

# The water-filled versus air-filled status of vessels cut open in air: the ‘Scholander assumption’ revisited

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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 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. Three different methods were used to estimate the length distribution of open vessels and compared it to the observed distribution of embolisms by the cryo-scanning electron microscope (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. In petioles of *Acer platanoides* and *Juglans regia*, 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 reason for this disagreement requires further study for a full elucidation.

*Key-words:* *Acer platanoides*; *Juglans regia*; embolism; open vessel-length; Scholander assumption.

## INTRODUCTION

What is the fate of water in a water-filled conduit of a plant when it is cut open in ambient air? The conduit in question can be a vessel or tracheid in a root, stem, petiole or rachis, and we will define ‘open conduit’ to mean a conduit cut open and its contents exposed to ambient air. There are three possibilities, i.e. water can flow out, it can stay in place or it can be drained away by some mechanism:

1 If the pressure of the water in the open conduit is greater than atmospheric pressure, water will flow for as long as the water-pressure remains above atmospheric. In some cases plant conduits can have water above atmospheric

pressure. Examples of this occur when water exudes from open conduits in roots driven by root pressure (Tyree & Zimmermann 2002; pp. 176–178). Water can also be ejected from the heartwood of trees; decay by *Methanobacteria* sp. can generate methane gas that puts water under positive pressure (Abell & Hursh 1931; Zeikus & Ward 1974). Lastly freeze–thaw cycles induce water movement in some trees and during the thaw cycle the water is often under positive pressure (Améglio *et al.* 2001; Ewers *et al.* 2001; Tyree & Zimmermann 2002; pp. 81–88) and will exude for a finite time.

- 2 If the pressure of the water is equal to atmospheric, the conduits should behave like a pipette filled with water with one end sealed off. It will not drain. However if the conduits are large enough and both ends are cut open, then gravity might overcome capillarity and the conduits should drain if held vertically.
- 3 If the plant is transpiring water at the time the conduit is cut open, the Cohesion–Tension theory predicts that the water will be under negative pressure, i.e. below atmospheric pressure prior to the cut. Continued transpiration theoretically should drain the water out of open conduits. Even in the absence of continued transpiration water will drain from the open vessels to rehydrate the surrounding tissue, since water in an open vessel is at a pressure equal to atmospheric pressure immediately after the cut because a flat meniscus is created at the surface of the open vessel.

As soon as the air–water interface is drawn into the conduit a curved meniscus should form. The meniscus will lower the pressure of the water in the xylem conduit ( $P_x$ ) to a value given by the so-called capillary equation:

$$P_x = -2T \cos(\theta)/r_c,$$

where  $T$  is the surface tension of water and  $\theta$  is the angle of contact between the water and the wall. The contact angle ( $\theta$ ) will equal zero in a hydrophilic (wettable) conduit wall so  $P_x$  equals  $-2T/r_c$  in this special case. However, lignified xylem conduit walls tend to be somewhat hydrophobic, hence  $\cos(\theta)$  may be somewhat less than one. This reduction in pressure is usually quite small, e.g.  $P_x \approx 15$  kPa for a conduit radius of  $10 \mu\text{m}$ , so it will slow down but not prevent transpiration from sucking the water out of the

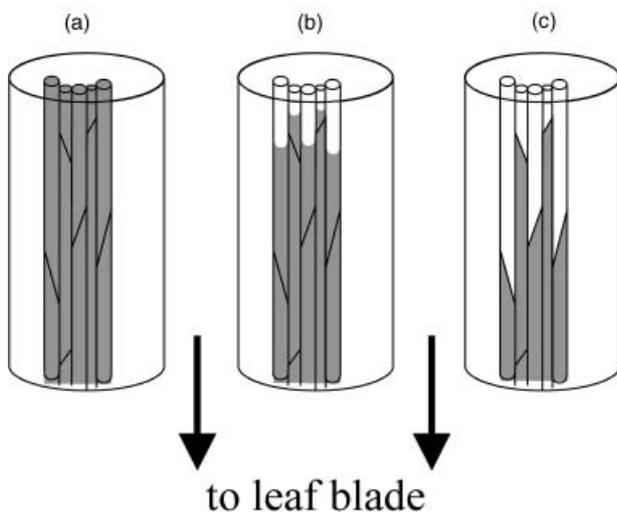
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conduit (Fig. 1). However, open conduits are of finite length and water flow to adjacent closed conduits must pass through the primary cell wall. The porosity of the primary cell wall is sufficiently small to generate quite a large negative water pressure as the meniscus breaks up into many small menisci and tries to pass through the pores of the primary wall. So in theory, water drainage by transpiration should stop temporarily at the primary wall until continued desiccation of leaf tissue generates a sufficiently negative pressure to draw at least one meniscus through the primary wall of the open vessel to induce cavitation of the adjacent closed vessel (Fig. 2).

