

PROFILING CRYOPRESERVATION PROTOCOLS FOR *RIBES CILIATUM* USING DIFFERENTIAL SCANNING CALORIMETRY

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Summary

DSC analysis was performed at three points in the cryopreservation process on encapsulated-dehydrated meristems of *Ribes ciliatum*. Meristems were excised from shoots pre-treated with either sucrose or glucose, encapsulated in alginate beads, dehydrated in sucrose solutions, air dried, and plunged in liquid nitrogen. Thermal analysis revealed glass transitions during cooling of air-desiccated meristems, however, on rewarming a small endothermic event was detected suggesting glass destabilization can occur. Interestingly, this did not occur in alginate beads or meristems when these components were cooled and rewarmed separately. The possibility exists that thermal and moisture gradients may arise within the alginate bead/tissue complex and we propose that the heterogeneous composition of the meristems and the surrounding alginate may promote ice nucleation on rewarming. The significance of this regarding the stabilization of glasses formed in alginate beads and their encapsulated meristems is discussed. This study also reports an approach to *Ribes* cryopreservation in which the pre-growth of shoots in 0.75M sucrose for 1 week can be used as a substitute for cold acclimation.

Keywords: Cryopreservation, water content, differential scanning calorimetry, desiccation, thermal analysis, vitrification, *Ribes*.

INTRODUCTION

Cryopreservation is the method of choice for the conservation of vegetatively propagated soft and top fruit germplasm (3, 21, 22). Cryopreserved germplasm collections are already established for apple (9) and pear (23). The successful cryopreservation of temperate fruit crop species is usually dependent upon the application of cold hardening treatments (1, 15, 20, 24) and these have been applied to germplasm from the genus *Ribes* (black currant and gooseberries). *In vitro*-grown shoots are exposed to cold-acclimation conditions for 1 wk before meristem excision (24). Following cold acclimation *Ribes* meristems survive controlled rate freezing, vitrification with PVS2 solutions, or encapsulation/dehydration

cryopreservation protocols (1,15). Post-storage survival can be genotype and method dependent (21) and tolerance to desiccation, chilling and freezing temperatures may be major determining factors.

During freeze/thaw cycles tissues are submitted to two major stresses, desiccation and ice crystal formation (17) which can be reduced in tissues exposed to high concentrations of sucrose (5, 7). Artificial and natural cold acclimation is correlated to various metabolic changes, including quantitative and/or qualitative variations in lipids (26), oligosaccharides (14, 25, 27) and proteins (13, 12, 19). It is also possible that sucrose treatments simulate cold hardening and this approach may be especially useful in developing cryopreservation methods for plants that are cold sensitive. Exposure of *R. nigrum* genotypes to high sucrose treatments prior to cryopreservation may substitute for cold acclimation (6). Shoots are grown on a 0.75M sucrose medium for 1 week, meristems are excised, encapsulated in calcium-alginate beads, osmotically and air dehydrated, and cryopreserved by direct plunging into liquid nitrogen. Survival rates of 90-95% were achieved in *R. nigrum* cultivars Ben Lomond and Ojebyn comparable to those obtained using cold acclimation (1, 6).

Vitrification-based cryopreservation methods (1) were developed for *Ribes spp* using two different approaches: {1} Chemical vitrification, involves the application of the highly concentrated, cryoprotectant solution, PVS2. This causes an increase in cellular viscosity, which in turn promotes glass formation on cryopreservation and {2} Encapsulation/dehydration (2) in combination with sucrose dehydration and evaporative desiccation. These treatments reduce the water content of cells in the meristem so that a glass is formed on exposure to liquid nitrogen. Differential Scanning Calorimetry (DSC) has been used to assist in the development of vitrification methods (1). Thus, using thermal analysis it is possible to profile the stages of a cryopreservation protocol and reveal water phase changes associated with ice nucleation, melting, glass transitions, relaxation, and devitrification events. Moreover, DSC can accurately determine the moisture status of tissues exposed to different cryoprotectants and low temperature treatments. In the case of cryopreservation methods based on vitrification it is important to optimize tissue moisture, such that on exposure to liquid nitrogen temperatures, glasses are formed and maintain their stability on re-warming (1).

