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Does sample length influence the shape of xylem embolism vulnerability curves? A test with the Cavitron spinning technique

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ABSTRACT

The Cavitron spinning technique is used to construct xylem embolism vulnerability curves (VCs), but its reliability has been questioned for species with long vessels. This technique generates two types of VC: sigmoid 's'-shaped and exponential, levelling-off 'r'-shaped curves. We tested the hypothesis that 'r'-shaped VCs were anomalous and caused by the presence of vessels cut open during sample preparation. A Cavitron apparatus was used to construct VCs from samples of different lengths in species with contrasting vessel lengths. The results were compared with VCs obtained using other independent techniques. When vessel length exceeded sample length, VCs were 'r'-shaped and anomalous. Filling vessels cut open at both ends with air before measurement produced more typical 's'-shaped VCs. We also found that exposing segments of 11 woody species in a Cavitron at the pressure measured *in planta* before sampling considerably increased the degree of embolism above the native state level for species with long vessels. We concluded that open vessels were abnormally more vulnerable to cavitation than intact vessels. We recommend restricting this technique to species with short conduits. The relevance of our conclusions for other spinning techniques is discussed.

Key-words: cavitation; Cavitron; centrifugation; embolism; technique; xylem anatomy; xylem physiology.

INTRODUCTION

Xylem sap is transported from the soil to the leaf under negative pressures. This metastable state is conducive to cavitation events, that is, sudden transitions to a stable vapour phase (Tyree & Zimmermann 2002). Cavitation ruptures the hydraulic continuum in the xylem conduits, which impairs leaf water supply and may eventually cause plant death (Tyree *et al*. 1994; Brodribb & Cochard 2009). Cavitation resistance is now considered as one of the most significant physiological traits involved in stomatal function (Jones & Sutherland 1991; Cochard 2002a), plant drought resistance (Hacke & Sperry 2001; Brodribb & Cochard 2009) and species distribution (Pockman & Sperry 2000; Kursar *et al*. 2009). Much effort is therefore being devoted to the identification of the precise mechanisms and fine structures responsible for cavitation in plants (Domec, Lachenbruch & Meinzer 2006; Choat, Cobb & Jansen 2008; Christman, Sperry & Adler 2009). In addition, with the development of time-saving centrifugation techniques, this trait has been proposed as a relevant criterion for the identification or screening of trees for extreme drought tolerance (Cochard, Casella & Mencuccini 2007; Cochard *et al*. 2008). The efficacy of such identification is partly determined by the reliability and accuracy of the techniques used to detect cavitation.

Vulnerability curves (VCs) are used to quantify xylem susceptibility to cavitation events. These curves are plots of xylem pressures versus an estimator of the degree of cavitation in the xylem conduits. The best and most relevant estimator of cavitation is the degree of embolism, that is, the amount of blockage to the sap flow caused by the presence of air in the conduits. Sperry, Donnelly & Tyree (1988) have developed a technique to estimate embolism based on the measure of the loss of hydraulic conductance in the xylem. They have dehydrated large cut branches and measured how the level of embolism increases with water stress. VCs obtained with this technique typically have a sigmoid shape (Fig. 1), hereafter called type 's'. In these VCs, cavitation occurs only when the xylem pressure falls below a critical value. This defines a 'safe' range of xylem pressures where cavitation does not occur. This physiological range has been found to correspond closely to the range of xylem pressures that species typically experience in their natural habitats (Hacke & Sperry 2001).

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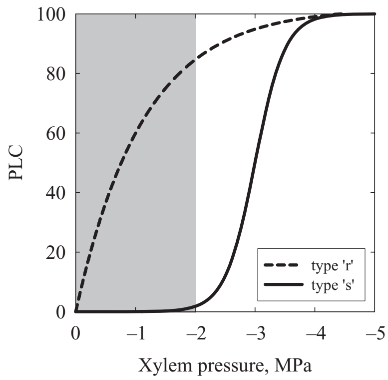

Figure 1. Schematic representations of xylem vulnerability curves showing the changes in segment loss of conductivity (PLC) versus pressure. The curves can be classified in two groups: 's'-shaped curves (solid line) are sigmoid, while 'r'-shaped curves (dashed line) show an exponential rise and levelling-off. A major difference between these two types of curve is that sigmoid curves display a 'safe' range of pressure (grey zone) where PLC values remain very low. We argue here that 'r'-type curves are anomalies produced by the centrifuge technique when samples contain open vessels.

The validity of this technique has been challenged by direct observations of xylem content (e.g. Canny 1997) and direct estimates of sap tension (e.g. Zimmermann *et al.* 1994). On the basis of their experiments, these authors concluded that xylem conduits were much more vulnerable to cavitation than predicted from these VCs. However, careful examination of the methods employed in these studies points to major technical artefacts (Sperry *et al.* 1996; Wei, Tyree & Steudle 1999; Cochard *et al.* 2000).

