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Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism

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ABSTRACT

We investigated the common assumption that severing stems and petioles under water preserves the hydraulic continuity in the xylem conduits opened by the cut when the xylem is under tension. In red maple and white ash, higher percent loss of conductivity (PLC) in the afternoon occurred when the measurement segment was excised under water at native xylem tensions, but not when xylem tensions were relaxed prior to sample excision. Bench drying vulnerability curves in which measurement samples were excised at native versus relaxed tensions showed a dramatic effect of cutting under tension in red maple, a moderate effect in sugar maple, and no effect in paper birch. We also found that air injection of cut branches (red and sugar maple) at pressures of 0.1 and 1.0 MPa resulted in PLC greater than predicted from vulnerability curves for samples cut 2 min after depressurization, with PLC returning to expected levels for samples cut after 75 min. These results suggest that sampling methods can generate PLC patterns indicative of repair under tension by inducing a degree of embolism that is itself a function of xylem tensions or supersaturation of dissolved gases (air injection) at the moment of sample excision. Implications for assessing vulnerability to cavitation and levels of embolism under field conditions are discussed.

Key-words: air injection; bench drying; diurnal recovery; refilling; xylem cavitation.

INTRODUCTION

The cohesion-tension theory posits that xylem sap is under tension whenever a plant is transpiring and that the tension in the xylem is a function of the height above the ground, soil water availability, the transpiration rate of the plant and the hydraulic resistance of the plant/soil pathway. Under periods of low soil water availability or high transpirational demand negative pressures in the xylem can 'seed' the water column with tiny bubbles pulled into conduits, which can nucleate cavitation of the metastable water in the xylem conduits (Zimmermann 1983). These cavitation events disable the conduits in which they occur by blocking them first with water vapour and eventually with air (Yang & Tyree 1992). Embolized conduits diminish the hydraulic capacity of the plant, which in turn can limit its photosynthetic capability (Sperry 2000). The tension at which cavitation begins to occur appears to be well tuned to the water availability and transpirational demands of a given habitat (Pockman & Sperry 2000; Lopez *et al*. 2005; Choat *et al*. 2012). Nonetheless, cavitation seems to frequently occur because of drought and periods of high transpirational demand (Cruiziat, Cochard & Améglio 2002).

Traditionally, the loss of conduits to cavitation was thought to permanently diminish the hydraulic capacity of a plant, with limited opportunities for returning embolized conduits to a functional state (Zimmermann 1983). Plants can grow new xylem and in some cases, where transpiration is low and the soil saturated, generate a positive pressure that propagates up the xylem by transporting osmotically active solutes into conduits in the root zone. Yet, while repair by root pressure may be common in guttating herbs (Kramer & Boyer 1995), in tall woody plants its importance for stem xylem hydraulic capacity is generally believed to be limited to seasonal recovery (Sperry *et al*. 1987, 1994; Ewers *et al*. 2001). The idea that woody plants might be able to reverse embolism on a diurnal basis even when tensions in the bulk xylem are large was first put forward in the 1990's with the discovery that the percent loss of conductivity (PLC) due to embolism in air-injected samples of *Laurus nobilis* declined with time after the stems were depressurized (Salleo *et al*. 1996). A new paradigm of xylem as highly dynamic, experiencing cycles of embolism and repair has become increasingly accepted, with the ability of plants to refill under tension considered an ecologically important behaviour that allows plants to operate near their hydraulic limits (Clearwater & Goldstein 2005). But to date no comprehensive hypothesis exists to explain how plants can hydraulically isolate, refill and then reconnect conduits while the rest of the xylem remains under tension (Holbrook & Zwieniecki 1999; Tyree *et al*. 1999; Zwieniecki & Holbrook 2009).

The initial intent of this study was to evaluate the conditions under which diurnal PLC cycles occur in the hopes of better understanding the process of embolism repair. We sought to build on previous measurements (Zwieniecki & Holbrook 1998) with a larger sample size and better temporal resolution.

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In the 1998 study, long branches were cut from the tree in air and the segments used to measure PLC were excised promptly from the branch under water. For the current measurements, better canopy access allowed us to make the initial cut of the branch from the tree under water, after which the branches were transported to the laboratory before excising the measurement sample. As a result, xylem tensions were largely relaxed at the time the segment for PLC measurement was cut from the branch. If cutting xylem conduits under water preserved the integrity of the water column regardless of the initial tension present, there should have been no difference in the results due to this change in protocol, and yet in red maple we did not find the pattern of diurnal PLC seen in 1998. This led us to investigate whether diurnal cycles in PLC could result from embolism formed during the excision of samples with large xylem tensions even though those cuts were made under water. We also investigated whether supersaturating water in stems that occurs during air injection could increase the likelihood of embolism formation when xylem conduits are severed.

MATERIALS AND METHODS

Plant material

Plant material included forest grown trees at Harvard Forest, Petersham, MA, USA (hereafter, HF), as well as specimens grown on the Cambridge Campus of Harvard University, Cambridge MA, USA (hereafter, CC). At HF, red maple (*Acer rubrum* L.), paper birch (*Betula papyrifera* Marsh.) and white ash (*Fraxinus americana* L.) were studied. The maples were mature canopy trees, approximately 30 m tall, while the birch and ash were in a 20 m tall mixed stand. All HF material was harvested using a 22 m tall canopy lift, which allowed access to the upper crowns of the trees. At CC, we employed material from a population of six red maple and five sugar maple (*A. saccharum* Marsh) trees grown in an experimental garden, with some additional red maple material collected from three irrigated ornamental trees growing nearby. All of the trees were between five and eight metres in height; upper canopy branches were sampled with pole pruners. We also used 4-year-old sugar maple and 2-year-old paper birch saplings (Lawyer's Nursery), which were potted in five gallon pots in Fafard 3b mixture (March 2012) and grown under supplemental lighting [14 h days, 550 μmol m^{-2} s photosynthetically active radiation (PAR)] in two greenhouses. These plants were transferred in their pots to the experimental garden in June 2012 and grown outdoors with supplemental water.

In all cases, current year extension growth from the uppermost branches exposed to full sun was sampled. We limited the measured material to current year growth to avoid embolism that originated from conditions prior to the growing season (i.e. no freeze-thaw embolism) and to facilitate the cleanest cuts possible, thus reducing the possibility that damage to the xylem during harvest would affect our results. In addition, vessels from previous years may be more vulnerable to cavitation compared to current year conduits (Melcher, Zwieniecki & Holbrook 2003).

Hydraulic parameters

Maximum vessel length was measured following the protocol of Greenidge (1952), xylem pressure was measured using a pressure chamber, and hydraulic conductance was measured onto a digital balance following Sperry, Donnelly & Tyree (1988). Vulnerability curves were generated for both red and sugar maple using the centrifuge method described by Alder *et al*. (1997). These methods are detailed in supplementary materials. In describing our experiments, we use 'relaxed tension' to refer to material supplied with water to bring xylem pressures to near zero prior to sample excision. We use 'native tension' to refer to samples excised without a previous rehydration treatment. Note that in all cases, measurement segments were excised under water.

