

Original Article

Excising stem samples underwater at native tension does not induce xylem cavitation

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ABSTRACT

Xylem resistance to water stress-induced cavitation is an important trait that is associated with drought tolerance of plants. The level of xylem cavitation experienced by a plant is often assessed as the percentage loss in conductivity (PLC) at different water potentials. Such measurements are constructed with samples that are excised underwater at native tensions. However, a recent study concluded that cutting conduits under significant tension induced cavitation, even when samples were held underwater during cutting. This resulted in artificially increased PLC because of what we have termed a ‘tension-cutting artefact’. We tested the hypothesized tension-cutting artefact on five species by measuring PLC at native tension compared with after xylem tensions had been relaxed. Our results did not support the tension-cutting artefact hypothesis, as no differences were observed between native and relaxed samples in four of five species. In a fifth species (*Laurus nobilis*), differences between native and relaxed samples appear to be due to vessel refilling rather than a tension-cutting effect. We avoided the tension-cutting artefact by cutting samples to slightly longer than their measurement length and subsequent trimming of at least 0.5 cm of sample ends prior to measurement.

Key-words: *Laurus nobilis*; cutting artefact; hydraulics; PLC; pressure head; relaxed tension; vessel refilling.

INTRODUCTION

Liquid sap within xylem conduits is transported under negative pressures in a metastable state (Dixon & Joly 1895). During high water demand or when soil water becomes depleted, negative pressure can lead to cavitation of water in xylem conduits when gas seeds through a pit membrane pore from an air-filled space (Sperry & Tyree 1988; Jarbeau *et al.* 1995). Freeze–thaw cycles can also nucleate cavitation when gas comes out of solution from the frozen sap and then expands upon thawing (Tyree & Sperry 1989; Davis *et al.* 1999a; Pittermann & Sperry 2003). High levels of emboli limit water supply to the leaves, reduce photosynthesis (Hubbard *et al.* 2001; Brodribb *et al.* 2002; Sperry *et al.* 2008), cause dieback (Davis *et al.* 2002) and are linked to drought-induced mortality (Pratt *et al.* 2008; Anderegg *et al.* 2012).

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Species widely differ in their resistance to cavitation and this is commonly evaluated by constructing vulnerability curves (VCs), which show the loss of xylem conductivity, as either a decline in specific conductivity (K_s) or an increase in the percentage loss in hydraulic conductivity (PLC), in relation to water potential. VCs are important tools for understanding plant ecology and physiology (Tyree & Sperry 1989; Sperry 2003; Vilagrosa *et al.* 2012). Several methods can be used for constructing VCs, which vary in the way that water stress is applied (e.g. dehydration, air injection, centrifugation) and cavitation evaluated (e.g. conductivity measurements, acoustic emissions, imaging).

Native measurements and bench-top dehydration are two of the measurements commonly identified as the standards by which to judge the accuracy of other methods (Sperry *et al.* 2012; Hacke *et al.* 2014). Both of these methods require the excision of a xylem segment from a larger sample while the xylem is under negative tension. Most studies assume that severing the xylem while the sample is held underwater preserves the hydraulic continuity of xylem conduits opened by the cut and thus provides an accurate measure of *in situ* cavitation levels; however, this idea has recently been challenged (Wheeler *et al.* 2013).

Wheeler *et al.* (2013) observed differences in PLC between samples excised from branches under native tension (negative pressure) and relaxed branches (rehydrated in order to bring xylem tension close to zero before sample excision). They suggested that cutting while samples were still under tension may nucleate cavitation in xylem conduits, which artificially increased the PLC of samples. They attributed the higher PLC of native samples to an experimental artefact, hereon referred to as the ‘tension-cutting artefact’.

The proposed tension-cutting artefact, if supported, would have broad implications for studies of cavitation and a series of opinion papers have highlighted this (Sperry 2013; Delzon & Cochard 2014; Rockwell *et al.* 2014). This artefact would potentially affect studies that have used dehydration-based curves to evaluate species cavitation resistance and studies that have used native or dehydration curves to verify other methods (e.g. Sperry & Tyree 1990; Cochard *et al.* 1992; Jarbeau *et al.* 1995; Pockman *et al.* 1995). Additionally, the refilling of xylem vessels during the course of water potential diurnal cycles or in response to rehydration has been an active area of research (reviewed in Zwieniecki & Holbrook 2009; Nardini *et al.* 2011). Many of these studies have relied on data showing that PLC are higher when stems are under

more negative pressure and lower under higher pressures as evidence for the existence of refilling (e.g. Zwieniecki & Holbrook 1998; Hacke & Sperry 2003; Nardini *et al.* 2008; Zufferey *et al.* 2011; Christman *et al.* 2012). On the basis of the hypothesized tension-cutting artefact, these findings have been questioned (Wheeler *et al.* 2013; Delzon & Cochard 2014; Rockwell *et al.* 2014); however, others have cautioned that this artefact should not be extrapolated to all species and studies (Sperry 2013).

