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CRYOPRESERVATION OF WINTER-DORMANT APPLE BUDS: II - TISSUE WATER STATUS AFTER DESICCATION AT -4°C AND BEFORE FURTHER COOLING

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Abstract

The established protocol for the cryopreservation of winter-dormant *Malus* buds requires that stem explants, containing a single, dormant bud are desiccated at -4°C, for up to 14 days, to reduce their water content to 25-30% of fresh weight. Using three apple cultivars, with known differences in response to cryopreservation, the pattern of evaporative water loss has been characterised, including early freezing events in the bud and cortical tissues that allow further desiccation by water migration to extracellular ice. There were no significant differences between cultivars in this respect or in the proportions of tissue water lost during the desiccation process. Differential Scanning Calorimetry (to -90°C) of intact buds indicated that bud tissues of the cultivar with the poorest response to cryopreservation had the highest residual water content at the end of the desiccation process and froze at the highest temperature

Keywords: *Malus,* cryopreservation, dormant bud, dehydration

INTRODUCTION

Freezing dormant winter bud explants, with recovery by direct grafting, is an established method for the cryoconservation of fruit trees based on a protocol for apple that was developed in the USA, in a continental climate (3). Under such conditions relatively severe winters induce significant cold hardiness, that aids survival following liquid nitrogen storage (3, 14). In maritime climates where relatively warm winters, by comparison, reduce the achieved levels of hardiness, the protocol has been adopted with variable success between seasons and cultivars (5, 6, 8, 12, 13, 20, 21). If the technique is to offer a reliable, low resource-cost alternative to cryopreservation of *in vitro* material (2) then such variation has to be understood in detail, so that it can be minimized and survival ensured at acceptable levels.

As the explants for dormant bud cryopreservation are from winter-hardened trees, extracellular freezing of stem and bud tissues, after a degree of supercooling, would be expected. This would provide the opportunity for water migration to extracellular ice in cortical tissues and to ice masses between primordial leaves and scales of dormant buds (1, 9,

10). However, previous studies have shown that, for buds, pre-freezing alone may not be sufficient for successful cryopreservation (11, 16, 17, 18). There is no published information, to date, that indicates the initial desiccation at -4°C is sufficient to sustain direct transfer to liquid nitrogen. Consequently, a further cooling step (typically slow cooling to -30°C) to allow further water migration is required for successful cryopreservation. There is practical value, therefore, in knowing how much freezable water remains in the buds after the -4°C pretreatment, as there will be a critical maximum for post-thaw survival (4, 19). The ability to influence water content in this regard is necessary if survival of dormant buds following cryopreservation is to be improved. Three apple (*Malus*) cultivars ('Holsteiner Cox', 'Maglemer' and 'Prima') were selected for study because of their different survival responses to cryopreservation (13). The water relations of the explants during the first step of the process, where samples are desiccated for an extended period at -4°C prior to further cooling and immersion in LN, have been investigated. Evaporative water loss from different surfaces/tissues of the explant has been measured and simple thermal analysis used to monitor nucleation events. Additionally, differential scanning calorimetry (DSC) has been used to quantify the freezable water in isolated buds before and after this desiccation period.

MATERIALS AND METHODS

Shoots of *Malus domestica* 'Holsteiner Cox', 'Maglemer' and 'Prima' were collected in January 2009 and 2010 after the trees were exposed for at least 72 consecutive hours to a mean daily temperature of -4.7°C. This was to ensure the natural cold hardiness of the buds was strongly developed (Fig. 1). 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.

Desiccation at -4°C

For each cultivar, samples of 10 randomly assorted explants were placed in large-mesh, plastic net bags (10 x 6 cm), placed in a fan-assisted incubator (Holm & Halby, Denmark) at -4°C for 11-14 days, and removed when the water content was reduced to c.30% of fresh weight (3). The bags were redistributed randomly in the incubator every second day to provide as uniform desiccation between the samples as possible

