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THE EFFECT OF COLD-ACCLIMATION ON THE WATER RELATIONS AND FREEZING TOLERANCE OF *Hordeum vulgare* L.

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Abstract

During a 5ºC and a 5/-1ºC cold acclimation (CA) regime there was a significant decline in the water potential of winter barley, and a concurrent decline in tissue water content of the 5/-1ºC CA plants. Results of carbohydrate analysis illustrated a significant (P<0.001) accumulation of sucrose, fructose and glucose in the 5/-1ºC CA plants, which was inversely correlated to water potential. Using an infrared imaging radiometer during a convection frost test the water release time (WRT) of 5/-1ºC CA was demonstrated to be significantly (P<0.001) longer than that observed in non-cold acclimated plants. This observation is consistent with visual analysis of exotherm curves where the rate of cellular water release to extracellular ice is reduced in the 5/-1ºC CA plants, compared to the non-cold acclimated plants. These biochemical and physiological changes were correlated to increased plant health following a non-lethal freezing test to -5ºC, where non-cold acclimated plants produced 2.3 ± 0.33 tillers and 5ºC and 5/-1ºC CA plants produced 2.4 ± 0.33 and 4.7 ± 0.67 tillers, respectively. Results from this study imply that cold acclimation leads to changes in the physical state of water that result in a less osmotically responsive cellular environment and subsequently significantly less damage to meristematic tissue.

Keywords: Water potential, water content, carbohydrate analysis, infrared imaging radiometer, water release time.

INTRODUCTION

During the process of cold-acclimation (CA) freezing tolerant plants such as winter cereals undergo a series of metabolic changes that result in increased frost resistance. These changes include the accumulation of cryoprotective compounds (osmolyte accumulation) and altered protein metabolism. The accumulation of cryoprotective compounds has been demonstrated both seasonally and *in-vitro* (7,18). Known cryoprotectants include proline, polyamines, sorbitol, glycine-betanie (11) and carbohydrates such as; sucrose, raffinose, sorbitol (11) and fructans in winter wheats (1). Sucrose is the most mobile and its concentration can increase 10-fold during exposure to low temperatures (11).

Changes in protein metabolism include the accumulation of soluble proteins (9,11,18) and the expression of novel proteins (dehydrins) and heat shock proteins (17,20). Both of

these changes in protein metabolism have been correlated to increased frost resistance in CA plants (11, 20).

There are several theories explaining how cryoprotective compounds and altered protein expression confer freezing tolerance to CA plants. Mechanisms of action proposed for novel proteins include chaperone activity and hydration replacement (5) and for cryoprotective compounds, theories include an increased osmotic effect (sugars decrease crystallisation of water and thus reduce freeze-induced dehydration) the metabolic effect, where the metabolism of sugars during CA produces unknown protective substances and the cryoprotective and glass effects (4) and finally the colligative effect of compatible solutes such as proline.

Another theory suggests that the mode of action of cryoprotective compounds during acclimation to desiccation stress is the replacement of water with hydrogen bonding compounds (13). The sum of these actions is the stabilization of the cytoplasm into a matrix that is less osmotically responsive to external osmotic gradients and thus leads to increased desiccation tolerance. Extracellular freezing is a form of induced cellular desiccation and has been increasingly included in desiccation theory.

There is considerable debate in the literature about the importance or even the existence of bound water (6,13,22), however nuclear magnetic resonance (NMR) studies have revealed that tissue water consists of several populations of water molecules with varying molecular mobilities that are influenced by external cues (13,22) including CA (11). Studies with wheat have shown that maximum freezing tolerance occurs when tissue water contents fall to around 65%, based on fresh weight (15), and illustrates the importance of cellular water retention versus migration to the extracellular ice, implicating that a degree of cellular water remains unfrozen. However this unfrozen fraction is insufficient to account for the difference in frost resistance between non-cold acclimated plants and cold acclimated plants (10).

Recent uses of thermal imaging technology have shown that it is possible to visualize and quantify the freezing rates of cellular water during freezing (3) furthermore with the use of thermal imaging technology it is now possible to time the removal of cellular water to the extracellular ice in a non-lethal freezing event (3). The aim of this paper is to evaluate the effect that CA has on the water relations, carbohydrate metabolism and freezing tolerance of winter barley.