The plant chosen for study was *Ribes ciliatum* Humb. & Bonpl. Luo and Reed (15) cryopreserved *in vitro* shoot cultures of this species by vitrification (using PVS2 cryoprotectants) with 70% survival. Micropropagated cuttings of these cultures were donated by BM Reed (United States Department of Agriculture, National Clonal Repository, Oregon) the *in vitro* cultures, of which, were used in the present study. Mexico is the centre of origin of *R. ciliatum*, and the original source plant was obtained from the north face of the Popocatepetl volcano (Tlalacas, Puebla state) at latitude 19, 10' N and longitude 98°, 40' W and an elevation of 3,960 m above sea level. Environmental extremes are prevalent at this location and this *Ribes* species may be exposed to 200 freeze/thaw cycles per year as well as temperatures as high as 35°C. Due to its location of origin in an extreme environment, *R. ciliatum* is an interesting subject for fundamental DSC investigations of desiccation and freezing behaviour. It is also important to apply cryopreservation to *R. ciliatum*, as it is a long-term and safe approach for the *ex situ* conservation of germplasm derived from wild species located in an at risk environment.

As desiccation tolerance is a major determining factor for the successful application of vitrification protocols, DSC may be used to aid the development of cryopreservation methods for which moisture status and the behaviour of freezable water is critical. This may be especially the case for desiccation sensitive genotypes. Thus, the objective of this study was to use DSC to investigate the behavior of water within calcium-alginate beads as well as in encapsulated and non-encapsulated meristems of *R. ciliatum* excised from two types of pretreated shoots. DSC profiles were assessed at various stages of the cryopreservation

protocol. The experimental aim was to construct and compare profiles of water content status and thermal behavior within freeze and desiccation sensitive and freeze tolerant meristems.

MATERIALS AND METHODS

Source plants

R. ciliatum is maintained as a field plant and *in vitro* at the USDA-ARS National Clonal Repository, Corvallis, Oregon. A culture was donated for study at the University of Abertay Dundee.

In vitro propagation

Shoots of *R. ciliatum* were sub-cultured monthly on RIB medium (24), a modified Murashige and Skoog medium (18), containing a third of the standard ammonium and potassium nitrate concentration, 50 mg.l⁻¹ ascorbic acid, 20 g.l⁻¹ glucose, 0.28 μM, N6-benzyladenine and 0.58 μM gibberellic acid, and solidified with 8 g.l⁻¹ agar. Plants were grown on 20 ml of medium in 200 ml glass jars at 25°C with 16 h light (25 μmol.m⁻².s⁻¹) photoperiod.

Pre-treatment of shoots

Shoots (1 cm) with leaves removed were cultured in Petri dishes containing either RIB medium or glucose-free RIB medium supplemented with 0.75 M sucrose (S-RIB) (1, 24).

Cryopreservation

Meristems were excised from pre-treated shoots and immediately transferred to RIB medium prior to further utilization. Meristems were immersed in calcium and cobalt salts-free, 3% (w/v) alginate made up in liquid S-RIB. Alginate droplets each containing 1 meristem were dispensed into 100 mM CaCl₂ prepared in liquid RIB medium and allowed to polymerise for 10 min (8). Beads were incubated in liquid S-RIB on a Gallenkamp shaker (setting 2-3) overnight (20-22 hours), then removed from the sucrose solution, blotted dry on sterile filter paper, transferred to an open, sterile Petri dish and desiccated for 4 hours under the sterile air flow. Desiccated beads were placed in cryotubes (Nunc, 2ml capacity, 5 beads per cryotube; 4-5 cryotubes were used for cryopreservation treatments) and directly plunged in liquid nitrogen. Cryotubes were thawed at ambient temperature for 20 min. Encapsulated meristems were transferred to solid RIB medium for recovery and sub-cultured weekly.

Differential Scanning Calorimetry

Thermal analyses of *Ribes* samples were undertaken using a DSC820 system (TA8000 Mettler-Toledo, GmbH, Schwerzenbach, Switzerland) incorporating a heat flux module, a liquid nitrogen cooling system and an Epson TAS811 workstation employing STAR^c software (Mettler-Toledo, GmbH, Schwerzenbach, Switzerland) for control and evaluation. The system was calibrated using indium (melting point 156.6°C, enthalpy 28.71 J g⁻¹) as an upper temperature and enthalpy standard, and dodecane (melting point -9.65°C) as a lower temperature standard. HPLC-grade water melting points were used as calibration checks. Samples were weighed (accuracy ±1 μg) and sealed in 40 μl aluminium pans. Water-state analyses were made on two components, meristems and alginate beads, frozen individually or conjointly. The following DSC protocol was used: *Freezing/cooling*: cooling rate of 10°C per min from 25°C to -100°C / 5°C per min from -100°C to -150°C and 10 min at -150°C. *Thawing/rewarming*: warming rate of 10°C per min from -150 up to 25°C.

