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TECHNICAL NOTES

Visualising the effect of freezing on the vascular system of wheat in three dimensions by in-block imaging of dye-infiltrated plants

David Livingston[1,2] | **Tan Tuong**[1] | **Ripley Tisdale**[1] | **Rich Zobel**[2]

[1]United States Department of Agriculture, Agricultural Research Service, Raleigh, North Carolina, USA

[2]Department of Crop and Soil Sciences, North Carolina State University, Raleigh, North Carolina, USA

Correspondence
United States Department of Agriculture, Agricultural Research Service and North Carolina State University, 840 Method Rd, Unit 3 Raleigh, NC, USA. 27606.
Email: dpl@ncsu.edu

Abstract

Infrared thermography has shown after roots of grasses freeze, ice spreads into the crown and then acropetally into leaves initially through vascular bundles. Leaves freeze singly with the oldest leaves freezing first and the youngest freezing later. Visualising the vascular system in its native 3-dimensional state will help in the understanding of this freezing process. A 2 cm section of the crown that had been infiltrated with aniline blue was embedded in paraffin and sectioned with a microtome. A photograph of the surface of the tissue in the paraffin block was taken after the microtome blade removed each 20 μm section. Two hundred to 300 images were imported into Adobe After Effects and a 3D volume of the region infiltrated by aniline blue dye was constructed. The reconstruction revealed that roots fed into what is functionally a region inside the crown that could act as a reservoir from which all the leaves are able to draw water. When a single root was fed dye solution, the entire region filled with dye and the vascular bundles of every leaf took up the dye; this indicated that the vascular system of roots was not paired with individual leaves. Fluorescence microscopy suggested the edge of the reservoir might be composed of phenolic compounds. When plants were frozen, the edges of the reservoir became leaky and dye solution spread into the mesophyll outside the reservoir. The significance of this change with regard to freezing tolerance is not known at this time.

Thermal cameras that allow visualisation of water freezing in plants have shown that in crops like wheat, oats and barley, ice forms first at the bottom of the plant and then moves upwards into leaves through water conducting channels. Leaves freeze one at a time with the oldest leaves freezing first and then younger ones further up the stem freeze later. To better understand why plants freeze like this, we reconstructed a 3-dimensional view of the water conducting channels. After placing the roots of a wheat plant in a blue dye and allowing it to pull the dye

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upwards into leaves, we took a part of the stem just above the roots and embedded it in paraffin. We used a microtome to slice a thin layer of the paraffin containing the plant and then photographed the surface after each layer was removed. After taking about 300 images, we used Adobe After Effects software to re-construct the plant with the water conducting system in three dimensions. The 3D reconstruction showed that roots fed into a roughly spherical area at the bottom of the stem that could act as a kind of tank or reservoir from which the leaves pull up water. When we put just one root in dye, the entire reservoir filled up and the water conducting channels in every leaf took up the dye. This indicates that the water channels in roots were not directly connected to specific leaves as we had thought. When plants were frozen, the dye leaked out of the reservoir and spread into cells outside. Research is continuing to understand the significance of this change during freezing. It is possible that information about this effect can be used to help breeders develop more winter-hardy crop plants.

KEYWORDS
3D reconstruction, Adobe After Effects, crown, grasses, leaves, microtome, midrib, reservoir, roots

1 | INTRODUCTION

Freezing injury is considered to be one of, if not *the* most limiting factor to the growth of crops worldwide.[1] Improving the ability of plants to withstand freezing conditions has been a well-documented effort for over a century.[2] To that end, understanding how freezing is initiated in plants and into which tissues ice progresses is an important aspect of improving winter hardiness.

Infrared (IR) thermography is a valuable technique for observing ice formation and propagation in plants and it has been used for that purpose in numerous species.[3,4,5,6] When wheat was monitored with IR thermography during a freezing event, under natural as well as controlled conditions,[6] it was demonstrated that freezing occurred in three stages. The first stage (lasting seconds) was a rapid freeze event that progressed from roots into the crown and acropetally through the vascular system of the leaves. After stage 1 freezing was completed and the tissue came to equilibrium, stage 2 freezing (lasting minutes) began and eventually encompassed the entire leaf. This stage of freezing appeared to begin in multiple regions of the leaf simultaneously and expanded stochastically throughout the leaf. Stages 1 and 2 never killed the plants if they were thawed after stage 2.[6]) Stage 3 freezing (lasting hours) was not visually apparent in IR as were stages 1 and 2. It was proposed that stage 3 freezing was the result of freeze-dehydration and/or adhesion[6]) and when taken to a low enough temperature, stage 3 freezing killed the plant without any visible exotherm.

To better understand freezing in wheat we wanted to visualise regions within plants where freezing was initiated. Previous studies confirmed that freezing began in the vascular system[6] and that the vascular system of winter cereals is especially complex where it transitions from roots into leaves.[7,8,9] We reasoned that a 3-dimensional tissue reconstruction would simplify this complex region and provide insight that would contribute to our understanding of freezing tolerance. In this paper, we describe a method for building a 3D volume of plants that simplifies a previously described 3D reconstruction technique.[10,11] A variation of block-face imaging[12] with several important differences allowed us to reconstruct the vascular system of dye-infiltrated winter cereals before and after freezing.

2 | MATERIALS AND METHODS

2.1 | Plant growth

Wheat seeds were sown into 1.2 cm × 12 cm cone-tainers in Farfard 4P soil mix (Sungro Horticulture, Agawam, MA) and grown in a growth chamber at 13°C. After 4 weeks, plants were transferred to a chamber at 3°C for cold acclimation (CA). Lighting was provided by LED's at 3500K for 12 h in both chambers at 500 μmol / m^2s^2. To test the effect of freezing on dye infiltration, CA plants were frozen to –12°C at 2°C per h and held for 3 h. Three days after thawing plants were subjected to the aniline blue treatment described below.