There is an alternative hypothesis to explain differences in PLC when samples are cut under native tension compared with relaxed tension, which is that emboli may reverse because of vessel refilling in relaxed samples. This hypothesis posits that lower PLC in relaxed tension samples is an artefact due to refilling, whereas PLC of samples cut under native tension describes what is happening *in situ* prior to rehydration. Trifilò *et al.* (2014) recently examined this alternative in an experiment performed with olive (*Olea europaea* L.) and bay laurel (*Laurus nobilis* L.). They determined that the lower PLC of relaxed samples was due to refilling and that the relaxed sample PLC were therefore not indicative of *in situ* cavitation levels of samples under tension. This 'refilling artefact' could be avoided by measuring native samples excised under native pressure or by girdling or chemically treating stems to inhibit refilling. Thus, in these two species the tension-cutting artefact was not supported. Additionally, another study that tested this artefact on leaf hydraulics in four species did not find support for the tension-cutting artefact (Scoffoni & Sack 2014).

We designed an experiment to further test the tension-cutting artefact in stems. We evaluated the effect of excising samples under native and relaxed tensions on PLC. Under the tension-cutting artefact hypothesis, we predicted that impurities and air dissolved in water could facilitate air bubble nucleation during sample excision, relaxation, or trimming. Therefore, we also tested the effect of performing these operations under normal tap water (TW) or a degassed solution (DS). If the tension-cutting artefact was related to solution-triggered nucleation, using filtered DS should reduce PLC values when samples were cut under tension. We sampled species that have both long and short vessels because, as suggested by Wheeler *et al.* (2013), the tension-cutting artefact was predicted to be greater in long-vesselled species. Finally, we examined the influence of girdling, which would prevent refilling, on PLC changes when samples were measured under native and relaxed tensions.

MATERIAL AND METHODS

Plant material

All samples were collected from irrigated plants grown on the campus of California State University, Bakersfield (CSUB), USA (35° 21' N, 119° 6' W). The study was performed with one short-vesselled species, red willow (*Salix laevigata* Bebb), and four longer-vesselled species, bay laurel (*L. nobilis* L.), glossy privet (*Ligustrum lucidum* Aiton), elderberry (*Sambucus nigra* L.) and white ash (*Fraxinus americana* L.).

All five species have vessels with simple perforation plates. Species selection was based on vessel characteristics and sample availability. Furthermore, white ash was studied by Wheeler *et al.* (2013) and bay laurel by Trifilò *et al.* (2014), which allowed for comparisons among studies.

Vessel length measurements

Maximum vessel length (VL_{max}) of the plants used for this study was determined using air injection (Greenidge 1952). Large branches (2.5–4.0 m) were collected, bagged in plastic, and transported to a laboratory in <1 h. A distal branch with a diameter of 3–7 mm was cut in the lab and a tube was attached to this distal branch. Nitrogen gas was injected at 100 kPa. During injection, the basal end was immersed in a water-filled tray and segments were cut from the basal end until the first bubbles travelling through the xylem were observed. The excised basal segments were 10 cm long when the distance to the distal end was greater than 2 m, 5 cm long when this distance was 2.0–1.5 m, and 1 cm long when the distance was smaller than 1.5 m to the distal end. Vessel length measurements were performed in March–April 2014.

Hydraulic measurements

Hydraulic conductivity (K_h) of stems was measured gravimetrically with a conductivity apparatus (Sperry *et al.* 1988). A low pressure head (1.5–2.0 kPa) was used in order to avoid displacing air from embolized vessels. K_h was calculated as the pressure-driven flow, corrected for background flows with no pressure head, divided by the pressure gradient. Background flows can occur as stems take up free water to fill depleted water stores and their correction increases accuracy (Hacke *et al.* 2000; Torres-Ruiz *et al.* 2012). Measurements were performed with a degassed (membrane contactor, Liqui-Cel Minimodule 1.7 × 5.5, Charlotte, NC, USA) and filtered (0.1 µm inline filter, GE Water and Process Technologies, Trevose, PA, USA) 20 mM KCl solution. This differs from Wheeler *et al.* (2013) study, which measured conductivity using a non-degassed solution (J. Wheeler, personal communication, 13 July 2014). The initial K_h of stems was measured for each treatment as described later. The stems were then flushed for 1 h at 100 kPa with the 20 mM KCl DS to remove emboli, which has been shown to effectively remove emboli (Hacke *et al.* 2014). Afterwards, maximum conductivity (K_{max}) was measured. The percentage loss of conductivity (PLC) of each stem was calculated as:

$$PLC = (1 - K_h / K_{max}) \times 100 \quad (1)$$

Stem segments were 1–4 years old and 3–7 mm diameter, and were always matched for diameter and location in the crown for different treatments.