The third possibility just described can be predicted from the capillary equation and the Cohesion–Tension theory, and this prediction has been called the Scholander assumption (Canny 1997a). However, the notion predates the predictions of Scholander *et al.* (1965). There has been some limited confirmation of the truth of this prediction for a long time. Anyone who uses a pressure bomb and a stereomicroscope can confirm it (Fig. 3). 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. However, it is quite difficult to confirm that the open conduits completely drain up to the primary wall surfaces bounding the entire conduit.

Canny (1997a) used a cryo-scanning electron microscope (SEM) method to test the Scholander assumption and failed to confirm it in sunflower petioles. The nature of the



**Figure 1.** A diagrammatic representation of the state of water in vessels of a stem or petiole after being cut in air according to the Scholander assumption. (a) Water fills all vessels at the instant the stem or petiole is cut. (b) Vessels are draining. (c) Vessels are drained to vessel ends. For details of the menisci in the vessel walls and what might happen with continued water loss see details in Fig. 2.

discrepancy was developed in a companion paper (Canny 1997b) as explained in the discussion. Canny's observation on sunflower is critical to pressure bomb theory and the Cohesion–Tension theory so it is important to know if other species violate the Scholander assumption. The purpose of this paper was to repeat Canny's experiment on leaves of other species while taking care to document the conditions of the leaves prior to introduction of air into the open vessels. The approach we used was: (1) to estimate the length of open vessels by three different methods (paint perfusion, microcasting of vessels with silicone rubber and hydraulic methods); and (2) comparing these independent estimates of open vessel length with the observed distance that air is sucked into vessels when frozen after cutting petioles in air.

## MATERIALS AND METHODS

Experiments were conducted on leaves of *Acer platanoides* L. and *Juglans regia* L. The mean diameter  $\pm$  SD of 12 petioles were  $1.64 \pm 0.21$  and  $3.40 \pm 0.28$  mm for *Acer* and *Juglans*, respectively. Diameters were measured at the midpoint between the cut surface and the leaf blade (or first leaf pair for *Juglans*) and are reported as the mean of the major and minor axes.

### Preparation of excised leaves under water

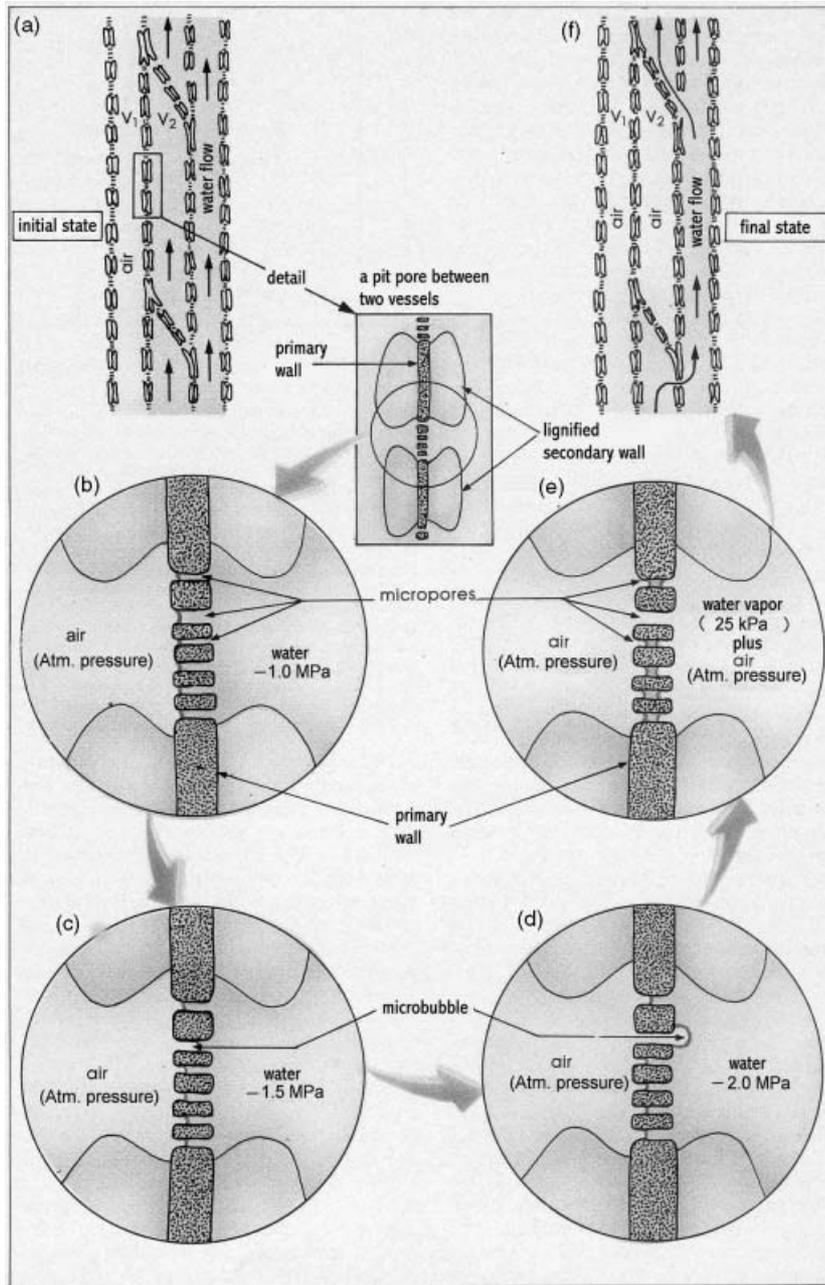
Branches of trees were excised in the field and immediately the stems were recut under water in a container and brought back to the laboratory, where the container was covered in a plastic bag and the branches were allowed to rehydrate for 2–18 h. The branches and leaves (after rehydration) were immersed in water and petioles excised near the petiole insertion region while held under water; the petioles were then recut beyond the major swelling near the base, that is 10–20 mm beyond the insertion. *Acer* petioles contain latex ducts and exuded latex for about 1 min following excision. This latex was disbursed by agitating the petiole under water and by cutting 1 mm sections of petiole from the cut base two or three times during agitation.