Determination of water content

After thermal analysis each aluminium pan was punctured and dried for 12 hours in a 60°C oven to determine absolute water content (g H₂O.g⁻¹ DW). Crystallinity, the percentage of

frozen water on a total water basis, was determined by evaluation of the resultant thermograms using STAR^e software. Measurements of crystallinity calculated from the melt endotherms were used for all experiments, where the melt onset was between 0 and -10°C and the heat of melt corresponded closely to 334.5 J.g⁻¹ water.

Note that preliminary experiments performed on meristems prior to, and post drying at 60°C for 12 hours showed that enthalpy changes had occurred (as water phase transitions) only in the hydrated samples. As 1g of water releases 334.5 joules when converted into ice (and vice versa, ice to water), the freezable water content of the sample (g of freezable water per g of dry weight) was calculated from the enthalpy changes recorded during cooling or warming. However, only the melt endotherms were used in this study as there was only slight variation in enthalpy between the samples at melt compared with the cooling/freeze exotherms. Moreover, in some instances ice formation was only detected during the thawing/rewarming process.

Statistical analysis and experimental design

Determinations of absolute water content and DSC thermal profiles (thermal event onset temperature and crystallinity) were performed on 2 to 5 samples. Where possible, treatment effects ($P < 0.05$) were compared using ANOVA (one way), after a Normal distribution check. DSC and water content analyses were assessed for three steps of the cryopreservation protocol:

Step 1: At 7 days pre-treatment of 1 cm shoots on either RIB (0.1M glucose) or S-RIB (0.75M sucrose). Experiments were performed on meristems excised from treated shoots.

Step 2: Step 1, followed by meristem encapsulation and treatment for 20 to 22 h in liquid S-RIB. Experiments were performed on encapsulated meristems, meristems excised from beads, and empty beads.

Step 3: Steps 1 and 2, followed by 4 hours desiccation of the encapsulated meristems. Experiments were performed on desiccated, encapsulated meristems; meristems excised from beads after desiccation, and desiccated empty beads.

Meristem re-growth was assessed after steps 2 and 3 and expressed as the percentage of meristems showing leaf expansion and shoot elongation at 4 weeks after thawing.

RESULTS

Thermal profiling and the evaluation of absolute and freezable water content

For comparative purposes, thermal profiles of treatments are shown in Tables 1-3. For clarity, representative thermograms (see Figs 1-3) are only shown for sucrose treatments.

Step 1: Preliminary studies were performed on meristems following 7-day treatments of the shoots with glucose or sucrose. The culture of *R. ciliatum* meristems for 7 days on S-RIB medium resulted in a decrease in water content from 12 to 1 g g⁻¹ dw (Table 1) and the capacity of the remaining water to freeze (as indicated by crystallinity) remained lower than in meristems excised from non-sucrose treated shoots (74% vs. 95%). Crystallization and melting events invariably occurred within the meristems at this stage of the cryogenic process.

While ice crystallisation occurred at similar (but not significantly) temperatures in both treatments, the melting event occurred at a higher temperature in the more hydrated glucose-treated meristems (Table 1). It was not possible to analyse melt phenomena as the data did not show Normal distribution. This preliminary study was thus primarily used to indicate the

status of the meristems before embarking on the subsequent cryoprotective treatments.

Table 1: Preliminary profiling of absolute water content, crystallinity, and thermal events during a freeze/thaw cycle of *Ribes ciliatum* meristems following a 7-day treatment of the shoots with glucose or sucrose.

Meristem treatment	Absolute water content (g.g ⁻¹ DW)	Crystallinity (%)	THERMAL EVENT			
			Cooling		Thawing	
			Nature	Onset T° (°C)	Nature	onset T° (°C)
0.1M glucose	12.5±0.15	95±0	cryst.	-21±1	melting	-2±0
0.75M sucrose	1.0±0.12	74±2	cryst.	-18±0.7	melting	-10±2

2 replicates per treatment (mean±standard deviation)
 cryst. = crystallization

Step 2: After encapsulation and 20-hours of liquid S-RIB treatment, the absolute water contents of meristems in both treatments were similar (Table 2). Empty alginate beads were the most hydrated components (2.7 g g⁻¹ dw). At this stage, differences in meristem crystallinity were no longer observed and ice crystallisation and melting occurred during the freeze/thaw cycle in both the meristem and the alginate bead, whether frozen individually or conjointly. See Figures 1a/b for representative thermograms. Similar observations were reported for the glucose treatment (data not shown). Ice nucleation occurred at a higher temperature in empty alginate beads (-11°C) than in encapsulated (-17°C) or non-encapsulated meristems (-20/22°C) probably reflecting the fact that the empty beads have higher water contents. This data (melt only) could not be statistically confirmed due to a non-homogeneous distribution of the melt onset temperatures.

