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Article

Published Version

CryoLetters **33** (2), 160-168 (2012)

CRYOPRESERVATION OF WINTER-DORMANT APPLE: III – BUD WATER STATUS AND SURVIVAL AFTER COOLING TO -30°C AND DURING RECOVERY FROM CRYOPRESERVATION

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Abstract

In a continuing study to improve the efficiency of dormant bud cryopreservation for tissues hardened in maritime climates, the water status of dormant buds was monitored between -4°C and recovery from liquid nitrogen (LN). Measurement of water content, simple thermal analysis and differential scanning calorimetry were employed. Buds did not lose water during cooling to, or holding at -30°C indicating that cryodehydration and/or other adaptive responses contributed during this essential step. A bud exotherm that was an artefact of warming was detected due to necessary handling at -4°C before cooling to -30°C. There were no significant differences between cultivars with respect to water status at -30°C or immediately upon rewarming from LN despite significant differences in post-LN survival. Buds rehydrated in 5 days, but up to 14 days may be needed for recovery for some cultivars. In some instances buds could be grafted without rehydration, taking up water across the early graft union.

Keywords: *Malus*, dormant bud, water status, cryopreservation

INTRODUCTION

An effective protocol for cryopreservation of dormant winter buds of woody plant species has been developed at Fort Collins, Colorado, USA, using material grown in the continental climate (10) of Geneva, New York (4, 5, 18, 19). Subsequently, the protocol has been transferred to locations in Europe where the climate is more maritime in nature and adaptive winter hardening often accumulates under less severe conditions than continental USA (6, 8, 13, 26). The European experience, in common with that in the USA, is that survival can vary significantly with season and cultivar but the minimal 40% recovery proposed as acceptable for genetic conservation (12) can be regularly achieved. A recent study (7) recording a 3 year average of explant survival indicated that, for some cultivars, the effect of different summer and winter environments may not be such a significant issue, but

the data was obscured by a holding period of 5-21 days at -5°C for the harvested samples. Further, quantitative study using a very wide range of genetic material was required. Inevitably, a more detailed understanding of the effects of the various stages of the Forsline (5) protocol would lead to protocol modifications that could optimize survival in a variable, maritime season. A central viewpoint is that the dormant bud cryo-technique avoids *in vitro* culture (3) or bud encapsulation (15) increasing utility in circumstances where access to resources and laboratory facilities are limited (11, 26), and reducing the costs and time needed for plant production from field germplasm collections (9).

Previous studies for *Malus*, and other woody species, have detailed the benefits of dehydrating dormant explants and buds at -4°C to reach a water content of c. 30% of fresh weight, in preparation for cryopreservation. This stage precedes cooling to around -30°C for up to 24 h before immersion in LN (5, 14, 16, 18, 20, 21, 22). Dehydration at -4°C alone will not produce survival from LN (26) and a study that suggested this step could be omitted from the formal protocol was based on material taken from extended storage at -4°C (17), essentially substituting for the first dehydration step.

Studies with *Malu*s grown in the east of Denmark (24, 25, 26) provided detail of the effects of the protocol, including post-cryopreservation survival, using ecodormant buds hardened during winters of differing severity. The initial, extended (c. 14 days) incubation at -4°C reduced bulk tissue water by evaporative loss and cellular water content by cryodehydration. The latter occurred, as there was, reproducibly, a freezing event in both the bud and stem tissues of the isolated explant within the first hour of incubation. The explant water content fell from a consistently typical level of 45% down to 30% moisture content (MC, fresh weight basis) during incubation, with no significant difference between explants regardless of cultivar or eventual survival. This earlier study also highlighted the contribution of secondary buds to recovery growth when the meristematic region of the primary bud was lethally injured during cryopreservation (25). As explants taken directly from the -4°C incubation did not survive to LN storage, the second protocol step, cooling to -30°C at 1°C h^{-1}, became essential for their recovery (25).