Gravimetrically determined water loss

The initial water content of 10 randomly selected, entire explants, and separated buds, was determined by drying at 90°C for 24 h (3 replications). The fresh weight of bagged samples of entire explants, and separated buds, was recorded at two day intervals to be able to estimate their reducing water content. The suitability of this estimation was confirmed by determining fresh and dry weights of twigs and buds (*n*=10) at two day intervals, when no significant differences between the methods were observed (*P*<0.05). To estimate water loss from explant surfaces, one, or both, of the cut stem ends and/or the bud surface were coated with a thin layer of Vaseline™ (petroleum jelly) before desiccation. Analysis of variance, using transformed data, was used to detect significant differences in water content, and a linear mixed model chosen to investigate patterns of water loss by cultivar. Analysis of covariance was used to test whether water loss differed between cultivars. Three water loss experiments, using different explant populations, were analysed statistically and in each case showed no significant difference between cultivars. The results for a single experiment are presented in Fig. 2a.
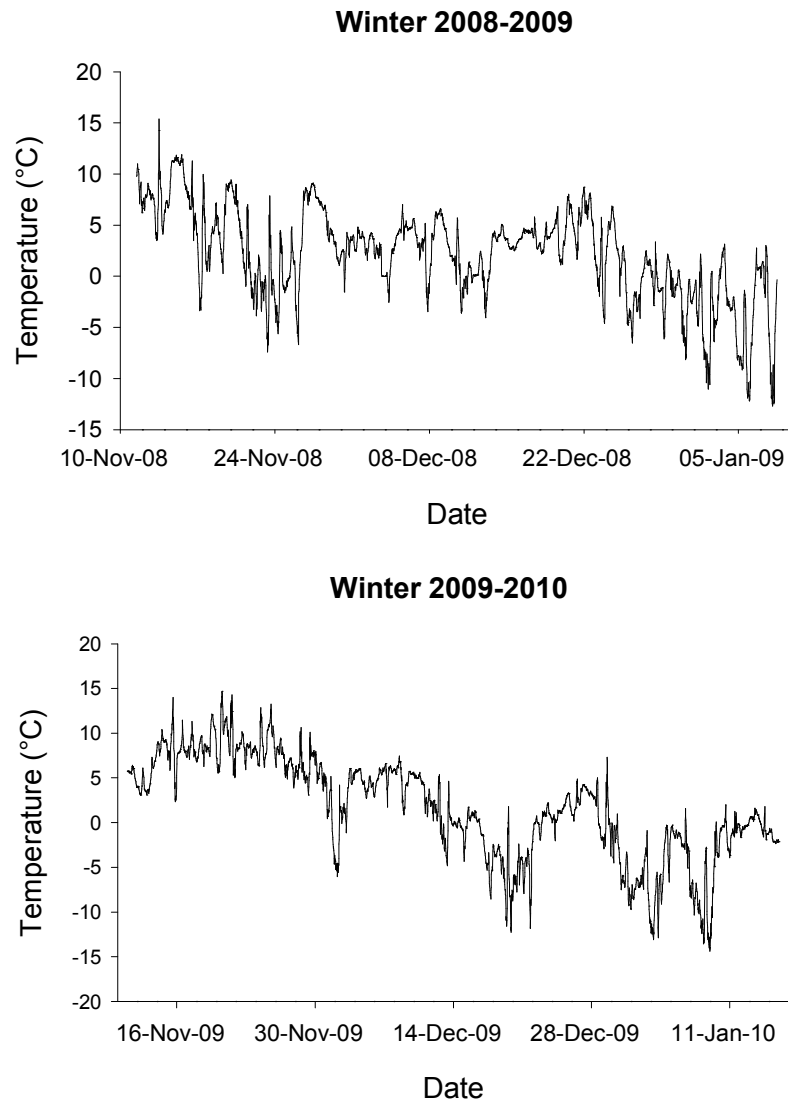
Figure 1. Continuous winter temperatures within the tree canopy at the experimental site at Taastrup , Denmark. Temperature was recorded every 20 min.

Simple Thermal Analysis (STA)

Beads of Type K thermocouples (RS Components, UK) were inserted, minimally invasively, into the tissues of the bud, immediately below the bark tissues, and at 3 mm into the exposed xylem cylinder of the explants. Material collected between November 2009 and January 2010 was taken through the standard desiccation procedure for 14 d with temperature recorded at 2 s intervals (Pico Technology TC-08 data-logger and software). Cultivar differences in nucleation time and temperature were evaluated using ANOVA. A minimum of 10 explants of each cultivar were used in each determination.

