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Limits to xylem refilling under negative pressure in *Laurus nobilis* and *Acer negundo*

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ABSTRACT

The ability of juvenile *Laurus nobilis* and *Acer negundo* plants to refill embolized xylem vessels was tested under conditions of soil drought when xylem sap pressure was substantially negative, thus violating the expected condition that pressure must rise to near atmospheric for refilling. Intact potted plants were dried to a stem water potential (Ψ_W) corresponding with approximately 80% loss of hydraulic conductivity (PLC) in shoots. Then plants were re-watered and kept at a less negative target Ψ_W for 1–48 h. The Ψ_W was measured continuously with stem psychrometers. Rewatered *L. nobilis* held at the target Ψ_W for 1 h showed no evidence for refilling unless Ψ_W was within a few tenths of a MPa of zero. In contrast, re-watered *L. nobilis* held for 24 and 48 h at water potentials well below zero showed a significant reduction in PLC. The recovery was highly variable, being complete in some stem segments, and scarcely evident in others. Embolism repair was accompanied by a significant but moderate decrease in the osmotic potential (Ψ_π) of the bulk xylem sap (Ψ_π = –67 kPa in recovering plants versus –31 kPa in controls). In contrast, embolized and re-watered *A. negundo* plants held for 24 h at target Ψ_W of –0·9 and –0·3 MPa showed no embolism reversal. The mechanism allowing *L. nobilis* plants to refill under negative pressure is unknown, but does not appear to operate in *A. negundo*, and is slower to act for drought-induced embolism than when embolism was artificially induced by air injection as previously shown for *L. nobilis*.

Key-words: embolism repair; osmotic potential; pit membrane osmosis; refilling mechanism; xylem cavitation; xylem parenchyma; xylem transport.

INTRODUCTION

Recent papers on a variety of species (summarized below) have suggested that embolized xylem conduits can refill despite significant negative pressure in the ambient xylem sap. This is a remarkable observation that has no mechanistic explanation, although ideas are not lacking. To date, there has been no systematic time course of the cavitation, refilling, and xylem pressure during a soil drought and re-watering cycle in species suspected of this novel refilling behaviour. In this paper, we supply this information for *Laurus nobilis* and *Acer negundo*. *Laurus nobilis* has been shown to exhibit novel refilling in response to air injection but not soil drought (Salleo *et al*. 1996; Tyree *et al*. 1999) whereas nothing is known of refilling in *A. negundo*.

Figure 1 illustrates the refilling conundrum. The typical vulnerability curve (solid line) shows how the percentage loss of hydraulic conductivity (PLC) in xylem increases from cavitation as the xylem pressure becomes more negative. The question is: what happens to the PLC as negative pressure return to atmospheric in intact plants, assuming sufficient time to equilibrate any change in PLC with pressure? The expectation, based on what is known of bubble dynamics in liquids, is shown by the dashed 'expected refilling' line (Fig. 1): no PLC recovery until xylem pressure (Ψ_{PX}) rises within several kPa of atmospheric (Yang & Tyree 1992). The precise recovery threshold depends on the gas contents in the sap and bubble, and bubble size. Briefly (see Yang & Tyree 1992), to collapse a water vapour bubble, the Ψ_{PX} must exceed $P_{wv} - 2T/r$, where P_{wv} is the vapour pressure of water, T is the surface tension (0·0728 N m^{-1} at 20 °C), and r is the radius of curvature of the gas–water interface of the embolus occupying the conduit (approximately the radius of the conduit). For $r = 8\ \mu$m, the minimum Ψ_{PX} allowing reversal of a vapour embolism will be approximately −118 kPa relative to atmospheric pressure at sea level (Fig. 1, left hand $-2T/r$ limit). If the embolus contains air, and the sap is air-saturated, the minimum Ψ_{PX} for the same size conduit rises to $-18\ \text{kPa} = P_a - 2T/r$, where P_a is atmospheric pressure (Fig. 1, right-hand $-2T/r$ limit).

Experiments on isolated stem segments under negative pressure in the laboratory have demonstrated refilling, but always within what we refer to here as the '$-2T/r$ limit'. When conditions were altered so that refilling would not be expected, none was observed (Borghetti *et al*. 1991; Sobrado, Grace & Jarvis 1992; Tyree & Yang 1992; Edwards *et al*. 1994).

In contrast, experiments on intact plants have suggested that embolism can be reversed when the ambient Ψ_{PX} is more negative than the $-2T/r$ limit. The extreme of this novel refilling behaviour is no hysteresis in the vulnerability curve (Fig. 1, dotted line, 'novel refilling'). The most extensive evidence is for *L. nobilis* (Salleo *et al*. 1996; Tyree *et al*. 1999). Embolism was induced artificially by air injection of

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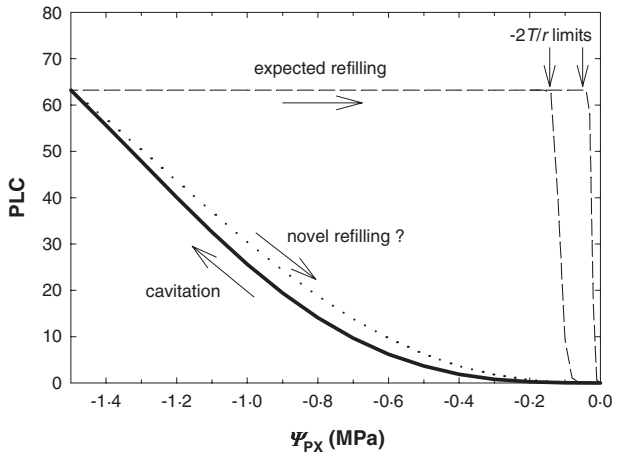

