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Ice and air: Visualisation of freeze-thaw embolism and freezing spread in young *L. tulipifera* leaves

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Summary

- Spring freezing is an unforgiving stress for young leaves, often leading to death, with consequences for tree productivity and survival. While both the plant water transport system and living tissues are vulnerable to freezing, we do not know whether damage to one or both of these systems causes death in young leaves exposed to unseasonal freezing.
- Whole saplings of *Liriodendron tulipifera* were exposed to freezing and thawing trajectories designed to mimic spring freezes in nature. We visualised freezing damage to the water transport system (xylem embolism) and living tissues (mesophyll freezing, decline in chlorophyll fluorescence).
- We 1.) provide the first visualisation of freeze-thaw embolism in leaves, 2.) reveal a predictable progression of ice formation within the mesophyll which is strongly influenced by leaf vein architecture, notably the presence or absence of bundle sheath extensions, and 3.) show that freeze-thaw embolism occurs only in the largest vein orders where mean vessel diameter exceeds 30µm.
- With evidence of both freeze-thaw embolism and damage to photosynthetic tissue, we conclude that this dual-mode lethality may be common among other wide-vesseled angiosperm-leaves, potentially playing a role in limiting distributions, and show that bundle sheath extensions may stall or even prevent freezing spread.

Key words: Embolism, Fluorescence, Freezing, Freeze-thaw embolism, Leaves, Optical imaging, Spring-freezing.

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Introduction

Freezing is one of the strongest determinants of plant distributions (Burke, Gusta et al. 1976, Loehle 1998, Sakai and Larcher 2012, Muffler, Beierkuhnlein et al. 2016), owing to the unforgiving and often lethal nature of freezing in plant tissues that are not specifically equipped to avoid, resist or compartmentalise ice crystal formation (Burke, Gusta et al. 1976, Stegner, Buchner et al. 2023). Some trees have adaptations to prevent or tolerate freezing within their tissues year-round, while others, such as those is temperate regions, are adapted only to seasonal winter-freezing (Burke, Gusta et al. 1976, Wisniewski and Fuller 1999, Sakai and Larcher 2012). Both the xylem and living leaf tissue are critical to consider when assessing the effects of freezing, as damage to plant water transport or the sites of photosynthesis jeopardise tree growth and survival (Brodribb, Brodersen et al. 2021).

Within branches and tree-trunks, xylem anatomy is known to influence the likelihood of freezing damage. During freezing, gas bubbles become suspended in the xylem sap as the crystallization process pushes dissolved gasses out of solution. Upon thawing, these bubbles either dissolve back into solution or expand to fill xylem conduits and block water flow depending on the pressure of the surrounding liquid (Hammel 1967, Sperry and Sullivan 1992, Hacke and Sauter 1996). Species with larger diameter xylem conduits have been shown to be more vulnerable to 'freeze-thaw embolism' (Sperry and Sullivan 1992, Feild and Brodribb 2001, Tyree and Zimmermann 2002, Cavender-Bares, Cortes et al. 2005, Sevanto, Holbrook et al. 2012, Li, Luo et al. 2024), with mean conduit diameter shown to decrease with increasing likelihood of freezing conditions (Sperry 1995). There are a number of possible explanations for this. Larger vessels have been shown to embolise earlier than smaller vessels exposed to freezing (Davis, Sperry et al. 1999, Cavender-Bares, Cortes et al. 2005). Additionally, smaller conduits have been shown, in some case, to freeze at higher velocity which lead to smaller air bubbles (Ewers 1985) which are more easily dissolved upon thawing (Sevanto, Holbrook et al. 2012).

Winter deciduousness allows many angiosperm trees (and some gymnosperms) to avoid leaf-level freezing damage, but does not protect spring growth, nor leaves that persist into autumn. In particular, freezes after bud-burst in spring can have severe consequences for deciduous trees (Burke, Gusta et al. 1976, Vitasse, Bottero et al. 2019). While winter-deciduousness allows deciduous trees to avoid winter frost-damage to leaves, spring frosts often kills or damages leaves (Burke, Gusta et al. 1976), which has been shown to cause a strong reduction in tree growth (Vitasse, Bottero et al. 2019).

Leaf senescence is not the only strategy that deciduous trees employ to protect their tissues against freezing damage: dormancy and other 'hardening' processes have evolved to

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prevent or limit the formation of ice in the living tissue of these trees, which can occur inside cells (intracellular) or outside cells (intercellular). Intracellular ice formation invariably leads to cell death by rupture of the cell membranes, caused by internal nucleation of piercing by external ice crystals (Mazur 1969, Burke, Gusta et al. 1976, Steponkus, Dowgert et al. 1983, Guy 1990). Extracellular ice formation can also result in cell death by creating a water potential gradient which draws out cellular water to feed the freezing-front (Guy 1990, Steponkus and Webb 1992), therefore imposing a desiccation stress resembling that caused by drought (Ruelland, Vaultier et al. 2009, Vitra, Lenz et al. 2017, Yang, Gerber et al. 2024). To combat ice formation within or between cells, beyond autumn, meristematic tissues in the buds of winter-deciduous trees enter dormancy when environmental conditions are unsuitable for growth (Lang 1987, Sapkota, Salem et al. 2023). During this period, cell activity within leaf and flower buds slows, with this delicate tissue encapsulated by protective bud-scales (Nilsson 2022).

Spring frost may be lethal in young leaves through at least two different modes that are not mutually exclusive. First, ice formation in or around the living tissues poses a significant threat because of the potential damage to cell walls and membranes leading to electrolyte leakage, damage to organelles and the generation of reactive oxygen species (Wise 1995, Lütz 2010). Second, embolism formation resulting from the freezing and thawing of the xylem sap could lead to complete failure of the leaf vein network thereby cutting off the supply of water to downstream tissues (Brodribb et al. 2021). While the effects of freeze-thaw embolism have been well documented in stems (Sperry, Donnelly et al. 1988, Feild and Brodribb 2001, Ashworth and Pearce 2002, Cobb, Choat et al. 2007, Mayr, Schmid et al. 2014, Robinson, Rennie et al. 2023), this has never been visualised in leaves.

The primary means of tracking the propagation of freezing damage through whole leaves has been with high temporal resolution using multiple forms of thermal imaging (Larcher, Meindl et al. 1991, Gusta, Wisniewski et al. 2004, Hacker and Neuner 2007, Kokin, Pennar et al. 2018, Zhang, Han et al. 2023). Hacker and Neuner (2007) in particular provide key, novel information about ice nucleation and freezing relative to leaf anatomy, showing ice nucleation beginning in the veins and spreading through the mesophyll tracking leaf venation, however this is one of few papers to consider the influence of leaf venation on ice-spread. While thermal imaging with very high temporal resolution (Hacker and Neuner 2007; 25 images per second) and some optical imaging (Kokin, Pennar et al. 2018) have been used to study leaf freezing; longer-term high resolution imaging of leaves during freezing and thawing has not been conducted. Given that ice crystallization is often lethal in living tissues and that leaf cells are also supplied by a vascular system that is vulnerable to freeze-thaw embolism, one of the goals of the present study was to provide a detailed characterisation of

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ice formation in leaf tissue by visualising freezing and thawing in the leaves of intact plants, a process has recently been undertaken in a freeze resistant species (Kane and McAdam 2024) but never in young, likely freeze-intolerant leaves. Using this information, we could assess the probable cause of leaf death (embolism, cell damage or a combination of both) due to spring frost.

Here we visualise the effects of freezing and thawing on both the living cells and the xylem in young leaves in *Liriodendron tulipifera*, a common deciduous tree in North America. To detect possible embolism and track ice crystal formation in leaves we used a combination of time-lapse imaging and chlorophyll fluorescence imaging to non-invasively study young leaves of *L. tulipifera* saplings exposed to freezing conditions, with minimum air-temperatures ranging from -1 to -5 °C. By capturing images of newly expanded leaves at regular intervals throughout freezing and thawing we aimed to elucidate the pattern of freezing spread in these leaves and capture any embolism that may occur using the Optical Vulnerability Method of Brodribb et al. (2017). We then measured chlorophyll fluorescence of leaf tissue before and after freezing as a proxy for tissue damage (Brodribb et al. 2021). To compare the effect of freeze-thaw embolism vs. drought-induced embolism, thereby partially isolating the effect of freezing on the living cells in the leaf, we tracked drought-induced embolism in leaves of the same age.

L. tulipifera has been shown to possess bundle sheath extensions (BSEs), structures that connect the bundle sheath and the epidermis and influence a range of physiological process in plants relating to both photosynthesis and hydraulics (Wylie 1943, Wylie 1952, Pray 1954, Esau 1960 ; Fig S1, Zwieniecki, Brodribb et al. 2007). Bundle sheath extensions compartmentalise the leaf lamina restricting the movement of gases therefore allowing gas exchange to occur independently across the leaf surface, with the leaves, and species possessing these, been termed heterobaric (Neger 1918). Assimilation has been shown to be higher in species with BSEs (Karabourniotis, Bornman et al. 2000), with these structures also shown to influence leaf hydraulics and drought tolerance (Kawai, Miyoshi et al. 2017). In leaves, upon exiting the xylem, water is transferred to the bundle-sheath (Trifiló, Raimondo et al. 2016). By connecting the veins and the epidermis, BSEs have been shown to assist in supplying water from veins to stomata (Zwieniecki, Brodribb et al. 2007) and also reduce the resistance to water flow between the bundle sheath and the epidermis (Buckley, Sack et al. 2011). Highly sclerified BSEs have also been shown to prevent the lateral prorogation of ice in the lamina in *Cinnamomum canphora*, yet this is the only species in which this has been documented (Hacker and Neuner 2007, Barbosa, Chitwood et al. 2019). In *L. tulipifera* BSEs extend to both the upper and lower epidermises in second order and third order veins (Pray 1954; Fig S1), while they extend only to the abaxial epidermis in

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higher order veins (Pray 1954).(Pieruschka, Schurr et al. 2006, Barbosa, Chitwood et al. 2019). It is possible that even the non-sclerified bundle-sheath extensions in *L. tulipifera* could act as a barrier to the ice crystallization front as it progresses across the lamina, which led us to attempt to determine their influence in this species.