Diurnal PLC sampling

During the 2011 summer growing season, branches of HF trees were collected in the afternoon (between 1300 and 1500 h) and again the following morning (between 0530 and 0630 h). Two individuals of paper birch, four of red maple and six of white ash were sampled. A split funnel was wrapped around the branch to be sampled, sealed using duct tape and filled with water. The branch was then cut under water using razor clippers (1 megaCut S, Wolfcraft, Germany), which minimized crushing of the xylem, and the cut end immediately transferred to a bucket of water. The leafy end of the branch was covered with a large plastic bag and the branch, with its cut end remaining in water, was transferred to the laboratory. It took 30 to 60 min to transfer the samples to the laboratory. Based on subsequent measurements, xylem tension in the branches would have relaxed substantially before the measurement section was excised.

In maple and birch, the initial cut was roughly 0.5 m from the region within the current year's extension growth that would subsequently be used for measuring PLC. When expressed in terms of vessel lengths, the distance between the initial cut and the measurement segment was at least four maximum vessel lengths for maple and at least two maximum vessel lengths for birch. In ash, the maximum branch diameter that could be severed within the water-filled funnel, coupled with much longer maximum vessel lengths (ca 1 m), resulted in collected branches in which the distance between the cut end and the measurement section was typically less than one maximum vessel length.

In 2012, diurnal measurements of PLC in red maple were repeated to directly compare the effect on PLC of cutting xylem conduits under tension. In this experiment, two branches were collected from each of the four trees twice a day, in the morning (0500 to 0600 h) and the afternoon (1300 to 1500 h), repeated over 4 d. One of the branches was collected by the method described above, while the other was collected by cutting the branch in air, a minimum of three maximum vessel lengths (0.4 m) from the intended measurement segment. Immediately after the latter branch was cut from the tree, the apical end of the branch was bent into a rectangular container ($20 \times 30 \times 15$ cm) and the measurement

segment excised under water, where it remained during the 30 to 60 min necessary to begin hydraulic measurements on both sets of samples. Note that the key distinction here is not whether the branches were cut off in air versus water, but rather the degree of tension at the time the measurement segment was excised under water.

PLC of crown-irrigated white ash

Because we were unable to harvest white ash branches that were of sufficient length to avoid the possibility of severing xylem conduits that extended into the portion of the branch used to measure PLC, we conducted an experiment at HF in which branches cut from the tree under water during the afternoon (native tension) were compared with branches cut under water following a 30 min period during which water was supplied to the crown through a cut side branch (relaxed tension). Two adjacent branches (0.6 to 1.1 m in length) per tree from nine separate trees were harvested under water from the main axis near the apex of a tree during the afternoon. After the native tension branch was cut, the upper two meters of the crown was covered with a reflective bag to suppress transpiration and water was provided through the cut branch stub with water filled tubing that was attached to the cut end while it remained under water. After 30 min, a second branch located within the bagged crown was cut under water (relaxed tension). Samples used to measure xylem pressure (sealed in foil covered bags on the tree three hours prior to the experiment) were collected at the time that each branch was sampled. To ensure that the removal of these leaves did not introduce embolism into the measurement segments they were collected from a nearby branch. This experiment was conducted at the end of the 2011 summer season (late August).

Bench drying vulnerability curves

We compared bench drying vulnerability curves for red maple, sugar maple and paper birch (CC) assembled from measurements on samples cut under relaxed versus native tensions. The red maple samples came from trees in both the experimental rain-fed garden and the irrigated population. The sugar maple and paper birch samples were taken from potted plants. Forked branches 0.6 to 1.2 m long were cut in air and immediately enclosed in plastic bags for transport to the lab. After a thirty minute to one hour equilibration time, a short leafy shoot (a leaf for paper birch) was removed from the branch, a minimum of 30 cm (greater than one maximum vessel length for all species) from the sample segment, to determine the initial xylem pressure. To achieve a wide range of xylem tensions, some branches were air dried under lab lights (<10 μmol m^{-2} s^{-1} PAR) for periods of up to 8 h and then bagged for a minimum of 1 h to allow xylem pressure to equilibrate across the branch.

While the branch xylem was under tension, one segment (1–2 internodes long) was cut under water near the apical end of one of the forks to measure PLC (native tension). Fifteen centimetres of the base of the original branch was then removed under deionized water in several 2–5 cm cuts, with approximately 10–15 s between each cut, and the branch allowed to rehydrate for 30 min to 2 h while the shoot end remained sealed inside a plastic bag. Branches with more negative xylem pressures required longer relaxation periods to achieve a final pressure greater than –0.5 MPa (typically >–0.3 MPa). After relaxation, a second short leafy shoot was harvested to estimate the remaining tension in the branch with the pressure chamber, and the segment for PLC determination (relaxed tension) was cut under water near the apical end of the second fork.

PLC in petioles of red maple

We sampled petioles from trees in the experimental garden (CC) following the same forked branch protocol described above to determine whether petioles would behave similarly to branches. To determine the degree of background embolism present, we sampled in the morning during rain when xylem pressures were near zero. To determine the effect of cutting under tension we sampled petioles at midday tension during clear sunny days, without supplemental dehydration in the lab (native tension) and compared these with petioles sampled only after xylem tensions were relaxed (relaxed tension). For these measurements all cuts were made under degassed water.

Rapid relaxation of stems for PLC measurement

We compared the PLC of red and sugar maple branches sampled under native tension versus sampled after rapid relaxation (<2 min). The goal of these experiments was to avoid the possibility of some form of refilling occurring during the >30 min rehydration intervals of the experiments described above.

Branches (>0.5 m) containing three consecutive internodes, each >5 cm in length, within the current year's extension growth were cut from the tree in air and dried in the lab for varying amounts of time (0.5–2 h) to reach a target xylem pressure range (–2 to –3 MPa for red maple and –2.5 to –3.5 MPa for sugar maple). The branches were subsequently sealed in large plastic bags for 30 min to 2 h to allow the water potential to equilibrate across the stem. After this equilibration period, xylem pressure was measured on a short leafy shoot excised from the branch a minimum of 0.4 m (more than three maximum vessel lengths) from the intended measurement segments. The entire branch was then submerged in water and the basal and apical ends of a three internode long segment were cut under water in quick succession using a pair of razor hand pruners, after which the attached leaves were sliced with a fresh razor blade between the tertiary veins to reduce the sinks for hydration and allow the xylem tensions to relax rapidly. After 2 min under water, the central internode was excised (relaxed tension) and the PLC compared to that of the segment where the first cut was made (native tension). The sampling protocol is diagrammed in Supporting Information Fig. S1.

PLC following air injection

To evaluate whether air injected samples might also be subject to embolism induced by sample excision, we examined PLC in samples excised from air injected cut branches of sugar and red maple, by excising the measurement segment either 2 min or 75 min after pressure release (detailed in supplemental methods and illustrated in Supporting Information Fig. S2). The latter time period was chosen, based on our observations of bubble evolution from air injected stems, as our estimate of the minimum time needed to allow the concentration of dissolved gases within the stem to return to levels near ambient. Comparisons were made at three different air injection pressures (0.1, 1 and 4 MPa). To avoid complications associated with cutting material at native xylem tensions, all experiments were conducted on cut branches rehydrated in the lab until xylem tensions were near zero.