Figure 1. The refilling question concerns the nature of the equilibrium (time independent) hysteresis in a xylem vulnerability curve. A 'dehydration' vulnerability curve (the solid line) shows how the percentage loss in the hydraulic conductivity of xylem (PLC) increases as xylem pressure (Ψ_{PX}) becomes more negative during water stress and causes cavitation. Conversely, a 'rehydration' vulnerability curve (broken lines) shows how the PLC changes as Ψ_{PX} becomes less negative during stress relief. The dashed 'expected refilling' rehydration curve predicts no vessel refilling and PLC recovery until Ψ_{PX} rises within several kPa of atmospheric as expected from the physics of gas bubble dissolution (Yang & Tyree 1992). The precise recovery threshold depends on gas contents in sap and bubble, and bubble size, but is delimited by the two vertical lines ($-2T/r$ limits). The dotted 'novel refilling' rehydration curve represents plants that can refill vessels to the cavitation point under substantially negative pressure by an as yet unknown mechanism. The extreme of this behaviour would be no equilibrium hysteresis in the vulnerability curve.

stems on intact plants. Despite a Ψ_{PX} estimated to be below −0·5 MPa (well below the $-2T/r$ limit), the injected plants showed a rapid (20 min) partial refilling based on a hydraulic method for measuring embolism. Apparently, the air injection process altered the refilling behaviour relative to embolisms induced naturally by water stress in this species. When substantial embolism was induced naturally by drought, there was no 20 min recovery – the rapid recovery was seen only when the embolism was induced artificially by air injection (Salleo *et al*. 1996). Similarly, an earlier study of pot-grown *L. nobilis* plants showed lack of recovery of droughted and embolized plants 24 h after irrigation (Salleo & LoGullo 1993). Thus, it is possible that the novel refilling in *L. nobilis* is a unique response to the air injection treatment.

Although novel refilling has been suggested for other species, the evidence is not as convincing as for *L. nobilis*. For example, Cochard and colleagues (Cochard *et al*. 2000) have exposed important flaws in the application of freezing-stage electron microscopy (cryo-SEM) to the refilling problem. Diurnal courses of cavitation and refilling, taken to show a novel refilling process at work in a variety of species (McCully, Huang & Ling 1998; McCully 1999; Facette *et al*. 2001; Melcher *et al*. 2001), were shown to be artifacts of freezing the xylem under transpirational conditions in walnut (*Juglans regia*) petioles. Other observations lack a full record of Ψ_{PX} measurements at the site of refilling and during refilling, so it is difficult to know whether the xylem pressure was below the $-2T/r$ limit throughout the refilling period (Sperry & Sullivan 1992; Sperry 1993; Sperry *et al*. 1994; Zwieniecki & Holbrook 1998; Holbrook *et al*. 2001).

In this article, we document the course of cavitation and refilling in *L. nobilis* subjected to a drought and re-watering cycle to determine whether novel refilling occurs following naturally induced cavitation. For comparison, we tested for novel refilling using the same protocol in *A. negundo*, a species where no refilling information is available. The procedure was to continuously monitor the stem water potential (Ψ_W) during the drought cycle while determining the amount of embolism at the site of the water potential measurement. Plants were held long enough at each Ψ_W to saturate the embolism response whether on the dehydration or rehydration phase of the vulnerability curve (Fig. 1). Embolism was measured with the hydraulic method (Sperry, Donnelly & Tyree 1988a). Osmotic potentials (Ψ_π) of the xylem sap were measured at key points to estimate Ψ_{PX} from Ψ_W ($\Psi_{PX} = \Psi_W - \Psi_\pi$).

METHODS

Plant material

Sixty plants of *Laurus nobilis* L. were purchased from nurseries and grown in the greenhouse of the University of Utah until they reached the desired size. The plants were 2–4 years old and approximately 1 m tall when used. *Acer negundo* saplings were propagated from cuttings collected in Red Butte Canyon near the University of Utah. Plants were approximately 1 m tall when used. Rapidly growing 1- to 2-year-old stems were used for the experiments.

Dehydration vulnerability curve and hydraulic conductivity measurements

Intact plants were dried in the laboratory under two 1000 W Na-vapour HID lamps at a photosynthetic photon flux density of approximately 350 μmol m^{-2} s^{-1} (at plant level). Dehydration proceeded for several days. The Ψ_W of a basal stem portion was monitored with a temperature-corrected stem psychrometer (PWS, Guelph, Ontario, Canada). The psychrometer calibration was frequently checked, and readings never differed more than 0·03 MPa from the water potential of NaCl solutions with known osmotic potential. Measurements were made in the laboratory where temperature differences between stem surface and psychrometer chamber were stable and within the correctable range using standard procedures for the instrument (Dixon & Tyree 1984; Comstock & Mencuccini 1998). At various stem Ψ_W a 12–16-cm-long, 1- to 2-year-old stem segment was cut from the plant under water. The initial hydraulic conductivity (k_i) of the segment was measured within 15 min of removal from the plant using a conductiv-

ity apparatus (Sperry *et al*. 1988a). The normal perfusion solution was filtered (0·2 μm) 50 mM KCl (Salleo *et al*. 1996; Tyree *et al*. 1999). After k_i had been determined, segments were flushed with the perfusion solution for 20 min at 125 kPa. Hydraulic conductivity was re-measured, and the flushing repeated until a stable plateau was reached. Usually, segments reached their maximum hydraulic conductivity (k_{max}) after the first 20 min flush. The percentage that k_i was below k_{max} gave the PLC. By plotting PLC versus the corresponding Ψ_W, as measured by the stem psychrometer, we obtained a vulnerability curve with respect to Ψ_W. A full dehydration curve was determined for *L. nobilis* (see Figs 3 & 4), whereas *A. negundo* plants were all dehydrated to a similar target Ψ_w of –2 MPa based on previously published dehydration vulnerability curves (Hacke *et al*. 2001).

