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Freezing in non-acclimated oats. II: Thermal response and histology of recovery in gradual and rapidly frozen plants

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ABSTRACT

Freezing in winter cereals is a complex phenomenon that can affect various plant tissues differently. To better understand how freezing affects specific tissue in the over wintering organ (crown) of winter cereal crops, non-acclimated oats (*Avena sativa* L.) were gradually frozen to −3 °C and tissue damage during recovery was compared to plants that had been supercooled to −3 °C and then frozen suddenly. Percentage of total water frozen, was the same whether crowns were frozen suddenly or gradually although the rate of freezing was considerably different. For example, all available water froze within 3 h in suddenly frozen crowns but it took more than 15 h for all available water to freeze in gradually frozen crowns. When plants were suddenly frozen, cells in the apical meristem were disrupted and apparently killed. In these plants re-growth was limited or non-existent. In contrast, the apical region of plants that were slowly frozen appeared undamaged but extensive vessel plugging was observed in cells of the lower crown, possibly from accumulation of phenolics or from microbial proliferation. These histological observations along with the calorimetric analysis suggested that the apical region was killed by intracellular freezing when frozen suddenly while the crown core was damaged by a process, which either induced production of putative phenolic compounds by the plant and/or permitted what appeared to be microbial proliferation in metaxylem vessels.

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1. Introduction

Winter survival of cereal crops such as rye (*Secale cereale* L.) wheat (*Triticum aestivum* L.) barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) involves numerous complex biological interactions with ice, within specific tissue of an overwintering organ called the crown [1–7]. These interactions with ice result in membrane and tissue disruption, intracellular freezing, adhesions, and freeze-dehydration [8–11].

The apical meristem was identified as the tissue within the crown that was most susceptible to freezing stress in non-acclimated wheat [7], oat [12] and barley [4]. After 3 weeks of cold-acclimation at 3 °C whole plant survival in oat increased dramatically. This increase in survival was primarily due to an increase in freezing tolerance of the apical region [12]. In fact, after cold-acclimation the apical meristem appeared undamaged in frozen and thawed plants while the lower portion of the crown, called the crown core, had completely degenerated [3,12].

The effect of freezing on specific tissue within organs of other grass species has also been documented. Mesophyll cells in maize had collapsed when frozen but bundle sheath and epidermal cells were apparently undamaged [13]. Changes in the ultrastructure of meristematic cells of tall fescue (*Festuca arundinacea*) that were frozen and thawed included "swelling and disruption of organelles, accumulation of osmophilic material and contraction of the nucleus" [14]. The apical meristem was the most freezing-tender part of the crown in orchardgrass plants that had been cold-acclimated [6].

This complexity of freezing in plants has made the results of many thermal analyses [9,15–19] difficult to interpret because of the inability to resolve numerous melting and freezing events which occur simultaneously within countless individual cells and multiple regions of tissue in the plant. In addition, hydration properties of water and interactions between water and macromolecules complicate interpretations of calorimetric data [20]. To help simplify the numerous individual thermal responses during freezing, Livingston et al. [3] separated the crown into the apical region and lower crown and found differences in percentage of water freezing and in carbohydrate redistribution during freezing [12] that were correlated to survival of the separate tissues. A

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further attempt was made to simplify freezing processes in winter cereals by investigating thermal responses of the least freezing tolerant winter cereal, oat under non-acclimated conditions, ostensibly in which a minimal number of protective mechanisms had an opportunity to develop [2].

In this study we froze oat crowns under conditions that would be likely to produce different means of equilibration within crowns. Our purpose was to document variability in tissue damage resulting from the different freezing conditions and provide a basis for further electron microscopic (EM) analysis of damage to ultra structure of cells within crown tissue of frozen winter cereal crops. Given the small scale of EM observation, efficiency could be improved significantly if observations were made on cells from tissue that ultimately died as a result of freezing. To avoid studying damage caused by freezing from which plants are able to recover [21] and which is not likely to contribute to death of the whole plant, we used bright field microscopy to observe recovering plants a few days after freezing. Specific tissue that warrants closer observation using EM can be identified in this way and may help identify mechanisms whereby plants are able to recover from freezing injury as compared to mechanisms whereby plants withstand freezing injury.

2. Materials and methods

2.1. Plant culture

Seeds of oat (*A. sativa* L., cv. 'Wintok') were planted in Scotts Metromix 510 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) in plastic tubes (2.5 cm diameter × 16 cm height) with holes in the bottom to allow drainage. Tubes were suspended in a grid which held 100 tubes. Plants were watered twice weekly with a complete nutrient solution [12] and flushed three times weekly with tap water. Non-acclimated plants were produced by growing them for 5 weeks at 13 °C day and 11 °C night temperatures in a growth chamber with a 12-h photoperiod at 400 μmol m^{-2} s^{-1} PAR (80% cool fluorescent and 20% incandescent; Philips Lighting Co., Somerset, NJ, USA), measured at mid-plant level.