### Vessel counts

Petioles were hand-sectioned with a razor blade and placed in a fluorescence microscope with surface illumination to visualize the lignified cell walls by their fluorescence. Vessels, tracheids and sclereids all have lignified cell walls. The vessels were discriminated on the basis of size and location and were confirmed by dye perfusions. However, the difference between small vessels and tracheids are indistinct. Vessel counts were made excluding the smaller vessels/tracheids for each species at 100–250× magnification.

### Open vessel length determination

Three methods were used to determine the length of open vessels.

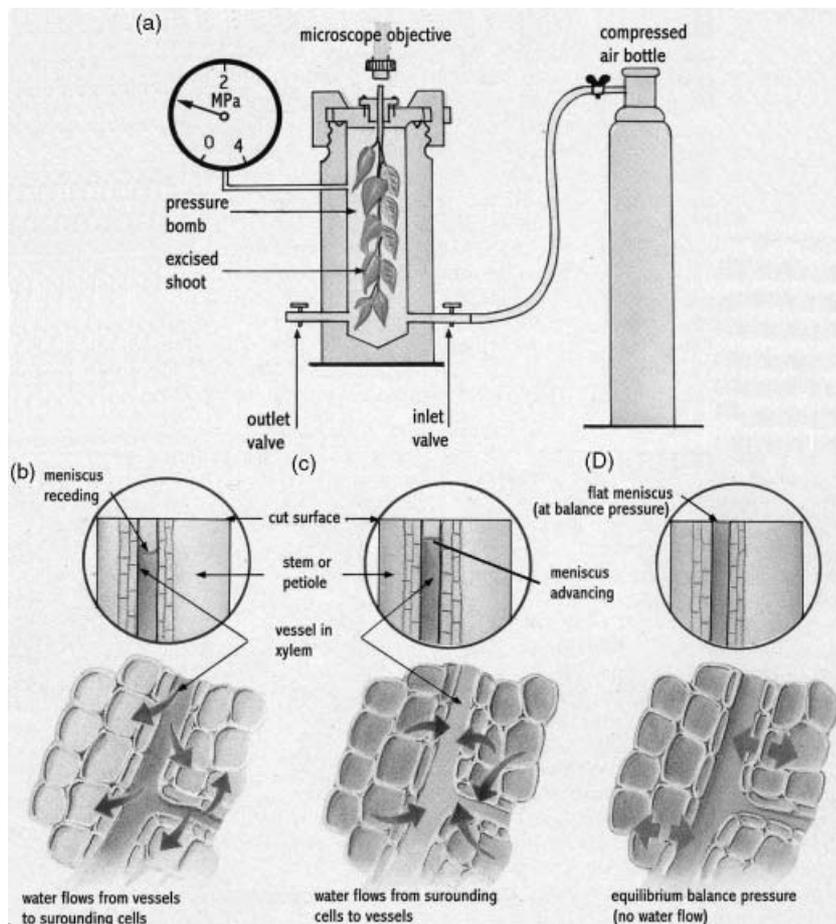


**Figure 2.** A detailed view of menisci in an open vessel is shown adjacent to a water-filled vessel. (a) After the open vessel has drained stable menisci are formed in pits. The open vessel is filled with air at atmospheric (Atm.) pressure plus water vapour. (b) Detail of a pit showing menisci in primary wall when adjacent water is at  $-0.5$  MPa. (c) If evaporation of water continues from the sample the menisci are sucked deeper into the primary wall. (d) At a critical xylem pressure the air is sucked through the largest pore in the primary cell wall (pit membrane). (e) and (f) The adjacent vessel is now embolized. The water vapour pressure will be more or less depending on temperature.

