Original Article

Are leaves 'freewheelin'? Testing for a Wheeler-type effect in leaf xylem hydraulic decline

Christine Scoffoni & Lawren Sack

Department of Ecology and Evolution, University of California Los Angeles, Los Angeles, CA 90095, USA

ABSTRACT

A recent study found that cutting shoots under water while xylem was under tension (which has been the standard protocol for the past few decades) could produce artefactual embolisms inside the xylem, overestimating hydraulic vulnerability relative to shoots cut under water after relaxing xylem tension (Wheeler *et al*. 2013). That study also raised the possibility that such a 'Wheeler effect' might occur in studies of leaf hydraulic vulnerability. We tested for such an effect for four species by applying a modified vacuum pump method to leaves with minor veins severed, to construct leaf xylem hydraulic vulnerability curves. We tested for an impact on leaf xylem hydraulic conductance (K_x) of cutting the petiole and minor veins under water for dehydrated leaves with xylem under tension compared with dehydrated leaves after previously relaxing xylem tension. Our results showed no significant 'cutting artefact' for leaf xylem. The lack of an effect for leaves could not be explained by narrower or shorter xylem conduits, and may be due to lesser mechanical stress imposed when cutting leaf petioles, and/or to rapid refilling of emboli in petioles. These findings provide the first validation of previous measurements of leaf hydraulic vulnerability against this potential artefact.

Key-words: cavitation; evaporative flux method; hydraulic resistance; xylem anatomy.

INTRODUCTION

The bulk of water movement through the plant occurs to replace the water lost through stomata during transpiration: stomata open to capture CO_2 for photosynthesis, and water is lost by diffusion to the dry atmosphere. When soil water supply becomes scarce, large tensions build up in the xylem, and cavitation may occur by air seeding: small air bubbles are pulled into xylem conduits, embolizing them and rendering them non-functional (Tyree & Zimmermann 2002). For the past several decades, scientists have quantified water movement through the plant under different water statuses by measuring hydraulic conductance in the laboratory. To do so, branches are typically dehydrated on the laboratory bench, and samples are then cut under water (with xylem still under tension) before being measured for hydraulic conductance,

which avoids opening conduits to further embolism such as what would happen if the branch was cut in air. However, a recent study challenged this methodological approach, arguing that cutting the sample under water while xylem is under tension can lead to additional embolism in the xylem conduits (Wheeler *et al*. 2013). Indeed, a century ago Dixon (1914) hypothesized that microbubbles might enter the xylem from the cutting knife (either because it is not completely wetted when the cut is made or because of small particles that could be found on a not perfectly clean knife causing bubbles to enter), and/or could be released from the apoplast when the cut is made (Wheeler *et al*. 2013), especially if the stem is subjected to strong mechanical pressure or bending that could compress xylem conduits or deform pits even transiently (Lopez *et al*. 2014; Mayr *et al*. 2014). Wheeler *et al*. (2013) tested for this artefact using stem samples on up to four temperate tree species depending on their treatments, by measuring stem hydraulic conductance of samples recut under water in the standard way (under tension) versus samples recut under water after the tension inside the xylem was relaxed by previously cutting the stem underwater upstream of the segment to be used for hydraulic measurement. Wheeler *et al*. (2013) found a significant impact of cutting the xylem under tension underwater, which they hypothesized to arise from bubbles entering the cut end, with their entry depending on the degree of tension and the xylem anatomy, although these mechanisms were not directly tested. Alternatively, a recent study reported that this effect could have arisen as an artefact, if relaxing the tension inside the xylem led to refilling of embolism in the xylem, which would overestimate the hydraulic conductance (Trifilo *et al*. 2014).

Although Wheeler *et al*. (2013) focused their study on the presence and impact of this artefact on stem and petiole hydraulic conductance measurements, they noted this could equally apply to hydraulic measurements for whole leaves and urged further testing. The aim of our paper was to test for this 'cutting artefact' in leaves.

Unlike in stems, water movement in leaves follows two pathways in series: xylem water transport in the leaf petioles and veins, and extra-xylary transport through the living bundle sheath and mesophyll cells to the sites of evaporation in the leaf. Thus, the leaf hydraulic conductance (K_{leaf}) is dependent on the hydraulic conductance of the xylem (K_x) and extra-xylem pathways (K_{ox}):

Correspondence: C. Scoffoni. E-mail: cscoffoni@ucla.edu

$$K_{\text{leaf}} = \left(K_{\text{x}}^{-1} + K_{\text{ox}}^{-1}\right)^{-1} \qquad (1)$$

The resistance to water movement in each pathway varies across species, from 12 to 89% in the xylem (Cochard *et al.* 2004; Sack *et al.* 2004, 2005). Thus, because the cutting arte-fact theory would only affect the leaf xylem, the impact on K_{leaf} would depend on the amount of hydraulic resistance allocated to the xylem, and further whether the embolism generated by the cutting artefact would noticeably impact measurements of K_{x}. Testing for such an artefact on whole leaves would be impossible, as rehydrating shoots prior to cutting to relax the xylem tension would most likely also rehydrate mesophyll cells, which would increase K_{ox} (Scoffoni *et al.* 2012, 2014) and create the impression that there might be a cutting artefact. Thus, to test for a cutting artefact on the hydraulic conductance of dehydrated leaves, it is necessary to test the impact on K_{x} directly. Methods used to measure K_{x} have all involved measuring the flow of water through veins under high or low positive pressures (Cochard *et al.* 2004; Sack *et al.* 2004; Nardini *et al.* 2008). Here, we directly tested this hypothesis on the leaf xylem by developing a new method to calculate K_{x} under subatmospheric pressures that would reduce artefactual refilling of embolized conduits during the measurement, with attention to simulating natural flow of water through the veins. We constructed leaf xylem vulnerability curves for four diverse species varying in leaf texture, allocation to xylem versus outside xylem conductance and drought tolerance, and tested whether measurements differed in treatments that relaxed the xylem tension in dehydrated leaves.

MATERIALS AND METHODS

Plant material

Four species with a wide diversity in phylogeny, growth form and drought tolerance traits were selected in and around the campus of University of California, Los Angeles, and Will Rogers State Park, Los Angeles, California (Table 1). Experiments were conducted from November 2013 to April 2014. Light-exposed shoots from three mature individuals per species were collected the night prior to the start of measurements and placed in a double layer of plastic bags filled with wet paper towels. They were directly transported to the laboratory where they were recut at least two nodes distal to the original cut under ultra-pure water (0.22 μm Thornton 200CR, Millipore, Molsheim, France) and rehydrated overnight at laboratory temperature (20–23 °C), covered in double layer of plastic bags filled with wet paper towels to halt transpiration.