Step 3: After 4-hours of evaporative desiccation, the absolute water content of meristem and beads (with and without a meristem) varied from 0.09 to 0.21 g.g⁻¹ DW (Table 3). The presence of one meristem within the bead may be responsible for a non-homogeneous desiccation of the surrounding alginate. Large disparities in water content were only observed in beads containing a single meristem. Crystallisation and melting events were not recorded (under our experimental conditions) in meristems desiccated for 4-hours, except in one out of 6 meristems excised from a sucrose-treated shoot (Table 3). In this latter sample, the amount of freezable water detected was very low (0.009 g g⁻¹ DW, data not shown) and consequently the average crystallinity was less than 2%. Representative thermograms showing the effects of cooling and rewarming on sucrose-treated, air desiccated, encapsulated and non-encapsulated *R. ciliatum* meristems are shown in Figures 2a and b (glucose thermograms are not shown). Whether a bead was empty or contained a meristem, glass transitions clearly took place during cooling in all the beads and it occurred at higher temperatures in empty beads (-46°C) than in those containing one meristem (-57 to -60°C).

Figure 1. DSC thermograms showing the effects of cooling and rewarming on *Ribes ciliatum* Meristems excised from shoots treated with 0.75M sucrose for 1 week. Treatments comprise: (a) an excised apex, encapsulated in alginate, osmotically dehydrated for 17 h in 0.75M sucrose and removed from the bead before DSC; (b) as for (a) but the apex is retained in the bead for DSC. The Y-axis represents relative scales of (a) 5mW and (b) 200mW. C = crystallization; M = melt.

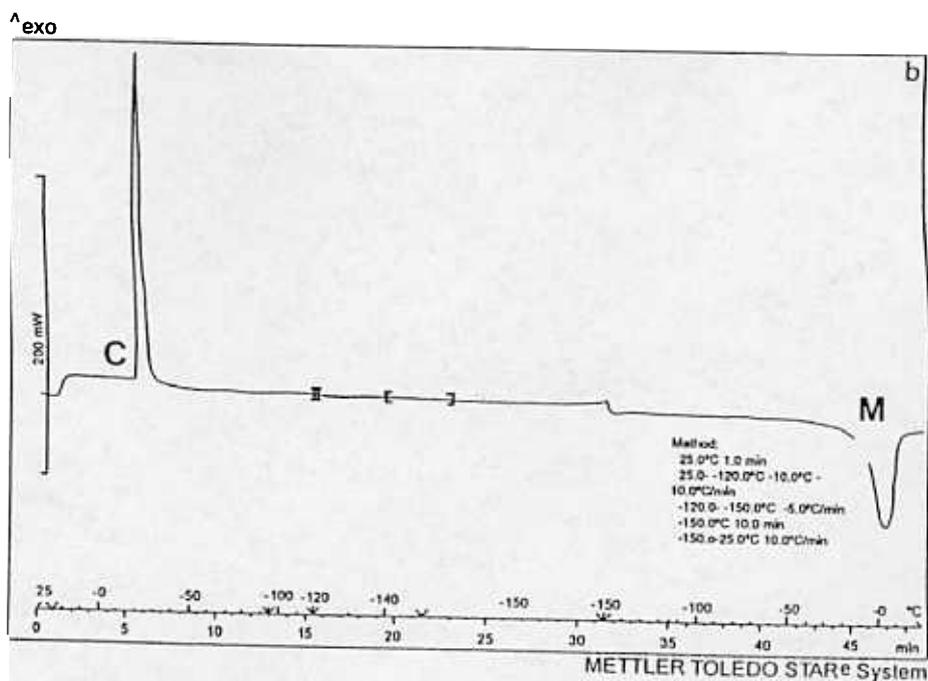
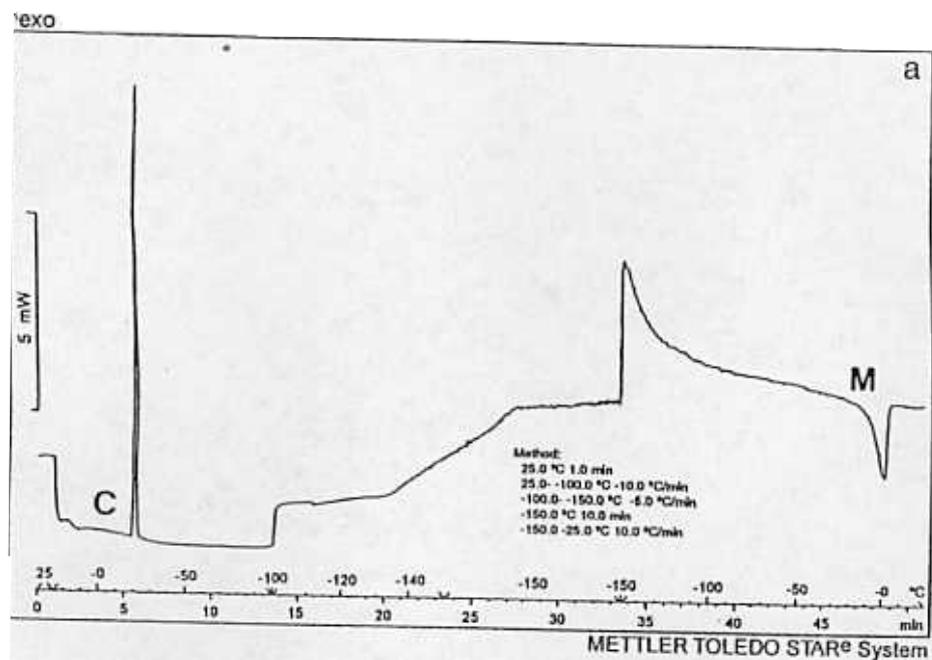