### Paint-perfusion method

The petioles were perfused with blue paint pigment; the pigment consists of insoluble plate-like crystals less than  $2\ \mu\text{m}$  in size. The pigment particles were 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 the paint should give an estimate of open vessel length. The paint pigment was a 3% (by weight) solution of the 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.

The excised leaves (collected as described above) were removed from water and placed in a pressure bomb with the base of the petiole protruding from the rubber seal (Fig. 3). The bomb pressure was increased by about  $0.2$  MPa above the balance pressure to ensure all vessels were water-filled. The balance pressure was usually  $<0.1$  MPa. A compression fitting was placed over the petiole and injected with the pigment suspension. The triangular petioles of *Juglans* were wrapped in multiple layers of parafilm wax sheets to round them out and hence improve the seal with the rubber seal of the compression fitting. The compression fitting was connected to a 1 m length of tubing also filled with the pigment suspension. The bomb pressure



**Figure 3.** These drawings show the state of menisci when plant tissue is placed in a pressure bomb and bomb pressure is raised or lowered. (a) Details of a pressure bomb showing a *Populus* shoot enclosed with the cut end extending through a rubber seal. The drawings that follow apply also if a single leaf is in the bomb with the petiole extending through the seal. (b) The meniscus is sucked into the open vessel when the bomb pressure is dropped below the balance point or when the leaf is initially cut in air; see also Fig. 1. As water drains from the vessel it flows into adjacent living cells. (c) The bomb pressure is being increased and the meniscus is being pushed to the cut surface. Water is moving from adjacent living cells to the vessel. (d) The bomb pressure is at the balance point and the meniscus is at the cut surface. Note: This drawing illustrates a branched vessel. This occurs in rare situations where a tri-perforate vessel element is formed. Although branched vessels have been observed in the past they are rather rare (André 2002).

was reduced to atmospheric pressure and pigment-filled tubing was connected to a source of compressed air at 0.25 MPa pressure and the paint was pressure perfused into the petioles for 2 h. During the perfusion 3–12 mL of pigment solution was perfused into the leaves and the leaf air spaces became filled with water.

Immediately after the perfusion period the petioles were cut into 5 or 10 mm segments and the number of paint-filled vessels counted versus distance from the infusion surface using a 70 $\times$  stereo-microscope and surface illumination provided by a fibre-optic light. The paint-filled vessels were open vessels. The 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. Data from seven to 10 leaves from each species were combined versus distance and plotted as a percentage of open vessels versus distance from the infusion surface.

#### Hydraulic method

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 embolized and hence should not conduct water as long as the bubbles remain in place during measurement.

The excised leaves collected as described above were placed in a pressure bomb and dehydrated under a pressure of 0.31 MPa for 10 min. During the dehydration the water exuded from the cut base of the petiole was blotted away. After 10 min of dehydration the balance pressure was determined and was found to generally be between 0.28 and 0.30 MPa. (Leaf blades had to be blotted dry prior to putting them in the pressure bomb, otherwise surface water is pressure-perfused through stomates into the leaf air spaces and hence more time is required to dehydrate the leaves to 0.28–0.30 MPa.)

The bomb pressure was released thus sucking air into the open vessels. The leaves were removed from the bomb and placed under water within 20 s. The petioles were cut into 10 mm sections and connected to a conductivity apparatus as described elsewhere (Cochard & Tyree 1990). Petiole segment hydraulic conductivity was measured under a pressure difference of 1 kPa (= 10 cm of water head) using the XYLEM apparatus (Cochard *et al.* 2000). This pressure difference was small enough to measure the hydraulic conductance 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 200 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 2 min duration was usually enough to achieve maximum conductivity ( $K_m$ ) because a second flush of 2 min did not change the conductivity of the petiole segments. Percentage loss of hydraulic conductance,  $PLC = 100(1 - K_i/K_m)$  was computed for each segment.

The PLC was also calculated on control segments to determine native state embolism. Excised leaves collected as described above were not dehydrated in the pressure bomb and were immediately cut into 10 mm segments and measured as described in the previous paragraph. The hydraulic method could not be used on *Acer* petioles because the latex ducts were also found to conduct water after a flush and accounted for most of the observed  $K_m$ .

### Silicone rubber-perfusion method

Silicone rubber casts were used to provide an independent measure of open vessel length. To our knowledge no one has ever compared the paint-perfusion method with an independent method of measuring open vessel length. More than 10 years of research summarized by André (2002) has confirmed that silicone rubber compounds can be vacuum-infused into open vessels and then reticulated into place. The reticulation involves formation of chemical cross links between the silicone polymer. The resulting microcasts conform to the interior surfaces of vessels revealing surface features to a resolution of  $<0.2 \mu\text{m}$ , but the silicone polymers do not pass through primary cell walls. Hence microcasting provides detailed images of open vessels. Jean-Pierre André kindly infused *Acer* petioles using his standard methods (André 2002). The resulting microcasts were then used to measure open vessel length. Although both latex ducts and vessels filled with silicone polymer, the microcasts of vessel were distinguished easily from ducts by diameter and surface features.

