressed air, with a flow rate of 
10 L min⁻¹. This treatment gave moisture contents ranging from 3.1 to 0.10 g g⁻¹ dry mass basis. Due to the very high variability of initial moisture levels of axes from recalcitrant seeds, the moisture content quoted following a particular flash drying treatment is best described by a range rather than a single value.

**Calorimetry**

To determine if the thermal characteristics of water were altered by cooling rate, DSC warming thermograms of whole axes of tea from −150 to +20 °C were recorded with a Perkin Elmer DSC-7 at a scanning rate of 10 °C min⁻¹. The DSC was calibrated for temperature using indium (156.6 °C) and methylene chloride (−96.5 °C) standards, and for energy using indium (28.45 J g⁻¹). Scans of empty aluminium pans were used to correct for baseline curvature.

Individual axes (sample size c. 1 to 2 mg) of varying moisture contents were cooled to −160 °C at 10 °C min⁻¹, c. 200 °C min⁻¹, or very rapidly. Samples cooled at 10 or 200 °C min⁻¹ were hermetically sealed in aluminium pans. The 10 °C min⁻¹ cooling rate was achieved through programmed cooling in the DSC. The 200 °C min⁻¹ treatment was achieved by precolling the sample holder of the DSC to −160 °C and then loading the sample; the sample came to temperature (−160 °C) in slightly less than 1 min. Very rapid cooling consisted of securing the axes to the lid of an open DSC pan by means of double-sided tape, rapidly immersing into liquid nitrogen (−196 °C) with forceps and immediately releasing the sample. (The latter was done so that the specimen did not act as a sink for heat originating from the forceps, and thus slowing the freezing rate). The pan was then assembled under liquid nitrogen, the sides of the bottom half of the pan being bent in order to secure good thermal contact with the specimen. The DSC pan was maintained immersed in the cryogen until it was transferred to the previously cooled sample holder of the DSC. Although it is difficult to ascertain an accurate rate for the cooling, these procedures generally give cooling rates of the order of thousands of degrees per second (Costello and Corless, 1978; Sitte et al., 1985). Immediately after a warming scan was recorded, the sample plus pan was weighed. The pan was then punctured and heated to 95 °C for 36 hours and reweighed. Moisture content is expressed on a dry weight basis.

Warming thermograms were analyzed for onset temperature and energy of the endothermic transitions. Onset temperatures were determined as the point of intersection between the baseline and the steepest portion of the peak. Energies of the transitions were determined from the area above the baseline. For each cooling rate, the enthalpy per dry weight was regressed against the moisture content. X-intercepts and slopes of the resulting lines were used to determine the amount of water that did not freeze, and the energy of the melting transition (on a per g of H₂O basis) for the water that did freeze (Vertucci, 1990).

In some cases, a sharp peak in the melting endotherm was apparent at about 0 °C. The presence of this peak has been reported previously and its analysis described (Vertucci et al., 1991; Berjak et al., 1992). Briefly, the area of this transition was calculated separately from the area of the main melting transition and expressed as a function of the water content of the sample. The amount of water associated with the sharp peak was then calculated for a given water content by assuming that the heat of fusion is 333 J g⁻¹ H₂O. In this way, the partitioning of water in the main melting transition and in the sharp peak could be determined as a function of the moisture content of the tissue.

**Electron Microscopy**

Freeze-fracture EM of tea axes was used to assess the degree of ultrastructural preservation attained by the different cooling rates. Embryonic axes, flash dried to various moisture levels, were mounted on gold planchets secured onto aluminium pins from a Reichert cryo-ultramicrotome. Rapid freezing consisted of propelling mounted samples into subcooled liquid nitrogen by means of compressed air generated by a simple spring-loaded piston device. Lowering the temperature of the liquid nitrogen from −196 to −210 °C was achieved by placing this cryogen under vacuum. The nitrogen flash thus obtained, delayed the onset of nucleation boiling around the sample (Leidenfrost phenomenon) which impairs the efficiency of the freezing step. The axes were covered with a thin film of glycerol seconds before freezing, since this has been reported to enhance freezing rates in cryogens showing nucleation boiling (Robards and Sleytr, 1985). The term rapid freezing is used here in preference to ultra-rapid freezing since the latter pertains to other methods where higher freezing rates can be attained (Sitte et al., 1985). An alternative cooling method consisted of using forceps to immerse mounted samples into melting Freon 22. The forceps are believed to provide a heat sink, so freezing rates using this method are considerably slower than the plunge method described above. The measurement of freezing rates is complicated by the fact that cooling rates will vary depending on the size of the thermocouple, the cryogen and also the method used (Costello and Corless, 1978).

