Rapid Microwave Processing of Winter Cereals for Histology Allows Identification of Separate Zones of Freezing Injury in the Crown

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ABSTRACT

In histological studies, microwave processing of tissue considerably shortens the time required to prepare samples for observation under light and electron microscopy. However, plant tissues from different species and different regions of the plant respond differently to microwave processing, making it impossible to use a single protocol for all plant tissue. The crown of winter cereals such as rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and oats (*Avena sativa* L.) is the below-ground portion of the stem that overwinters. It is composed of numerous types of cells with an organizational pattern that is similar to other grasses. When we used microwave protocols that were developed for other plant tissues, winter cereal crown tissue shattered and crumbled when sectioned. This study reports a procedure developed to process winter cereal crowns for histological observations. Using this microwave protocol, samples were prepared in 1 d as compared to 2 wk using traditional protocols. This enabled many more samples to be processed and allowed us to identify four overlapping zones of response to freezing within the crown. Results of varying time, temperature, and microwave wattage during fixing, dehydrating, and embedding in paraffin are described. High quality sections from the crowns of oat, barley, wheat, and rye indicate that this procedure is valid for all winter cereals. Since crown tissue is similar across all grass species, we predict that the protocol will be useful for other grasses as well.

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Abbreviations: Ethanol, EtOH; recombinant inbred line, RIL; tertiary butyl alcohol, TBA.

Traditional preparation of tissues for microscopy is time consuming because it requires passive infusion of solvents. Traditional methods for preparation of crowns of winter cereals for histology often require up to two weeks before samples can be sectioned and examined (Livingston et al., 2005). Once plants are removed from growing medium three processes are involved in tissue preparation: (i) fixation, (ii) dehydration, and (iii) embedding. Fixation is done to preserve tissue/cellular structure and may require up to two days by standard protocols. Dehydration replaces the water in tissues with a solvent that is miscible with embedding media such as paraffin or resin. Depending on the exact protocol, dehydration often requires five days or more. Infiltration with embedding medium often requires three to seven more days (Johansen, 1940; Ruzin, 1999; Livingston et al., 2005). Although smaller plant tissues such as young leaves or roots can be processed in a shorter time, the penetration of solvents into crown tissue from winter cereals is more difficult. Certain regions of the crown contain dense meristematic tissues with small cells and

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trapped air (Livingston et al., 2005), making them more resistant to penetration of solvents than leaves or roots.

In winter cereal crops the crown is the lower 5 to 10 cm of the stem, and it has routinely been used in freeze-testing as a representative of the whole plant (Livingston, 1996). The bottom 4 mm of the crown has been called the crown-meristem complex because it contains shoot apical meristems that later produce the inflorescences of the tillers (Evert, 2006). Subjacent to the shoot apical meristem (or shoot apex) is a larger meristematic zone, called the transition zone. Below this area is the "crown-core", which produces a conductive interface with the root system (Livingston et al., 2005; Aloni and Griffith, 1991). The crown-core is composed of an intertwining series of vascular sclerenchyma and parenchyma cells in a generally inverted cone shape. Therefore, crown tissue is complex with many different cell types (Pearce et al., 1998), presenting a challenge for microscopic sample preparation to generate high-quality images.

While the exact mechanism of microwave-assisted fixation is not known, rapid processing of samples is thought to be through the synergistic action of: (i) the brief microwave energy that makes polar molecules, especially in membranes, oscillate at about a billion times per second, and (ii) the effects of mild heating (generally 25°C–45°C). This synergistic action increases the rate of diffusion of histo-chemicals into tissue. The use of microwave technology to speed the process of fixing, dehydrating, and embedding selected animal and plant tissues for light as well as electron microscopy has been reviewed extensively (Giberson and Demaree, 2001), although it is still not commonly practiced for plant specimen preparation.