MATERIALS AND METHODS

Winter barley (*Hordeum vulgare*) cv Pastoral was sown in standard seed trays using a mix of moist multipurpose peat-based compost and John Innes No 2 (60:40 by volume). Germination was established on a heated mist bench. Seedlings were then transferred to a heated greenhouse with a 20/10ºC day/night temperature and a 16 h photoperiod, extended by supplementary sodium vapour lighting, (PAR 145 µmol m^{-2} s^{-1}). Seed trays were placed on capillary matting and base watered. Two cold acclimation regimes were employed: one at a constant 5ºC for 14 d with a 9 h photoperiod (PAR 9.03 ± 0.115 x 10 µmol m^2 s^{-1}) and a second regime, where plants were held at 5ºC for 7 d and then transferred to -1ºC for a further 7 d, with a 9 h photoperiod (PAR 8.93 ±0.115 x 10 µmol m^2 s^{-1}). Non-acclimated plants were held at 20/10ºC day/night temperatures in a growth chamber with a 16 h photoperiod (PAR 8.57 ±0.257 x 10 µmol m^2 s^{-1}).

Water Potential

The water potential of the second youngest leaf of plants, at decimal growth stage 15, was measured with a J14 press (2). Measurements commenced after the 5/-1ºC CA plants had been subjected to 24 h equilibration at the 5ºC acclimation temperature and continued at 24 h

intervals for 12 days. All water potential measurements were obtained in the last 2 h of the photoperiod. A completely randomised experimental design was employed with 6 replicates for each culture temperature and sampling period and the data were subjected to one-way analysis of variance.

Carbohydrate Analysis

Three replicate samples were prepared by taking 1.5 g of fresh crown material (leaf base and shoot meristem) and grinding into a paste with 5.5 ml of ddH_2O. Samples were then centrifuged at 13,000 G for 5 minutes and then the supernatant was decanted into fresh microfuge tubes. Samples were diluted 20µl in 980µl of ddH_2O and analysis was performed on a BioLC amino acid analyser (Dionex Inc). The system consisted of a Dionex GS50 gradient pump, Dionex AS50 auto sampler (5µl sample loop) and column heater. Detection was by a Dionex ED50 electrochemical detector (integrated amperometry) separation was achieved using an AminoPac PA10 analytical column (i.d. 2 x 250mm). A guard column, AminoPac PA10 (i.d. 2 x 50mm) was situated in front of the analytical column. The guard and analytical columns were maintained at a constant 30ºC. Compounds were eluted using water (A), 200mM sodium hydroxide (B) (carbonate free) and 1M sodium acetate (C) (carbonate free) gradient systems (Table 1). Helium gas was used to pressurise the headspace of eluant bottles reducing carbon dioxide absorbance. Anion traps (ACT-1, i.d. 9 x 24mm, Dionex Inc) were used to remove trace amounts of metal contamination in the eluants prior to use. The flow remained constant at 0.25ml min^{-1}.

Table 1: Gradient conditions used in elution of carbohydrates. A = water, B = 200mM sodium hydroxide and C =1M sodium acetate.

Time (min)	A (%)	B (%)	C (%)
0	80	20	0
2.0	80	20	0
12.0	80	20	0
16.0	68	32	0
24.0	36	24	40
40.0	36	24	40
40.1	20	80	0
42.1	20	80	0
42.2	80	20	0
62.0	80	20	0

Glucose, fructose and sucrose were quantified using a standard reference compound at various concentrations. Data capture was by Chromeleon software (Dionex Inc) on an IBM PC.

Water Content

The relative water content (RWC) of winter barley was determined using three replicate samples of 10 plants for each sample. Plants were removed from the soil and weighed, (fw) and then dried to a constant weight in a fan-assisted oven at 80°C for 48 h and then reweighed (dw) and RWC% calculated as 1 –dw/fw x 100.

Anatomical Measurements

The crown diameter of plants was measured at the end of each acclimation regime, just prior to freezing, using a micrometer. Seven plants from each acclimation temperature were measured 5 mm above the union of the roots and shoots.