The present study examines the effects of this second step on the water relations of the explants and their eventual survival after cryopreservation. The issue of interim warming is also considered as the explants, cooled in air at -4°C, are next grouped in containers for further cooling to -30°C prior to immersion in LN. This handling procedure may take more than 2 min to complete, with inevitable warming. Thermal history, tissue water content and survival during cooling from -4°C to -30°C have been investigated, together with the effects of post-rewarming rehydration in a moistened substrate, such as peat or sand, for up to 15 days (5, 26). Differential scanning calorimetry (DSC) has been used to investigate the effect that changes in bud water content during preparative desiccation and cooling have on glass transitions during further cooling beyond -30°C.

MATERIALS AND METHODS

Cryopreservation

Winter-hardened shoots of *Malus domestica* 'Holsteiner Cox', 'Maglemer' and 'Prima' were collected in January 2009 and 2010 following a minimum of 4 days with a mean temp below -4°C. Explants were prepared from wood produced in the previous growing season, each comprising a 3.5 cm stem segment bearing a single bud close to the midpoint. These were desiccated at -4°C as previously described (25). When the water content fell to 29.5±1% MC (c. 14 days), batches of 10 explants were sealed into 50 ml polypropylene centrifuge tubes and cooled to -30°C at 1°C h^{-1}. The vials were held at this temperature for 24 h and then

plunged directly in LN. To recover the explants the tubes were removed from LN, uncapped and held at 5±1°C for 24 h (0.8°C min^{-1} average rewarming rate from LN to 5°C) before transfer to moist peat for up to 14 days, also at 5±1°C.

Recovery and survival

Shoots collected in January 2010 were rewarmed from LN in March 2010. Twenty-four hours after rewarming (and without rehydration) a randomly selected sample of explants was grafted onto M7 rootstocks. The remainder were placed in containers with moist peat (10 explants in a container of 280 ml, 95 mm diameter) and placed at 5±1°C for hydration. After 5, 9 and 14 days of rehydration, survival was recorded and evaluated using regression analysis. The procedure was repeated twice.

Water content

For samples harvested in 2009, buds were separated from the explant with a single, basal cut and the MC of isolated buds and entire explants determined gravimetrically immediately before, and after, cooling to -30°C and after recovery from LN (24). This was repeated in 2010 for buds and the MC of explants estimated by recording the fresh weight of bagged samples of entire explants, and separated buds at 2-day intervals (24). Bud MC was determined gravimetrically after 0, 5, 9 and 14 days of rehydration in moist peat.

ANOVA was used to detect significant differences in the MC of explants and buds within a cultivar and for the different steps of the protocol ($P \leq 0.05$). Both a linear mixed model and analysis of covariance were used to identify significant differences in rates of water loss and uptake.

Simple thermal analysis

Shoots were gathered in December 2009 following a period of 3 days when the mean daily temperature was below 0°C, and the buds were ecodormant. Type K thermocouples (RS Components, UK) were inserted 2-3 mm into bud tissues [n=16 (24)] to be able to monitor tissue freezing events.

To avoid warming due to handling, explants were incubated for 14 days at -4°C and then immediately cooled (in the same freezing chamber) at 0.5°C min^{-1} to -30°C. The experiment was repeated twice.

A second experiment simulated the rewarming that results from moving explants from -4°C into tubes for cooling to -30°C. Explants were taken following desiccation at -4°C and thermocouples inserted into the buds on a work surface at 0°C. The insertions were timed to simulate the handling events required by the protocol. Subsequently, the explants were cooled at 0.5°C min^{-1} to -30°C (n >15 per cultivar).

Temperature was recorded at 2 s intervals using a Pico Technology TC-08 data-logger software (Picotechnology, Cambridge, UK) and significant differences in nucleation temperature were identified using ANOVA.

Differential scanning calorimetry (DSC)

Explants were prepared and processed as described by Vogiatzi *et al.* (24) and isolated buds analysed from explants after: cooling to -30°C; cooling to -30°C + 24 h at -30°C; removal from LN following the complete cryopreservation protocol; 24 h post-rewarming at 5±1°C. In this last treatment no additional water was available to the explants, limiting cellular rehydration to uptake from the extracellular matrix. The DSC outputs were processed using TA Universal Analysis software (TA Instruments, UK) and the results combined with the gravimetrically determined fresh and dry weights to provide the proportion of water crystallized (g water per g fresh weight).