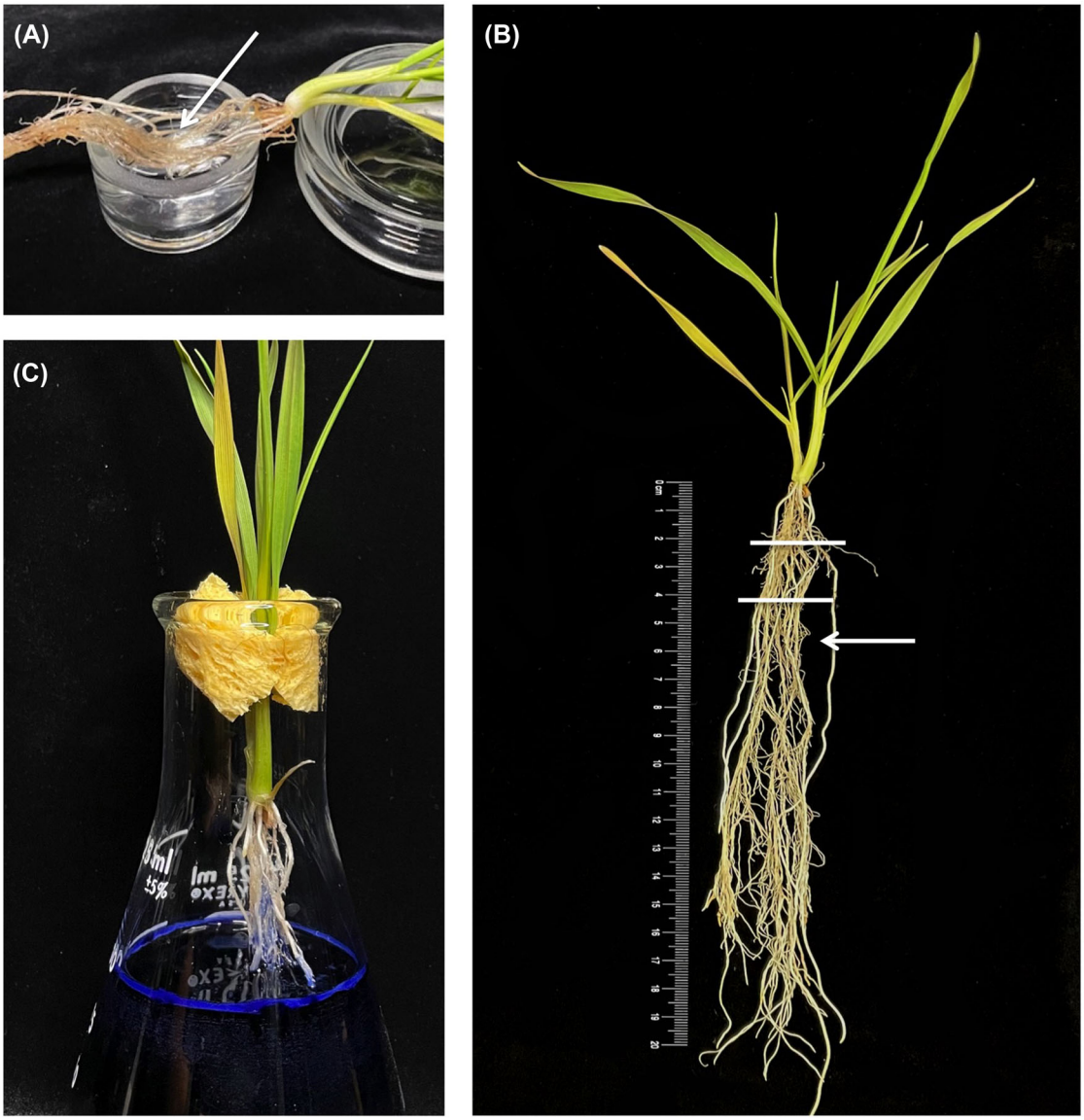

FIGURE 1 Preparation of a wheat plant for aniline blue dye infiltration. (A) A portion of the root below the crown that is submerged in mineral oil (arrow) to prevent dye from infiltrating the apoplast of crown tissue and interfering with the 3D reconstruction. (B) Complete wheat plant with adjacent ruler. The 2 white lines across the roots are the region that was submerged in mineral oil. The arrow shows where the roots were cut before placing in dye solution. (C) Wheat plant with the cut end of the roots submerged in aniline blue dye solution

2.2 | Aniline blue uptake

After 3 weeks in CA plants were removed from tubes and soil was gently washed from about 5–6 cm of roots below the crown. A 3 cm length of the cleaned roots was submerged in mineral oil for 15 min in a shallow dish that minimised bending of roots (Figure 1A). Without the mineral oil treatment, aniline blue was radially transported through the apoplast and cell-to-cell pathways from the root endodermis and it infiltrated the apoplast of the outer leaves of the crown. This made it impossible to create a clean 3D reconstruction from the images of the surface of the block.

Roots were cut below the area submerged in mineral oil (Figure 1B) and the plant was quickly suspended above a beaker containing 1% aniline blue dissolved in pH 7 phosphate buffer with 5% acetic acid (Figure 1C). One centimetre of the cut surface of the roots was submerged in the dye solution; suspended plants were then placed under fluorescent lighting at room temperature for 4 h to encourage photosynthesis during dye infiltration. Interestingly, if uncut roots were submerged in dye, the plant did not pull dye into roots.

After rinsing excess dye solution from roots, all roots about 2 mm from the base of the crown were removed with a razor blade. A 1 cm long segment that included

the crown and leaf bases was partially fixed in a solution of 0.1% Safranin, 10% glycerol, 10% acetic acid, and 80% methanol for 16–18 h at room temperature. Fixing the tissue was necessary to prevent rapid expansion of leaves as they were being sectioned; this expansion interfered with the sequential aligning of the tissue and distorted the 3D reconstruction. While methanol in the fixative likely had a dehydrating effect, complete dehydration and paraffin penetration of cells were avoided in order to minimise tissue and cell distortion. Safranin was added to the fixative to help provide contrast to images and allow differentiation of vascular bundles from other tissue in Adobe After Effects. The brief fixing treatment did not appear to affect the ability of the various tissues to keep dye solution from bleeding into surrounding tissues.

