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TECHNICAL FOCUS

Evaluation of a new centrifuge technique for rapid generation of xylem vulnerability curves

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A new technique for generating xylem cavitation and vulnerability curves was evaluated. The centrifugal force was used to lower the negative pressure in a xylem segment and to induce a positive pressure difference between sample's ends. This enabled the determination of sample hydraulic conductance during centrifugation and, hence, its variation with decreasing xylem pressures. The centrifuge technique was compared with standard methods on a large number of species including conifers, diffuse-porous and ring-porous woody angiosperms. A very good agreement was found for coniferous and diffuse-porous species. However, the technique was not appropriate for ring-porous species, probably because many vessels were cut open in the centrifuged xylem segments. The main advantage of this technique is its rapidity, the vulnerability curve of a xylem segment being constructed typically in less than half an hour. This will greatly facilitate the study of xylem cavitation in ecological or genetic researches.

Introduction

Studies on xylem cavitation during the past two decades have considerably contributed to the understanding of plant water relations during water stress. Indeed, species adapted to drier habitats exhibit higher resistances to cavitation, which is consistent with the general finding that between species, the onset of cavitation correlates with the point of stomatal closure (Cruiziat et al. 2002). Therefore, cavitation resistance seems a sound criterion for evaluating species performance under drought conditions. It can be predicted that cavitation analysis will be more and more incorporated in ecological studies and, even, in breeding programs. Substantial variations in cavitation resistance have indeed been found between genotypes within the same species (Neufeld et al. 1992, Sangsing et al. 2004), and breeders are now willing to use this criterion to screen their genotypes for drought resistance. However, the major limitation for the expansion of cavitation studies is technical, as the current methodologies remain laborious and time consuming, limiting the analysis to a few species or genotypes. Therefore, there is a need for a rapid and reliable method to generate vulnerability curves.

Several techniques have been developed to measure xylem cavitation and embolism. Cavitation events have been detected by mean of ultrasonic acoustic emissions (Tyree and Dixon 1983). The degree of xylem embolism is usually detected by its effect on loss of hydraulic

Abbreviations – F, water flow; R, distance from water level in the downstream reservoir to axis of rotation; r, difference of water level between reservoirs; s, cross sectional area of water in the reservoir; ρ, water density; ω, angular velocity; P, xylem pressure in the middle of the sample; ΔP, difference of pressure between sample's end; PLC, percent loss of conductance.

conductance (Sperry et al. 1988). These techniques are used to generate xylem-vulnerability curves, i.e. the relationship between the degree of embolism and the xylem pressure that provoked the embolism. For this, different branches are dehydrated to different xylem pressures, and the amount of embolism is determined. Typically, it takes several days to generate a whole vulnerability curve with the standard hydraulic technique and several hours with the air pressurization method (Cochard et al. 1992a). The objective of this study was to develop a technique enabling the construction of a vulnerability curve in less than 1 h.

Recently, one of us has developed a centrifuge technique to measure xylem conductance under high negative pressures (Cochard 2002). Centrifugal forces have long been used to induce negative pressure and xylem embolism in plants (Alder et al. 1997, Holbrook et al. 1995). It is one of the most efficient and rapid way of inducing embolism. However, in these studies, embolism was still measured with the conventional methods. The technique developed by Cochard (2002) permits both to induce and to measure the amount of xylem embolism in a shoot. The principle is to use the centrifugal force to lower the xylem pressure in the sample and to induce a positive pressure gradient between sample ends and, hence, a water flow through the sample. By measuring this flow, it is then possible to determine how the sample hydraulic conductance varies, while the centrifugal force is increased. In our previous study (Cochard 2002), we have demonstrated with this technique that xylem conduits were capable of maintaining intact xylem conductances even in the presence of high negative pressures. However, in this work, the potential of the technique for generating vulnerability curves was not evaluated nor compared with the standard hydraulic methods. In this study, we have used the spinning technique on a large number of species having different xylem anatomies (conifer, diffuse porous and ring porous). This enabled us to conclude about the feasibility of a rapid generation of vulnerability curves with this technique.

