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REVIEW PAPER

Methods for measuring plant vulnerability to cavitation: a critical review

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Abstract

Xylem cavitation resistance has profound implications for plant physiology and ecology. This process is characterized by a 'vulnerability curve' (VC) showing the variation of the percentage of cavitation as a function of xylem pressure potential. The shape of this VC varies from 'sigmoidal' to 'exponential'. This review provides a panorama of the techniques that have been used to generate such a curve. The techniques differ by (i) the way cavitation is induced (e.g. bench dehydration, centrifugation, or air injection), and (ii) the way cavitation is measured (e.g. percentage loss of conductivity (PLC) or acoustic emission), and a nomenclature is proposed based on these two methods. A survey of the literature of more than 1200 VCs was used to draw statistics on the usage of these methods and on their reliability and validity. Four methods accounted for more than 96% of all curves produced so far: bench dehydration–PLC, centrifugation–PLC, pressure sleeve-PLC, and Cavitron. How the shape of VCs varies across techniques and species xylem anatomy was also analysed. Strikingly, it was found that the vast majority of curves obtained with the reference bench dehydration-PLC method are 'sigmoidal'. 'Exponential' curves were more typical of the three other methods and were remarkably frequent for species having large xylem conduits (ring-porous), leading to a substantial overestimation of the vulnerability of cavitation for this functional group. We suspect that 'exponential' curves may reflect an open-vessel artefact and call for more precautions with the usage of the pressure sleeve and centrifugation techniques.

Key words: Cavitation, embolism, review, technique, xylem.

Introduction

Water transport in trees has fascinated generations of physiologists and physicists. Trees are able to extract water from relatively dry soils, and transfer it tens of metres above where it is evaporated by the foliage. The most amazing aspect of this process is that it relies on the performance of a very unstable mechanism that we know as the 'cohesion–tension' (CT) theory (Dixon and Joly, 1895; Angeles *et al.*, 2004; Cochard, 2006). The tree plumbing system consists of tiny conduits (vessels and tracheids) that form continuous water columns between the soil and the leaves. When water evaporates from the leaves, tension develops at the site of evaporation and acts to pull up the entire water columns due to the huge cohesive strength of the liquid water. Sap is hence transported under negative pressures (tension). Van den Honert (1948) proposed a very simple but effective model where the pressure in the xylem sap (P_x) depends

Abbreviations: CT, cohesion–tension; PLC, percentage loss of conductivity; VC, vulnerability curve.

on the pressure of the water in the soil (P_{soil}), the hydraulic conductance of the sap pathway (K_{tree}) and the sap flow (F): $P_x = P_{soil} - F/K_{tree}$.

From this relationship, it is clear that when the soil is dry (more negative P_{soil}) or when the sap flow is high, P_x can be under a considerable negative pressure. Typical P_x values are found in the range of –1 to –3 MPa but much more negative pressures can develop under drought conditions. Under such negative pressures, liquid water is in a physically metastable state and susceptible to sudden phase change to a more stable gaseous phase by a phenomenon called 'cavitation'. Cavitation is the Achilles' heel of sap transport in trees. If cavitation occurs, the integrity of the water columns is disrupted and the mechanism of sap ascent is interrupted. Leaves are then no longer supplied with water and the plant may dehydrate to lethal levels. The consequence of cavitation is hence the blockage of the sap flow by the presence of an air bubble in a vessel lumen. This blockage is named an *air embolism* by plant physiologists. 'And, of course, under the stress, a lumen in which rupture has occurred at once becomes waterfree and useless' (Dixon and Joly, 1895). It is critical to know how xylem sap transport dysfunction varies as a function of water stress. The curve describing this dependence is called a xylem 'vulnerability curve' (VC) to cavitation or an embolism (Fig. 1).

The physics of cavitation in plants is relatively well understood (Pickard, 1981; Cochard, 2006). Two possible mechanisms could explain the induction of cavitation: a loss of cohesion between water molecules in the volume of xylem conduits (homogeneous cavitation), or a loss of adhesion between water and conduit walls (heterogeneous cavitation). The rupture of cohesive forces between water molecules is known to occur only at pressures below –20 MPa (Caupin and Herbert, 2006), i.e. much below the most negative pressures recorded in xylem sap (around –15 MPa). Therefore, the hypothesis of homogeneous cavitation in trees is usually rejected. Rather, cavitation is heterogeneous and caused by the capillary rupture of the air–water meniscus located on a pore through the conduit wall (presumably at the level of intervessel pits).

There is now considerable evidence that cavitation is a fundamental aspect of plant water relations and has multiple implications in their anatomy, physiology, and ecology. For instance, stomata close during the early stage of a water shortage to prevent the induction of cavitation (Jones and Sutherland, 1991; Cochard *et al.*, 2002). In addition, the accumulation of cavitation events during drought leads to plant death (Brodribb and Cochard, 2009; Brodribb *et al.*, 2010). Therefore, cavitation resistance is now seen as one of the major physiological factors driving reductions in forest productivity and drought-induced mortality in trees (Anderegg *et al.*, 2012; Choat *et al.*, 2012). It is expected that studies on cavitation resistance will show considerable developments in the near future as this trait start to be implemented into models predicting either plant productivity (ecophysiological models) or species distribution (biogeographical models).

The techniques for measuring cavitation and constructing VCs are numerous and very diverse. They differ by the way xylem transport capacity is measured and by the way xylem water stress is assessed or induced. In this review, we will focus on methodological aspects and will provide an overview of the most frequent methods used for establishing VCs in plants. Unfortunately, it is becoming clear that not all these techniques meet the criterion of reliability. Methodological issues have always been central in the discipline because of the great risk of artefacts. Indeed, because the sap is under a highly metastable state, any perturbation or injury is likely to seed cavitation or the entry of air into xylem conduits. The unique and broad dataset used here allowed us to weigh the advantages and disadvantages of each option, and to discuss new avenues for future work.