Figure 2. DSC thermograms showing the effects of cooling and rewarming on *Ribes ciliatum* Meristems excised from shoots treated with 0.75M sucrose for 1 week. Treatments comprise: (a) an excised apex, encapsulated in alginate, osmotically dehydrated for 17 h in 0.75M sucrose, air desiccated for 4 h and removed from the bead before DSC; (b) as for (a) but the apex is retained in the bead for DSC. The Y-axis represents relative scales of (a) 5mW and (b) 200mW. GT = glass transition

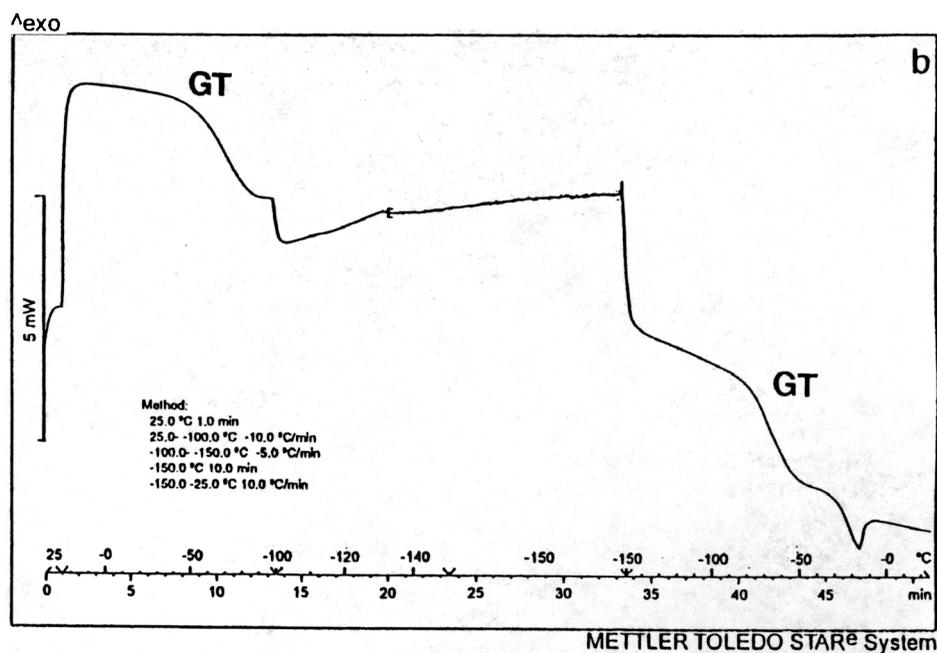
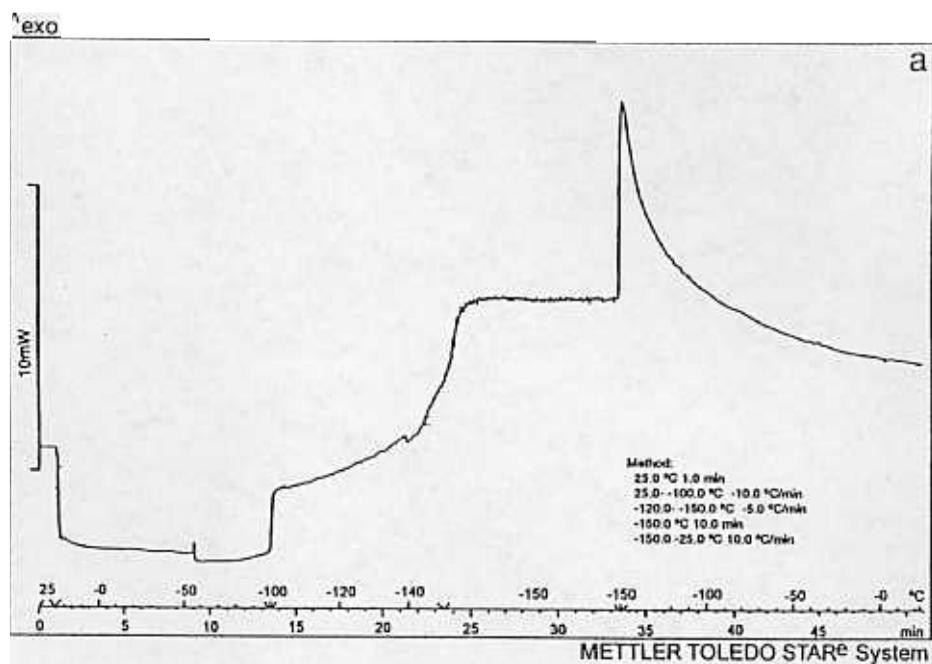