Based on the known physical processes that govern ice nucleation and growth in laminar systems that superficially resemble leaves, we expected ice to first form in leaf regions with low solute content, and therefore a higher freezing temperature, such as the midveins containing numerous xylem conduits loaded with xylem sap. Ice would then propagate outward across the lamina as temperature decreases below the freezing point of the surrounding tissues with higher solute content. Given that the BSEs represent a physical barrier that would slow the movement of water toward the ice nucleation front, we expected BSEs to significantly influence the spatial pattern and progression of ice across the lamina, which would be most noticeable in the higher order veins where BSEs span both the upper and lower epidermis. We developed two possible working hypotheses for freeze-thaw embolism: 1.) Freeze-thaw embolism would occur upon thawing in a pattern resembling embolism caused by drought, where the lower vein orders embolize first, followed by the higher order veins; or 2.) Embolism would occur only in veins with large vessel diameters (equal to or > 30 µm) based on research in stems which shows a steep increase in freeze thaw embolism in conduits above this threshold (Pittermann and Sperry 2006). Finding this would suggest that conduit size may govern the occurrence of freeze-thaw embolism across organs. We predicted that ice crystal formation would lead to damaged cell walls and membranes and thus irreparable damage to the mesophyll, made visible by significant decreases in chlorophyll fluorescence shortly after thawing. If freeze-thaw embolism occurred, it would lead to cell death in any remaining un-frozen tissue but over a longer time period. The combined effects of these processes may explain the universality of leaf death due to spring frost across broad-leaved tree species.

Materials and Methods

Freezing experiment

Saplings of *Liriodendron tulipifera* (~ 2 years old) were grown under glasshouse conditions (day temperature of ~25-30 C and night temperatures of ~ 15-20 C) for 1.5 months, until there were at least two fully expanded leaves per tree, before experimentation (conducted between March and May 2022). A total of 13 trees were frozen to one of five minimum freezing temperatures (-1, -2, -3, -4 or -5 ˚C). We selected this range of minimum temperatures to both determine the temperature needed to fully freeze leaves, but also to

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test the effects of cold temperatures that did not induce freezing. We used the Optical Vulnerability Method (OVT) of Brodribb et al. 2017 to monitor both the formation of embolism but also the changes in optical density of the mesophyll associated with freezing. We imaged a total of 24 leaves and one main stem. Each tree was frozen as described below.

Saplings growing in pots were placed in an upright freezer (Model: FFFH20F2QWC, Electrolux home products Inc.) which was attached to a thermostat (Model: ITC-310T-B, Inkbird dual relay) which was used to control the freezing trajectory. Two adjacent fully expanded leaves were placed inside 'cavicam' imaging clamps (docs.cavicams.com, Fig. 1). Briefly, a digital raspberry pi camera (V2, 9132664) was positioned to observe the adaxial leaf surface and transmitted light is provided by a digitally controlled set of LED lights that only illuminate during image capture (Brodribb, Bienaime et al. 2016). Cameras were set to capture images every 5 minutes during freezing and thawing, a process that was programmed to occur over 20 hours (see details below). A custom-built temperature logger 'envirologger' run by a data-logger (Adafruit feather M0 Adalogger) was placed in the freezer to track the internal conditions with a temperature/humidity sensor (Model: AM2315 12C, Adafruit).

The freezing sequence and trajectory were based on winter/spring temperature data from a weather station (Campbell Scientific CR300) monitoring windspeed, air temperature, relative humidity, precipitation, soil moisture and soil temperature, averaged hourly over the measurement period) at nearby Yale Myers Forest (Union, CT, USA; 41° 57' 8.2944" N, 72° 7' 26.688" W), where *L. tulipifera* trees are known to grow, in order to mimic the freezing conditions experienced by this species under natural conditions. A representative night in 2019 where temperatures reached -5 ˚C was chosen as a reference. The temperature data from Yale Myers Forest (recorded half-hourly) was averaged and rounded to the nearest whole number to determine mean hourly temperatures. These temperatures where then used to inform a 12-step programmed freezing sequence. This resulted in a stepped freezing sequence that ran for approximately 20 hours (including an ~ 6 hour period at a positive temperature at the end) mimicking an overnight freezing event (Fig S2).

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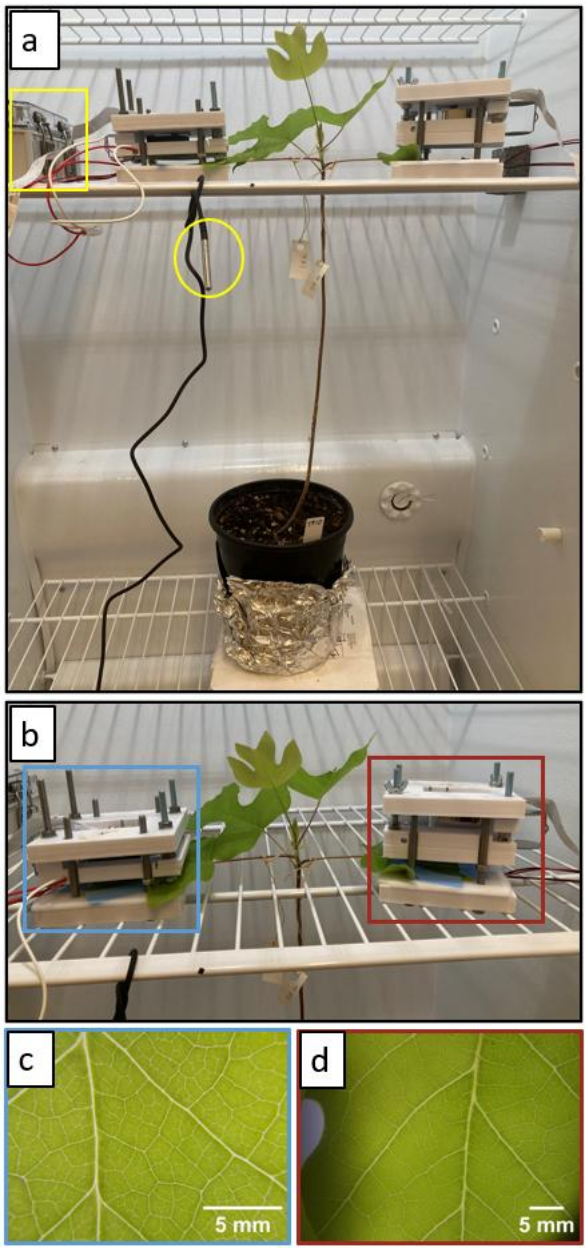

Figure 1: The set-up for monitoring freeze in *L. tulipifera* trees. Panel (a) shows the sapling inside the freezer with cameras attached. The unit for measuring freezer air temperature is denoted by a yellow box while the temperature probe attached to the device controlling the freeze trajectory is circled in yellow. In panel (b) the two cameras are distinguished by boxes, with the higher magnification camera in blue and lower magnification camera in red. Corresponding images taken by each camera are shown below the respective cameras, in boxes of the same colours (blue; higher magnification and red; lower magnification) with a 5mm scale bar in the bottom right-hand of each image.

Chlorophyll Fluorescence Imaging

To determine the physiological effects of freezing temperatures on leaves we used chlorophyll fluorescence imaging, which provides a measure of the status of the photosynthetic apparatus and its capacity for processing light. We performed dark-adapted

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Fv/Fm imaging on leaves both before freezing to provide a baseline reference measurement, and then again after freezing to determine how much the photosystems were damaged (Mini Imaging-PAM M-series, Walz GmbH, Germany). Before fluorescence measurements, a small piece of opaque tape was applied to a small section (~ 0.5x 0.5 cm) of the leaf, then the leaves were wrapped in aluminium foil to exclude light. The patch of opaque tape was used because initial testing showed that, as removal of aluminium foil was required to align the leaf under the imaging PAM, an initial measure was needed to maintain some leaf tissue in the dark-adapted state required for Fv/Fm measurements. The tape was removed seconds before measurement to ensure that the leaf tissue beneath the tape remained fully dark adapted until fluorescence was measured.

Freezing and thawing in the leaf mesophyll

Image sequences were processed in FIJI software (Schindelin, Arganda-Carreras et al. 2012) to determine the spread and extent of ice crystallization during the experiments. The tracing tool in FIJI was used to quantify the total leaf area frozen at each time step, which was indicated by a change in the colour intensity of the pixels. Freezing was detected as patches of brighter pixels in the leaf lamina, typically bounded by higher order veins.