The technique introduced by Sperry *et al.* (1988) is reliable but time-consuming. Efforts have therefore been made to develop faster methods for constructing VCs. Today, the two most frequently employed methods are air injection and centrifugation. The air injection technique (Cochard, Cruiziat & Tyree 1992b; Salleo *et al.* 1992) consists in injecting compressed air into a pressure sleeve covering a portion of a cut xylem segment, forcing air into the xylem conduits. The change of sample conductance with increasing air pressure allows the sample VC to be constructed (Cochard *et al.* 1992b). Centrifugation techniques use the centrifugal force to lower the xylem pressure in the conduits of a cut segment. Again, the variation of conductance with pressure yields a VC. The different spinning techniques differ slightly in the way VCs are constructed. For instance, Pockman, Sperry & O'Leary (1995) and Alder *et al.* (1997) have used the centrifuge to expose segments successively to negative pressures and measured the impact on segment conductance with a low pressure flow meter. Cochard (2002b) and Cochard *et al.* (2005) have modified the technique so that the segment conductance is determined during centrifugation, which substantially speeds up the procedure. A noteworthy distinction between our 'Cavitron' and previous spinning methods is that water flows through the segment during centrifugation.

When the air injection or centrifugation techniques are employed, VCs occasionally show an exponential rise and levelling-off, hereafter termed 'r'-shaped (Fig. 1). This type of curve is apparently not found in conifer species (Cochard 2006) but most often in species with long vessels (Cochard *et al.* 2005; Choat *et al.* 2010). Species with 'r'-type VCs have no 'safe' zone because cavitation occurs as soon as the xylem pressure falls below the atmospheric value. In other words, species with 'r'-type curves must experience frequent and cyclic cavitation events. As a corollary, they must be able to refill their conduits overnight with water when sap pressure is less negative. The mechanism by which xylem can refill under negative pressure may involve plant metabolism (Salleo *et al.* 2009), but remains largely unknown (Holbrook & Zwieniecki 1999). This behaviour could be beneficial to the plant, as the water freed by cavitation could transiently improve plant water status. However, this effect is probably marginal (Hölttä *et al.* 2009).

We recently speculated that 'r'-type VCs obtained with the Cavitron technique were anomalies caused by the presence of conduits cut open in the xylem segments (Cochard *et al.* 2005). The objective of this study was to seek experimental support for this hypothesis. Accordingly, we analysed the VCs obtained from samples of different lengths on four species with contrasting conduit lengths. We hypothesized that species with long vessels should exhibit more pronounced 'r'-shaped curves as well as VCs constructed with shorter segments. We tested also this hypothesis by constructing VCs with samples that had only intact xylem conduits, for which we predicted an 's' shape. Finally, we compared the native degree of embolism measured *in planta* at midday when the xylem pressure is the most negative to the degree of embolism induced by centrifugation at the same prevailing pressure. We hypothesized that the level of embolism greatly increased for species with long vessels. The results of our experiments largely support these hypotheses.

MATERIALS AND METHODS

Plant material

Experiments were performed on different tree species from the INRA-Crouël campus in Clermont-Ferrand (central France). Most of the experiments were conducted on four species with contrasting xylem anatomies and vessel lengths. Oak (*Quercus robur* L.) is a ring-porous species with very long vessels. Birch (*Betula pendula* Roth) is a diffuse-porous species with very short vessels. Peach (*Prunus persica* (L.) Batsch) has vessels of intermediate length. Scots pine (*Pinus sylvestris* L.) is a coniferous species with tracheids. For these species, the experiments were conducted on non-ramified terminal shoots less than 3 years old. Shoots longer than maximum vessel length were cut in the morning and brought to the laboratory where they were analysed the same day. For the 'native state' experiments, we selected 11 woody angiosperms with widely different xylem anatomies: *B. pendula* Roth, *Carpinus betulus* L., *Castanea sativa* Mill., *Clematis vitalba* L., *Fraxinus excelsior* L., *Juglans regia* L., *Ligustrum vulgare* L., *Olea europaea* L., *P. persica* (L.) Batsch, *Robinia pseudacacia* L. and *Vitis vinifera* L.

Maximum length of xylem vessels

We used the air infiltration technique (Zimmermann & Jeje 1981; Ewers & Fisher 1989) to measure maximum vessel length. Briefly, long samples ($n = 6$) were cut in the air and infiltrated with compressed air (0.15 MPa) at about 20 cm from their shoot apex. Their basal end was immersed in water. The stem was successively shortened by 0.02 m at its base until bubbling was observed. Because compressed air cannot pass through vessel end walls, this bubbling indicated that a vessel was cut open at both ends. The maximum vessel length was defined as the remaining shoot length.

Centrifugation-induced cavitation

We used the 'Cavitron' technique (Cochard 2002b; Cochard *et al.* 2005) to construct xylem VCs. This technique is derived from the centrifuge method first proposed by Pockman *et al.* (1995). In short, the technique consists in spinning xylem segments centred on a dedicated rotor to expose them to large negative pressures and then measure the effect on their hydraulic conductivity *K*. The Cavitron differs from previous spinning techniques by the fact that *K* is measured during centrifugation. Both sample ends are inserted in transparent vials containing ultrapure water with 10 mM KCl and 1 mM $CaCl_2$ filtered to 0.2 μm. We recently established that the presence of ions in the solution had no influence on species VCs (Cochard *et al.* 2010). The solution in the vials was degassed at the start of the measurement but remained in contact with the atmosphere during centrifugation. The water level in the vial where the basal segment end was inserted was adjusted to 1.5 cm before each *K* determination. The water level in the other vial was constant and set to 1 cm. The difference in water levels creates a pressure gradient, and hence a water flow through the sample, which allows *K* to be estimated (see Cochard 2002b and Cochard *et al.* 2005 for more details on this technique). The maximum sample conductivity (K_{max}) was measured at low speed and high pressure (–0.1 or –0.25 MPa). Dividing K_{max} by sample basal wood area yielded sample specific conductivity. Xylem pressure was then lowered stepwise by increasing the rotational velocity, and *K* was determined anew. Sample loss of conductivity [sample percent loss of conductivity (PLC)] was computed as:

