Freezing in nonacclimated oat: thermal response and histological observations of crowns during recovery

David P. Livingston III, Shyamalrau P. Tallury, Shirley A. Owens, Jesse D. Livingston and Ramaswamy Premkumar

Abstract: The complex nature of freezing in plants may be easier to understand if freezing is studied in nonacclimated plants at temperatures just below freezing. Thermal patterns of model systems frozen at –2.6 °C were compared with those of crown tissue from oat (*Avena sativa* L.). Thermal patterns of live crowns more closely resembled those of fructan and sugar solutions with filter paper than of plain water or a BSA solution. When the percentage of water freezing in nonacclimated plants at –2.6 °C was manually limited to 10%, the survival was reduced from 100% in supercooled plants to 25%. During cold acclimation, the percentage of water freezing at –2.6 °C went from 79% to 54% after 3 weeks of cold acclimation and resulted in 100% survival. The nucleus of cells in the primary apical meristem of nonacclimated plants appeared to have disintegrated, an effect that was not observed in any cold-acclimated (unfrozen controls) plants. Nuclear pycnosis was observed in leaf sheaths surrounding the meristem and in cells directly below the meristem. Cells of secondary meristems and in the crown core appeared undamaged, but vessels in plants frozen for as little as 30 min were ruptured and appeared plugged. The distinctive nature of injury in the apical meristem and the rapid ability of the plant to acclimate during cold to the stress causing this injury indicate that specific tissue, namely the apical region of the crown, should be the focus of attention when attempting to determine cause and effect between genetics or metabolism and cold acclimation in winter cereals.

Key words: freezing tolerance, cold acclimation, nuclear pycnosis, meristem, crown, winter cereal, oat (*Avena sativa*), calorimeter.

Résumé : La nature complexe de la congélation chez les plantes pourrait être plus facile à comprendre si on étudiait le gel chez des plantes non-acclimatée, à des températures juste sous le point de congélation. Les auteurs ont comparé les patrons thermiques de systèmes modèles à –2,6 °C avec ceux des tissus du collet, chez l'avoine (*Avena sativa* L.). Les patrons thermiques des collets vivants ressemblent plus à ceux des solutions de fructan et de sucre sur papier filtre, que ceux de l'eau pure ou du BSA. Lorsque le pourcentage de l'eau qui gèle chez des plantes non-acclimatées à –2,6 °C est manuellement limité à 10 %, la survie est réduite de 100 % chez les plantes en surfusion à 25 %. Au cours de l'acclimatation au froid, lorsque le pourcentage d'eau gelant à –2,6 °C passe de 79 % à 54 %, après 3 semaines d'acclimatation au froid, on obtient une survie de 100 %. Le noyau des cellules du méristème apical primaire des plantes non-acclimatées semble s'être désintégré, un effet qu'on n'observe pas chez les plantes acclimatées au froid (témoins non gelés). Une pycnose nucléique se manifeste dans les gaines foliaires entourant le méristème et dans les cellules immédiatement sous le méristème. Les cellules des méristèmes secondaires et du centre du collet ne semblent pas endommagées, mais les vaisseaux dans les plantes gelées pendant aussi peu que 30 min, sont rupturés et apparaissent bouchés. La nature distincte des blessures dans les méristèmes apicaux et la capacité des plantes à s'acclimater au froid contre les stress causant des blessures indiquent qu'un tissu spécifique, nommément la région apicale du collet, devrait être le centre de notre attention, lorsqu'on tente de déterminer la cause et l'effet entre la génétique ou le métabolisme, et l'acclimatation au froid, chez les céréales.

Mots clés : tolérance au froid, acclimatation au froid, pycnose nucléique, méristème, collet, céréale d'hiver, avoine (*Avena sativa*), calorimètre.

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Introduction

Freezing in plants is a complex process involving the dynamics and kinetics of water as it interacts with various tissues. The ability of plants to withstand stresses caused by ice formation is determined by numerous physiological characteristics of plants, which are regulated by the genetics of the species under consideration as well as by the environment in which it grows.

Many studies have demonstrated an enhanced ability of plants to survive freezing temperatures by exposure to low but above freezing temperatures, a process called cold acclimation. Cold acclimation induces an increase in cytosolic sugar as well as numerous other metabolites and cold-regulated genes (Uemura and Steponkus 1999; Thomashow 2001; Cook et al. 2004; Kaplan et al. 2004), which produce an increase in the cryostability of plasma membranes and cell walls. These changes allow plants to withstand damage produced by freezing stress, including mechanical disruption of cell walls and membranes, adhesions, jump lesions, and desiccation.

In addition to acclimation resulting from exposure to low, above-freezing temperature, freezing tolerance is conferred by exposure of cold-acclimated plants to temperature slightly below freezing but before freezing injury occurs (Livingston 1996). This acquisition of freezing tolerance beyond that achieved by cold acclimation has been called "second-phase hardening" and is here called subzero acclimation. One of several metabolic changes implicated in subzero acclimation is fructan hydrolysis (Trunova 1965) and a concomitant increase in sugars, particularly in the apoplast (Olien 1984; Livingston and Henson 1998). Castonguay et al. (1995) reported an increase of raffinose and stachyose in alfalfa during subzero acclimation and suggested that these sugars may have a protective effect on plant tissues similar to that proposed for sucrose, glucose, and fructose. Yoshida et al. (1997) described a change in the physical state of water in crown tissue, which is signified by a decrease in spin-lattice and spin-spin relaxation times of protons in plants at –3 °C. This change results in a decrease in the amount of weakly bound water as well as an increase in water binding strength that is critical for freezing survival of plants (Yoshida et al. 1997). More than 50 proteins either increased or decreased in spot volume in a protein analysis of wheat during subzero acclimation (Herman et al. 2005).

In addition to the interaction of complex systems in both cold and subzero acclimation, the differential acclimation of tissue within specific regions of plants (Tanino and McKersie 1985) has made freezing tolerance difficult, if not impossible, to definitively characterize. If individual stages of freezing can be separately investigated within specific regions of plants, it may be possible to provide a more comprehensive understanding of how plants withstand freezing stresses. The formation of ice just below freezing, at approximately –2 °C, is clearly an event during which a minimum number of protective systems are necessary for a plant to survive freezing injury; many temperate-region plants with little cold acclimation can survive this temperature.