RESULTS

Diurnal PLC in Harvard Forest trees

The original goal of this study was to document diurnal patterns in PLC in forest trees to better understand the physiological constraints on embolism repair. We sampled three species, two of which (red maple and white ash) had been shown in a previous study to exhibit diurnal variation in PLC (Zwieniecki & Holbrook 1998). The current study differs from the previous not only in that all branches were cut from the tree under water, but more importantly that xylem tensions were allowed to relax before the segments used to measure PLC were excised.

We found that the PLC of the uppermost branches of red maple and paper birch trees sampled in this way was low and there was no significant difference between samples collected in the morning and at midday (Fig. 1). The low midday PLC in red maple found in this study cannot be attributed to a lack of xylem tension. At midday the xylem pressures measured in red maple during the sampling days ranged from –1.8 to –2.1 MPa, similar to values reported by Zwieniecki and Holbrook (–1.1 to –2.1 MPa). Birch midday xylem pressures ranged from –1.2 to –1.7 MPa.

In 2012, we repeated the diurnal measurements of PLC in red maple trees (HF), with the difference that segments for measurement of PLC were excised at both native and relaxed xylem tensions in the morning and the afternoon. As in 2011, red maple branches cut from the tree under water and then allowed to relax fully before excision exhibited low values of PLC that were not different between morning and midday (Fig. 2; $P = 0.26$ independent samples one tailed t-test). In contrast, branches cut in air (more than three maximum vessel lengths from the region to be measured) and from which the measurement segment was immediately excised under water (i.e. at native tension) had significantly higher PLC in the afternoon than the following morning (Fig. 2; $P = 0.006$ independent samples one tailed t-test). We interpret these results as evidence that diurnal variation in PLC in red maple can be the result of embolism introduced during sample collection. We therefore believe that the measurements of PLC made on relaxed branches reflect the state in nature and thus that the red maple trees at HF have low levels of embolism that do not fluctuate on a diurnal basis.

In contrast to red maple and paper birch, white ash had higher levels of PLC at midday (Fig. 1c; ca. 60%, xylem pressures –2.5 to –1.4 MPa) than the following morning (ca. 30%, 1.5 to –0.3 MPa), consistent with data reported in Zwieniecki & Holbrook (1998). However, for long-vesseled taxa such as ash (maximum vessel length 1 m, versus 18 cm for birch, and 9 cm for maple), the two sampling protocols would effectively have been the same, as the relaxed samples on which PLC was measured likely contained conduits opened while still under tension during the first cut from the tree. The much shorter vessels in maple and birch meant that the initial cuts, made when xylem tensions were at their native values, occurred several maximum vessel lengths away from the measurement segment.

Irrigating the crowns of white ash trees resulted in a significant reduction in afternoon PLC (Fig. 1d; $P = 0.007$ one tailed, paired samples t-test). The average PLC of branches cut at native tensions was 46%, while the branches sampled following crown irrigation for 30 min had a PLC of 21%. Xylem pressure measured prior to irrigation was –1.0 MPa ($\pm$0.1 MPa se), substantially wetter than measured during mid-summer. The xylem pressure following 30 min of crown irrigation was not significantly different from that measured prior to irrigation ($P = 0.41$ one tailed, paired samples t-test). We believe this may result from the large capacitance of the crown and the distance between the water supply and the leaves used to measure xylem pressure, which was much greater than the distance between the water supply and the branch from which the relaxed sample was collected.

Vulnerability curves sampled at native and relaxed tensions

We then set out to examine systematically whether severing conduits under water affects measurements of PLC by constructing vulnerability curves using the bench drying method. In one set of samples (dried to the target xylem pressure on the bench), the measurement segments were excised under water after the imposed xylem tension had been relaxed, while in a second set of samples segments were excised at native xylem tension. These measurements were made only on the two short-vesseled species (red maple and paper birch). The long vessels in ash precluded working with individual branches and we did not have access to a sufficient number of whole trees that could be sampled to construct the two vulnerability curves.

In paper birch, we found no evidence of a sampling artifact associated with the presence of xylem tensions (Fig. 3). Vulnerability curves in which the measurement segments were excised at native versus relaxed xylem tensions overlapped. In contrast, red maple showed a marked discrepancy between the vulnerability curve in which the measurement segments were excised at native xylem tensions versus the curve in which tensions were relaxed prior to sample excision (Fig. 4). In the relaxed curve, PLC remained low until xylem