Measuring leaf xylem hydraulic decline using the vacuum pump method

The vacuum pump method was first developed to measure whole shoot and root hydraulic conductance (Kolb *et al.* 1996) and later modified to measure leaf hydraulic conductance (Martre *et al.* 2001; Nardini *et al.* 2001; Sack *et al.* 2002;

Table 1. Study species, family and mean ± SE for drought tolerance leaf traits and hydraulics

Species	Family	Growth form	LA (cm^2)	TLP (−MPA)	LMA (g m^{-2})	K_{max} (mmol m^{-2} s^{-1} MPa^{-1})	%R_{x}	Maximum leaf xylem conduit length (vein order)	Maximum stem vessel length (cm or vein order)	Leaf P_{80} (MPa)	Leaf xylem $P_{x_{50}}$ (MPa)
Conarostaphylis diversifolia	Ericaceae	Tree	9.64 ± 0.42	3.45 ± 0.34	253 ± 16.9	2.96	8.4	2° (2/5 leaves)	30 cm	−4.56	−5.59
Hedera canariensis	Araliaceae	Shrub	81.0 ± 4.52	1.98 ± 0.09	84.1 ± 11.0	5.73	23	3° (3/10)	25 cm	−1.18	−1.89
Quercus agrifolia	Fagaceae	Tree	9.41 ± 0.42	3.00 ± 0.12	188.0 ± 7.53	3.96	14	2° (3/3)	91 cm	−3.83	−5.43
Salvia canariensis	Lamiaceae	Herb	20.7 ± 2.28	1.18 ± 0.07	41.4 ± 6.01	52.9	77	2° (2/5)	2° (2/11 leaves)	−0.59	−0.89

Data for TLP, LMA, K_{max} and leaf P_{80} are from previous papers (Scoffoni *et al.* 2012, 2014), except for K_{max} and leaf P_{80} of *S. canariensis*, which were obtained in this study. Maximum leaf xylem conduit lengths are presented as the highest vein order in which conduits ended within the leaf; maximum stem xylem conduit lengths are presented as length of stem, or for *S. canariensis*, the highest vein order in the leaf to which stem conduits extended. %R_{x}, percentage hydraulic resistance in the xylem; K_{max}, leaf hydraulic conductance at full turgor; LA, leaf area; leaf P_{80}, water potential at which 80% of leaf hydraulic conductance is lost; LMA, leaf mass per unit leaf area; TLP, turgor loss point; leaf xylem $P_{x_{50}}$, xylem water potential at which 50% of the leaf xylem hydraulic conductance is lost.

Brodribb & Holbrook 2003; Lo Gullo *et al.* 2003). In this method, the hydraulic conductance is determined as the slope of the change in flow rate over the change in vacuum level. Here, we modified this technique to measure K_x.

Shoots with at least three leaves were cut under water from the larger rehydrated shoots and were allowed to dehydrate on the bench (or on a fan) to achieve a wide range of water potentials. Then, the leaves on the shoots were individually sealed in bags (Whirl-Pak, Nasco, Fort Atkinson, WI, USA), which had been previously exhaled in, and then the entire shoot with bagged leaves was placed inside a larger sealable bag with wet paper towel, to equilibrate for at least 30 min. For the measurement of the maximum K_x, rehydrated shoots were used directly after this procedure. After equilibration was reached, the top and bottom leaf from each shoot were excised and measured for leaf water potential (Ψ_{leaf} = leaf xylem pressure since leaves were equilibrated; Tyree & Zimmermann 2002) using the pressure chamber (Model 1000, Plant Moisture Stress, Albany, OR, USA). If the values differed by more than 0.2 MPa (or 0.3 MPa in very dehydrated leaves), the shoot was discarded.