ANOVA was used to identify significant differences ($P \leq 0.05$) in exotherm initiation temperatures, glass transition temperatures and residual water contents, calculated from the DSC outputs.

RESULTS AND DISCUSSION

Water content, recovery and survival

The study of water content indicated that there was no loss from explants or buds during cooling from -4°C to -30°C (Table 1) and there was no statistically significant difference in water content ($P<0.05$) within or between cultivars at any stage in the process. However, this uniformity of water status was not reflected in the post-cryopreservation survival of the cultivars, which ranged from 10 to 95% (Table 2). Vertucci and Stushnoff (23) considered that buds of *Malus domestica* and *Amelanchier alnifolia* (Saskatoon berry) that withstood freezing at -45°C were those that had become desiccated to, but not beyond, the critical MC for desiccation damage, and that failure to survive related to exceeding this limit. Yet such damage is unlikely to be as critical an issue in this study, as the poor survival of 'Holsteiner Cox' followed LN immersion and was not seen at -30°C, where performance was comparable to the other cultivars (26). Using nuclear resonance spectroscopy (NMRS), Tyler *et al*. (22) achieved comparable desiccation to the levels seen in this study by progressive cooling, attaining optimal levels of desiccation between -30°C and -40°C, depending upon cultivar. However, in the present study and using the Forsline *et al.* (5) protocol and material acclimated under maritime conditions, all of the desiccation occurred during the extended incubation at -4°C that precedes cooling to -30°C. The data in Table 1 showed no significant change in MC of explants during cooling between -4°C and -30°C that is critical for survival (26). This suggests that water movement from unfrozen cytoplasm to the frozen, extracellular matrix (cryodehydration) and, possibly, other cellular adaptations in response to reducing temperature and increasing osmotic stress are involved (13). There was no change in MC of explants or buds rewarmed from LN when compared to the -30°C levels.

Table 1. Mean moisture content (% fresh weight ± sem**) of explants and buds of the *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' cooled from -4°C to -30°C at 1°C/h^{-1}, after 24 h at -30°C and after rewarming from LN (*n* =30 in 2008-9, >3 in 2009-10).

Treatment stage	Winter 2008-2009 collection					
	Cultivar					
	Holsteiner Cox		Maglemer		Prima	
	Explant	Buds	Explant	Buds	Explant	Buds
At -4°C	30.2± 0.3	28.7± 0.4	30.0± 0.3	26.7± 0.4	29.6± 0.3	28.6± 0.6
At -30°C	30.0± 0.4	26.8± 0.5	30.4± 0.2	26.8± 0.4	29.7± 0.3	26.8± 0.4
After LN	29.6± 0.3	26.7± 0.4	29.4± 0.2	26.5± 0.4	29.3± 0.3	28.1± 0.4
	Winter 2009-2010 collection					
At -4°C	31.6*	33.5±0.5	30.1*	29.1±1.7	31.6*	21.2±1.8
At -30°C	31.3*	31.4±1.0	29.6*	28.9±0.2	31.1*	24.6±0.8
24 h at -30°C	31.2*	31.1±1.2	29.3*	28.0±0.8	30.8*	23.6±0.1
After LN	30.9*	31.4±1.2	30.2*	27.6±0.7	30.6*	22.6±0.9

*calculated values, see M&M and (24); ** sem – standard error of the mean.

After a 5-day incubation in moist peat (Table 2) the explants had rehydrated back to their MC at harvest and did not increase significantly thereafter up to 14 days ($P \leq 0.05$). The linear

mixed model showed no significant differences in MC or rate of rehydration between cultivars and there was no indicative relationship between MC and survival. Given the very large difference in survival between 'Prima' and 'Holsteiner Cox' on recovery from LN, it suggests that the initial uptake into the tissues from the external environment is not strongly influenced by lethally damaged cells and results, at least in part, from non-osmotic differences in water potential not directly related to viability. This section of the study demonstrated that water status during cooling from -4°C, and during rehydration, was not a reliable indicator of eventual survival following cryopreservation.

