

# Freeze/thaw-induced embolism depends on nadir temperature: the heterogeneous hydration hypothesis

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## ABSTRACT

**Freeze/thaw-induced embolism was studied in leaves of field-grown snow gum (*Eucalyptus pauciflora*) subject to frequent morning frosts. Juvenile trees were grown in buried pots, brought to the laboratory at different stages of acclimation and subjected to simulated frost-freezes (at 2 °C h<sup>-1</sup>) to nadir temperatures of -3 or -6 °C, which snow gums commonly experience. Frost-frozen and subsequently thawed leaves were cryo-fixed to preserve the distribution of water and were then examined by cryo-scanning electron microscopy. No embolisms were found in leaves frozen to -3 °C and thawed. In contrast, 34% of vessels were embolized in thawed leaves that had been frozen to -6 °C. This difference was seen also in the extent of extracellular ice blocks in the mid-vein expansion zones in leaves frozen to -3 and -6 °C, which occupied 3 and 14% of the mid-vein area, respectively. While the proportion of embolism depended on nadir temperature, it was independent of season (and hence of acclimation state). From the observation that increased embolism at lower nadir temperature was related to the freeze-induced redistribution of water, we hypothesize that the dehydration of cell walls and cells caused by the redistribution exerts sufficient tension on xylem water to induce cavitation on thawing.**

*Key-words:* Cell wall dehydration and rehydration; cryo-SEM; *Eucalyptus pauciflora*; expansion zones; extracellular ice; frost acclimation.

## INTRODUCTION

Low temperatures are important determinants of species distributions, with freezing presenting major challenges to evergreen species in frost-prone climates (Sakai & Larcher 1987). Woody plants that tolerate freezing undergo seasonal change in acclimation which enables living cells to tolerate the dehydration that accompanies extracellular ice formation (Xin & Browse 2000). An additional problem occurs with disruption of hydraulic function when xylem conduits become embolized following freeze/thaw events

(Hacke & Sperry 2001). Such loss or reduction in water transport reduces the capacity of a plant to take advantage of warm conditions, particularly when growth recommences in spring. Hence, interspecific differences in the vulnerability of woody evergreens to freeze/thaw-induced embolism could influence growth, competitive ability and distribution along gradients in minimum temperature. Although freeze/thaw-induced embolism has been studied in a variety of species (e.g. Sperry *et al.* 1994; Langan, Ewers & Davis 1997; Pockman & Sperry 1997; Cavender-Bares & Holbrook 2001; Feild & Brodribb 2001), the mechanism is not well understood.

The mechanism of freeze/thaw-induced embolism differs from that caused by tension (Hacke & Sperry 2001). Ice is crystalline and the solubilities of gases in ice are much lower than in liquid water. Consequently, when water freezes, gases dissolved in water can be forced out of solution, forming bubbles surrounded by ice. When frozen xylem sap thaws, bubbles formed during freezing can either dissolve (in water released by melting ice) or expand until the xylem conduit is filled with gas, a process known as cavitation. Initially, a cavitated vessel is filled largely with water vapour but becomes embolized or filled with air at atmospheric pressure as gases diffuse from surrounding tissues. According to the Young–Laplace equation, whether a bubble dissolves or nucleates a cavitation depends on its internal pressure ( $P_B$ ), which is a function of its radius ( $r$ ), the surface tension of xylem sap ( $\gamma$ ) and the pressure of the xylem sap ( $P_X$ ), where

$$P_B = (2\gamma/r) + P_X$$

Small bubbles or those subject to large positive external pressure tend to collapse or dissolve, so the probability that a bubble will nucleate a cavitation increases with increase in the radius of curvature of the bubble and the tension ( $-P_X$ ) in the xylem stream. These two components are highly variable. Values of  $r$  are thought to depend on the size of xylem conduits; larger conduits contain more water and hence more air is forced out of solution during freezing, thereby increasing the probability that a bubble with a critical radius for nucleation will form on thawing (Sperry & Sullivan 1992). Indeed, many studies (but see Cavender-Bares & Holbrook 2001) have reported a correlation between vessel diameter and loss of hydraulic conductivity

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on thawing (Sperry & Sullivan 1992; LoGullo & Salleo 1993; Langan *et al.* 1997; Davis, Sperry & Hacke 1999; Pit-terman & Sperry 2003). The magnitude of  $P_x$  depends on several factors, including the transpiration rate and soil water content during thawing. Indeed, a decrease in  $P_x$  due to drought stress increases loss in hydraulic conductivity during thawing (Sperry & Sullivan 1992). However, the expansion of water on conversion to ice may increase the xylem pressure, thereby enhancing the dissolution of bubbles during thawing (Hammel 1967; Robson, McHardy & Petty 1988; Tyree & Yang 1992).

Xylem contains few solutes, so its equilibrium freezing temperature is close to 0 °C (and depends only weakly on pressure). If freeze/thaw-induced embolism results solely from the exclusion of air during ice formation as described above, then there should be no dependence on nadir temperature other than the minimum temperature required to induce freezing. However, recent studies have reported a greater loss in hydraulic conductivity on thawing of stems frozen to lower nadir temperatures (LoGullo & Salleo 1993; Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). Pockman and Sperry noted that these results challenged the widely held assumption that freeze/thaw-induced embolism is independent of nadir temperature, and speculated that effects of lower nadir temperatures might have been caused by damage to living cells.

However, there are also sound physical reasons to expect freeze/thaw-induced embolism to be affected by the nadir temperature during freezing (Wolfe & Bryant 1999). The formation of an ice embryo and the attainment of critical size for crystal growth are probabilistic, with the chance of nucleation dependent on the volume of available water, the concentration of nucleators and the degree of supercooling. Freezing is expected to occur first in apoplastic water because it contains fewer solutes, and hence has a higher equilibrium freezing temperature, and becomes supercooled at warmer subzero temperatures than cytoplasmic or vacuolar water. Freezing is likely to start in the water filling the lumen apoplast of large vessels (Sakai & Larcher 1987) because the apoplastic spaces within walls (Canny 1995) are usually much too small for crystal growth (Wolfe & Bryant 1999). Indeed, infrared video thermography revealed that freezing of intact, attached leaves of snow gum (*Eucalyptus pauciflora*) begins in the thickest region of the mid-vein where the greatest concentration of large xylem vessels occurs (Lutze *et al.* 1998; Ball *et al.* 2002b). Following ice nucleation, freezing spreads rapidly through the leaf as a thin layer of extracellular ice grows over the walls of mesophyll cells (Ball *et al.* 2002b) and fibres lining the upper and lower expansion zones in veins (Ball *et al.* 2004). This extracellular ice can grow at the expense of water contained in cells and walls because ice has a lower vapour pressure and chemical potential than pure liquid water at the same subzero temperature (and a similar pressure). Specifically, consider the water potential, defined as the difference in chemical potential with respect to pure, liquid water at the same temperature and atmospheric pressure, divided by the volume of unit mass of liquid water.

The water potential of ice declines by approximately 1.2 MPa for each 1 °C below the equilibrium freezing temperature. Once the temperature of extracellular ice is lower than the freezing-point temperature of an intracellular solution, water diffuses from the cell to sites of ice formation, thereby dehydrating the cell and its surrounding wall (Wolfe & Bryant 1999). Thus, the extent of freeze-induced dehydration can depend strongly on nadir temperature.

In snow gum leaves, as in many species (see Levitt 1972), extensive blocks of ice form in the expansion zones next to veins (Ball *et al.* 2004; McCully, Canny & Huang 2004), reflecting major redistribution of water as walls, and living cells become dehydrated during freezing of hydraulic tissues. Rehydration of these tissues requires long-distance diffusion of water liberated by thawing of remote ice blocks. If thawing of ice in vessels occurs before rehydration is complete, then we hypothesize that dehydrated tissues may lower  $P_x$ , thereby increasing the probability of cavitation during thawing. In this way, freeze-induced dehydration could provide a source of tension in the xylem leading to dependence of freeze/thaw-induced embolism on nadir temperature.

We explore this hypothesis by examining seasonal variation in freeze/thaw-induced embolism as a function of nadir temperature in field-grown snow gum. This species is one of the most freeze-tolerant of eucalypts (Harwood 1981; Sakai, Paton & Wardle 1981). The snow gum dominates forest canopies in subalpine areas in Australia as well as at lower elevations around the floor of valleys receiving cold air drainage (Austin, Nicholls & Margules 1990). In these frost-prone areas, minimal air temperatures can be as low as -15 °C in winter, with leaf temperatures ranging from 1 to 4 °C colder than air temperatures (Leuning & Cremer 1988; Ball *et al.* 1997; Blennow *et al.* 1998). Frost episodes on the Southern Tablelands of New South Wales are frequent but largely restricted to nights. Air temperature rises above freezing during the day; the leaves thaw and resume their physiological activities. Hence, we use the term 'frost' to mean a relatively short, nocturnal freezing event, and our simulated frost experiments are similar to field events with freeze/thaw cycles of a few hours.

## MATERIALS AND METHODS

### Definitions

The slow freezing that occurs during natural or simulated freezing events will be called a frost-freeze. The rapid freezing with cryo-pliers to preserve the distribution of water in either frozen or unfrozen tissue will be called a cryo-freeze, and cryo-frozen samples may be referred to as cryo-fixed or cryo-preserved.

### Plant material

Seeds of *E. pauciflora* Sieb. ex Spreng. were collected from trees growing along the floor of the Orroral Valley at an elevation of 850 m in New South Wales, Australia. Seeds

were cold-stratified under moist conditions at 3 °C for 6 weeks before germinating on sand flats in a mist house. Seedlings of similar size were transferred to individual containers and grown out of doors (in Canberra, elevation 600 m) for 3 months, after which they were transferred to the field site, a flat, fully exposed pasture (elevation 700 m) near Bungendore, New South Wales, Australia (latitude 35°15'S, longitude 149°27'E). There, the seedlings were subjected to seasonal variations in irradiance, rainfall, temperature and frost, and the natural cycles of acclimation and deacclimation to freezing temperatures. The seedlings were transplanted to larger tubes (15 × 40 cm), which were buried in the ground. These tubes were closed at the bottom with shade cloth to prevent roots escaping into the soil beneath, and allowed intact, field-grown plants to be transported to the laboratory for controlled freezing experiments. Snow gum leaves are produced mainly in summer and can survive for several years. The leaves used in the experiments were approximately 6–12 months old and were of the juvenile form typical of establishing seedlings, with a mid-vein and divergent branch veins, not the almost parallel venation attained by leaves on mature trees.