After the 5-week growth period, plants were transferred to a similar chamber at 3 °C (Environmental Growth Chambers, Model M36, Chagrin Falls, OH, USA) with a 10-h photoperiod at 300 μmol m^{-2} s^{-1} measured as above. For cold-acclimation, plants were kept for 3 weeks at 3 °C.

2.2. Freeze tests and thermal analysis

Roots and shoots were trimmed from each plant after the respective growth treatments and a 2.5-cm portion of the base of the stem (crown) was used for calorimetric analysis. Crown tissue was studied in a Calvet Isothermal Calorimeter (model MS 80 Setaram, Saint-Cloud, France,) inside a small, refrigerated-room at −15 °C. The calorimeter was maintained from −1 to −3 °C by precisely heating the thermopile. It took 24 h for the calorimeter to come to equilibrium once the temperature was changed. Plant tissue (crowns) could not be supercooled below −3 °C without spontaneously freezing so they were tested only at −1, −2 and −3 °C. Plants were harvested about an hour before placing them into the calorimeter on the day they were to be used. At full sensitivity (Seebeck circuit), 1 mV output from the calorimeter equaled 17.6 mW displacement from baseline.

Crown samples were allowed to equilibrate at their respective freeze-temperatures (in a supercooled condition) until the baseline of the calorimeter stabilized (6 h). Plant samples at −1, −2 and −3 °C were induced to freeze (sudden freeze) 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 samples were located. Heat generated from inserting the wire was below limits of detection for settings used in these experiments.

Crowns that were frozen gradually were treated similarly except that they were placed in the calorimeter at +0.5 °C and then the temperature was lowered to approximately −3 °C. The calorimeter was cooled in a passive manner by turning off the heat and allowing the core to come to equilibrium with the temperature of the refrigerated room in which it was kept. Crowns were inoculated with the wire described above when the core temperature was −0.3 °C. As the temperature approached the set point, heat was automatically pulsed to the thermopile, bringing the core temperature to the set point in a gradual manner. This method of cooling is characteristic of an isothermal calorimeter which is known for precise temperature control at a specific temperature and not for a precise rate of cooling. For the first 10–12 h, rate of cooling was approximately 0.5 °C h^{-1} and during the next 10–12 h it was approximately 0.1 °C h^{-1}. Plants were removed approximately 10 h after they had equilibrated at −3 °C.

To determine the accuracy of using latent heat to measure amount of water freezing, the freezing point depression equation ($\Delta T = -1.86$ m) was expanded and solved for expected percentage of water remaining unfrozen as a function of molality and equilibrium temperature [22]. Sucrose solutions were frozen at varying temperatures and using latent heat determinations, amount of remaining liquid was calculated and compared to expected values. The values obtained by using calorimetric measurements of latent heat to determine percent water remaining liquid in partially frozen solutions were within 1% of expected values [22].

As samples froze, release of latent heat was recorded on a strip chart recorder and areas under curves were measured using a hand-held planimeter. The average of 3 measurements (less than 3% variation was observed between measurements) was used in all calculations. Area under curves was related to calories using latent heat of freezing from −1 to −6 °C for known quantities of pure water [22]. A standard curve (not shown) with varying amounts of water indicated a linear relationship between g 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 latent heat was recorded, crowns were removed from the calorimeter when they had completely equilibrated (16 h, for the sudden freeze). Crowns were planted into the same soil mix in which they were grown initially and allowed to recover under the controlled conditions at 13 °C described above.

2.3. Histology

Crown tissue was observed 5 days after freezing to avoid confusion with tissue changes during freezing from which plants are able to recover and are therefore not likely to be relevant to survival of the whole plant.

Recovering plants were randomly selected and one to three cm of the lower part of the stem from 7 plants was placed in fixative containing 18:1:1 parts of 70% ethyl alcohol, glacial acetic acid and formaldehyde, respectively. The collected samples were kept at room temperature for 48 h and transferred to 70% alcohol and kept at 3 °C until they were processed for dehydration and embedding.

Samples were dehydrated according to procedures detailed by Johansen [23] 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 rotary microtome (Reichert-Jung 2050, Cambridge Instruments, Buffalo, NY, USA) at a thickness of 15 μm. The resulting paraffin ribbon containing serial sections was placed on a glass slide coated with Haupt's adhesive [23], flooded with 3%

formalin, and transferred to a slide warmer at 41 °C. Dried slides were stored at room temperature until stained. Slides were left overnight in dishes containing Xylene to remove paraffin before sections were stained with a triple stain of Safranin, Fast green and Orange G [23]. A cover-glass was then added to slides with 1 or 2 drops of Permount.