The lamina of a third leaf (still connected to the shoot) was then placed under ultra-pure water over a white light transilluminator table (Model TW, UVP, Upland, CA, USA), allowing visualization of minor veins (fourth order and higher). Cuts were made to open up the bulk of the minor vein network using a scalpel with cuts between approximately 95% of tertiary veins throughout the leaf, so that the number of cuts per leaf area ranged 7.3–34.0 cuts cm^{-2} depending on species, enough such that water would move directly out of the cut minor veins, 'shorting' out the resistance outside the xylem (Sack *et al.* 2004). Because species with larger leaves have their tertiary veins spaced further apart than smaller leaves (Sack *et al.* 2012), fewer and longer cuts were made in larger leaves (length of cuts ranged 0.8–4.0 mm depending on leaf size). Special care was taken that no major veins (first, second and third orders) were severed in the process. Cuts were made within 5–15 min. Once all the cuts were made, the leaf was excised from the shoot at the base of the petiole under ultra-pure water, and stretched parafilm was quickly wrapped around it before it was recut at the end (≤1 mm) under water using a fresh razor blade, and connected under ultrapure water to silicone tubing (Cole-Parmer, Vernon Hills, IL, USA) or compression fitting (Omnifit A2227 bore adaptor; Omnifit, Cambridge, UK) connected to hard tubing running to a water source on a balance (models XS205 and AB265, ±10 μg sensitivity; Mettler Toledo, Columbus, OH, USA). The water was degassed overnight using a vacuum pump (Gast, Benton Harbor, MI, USA) and refiltered (0.2 μm; syringe filter; Cole-Parmer). For species with smaller petioles for which silicone tubing was preferred (*Comarostaphylis diversifolia* and *Quercus agrifolia*), vacuum-tight seals between the petiole and the tubing were obtained by tightening the tubing around the petiole with zipties and sealing the exposed end of the tubing to the petiole using super glue (Loctite 409 glue; McMaster-Carr, Los Angeles, CA, USA) with accelerator (Loctite 712
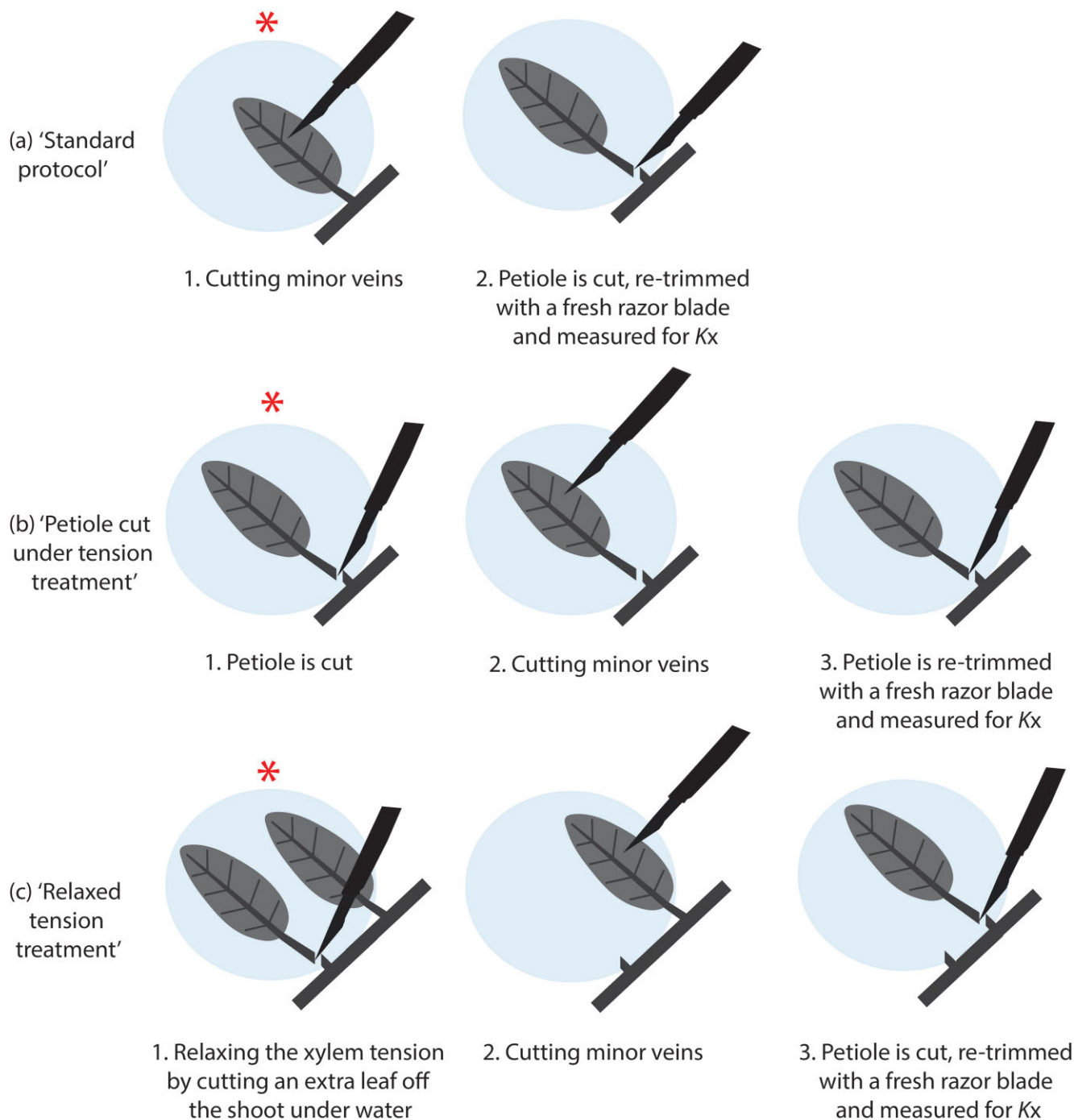
accelerator). Leaves were then placed along with a thermocouple (Cole-Parmer) in vacuum flasks connected by a four-way valve to a vacuum pump (Gast) and a pressure gauge (±0.002 MPa; J4605 Marsh/Bellofram; Marshall Instruments Inc., Anaheim, CA, USA).

Five vacuum levels were applied, starting at approximately 0.06 MPa and then reducing by 0.01 MPa intervals until 0.02 MPa was reached. The mass of water on the balance was logged to a computer every 30 s for the calculation of flow rate through the leaf xylem. Leaves were left under a given vacuum until stable flow rate was achieved, with a coefficient of variation <5% for at least 5 min. Because the leaf venation extends across a laminate surface with a hierarchical and reticulate structure, and because of the low pressure driving force for flow, 30 min to 2 h was typically required for the flow rate to stabilize under the first vacuum level depending on the species and leaf size (the larger the leaf, the longer the equilibration times). For the subsequent vacuum levels, stabilization time ranged from about 10 min to 1 h. Once the flow was stable, the vacuum level inside the flask was recorded from the pressure gauge, as well as the leaf temperature from the thermocouple (20–25 °C for all experiments). Once all five points of flow rates versus vacuum pressure were measured, we tested for possible leaks from the system by cutting the leaf lamina off under water, and sealing the petiole end with superglue. Leak flow rate for given vacuum levels was measured in the same way as described above. Only 11% (18/161) of measurements across all leaves and species were found to have small leaks during the measurement. Because flow through the leak would be in parallel with that of the leaf, the leak hydraulic conductance values were subtracted from K_x. Leaf xylem hydraulic conductance (K_x) was calculated as the slope of the flow rate against vacuum pressures, normalized for leaf area and for the effect of temperature on the viscosity of water (Weast 1974; Yang & Tyree 1993; Sack *et al.* 2002). All plots of flow against pressure were linear ($r^2 = 0.79$–0.999; Supporting Information Fig. S1). To construct leaf xylem vulnerability curves, we plotted K_x values against the average of the two Ψ_{leaf} values determined at the start of the experiment for a given shoot. To test whether measurement time might correspond to xylem refilling and thus influence the determination of vulnerability curves, we plotted the residuals of K_x versus Ψ_{leaf} at mild dehydration levels (0 to −2 MPa in *C. diversifolia*, 0 to −1.2 MPa in *Hedera canariensis*, 0 to −4 MPa in *Q. agrifolia* and 0 to −0.4 MPa in *Salvia canariensis*) against the total time the leaf was on the system; we found no significant trend (Supporting Information Fig. S2).

We note that, while we found this method to be highly effective, leaf xylem hydraulic vulnerability curves can take 2 weeks to a month to construct with three systems running in parallel, depending on the species.

