Methods

Xylem resistance to embolism: presenting a simple diagnostic test for the open vessel artefact

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Summary

- Xylem vulnerability to embolism represents an essential trait for the evaluation of the impact of hydraulics in plant function and ecology. The standard centrifuge technique is widely used for the construction of vulnerability curves, although its accuracy when applied to species with long vessels remains under debate.
- We developed a simple diagnostic test to determine whether the open-vessel artefact influences centrifuge estimates of embolism resistance. Xylem samples from three species with differing vessel lengths were exposed to less negative xylem pressures via centrifugation than the minimum pressure the sample had previously experienced. Additional calibration was obtained from non-invasive measurement of embolism on intact olive plants by X-ray microtomography.
- Results showed artefactual decreases in hydraulic conductance (*k*) for samples with open vessels when exposed to a less negative xylem pressure than the minimum pressure they had previously experienced. X-Ray microtomography indicated that most of the embolism formation in olive occurs at xylem pressures below −4.0 MPa, reaching 50% loss of hydraulic conductivity at −5.3 MPa.
- The artefactual reductions in *k* induced by centrifugation underestimate embolism resistance data of species with long vessels. A simple test is suggested to avoid this open vessel artefact and to ensure the reliability of this technique in future studies.

Introduction

The continuous water column that connects the soil and the upper portions of the plants through the xylem is exposed to negative pressures induced by water evaporation at the leaf surface. Water can be pulled up from the soil through the entire plant system as a result of the strong cohesive forces between water molecules (Dixon, 1914). Under water deficit, the relative water content of the soil decreases, inducing a more negative soil water potential, and the negative pressure inside the water-conducting xylem tissue may become interrupted by gas bubbles. These bubbles (emboli) can expand to block conduits and reduce the long-distance vascular transport of water. As the number of gas-filled conduits increases, the xylem hydraulic conductance (*k*, $\text{kg s}^{-1}\,\text{MPa}^{-1}$) decreases until water flow stops, causing desiccation of plant tissues and, eventually, plant death under severe and prolonged droughts. Failure of the plant hydraulic system is now considered to be the principal mechanism of drought-induced plant mortality (Brodribb & Cochard, 2009; Brodribb *et al.*, 2010; Urli *et al.*, 2013; Salmon *et al.*, 2015; Anderegg *et al.*, 2016). Given the narrow hydraulic safety margins within which many plants operate (Choat *et al.*, 2012), the evaluation of the resistance to embolism across plant species is essential to predict future mortality events and shifts in species distribution ranges as a result of climate change with more intense and severe drought events (IPCC, 2014).

The resistance to xylem embolism is typically assessed by the construction of vulnerability curves, which represent the variation in specific hydraulic conductivity (K_s, kg s^{-1} MPa^{-1} m^{-1}) as a function of xylem water potential (Sperry *et al.*, 1988; Cochard *et al.*, 2009; Torres-Ruiz *et al.*, 2017). During the last few decades, a variety of methods have been developed to determine the loss of K_s in xylem samples exposed to increasing levels of water stress (for a review, see Cochard *et al.*, 2013). Although there is general consensus about centrifuge methods for the construction of vulnerability curves for species with short conduits (e.g. conifer species), the application of appropriate methods for species with long vessels remains a subject of debate, especially with regard to the standard centrifuge technique (Alder *et al.*, 1997; Jacobsen & Pratt, 2012; McElrone *et al.*, 2012; Tobin *et al.*, 2013; Torres-Ruiz *et al.*, 2014; Hacke *et al.*, 2015). This technique is based on the spinning of xylem samples in a custom-built rotor that induces a wide range of pressures at the centre of a sample. In contrast with the Cavitron method (Cochard *et al.*, 2005), no water flow is induced through the sample during spinning in the standard centrifuge technique. Each pressure applied may induce a certain loss of conductivity that is determined gravimetrically with a custom-built device (see, for example, Torres-Ruiz *et al.*, 2012) or with a commercial instrument relying on Coriolis-type mass-flow meters (e.g. Xylem embolism meter; Bronkhorst, Montigny-les-Cormeilles, France). The debate is centred on the high losses of K_s induced by the centrifuge technique at relatively mild xylem pressures for species with long vessels, which results in exponential vulnerability curves (so-called 'r-shaped' curves). These curves have been interpreted as a methodological artefact resulting from the presence of open vessels, i.e. vessels that have no vessel end within the stem segment (Choat *et al.*, 2010; Cochard *et al.*, 2010; Torres-Ruiz *et al.*, 2014). However, other authors consider these curves to be a reliable estimate of embolism resistance in these species, i.e. the occurrence of significant embolism within very moderate ranges of water stress (Christman *et al.*, 2012; Sperry *et al.*, 2012; Hacke *et al.*, 2015). The discrepancy in these two interpretations impedes our progress in understanding the role of hydraulics in plant function (Delzon & Cochard, 2014) and generates confusion regarding the results and conclusions reached in the field of plant hydraulics. Much effort has been devoted to the clarification of whether there is an open-vessel artefact with the standard centrifuge technique, but contradictory conclusions have been reached, even when working with the same plant species. For example, contrasting vulnerability curves and resistances to embolism have been observed for olive (*Olea europaea*) depending on the technique used for the assessment of the degree of embolism. Although observations with high-resolution computed tomography indicate that embolism does not occur in olive stems until a certain threshold in xylem pressure is reached (approximately −3.0 MPa) and that vulnerability curves of this species show sigmoidal shapes (i.e. a so-called 's-shaped' curve; Torres-Ruiz *et al.*, 2014), a recent study reported significant decreases in K_s at relatively high xylem water potentials (Hacke *et al.*, 2015), suggesting r-shaped behaviour and the high embolism vulnerability of this species.