### Cryo-SEM observations

Cryo-SEM methods similar to those of Canny (1997a) were used to determine the air- versus ice-filled state of vessel frozen in liquid nitrogen ( $\text{LN}_2$ ). Since Cochard *et al.* (2000) have reported freezing-induced embolism in *Juglans* when xylem pressure was below  $-0.5 \text{ MPa}$ , we decided to dehydrate all leaves in a pressure bomb to a balance point ( $-$  xylem pressure) of about  $0.3 \text{ MPa}$  to reduce this artifact. 'Experimental' leaves consisted of excised leaves excised under water as described above and placed in a pressure bomb and pressurized at  $0.31 \text{ MPa}$  for 10 min to obtain leaves at a balance pressure between  $0.28$  and  $0.30 \text{ MPa}$ . The pressure was released and the leaves were removed and frozen in  $\text{LN}_2$  within 20 s. This 20 s exposure to air was sufficient to suck air into the open vessels of the experimental leaves; water drained from the vessels rehydrated the leaf cells. The leaves probably had a minimal transpiration rate because they were in the dark pressure-bomb just prior to freezing.

'Controls' were prepared in the same manner as the experimental leaves, namely excised under water and dehydrated in a pressure bomb to a balance pressure of  $0.28$ – $0.30 \text{ MPa}$ . After determination of the balance pressure, rubber tubing was placed over the petiole and the bomb pressure was increased to  $0.5 \text{ MPa}$  briefly to refill all open vessels. Then water was injected into the tubing to cover the open vessels with water and the pressure was released from the bomb. Hence the dehydrated leaves would suck in water rather than air while they were removed from the bomb. The leaves were then immersed in water and the tubing removed. Leaves were removed from the water bath with the petioles pointing down. We noted that this procedure retained a drop of water over the cut surface of the petioles and allowed water to flow down the surface of the petiole to add to the drop of water covering the cut surface while out of the water and thus prevented air intake into the open vessels. The leaves thus removed were then frozen immediately in  $\text{LN}_2$ .

Preliminary results from these controls indicated that embolism counts increased from base to apex of the petioles of *Acer* and we thought this might be an artifact of pressurization of the leaves in the pressure bomb or a consequence of freezing petioles with leaf blades attached. So two other controls involved leaves that had never been placed in the pressure bomb: (1) leaves were cut under water the petioles were frozen with the leaf blades attached (2) as in (1) but the petioles were excised from the blades under water prior to freezing the petioles.

All frozen petioles were detached from the blades and transferred under  $\text{LN}_2$  in a freezer at  $-80 \text{ }^\circ\text{C}$ , kept overnight, and examined the next day in the cryo-SEM. The frozen samples were transferred to the cryo-SEM while immersed in liquid nitrogen. Most samples were freeze fractured under  $\text{LN}_2$  and transferred to the cryo-SEM vacuum chamber. This procedure caused more frost to be deposited from the laboratory air during transfer compared with 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 that had been freeze-fractured at different known distances from the cut surface that existed prior to freezing in  $\text{LN}_2$ . Counts of air-filled vessels were generally done at  $200$ – $300\times$  but in some cases the magnification had to be increased up to  $900\times$  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  $5$ – $8 \mu\text{m}$  in diameter were occasionally observed but were not counted since conduits of this size would have been ignored in the paint-perfused conduits described above.

## RESULTS

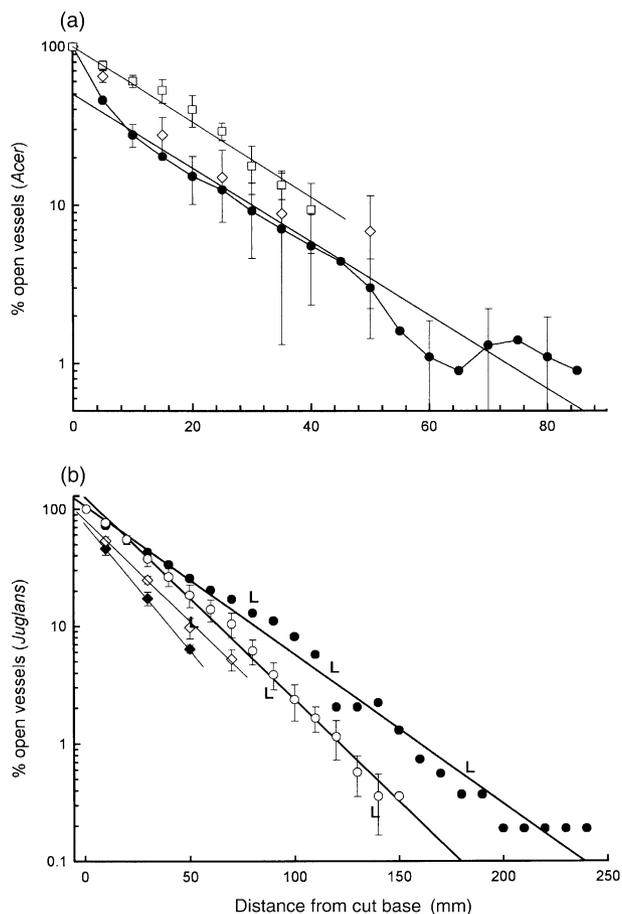
Vessel counts  $\pm$  standard error of the mean (SE) were  $274 \pm 8$  ( $n = 10$ ) for *Acer* and  $384 \pm 11$  ( $n = 6$ ) for *Juglans*. Vessel counts were fairly uniform along the petioles of *Acer* but declined along *Juglans* after the first and second leaflet pair along the rachis; the counts were  $292 \pm 15$  and  $223 \pm 11$  after the first and second leaflets, respectively ( $n = 6$ ). Since our objective is to estimate the distribution of length of open vessels from the cut base of the petiole, vessel number declines after the leaflets are irrelevant for future comparisons.