A prolonged rehydration phase appeared to be beneficial for survival in some instances e.g. the cultivar 'Prima' showed significant, increasing survival with rehydration time, indicating a process of repair and recovery in explants that were damaged, but not lethally so [Table 2; (1, 2)]. No similar, beneficial effect was seen for 'Holsteiner Cox' indicating that the damage suffered by these explants was irreparable. In some instances, an extended rehydration period can reduce survival (17). Notably, some buds from the 2009-10 harvest of cultivars 'Prima' and 'Holsteiner Cox' that were grafted 24 h after rewarming without rehydration were viable (55 and 20% respectively, Table 2) indicating effective water uptake across the early graft union. The potential significance of these results indicates that a larger, quantitative survival study dealing with the role and impact of the rehydration step in the protocol is necessary.

Table 2. Mean moisture content (MC, % fresh weight ± sem**) of buds of the *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' after rewarming from LN for 24 h and after 5, 9 and 14 days of rehydration. Survival expressed as successful grafts (mean of 2 replicates., minimum n = 10).

Treatment stage	Cultivar				
	Holsteiner Cox		Maglemer*	Prima	
	MC (% fwt)	Survival (%)	MC (% fwt)	MC (% fwt)	Survival (%)
Rewarmed 24h	35.4±2.4	3/20 (15)	27.3± 2.0	27.0±1.9	11/20 (55)
5 days rehydration	45.2±0.5	3/30 (10)	44.7± 0.6	45.4±0.9	12/30 (40)
9 days rehydration	42.8±0.7	4/30 (13)	42.9± 0.6	44.6±0.6	12/30 (40)
14 days rehydration	45.3±0.7	2/20 (10)	45.3± 0.7	46.8±0.6	19/20 (95)

*survival data not recorded for this cultivar; **sem – standard error of the mean

Simple thermal analysis

When explants were cooled directly from -4°C to -30°C without intermediate warming there were no exothermic events recorded in bud tissues (n =24). However, when explants from -4°C were being sealed into tubes for the subsequent cooling steps, then bud tissues warmed at a mean of 3.18°C min^{-1}. They reached above 0°C, and up to 5°C, within the 2 min realistically required for handling. Subsequent cooling to -30°C produced a single exotherm in bud tissues, indicating that ice had indeed melted during handling and was readily refrozen (Table 3).

The absence of a bud exotherm during uninterrupted cooling after desiccation at -4°C and down to -30°C suggested there was relatively little water in the bud tissues after desiccation that was not either frozen, or in a localized, vitrified condition. This cooling step to -30°C was

essential for survival (26) and any further, small freezing and/or vitrification events that occurred would be recorded using the techniques employed here. However, although the exotherm measured during cooling to -30°C was an artefact, it provided information about the thawed solutions in the extracellular matrix of the bud tissues. When comparing cultivars, the exotherm initiation temperature for Holsteiner Cox (collected winter 2008-09) was significantly higher ($P \leq 0.05$) than for the other two cultivars (Table 3). This was the cultivar with the lowest survival for that winter harvest [28% *vs.* 88% for Maglamer and 84% for Prima; (25)] and the higher initiation temperature may indicate lower solute concentrations in the extracellular matrix, perhaps reflecting inadequate hardening.

Table 3. Mean exotherm initiation temperature (°C± sem) and proportion of nucleating buds of the *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' cooled at 0.5°C min^{-1} following sample transfer from desiccation at -4°C.

	Cultivar		
Exotherm	Holsteiner Cox	Maglemer	Prima
Mean nucleation temperature (°C)	-10.2 ± 0.6[a]	-12.5 ± 0.2[b]	-11.9 ± 0.6[b]
Nucleated buds	13/15 (87%)	15/17 (88%)	10/15 (67%)

Different superscript letters indicate significant differences between cultivars; sem – standard error of the mean