For a subset of measurements on *L. nobilis* we used a 200 mM KCl perfusing solution instead of the usual 50 mM concentration. The xylem conductivity increases significantly with KCl concentration in *L. nobilis* (Zwieniecki, Melcher & Holbrook 2001). If the concentration of the measuring solution is not at least as high as in the native sap, the PLC due to embolism could be under-estimated. When switching the KCl concentration from high to low, it takes several minutes of perfusion for the consequent drop in conductivity to occur (Ieperen, Meeteren & Gelder 2000; Zwieniecki *et al*. 2001). If the xylem sap KCl concentration was higher than that of the measurement solution, the k_i could be too high (being measured within minutes and thus influenced by the high xylem sap KCl concentration) relative to the k_{max} which was measured after prolonged flushing with the lower KCl concentration. At 200 mM KCl, we could be reasonably sure that the measuring solution was at a higher concentration than the xylem sap. The conductivity response to an increase in KCl concentration is nearly instantaneous (as opposed to the delayed response to decreasing concentration), meaning that both the k_i and k_{max} would reflect the same KCl concentration and the only difference would be in the amount of embolism present.

We also expressed k_{max} relative to the area of a stem cross-section (including bark, $k_{s\text{-}max}$). This was carried out to check whether there was any reduction in the maximum hydraulic conductivity due to tylosis formation during the course of the refilling experiments. If present, this could give the impression of refilling over time (a reduction in PLC during the rehydration phase) when none was actually occurring.

Air injection vulnerability curve

Vulnerability curves of *L. nobilis* were also measured with the air injection method (Cochard, Cruiziat & Tyree 1992; Salleo *et al*. 1992; Sperry & Saliendra 1994). The principle of the method is to measure the PLC while embolism is induced by forcing air across intervessel pits into the xylem at elevated air pressure. Although this method may alter the refilling response, the air injection technique has been shown to provide reliable vulnerability curves in many species (summarized by Sperry *et al*. 1996). Previous studies have shown that hydraulic conductivity decreases with increasing air pressure in the same manner as it decreases with decreasing Ψ_{PX} (Salleo *et al*. 1996; Sperry *et al*. 1996). After segments 20 cm in length had been flushed to k_{max}, they were mounted in a double-ended pressure bomb as described in Sperry & Saliendra (1994). The bomb was first pressurized to 0·1 MPa, and flow was measured as a reference value. The bomb pressure was then raised by a specified amount (usually in 0·5 MPa steps) and held for 10 min. The bomb was then depressurized to 0·1 MPa, and hydraulic conductivity was re-measured. The percentage that conductivity was below the original reference value gave the PLC induced by air pressure.

Rehydration vulnerability curve

Potted plants of *L. nobilis* were droughted to stem Ψ_W ~ – 2·7 MPa, which was low enough to cause a predictable and substantial PLC, and yet is also within the Ψ_W range *L. nobilis* experiences in nature (Duhme & Hinckley 1992). Potted *A. negundo* plants were droughted to approximately –2 MPa, which corresponds to severe drought stress for this species (Dina & Klikoff 1973). A subset of these stressed plants was harvested for PLC measurements. The 'stressed' PLC average was used to determine the extent of refilling (= PLC reduction) in the re-watered plants. Plants were re-watered while Ψ_W of the stem was continuously monitored with a stem psychrometer. Usually, plants were re-watered in the afternoon, and had established a relatively constant Ψ_W plateau (the 'target Ψ_W') by the next morning (e.g. Fig. 2). *Laurus nobilis* plants remained at their target Ψ_W for holding periods of 1, 24 or 48 h. *Acer negundo* plants were held for 24 h. During the holding period plants were

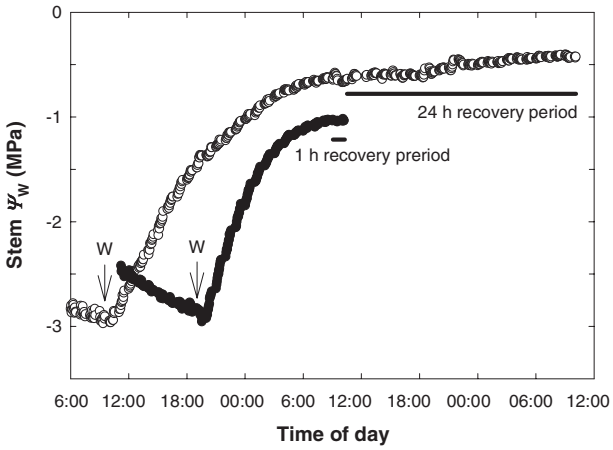

Figure 2. Stem water potential (Ψ_W) of two potted *Laurus nobilis* plants as measured with stem psychrometers during typical drying–re-watering experiments. The Ψ_W increased steeply within 30–60 min after a plant was re-watered (W). The Ψ_W reached a plateau after 12–24 h of watering. The Ψ_W of this plateau could be controlled somewhat by the amount of water added. Plants were held at a more or less constant 'target Ψ_W' for either 1 h (●), 24 h (○), or 48 h (not shown). The reported target Ψ_W was averaged over the holding period.

left unbagged under low light conditions in the laboratory. At the end of the holding period segments, 12–16 cm long, were cut under water, and their PLC was determined. The extent to which the PLC of these re-watered plants was below the stressed PLC indicated the amount of refilling, assuming the control experiments described above showed no KCl or tylosis artefact.