2.3 | Embedding

The fixed crowns were placed in a small beaker of melted paraffin and stearate (50:50 w:w) and then moved quickly under a vacuum bell before the paraffin could solidify. Stearate was added to paraffin to increase the opacity of paraffin which reduced colour reflections within the paraffin; colour reflections interfered with image processing in Adobe After Effects. Vacuum was applied 3 times down to approximately 100 mbar to fill empty voids within the plant with paraffin/stearate (P/S). The crown was placed, roots down, in a stainless steel mould, filled with P/S and a plastic embedding ring was added. After completely filling the mould and ring with P/S, the embedded tissue was placed under vacuum again to remove air bubbles adhering to the crown.

2.4 | Sectioning and image capture

A Nikon SMZ-745t dissecting microscope (Nikon instruments Inc, Melville, NY) and DS-Fi3 camera with a Nii-LED light source and fibre optic ring-light (all Nikon products) were placed in front of the P/S block being sectioned (Figure 2). Blocks were sectioned with a Leica RM2255 rotary microtome (Leica Microsystems, Buffalo Grove IL) at 20 μm. After each section was cut, the surface of the exposed tissue, within the block, was photographed at a magnification of 20× and saved as a JPEG2000 image using Nikon-elements Basic Research software. During sectioning in manual mode, the specimen holder was locked at the exact same position before a picture of the block surface was taken. After each manual rotation of the cutting blade, the block was automatically advanced (towards the blade) by the microtome. The precise stopping and 20 μm advancement by the microtome insured that the surface of the block remained focused and aligned throughout sectioning. About 300 images were captured in approximately 45 min.

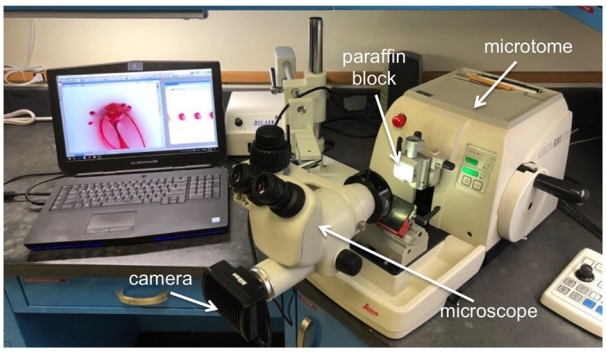

FIGURE 2 Arrangement of the components used for in-block imaging. The microscope is mounted on a stand, which allows the lens to be aimed at the surface of the paraffin block. The cutting blade of the microtome is under the white paraffin block and cuts a variably thick section (in this case 20 μm) with an upward motion. The microscope remains in a fixed position and after cutting each section from the block the microtome automatically shifts the block forward. This means the surface of the block, containing internal plant tissues does not need to be re-focused; so unless the microscope or microtome is moved, every section will be precisely aligned. Individual images were captured by the camera mounted on the microscope and saved to Nikon software on a laptop computer. All images were transferred to Adobe After Effects where they were processed for 3D reconstruction (see Section 2)

To determine fluorescence in fresh, unfixed tissue, a Nikon Eclipse 50i microscope was used to photograph hand cut thin sections. Autofluorescence was photographed at a shutter speed of 1 s using a Nikon Elipse 50i fluorescence microscope at 200× using a Nikon GFP/F Long Pass filter set with an excitation wavelength of 450–490 nm and barrier at 500 nm.

2.5 | Image processing

JPEG2000 images for 3D reconstruction were imported into Adobe After Effects as a sequence and processed as described[10] except that images did not need to be aligned.

A 3D volume of aniline blue-infiltrated tissues was combined with a 3D volume of the crown and transparency of the crown was adjusted to visualise the dye-infiltrated vascular system within the crown in three dimensions (Supplemental Video 1).

Identical plant and image processing was used for frozen plants.

Percentages of dye were calculated by determining the number of pixels that were blue (from aniline blue) and dividing by the total number of pixels in each image.

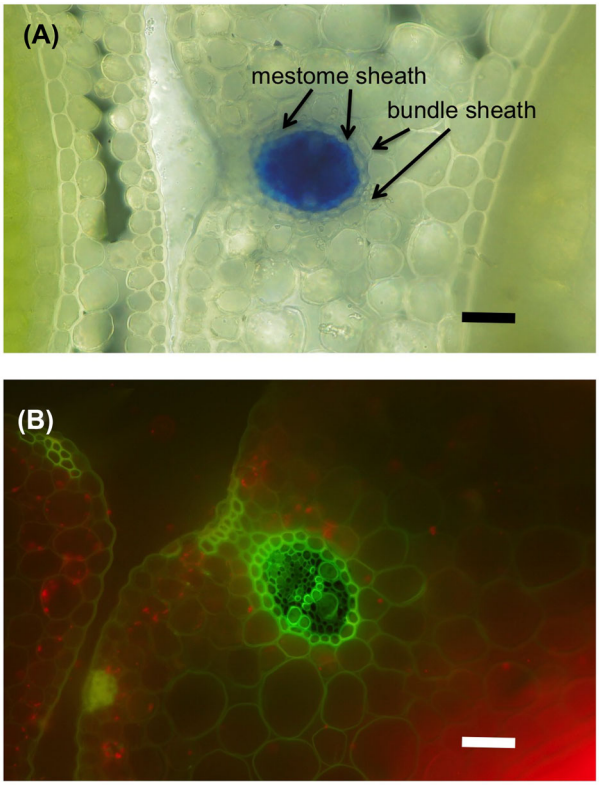

FIGURE 3 (A) Fresh cross section of a vascular bundle from a leaf of a wheat plant whose roots were cut and placed in an aniline blue dye solution (see Section 2). (B) Fluorescent image of a similar vascular bundle from a leaf. Note the prominent fluorescence of the mestome sheath, suggesting the presence of suberin. The bar represents 10 μm

The total consisted of the vascular system (blue) plus the remainder of the tissue (pink/red from the Safranin stain). Sigma Scan Pro (Systat Software Inc, San Jose CA, USA) was used to determine the number of pixels of a specific colour in each image.