To quantify the colour change in both mesophyll tissue that did and did not freeze, circular ROIs of 40x40 pixels were selected in regions of both mesophyll that froze and that which did not in all leaves frozen to -4 ˚C. The mean grey scale value in these ROIS was calculated at three time-points, at the start of image capture, at -4 ˚C and immediately after thawing. The values at -4 ˚C and after thawing were expressed as a percentage of the initial mean greyscale value (from the start of image capture) and the average percentage of the initial value was calculated for both frozen and unfrozen mesophyll.

A similar method, utilising circular ROIs in FIJI, was used to track the change in mean greyscale area in the midveins and mesophyll of all leaves which experienced freezing. In each leaf, ROIs which fit within the diameter of the midvein were selected and added to the ROI manager. This was repeated across the image stack or up to 250 images (where more images were recorded), with the ROI shifted using ‘edit>selection>specify’ when the leaf moved. The ‘Interpolate ROI’s’ option through the ROI manager was then used to interpolate between the selected ROIs for all images. The ‘measure’ function was then used to calculate the mean greyscale area in the selected ROI for each image. This was then repeated for a section of mesophyll adjacent to the midvein, using an ROI of the same area. The mean greyscale values across the images were expressed as a percentage of the initial (unfrozen) mean greyscale value in order to generate lines plots of changes in mean grey scale value before, during and after freezing.

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The freezing extent was calculated in the image where the maximum amount of tissue was frozen, which corresponded with the minimum air temperature to which the leaves were exposed. The frozen regions were selected using the tracing tool in FIJI (Schindelin, Arganda-Carreras et al. 2012) and expressed as percentage of the total visible leaf area within the field of view to calculate a percentage.

Embolism upon thawing

We monitored the leaf vein network for the formation of embolism using the OVT (Brodribb, Bienaime et al. 2016). Freeze-thaw embolism was analysed using the same methodology which is used to highlight drought-induced embolism with leaf images analysed via an image subtraction, where each image is subtracted from the one before to reveal the changes between the images. As there is a change in light transmission as air rapidly replaces water in the xylem due to embolism, the image subtraction reveals these changes which can then be visualised through time. A detailed description of the methodology used to process images can be found here: https://www.opensourceov.org/. While embolism was expected to occur upon thawing, cameras remained attached to plant organs for ~6 hours to ensure that all embolism was captured.

Drought embolism

Optical analysis of embolism was undertaken in a total of six leaves from branches of adult trees, one leaf was sourced from a tree located on the Yale University campus, New Haven, Connecticut, USA (41°19'16.6"N 72°55'25.8"W), and the remaining five leaves were sampled form across four trees located in North Hobart, Tasmania, Australia (42°52'07.6"S 147°18'57.8"E). Young, fully expanded leaves (as a similar developmental stage as those used in the freezing experiment) were chosen for imaging. One leaf from each branch was placed within an imaging clamp and set to take images at 5-minute intervals as the branches dried. Embolism was determined to have finished after 12 hours without embolism. Images were analysed using an image subtraction, as described above.

Analysis of embolism

The timing and extent of embolism were determined both in leaves exposed to freezing and those exposed to drought. To visualise embolism through time, embolism was colour coded according to time in both a leaf exposed to freeze and a leaf exposed to drought. This was done using FIJI (Schindelin, Arganda-Carreras et al. 2012) using the ‘colour slices’ function in the ‘OSOV toolbox’, the toolbox and instructions are available here: https://www.opensourceov.org/.

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Embolism and vein order

To determine how much the vein network embolised, the total length of the visible venation network was calculated along with the total area of the embolised vein network using the 'segmented line' tool in image J and the embolised vein area was then expressed as a percentage of the vein network. Total vein area was calculated from a raw image of each leaf taken from the stack of images which captured during the drought or freezing treatment while total embolised vein area was calculated form the 'Z- stack' showing all the embolism which occurred during the treatment. Total embolised vein area was then expressed as a percentage of total vein area visible with the optical cameras. As embolism was only observed in the mid-rib and major veins in response to freeze, only these vein orders were included in calculations of vein length and embolised vein length for frozen leaves. The raw images of the vein network and Z-stacks of embolism from leaves exposed to drought were segmented into four even quadrants using a macro in FIJI and the top right corner was analysed for each leaf in order to provide a random sample of the vein network.

In *L.tulipifera* leaves, the midvein, second order and tertiary veins are deemed the 'major veins' while the fourth, fifth and sixth and seventh order veins are considered the 'minor' veins and make up the majority of the venation network (Pray 1954).

To quantify the contribution of the midvein and second order veins to the earliest embolism is leaves exposed to drought, 25% (embolised area) of the total drought-induced embolism was chosen as a reference value. This was in order to determine whether embolism observed due to freezing and thawing matched early embolism during drought. At this percentage embolism, in all droughted leaves, we calculated both the area contribution of these veins compared to higher vein orders, and the total length of midvein and second order veins embolised as a percentage of the total embolised area of these veins at 100% embolism. For area, the 'freehand sections' tool in FIJI was used to 'circle' all vein orders expect for the midvein and second order veins and the area of these veins was calculated using the 'measure' function and expressed as a percentage of the total cumulative embolised area at 25% embolism. The resulting percentage of higher-order-veins was subtracted from 100% to reveal the percentage contribution of the midvein and second order veins to the embolised area at 25 % embolism. For vein length, the 'segmented line' tool was used to calculate the length of the total embolised area of the midvein and second order veins in a Z-stack of the cumulative embolism at 25% embolism. This was expressed as a percentage total embolised midvein and second order vein length at 100% embolism.

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High resolution freezing

Branches of adult *L. tulipifera* trees were excised from individuals on the EPFL campus, Lausanne, Switzerland (46.5191° N, 6.5668° E) and transported to the ETH Honggerberg Campus in Zurich. Small leaves were mounted between two glass microscope slides and placed on a customised freezing stage beneath a compound microscope using confocal microscopy (Yokogawa Spinning Disk and Nikon Eclipse, set up &software by 3i imaging). The set-up used is described in (Gerber, Wilen et al. 2023) and the documentation is available at: https://github.com/dogerber/temperature_gradient_microscopy_stage/tree/main. A CF640 filter was used to visualise eaves, capturing the autofluorescence of chlorophyll therefore highlighting the cells in the images.

Xylem conduit diameter analysis

Young *L. tulipifera* leaves were collected from trees and brought to the lab. Vein samples from three leaves were used for the conduit diameter analysis. Midvein samples were collected 1cm from the petiole lamina junction. Second and third order veins were collected 0.5 cm from the junction with the subtending lower order vein. Samples were mounted to a freezing stage (BFS-3MP, Physitemp Instruments, USA) on a sliding microtome encased in a dilute glucose solution. Thin sections were stained with a 0.5% toludine blue solution, rinsed in DI water, and then mounted in water. Images were captured with a digital camera mounted to a compound light microscope (BX43, Olympus Inc, Japan) using a 4x or 10x objective lens. Images were then used to measure xylem vessel diameter in FIJI. Vessel diameter was measured as the longest distance across the lumen.

Data analysis

Data were plotted and analysed using SIGMAPLOT v.12.5 (Systat software 2003) and RStudio was used to perform Single factor ANOVAs to determine statistical differences between groups in RStudio (2016). Xylem vessel diameter analysis was performed in RStudio using the dplyr package (Yarberry and Yarberry 2021).

Results

Pattern of Freezing and thawing

We observed mesophyll freezing in all leaves which were exposed to minimum air temperatures of -4˚ C or -5˚ (total of 18 leaves). At -4˚ C greyscale pixel values increased in the freezing regions that can be interpreted as a change from air to water in the intercellular space as water is drawn out of the mesophyll cells to feed the freezing front (Fig. 2).

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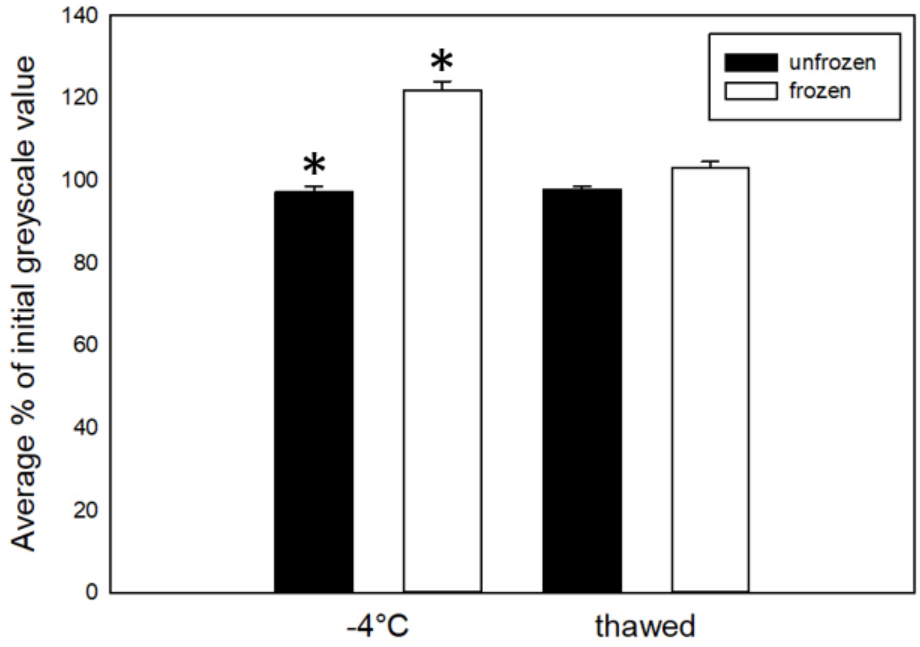

Figure 2: The average percentage of initial (pre-treatment) mean greyscale value (brightness) for all 18 leaves that froze, comparing tissue that did and did not freeze within the same leaves. These values were calculated at -4 ˚C and immediately after thawing. Unfrozen tissue is shown in black, while frozen tissue in shown in white. This was calculated for all 18 leaves that froze. Asterix's indicate a statistically significant difference (P<0.05).