### Planting design

The field plot was divided into five blocks. A pre-existing, 1-year-old seedling growing in each block was designated for measurements during a natural freeze/thaw event. A snow gum seedling was planted in the centre of each of eight additional planting squares (1 × 1 m) in each block, as described earlier. Six experimental treatments were randomly assigned to the eight seedlings planted in the squares in each block, with two seedlings being designated as spares if additional analyses were required. Thus, in each block two seedlings were allocated to assays of freeze tolerance in autumn or winter, and four seedlings were allocated to simulated frost-freezes of either –3 or –6 °C during either autumn or winter.

### Microclimate

Temperatures were measured in a weather screen at 1.2 m above ground located adjacent to the study site. Air temperature was measured with a PT-100 platinum resistance thermometer every 1 min, and a 30 min average was recorded on a DT 500 datalogger (Data Electronics, Victoria, Australia).

### Freeze tolerance

Electrolyte leakage from leaf cells was determined according to Webb *et al.* (1996) with a few modifications. Measurements were made on five replicate, fully expanded leaves of similar age, one leaf from each of five plants during autumn and winter. Eight disks (8 mm diameter) were cut from the lamina of each leaf and randomly assigned to eight temperature treatments, giving five replicates (i.e. one disk from each leaf) for each treatment. The disks were placed

in individual test tubes cooled to 5 °C in a computer-controlled, refrigerated glycol/water bath (Julabo, Seelbach, Germany). The temperature was lowered to –2 °C for 30 min before nucleation was initiated by addition of a small amount of ice. The disks were incubated at –2 °C for 60 min before measurements began. Temperature was then decreased in 1 °C steps, allowing 5 min to reach each new temperature. This was followed by incubation at the designated temperature for 30 min. Five leaf disks were removed after 30 min at each 1 °C step in temperature, and thawed in the dark for 10 min at room temperature before addition of deionized water. Intactness of leaf cells was assayed as the percentage change in electrical conductivity following dark incubation of disks in deionized water at 3 °C for 24 h before and after breakage of cells by immersion of leaf disks in liquid N<sub>2</sub> (Webb *et al.* 1996).

### Natural frost-freezing

Intact, attached snow gum leaves on acclimated plants were cryo-preserved under field conditions during a natural freeze/thaw event in mid-winter, and examined with cryo-scanning electron microscopy (SEM). Paired samples of naturally frozen and thawed leaves were collected from each of five replicate seedlings. Frozen leaves were collected before dawn when air temperature was –6 °C, and thawed leaves were collected after air temperatures had been above 0 °C for 30 min. The samples were held in cryo-storage at liquid nitrogen temperature until examined in the cryo-SEM.

### Simulated frost-freezing

Measurements were made on unacclimated plants in early autumn and on acclimated plants in late winter. Intact, attached leaves of five field-grown snow gum seedlings were frozen at natural rates (approximately 2 °C h<sup>-1</sup>) to –3 or –6 °C, two temperatures well within the natural range of autumn and winter freezing temperatures (Ball *et al.* 2002a). The 10 plants in their pots were extracted from the ground in the late afternoon on the day before measurements were made, transported to the laboratory and stored for the night in a cold room (4 °C) until the following morning. Three leaves were selected on each plant and allocated to one of three treatments: control (i.e. unfrozen), frozen or thawed. The leaves remained intact and attached until samples were cryo-frozen for analysis by cryo-SEM as described later. Samples of control leaves at room temperature were collected by cryo-fixing with cryo-pliers cooled to –196 °C. The remaining leaves were enclosed with a weight in individual, water-tight plastic bags and gently submerged in a computer-controlled, stirred bath of ethylene glycol/water at 5 °C. The bath temperature was then decreased for 30 min to –2 °C. A small amount of ice was sprinkled over the leaves in each bag to induce freezing, and the leaves were incubated at –2 °C for a further 30 min. This protocol was followed to ensure that freezing of the xylem conduits occurred under the same conditions,

although the leaves were subsequently cooled to different nadir temperatures. The temperature was then lowered in step changes of 1 °C, taking 5 min to reach the step temperature, followed by 30 min incubation at that temperature before initiating the next step change in temperature. Cooling continued until the leaves had been incubated at the designated nadir temperature of either -3 or -6 °C for 30 min. The frozen, bagged leaves were then removed from the bath and either immediately cryo-fixed or allowed to thaw in air at room temperature for up to 30 min before being cryo-fixed. The samples were held in cryo-storage at liquid nitrogen temperature until examined in the cryo-SEM.

### Cryo-fixation

Cryo-fixation (Canny 1997) was used to preserve the distribution of liquid water, ice and gas in the leaves before frost-freezing, during frost-freezing at -3 and -6 °C and after thawing for 30 min (Ball *et al.* 2002b, 2004; McCully *et al.* 2004). There is debate about use of the technique in assessing the distribution of emboli due to cavitation of vessels under tension in rapidly transpiring leaves (Cochard *et al.* 2000; Canny, McCully & Huang 2001; Kikuta & Richter 2003). However, the possibility of such artefacts was minimal in the present study in which the plants were well watered, and cryo-preservation of leaf material occurred when leaves were already frost-frozen or when transpiration would have been minimal in control and thawed leaves under dim light.

The cooling rate of tissues in liquid nitrogen is not very fast because of insulation by the evolution of boiling gas. Cryo-fixation is best achieved by some non-volatile liquid or by polished copper surfaces that have been cooled in liquid nitrogen. The snow gum leaf mid-veins were cryo-fixed by gently clamping the base of the leaf in the massive polished copper jaws of a pair of cryo-pliers at liquid nitrogen temperature (McCully *et al.* 2000). The frozen leaf was then trimmed in a bath of liquid nitrogen to a strip  $\approx$  5 mm wide by 30 mm long, which contained the mid-vein and parts of the adjoining lamina. The strip was stored in a cryovial and held at liquid nitrogen temperature until it was prepared for microscopy.

### Cryo-SEM

Details of the preparation of specimens and of the microscope settings are given in McCully *et al.* (2000). At liquid nitrogen temperature the central part of a strip of mid-vein was excised and mounted in a slot in a stub, and a transverse face was planed with a glass knife (followed by a diamond knife) at -80 °C in a cryo-microtome. The specimen was transferred to the preparation chamber of a Cambridge S360 SEM, etched for a few minutes at -90 °C to reveal cell outlines, then cooled to -160 °C before coating with gold and examined while held at -160 °C. Micrographs were captured as digital images. Unless otherwise stated, the

features presented in micrographs were found in all five replicate samples of each treatment.

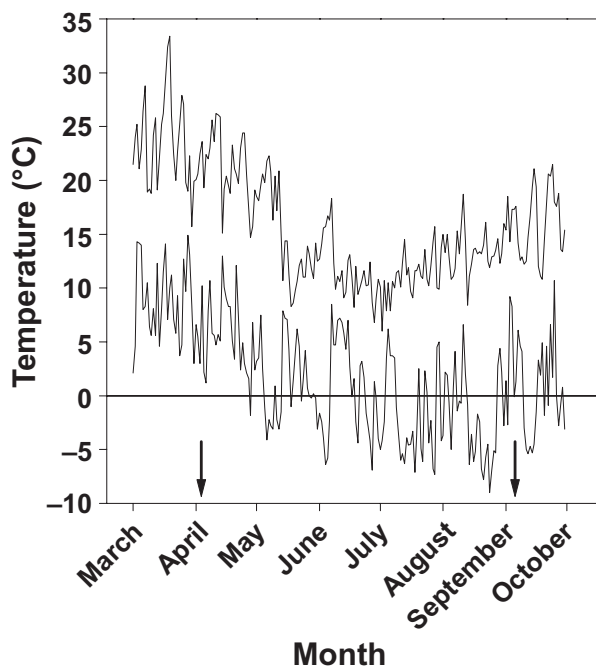
The freezing point of field-grown leaves of well-watered *E. pauciflora* seedlings is -0.5 °C. In the absence of surface ice, these leaves supercool by approximately 2.5 °C, with ice nucleation occurring at -3 °C in the mid-vein area and spreading rapidly to the rest of the leaf (Ball *et al.* 2002b). In the present study, freezing was induced by addition of ice when leaf temperatures reached -2 °C, a temperature below the freezing point and routinely used to initiate freezing in assays of freezing tolerance (Ball *et al.* 2002b). Each set of micrographs from frost-frozen and thawed leaves was scrutinized for evidence that freezing had occurred. Freezing was demonstrated by three criteria: the texture of ice in vessels, the presence of extracellular ice in mid-vein expansion zones, and in intercellular spaces in the mesophyll. A detailed explanation of the differences in ice texture due to slow frost-freezing or rapid cryo-fixing is given in results describing eutectic domains. It was clear from these criteria that freezing had occurred in all but one set of leaves incubated for 30 min at -3 °C, and those unfrozen leaves were excluded from analyses.

### Freeze-induced water uptake

Leaves were collected at sundown from field-grown plants during winter. The petioles were severed from stems by cutting under water, and the leaves were transported with the petioles in water to the laboratory. The leaves were weighed, covered with a plastic bag and kept in darkness with petioles in water at 3 °C overnight. In the morning, the bases of the petioles were recut under water, and the leaves were weighed before inserting the petioles into tightly fitting plastic tubing filled with cold water. A group of three similar leaves was fixed with double-sided tape to a temperature-controlled, brass cold plate. The leaves were covered with a metal plate and a layer of insulation to ensure even cooling with minimal frost formation on leaf surfaces. The temperature was lowered from 20 to 0 °C in 30 min and then from 0 to -3 °C in 30 min, with the covering briefly removed at -2 °C to allow sprinkling of a few ice crystals over leaf surfaces. Once leaf temperatures reached -3 °C, the rate of cooling was reduced to 2 °C h<sup>-1</sup> and cooling proceeded to -6 °C. The leaves were kept at this nadir temperature for a further 1 hour. The position of menisci in the tubes was recorded when leaf temperature was 0 °C, -3 °C and thereafter at 30 min intervals. Leaves were then quickly removed from the plate and allowed to thaw at room temperature for 10 min, after which the position of the menisci was again recorded. Uptake of water was calculated from the retreat of the menisci.

### Statistics

Genstat 5, version 4.1 (VSN, Hemel Hempstead, UK) was used to analyse data by ANOVA. As already described, the experiments were conducted on plant material in a randomized block design that satisfied the assumptions for ANOVA.