The specimens were fractured and replicated at −105 °C with a Balzers BAF 301 freeze-etching system. Unidirectional shadowing with platinum and rotary carbon coating were done immediately following fracture, since etching was not required. Specimens were then thawed, and the replicas cleaned by placing in an aqueous chromic acid-sulphuric acid solution (50% v/v) overnight, followed by full strength solution for no less than 2 h. Replicas were collected on 600 mesh copper grids and viewed with a JEOL 100 C transmission electron microscope.

**Tissue Culture**

To determine if rate of cooling to liquid nitrogen temperatures affected survival, tea axes flash dried to different moisture levels and cooled at different rates were placed in culture and assayed for germination. As detailed above, axes were cooled at 10 °C min⁻¹ and 200 °C min⁻¹ in the DSC, while rapidly cooled axes were frozen in subcooled nitrogen. All axes were thawed in liquid culture medium (Murashige and Skoog, 1962) at room temperature. Axes were allowed to rehydrate for 30 minutes, after which they were surface-sterilized in a solution containing 1.0% sodium hypochlorite and 0.2% hibitane (Hibiclens; Stuart Pharmaceutical, Wilmington, DE) for 10 minutes, rinsed twice in sterile distilled water and placed on a supplemented, solid MS medium (after Pittcchard and Frendergast, 1986). Survival, as indicated by greening and organ development, was assessed at weekly intervals.

**Results**

The thermal characteristics of water in embryonic axes of tea flash dried to various moisture contents and then cooled at slow (10 °C min⁻¹), intermediate (200 °C min⁻¹) and rapid rates were determined by DSC. Melting endotherms of representative samples dried to different levels and then cooled at different rates are given in Figs. 1, 2 and 3. Thermograms can be divided into three categories: those that lack an endothermic peak (moisture contents less than 0.23 g g⁻¹), those with a broad endothermic peak with an onset temperature between −7 and −50 °C, and those with an additional sharp peak with onset temperature of about −2 °C. The onset temperature of the broad endothermic peak was similar.
-8 °C) for axes with water contents greater than 0.6 g g⁻¹ (Fig. 4). At moisture levels less than 0.6 g g⁻¹, onset temperature decreased sharply with water content. There appeared to be no differences in the temperature of the transition for axes of similar moisture levels that were cooled at different rates. The onset temperature of the sharp melting transition was -2 °C and did not vary with moisture content (Fig. 4).
This peak was more prevalent in axes cooled at 10 °C min⁻¹ than it was for axes cooled at faster rates.

To determine the amount of water that did not freeze and the heat of fusion of the water that did freeze, the enthalpy of the melt of tea axes was regressed against the water content (Fig. 5). As reported previously for other species (Vertucci, 1989a, 1990; Pammenter et al., 1991; Vertucci et al., 1991; Berjak et al., 1992), the relationships were linear (r² = 0.997, 0.994, and 0.972 for 10 °C min⁻¹, 200 °C min⁻¹ and rapid cooling, respectively), and the lines were indistinguishable for the different cooling rates (slope = 303, 304, and 338 J g⁻¹ H₂O, respectively). The moisture content at which water was not observed to undergo a melting transition was also similar among the cooling treatments (x intercept = 0.22, 0.23, and 0.21 g H₂O g⁻¹ dw, respectively). Small endothermic transitions with energies ranging from 0.2 to 1.0 J g⁻¹ dw were often observed at about -120, -80 and -60 °C (data not shown). However, there was no apparent change in the energy or temperature of these transitions with either moisture content or cooling rate.

In view of the similarity among the temperature and energies of the melting transitions, and the moisture content below which these transitions were not apparent, it might be assumed that there were no differences in the calorimetric properties of the water in samples cooled at different rates. However, there were differences in the shape of the endotherms for axes cooled at different rates. There was a sharp peak at c. -2 °C that was most prominent in fully hydrated axes cooled at 10 °C min⁻¹ and that was not present in rapidly-cooled axes (compare Figs. 1 and 3). The sharp peak was less pronounced as axes were dried to lower moisture levels or cooled at faster rates (Figs. 1, 2, and 3). These trends are expressed as the change in the ratio of water associated with the sharp and broad peaks (Fig. 6). In axes cooled at 10 °C min⁻¹, the ratio declined from 0.075 at high water contents to zero at a water content of 0.44 g g⁻¹. In samples cooled at 200 °C min⁻¹, the maximum value of the ratio of water in the sharp peak relative to the broad peak was 0.01, and no sharp peak was present at water contents below 0.8 g g⁻¹. There was no sharp peak in the melting endotherms of axes that were rapidly cooled, irrespective of the moisture content. Thus, the ratio of water in the sharp peak relative to the broad peak remained constant at 0.

