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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%