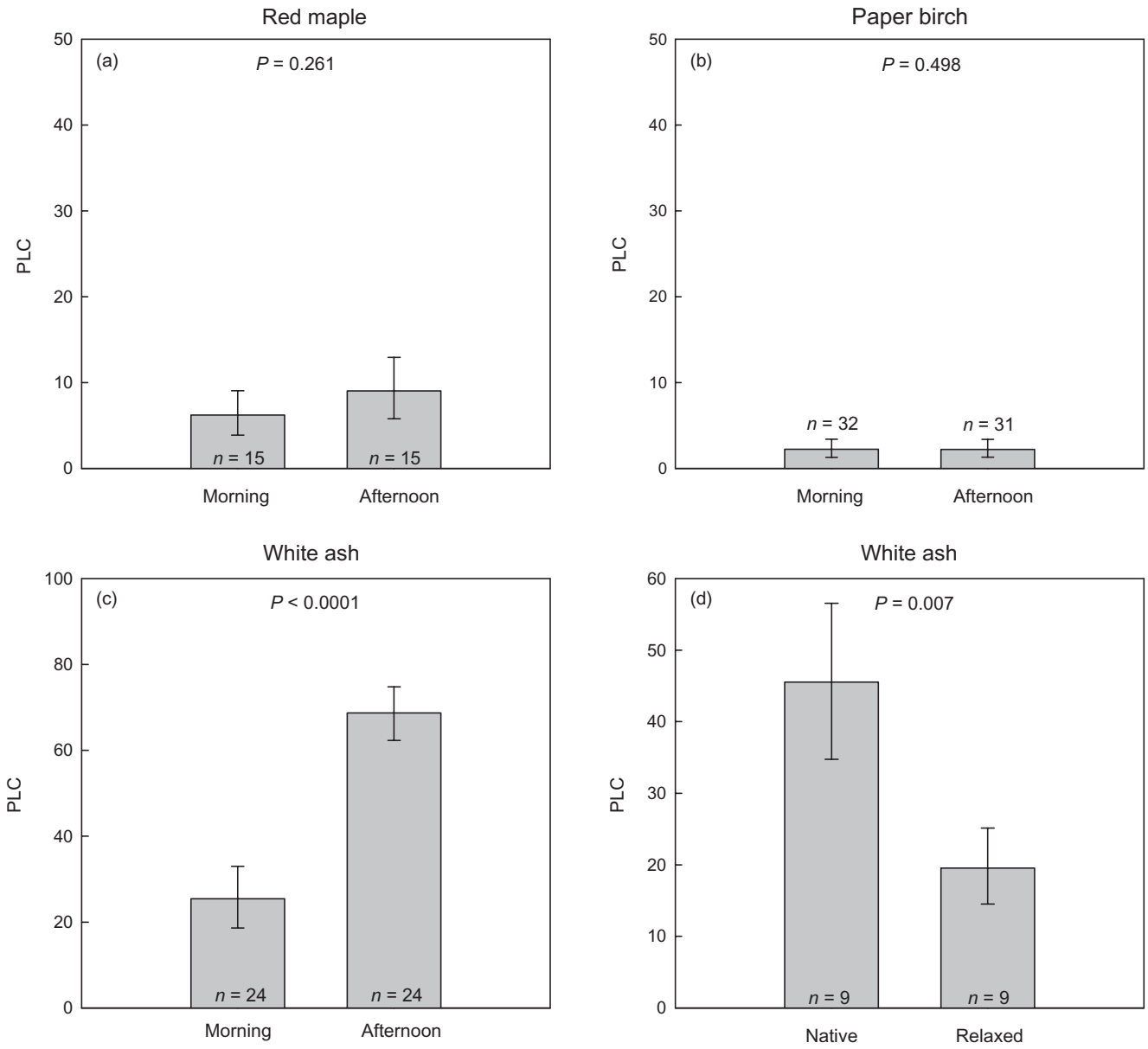

Figure 1. Mean (± standard error) percent loss of conductivity (PLC) measured on current year growth of branches collected from the upper canopy of red maple, (a) paper birch (b) and white ash (c) trees at Harvard Forest in the morning (0530–0630 h) and afternoon (1300–1500 h) during summer 2011. (d) PLC of white ash branches collected before (native) and after (relaxed) supplying water to the crown through a cut side branch for 30 min.

pressures reached approximately –2 MPa and then rose to near 100% as branches were dried to xylem pressures more negative than –3.3 MPa. In the vulnerability curve constructed from segments excised at native tensions, PLC values above background levels were observed at xylem pressures as wet as –1.2 MPa. Furthermore, between –1.2 and –2 MPa the measured PLC was highly variable, with some samples coinciding with the relaxed curve, while others exhibited PLC as high as 58%. At xylem pressures more negative than –3.3 MPa the correspondence between the native and relaxed curves was relatively good, with large (80–97%) PLC's measured in both curves.

The fact that cutting under water at native tensions inflates measurements of PLC in red maple, but not in paper birch led us to question the basis for this difference. Our initial hypothesis was that paper birch cavitates at lower xylem tensions than are required to observe a discrepancy between the PLC of segments excised at native versus relaxed tensions. This motivated us to examine a second species, sugar maple, which we knew from previous work had both short vessels (<10 cm) and was more resistant to cavitation than birch. A comparison of the relaxed versus native bench drying vulnerability curves in sugar maple shows a significant effect based on sampling method (Fig. 5), although the

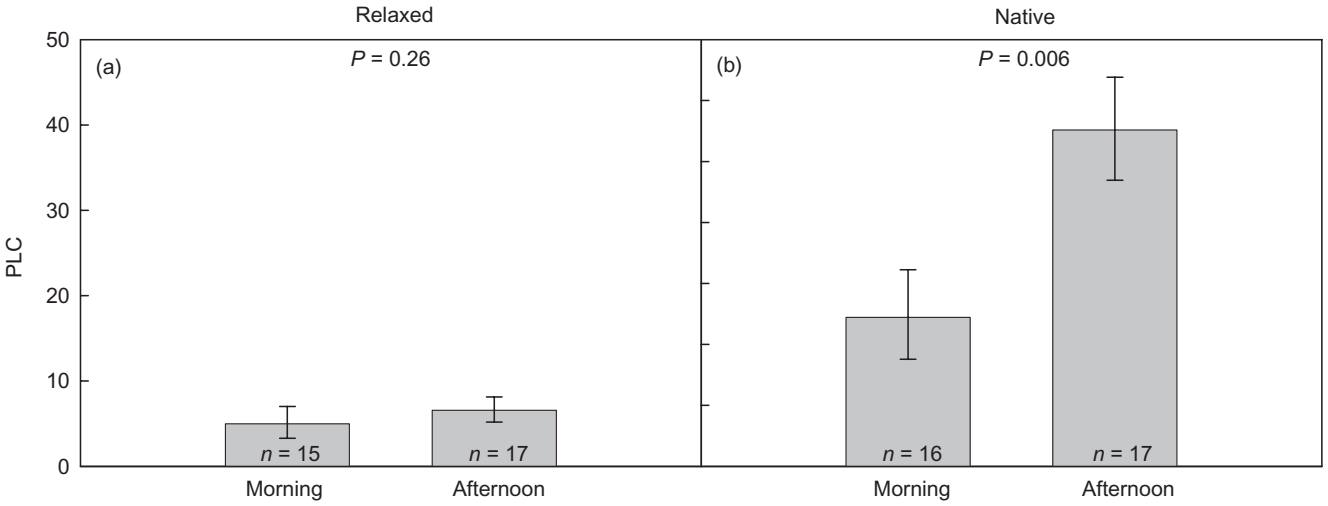

Figure 2. Mean (± standard error) percent loss of conductivity (PLC) for red maple branches collected in the morning and afternoon by two different methods during summer 2012; those cut from the tree under water (a, relaxed) and those initially cut from the tree in air (more than three vessel lengths from the sample) and then re-cut under water (b, native).

overall impact is not as large as in red maple. Furthermore, PLC's of sugar maple sampled at native tensions were not nearly as variable as in red maple. This demonstrates that the potential for artifacts arising from cutting under water when the xylem is under tension is not limited to red maple. However, further work will be needed to determine what structural features of stems make one species more susceptible than another to such sampling artifacts.

We also note that the centrifuge data and relaxed bench drying data for sugar maple diverge at xylem pressures more negative than –3 MPa. This observation combined with the slow rate at which the xylem tension of highly cavitated stems

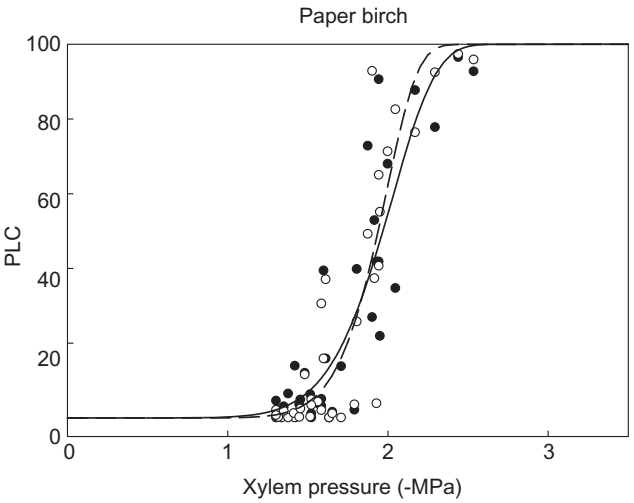

Figure 3. Paper birch PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2 = 0.90$; relaxed: dashed line, $r^2 = 0.91$).