Electron microscopy of freeze-fracture replicas demonstrated the significance of freezing rates on the ultrastructural preservation of frozen axes. In order to maintain cellular ultrastructure free from freezing artefacts, the time spent by the sample between the critical temperatures of c. -40 and -133 °C should be minimized. If this is not met, severe ultrastructural distortions are introduced as a consequence of ice crystal growth (Robards and Sleytr, 1985).

Fig. 5: The relationship between water content and enthalpy of the melting transition of tea axes dried to different levels and frozen at three cooling rates. Lines are drawn from the least squares fit of the data collected from each cooling rate.

Fig. 6: The ratio of the amount of water represented in the sharp peak of the melting endotherm relative to the broad peak for axes dried to different levels and frozen at three cooling rates.

Fig. 7: Freeze-fracture replica of a tea axis flash-dried for 40 min and frozen (while held with forceps) in Freon 22. Cells show the presence of damage resulting from the formation of ice crystals. The severe and extensive ice damage is representative of partially hydrated axes frozen at intermediate rates. cw, cell wall; *, unidentified organelle.

Fig. 8: Freeze-fracture replica of a fully hydrated tea axis that was rapidly frozen. The rough appearance of the cytomatrix is a result of the presence of ice crystals of c. 125 nm (circled), which can be seen in places to fuse into larger structures (arrowheads). The bounding membrane of an organelle (arrows) shows evidence of ice deformation.

Fig. 9: Freeze-fracture replica of a tea axis flash-dried for 20 min and then rapidly frozen. There is no evidence of ice crystal formation or intracellular damage. N, nucleus; er, endoplasmic reticulum; mf, microfilaments; vac, vacuole.

Fig. 10: Freeze-fracture replica of a tea axis flash-dried for 60 min and then rapidly frozen. There is no evidence of ice crystal formation or intracellular damage. N, nucleus; vac, vacuole; v, vesicle.
Substantial damage was associated with the freezing of axes held with forceps in Freon 22, although this is reported to result in adequate preservation of cryo-protected preparations for freeze fracture (Fineran, 1978). In the present circumstances, axes dried to water contents of 0.7 to 1.1 g g⁻¹ showed severe ice damage (Fig. 7). The effects of disruptive hexagonal ice are visible where intracellular material appears as ridges around depressions caused by the ice crystals. Identification of cellular detail other than cell walls was difficult. This was attributable to the slower cooling rates due to heat transfer from the metal forceps to the specimen. Although the freezing rate of the axes frozen in melting Freon 22 could not presently be ascertained, it may be regarded as being intermediate between the 10 °C min⁻¹ and the rapid freezing method. The degree of preservation shown by these axes may represent the extent of cell damage caused by the 200 °C min⁻¹ cooling treatment at water contents of about 1.1 g g⁻¹.

The amount of damage observed was substantially reduced by the rapid freezing method, although samples at water contents above 1.6 g g⁻¹ showed evidence of ice deformation (Fig. 8). The presence of a large number of ice crystals, of c. 125 nm throughout the cells obscured fine detail: nuclei or vacuoles could be identified, but extensive damage to membranes was evident. The freezing injury shown is closely matched by the low survival record of such axes in culture (Table 1). The rapid freezing method resulted in good preservation at moisture contents at or below 1.6 g g⁻¹, achieved by flash drying materials for 20 min (Fig. 9), or longer (Fig. 10 gives an example for 60 min of flash drying); however, the cytomatrix became increasingly compacted as water contents decreased. There was no visible sign of ice damage, comparable to that observed in axes frozen at high moisture levels or slower cooling rates, in any of the rapidly frozen material which had been flash dried for at least 20 minutes.