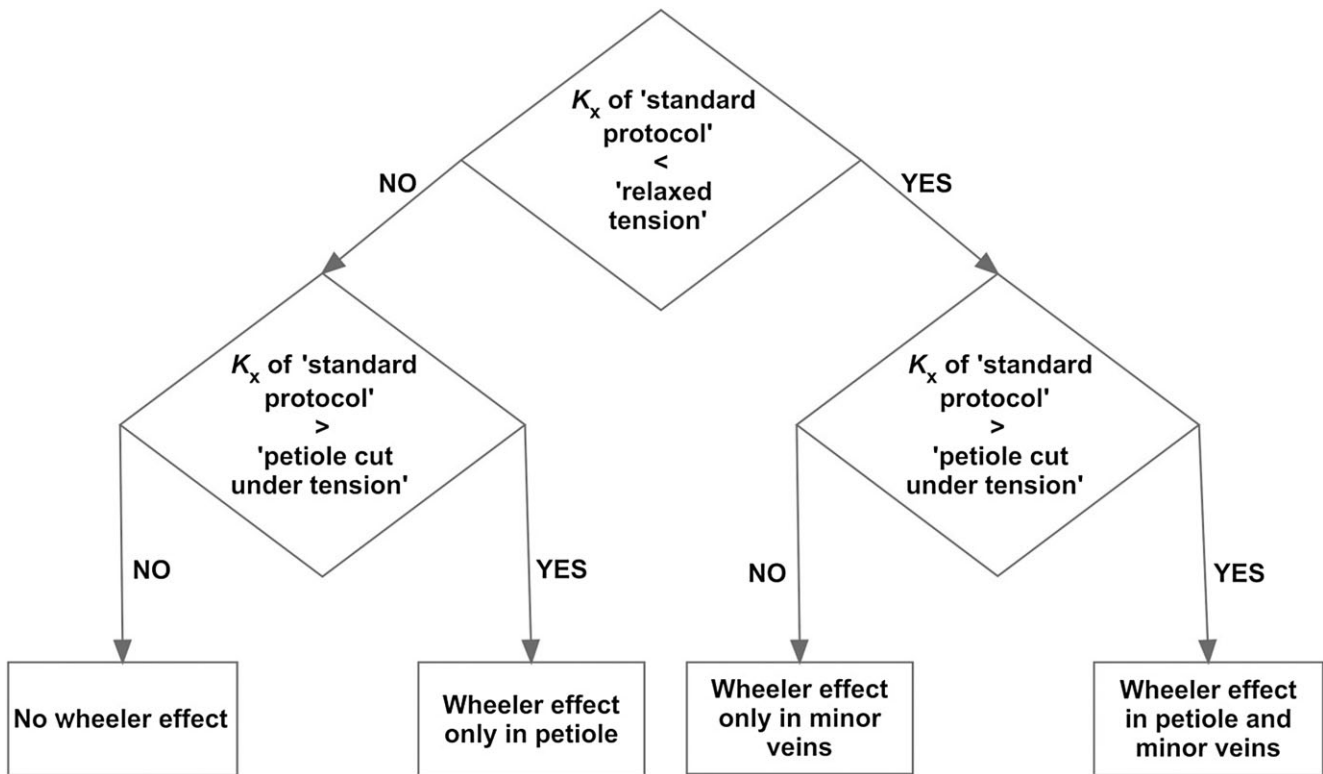## Testing for a cutting artefact on leaf xylem hydraulic conductance

We designed a 'standard protocol' for measuring K_x for vulnerability curves, reducing the time the petiole is in contact

Figure 1. Experimental design: (a) 'Standard treatment': minor vein cuts were applied on leaves with xylem under tension before the petiole was cut under water, for measurement of xylem hydraulic conductance (K_x). (b) 'Petiole cut under tension treatment': the petiole was cut under water for a leaf with xylem under tension before minor vein cuts were applied and the leaf measured for K_x. (c) 'Relaxed tension treatment': another leaf on the shoot was cut under water releasing the xylem tension, before minor vein cuts were made for the measurement leaf, and the petiole was cut under water for measurement of K_x. Red asterisk indicates when the xylem is cut under tension: in treatments (a) and (b), cuts could potentially cause an artefact in petioles and minor veins; in treatment (c), no 'cutting artefact' would be observed.

with water before measurement. We cut the leaf from the shoot under water after having previously made the cuts to the minor veins with the lamina under water (Fig. 1a). According to Wheeler *et al*. (2013), this standard protocol

could potentially create artefactual embolisms in the leaf minor veins by a cutting artefact as minor veins are cut under water while the leaf is under tension. Alternatively, because conduit sizes in the minor veins are very small, and thus

Figure 2. Flow chart presenting the interpretation of differences between the 'Standard protocol treatment' and the two other treatments depicted in Fig. 1 ('Relaxed tension' and 'Petiole cut under tension').

potentially less prone to be affected by artefactual bubble formation at the cut ends, this standard protocol might instead be simply relaxing the tension in the leaf xylem before the leaf is cut from the shoot, thus acting to prevent the cutting artefact from occurring. Thus, we applied two additional treatments to determine whether a cutting artefact might influence leaf xylem conductance (K_x) (Fig. 2).

We first tested a 'petiole cut under tension' treatment – that is, whether excising the leaf under water while the shoot xylem is still under tension could induce an embolism artefact in the petiole that would impact K_x (Fig. 1b). This treatment parallels most closely the scenario experienced by the leaf xylem for leaves measured for K_{leaf} during the construction of typical whole-leaf hydraulic vulnerability curves. Here, we prepared five to six shoots per species of at least three leaves dehydrated to approximately the xylem water potential values corresponding to 50% loss of K_x (see the Result section 'Leaf xylem hydraulic vulnerability curves: results from maximum likelihood analysis'; Table 1) as assessed from the Ψ_{leaf} of the top and bottom leaf on the shoot. For this treatment, the measurement leaf was excised under water while shoot xylem was under tension, prior to making the minor vein cuts with the leaf under water. Here, we might have expected the cutting artefact to occur at the petiole, which was cut under strong negative pressures (Fig. 2). Once all the minor vein cuts were made, the leaf was connected to the system, and K_x determined in the same way as described for the standard protocol.

We then tested a 'relaxed tension' treatment (Fig. 1c). To do so, we prepared five to six shoots per species of at least four leaves dehydrated to approximately the xylem water potential values corresponding 50% loss of K_x based on the K_x vulnerability curves (see Result section 'Leaf xylem hydraulic vulnerability curves: results from maximum likelihood analysis'; Table 1) as assessed from the Ψ_{leaf} of the top and bottom leaf on the shoot. Then, before making cuts to the leaf veins under water, we first cut a different leaf from the shoot under water, relaxing xylem tension throughout the whole shoot, and then 1–2 min later we performed the cuts to the veins of the leaf to be measured for K_x. Once the cuts were made, the leaf was excised and cut under water, and K_x was measured as described by the standard protocol. This treatment was designed to prevent any cutting artefacts from occurring either when making the cuts to the veins or when excising the leaf petiole under water before it is connected to the system (Fig. 2).

In designing these treatments, we assumed that cutting the shoot or petiole under water would relax the tension in the xylem. Even though the dehydrated leaves on the shoot would continue to rehydrate, and thus drive a flow through the shoot causing a pressure drop across the hydraulic pathways declining to atmospheric pressure at the cut petiole end, we expected based on previous work that the bulk of the resistance is in the mesophyll and vein xylem, especially in dehydrated leaves (Cochard *et al*. 2004; Sack *et al*. 2004; Scoffoni *et al*. 2014), and the petiole xylem pressure would

thus relax to a value close to atmospheric pressure. To confirm this, we directly measured the xylem pressure in the petiole after cutting treatments. On dehydrated shoots (one from each of three individuals of *C. diversifolia* and *Hed. canariensis*), we excised a leaf under water and allowed to rehydrate 1 min, and then measured the xylem pressure at the cut petiole end directly using a pressure transducer previously calibrated at a range of subatmospheric pressures (PX26-005GV, Omega, Stamford, CO, USA). The shoot, xylem pressures were –0.004 MPa or less, indicating that excising the leaf under water relaxed the tension in the petiole xylem as expected.