To observe differences among treatments, sections were viewed under a wide angle dissecting microscope (Wild Heerbrug, Gais, Switzerland) with bottom lighting and on a compound microscope (Zeiss, photomicroscope III, Jena, Germany). Photographs were taken of sections that best represented damage that was typical of freezing injury with a digital camera (Sony DSC707, Tokyo, Japan) attached to the microscope.

3. Results and discussion

3.1. Thermal patterns in partially frozen crowns

The most obvious difference between freezing pattern of plants that were suddenly frozen and those gradually frozen was the intensity (maximum mW of signal detected) of freezing (Fig. 1). The output in gradually frozen plants reached a maximum of 0.95 mW after about 7 h while the output in suddenly frozen plants was 10-fold higher at nearly 9.5 mW g^{-1} fresh wt within the first hour after freezing (Fig. 1). Despite this difference in freezing intensity the total percentage of water freezing in the crowns was nearly identical with 74% of the total freezing in suddenly frozen vs. 73% in gradual frozen plants and was not significantly different at $p = 0.05$.

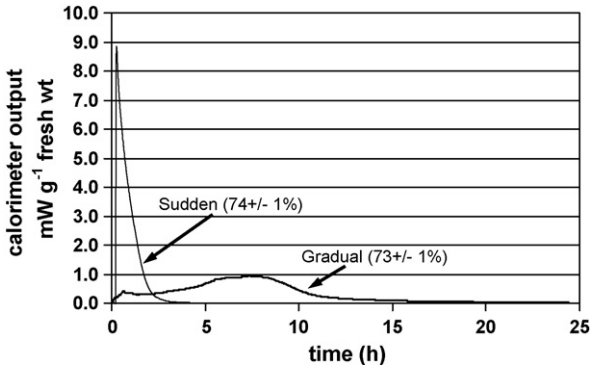
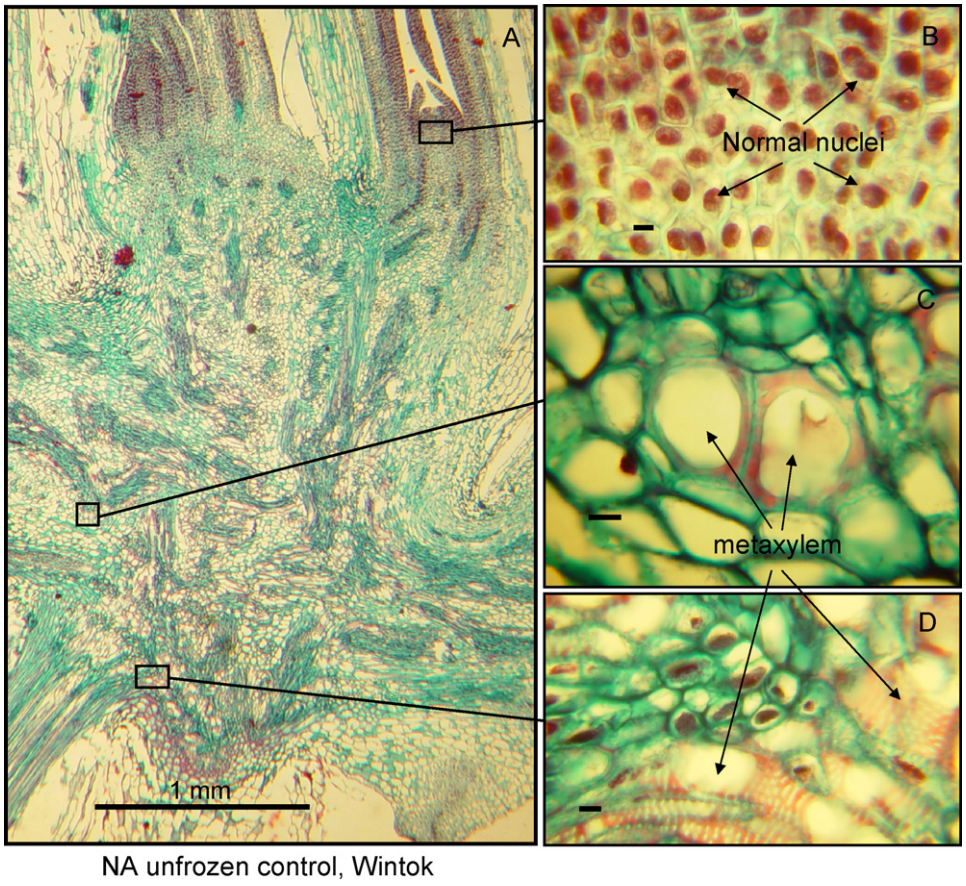


Fig. 1. Thermal output from 10 non-acclimated oat plants that were frozen suddenly and gradually to −3 °C. The percentage of water freezing in the plants in shown in parenthesis.