The possibility of thermocouple-induced nucleation was rejected as no significant differences in nucleation temperature were observed when the thermocouple bead was fixed externally, directly behind the bud. This configuration was not routinely adopted as it was less robust during experimental handling.

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Differential Scanning Calorimetry (DSC)

Explants were collected in January 2010 in Taastrup, Denmark and wrapped in moist paper and polythene film to prevent water lo. These were stored at 4°C for a maximum of 28 days before -4°C desiccation. Individual buds were excised from explants (on ice), weighed as rapidly as possible and sealed into Tzero Hermetic Aluminium pans (45 μl, TA Instruments, UK). Samples were examined in a Q2000 Differential Scanning Calorimeter (TA Instruments, UK). The samples were held at +5°C for 3 minutes to become isothermal, then cooled to -90°C at 5°Cmin^{-1}. After equilibration at this temperature they were re-warmed at the same rate. After warming the sample-pans were perforated and heated to constant weight at 90°C to determine sample dry weight. The DSC outputs were processed using TA Universal Analysis software (TA Instruments, UK), using peak area estimation to determine the amount of water that froze, 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 evaluate differences in the amount of water crystallized in buds at the start, and end, of the -4°C procedure.

RESULTS AND DISCUSSION

Gravimetrically determined water loss

There was no significant difference in initial water content of entire explants of the three cultivars ($P< 0.05$; Table 1), and in each case the water content of the entire explant was higher than that of the isolated bud ($P<0.05$). This supports the view that explant water contents, prior to desiccation, are poor indicators of bud water content. However, following desiccation they may have some predictive value with regard to survival after cryopreservation (Table 2).

Table 1. Mean initial water content (% fwt ± SEM) of 3.5 cm explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' from winter 2008-09 (*n*= 60 for explants, 30 for buds). Survival after cryopreservation (%) is shown and significant differences within a row are indicated by different superscript letters

		Water content (% fwt)		
		Holsteiner Cox	Maglemer	Prima
Entire explant	2008-2009	47.8 ± 0.3[a]	45.9 ± 0.2[a]	48.3 ± 0.2[a]
Bud	2008-2009	45.0 ± 0.6[ab]	43.8 ± 0.6[a]	46.8 ± 0.7[b]
Survival (%)*		72	97	60

*survival data from this study previously published in Vogiatzi *et al*. (22)

Table 2. The mean water content (% fwt ± SEM) of 3.5 cm explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' from winter 2009-10 at the start, and end, of -4°C desiccation (*n*=5). Survival after cryopreservation (%) is also shown.

	Water content (%fwt)		
	Holsteiner Cox	Maglemer	Prima
Start -4°C	46.4 ± 0.3	43.5 ± 0.4	47.1± 0.4
End -4°C*	31.6	30.4	31.6
Survival (%)	28	88	84

