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Vessel contents of leaves after excision: a test of the Scholander assumption

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Abstract

When petioles of transpiring leaves are cut in the air, according to the 'Scholander assumption', the vessels cut open should fill with air as the water is drained away by tissue rehydration and/or continued transpiration. The distribution of air-filled vessels versus distance from the cut surface should match the distribution of lengths of 'open vessels', i.e. vessels cut open when the leaf is excised. A paint perfusion method was used to estimate the length distribution of open vessels and this was compared with the observed distribution of embolisms by the cryo-SEM method. In the cryo-SEM method, petioles are frozen in liquid nitrogen soon after the petiole is cut. The petioles are then cut at different distances from the original cut surface while frozen and examined in a cryo-SEM facility, where it is easy to distinguish vessels filled with air from those filled with ice. The Scholander assumption was also confirmed by a hydraulic method, which avoided possible freezing artefacts. In petioles of sunflower (*Helianthus annuus* L.) the distribution of embolized vessels agrees with expectations. This is in contrast to a previous study on sunflower where cryo-SEM results did not agree with expectations. The reasons for this disagreement are suggested, but further study is required for a full elucidation.

Key words: Embolism, *Helianthus annuus*, open-vessel length, Scholander assumption.

Introduction

According to the Scholander assumption (Scholander *et al*. 1964, 1965), the water contents of xylem conduits (vessels or tracheids) should be displaced by air when leaves or stems are cut in air, provided that the leaf is transpiring and/or dehydrated at the time the stems or petioles are excised from the plant. When the water column is cut, the pressure of the water column is increased to atmospheric pressure when the meniscus is flat. As the meniscus is drawn into the cut conduit the meniscus develops a radius of curvature that is ⩾the radius of the open conduit. Capillarity will then put the water column under subatmospheric pressure equal to a few kPa below atmospheric pressure, but water will continue to drain from the cut conduit as long as the water potential of the leaf cells remain more negative than that in the cut conduit.

Anyone who uses a pressure bomb and a stereomicroscope can confirm that water drains out of sight in cut conduits. With a microscope at 70× you can see into conduits to a distance equal to one or two conduit-diameters and you can observe that open vessels contain no water when transpiring shoots are cut in air. Furthermore, when the shoot is placed in a pressure bomb and is pressurized to the balance point, the meniscus can be seen to Return to the cut surface of the open conduits. But it is quite difficult to confirm that the open conduits completely drain up to the primary-wall surfaces bounding the entire conduit. According to the Scholander assumption, open vessels, i.e. vessels cut open, should drain at least as far as the vessel ends as long as the surrounding living cells are at a more negative pressure than say –50 kPa below atmospheric. Although Scholander recognized that the

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menisci hang up on pit membranes due to surface tension, he did not discuss the corollary of the assumption, i.e. that if the pressure difference across the menisci becomes too large then at least one meniscus will pass through the membrane. Xylem cavitation was not a research topic in the 1960s, hence there was no reason for Scholander to dwell on this issue. But today it is known that the vessels should drain beyond the vessel ends when living cells are very dehydrated (see Tyree *et al*., 2003, for more details).

Canny (1997*a*) tried to confirm the Scholander assumption by cutting the petioles of transpiring sunflower leaves and observing the state of open vessels after freezing the cut leaves in liquid nitrogen (LN$_2$). The petioles were sectioned while frozen and were observed in a cryo-scanning electron microscope (cryo-SEM). While some embolized (air-filled) vessels were observed, many more ice-filled vessels were observed than might be predicted from the probable length of open vessels in the petioles of sunflower. The number of embolized vessels was near zero in the morning and late afternoon and appeared to increase to 40% around noon. The number of embolized vessels generally did not increase with the time (0.2–16 min) between excision and freezing in LN$_2$. Although Canny (1997*a*) did not characterize the transpiration rate or water potential of the leaves at the time of excision, there is little doubt in the authors' minds that vessels should have drained in less than a minute. For example, experiments have been done on potted sunflower plants under low light and low transpiration conditions in a laboratory, leaves were cut under water and the petioles immersed in Phloxine B dye (Cyanosine). Within 1 min the dye was observed in the minor veins of the leaf blade (personal observation). If the dye can move rapidly in a leaf, then air should displace water with equal speed within the open vessels.