Calculation of the percentage resistance in the xylem and whole-leaf hydraulic vulnerability

We calculated the percentage hydraulic resistance in the xylem for each species as the maximum leaf xylem hydraulic conductance (i.e. at full turgor; $K_{x, max}$) and the maximum whole-leaf hydraulic conductance ($K_{leaf, max}$) (see 'Statistics' section). For three of the four species, we used values of $K_{leaf, max}$ and further reported the water potential at 80% loss of hydraulic conductance reported in a previous paper for the same species and individuals (Scoffoni *et al.* 2012). These values were determined for *S. canariensis* by constructing a leaf hydraulic vulnerability curve using the same evaporative flux method as previously used for the other species, following the previously described and filmed protocol (Sack & Scoffoni 2012). An exponential function $\left(K_{leaf} = -0.82 + 53.7 \times e^{-2.63\Psi_{leaf}}\right)$ was found to best fit the data using maximum likelihood (Supporting Information Fig. S3).

Measurement of maximum xylem conduit length

To test whether maximum xylem conduit length would explain our findings for the cutting artefact, we selected 3–10 leaves from shoots of three individuals per species that had been rehydrated overnight as described above. Leaves were connected by silicone tubing to a four-way valve connected to a syringe. Zipties were applied around the tubing and petiole to ensure a tight seal. Air pressure was applied using a caulking gun while the leaf was placed under water, over the transilluminator table. Using a scalpel, cuts were made throughout the leaf beginning with the highest order veins, and progressively to lower order veins, and finally along the midrib towards the leaf base, until air bubbles first emerged from the xylem, indicating maximum conduit length.

As another measure of conduit length in leaf xylem, we further assessed whether the xylem conduits in the stems extended into the leaves. For 3–11 samples per species, we applied the air pressure method described above to the cut end of stems, and using a scalpel progressively made cuts through leaves from highest to lowest order veins, then petiole; and subsequently removed 1 cm at a time from the stem using a razor blade, until air bubbles first emerged from the xylem, indicating maximum conduit length.

Statistics

Maximum likelihood was used to select the best-fit function for each species' leaf xylem hydraulic vulnerability data using the *optim* function in R 3.0.0 as previously described in the literature (http://www.r-project.org; Burnham & Anderson 2002, 2004; Scoffoni *et al.* 2012). Five functions were tested (Scoffoni *et al.* 2012, 2014): linear $(K_x = a\Psi_x + y_o)$, two-parameter sigmoidal $\left(K_x = \dfrac{100}{1 + e^{(a(\Psi_x - b))}}\right)$, three-parameter sigmoidal $\left(K_x = \dfrac{a}{1 + e^{-\left(\frac{\Psi_x - x_o}{b}\right)}}\right)$, logistic $\left(K_x = \dfrac{a}{1 + \left(\dfrac{\Psi_x}{x_o}\right)b}\right)$ and exponential $(K_x = y_o + ae^{-b\Psi_x})$. For each data set, functions were compared with the Akaike information criterion (AIC) corrected for low n, and the function with the lowest AIC score and highest r^2 value was chosen. From the best-fit function, we calculated the maximum K_x ($K_{x,max}$) for each species. The percentage resistance in the xylem was then obtained as the inverse of $K_{x,max} / K_{leaf,max}$ (as hydraulic resistance = 1/hydraulic conductance).

To test whether K_x values differed across treatments, we calculated the average and standard deviation of K_x and leaf xylem water potential for the leaves in each treatment and compared them with the K_x obtained from the species' K_x vulnerability curve at that same water potential, that is, the 'standard protocol', using a one-sample two-tailed *t*-test (Minitab Release 16, State College, PA, USA).
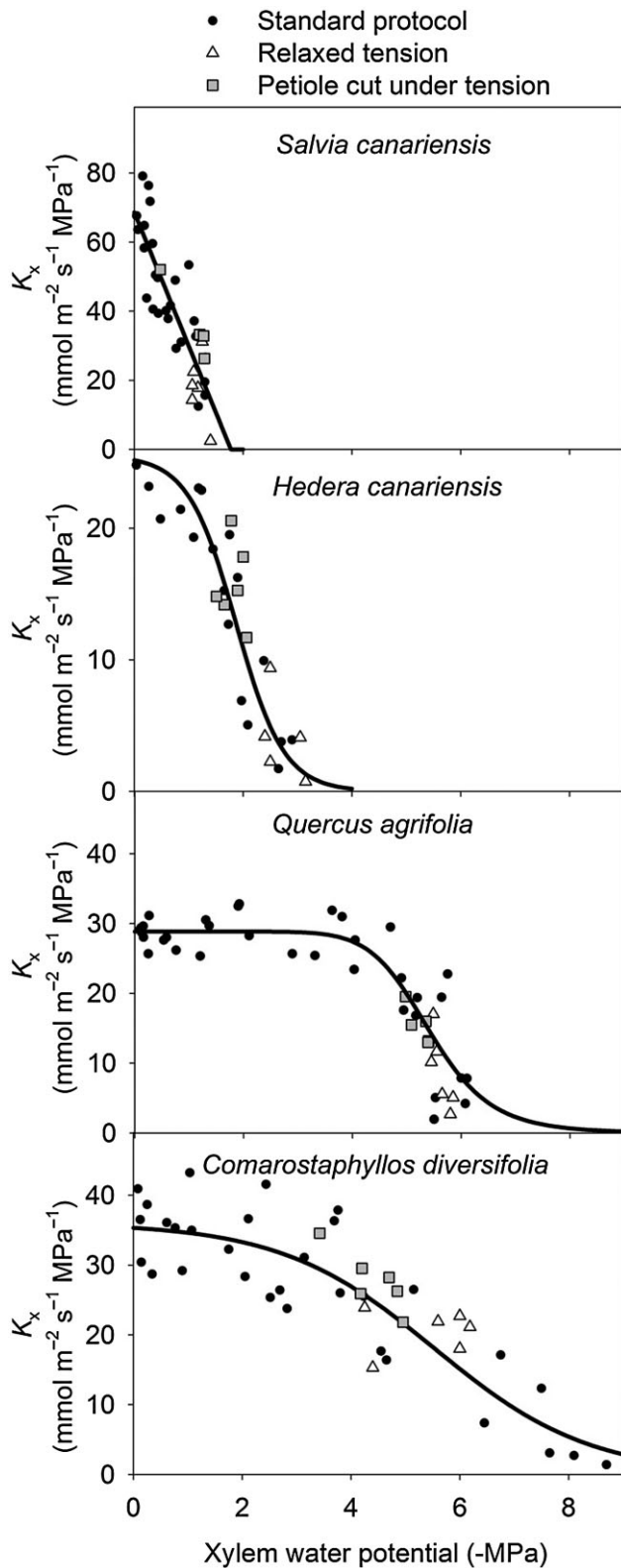
RESULTS