Contrasting conclusions with regard to the reliability of the standard centrifuge technique have also been attributed to differences in the centrifuge rotor design used for the measurements (Hacke *et al.*, 2015). Briefly, there are two custom rotor designs in use: the original rotor of Alder *et al.* (1997) and the Cavitron rotor (Cochard *et al.*, 2005). These designs differ mainly in the way in which samples are secured and how sample ends are immersed in solution during spinning. The possible effects of the rotor design on vulnerability curves have been evaluated by Torres-Ruiz *et al.* (2014), who concluded that both designs produce biased results (overestimated vulnerability to embolism) when samples contain open vessels. Samples in Torres-Ruiz *et al.* (2014) were cut into two pieces for the measurement of K_s gravimetrically after spinning, and the appropriateness of this protocol has been questioned by some authors (Hacke *et al.*, 2015). Although some intraspecific variation in resistance to embolism could be expected between studies as a result of the use of different plant material, varieties, seasonality, growth conditions, etc., the large discrepancies observed for species such as olive warrant further investigation.

Typically, resistance to embolism has been assessed by the induction of decreasing xylem pressures and determination of the corresponding losses in *k*. However, when using a centrifuge method with stem or root segments, it is not possible to determine with certainty whether such losses in *k* reflect patterns of embolism that occur *in situ* or whether they come from an artefact caused by the presence of open vessels (Fig. 1). By using alternative techniques that allow the direct observation of xylem embolism in a non-invasive manner, such as MRI (Holbrook *et al.*, 2001; Kaufmann *et al.*, 2009; Choat *et al.*, 2010) and X-ray microtomography (micro-CT; Cochard *et al.*, 2015; Torres-Ruiz *et al.*, 2015a, 2016; Bouche *et al.*, 2016; Choat *et al.*, 2016), the

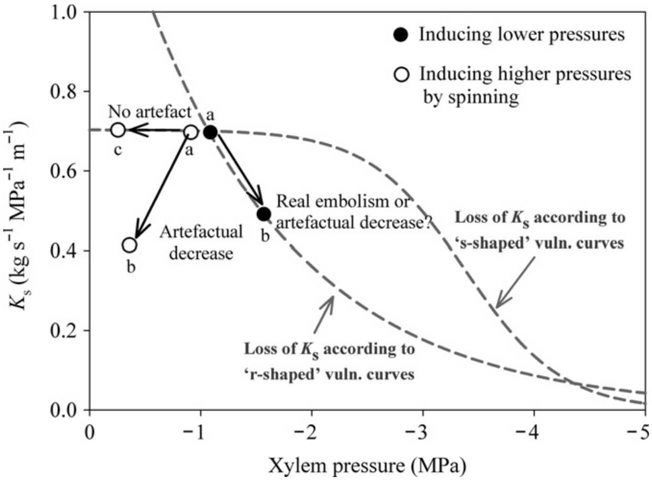

Fig. 1 Two contrasting curves showing the predicted loss in mean specific hydraulic conductivity (K_s) according to the 's-shaped' and 'r-shaped' scenarios. Resistance to xylem embolism has typically been evaluated by inducing lower xylem pressures progressively and determining their corresponding losses in K_s (closed circles), which makes it difficult to distinguish between real and artefactual losses in K_s (closed circle 'a' to 'b'). By exposing a sample to a less negative pressure, i.e. nearer to zero, than the minimum pressure previously experienced, a decrease in K_s can be considered as artefactual (open circle 'a' to 'b') as less negative pressures cannot induce embolism (open circle 'a' to 'c').

open-vessel artefact can be avoided and the process of embolism formation evaluated with greater certainty. As access to some of these non-invasive techniques can be restricted, we propose here a simple alternative test to diagnose the probability of an open-vessel artefact occurring, and thus ensuring the accuracy of the standard centrifuge technique for a given species. This consists of spinning xylem samples to a less negative pressure than the minimum xylem pressure (i.e. water potential) experienced previously under native conditions or during the progressive dehydration of the plant (Fig. 1). As it is unlikely that embolism will be induced at a less negative pressure, any decrease in K_s before and after the spinning of the sample should represent an experimental artefact.

The aim of this study was to test whether K_s values resulting from the standard centrifuge technique are influenced by the presence of open vessels in stem samples with a different vessel anatomy. For this, xylem samples from three vessel-bearing species with contrasting proportions of open vessels were progressively dehydrated to a given xylem pressure. After measuring their K_s gravimetrically, they were exposed to a less negative xylem pressure using the standard centrifuge technique, and their K_s values were measured again. Measurements were undertaken with both rotor designs ('original design' of Alder *et al.*, 1997 and 'Cavitron design' of Cochard *et al.*, 2005) to evaluate the effects of the rotor type on the results. In addition, embolism resistance in olive trees was determined in intact plants using micro-CT and compared with earlier centrifuge measurements for this species.

Materials and Methods

Plant material

Three species with different vessel length distributions were used in this study: two species with relatively long vessels, *Olea europaea* Linnaeus and *Quercus palustris* Muenchh, and one species with relatively short vessels, *Betula pendula* Roth (Ennajeh *et al.*, 2011; Torres-Ruiz *et al.*, 2014). Samples from *Q. palustris* and *B. pendula* were collected from mature individuals located near the campus of the University of Bordeaux (France) for measurements with the Cavitron rotor design, and at the Blue Mountains Botanic Garden (Mount Tomah, Australia) for measurements with the Alder rotor design. For *O. europaea*, 2–3-yr-old saplings were ordered, repotted into 1.5-l pots and grown for 2 months under well-watered conditions and at ambient light and temperature at the University of Bordeaux (France) and at the Hawkesbury Institute for the Environment (NSW, Australia). A subset of these olive saplings was transported to the SOLEIL synchrotron (Paris, France) in April 2015 for direct micro-CT visualization of embolism formation whilst the saplings were progressively dehydrated.