In a companion paper, Canny (1997*b*) froze sunflower leaves and petioles in LN$_2$ before excising them from the plants. Canny documented the transpiration rate and balance pressure of adjacent leaves prior to freezing. Figure 11 in Canny (1997*b*) shows a poor correlation between the percentage of embolized vessels and the balance pressure, which should equal minus the negative pressure of the xylem fluid (Wei *et al*., 1999). Nevertheless, Canny concluded that vessels embolize and refill while xylem-water pressure is in the range of –0.2 to –0.6 MPa. By comparing Figs 10 and 11 of Canny (1997*a*) the reader might also conclude that there was a weak correlation between xylem pressure and transpiration rate. Canny (1997*b*) felt that his experiment 'negates all the assumptions and evidence of the Cohesion–Tension theory.'

Considering the importance of the Scholander assumption as a corollary of the Cohesion–Tension theory, Tyree *et al*. (2003) recently tried to confirm the Scholander assumption on the excised leaves of two woody species.

The Scholander assumption was confirmed on leaves of *Acer* and *Juglans*. This paper reports the second step of the research. It was assumed that Canny's results on sunflower (Canny, 1997*a*, *b*) leaves would be repeatable so a hypothesis was formulated as to why vessels might refill during the kinetics of freezing sunflower petioles in LN$_2$. Surprisingly, however, it was not possible to repeat Canny's observations because the results exhibited rather good agreement with the Scholander assumption. These results are presented below and in the discussion the authors speculate as to why Canny may have reached a contrary answer.

Materials and methods

Helianthus annuus L. cv. LG-5660 seeds were sown in a commercial potting mix in 2.0 l pots and grown in a growth chamber at 25/18 °C day/night temperature and 12/12 h light/dark cycles at 500 µmol s^{-1} m^{-2} PAR. Leaves were harvested when plants were 1.1–1.5 m tall and flower buds were beginning to open. Mature leaves were selected with petioles 110–130 mm in length. Petioles in cross-section were approximately hemi-circular to triangular with a major and minor axis ±SD of 4.29±0.22 and 3.66±0.41, respectively, measured at the midpoint of the petioles.

Determination of open vessel lengths

The paint-perfusion method was used as an independent method to estimate open-vessel length, i.e. the distribution of the length remaining of vessels cut open when the petioles were severed about 10–15 mm from the stem insertion. All leaves in all experiments were cut 10–15 mm from the stem insertion. Preliminary air-perfusion experiments confirmed that the longest vessels in sunflower stems were 0.5 m and that vessels frequently extended from the stem through the petiole insertions and through the petioles. Hence, plants cut from roots in air might seed unwanted embolisms in petioles. To prevent the introduction of extra embolisms, all pots were immersed in water and stems were cut under water and the shoots were transferred to a bucket of water while keeping the cut base of the stems in a beaker of water. Most of the leaves of the excised shoots were in the laboratory air and transpiring under laboratory lights. Leaves were then harvested from shoots in water either while the petioles were or were not held under water as described below.

Paint-perfusion method

Petioles were perfused with blue paint pigment; the pigment consists of insoluble plate-like crystals <2 µm in size. The pigment particles are assumed to be small enough to pass through vessel lumina, but too big to pass through primary cell walls into adjacent vessels. Hence the distance of penetration of paint should give an estimate of open vessel length. The paint pigment was a 3% (by weight) solution of the blue pigment-concentrate used to colour latex paint. The solution was allowed to sediment for 24 h and the particles left in suspension were drawn off with a syringe and used for perfusions.

Leaves were cut in air and a 10×200 mm strip of Parafilm was tightly wrapped around the petiole near the cut surface to make a more circular surface to seal for paint perfusion. The leaf was placed in a pressure bomb and the bomb pressure was increased about 200 kPa above the balance pressure to refill all vessels with water. The balance pressure was usually 220±50 kPa. A compression fitting was placed over the petiole and injected with the pigment suspension. The compression fitting was connected to a 1 m length of

tubing also filled with the pigment suspension. The bomb pressure was reduced to atmospheric pressure and pigment-filled tubing was connected to a source of compressed air at 220 kPa pressure and the paint was pressure perfused into the petioles for 1–2 h. During the perfusion 8–12 ml of pigment solution was perfused into the leaves and leaf air-spaces became partly filled with water.