The precision of an isothermal calorimeter is dependant on a high degree of temperature control which necessitates a lengthy time to reach equilibrium. In addition, it was not possible to reach −3 °C any quicker than 16–18 h in the gradual freeze because the cooling phase uses a passive means to cool (see Section 2). During that time metabolic effects such as fructan hydrolysis to simple sugars [24] could have induced melting, an *endothermic* process that would affect the baseline of gradually frozen plants by absorbing heat. In addition, since respiratory rates are significant in crowns, even down to −3 °C [25], this *exothermic* process could have affected



NA unfrozen control, Wintok

Fig. 2. Paraffin embedded section of non-acclimated Wintok oat crown that was not frozen. Plants under these conditions were used as controls to evaluate the ability of frozen plants to recover from freezing damage. The section was triple stained with Safranin, Fast Green and Orange G. (A) Section of the whole crown showing normal tissue in both the crown core and the apical region. Note the absence of disintegrated cells and pycnotic nuclei as seen in Fig. 3 and note the absence of vessel plugging as seen in Fig. 4. (B) A closer view of the center of the apical meristem showing normal cells. (C) A cross-section of normal metaxylem vessels. (D) A longitudinal view of metaxylem vessels. Unlabeled bars in sub panels (B), (C), and (D) represent 10 μm.

the baseline in the opposite direction, which would counteract heat absorbed by ice being melted from fructan hydrolysis.

3.2. Freeze damage: unfrozen controls

Unfrozen (control) crowns from oats that have been sectioned, triple stained and observed under brightfield microscopy have been extensively described [3]. Briefly, the dark red to purple nuclei of cells in the apical region of unfrozen crowns were easily discernable (Fig. 2). The cell walls were intact and were a light green color. Cells in the lower portion of the crown (termed the "crown-core") were a mixture of metaxylem, phloem, fiber and mesophyll [3]. Nuclei of cells in the crown core from unfrozen crowns were all clearly visible with the same color as those in the apical region. Mesophyll cells in the crown core were 2–3 times the size (approximately 15–20 μm in diameter) as those in the apical region; their walls were intact and were a brilliant green. The cell walls of metaxylem vessels were stained red from Safranin and always appeared empty (Fig. 2).

3.3. Rapidly frozen crowns

The most obvious difference between unfrozen crowns (Fig. 2) and those that were rapidly frozen at −3 °C (Fig. 3) were cells within the apical region that appeared ruptured with no discernable nucleus. This difference from controls was not observed in crowns that were frozen to −2 °C (Fig. 4), or −1 °C (not shown). This damage to the apical region was observed in all rapidly frozen crowns (from 7 plants) at −3 °C but never in unfrozen controls (Fig. 2). This confirms previous reports of the lower level of freezing tolerance in the apical region, as compared to the crown core, of non-acclimated wheat [7] and oat [3].

The rapid freeze at −3 °C would have affected both amount as well as type of stress on individual cells and tissue. The freezing intensity of the rapid freeze (Fig. 1) would disrupt equilibrium within the crown which would induce water potential gradients that could produce significant tissue damage (Fig. 3), like that in the apical region, as equilibrium in the crown is re-established.

3.4. Gradually frozen crowns

In contrast to suddenly frozen crowns, little damage to the apical region was observed in crowns gradually frozen to −3 °C (Fig. 5). Out of 7 crowns only 2 had one or two tillers with dead apical regions (not shown); most sections resembled the unfrozen crown (Fig. 2) with the apical region showing no apparent damage.