Presence of open vessels

The presence of open vessels was determined in similar samples (i.e. similar length, diameter, age and position in the tree) collected from the same branches (for *B. pendula* and *Q. palustris*) or similar saplings (for *O. europaea*) to those used for the evaluation of the open-vessel artefact in both rotor designs (i.e. 15 or 40 cm in length depending on the rotor design). Samples were injected with air at low pressure (50–100 kPa) whilst their apical ends were immersed in water (Greenidge, 1952; Zimmermann & Jeje, 1981) to check for the presence of open vessels. In those species for which a continuous flow of air bubbles was detected, the percentage of open vessels was determined by application of the silicone injection method (Hacke *et al.*, 2007). New samples, and not those used for the evaluation of the open-vessel artefact, were employed to perform these measurements to avoid the effect of any potential damage to the vascular system caused by the centrifugation itself on the final results. Xylem samples were first flushed basipetally with 10 mM KCl and 1 mM $CaCl_2$ solution at 0.15 MPa for 45 min to remove embolism. Samples were then injected under 30–50 kPa pressure overnight with a 10 : 1 silicone : hardener mix (RTV-141; Rhodia, Cranbury, NJ, USA). A fluorescent optical brightener (Ciba Uvitex OB; Ciba Specialty Chemicals, Tarrytown, NY, USA) mixed with chloroform 1% (w/w) was added to the silicone (one drop per gram) for the detection of silicone-filled vessels in transverse sections under fluorescence microscopy (NanoZoomer; Hamamatsu Photonics, Shizuoka, Japan). After 2–3 d to allow the silicone to harden, the two sample ends were sectioned with a sliding microtome at 5 mm from each end. As the silicone mixture does not penetrate pit membranes, but fills entirely the vessel lumina starting from the injection site, the amount of open vessels through the sample was determined by counting the fraction of silicone-filled vessels at both sample ends.

Evaluation of the open-vessel artefact

As embolism cannot, theoretically, be induced via air seeding by exposing the xylem to less negative water potentials than the minimum water potential previously experienced, the open-vessel artefact was evaluated by spinning xylem samples at a velocity that corresponded to a less negative pressure than the pressure reached by stem branches during a previous drought treatment. Two different rotor designs, the original rotor design (Alder *et al.*, 1997) and the Cavitron (Cochard *et al.*, 2005), were used to check whether the type of rotor could have an effect on the results. Several branches (≥ 2.0 m in length) of *B. pendula* and *Q. palustris* were collected in the early morning and transported to the laboratory, where they were allowed to dehydrate progressively (up to 5 h, depending on the species). For *O. europaea* (var. *arbequina* for the Cavitron design and *manzanilla* for the original design), saplings were exposed to increasing water stress by withholding irrigation. The xylem pressure of each branch and sapling was monitored regularly by measuring the leaf water potential with a Scholander-type pressure chamber (DGMeca, Gradignan, France) on two leaves per branch or sapling that had been previously bagged up (at least 1 h before taking the water potential measurements). Once the xylem pressure reached values of −1.8 MPa or lower, a xylem sample was immediately collected for K_s measurements. Samples were collected according to the experimental protocol of Torres-Ruiz *et al.* (2015a) to avoid the excision artefact described by Wheeler *et al.* (2013). Briefly, the entire branch or sapling was progressively recut under water so that the xylem samples were excised once the xylem pressure was released. Depending on the species and the diameter of the rotors used, different sample lengths were used to avoid/ensure the presence of open vessels in the samples, which allowed us to check their effect on K_s after spinning. Thus, for the species with long vessels (*O. europaea* and *Q. palustris*), excised samples were 15 cm in length for both rotor designs, whereas, for the species with short vessels, *B. pendula*, samples were 40 cm in length for the Cavitron design and 15 cm in length for the original design (40-cm-diameter original rotor not available). K_s for each sample was determined by attaching the samples to a tubing apparatus (Sperry *et al.*, 1988; Torres-Ruiz *et al.*, 2012) filled with a degassed and ultrapure 10 mM KCl and 1 mM $CaCl_2$ solution. All samples were *c.* 3–9 mm in xylem diameter (i.e. without bark) and included growth rings from previous years. However, no air-seeding fatigue is expected as embolized conduits were not flushed. Before taking the measurements, both sample ends were debarked and slightly trimmed under water with a sharp razor blade to adjust to the desired length and to clear any accidentally blocked vessels. To account for possible passive uptake of water by the sample, K_s was calculated as the slope of the flow, which was calculated from the linear regression of the pressure gradient for three different pressure heads (between 2.0 and 3.5 kPa), as described by Torres-Ruiz *et al.* (2012). Previous tests had shown that the pressure gradients used for our measurements were sufficiently low to avoid the displacement of air from embolized vessels open at both ends of the segment. Samples were installed in a custom-built rotor of the Alder *et al.* (1997) design (15 cm in diameter) or the Cochard *et al.* (2005) Cavitron design (40 cm). They were then spun for 5 min, with their ends immersed in the same solution as used for K_s measurements, at a velocity equivalent to a xylem pressure of −1.5 MPa and without inducing any flow through the samples during spinning. To avoid desiccation and to keep the solution in contact with the sample ends when the rotor was stopped, foam pads soaked in the solution used for the measurements were placed in the reservoirs of both rotor designs (Tobin *et al.*, 2013). After spinning, samples were removed from the rotor and K_s was measured again. Samples were spun only once (‘single-spin centrifuge method’, Hacke *et al.*, 2015) to avoid any effect associated with repeated spinning on the same sample. Sapwood area-specific conductivity (K_s), instead of percentage loss of hydraulic conductivity (PLC), was reported to avoid any possible drift by errors when calculating the maximum K_s (Sperry *et al.*, 2012). The native K_s of five extra olive saplings was also measured in order to test for possible changes in K_s at relatively high water potential.