Microwave processing of tissues for histology can shorten processing time, but several parameters must be adapted for a particular tissue involved. These are: (i) type and concentration of solvents used in the various steps, (ii) time at each step, (iii) temperature to which tissue is heated, and (iv) wattage of the oven. The present study was undertaken because preliminary experiments showed that there is no generalized protocol for processing plant tissue using microwaves. We found that several published protocols (Ruzin, 1999; Schichnes et al., 2005) resulted in crown tissue that shattered and tore when sectioned. It was likely that the crown tissue was either not sufficiently dehydrated and/or that infiltration with paraffin was incomplete. In initial trials to modify published protocols, simply lengthening times for the various steps did not produce acceptable results.

This paper describes the results of our further trials, which ultimately generated a microwave processing protocol that efficiently delivers high quality results for anatomical studies of the crown tissues of rye, wheat, barley, and oats. Because the crown contains root and shoot meristems, it is vital for the cold survival of winter cereals even though "not all cells in a crown contribute equally to survival of that crown" (Chen et al., 1983). To illustrate the utility of this protocol, we generated a sequence of 280 sections from an oat crown that was frozen and allowed to recover for ten days. The sections were aligned and processed in animation software that allows a sequential, continuous examination as a video of the internal anatomy of the crown from the root-shoot junction to the shoot apex.

MATERIALS AND METHODS

Plant growth, Including Freezing, Thawing, and Regrowth

Seeds of rye (cv. Rosen), wheat (cv. Jackson), barley (cv. Dictoo), and oat (cv. Wintok) were planted and grown under controlled conditions as described (Livingston et al., 2005). Briefly, plants were grown in cone-shaped containers (2.5 cm in diameter by 16 cm high) at 13°C with a 12-h daylength for 5 wk, then cold acclimated at 3°C for 3 wk.

Plants were removed from containers and washed free of soil. Roots and shoots were trimmed to a total length of 5 cm. Plants that had been trimmed (crowns) and were to be frozen were inserted into slits made in circular moist sponges, placed in a plastic bag to prevent desiccation, inoculated with ice shavings to initiate freezing and prevent supercooling and then placed in a freezer at –3°C. Once the sponges had frozen (approximately 6 h) the temperature in the freezer was reduced to –10°C at 1°C per hour and held at this temperature for 3 h. The temperature was then raised to 2°C at 2°C per hour. The crowns were transplanted into soil mix and allowed to grow for 7 d at 20°C.

Histological Methods, Microscopic Observations, and Compiling of Sections

The bottom 1 cm of the crown consisting of the base of three stems (or tillers) was removed and subjected to fixation, dehydration, and embedding as shown in Table 1 using a laboratory microwave with integrated vacuum (Pelco Biowave Pro Tissue Processing System, Ted Pella Inc, Redding, CA). The key points involved in development of the protocol are described under Results.

A rotary microtome (Model RM2255, Leica, Wetzlar, Germany) was used to make 15 μm thick sections of crowns embedded in paraffin (Paraplast Plus, Oxford Labware, St. Louis, MO). Ribbons were placed on slides with Haupts A solution (Ruzin, 1999) as an adhesive and floated on 3% formalin at 48°C; the elevated temperature was crucial to prevent wrinkling of sections from the crown core. Sections of this region of the crown, which requires 10 to 20 sections if sectioned completely, are particularly susceptible to wrinkling as well as shattering. As soon as the paraffin ribbon had expanded and sections were wrinkle free (~2 min), they were transferred to a 40°C slide warmer to finish drying.

The dried slides were submerged in xylene for 30 min to dissolve paraffin before sections were triple stained with Safranin, Fast Green, and Orange G (Fisher Scientific, Pittsburgh, PA) as described (Ruzin, 1999). A cover-glass was added to slides with Permount adhesive (Fisher Scientific, Pittsburgh, PA).

Sections were viewed with a wide-angle dissecting microscope (Wild Heerbrugg, Gais, Switzerland) with transmitted light and with an upright microscope (Zeiss photomicroscope III, Zeiss, Thornwood, NY). Sections were photographed with a digital camera (Sony DSC707) attached to the microscope.