Table 2: Profiles of absolute water content, crystallinity and thermal events during a freeze/thaw cycle of alginate beads and encapsulated and non-encapsulated *Ribes ciliatum* meristems following a 7-day treatment of shoots, alginate encapsulation and a 20-22h pre-treatment in 0.75M sucrose

Meristem treatment	water content ($\dot{g}\cdot g^{-1}DW$) Absolute	% Crystallinity	THERMAL EVENT			
			cooling		thawing	
			ice	onset T ^o (°C)	melt	onset T ^o (°C)
0.1M glucose	1.3 ^a ±0.17	84 ^a ±6	3/3	-22 ^a ± 0.70	3/3	-10 ± 2.08
0.1M glucose and encapsulated	2.2 ^{cb} ±0.35	88 ^a ±2	3/3	-17 ^a ± 6.24	3/3	-5 ± 3.05
0.75M sucrose	1.7 ^a ±0.18	90 ^a ±1	3/3	-20 ^a ± 1.41	3/3	-8 ± 0.81
0.75M sucrose and encapsulated	2.2 ^b ±0.02	85 ^a ±3	3/3	-17 ^a ± 3.05	3/3	-9 ± 1.58
alginate bead	2.7 ^c ±0.1	84 ^a ±2	3/3	-11 ^b ± 1.41	3/3	-7 ± 0.09

3-4 replicates were used per treatment (mean±standard deviation), Values within columns with different superscripts differ significantly ($P<0.05$). For thermal events, statistical analysis using was only performed with the onset temperature of the glass transition. Ice and melt events were recorded for each of the beads and/or meristems.

The beads containing one meristem underwent a glass transition, from -71 to $-81^{\circ}C$, upon rewarming, which was invariably followed by an endothermic event between -30 and $-26^{\circ}C$. The freezable water content determined from this last event was very low (0.01 to $0.03 g g^{-1} DW$, data not shown) and showed a large disparity from one sample to another. As a direct consequence the crystallinity of the alginate beads containing one meristem was also variable (Table 3). Rewarming profiles of empty beads desiccated for 4 hours revealed glass transitions, which took place at a higher, but not significantly different temperature ($-58^{\circ}C$) compared to the beads containing one meristem. However, at this stage, the empty beads no longer contained freezable water (Table 3). A representative thermogram of a sucrose-treated, empty alginate bead is shown in Fig. 3.

Recovery after cryopreservation

Most (90%) of the meristems excised from sucrose treated shoots survived dehydration in sucrose and 70% regrew after evaporative desiccation (Table 4). After cryopreservation 45% of the meristems had normal growth within 4 weeks of recovery. In contrast, over 50% of the meristems excised from glucose treated shoots) were killed by the sucrose pre-treatment. The evaporative desiccation step was lethal to 90%, and none survived cryopreservation.

Table 3: Profiles of absolute water content and thermal events recorded during a freeze/thaw cycle of encapsulated and non-encapsulated *Ribes ciliatum* meristems performed after Step 3 of the cryopreservation protocol.