While an increase in mean greyscale value, indicating freezing, was always detected in the mesophyll, this was not consistently observed in the midvein (Fig 3). Freezing in the mesophyll was evident in a 10-20% increase in mean greyscale brightness while freezing in the veins incurred an increase in mean greyscale of <5% (Fig 3). Only one of the eight leaves frozen to -4°C showed a signal of freezing in the midvein, while six of the ten leaves frozen to -5°C showed this signal of freezing.

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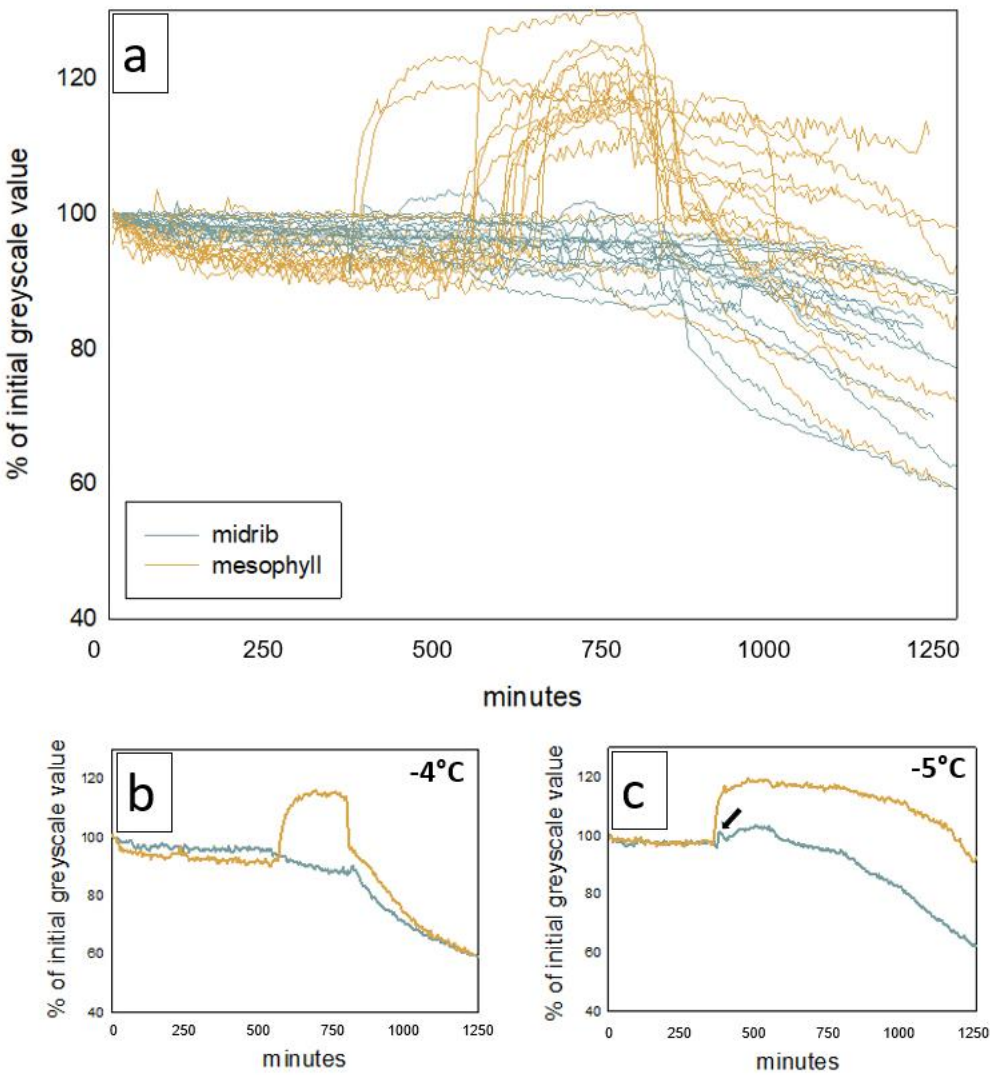

Figure 3: Percentage of initial greyscale value against time for the midvein (midrib) and mesophyll of all *L. tulipifera* leaves frozen to -4°C or -5°C. The mean greyscale values for leaf midveins (blue) and mesophyll adjacent to the midvein (orange) across 250 images (~20 hours) for all leaves frozen to -4°C or -5°C (a). Increases in mean greyscale brightness in the mesophyll (orange) indicate mesophyll freezing. Panel (b) shows the midvein and mesophyll mean greyscale traces for a representative single leaf frozen to -4°C and panel (c) shows the same for a leaf frozen to -5°C with a black arrow denoting the increase in mean greyscale brightness indicating freezing in the midvein.

Freezing was first detected in mesophyll immediately adjacent to the midvein and 2˚ veins, followed by ice propagation away from the vein into areoles bounded by higher order vines (3-5˚ veins) (Fig. 4). Freezing began at ~ -4°C continuing until 0°C when thawing occurred (Fig. 4). Freezing advanced across the leaf surface in a stepwise pattern between adjacent areoles in discrete patches. Freezing was in some cases not perfectly spatially homogenous, and in all but two leaves where 100% of the area frozen (16 out of 18 leaves) some areoles bounded by third order veins remained unfrozen (Fig. 4, Vid. 1, Fig. S3).

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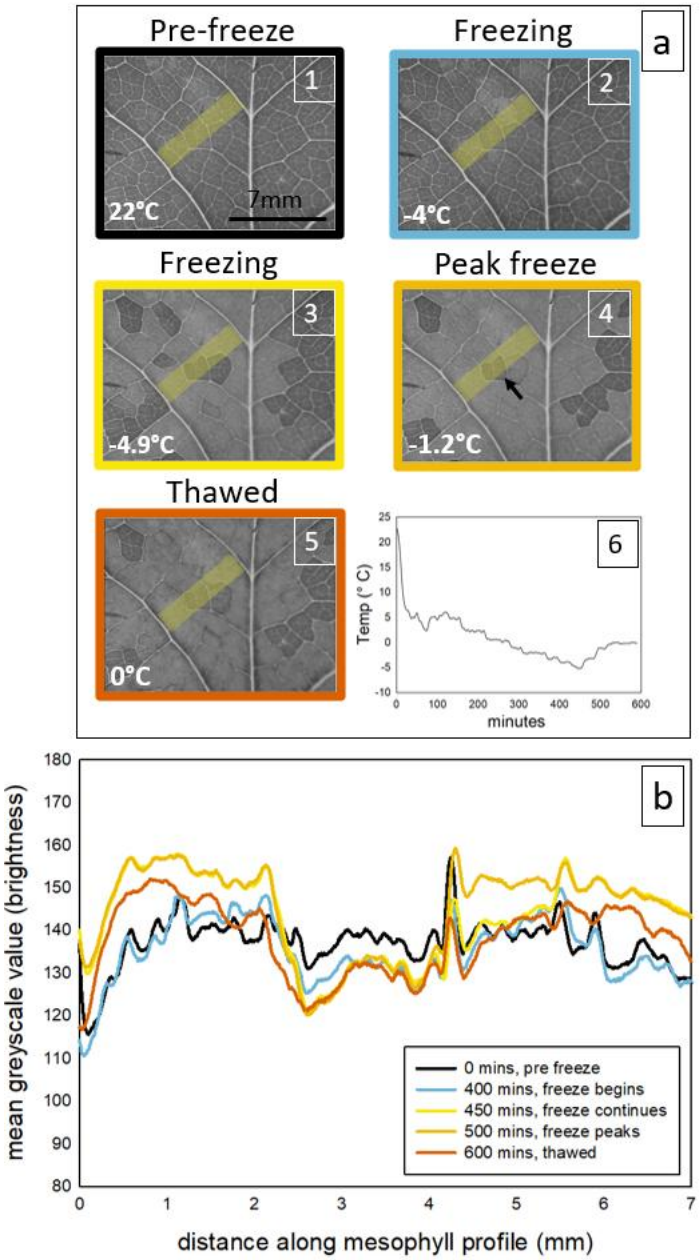

Figure 4: Representative freezing and thawing sequence including images, mean greyscale value and air temperature for a representative *L. tulipifera* leaf visualised using time-lapse imaging. Panel (a) showed greyscale images of the leaf surface including the midvein and surrounding higher order veins with the transect used to calculates mean greyscale values denotes by a yellow rectangle. Mean greyscale values (b) across a 7mm mean greyscale profile between the midvein and a major vein of *L. tulipifera* leaves measured at 5 stages of freezing in a leaf frozen to -5°C [pre-freeze (1): black, freezing; blue (2) and yellow (3), peak freeze: light orange (4), thawed; terracotta (5)]. The temperature at each stage is in the bottom right-hand corner of each image and panel (6) shows time (minutes) vs temperature (°C). This figure shows the distinctive colour change which occurs with freezing visually (a) and numerically (b), illustrating a situation where a patch in the centre of the

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mean greyscale profile remains unfrozen (denoted by a black arrow in the 'Peak freeze' image in panel (b).