Regardless of how much water was added *L. nobilis* plants never achieved a stem Ψ_W higher than approximately −0·3 MPa by themselves. To test for refilling at higher Ψ_W (values closer to zero) in *L. nobilis* we used a root pressure bomb (Saliendra, Sperry & Comstock 1995). This was carried out after stem Ψ_W had reached a plateau of approximately −0·5 MPa. The potted root system was sealed within the bomb with the shoot emerging. Bomb pressure was slowly raised while Ψ_W was recorded on a protruding stem portion with a stem psychrometer. The psychrometer had previously been calibrated for the Ψ_W range between 0 and −0·5 MPa.

Laurus nobilis plants tested for refilling after a 1 h holding period had only one major stem, so the 1 h data points in Fig. 4a (open circles) refer to one plant and one segment. *L. nobilis* plants tested for refilling after a 24 and 48 h holding period had multiple (10–15) stems. Each PLC measurement shown for these plants is the mean of three to four stems per plant (open symbols in Fig. 4b). The root pressure chamber was not used for these multiple-stemmed plants. *Acer negundo* plants were only tested for refilling after 24 h at two holding pressures, with two to four segments per plant and treatment harvested for a total of six plants. We tested for differences in PLC between basal and distal segments of long shoots. However, results for distal and basal segments were identical at all pressures (*t*-test), so data was pooled. The mean PLC for each holding pressure is shown for all segments from all plants in Fig. 7.

Osmotic potential of xylem sap

To test whether refilling in *L. nobilis* was associated with an increase in the xylem sap solute concentration, five plants were dried to an average stem $\Psi_W = -2{\cdot}7 \pm 0{\cdot}2$ MPa (mean ± SD), and subsequently re-watered. Plants were held for 24 h at a target Ψ_W of $-0{\cdot}5 \pm 0{\cdot}1$ MPa. Xylem sap was then extracted in the following manner: One long (75 cm) shoot was cut from a plant. About 2·5 cm of bark, phloem, and cambium were removed from the cut end of the shoot to prevent contamination of the sap. The cut end was then trimmed with a razor blade and cleaned with deionized water and tissue paper. The shoot was inserted into a long custom-made pressure chamber with the cut end protruding. The air pressure in the chamber was raised until xylem sap occurred at the cut surface (balancing pressure). To prevent dilution of xylem sap by hyperfiltered cellular water, only a low over-pressure of approximately 0·3 MPa was used for sap extraction (Berger, Oren & Schulze 1994). The over-pressure was defined as the additional air pressure applied to the pressure chamber after the balancing pressure had been established. The first drops of xylem sap were collected in plastic tubing attached to the cut end of the shoot. The sap was stored in Eppendorf caps in a freezer. Xylem sap was collected in the same manner from five control plants, which had always been kept in moist soil. The osmotic potential (Ψ_π) of the xylem sap was measured with an isopiestic psychrometer (Boyer 1995; Isopiestics Co., Lewes, DE, USA). Deionized water was placed on tissue paper in the cup of the psychrometer. Known sucrose solutions were placed on the thermocouple with a syringe, and their output was measured and plotted. Xylem sap of unknown Ψ_π was then placed on the thermocouple and the Ψ_π was determined from the calibration plot.

Vessel diameters

For a general microscopic analysis of xylem cross-sections, lignified cell walls were stained with safranin. To determine the mean vessel diameter in laurel stems, cross-sections were prepared from stems that had previously been used for hydraulic measurements. Inner diameters of vessels were measured in radial sectors of $n = 5$ stems. A total of 300 vessels was measured using a light microscope interfaced with a bit pad (Sperry & Saliendra 1994).

Statistics

A one-way analysis of variance (ANOVA) combined with a *post-hoc* Tukey test was used to evaluate differences in the PLC of stressed versus stressed and re-watered plants. An unpaired *t*-test was used to test whether Ψ_π and $k_{s\text{-}max}$ differed between treatments. SPSS software (SPSS Inc., Chicago, IL, USA) was used for the analyses.

RESULTS

Laurus nobilis

Figure 3 shows vulnerability curves of *L. nobilis*. Curves obtained with the air injection method (Fig. 3, open circles) were similar to the natural dehydration curve (Fig. 3, closed circles), although the air injection method gave slightly higher PLC values at the high air pressure (low xylem pressure) end of the curve. The stem Ψ_W associated with a 50% loss of hydraulic conductivity (P50) was −1·66 and −1·49 MPa according to the air-dehydration and air-injection methods, respectively. Well-watered plants had native PLC between 0 and 40%, and their xylem pressure was between −0·5 and −1 MPa under laboratory conditions.

Plants stressed to approximately −2·7 MPa averaged between 75 and 80 PLC (Figs 3 & 4). When the stressed plants were re-watered, there was a 30–60 min lag before stem Ψ_W increased relatively rapidly and levelled at different target Ψ_W values depending on the amount of water given (Fig. 2). At the plateau, Ψ_W usually did not change more than 0·1 MPa h^{-1} (Fig. 2). The target Ψ_W was taken as the average of 5 min stem Ψ_W readings over the holding period.