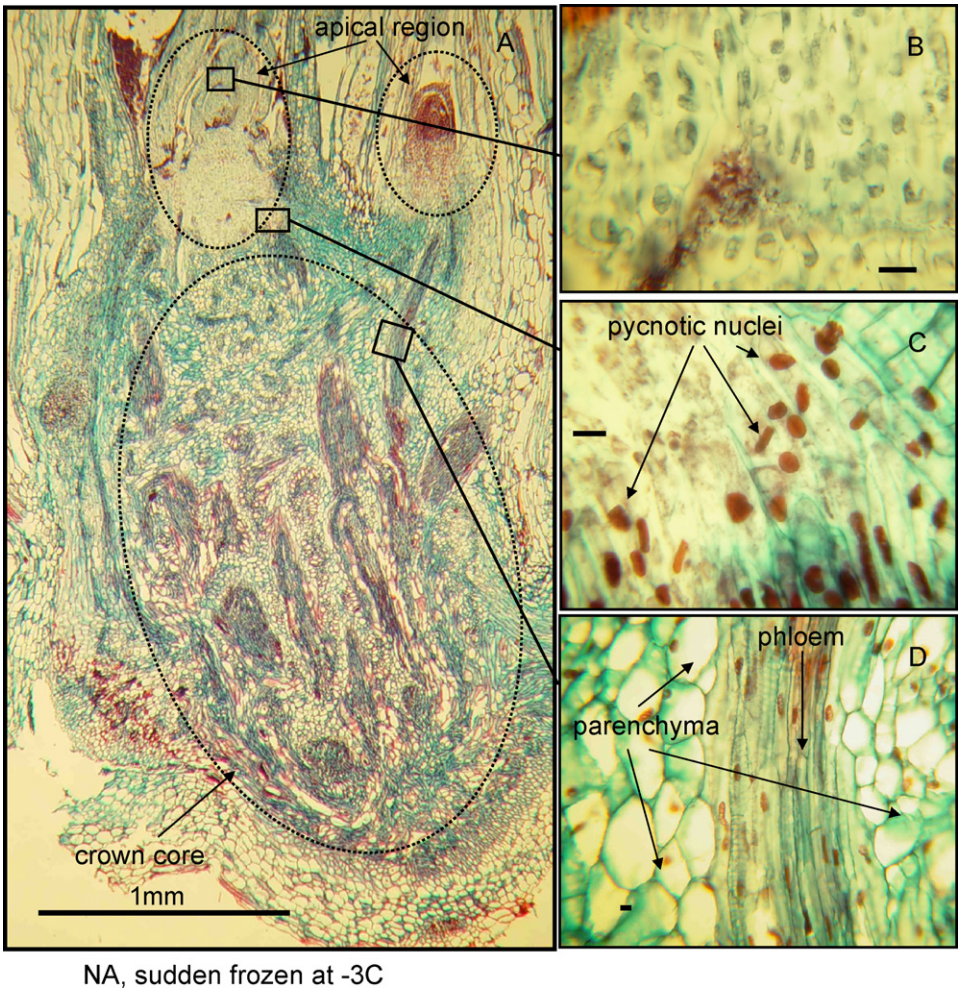


Fig. 3. Paraffin embedded section of non-acclimated Wintok oat crown supercooled for 6 h and then suddenly frozen at −3 °C and allowed to recover for 7 days under normal growing conditions. The section was triple stained with Safranin, Fast Green and Orange G. (A) Section of the whole crown showing the relatively normal crown core and degenerated tissue in the apical region of 2 tillers. (B) A closer view of the center of the apical meristem showing the degenerated cells (compare to Fig. 2B). (C) A closer view of the edge of the apical region and upper part of the crown core showing nuclear pycnosis in the cells. (D) Closer view of relatively normal vessels in the upper part of the crown core as well as apparently undamaged (and/or fully recovered) parenchyma cells. Unlabeled bars in sub panels (B), (C), (D) represent 10 μm.