Meristem treatment	THERMAL EVENTS				
	water content g.g ⁻¹ DW absolute	cooling		thawing	
		nature	onset T ^o (°C)	nature	onset T ^o (°C)
0.1M glucose	0.17 ^a ±0.05	NE	/	NE	/
0.1M glucose and encapsulated	0.09±0.06 ^a	3/3 ^{GT}	-60±3 ^a	3/3 ^{GT}	-81±4 ^a
				3/3 ^E	-30±2
0.75M sucrose	0.21±0.028 ^a	1/3 ^C	-54	1/3 ^M	-2
		2/3 ^{NE}	/	2/3 ^{NE}	/
0.75M sucrose and encapsulated	0.10±0.12 ^a	3/3 ^{GT}	-57±3 ^a	3/3 ^{GT}	-71±12 ^{ab}
				3/3 ^E	-26±3
alginate bead	0.23±0.03 ^a	4/4 ^{GT}	-46b±2 ^b	4/4 ^{GT}	-58±7 ^b

3 to 4 replicates were used per treatment (mean±standard deviation). Values within columns with different superscripts differ significantly, (P<0.05). Ice and melt events were recorded for each of the beads and/or meristems. Thermal events are designated as ice crystallisation nucleation (C); glass transition (GT); melt (M); endothermic event (E); no event detected (NE).

Figure 3. DSC Thermogram showing the effects of cooling and rewarming on a sucrose-treated (empty) bead after 4 h desiccation. GT = glass transition

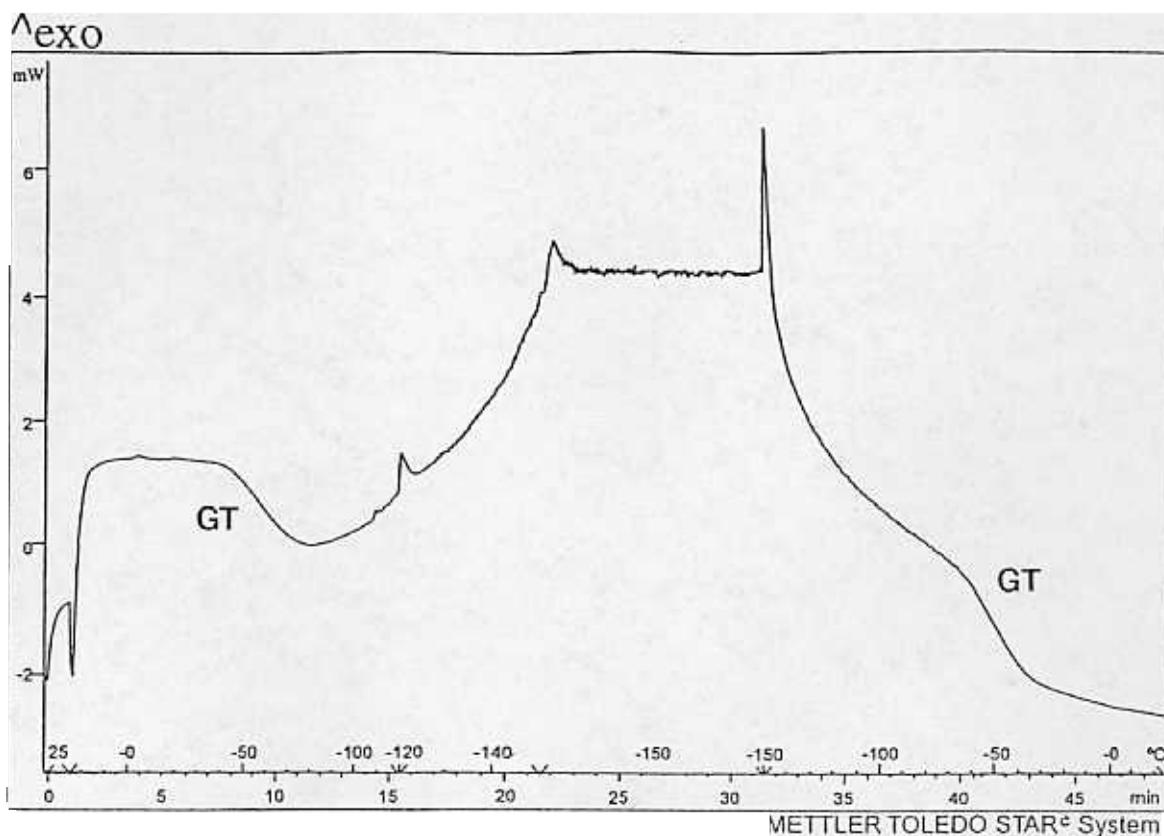


Table 4: Survival of encapsulated meristems of *R. ciliatum* after exposure to different stages of the cryopreservation protocol

TREATMENT	% SURVIVAL		
	20-22 hr 0.75M sucrose	4-hr desiccation	cryopreservation
0.1M glucose	6/14 (42%)	2/20 (10%)	0/21 (0%)
0.75M sucrose	9/10 (90%)	14/20 (70%)	9/20 (45%)

Meristems were excised from either sucrose (0.75M) or glucose (0.1M) treated shoots at each of the 3 stages of the cryopreservation protocol. Total survival is as shoot regeneration for the total number of shoot-meristems cryopreserved in 4 replicate tubes each containing 5 encapsulated meristems.