Checking for possible embolism repair during xylem relaxation

The relaxation of xylem pressure carried out to avoid the excision artefact could favour embolism repair in some species (Trifilò *et al.*, 2014). If this was the case for our species, K_s measured for the dehydration treatment would be higher than K_s measured after centrifugation, only because partial refilling had occurred with xylem relaxation treatment. In this case, lower K_s after spinning could be erroneously interpreted as an artefact. In order to eliminate this possibility, we performed an additional phloem girdling treatment, which has been reported to be an effective method to inhibit xylem refilling (Trifilò *et al.*, 2014). K_s from girdled and non-girdled samples was measured after xylem relaxation following the same protocol as described for the evaluation of the open-vessel artefact. Thus, once the plant material reached a xylem pressure of −1.8 MPa or lower, a group of five to six samples per species was girdled for their entire length by removing 5–10-mm-wide bark rings at 10–15-cm intervals, whereas a second group of five to eight samples was left intact (control). To prevent desiccation, the exposed wood was immediately covered with a thin layer of silicone grease. For collection of the girdled and control samples, the entire branches were progressively recut under water and the samples excised once the xylem pressure was released. Samples were then attached to the tubing apparatus and K_s was measured gravimetrically as described above to check for possible repairing processes (i.e. higher K_s values) occurring in the non-girdled samples.

To determine whether the process of connection and disconnection or perfusion of the samples with the measurement solution could account for a possible decline in conductance after centrifugation, K_s was measured for one of the species with long vessels (*Olea europaea*) before (K_{s1}) and after (K_{s2}) disconnection of the samples. For this purpose, samples were kept in water for a similar time as the duration of the centrifuge spinning (i.e. 5 min) and then reconnected to the tubing of the conductivity apparatus.

Resistance to embolism in intact olive saplings: micro-CT

Synchrotron-based micro-CT was used to visualize embolized and water-filled conduits in the current year shoots of intact olive saplings (*Olea europaea*, var. *arbequina*). Fifteen olive saplings were scanned between 8 and 12 April 2015 at the French synchrotron facility SOLEIL (Paris, France) using the micro-CT beamline (PSICHE). Two weeks before the scans, irrigation was withheld progressively in different saplings in order to generate a wide range of xylem water potentials at the time of the scanning.

Once at the synchrotron and 1.5 h before each scan, two leaves, located 20 mm below the scanned area, were wrapped in a plastic bag and covered with aluminium foil to prevent transpiration and to allow equilibrium to develop between leaf and stem water potential. Water potential was then measured on these bagged leaves immediately before the scan with a Scholander-type pressure chamber (Precis 2000, Gradignan, France). Current year shoots were scanned using a high-flux (3.1011 photons mm^{-2}), 25-keV monochromatic X-ray beam. The projections were recorded with a Hamamatsu Orca Flash sCMOS camera equipped with a 250-µm-thick LuAG scintillator and visible light optics providing an effective pixel size of 3 µm. The complete tomographic scan included 1500 projections, 50 ms each, for a 180° rotation. Samples were exposed for 75 s to the X-ray beam. Tomographic reconstructions were performed using PyHST2 software (Mirone *et al.*, 2014) employing the method of Paganin (2006).

The loss of theoretical conductance (k) was determined from a transverse two-dimensional micro-CT slice taken from the centre of the scan volume by estimating the theoretical k of each sapling stem based on the conduit dimensions of embolized and functional conduits. A final scan was carried out on each shoot after cutting it in air at *c.* 5 mm above the scan area. Because the xylem was still under negative pressure, we therefore induced embolism in all remaining functional vessels in order to create a reference scan ('cutting artefact', Wheeler *et al.*, 2013; Cochard *et al.*, 2015; Torres-Ruiz *et al.*, 2015a; Choat *et al.*, 2016), whilst distinguishing, at the same time, a small amount of living vessels (i.e. not fully developed), which were excluded from our calculations. The maximum theoretical k (k_{max}, kg s^{-1} MPa^{-1}) of each sapling was then calculated according to the Hagen–Poiseuille equation:

$$k_{\text{max}} = \sum \pi D^4/128\eta$$

where D is the conduit diameter and η is the viscosity of water. The k value for each sapling and xylem pressure was calculated by subtracting the total k of all the embolized vessels from k_{max}, with the percentage loss of k (PLC) calculated as:

$$\text{PLC} = 100 \times (1 - k/k_{\text{max}})$$

To account for a small amount of embolized vessels that are typically observed at high xylem water potentials, the vulnerability curve was fitted with a modified Weibull function (Neufeld *et al.*, 1992) to include an additional independent parameter (Torres-Ruiz *et al.*, 2015b):

$$\text{PLC} = (100 - y_0)(1 - e^{-\left(\frac{x}{b}\right)^c}) + y_0$$

where x is the xylem pressure, b is the xylem pressure for a PLC of 63%, c is a dimensionless parameter controlling the shape of the curve and y_0 is the PLC at a xylem water potential of 0 MPa. The air entry pressure (P_e), indicating the threshold xylem pressure at which loss of conductivity begins to increase rapidly (Meinzer *et al.*, 2009), was computed from the x-intercept of the tangent through the midpoint of the vulnerability curve.

Statistics

Differences within a species in mean K_s values measured in both girdled and non-girdled (control) samples, and before and after spinning, were tested with paired t-tests after testing for normality and homogeneity of variances. The tests were made at a probability level of 5%. All analyses were performed using SIGMAPLOT (SPSS Inc., Chicago, IL, USA).

Results

The air injection method confirmed the presence of open vessels in 15-cm-long samples for the two species with long vessels (*Q. palustris* and *O. europaea*), whereas no open vessels were detected for *B. pendula* in the 15- and 40-cm-long stem segments. Results from the silicone injection technique showed that the percentages of open vessels for the 15-cm-long samples of *Q. palustris* and *O. europaea* (Fig. 2) were 70.7 ± 7.3% (n = 4) and 27.0 ± 8.4% (n = 4), respectively.

The progressive dehydration induced mean xylem pressures between −1.8 and −2.5 MPa before taking K_s measurements (Table 1) for the three species studied. A significant decrease in K_s was observed for the two species with long vessels when samples were exposed in a centrifuge to a less negative xylem pressure than the minimum pressure previously experienced by the entire plant or large branches (i.e. −1.5 MPa, Fig. 3), independent of the rotor design. Thus, *O. europaea* showed mean decreases in K_s of 46.8 ± 8.1% and 54.3 ± 4.9% using the Cavitron and the original rotor, respectively, whereas, for *Q. palustris*, these decreases reached 66.6 ± 9.1% and 61.4 ± 6.7%, respectively. By contrast, samples of the species with short vessels, *B. pendula*, showed similar conductances before and after spinning for both rotor types.