Immediately after the perfusion period the petioles were cut into 5 mm segments and the number of paint-filled vessels counted versus distance from the infusion surface using a 50× stereo-microscope and surface illumination provided by a fibre-optics light. Paint-filled vessels are open vessels. Paint-filled vessels were also counted at a distance of 0.5–0.8 mm from the cut surface and are referred elsewhere as the count at <1 mm.

Preparation of leaves prior to freezing in LN$_2$

Cryo-SEM methods similar to those of Canny (1997*a*) were used to determine the air-filled versus ice-filled state of vessel frozen in LN$_2$. Since Cochard *et al.* (2000) have reported freezing-induced embolism in *Juglans* when xylem pressure was below –500 kPa, it was decided to dehydrate all leaves in a pressure bomb to a balance point (–xylem pressure) of about 300 kPa to reduce this artefact. It was felt that this was preferable to excising leaves from plants without knowing the leaf water potential. 'Experimental' leaves consisted of leaves excised in air as described above and placed in a pressure bomb and pressurized at 350 kPa for ~1 min to obtain leaves at a balance pressure between 280–300 kPa. The pressure was released and the leaves were removed and held in air for 60–80 s and then immersed in water. The leaf blades were excised under water to reduce negative pressure in the xylem and the petioles frozen in LN$_2$. This 60–80 s exposure to air was sufficient to suck air into open vessels of the experimental leaves; water drained from the vessels and rehydrated leaf cells and/or evaporated from the leaf. Leaves probably had a minimal transpiration rate because they were in the dark pressure-bomb and laboratory-light just prior to freezing.

'Controls' were prepared as the experimental leaves, i.e. excised in air and dehydrated in a pressure bomb to a balance pressure of 280–300 kPa. After determination of the balance pressure the bomb pressure was increased to 500 kPa briefly to refill all open vessels. Then the cut surface was covered with water and the pressure was released from the bomb. Hence the dehydrated leaves could suck in water rather than air while they were removed from the bomb. The leaves were then immersed in water and the leaf blades excised. The petioles were then frozen immediately in LN$_2$.

All frozen petioles were transferred under LN$_2$ in a freezer at –80 °C and kept until examination in the cryo-SEM. The frozen samples were transferred to the cryo-SEM while immersed in liquid nitrogen. Samples were scored around the circumference with a diamond knife to freeze-fracture in cross-section under LN$_2$ and transferred to the cryo-SEM vacuum chamber. This procedure caused more frost to be deposited from the laboratory air during transfer compared to samples freeze-fractured in the cryo-vacuum chamber, but frost formed even in the cryo-vacuum chamber and the time to sublimate the frost was about the same regardless of where they were fractured.

After sublimation of surface frost, the number of embolized vessels was counted in petioles freeze-fractured at different distances from the cut surface that existed prior to freezing in LN$_2$. Counts of air-filled vessels were generally done at 200–300×, but in some cases the magnification had to be increased up to 900× to distinguish ice-filled from air-filled vessels. Vessels were counted as embolized even if only part of the vessel was air-filled; but vessels filled with a mixture of ice and air were rare (<5% of the vessels counted as embolized). Vessels were distinguished from tracheids and sclereids based on size and position and, again, small vessels could not be distinguished from tracheids. Embolisms in very small

vessels/tracheids less than about <10 μm in diameter were occasionally observed, but were not counted since conduits of this size are not easily filled with pigment in the paint-perfused conduits described above.

Hydraulic method of testing the Scholander assumption

According to the Scholander assumption, an excised leaf should suck air into open vessels if it is dehydrated at the time of excision. Open vessels filled with air are embolised and hence should not conduct water as long as the bubbles remain in place during measurement. The hydraulic method allows the Scholander assumption to be tested without potential artefacts caused by freezing, because freezing of samples might cause water movement inside petioles while there is a mix of water and ice.

Excised leaves cut in air were placed in a pressure bomb and dehydrated under a pressure of 350 kPa for 1–2 min. During dehydration, the water exuded from the cut base of the petiole was blotted away. After dehydration, the balance pressure was determined and was found to generally be between 280–300 kPa.