Water Release Time

Water release time (WRT) is a concept associated with freezing that has been derived from observations using infrared thermal imaging and relates to the time taken for the main exotherm of the tissue which is equated to cellular freeze dehydration (16). Plants were raised and CA for 14 d as described previously prior to slow freezing (3°C h^{-1}) in a convection frost chamber to a non-lethal freezing temperature of -5°C. Intact plants were removed complete with a root ball of soil and were placed on cork tiles with the roots thermally insulated using foam rubber. Ice nucleation of the shoots was controlled by using a 10 µl droplet of a suspension of *Pseudomonas syringae* strain Cit 7 with a mean nucleation temperature of –2.95°C. Plants were frozen for 1 h after the initial observation of freezing of the last nucleated plant to ensure that all exothermic events had completed. During freezing plants were observed using an infrared thermal imaging radiometer (Model 760, Inframetrics, North Billerica, MA) as previously described by (8). Infrared images were recorded on videotape for subsequent analysis using custom software Thermagram™. The temperature span of the radiometer was set at 2°C in order to detect the small exothermic events associated with plant freezing (21).

Following freezing plants were allowed to thaw for 24 h at 4°C. Plants were then potted into standard seed trays and grown on for 14 d in the glasshouse using the standard culture conditions described above. Plant health was assessed as tiller counts at 14 d.

Analysis

All quantitative data were analysed using Analysis of Variance using Minitab v. 13.

RESULTS

Water potential of NA plants remained relatively constant throughout the experiment (-0.09 ± 0.024 MPa) however; when plants were transferred to 5°C CA temperature there was a significant (P<0.001) decline in the water potential. This decline continued for a further 48 h but after 72 h, at 5°C, plant water potential started to increase and at the end of the experiment (12 d) the water potential of 5°C CA plants was not significantly different to that observed in NA plants. The water potential of 5/–1°C CA plants was significantly less (P<0.001) than both the NA and the 5°C CA plants. As acclimation progressed in this treatment the water potential of these plants rose in parallel to the 5°C response but remained significantly lower throughout the experiment (Fig. 1)

Relative water content was significantly lower (P<0.001) in the 5/-1°C CA plants (80.5% ± 0.79%) compared to the 5°C CA (90.03% ± 0.15%) and the NA plants (88.97% ± 0.59%). There was no significant difference (P = 0.173) in the anatomical character with an overall average crown diameter of 1.978 ±0.126mm.

Water release time (WRT) was increased by CA and was significantly (P<0.001) longer in plants subjected to the 5/-1°C CA regime than either 5°C CA and NA plants (Fig. 3). This observation is consistent with exotherm curves plotted from data captured using Thermagram™ software (Fig. 4). Water release time and exotherm data correlated (r = 0.80; P<0.01) with increased plant health following freezing (Fig. 5) and negatively correlated (r = -0.84; P<0.01) with plant water potential.

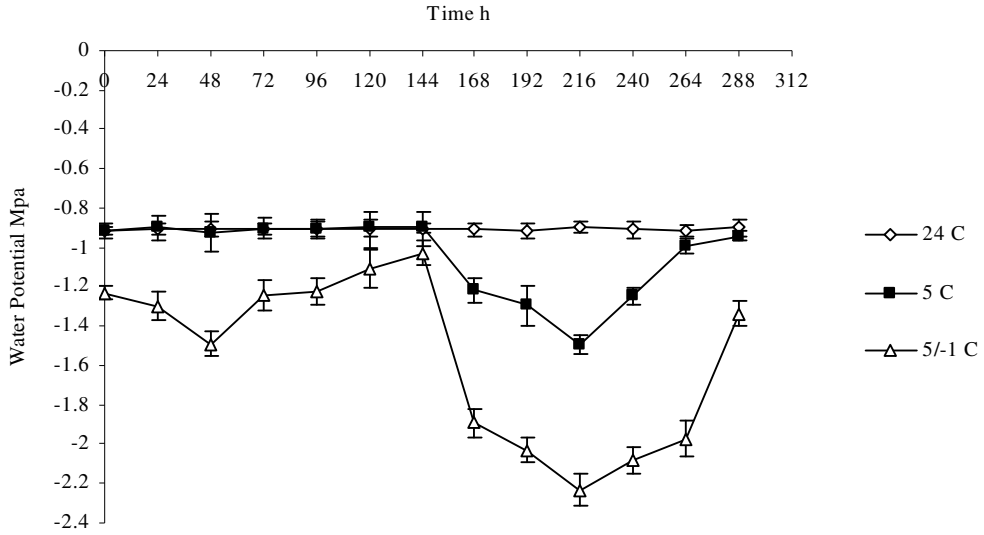

Figure 1. Water potential profile of NA and CA winter barley plants, bars represent standard error, n = 6.