There was no evidence that the relaxation of the xylem pressure carried out to avoid the excision artefact resulted in embolism repair; similar K_s values were observed in girdled and control xylem-relaxed samples for the three species evaluated (Fig. 4a). Differences in K_s values reported in Figs 2 and 4 are a result of the fact that both sets of measurements were carried out at different times. No differences in K_s were found between control samples that were kept in water for 5 min and then reconnected to the conductivity apparatus (Fig. 4b).

Micro-CT images from drought-stressed olive saplings showed that the PLC remained relatively low (12.6%) when the xylem pressure decreased from −0.4 to −4.0 MPa (Fig. 5). However, images showed a significant loss of hydraulic conductance (PLC = 75.7%) when the xylem pressure reached −6.2 MPa. Thus, the vulnerability curve for olive obtained by direct micro-CT observation showed only a slight increase in PLC up to

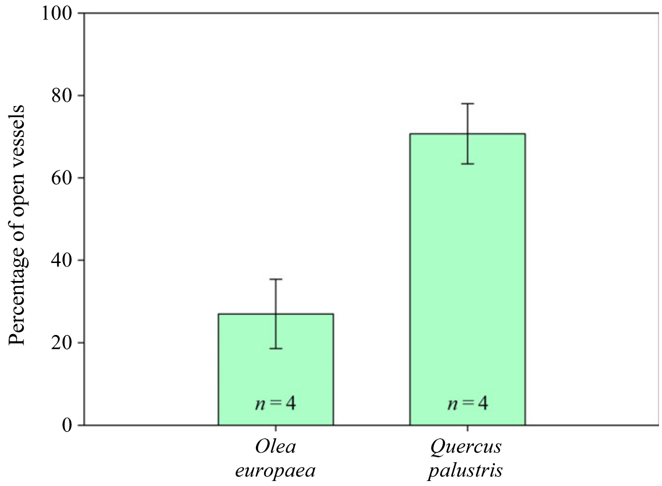

Fig. 2 Percentage of open vessels (i.e. open at both sample ends) in 15-cm-long samples of *Olea europaea* and *Quercus palustris*. There are no data for *Betula pendula* as no open vessels were detected in either the 15- or 40-cm-long samples for this species. Each column is the average of four samples per species and vertical bars represent ± SE.

−4.0 MPa (Fig. 6). As the P_e values indicated, at xylem pressures below −4.0 MPa, the loss of *k* started to increase steeply, reaching 50% loss of *k* (P_{50}) at −5.3 MPa.

Discussion

All xylem samples with some proportion of their vessels open at both cut ends showed a decrease in K_s when they were exposed to a less negative xylem pressure than the minimum pressure previously experienced during the progressive dehydration of the branch samples (*Q. palustris*) or the intact plant (*O. europaea*). This observation was found by application of the standard centrifuge for both the Alder and Cavitron rotor designs. Thus, *Q. palustris* and *O. europaea* showed a mean decrease in K_s of 70.8 and 49.6%, respectively, when using the Alder rotor design, and 61.4 and 54.3%, respectively, when using the Cavitron design.

Table 1 Overview of the species studied with reference to their sample length, mean native xylem water potentials before taking specific hydraulic conductivity measurements, and the pressure induced by centrifugation using the Cavitron or the original rotor design (i.e. Alder *et al.*, 1997 design)

	Sample length (cm)		Native xylem water potentials (MPa, mean ± SE; *n*)		
Species	Cavitron	Original	Cavitron	Original	Induced pressure (MPa)
Betula pendula	40	15	−2.1 ± 0.1; 6	−1.8 ± 0.0; 6	−1.5
Olea europaea	15	15	−2.4 ± 0.1; 7	−2.0 ± 0.1; 6	−1.5
Quercus palustris	15	15	−2.5 ± 0.2; 6	−2.0 ± 0.1; 6	−1.5

Values indicate mean ± standard error.