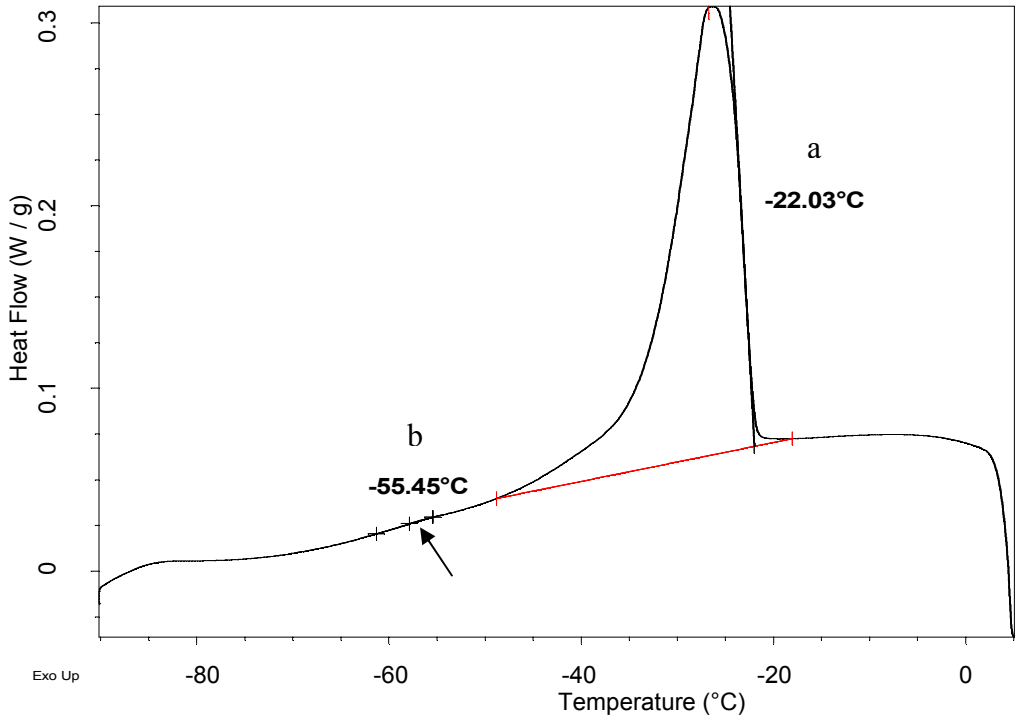

Figure 1. An illustrative DSC trace showing thermal events from a bud of 'Prima' 24 h after recovery from LN. The initiation temperature of the exotherm peak is indicated (a), together with that for the start of the glass transition (b). The vertical lines on the trace (arrow) indicate the start, inflection and end points of the transition, calculated by the DSC software.

Differential scanning calorimetry (DSC)

Preparation of previously frozen explants for DSC analysis had the unwelcome consequence of tissue warming, as there is a 3 min equilibration at 5°C. The consequence in this study was that subsequent analysis detected an exotherm that was again an artefact of preparation (Table 4), comparable to that seen during the cooling step to -30°C (above). There were no significant differences in exotherm initiation temperature within the cultivars when different stages of the protocol were examined but there were differences between them (Table 4). As seen with the exotherm detected by thermal analysis, 'Holsteiner Cox' (the lowest surviving cultivar) had a significantly higher ($P \leq 0.05$) exotherm initiation temperature than the other two cultivars (Table 4). The previous suggestion that this resulted from lower solute concentrations in the bud extracellular matrix and was indicative of an inadequate level of winter hardiness could be considered again here.

Table 4. Mean exotherm initiation temperature (E^i ± sem) and glass transition inflection temperature (Tg^i ± sem) for buds of the *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' sampled during the cryopreservation protocol, after desiccation at -4°C (minimum *n* =3).