Materials and methods

Plant materials

The centrifuge technique described below was tested on a relatively large number of woody species including two conifers [*Picea abies* (L.) Karst, *Pinus sylvestris* (L.)], eight diffused porous species [*Fagus sylvatica* L., *Carpinus betulus* L., *Betula pendula* Roth, *Amelanchia ovalis* Med., *Juglan regia* L., *Populus nigra* L., *Salix caprea* L., *Cytisus scoparius* (L.) Link] and five ring-porous species (*Fraxinus excelsior* L., *Quercus robur* L., *Quercus ilex* L., *Celtis australis* L., *Vitis vinifera* L.). The different species were collected in the vicinity of Clermont-Ferrand (France) except *C. australis* and *Q. ilex,* which were sampled in Montpellier (southern France). Large leafy shoots were cut from the plants and brought to the laboratory enclosed in plastic bags to minimize shoot dehydration. In the laboratory, 28-cm-long xylem segments were excised under tap water. The samples were generally taken from the main shoot axis. When present, leaf petioles and lateral twigs were removed. The bark was usually left, but on some occasion, it was removed and the segment was then wrapped with plastic tape to prevent evaporation. Sample ends were trimmed under tap water with a fresh razor blade and inserted in two polycarbonate tubes filled with distilled water and 10 mmol KCl. The segment and the tubes were rapidly placed on the rotor of the centrifuge and the tubes filled again to replace the water that spilled out of the tubes during the installation.

Centrifuge technique

The principle and the theory of the centrifuge technique employed in this work have been described in detail by Cochard (2002). A dedicated rotor was machined from a solid block of aluminium AU4G (2017A) and adapted to a Sorvall SS1 centrifuge (Fig. 1A). The rotor was successfully tested at a maximum speed of 14500 *g* (corresponding to a minimum xylem pressure of−9.3 MPa), but we cannot certify that the design of our rotor is explosion-proof or that it complies with the current safety rules. The xylem segment was centred on the axis of a centrifuge with its ends immersed in water contained in two plastic reservoirs (Fig. 1B). The maximum water level in each reservoir was determined by the position of a hole in the wall. Water could be forced through a tube place under the rotor lid to refill the reservoirs. The hole in the downstream reservoir was placed at a distance $R = 13$ cm from the axis of rotation. The upstream reservoir was located 1 cm closer to the axis of rotation, therefore creating a positive pressure difference (ΔP) and a water flow (F) from the upstream and downstream reservoirs through the sample. The water level in the downstream reservoir was constant because water was expunged through the hole. The water level in the upstream reservoir decreased over time proportionally to F. The difference of water level (r) between the reservoirs was determined optically during centrifugation by measuring the distance between the air and water menisci in the reservoirs. The illumination of the reservoirs in their axis of rotation with a light source facilitated the location of the menisci