The bomb pressure was released, thus sucking air into open vessels. The leaves were removed from the bomb and placed under water 60–80 s later. The petioles were cut into 12 mm sections and connected to a conductivity apparatus as described elsewhere (Cochard and Tyree, 1990). Petiole-segment hydraulic conductivity was measured under a pressure difference of 2 kPa (= 20 cm of water head) using the XYL'EM apparatus (Cochard *et al.*, 2000). This pressure difference was small enough to measure the hydraulic conductivity of vessels filled with water, but too low to displace bubbles in embolized vessels. A pressure of >4 kPa is generally needed to displace bubbles. After the initial conductivity measurement (K_i) the petiole segments were flushed with water at a pressure difference of 110 kPa to displace embolisms in vessels cut open on both sides and to dissolve embolisms in vessels closed on one or both sides. A flush of 30 s duration was usually enough to achieve maximum conductivity (K_m) because a second flush of 30 s did not change the conductivity of the petiole segments. The percentage loss hydraulic of conductivity, $PLC = 100(1 - K_i/K_m)$ was computed for each segment.

Initially, the petiole segments were wrapped in Parafilm and inserted into 6 mm ID tubing and clamped to provide a seal for conductivity measurements. But it was found that the tubing had to be clamped so hard to achieve a seal that the petiole was being compressed enough to reduce K_i and K_m. A better seal was achieved with Terostat-VII (Henkel Teroson, Heidelberg), which was wrapped around the petiole and stuffed into the 6 mm ID tubing using a small screwdriver while holding everything under water. When a hose-clamp was tightened around the tubing the Terostat would deform to fill in gaps and seal without compressing the petiole segment.

PLC was also calculated on control segments to determine native state embolism. Leaves were excised under water were not dehydrated in the pressure bomb and were immediately cut into 12 mm segments and measured as described in the previous paragraph.

Results

The anatomy of this cultivar of sunflower was very similar to that described by Canny (1997*a*) except that the petioles were about half the diameter of the Russian mammoth cultivar used in Canny's study. There were 141±6 vessels (mean ±SD) per petiole >15 μm diameter, when counted in a fluorescence microscope at 100–250× magnification. Vessels in cross-section were round to elliptical; the largest
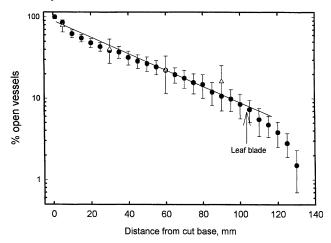
Fig. 1. Solid circles are a plot of open-vessel length distribution in sunflower petioles obtained by paint perfusion. The error bars are standard error values $n=7$. Open triangles are from embolized vessel counts in the cryo-SEM at distances of 5, 30, 60, and 90 mm. The error bars at these distances are the standard error of the cryo-SEM counts.

Fig. 2. The hydraulic method was used to measurement of percentage loss of hydraulic conductivity (PLC) of 12 mm long petiole segments. PLC estimates the hydraulic impact of air-filled vessels and provides qualitative information on the distance air is sucked into open vessels. Open circles are controls showing the native-state PLC. Solid circles are the experimental petioles where air has been sucked into the petioles through the cut surface. All error bars are standard error with $n=5$–6.

were elliptical and 60×100 µm and the smallest were 10–15 µm and approximately round.

The number of vessels containing paint at a distance of <1 mm from the cut surface was generally about 60% the number of total vessels; 86 ± 11 vessels were paint filled at <1 mm. Failure of paint to enter a substantial fraction of the perfused vessels is a common occurrence, but rarely commented on in the literature concerning vessel length distributions measured by paint perfusion. Paint might penetrate large vessel more than small so the open-vessel length distributions shown in Fig. 1 might be biased towards the length of larger diameter vessels. The percentage of open vessels declined in a log-linear function from the cut surface as in a previous report on *Acer* and *Juglans* (Tyree *et al.*, 2003). About 8% of the vessels extended into the three major veins of the leaf blade.

The hydraulic method for determining the distance air can advance into open vessels agreed qualitatively with the paint method (Fig. 2). In control plants there is a native level of percentage loss of hydraulic conductivity (*PLC*) of ~20% which is more or less constant along the length of walnut petioles. The *PLC* of experimental petioles was higher over the entire length indicating that many vessels extended >96 mm. In theory, hydraulic conductivity is not proportional to the open-vessel count. Vessel conductivity should increase approximately with the 4th power of their diameter (Martre *et al.*, 2000). Since large diameter vessels tend to be long vessels (Tyree and Zimmermann, 2002), the hydraulic method suggests longer 'hydraulic' lengths than the paint perfusion method.