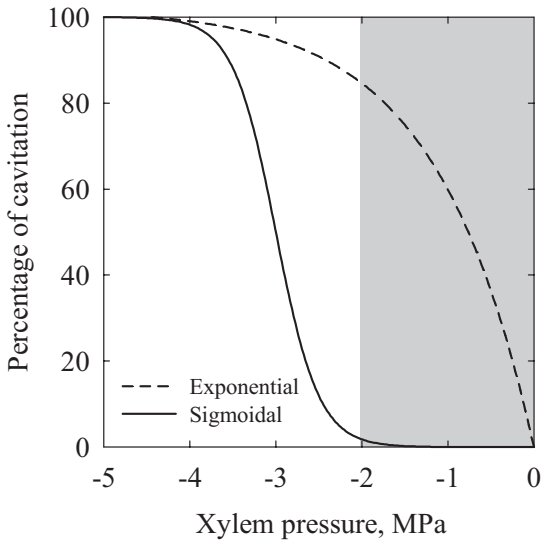

Fig. 1. Schematic xylem VCs showing the relative changes in the degree of cavitation as a function of xylem pressure. VCs in the literature have two extreme shapes, 'sigmoidal' (solid line) or 'exponential' to 100% (dashed line). A major distinction between these two types of curve is that sigmoidal curves display a 'safe' range of pressure (grey zone) where cavitation remains very low. Our literature survey indicates that 'exponential' curves are specific to VCs established with the centrifuge or pressure sleeve techniques on short xylem segments. We suspect here that 'exponential' curves are vitiated by an open-vessel artefact.

Methods to induce xylem cavitation by water stress

To construct a VC to a water stress-induced embolism, the samples should be exposed to a known and gradual level of dehydration. The only relevant measure of water stress for the construction of VCs is the xylem pressure potential (P_x, MPa), the key variable that determines the induction of cavitation during water stress (Sperry and Tyree, 1988). In this section we will review the different procedures to expose xylem vessels to different pressure potentials in order to induce cavitation.

Bench dehydration

This is the most straightforward and natural way of inducing cavitation in plants. Whole, intact plants are allowed to

dehydrate in pots (Tyree *et al*., 1992) or *in situ* (Bréda *et al*., 1993). The xylem pressure can be estimated with a pressure chamber on non-transpiring covered leaves or with stem psychrometers. The relevant pressure is the most negative pressure the plants have experienced during the drought treatment. It is usually taken at midday on the driest day. Experience shows that weeks of water stress are required to induce cavitation in intact trees. Therefore, more often only a large branch segment (typically a leafy branch >1 m long) is cut from an intact plant and allowed to dehydrate freely in the air. Very fast dehydration should be avoided because it can induce a high heterogeneity of water stress in the branch. The branches are best placed on the bench of a laboratory under ambient light conditions. Xylem pressure is measured as above. Tyree *et al*. (1992) demonstrated that VCs obtained from intact plants and cut branches are similar. The latter procedure is preferable because branch water status is better controlled.

Kikuta *et al*. (2003) used a different experimental setup to dehydrate small xylem samples. They enclosed these samples in a chamber where the air humidity was controlled by a saturated solution of different salts. Upon equilibrium, the xylem pressure in the sample equalled the air water potential.

Air pressurization

According to the 'air seeding' hypothesis, air entry in a xylem conduit is caused by a capillary rupture for both angiosperms (Sperry *et al*., 1988) and conifers (Cochard *et al*., 2009; Delzon *et al*., 2010). The rupture occurs when the pressure difference (P_{air}–P_x) across an air–water meniscus located on xylem walls exceeds a critical value. Therefore, decreasing P_x by dehydration under constant atmospheric pressure (P_{air}=0) or increasing P_{air} while maintaining the xylem pressure close to 0 MPa has the same effect on embolism induction. This was first demonstrated by Crombie *et al*. (1985*a*) and Sperry and Tyree (1990) who induced an embolism by forcing air into a xylem segment inserted at one end into a standard Scholander pressure chamber. Melcher *et al*. (2003) and Choat *et al*. (2005) miniaturized this method to detect the cavitation threshold in a single vessel. Cochard *et al*. (1992*a*) and Salleo *et al*. (1992) independently developed the 'pressure sleeve' method. Here, a short (typically <0.3 m) xylem segment cut at both ends is inserted into a pressure chamber with the two ends protruding out of the chamber. Cavitation is induced by increasing the air pressure inside the chamber while the water flow through the sample is measured simultaneously. It is important to notch the segment (Sperry and Saliendra, 1994) or to remove the bark inside the chamber (Ennajeh *et al*., 2011) to facilitate the entry of air into the xylem. Cochard *et al*. (1992*a*) also used air pressurization to induce an embolism in a different way. The principle is to dehydrate a long leafy branch (about 0.8 m) in a large Scholander chamber in a similar way to how pressure–volumes curves are constructed (Tyree and Hammel, 1972). The branch is inserted into the chamber with the cut end protruding and the pressure in the chamber is maintained to the target value P_{air} until sap has ceased to exude from the cut end. At this point, cavitation has been induced as if the branch was air dried to $-P_{air}$ (Cochard *et al*., 1992*a*,*b*).

The main advantage of the air injection technique is that the water constraint can be manipulated with great accuracy and applied to the sample within minutes. Moreover, the pressure sleeve technique enables the construction of a whole VC on one sample within a few hours, a substantial improvement over the bench dehydration method. This probably explains the popularity of the technique. The presence of pressurized air inside the intercellular spaces or in the embolized vessels can disturb the measurement of its hydraulic conductivity. It is then necessary to wait until this air pressure is relaxed to atmospheric pressure. Recently, Choat *et al*. (2010) and Ennajeh *et al*. (2011) reported an 'open vessel' artefact with the pressure sleeve technique. This artefact was probably due to the fact that xylem sap was oversaturated with gas in the chamber and particles not filtered out by the vessel ends nucleated cavitation,in the same way that defects nucleate bubbles in a glass of champagne (Liger-Belair *et al*., 2005; Wheeler *et al*., 2013). This very critical open-vessel artefact will be discussed later.

Centrifugation

Physicists have long used the centrifugal force to expose water columns to large negative pressures (Briggs, 1950). Holbrook *et al*. (1995) demonstrated that the same applied for a xylem segment, and Pockman *et al*. (1995) demonstrated that this technique can be employed to induce cavitation. The principle is to spin a short (<0.3 m) branch segment in a centrifuge to lower the xylem pressure in the middle part of the sample at a target value. Within minutes, cavitation is induced in the xylem. The percentage loss of conductivity (PLC) value is then determined gravimetrically in a central segment of the branch (Pockman *et al*., 1995) or on the whole sample if the ends are maintained under water during centrifugation (Alder *et al*., 1997). The PLC can also be determined during centrifugation (see below, 'Cavitron').