3 | RESULTS AND DISCUSSION

3.1 | Dye movement within the plant

One reason in-block imaging of the vascular system was successful is that during infiltration, dye (and by implication water –[7,13,14] is initially restricted to vascular bundles. After cutting and immersing roots, dye solution was pulled acropetally through vascular bundles into the base of the crown and finally into leaves. Vascular bundles of plants typically contain meta- and protoxylem vessels plus phloem cells.[13] The bundles in grass leaves are surrounded by an inner layer of cells called the mestom sheath (Figure 3A) and an outer layer called simply the bundle sheath or parenchyma sheath. The inner surface of the mestome sheath is composed of a thickened wall

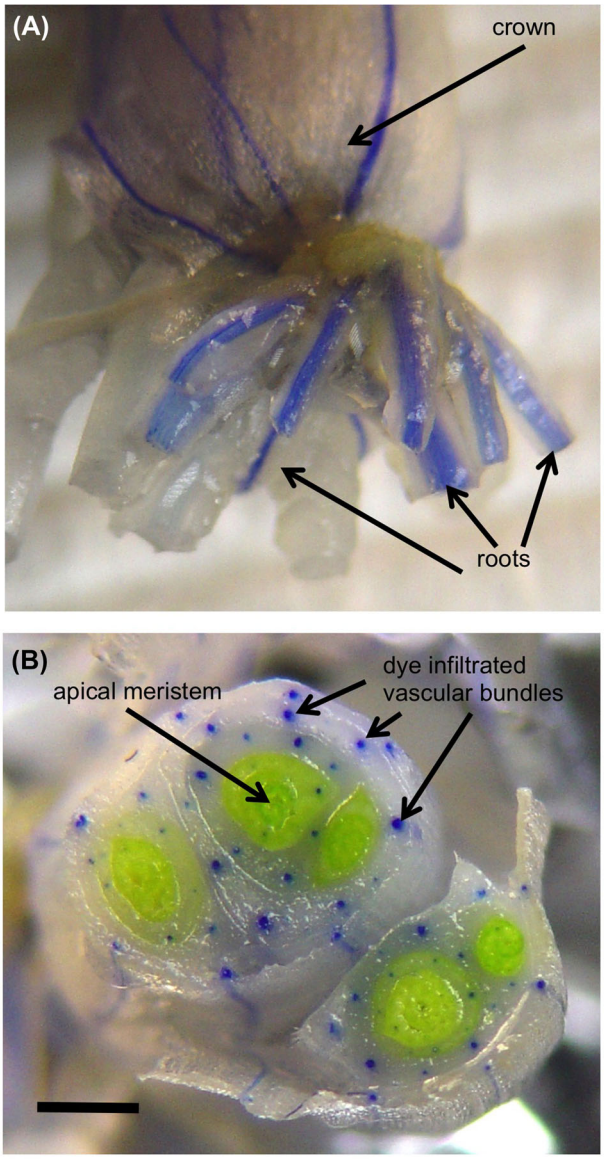

FIGURE 4 (A) Crown and roots of a wheat plant showing aniline blue that the plant pulled through the vascular system. (B) Exposed section of the crown cut in cross section approximately 2 cm above the root-shoot junction. The bar represents 1 mm

that has been called 'suberin lamella'[14] which exhibits prominent autofluorescence (Figure 3B); it is this layer that reportedly restricts lateral flow of water out of the vascular bundle.[15] The dye in Figure 3 was clearly contained within the vascular bundle and did not pass through the mestome sheath. In fact, the dye appeared to be constrained at the edge of the inner wall of the mestome sheath (Figure 3A).

While dye appears restricted to vascular bundles over the time period used for infiltrating in this study, water is clearly transported throughout every cell in the plant. Using dye solutions[7] demonstrated that the bulk of water moves primarily in the lateral veins of leaves to leaf tips and that transverse veins provide water to

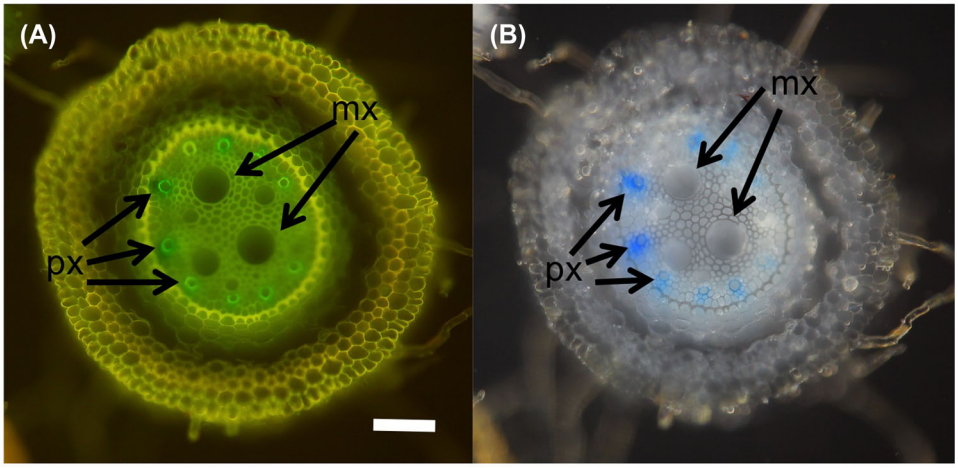

FIGURE 5 Hand cut section of a fresh, cold-acclimated wheat root. (A) Under fluorescence. (B) The same section but under visible light. Note that dye is present only in several proto xylem vessels. Dye did not present in metaxylem of roots. mx = metaxylem, mx = protoxylem. The bar represents 100 μm

mesophyll cells. After infiltrating plants for longer than 4 h, we also observed dye in transverse veins of leaves (not shown).