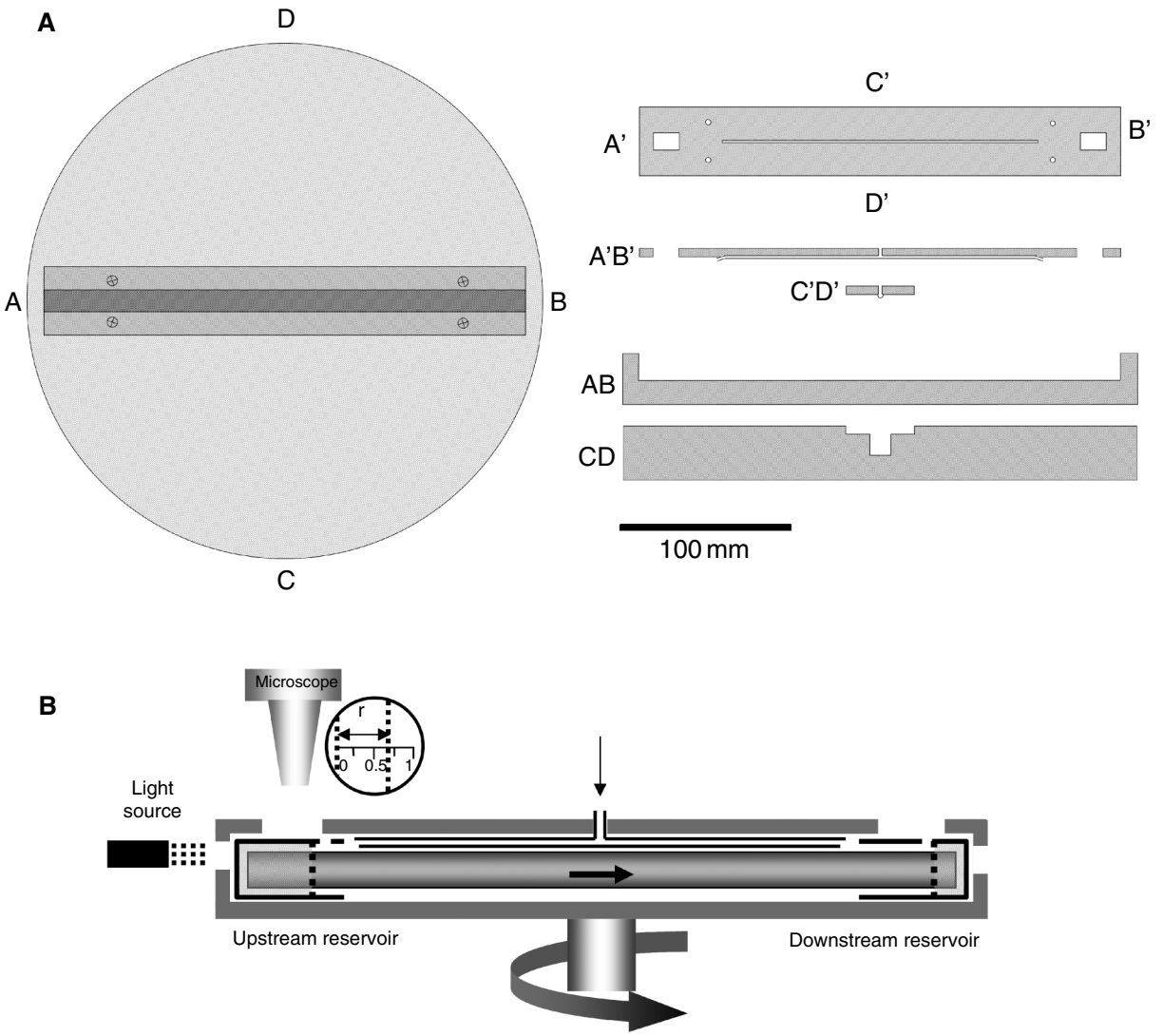

Fig. 1. (A) On scale drawing of the rotor used to centrifuge xylem segments. ABCD is a view from above of the rotor with the lid A'B'C'D' removed. AB, CD and A'B', C'D' are two axial and orthogonal cross-sections of the rotor and the lid, respectively. (B) Schematic drawing showing the principle of the centrifuge technique for constructing vulnerability curves. The sample is centered on the axis of a centrifuge with its ends immersed in water contained in two plastic reservoirs. The maximum water level in each reservoir is determined by the position of a hole in the wall. The hole in the upstream reservoir being located closer to the axis of rotation, a pressure difference develops during centrifugation and water flows from the upstream and downstream reservoirs through the sample. The water level in the upstream reservoir decreases over time at a speed determined optically during centrifugation by measuring the distance between the air and water menisci in the reservoirs. The light source is used to highlight the position of the menisci. A 2-mm diameter tube under the lid can receive a needle through a hole on the axis of rotation to refill the two reservoirs (Cochard, 2002).

under the microscope. We did not measure F directly. Rather, we measured the time (dt) for the meniscus in the upstream reservoir to cover a given distance (dr). F was inversely related to dt according to:

$$F = \frac{d rs}{dt}, \qquad (1)$$

where s is the cross-sectional area of water in the reservoir (assumed constant). ΔP was computed according to the following formula (Cochard 2002):

$$\Delta P = 0.5\rho\omega^2\{R^2 - (R - r)^2\}, \qquad (2)$$

where ρ is the density of water (1000 kg m^{-3}), ω the angular velocity (rad s^{-1}), R the distance (m) from the rotational axis to the distal (downstream) reservoir and r the difference of water level (m) between the two reservoirs (counted positive).The sample hydraulic conductance (K) was then computed as $K = F/\Delta P$. The

minimum xylem pressure (P) in the middle of the sample was adjusted by varying the rotational velocity according to the equation:

$$P = -0.25\rho\omega^2\{R^2 + (R - r)^2\}. \quad (3)$$

More details about the validity and the precision of this technique will be found in Cochard (2002). The sample vulnerability curve was given by the dependence of K on P. A thermocouple was in contact with the centrifuge rotor to account for the temperature dependence of K. However, we probably recorded more the air temperature inside the centrifuge rather than the exact sample temperature and the thermal correction may not have been very precise. The use of a refrigerated centrifuge might be recommendable. A software was developed to log the rotational velocity and temperature and to compute P, ΔP, F and K. Logistic functions were fitted to the different vulnerability curves according to Pammenter and Vanderwilligen (1998).