**Figure 1.** Daily maximum and minimum air temperatures from autumn through winter 2002 at Bungendore, Australia. Arrows indicate when in autumn and late winter field-grown plants were brought into the laboratory for freezing studies.

## RESULTS

### Seasonal change in air temperature at the study site

Snow gum (*E. pauciflora*) seedlings were grown under field conditions at a site within their natural habitat, where severe frosts frequently occur, particularly during winter (Fig. 1). Autumn air temperatures during the present study were mild, with no frosts recorded until late April. The frequency and severity of frosts increased with the progress of the seasons, and the lowest minimum air temperature ( $-9^{\circ}\text{C}$ ) was recorded in late August. Frosty nights continued as temperatures rose during spring.

### Seasonal change in freeze tolerance

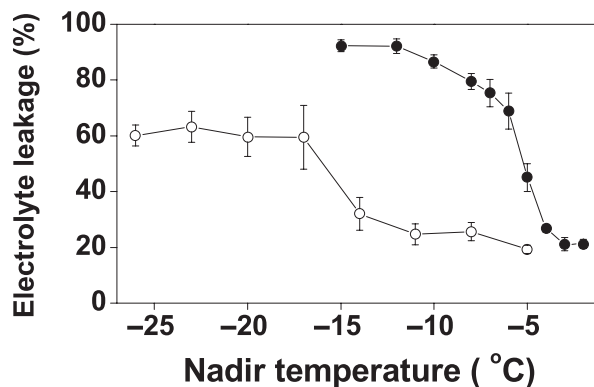
Freeze tolerance of snow gum seedlings differed between autumn and winter (Fig. 2). In mid-autumn (April), there were no obvious effects of freezing at  $-2$  or  $-3^{\circ}\text{C}$  on electrolyte leakage from leaf tissues. Electrolyte leakage began to increase at  $-4^{\circ}\text{C}$ , with 50% of maximal leakage occurring at approximately  $-5^{\circ}\text{C}$ . In contrast, the nadir temperature inducing 50% loss of electrolytes was approximately  $11^{\circ}\text{C}$  lower in winter (August) acclimated tissues ( $-16^{\circ}\text{C}$ ).

### Changes in leaf structure during a natural freeze/thaw event

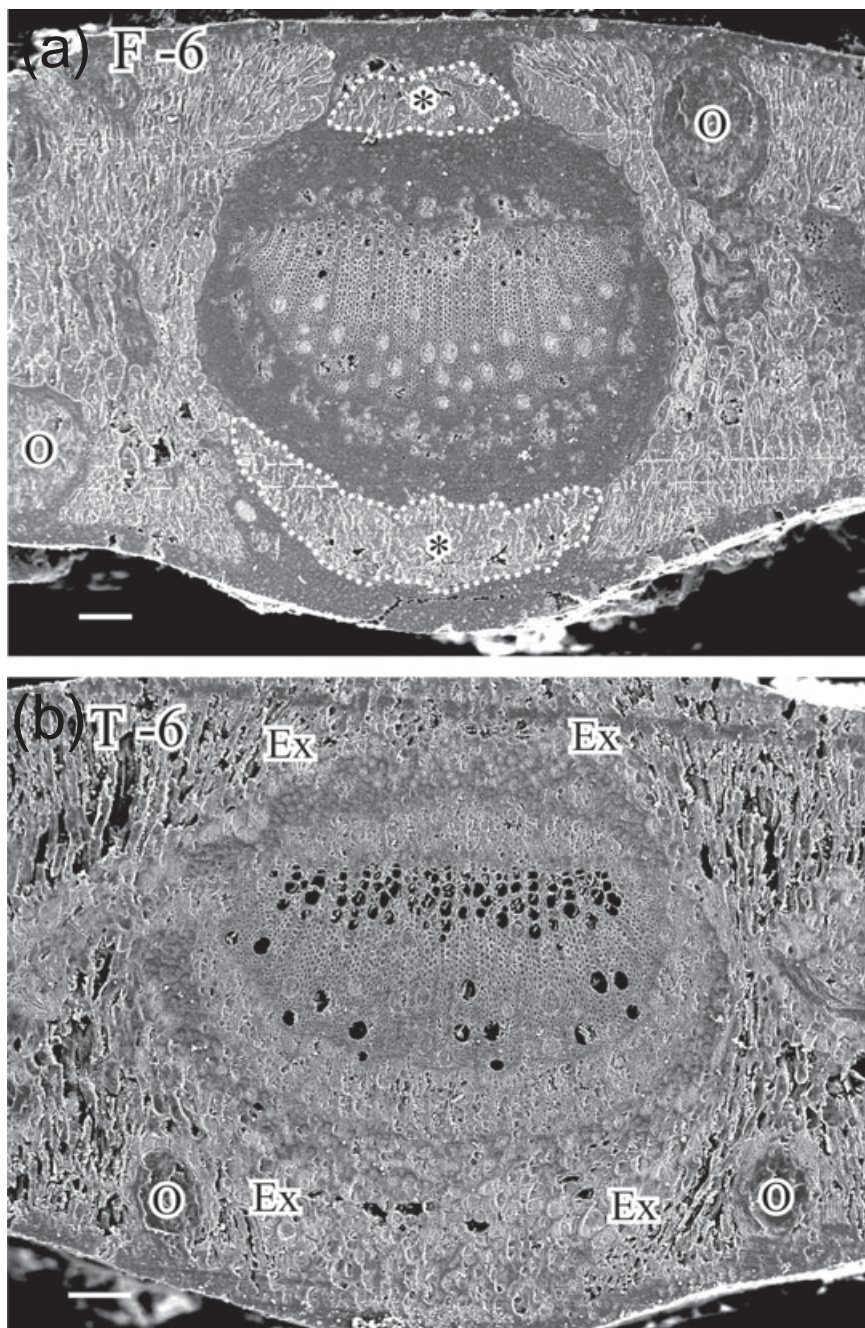
Cryo-SEM of naturally frost-frozen, winter leaves revealed considerable quantities of extracellular ice in three regions:

expansion zones in the mid-vein, the luminal spaces of vessels and tracheids in xylem tissue, and the intercellular gas spaces of the mesophyll (Fig. 3). First, in the mid-vein tissue, large blocks of ice formed in abaxial and adaxial expansion zones bounded by fibres (Fig. 3a). Adjacent living cells were shrunken in appearance, consistent with diffusion of water to sites of ice formation. Second, the luminal spaces of the primary and secondary xylem were largely filled with ice (Figs 3a & 4a). In snow gum, the primary xylem begins to lose its water contents during autumn. Once emptied, the embolized cells do not refill. However, cryo-SEM images of all five leaves examined revealed annuli of ice surrounding air-filled spaces within the lumen of primary xylem vessels (arrows in Fig. 4a). In contrast, freezing of the secondary xylem produced vessels that were completely filled with either ice or air (arrowheads in Fig. 4a). Finally, ice formed in the intercellular gas spaces of the mesophyll (Figs 3a & 5a). In the frozen leaf lamina, photosynthetic cells of the mesophyll were greatly reduced in volume, as liquid water had diffused to extracellular sites of ice formation (Fig. 5a). Most of the spaces between cells were filled with ice. Air bubbles commonly occurred in spaces beneath stomatal cavities, apparently marking paths of air forced out of the leaf by growing ice masses. [For more detailed views of frost-frozen mesophyll, see Ball *et al.* (2004)]. No ice was found within stomatal cavities, despite the proximity of ice that filled intercellular spaces in the mesophyll tissue or the presence of frost on the external leaf surface.

After thawing, the internal leaf structure appeared normal (Fig. 3b). In the mid-vein, ice blocks had melted and an archipelago of small air-filled spaces was all that marked the location of expansion zones (Ex in Fig. 3b). Figures 3b and 4c show that the vessels in the primary xylem returned to their characteristically air-filled state in thawed leaves, so the freeze-induced filling of embolized vessels in the primary xylem must have been short-lived. In contrast, effects of freezing were apparent in the secondary xylem,



**Figure 2.** Seasonal change in the freeze tolerance of field-grown snow gum (*Eucalyptus pauciflora*) seedlings as measured by electrolyte leakage of leaf tissue collected in mid-autumn and mid-winter (closed and open circles, respectively). Values are means  $\pm$  SE,  $n = 5$  independent leaf samples.



**Figure 3.** Cryo-scanning electron micrographs of snow gum (*Eucalyptus pauciflora*) leaves collected during a natural freeze ( $-6^{\circ}\text{C}$ ) and thaw event under winter field conditions. Here, and in all successive figures, freeze and thaw treatments are indicated by F and T, respectively, followed by the nadir temperature during freezing (i.e.  $-3$  or  $-6^{\circ}\text{C}$ ). Oil glands (O) are indicated in both leaves. (a) Transverse section through a frost-frozen leaf. Ice fills the intercellular spaces in the mesophyll and expansion zones in the mid-vein (\*). Most of the secondary xylem vessels contain frozen sap, and ice has developed on the interior walls of embolized vessels in the primary xylem, the dark spots indicating air surrounded by ice. These features are better illustrated in Figure 4. Scale Bar =  $100\ \mu\text{m}$ . (b) Transverse section through the mid-vein region of a thawed leaf, showing the positions of the expansion zones (Ex) that were filled with ice, and embolized vessels (black) in the primary and secondary xylem. Scale Bar =  $100\ \mu\text{m}$ .

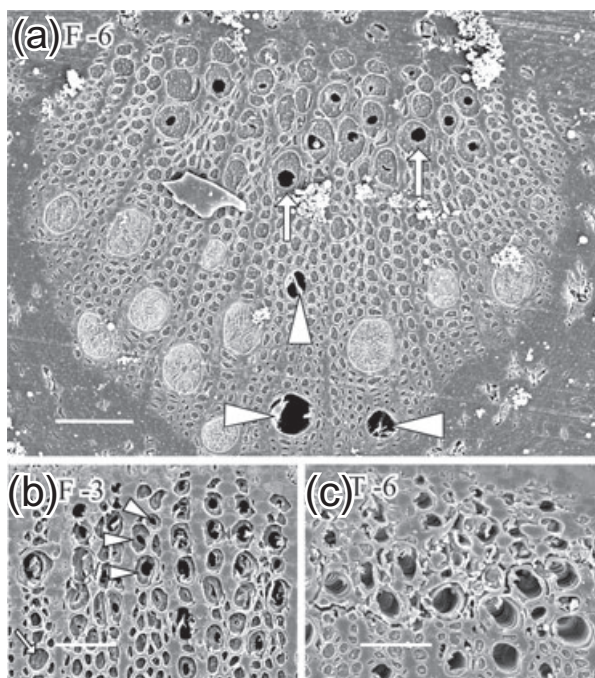
where the proportion of embolized secondary xylem vessels was greater in thawed leaves than in frozen leaves (compare Fig. 3a & b). Finally, the mesophyll cells regained their volume and shape, showing that water from thawing ice had been resorbed (Fig. 3b & 5b).