Freezing at high magnification revealed a similar pattern, where a freezing-front propagated through individual areoles, but then stalled upon reaching a third-order vein boundary (Vid. 2). The freezing front can be observed progressing from the bottom left corner towards the middle of the field of view where it can be seen moving through cells before reaching a third-order vein boundary and then progressing to the bottom right and moving towards the middle before reaching the vein boundary in the same manner (Video 1)

Thawing, by contrast, did not appear to occur as a staged process, occurring over 1-2 images (where images were taken at 5 minutes intervals) when the temperature reached a positive value (0 °C, Fig 4). Thawing resulted in a 'darkening' of the previously lighter frozen patches under the optical cameras which used transmitted light (Fig 2, Fig 4), while thawing corresponded with lightening in the high-resolution video of thawing as this set-up utilised reflected light (Vid. 3). In the hours following thawing, frozen tissue was observed to become red (Fig 5).

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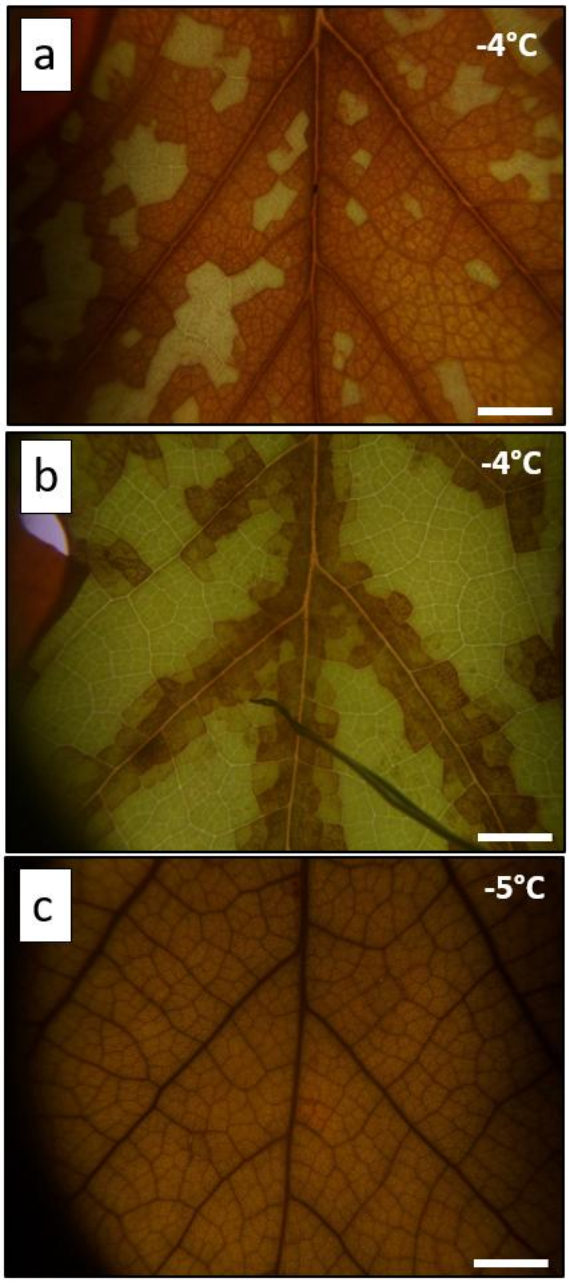

Figure 5: L. *tulipifera* leaves ~ 10 hours after thawing, demonstrating the reddening of the tissue that occurred in frozen tissue. This reddening corresponding to the area which was frozen. In leaves frozen to -4° C the patchy-freezing resulted in patchy redness which varied between leaves as demonstrated in (a) and (b), while freezing and therefore reddening affected the entire visible lamina in leaves frozen to -5 ° C (c). Bars = 5mm**.**

Freezing extent

Freezing began at an air temperature of -4 °C, with no visible evidence of freezing in leaves exposed to air temperatures of -1°C, -2°C and -3°C. In leaves frozen to -4°C (n = 8), an 58%± 5% of the leaf froze within the camera's field of view, while this increased to 93% ±2% in the ten leaves frozen to -5°C (; P< 0.000008, ANOVA; Fig. 6). Beginning as quickly as 2

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hours following thawing, veins and frozen mesophyll tissue transitioned to a 'red' colour, further highlighting where freezing had occurred (Fig 5).

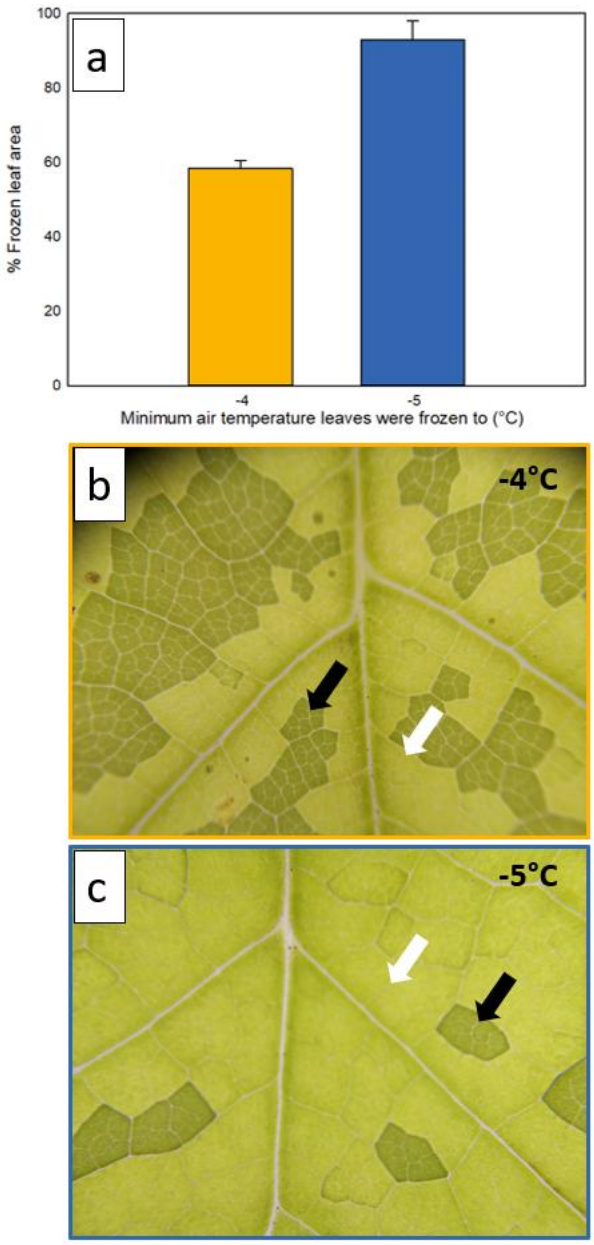

Figure 6: The percentage frozen leaf area in leaves frozen to air temperatures of -4°C and - 5°C. panel (a) shows to total percentage frozen leaf tissue in all leaves frozen to -4°C (gold) and -5°C (blue). Representative images of the freezing extent are also shown in both a leaf frozen to -4°C (b) and a leaf frozen to -5°C (c). Arrows mark unfrozen tissue (black arrows) and frozen tissue (white arrows) in both (b) and (c).

Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence measured ~6 hours after freezing and thawing invariably decreased in frozen tissue and largely remained high (close to 0.8 Fv/Fm) in tissue which did

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not freeze (Fig. 7). Mean Fv/Fm for frozen tissue (0.349 ± 0.06) was significantly lower than that for unfrozen tissue (0.733 ± 0.21; P< 0.000003, ANOVA). In leaves exposed to -1°C - 2°C or -3°C, where we observed no freezing of the lamina, fluorescence remained > 0.7 Fv/Fm (Fig S four). Evidence of both Fv/Fm depression and high Fv/Fm values in frozen and unfrozen tissue, respectively, were measured in leaves which experienced sub-freezing temperatures (i.e. those exposed to temperatures of -4°C or -5°C). The ‘patchy’ nature of freezing in the leaf mesophyll visible with the optical camera (Fig.6) was also visible through chlorophyll fluorescence imaging (Figs. 7b, S5).