The centrifugation technique uses the advantages of the air injection method without the inconvenience associated with the presence of pressurized air in the sample. Moreover, it can be applied to tiny herbaceous species such as *Arabidopsis*, which cannot be done with air injection method (Tixier *et al*., 2013). However, the possibility of an open-vessel artefact with this method has been discussed recently in the literature (Li *et al*., 2008; Cochard *et al*., 2010).

Methods to measure xylem cavitation and embolisms

The methods for measuring xylem cavitation and embolisms can be grouped into three general categories. The first group of techniques is based on the production of acoustic emissions (AEs) during a cavitation event. In the second category,

the xylem or the conduits contents (i.e. air versus sap) are observed directly. In the last group, the impact of cavitation on the xylem hydraulic conductivity is measured.

Acoustic detection of cavitation

When a cavitation event occurs, the large negative xylem pressure prevailing in the conduit suddenly increases to 0 MPa. Part of the energy relaxed is dissipated as AEs. AEs occur over a very broad spectrum of frequencies, from audible to ultrasonic (UAEs). Milburn and Johnson (1966) were the first to detect cavitation *in planta* with this approach. They used a record player pick-up head connected to an amplifier and a loudspeaker and demonstrated that audible 'clicks' were produced by dehydrating leaves. Working with AEs in the audible range has been very constraining, and AEs were later detected in the ultrasonic range by Tyree *et al.* (1984). A drought stress monitor (model 4615 DSM; Physical Acoustic Corp., Princeton, NJ, USA) has been developed to detect ultrasonic AEs from the xylem (Tyree and Sperry, 1989).

The main advantage of the techniques based on the detection of AEs is that they are non-destructive and non-invasive and can even be used under field conditions. A sensor is simply clamped to a branch or a leaf, and AEs are recorded automatically. These techniques also have a very high temporal resolution and can thus detect the time of occurrence of a cavitation event with great accuracy.

The limitations for these methods are numerous. First, UAEs are not produced by cavitation events in xylem conduits only (Kikuta, 2003). In oak, for instance (Cochard and Tyree, 1990), thousands of UAEs are recorded before any detectable change in xylem hydraulic conductance. Accordingly, the number of UAEs greatly exceeds the number of conduits in the recordable volume of the acoustic sensor. Better results have been obtained with audible AEs in this regard (Ritman and Milburn, 1988). Secondly, the method is more qualitative than quantitative, unless the total number of possible 'clicks' is known. Finally, the method is 'amnesic' in the sense that the number of cavitation events that have occurred before the onset of the recording is unknown (samples totally hydrated or totally dry both produce no AEs).

The greatest merit of the acoustic techniques is historical because they provided the first demonstration that plants were living under the threat of cavitation. The bloom of studies on plant hydraulics in the 1980s owes a lot to these pioneering researches. There is currently a renewed interest in these techniques thanks to the commercialization of a new generation of acoustic systems that allow a much more advanced analysis of acoustic signals (Mayr and Rosner, 2011; Wolkerstorfer *et al.*, 2012). It is now becoming possible to filter out all the irrelevant UAEs and count only the emissions specific to cavitation events in xylem conduits.

Observations of xylem water content

Direct observations The first evidence for embolized conduits in plants were obtained by noting the presence of air bubbles in the xylem lumens of thin axial wood sections observed by eye or under a light microscope (Richard, 1838; Dixon, 1914; Haines, 1935; Sperry, 1985;Sperry *et al.*, 1988; Brendel and Cochard, 2011). Although very simple in principle, it is actually difficult to obtain reliable observations with this technique. The main difficulty is in the preparation of thin wood sections without causing the entrance of air into the xylem conduits. The samples have to be cut and prepared under water and observed rapidly because air bubbles dissolve with time (Lewis *et al.*, 1994). Another constraint is the very limited field of observation, which prevents a quantitative estimation of the total xylem dysfunction with this technique. In recent years, a number of more sophisticated technologies have also been used for direct observations of xylem content. We will review three of them here.

First, Canny (1997) used a scanning electron microscope equipped with a cryogenic stage to observe the vessel content in snap-frozen xylem cross-sections. With this methodology, air-filled conduits are very easily distinguished from ice-filled and functional ones. The method is laborious and access to such equipment is limited, but it surpasses all the other techniques in terms of spatial resolution. The water content of tiny tracheids in conifer needles (Cochard *et al.*, 2004), wood fibres (Utsumi *et al.*, 1998) and protoxylem conduits (Cobb *et al.*, 2007) can be studied with this technique. This method was also applied recently to distinguish conductive and non-conductive imperforate tracheary elements in wood (Sano *et al.*, 2011). However, Cochard *et al.* (2000) demonstrated that cavitation can occur if the sap is under negative pressure when samples are immerged in liquid nitrogen. More reliable results were obtained when the xylem pressure is relaxed to atmospheric pressure by first cutting the samples under water before freezing (Cochard *et al.*, 2000).

Secondly, Holbrook *et al.* (2001) successfully used magnetic resonance imaging technology to visualize the vessel water content of a *Vitis* stem. The main advantage of this method is that it enables a non-invasive and unbiased three-dimensional observation of vessel contents *in vivo.* Water movements are also measurable with this method (Köckenberger *et al.*, 1997). However, the accessibility of the equipment and its low spatial resolution (~20 μm) significantly limit the usage of this technique.

Finally, Fromm *et al.* (2001) used a high-resolution X-ray computed microtomography system to measure the xylem water content in spruce and oak trees. This technology presents the same advantages as magnetic resonance imaging, but the spatial resolution is much higher (<1 μm), which makes it much more appropriate for the study of xylem water content. X-ray beamlines from large synchrotron facilities were first used (Brodersen *et al.*, 2010), which considerably constrains the usage of this technology. Recently, Charra-Vaskou *et al.* (2012) successfully used a laboratory computed microtomography system apparatus (Nanotom 180 XS; GE, Wunstorf, Germany) to study the xylem water content in conifer needles. As the methodology is becoming more accessible, it is predictable that it will generate considerable interest in the future. It is not possible to use these high-technology methods for the routine study of xylem embolisms in plants.