To generate the video file showing successive cross-sectional areas of the crown, micrographs of 280 sections were manually aligned in Adobe Photoshop (Adobe Inc., San Jose, CA). Transitions between sections as well as rendering was done in Adobe After Effects. Each frame of the video is a single 15-μm section. The video then is a sequential, internal observation of the bottom 4.2 mm (0.015 mm × 280 sections) of the plant.

RESULTS AND DISCUSSION

The parameters that were particularly important in generating the final protocol are highlighted here. Although vacuum infiltration does not necessarily improve results in all tissues (Ruzin, 1999), it was required during fixation and paraffin embedding of crown tissue. Apparently, crown tissue contains air space because most crowns floated in the fixative and bubbles frequently came out as vacuum was applied.

Table 1. Microwave fixation, dehydration, and paraffin embedding protocol used to successfully process crown tissue from oat, wheat, barley and rye. Note that Meth-FAA* used for fixation substitutes methanol for EtOH.

Step	Chemical medium	Time†	Temperature	Wattage
Fixation				
	Meth-FAA‡	1 h	30°C	200 with vacuum
Post Fixation				
	60% EtOH	2 h	77°C	200
Dehydration				
	70% EtOH 50% EtOH	5 min	77°C	200
	50% isopropanol	5 min	77°C	200
	100% isopropanol	5 min	77°C	200
	100% isopropanol	5 min	77°C	200
Embedding				
	50% isopropanol 50% paraffin	10 min	65°C	200
	100% paraffin	1 h	65°C	200 with vacuum
	100% paraffin	3 h	65°C	200 with vacuum

†Total time 7 h 30 min.

‡"Meth-FAA" is 45% methanol, 10% formaldehyde, 5% glacial acetic acid, 40% water.

Fixation

A major objective when developing the microwave protocol was preventing shattering or crumbling of sections (Fig. 1A). Many variations of time, temperature, and wattage for fixing, dehydrating, and embedding steps were tested without visible improvement. Then it was discovered that a post-fixation treatment of the tissue in 60 to 70% ethanol (EtOH) completely eliminated crumbling during sectioning (Fig. 1B). The concentration of EtOH was important; post-fixation with 95% EtOH resulted in complete crumbling. In addition, microwave time was also a factor with optimum results at two to four hours. Differing temperatures were not evaluated.

One advantage of a microwave oven designed for use in microscopy/histology is that the temperature and rate of heating of the sample can be adjusted by altering the amount of energy put into the sample rather than using one energy level and cycling the magnetron on and off as in a home microwave. Microwaving 70% EtOH caused the temperature to increase at 3.7, 6.7, or 10.4 °C/min at 200, 450, and 650 W, respectively. In addition, when the sample was heated to the same temperature, but at a lower wattage, the magnetron cycled more often after reaching the set temperature than when a higher wattage was used. There was no correlation between the wattage and shattering of sections, so the lowest wattage (200 W) that would still give good results was selected.

Dehydration

Solvents that are commonly used to dehydrate plant tissues include EtOH and tertiary butyl alcohol (TBA). Most protocols begin dehydration with 100% EtOH (v/v) and gradually increase the TBA concentration to 100%. Use of this combination in traditional protocols resulted in excellent sections (Livingston et al., 2005), but the procedure is time consuming. In initial trials, dehydration beginning with 25% EtOH/25% TBA followed by gradual conversion to 100% TBA (microwaving for 30 min, 50°C, 450 W at each step) produced sections that shattered badly. A gradual change from EtOH to isopropanol, which reportedly works well with leaf and stem tissue (Schichnes et al., 2005), also produced sections that shattered. Temperature, time, and