Treatment stage	Cultivar					
	Holsteiner Cox		Maglemer		Prima	
	E^i (°C)	Tg^i (°°C)	E^i (°C)	Tg^i (°C)	E^i (°C)	Tg^i (°C)
At -4°C	-15.0±0.2 ^{a}	-54.3±1.4^{x}	-22.0±1.1^{ab}	-55.9±0.8^{x}	-24.6±0.9^{b}	-62.4±1.7^{y}
At -30°C	-12.9±0.3 ^{a}	no Tg	-20.7±0.5^{b}	-59.4±1.2^{y}	-22.4±0.6 ^{b}	-63.9±1.0^{y}
24 h at -30°C	-14.8±0.6 ^{a}	-59.1±0.6^{y}	-18±1.8^{ab}	-59.1±0.5^{y}	-22.4±0.8 ^{b}	-60.0±0.6^{y}
After LN	-18.5±1.8 ^{a}	-55.0±2.6.^{y}	-19.8±0.6^{a}	-55.7±0.8 ^{y}	-26.4±1.7 ^{b}	-60.0±0.9^{y}
Thawed 24 h	-14±1.9 ^{a}	-50.3±1.6^{x}	-21.7±2.2^{b}	-59.8±1.9^{y}	-20.9±1.3^{b}	-57.5±1.2^{y}

Different superscript letters indicate significant differences between cultivars - ^{ab} exotherm initiation, ^{x,y} glass transition.; sem – standard error of the mean.

The DSC data also showed no significant differences in glass transition temperature (Tg^i) between the cultivars at the point (-30°C) where they were plunged in LN and this uniformity was not reflected in post-rewarming survival. When rewarmed buds were taken directly from LN before analysis there was no significant change in exotherm or glass transition initiation temperature, as might be expected. However, when rewarmed buds were taken after 24 h incubation at 5±1°C the lowest surviving cultivar ('Holsteiner Cox') did show significant differences from the other two. In this case dying, or dead, tissues will influence the properties of the solutions involved in the freezing event recorded as an exotherm.

The DSC outputs indicated that a relatively small proportion of the bud water was involved in the exotherm (<10%) and that the bulk of the non-frozen water in the bud at -30°C contributed to the later glass transition. Table 5 supports this with a comparison of the bud water (% fresh weight) contributing to the glass transition, presented both as the calculated DSC output and the gravimetrically determined total water content. The DSC data indicated limited differences between cultivars after the -4°C treatment that were not evident at-30°C.

This study has shown that the necessary desiccation for survival following cryopreservation of *Malus* dormant buds from a maritime climate could be achieved wholly by desiccation at -4°C. Cryodehydration that occurred during further cooling to -30°C was necessary for survival, perhaps with other adaptive responses. The possibility of achieving the required cryodehydration at higher temperatures, e.g. -20°C, with a longer duration, will be investigated, as this might allow the use of readily-available, conventional freezers for cooling prior to immersion in LN.

Table 5. Bud water (% fresh weight ± sem) contributing to the glass transition in buds of the *Mal*us cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' measured gravimetrically or calculated as an output from DSC during cooling at 5°C min^{-1} to -90°C (minimum *n* =3).

Treatment	Holsteiner Cox		Maglamer		Prima	
	DSC	Gravimetric	DSC	Gravimetric	DSC	Gravimetric
Desiccated at -4°C	29.0±0.5 [a]	28.7± 0.4	27.2±1.8 [ab]	26.7± 0.4	20.4±1.6 [b]	28.6± 0.6
Cooled to -30°C	27.4±0.5	26.8± 0.5	26.5±0.2	26.8± 0.4	23.4±0.7	26.8± 0.4

Different superscript letters indicate significant differences between cultivars; sem – standard error of the mean.

Exotherms seen during cooling to -30°C were artefacts resulting from unavoidable sample handling that allowed warming, yet the percentage of water that thawed was low and did not alter significantly the amount of bud water that vitrified between -50°C and -60°C. The changes in water content did not reflect eventual survival following cryopreservation, there being no significant differences between high- and low-surviving cultivars.

Rehydration of explants in a moistened medium such as peat may not be essential for survival, as some buds survived after grafting directly after rewarming from LN. It is probable, however, that many cultivars will benefit from a period for damage repair, particularly where high levels of viability are required and an optimum period can only be determined empirically. A non-destructive, post-cryopreservation viability test would be of great value in this situation and an assay based on a rapid method to measure bud respiration is under development.

Acknowledgements: The authors thank Claire Rawlinson-Malone and Rebecca Green, University of Reading School of Pharmacy, for access to their DSC equipment and Milos Faltus, Crop Research Institute, Prague, Czech Republic for advice on DSC analysis.

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Accepted for publication 13/2/2012