Leaf xylem hydraulic vulnerability curves: results from maximum likelihood analysis

The vacuum pump method was effective for the construction of leaf K_x vulnerability curves (Fig. 3). Species differed in the shape of their K_x vulnerability curves (Fig. 3; Supporting Information Table S1): *S. canariensis* showed a linear decline whereas the other three species showed a non-linear decline (sigmoidal functions were selected by maximum likelihood for *C. diversifolia* and *H. canariensis* and logistic function for *Q. agrifolia*). Species also differed in their percentage leaf hydraulic resistance in the xylem at full hydration, ranging from 8.4% in the California chaparral species *C. diversifolia* to 77% in the herb *S. canariensis* (Table 1). Leaf xylem water potential at 50% loss of K_x ranged from –0.89 MPa in *S. canariensis* to –5.59 MPa in *C. diversifolia* (Table 1).

Testing for a cutting artefact in leaf xylem hydraulic conductance

No significant differences in the K_x of dehydrated leaves were observed when applying the three cutting treatments for any of the four species (Table 2; Fig. 3). Indeed, neither mean K_x values from leaves of shoots with relaxed tension (white triangles in Fig. 3) or from leaves with petioles cut under tension (grey squares in Fig. 3) differed statistically from the predicted K_x value from the best-fit function through data

Figure 3. Leaf xylem hydraulic vulnerability curves for four species diverse in phylogeny, growth form, drought tolerance and xylem anatomy, ordered from most vulnerable on top to least on bottom. The line fitted through the black dots (obtained using the standard protocol) was selected as best-fit function using maximum likelihood (see Methods and Supporting Information Table S1). A linear function was selected for *Salvia canariensis*: $K_x = -38.6 \times \Psi_x + 68.6$), a three-parameter sigmoidal function was selected for *Hedera canariensis* and *Comarostaphyllos diversifolia*:

$$\left(K_x = \frac{25.5}{1 + e^{-\left(\frac{\Psi_x - 1.87}{-0.44}\right)}} \right) \text{ and } \left(K_x = \frac{36.0}{1 + e^{-\left(\frac{\Psi_x - 5.54}{-1.42}\right)}} \right), \text{ respectively. Finally,}$$

a logistic function was selected for *Quercus agrifolia*:

$$\left(K_x = \frac{28.8}{1 + \left(\frac{\Psi_x}{5.43}\right)^{9.67}} \right).$$

Species variation in maximum xylem conduit length

Vein orders in which the longest xylem conduits from the petiole ended differed across individuals even for given species. For all leaves of all species, the longest xylem conduits from the petiole had ended by the secondaries or tertiaries, that is, before the minor vein network (Table 1). For three out of four species, no xylem conduits from the stem extended into the leaves. However, for *S. canariensis*, 6/11 of the measured shoots had xylem conduits extending into the leaves: for 2/6 shoots, at least one conduit in the stem ended in the petiole; for 2/6 at least one conduit ended in the midrib; and for 2/6 at least one ended in a secondary vein (Table 1).

DISCUSSION

> Don't think twice, it's alright.
> – Bob Dylan, The Freewheelin' (1963)

This study demonstrated that the cutting artefact had no impact on leaf xylem hydraulic conductance measured for dehydrated leaves. These findings provide the first validation of previous measurements of leaf hydraulic vulnerability against this potential artefact. Indeed, our results showed no significant differences in leaf xylem hydraulic conductance (K_x) of leaves cutoff from shoots after relaxing the xylem tension relative to shoots with high xylem tensions, for four diverse species differing in their initial percentage allocation to xylem versus outside xylem conductance.

Our finding for leaves contrasted strongly with the effect shown to occur for the stem xylem for some species (Wheeler *et al*. 2013). For two maple species, Wheeler *et al*. found that stem percentage loss of hydraulic conductance (PLC) was 43–71% lower when shoot xylem tension was relaxed prior to cutting after shoots were dehydrated to around water potentials at 50% loss of hydraulic conductance, thus indicating that these species were slightly less vulnerable to cavitation than previously thought. However, no significant difference was found in *Betula papyrifera* stem vulnerability curves for relaxed versus non-relaxed shoots, suggesting that for stem tissue this cutting artefact is

points obtained from the 'standard protocol' in which minor veins were cut under tension before cutting the petiole from the stem under water ($P = 0.097$–0.56 across treatments and species; Table 2; Fig. 3).

Table 2. Testing the 'cutting artefact': number of samples (*n*), xylem water potential ± standard deviation (MPa), predicted xylem hydraulic conductance (K_x); mmol m-2 s-1 MPa-1 for leaves of treatments depicted in Fig. 1, based on the mean xylem water potential obtained from the leaf xylem vulnerability curve using the 'standard protocol treatment', along with the expected and resulting trends and *P*-value from one-sample *t*-test

Treatment	'Relaxed tension treatment'				'Petiole cut under tension treatment'			
Species	*n*	Ψ_x ± SD	Predicted K_x from the 'standard protocol'	Mean K_x ± SD (*P*-value for comparison with 'standard protocol'	*n*	Ψ_x ± SD	Predicted K_x from the 'standard protocol'	Mean K_x ± SD (*P*-value for comparison with 'standard protocol'
Comarostaphylis diversifolia	6	−5.41 ± 0.35	(18.8)	20.5 ± 1.13 (0.25)	6	−4.38 ± 0.23	(25.7)	27.7 ± 1.73 (0.30)
Hedera canariensis	5	−2.72 ± 0.16	(3.21)	4.13 ± 1.46 (0.56)	6	−1.82 ± 0.09	(13.6)	15.7 ± 1.26 (0.16)
Quercus agrifolia	6	−5.63 ± 0.07	(12.0)	8.70 ± 2.15 (0.19)	5	−5.24 ± 0.09	(16.9)	15.4 ± 1.18 (0.27)
Salvia canariensis	6	−1.17 ± 0.05	(23.4)	18.4 ± 3.89 (0.26)	5	−1.10 ± 0.16	(26.1)	35.5 ± 4.35 (0.097)

A $P > 0.05$ is considered non-significant.
SD, standard deviation.

dependent on species and potentially depends on xylem anatomical traits, such as conduit diameter, length and/or presence of perforation plates (Wheeler *et al*. 2013; Rockwell *et al*. 2014).