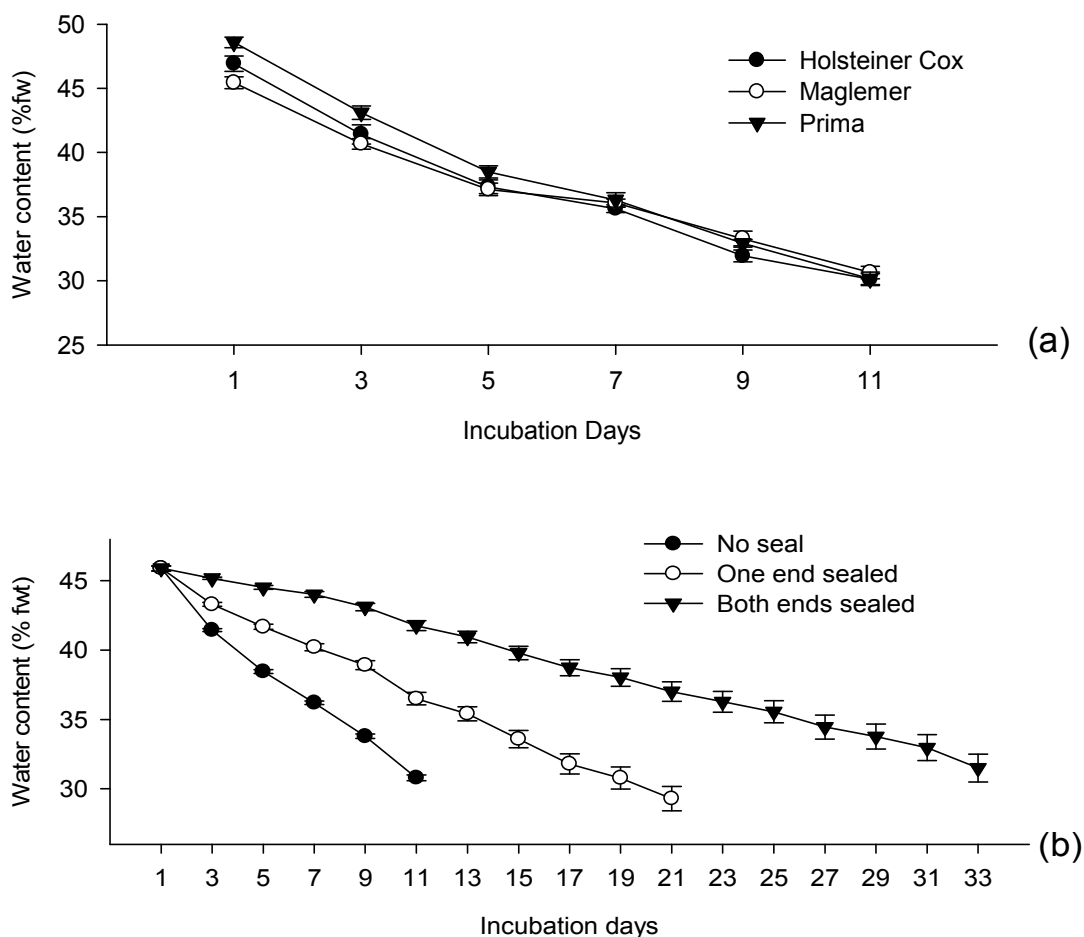*calculated values, see Materials and Methods

Figure 2. (a) Water content (% f.wt) of acclimated 3.5 cm explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' during the -4°C desiccation procedure (*n*=10) (b) Contribution to desiccation by water loss from the cut ends of the explant (data from 'Prima', n=10). Bars indicate SEM.

Analysis of covariance showed no significant differences between the cultivars with respect to water loss during the desiccation process (Fig. 2a). When examining the extent of water loss

from different explant surfaces (Fig. 2b) it is clear that there are significant differences between the surfaces, and the mixed model showed no significant difference between cultivars with respect to the relationships between the explant surfaces in the pattern of loss. As would be expected, the cut ends of the explant would be a major site for bulk water loss and sealing them will slow desiccation of the explant overall, as bulk loss has to through alternative, intact surfaces. Considering survival data (Tables 1 and 2), it becomes evident that simple measures of water content and loss from entire explants are helpful as an indicator of an explant status conducive to survival following cryopreservation, but it cannot be used as any kind of precise, predictive indicators of successful recovery. When recording water content for the population of ten samples, the standard error of the mean increased, for each cultivar, as the desiccation proceeded. This suggests an increasing divergence in the pattern of water loss between individual explants, with time, that requires further investigation.

Considering the bud rather than the entire explant, a statistically significant difference in water content ($P<0.05$) was observed between excised buds of 'Maglemer', the high-surviving cultivar and 'Prima', the lowest surviving one (Table 1), yet there was no significant difference between the water content of 'Holsteiner Cox' and the other two cultivars. It is possible this relates to different states of hardiness that influences survival but, in this instance, the size of the observed differences is so small that any predictive value of the data is questionable.