Figure 3 shows the cryo-SEM counts of embolized vessels versus distance from the cut surface of controls and
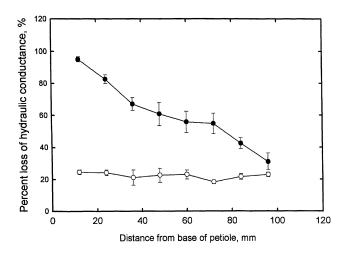
experimental plants. The high count of embolized vessels in controls is consistent with the ~20% loss of hydraulic conductivity found in Fig. 2. At all distances, the number of embolized vessels in the experimental petioles was significantly above the controls. The difference between the two curves in Fig. 3 represents the number of embolized vessels that can be used to test the Scholander assumption. This difference can be expressed as a percentage of water-filled vessels in the controls and has been plotted as open triangles in Fig. 1. The results are not significantly different from what would be predicted by the Scholander assumption.

There was a non-significant trend in embolized-vessel count in controls from high counts near the base to low counts near the leaf blade (Fig. 3). In fact, some controls had relatively low counts of 5–10 without a trend with distance (three cases) and other started at about 50 and declined to 15 (four cases). The leaves were cut in air and the pressure bomb was used to refill the vessels; in some cases a good seal was achieved between the petiole and rubber seal in the pressure bomb and little air passed through. In other cases a lot of air passed through because of a poor seal. The petioles were flooded with water before releasing the pressure and when bubbling was vigorous some air may have passed into some vessels when the bomb pressure was released. The hypothesis was tested that agreement between cryo-SEM counts and paint-counts of open-vessel length could be obtained by a reanalysis of Fig. 3, assuming that the control counts should have been a constant value, independent of distance equal to 5 or 10 or 20 vessels. Fits as good or better were obtained between
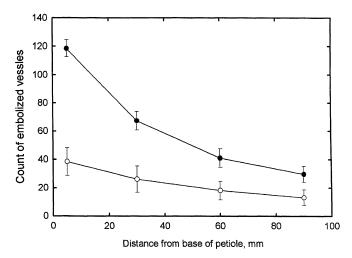
Fig. 3. Cryo-SEM counts of the number of embolized vessels versus distance. Open circles are controls and closed circles are experimental segments. Error bars are standard errors with $n=6–7$. The difference in counts of experimental minus control segments is plotted as a percentage of water-filled vessels in controls in Fig. 1 (triangles).

cryo-SEM counts and paint-counts (circles in Fig. 1) of open-vessel length versus distance (data not shown). A constant count of embolized vessel in controls would be consistent with the PLC controls in Fig. 2.

Discussion

The observations of Canny (1997*a*) on the Russian mammoth cultivar of sunflower are not reproducible in the LG-5660 cultivar used in this study. The pattern of percentage embolized vessels from cryo-SEM counts is as expected from the Scholander assumption. There is a log-linear decline in the percentage of embolized vessels from cryo-SEM counts as well as from paint-perfusion counts and the slopes are not significantly different.

In previous studies on *Acer* and *Juglans* (Tyree *et al.*, 2003), the absolute percentages do not completely agree, but this may be due to the difficulty in determining the reference counts for vessels at the cut surface used to compute the percentages. For the paint-perfusion method the reference count was the number of paint-filled vessels at about 1 mm from the surface. This is the standard reference count used in all other studies where the paint-perfusion method is used, even though only about half the vessels are filled with paint at 1 mm. By contrast, in the cryo-SEM method the reference count is the number of vessels counted in fluorescence-light microscopy, and the problem with this microscopy-method is distinguishing vessels from tracheids and sclereids in cross-sections. Since there are many small tracheids and/or sclereids in *Acer* and *Juglans* there is considerable uncertainty in the reference counts at the cut surface. So there is an argument

that the congruence of slopes is a more powerful test of the Scholander assumption than absolute counts.