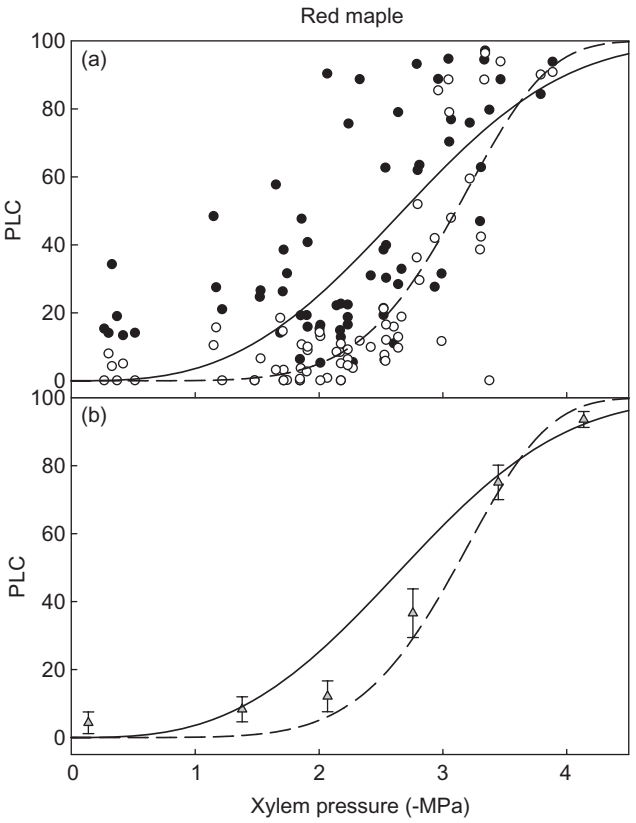

Figure 4. (a) Red maple PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2 = 0.66$; relaxed: dashed line, $r^2 = 0.72$). (b) A vulnerability curve determined by the centrifuge method (gray triangles) is plotted as a reference (mean and standard error, $n = 10$) along with the Weibull curves fit to the data in (a).

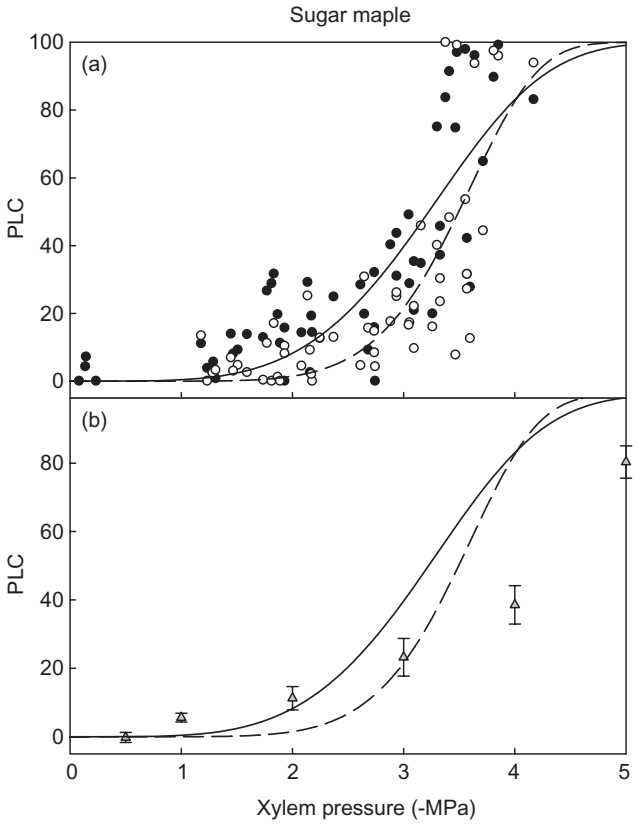

Figure 5. (a) Sugar maple PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2 = 0.82$; relaxed: dashed line, $r^2 = 0.75$). (b) A vulnerability curve determined by the centrifuge method (gray triangles) is plotted as a reference (mean and standard error, $n = 8$) along with the Weibull curves fit to the data in (a).

relaxed to near zero implies that the hydraulic pathway within the stem can become sufficiently limiting as to effectively decouple the xylem pressure of the leaves from that in the stems. At some point all hydraulic connections between the leaves and the stem may be severed and any water remaining in some leaves will no longer indicate the true stem xylem pressure. In addition, any stem cavitation that occurs will release water, which the leaves will take up, resulting in pressure bomb readings that indicate a less negative xylem pressure than actually exists in the stem. This suggests that in the range of xylem pressures where stems are severely cavitated, stem psychrometres may be a more reliable method of estimating xylem pressure. Specifically, we suggest that better estimates of xylem pressure at the drier end of our relaxed bench dried vulnerability curves could help resolve differences with the centrifuge curves.

At xylem pressures less negative than the P50, the point at which PLC = 50, there was good agreement between the relaxed vulnerability curve generated using bench drying and the spinning vulnerability curve in both red and sugar maple. This is consistent with the idea that severing conduits while the xylem is under tension can create artifacts that inflate PLC values above *in vivo* levels of cavitation. Nevertheless, to address the possibility that native embolism was reversed during the 30 or more minutes when the cut branches were allowed to rehydrate, we conducted a parallel set of experiments in which xylem tensions were relaxed rapidly. We dehydrated cut branches to a target range of xylem pressure (–1.91 to –2.79 MPa for red maple and –2.65 to –3.57 MPa for sugar maple) and compared the PLC in these samples to that in which tension was relaxed for two minutes (Fig. 6). If cutting under water always preserved the hydraulic continuity of the xylem, we would not expect to see a difference in the PLC of adjacent segments. Yet, in both maple species, the PLC in the relaxed segments was significantly lower compared to the segments cut under native xylem tension (Fig. 6;