Oat (*Avena sativa* L.) is the least hardy of the winter cereals and therefore may be a species in which freezing tolerance is achieved using fewer mechanisms than more hardy cereals such as rye or wheat. Our purpose was to see if survival at –2 °C in nonacclimated oat had any relationship to calorimetrically measured freezing patterns and (or) to histological damage within crowns during recovery from freezing.

Materials and methods

Plant culture

Seeds of oat ('Wintok') were grown in Scotts Metromix 510 (Scotts-Sierra Horticultural Products Co., Marysville, Ohio) in plastic tubes (2.5 cm diameter × 16 cm height) with holes in the bottom to allow drainage. The tubes were suspended in a grid that held 100 tubes. Plants were watered three times weekly with a complete nutrient solution (Livingston 1991) and flushed three times weekly with tap water. Nonacclimated plants were produced by growing them in Environmental Growth Chambers (model M18) (Chagrin Falls, Ohio) for 5 weeks at 13 °C day and 11 °C night temperatures with a 10-h photoperiod at 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR (measured at mid-plant; 80% cool fluorescent (Philips Lighting Co., Somerset, New Jersey) and 20% incandescent (Philips Lighting Co.)).

After the 5-week growth period, plants were transferred to a similar chamber at 3 °C with a 10 h photoperiod at 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (lamps as above). Plants were sampled at days 1, 2, 3, 8, and 21. Plants receiving the 21 d treatment constituted fully cold-acclimated plants.

Freeze tests/thermal analysis

After the respective growth treatments, crowns were separated and removed from each plant by trimming roots and shoots resulting in a 2.5 cm portion of the base of the stem. Model solutions (Table 1) and plant tissues were studied in a Calvet isothermal calorimeter (model MS 80; Setaram, Saint-Cloud, France) inside a small refrigerated room at –10 °C. Whatman (Maidstone, England) 42 ashless filter paper that had been thoroughly rinsed in deionized water (to remove salts) was added to water, sugar, bovine serum albumin (BSA), and fructan solutions to approximate dry weight percentages of plant crowns. The calorimeter was maintained at –2.6 °C by precisely heating the core. At full sensitivity (Seebeck circuit), 1 mV output from the calorimeter equaled 17.6 mW displacement from baseline.

The successful measurement of latent heat of freezing at a particular temperature is dependent on the ability both to supercool the sample and to initiate freezing without inputting or removing heat. Wheat, oat, and *Arabidopsis* all froze spontaneously at around –3 °C, which was similar to results for evergreen leaves (Ball et al. 2002). Therefore, –2.6 °C was selected because plants were consistently supercooled at that temperature without freezing. Model solutions and oat crowns were allowed to equilibrate at –2.6 °C until the baseline of the calorimeter stabilized (6 h). Unfrozen water, solutions, and plant samples at –2.6 °C were induced to freeze (heterogenous nucleation) with a few ice crystals adhering to the end of a narrow-gauge wire (guitar string) inserted into the core of the calorimeter where the tissue samples were located. The heat generated from inserting the

Table 1. Various parameters of model systems frozen at –2.6 °C in an isothermal calorimeter. A recording of the thermal output of each system (except KCl) as it froze is shown in Fig. 1.

System	Water (%)	Concentration (g/L)	Molality (mol/kg)	Water freezing (%)*
Water	100	na	na	100 (1.4)
Water + fpaper[†]	78	na	na	93 (1.4)
BSA	100	48	0.0007	92 (1.1)
BSA + fpaper[†]	62	48	0.0007	83 (nd)
Sugar (g + f) [‡]	100	54	0.3	62 (2.4)
Sugar + fpaper[†]	66	54	0.3	61 (0.8)
fn[§]	100	57	na	47 (0.5)
fn[§] + fpaper[§]	63	57	na	52 (nd)
KCl[‖]	99	7.45	0.1	84.6 (0.2)

*SD is given in parentheses.

[†]fpaper, Whatman No. 42 ashless filter paper washed to remove salts.

[‡]g+f, glucose plus fructose.

[§]fn, fructan, a mixture of size classes extracted from oat plants (Livingston 1990).

[‖]KCl was frozen at –2.0 °C.

wire was below the limits of detection for the settings used in these experiments.

As the sample froze, the release of latent heat was recorded on a strip chart recorder and areas under curves were measured using a handheld planimeter. The average of three measurements (less than 3% variation was observed between measurements) was used in all calculations. Area under curves was related to calories using the latent heat of freezing at –2.6 °C for known quantities of pure water. A standard curve (not shown) with varying amounts of water indicated a linear relationship between grams of water and curve area, up to the largest peak area measured, with a correlation coefficient of 0.999. This standard curve was used to quantify total energy in all subsequent measurements.

After the latent heat was recorded, crowns were removed from the calorimeter at varying times up to 16 h, at which time frozen crowns had equilibrated with the calorimeter. Crowns were planted into the same soil mix in which they were grown initially and allowed to recover under controlled conditions described above. After 3 weeks, crowns were rated for regrowth on the basis of new roots and shoots. Percent survival consisted of the number of plants surviving the –2.6 °C freeze divided by the total number of plants in the experiment.

Histology

Plants were grown at 13 °C for 5 d after freezing, after which 1–3 cm of the lower part of the stem was placed in fixative containing 18:1:1 parts of 70% ethyl alcohol – glacial acetic acid – formaldehyde. The collected samples were kept at room temperature for 48 h and transferred to 70% alcohol and kept at 4 °C until they were processed for dehydration and embedding.

Dehydration, infiltration, embedding, and staining

Samples were dehydrated according to procedures detailed by Johansen (1940) using a series of ethanol and tertiary butyl alcohol solutions. Fully infiltrated tissues were embedded in Parapast Plus and kept refrigerated until sectioned. Embedded sample blocks were sectioned in a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Buffalo, New York) at a thickness of 15 μm. The resulting paraffin ribbon containing serial sections was placed on a glass slide coated with Haupt's adhesive (Johansen 1940), flooded with 3% formalin, and transferred to a slide warmer at 41 °C. Dried slides were stored at room temperature until stained. The slides were left overnight in dishes containing xylene to remove paraffin before sections were stained. A triple stain with Safranin, Fast Green, and Orange G was used as described by Johansen (1940). A cover-glass was added to slides with one or two drops of Permount.

Observation of sections

To observe differences among treatments, sections were viewed under a Wild Heerbrug (Gais, Switzerland) wide-angle dissecting microscope with bottom lighting and on a Zeiss (Jena, Germany) photomicroscope III. Observations were made on three separate plants for each treatment. Photographs of sections that best represented damage typical of freezing injury were taken with a Sony (Tokyo, Japan) DSC848 digital camera attached to the microscope. Control sections of unfrozen plants were not included here because they have been published elsewhere (Livingston et al. 2005*a*).