The sugar concentration in the 5°C CA plants were slightly lower than the NA plants although only glucose concentration was significantly reduced (P<0.001). With the 5/-1°C CA there were significant (P<0.001) increases in the concentration of glucose, fructose and sucrose (Fig. 2) with sucrose concentration increasing by more than 4 times that observed in NA plants.

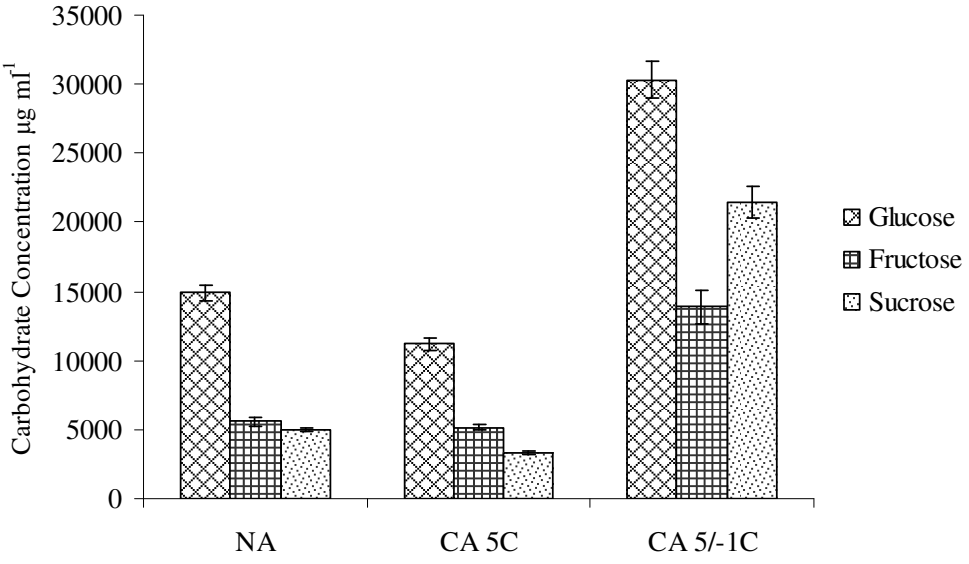

Figure 2. Carbohydrate accumulation of NA and CA winter barley, n = 3.

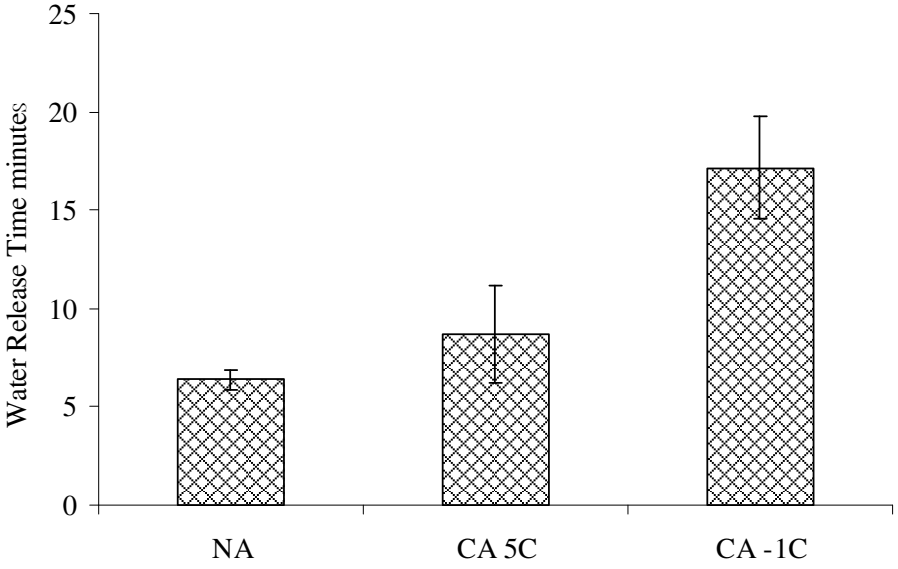

Figure 3. Water release time for winter barley subjected to a slow convection freezing test. Bars represent standard error, n = 6.