Thus, freezing of leaves during a natural frost event induced massive redistribution of water within the leaf tissues of winter-acclimated plants. The structural changes associated with freeze-induced redistribution of water were largely reversible. The only obvious legacy of the freezing event was an increase in the fraction of embolized vessels in the secondary xylem of thawed leaves.

### Effects of acclimation on changes in leaf structure during freezing and thawing

#### *Freeze/thaw of unacclimated leaves*

Freezing was further explored with simulated frosts under laboratory conditions. During autumn, there was a high degree of variability both within and among unacclimated leaves in the functional status of primary xylem vessels. Some were full of water while others were full of air when leaves were frozen, as shown in Fig. 6a. Similarly, there was considerable variability among leaves in whether or not freezing at  $-3^{\circ}\text{C}$  induced filling of embolized primary

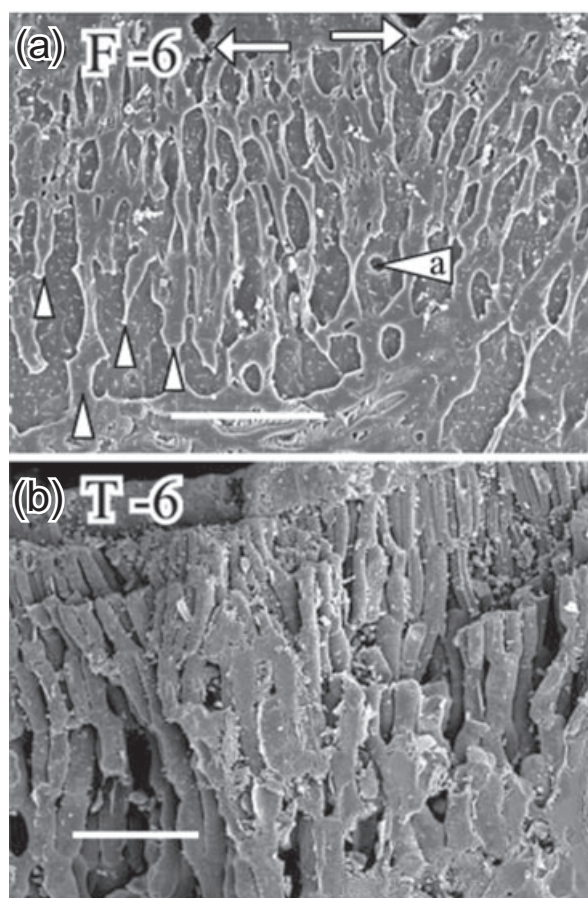


**Figure 4.** Cryo-scanning electron microscope images of vessel contents in frozen and thawed xylem. (a) Transverse section through xylem tissues in the mid-vein of a frost-frozen leaf. Primary xylem vessels that were embolized before the freezing event were filled with ice, which surrounded and compressed trapped air (arrows). Secondary xylem vessels were either embolized (arrowheads) or filled with ice that formed from the frost-freezing of water-filled conduits. Note differences in the patterns of eutectic domains in ice contained in secondary vessels as compared with that in the primary vessels and tracheids. Scale Bar = 50  $\mu\text{m}$ . (b) Freeze-induced filling of embolized vessels in the primary xylem of an intact, attached frost-frozen leaf cooled to  $-3^\circ\text{C}$ . Arrowheads point to three places where ice formed on the lumen walls of embolized vessels, trapping and compressing air that occupied the vessels before freezing. Some primary vessels were water-filled at the time of cryo-freezing (arrow). Scale Bar = 50  $\mu\text{m}$ . (c) After thawing from  $-6^\circ\text{C}$  primary xylem vessels returned to their previous gas-filled state, revealing the characteristic spiral structure of the primary walls. No water is visible on the lumen walls. In contrast, the tracheids are filled with water. Scale Bar = 50  $\mu\text{m}$ .

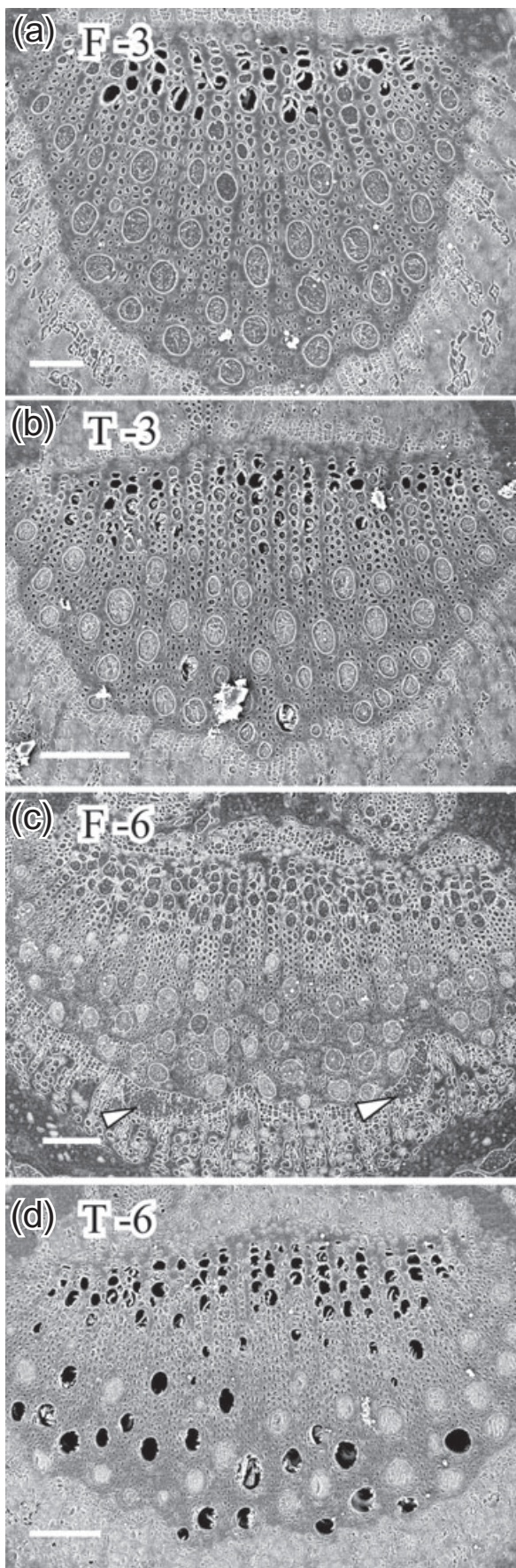
xylem vessels with ice. No such filling was apparent in the frozen leaf shown in Fig. 6a, but ice annuli developed in some other samples (Fig. 4b). As was typical of the autumn leaves, there were no embolized vessels in the secondary xylem of the frozen leaf shown in Fig. 6a, and freezing to  $-3^\circ\text{C}$  produced a relatively small amount of ice in mid-vein expansion zones (Fig. 7a). On thawing, there was little change in the condition of primary and secondary xylem vessels (Fig. 6b). The only obvious legacies of freezing were the small pools of extracellular water in the closed expansion zones (arrowheads in Fig. 7b) 30 min after thawing began.

Freezing unacclimated autumn leaves to  $-6^\circ\text{C}$  induced damage to the living tissues of the mid-vein (Fig. 6c). Intra-

cellular ice formed in the thin walled cells of the cambium (arrowheads in Fig. 6c; greater detail in Fig. 10c). Here the walls were ruptured, which evidently had allowed the ice masses to coalesce and grow, causing considerable tissue displacement. In many cells of the phloem, where thick walls contained the ice, intracellular freezing must also have occurred. Many of these cells contained calcium oxalate crystals that apparently formed during intracellular freezing (arrowheads in Fig. 10c), as previously reported (Ball *et al.* 2004). Freezing to  $-6^\circ\text{C}$  also induced formation of large ice blocks in expansion zones (compare Fig. 7a & c). On thawing, a large proportion of both primary and



**Figure 5.** Cryo-scanning electron microscope images of leaf lamina tissue. (a) Transverse section of a frost-frozen leaf. The palisade mesophyll cells (arrowheads) were shrunken, and their lost water accumulated as ice (darker background matrix) filling the enlarged intercellular air spaces. The ice often contained bubbles of gas (arrowhead a), which were remnants of the intercellular gas of the unfrozen leaf trapped by the growth of the ice. The stomatal cavities (arrows) were always air-filled and showed no signs that ice had entered the leaf through them. Scale Bar = 100  $\mu\text{m}$ . (b) Palisade mesophyll of a thawed leaf, in a region where the planed face has broken, revealing the three-dimensional appearance of the surface of the cells and intercellular spaces, as well as the cell contents revealed by the planing. The cells have absorbed the water from the melted ice and regained their turgor, while the intercellular spaces again contain air. Scale Bar = 100  $\mu\text{m}$ .