Scanning electron microscopy

Samples were placed in the fixative described above, washed with several changes of 50% ethanol, dehydrated in graduated concentrations of ethanol, dried to the critical point in CO_2, mounted on stubs, and sputter-coated with gold. Samples were observed and imaged using a model JSM-6400V (JEOL, Peabody, Massachusetts), scanning electron microscope at 10–12 kV of accelerating voltage.

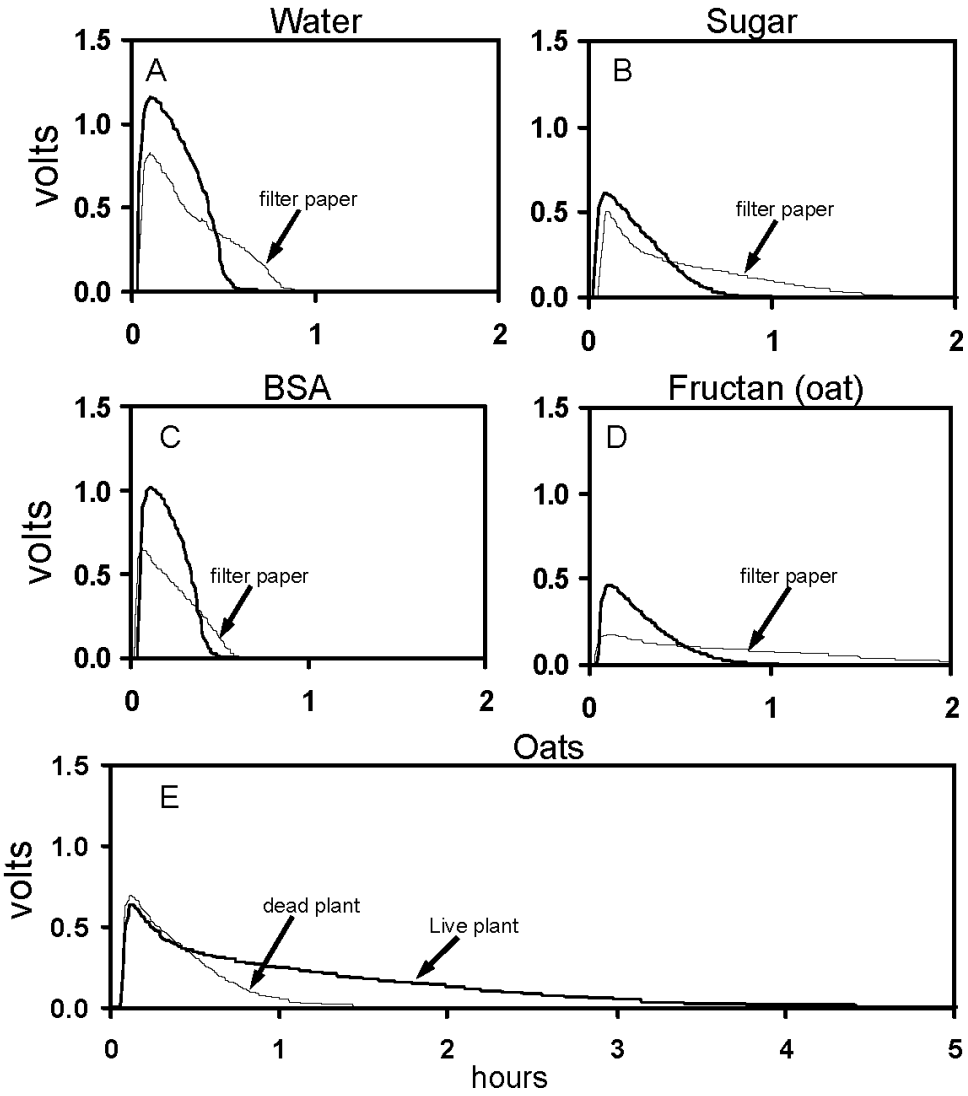
Results and discussion

Freezing in simple systems

As water freezes, the heat capacity of the liquid–solid system changes rapidly because the specific heat capacity of liquid water is different from that of ice. This change can affect the net amount of heat detected. However, calibrating the system with water as it freezes takes into account this change in heat capacity. Therefore, assuming that the freeze-induced change in the heat capacity of water in plants and model systems is similar to that of pure water, this error would be negligible. Mazur (1963) reported the error in calorimetrically determined latent heat measurements due to heat capacity changes during freezing to be 1.2% at –2 °C. Williams and Meryman (1965) calculated this error to be about 0.1%. Both of these error estimates were less than the variability that we found between replicated measurements of model systems and plant samples. To confirm the accuracy of our measurements, the amount of water freezing at –2.0 °C in a 0.1 mol KCl/L solution was measured. Using data from the International Critical Tables, Williams and Meryman (1965) calculated that 81%–83% of the water in a 0.1 mol KCl/L solution should freeze at –2 °C. Our measurement of 84% (Table 1) was within 1% or 2% of the value they reported (Williams and Meryman 1965).

Assuming that other sources of heat were insignificant compared with that generated by water freezing, and interactions between ice and hydrophilic compounds such as mem-

Fig. 1. Thermal output of four different model systems (A–D) during freezing at –2.6 °C in an isothermal calorimeter and thermal output (E) during freezing of live oat crowns compared with those killed in an oven and then reconstituted with water. The various parameters of each system are listed in Table 1.



branes did not influence latent heat to an appreciable extent, the heat generated by water freezing at –2.6 °C provided an estimate of the net amount of water that froze (Vali 1995).

Pure water froze and reached equilibrium at –2.6 °C within 30 min (Fig. 1A), but when filter paper, equivalent to the dry weight of cold-acclimated oat (Livingston et al. 2005*b*), was added to the water sample, it took nearly an hour to equilibrate. In addition to altering the shape of the freezing curve, the amount of water freezing was reduced to 93% by inclusion of filter paper (Table 1). Since the only difference in the two samples was filter paper, the change in the shape of the freezing curve (rate of freezing) was likely due to an interaction of water with the filter paper, which is a matric effect. Matric forces are a result of water adsorption to various surfaces such as colloids, proteins, and cell walls and can be particularly strong in cases where water has a close association with a hydrophilic surface (Boyer 1969) such as cellulose fibers in filter paper. Olien (1981) reported that water structure in an interface with hydrophilic colloids is influenced for a distance of "at least several water molecules beyond the tightly bound unfreezable water". Matric forces can therefore affect not only the total amount of water freezing but also the rate at which the water is frozen.