For most experiments, P was first set to -0.5 MPa, and the initial xylem conductance was determined (K_0). P was then decreased to a more negative value and maintained constant for about 1 min. Preliminary experiments revealed that cavitation formed in a few second when the sample was exposed to P. P was then returned to -0.5 MPa and K was determined anew. The percentage loss of xylem conductance was the determined by $PLC = 100 \times (1 - K/K_0)$. The sample was then exposed to a more negative pressure, and the PLC values were determined as above. The experiment was stopped when near 100% PLC was measured. A second procedure consisted in measuring K, while the sample was still exposed to a given negative pressure, i.e. without increasing it repeatedly to -0.5 MPa.

Evaluation of the centrifuge technique

The vulnerability curves constructed with the centrifuge technique described above were compared with standard techniques, i.e. bench dehydration (Sperry et al. 1988) and pressure bomb dehydration (Cochard et al. 1992a). We used previous data published by our group for *Picea abies*, *Pinus sylvestris* (Cochard 1992), *Fagus sylvatica* (Cochard et al. 1999), *Juglans regia* (Tyree et al. 1993), *Fraxinus excelsior* (Cochard et al. 1997), *Quercus ilex* (Tyree and Cochard 1996) and *Quercus robur* (Cochard et al. 1992b). For *Betula pendula*, we constructed two new curves based on the pressure bomb dehydration technique on mature and potted trees. With the exception of *Juglans regia* and mature *Betula pendula*, comparison between the centrifuge and standard techniques were based on different plant materials. The correlation between the two techniques for the species listed above was statistically evaluated by a regression analysis between the xylem pressures provoking 50% loss of conductance (Ψ_{PLC50}). Ψ_{PLC50} was determined for each species and each method by fitting a logistic function to the different vulnerability curves (Pammenter and Vanderwilligen 1998).

For *Carpinus betulus*, *Amelanchia ovalis*, *Populus nigra*, *Salix caprea*, *Cytisus scoparius*, *Celtis australis* and *Vitis vinifera*, standard vulnerability curves were not available to us. Therefore, our evaluation of the centrifuge technique for these species was based on the repeatability of the results and their consistence with the species ecological preference. For instances, we expected *Amelanchia ovalis* and *Celtis australis*, two drought tolerant species, to be less vulnerable than more drought sensitive species like *Populus nigra* or *Salix caprea*.

Vessel-length determination

It became rapidly apparent to us that vessel length was a key factor to explain abnormal results on some vessel-bearing species. The air-injection technique (Cohen et al. 2003) was employed to detect the presence of vessels cut open in the above 28-cm-long segments. One end of the segment was perfused with air at a pressure of 0.1 MPa, and when air bubbles were conspicuously released at the other end, we assumed that some vessels were longer than sample length. For two species (*Juglans regia*, a diffuse porous, and *Fraxinus excelsior*, a ring porous), vessel-length distribution was assessed with the hydraulic technique proposed by Cochard et al. (1994). Four representative 28-cm-long xylem segments were first flushed with distilled water at 0.15 MPa to remove possible native embolism. Each sample end was then flushed with air at 0.1 MPa to empty the vessels cut open. The percentage loss of xylem conductance (PLC) was then determined with a XYL'EM Apparatus (Bronkhorst France, Montigny les Cormeilles) on five 2-cm-long samples regularly distributed along the sample (Fig. 3) and excised under water to prevent accidental air entry in the samples. High PLC values indicated the presence of a large number of air-filled vessels, i.e. cut open.

Results and Discussion

On Fig. 2, xylem-vulnerability curves constructed with the centrifuge and reference techniques are compared for four species including one conifer (*Picea abies*), two