The water content of the explants was markedly reduced during the desiccation step, and the cultivars reached comparable levels close to the 30% of fresh weight that is recommended in the standard protocol. When the proportion of water lost during desiccation was considered (from Table 2), the only significant difference ($P<0.05$) was found between 'Maglemer' and 'Prima', suggesting that this parameter is not a reliable indicator of post-cryopreservation survival at an acceptable level (9)

The protocol has been applied, with varying levels of success, in several, different maritime climates (Denmark, Spain and UK) using a water content after -4°C desiccation of between 25 and 30% of fresh weight (8, 13; A.Wetten, National Fruit Collection, UK pers. comm., 2010).

These experiences, together with the data from the current study, suggest that no narrow range of post-desiccation water content can be prescribed for successful cryopreservation of dormant buds, and that the eventual level for successful preservation is strongly influenced by season and genotype. There would be considerable benefit if a relationship between initial water status after hardening and final water content after desiccation could be found that would allow a prediction of the required water content for survival of 40%, or more, of the frozen explants

Simple Thermal Analysis

Regardless of cultivar, the vast majority of explants (179 of 181) nucleated during the first hour of incubation at -4°C with the remaining two nucleating between 60 and 90 minutes. There was no significant difference between cultivars with respect to the time taken to nucleation during that first hour ($P<0.05$). Near-simultaneous exotherms were detected in the vascular cylinder, below the cortical tissues and the dormant bud (Fig. 3). The raw data from the data logger (2 s intervals) indicated that the nucleation events in each explant were separated by, at most, 4 s, suggesting that a single nucleation event within the explant induced the subsequent nucleation of the other tissues, and that there was continuity between the bulk water in the different tissues. Excised buds also nucleated at -4°C within 30 min of exposure.
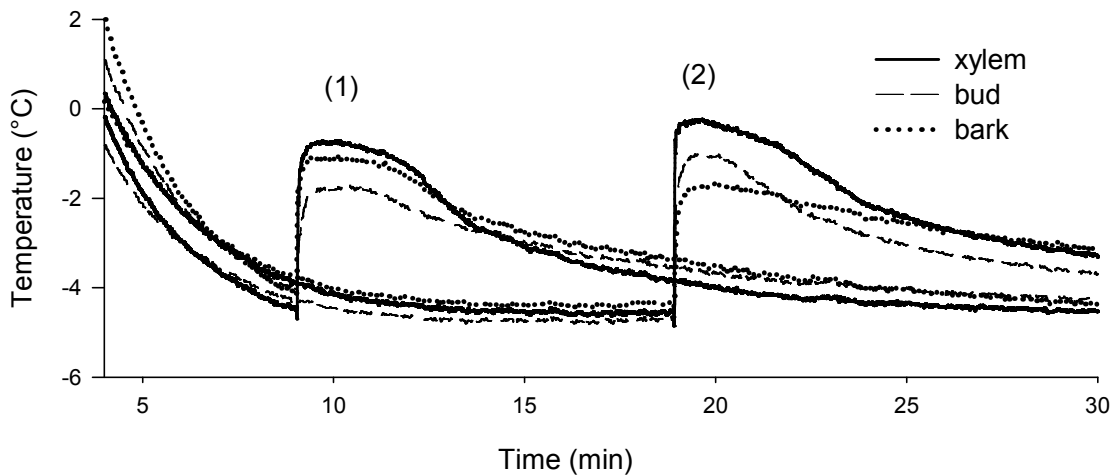
Figure 3. Typical exotherms indicating ice nucleation during desiccation at -4°C in acclimated explants 1 and 2 of the *Malus* cultivar 'Prima' harvested in the winter of 2009-10.

The apparent trend with respect to mean time to nucleation was, overall, to increase with later collection dates (November to January; Fig. 4), that would reflect cultivar differences in hardening responses, including solute accumulation and the availability of nucleation sites. However, when comparing time to nucleation between the first and last sampling dates only Maglemer showed a statistically significant difference ($P<0.05$). Survival was recorded for the last sampling date only (Holsteiner Cox 28%, Maglemer 88%, Prima 84%), and at that point there was no correlation with time to nucleation, as there was no significant difference in the times recorded for each cultivar ($P=0.59$). A relatively high nucleation temperature after desiccation, as shown by the DSC data (Fig.5) does, however, correlate with low survival.