3.2 | Vascular anatomy of the plant in three dimensions

3.2.1 | Roots

Because movement of dye solution from the epidermis and along root cell layers was blocked by a coating of mineral oil (see Section 2), the dye was taken up from the cut ends of roots (Figure 4A) into vascular bundles and into bundles in leaves (Figure 4B). Fluorescent imaging suggested that suberin lamellae acted as a barrier to restrict the radial flow of dye solution from xylem vessels (Figure 5A). Although non-suberised passage cells occurring in the endodermis near protoxylem vessels provide lower resistance for water movement,[16] these cells did not frequently present dye in the mature root regions. Interestingly, the dye solution was only present in some protoxylem vessels whereas the metaxylem always remained clear (Figure 5B). It is possible that embolisms were created in metaxylem vessels when roots were excised, preventing the uptake of dye solution.[17] demonstrated that embolisms are introduced more frequently in metaxylem than in protoxylem in cut maize leaves, and comparability between leaf and root xylem is assumed.

In addition to differential dye uptake by vessels within roots, some roots did not take up dye at all (Figure 6). Plant root taxonomy[18] projects four developmental-genetic classes of root in the wheat crown: elongated radicle, basal (seminal), coleoptile and shoot-borne roots. Although these different classes of root are developmentally different, few functional differences have been demonstrated.[18] Differential uptake (Figure 6) of dye solution by the radicle and basal roots, compared to the coleoptile and shoot-borne roots, might be one functional difference.

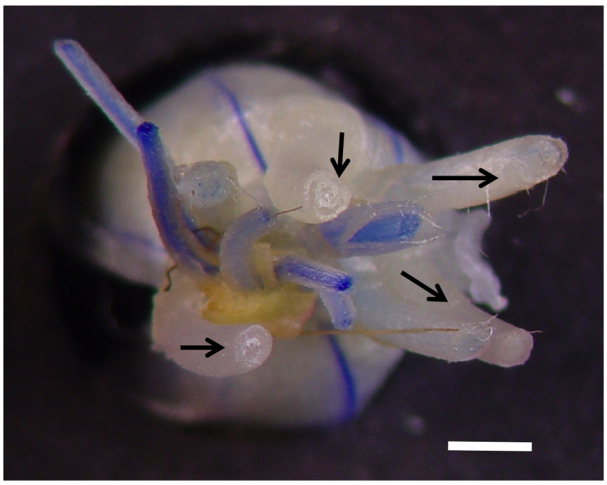

FIGURE 6 Cut end of roots after dye infiltration. Note that dye is present only in small seminal roots. Arrows point to larger shoot-born roots that do not present dye. Bar represents 1 mm

Another possibility for differential uptake of dye is that young shoot-borne root zones (i.e. the rapidly elongating and maturing portions of shoot-borne roots) retain cell wall membranes in their xylem, thus preventing the flow of water, through these roots, to the shoot.[19,20,21] In Figure 6, the acropetal initiation of roots as the seedling develops can be seen, with larger, later-developed shoot-borne roots more apical than the thinner seminal roots (radicle plus basal roots). The larger (shoot-borne) roots were much shorter than the thinner roots and could have still been maturing, such that the protoxylem was not yet permeable to water. These two explanations are not mutually exclusive and further experimentation, outside the scope of this

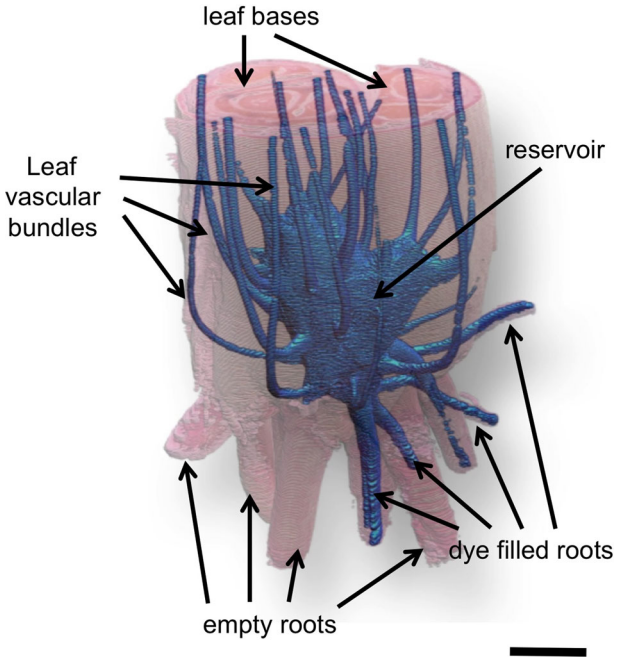

FIGURE 7 3D reconstruction of the dye-infiltrated vascular system inside a wheat crown. This image is a still frame from supplemental video 1. The semi-transparent pink colour of the leaf tissue is from safranin in the fixative solution (see Section 2). The bar represents 1 mm

study, should be able to determine if either one or the other, or both are valid.

3.2.2 | Crown

Vessels in roots did not appear to lead through the crown to individual leaves but to a dye-filled volume of cells that in three dimensions resembled a roughly sphere-shaped region to which vascular bundles, leading to leaves, were attached (Figure 7 and see supplemental video 1 for a 3D perspective of the region). A boundary layer, or sheath, at the edge of the volume appeared to prevent dye solution from penetrating into mesophyll and chlorenchyma tissue outside the volume (Figures 8 and 9) creating an area that resembled a reservoir (Figure 7).