However, their usage as a 'gold standard' to validate other methods is invaluable.

Indirect observations Since the work of Hales (1727), it has been known that coloured fluids can be used to trace sap pathways in xylem tissues. According to the CT theory, water can move only in water-filled conduits, and the presence of air blocks its movement. If water is coloured with a dye, it is possible to visualize the percentage of functional conduits in the xylem. Dye coloration is performed on whole branches by absorption induced by leaf transpiration, or by infiltration through a xylem segment with a small gravimetric pressure (Sperry, 1986). Safranine, basic fuchsine or phloxine are frequently used in these experiments. Dye coloration is a simple but effective way of visualizing the presence of an air embolism in a xylem tissue, and good agreements with other more sophisticated techniques are usually found (Cobb *et al.*, 2007; Hietz *et al.*, 2008). It is always good practice to stain a few segments to confirm the presence of an embolism or to localize the presence of non-functional rings, for instance. Cai and Tyree (2010) combined staining and centrifugation techniques to demonstrate the effect of vessel diameter on their vulnerability to cavitation. However, to make this method quantitative is very laborious, and other techniques are preferred.

Whole-xylem water content can be an indication of xylem lumen water content. However, this parameter is also influenced by the water content of the wood symplast. Water content has been measured gravimetrically (Hietz *et al.*, 2008), by time domain reflectometry (Holbrook *et al.*, 1992) and by γ-ray attenuation (Edwards and Jarvis, 1983). These methods are more appropriate for assessing the xylem water storage capacity rather than its transport capacity.

Hydraulic detection of embolisms

At the beginning of the 20th century, Ewart (1905) noted that 'stems saturated with water allow much more water to pass than stems in which the vessels contain numerous air bubbles, the presence of the latter considerably increasing the resistance to flow'. The principle of these techniques is hence to measure the relative decrease in xylem transport efficiency caused by the presence of air in the conduits. The xylem hydraulic conductivity (K_x) is the best estimator of this capacity. It is defined as the mass flow (F, mmol s^{-1}) of water passing through a segment exposed to a positive gravimetric pressure gradient P/l (MPa m^{-1}), where l is the sample length (m). The unit for K_x is hence mmol m s^{-1} MPa^{-1}. The degree of embolism in a xylem segment can then be quantified as the PLC. Ewart (1905) and later Zimmermann (1978) provided the first estimates of wood conductance, but it was only later that this value was used to quantify the amount of embolism. Stuart Crombie was probably the first to measure the impact of embolisms on xylem conductance (Crombie, 1983). Sperry (1985, 1986) and Tyree and Dixon (1986) used a similar approach later. The methodology that we use today was finally published by Sperry *et al.* (1988). In contrast to other techniques, this method is quantitative, i.e. it quantifies the amount of loss of xylem functionality at any given time. It is also relatively simple to implement and operate. This probably explains why it was rapidly adopted as the most common method for measuring embolism in plants. Since 2001, Bronkhorst has commercializes a system specifically designed to measure xylem embolisms on the basis of this technique (Xyl'em; Bronkhorst, Montigny-les-Cormeilles, France). Because of its significance, it is important to discuss in more detail the different problems associated with this hydraulic technique.

Let us assume a xylem segment (such as a petiole, shoot internode, or root segment) that is fully functional (no embolism), having a hydraulic conductivity equal to K_x. If cavitation occurs and an embolism forms, K_x is reduced to K_x'. The PLC can then be computed as: $PLC=100\times(1-K_x'/K_x)$. If PLC=0%, then $K_x'=K_x$ and none of the conduits were embolized. If PLC=100%, then $K_x'=0$ and all the conduits were embolized. Therefore, to compute PLC, measures of K_x and K_x' are needed. However, it is usually not possible to measure K_x for the trivial reason that the technique is destructive. When a sample is collected on an experimental plant, it is likely to contain a native embolism. Hence, K_x' is measured first. To estimate K_x, the sample is saturated in order to dissolve any air bubbles that may have formed during the treatment. To remove the embolism, the segment is either vacuum infiltrated, pressurized (Sperry, 1985) or perfused ('flushed') with pressurized and degassed water (Sperry *et al.*, 1988). After full saturation, the conductivity of the xylem segment is K_x''. The PLC is then computed as: $PLC=100\times(1-K_x'/K_x'')$

It is easy to see that the technique will meet our expectation only if $K_x''=K_x$, i.e. if the xylem conductivity of the saturated segment equals the conductivity of the same segment before treatment. Our experience with many woody species and several herbaceous ones shows that this condition is generally satisfied. However, situations exist where PLC values might be misleading.

Possible problems with K_x' K_x' should correspond to the hydraulic conductance of the xylem segment *in planta*, i.e. before it was excised. By the time the sample was collected and measured, no embolism must have formed or dissolved, or the value will be altered artefactually. Xylem vessels *in planta* are usually exposed to large negative pressures. Therefore, when a cut is made in the xylem, water in the open vessels is exposed to atmospheric pressure and will thus be sucked upwards and downwards into the xylem in a few seconds. The capillary pressure that develops at the cut end of the conduits is far too small to maintain the air–water meniscus intact. Sap will be sucked back along the entire length of the conduits. This length represents a few millimetres for tracheids but several metres in some large vessel-bearing plants (ring-porous trees for instance). Therefore, it is essential to know the maximum vessel length if a xylem segment is to be cut in air and to recut this entire length under water. It is common practice to excise xylem segments under water directly on the tree or on large dehydrating branches to circumvent this open-vessel problem. However, in a recent report, Wheeler *et al.* (2013) demonstrated that this procedure is biased and

greatly overestimates the PLC value when the prevailing xylem pressure is largely negative. As already mentioned by Zimmermann (1978), it is critical to release any residual tension in the xylem before measuring K_x'. This can easily be done by cutting large branches in the air, immerging the cut base in water and by successively cutting segments under water from the cut end. The PLC can then be determined on segments excised under water from the remaining distal part of the branch.