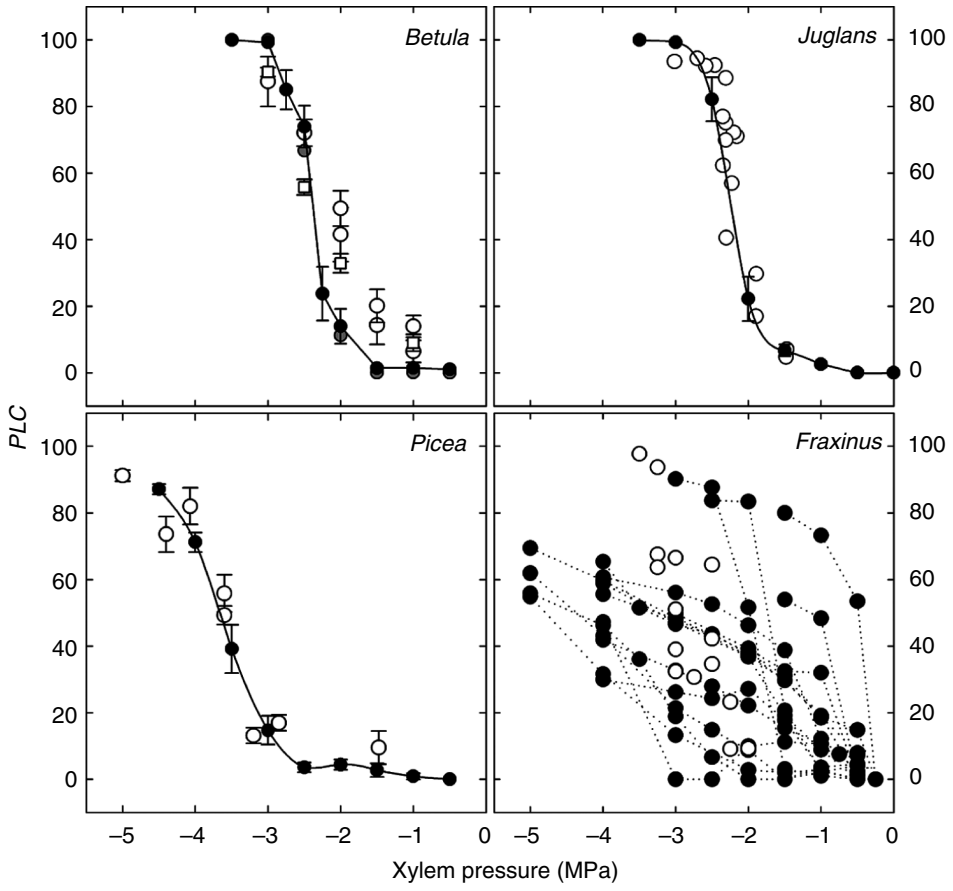

Fig. 2. Vulnerability curves obtained with the centrifuge technique (close symbols) compared with a standard technique (open symbols). For *Betula verrocosa*, the mean centrifuge data (n = 8) are for mature trees. The reference curves were obtained with the pressure bomb dehydration technique on mature trees (open squares) and potted saplings (open circles). Grey circles refer to a sample stained with phloxine B before centrifugation. For *Juglans regia*, the mean centrifuge data (n = 14) was established on potted trees. The reference curve was obtained on shoots, air dehydrated on a similar plant material (replot from Tyree et al. 1993). For *Picea abies*, the mean centrifuge data (n = 3) are for mature trees and the reference curve was obtained on air-dehydrated shoots (replot from Cochard 1992). For *Fraxinus excelsior*, the centrifuge technique was performed on 16 different segments (different dashed lines) and the reference curve was obtained on pressure bomb dehydrated shoots (replot from Cochard et al. 1997). Error bars represent one standard error.

diffused porous (*Juglans regia* and *Betula pendula*) and one ring porous (*Fraxinus excelsior*). The results obtained on these species were representative of the results obtained for conifers, diffuse-porous and ring-porous species, respectively (Table 1). As a rule, a close agreement was found between the different techniques only for conifers and diffuse-porous species. The correlation was highly significant ($R^2 = 0.996$; $P = 0.0001$), and the slope not significantly different from 1. On these species, the curves were always sigmoid, i.e. embolism developed only when the xylem pressure dropped below a species-specific threshold P_{cav}. For these species, the variance around P_{PLC50}, the pressure provoking 50% loss of conductance was small between replicates, indicating a high degree of repeatability (Table 1). The results for diffuse species with unknown vulnerability curves were consistent with their ecological preferences. For instance, for *Salix caprea*, a species from wet sites, P_{PLC50} equalled −2.2 MPa, whereas for *Amelanchier ovalis*, a species from very dry sites, no detectable change in conductivity was noticed above −6 MPa.

By contrast, the results obtained on the five ring-porous species we have evaluated were inconsistent and unrepeatable as illustrated by the data for *Fraxinus excelsior* on Fig. 2. The correlation between the two techniques was poor ($R^2 = 0.38$) and non-significant ($P = 0.58$). Most of the vulnerability curves were like exponential rises to a maximum value, i.e. with a threshold P_{cav} value above −0.5 MPa, in contradiction with the previously published vulnerability curves obtained with the standard technique for *Fraxinus excelsior*, *Quercus robur*, *Quercus ilex* and the drought adaptation of *Celtis australis*.