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

We used three rotors with different sizes (diameter 0.2, 0.3 and 0.4 m) to obtain VCs on xylem segments of various lengths (0.175, 0.275 and 0.375 m, respectively). It was not possible to expose 0.175-m-long segments to pressures below –2.5 MPa as the maximum rotational velocity of our Cavitron was limited to 8500 rpm. The basal segment end was inserted in the upstream reservoir of the Cavitron for the water to flow in the natural direction through the sample.

Shoot segments were prepared in two different ways (Fig. 2). In the first case (control, Fig. 2a), the segments ($n = 4$) were cut from the branch under tap water to the desired length and rapidly placed in the Cavitron. This prevented air from entering the cut vessels at both sample ends (see Results). The samples were not flushed with water before measuring K_{max}. In the second case ($n = 4$), the segments were cut in the air and successively infiltrated with compressed air (0.15 MPa) at each end until sap stopped flowing out of the segment at the other end. This had the effect of emptying all the lumens of the vessels that were cut open at each end (see Results, Fig. 2b). The pressure causing

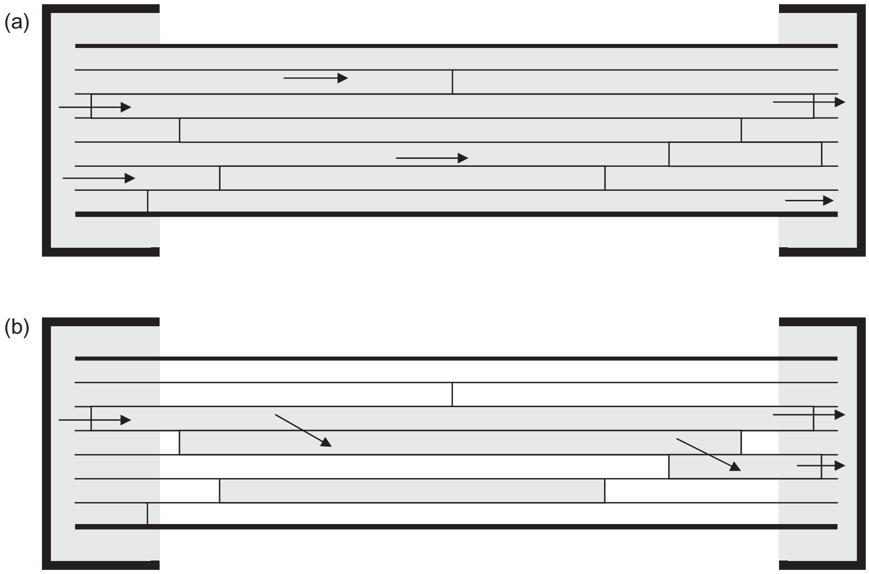

Figure 2. Hypothetic xylem sap pathways in centrifuged xylem segments. The upstream reservoir is on the left. Sample (a) represents a control sample cut under water. All the vessels are filled with sap and conduct water. Sample (b) was infiltrated with air at both ends before centrifugation. This filled the lumens of all the cut open vessels with air. During centrifugation, water flows only through the intact vessels.

50% loss of conductivity (P_{50}) was computed graphically to compare the results of the different treatments. For *Pinus*, a tracheid-bearing species, this treatment was irrelevant.

To gain more insight into this centrifugation technique, we measured the amount of embolism in different portions of samples treated with the centrifuge method. We used 0.275-m-long peach segments for this set of experiments. Control samples ($n = 3$ for each treatment), cut under water, were prepared as described above and exposed for 10 min at –0.2, –3 or –5 MPa. We also exposed samples infiltrated with air at both ends before centrifuging them for 10 min at –0.2 MPa ($n = 3$). After high-speed centrifugation, the rotational velocity was reduced to expose the samples for 10 min more at –0.2 MPa to relax xylem pressure. The samples were then rapidly removed from the Cavitron and immersed in tap water. This procedure prevented air from entering at both sample ends (see Results, Fig. 5). Five 2-cm-long segments were cut under water at regular intervals on the shoots, and immediately connected to a Xyl'em apparatus (Bronkhorst, Montigny-les-Cormeilles, France). This apparatus measures the percentage loss of hydraulic conductance due to the presence of air-filled conduits in the segments (Cochard *et al*. 2002). We also perfused with a 1 g L^{-1} safranine solution 5-cm-long segments cut under water from the middle part of samples air infiltrated and centrifuged for 10 min at –0.2 MPa ($n = 5$). On cross sections, we measured the diameter distribution of the stained (i.e. conductive) and unstained vessels.