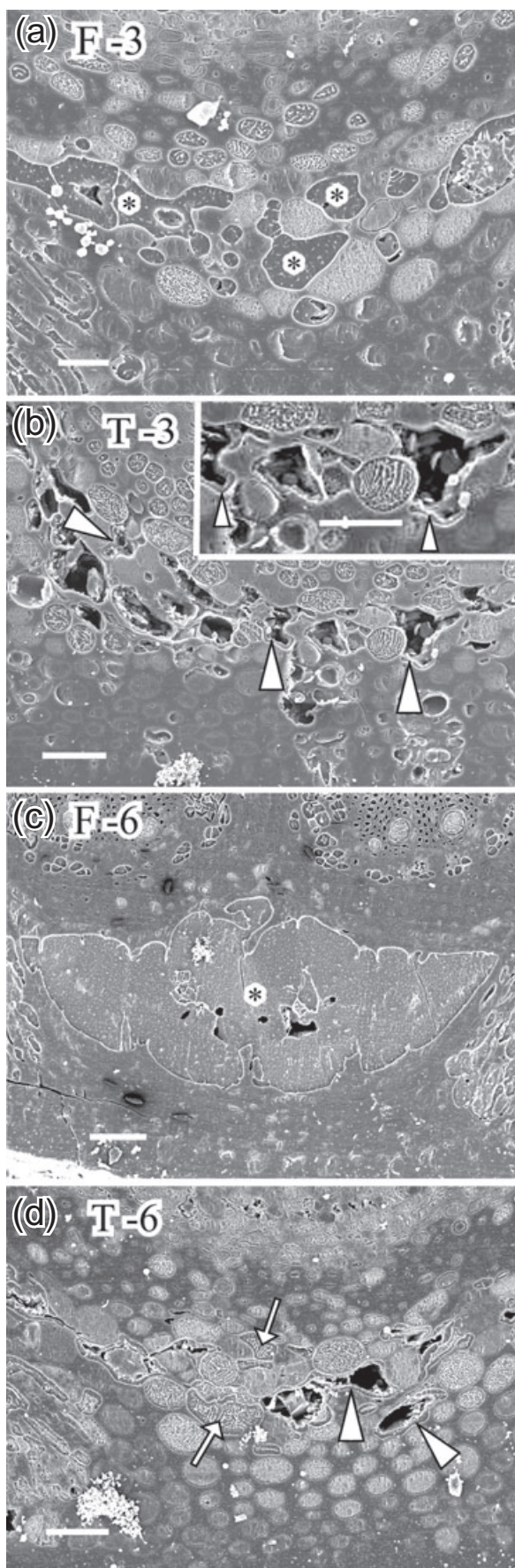
secondary xylem vessels were embolized (Fig. 6d). Most of the extracellular water derived from melting ice in expansion zones must therefore have been resorbed, but pools of water were conspicuous 30 min after thawing (arrowheads in Fig. 7d). Some parenchyma cells surrounding expansion zones had a bloated, watery appearance suggesting that they had lost cellular integrity (arrows in Fig. 7d).

#### *Freeze/thaw of acclimated leaves*

In late winter, the primary xylem was fully embolized and the secondary xylem typically contained a higher fraction of embolized vessels than in autumn leaves (Fig. 8a & c). Freezing acclimated winter leaves to  $-3^{\circ}\text{C}$  produced images with a relatively small amount of ice formed in expansion zones (Fig. 9a), and frequently induced some filling of embolized vessels in the primary xylem with ice (Fig. 8a). On thawing, primary xylem vessels returned to their previously embolized state, and the proportion of embolized vessels in the secondary xylem remained unchanged (Fig. 8b). Most water from melting ice in expansion zones was resorbed, with surrounding cells exhibiting the lacy eutectic domains of intact, healthy cells (Fig. 9b). Freezing winter leaves to  $-6^{\circ}\text{C}$  also induced filling of embolized primary xylem vessels with ice (Fig. 8c), but caused no intracellular freezing to living cells in the cambium and phloem, and large ice blocks formed in expansion zones (Fig. 9c). After thawing, the primary xylem vessels returned to their embolized state, and there were many more embolized vessels in the secondary xylem than when frozen (Fig. 8d). Thus, a major effect of the lower nadir temperature was an increase in the proportion of embolized vessels after thawing. Finally, water from the melting of the large ice blocks was largely resorbed, with surrounding cells revealing no signs of damage (Fig. 9d). Note, for example, the flattened, shrunken fibres in the frozen leaf (Fig. 9c) and the rounded, rehydrated fibres in the thawed leaf (Fig. 9d).

These characteristics observed in field-grown, acclimated, winter leaves frozen to  $-6^{\circ}\text{C}$  under laboratory conditions were fully consistent with those observed in a natural freeze to  $-6^{\circ}\text{C}$  under field conditions (Figs 3–5). This gives us confidence that the rates of freezing and thawing in the experimental treatments did not induce artefacts. The images clearly show that the occurrence of emboli in

**Figure 6.** Cryo-scanning electron microscope images of the effect of nadir freezing temperature on the distribution of air and liquid water in xylem vessels of intact, attached leaves of unacclimated *Eucalyptus pauciflora* in mid-autumn. The images are transverse sections through mid-veins of leaves that were cryo-preserved after slow frost-freezing and after thawing for 30 min. (a) Mid-vein during freezing at  $-3^{\circ}\text{C}$ . Note gas-filled (black) primary xylem vessels. Scale Bar =  $50\ \mu\text{m}$ . (b) Mid-vein after thawing from  $-3^{\circ}\text{C}$ . Scale Bar =  $100\ \mu\text{m}$ . (c) Mid-vein during freezing at  $-6^{\circ}\text{C}$ . Arrowheads indicate regions of cambium disrupted by ice. Scale Bar =  $100\ \mu\text{m}$ . (d) Mid-vein after thawing from  $-6^{\circ}\text{C}$ . Scale Bar =  $100\ \mu\text{m}$ .



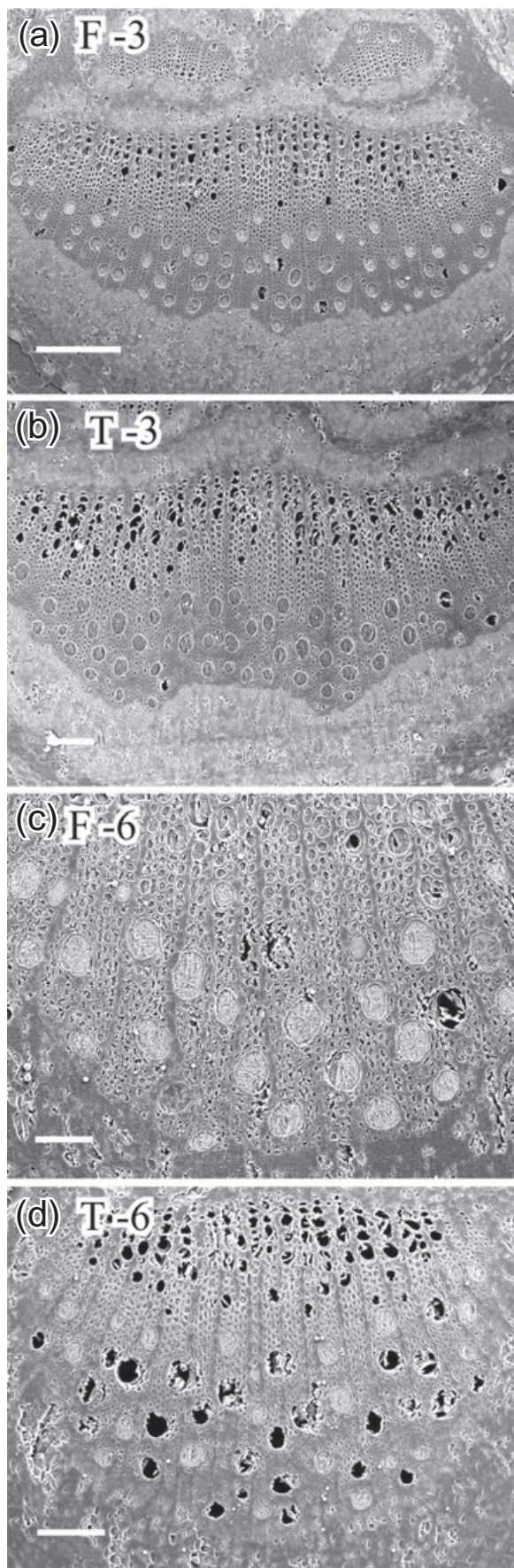
secondary vessels of thawed leaves depended on the nadir temperature during freezing, and these data are quantitatively analysed below.

### Eutectic domains in ice and the status of water in xylem tissues

The cryo-SEM micrographs can provide some insight into the history and composition of liquids that have been cryo-fixed, because the textural patterns caused by eutectic domains in ice can vary with both rates of freezing and the solute contents of the water that freezes (McCully *et al.* 2000; Yamada *et al.* 2002). As is the case with granite and basalt, the different cooling rates produce different crystal sizes and therefore different textures. When a sample is cooled slowly, ice crystals form at a relatively small number of nuclei and have time to form large, visible crystals. Meanwhile the remaining unfrozen phase is concentrated. In some cases, it reaches its eutectic point, that is, the point on a phase diagram of maximum solution concentration and maximum freezing point depression. Below this temperature, ice and crystals of solutes may coexist and ice crystals usually stop growing. This produces a characteristic coarse texture (like granite) in the eutectic domains visible in a micrograph. In contrast, cooling at rapid rates, such as are achieved with cryo-pliers, produces substantial supercooling; freezing nucleates at a much greater number of sites, producing much smaller crystals (like basalt) and a corresponding fine texture to the eutectic domains in the ice. These effects were demonstrated experimentally by McCully *et al.* (2000), who showed that slowly frozen solutions in xylem vessels have thicker, coarser eutectic domains than rapidly frozen solutions, and that the densities of the domains indicate the concentration of solutes in solution.

Unfrozen, control leaves at room temperature were rapidly cryo-frozen to preserve the distribution of water and were then examined with cryo-SEM (Fig. 10a). Most of the secondary vessels and all of the tracheids were filled with liquid water at the time of freezing. The ice produced by rapid cryo-freezing of liquid water was similar in appearance in both secondary vessels and tracheids. These ice

**Figure 7.** Cryo-scanning electron microscope images showing the effect of nadir freezing temperature on the distribution of ice and liquid water in mid-vein expansion zones of intact, attached leaves of unacclimated *Eucalyptus pauciflora* in mid-autumn. The images are transverse sections through mid-veins of leaves that were cryo-preserved during slow frost-freezing and after thawing for 30 min. Symbols indicate extracellular ice masses during freezing (\*), extracellular liquid water after thawing (arrowheads) and freeze-damaged cells (arrows). (a) Expansion zone during freezing at  $-3^{\circ}\text{C}$ . Scale Bar =  $25\ \mu\text{m}$ . (b) Expansion zone after thawing from  $-3^{\circ}\text{C}$ . (Inset) Detail showing residual water not completely absorbed from the expansion zone space. Bars =  $50\ \mu\text{m}$ . (c) Lower expansion zone during freezing at  $-6^{\circ}\text{C}$ . Scale Bar =  $50\ \mu\text{m}$ . (d) Lower expansion zone after thawing from  $-6^{\circ}\text{C}$ . Scale Bar =  $50\ \mu\text{m}$ .