The freezing pattern of a physiologically relevant sugar solution (Table 1) was somewhat different from that of pure water, both in the amount of water freezing and in the rate of freezing (Fig. 1B). Addition of filter paper to the sugar solution had a negligible effect (62% versus 61%) on the amount of water freezing (Table 1) but had a dramatic effect on the shape of the freezing curve (Fig. 1B); the interaction between the sugar and filter paper nearly doubled the time that it took the water to freeze. Therefore, in this system, addition of filter paper resulted only in altering the rate of freezing and had no effect on the total amount of water freezing.

The colligative effect of freezing is normally considered in terms of freezing point depression; however, under isothermal conditions at a single temperature (–2.6 °C), colligative effects can be demonstrated by comparing the freezing

Fig. 2. Percent survival of nonacclimated plants that were removed from the calorimeter when the indicated percentages of water were frozen. The time after freeze initiation is shown directly on the graph.

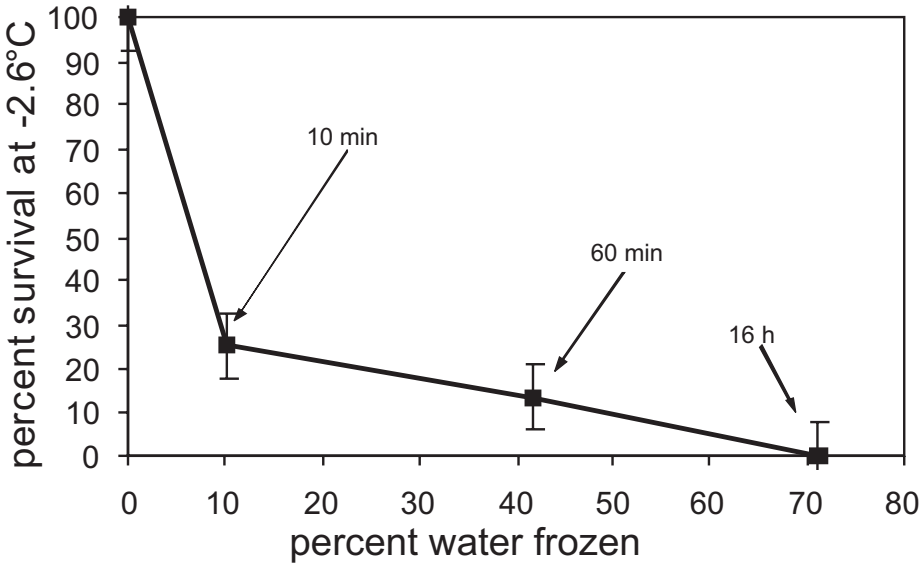
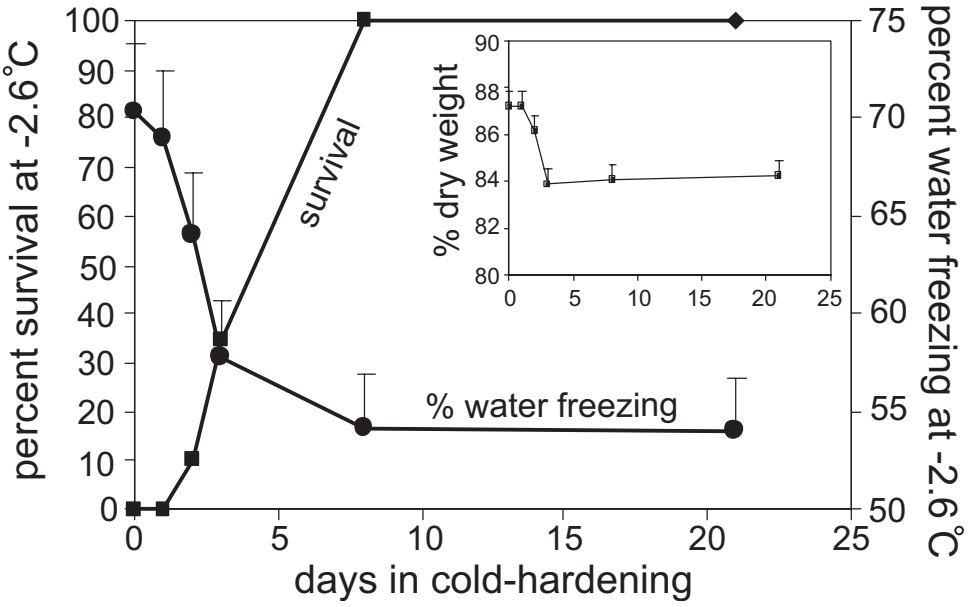


Fig. 3. Increase in percent survival of 'Wintok' oat during cold hardening after being frozen at –2.6 °C for 6 h in an isothermal calorimeter. Included on the same graph (*y*-axis on the right) is the change during cold hardening in the amount of water freezing at –2.6 °C. The inset is the change in percent water of crown tissue during cold acclimation.



patterns of pure water, a sugar solution, and a solution of BSA (Figs. 1A–1C). The amount of BSA in solution was nearly identical by weight to the amount of simple sugar in solution (Table 1), but due to its size, the BSA solution had a fewer number of molecules and therefore a lower molality. Since colligative properties of solutions are affected only by the number of solute molecules present (Chang 1981) and not by the size or mass of the solute, as the water in the BSA solution froze (Fig. 1C), its thermal pattern resembled that of pure water (Fig. 1A) more than it did that of the sugar solution (Fig. 1B). Ninety-two percent of the water in the BSA solution froze at –2.6 °C within 30 min; the addition of filter paper reduced the amount of water freezing to 83% (Table 1) and slightly increased the time to freeze completely (Fig. 1C). The differences in freezing pattern when filter paper was added suggest an interaction between BSA and filter paper. Without further analysis, speculating on the nature of the BSA – filter paper – water interaction would be premature.