Reference xylem VCs

Reference VCs based on the original bench drying technique (Sperry *et al*. 1988) had been obtained in the past by our group on different plant materials for *Q. robur* (Cochard *et al*. 1992a) *B. pendula* (Cochard *et al*. 2005) and *P. sylvestris* (Cochard 1992). The reference VC for *P. persica* was constructed for this study. Shoots 1 m long were cut from different trees early in the morning and dehydrated on a bench to obtain a range of xylem pressures from –0.6 to –5.7 MPa. Xylem pressure was measured with a pressure chamber on bagged leaves. The percent loss of xylem conductance due to embolism was measured with a Xyl'em apparatus on shoot internodes as described before. In all, 27 different shoots were used to construct the VC.

'Native state' experiment

The objective of this experiment was to compare, on a large panel of woody species, the native states of embolism (PLC_{native}) and xylem pressure (P) with the PLC_{centri} values obtained after centrifugation at the same xylem pressure. Shoots longer than the maximum vessel length were collected in the field during August and September at midday and immediately enclosed in a black plastic bag to stop transpiration and allow leaf water potentials to equilibrate with xylem pressures. After 1 h, P was measured with a pressure chamber on 2–3 leaves per shoot. For most species, P typically ranged between –1 and –1.5 MPa. Segments 0.275 m long were then cut under water from different current-year terminal parts of each shoot. The segments were randomly allocated to three different treatments. On a first set of segments ($n > 4$), we measured the PLC_{native} values on two consecutive internodes cut under water from the middle of each segment. The second set of segments ($n > 4$) was infiltrated with compressed air (0.15 MPa) at both ends as described before. The PLC_{air} values in the middle of each segment were then determined as above. The last set of segments ($n > 4$) was treated by centrifugation. The segment ends were inserted in two similar reservoirs containing a constant level of water (1 cm) and the segment was rapidly placed in the Cavitron. The central part of the segment was then exposed for 5 min at the native pressure previously determined for each species. As the water level was similar in both reservoirs, no water flowed through the segment during centrifugation. The xylem pressure was then raised to –0.2 MPa for 3 min and the Cavitron stopped to relax the xylem pressure. The segment was finally removed from the centrifuge, rapidly immersed in water, and the PLC_{centri} values were determined in the central part of the segment as already described.

RESULTS

Maximum vessel length

Vessel length differed considerably across species. Oak had the longest vessels [1.34 m standard deviation (SD) = 0.38, $n = 6$] and birch the shortest (0.16 m SD = 0.04, $n = 6$). The other species studied had intermediate vessel lengths (see Fig. 7).

VCs of control samples

Figure 3 (closed symbols) shows the VCs established with the centrifuge technique on control samples (i.e. cut under water) of various lengths. For *Pinus*, a species with tracheids, and *Betula*, a species with very short vessels, all curves had an 's' shape and sample length had no visible effect on VCs. In *Prunus*, a species with intermediate vessel length, sample length very strongly influenced the shape and the P_{50} of the VCs. Short samples showed 'r'-type curves with very high P_{50} values (–1 MPa), whereas long segments produced more 's'-shaped curves with much more negative P_{50} values (–4.5 MPa). In *Quercus*, a ring-porous species with very long vessels, sample length had a moderate impact on VCs. All VCs were strongly 'r'-shaped, with high P_{50} values (above –1 MPa).

VCs of air-infiltrated samples

Infiltrating air at low pressure in the sample ends before establishing VCs significantly decreased sample hydraulic conductivity (Fig. 4) for all the species ($P < 0.01$). The reduction was more pronounced on species with long