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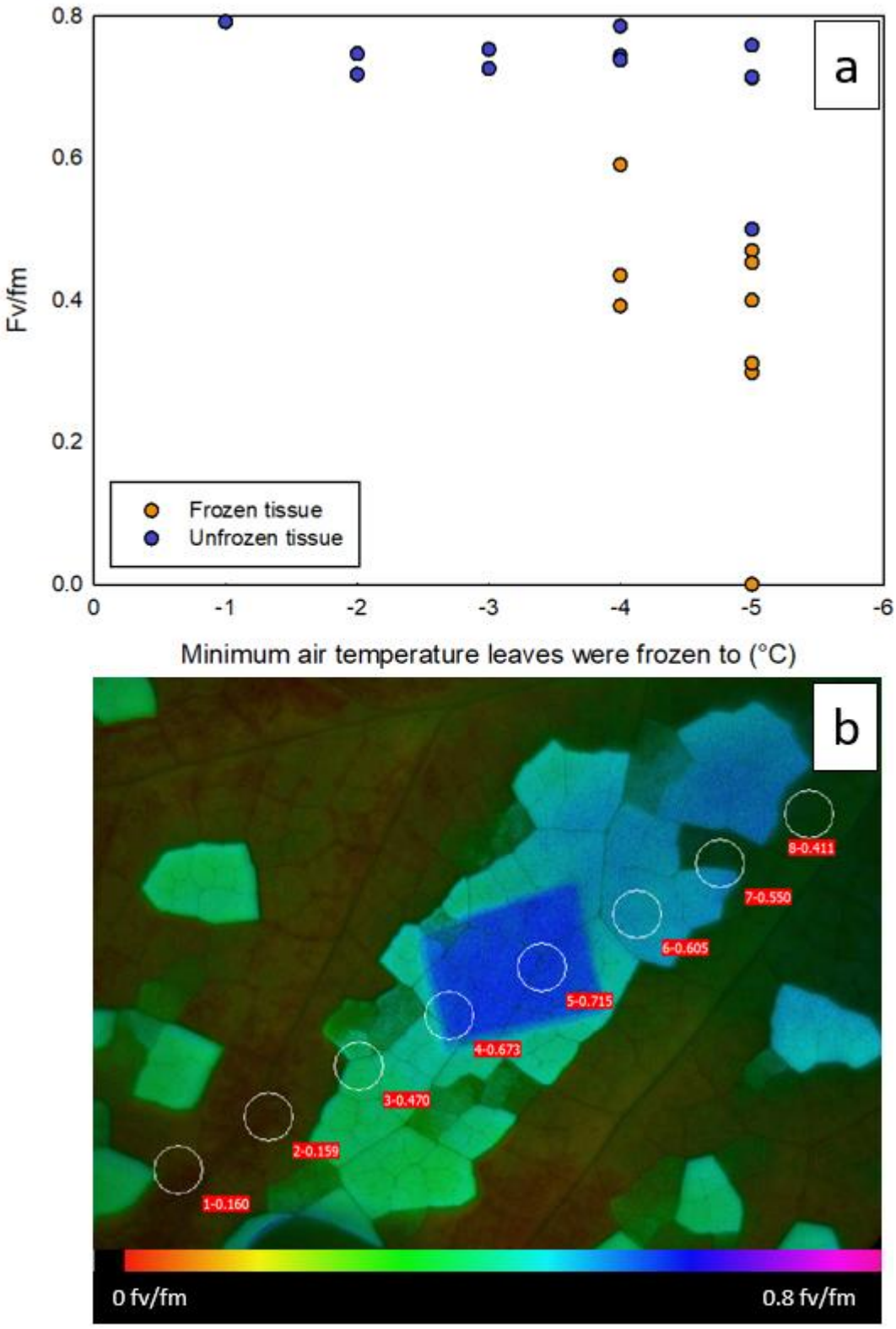

Figure 7: Changes in chlorophyll fluorescence in response to freezing temperatures in leaves of *L. tulipifera*. Panel (a) shows Fv/Fm (photosynthetic capacity) in frozen (orange) and unfrozen (blue) leave tissue against the minimum air temperature leaves were frozen to, along with a visual representation of fluorescence in a leaf post-freeze (b). The dark blue rectangle in (b) shows the patch of unfrozen tissue which was 'dark-adapted' before the fluorescence measurement, with a value

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of 0.715 Fv/Fm. Unfrozen tissues outside of this dark-adapted area are turquoise and light green, indicating a higher fluorescence than the surrounding, darker unfrozen tissue. Point values of Fv/fm are shown in eight circular areas of interest arranged diagonally across the image. The colour-scale beneath panel (b) indicates fluorescence values.

Tree Recovery

While leaves frozen to -4°C or -5°C invariably died, all but one of the 13 trees exposed to these temperatures, which displayed 100% leaf loss, resprouted leaves (Fig. S8). The tree which did not exhibit resprouting was not that which demonstrated the highest percentage of lamina freezing. Resprouting occurred from dormant buds below the leaves frozen during the experiment (Fig. S8)

Freeze-thaw vs. drought-induced embolism

Freeze-thaw embolism was seen in six of the eight leaves frozen to -4°C, but not in any of the leaves frozen to -5°C. Embolism after freezing occurred in 1-3 events and were isolated to the midvein and 2˚ veins (Fig. S6 Fig.8). We observed no freeze-thaw embolism in the higher order veins, which was distinctly different than the pattern of embolism spread resulting from drought (Fig. 8).

Freeze-thaw embolism was confined to the midvein and second order veins, while drought induced embolism was observed in all vein orders (Fig. 8). In leaves where freeze-thaw embolism was observed, 77.6 ± 11.8 % of the midvein and second order veins embolised, compared to 100% of these veins in drought-exposed leaves. In response to drought, the percentage of embolism in tertiary veins and above was a similar to the percentage of freeze-thaw embolised midveins and major veins 74.25 ± 7 %, including all vein orders in drought-exposed leaves (adding major and second order veins) did not alter the percentage of the vein network that was embolised substantially, with 78%± 1.4% of the total vein network found to embolise in leaves exposed to drought. There were far fewer frames which contained embolism in response to freezing and thawing (1-3) than in response to drought (average of 127 ± 24 frames). The midvein and second order veins often embolised first in response to drought embolism (representing 83.56% ± 4% of the total embolised area at P_{25} and 76% ± 34% of the total embolised length of midveins and second order veins at P_{25} as a percentage of the total length at P_{100}). However, embolism in these veins was accompanied, or shortly followed, by embolism in the minor veins as well (Fig. 8) unlike in leaves exposed to freezing and thawing. The timescale across which drought embolism and freeze thaw-embolised were also very different. While freeze-thaw embolism events occurred within 5-15 minutes, drought induced embolism occurred over a range of 1- 5 days (Fig. 8).

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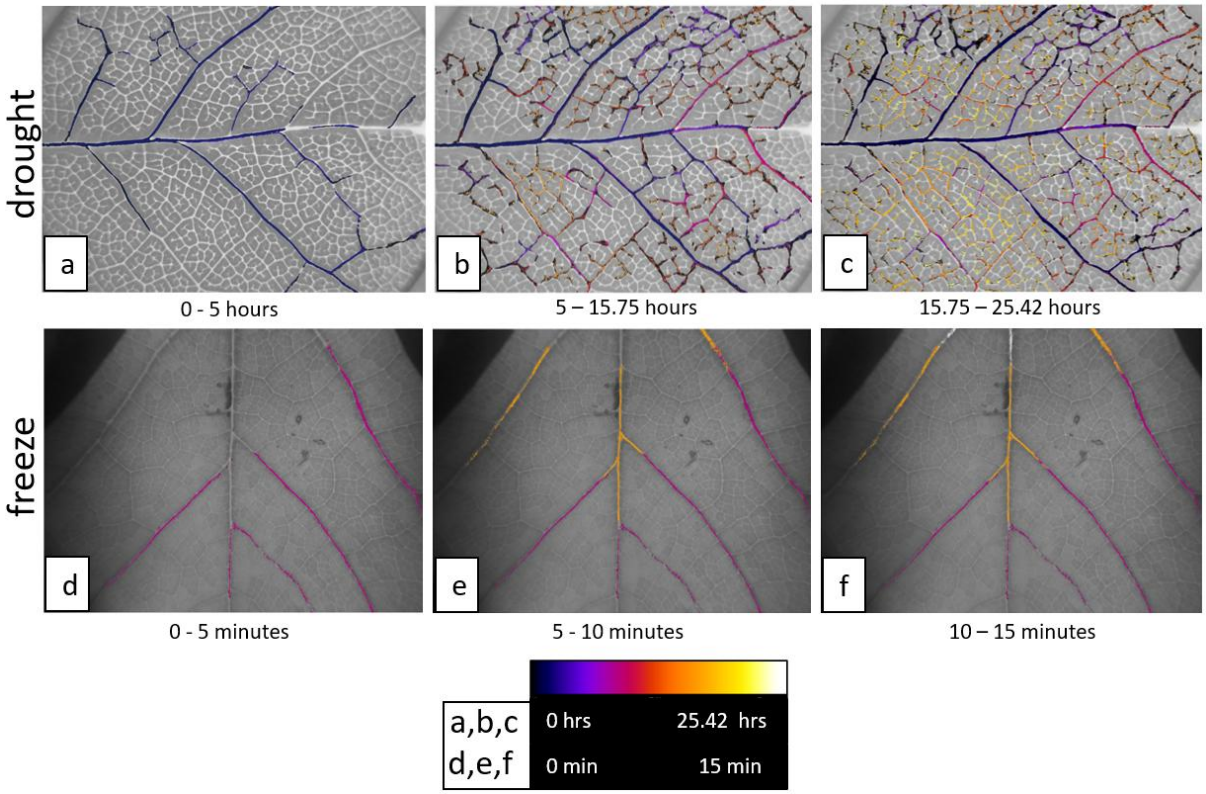

Figure 8: Representative image sequences showing the difference between drought- and freeze-thaw-induced embolism in *L. tulipifera* leaves. Drought-induced embolism spread across 25.42 hours (a, b, c) and freeze-thaw embolism over 15 minutes (d,e,f) in *L. tulipifera* leaves. Panels a, b and c show the progression of drought-induced embolism in four steps of ~23.5 hours each, while the three images in panel (b) show the progression of freeze-thaw embolism in three steps of 5 minutes, where thawing begun at 0 minutes. Embolisms are colour coded according to when they occurred, as indicated by the colour scale beneath each set of images in panels (a) and (b).

Freeze-thaw vs. drought-induced embolism: Stem

A camera placed on the main stem of a tree exposed to a minimum air temperature of -5°C revealed no embolism, though there was evidence of partial freezing in the hydrogel applied to the surface (Fig. S7).