The number of vessels containing paint at a distance of  $<1$  mm from the cut surface was generally about half the number of total vessels. 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. The question of what happens in the first millimetre is resolved by the microcasting data in Fig. 4a. The number of microcast vessels in the first millimetre was  $282 \pm 10$  ( $n = 3$ ) did not differ significantly from the number counted by fluorescence microscopy. The percentage of open vessels (micro-cast) declined in a log-linear relationship with distance from the cut surface indicating that the open-vessel length distribution declined exponentially with distance by all methods used in this paper. The plot in Fig. 4 can be viewed as a frequency distribution of the fraction of open vessels at least as long as the length on the  $x$ -axis.

As less than half the vessels were paint-filled at  $<1$  mm and the decline was steeper than log-linear in the first 5 mm (in *Acer*), we concluded that paint tends to clog vessels near the cut surface in *Acer* petioles. However, if the vessels remain unclogged after 5 mm they remain unclogged for the entire distance otherwise the slope of the paint-perfusion curve would be different from that measured by the other methods. The slopes are not significantly different between the paint-perfusion and microcasting methods, but the percentage of open vessels from the microcasting technique was significantly higher than from the paint-perfusion technique at each plotted distance.

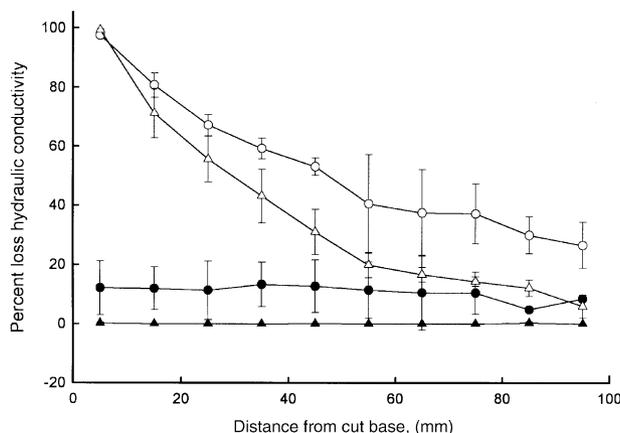
The hydraulic method for determining open vessel length shows a different pattern for *Juglans* (Fig. 5). In control plants there is a native level of percentage loss hydraulic conductivity which is more or less constant along the length of walnut petioles. Generally the hydraulic lengths are longer than those determined by paint perfusion because, in theory, hydraulic conductivity is not proportional to the vessel count. Vessel conductivity should increase approximately with the fourth power of their diameter (Martre, Durand & Cochard 2000). As large diameter vessels tend to be long vessels (Tyree & Zimmermann 2002), the hydraulic method suggests longer 'hydraulic' lengths than the paint-perfusion method.

The air-filled vessels were counted on frozen samples at 5 mm from the cut surface in *Acer* and were found to be significantly higher in experimental than control petioles, namely  $195 \pm 12$  (mean  $\pm$  SE,  $n = 10$ ) versus  $23 \pm 7$  ( $n = 8$ ).



**Figure 4.** The percentage open vessel lengths were determined by various methods (circles, paint-perfusion method; squares, silicone microcasting; diamonds, cryo-SEM). The number of paint-filled vessels were counted at different distances from the cut surface and expressed as a percentage of those at the surface (estimated from a cut at 0.6–0.8 mm from the surface). For the other methods percentages were based on mean vessel counts presented in results. (a) Percentage of open vessels versus distance (mm) from cut surface in *Acer* petioles. Points are means and SE ( $n = 7$ , for the paint method;  $n = 4$  for the cryo-SEM method; and  $n = 3$  for the microcasting method.); error bars are shown only for every other point for the paint-perfusion method. (b) Percentage of open vessels versus distance (mm) in *Juglans* petioles. Open circles are for leaves collected from mature trees in the field; closed circles are for leaves collected from 3-year-old trees grown in a greenhouse. The bigger greenhouse leaves had longer open vessels. All other experiments were carried out on leaves from mature trees. Shown are means and SE ( $n = 7$ , for the paint method;  $n = 6$  for the cryo-SEM method). For the cryo-SEM method only values significantly different from zero are plotted and open diamonds are for leaves dehydrated to 0.38 MPa and allowed to suck air for 70–80 s whereas closed diamonds are for leaves dehydrated to 0.28 MPa and allowed to suck air for 10–20 s before freezing. The 'L' symbols indicate the approximate location of leaflet pairs in the leaves.