Why should leaf xylem show no sensitivity to a Wheeler-type effect? Although no mechanistic explanation has been tested to explain this effect in stems, several hypotheses have been proposed. The main goal of our paper was to test whether this artefact has an effect in leaf hydraulics measurement in the way shown for stems. Because no effect was found, we discuss the possible explanations for why leaves were found to differ from stems.

Although the cutting artefact was found for petioles in one species (*Acer rubrum*) by Wheeler *et al*. (2013), the methodology used was different from ours.

A first possibility for the difference between stem and whole leaves in the occurrence of the cutting artefact might be due to xylem conduits in petioles being narrower than those in stems, given that air bubbles may form more rapidly or enter more easily into wider conduits (Wheeler *et al*. 2013; Rockwell *et al*. 2014). We do not have data on petiole xylem conduit diameters, but values are available for mean midrib xylem conduit diameters for the same plants of these species, and these midrib conduit diameters would be yet narrower than those in the petiole: 27.8, 59.5, 27.2 and 198 μm for *C. diversifolia*, *Hed. canariensis*, *Q. agrifolia* and *S. canariensis*, respectively (John *et al*. 2013). This range of diameters encompassed that for stem mean vessel diameters previously reported for *A. rubrum* and *B. papyrifera* used in the study by Wheeler *et al*. (45.3 and 34.0 μm, respectively; Jacobsen *et al*. 2012). If xylem conduit diameters were an important factor in determining the cutting artefact, then we would have expected *S. canariensis* to show a strong effect, given its midrib conduit diameter was over fourfold greater than that of *A. rubrum*, which had showed the strongest cutting artefact (Wheeler *et al*. 2013).

A second possibility for the lack of a cutting artefact in leaves is that it could be due to shorter xylem conduit lengths in leaves. Indeed, in shorter conduits, any embolism created artefactually by cutting under tensions would be expected to

be stopped at end walls close to the cut, and thus these might have been removed when trimming the petiole with a clean cut before it is placed on the system (Wheeler *et al*. 2013; Rockwell *et al*. 2014). Maximum conduit lengths have been reported to be of 0.14 m in *A. rubrum*, and mean vessel lengths of 0.03, 0.03 and 0.02 m in *A. rubrum*, *A. saccharum* and *B. papyrifera* (Jacobsen *et al*. 2012). We found maximum conduit lengths of 0.25–0.91 m in stems for *H. canariensis* and *Q. agrifolia*, and that conduits sometimes extended from the stems to leaf secondary veins in *S. canariensis* (Table 1). Further, although not as long as vessel lengths in stems, xylem conduits in leaves of our study species showed continuity up to third-order veins (Table 1). Of course, mean conduit length might play a more important role than maximum conduit length; however, given our findings, it is unlikely that xylem conduits in the petiole would be on average ≤1 mm long (the length of the final cut we made before connecting the leaf on the system), enabling the removal of any artefactual embolism that might have formed in these small enclosed conduits. Further studies of xylem conduit distributions in petioles and leaf veins are needed to fully verify our rejection of the idea that conduits are so short that trimming the petiole would remove embolisms that entered during cutting.

If neither conduit diameters nor lengths play a role in explaining the lack of a cutting artefact in leaves, we posit three other possible explanations for consideration. First, a cutting artefact that might occur in a petiole, as reported for one species by Wheeler *et al*. (2013), might not have scaled up to an influence on K_x, given that only some xylem conduits from the petiole extend into the leaf veins, and the petioles account for a minority of the xylem hydraulic resistance. Second, it is possible that the Wheeler effect occurs in stems of some species in part or entirely due to air entering from airspaces within the stem aggravated by the mechanical disturbance generated by the act of cutting stems, especially as this would generate high and low pulses of pressure by deforming conduits (Lopez *et al*. 2014; Mayr *et al*. 2014). Such pressure pulses within the xylem would be expected to cause transient expansion of vapour spaces

within xylem conduits as the conduits shrink and stretch and/or might induce air seeding across pit membranes. Any such effects due to physical disturbances during cutting would be much reduced in a petiole relative to the stem given their reduced diameters, densities and mechanical strength. It seems indeed likely that the xylem conduits and their pit membranes within leaves would be adapted to cope with the effects of transient pressures in the xylem caused by mechanical disturbances of a magnitude like that imposed by cutting the petiole with a sharp blade, whereas stems might not be adapted to the effects of disturbances as severe as those imposed when cutting them for hydraulics measurements. Indeed, leaves frequently suffer major mechanical stresses such as when they are blowing in the wind, or if a hard rain is to fall on them.

A third possible explanation for the lack of a cutting artefact in leaves is that this artefact in stems arises itself from an artefact of hastening xylem refilling when xylem tension is relaxed (Trifilo *et al*. 2014). Indeed, the cutting artefact for stems disappeared in two Mediterranean tree species, *Laurus nobilis* and *Olea europaea*, when stems were girdled or exposed to sodium orthovanadate (Na_3VO_4) prior to measurements, both of which treatments would impede xylem refilling. The authors concluded that relaxing the stem xylem tension before cutting, as proposed by Wheeler *et al*., could in fact be inducing xylem refilling before the sample is placed on the system, and thus under estimating PLC values. If that hypothesis is true, we would expect to find a Wheeler effect only in those species in which xylem refilling occurs. In leaves, recovery of hydraulic conductance with rehydration was found for four species with petioles under water for up to 1 h including *C. diversifolia*, which recovered completely in K_{leaf} after 1 h (Trifilo *et al*. 2003; Scoffoni *et al*. 2012). However, this could be due to recovery in K_{ox} rather than K_x (Scoffoni *et al*. 2014). Only one study to our knowledge, for *Helianthus annuus*, has shown that embolism refilling might occur in the leaf veins; in rehydrated leaves, vein staining from infiltration with Phloxine B was greater than in dehydrated leaves, suggesting embolism refilling had occurred in the leaf veins, although the time scale of the process was not clear (Trifilo *et al*. 2003). More studies are needed to confirm potential embolism recovery in leaves after rapid rehydration.

More studies will be needed of stem xylem tissue to fully understand the mechanism(s) underlying the cutting artefact in stems. For leaves, based on this first detailed study, until an artefact is shown, one may consider the previous literature on leaf hydraulic vulnerability to be validated against this effect.

ACKNOWLEDGMENTS

REFERENCES

Brodribb T.J. & Holbrook N.M. (2003) Changes in leaf hydraulic conductance during leaf shedding in seasonally dry tropical forest. *The New Phytologist* **158,** 295–303.