Canny (1998) argued that the hydraulic determination of xylem embolisms greatly underestimates the actual levels *in planta* for the reason that xylem pressure has to be released to atmospheric or supra-atmospheric values during measurements. The problem could be potentially acute if xylem conduits remained saturated with water vapour (not air) for a long period. Using a spinning experiment, Cochard *et al.* (2000) and Cochard (2002) demonstrated that this underestimation was very unlikely. However, for tiny samples (leaf petioles or leaf veins, for instance) with small-diameter lumens (and thus high capillary pressures), one may observe a rapid embolism dissolution under near-zero xylem pressure. This will translate into a progressive increase in sample conductivity during the initial K_x' measurement. One way around this problem is to place the sample above the inlet reservoir in order to expose it to a negative pressure during measurement (Tyree and Yang, 1992).

The major risk of embolism dissolution is not this passive refilling during measurement. Rather, the critical phase is when samples are inserted into the hydraulic circuit. Indeed, it is easy to displace the air bubbles trapped in an open vessel during this phase. A water head of 60 cm (6 kPa) is sufficient to refill an embolized open vessel with a 50 µm lumen diameter. For larger conduits, the threshold pressure can be as low as 1 kPa (i.e. 10 cm). Therefore, one must be sure that the water head used for the initial K_x' estimate is lower than this threshold value. Working with longer segments decreases the number of open vessels and thus the risk of refilling described above. However, longer samples are more difficult to saturate and more liable to clogging.

This raises frequent questions regarding the impact of segment length on PLC values. This question does not seem to have been answered so far. The hydraulic conductivity of short segments is obviously higher because they contain more open vessels. However, as the PLC is a ratio of conductivity, the impact of sample length is usually considered to be minimal. We have treated this problem mathematically. Let us assume a sample of length L containing N vessels. For simplicity, we consider that all these vessels have the same diameter and length ($=L$) and that the vessel ends are distributed randomly. The hydraulic conductance of each vessel is determined by its end-wall conductance k_w and its lumen conductance k_l. When the sample is shortened to αL (with $0<\alpha\leq 1$) the number of open vessels is $(1-\alpha)N$ and the maximum sample conductance, k_{max}, is:

$$k_{max} = N\left[\frac{1-\alpha}{\alpha}k_l + \frac{\alpha k_l k_w}{\alpha k_w + k_l}\right].$$

Let us assume now that βN vessels are embolized (with $0\leq\beta\leq 1$). If cavitation occurs randomly across open and close vessels, then the sample conductance is reduced to k_{min} with:

$$k_{min} = N(1-\beta)\left[\frac{1-\alpha}{\alpha}k_l + \frac{\alpha k_l k_w}{\alpha k_w + k_l}\right].$$

The sample PLC is then equal to:

$$\text{PLC} = 100\left[1-\frac{k_{min}}{k_{max}}\right] = 100\beta.$$

In this case, the segment length has no effect on the PLC value. We can also consider the situation where open or longer vessels embolize first. In this case, we have:

$$\text{If } \beta > (1-\alpha), \text{then } k_{min} = N(1-\beta)\left[\frac{\alpha k_l k_w}{\alpha k_w + k_l}\right] \text{ and}$$

$$\text{if } \beta < (1-\alpha), \text{then } k_{min} = N\left[\frac{1-\alpha}{\alpha}k_l(1-\alpha-\beta) + \frac{\alpha k_l k_w}{\alpha k_w + k_l}\right].$$

In this situation, the sample PLC is no longer equal to 100β and can substantially be higher especially if k_w is large relative to k_l and if α is close to 0.5 (Fig. 2). The correct PLC values are thus obtained when the vessels are all closed ($\alpha=1$) *or* all open (α reaching 0).

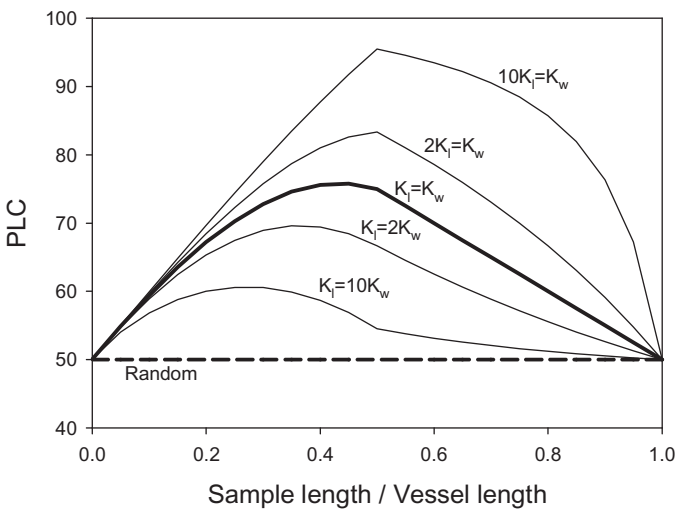

Fig. 2. Theoretical effect of segment length on the PLC for a sample having 50% embolized vessels. Segment length is expressed relative to the vessel length (*x*-axis),with a shorter segment containing more open vessels. If embolism is distributed randomly across open and close vessels, segment length has no impact on the PLC value (dashed line). By contrast, if open vessels are embolized first, PLC is influenced by segment length, more strongly when the vessel end-wall conductance, k_w, is high compared with its total lumen conductance, k_l. Correct PLC estimates are obtained for segments much shorter or longer than the vessel length.

Possible problems with K_x'' K_x'' is the xylem conductance of the saturated segment. K_x'' is supposed to be an estimate of K, the conductance of the same segment before treatment. However, under certain circumstances, or for several species, K_x'' can be very different from K_x.

Situations exist where K_x'' greatly overestimates K_x. In *Festuca* lamina (Martre *et al*., 2001), intercellular air spaces are well developed and form continuous pipes in the axial direction. When leaves are flushed, the air spaces become water filled and conduct water. Therefore, very high PLC values were noted in the leaf lamina of this species, even for control plants, but this was because K_x'' was overestimating K_x. A more common overestimation of K_x is found in species having a high native state of embolism. Temperate ring-porous species are illustrative of this problem. The large early-wood vessels of these species that embolize by frost-induced embolism during winter are never refilled (Cochard and Tyree, 1990; Cochard *et al*., 1997). Therefore, large vessels conduct water only in the current-year ring in such species. Older rings contain non-functional air-filled vessels. When such samples are flushed, these >1-year-old vessels are refilled and conduct water. The overall PLC value can then be quite high, especially for old samples (Cochard *et al*., 1997), but the functionality of the current-year ring is nevertheless maximal. To overcome this problem, it is recommended to work only with current-year shoots or to glue shut older growth rings before measurements are made.