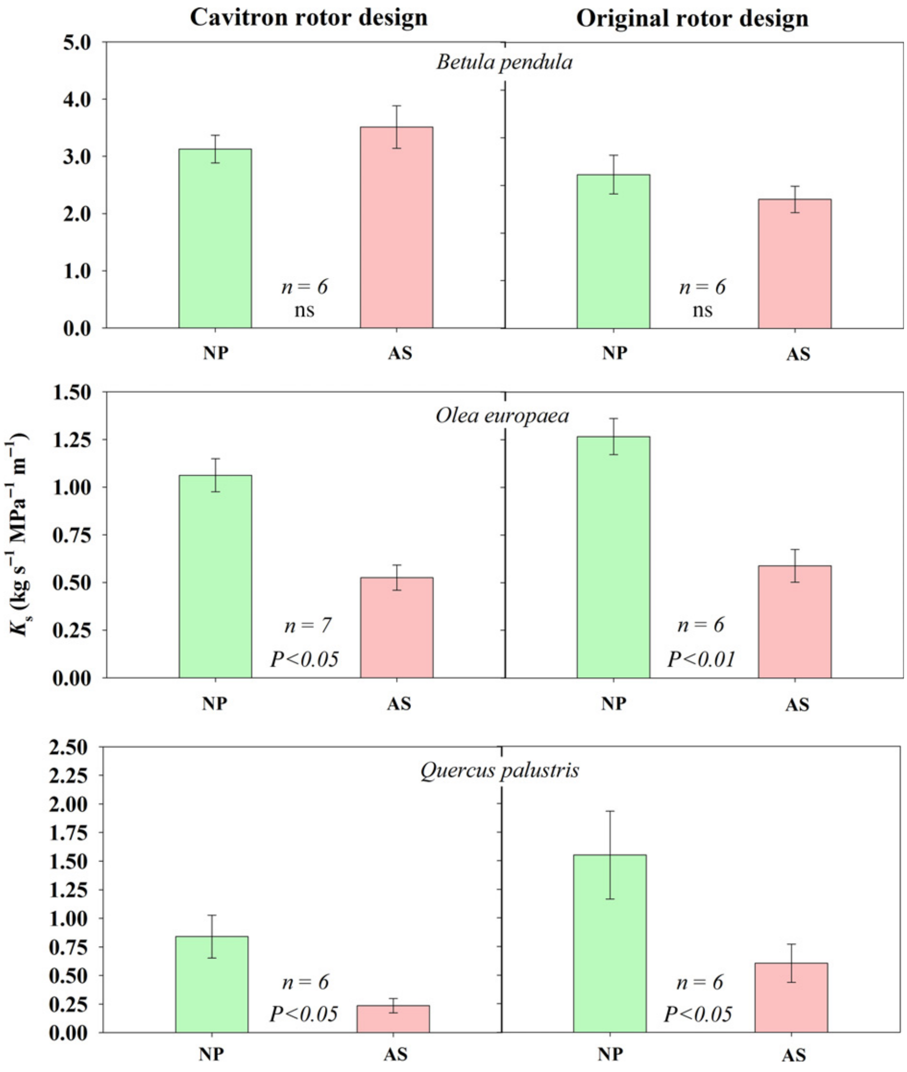

Fig. 3 Mean specific hydraulic conductivity (K_s) for *Betula pendula*, *Olea europaea* and *Quercus palustris* xylem samples before (NP, 'native xylem pressure') and after spinning in a rotor according to the Cavitron (left panels) or the original rotor design (Alder *et al.*, 1997; right panels) to induce a less negative xylem pressure (−1.5 MPa, AS, 'after spinning') than the minimum pressure previously experienced by the plant. Each column is the average of *n* samples per species and vertical bars represent ± SE. Significant differences between NP and AS are indicated as $P < 0.05$ or $P < 0.01$; ns, no significant differences.

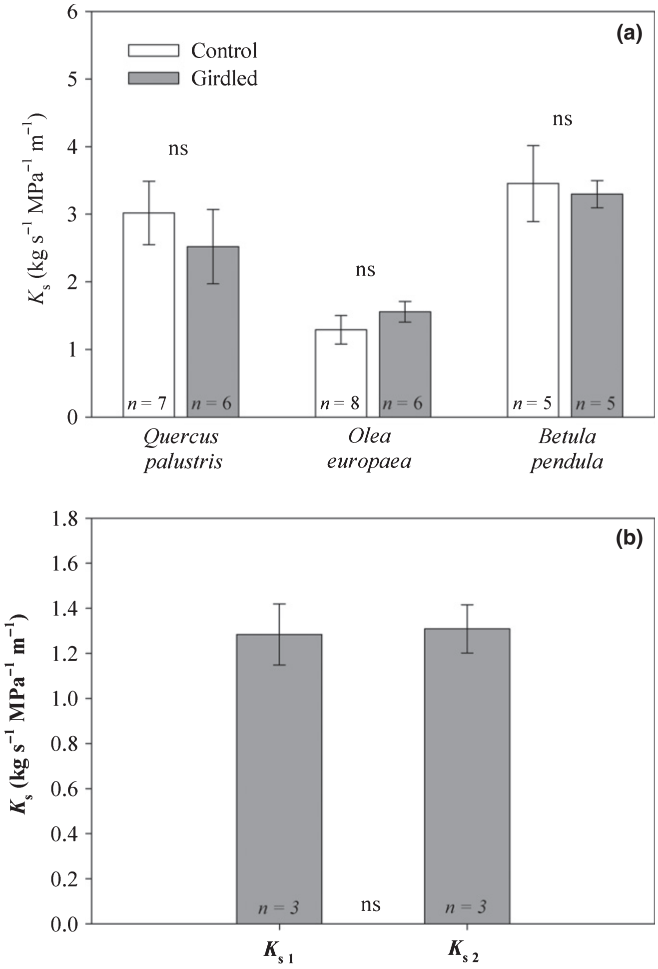

Fig. 4 (a) Mean specific hydraulic conductivity (K_s) for *Betula pendula*, *Olea europaea* and *Quercus palustris* girdled and control (i.e. non-girdled) xylem samples after xylem pressure relaxation. (b) Specific hydraulic conductivity (K_s) for *Olea europaea* measured before (K_{s1}) and after (K_{s2}) disconnection of the samples, leaving them to sit in water for an equivalent time as those that were spun (i.e. 5 min) and reconnecting them to the tubing conductivity apparatus. Each column is the average of *n* samples per species and vertical bars represent ± SE. ns, no significant differences (i.e. $P > 0.05$).

As embolism should not be induced by exposing the xylem to less negative pressures, the observed reductions in K_s are probably caused by an experimental artefact. Indeed, the control test showed that such reductions were not caused by wounding responses, perforation plate morphology, clogging or embolism induction whilst samples were loaded and unloaded in the tubing conductivity apparatus, or by other artefacts during handling and sample preparation. In addition, direct micro-CT observations show little embolism formation in intact plants at a xylem pressure of -1.5 MPa, providing further evidence that this artefact was the main cause of reductions in K_s after centrifugation. Samples of *B. pendula* that did not contain any open vessels exhibited no change in K_s before and after being spun in both rotors. These results are consistent with the hypothesis that the experimental artefact causing significant embolism at xylem pressures near zero is associated with the presence of open xylem vessels in the test segment. This artefact has been reported previously on application of the standard centrifuge technique to species with long vessels (Choat *et al.*, 2010, 2016; McElrone *et al.*, 2012), with evidence showing that the shape of the vulnerability curves