DISCUSSION

In this study, an extended 0.75M sucrose treatment was applied to shoots of *R. ciliatum*, rather than to the encapsulated meristem only, as is generally the procedure (4, 8, 16, 11). The 1-week exposure to high concentrations of sucrose was initially used as a substitute for cold acclimation and is crucial to enhance the tolerance of meristems to desiccation and consequently cryopreservation.

The most obvious difference between meristems treated with sucrose or glucose is the response to the 20-hr sucrose dehydration. Indeed, after this osmotic stress, the meristems excised from glucose-treated shoots died even though both types of treated meristems had similar absolute and freezable water contents.

It is possible that the dynamics of water loss and/or uptake plays a crucial role in desiccation and consequently cryopreservation tolerance. Slow desiccation treatments allow tissues to reach a lower water content which is commensurate with the maintenance of viability for some plant tissues (5, 10). However, for other, highly desiccation-sensitive tissues the opposite conclusion can be drawn (2). It is conceivable that during the 7-days exposure of *R. ciliatum* shoots to high sucrose concentrations meristems develop adaptive mechanisms against further osmotic stress.

DSC studies revealed differences in absolute water content (Table 1) between cryo-tolerant (sucrose-treated shoots) and cryo-sensitive (glucose-treated shoots) meristems. Thus, supporting the importance of using sucrose in pre-treatments.

Glass transitions were always recorded in the 4-hr desiccated encapsulated meristems during cooling, however, a small endothermic event was invariably recorded in these samples during re-warming. This may suggest that ice was formed either during cooling or re-warming which was not detected by the DSC technique applied. However, the glass formed during the cooling step of the encapsulated meristems appears not to be stable during re-warming. Interestingly, no sign of glass destabilisation was recorded either in the alginate bead or meristem when these components were cooled/re-warmed separately. It is possible to speculate (albeit that the DSC evidence in this study is not conclusive) that the instability of the glass formed within beads containing a single meristem may be due to thermal gradients within the sample. This could be a result of the heterogeneous composition of the alginate bead and the meristem and this situation may promote ice nucleation. This may be a consequence of the differences between the thermal properties of the two components. However, other possibilities must not be overlooked, thus, a differential moisture gradient may exist between the tissue and the bead which could promote glass destabilization events on re-warming. It is also possible to speculate that, the bead and the meristem exhibit

differential glass relaxation properties which could also promote ice nucleation. Clearly, more detailed studies of the thermal events that take place in the alginate-plant system would be useful. These studies indicate that the meristem-alginate complex appears to behave differently to the independent system components. Thus, glass destabilization on rewarming may cause additional damage to meristems and consequently reduce survival to cryopreservation. This factor may not be of significance for genotypes which are amenable to cryopreservation, but it may be important for storage-recalcitrant species and cultivars.

Encapsulation obviously facilitates meristem handling and may advantageously reduce the rates of desiccation (osmotic and evaporative) and re-hydration. However, practically, it may be important to examine, in more detail, the re-warming stage of the protocol. Two-step rewarming (slow warming, followed by rapid warming) of encapsulated meristems may offer the advantage of stabilizing the glass as it passes through relaxation events and the glass transition temperature. In the case of *Ribes* meristems frozen in the presence of a vitrification solution (PVS2), ice crystal formation always occurred during the freeze/thaw cycle (1). In contrast, only glass transitions were recorded when the meristems loaded with PVS2 were cryopreserved after removal from the vitrification solution. Our further work will investigate the re-warming step of the encapsulation protocol.

We consider that this modified *Ribes* cryopreservation procedure may offer a useful alternative to cold acclimation. The approach may be especially useful for chill-sensitive genotypes and it employs a very simple pre-growth step (0.75M sucrose for one week) for which controlled, low temperature, environment facilities are not required. By using this modified method we were able to cryopreserve a wild species, *R. ciliatum*. Using the same method 90-95% survival was obtained with commercial *R. nigrum* cultivars, Ben Lomond and Ojebyn (6) and these survival responses were comparable to those obtained using cold acclimation (1, 6). We are now applying the method to a wider range of *Ribes* genotypes with a view to establishing a cryopreserved genebank of *Ribes* germplasm in Scotland (22).

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