Ring-porous species differ from diffuse porous and conifers by the great length of their vascular conduits (Tyree and Zimmermann 2002). Accordingly, the air-injection technique revealed that diffuse-porous species had vessels shorter than the length of the segment centrifuged (28 cm). By contrast, many vessels were cut

Table 1. Xylem pressure provoking 50% loss of conductance (Ψ_{PLC50}, MPa) as assessed with standard techniques and the centrifuge technique evaluated in this work.

Anatomy	Species	Ψ_{PLC50} standard	Ψ_{PLC50} centrifuge
Coniferous	*Picea abies*	−3.66 ± 0.08	−3.67 ± 0.10
	Pinus sylvestris	−3.20 ± 0.09	−3.16 ± 0.05
Diffuse porous	*Betula pendula*	−2.31 ± 0.12	−2.34 ± 0.03
	Juglans regia	−2.10 ± 0.07	−2.22 ± 0.02
	Fagus sylvatica	−3.15 ± 0.18	−3.16 ± 0.14
	Carpinus betulus	–	−3.95 ± 0.06
	Populus nigra	–	−2.95 ± 0.20
	Salix caprea	–	−2.22 ± 0.05
	Cytisus scoparius	–	−3.62 ± 0.43
	Amelanchier ovalis	–	−6.70 ± 1.08
Ring porous	*Fraxinus excelsior*	−2.86 ± 0.67	−3.84 ± 2.27
	Quercus ilex	−5.52 ± 0.96	−4.13 ± 7.45
	Quercus robur	−2.83 ± 0.08	−2.08 ± 0.67
	Vitis vinifera	–	−0.75 ± 0.19
	Celtis australis	–	−1.65 ± 0.54

open in ring-porous samples. The change in *PLC* values with distance to cut end for *Juglans regia* (diffuse porous) and *Fraxinus excelsior* (ring porous) was consistent with their vascular anatomy (Fig. 3). These results suggested that approximately half of the vessels were cut open in the middle of the sample for *Fraxinus* compared to approximately 10% for *Betula*. Clearly, the centrifuge technique we have used was problematic when vessel length exceeded sample length. Vessels cut open seemed much more prone to cavitation than intact vessels. We have no clear explanation for this observation, but one may hypothesize that when water does not cross pit membranes at vessel ends, microscopic particles or air bubbles are not filtered out, and hence, seed cavitation in the middle of the samples. Also, we do not know if centrifuging much longer samples would yield better results on these species. Meanwhile, we recommend restricting the usage of our centrifuge technique to species having vessel much shorter than sample length. The centrifuge technique we have described here is in essence similar to the ones used in previous studies (e.g. Hacke et al. 2001), so our recommendation may also hold true for these methods. We have in the past obtained inconsistent data with the double-end pressure bomb technique to construct vulnerability curves (Cochard, unpublished). These results were also collected on relatively short samples containing many cut open vessels so that it can be hypothesized that the problem we have identified with our centrifuge technique may also concern other techniques.

For conifers having short tracheids (a few millimetres), the problem we have noted for ring porous species is thus irrelevant for conifers. There is however, a potential risk of embolism underestimation with our centrifuge technique. This underestimation results from the fact that during centrifugation, xylem pressure is minimum in the middle of the sample and null at the extremities (Fig. 4). Therefore, a gradient of embolism along the sample is predicted (Fig. 5). With our technique, we measured the loss of conductance of the whole sample, an underestimation of the degree of embolism in the middle of the sample. To evaluate this problem, we have considered two theoretical

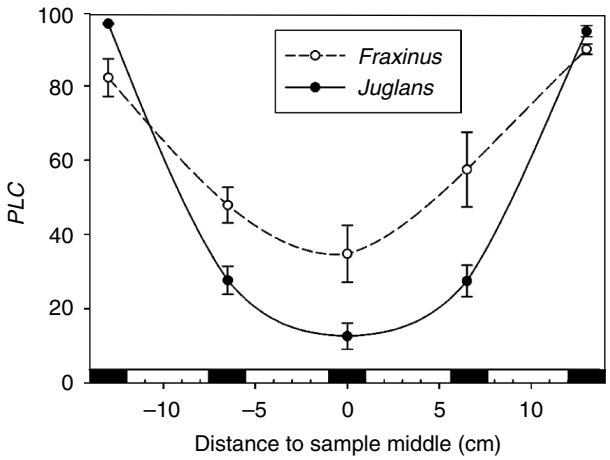

Fig. 3. Profiles of percentage loss of xylem conductance in 28-cm-long xylem segments for *Fraxinus excelsior* and *Juglans regia*. The shoots were first saturated with water, then perfused with air at their two ends. The black bars on the *x*-axis indicate the position where the 2-cm-long segments were sampled. The *PLC* value is a proxy of the number of open vessels. Vessels were longer in *Fraxinus*, a ring-porous species.