Stressed plants that were re-watered and held for 1 h at

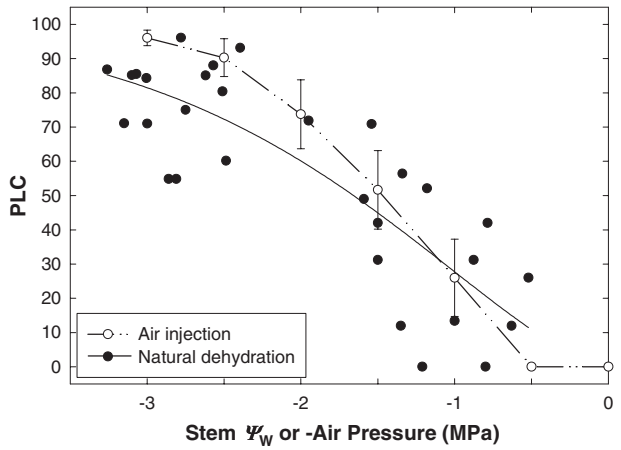

Figure 3. Vulnerability curves for 1- to 2-year-old *L. nobilis* stems, showing the percentage loss of hydraulic conductivity (PLC) with decreasing stem water potential (Ψ_W) or increasing air pressure. Curves were measured with the air injection method (○, -air pressure on *x* axis) and by air-drying potted plants to various water potentials (●, Ψ_W on *x* axis). Error bars are SE, $n = 5$ stem segments. Each closed circle represents one plant.

their target Ψ_W showed no refilling unless the target Ψ_W was above (less negative than) −0·3 to −0·2 MPa (Fig. 4a, open circles). The average PLC for re-watered plants with a target Ψ_W more negative than −0·3 MPa was no different from that of the corresponding stressed plants (Fig. 4a, compare stressed versus 50 mM 1 h holding). Only when the target Ψ_W was raised above approximately −0·3 MPa with the aid of the root pressure chamber did the PLC decline from the stressed value of approximately 80 to a minimum below 20. Thus, in the 1 h holding treatment there was a considerable hysteresis in the vulnerability curve and no evidence of any refilling unless Ψ_W was within a few tenths of an MPa of zero.

The calculated minimum average $-2T/r$ limit was $\Psi_{PX} = -0{\cdot}1$ MPa (relative to atmospheric), assuming $T = 0{\cdot}0728$ N m, a gas bubble of pure water vapour at 2·34 kPa, and an atmospheric pressure of 0·086 MPa at our laboratory in Salt Lake City, Utah. This corresponds to the average vessel diameter in stems of $20{\cdot}0 \pm 0{\cdot}9$ μm (mean ± SD), for an *r*-value of 10 μm.

Re-watered plants held for 24 h showed different refilling behaviour. These plants were all given similar amounts of water and reached a similar target Ψ_W averaging $-0{\cdot}73 \pm 0{\cdot}12$ MPa (mean ± SD). This target Ψ_W was more negative than the refilling threshold observed in the 1 h results. However, the longer holding period of 24 h resulted in a partial reversal of the PLC (Fig. 4b, open squares). The mean PLC for the plants held 24 h was near 40, which was significantly less than the approximately 80 PLC measured in the corresponding stressed plants (Fig. 5, compare 50 mM stressed versus 50 mM, 24 h holding). The same trend (although not significant) was observed if we repeated the 24 h holding experiment using a 200 mM KCl measuring solution instead of the 50 mM concentration (Fig. 4b, triangles versus squares; Fig. 5, compare 200 mM stressed versus 200 mM 24 h holding). This suggested that the decline in PLC was not an artefact of the KCl effect on xylem conductivity (see Methods). To test whether additional refilling would occur with a longer holding time, we re-watered another set of stressed plants and held them for 48 h. As for the 24 h experiments, we gave all plants similar amounts of water, and they reached a similar target Ψ_W averaging $-0{\cdot}49 \pm 0{\cdot}08$ MPa. The results were similar to the 24 h experiment: the PLC in re-watered plants held for 48 h was significantly less than for the corresponding stressed plants, but no less than for re-watered plants held for 24 h (Fig. 4b, open diamonds; Fig. 5, compare 200 mM stressed with 200 mM 48 h holding). This result suggested that the drop in PLC was not progressive over time, but was completed within 24 h.