The hydraulic method provides strong qualitative evidence for how far air can be sucked into petioles cut in the air. Air entering open vessels results in a >96% loss of hydraulic conductivity in the first 12 mm segment and the percentage loss of hydraulic conductivity (*PLC*) is still significantly above the controls at a distance of 96 mm from the cut surface. The profile of *PLC* cannot be related directly to vessel counts since the conductivity of vessels increases with the 4th power of the vessel diameter. However, the hydraulic method provides strong support for the Scholander assumption and cannot be questioned because of possible artefacts caused by movement of air or water in petioles while freezing proceeds. Similar hydraulic confirmation of the Scholander assumption has been reported in the past (Cochard *et al.*, 1994) and has been used commonly as a method of determining maximum open-vessel length in the experimental design for new species, for example, maize leaves (Cochard, 2002, and unpublished methods used frequently by Cochard and Tyree).

Why did Canny (1997*a*) get such different results? There were differences in methods used, but at best there can only be speculation. Canny had very poor success with estimating open-vessel length by paint infusion, i.e. only about 20 vessels were filled with paint near the base of the petioles (table 1 in Canny, 1997*a*). This is explained because Canny used green latex paint rather than pure paint-pigment. Latex particles are much bigger and stickier than the pigment particles hence latex tends to block vessels before they are completely filled with latex paint. Paint infusion in Canny's paper was by the suction induced by transpiration in leaves that eventually wilted and hence vessels were further blocked by cavitation. In this study the petioles were pressure-perfused at 220 kPa while the leaf blades were enclosed in a pressure chamber, hence transpiration was low and leaves did not wilt. Some of the leaf air-spaces were filled with water so cavitation could not have contributed to the blockage of vessels.

Canny (1997*a*) did not measure the water potential, Ψ, of his sunflower leaves prior to excision. Furthermore, the leaves were enclosed in plastic bags to reduce transpiration 1 min prior to cutting them from the plants. The leaves remained in the bags for periods of 0.2–16 min before they were frozen in LN_2. Hence it is not clear that there was enough tissue desiccation and transpiration to drain the vessels. The advantage of the procedure used here was that the water stress was controlled in excised leaves to be sure that the water potential was negative enough to suck in air, but not so negative that other artefacts might occur (see below).

Finally, there is a possibility that embolized vessels might have refilled more during freezing of Canny's samples than in this study's samples. Little attention has

been paid to devising models that might predict the direction and magnitude of water flow into or out of cells as they freeze. Since water expands 9% in volume as it freezes, most would assume that freezing will tend to push water into empty vessels, which would then freeze in place. By contrast, Cochard *et al*. (2000, 2001*a*, *b*) argue that vessels can be drained of water while freezing in sufficiently dehydrated samples. What processes can make water flow into or out of vessels while samples freeze?

There must be another process that draws water from vessels to living cells that exceeds the capacity of water to grow 9% in volume as it freezes. First, only the effect of water volume change as ice forms will be considered, ignoring other effects. In soft tissue the volume change of living cells from full turgor to the turgor loss point is often 10–20%. So if a cell has lost 20% of its volume and is at the turgor loss point before it freezes, the water potential is negative and will stay negative while all the water freezes and the volume of the cell increases by 9% from ice formation. Even though the expanding ice will raise the turgor pressure of the unfrozen cell sap the volume increase will not be enough to raise the turgor pressure to equal the osmotic pressure, hence water potential will remain negative during the entire freezing process. Before freezing, the water pressure in adjacent vessels will be at negative pressure and in approximate equilibrium with living cells, but as soon as ice is seeded somewhere in the vascular system air-bubbles will come out of solutions seeding a cavitation event and releasing the negative pressure. Hence water can flow from the unfrozen portion of vessels to the unfrozen portion of living cells while freezing progresses if the living cells were dehydrated by more than 9% in volume prior to the freeze. The net effect will be the formation of air spaces in vessels previously filled with water.

On the other hand, water could flow into a previously empty vessel if the adjacent living cells were dehydrated by less than 9% in volume prior to freezing. For example, if the living cells were dehydrated 4.5% prior to freezing then once the cell is more than half frozen, the turgor pressure would be raised above the osmotic pressure by the expanding ice forcing water out of the cell for the remainder of the freezing episode.

Water movement will be induced by more than just the volume change of water as it freezes because water potential (Ψ) also depends on temperature, $\Psi = RT\ln(\alpha) + P_t$, where R is the gas constant, T is the absolute temperature, α is the activity of water, and P_t is the turgor pressure. As water cools from say 26 °C to 0 °C, T will decline by 10% (301 to 276 °K) and P_t can change by 10–20% because temperature affects the elasticity of cell walls (see Tyree *et al*., 1974, for details). Finally, in non-ideal solutions α will also depend on temperature, sometimes increasing or decreasing depending on solute. Hence it is not easy to predict which way water will flow nor how much water flows as a petiole cools and freezes.