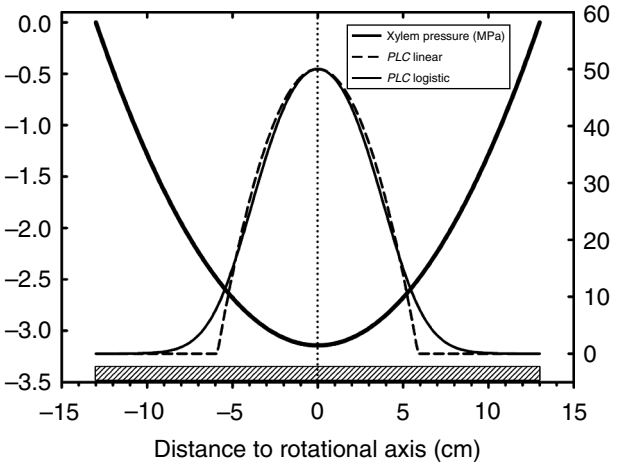

Fig. 4. Pressure profile during centrifugation in a xylem segment exposed to a minimum pressure of −3 MPa (thick plain line). The thin plain and dashed lines are the expected profiles of percent loss of xylem conductance assuming the theoretical vulnerability curves shown in Fig. 5.

vulnerability curves (Fig. 5, plain line) with different shapes (linear vs sigmoid), discretized the 28-cm-long sample in 1 mm segments and computed the vulnerability curve, as it would be detected by our technique. We assumed that all segments had the same initial resistance R_i. The whole initial sample resistance was then $R = \Sigma R_i$. The *PLC* value of each segment (PLC_i) was computed according to their actual negative pressure and the given vulnerability curves which enabled the calculation of the final segment resistance $R'_i = R_i/(1 - PLC_i)$. The whole sample final resistance was then $R' = \sum R'_i$ and the whole sample *PLC* value computed as: $PLC = 1 - R/R'$.

The result (Fig. 5, dashed lines) shows a substantial shift in the vulnerability curves (about 0.5 MPa). However, the good agreement we found between the centrifuge and standard techniques for the conifers we have evaluated suggest that this potential problem is relatively minor. It is possible that the effect of cavitation on xylem loss of conductance exceeds the length of one individual tracheid, which should minimize the problem. Indeed, if one assumes the extreme situation where tracheids lie in series in the axial direction with null conductances in the radial direction, then the cavitation of one tracheid will impair the flow of the whole line like in vessel-bearing species.

We have used two different procedures to generate vulnerability curves: either samples were exposed to increasingly negative xylem pressures and returned to −0.5 MPa for conductance determination (procedure 1) or the conductance was measured at the negative pressures that induced the embolism (procedure 2). The two procedures yielded similar results, but procedure 2 was faster. Fig. 6 shows a typical trial with this procedure demonstrating that an entire vulnerability curve could be determined in less than 10 min for a *Betula* sample. However, it must be pointed out that with procedure 2, the different conductance measurements are not done in the exact same conditions. For instance, the pressure difference between sample ends and temperature increased significantly with angular speed. Procedure 1 was slower (about 30 min for an entire curve), but tended to be more repeatable because sample conductance was always measured in the same conditions, which might be preferable. In a previous study (Cochard 2002), we have demonstrated that xylem refilling was not occurring when pressure was returned to a less negative pressure.

Contrary to the hydraulic method introduced by Sperry et al. (1988), our method is insensitive to the occurrence of native embolism in branch segments. In