To test whether the drop in PLC after 24 and 48 h was

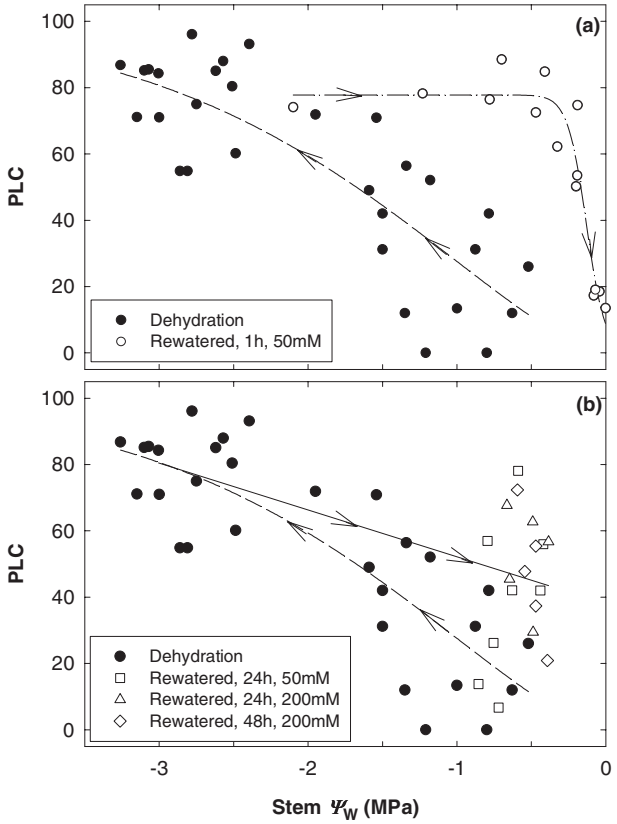

Figure 4. (a) Hysteresis in the refilling of *L. nobilis* xylem vessels under negative pressure. The dehydration curve (solid symbols) is for plants dried to various water potentials (Ψ_W), and is the same data shown in Fig. 3. The rehydration curve (open symbols) is for plants dried to approximately −2·7 MPa, re-watered, and held for 1 h at their target Ψ_W (e.g. Fig. 2). Each data point represents one plant. (b) Percentage loss of hydraulic conductivity (PLC) in dehydrated plants (●, same data as in Figs 3 and 4a) versus plants that had been stressed to approximately −2·7 MPa and re-watered with equivalent amounts of water (open symbols). After re-watering, all plants reached a similar target Ψ_W and were held for either 24 h (□ = 50 mM KCl; △ = 200 mM KCl) or 48 h (◇ = 200 mM KCl). The concentrations refer to the KCl solution used to measure the PLC.

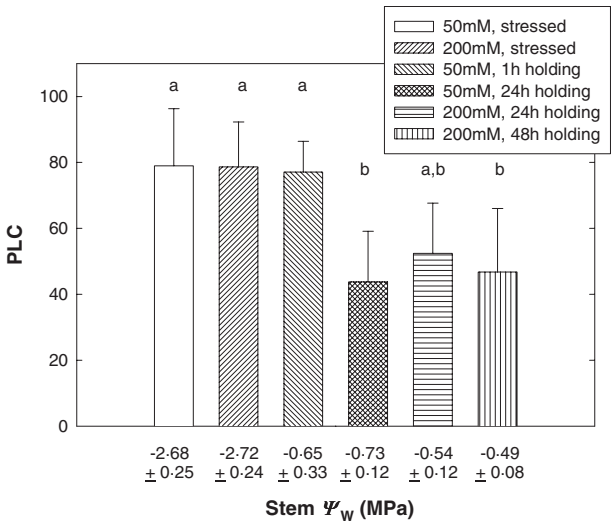

Figure 5. Summary of the refilling experiments, showing the percentage loss in hydraulic conductivity (PLC) in stems. The legend gives the KCl concentration used to measure PLC for each experiment. Below each column is shown the average target stem water potential (Ψ_W) for each treatment. The first two columns (left to right) are plants stressed to approximately −2·7 MPa as indicated, but not re-watered. The remaining columns are plants stressed to approximately −2·7 MPa, re-watered, and held for the indicated times. The data for the last three columns (24 and 48 h experiments) are the averages from the individual data points shown in Fig. 4b. The data for the 1 h holding time (third column) are mean values for the data in Fig. 4a, considering values between −1·2 and −0·3 MPa only. Values are means ± SD, $n = 5$–6. Treatments with same letters represent homogenous subsets of means determined with a Tukey test ($P > 0·05$).

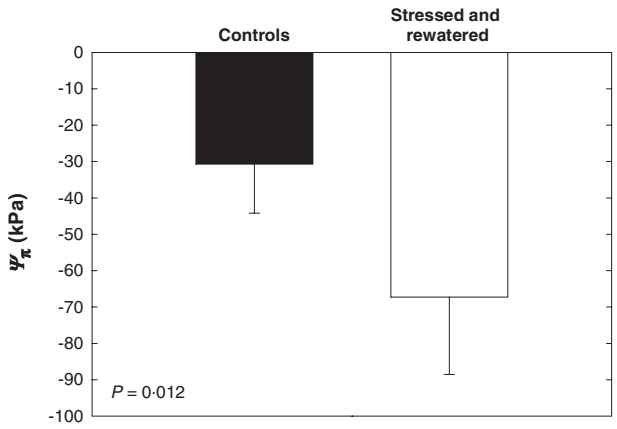

Figure 6. Osmotic potential (Ψ_π) of the xylem sap in continuously well-watered control plants versus plants that were droughted to approximately −2·7 MPa, re-watered, and held for 24 h at their target Ψ_W. Mean and SD, $n = 5$. Plants undergoing a drying-re-watering cycle showed more negative Ψ_π, $P = 0·012$ (*t*-test).

an artefact of a drop in maximum hydraulic conductance (k_{max}) due to tylosis formation in embolized vessels, we compared the k_{max} on an area basis ($k_{s\text{-}max}$) between stems that were dehydrated and not re-watered versus stems that had been dehydrated, re-watered, and held at their target Ψ_W for 48 h.

There was no basis for suspecting a PLC artefact due to tylosis development. The $k_{s\text{-}max}$ did not decrease as a result of the holding period (0·29 ± 0·13 kg s^{-1} MPa^{-1} m^{-1} in dehydrated plants versus 0·27 ± 0·12 kg s^{-1} MPa^{-1} m^{-1} after a 48-h holding period, $n = 5$ plants, means ± SD, *t*-test: $P > 0·05$).