Control petioles had a native level of embolism due to previous water stress experienced in the field. The experimental petioles appeared to suck air into more vessels than were air-filled in the native state of controls. Similar com-



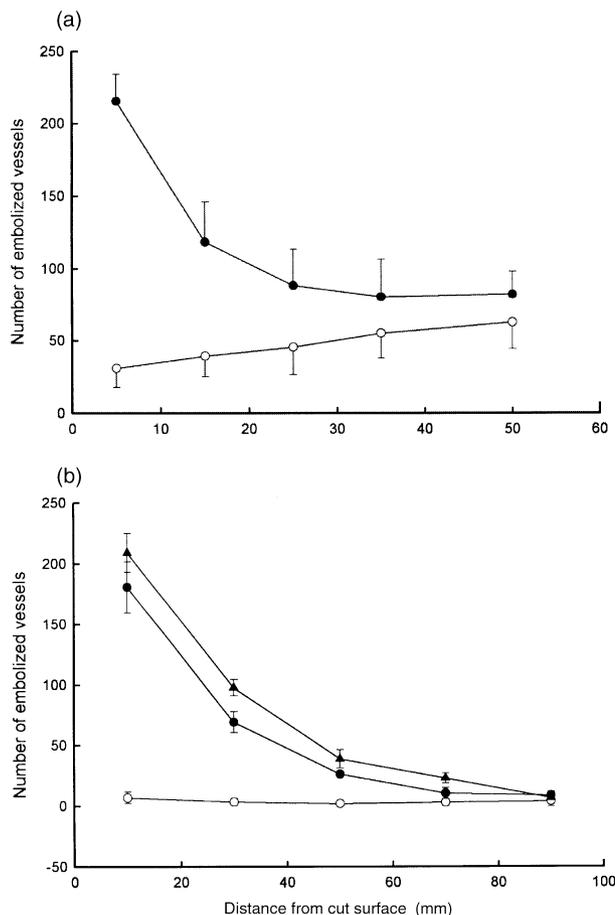
**Figure 5.** Percentage loss hydraulic conductivity in petiole segments of *Juglans*. Each point is the mean and SE ( $n = 4$ ) of 10 mm long petiole segments measured from the cut surface. When petioles are cut in the air (open symbols) more embolism is present than when the petiole is cut under water (closed symbols). Circles are for 3-year-old greenhouse trees with leaves that were larger than the field trees (triangles). These measurements give a hydraulically weighted estimate of open vessel length. See text for more details.

comparisons in *Juglans* were made at 10 mm from the cut surface. The two experiments differed in the amount of dehydration of the leaves in the bomb and the time exposed to air prior to freezing. When leaves were dehydrated to 0.28 MPa and exposed to air for about 20 s the number of embolized vessels at 10 mm was  $181 \pm 21$  ( $n = 6$ ) but when the leaves were dehydrated to 0.38 MPa and exposed to air for about 80 s prior to freezing the number of embolized vessels at 10 mm was  $209 \pm 16$  ( $n = 6$ ); the means were not significantly different between the two experimental treatments. The controls had  $7 \pm 5$  embolized vessels ( $n = 6$ ). The significantly higher incidence of embolized vessels in experimental petioles than in controls is consistent with the Scholander assumption.

In a smaller number of replicates, air-filled vessel counts were made versus distance from the cut surface for experimental and control petioles. Data for *Acer* are presented as raw counts of number of embolized vessels versus distance for control and experimental petioles (Fig. 6a); the control and experimental petioles were both pressurized in the pressure bomb. The increase in number of embolisms from the base to the apex may have been caused by the pressurization in the bomb or due to freezing of leaf blades with petioles. So we did two more 'controls' that were never in the pressure bomb and were frozen with or without the leaf blades attached. The number of embolized vessels at 50 mm was  $81 \pm 12$  ( $n = 5$ ) and  $77 \pm 12$  ( $n = 5$ ) when petioles were frozen with and without leaf blades attached, respectively. The count of embolized vessels in the profiles in Fig. 6a at 50 mm was not significantly less. We tentatively conclude that the distal end of *Acer* petioles is more vulnerable to cavitation than the basal end or that there is a significant gradient in water potential during transpiration

that accounts for the gradient in native embolism. In *Juglans* there were low numbers of embolized vessels at all positions for the control petioles (Fig. 6b), but there were significantly higher numbers of embolized vessels at 10, 30, 50 and 70 mm.