The situations where K_x'' underestimates K_x are of common occurrence. Segment conductivity may decline continuously during K_x measurements or after each flush, because the sample gets clogged by tiny particles in the solution or because of the interaction between ions in the solution with vessel walls (Van Ieperen *et al*., 2000). Species with resin or latex in their xylem will also cause plugging problems. Another source of plugging is the formation of tyloses or gums in embolized vessels. Tyloses are invaginations of contact cells into vessel lumens (usually only in large vessels). Vessels probably have to be embolized for tyloses to form (Cochard and Tyree, 1990; but see Sun *et al*., 2007). Tyloses will obviously lower the hydraulic conductance of these vessels once refilled. The extreme situation is found when all embolized vessels are entirely filled with tyloses, the PLC values for such samples being close to zero, whatever the actual degree of embolism *in planta*. A similar situation is found in conifers where pit membranes may remain permanently aspirated against the cell walls of embolized tracheids. These membranes cannot return to their initial position, and flow through these tracheids is permanently disrupted. To identify and then overcome these problems is rather difficult. It is always good practice to confirm these PLC data with an independent technique (dye coloration, for instance). It is also important to verify that the xylem-area-specific hydraulic conductivity of the saturated samples remains constant during sample dehydration, otherwise the actual PLC can be greatly underestimated. It is possible to correct these values if the specific conductivity of control segments is known (Cochard, 1992).

Although the number of possible biases may seem large, the reliability of the hydraulic technique is high when used properly. It should be regarded as a reference method and should be preferred in hydraulic studies.

Cochard *et al*. (2000) and Cochard (2002) proposed a method for measuring the hydraulic conductivity of a xylem segment under the same large negative pressure measured *in planta*. The negative pressure is generated by a centrifuge as describe above and the water flow rate through the segment is measured during centrifugation. This 'Cavitron' method enables the construction of an entire VC on one sample typically in less than half an hour. It permitted for the first time the evaluation of genetic and phenotypic variability of this trait on a large scale (Lamy *et al*., 2011; Wortemann *et al*., 2011). An open-vessel artefact has also been reported with this technique (see below).

Methods of constructing VCs

A VC is a two-dimensional graph showing how the percentages of cavitation or embolism in a xylem tissue vary with its xylem pressure. Any of the methods described above to measure cavitation can be combined with any method to induce water stress to construct a VC. We propose a nomenclature for VCs based on these two entries (Table 1). For instance, bench dehydration–PLCg means: cavitation induced by dehydrating cut branches on a bench and embolism measured gravimetrically by the loss of xylem hydraulic conductivity.

In a recent survey of cavitation resistance in trees, Choat *et al*. (2012) constructed a database of more than 1200 published VCs. This Xylem Functional Traits (XFT) database is probably not exhaustive but contains the vast majority of VCs published so far worldwide. We have used this database to statistically evaluate the methods to construct VCs. Nearly 96% of the curves were obtained with only four techniques: centrifugation–PLCg (33.6%); bench dehydration–PLCg (24.4%); pressure sleeve–PLCg (21.7%), and centrifugation–PLCc (Cavitron) (15%). The pressure chamber dehydration–PLCg technique has been employed in 2% of the studies and all the other techniques <1%. However the proportion of curves obtained with the pressure sleeve and centrifugation techniques has considerably increased over time (Fig. 3). These techniques are attractive because they are much more rapid and consume less plant material than the classical bench dehydration method. Unfortunately, we suspect that this gain in time efficiency is probably associated with a loss of reliability.

How reliable are the methods used to construct VCs?

We mentioned in the introduction of this review that the physics of water transport in plant is so peculiar that the risk of artefacts in the measurement of cavitation resistance is high. Many studies have compared techniques head to head but always on a very limited number of species (e.g. Li *et al*., 2008; Cochard *et al*., 2010; Choat *et al*., 2010; Ennajeh *et al*.,

Table 1. *Classification of the different methods used to construct xylem vulnerability curves*

The methods differed by the way cavitation is induced (columns) and the way cavitation is measured (rows).

		Method used to induce cavitation				
		Bench dehydration (BD)	**Pressure sleeve (PS)**	**Single end air injection (AI)**	**Pressure chamber dehydration (PC)**	**Centrifugation (CE)**
Method used to measure cavitation	PLC gravity (PLCg)	BD-PLCg (Crombie, 1983; Sperry *et al.*, 1988)	PS-PLCg (Cochard *et al.*, 1992*a*)	AI-PLCg (Crombie *et al.*, 1985*a*; Sperry & Tyree, 1990)	PC-PLCg (Cochard *et al.*, 1992*b*)	CE-PLCg (Pockman *et al.*, 1995)
	Acoustic emission (AE)	BD-AE (Milburn & Johnson, 1966)	PS-AE (Salleo *et al.*, 1992)	AI-AE (Crombie *et al.*, 1985*a*)	PC-AE (Tyree *et al.*, 1984)	
	PLC centrifugation (PLCc)					CE-PLCc (Cochard *et al.*, 2000; Cochard, 2002)

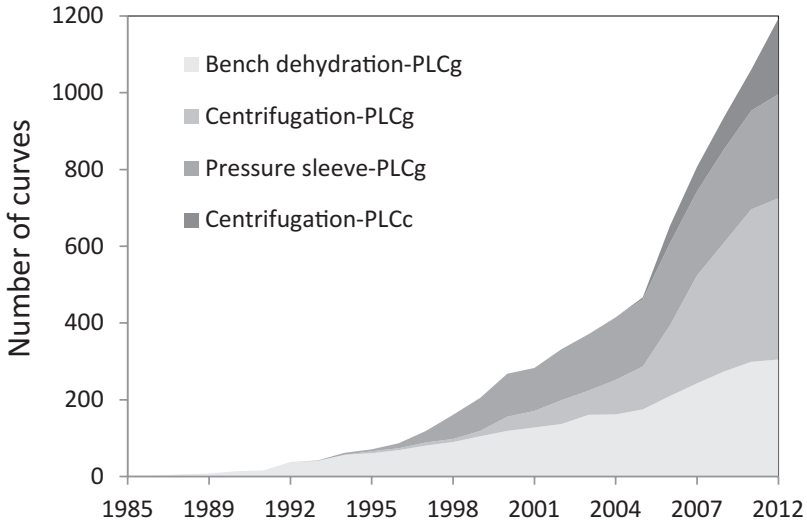

Fig. 3. Temporal evolution of the number of xylem VCs published in the literature by each of the four more popular techniques. In recent years, the number of curves obtained with more modern methodologies has increased exponentially.