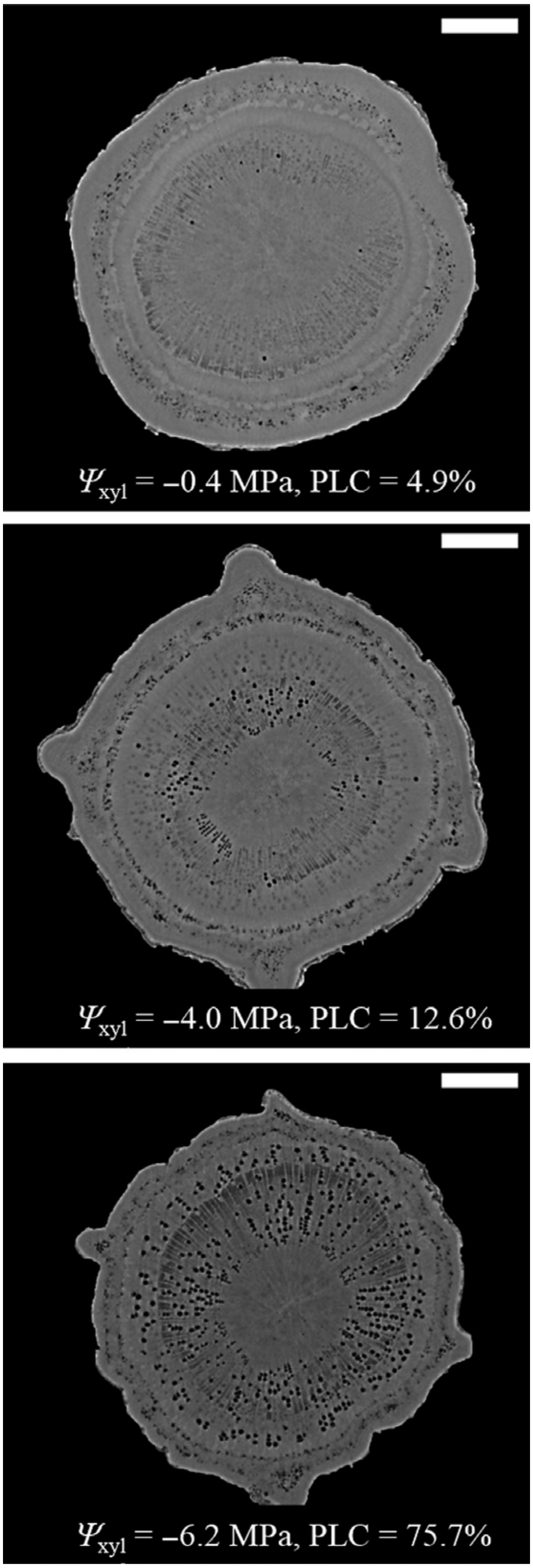

Fig. 5 Transverse X-ray microtomography (micro-CT) images of *Olea europaea* at different xylem water potentials (Ψ_{xyl}, bottom of each photograph). Embolized vessels are observed as black. The percentage loss of hydraulic conductivity (PLC) is indicated for each sample. Bars, 500 μm.

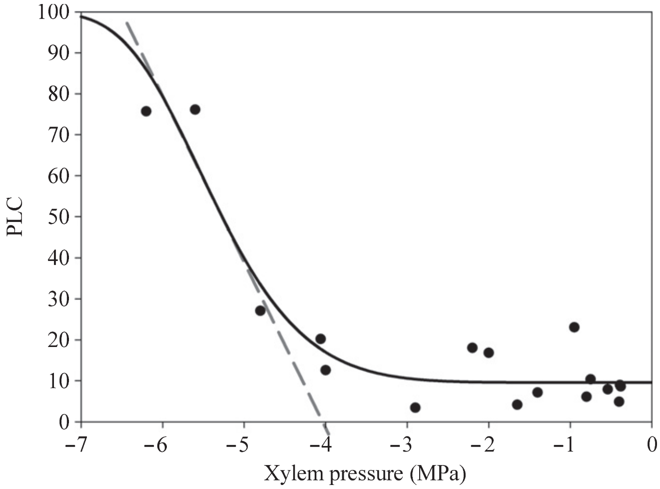

Fig. 6 Vulnerability curve obtained by direct observation (X-ray microtomography, micro-CT) from intact olive plants (black dots correspond to individual PLC measurements based on scans, and the solid black line to the Weibull fitting). The grey dashed line represents the tangent through the midpoint of the vulnerability curve, and its *x*-intercept represents the air entry pressure (P_e) according to Meinzer *et al.* (2009).

obtained using this method varies with the amount of open vessels (Torres-Ruiz *et al.*, 2014). A possible explanation of the observed decreases in K_s after spinning could be the ease with which water can drain from open vessels during spinning because of the asymmetric vessel morphology. In particular, it is well known that the inner diameter of vessel lumina shows considerable variation along its axis (Akachuku, 1987; Balaz *et al.*, 2016). Moreover, xylem vessels are not ideal 'pipes' running perfectly straight, but show some tortuosity (Braun, 1959; Tyree & Zimmermann, 2002). Therefore, it is likely that, during spinning, minor pressure differences occur between both ends, with air entering one end and water draining out of the other.

The present study provides additional evidence that the standard centrifuge technique is prone to the underestimation of resistance to embolism in samples with open vessels, regardless of rotor design. This is consistent with a survey of more than 1200 vulnerability curves (Cochard *et al.*, 2013), illustrating that exponential-shaped curves are remarkably frequent for species with long xylem vessels when they are obtained with centrifuge methods, i.e. both the standard centrifuge and the Cavitron method.