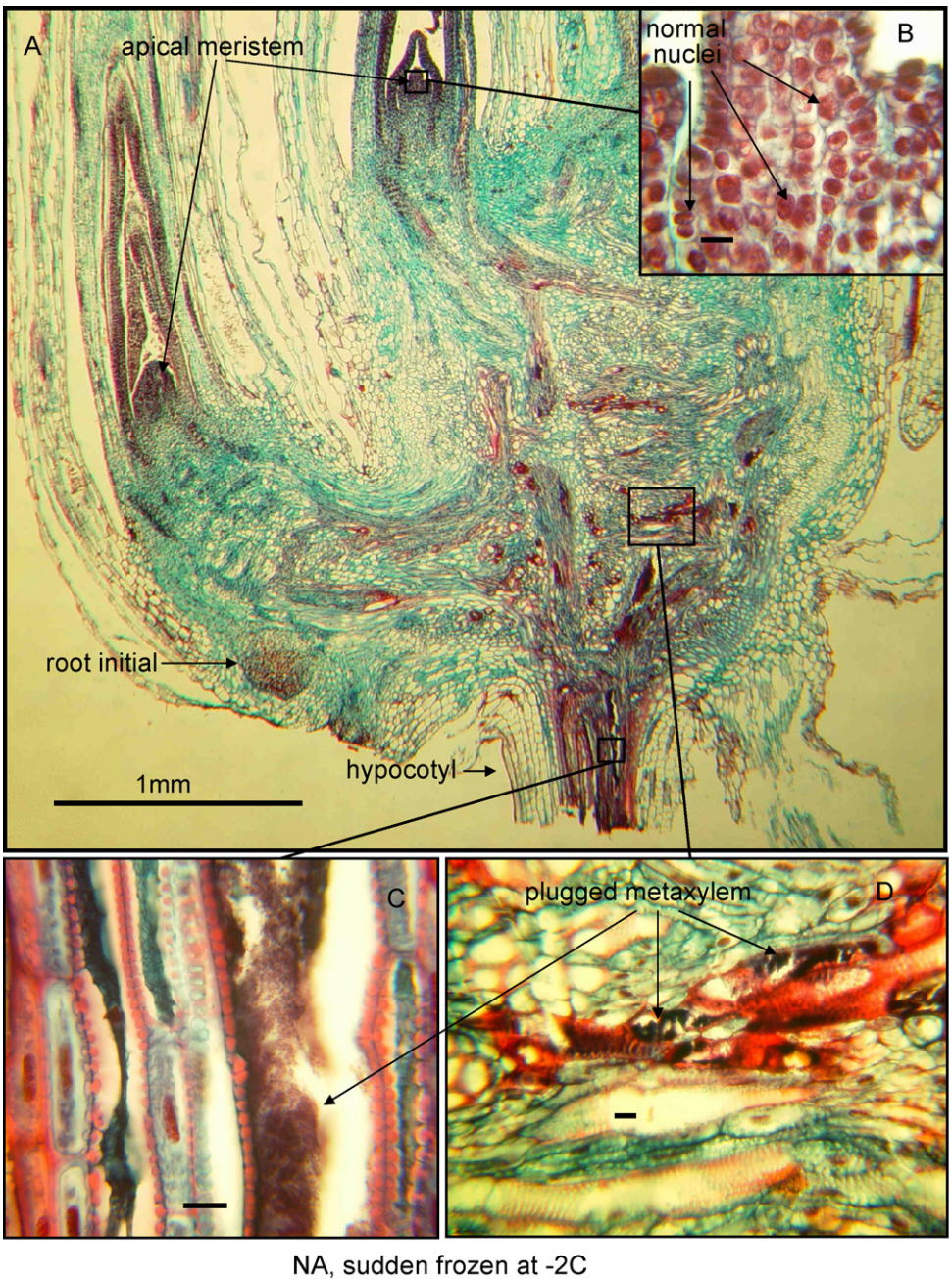


Fig. 4. Paraffin embedded section of non-acclimated Wintok oat crown supercooled for 6 h and then suddenly frozen at −2 °C and allowed to recover for 7 days under normal growing conditions. The section was triple stained with Safranin, Fast Green and Orange G. (A) Section of the whole crown showing scattered plugged vessels in the crown core and normal tissue in the apical region. (B) A closer view of the center of the apical meristem showing normal cells. (C) A closer view of plugged metaxylem vessels in the upper region of the hypocotyl. (D) Closer view of plugged metaxylem vessels in the center of the crown core. Unlabeled bars in sub panels (B), (C), (D) represent 10 μm.

The only evidence of damage in gradually frozen crowns was what appeared to be extensive vessel plugging at −3 °C (Fig. 5). This putative plugging also occurred in rapidly frozen crowns at −2 °C in which the apical region was not damaged (Fig. 4).

This presumed vessel plugging could have been a result of the proliferation of microbes [3] but this will need to be confirmed. Bacteria belonging to the genus *Pseudomonas* were isolated from frozen and thawed barley roots [26]. These organisms reportedly move from roots into the crown during recovery from freezing and secrete a toxin that is responsible for tissue death within the crown [26]. Various fungal species were also found to proliferate in plants recovering from freezing [26]. The colonization of the apoplast with endophytic bacteria was demonstrated in maize using electron microscopy in a study where the apoplast was referred to as a "habitat for microorganisms" [27].

The darkly staining material appeared to be confined to xylem, metaxylem and fiber in the crown core. It was neither observed within parenchyma or phloem cells nor in the apical region. However, if plants were given more time to recover, it is possible that this supposed infection would have spread to surrounding tissue. The reddish coloration adjacent to infected vessels suggests that after 7 days of recovery infection may have spread into intercellular spaces (Fig. 5).

Preliminary observations under fluorescence microscopy (not shown) indicated that the material plugging vessels, fluoresces in the visible spectrum (yellow-brown) when excited with UV light.