Xylem vessel diameter

We measured vessel lumen diameter distributions in the three lowest vein orders to determine whether this trait might influence vulnerability to freeze-thaw embolism based on the average conduit diameter of 30µm diameter, beyond which a steep increase in freeze-thaw embolism has bene observed (Pittermann and Sperry 2003). We found a mean vessel diameter of 34.4± 6.96, 27.4± 6.95, and 7.45 ±1.85 for the midveins, second order veins, and third order veins, respectively (Fig. 8). We found that 72% and 33.3% of the vessels

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were greater than 30µm in the midveins and second order veins, respectively, but no vessels >30µm were found in high order veins.

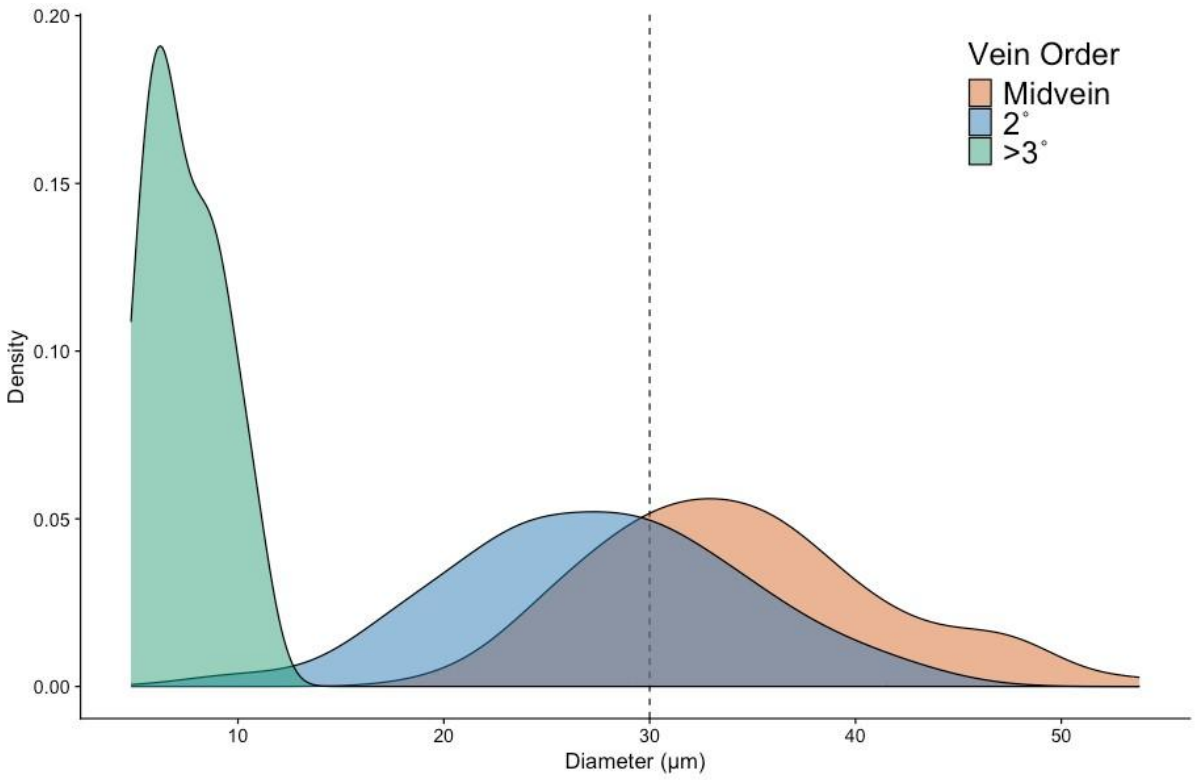

Figure 8: Xylem vessel lumen diameter distributions for leaves of *L. tulipifera*. Distributions are shows for different vein orders (blue = midveins, green = second order veins, orange = high order veins). n = 3, mean diameter is statistically different for each group ($p<0.05$). Vertical dashed line shows the 30µm threshold, where conduits below this threshold are known to be less vulnerable to freeze-thaw embolism.

Discussion

Freezing young *L. tulipifera* trees to air temperatures of -4 °C or -5 °C invariably led to visible freezing in the mesophyll, cell damage detected after thawing, and ultimately leaf death. Our visualisations of ice formation in *L. tulipifera* leaves revealed that mesophyll freezing began adjacent to the midveins and second order veins before progressing in a step-wise manner across areoles bounded by third order veins. These freezing patterns support our hypothesis that bundle-sheath extensions (BSEs) strongly influence how ice propagates and spreads through the lamina of these leaves, and possibly all leaves with BSEs. Freeze-thaw embolism occurred only in veins containing vessels with radii 30 µm or > , in contrast to drought-induced embolism which occurred across all vein orders. This supports our second

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hypothesis that freeze-thaw embolism is driven by conduit size, agreeing with research in stems, and possibly suggesting that conduit diameter is a major driver of freeze-thaw embolism across all plant organs.

Mesophyll freezing and thawing

Mesophyll freezing, detected with optical cameras, was associated with an increase in pixel brightness, as also recently shown by Kane and McAdam (2024). This increase in brightness can likely be explained by a transition from air to water in the intercellular spaces of the leaf during freezing. This is the same principle that underpins the Optical Vulnerability Technique (OVT) for the observation of drought-induced embolism (Brodribb, Bienaime et al. 2016, Brodribb, Carriqui et al. 2017). The OVT, the original application of the optical cameras used here, detects embolism in leaves as a colour-change from light-coloured translucent xylem (water-filled) to darker air-filled xylem (Brodribb, Carriqui et al. 2017). Therefore, the transition from darker to lighter tissue at freezing temperatures (Kane and McAdam 2024) can likely be explained by the movement of water/ ice into previously air-filled spaces. This idea would also account for the brightening of leaf tissue which remained upon thawing (Fig. 4). As the refractive indexes of liquid water (1.33) and ice (1.31) are very similar, the flooded intercellular space would appear brighter even after thawing, as we saw here. This colour change was reversed in the high-resolution freezing, whereby freezing caused a darkening of tissue (Vid. 2). This can be explained by the use of reflected light, rather than transmitted light, further supporting our explanation. The occurrence of intercellular freezing is in-line with the idea of freezing as a dehydration stress in plants (Guy 1990, Steponkus and Webb 1992, Ruelland, Vaultier et al. 2009, Vitra, Lenz et al. 2017, Yang, Gerber et al. 2024) whereby water is pulled out of living cells to feed the freezing front in the spaces between cells. In the absence of intracellular freezing, this process is thought to cause tissue death (Steponkus and Webb 1992).

The difference in the wave-like spread of freezing from around the midvein and second order veins and the patchy freezing that occurred in mesophyll bound by tertiary veins (Vid 1, Vid 2) is likely explained by Bundle-sheath extensions (BSEs). This agrees with research utilising thermal imaging which showed that freezing initiated close to the midvein and second order veins in angiosperm leaves, though freezing could only be tracked dup to third order veins in this study (Hacker and Neuner 2007). BSEs have been shown to have numerous functional roles in leaves, including light processing (Barbosa, Chitwood et al. 2019), gas exchange (Buckley, Sack et al. 2011) and water relations (Zwieniecki, Brodribb et al. 2007, Kawai, Miyoshi et al. 2017). Our research suggests an additional functional role of these structures, in freezing.

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Bundle sheath extensions extend to both the upper and lower epidermis in tertiary veins in *L. tulipifera* (Fig. S1), but are not present in the midvein or second order veins. BSEs have also been shown to be discontinuous or extend to only one leaf surface in fourth order veins and higher in *L. tulipifera* (Pray 1954). The absence of BSEs in the two lowest vein orders likely accounts for the apparently uninhibited spread of ice adjacent to the midvein and second order veins while their presence may explain the independent and patchy freezing or areoles bounded by tertiary veins (Fig S3). The delay to freezing spread at tertiary vein boundaries in some cases (Vid. 1, Vid. 2), and complete blockage in others is likely explained by BSEs acting as physical barriers to the movement of the ice crystallisation. This suggests that it is not only highly sclerified bundle sheaths like those in *Cinnamomum canphora* that can influence ice propagation (Barbosa, Chitwood et al. 2019).

Simultaneous thawing of the leaf tissue also suggests that the physical component of this barrier is more important than chemical differences. If variable solute concentrations in the leaf were governing the pattern of freezing, we might expect thawing of some regions before others. The change to a darker colour upon thawing (Fig. 3), followed by a reddening of affected tissue (Fig. 5) was likely caused by cell lysis (i.e. due to the intrusion of ice crystals) and leakage of electrolytes and other toxic cellular components (Burke, Gusta et al. 1976). Permanent damage to cells after freezing and thawing was clearly evident in the depressed fluorescence (Fig. 7).

Freeze-thaw xylem embolism

Here we present visualisation of freeze-thaw embolism in leaves and evidence of freeze-thaw embolism in real-time, finding that perhaps the most convincing driver of freeze-thaw embolism is conduit size. Freeze-thaw embolism in the lamina was confined to the two lowest vein orders (midvein and second order veins), which also possess the largest vessels (Fig. 8). We found vessel lumen diameters exceeding 30µm in both of the vein orders also showing embolism, with no vessels >15µm in diameter in the third order veins. This aligns with research in conifer stems which shows a sharp increase in vulnerability to freeze-thaw embolism when conduits diameter exceeds 30µm (Pittermann and Sperry 2003) and is consistent with the large body of research in woody plants which shows that larger diameter conduits are at greater risk (Sperry and Sullivan 1992, Sperry, Nichols et al. 1994, Feild and Brodribb 2001, Tyree and Zimmermann 2002, Pittermann and Sperry 2006, Choat, Medek et al. 2011, Robinson, Rennie et al. 2023). This has been theorised (Sevanto, Holbrook et al. 2012) and been inferred to be the case in leaves (Ball, Canny et al. 2006) but has, until now, not been visualised in leaves.