The results of the survival studies are shown in Table 1. The negligible survival of axes frozen at 10 °C min⁻¹ contrasts with the higher recovery of axes frozen at faster rates. As described for Landoiphia kirkii (Vertucci et al., 1991), the responses of embryonic axes in culture could be divided into three broad categories: a. those axes which became necrotic and died within 48 to 72 hours of culturing; b. those which showed no signs of necrosis, but did not green within a period in excess of 2 months; c. those axes which became green and developed roots and leaves with 4 weeks in culture. The viability of Camellia sinensis axes flash-dried to different moisture levels and not frozen (control), declined as moisture content approached 0.4 g g⁻¹. In this treatment, axes which were scored as non-surviving failed to develop in culture but showed no necrosis (response b). Axes cooled at 10 °C min⁻¹ became necrotic (response a) unless dried to the minimum tolerable moisture levels, where only 14% survival was recorded. Following cooling at 200 °C min⁻¹, there was an increasing survival of flash-dried axes as water content was lowered from 1.1 to 0.4 g g⁻¹. There was a lower incidence of early necrosis in axes which were scored as non-surviving compared with comparable axes cooled at 10 °C min⁻¹. When rapidly frozen, the range of water contents of axes that survived the freezing process broadened to 0.4 to 1.6 g g⁻¹. Axes flash-dried for 20 minutes to water contents of between 1.6 and 1.1 g g⁻¹ showed 100% survival when rapidly frozen, with the first instance of greening and expansion occurring after the relatively short period of 3 weeks in culture. Rapid freezing of axes dried to lower moisture contents resulted in relatively slower recovery in culture and also decreased survival. In some cases, the epicotyl portion of these axes turned brown (distinguished from the early necrosis described above).

### Discussion

In this study, calorimetry, EM and tissue culture techniques have been used in order to understand the combined effects of water content and cooling rate on freezing tolerance or sensitivity of embryonic axes of Camellia sinensis. The parameters required for vitrification of biological material, or at least to minimize the size of ice crystals formed, were borne in mind when designing the rapid cooling method utilized here. The results indicate that maximum survival of axes is achieved if axes are dried to moisture contents between 1.6 and 1.1 g g⁻¹ and then rapidly frozen (Table 1).

DSC studies indicate that comparable amounts of water froze in tissues at similar moisture contents irrespective of the cooling rate applied (Fig. 5). However, the presence of a sharp peak in melting thermograms appeared to be particular to the 10 °C min⁻¹ treatment at water levels above 0.44 g g⁻¹, and to axes above 0.80 g g⁻¹ if frozen at 200 °C min⁻¹. Tissue culture of similarly-treated axes resulted in very low survival, while freeze-fracture analysis showed axes of c. 1.1 to 0.7 g g⁻¹ frozen at an intermediate cooling rate to have poor preservation of subcellular detail (Fig. 7). Comparison of these findings with DSC results are in agreement with those for Landoiphia kirkii (Vertucci et al., 1991), and supports the hypothesis that the presence of a sharp peak at about 0 °C in the melting endotherm is associated with lethal freezing injury. Freezing conditions similar to the ones which give rise to sharp peaks in melting thermograms of frozen axes have also been found to be conducive to the presence of ice crystals observed with freeze-fracture microscopy. It is suggested that the presence of

<table>
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<th>Water content (g H₂O g⁻¹ dw)</th>
<th>Control</th>
<th>10 °C min⁻¹</th>
<th>200 °C min⁻¹</th>
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<tr>
<td>&gt; 1.6</td>
<td>100% (9/9)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
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<tr>
<td>1.6 - 1.1</td>
<td>ns (0/10)</td>
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<tr>
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<td>100% (10/10)</td>
<td>0% (0/10)</td>
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<tr>
<td>0.7 - 0.4</td>
<td>30% (20/65)</td>
<td>5% (1/19)</td>
<td>60% (6/10)</td>
</tr>
<tr>
<td>0.4 - 0.3</td>
<td>67% (10/15)</td>
<td>14% (4/28)</td>
<td>ns (ns)</td>
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Numbers in parentheses represent the number of axes surviving over the total number of axes treated in the indicated way, which remained sterile in culture. Axes were scored as surviving if they expanded, greened and developed roots; ns = not studied.
sharp peaks at 0°C may arise from the melting of pure water, which could have been distilled during freezing at relatively slow rates. Depending on the freezing rate, the size of the crystals thus formed could be large enough to be detected by DSC as a sharp peak and also to disrupt cellular integrity and cause loss of viability either by mechanical damage or severe dehydration. Our data suggest that a range of cellular tolerance to freezing injury exists, and support the observation that the presence of intracellular ice crystals is not necessarily lethal (Vertucci, 1989a; Vertucci et al., 1991). The extent to which cellular repair mechanisms can respond to ice damage following freezing will determine the survival of axes following exposure to liquid nitrogen. The presence of the sharp peak may indicate the inability of the cells to repair.