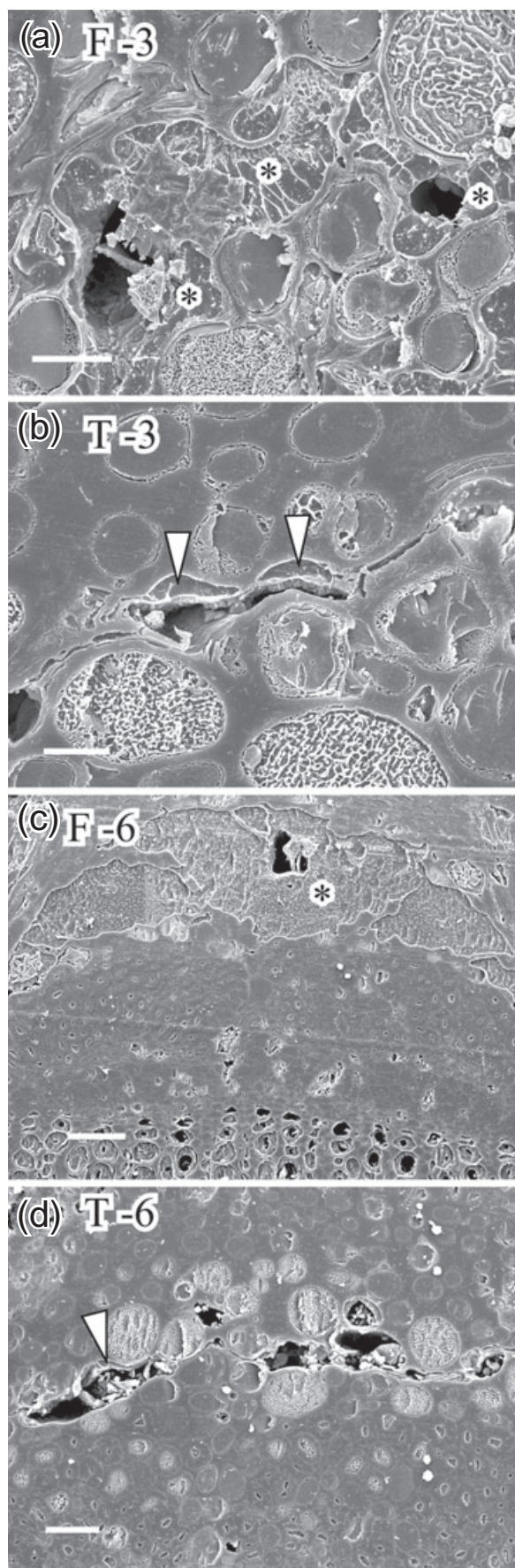
masses were characterized by a sparse population of small, mostly globular, eutectic domains, indicating a low concentration of solutes in the xylem sap, and appeared dark (non-electron emissive) in the micrographs.

Slow frost-freezing of unacclimated leaves to  $-6^{\circ}\text{C}$  caused greater diversity in the appearance of ice in water-filled xylem conduits (Fig. 10b). The appearance of ice in the tracheids of these slowly frost-frozen leaves was similar to that in rapidly cryo-frozen control leaves, indicating low solute levels in water filling these conduits. In contrast, the water-filled secondary vessels of frost-frozen leaves were not all the same. Ice in the cryo-fixed vessels was of two types: dark (non-electron emissive) ice with few eutectic domains similar to that found in cryo-fixed control (i.e. unfrozen) leaves (arrow in Fig. 10b and vessels of Fig. 10a, respectively), and paler (electron emissive) ice with coarse patterns of eutectic domains (most vessels in Fig. 10b). We take this as evidence that the water in vessels with dark ice was rapidly frozen by cryo-fixation (i.e. the sap was still liquid before fixation), while the vessels with pale ice had been slowly frost-frozen before cryo-fixation. Thus, a small proportion of secondary vessels in a freezing leaf contained unfrozen liquid water even after leaf temperature had been  $-6^{\circ}\text{C}$  for 30 min. Finally, a few secondary vessels were filled with gas (Fig. 10c). In these vessels, the luminal walls were dry and, unlike in the primary xylem, there was no evidence of freeze-induced filling of embolized secondary vessels with ice.

In unacclimated leaves that had been thawed after frost-freezing to  $-6^{\circ}\text{C}$ , a third state of the original solution could be distinguished in the cryo-fixed tissue. The eutectic domains were much denser and coarser than in either the control or frost-frozen leaves and were present in the sap of both vessels and tracheids (Fig. 10d). This is evidence of higher solute content in the sap. These solutes must have been released from living cells killed by frost-freezing, and then diffused into the liquid contents of the xylem. Had there been no solute leakage, the ice formed during cryo-fixing of thawed leaves should have resembled that of the control leaves (Fig. 10a).

Finally, there was a fine texture in ice masses that formed slowly during frost-freezing as liquid water diffused through walls to sites of ice formation in the intercellular spaces of the mesophyll (Fig. 5a), the expansion zones of the mid-vein (Figs 7a, c & 9a, c) and the luminal space of embolized vessels in the primary xylem (Fig. 4a & b). The

**Figure 8.** Cryo-scanning electron microscope images showing the effect of nadir freezing temperature on the distribution of air and liquid water in xylem vessels of intact, attached leaves of acclimated *Eucalyptus pauciflora* in late winter. The micrographs show transverse sections through mid-veins of leaves that were cryo-preserved during slow frost-freezing and after thawing for 30 min. Embolized vessels appear black. (a) Mid-vein during freezing at  $-3^{\circ}\text{C}$ . Scale Bar =  $200\ \mu\text{m}$ . (b) Mid-vein after thawing from  $-3^{\circ}\text{C}$ . Scale Bar =  $100\ \mu\text{m}$ . (c) Mid-vein during freezing at  $-6^{\circ}\text{C}$ . Scale Bar =  $50\ \mu\text{m}$ . (d) Mid-vein after thawing from  $-6^{\circ}\text{C}$ . Scale Bar =  $100\ \mu\text{m}$ .



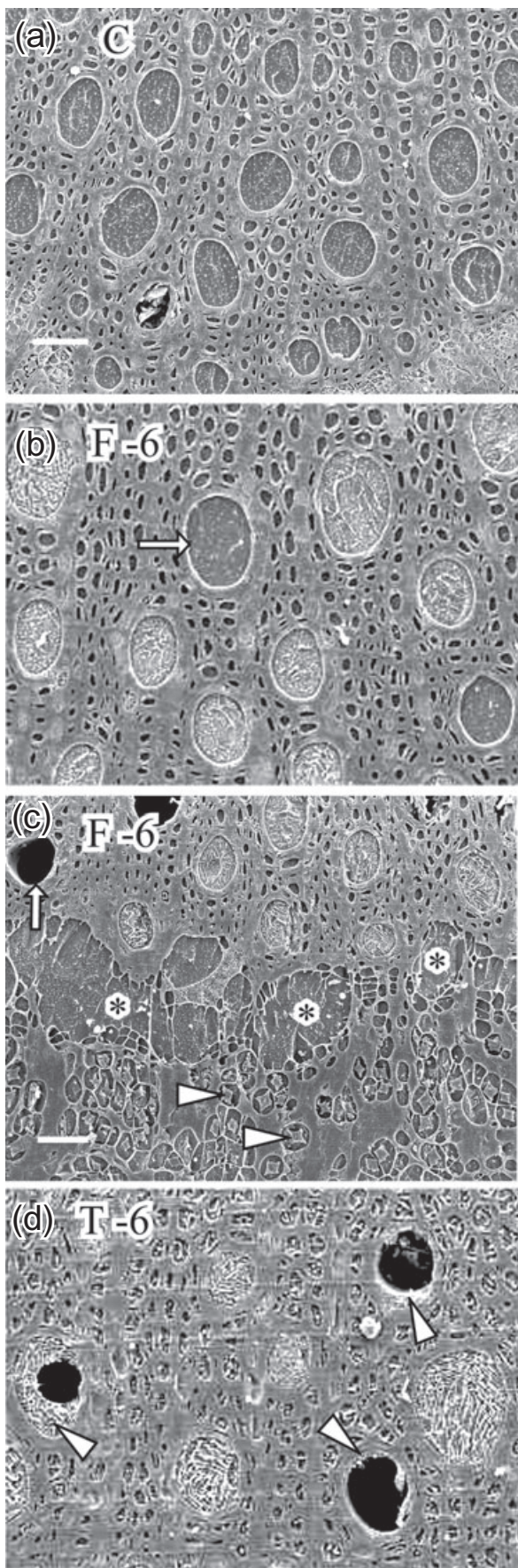
texture of these ice masses resembled that of the rapidly cryo-frozen liquid water described earlier (Fig. 10a), with a sparse population of small, mostly globular, eutectic domains indicating a low concentration of solutes in the freezing water, and a dark (non-electron emissive) appearance in the micrographs.

### Dependence of freeze/thaw-induced embolism on nadir temperature

The percentage of embolized secondary xylem vessels was determined from electron micrographs of mid-vein tissue cryo-preserved before freezing, during freezing and after thawing of intact, attached leaves cooled to nadir temperatures of either  $-3$  or  $-6$  °C. Counts of embolized vessels were restricted to the secondary xylem because primary xylem vessels naturally become filled with air as fully expanded leaves of snow gum age. The results showed that the extent of embolism on thawing increased significantly ( $P < 0.005$ ) with decrease in nadir temperature during freezing (Fig. 11).

In autumn, an average of  $3 \pm 1\%$  of secondary vessels were embolized in control leaves collected at room temperature, before freezing. This native or background level of embolism significantly ( $P < 0.01$ ) increased to  $27 \pm 9\%$  in control leaves collected in late winter. In both autumn and winter, freezing leaves to either  $-3$  or  $-6$  °C caused no significant increase in the percentage of embolized vessels in frozen leaves relative to control, unfrozen leaves. Similarly (Fig. 11c), there was no significant increase in the percentage of embolized vessels in thawed leaves that had been frozen to  $-3$  °C. In contrast, the percentage of embolized vessels increased significantly ( $P < 0.005$ ) in thawed leaves that had been frozen to  $-6$  °C, averaging 24 and 43% in autumn and winter leaves, respectively. However, we observed no significant ( $P = 0.671$ ) interaction between season and nadir temperature. Thawing after freezing to  $-6$  °C induced a similar increase in the percentage of embolized vessels in leaves during either autumn or winter, with the difference between seasons in absolute values being due to differences in background levels of embolism. These results showed that freeze/thaw-induced embolism was dependent on the nadir temperature during freezing and was not

**Figure 9.** Cryo-scanning electron microscope images showing the effect of nadir freezing temperature on the distribution of ice and liquid water in mid-vein expansion zones of intact, attached leaves of acclimated *Eucalyptus pauciflora* in late winter. The micrographs show transverse sections through mid-veins of leaves that were cryo-preserved during slow frost-freezing and after thawing for 30 min. Symbols indicate extracellular ice masses during freezing (\*) and extracellular liquid water after thawing (arrowheads). (a) Lower expansion zone during freezing at  $-3$  °C. Scale Bar = 20  $\mu\text{m}$ . (b) Lower expansion zone after thawing from  $-3$  °C. Scale Bar = 20  $\mu\text{m}$ . (c) Mid-vein during freezing at  $-6$  °C. Scale Bar = 50  $\mu\text{m}$ . (d) Lower expansion zone after thawing from  $-6$  °C. Scale Bar = 25  $\mu\text{m}$ .



strongly dependent on season (and hence acclimation state).