In fructan solutions, the amount of water freezing was reduced (Table 1), but it took almost the same length of time to freeze as did the water in the sugar solution (compare Figs. 1D and 1B). The fructan used in these experiments was a mixture of large DP fructans extracted and purified from oat with an average degree of polymerization of 12 (Livingston 1990). While the exact molality of this solution was not known due to the mixture of size classes, clearly the molality was less than that of the sugar solution and on a

Fig. 4. Paraffin-embedded section of nonacclimated 'Wintok' oat crown frozen at –2.6 °C for 1.5 h and allowed to recover for 5 d. The section was triple stained with Safranin, Fast Green, and Orange G. (A) Section of the whole crown showing relatively normal tissue in the crown core region. (B) Closer view of the apical meristem of a secondary tiller indicating no damage to nuclei. Inset: normal nuclei adjacent to a cell with nuclear pycnosis. (C) Closer view of the apical meristem and subjacent tissue of the primary tiller. Note the apparent degeneration of the nucleus in cells of the apical meristem and slightly below. Note the area below the apical meristem with nuclear pycnosis and normal nuclei separated by a layer of condensed parenchyma cells that will likely differentiate into a node. Inset: apparent barrier between cells with disintegrated nuclei and pycnotic nuclei in the leaf. The effects in this figure were never observed in unfrozen plants (Livingston et al. 2005). Scale bars = 100 μm in Figs. 4B and 4C; scale bars = 10 μm in insets in Figs. 4B and 4C.

colligative basis it should have frozen more quickly than the sugar solution (as the BSA solution did; Fig. 1C). Therefore, the similarity in thermal pattern to the sugars (compare Figs. 1B and 1D), despite their lower molality, must have been due to an interaction between fructan and water. Addition of filter paper had a dramatic effect on the rate of freezing and appeared to have a closer resemblance to the freezing rate of live plants (compare Figs. 1D and 1E) than any other model system. The finding that *more* water froze when filter paper was added (Table 1) suggests that fructan may have interacted with filter paper to a greater extent than it did with water such that the effective concentration of fructan in solution was reduced, lowering the molality of the freezable solution and allowing more water to freeze.

To see if it would be possible to duplicate freezing patterns in plants to those in filter paper systems (Figs. 1 A–1D), plant tissue was made as inert as possible by heat-killing in an oven and then reconstituting with the same amount of water lost during drying. Under these conditions, a considerably different pattern of freezing resulted than in live plants (Fig. 1E). It took over 6 h for all of the water to freeze in live plants, while dead plants, reconstituted with water, took just over an hour. In live plants, after free water is frozen, continued freezing results as compartmentalized water relatively slowly diffuses through cell walls to regions of ice growth. Ball et al. (2002) suggested that the first part of an exotherm in plants, which consists of a rapid freeze (corresponding approximately to the first 10 min in our freeze curves (Fig. 1)), indicated freezing of water vapor plus apoplastic water, while the subsequent slower release of heat was due to symplastic water moving to extracellular sites. Due to considerable cellular disruption, a heat-killed plant reconstituted with water undoubtedly possesses a more or less continuous aqueous space that will result in more rapid freezing than in live plants. In fact, the freezing pattern of heat-killed plants resembled that of sugar with filter paper (compare Figs. 1B and 1E). Since carbohydrates in live plants were still present after being heat-killed, albeit with some altered tissue interactions, it seems reasonable that the freezing pattern of heat-killed plants should be similar to that of sugar with filter paper. Another factor contributing to the freezing rate in live plants could be the effect of numerous microcapillaries within cell walls (Ashworth and Abeles 1984), which were shown to affect freezing behavior in polycarbonate membranes and controlled glass pores. Many of these microcapliaries could have been destroyed in the heat-killing treatment.

Freezing in oat crowns

Nonacclimated plants that were supercooled (but not frozen) to –2.6 °C for up to 23 h completely survived (data not shown). The absence of ice formation was confirmed by the lack of a freezing curve during continual calorimetric monitoring as well as by visual inspection of crowns for the presence of ice after removal from the calorimeter. After heterogenous nucleation, crowns were removed from the calorimeter at varying times in the freezing process (Fig. 2). Ten minutes after nucleation, only 10% of the water had frozen, but survival was reduced to 25% (Fig. 2). As the percentage of water freezing increased, percent survival decreased (Fig. 2); by 16 h, 70% of the water had frozen and survival was 0%.

Tolerance of plants to freezing is known to increase after exposure to cold but nonfreezing conditions (cold acclimation). In nonacclimated oat, 70% of total water froze and plants did not survive a freeze at –2.6 °C (Fig. 3). Within 4 d of cold acclimation, 50% of plants survived –2.6 °C (Fig. 3), and by day 8, the amount of water freezing was reduced to 54% with survival at 100%. A close relationship between killing temperature and moisture content was reported in wheat and barley (Metcalf et al. 1970), but at low temperatures and with fully cold-acclimated plants, the net amount of water freezing in wheat (Gusta et al. 1975) or in oat (Livingston et al. 2005*c*) was not correlated with survival. This was because the net amount of water freezing in crowns is a cumulative result of all melting and freezing events produced by numerous protective systems that develop during cold acclimation.

Uemura and Steponkus (1994) described significant changes in membrane composition of oat protoplasts within 1 week of cold acclimation, some of which could help prevent cell rupture when frozen and reduce the amount of water available for freezing. Kaplan et al. (2004) suggested that a multiplicity of primary metabolites, many of which increase in number within the first 48 h of cold acclimation, could act collectively as osmoprotectants,. In a study with *Arabidopsis*, as many as 325 metabolites were found to increase in concentration during cold acclimation (Cook et al. 2004).

The percent moisture of crowns also became lower during cold acclimation (Fig. 3 inset), possibly due to an increase of lignin-like components of cells (Livingston et al. 2005*b*), and may partially explain the gradual reduction during cold acclimation in the amount of water freezing (Fig. 3). Matric forces are a result of water adsorption to various components in plants, including colloidal substances, protein, and starch (Olien 1981), but the largest matric force is reportedly associated with cell walls (Boyer 1969). Therefore, it is possible that as the surface area of the cell wall increases, the matric effects on freezing will also increase. Gusta et al. (1975) reported that the percentage of total water freezing in fully cold-acclimated wheat is as low as 18% and as high