2011). The conclusions of these studies are contradictory, but these contradictions are probably the consequence of a strong dependency of the reliability of the technique on the anatomy of the species evaluated.

Here, our contribution to this debate uses a different approach. We have used the large XFT database (Choat *et al.*, 2012) to conduct the first exhaustive and statistical analysis of the performance of these techniques. We have extracted from this database all the curves for which we knew the technique, the shape of the curve, and the xylem anatomy (867 curves in total). For the sake of simplicity and statistical representativeness, we focused on the three most frequently used techniques listed above. We excluded the Cavitron technique from the analysis because the contributors of data for this method (H.C. and S.D.) have deliberately eliminated many suspicious curves from their database. We classified the shape of the curves as 'sigmoidal', 'exponential rise to max', and 'intermediate'. 'Sigmoidal' and 'exponential' curves differ by the way the embolism increases at the onset of dehydration: in the former case, the embolism remains low until a threshold pressure is reached, while in the latter situation, the embolism increases sharply as soon as the pressure is lowered (see Fig. 1). In some cases, the curves were more or less linear and were classified as 'intermediate'. Three categories were used for defining the wood anatomy of the species: vessel-less (i.e. coniferous), diffuse porous, and ring porous. Semi-ring-porous species were included with diffuse-porous species. These categories reflect a range of xylem conduit dimensions. In Fig. 4, we show the proportion of the different curve shapes per technique and per xylem type. From this graph, it is clear that curve shapes contrast sharply across the techniques, depending on species anatomy. Whatever the species anatomy, the majority of curves (>80%) constructed with the bench dehydration–PLC technique were 'sigmoidal'. By contrast, the proportion of 'exponential' or 'intermediate' curves obtained with the pressure sleeve–PLCg and centrifugation–PLCg could be high (>50%). This proportion was clearly influenced by the xylem anatomy: the vast majority of curves were 'sigmoidal' for tracheid-bearing species, whereas 100% of the curves obtained with these two techniques on ring-porous species were 'exponential' or 'intermediate'. In addition, all 31 curves obtained with the pressure chamber

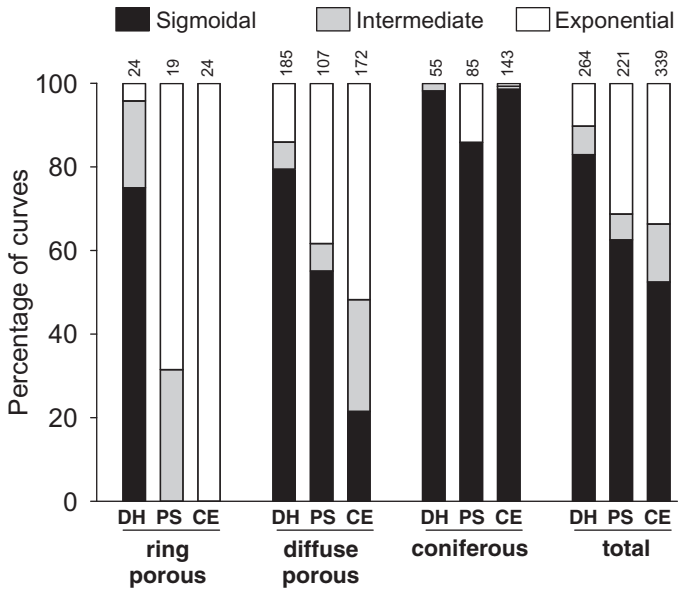

Fig. 4. Literature survey of the shape of 867 VCs distributed across techniques (DH, dehydration–*PLCg*; PS, pressure sleeve–*PLCg*; CE, centrifugation–*PLCg*) and xylem anatomies (ring-porous, diffuse-porous, or coniferous). Curves obtained with the reference dehydration–*PLCg* technique are 'sigmoidal' as a rule. By contrast, 'exponential' curves are strongly associated with the pressure sleeve and centrifugation methods and the presence of large conduits in the xylem tissue.

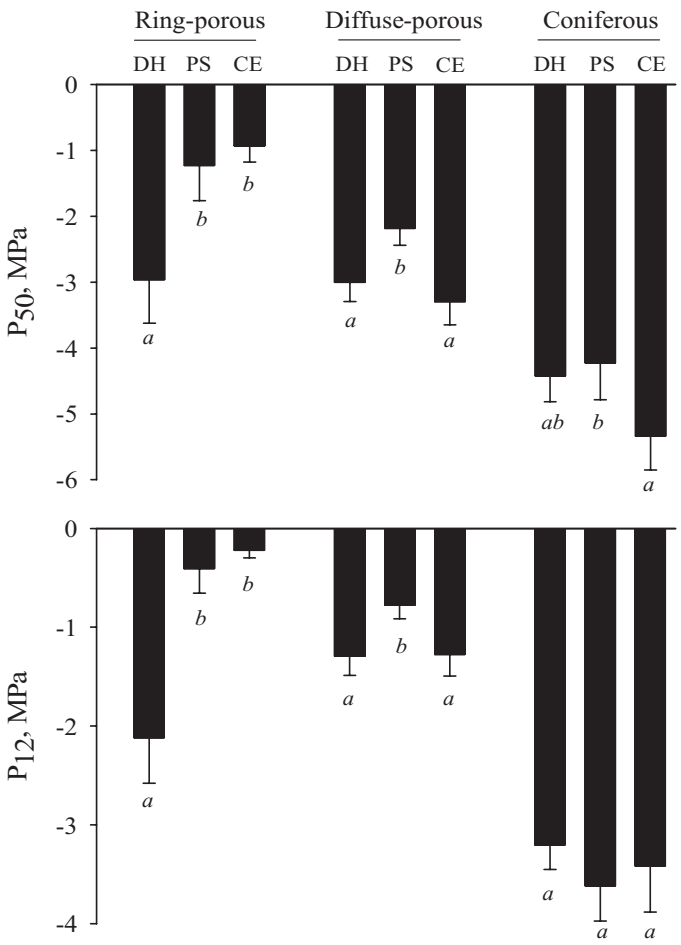

Fig. 5. Literature survey of the impact of techniques and xylem structure on the mean xylem pressure provoking 50% (P_{50}, upper panel) or 12% (P_{12}, lower panel) embolism. See Fig. 4 for abbreviations. Within each xylem type, methods having a letter in common are not statistically different at $P<0.05$. Methods producing 'exponential' curves tend to overestimate xylem vulnerability to cavitation.