Burnham K.P. & Anderson D.R. (2002) *Model Selection and Multimodel Inference*, 2nd edn. Springer, New York, NY, USA.

Burnham K.P. & Anderson D.R. (2004) Multimodel inference – understanding AIC and BIC in model selection. *Sociological Methods & Research* **33,** 261–304.

Cochard H., Nardini A. & Coll L. (2004) Hydraulic architecture of leaf blades: where is the main resistance? *Plant, Cell & Environment* **27,** 1257–1267.

Dixon H.H. (1914) *Transpiration and the Ascent of Sap in Plants*. MacMillan and Co., London, UK.

Jacobsen A.L., Pratt R.B., Tobin M.F., Hacke U.G. & Ewers F.W. (2012) A global analysis of xylem vessel length in woody plants. *American Journal of Botany* **99,** 1583–1591.

John G.P., Scoffoni C. & Sack L. (2013) Allometry of cells and tissues within leaves. *American Journal of Botany* **100,** 1936–1948.

Kolb K.J., Sperry J.S. & Lamont B.B. (1996) A method for measuring xylem hydraulic conductance and embolism in entire root and shoot systems. *Journal of Experimental Botany* **47,** 1805–1810.

Lo Gullo M.A., Nardini A., Trifilo P. & Salleo S. (2003) Changes in leaf hydraulics and stomatal conductance following drought stress and irrigation in *Ceratonia siliqua* (Carob tree). *Physiologia Plantarum* **117,** 186–194.

Lopez R., Badel E., Peraudeau S., Leblanc-Fournier N., Beaujard F., Julien J.-L., . . . Moulia B. (2014) Tree shoot bending generates hydraulic pressure pulses: a new long-distance signal? *Journal of Experimental Botany* **65,** 1997–2008.

Martre P., Cochard H. & Durand J.-L. (2001) Hydraulic architecture and water flow in growing grass tillers (*Festuca arundinacea* Schreb.). *Plant, Cell & Environment* **24,** 65–76.

Mayr S., Bertel C., Damon B. & Beikircher B. (2014) Static and dynamic bending has minor effects on xylem hydraulics of conifer branches (*Picea abies, Pinus sylvestris*). *Plant, Cell & Environment* **37**, 2151–2157.

Nardini A., Tyree M.T. & Salleo S. (2001) Xylem cavitation in the leaf of *Prunus laurocerasus* and its impact on leaf hydraulics. *Plant Physiology* **125,** 1700–1709.

Nardini A., Ramani M., Gortan E. & Salleo S. (2008) Vein recovery from embolism occurs under negative pressure in leaves of sunflower (*Helianthus annuus*). *Physiologia Plantarum* **133,** 755–764.

Rockwell F.E., Wheeler J.K. & Holbrook N.M. (2014) Cavitation and its discontents: opportunities for resolving current controversies. *Plant Physiology* **164,** 1649–1660.

Sack L. & Scoffoni C. (2012) Measurement of leaf hydraulic conductance and stomatal conductance and their responses to irradiance and dehydration using the evaporative flux methods (EFM). *Journal of Visualized Experiments* **70,** e4179, doi: 10.3791/4179.

Sack L., Melcher P.J., Zwieniecki M.A. & Holbrook N.M. (2002) The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of Experimental Botany* **53,** 2177–2184.

Sack L., Streeter C.M. & Holbrook N.M. (2004) Hydraulic analysis of water flow through leaves of sugar maple and red oak. *Plant Physiology* **134,** 1824–1833.

Sack L., Tyree M.T. & Holbrook N.M. (2005) Leaf hydraulic architecture correlates with regeneration irradiance in tropical rainforest trees. *The New Phytologist* **167,** 403–413.

Sack L., Scoffoni C., McKown A.D., Frole K., Rawls M., Havran C., . . . Tran T. (2012) Developmentally-based scaling of leaf venation architecture explains global ecological patterns. *Nature Communications* **3,** 837.

Scoffoni C., McKown A.D., Rawls M. & Sack L. (2012) Dynamics of leaf hydraulic conductance with water status: quantification and analysis of species differences under steady-state. *Journal of Experimental Botany* **63,** 643–658.

Scoffoni C., Vuong C., Diep S., Cochard H. & Sack L. (2014) Leaf shrinkage with dehydration: coordination with hydraulic vulnerability and drought tolerance. *Plant Physiology* **164,** 1772–1788.

Trifilo P., Gasco A., Raimondo F., Nardini A. & Salleo S. (2003) Kinetics of recovery of leaf hydraulic conductance and vein functionality from cavitation-induced embolism in sunflower. *Journal of Experimental Botany* **54,** 2323–2330.

Trifilo P., Raimondo F., Lo Gullo M.A., Barbera P.A., Salleo S. & Nardini A. (2014) Relax and refill: xylem rehydration prior to hydraulic measurements favours embolism repair in stems and generates artificially low PLC values. *Plant, Cell & Environment*. doi: 10.1111/pce.12313

Tyree M.T. & Zimmermann M.H. (2002) *Xylem Structure and the Ascent of Sap*. Springer, Berlin, Germany.

Weast R.C. (1974) *Handbook of chemistry and physics*, 54th edn. CRC Press, Cleveland, OH, USA.

Wheeler J.K., Huggett B.A., Tofte A.N., Rockwell F.E. & Holbrook N.M. (2013) Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism. *Plant, Cell & Environment* **36**, 1938–1949.

Yang S.D. & Tyree M.T. (1993) Hydraulic resistance in *Acer saccharum* shoots and its influence on leaf water potential and transpiration. *Tree Physiology* **12**, 231–242.

SUPPORTING INFORMATION

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

Figure S1. Typical plots of flow versus vacuum pressure in four species at high (−0.04 to −0.58 MPa; left) and low (−1.1 to −5.9 MPa; right) leaf water potentials. The slope (in bold) is equal to leaf xylem hydraulic conductance (K_x).

Figure S2. Residuals of leaf xylem hydraulic conductance (K_x) versus leaf water potential against measurement time on the vacuum system. No significant correlation was found for any of the four species, indicating no effect of rehydration time on measured K_x.

Figure S3. Leaf hydraulic vulnerability of *Salvia canariensis* obtained using the evaporative flux method. Best-fit function selected by maximum likelihood is fitted through the points: ($K_x = -38.6 \times \Psi_x + 68.6$).

Table S1. Parameters (and standard errors) for the decline of xylem hydraulic conductance with dehydration for four species fitted with five different functions. Values in bold indicate the best-fit model for each species, r^2 for observed values plotted against predicted values, and values for the Akaike information criterion (AIC) corrected for low *n*.