Experiment 1: native versus relaxed for a short-vesselled species

In October 2013, three deeply forked branches 2.5–3.5 m long of one red willow tree were cut in air in the morning and

immediately transported to a laboratory. We chose to sample large forked branches because it enabled us to induce both of our treatments within this larger branch to minimize variability that occurs between branches (Supporting Information Fig. S1). Nine leaves were covered with small resealable plastic bags for water potential measurements. The branches were triple bagged in large plastic bags and covered with a moist sheet for 3 h, so that the water potential within each branch equilibrated and leaf measures would be indicative of the xylem water potential of stems (Ψ_x).

Hydraulic conductivity was measured on stem samples exposed to two different treatments: (1) 15–17 cm stem segments were excised with clippers underwater at the branch's native Ψ_x , shaved-down to 14 cm with fresh razor blades, and K_h determined. We refer to this treatment as 'native' (N). We did not excise exact 14 cm segments and shaved only a few micrometres as Wheeler *et al.* (2013) as we intended to cut samples in the manner that natives are commonly collected; (2) The Ψ_x was brought to near zero prior to stem segments excision by immersing the cut base underwater and cutting a 10 cm increment from the base underwater every 10–20 s. When these cuts neared the 15–17 cm stem segment to be excised, this process was performed both from the apical and basal end alternatively. This process was completed in 5–10 min. The segments were shaved with a fresh razor blade to 14 cm and K_h was measured. We refer to this treatment as 'relaxed' (R). Following K_h measurements, K_{max} was measured for all samples and PLC calculated. Within each large branch, first the three native stems were collected from one side of the main fork, and afterwards, three relaxed stems were collected from the other side of the fork (Supporting Information Fig. S1). Native samples were collected within 10 min. The order of collection of the natives was registered, and there was no trend or difference in PLC related to this factor ($P = 0.334$). All stem segments were collected at a distance greater than twice the mean VL_{max} from where the large branch was cut in air. Using such a long branch should have minimized air entry into the region of the crown containing the branches we targeted for measurements; however, because our native and relaxed treatments were replicated on the same large branches, any errors from air entry should have affected both our native and relaxed treatments, thus not affecting the main comparisons of our study.

Prior to native sample excision, six of the previously covered leaves (three from each side of the main fork) were collected and measured to estimate the native Ψ_x using a pressure chamber (model 2000, PMS Instrument Co., Albany, OR, USA). To ensure the effectiveness of the relaxed treatment, just before the final stem segment excision the final three covered leaves were collected and their Ψ measured as an estimated of the relaxed Ψ_x .

Experiment 2: TW versus DS in four long-vesselled species

This experiment was performed on bay laurel, glossy privet, elderberry and white ash in March–April 2014. At midday, large forked branches (2.5–4.0 m) were cut in air and trans-

ported to a laboratory. For each branch, eight leaves (four on each side of the main fork) were covered with small resealable plastic bags for Ψ_x determination. The large branches were then triple bagged in large plastic bags, covered with a moist sheet, and left to equilibrate overnight. The following morning, hydraulic measurements were performed with three stems excised under native and three under relaxed tension within each branch, as described earlier. The distance from the base of the cut branch to the base of each of the segments was registered. Within each species, half of the branches were relaxed with TW, and the other half with the filtered and degassed 20 mM KCl solution. The measured stems segments collected from each branch were shaved in the same liquid in which they were relaxed. We refer to these treatments as TW and DS. The DS was used within 1 h from its preparation, but could have contained some gas as it was poured into a tray for the relaxation treatment, but the amount of gas and nucleating particles it contained would likely be lower than TW.