dehydration–PLCg technique were 'sigmoidal', even for ring-porous species like oak (Cochard *et al.*, 1992*b*) or ash (Cochard *et al.*, 1997), suggesting that it is not the pressurization *per se* that might alter curve shapes but more the length of the segments exposed to air pressure.

From this first analysis, we can conclude that there is a bias for the shape of VCs associated with the different techniques. However, this does not necessary signify that there is a bias induced by techniques in the estimators of species vulnerability to cavitation. Therefore, we computed the average xylem pressure provoking 50% (P_{50}) and 12% (P_{12}) embolism for the different techniques and xylem anatomies (Fig. 5). Mean P_{12} and P_{50} values agreed well across techniques for coniferous and diffuse-porous species, although the pressure sleeve–PLCg method yielded significantly higher values for diffuse-porous species. For ring-porous species, the situation was drastically different as we found significantly higher vulnerabilities to cavitation with the centrifuge and pressure sleeve methods, with a non-negligible number of unrealistic P_{50} values ranging between 0 and –0.5 MPa. The discrepancy was considerable. For instance, a tenfold increase in P_{12} value was found between the centrifuge–PLCg and the reference bench dehydration–PLCg methods.

From these statistics, we can conclude that: (i) VCs obtained on long branches (bench dehydration–PLCg and pressure chamber dehydration–PLCg) are 'sigmoidal' in shape as a rule, and more exceptionally 'exponential'; and (ii) VCs obtained on smaller branch segments (centrifugation–PLCg, pressure sleeve–PLCg and centrifugation–PLCc) are more prone to exhibit 'exponential' shapes and to display higher P_{50} and P_{12} values, the presence of long vessels in the xylem tissue dramatically exacerbating the problem.

Choat *et al.* (2010) and Ennajeh *et al.* (2011) have recently identified an open-vessel artefact with the pressure sleeve–PLCg method. Indeed, VCs constructed with samples longer than the maximum vessel length have a typical 'sigmoidal' shape whereas working with shorter samples gave 'exponential' VCs. The same conclusion was obtained by Cochard *et al.* (2005, 2010) for the centrifugation–PLCc technique (Cavitron method). For a reason that remains to be elucidated, open vessels are much more vulnerable to cavitation than intact ones and the VCs obtained with such samples greatly overestimate their true vulnerability. Our results here suggest that 'exponential' curves obtained with the centrifugation or pressure sleeve techniques on short xylem segments that are likely to contain open vessels may overestimate the actual species vulnerability to cavitation. The meta-analysis of the XFT database suggests to us that the 'open-vessel' artefact is probably more global than previously thought

and may concern up to 20% of all curves published so far. Cai *et al*. (2013) have recently argued that 'recalcitrant' VCs obtained with centrifugation methods are associated with different hydraulic connections between vessels. However, the more parsimonious hypothesis that open vessels embolize first has not been evaluated in this study. Nevertheless, it is possible that some of these 'exponential' curves are correct, especially for highly vulnerable hygrophilous species, but it will be probably necessary to validate these suspicious data with more robust methods in the future.

Concluding remarks

The diversity of methods that have been proposed to study xylem vulnerability to cavitation in plants probably reflects the necessity to develop more efficient and less-time-consuming technologies. This is an inevitable consequence of the necessity of exploring more species, more treatments, more phenotypes, and more genotypes. It is predictable that this tendency will be exacerbated in the future as ecological, genetic, and genomic studies are likely to develop in the field of plant hydraulics. It is of paramount importance to ground these future studies on a sound methodological basis. This review of the different methods available to characterize xylem vulnerability to cavitation suggests that two of the most popular techniques routinely used today in many laboratories may give erroneous results for many species. We urge cross-validation with independent methods for curves having an 'exponential' shape.

It is important to realize that, beyond the 'exponential' versus 'sigmoidal' shape debate, the physiological and ecological significances of cavitation are also involved. Under the 'sigmoidal' hydraulic paradigm, cavitation forms only when the xylem pressure drops below a threshold value, P_{cav} (Fig. 1). This threshold is typically below the minimal xylem pressure a plant experiences under optimal growth conditions, below the leaf turgor loss point and below the point of stomatal closure (Crombie *et al*., 1985*b*; Jones and Sutherland, 1991). Cavitation then appears as an exceptional process that occurs only when a safety margin has been crossed after prolonged water stress. Increasing xylem resistance to cavitation may be seen as a strategy to increase species tolerance to drought, and our research efforts should be directed, for instance, towards the identification of more cavitation-resistant genotypes, towards understanding the mechanism of cavitation induction and recovery, and towards the discovery of its genetic and genomic basis.

By contrast, under an 'exponential' hydraulic paradigm, cavitation forms as soon as the xylem pressure drops below zero and plants are exposed on a daily basis to high levels of embolism, even under well-watered conditions. As a corollary, the embolism must refill at night when xylem pressure is higher by a mechanism that remains 'miraculous' (Holbrook and Zwieniecki, 1999). Here, cavitation must be seen as a process having a positive impact on plant performance, possibly through the release of water into the transpiration stream. Drought tolerance may then be linked to plant capacity to refill the embolism, and research efforts should focus on this mechanism. Considering that (i) 'exponential' curves are largely artefactual (this study), and (ii) experimental evidence for 'miraculous' refilling are also probably artefactual (Wheeler *et al*., 2013), we conclude that this alternative paradigm for plant hydraulics was constructed on the basis of faulty methods and data. It is therefore crucial to consolidate the methods for measuring cavitation in order to guide research on plant hydraulics in the most relevant and fruitful directions.

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