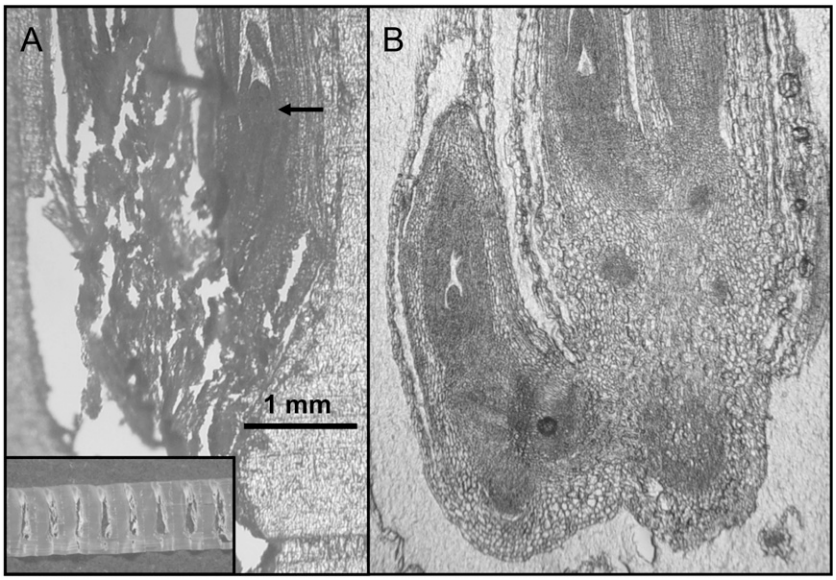


Figure 1. A. Paraffin embedded section (before paraffin removal and staining) of oat crown processed using a microwave processing protocol for leaves. Note shattering of the central crown region but relatively intact apical region (arrow). The inset shows shattering of crown tissue within a ribbon. B. An intact section using the protocol outlined in Table 1.

wattage were all adjusted in numerous combinations, none of which produced acceptable results. In contrast, when the dehydration step was simplified with a gradual conversion from 70% EtOH to 100% isopropanol (Table 1) at 77°C and 200 W, sections were smooth and intact. The time at each step was reduced until sections began to shatter. Five minutes was the minimum time necessary at each step for complete dehydration of crown tissue.

Embedding

The only difficulty encountered in this step was that because paraffin does not absorb microwave energy, it did not heat very well in the microwave oven. Therefore, to keep the paraffin in a liquid state during embedding, the paraffin had to first be melted in a convection oven at 65°C. Then the container of melted paraffin was placed in a water bath, which was maintained at 65°C in the microwave oven.

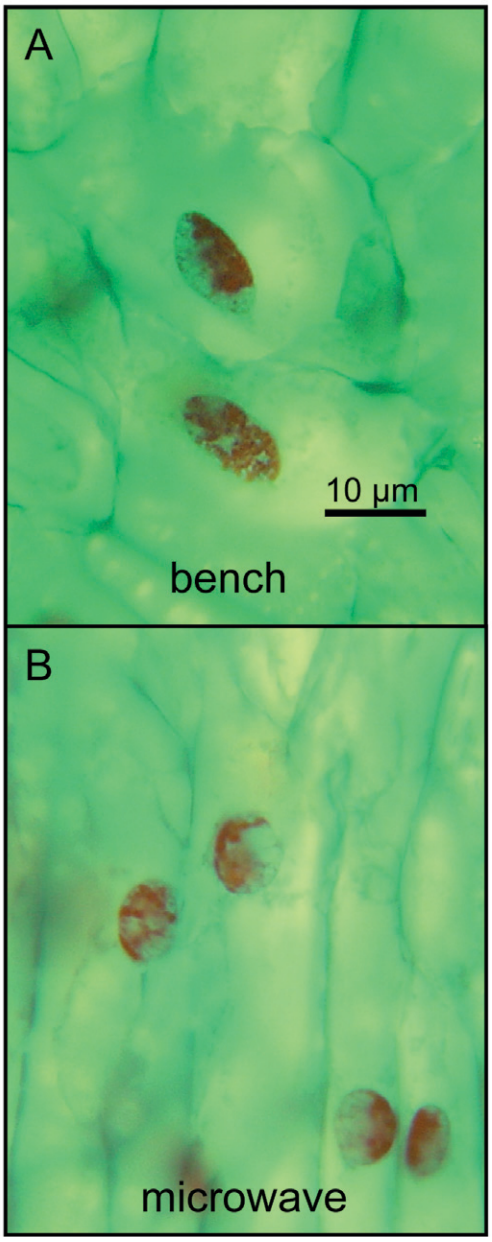


Figure 2. Nuclei of cells within the crown core region of oats comparing bench (A) and microwave (B) processed crown tissue. Note that red-stained chromatin remains dispersed within the nucleus in both protocols.