Autofluorescence was also observed in fresh-sectioned tissue between the sheath bordering the reservoir (Figures 8 and 9) as well as the mestome sheath in vascular bundles of leaves (Figure 3B). It is possible that a layer of suberised lamella, which is adjacent to the mestome sheath in leaves,[13,14,15] may also prevent leakage of dye solution through the 'walls' of the reservoir.[5] review several ice barriers within plants; some of these could also limit the movement of liquid water and in this case dye solution. This sheath occupied a similar position within the crown as a barrier described in crowns of oat (*Avena sativa*) plants recovering from freezing.[22] In that study, it

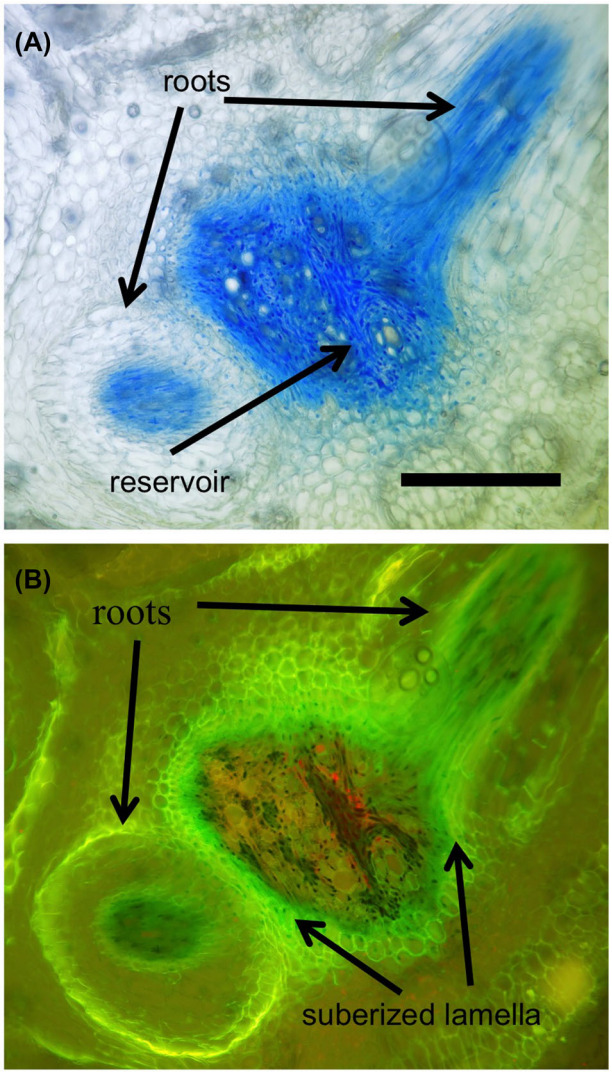

FIGURE 8 Fresh hand section from the base of the aniline blue filled reservoir shown in Figure 6. (A) Note one root entering the reservoir and another root approaching the reservoir. (B) Fluorescent image of 'A' showing the putative suberised barrier of the reservoir. The bar represents 100 μm

was suggested that the barrier was composed of phenolic compounds due to its autofluorescence and staining with Safranin.[22]

As a reservoir, this volume of cells could provide redundancy for the plant with regard to water supply. To confirm this, a single root was cut and submerged into a dye solution with safranin; all other roots were submerged in water. Despite the plant being fed with dye by only a single root (Figure 10) the entire reservoir filled with dye and within 4 h nearly every leaf had taken up dye to their tips. This means that if a root was damaged and its ability to transport water was compromised, any particular leaf would not be negatively affected since the root simply fed the entire reservoir rather than a single leaf. Any one (or more) of the attached roots could keep the reservoir filled, which would

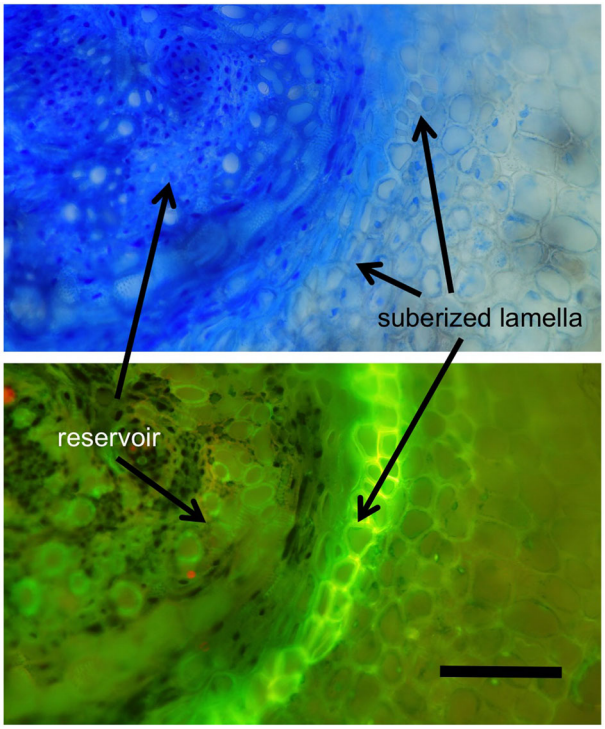

FIGURE 9 Fresh hand section of a dye-infiltrated wheat crown in the middle of the reservoir. (A) Note slight leakage of aniline blue outside the reservoir. (B) Fluorescent image of 'A' showing the putative suberised barrier. The bar represents 100 μm

FIGURE 10 Cold acclimated wheat plant with a single root (arrow) placed in safranin dye solution. Note that vascular bundles from all visible leaves are filled with dye solution, despite only one root pulling up dye. The asterisk points to one vessel bundle of an outer leaf containing safranin. A cross section of this plant (not shown) indicated that the reservoir was also filled with dye

provide a source of water to all the leaves. The model of water distribution in wheat described by Altus and Canny[7] does not include the effect of water movement into the crown and it seems likely that the presence of a putative distribution centre or reservoir within the crown would impact how the flow (of water) in wheat leaves is understood.