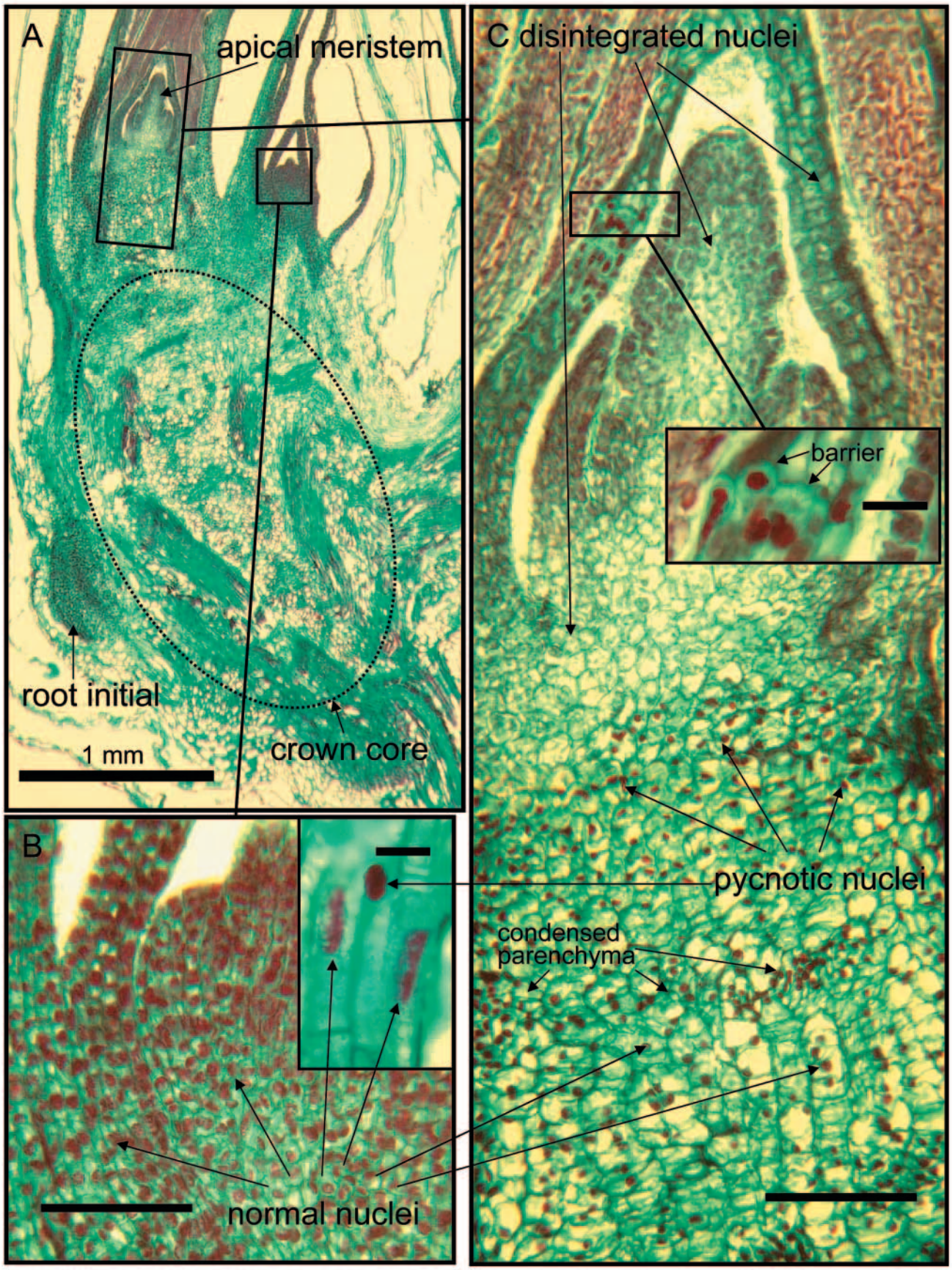


Fig. 5. Paraffin-embedded sections of nonacclimated 'Wintok' oat crowns frozen for 30 min (A) and 5 h (B) at –2.6 °C and allowed to recover for 5 d. Sections were triple stained with Safranin, Fast Green, and Orange G. (A) Note the apparent vessel plugging in the crown core (see Fig. 4A for location of crown core) but normal primary and secondary apical meristem. (B) Section with nuclear pycnosis in leaf base tissue. (C) Closer view of vessel plugging adjacent to areas of tissue disruption, presumably by ice formation. Despite this damage, note the absence of nuclear pycnosis as found in Figs. 4B inset and 4C. (D) Scanning electron micrograph from the top of an unfrozen 'Wintok' oat crown showing the apical region (also shown in Fig. 5A) exposed by removal of the stem base (see Livingston et al. (2005*b*) for details of the crown fractionation procedure used to expose this region of the crown). (E) Closer view of nuclear pycnosis in leaf base tissue to the side and directly above the apical meristem but not in the apical meristem itself or in the outer leaf as was found in the section in Fig. 1. The effects in this figure were never observed in unfrozen plants (Livingston et al. 2005*b*). Scale bars = 100 μm in Figs. 5C and 5E.

as 79%. From 59% to 77% of the total water freezes in most cultivars (Gusta et al. 1975). These values are higher than those that we measured in cold-acclimated oat (Fig. 3, 21 d in cold acclimation and Livingston et al. (2005*b*)).

During cold acclimation, carbohydrates, primarily fructan, are known to accumulate in oat (Livingston 1991) and other grasses and this could also explain the reduction in the amount of water freezing. Levitt and Scarth (1936) reported that the effect of sugars in reducing the amount of ice formed in plants is more important than overall freezing point depression. While Chen et al. (1976) found that hardier species can tolerate more ice in their tissues, Gusta et al. (1975) found no relationship between the amount of water freezing and freezing tolerance within species. Clearly, the relationship between the amount of frozen water and freezing tolerance is complex and would likely be better understood at a cellular level. Ball et al. (2002) found marked spatial variation of water content in snow gum (*Eucalyptus pauciflora* Sieber ex Sprengel) leaves, which has a significant effect on homogenous nucleation, duration of freezing, and rates of cooling, all which contribute to temperature differences of as much as 4 °C over leaf areas. These differences affect the survival of specific regions of tissue to a different extent (Ball et al. 2002). Olien (1981) stated that major differences between patterns in water transition "occur simultaneously in different tissue of a single plant", presumably because of differences between tissues in hydrophilic compounds such as sugars, proteins, and nucleic acids (Gusta et al. 1979).