### Dependence of extracellular ice formation on nadir temperature

The electron micrographs showed formation of large ice blocks in expansion zones located in fibrous regions of the mid-vein (Figs 7a, c & 9a, c). The area covered by ice in these expansion zones was measured and compared with the total area of the mid-vein. There were no significant effects of season on the size of ice blocks. However, the average area occupied by ice blocks increased significantly ( $P < 0.01$ ) from 3 to 14% of the total mid-vein area of frozen leaves with decrease in nadir temperature from  $-3$  to  $-6$  °C, respectively.

### Freeze-induced water uptake by leaves

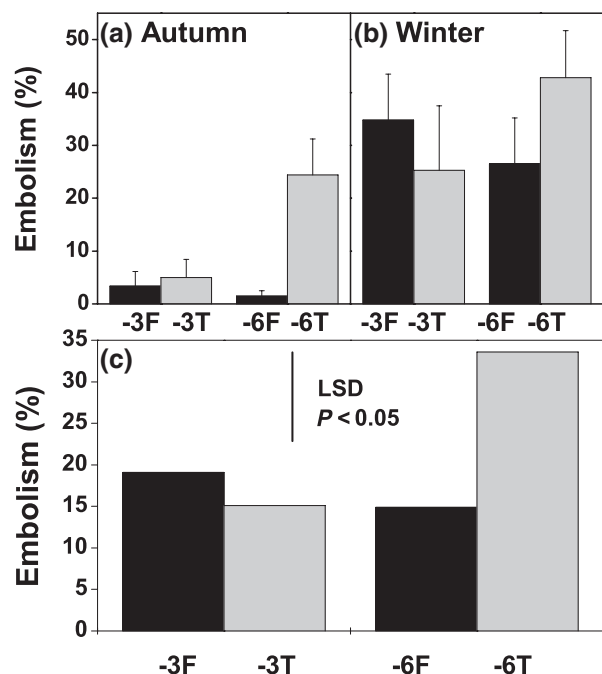
What is the source of water that forms ice in the expansion zones? Most must be drawn from the symplast and from apoplastic wall spaces of adjacent cells and tissues. However, water could also be imported into a freezing leaf if even a few xylem vessels were to remain unfrozen, as suggested by the appearance of eutectic domains in a few xylem vessels. Indeed, intact detached leaves absorbed water during freezing as leaves were slowly cooled in the dark to  $-6$  °C (Fig. 12). Water uptake increased with decrease in nadir temperature to a maximum rate of  $20 \mu\text{L h}^{-1}$ , which was sustained for 90 min after leaf temperature declined below  $-5$  °C. The frozen leaves were removed from the cold plate and allowed to thaw at room

**Figure 10.** Cryo-scanning electron microscope images showing the variation in the appearance of secondary xylem vessels in the mid-vein of an intact, attached leaf of unacclimated *Eucalyptus pauciflora* cryo-preserved before, during and after frost-freezing to  $-6$  °C in mid-autumn. (a) Unfrozen, control leaves. Tracheids and most vessels were filled with liquid water that upon rapid cryo-freezing contained scattered eutectic domains that appear as white specks in the grey ice. An embolized vessel containing some debris from the planing knife is visible in the lower left. Scale Bar =  $25 \mu\text{m}$ . (b & c) Frost-frozen leaf. (b) The ice in most vessels contains a dense pattern of eutectics apparently formed during slow, frost-freezing of the leaf. However, eutectic patterns in a few vessels (arrow) resemble that in the unfrozen vessels of control leaves as shown in (a). Scale Bar =  $25 \mu\text{m}$ . (c) Some secondary xylem vessels were embolized and filled with gas (arrow) while slow frost-freezing of water-filled vessels produced ice with dense populations of eutectic domains. Intracellular freezing occurred in thin-walled cells of the cambium, where the expanding ice broke the walls and coalesced into a large ice mass (\*), which caused substantial tissue displacement. Intracellular ice also formed in parenchyma cells of the phloem, where it led to formation of calcium oxalate crystals (arrowheads), but was largely contained by strong cell walls. Scale Bar =  $25 \mu\text{m}$ . (d) 30 min after thawing. Cryo-preservation of water-filled vessels and tracheids reveals dense patterns of eutectic domains. Some vessels were embolized or contained macro-bubbles (arrow heads). Scale Bar =  $25 \mu\text{m}$ .

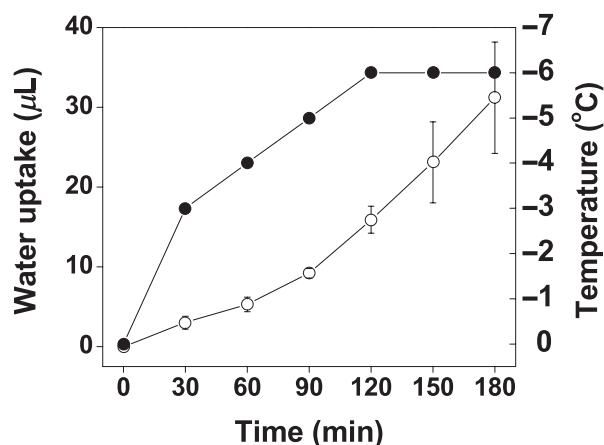
temperature. Within minutes, the thawing leaves rapidly absorbed an additional  $11.0 \pm 4.7 \mu\text{L}$  for a total average uptake of  $45.7 \pm 8.2 \mu\text{L}$ . This freeze/thaw-induced water uptake increased the average fresh weight of the leaves by  $3.4 \pm 0.6\%$ , raising the percent water content of the leaves above the fully saturated level of  $65\%$  by  $5.2 \pm 0.9\%$ .

## DISCUSSION

The results of the present study show that freeze/thaw-induced embolism depends on the nadir temperature during freezing. There is no evidence of freeze/thaw-induced embolism in leaves that had been frozen to  $-3^\circ\text{C}$ . The cryo-SEM micrographs of frozen leaves clearly show eutectic domains in the ice that formed from freezing of liquid water contained in xylem conduits. We call these eutectic domains because they are not simply air bubbles, but an unknown mixture of gases, solutes and unfrozen water. It follows that neither the expulsion of air during freezing nor the rate of thawing induced cavitation. It is possible that expansion of hydrated walls surrounding frozen vessels may have exerted sufficient pressure to redissolve air bubbles in the thawing, degassed water (Hammel 1967; Robson *et al.* 1988; Tyree & Yang 1992) when leaves had been frozen to  $-3^\circ\text{C}$ . However, the greater occurrence of embolized vessels in leaves frozen to  $-6^\circ\text{C}$  and then thawed requires a different explanation.



**Figure 11.** Percentage of embolized secondary xylem vessels in frozen or thawed mid-veins of intact, attached leaves of snow gum (*Eucalyptus pauciflora*) subjected to nadir temperatures of either  $-3$  or  $-6^\circ\text{C}$  during autumn or late winter. Values in panels (a) and (b) are means  $\pm$  SE,  $n = 5$  for the full experiment. Values in panel (c) show the significant ( $P < 0.005$ ) effects of nadir temperature on the percentage of embolized vessels averaged over both seasons. Bar indicates the least significant difference between means.



**Figure 12.** Freeze-induced water uptake by leaves. Uptake of water through the petiole ( $\circ$ ) and change in leaf temperature ( $\bullet$ ) with time for detached, fully expanded leaves of snow gum (*Eucalyptus pauciflora*). Values are means  $\pm$  SE,  $n = 3$ .

## Does freeze damage to living cells increase embolism on thawing?

One possibility is that the lower nadir temperature may have induced damage to living cells, which in turn contributed to the greater occurrence of embolism on thawing (Pockman & Sperry 1997; Martinez-Vilalta & Pockman 2002). The snow gum, like other temperate evergreens, undergoes seasonal changes in acclimation to freezing which enable cells and tissues to tolerate, within species-specific limits, freeze-induced dehydration followed by rapid rehydration during thawing. Here an  $11^\circ\text{C}$  shift in the nadir temperature inducing 50% loss in electrolytes (Fig. 3) was consistent with seasonal change in growth temperatures (Fig. 2). In autumn, when acclimation to freezing was low, the leaves tolerated freezing to  $-3^\circ\text{C}$  but showed irreversible damage at  $-6^\circ\text{C}$ , as in previous studies (Ball *et al.* 2004). The damage was due to intracellular freezing and loss of osmotic responsiveness during thawing, consistent with destabilization of the plasma membrane as established in studies on other species (Steponkus & Lynch 1989). In such freeze-damaged tissues, the death of phloem and xylem parenchyma cells that play a role in recovery from cavitation (Canny 1997; Salleo *et al.* 2004) would have long-term effects on the maintenance of hydraulic function, but it is not clear how their death would increase the fraction of embolized conduits on thawing. Martinez-Vilalta & Pockman (2002) suggested that the release of cellular material from lysed cells might provide nuclei for cavitation in thawed vessels under tension. In the present study, large bubbles were found in vessels laden with solutes that had apparently leaked from ruptured cells (Fig. 10d). However, we conclude that these large bubbles failed to cavitate the vessels where tension in the xylem was reduced with loss in osmotic gradients generated by living cells. In this way, damage caused to living cells by freezing might reduce, not increase, the propensity for cavitation during thawing. Whatever the effects of cell death, freezing to  $-6^\circ\text{C}$

induced the same fractional increase in embolized vessels in thawed leaves regardless of season and hence also of acclimation state (Fig. 11). Thus, the increase in freeze/thaw-induced embolism with decrease in nadir temperature is not a consequence of effects on living cells, and is more related to physical than biological processes.