OBSERVATIONS

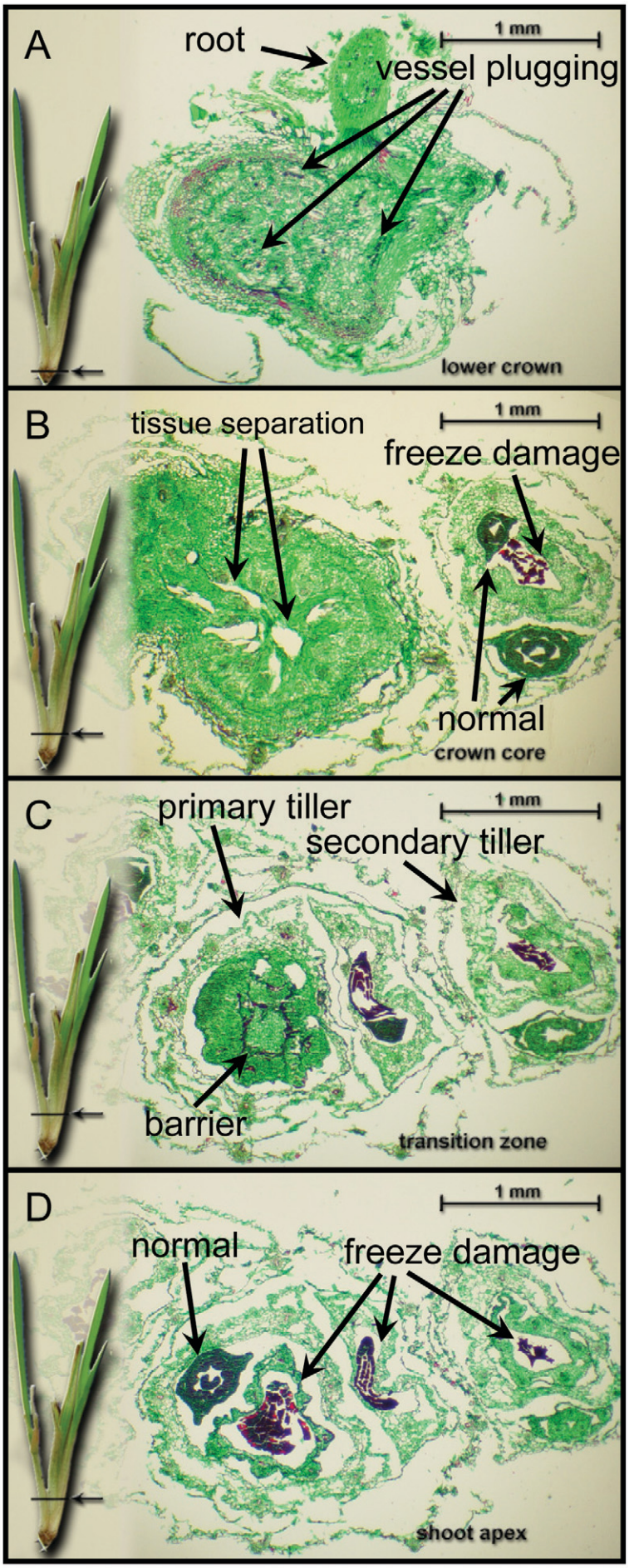
Intracellular

The only prominent intracellular structure visible under bright-field microscopy in oat crowns is the nucleus. Safranin stains chromatin a deep red color making the internal structure of the nucleus visible under high magnification (630X) (Fig. 2). The same apparently random distribution of chromatin throughout the nucleus was found in mesophyll cells in both bench and microwave processed tissue. Size and shape of nuclei as well as cell color was a function of where the cell was located in the crown and not a processing-induced difference.