Individual xylem vessels inside the reservoir could not be differentiated with this technique. While conventional triple-staining revealed a seemingly stochastic but interconnected arrangement of vessels within the crown[23] when the plant was infused with dye, the entire reservoir filled with aniline blue solution suggesting xylem vessels within the region were leaky and thus prevented the tracing of individual vessels. This might explain the susceptibility of the reservoir to freezing damage if it is a region of high water content due to the leaky nature of vessels within the region.

3.2.3 | Leaves

Each leaf was attached to the crown at a different position, which naturally affected where vascular bundles within the leaves were joined to the reservoir. Vascular bundles in leaves 1 and 2 (the 2 oldest leaves, i.e. the first to emerge) were attached at the lowest point (closest to the roots) and were oriented slightly downwards before reversing direction and pointing acropetally (Figure 7 and Supplemental video 1). The attachment of leaves 3 and 4 were higher on the side of the reservoir and their vascular bundles initially protruded horizontally before curving acropetally. Vascular bundles leading to leaves 5 and 6 extended vertically out of the top of the reservoir.

The point of attachment of the leaves on the reservoir apparently had an effect on when dye made its appearance in a particular leaf. For example, dye was first detected in leaf 1, the first leaf to emerge (oldest leaf) which was also the lowest leaf (the most basipetal) on the reservoir. In fact, dye quickly filled leaves in what appeared to be an age dependent manner with the youngest leaves (leaves 5 and 6) at the top of the reservoir taking up little or no dye solution (not shown).

Determined that when wheat plants were frozen, leaf 1 (oldest leaf) was the first leaf to freeze and leaf 6 the last.[6] What appeared to be age-dependent infiltration of

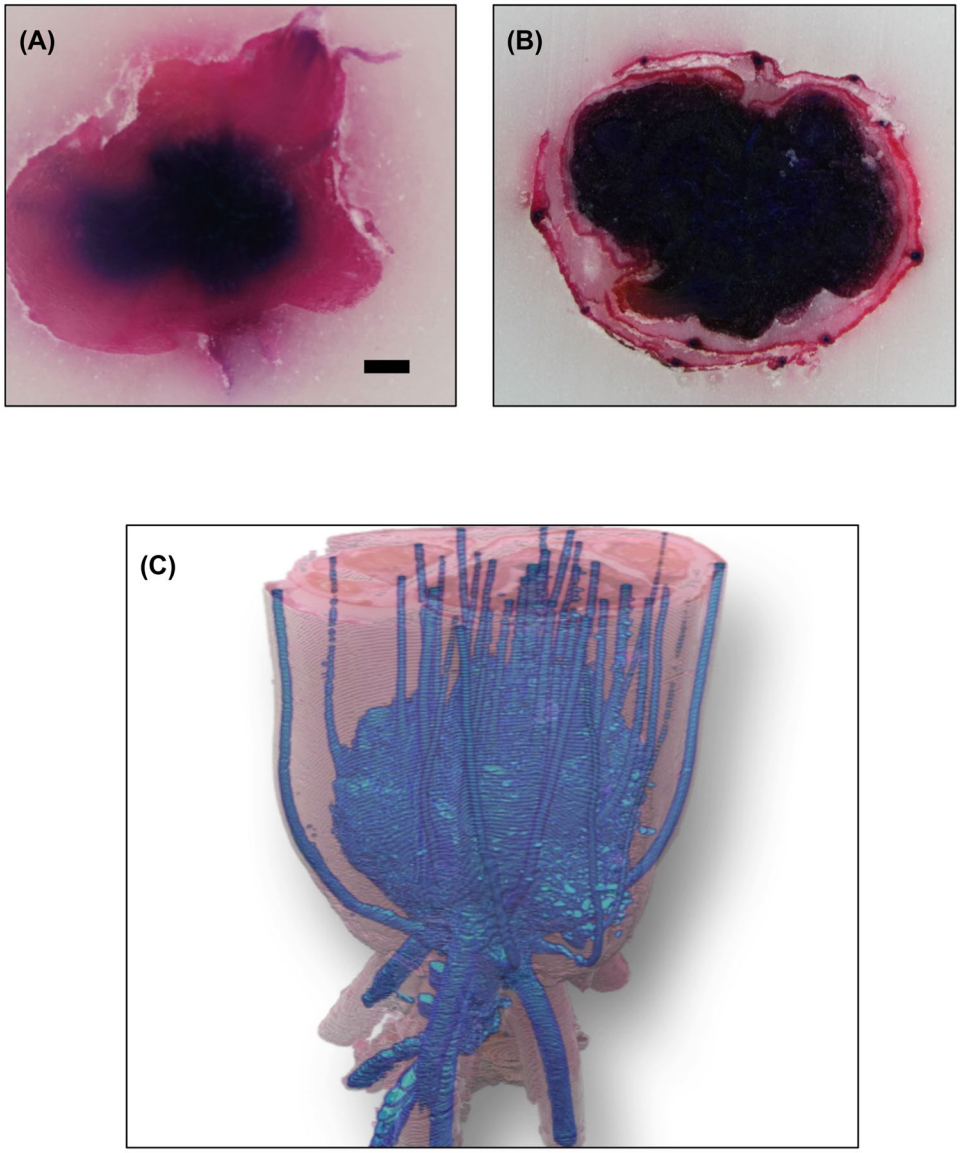

FIGURE 11 Aniline blue infiltrated wheat crown. (A) Cross section from a cold acclimated (non-frozen) plant. (B) Cross section through the centre of the reservoir of a plant that was frozen at –12°C and allowed to recover for 3 days. Note extensive leakage of dye solution outside the reservoir into the mesophyll as compared to the edge of the reservoir in 'A'. (C) 3D reconstruction of a dye infiltrated wheat plant that had been frozen. Note the expanded reservoir compared to the reconstruction of an unfrozen plant in Figure 6. Also note that despite the expanded reservoir, the vascular bundles above and to the side of the reservoir did not appear to be altered by the freeze treatment. The bar in 'A' is 1 mm and also applies to 'B' and 'C'

dye solution could confirm the suggestion[24] that leaf position in the crown may at least partially explain age dependent freezing in wheat. Ice formation was initiated in roots and progressed acropetally from the crown into leaves with the youngest leaves attached to the top of the reservoir freezing last.[24]