For this experiment, two to four branches were measured per day and six to 10 branches were collected per species. Sample Ψ_x were estimated by measuring four leaves prior to native excision and four leaves collected after relaxation for each branch. All stem segments were collected at a distance greater than $1 \times$ the mean VL_{max} from where the branch was cut in air. The only exception to this was white ash, which had very long vessels, and we could not collect all branches so that they were $1 \times$ the mean VL_{max} . Of 48 samples collected for this species, 25 were collected at 100–150 cm from the basal cut, and the 23 remaining were at a distance >150 cm. To test for how this affected our PLC, we measured the distance between the point of where we cut our large branch to where we sampled our native (N) and relaxed (R) side branches. We included this distance as a covariate in an analysis of covariance model to assess if the distance to an open vessel affected PLC measures. The distance did not significantly affect our measured PLC ($F_{1, 13.92} = 0.0350$, $P = 0.8542$) and this factor was therefore excluded from our final analyses.

Experiment 3: tension-cutting versus vessel refilling

This experiment was performed as a follow-up experiment using only bay laurel in order to test if the PLC differences, which were observed between native and relaxed stems only for this species in experiment 2, were due to a tension-cutting artefact (Wheeler *et al.* 2013) or to vessels refilling during relaxation (Trifilò *et al.* 2014). To test for this we included girdled stems as an additional treatment. 'Novel refilling' is associated with phloem function (Salleo *et al.* 1996; Nardini *et al.* 2011), and girdling should avoid vessel refilling (Trifilò *et al.* 2014).

In April 2014, 15 large branches (3–4 m long) with three main shoots each were selected. One of the shoots (longer than 1 m) was repeatedly girdled along its length (Supporting Information Fig. S2). The bark and phloem were carefully removed from 5-mm wide rings every 10–15 cm, two times above (distal end) and four to six times below (basal end) the

stem segment that would later be excised for hydraulic measurements (Trifilò *et al.* 2014). The exposed xylem was immediately covered with a thin layer of grease (High Vacuum Silicon Grease, Dow Corning Corporation, Midland, MI, USA) to avoid stem desiccation. Girdling was performed in the morning (09:00–10:00 h). The branches were cut in air within an hour after being girdled, and transported to a laboratory (two to four branches per day). Three leaves per shoot (i.e. nine leaves per branch) were covered with small resealable plastic bags for later Ψ_x measurements, and the branches were triple bagged in large plastic bags and covered with a moist sheet for 2 h so that they equilibrated.

Three treatments were applied in each branch. First, one stem segment from an intact shoot was excised underwater, following the native treatment previously described. Then the remaining shoots were relaxed in water, as described earlier, in order to bring their xylem tension close to zero before excision. The excised stem from the intact branch was the relaxed treatment (as described earlier), and the stem segment obtained from the relaxed and girdled shoot was referred to as the 'girdled' (G) treatment. All stem segments were collected at a distance greater than 85 cm from where the branch was cut in air, which is greater than the mean VL_{max} for this species. Bagged leaves for native Ψ_x were collected just before the first stem excision, and leaves from the relaxed and girdled shoots just before the last relaxation cuts. As described earlier, K_h and K_{max} were measured and PLC calculated.

Since the tension of girdled stems was brought close to zero before excision, two possible outcomes were predicted: (1) if girdled PLC equalled native PLC, this would indicate that the lower PLC of relaxed samples was due to refilling; and (2) if girdled PLC equalled relaxed PLC, this would support to the tension-cutting artefact.

Experiment 4: native versus positive pressure relaxation (R_p)

It is possible that even though we thoroughly relaxed the tension in R treatment stems, any tension could lead to a cutting artefact. To test for this we put stems under positive pressure before cutting the xylem and we compared the PLC of these samples with those from stems cut under native tension.

In April 2014, four large forked glossy privet branches (3–4 m long) were cut in air at midday and immediately transported to the laboratory. Six leaves were covered with small resealable plastic bags for Ψ_x measurements. The branches were triple bagged in very large plastic bags and covered with a humid sheet to equilibrate overnight. The next morning, three stem segments were excised following the native treatment described previously, and their K_h was measured. Then 80 cm (approximately $1 \times$ the mean VL_{max}) were removed from the basal end of the branches by successive 10 cm cuts underwater every 10 s. The base was shaved with a new razor and a reservoir with 20 mM KCl filtered DS was connected to the branch. This reservoir was placed 35 cm above the branch, creating a positive pressure of 3.4 kPa, and

the branch was covered with a dark plastic (Supporting Information Fig. S3). After 15 min, three stem segments (15–17 cm) were excised underwater while the reservoir was still connected, and then shaved to 14 cm for K_h measurement. We refer to this treatment as R_p . All stem segments were collected at a distance greater than $2 \times$ the mean VL_{max} from where the branch was initially cut in air. Finally K_{max} was measured for all stems and PLC calculated. For Ψ_x measurements three leaves were collected just before the native stems were excised, and the remaining three after the 15 min R_p .