If this is true, then the analysis of water partitioning in the sharp and broad peaks of the melting endotherms may be a predictive tool for determining the range of moisture levels which maximize the cryopreservation of recalcitrant germplasm (Vertucci et al., 1991). The moisture content at which desiccation damage occurs in the absence of low temperature stress, will represent the lower water limit. At the opposite extreme, the presence of the sharp peak at about 0°C in the DSC warming endotherm will determine the upper limit of permissible moisture levels. Therefore, if tea axes suffer desiccation-induced damage at moisture levels below 0.40 g g⁻¹ (Berjak et al., unpublished data) and a sharp peak is observed at moisture contents greater than 0.44 g g⁻¹ when axes are cooled at 10°C min⁻¹ (Fig. 6), then the predicted permissible water contents extend between 0.40 and 0.44 g g⁻¹, representing an exceedingly small window. Increased cooling rates resulted in the sharp peak occurring only at higher moisture contents. According to the present hypothesis, the allowable moisture levels should range from 0.4 to 0.8 g g⁻¹ in axes cooled at 200°C min⁻¹ and greater than 0.4 g g⁻¹ for axes cooled rapidly. Axes surviving in situ after cooling at 200°C min⁻¹ to −160°C, had an extended range of water levels from 0.40 to 0.7 g g⁻¹. Survival of axes at the drier end of this range was 60%, this value decreasing to 0% at water levels above 1.10 g g⁻¹. Rapid cooling allowed this window of optimal moisture levels to be further extended to 1.6 g g⁻¹. Maximum survival was recorded for axes flash-dried for 20 minutes to c. 1.6 g g⁻¹ (Table 1).

Freeze-fracture replicas of rapidly-frozen partially-dried axes confirmed a high degree of ultrastructural preservation. There was fine subcellular detail and no signs of ice crystal damage in rapidly frozen axes (Figs. 9, 10). However, rapid freezing of fully hydrated axes resulted in the formation of a large number of nucleation events throughout the cell cytoplasm, which, in this instance, resulted in the formation of crystals of approximately 125 nm (Fig. 8). Sharp peaks in the warming thermograms of fully hydrated axes were not apparent, as would be predicted by the present hypothesis, perhaps because they were masked by the very large broad peak of the endotherm. In terms of freezing theory, a low rate of heat exchange between specimen and cryogen occurs in fully hydrated axes because of the high heat capacity and the latent heat of fusion of water. This means that the specimen will take longer to traverse the critical cooling region (−40 to −133°C) allowing the growth of ice crystals formed upon freezing. Flash drying reduces the water content of an axis, thereby increasing the rate of heat loss, minimizing ice growth and consequent damage during freezing.

Although the likelihood of ice damage is reduced as moisture content decreases, survival after rapid freezing of axes dried for longer than 20 min is diminished (Table 1). This suggests the existence of stresses other than those induced by ice damage alone. Tissue culture showed epicotyl browning after several weeks to be common in these axes and that regeneration took place once growth was resumed. The large surface to volume ratio of the primary leaves may have resulted in a greater degree of water loss during flash drying than occurred in the rest of the same axis. This may have introduced a desiccation-induced stress. No browning occurred in 20 minute flash dried, cryopreserved axes or in axes flash dried for longer times in the absence of freezing. It is possible that other cellular components of severely dehydrated epicotyl tissues may have been prone to phase transitions of molecules other than water during the freezing and thawing processes. It is well documented that the transition temperatures of polar lipids in membranes increase with decreasing moisture level (Crowe and Crowe, 1988), and that seed lipids in situ have transitions at temperatures as low as −90°C (Vertucci, 1989a, b). It has been suggested that lipid transitions may be an important factor in the viability of desiccation sensitive tissues (reviewed by Crowe and Crowe, 1986; Pammenter et al., 1991; Vertucci et al., 1991). Embryonic axes of Landolphia kirkii dried to moisture levels where water transitions were not observed, also had a lower survival rate (40%) when frozen to −70°C compared with controls, which were not given the freezing treatment (Vertucci et al., 1991). In addition, axes from that species did not survive exposure to −150°C at any moisture content when cooled slowly (data not published). Similarly, an additional decline in survival following freezing was observed for embryonic axes of some temperate tree species that were dried to moisture contents of about 0.2 g g⁻¹ (Pence, 1992). Those observations are consistent with the suggestion that phase transitions of cellular macromolecules may be responsible for damage in partially dried and frozen tissues, and that such transitions occur at very low temperatures.

In conclusion, cooling rates have been shown to affect the properties of water in embryonic axes of tea. The presence of a sharp peak in the melting thermograms of frozen axes was associated with necrosis and death in culture, and this signal was related to the formation of large ice crystals. Sharp peaks were eliminated from thermograms by either increasing the cooling rate and/or flash drying the axes. Both these treatments resulted in increasing the survival of axes in culture. Although survival is largely dependent upon the rate of cooling and not the liquid nitrogen temperature per se, the possibility of temperature-related changes introduced in the lipid component of axes subjected to longer flash-drying treatments cannot be ruled out.

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