How much water flows in or out will depend on the hydraulic conductance of the cell membranes (L_p), the time-averaged difference in water potential between the freezing cell and the adjacent unfrozen tissue ($\Delta\overline{\Psi}$), and the length of time (Δt) the freezing cell retains some liquid water. So the volume of water, V, displaced over a given cell surface area (A) during freezing is:

$$V = AL_p\Delta\overline{\Psi}\Delta t \qquad (1)$$

Now consider what happens at the tissue level as a petiole freezes. The petiole will be encased in a rigid ice-chamber, once the outer cell layers have frozen, so further expansion of ice will force water out of living cells towards any empty air space, i.e. in vessels. So, as the core freezes, the outer ice layer will be pushed from the inside putting the outer annulus of ice under tangential tension. These physical forces were confirmed by axial splitting of *Juglans* petioles when they were frozen (unpublished observation). In one dramatic incident a sunflower petiole exploded while quiescently settling at the bottom of a pool of LN_2; the petiole split axially into three sections! Frequently, when an attempt was made to freeze-fracture sunflower cross-sections they were also split axially into several fragments, which is again evidence of positive pressure developing in the core and corresponding tension along the surface of the petiole.

The kinetics of heat diffusion is much like the kinetics of diffusion of molecules. Heat must diffuse away for water to get cold enough to freeze and to remove the latent heat of fusion. But the time for heat to diffuse away will increase with the square of the distance that heat has to diffuse to get past the outer layer of ice. The petioles of the LG-5660 cultivar were only about half the diameter of the Russian mammoth cultivar. So vessels might be expected to be about twice as far from the surface of Canny's petioles than petioles in this experiment, hence living cells adjacent to vessels would take about four times longer to freeze and hence four times as much water might be squeezed out of these cells while they are freezing.

More work needs to be done to quantify the model above to see if it might explain the different findings between Canny (1997*a*) and this work. Petioles about 4 mm thick can take about 10 s to freeze to the centre (Cochard *et al*., 2000). But it is necessary to know how long it takes an ice-front to reach any given conduit and how long the adjacent cells remain partly frozen to begin evaluating the magnitude of water flow in or out according to equation (1). The influence of the kinetics of freezing on the state of the resulting mix of air and ice must be understood if cryo-SEM images relating to embolisms in xylem conduits are to be properly interpreted. But for now, the Scholander hypothesis can be confirmed in three species, for example,

Helianthus (this paper) and *Acer* and *Juglans* (Tyree *et al*., 2003).

Canny (1997*a, b*) and related papers together with some early pressure probe work (Balling and Zimmermann, 1990; Benkert *et al*., 1995) precipitated a watershed in Martin Canny's thinking regarding the motive force for transpiration. Hence Canny sought an alternative to the Cohesion–Tension theory and proposed a concept of 'compensating tissue pressure' to explain how water movement can occur in xylem conduits with little or no negative pressure and how embolized conduits might refill while transpiration continues. A full discussion of these issues is not appropriate in a short research paper, but readers might want to refer to some related literature. For example, Tyree (1999) and (Comstock (1999) have argued that Canny's concept of tissue pressure is physically impossible, i.e. it violates some basic physical principles. Tissue pressure can have influence for only transitory periods of a few seconds and cannot explain refilling in laurel (Tyree *et al*., 1999). Other researchers have failed to find experimental confirmation of the predictions of the compensating-pressure concept (Stiller and Sperry, 1999). Others have shown that the appearance of embolisms in frozen samples is an artefact of freezing samples with xylem pressure below about –500 kPa (Cochard *et al*., 2000, 2001*a, b*; Canny *et al*., 2001; Richter, 2001). New experimental evidence with the cell-pressure probe has provided strong experimental support for the Cohesion–Tension theory (Wei *et al*., 1999, 2001; Tyree *et al*., 2003). Finally, this paper and Tyree *et al*. (2003) have verified the Scholander assumption that can be taken as a corollary of the Cohesion–Tension theory.

Acknowledgements

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