The Ψ_π of well-watered, nonstressed plants averaged −30·8 ± 13·4 kPa (Fig. 6). Plants stressed to −2·7 ± 0·2 MPa and subsequently re-watered to −0·5 ± 0·1 MPa for 24 h exhibited a Ψ_π of −67·3 ± 21·2 kPa (means ± SD, $n = 5$). This was significantly more negative than the controls (Fig. 6). Subtracting the Ψ_π of re-watered plants from the target Ψ_W measured with the psychrometer gives an estimate of the xylem pressure (Ψ_{PX}) during xylem refilling. The minimum average target Ψ_W during which we measured a reduction in PLC was −0·73 MPa (Fig. 5, 50 mM, 24 h holding). The Ψ_{PX} would have been approximately −0·66 Mpa, which was considerably below the minimum $-2T/r$ limit of $\Psi_{PX} = -0·1$ MPa.

Acer negundo

In contrast to *L. nobilis*, *A. negundo* plants did not show significant refilling, even after 24 h (Fig. 7). *Acer negundo* plants stressed to a stem Ψ_W of −1·99 ± 0·04 MPa were between 70 and 80% embolized (Fig. 7 'stressed'). Plants re-watered to either −0·91 ± 0·04 or −0·29 ± 0·02 MPa and held for 24 h showed no change in PLC (Fig. 7, 're-watered').

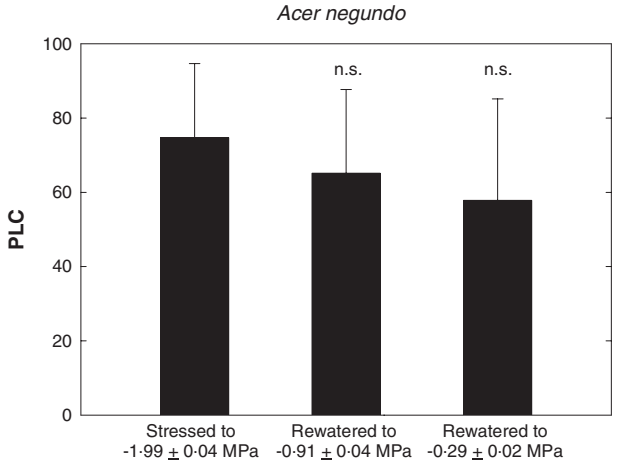

Figure 7. Percentage loss of hydraulic conductivity (PLC) in stems cut from *Acer negundo* plants at different points in a drought-re-watering cycle. 'Stressed' stems were harvested at the extreme of the drought when xylem pressures averaged near −2 MPa. 'Rewatered' stems were from stressed plants that were rehydrated and held for 24 h at an average target stem Ψ_W of −0·91 or −0·29 MPa. Grand means of 12 PLC measurements (six trees per treatment with basal and distal segments pooled) ± SD. A one-way ANOVA was not significant across treatments showing no refilling in re-watered plants.

DISCUSSION

We found no evidence of novel refilling following relief of soil drought in *Acer negundo*. Although this species may show seasonal refilling in association with near-atmospheric or positive xylem pressures as do other members of the genus (Sperry, Donnelly & Tyree 1988b; Hacke & Sauter 1996), it did not refill under the negative pressures tested in this study (Fig. 7). However, there was a non-significant trend towards lower PLC with higher Ψ_W in the re-watered plants (Fig. 7, compare stressed versus re-watered). The imposed drought of −2 MPa was severe for this species, causing considerable leaf dieback. To the extent a novel refilling process is dependent on an active plant metabolism, it could be impaired by excessive drought.

In *L. nobilis*, there was no clear indication of novel refilling in plants held for only 1 h at their target Ψ_W (proceeded by a nearly 12 h rehydration phase, Fig. 2). The Ψ_W threshold for refilling in these plants was quite close to the minimum $-2T/r$ limit of −0·1 MPa. Taking −0·25 MPa as the Ψ_W threshold (Fig. 4a, open symbols), and assuming a xylem sap Ψ_π of −0·067 MPa (as measured in 24 h plants, Fig. 6), the Ψ_{PX} threshold would be 0·18 MPa. Although this is slightly lower than the minimum $-2T/r$ limit, given the increasing error in conventional psychrometry as Ψ_W approaches within 0·2 MPa of zero (Boyer 1995), it is not low enough to unambiguously violate the $-2T/r$ limit. This result is consistent with Cochard's observation of no short-term refilling in *L. nobilis* stems cavitated and brought to near zero xylem pressures by centrifugation (Cochard 2002).

However, the results from *L. nobilis* plants held for 24 and 48 h at their target Ψ_W did exhibit novel refilling. The target Ψ_W for the 24 and 48 h experiments was well below the $-2T/r$ limit, and yet we observed a significant reduction in PLC. This could not be attributed to negative xylem sap Ψ_π, or artefacts associated with tylosis formation or KCl concentration. The recovery was very erratic, being complete in some stem segments and scarcely evident in others. The recovery reached a plateau after the 24 h holding period. Doubling the holding period did not increase embolism reversal (Figs 4b & 5).