### Heterogeneous hydration and the enhancement of freeze/thaw-induced embolism

According to the Young–Laplace equation, the increase in vulnerability to cavitation in the absence of differences in the surface tension of water or the size distribution of bubbles requires an increase in the tension of the xylem sap. This tension could increase with temperature-dependent dehydration during freezing. As shown in the present study, the distribution of water in a freezing leaf is very different from that in a fully hydrated, unfrozen leaf, and the differences become greater as the temperature of the ice decreases. Although freezing of water within xylem vessels effectively immobilizes that water, unfrozen water in solute rich cells and in walls surrounding frozen vessels (Wolfe, Bryant & Koster 2002) can diffuse down chemical potential gradients to distant sites of ice formation, dehydrating the cells and walls and lowering the chemical potential of water remaining in them (Yoon, Pope & Wolfe 2003). Such freeze-induced redistribution of water and its effect on the chemical potential of liquid water in partially dehydrated walls and living cells may well produce the tension that causes cavitation in xylem vessels during both the freezing and the thawing of leaves.

### Freeze-induced filling of embolized primary xylem vessels with ice

The results also reveal that freezing in vein tissue is more complex and diverse than expected. In acclimated leaves, freezing begins in apoplastic water, with potential nucleation sites occurring on wet surfaces of walls facing gas spaces and in the water-filled luminal spaces of xylem conduits. Walls exposed to gas spaces include those of mesophyll cells bordering intercellular gas spaces in the leaf lamina, and those of fibres and parenchyma cells bordering expansion zones in veins. A third location is the inner wall of embolized xylem conduits such as the primary xylem vessels in snow gum. These vessels remain embolized once cavitation occurs during normal seasonal progression from late summer to winter. However, hydration of the walls would reflect the water status of the leaf, and the walls would be wet if leaf water content were high. During frost events, ice nucleates on the wet luminal surface of these embolized vessels, possibly during condensation of water vapour on the wall as the leaf cools below freezing temperatures. The ice forms a hollow cylindrical shape, appearing like an annulus in cross section, as water diffuses from the wall to sites of ice formation on the wall surface. As the ice cylinder grows inwards from the wall, the gas volume at the centre of the embolized vessel becomes smaller, compress-

ing the trapped air that previously filled the vessel. The surface between ice and air changes as the ice mass grows, with sublimation of ice and condensation of vapour reworking the interface, producing a smooth surface of minimal area relative to the volume of trapped air. In this way, the trapped, pressurized air would tend to occupy a spheroidal or cylindrical volume surrounded by ice. Such ice formation was predicted by O'Malley & Milburn (1983) to occur in the embolized fibres of sugar maple, with implications for the generation of positive pressure in stems on thawing. The freeze-induced filling of embolized vessels with ice may explain previous observations (Sucoff 1969; Robson *et al.* 1988; Utsumi *et al.* 1998) that were interpreted as bubbles trapped by exclusion of air during centripetal ice formation in water-filled vessels.

### Freezing, supercooling and cavitation in the secondary xylem of freezing leaves

The behaviour of water-filled vessels during freezing in the vein is more variable than expected. Based on infrared thermography, a previous study (Ball *et al.* 2002b) showed that freezing in intact, attached snow gum leaves started and propagated rapidly through veins, presumably in large water-filled vessels. The present study reveals greater complexity. Not all vessels froze, implying that ice nucleation occurs independently in different vessels. This has major implications both for transport of water into a freezing leaf and for the vulnerability of supercooled vessels to cavitation while freezing occurs elsewhere within a leaf.

Vessels whose contents remained supercooled would have been major conduits for water uptake during freezing in leaves. The water thus imported would have contributed to temperature-dependent growth of ice masses in the expansion zones of veins and the intercellular gas spaces of mesophyll tissues, and may account for previous observations of unexpectedly prolonged durations of freezing in intact, attached leaves of snow gum (Ball *et al.* 2002b). It is noteworthy that the average rate of water uptake by freezing leaves reached a maximum of  $20 \mu\text{L h}^{-1}$  as temperature declined from  $-3$  to  $-6$  °C, with an associated drop in the water potential. The implication is that the rate of uptake was limited by the size and number of unfrozen conduits and by the rate of diffusion through the increasingly viscous aqueous phases of cell walls and symplast. This could have major implications for freeze/thaw-induced cavitation in stems of evergreens. As leaves cool at faster rates than stems, leaves could freeze ahead of stems and withdraw considerable amounts of water from them. In the present study, for example, 10 leaves freezing at  $-5$  °C could potentially withdraw  $200 \mu\text{L h}^{-1}$  from an unfrozen stem. This could induce cavitation in the stem if the supply of water to the stem were limited by frozen or drought-dried soil, or by hydraulic conductance of the roots at low temperatures. Freeze-induced water uptake has been reported in other freeze-tolerant species (Johnson & Tyree 1992), but its potential role in freeze-induced embolism of overwintering evergreens requires further study.

Some supercooled vessels may have cavitated. Unfrozen vessels would become subject to increasing tension as freezing elsewhere caused dehydration of tissues, leading to lower water potentials. As tension increased with decrease in freezing temperatures, so too would the probability of cavitation in unfrozen vessels. Vessels embolized in this way would be unlikely to fill with ice like those in the primary xylem. Upon cavitation, water menisci would retreat into the walls. The unfrozen water would diffuse towards distant sites of ice growth, dehydrating the wall and hence lowering the water potential of unfrozen water in the wall. This would reduce the probability of ice growth on luminal walls of vessels that cavitated under freeze-induced tension. These embolized vessels would be expected to remain filled with gas while freezing progressed elsewhere in the vein.

Although it is possible that supercooled vessels cavitated while freezing occurred elsewhere within the vein, no significant differences were found between the average percentage of embolized vessels measured in veins before or during frost-freezing. This suggests that most of the secondary vessels that were embolized when frost-freezing leaves were cryo-preserved had accumulated before the frost-freezing event. The increase in the proportion of embolized vessels in unfrozen leaves from 3% in autumn to 27% in late winter may have been due to increasing exposure to extreme frost events, with embolized vessels accumulating as successive frost events produced more of them.

Regardless of the mechanism, the occurrence of embolized secondary vessels in unfrozen leaves invites the question: why did embolized vessels in the secondary xylem not fill with ice as did those in the primary xylem when leaves were frozen to  $-6^{\circ}\text{C}$ ? The differences may reflect effects of wall structure on the movement of water through walls. The most permeable part of a vessel wall is the surviving primary wall, which must be many times more capable of conducting water by flow or diffusion than those parts of the wall reinforced by secondary wall substances. In the vessels of the primary xylem, most of the surface consists of primary wall, with only isolated strips of secondary wall in hoops or helices. However, in secondary vessels, most of the wall surface is secondary wall, interspersed by small areas of primary wall in pits. Thus, the area available for water exchange is much greater in the primary vessels than in the secondary vessels, which possibly explains the difference in ice accumulation between embolized vessels in primary and secondary xylem.

### *Heterogeneous hydration during freezing and thawing*

As freezing progresses, the distribution of water becomes increasingly different from that in a fully hydrated, unfrozen leaf. In the present study, by the time thawing began after freezing to  $-6^{\circ}\text{C}$ , primary xylem vessels that had previously been embolized, had partly filled with ice surrounding and compressing trapped air, whereas secondary xylem vessels that had previously been embolized remained filled

with gas. Secondary xylem vessels that had previously been filled with liquid water were either frozen, supercooled or embolized; living cells and walls were dehydrated; blocks of ice occupied expansion zones; and leaves contained as much as 3–5% more water than at the onset of freezing. On thawing, the gradients in water potential which had driven the redistribution of water within the freezing leaf would change direction. Liquid water, produced by melting ice, must diffuse down these gradients through vessels, walls and symplasm to recently dehydrated regions of low water potential. In mesophyll tissues, where ice forms on the exterior walls of the cells as they undergo cytorrhysis, the path of diffusion from the melting ice to the original apoplastic and symplastic sources of the water is short, and water would be rapidly resorbed by the walls and cells from whence it came. However, the path of diffusion for water from ice melting in expansion zones to the dehydrated walls and living cells of the xylem tissue is much longer. Such diffusion takes time, and as the resistance to water movement through extravascular tissue can be very high (Gasco, Nardini & Salleo 2004), hydration of the vein tissues can be highly variable during thawing. Indeed, despite containing a surplus of water on a bulk leaf basis, thawing leaves rapidly absorbed 10  $\mu\text{L}$  of water through their petioles during the first minutes of thawing. Even after 30 min at room temperature, evidence of spatial variation in hydration was apparent. Extracellular water in expansion zones suggested that the surrounding, plump cells were saturated with water while the shrunken appearance of some clusters of cells farther away implied that they had not yet regained turgor. This heterogeneity in hydration has major implications for dependence of freeze/thaw-induced embolism on nadir temperature. If cell walls were to become dehydrated during freezing of xylem tissue, then with rapid thawing of the tissue, the partially dehydrated walls could exert sufficient tension on xylem water to induce cavitation of the vessels. As dehydration would be more severe with lower nadir temperature during freezing, the probability of a tension-induced cavitation on thawing would also increase with decrease in nadir temperature.

### CONCLUSION

The phrase, 'freeze-induced embolism', is commonly used to denote the occurrence of gas-filled vessels following a freeze/thaw event. However, it is apparent from the complexity revealed in the present study that this designation is misleading. Nucleation of freezing in water-filled vessels was variable, with some vessels supercooling to much lower temperatures than others. We found no evidence that the freezing of xylem water *per se* induced cavitation either during freezing or during thawing. Cavitation on thawing required cooling to a lower temperature during freezing than is required to freeze water in the vessels. We also found no evidence of centripetal ice formation in freezing of water-filled conduits; eutectic domains were dispersed when water froze rapidly and clustered along numerous channels formed between dendritic sheets of developing ice

crystals when water froze at slow natural rates. In contrast, freezing induced the centripetal filling of embolized primary vessels with ice formed by freezing of water withdrawn from the walls and neighbouring tissues. Most cavitations occurred during thawing, but in apparent response to effects of freeze-induced dehydration of walls and tissues on the tension of liquid water in thawing xylem conduits. This might be more accurately termed dehydration-induced cavitation on thawing (DICoT), a process that would be highly dependent on the nadir temperature during freezing.

## ACKNOWLEDGMENTS

The authors thank Wayne Phippen for expert technical assistance and Paul Sillis for permission to conduct experiments on his farm.

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Received 9 March 2005; received in revised form 22 June 2005; accepted for publication 6 August 2005