Statistical analyses

We sampled large forked branches and subsampled side branches of the large branch to control for inter-branch variation. The PLC data were analysed using linear mixed models by restricted maximum likelihood with JMP 9.0.0 (SAS Institute, Inc., Cary, NC, USA). The treatment factors for experiments 1, 3 and 4 were branch (random) and a fixed treatment factor (N, R, G and/or R_p). The PLC data of experiment 2 were analysed using a linear mixed model with branch (random), the fixed treatments of N and R, a second fixed treatment of DS and TW, and possible interactions (Supporting Information Table S1). Assumptions of parametric models were verified.

RESULTS

The mean VL_{max} was widely different among the sampled species with the shortest VL_{max} in red willow (40 cm) and the greatest (154 cm) in white ash (Table 1).

There were no differences in PLC between native and relaxed treatments in any of our sampled species (Fig. 1), except for the bay laurel (discussed later). This is contrary to what would be expected if there were a tension-cutting artefact (Fig. 1a). This result cannot be explained by insufficient relaxation of xylem tensions. The Ψ_x of relaxed branches significantly increased with relaxation in all species of experiments 1 and 2. The Ψ_x rose approximately 1 MPa in red willow, elderberry and white ash, and 2 MPa in bay laurel and glossy privet. The relaxed Ψ_x was approximately -0.5 MPa for all species except bay laurel, whose relaxed Ψ_x was -0.9 MPa (Fig. 1). We also did not find any difference in PLC between TW and DS treatments, contrary to tension-cutting artefact predictions (Fig. 1).

Although our relaxed samples did not become fully hydrated (0 MPa) for the experiments described earlier, our results cannot be explained by inadequate relaxation because our R_p treatment, which was used to eliminate the tension in the xylem for glossy privet, also did not result in significant differences in PLC (Fig. 2). In this treatment, leaves had significantly greater Ψ_x than our R treatment (compare Figs 1d & 2a). Seven relaxed leaves (out of 14) had ≥ 0 MPa as a Ψ_x and exuded water after excision. In spite of the xylem being under positive pressure, there were no PLC differences between native and R_p (Fig. 2b). Therefore, these results are also contrary to the tension-cutting hypothesis for which a higher PLC for native treatment is predicted.

Species	n	Stem diameter (mm)			Maximum vessel length (cm)		
		Mean \pm SE	Min	Max	Mean \pm SE	Min	Max
Red willow	20	4.0 \pm 0.1	2.9	4.7	40.8 \pm 5.3	14.6	99.6
Bay laurel	16	5.0 \pm 0.2	3.7	6.9	61.2 \pm 3.7	34.9	82.0
Glossy privet	11	4.2 \pm 0.2	3.2	5.9	77.5 \pm 6.7	51.3	124.7
Elderberry	17	5.3 \pm 0.1	4.3	6.4	135.8 \pm 14.9	15.4	222.0
White ash	12	5.1 \pm 0.2	4.1	6.5	153.5 \pm 11.3	91.7	215.0

Stem diameter (including bark) was measured at the distal end of the branch where it was injected with nitrogen.
SE, standard error.

Bay laurel was the only species that showed a significant difference in PLC for the relaxed and native treatments (Fig. 1c). To test if this difference was due to the tension-cutting artefact or because of refilling, we performed a girdling treatment. Relaxed PLC was significantly lower than both native and girdled PLCs (Fig. 3b). Moreover, native and girdled PLCs did not differ (Fig. 3b). The Ψ_x of native branches was -2.2 MPa, and significantly increased to -0.5 MPa for relaxed and girdled branches (Fig. 3a). The girdled results follow the pattern of the refilling predictions and not tension-cutting artefact predictions.

DISCUSSION

Our results do not support the existence of a tension-cutting artefact. We found no evidence for tension-cutting artefact in any of the five species that we examined. Our experiment included one species, white ash, which Wheeler *et al.* (2013) reported to display evidence of the tension-cutting artefact; however, we did not find evidence for this artefact in this species.

Of the four species examined in Wheeler *et al.* (2013), they did not observe the tension-cutting artefact in paper birch (*Betula papyrifera* Marsh.) and they hypothesized that this may be due to limited spread of artefact-related emboli because of scalariform perforation plates. They also hypothesized, based on the species in which they observed the artefact, that species with long vessels were more prone to the artefact. In designing our experiments, we intended to test these hypotheses; however, we started our experiments by sampling species that should have been most prone to the artefact, that is, those with long vessels and simple perforation plates. We did not further evaluate short-vesselled species or species with scalariform perforation plates because we did not find evidence for the tension-cutting artefact among long-vesselled species predicted to be most susceptible to this potential artefact.