These results are in general agreement with a previous study that found no short-term recovery from xylem embolism in *L. nobilis* when the embolism was induced naturally by negative xylem pressure during soil drought (Salleo & LoGullo 1993). In contrast, we found no evidence of the rapid recovery that was detected for embolism induced by air-injection while the Ψ_{PX} was substantially negative (Salleo *et al*. 1996; Tyree *et al*. 1999). Instead, we found a longer-term recovery process at work for naturally induced embolism. Perhaps the confusion will be resolved once more is learned of the mechanism for the novel refilling process. Induction of embolism by drought is necessarily accompanied by prolonged stress which could reduce energy stores required for driving a rapid refilling process.

There has been considerable speculation concerning the mechanism of novel refilling. Presumably the Ψ_{PX} in the refilling conduits must rise above the $-2T/r$ limit, whereas neighbouring conduits remain under lower pressure, probably via local osmotic action (Tyree *et al*. 1999). Holbrook & Zwieniecki (1999) have proposed what we call the 'pit valve' hypothesis. The xylem parenchyma cells adjacent to an embolized vessel secrete solutes that create an osmotic gradient for water movement into the embolized conduit. The water is prevented from draining into the neighbouring transpiration stream by persistent air pockets trapped by capillary forces in the pit chambers between the embolized and functional conduits. The pits act as valves allowing a build up of positive Ψ_{PX} in the embolized vessel. In this way, the Ψ_{PX} within the embolized conduit rises above the $-2T/r$ limit and dissolves the gas. The valves have been shown to work (see also Zwieniecki & Holbrook 2000), and metabolic poisons apparently reduce the refilling activity (Zwieniecki *et al*. 2000). However, the local osmotic potential of xylem sap due to inorganic ions as estimated in refilling vessels of *L. nobilis* (via microprobe analysis in cryo-SEM preparations) was not sufficiently negative to drive pressurization (Tyree *et al*. 1999). Moreover, for the process to be completed, the air pockets in the pit chambers must all dissolve simultaneously to re-connect the refilled vessel at positive Ψ_{PX} with the functional vessels at negative Ψ_{PX}. If an air pocket in one pit chamber dissolved before the rest, the remaining air bubbles would expand to re-embolize the vessel. Thus, although this is an interesting hypothesis, it has some unparsimonious complications, and the evidence is incomplete.

We introduce another idea for consideration (Fig. 8), for which there is no more or no less evidence, but which has

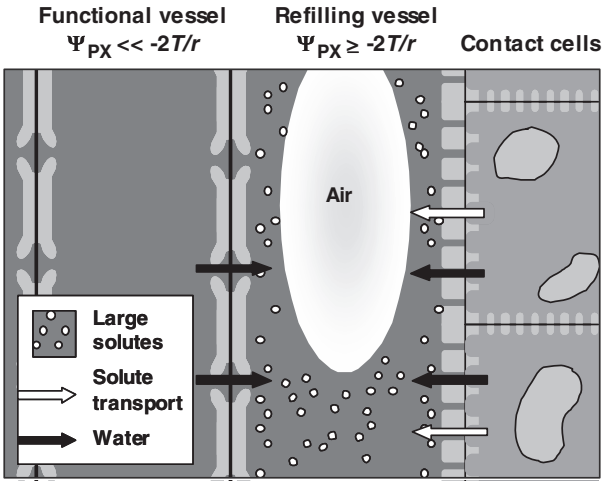

Figure 8. The 'pit membrane osmosis' hypothesis for vessel refilling. Living contact cells release solutes into a cavitated vessel, thereby locally lowering Ψ_π. The solutes are large enough to be held back in the refilling vessel by interconduit pit membranes, which therefore act as osmotic membranes. The high solute concentration in the refilling conduit attracts water both from parenchyma cells and from the transpiration stream, generating a positive Ψ_{PX} in the embolized vessel analogous to turgor pressure in a living cell. The refilling vessel remains hydraulically connected to the transpiration stream.

the virtue of eliminating the complication of hydraulic isolation of the refilling conduit from the surrounding sap stream. As in the pit valve hypothesis, an osmotic gradient is responsible for pulling water into the embolized vessel. As in the pit valve hypothesis, the negative Ψ_π is localized to the refilling vessel, becoming diluted when bulk samples are expressed for conventional Ψ_π measurement. However, unlike the pit valve hypothesis, this osmoticum is of large enough size to be impermeable to the pit membrane. A large organic solute would escape notice by microprobe methods that detect only inorganic ions (Tyree *et al*. 1999). Such a solute, being trapped within conduits, would also not be present in xylem sap extracts and would be missed by the methods used in our study (i.e. Fig. 6). With the pit membrane acting as an osmotic membrane, water can be pulled across the membrane from the transpiration stream at negative Ψ_{PX}. The embolized conduit under positive Ψ_{PX} remains connected to the sap stream while the air is dissolved. Semi-permeable cell walls are known to act as osmotic membranes (Oertli 1993) and osmosis across cell walls in response to a sucrose gradient has been implicated in maple sap flow (Johnson & Tyree 1992), so there is precedent for osmosis across cell walls. This 'pit membrane osmosis' mechanism would only work for species with relatively fine-textured pit membranes capable of excluding high molecular weight solutes – polysaccharides, for example.

At present there is no conclusive evidence for any particular mechanism – only that a novel refilling process exists and seeks an explanation. The protocol we used in our experiments is straightforward and provided good resolution of Ψ_{PX} for distinguishing between expected versus novel refilling for the most interesting case of drought-induced cavitation. The results suggest that novel refilling is not universal across species, being absent in *A. negundo*, and is slow and variable in soil-droughted *L. nobilis*. The approach can be readily extended to test the effect of experimental treatments on refilling behaviour, bringing us closer to an answer to this intriguing puzzle in plant water relations.

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