For acclimated woody tissues, nucleation occurs commonly at -8 to-10°C (1) or lower (7), yet a number of the explants in this study nucleated at temperatures as high as -2°C. Moving air in the incubator chamber, or intermittent compressor vibration, may have limited undercooling, as explants cooled in a -20°C freezer without fan assistance nucleate at temperatures clustered around a more typical -8°C ($n=35$, unpublished data). It is widely accepted that early nucleation is, at worst, not disadvantageous in cryopreservation and in this instance it may be that it provides the opportunities for cryodehydration that may be beneficial. The data from this study indicates the contribution of the desiccation step to eventual survival following cryopreservation, where it allows for bulk water reduction by evaporative water loss and water redistribution at the tissue and cellular levels due to extracellular ice and cryodehydration

Notably, the 2005 study by Towill and Bonnart (15) suggested that the -4°C desiccation step might be omitted without affecting survival following cryopreservation. They worked with material gathered under typical, continental winter conditions, yet stored this in plastic bags at -4°C for 3-5 months before use. They did not monitor explant nucleation, omitted the -4°C desiccation step from the standard protocol and recovered explants from 8 of 12 accessions of *Malus* following cryopreservation. To enable recovery of all of the accessions, they reduced the cooling rate of the second stage of the protocol (-4°C to -30°C) from 1°C to 0.21°C h^{-1}. This study questions the value of the -4°C desiccation step, yet the extended pre-experiment storage given to their material at just this temperature could readily replicate a significant part of the desiccation step typically included in the protocol. Slowing the cooling rate used to

reach -30°C, the procedural step that follows -4°C desiccation, would extend significantly the time available for redistribution of water as a result of cryodehydration.
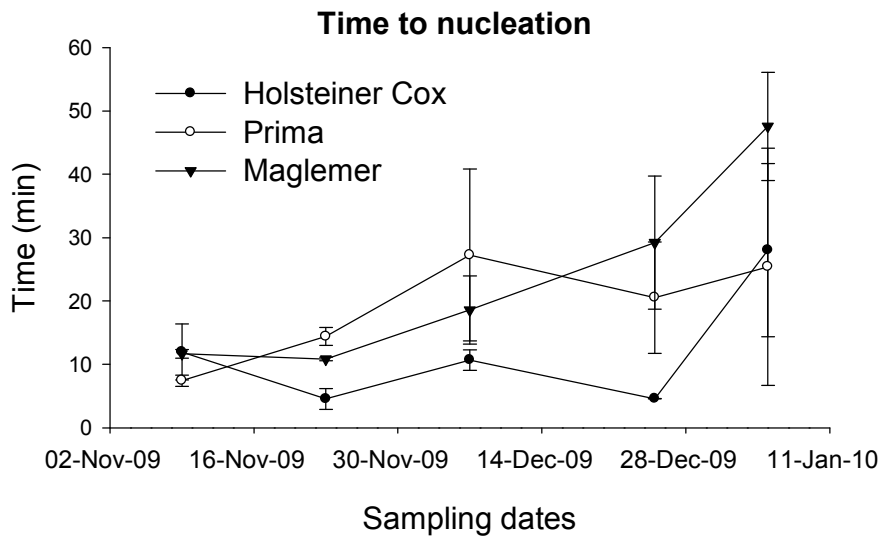
Time to nucleation

Figure 4. Time to the first nucleation event at -4°C in acclimated 3.5 cm explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' collected at various times during winter hardening. Bars indicate standard error of the mean, n =10.

Differential Scanning Calorimetry

Significant differences ($P<0.05$) in the crystallization temperature and the amount of water that crystallized within excised buds were recorded between cultivars sampled at both the start and end of the -4°C desiccation step. At both the beginning and end of desiccation the cultivar with the lowest survival following cryopreservation ('Holsteiner Cox'), had the highest crystallization temperature ($P<0.05$, Fig. 5a) and the highest amount of freezable water ($P<0.05$, Fig. 5b). This underlines the value of the early desiccation step in water reduction to the required level, dependant upon cultivar and season, for relatively high levels of survival following cryopreservation to be achieved.