Some of the results of Wheeler *et al.* (2013) may be explained by the refilling of conduits within their relaxed samples. While we relaxed samples with their cut ends in water for 5–10 min, in most of their experiments Wheeler *et al.* (2013) relaxed them for 30 min or longer and this could have led to refilling. We chose not to relax for this long because we wanted to avoid refilling and our shorter relaxa-

Table 1. Maximum vessel length of stems collected from the same plants as those used for percentage loss in conductivity (PLC) measurements

tion times were highly effective. It should be noted that Wheeler *et al.* (2013) also tested a ‘rapid’ (<2 min) relaxation of samples for two species, where they also found a difference between native and relaxed samples. It is possible that the cutting of samples without further trimming (described later) contributed to this difference.

Wheeler *et al.* (2013) argued that cutting large branches retarded phloem function, thus phloem involvement in refilling was unlikely within large collected branches. We only observed refilling in bay laurel, which was significantly mitigated by girdling, suggesting the phloem was involved in the refilling response and that it may occur in large cut branches like those we sampled. It also suggests that this refilling can happen quite rapidly (Trifilò *et al.* 2003). Our results are consistent with those reported by Trifilò *et al.* (2014) who also reported evidence of refilling in bay laurel when stems were not girdled or metabolically blocked with chemicals.

It may be possible that the higher PLC in girdled samples compared with the PLC of relaxed samples was due to cavitation induced by girdling. However, this does not seem the most parsimonious explanation as girdled PLC was not different from native PLC in our study and a previous study (Trifilò *et al.* 2014) and because of two additional experiments conducted by Trifilò *et al.* (2014) on *L. nobilis*. In their study, Trifilò *et al.* (2014) used sodium orthovanadate to chemically retard the refilling process in relaxed stems, which resulted in PLCs not different from stems cut under tension. Moreover, they compared PLCs of girdled and ungirdled stems sampled at native tensions and found that they were not significantly different (see Fig. 2 of Trifilò *et al.* 2014). If girdling was inducing cavitation, then the PLC of girdled samples would have been greater than ungirdled samples when sampled under tension; however, cavitation induced by girdling may be an alternative explanation for our results.

Novel refilling does not occur in all species, thus refilling is an unlikely explanation for all of the differences between relaxed and non-relaxed stems reported by Wheeler *et al.* (2013). Instead, the differences are likely due to sampling methods. Wheeler *et al.* (2013) cut non-relaxed stems to exactly 14 cm and only trimmed off micrometre thin sections from the cut ends with razor blades following initial cuts. By contrast, in our study, we cut native stem segments that were 1–3 cm longer than their eventual measurement length and