Freeze Damage

Using this protocol in conjunction with software to align photographs of individual sections and produce a sequential animation, we identified four zones within the crown that differ in response to freezing (Fig. 3A to 3D and accompanying Supplementary Data Video, Fig. 3E). The video (3E) is a continuous sequential view of approximately 4 mm from the bottom of the plant to the shoot apex. While the zones of freezing damage overlap to a certain degree, when a large number of plants was observed, a general pattern of response to freezing was noted in oat crowns that were frozen at –10°C. (i) The bottom of the crown, just above the root-shoot junction, called the "lower crown" (Fig. 3A), had more vessel plugging than any other region. Vessel plugging was seen as xylem vessels that appeared closed and were stained dark-red throughout. This apparent plugging was never seen in unfrozen controls (Fig. 4). Vessel plugging in plants recovering from freezing was first described in oats (Livingston et al., 2005) and was recently proposed to be a result of the production of phenolic compounds because of its auto-fluorescent properties (Livingston and Tallury, 2008). The auto-fluorescence was similar to a stress response of phenol production often seen in plants exposed to disease (Hutzler et al., 1998), but additional experiments will be necessary to confirm that the vessel plugging that we have observed is from phenolic compounds. (ii) Tissue separation was more prevalent within the central part of the crown, called the "crown core" (Fig. 3B) than in other parts of the crown. Olien (1981) described this kind of damage in barley and attributed it to the growth of large ice crystals during freezing. (iii) Just above the crown core but below the shoot apex is the transition zone (Fig. 3C). A prominent feature in this region was an apparent semi-circular "barrier" that stained in a similar manner to the plugged vessels in the lower crown. In most plants, cells outside this barrier had no visible damage as judged by intact cell walls and normal (non-pycnotic) nuclei. This barrier was

Figure 3. A-D Cross-sections of four regions of an oat crown, progressing from the bottom (A) to the top (D), showing differences in freezing injury. After freezing at –10°C, the plant recovered for 10 d before fixation. Images A to D are freeze-frames taken from the video in 3E. The arrow on the left shows the position in the crown of the frozen plant where the cross-section was taken. A) The lower crown region. Arrows point to dark red spots that reflect xylem vessels plugged with putative phenolic compounds. B) The crown-core of the plant where tissue separation becomes the prominent form of freezing injury. Tissue separation shown here is in the primary tiller only. Arrows designate freeze-damaged and normal apices of the secondary tiller. C) The vascular transition zone in which a dark-staining region that appears to be a circular barrier is the major indication of injury. Arrows designate the primary and secondary tillers. D) The shoot apex of the primary tiller and two other apices showing what appears to be complete deterioration within those apices. However, note the adjacent apex that appears normal. Meristems giving rise to this apex possibly escaped from or were able to withstand freezing and produced a new shoot apex during recovery from freezing. E) (See Supplemental Data Video) Forty-five s video compiled from 280 serial sections of the crown. Successive frames in the video show the interior of the crown from the root-shoot junction at the bottom of the crown to the top of the shoot apex (approximately the bottom 4 mm of the stem). Arrows are used to indicate each of the four zones within the primary tiller only. Individual sections were aligned in the primary tiller region which meant that secondary and tertiary tillers shifted somewhat randomly during slide preparation once they became separated from the primary tiller. The arrow to the right of the plant in the lower left moves gradually upward showing the location in the crown from where the section was taken.



also first described in oats (Livingston et al., 2005). (iv) The fourth zone at the top of the crown consists of the shoot apex or shoot apical meristem (Fig, 3D) with attending leaf initials as well as the base of more mature leaves. Freeze damage to this region resulted in apparent complete deterioration of cell structure within a particular shoot apex. However not all apices were damaged (See arrows in Fig, 3D for examples of undamaged leaf initials in some shoot apices). Presumably these were meristematic regions that escaped or withstood lethal freezing and during recovery after freezing were able to generate new leaf tissue.