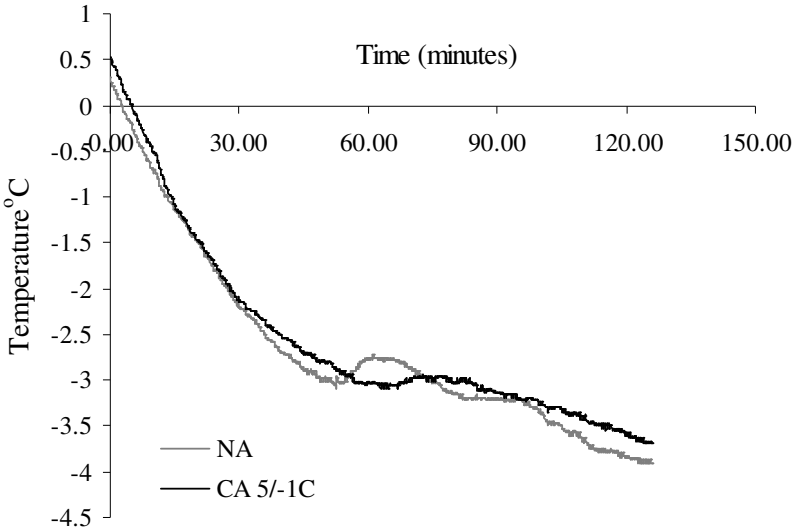

Figure 4. Exotherm curves for NA and 5/-1ºC CA winter barley plants subjected to a -5ºC freezing regime, illustrating the steep and large exotherm typically observed in NA plants and the smaller shallower exotherm observed in 5/-1ºC CA plants.

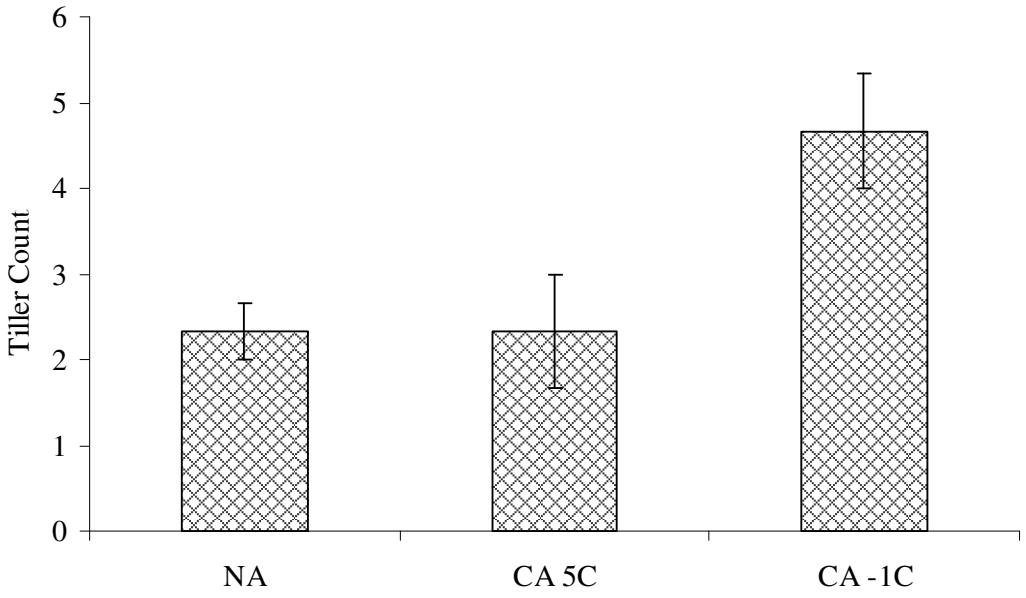

Figure 5. Assessment of plant survival 14 days post freezing. Error bars are standard error, n = 6.

DISCUSSION

The observed decrease in water potential of winter barley plants, when subjected to a 5ºC treatment, is consistent with observations in CA cultivars of wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) (7). Water potential data presented here for the 5ºC CA plants also illustrated a return to normal levels within the duration of the experiment, as seen in winter wheat or rye (7). Further cold acclimation at -1ºC resulted in a further, significant, decline in leaf water potential compared to the 5ºC CA plants and the NA controls, and remained significantly less than either the NA or 5ºC CA plants for the duration of the experiment. The initial and transient decline in water potential in 5ºC CA plants is interpreted as a cold shock response as a result of changing ionic strength in the cytoplasm (quick response) and once equilibrium has been reached water potential returns to that observed in NA plants. The second drop in water potential at –1ºC is interpreted as the result of solute synthesis such as compatible solutes, carbohydrates and proteins which is a slower process than the accumulation of ions and thus results in a further decline in plant water potential and a slower return to normal water potential values.