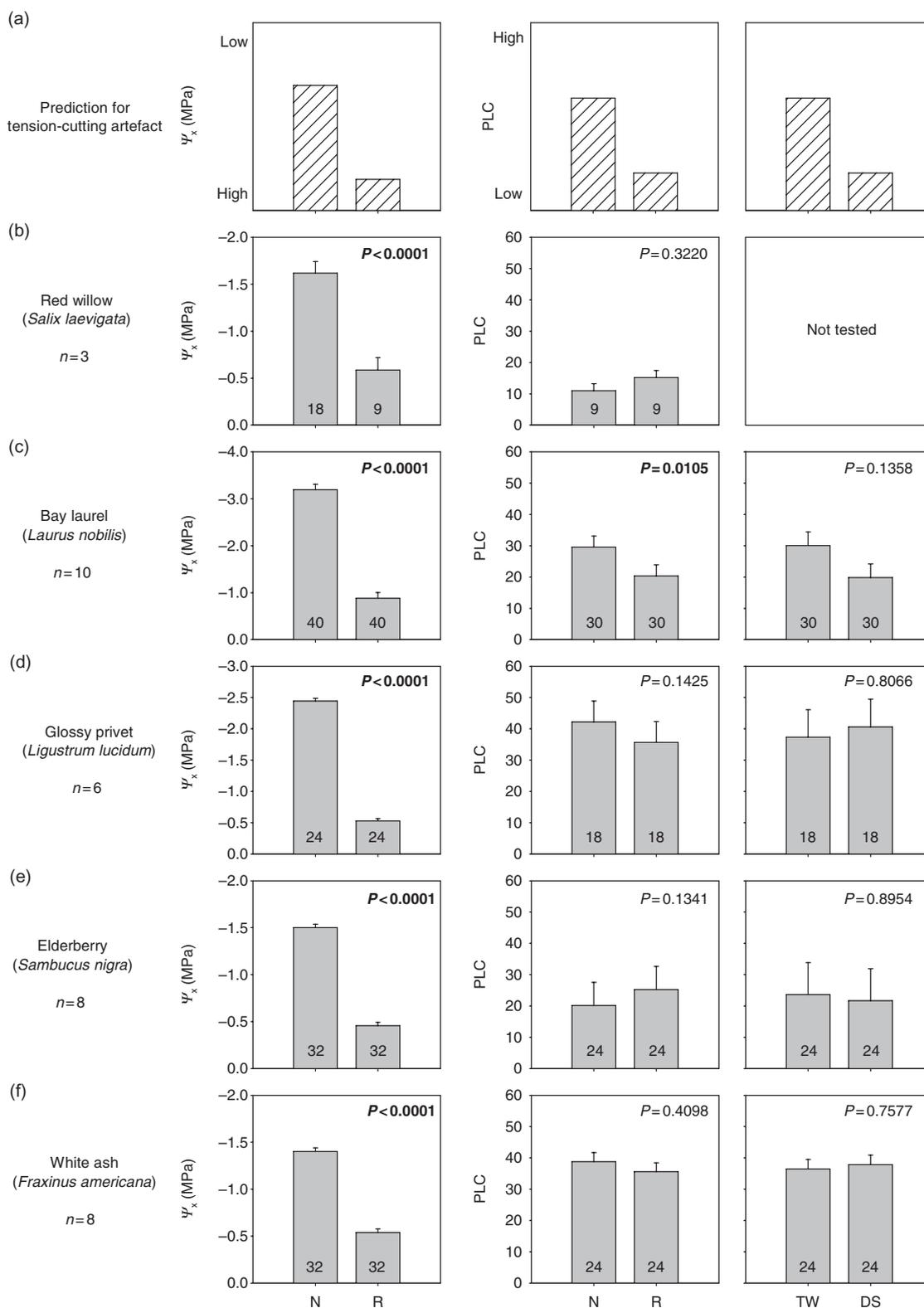


Figure 1. Water potential (Ψ_x) of native (N) and relaxed (R) branches, percentage loss of conductivity (PLC) for samples excised under native (N) or relaxed (R) tension, and PLC of samples excised under tap water (TW) or filtered (0.2 μ m) degassed 20 mM KCl solution (DS). Predictions for tension-cutting artefact (a) and least square means \pm 1 standard error for the five studied species (b, c, d, e, f). Species are arranged from the species with the shortest vessels (b) to the longest (f), and maximum vessel lengths for each species are included in Table 1. Under each species name the number (*n*) of independent replicates (large branches) are reported. The number within each box is the number of subsamples (observations) per treatment. The statistical significance of differences between treatments is reported within each panel (*P*-value).

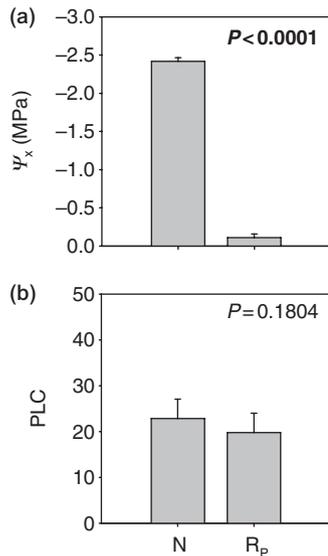


Figure 2. Glossy privet (*Ligustrum lucidum*) water potential (Ψ_x) and percentage loss of conductivity (PLC) for stem samples excised under native tension (N) and positive pressure (R_p). Least square means ± 1 standard error and significance of differences between treatments are reported. Results obtained from four independent replicates (large branches) and 12 subsamples (observations) per treatment.

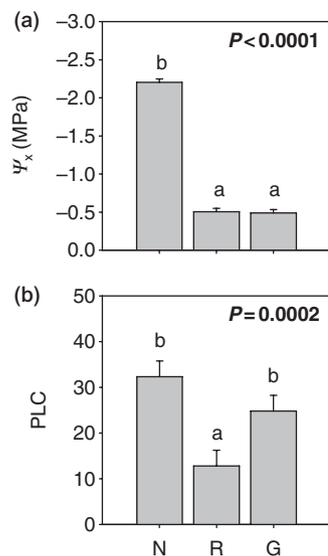


Figure 3. Bay laurel (*Laurus nobilis*) water potential (Ψ_x) and percentage loss of conductivity (PLC) for stem samples excised under native tension (N), after tension being relaxed (R), and after multiple-girdling and relaxation (G). Least square means ± 1 standard error and significance of differences between treatments are reported. Results obtained from 15 independent replicates (large branches), with 15 subsamples (observations) per treatment level. Bars with the same letters are not significantly different at 95% confidence level (Tukey's honestly significant difference homogeneous groups).