Portability of the Protocol

To see if the protocol could be used on other cereal crops, cold-acclimated crowns of wheat, barley, and rye were fixed, dehydrated, and embedded in paraffin as described in Table 1. Sections from the other three species were identical in quality to those of oats (Fig, 4). Since all grasses have crowns that generate new growth in the spring or after grazing, we expect that this protocol will be useful for other grass species as well.

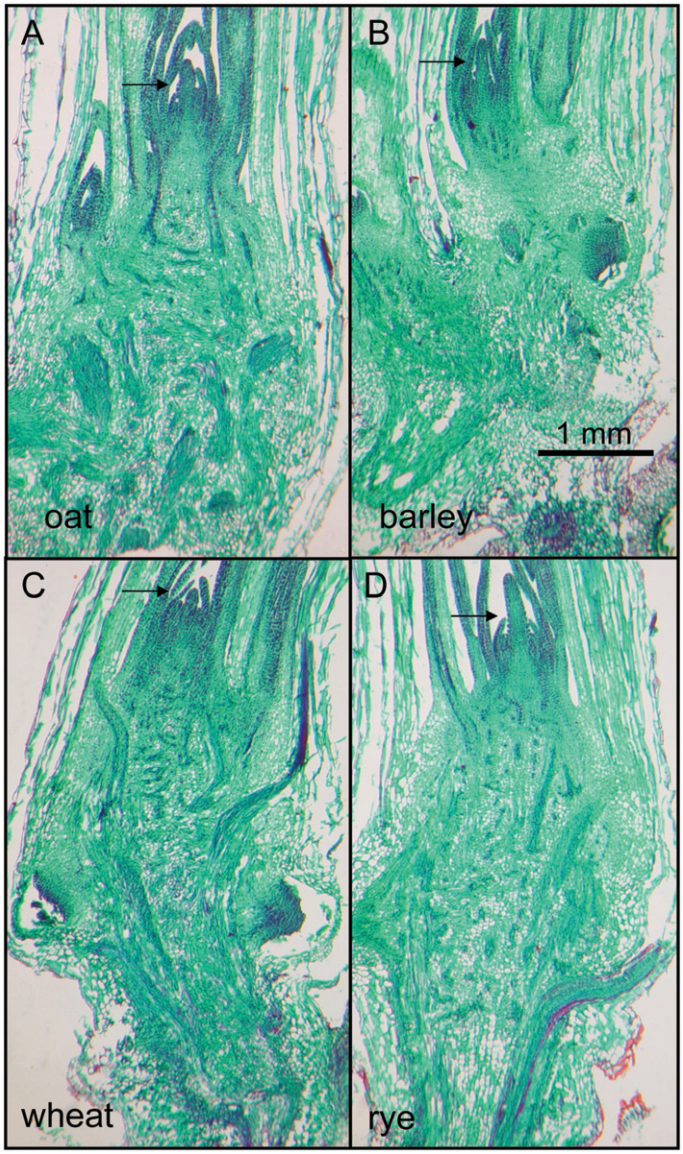


Figure 4. Triple-stained, longitudinal sections (15 μm thick) of unfrozen oat (A), barley (B), wheat (C), and rye (D) crowns showing the shoot apex (arrows) of one tiller. Tissue was fixed, dehydrated and embedded in paraffin in 1 d using the protocol in Table 1. The magnification bar applies to all the micrographs.

CONCLUSION

Drawing valid conclusions from observations of internal damage caused by freezing requires processing and examining many samples. For example, a histological analysis of freezing damage in a recombinant inbred line (RIL) population would involve observation of more than 100 lines, each with three to five plants. DNA markers in a freezing tolerant × non-freezing tolerant oat RIL population were correlated with the freezing survival of individual lines (Santos et al., 2006). To determine how survival of whole plants may be related to survival of tissue within the crown that may be linked to genetic markers will require analysis of many plants. Using the protocol described here will allow rapid processing of plants for histological observations and make these kinds of experiments considerably easier in the future.

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