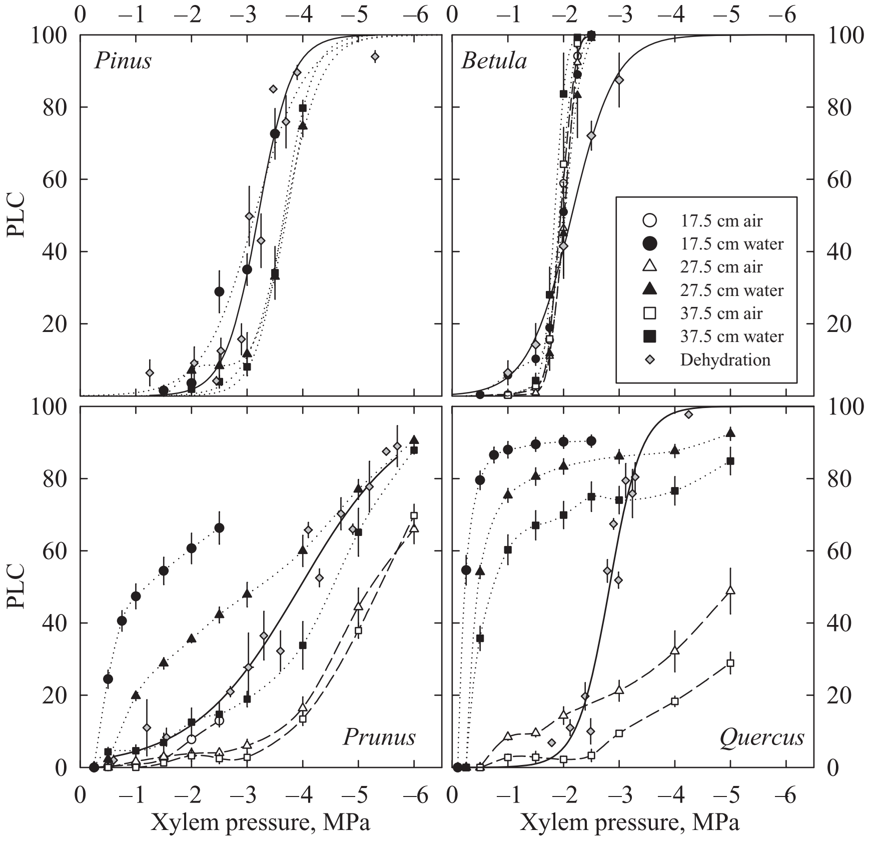

Figure 3. Xylem vulnerability to cavitation of four species plotted with different techniques. Closed symbols represent samples of various lengths (different symbols) cut under water and centrifuged with the Cavitron technique. Open symbols are for samples treated similarly but infiltrated with air at both ends before centrifugation. The grey symbols show vulnerability curves obtained with the reference bench dehydration technique. Error bars represent one SE ($n = 4$).

vessels. It was not possible to determine the VC of 0.175-m-long oak segments because the flow through the segments was not detectable with our technique. Air injection had no effect on birch VCs, whatever the length of the samples (Fig. 3, open symbols). In peach, injecting air strongly reduced the P_{50} values (c. –5.2 MPa), and VCs for 0.275 m and 0.375 m segments were very similar and 's'-shaped. We could not construct the entire curve for 0.175-m-long segments for this species. But at –2.5 MPa, the loss of conductivity was only 10%. In oak, this treatment yielded very flattened 's'-shaped curves, with P_{50} values below –5 MPa. For the longest segments, PLC values increased only for pressures below –2.5 MPa.

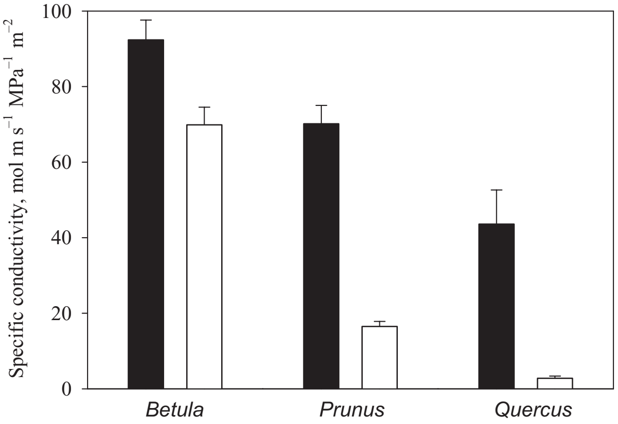

Figure 4. Initial specific hydraulic conductivity of 0.275-m-long xylem segments cut under water (black bars) or infiltrated with air at both ends (open bars). Error bars represent one SE ($n = 4$).

Reference VCs

The reference VCs obtained by the bench dehydration technique are shown with grey symbols on Fig. 3. All the species gave 's'-type curves. P_{50} values ranged widely across species (from –2.1 MPa for *Betula* to –3.9 MPa for *Prunus*).

Embolism formation in centrifuged samples

When 0.275 m control peach samples were cut under water and centrifuged at –0.2 MPa, the degree of embolism was less than 5% in all parts of the segment (Fig. 5). By contrast, control segments exposed to –3 MPa showed high levels of embolism mainly in the basal and upstream end. Exposing control segments to –5 MPa further increased the PLC only in the central part of the segments. Air infiltration at both sample ends before centrifugation at –0.2 MPa caused high levels of embolism near both ends, but 80% of the vessels remained conductive in the middle of the sample. However, dye staining experiments suggested that unstained vessels tended to have larger diameters (Fig. 6).

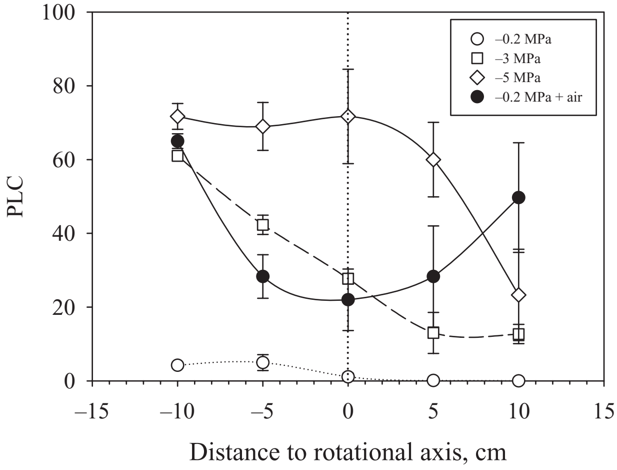

Figure 5. Loss of conductivity due to embolism in the different portions of 0.275-m-long peach segments exposed to centrifugation. The segments were cut under water and spun at –0.2 MPa (open circles), –3 MPa (open squares) and –5 MPa (open diamonds), or infiltrated with air and spun at –0.2 MPa (closed circles). The arrow indicates the direction of the water flow during centrifugation. Error bars represent one SE ($n = 3$).