Our findings contrast with the conclusion that the standard centrifuge technique accurately measures vulnerability curves of species with long vessels, such as grapevine and olive (Jacobsen & Pratt, 2012; Hacke *et al.*, 2015; Pratt *et al.*, 2015). This conclusion was based on the agreement between native K_s and PLC values from dehydration-based measurements and from single-spin centrifugations (Fig. 6 in Hacke *et al.*, 2015). However, no native K_s values or direct observations were reported in Hacke *et al.* (2015) for xylem pressures between 0.0 and −1.0 MPa, i.e. the pressures at which the largest changes in K_s are expected according to their exponential-shaped curve. Also, their measurements were conducted in September and October, i.e. late summer. As the trees sampled by Hacke *et al.* (2015) could have been exposed to lower water potentials during mid-summer, they could have experienced some embolism that would not correspond to the water potential measured at the time of the K_s measurements. These underestimated K_s values at relatively high water potentials could agree with the reduced centrifuge K_s values resulting from an open-vessel artefact and, therefore, be erroneously considered as evidence for the rejection of the occurrence of an open-vessel artefact. Moreover, no effect of the amount of open vessels on the shape of the curve was reported when using 13.5- and 27-cm-long samples with different amounts of open vessels (Fig. 4 in Hacke *et al.*, 2015). If the amount of open vessels is relatively high (i.e. > 50%) for both stem sizes, vulnerability curves based on samples can be equally biased (Fig. 3 in Hacke *et al.*, 2015). Moreover, a control experiment with samples showing 0% of open vessels is required to confirm that the amount of open vessels has no effect on a vulnerability curve that is based on centrifuge techniques. Similarly, low resistances to embolism were observed for 1-yr-old grapevine stems with different percentages of open vessels (Jacobsen & Pratt, 2012). Although some differences in P_{50} could be expected between varieties of grapevine, independent studies carried out on intact grapevine plants by micro-CT have reported considerably higher stem resistances to embolism for *Vitis vinifera* (McElrone *et al.*, 2012; Charrier *et al.*, 2016).

It is important to highlight that particular attention was paid to address previous criticisms from similar tests on the open-vessel artefact, following the latest recommendations and accounting for various sources of error in hydraulic measurements: the use of foam pads to avoid the draining of open vessels (Tobin *et al.*, 2013); single centrifugation of samples ('single-spin' protocol, Hacke *et al.*, 2015); accounting for passive water uptake by samples (Torres-Ruiz *et al.*, 2012); releasing the xylem pressure before excising samples (Wheeler *et al.*, 2013; Torres-Ruiz *et al.*, 2015a); and hydraulic quantification based on K_s values instead of PLC (Sperry *et al.*, 2012). In addition, no evidence for embolism repair was detected in any of the three species evaluated, showing similar K_s values between girdled (i.e. samples with their possible refilling mechanism inhibited, see Trifilò *et al.*, 2014) and non-girdled (control) samples after relaxing their xylem pressure. Indeed, there is growing evidence from various research groups that the water transport system of plants is generally resistant to air entry for the normal, daily range of pressures experienced by the xylem (McElrone *et al.*, 2012; Martin-StPaul *et al.*, 2014; Bouche *et al.*, 2016; Brodribb *et al.*, 2016; Choat *et al.*, 2016; Lens *et al.*, 2016). However, a challenge ahead, which is at least as important as technical progress in the quantification of xylem embolism resistance, includes a better understanding of the actual mechanisms behind drought- and frost-induced embolism formation in plant xylem, in particular air seeding (Charrier *et al.*, 2014; Jansen & Schenk, 2015; Schenk *et al.*, 2017).

Our results do not undermine the standard centrifuge technique, but highlight the importance of conducting simple checks on plant material in order to ensure that embolism resistance is measured accurately. These checks are not necessary for tracheid-bearing species, but are advisable for angiosperm species, particularly those known to have long xylem vessels. Only when K_s

remains similar before and after spinning at a less negative pressure than the minimum pressure previously experienced by the material can vulnerability curves based on the centrifuge technique be considered as accurate. Indeed, the test proposed here would probably have avoided the discrepancy with regard to the shape of the response of k to xylem pressure for olive (Torres-Ruiz *et al.*, 2014; Hacke *et al.*, 2015). The direct observation of embolism formation in intact olive trees carried out in this study, however, confirms that embolism formation occurs when xylem water potential falls below a threshold value that is reached under severe drought conditions, supporting its designation as a drought-tolerant species (Lo Gullo & Salleo, 1988; Fernández & Moreno, 1999; Connor & Fereres, 2005; Diaz-Espejo *et al.*, 2012) and the sigmoidal shape of the vulnerability curves of this species (Torres-Ruiz *et al.*, 2014), which is consistent with the paradigm of high embolism resistance proposed by Delzon & Cochard (2014).

The use of different varieties, growing conditions, plant sizes, sampling period, etc. is likely to explain some degree of intraspecific variability in P_{50} between studies (this study; Ennajeh *et al.*, 2008; Torres-Ruiz *et al.*, 2013), but not the observation of dramatically contrasting embolism resistance strategies (Torres-Ruiz *et al.*, 2014). Indeed, the low variability in P_{50} typically observed within a single species (Martínez-Vilalta *et al.*, 2004; Lamy *et al.*, 2014) and the fact that 'exponential' curves are typically associated with centrifuge methods and angiosperm species with long vessels (Cochard *et al.*, 2013) casts further doubt over measurements indicating a low resistance to embolism for olive.

Conclusions

This study demonstrates that the standard centrifuge technique does not accurately measure resistance to embolism in xylem samples with some proportion of open vessels, regardless of the rotor design used. Wood samples with open vessels showed a significant decrease in K_s after spinning at a less negative xylem pressure than the minimum pressure previously experienced by the plant material, which was not a result of embolism formation. Micro-CT observations of intact olive plants confirmed the high embolism resistance strategy for olive, with almost no embolism formation until the water potential reaches a certain threshold value. Overall, the findings from this study are in line with earlier evidence indicating that embolism resistance in plants, especially for species with long vessels, is not as low as previously suggested.

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Author contributions

J.M.T-R. designed the experiment, carried out the measurements, performed the data analysis and wrote the first manuscript draft. J.M.T-R., I.T., R.L., S.D., E.B. and C.M.P-D. collected the plant material and carried out some of the measurements. J.M.T-R., H.C., S.D., B.C., S.J., E.B., R.B., A.K., N.L. and N.K.S-P. assisted with the setting up of the Synchrotron scans. J.M.T-R., S.J., B.C., H.C. and S.D. contributed ideas and assisted substantially with manuscript development.

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