In leaves, dye solution was not confined to xylem vessels but as stated above, was contained within the mestome sheath of the vascular bundle (Figure 3). The mestome sheath in roots as well as leaves apparently prevented movement of dye solution outside the vascular bundle. Vascular bundles in leaves 4, 5, and 6 appeared to lack a suberised lamella layer (not shown) but this did not seem to have an effect on leakage; the dye solution was contained within the vascular bundles in leaves 4, 5 and 6 as well as it was in leaves 1, 2 and 3 (not shown).

3.3 | Effect of freezing on the vascular system

For a variety of reasons, different tissues within the same plant are better able to withstand freezing stress than others.[9,23] In-block imaging described here to visualise

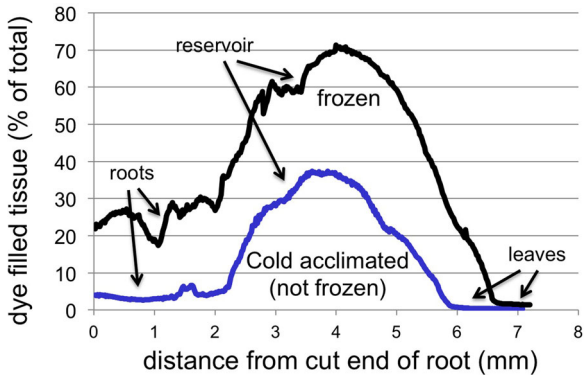

FIGURE 12 The percentage of dye within each 20 μm section from the cut end of the root (at zero on the *x*-axis) to the top of the crown. Note the considerably larger percentage of dye in the area of the graph designated 'reservoir' in the frozen crown as compared to the cold acclimated crown. While the roots also are showing more % dye in the frozen crown, this may be due to differences between the number of roots taking up dye rather than an effect of freezing

the vascular system of wheat plants that had been frozen revealed that the freeze event changed the ability of the reservoir to contain dye solution (compare Figure 11A and B and compare to Supplemental video 1 to Supplemental video 2). The reservoir was noticeably enlarged 3 days after freezing (compare Figure 7 to Figure 11C and compare Supplemental video 1 to Supplemental video 2), suggesting that cells surrounding the reservoir were damaged or altered in some way, which allowed dye to move into the mesophyll outside the reservoir. The percentage of dye that was contained in the crown (Figure 12) was higher ($p = 0.17$) in frozen plants, with 32% of the crown containing dye in frozen plants and 13% in non-frozen. Plant-to-plant variability in percentage of dye was very high in these examples (hence the poor level of statistical significance) because the overall size of the reservoir differed considerably between plants. While roots of frozen plants appeared to contain a higher percentage of dye (Figure 12) the difference from the CA plants may simply have been a result of more individual roots taking up dye, rather than increased leakage of dye outside vascular bundles.

There was no difference in leakage of dye from the reservoir whether plants were frozen to –3°C (not shown) or –12°C. This suggests that ice formation in the crown, between –2°C and –4°C, was the event that caused a change in the ability of cells to contain dye, rather than prolonged freeze dehydration or adhesion when the temperature was reduced to –12°C.

Reported that the mestome sheath of rye (*Secale cereale*) became thicker in CA plants due to increased deposition of suberin.[25] The difference in freeze response between vascular bundles in leaves and the reservoir indicates that the composition of the lamellar layer is not the same for both barriers. The reservoir barrier may be composed primarily of phenolic compounds as was suggested for a similar region in oats.[22] But more research would be necessary to confirm this as well as to determine if freezing affects suberin differently than it does phenolic compounds.

Three weeks after freezing and thawing, some leakage still occurred into the mesophyll outside the reservoir (not shown) but not to the extent it did 3 days after freezing (Figure 11B). This indicates *some* restoration of the reservoir occurred but not to its pre-frozen condition.

After 2 weeks of recovery from freezing, some of the leaves pulling solution from the reservoir appeared to be disconnected since they were not able to pull dye into their vessels (not shown); these were mostly older leaves with large vessel diameters that quickly died after freezing. It is likely that embolisms were the cause of this apparent disconnection of the vascular system.[26,27] Younger tissues closer to the centre of each tiller with smaller diameter vessels and connected at the top of the reservoir were still able to pull up dye solution and did not die after freezing at –12°C.

4 | CONCLUSION

The 3D reconstruction technique described here is well suited for biological tissues into which dye can be infiltrated prior to processing for sectioning. It is ideally suited to studies where minimal fixation is important such as determining in situ anatomy because it eliminates the need for complete tissue dehydration and infiltration with paraffin. A major advantage over images that are reconstructed after sectioning and staining[10] is that alignment of individual images is not necessary; this avoids the problem of section distortion. A disadvantage is that in vivo staining is required which can be limited by the ability of the dye to uniformly infiltrate tissue of interest prior to embedding. This issue was discussed in detail[12] in a description of block-face imaging. In our case, the vascular system was ideally suited for in-block imaging since dye solution was readily drawn into water conducting vessels.

In this study, a dye-filled volume was identified that appeared to act as a reservoir for the plant. In fact, an in-block image analysis of rye (*Secale cereale*) and two oat (*Avena sativa*) cultivars revealed a similar reservoir-like volume in the centre of the crown (not shown). The presence of a reservoir within the crown will likely influence the understanding of water relations in small grains. When wheat plants were frozen, the integrity of the reservoir was disrupted. How this disruption might affect freezing tolerance is not known at this time.

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SUPPORTING INFORMATION

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