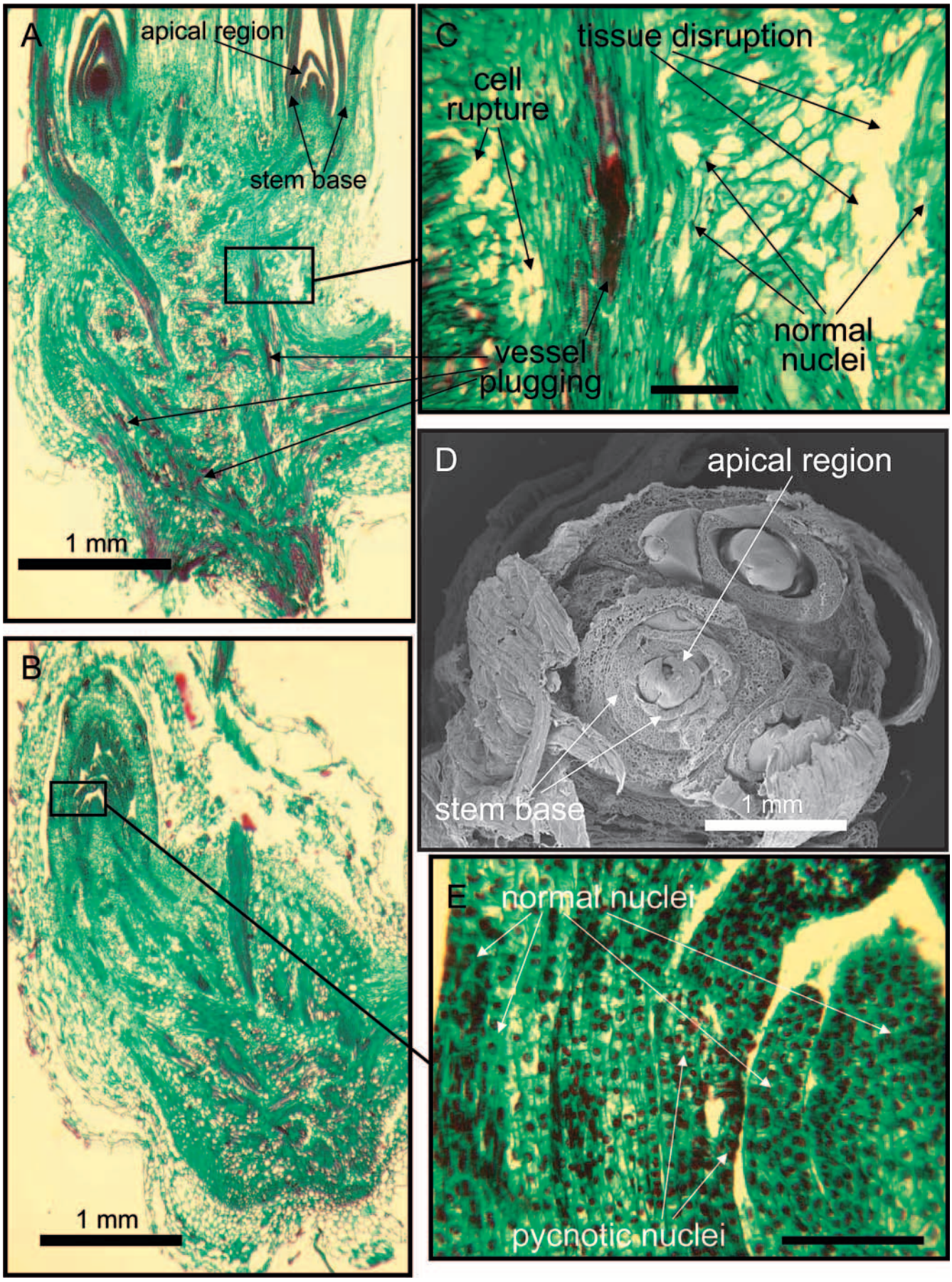
In addition to colligative effects, fructan as well as other sugars are known to stabilize membranes and have been implicated in protective mechanisms in plants under drought stress as well as freezing (Crowe et al. 2001; Hincha et al. 2002; Ottenhof et al. 2003; Hincha and Hagemann 2004). During cold acclimation, the concentration of fructan in crown tissue increases (Livingston 1991), but during subzero acclimation, fructan hydrolyses to simple sugars (Olien 1984; Livingston and Henson 1998) to a greater extent in the base of oat crowns than in leaf bases (Livingston et al. 2005*a*). This increase of sugars during a mild freeze induces melting, which is an endothermic event that would counteract the exothermic process of water freezing and reduce the net energy of freezing (Olien 1984). Another melting event that would offset the energy of freezing is CO_2 dissolving in liquid water (Livingston et al. 2000).

Histology of oat during recovery from freezing

Freezing damage was assessed by comparison of frozen tissue after it had recovered for 5 d with sections from plants that had not been frozen (unfrozen tissue not shown; see Livingston et al. 2005*b*). Chen et al. (1995) stated that "in tender plants freezing injury occurs when ice forms in the tissue. . .and results in either mechanical damage and or dehydration injury to the tissue". Damage in nonacclimated oat resulted in apparent nuclear degeneration (Figs. 4A and 4C), cell rupture (Figs. 5A and 5C), tissue disruption (Fig. 5C), vessel plugging (Figs. 5A and 5C), and nuclear pycnosis (Figs. 4B inset, 4C, and 5E). None of these effects are found in unfrozen (control) plants (Livingston et al. 2005*b*). With the exception of apparent nuclear degeneration (Fig. 4C), the injury observed in plants that are not cold acclimated (Figs. 4 and 5) is similar to injuries observed in cold-acclimated plants (Livingston et al. 2005*b*). However, the injuries shown here in nonacclimated plants were caused at a warmer temperature (–2.6 °C) than those in cold-acclimated plants. In fact, cold-acclimated plants that were subzero acclimated at –3 °C for 3 d have no observable freeze damage (Livingston et al. 2005*b*).

The initial 10 min of the freezing process produced only a small percentage (10%) of the total energy released during freezing, but it was also the time period with the most intensity (highest point of the curve, Fig. 1). Because the extent of damage did not appear to increase appreciably after the first 10 min of freezing, it is tempting to suggest that much of the tissue damage shown in Figs. 4 and 5 was a result of the apparent high freezing intensity during the initial freezing event. While the freeze resulted from only 2.6 degrees of frost, this was in fact a nonequilibrium freeze, which provides energy for "disruptive formation of ice crystals" (Olien 1981) and likely resulted in intracellular freezing. Clearly, not all of the tissue in these plants was damaged by this form of stress (Figs. 4 and 5).