The number of embolisms due to air being sucked into vessels in the experimental petioles was computed from (the embolized vessel count of the experimental petioles) – (the embolized vessel count of control petioles). The difference, expressed as a percentage, was plotted on the same graphs with open-vessel length determinations by the paint-perfusion method in Fig. 4a for *Acer* and Fig. 4b for *Juglans*. The slopes obtained by using the cryo-SEM and paint-perfusion methods were not significantly different.



**Figure 6.** The determination of open vessel length by the cryo-SEM method are shown with means and SE for controls and experimental petioles versus distance from cut surface, i.e. 0 mm is the base of the petiole. (a) *Acer*  $n = 4$ , closed circles (experimental) show the number of air-filled vessels in experimental petioles when air is allowed to enter the cut surface. Open circles (control) show the number of air-filled vessels in control petioles when air is not allowed to enter the cut surface. (b) *Juglans*  $n = 6$ , closed circles (experimental) show the number of air-filled vessels in experimental petioles when air is allowed to enter the cut surface. Open circles (control) show the number of air-filled vessels in control petioles when air is not allowed to enter the cut surface. Closed circles and triangles are for field-grown versus greenhouse-grown leaves, respectively.

## DISCUSSION

Canny (1997a) 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 LN<sub>2</sub>. The petioles were sectioned while frozen and were observed in a cryo-scanning electron microscope (cryo-SEM). Although 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 (1–16 min) the excised leaves were allowed to transpire before freezing in LN<sub>2</sub>. Although Canny (1997a) did not characterize the transpiration rate or water potential of the leaves at the time of excision, there is little doubt in our minds that vessels should have drained in less than a minute. For example we have carried out experiments on potted sunflower plants under the low light and low transpiration conditions in a laboratory and have cut leaves under water and immersed the petioles in Phloxine B dye (Cyanosine; Sigma Chemicals, St Louis, MO, USA). Within 1 min the dye was observed in the minor veins of the leaf blade (personal observation). If the dye can move that distance in a minute, then air should displace water with equal speed.

In a companion paper Canny (1997b) froze sunflower leaves and petioles in LN<sub>2</sub> before excising them from the plants. Canny documented the transpiration rate and balance pressure of adjacent leaves prior to freezing. Figure 11 in Canny (1997b) 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 he 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 (1997a) the reader might also conclude that there was a weak correlation between xylem pressure and transpiration rate. Canny (1997b) felt that his experiment ‘negates all the assumptions and evidence of the Cohesion–Tension theory’. Subsequently several papers have appeared to confirm the Cohesion–Tension theory and readers interested in this story should read Tyree & Zimmermann (2002 pp. 49–81) and literature cited therein. Furthermore, the studies described in Cochard *et al.* (2000) and Cochard, Améglio & Cruiziat (2001a, b) have shown that freezing water-filled, closed vessels while under negative pressure can induce embolism when the water pressure is below –0.5 MPa but they remain ice-filled when frozen at less negative pressure. Hence Cochard *et al.* (2000) may have explained why cryo-SEM studies show few embolized vessels in the morning and afternoon but many at peak transpiration around mid-day. Apparently cryo-SEM studies cannot be relied upon to prove that vessels embolize and refill while pressures remain somewhat negative (see also Cochard *et al.* 2001a, b; Canny, Huang & McCully 2001; Richter 2001).

The observations of Canny (1997a) on sunflower are not reproducible in *Acer* and *Juglans*. 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 between methods in *Acer* and *Juglans* petioles. In *Acer* the slope from the silicone microcast count is also the same as that obtained from the cryo-SEM and paint-perfusion methods.

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 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. In 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 sclereids compared to vessels there is considerable uncertainty in the reference counts at the cut surface. So we argue 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 >99% loss of hydraulic conductance in the first segment and the PLC is still significantly above the controls at a distance of 90 mm from the cut surface in *Juglans*. The profile of PLC cannot be related directly to vessel counts since conductance of vessels increases with the fourth power of the vessel diameter. However, the hydraulic method provides strong support for the Scholander assumption and cannot be questioned because of possible artifacts 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, Ewers & Tyree 1994) and has been used commonly as a method of determining maximum open vessel length in the experimental design for new species such as maize leaves (Cochard 2002; and unpublished methods used frequently by Cochard and Tyree).

Clearly, the reason for the different behaviour of sunflower (Canny 1997a) needs to be addressed in further studies. However, we feel that our study has better control of the conditions and expectations of the Scholander assumption because: (1) Canny (1997a) did not know the water-potential status of leaves at the time they were cut in the air whereas we dehydrated our leaves in a pressure bomb so that all leaves were at the same water potential prior to exposure of the cut surfaces to air or water; and (2) we used more methods to estimate open-vessel length distribution than did Canny (1997a) and hence we have a better predic-

tor of embolism profiles according to the Scholander assumption.

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