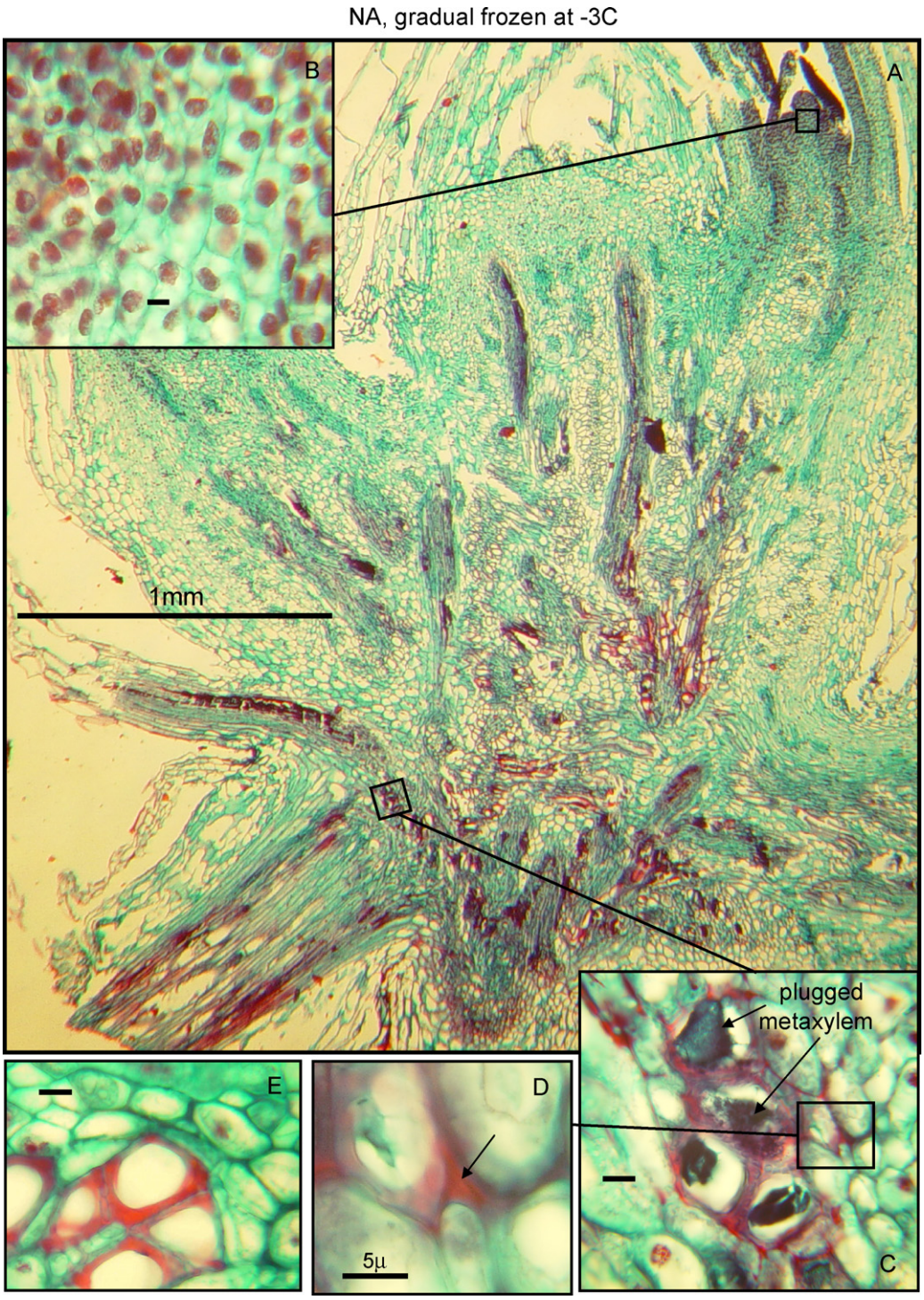


Fig. 5. Paraffin embedded section of a non-acclimated Wintok oat crown gradually frozen to −3 °C and allowed to recover for 7 days under normal growing conditions. The section was triple stained with Safranin, Fast Green and Orange G. (A) Section of the whole crown showing scattered but significant vessel plugging in the crown core and apparently normal cells within the apical region. (B) A closer view of the center of the apical meristem showing normal nuclei in the cells. (C) A closer view of metaxylem cells in cross-section at the root-crown junction that was plugged. (D) A closer view of the intercellular space adjacent to the plugged metaxylem vessel showing red staining that is not present in unfrozen metaxylem vessels as shown in (E). (E) Closer view of metaxylem in cross-section that had not been frozen showing no red staining in intercellular space as in (D). Unlabeled bars in sub panels (C) and (E) represent 10 μm.

Phenolic compounds are commonly localized in plant tissues and fluoresce with UV light [28]. It is possible that the plant produced phenolic compounds in response to freezing stress as part of a cascade of wound responses [29]. We are attempting to identify this material and determine how/if it is involved in helping the plant recover from freezing.

The concentration of degree of polymerization >5 (DP > 5) fructan in the apical region of crowns from non-acclimated oat plants was twice as high on a dry weight basis as it was in the lower crown [2]. It is possible that fructan had a protective effect on membranes of cells [30,31] within the apical region, resisting stresses induced by slow freezing (such as desiccation) or by a lower intensity freeze

(at −1 °C or −2 °C) but was not able to provide protection from a rapid, high intensity freeze at −3 °C. The more rapid return to baseline of crowns frozen at −3 °C in comparison to those frozen at −1 and −2 °C (Fig. 6A) despite the larger percentage of water freezing at −3 °C supports this possibility, although this thermal analysis was done on whole crowns not just the apical region.

3.5. Cold-acclimated plants

Twice as much water froze at −3 °C in non-acclimated plants as did in cold acclimated plants (Fig. 6A and B). This difference in the amount of water freezing was correlated with freezing survival of the plant with survival in non-acclimated plants at 0%, while it was 100% in cold-acclimated plants. In another study, 75% of total water in crowns from non-acclimated plants froze at −3 °C [22] and the apical region was killed resulting in death of the plant. Within the same oat cultivar, frozen at the same temperature, the amount of water freezing was reduced to 55% after cold-acclimation [22]. The apical region of these crowns completely survived this temperature and the whole plant survived.