Native state experiment

Across species, the native pressure measured at midday under field conditions ranged from –1 to –2 MPa and the corresponding native level of embolism was found to vary between 2 and 27% (Fig. 7a,b). PLC_{native} was not correlated with vessel length across species ($R^2 = 0.002$; $P = 0.9$). The air injection and centrifugation treatments significantly increased the PLC values in the middle of the segments of all the species ($P < 0.05$) except for *Betula*, *Carpinus* and *Juglans*, three species with very short vessels. A significant positive correlation was found between the PLC_{air} increase and species vessel length ($R^2 = 0.4$; $P = 0.03$) (Fig. 8b).

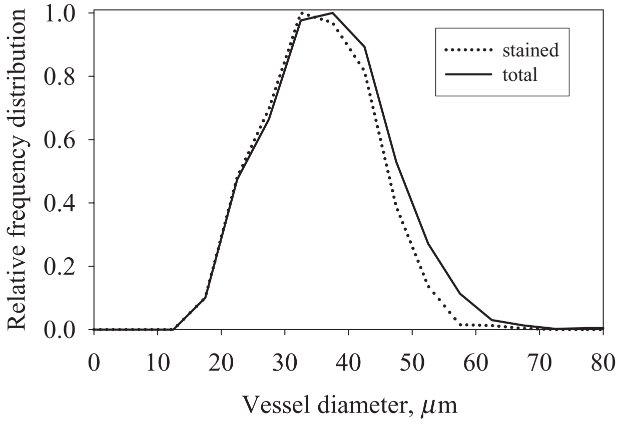

Figure 6. Relative vessel diameter frequency distribution in the middle of 0.275-m-long peach samples infiltrated with air at both ends and centrifuged at –0.2 MPa. The plain curve is the distribution for all the vessels. The dotted curve represents the distribution for the vessels stained with safranine, that is, that remained conductive after air infiltration.

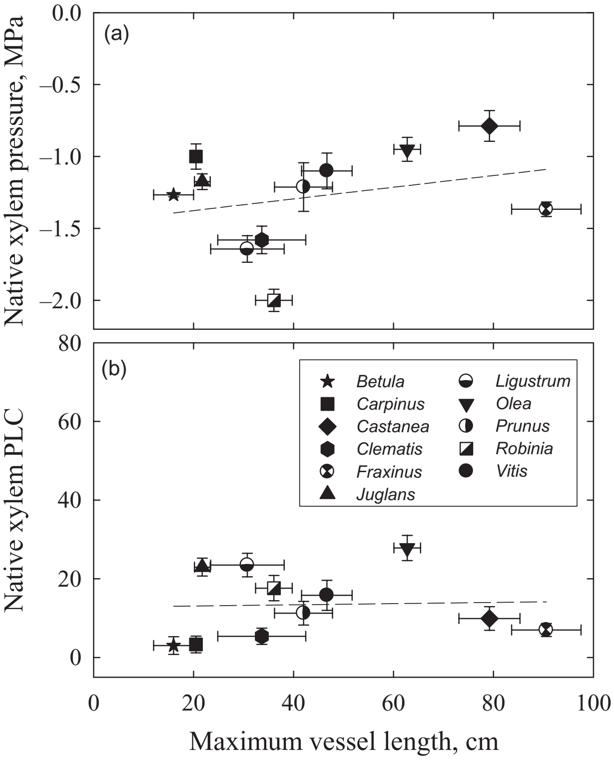

Figure 7. Relations between species maximum vessel length and midday xylem pressure (a), and midday native state of xylem embolism (b) for a panel of species with contrasting vessel lengths. Error bars represent one SE ($n > 4$), and dashed lines are near linear regressions through the data.

Similarly, the increase in PLC_{centri} values was significantly correlated with vessel length (Fig. 8a; $R^2 = 0.5$; $P = 0.02$). A very strong, close to 1:1 correlation ($R^2 = 0.9$; $P < 0.0001$) was found between the increase in PLC_{air} and PLC_{centri} values. In other words, the centrifugation and air injection treatments caused similar changes in the degree of xylem embolism in the middle of the segments.

DISCUSSION

The centrifuge technique we have used in this study produced very different VCs when sample length was varied, or when samples were measured intact or infiltrated with air at both ends before centrifugation. In addition, the results ranged widely across species according to their xylem anatomies. These findings shed light on the reliability of the technique. We will argue here that this Cavitron technique appropriately measures the vulnerability of only intact xylem vessels, that is, vessels whose two ends are included in the spun segment.

Xylem VCs for *B. pendula* (Cochard *et al.* 2005; Barigah *et al.* 2006), *P. sylvestris* (Cochard 1992) and *Q. robur* (Cochard *et al.* 1992a; Bréda *et al.* 1993) have already been obtained with the field, bench or pressure-bomb dehydration techniques. These techniques have proved very robust and consistent, and we can consider these VCs as true

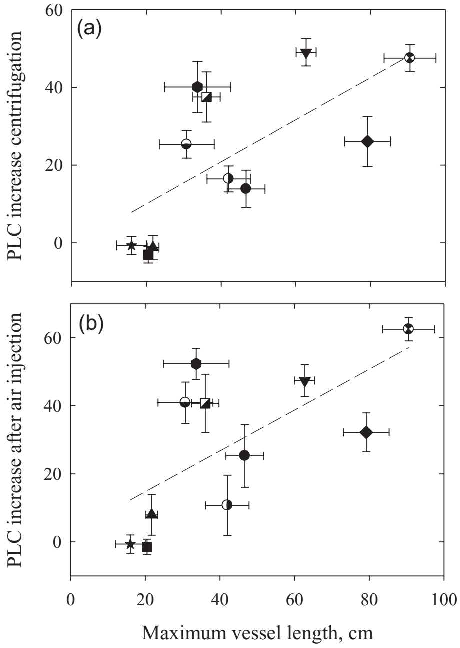

Figure 8. Increase in losses of xylem conductance in the central portions of 0.275-m-long segments of different species after centrifugation at the pressure previously measured *in planta* and after injection of air at low pressure at both segment ends. Error bars represent one SE ($n > 4$) and dashed lines are near linear regressions through the data. Same symbols as in Fig. 7.