then used fresh razor blades to trim off between 0.5 and 1.5 cm from each stem end after the initial cut prior to conductivity measurements. Excising stems at native tensions underwater could lead to a small volume of non-degassed water and microbubbles being sucked into the stem xylem vessels during the first cut. The amount of water taken in would depend on the tension of the stem and on the volume of discharged capacitance in the tissues fed by the xylem. Larger and greater quantities of microbubbles could travel farther into stems with xylem that has longer vessels and simple perforation plates. As Wheeler *et al.* (2013) only shaved a few micrometres off sample ends, any microbubbles that were taken up during cutting remained and would have led to elevated losses in conductivity, especially for longer-vesselled species. In cases where tension-cutting effects were not observed, in short-vesselled species and those with scalariform perforation plates, shaving off only micrometres was apparently sufficient to remove blockages. By contrast, in our study, shaving off greater amounts of xylem 0.5–1.5 cm removed the microbubbles and avoided a tension-cutting artefact even in our long-vesselled species.

The tension-cutting artefact described by Wheeler *et al.* (2013) is not likely to have broad implications for previous studies of native embolism as has been suggested in several papers (Wheeler *et al.* 2013; Delzon & Cochard 2014; Rockwell *et al.* 2014). It is more time consuming to cut a stem from a plant underwater to a precise length in the field, which would limit that number of stems that could be sampled for a given time point. Also, we have always been concerned that our initial cut could lead to xylem damage or cavitation as described by Wheeler *et al.* (2013). Because it is more efficient and safer to initially excise an unmeasured longer segment and then to subsequently trim to size underwater, we and colleagues have usually sampled in this manner (e.g. Davis *et al.* 1999b; Ewers *et al.* 2004; Jacobsen *et al.* 2007). Trimming to size, especially with a fresh and sharp razor blade, has the additional advantage of ensuring a clean cut to the xylem. Those studies that have sampled longer branches and subsequently trimmed them to their measurement length would likely have not have been at risk of a tension-cutting artefact.

Another prediction of the hypothesized tension-cutting artefact was that the artefact would be more likely to occur if samples were excised and trimmed in TW, with atmospheric gas concentrations, rather than in a filtered DS (Rockwell *et al.* 2014). The rationale for this was that more nucleation particles or microbubbles that could trigger cavitation are present in TW. However, it is also possible that air on the cutting blade or within the bark, xylem, and pith could be nucleation agents. As many prior studies report excising samples underwater, we specifically tested the effect that this could have on PLC values. Our results show that there are no differences between relaxing, excising and preparing samples in TW or in a DS. Preparing a DS just before sampling is time-consuming, and our results suggest, unnecessary. We did not test if using degassed water eliminates a cutting artefact when samples are cut to their precise size as was done by Wheeler *et al.* (2013).

In summary, when samples are initially cut slightly longer than the measurement segment length we did not find any evidence supporting the tension-cutting artefact described by Wheeler *et al.* (2013). There appears to be little reason to question the validity of studies that report daily changes in PLC or VCs constructed with samples excised under TW at native tension in this manner. Moreover, we caution that branch relaxation, particularly over longer time periods, may lead to vessel refilling, as shown for bay laurel and olive (Trifilò *et al.* 2014; present study). We recommend that branch relaxation not be used or that it should be used cautiously and steps should be taken, such as shorter relaxation times and stem girdling, to ensure that refilling is not occurring in relaxed samples. Finally, we recommend that segments initially excised for native measurements should be at least 1–3 cm longer than the desired measurement length so that more than 0.5 cm are shaved off each end prior to measurement.

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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. Diagram depicting the location of measured stem segments within larger harvested shoots for experiments 1 and 2, in which native and relaxed samples were collected from large forked branches.

Figure S2. Diagram depicting the location of measured stem segments within larger harvested shoots for experiment 3, in which large triple-branched shoots were sampled for examining the effect of harvesting under native, relaxed, and relaxed and girdled conditions.

Figure S3. Diagram showing the experimental set-up and sampling procedure for experiment 4, in which samples were harvested under native and positive pressure relaxation conditions.

Table S1. Diagrams of the models to which the PLC data were fit for each experiment.