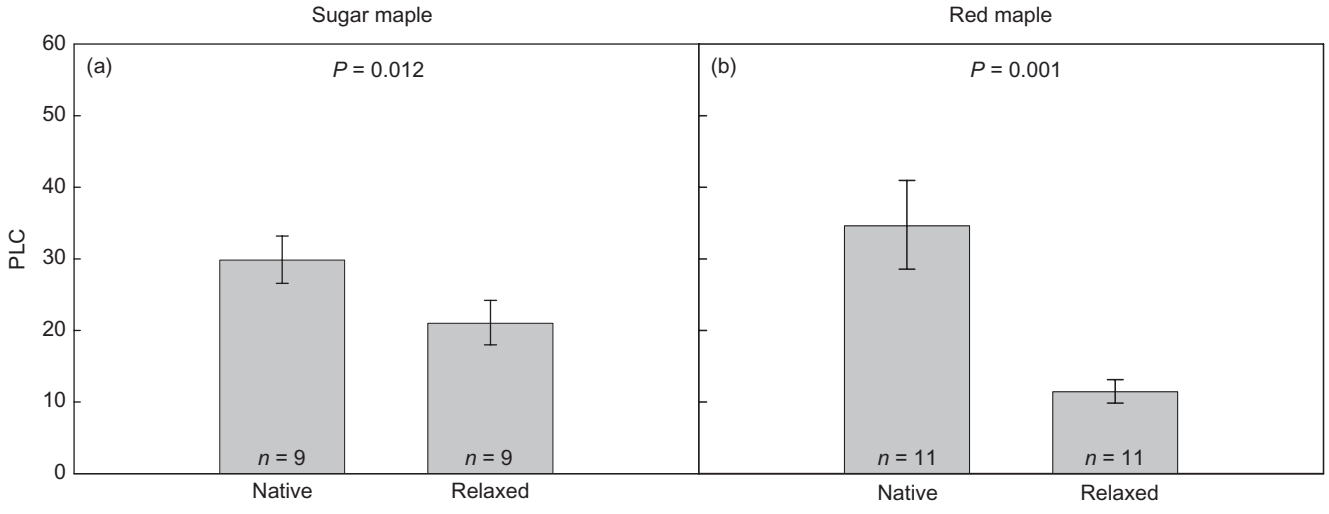

Figure 6. Mean and standard error PLC for sugar maple (a) and red maple (b) branches cut at target tension (native) and after a 2 min rehydration (relaxed).

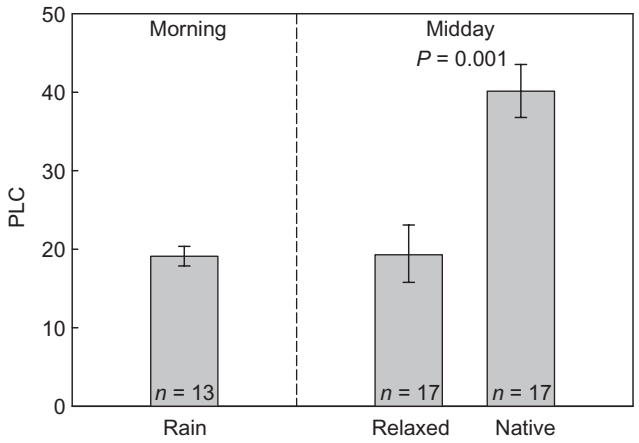

Figure 7. Mean and standard error PLC for red maple petioles from branches cut in the morning and at midday. Native petioles were cut under water from branches still under tension. Relaxed petioles were cut under water from branches allowed to relax to xylem pressures >–0.2 MPa. Background petioles (provided for reference) were cut under water from branches collected in the morning in the rain, xylem pressures >–0.3 MPa.

one-tailed, paired samples t-test: sugar maple $n = 9$ $P = 0.012$; red maple $n = 11$ $P = 0.001$). The difference between the native and relaxed values was greater in red maple than in sugar maple, as was found when longer rehydration times were used (Figs 4–6).

Although the focus of this study was on stems, we wanted to know whether cutting petioles under water at native xylem tensions could also lead to inflated values of PLC. To this end, we compared PLC of red maple petioles collected at midday and excised under water while the branch remained at the midday xylem pressure (–1.5 to –0.8 MPa) with petioles cut after xylem pressures had been relaxed to >–0.2 MPa. The PLC of petioles cut under water at native xylem tensions was significantly greater than the PLC of petioles where the tension was relaxed prior to sample excision (Fig. 7; $P = 0.001$ one-tailed, paired samples t-test). The PLC of petioles sampled after xylem tensions had been relaxed was not different from the background levels of PLC recorded from petioles cut early in the morning in rain ($P = 0.467$ one-tailed, independent samples t-test).

PLC following air-injection

The final part of our study evaluated whether the excision of samples following radial air injection might also inflate values of PLC due to the potential for dissolved gasses to come of out solution when a recently pressurized stem is cut. We used three different pressures, two of which we predicted, based on the centrifuge vulnerability curves, would have no (0.1 MPa) or minimal (1.0 MPa) impact on PLC. In contrast, we expected the third pressure (4.0 MPa) to cause PLC >90%.

Branches exposed to 1.0 MPa and then cut 2 min after the pressure had been released exhibited PLC's of 60% in red maple and 50% in sugar maple (Fig. 8b,e), markedly higher than levels predicted from the vulnerability curves measured for each species (Figs 4 & 5; <20% for each species). The PLC of segments excised 75 min following chamber depressurization were significantly lower than in the segments sampled at 2 min. A significant effect was present, but less pronounced, for branches exposed to 0.1 MPa (Fig. 8a,d). Branches pressurized to 4 MPa exhibited high PLC (Fig. 8c,f; 75 to 90% for red maple and >90% for sugar maple) that was independent of the time interval between chamber depressurization and sample excision. Background PLC for all samples used in all treatments was consistently less than 10%.

DISCUSSION

> '*When a branch is cut, even under water, it is possible that bubbles are formed in the tracheae by the act of cutting. Bubbles may be formed anywhere close to the knife, but naturally mostly in the tracheae in contact with the knife on either side, as the knife introduces a discontinuity, and the water adheres feebly to it. Probably some of the bubbles observed were thus formed at the moment of making the preparation for examination, and were non-existent when the plant was transpiring.*' Dixon 1914, p. 94.

Dixon's prescient comments notwithstanding, for the past several decades the working assumption in plant hydraulics has been that cutting under water preserves the hydraulic continuity that existed prior to cutting. Our data demonstrate that this is not always the case. In red maple, whether midday PLC was greater than that measured at first light depended entirely on how the samples were collected (Fig. 2). When we relaxed the tensions in the xylem before excising the measurement segment, we found no diurnal variation in PLC. However, when the measurement segments were excised while there was significant tension in the xylem, we found elevated PLC at midday, even though the cuts were made under water.

We interpret this as evidence that embolism can be introduced during the act of severing the vessels if the xylem tension is sufficiently large and thus that the higher midday PLC of branches sampled at native tension, even though they were cut under water, is an artifact. An alternative explanation for the differences in PLC between samples cut under relaxed and unrelaxed (i.e. native) tensions is that refilling of cavitated xylem conduits occurred in the relaxed treatment during the 30 min or longer period that branches were supplied with water. We questioned this possibility for two reasons. First, the refilling under tension phenomena as currently documented appears to vanish when the phloem is damaged (Salleo *et al*. 1996; Tyree *et al*. 1999; Bucci *et al*. 2003), let alone the whole stem is cut. And second, refilling by capillarity in conduits of the size found in the species examined here is too slow (Yang & Tyree 1992) to account for the differences in PLC observed.