physiological curves. The curves obtained with these techniques have a typical sigmoid shape, and the VC for *Prunus* we found in this study confirms this finding. Such VCs are highly consistent with other physiological parameters, such as the variation of xylem pressure and stomatal conductance during drought (Bréda *et al.* 1993; Cochard, Bréda & Granier 1996; Cochard *et al.* 2002). The native state survey of embolism we have conducted here also confirms that midday embolism remains low (<30%) in the xylem of the current-year growth near the end of a relatively wet summer. These findings clearly contrast with the results of the centrifuge technique.

VCs can vary substantially within species among individuals growing under different environmental conditions (Cochard, Lemoine & Dreyer 1999; Martínez-Vilalta *et al.* 2009; Awad *et al.* 2010). This may explain slightly different VCs across techniques for *Pinus* and *Betula*, as the experiments were not conducted on the same plant material. However, the differences were so pronounced for *Quercus* and the shapes of the curves were so different that other explanations are needed to account for these discrepancies for this long-vessel species.

The first experimental evidence that substantiates our hypothesis of a vessel-length artefact is the finding that VCs obtained with our centrifugation technique agreed with reference VCs when segments were longer than the maximum vessel length. For *Betula* and *Pinus*, the xylem conduits were very short and all the samples we measured were longer than conduit length. Therefore, sample length had no effect on VCs, and so, all these VCs were 's'-shaped and in close agreement with the reference VC. In line with this, conifer species with very short tracheids always display 's'-shaped VCs (Cochard 2006). For oak, all the samples were much shorter than vessel length because vessels are extremely long (1.34 m) in this ring-porous species. Increasing sample length, therefore, changed their VCs only marginally. All the VCs were strongly 'r'-shaped and showed much greater vulnerability than the reference VCs. Considerable embolism occurred as soon as pressure fell below the initial pressure. We can further suspect that substantial embolism occurred between 0 and this initial pressure (–0.1 or –0.25 MPa), explaining why the conductivity of control oak segments was relatively low compared with that of birch (Fig. 4). Peach was the most instructive species because we were able to establish VCs on samples shorter than maximum vessel length and on samples close to that vessel length. Here, we found that sample length drastically changed the shape and the P_{50} of the VCs. A clear shift from 'r'- to 's'-shaped VCs was noted when sample length approached maximum vessel length. From this first set of experiments, we concluded that 'r'-shaped VCs with high P_{50} were typical of curves obtained on samples shorter than species vessel length. By contrast, samples longer than vessel length give 's'-shaped VCs with much lower and consistent P_{50} values.

We can thus hypothesize that the vulnerability of cut open vessels was abnormally increased when measured with our centrifuge technique. To test this hypothesis, we conducted two experiments. Firstly, we constructed VCs on segments infiltrated with air at low pressure at both ends. This caused all the vessels cut open at both ends to fill with air, as demonstrated by the high distal and proximal PLC values in these samples (Fig. 5; see also Tyree, Cochard & Cruiziat 2003). Hence, only intact vessels whose two ends were included in the sample remained filled with water and could transport water through the segment. In the centrifuge, sample ends are immersed 1 cm under water at supra-atmospheric pressure. This probably refilled the lumens of all the immersed vessels and hence re-established the hydraulic continuity with the vessels with their terminal ends in this portion of the sample (see Fig. 2b). Of course, the longer the vessels, the fewer are re-connected. This explains why the hydraulic conductivity of air-infiltrated samples, compared with non-infiltrated samples, was reduced much more in oak than in birch (Fig. 4). This treatment had no effect in birch, but lowered the P_{50} values very strongly for the two other species. In peach, sample length no longer had any effect, suggesting that this effect was caused by the presence of more numerous open vessels in short segments. VCs obtained with this treatment were actually relatively close to the curve obtained with the longest segments cut under water. Furthermore, for all the species, the onsets of cavitation that could be derived from these air-infiltrated VCs were consistent with reference VCs.

The second line of evidence comes from the pattern of embolism formation inside centrifuged peach segments. Firstly, it is important to note that PLC in samples cut under water and centrifuged at –0.2 MPa showed little embolism, indicating that embolism did not occur during sample manipulation. Xylem pressure during centrifugation is lowest in the middle of the sample and nil at its ends (Cochard *et al*. 2005). Because cavitation is driven by sap tension, we expected to find more embolisms close to the axis of rotation. However, we found that embolism at –3 MPa occurred first in the basal and upstream portion of the segment (Fig. 5) where the pressure was highest. The PLC levels in these samples were actually similar to those caused by air infiltration at the cut ends. This suggests that the effect of centrifuging control peach segments at –3 MPa was to empty the vessels cut open in the basal portion of the segment while leaving most of the vessels in the other parts of the segments filled with water. When the same analysis was conducted on a segment centrifuged at –5 MPa, that is, below the presumed point of 'true' cavitation, we found that embolism was significantly increased only in the central part of the segment, as expected.