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A reduction in xylem conduit diameter was one of the key adaptations that allowed flowering trees to radiate into freezing environments (Zanne, Tank et al. 2014) and decreasing conduit diameter with increasing probability of freezing temperatures has been observed at a global scale (Sperry 1995, Sevanto, Holbrook et al. 2012). With a large population of vessels <30µm, it is possible that if embolism of the lower vein orders was incomplete, the smaller conduits which are less vulnerable to freeze-thaw embolism could maintain water supply to these leaves. This may allow *L. tulipifera* leaves to resist freeze provided that the photosynthetic tissue are 'hardened' to freezing temperatures. The extent of freezing damage in photosynthetic tissues may, however, outweigh any effects of embolism, at least early in the growing season when leaves are not acclimated for cold temperatures.

A mean greyscale signal of freezing in the midvein was observed in six out of the 10 leaves frozen to -5°C but only one out of the 8 frozen to -4°C. This is contrary to evidence from thermal imaging which showed that freezing occurs first in the veins (Hacker and Neuner 2007). It is possible that vein freezing is stochastic, occurring base on spontaneous ice nucleation which does not always occur. This may be explained by the nucleation by substances other than water such as macromolecules or dust, termed 'heterogenous nucleation'(Kanji, Ladino et al. 2017, Shardt, Isenrich et al. 2022). Another possibility is that the inconsistency in the detection of freezing in the midvein may be related to the already high transparency of the leaf venation in optical images making the subtle change in the reflective index when water freezes more difficult to detect.

A notable factor that we did not test in this study is the speed of freezing and thawing. We opted for a slow freeze-thaw trajectory which mimics field conditions but did not test different speeds of freezing or thawing. Slower freezes are thought to increase the likelihood of freeze-thaw embolism (Sevanto, Holbrook et al. 2012) but slower thawing is thought the decrease the loss of conductivity in the xylem (Langan, Ewers et al. 1997). Given the large increase in percentage frozen area between -4°C and -5°C degrees it's possible that that rapidity of freezing over this one degree temperature range precluded embolism formation and that a slower freeze or thaw may have produced different results.

Freeze-thaw vs. drought induced xylem embolism

Differences in the mechanism behind embolism induced by drought vs. freeze-thaw embolism in the leaf lamina likely explains the contrasting extent of embolism observed in *L. tulipifera* leaves exposed to freezing and drought. Importantly, the clear visibility of embolism in higher order veins in leaves exposed to drought means that we can be sure that the lack of embolism in their higher order veins in frozen leaves was not a failure of the optical cameras to detect these events, but an absence of embolism in these vein orders. The

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vulnerability of xylem conduits to air-seeding under drought conditions has been linked anatomical characteristics such as the thickness of the pit membranes in the xylem wall (the site at which air enters, causing embolism due to drought;Thonglim, Delzon et al. 2021, Thonglim, Bortolami et al. 2023) and connectivity of the xylem network (Brodersen and Roddy 2016, Johnson, Brodersen et al. 2020, Mrad, Johnson et al. 2020). Combined, these anatomical features likely explain the pattern of drought-induced embolism we observed in young *L. tulipifera* leaves whereby embolism progressed through the major vein orders terminating in the higher order veins, a pattern which is consistently observed across the leaves of woody and herbaceous angiosperms (Johnson, Jordan et al. 2018, Tonet, Carins-Murphy et al. 2023). In contrast, freeze-thaw embolism in the lamina, likely caused by gas segregation and subsequent bubble expansion (Sevanto, Holbrook et al. 2012), is thought to be determined primarily by xylem diameter (Pittermann and Sperry 2006). This, as described above, may explain why freeze-thaw embolism occurred only in the largest veins in *L. tulipifera*, where we found that 72% and 33.3% of the vessels in the midveins and second order veins, respectively, were above the 30µm threshold. Conduit size is shown to be a poor predictor of drought-induced xylem embolism resistance (Lens, Gleason et al. 2022) and connectivity and xylem-pit-properties unlikely to have any influence on the formation of freeze-thaw embolism. It is therefore likely that differences in the drivers of drought and freeze-thaw embolism cause the differences we observed in the extent of embolism in *L. tulipifera* leaf venation networks. This evidence strongly supports that idea that conduit diameter plays a mechanistic role in freeze-thaw embolism.

What kills L. *tulipifera leaves during a freeze? Freezing as a drought stress*

The freeze-induced embolism and damage to living cells detected here highlights that freezing can affect both the water transport and photosynthetic systems in young *L. tulipifera* leaves. It should also be noted however, that regardless of whether embolism occurred, all leaves which showed evidence of mesophyll freezing subsequently died. Cell damage and death was evident in all leaves that froze. All leaves in trees exposed to temperatures below -4˚C died after thawing, clearly evident in leaf browning within days of freezing and thawing (Fig. S8). This lethal damage was also evident in the notable depression of fluorescence in frozen mesophyll (Fig. 7), and the ‘red’ appearance of the leaves in the hours following thawing indicating cell-rupture (Fig.5). Additionally, *L. tulipifera* leaves presented ‘wilted and wet’ after thawing, a state described by Burke and Gusta et al (1976) in observations of young spring leaves after frost events, attributed to the loss of semi permeability in the cell membranes. Importantly, a lack of fluorescence decline in leaves where freezing was not observed, (those frozen to -1°C, -2°C and -3°C) indicates that fluorescence values were not impacted by the time spent in the freezer or the placement of cameras on leaves (Fig. S4).

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The absence of embolism in the lamina of leaves frozen to -5°C indicates that embolism in the lamina is not necessary to induce leaf-death.

The fate of L. *tulipifera leaves exposed to freeze*

The wide-vesseled, soft, broad leaves of winter-deciduous *L. tulipifera* trees are clearly not adapted to survive freezing in spring. Our results suggest that onset of freezing damage represents the point of no-return in these leaves, with freezing found to progress over a narrow air-temperature range expanding from 60% to nearly 100% of the leaf area frozen across just 1°C. While the death of living cells was ultimately the cause of mortality in these leaves, the water transport system is inherently compromised because of freezing. With xylem conduit size fixed at the time of leaf development, the large diameter vessels of the midvein and second order veins in the leaf lamina likely make *L. tulipifera* leaves susceptible to freeze thaw embolism throughout the growing season. However, high redundancy of conduits in the two lowest vein orders, and a large proportion of vessels below the 30µm threshold, may allow leaves to continue to function provided that the mesophyll and other tissues is frost-hardened (Vitra, Lenz et al. 2017).

Conclusions

Freezing invariably killed *L. tulipifera* leaves. Death of living tissue was a constant, sometimes accompanied by freeze-thaw embolism which occurred rapidly and was confined to the veins with the largest conduits, in stark contrast to drought-induced embolism. This supports research in stems showing that conduit diameter drives freeze-thaw embolism vulnerability. The progression of freezing through the mesophyll in patches bounded by tertiary veins implicates BSEs in driving lateral ice propagation. While freezing in the mesophyll conclusively led to death in *L. tulipifera* leaves, a significant proportion of xylem vessels were inherently vulnerable to freeze-thaw embolism, showing that spring frost results in a dual mode of lethality in *L. tulipifera* leaves.

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Author contributions

KMJ and CRB conceived and designed the study. KMJ and CRB conducted experiments and measurements using optical cameras and microscopy, while KMJ and MS designed and conducted the collection of high-resolution imagery with guidance from DG, RWS and ERD. KMJ and CRB wrote the manuscript with contributions from all authors.

Competing Interests

None declared

Data availability

The data will be made available by the corresponding authors upon reasonable request.

Supporting Information

Figure S1: Transverse light microscope section of the lamina of a *Liriodendron tulipifera* leaf highlighting a bundle-sheath extension.

Figure S2: Comparison of natural and experimental freezing trajectories.

Video 1: The pattern of freezing and thawing detected in a leaf frozen to -5 ° C with time-lapse imaging.

Figure S3: Examples of the 'patchy' nature of freezing observed in *Liriodendron tulipifera* leaves frozen to air temperatures of both -4 ° C and -5 ° C.

Figure S4: Chlorophyll fluorescence (Fv/Fm) in *Liriodendron tulipifera* leaves which were placed in the freezer but did not show visible signs of freezing.

Figure S5: Comparison of optically resolved freezing and cell damage shown through imaging fluorescence in *Liriodendron tulipifera leaves*.

Figure S6: The total embolism overlayed onto initial images of each the six *Liriodendron tulipifera* leaves in which embolism was observed.

Figure S7: The changes to the tensive hydrogel applied to the surface of the stem of a *Liriodendron tulipifera* tree during freezing.

Video 2: High resolution freezing in a *Liriodendron tulipifera* leaf.

Video 3: High resolution GIF of thawing of a *Liriodendron tulipifera* leaf.

Figure S8: *Liriodendron tulipifera* trees shortly after they were exposed to -4°C or -5°C freeze treatments.

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