Nevertheless, to explore the possibility that an active (i.e. non-capillary) refilling process does indeed occur in cut branches, we undertook the rapid-relaxation experiments. In these experiments, a three-internode segment was cut at both

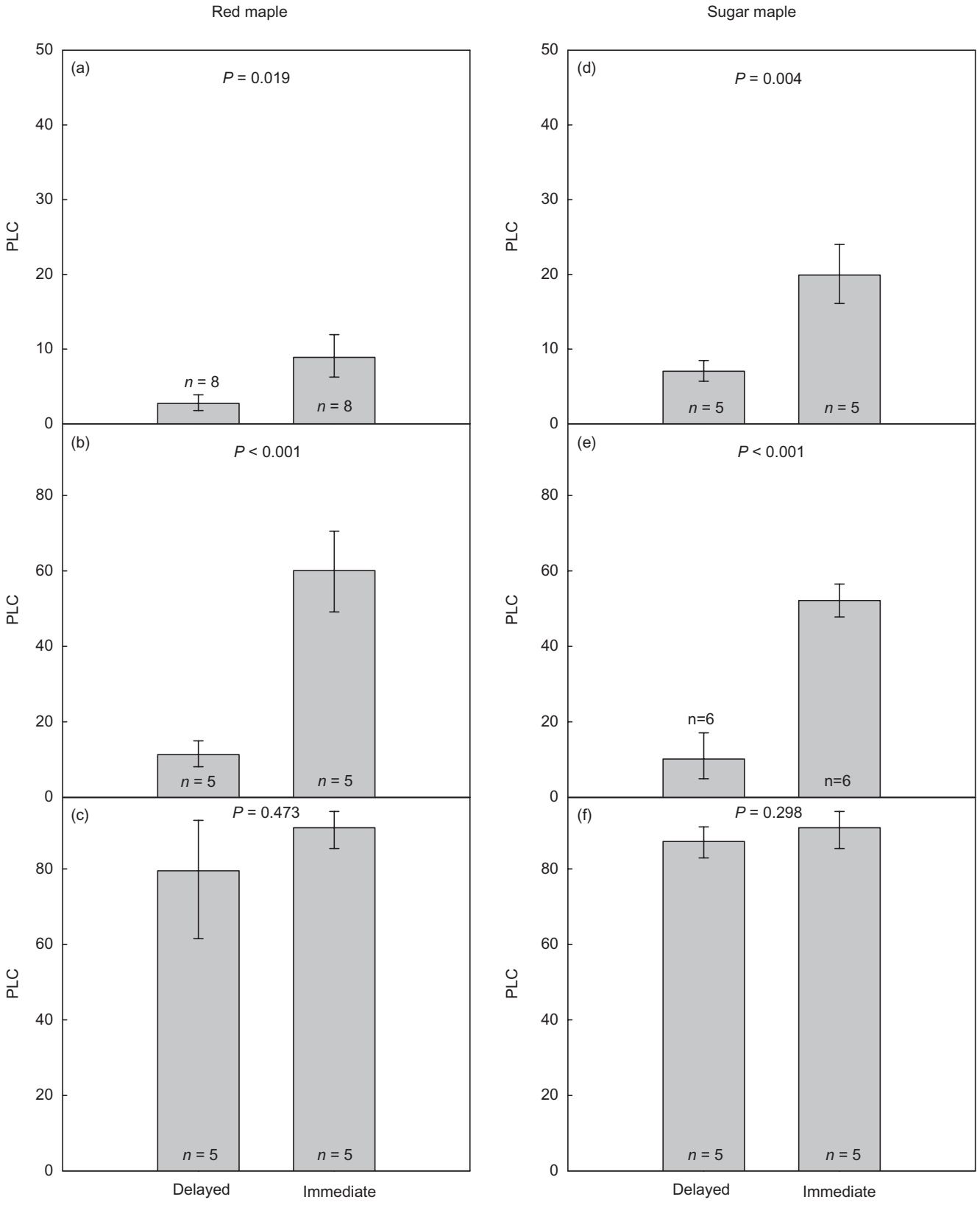

Figure 8. Mean and standard error PLC of red maple (a,b,c) and sugar maple (d,e,f) comparing the effects of immediate or delayed sample collection following air injection at three different pressures (a,d = 0.1 MPa, b,e = 1 MPa, c,f = 4 MPa). Note that the scale for the 0.1 MPa injections has been changed.

ends under water at native tension, and after 2 min the middle internode was excised. We then compared the PLC of the middle internode (relaxed) to the segment with the end that was first cut (native), and found essentially the same difference as in the longer relaxation experiments on whole branches: higher PLC in the segment cut at native tension compared to the middle internode in which the tensions were relaxed prior to excision (Fig. 6).

We are aware of only two material differences between the two treatments in the rapid relaxation experiment to explain the differences in observed PLC: first, the tension in the xylem at the time of the cuts; and second, the middle internode would have a greater proportion of conduits intact during the two minutes of relaxation. We considered the possibility that osmotica suspended in droplets on the walls of embolized conduits (e.g. Brodersen *et al*. 2010) could provide a driving force to dissolve previously existing emboli in intact vessels, but not in opened conduits, and that this then explains the difference between the two samples. However, this hypothesis requires (1) that the driving force for refilling not be dissipated before all of the air is forced into solution (i.e. that the reconnection problem be solved; Zwieniecki & Holbrook 2009) and (2) that the emboli be dissolved within only 2 min, substantially quicker than time scales for refilling reported in the literature (Hacke & Sperry 2003; Brodersen *et al*. 2010). Furthermore, if cut segments containing a preponderance of closed vessels are able to refill those conduits within 2 min, it would not be possible in general to measure PLC on species with short vessels. Indeed, we should not have been able to generate vulnerability curves by bench drying for either sugar or red maple. We therefore interpret the differences in PLC observed in our rapid relaxation experiments as the result of air introduced into the xylem at the moment the vessels were severed.

Our measurements of white ash are complicated by the fact that the maximum vessel length was longer than the branches available for sampling. As a result, we were unable to cut branches off under water that were more than one maximum vessel length away from the current year's extension growth. Nevertheless, when we supplied water to the stem before sampling at midday, we found significantly lower PLC, comparable to morning values (Fig. 1d), although we were not able to document the degree of relaxation achieved in the stem xylem. These results, however, are consistent with the hypothesis that the elevated PLC that we observed at midday when sampling prior to supplying water to the stem was due to embolism introduced into the measurement segment by cutting the branch off under water.

Narrowly construed, these results simply call into question the idea that red maple and white ash undergo diurnal variation of PLC *in vivo*. However, we think such artifacts have the potential to be widespread. When stems are cut under water, fluid accelerates into the open conduits. Microbubbles present in the reservoir fluid, released from the apoplast upon cutting or arising from imperfectly wetted defects on the cutting surface (as suggested by Dixon), can be drawn into open conduits. How far a bubble penetrates into an open vessel will depend upon the rate at which the xylem tensions are relaxed, which in turn will be affected by the initial xylem tension, the conductance of the xylem, and the size of the sinks for water uptake (e.g. living cells in leaves and stems). If the tension in the water is sufficiently large, the bubbles could expand to fill the entire conduit (Supporting Information Fig. S3). In this case, cutting is akin to air-seeding and the open vessels would essentially cavitate.