The 'native state' experiment provided a final line of evidence in support of our hypothesis. All the species we evaluated in this survey had a low native state of embolism at midday, although the prevailing xylem pressure was relatively negative (<–1 MPa). In other words, *in planta*, xylem conduits are routinely exposed to negative pressures that do not cause extensive degrees of embolism. By contrast, similar samples exposed to the same negative pressure with a centrifuge exhibited much higher levels of embolism. This increase correlated with the length of the xylem conduits and was very similar to the degree of embolism caused after the injection of air at both ends. We can thus deduce that centrifugation artificially embolizes vessels cut open at both sample ends. Choat *et al*. (2010) recently came to the same conclusion on a study of cavitation in *Vitis*. They measured the PLC and the proportion of air-filled vessels by a non-invasive nuclear magnetic resonance technique in the xylem of dehydrated plants. Their results suggest that cavitation forms *in planta* only at xylem pressures below –2 MPa which contrasted sharply with the VCs obtained with the centrifuge and air-injection techniques on the same material. However, the results of our experiments contrast with those of Li *et al*. (2008) or Taneda & Sperry (2008), who found a close agreement between the native state of embolism and the prediction from VCs. However, it is difficult to compare the two studies because the native PLC values were always low in our studied species and we did not remove this native embolism before experimentation. An artefact associated with a cavitation 'fatigue' problem (Hacke *et al*. 2001) was thus very unlikely in our study.

From this set of experiments, we conclude that the vulnerability of open vessels to cavitation is abnormally high and does not represent their true vulnerability *in planta*. This was clearly an artefact of the centrifuge technique we used in this study. The reason why the open vessels are more vulnerable is still unknown. However, we can speculate that pits located at the vessel ends act as filters that eliminate tiny particles or bubbles that may nucleate cavitation when they migrate near the middle of the stem. Filling all open vessels with air before their insertion in the Cavitron offered a way to obtain more reliable VCs with this technique, although not for oak, where VCs still strongly diverged from the reference VCs. Here, it is probable that air infiltration artificially emptied all the large vessels, and flow occurred only through the few tiny vessels or tracheids that remained conductive. This is consistent with the very low conductivity that we measured after this treatment. Hence, the air infiltration procedure may underestimate vulnerability if it eliminates one category of xylem conduits, longer and larger conduits tending to be more vulnerable in the same xylem segment. For peach, this underestimation was noteworthy because the VCs of air-infiltrated segments yielded more negative P_{50} values than with the reference VC. Consistently, we found that air infiltration changed the diameter distribution of the vessels that remained conductive in the middle of the sample. Thus, it may be advisable to fill open vessels with air before constructing VCs with the Cavitron on diffuse porous species. A measure of the PLC in the middle of the segment or a determination of the diameter distribution of the conductive vessels should be performed to evaluate the validity of such VCs. Finally, we do not recommend the use of this technique with plants bearing long vessels, such as ring-porous or vine species.

The relevance to other centrifuge techniques (e.g. Alder *et al*. 1997) of the criticisms of our Cavitron technique made here is a matter of debate. We have pointed out that one specific feature of the Cavitron method is that water flows through the sample during centrifugation and this may be responsible for the artefact we report in this study. However, we do not think this is the case for two reasons. Firstly, Li *et al*. (2008) have thoroughly compared the Cavitron with another centrifuge technique and clearly established a close agreement between the two methods across many species. For instance, all the techniques consistently produced strongly 'r'-shaped curves with very high P_{50} values for ring-porous species such as oak or ash, in agreement with our observations. The second argument is that when water did not flow through the segment during centrifugation in our experiments (Fig. 7), the results were similar. From this, we can conclude that the principle of the Cavitron technique itself is not responsible for the problem we have identified here. We do not think either that it is linked to a difference in rotor design across laboratories, although this point has not yet been fully explored. Even so, the 'r'-shaped curves and extremely high P_{50} values we obtained here with our Cavitron on ring-porous species are similar to the curves reported by Li *et al*. (2008) for similar species but with another rotor design. The conclusions would be very different if the curves were 'r'-shaped only with our apparatus. When we compared our centrifuge curves with reference VCs obtained with the bench dehydration technique, we concluded that there was a very marked overestimation of xylem vulnerability to cavitation for species with long vessels with the spinning method. Unfortunately, Li *et al*. (2008) did not provide reference VCs for all the species that they evaluated in their study, so no firm conclusion can be drawn. Furthermore, there is experimental evidence (Choat *et al*. 2010; Ennajeh *et al*. unpublished results) for similar vessel-length artefacts with the pressure sleeve technique, so this method cannot be used for cross-validation with other methods.

In conclusion, our study and the study of Choat *et al*. (2010) suggest that VCs constructed from data obtained using the Cavitron and other techniques on species with long conduits are flawed. Our findings may be relevant to other centrifuge techniques, but this point remains to be thoroughly evaluated. Careful reassessment of the different techniques for assessing xylem vulnerability seems necessary in order to identify the mechanism of cavitation and the functional and ecological significance of this process on a sound basis.

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