Survival of nonacclimated plants was related to the length of time that plants were frozen at –2.6 °C (Fig. 2), but whole-plant survival was not necessarily related to damage in any particular tissue. For example, while the primary apical meristem was clearly dead (Fig. 4C) in plants frozen for 1.5 h, secondary meristems had survived (Fig. 4B) and could have resulted in the development of a normal plant. Figures 5B and 5E illustrate a primary apical meristem that appeared undamaged but with nuclear pycnosis in cells of the leaf base surrounding it. This discrepancy between whole-plant survival and survival of specific tissue within the crown confirms previous findings of apical meristem production from apparently undamaged meristematic tissue in an oat plant killed by freezing (Livingston et al. 2005*b*). Conversely, extensive damage to the crown core was observed in plants that completely survived. Differential survival of specific tissue within crowns has also been reported for barley (*Hordeum vulgare* L.) (Olien 1964; Pearce et al. 1998),



orchardgrass (*Dactylis glomerata* L.) Shibata and Shimada 1986), and wheat (Tanino and McKersie 1985).

Tanino and McKersie (1985) reported that the apical meristem in wheat is the region most susceptible to freeze injury within the crowns of nonacclimated plants. The section in Figs. 4A and 4C confirms that in nonacclimated oat plants, the apical meristem (specifically the primary apical meristem) was less freezing tolerant than other regions of the crown.

Cell rupture and tissue disruption (Fig. 5C) were likely a direct result of physical damage by ice (Olien 1961) and did not occur concomitantly with other types of damage. For example, apparent nuclear degeneration occurred without visible cellular or tissue disruption (Figs. 4A and 4C) and cellular rupture and tissue disruption occurred without pycnosis (Fig. 5C). This difference in damage suggests that different freezing stresses occurred within the whole crown and implies that even in nonacclimated plants, measurements of various physical properties such as those shown in Figs. 1–3 may need to be determined in specific tissue and possibly even in specific cells. Indeed, Mazur (1963) showed that at a given rate of cooling, larger cells retain a higher percentage of their water than do smaller cells, suggesting that differences exist between cells with regard to their ability to withstand desiccation injury.

Vessels that appeared to be plugged (Figs. 5A and 5C) are likely the result of microbial proliferation (Olien 1981; Livingston et al. 2005*b*) after freezing. Plugged vessels are not observed in unfrozen plants (not shown) and are observed in metaxylem vessels as early as 3 d after freezing at –11 °C (Livingston et al. 2005*b*). In this study, extensive plugging was observed in plants that were frozen for as little as 30 min (Figs. 5A and 5C); however, these plants had about 20% survival (Fig. 2), so this particular damage does not always result in plant death. Redundancy of various tissues in the crown may help the plant circumvent damaged regions and allow meristems to obtain material support from roots and leaves. The extensively plugged and degenerated tissue in the crown core of plants that completely survives freezing (Livingston et al. 2005*b*) supports this suggestion.

Nuclear pycnosis in frozen plants was first described by Shibata and Shimada (1986) in orchardgrass. Tzinger and Petrovskaya-Baranova (1970) described nuclear damage in frozen wheat; it resembled nuclear pycnosis, although that term was not used. Nuclear abnormalities have been reported in mammalian tissue exposed to various sources of radiation, high temperatures, severe dehydration, anesthetics, and different carcinogens (Torres-Bugarin et al. 2003; Cerqueira et al. 2004). Some of the abnormalities are described as "condensed chromatin and pycnosis" and appear equivalent to the nuclei shown in Figs. 4B inset and 4C. The possibility that nuclear pycnosis in frozen cells is due to freeze dehydration is being investigated. It is possible that nuclear pycnosis is an initial or intermediate stage leading to complete nuclear degeneration due to the loss of intracellular compartmentation either from freezing or from thawing. In that case, waiting longer than 5 d to sample tissue would result in more extensive nuclear degeneration than that seen in Fig. 4. However, this could not be confirmed because of the destructive sampling procedure used to visualize pycnosis.

Single and Marcellos (1981) stated that survival of the apical region depends on supercooling due to nodal blockage of ice formation into the apical region. Despite the ability of xylem vessels to continuously transport water throughout the plant, discontinuities between cells can form barriers to the progression of ice growth (Single and Marcellos 1981). Pearce and Fuller (2001) suggested that nodal segmentation in stems delays or prevents the spread of freezing, particularly into the crown. The apparent barriers within crown tissue described by Livingston et al. (2005*b*) support this possibility. The layer of somewhat condensed parenchyma cells separating those with nuclear pycnosis from normal cells (Fig. 4C) is probably the precursor to a node and seems to confirm the nodal blockage theory (Single and Marcellos 1981; Pearce and Fuller 2001). However, in this case, the more severe freezing damage was above the node, whereas previously, it was below the putative barrier in cold-acclimated plants (Livingston et al. 2005*b*).

Canny (1995) suggested that solutes tend to accumulate in regions of plants called sumps. In these regions, sugar concentrations could be much higher and may provide the critical margin to allow specific cells or tissues to withstand certain types of freezing stress. It is possible that the nodal region such as that shown in Fig. 4C may be a sump and that solute accumulation in this region prevented ice formation into the crown core, whereas in fully acclimated plants (Livingston et al. 2005*a*), the sump prevented ice growth into the apical regions.

Conclusions

The sensitivity to freezing of the apical region of nonacclimated plants may be due to inadequate development of a nodal block (Single and Marcellos 1981) or to the insufficient development of membrane stability of cells in the apical meristem (Tanino and McKersie 1985; Uemura and Steponkus 1999). Both are probably true. Also, the amount of water freezing may be causally related to nuclear degeneration or pycnosis in apical meristems of nonacclimated crowns, but it will be important to measure physiological, biochemical, and genetic differences between tissues within the crown before suggesting possible causes and effects.

It seems clear that whole-plant survival, in oat at least, is based on the hardiness of a relatively few number of cells in strategic locations in the crown. This calls into question the validity of correlations between plant survival and physiological, biochemical, and (or) genetic measurements taken on whole plants or even whole tissues.

The aim of this study was to see if freezing stresses would be limited under mild freezing in nonacclimated plants in the least freezing tolerant winter cereal so that cause and effect relationships between specific measurable events and visible tissue damage would be more easily established. However, the complex nature of freezing resistance involving the interaction of specific tissue and even specific cells precluded any clear association between the percentage of water freezing and visible tissue damage. It is probable that freezing tolerance in acclimated winter cereals is more complex than supposed so that whatever simplification achieved in our study was simply not apparent.

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