A more likely scenario, in our opinion, is that as bubbles become entrained in the water flowing into the vessel they expand and coalesce until they become lodged against the conduit walls. In some cases bubbles may be carried as far as the vessel end wall, while in other cases, bubbles may penetrate only partway down an open conduit before becoming hung up on the vessel wall. Indeed, the absence of a cutting artifact in paper birch (Fig. 3) may be due to their scalariform perforation plates, which may trap any entering bubbles so close to the entry-point that they were removed when the stem surface was shaved with a clean razor blade prior to measurement (John Sperry, personal communication). If the scenario of artifactual PLC arising from translating bubbles is correct, then the rate at which large volume sinks for flow (such as leaves) are severed could make the difference between whether air introduced during cutting is isolated at the cut ends, where it may be trimmed off, or penetrates far into the measured sample.

Bench drying is the original method used to assess vulnerability to cavitation and it is often looked to as a reference (Christman, Sperry & Smith 2012). Our data indicate that bench drying vulnerability curves, generated without relaxing the xylem tension in the material prior to sample excision, may indicate that plants are more vulnerable to cavitation than they truly are. In particular, we note that this artifact can lead to an underestimation of P50 and an appearance of significant levels of cavitation at moderate water potentials (Figs 4 & 5). Data from species with long vessels may be particularly at risk for bias, as the possibility of trimming out embolism caused during sample excision would be much less than in species with shorter vessels. The substantial scatter in our PLC measurements in segments cut underwater at moderate xylem tensions (Figs 4 & 5) suggests that the introduction of air during cutting may be a somewhat stochastic process affected by aspects of xylem anatomy such as conduit diameter and the prevalence of air-filled fibres or variation in cutting surfaces. Given this uncertainty, the only way to be sure that air bubbles introduced during cutting do not affect measurements of PLC is to first relax tensions and then trim off a maximum vessel length. However, Choat *et al*. (2010) were able to construct a vulnerability curve by bench drying for *Vitis vinifera* that was validated by both magnetic resonance imaging (MRI) and high resolution computed tomography (HRCT) imaging (McElrone *et al*. 2012). In this case, once the stem had reached the target potential, leaves and lateral appendages were apparently trimmed off under water, relaxing stem xylem tensions prior to excision of the measurement sample. We are therefore optimistic that relaxation of stems by hydration through lateral appendages (as in our ash experiments) may prove a reliable method for sampling long-vesseled species.

It has not escaped our notice that artifactually induced embolism in petioles (and likely extending into leaf veins) (Fig. 7) may inhibit the ability to correctly evaluate leaf vulnerability to embolism as measured by either rehydration kinetics (Brodribb & Holbrook 2003) or the evaporative flux method (Scoffoni *et al*. 2012). However, we lack sufficient information to determine whether the resistance generated by this embolism can dominate the greater resistance of the extraxylary hydraulic pathways in the leaf. Further study will be necessary to clarify whether leaf vulnerability to cavitation can be determined accurately using existing methods.

A decrease in PLC following air-injection has been interpreted as providing a second line of evidence for refilling under tension (Salleo *et al*. 1996; Tyree *et al*. 1999; Secchi & Zwieniecki 2011). On face value, such experiments might be seen as immune from the cutting artifact described above because xylem tensions do not vary over the time period of the experiments. However, we found that injecting stems with gas, even at pressures too low to induce cavitation, inflates measurements of PLC when the vessels to be measured are severed soon after pressurization is completed (Fig. 8). We believe that this occurs as a result of the water within stems becoming supersaturated with gases, increasing the likelihood that severing the xylem, even under very slight tensions, results in the formation of stable emboli. In other words, recently pressurized stems behave no differently than does a carbonated beverage when opened. We believe that this is why the measured PLC disappears after sufficient time is allowed for the concentration of dissolved gases to return to a level at equilibrium with the ambient air pressure. We expect that this effect is, at least to first order, independent of xylem structure and that the degree of sampling-induced embolism will be exacerbated by the presence of tension in the xylem. As a result, we feel that all refilling data that is based on air-injection should be treated with caution.

Taken together, the artifacts documented here provide an alternative to embolism repair under tension as an explanation for changes in PLC in relation to diurnal variation in water potential or following air injection. In our minds, this calls into question the idea that embolism repair occurs concurrent with the xylem tensions found in actively transpiring plants. Whether mechanisms other than root pressure serve to reverse embolism in plants with moderate water potentials will require further work combining advanced imaging and more detailed physiological measurements on species that do not make root pressure (Brodersen *et al*. 2010).

In summary, we find that the most parsimonious explanation for our results is that severing xylem vessels that are under tension, even if the cuts are made under water, can introduce embolism into the xylem, and that the degree of embolism appears to scale with the existing tension in the xylem. Severing vessels shortly after air injection, at tensions where no embolism should occur, also appears to introduce embolism. These findings have the potential to alter our understanding of the frequency of embolism in plants in nature and the methods employed to quantify vulnerability to cavitation. If the sampling artifacts described here occur widely across species, we may need to reassess the idea that many plants operate with substantial xylem dysfunction under typical midday water potentials (e.g. Zufferey *et al*. 2011). This would call into question the idea that plants routinely operate at the brink of hydraulic failure and shift our thinking back towards the idea that, for many species, embolism may only occur to any significant degree under conditions of substantial soil drying or due to winter freezing (Zimmermann 1983).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Rapid relaxation protocol: (a) a branch dried to the target pressure range was sealed in a plastic bag for 30 min to equilibrate and then xylem pressure was measured. (b,c) The branch was cut under water at both ends of the three internode sample segment, the leaves sliced between the tertiary veins, and the segment relaxed for 2 min. (d) After the 2-min relaxation period, the relaxed sample was excised and the PLCs of the tension sample and the relaxed sample were measured.

Figure S2. Air-injection protocol: (a) branches removed from a tree are sampled for initial xylem pressure. (b) The branch base was trimmed under water (20 cm) and the tension relaxed with the branch in a plastic bag for 30 min, after which the xylem pressure was measured. (c) The fork containing the background sample was removed under and covered with plastic bags to ensure that no changes occurred in the samples during the time necessary for air injection. A pressure sleeve was attached to the air-injection fork and pressure was applied for 20 min. (d) Following depressurization, xylem pressure was measured, and the sample segment was excised from the stem and allowed to rest for either 2 min or 75 min before measuring PLC. Both the background sample and the air-injected sample were measured after the resting period.

Figure S3. Illustration of the potential outcomes of an air bubble entrained by fluid rushing into the cut surface (red arrows) of a xylem vessel excised under water. (a) The tension in the xylem fluid is sufficient to cause the bubble to expand and fill the opened vessel to the end wall where intervessel pit membranes prevent the spread of gas throughout the intact xylem. (b) The tension in the fluid is insufficient to cause the bubble to expand, but it is drawn the length of the vessel and lodges against the end wall. (c) The bubble is drawn only partly into the opened vessel and may be excised if a sufficient length of the stem is trimmed before measurement. (d) Scalariform perforation plates (as in paper birch) may prevent bubbles from travelling deep into the opened vessel and thus may be easily removed by shaving the ends before measurement.