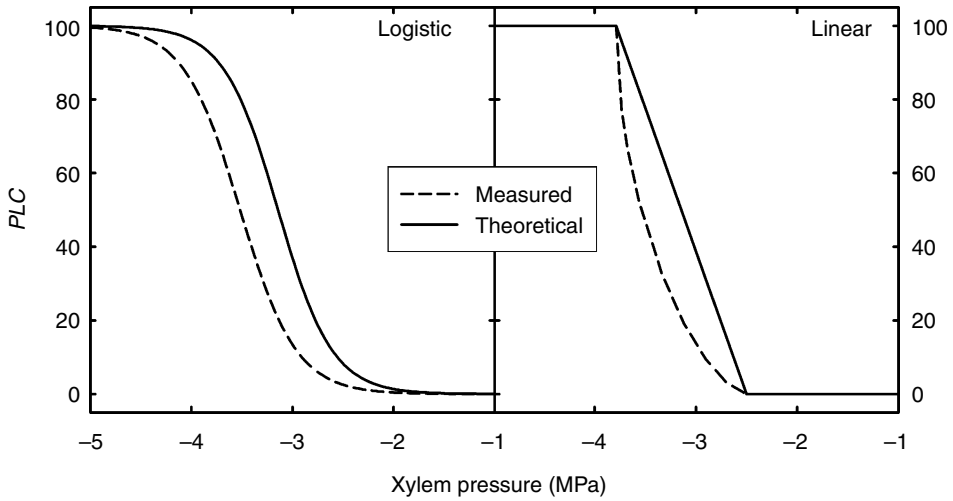

Fig. 5. Theoretical underestimation of actual loss of xylem conductance with the centrifuge technique for coniferous species. Assuming two theoretical vulnerability curves with different shapes (plain lines), the percent loss of conductance for the whole segment (dashed lines), as recorded by the centrifuge technique, should be, in theory, substantially lower than the actual value in the middle of the sample. However, the actual values were not different.

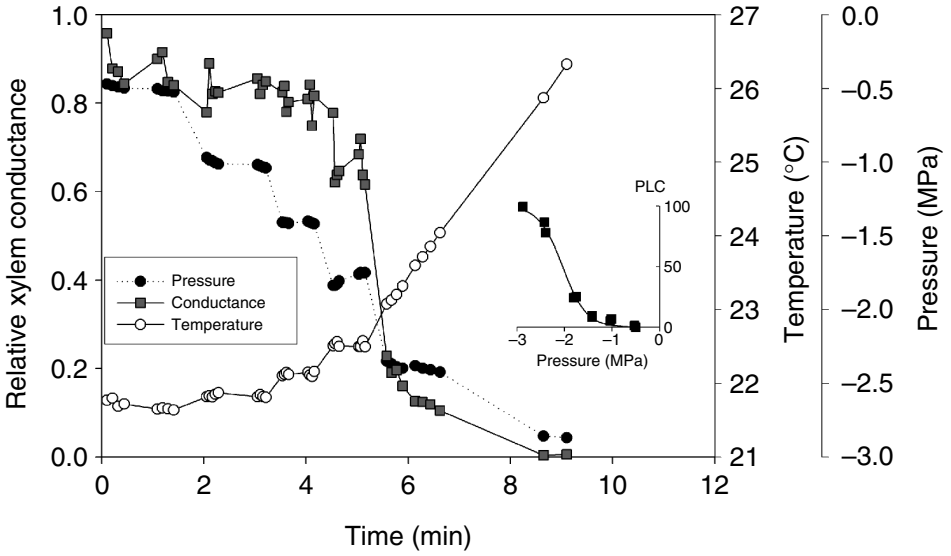

Fig. 6. Typical time courses of minimum xylem pressure (close circles), whole-segment conductance (grey squares) and temperature (open circles) during a spinning experiment with a *Betula pendula* sample. The resulting vulnerability curve is shown in the insert. Note that the whole curve was constructed in less than 10 min.

the more than 1-year-old shoots, substantial level of embolism can exist, usually as a consequence of unresorbed winter embolism. Thus, the *PLC* value of such shoots can be noisy for high xylem pressures. Our centrifuge technique records only the change in native xylem conductance, independently of the actual shoot *PLC* value. This probably explains the small discrepancies between the two techniques for high xylem pressures in Fig. 2. However, if embolism was induced by drought before sample collection, the threshold pressure for embolism induction detected with our technique will correspond, in theory, to the most negative xylem pressure the sample was exposed to during the drought episode. It might therefore be useful to estimate the degree of native embolism in this situation. We found that it was possible to colour the functional conduits during the first conductance measurement at −0.5 MPa with a solution of phloxine B without any detectable effect on the vulnerability curves (see grey circles for *Betula* on Fig. 2). This may provide a coarse way to estimate the degree of native xylem functionality, but a more precise estimate with the XYL'EM technique on similar samples is preferable.

In conclusion, the centrifuge technique we have evaluated is a very promising tool for rapid evaluation of xylem vulnerability to embolism. This is the first technique that enables the construction of a vulnerability curve in, typically, less than half an hour, more than 10 times faster than the other methods we have used so far. This will enable us to include cavitation studies in genetic or ecological surveys that usually require the analysis of a great number of individuals. However, the technique is currently limited to species having relatively short vessels and conifers.

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