The decline in relative water content of –1ºC CA plants is consistent with the observation of increased solute concentration and correlates with the observed increase in glucose, fructose and sucrose. These sugars increased significantly in the -1ºC CA plants compared to the NA and 5ºC CA plants and are consistent with increases in soluble sugars observed in barley and oats, which were correlated to an increase in freezing tolerance (14). Similar observations have been reported for cabbage seedlings (18). Conversely the decrease in the concentration of sugars in the 5ºC CA plants may be a result of decreased photosynthesis as a function of decreased day length and temperature in the growth chamber, culminating in a reduced rate of photosynthesis. The comparative similarity in RWC of NA and 5ºC CA plants is consistent with the theory of osmotic adjustment as a function of ion concentration. Accumulation of ions (Na^+, Ca^{2+} and K^+) would not result in a significant decline in RWC

water content compared to the accumulation of solutes whereas the accumulation of solutes at –1ºC could account for the decrease in RWC observed in this treatment.

The significant increase in plant health of 5/-1ºC is correlated to observed changes in water potential, increased carbohydrate concentration and subsequent changes in RWC of these plants. The implication from this study is that the observed biochemical and physiological changes during the 5/ -1ºC CA has resulted in reduced damage to meristematic cells in the crown tissue of 5/-1ºC plants and thus increased tiller production following freezing to –5ºC.

The water relations data presented here implies a change in the biochemistry of 5/-1ºC CA cells, where increases in cellular carbohydrates are correlated to a decrease in leaf water potential and cellular water content and an increase in plant survival, following a slow convective freezing test. These changes are analogous to those observed in winter wheat (7) where a decrease in leaf water potential and crown osmotic potential (7) were correlated to an increase in dehydrin gene expression and increased frost resistance, similar trends in protein expression and cell water relations were observed in spinach (12). It is well documented that CA plants accumulate carbohydrates and proteins and these observations have consistently been correlated to increased freezing resistance. Their mechanisms of action on cellular water and freezing tolerance are however, speculative. Recent work on spin-lattice and spin-spin relaxation times of protons have been shown to decrease in crown tissue of winter wheat with increased freezing resistance (22) and the implication is that the solute changes influence water behaviour. The data presented by Yoshida *et al.* (22) are similar to those published for desiccation tolerance in plants by Hoekstra *et al.* (13) where the storage longevity of anhydrobiotic plants was inversely correlated with the molecular mobility of the cytoplasm (13). Cell survival in anhydrobiotic conditions is now considered to rely on the accumulation of carbohydrates and stress proteins such as dehydrins. The integration of these observations predicts an increase in the time it takes for intracellular water to migrate to the extracellular ice in CA plants. Such measurements have hitherto been difficult to record and analyse since the existing thermocouple technology does not facilitate accurate measurements without the introduction of artefacts associated with the attachment of the probes. Infrared thermography, which is a non-invasive technique, overcomes this difficulty and in conjunction with specialist software (Thermagram ™) a wide range of points on a plant can be monitored for temporal temperature changes.

Observations showed that exotherms in NA plants are typically larger, which in turn has a characteristically large initial release of water giving rise to a steep slope observed in the linear phase of the exotherm and a subsequent rapid fall in temperature as cellular water is frozen out. Conversely CA barley plants exhibit a smaller exotherm event and consequently the rate of the slope is reduced, furthermore there is a slower rate of decline in the temperature of the exotherm as cellular water is frozen (Fig. 4). Both NA and CA plants frequently show a preliminary small exotherm, which has been interpreted as the freezing of free water in the apoplast (16). In an attempt to quantify the exotherm characteristics the concept of water release time (WRT) has been devised.

Measurements of water release time, observed in this study, support the prediction that CA leads to a slower release of intracellular water to the extracellular ice (Figs. 3 & 4). These observations imply that cellular water in –1ºC CA plants is either osmotically restricted or structured and the inference is that this is due to macromolecule-water interactions (19). The WRT data presented in this study imply that a large proportion of the cell's aqueous environment is either osmotically restricted or structured due to macromolecule-water interactions. Water release time data cannot reveal whether this structuring is due to water interactions with proteins alone or a combination of protein and saccharide water interactions. The absence of any significant differences in crown diameter size negates the possibility that

the observed delay in the release of cellular water to the extracellular ice was due to differences in plant size. The data presented here are analogous to the observation that the molecular mobility of crown tissue water in winter wheat decreases with seasonal acclimation to freezing, increased crown sugar content and increased freezing tolerance (1,22).

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