The data from this study support the view that desiccation at relatively high sub-zero temperatures is a prerequisite for the successful cryopreservation of winter-hardened, dormant buds of *Malus*. A significant amount of this is achieved, using the standard protocol, by evaporative water loss through exposed surfaces of the explant. Additionally, we suggest that the water content of the bud is reduced by the migration of water to the extracellular ice resulting from nucleation events early in the incubation period at -4°C, and is a key element in the success of the standard protocol.
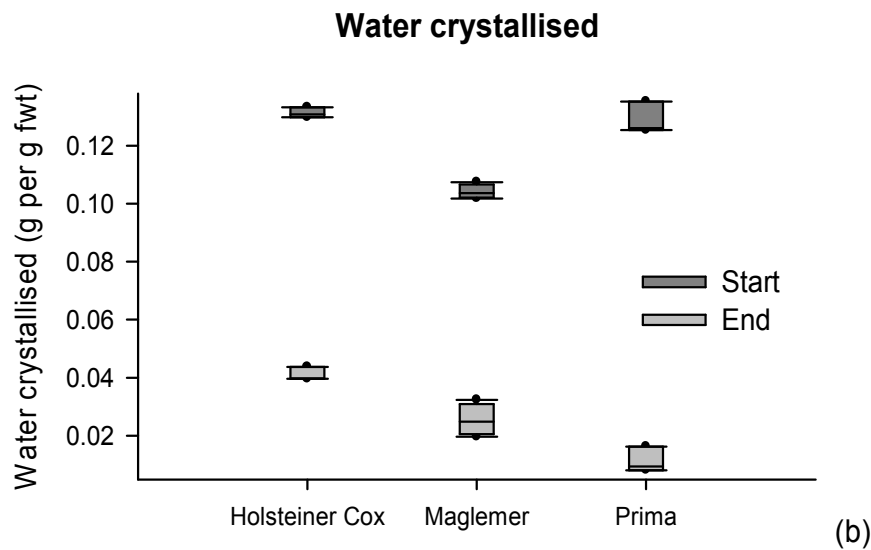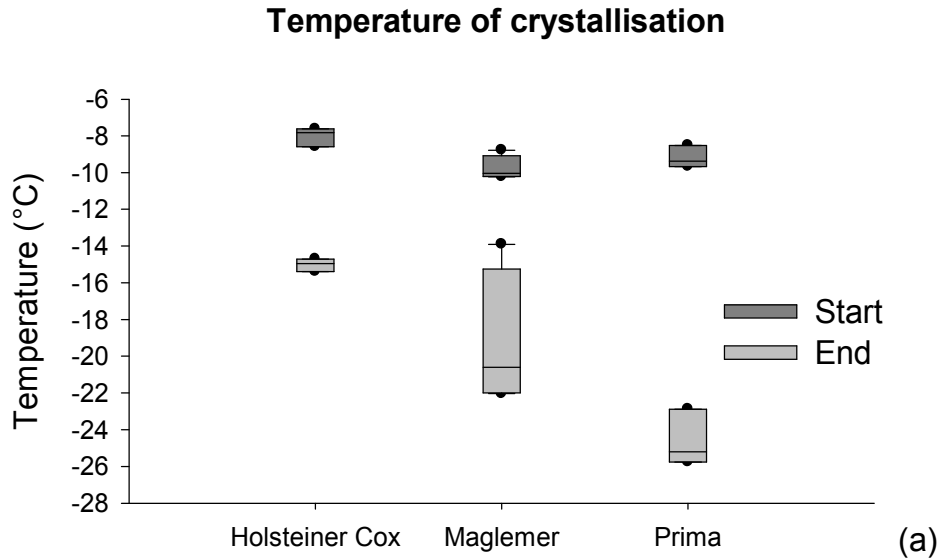
Temperature of crystallisation

Water crystallised

Figure 5. (a) Crystallisation temperatures of excised, dormant buds of 'Holsteiner Cox', 'Maglemer' and 'Prima' at the beginning and end of the -4°C desiccation process. (b) Freezable water (g/g fwt) in the dormant buds of 'Holsteiner Cox', 'Maglemer' and 'Prima' at the beginning and end of the desiccation process. Explants were gathered in the winter 2009 -10.

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