The change in the amount of water remaining unfrozen during cold-acclimation could have been a result of colligative, or matric effects [32] or by partitioning of water through alteration of membrane composition, including alteration of water channels [33,18]; this would prevent ice penetration and effectively make intracellular water unavailable for freezing. Alternatively, solutes in a pure solution are excluded from ice as it freezes [34]. If a similar effect occurs in crowns, then as solutes retreat from the expanding ice lattice they would concentrate and could eventually form a barrier to freezing like those allegedly found in oat crowns [3] and postulated by Aloni and Griffith [35].

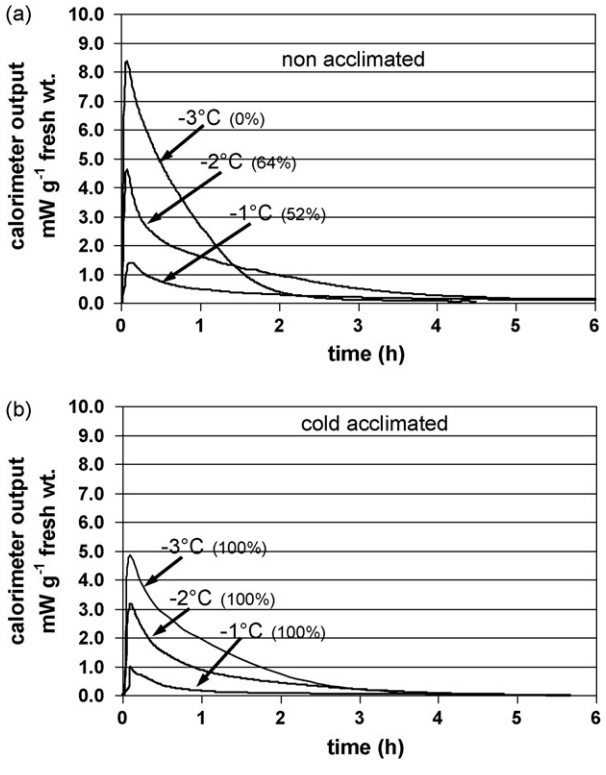


Fig. 6. (A) Thermal output from 10 non-acclimated oat plants suddenly frozen at 3 temperatures. The percent survival of the plants is in parentheses next to its respective freeze curve. (B) Thermal output from oat plants that were cold acclimated for 3 weeks at 3 °C and then suddenly frozen in an identical manner to (A). Percent survival is in parentheses.

Numerous other survival mechanisms induced by cold acclimation have been described [10,20,33,36–38] and would enable plants to withstand stresses generated by a sudden freeze at −3 °C. Indeed, when cold-acclimated plants are suddenly frozen at −3 °C no damage in recovering plants is observed from either a histological point of view or from survival percentages ([3] and Fig. 6B).

4. Conclusions

Freezing in plants initially produces a chemical potential gradient within the system. Equilibrium will be re-established within the plant when tissue disruption (such as membrane rupture) reduces chemical potential differences between regions of tissue. The weakest barrier that upon initial freezing maintains a disequilibrium, will likely be the first to be disrupted. If that disruption (for example, a breach in the membranes of non-acclimated cells in the apical region) allows equilibrium to be reestablished at a particular temperature then no further changes (i.e. damage) are required to re-establish equilibrium until the temperature is lowered. The magnitude of the gradient clearly has a significant effect on the type of adjustment (which under these conditions resulted in injury) that promotes re-establishment of equilibrium.

In addition to total *amount* of water freezing, the *rate* at which water freezes also determines the type of stresses and subsequent damage that occurs within plants. Intracellular freezing has been implicated under sudden freezing conditions while slow freezing reportedly results in dehydration stress [9,13,37]. Although, as early as 1886, Muller-Thurgau (cited in [17]) stated that sudden freezing may also cause sudden dehydration which can be as damaging to plant tissue as intracellular freezing and thawing.

Because freezing method (gradual vs. sudden freezing) did not result in a difference in percentage of water freezing, the total amount of water freezing is probably not the issue that determined tissue survival at least in non-acclimated plants. The freezing rate, on the other hand, determines the speed at which water is withdrawn from cells which apparently determines which tissue within the crown will survive the freeze. To that end, 2 regions of non-acclimated crowns have been identified for more detailed study: cells within the apical region which under conditions of rapid equilibration (sudden freeze) appear to rupture and die, and cells within the lower crown which under slow freezing conditions appear to accumulate a darkly staining material that could be a wound-induced accumulation of phenolic compounds.

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