Visual and hydraulic techniques produce similar estimates of cavitation resistance in woody species

Alice Gauthey1, Jennifer M. R. Peters1, Madeline R. Carins-Murphy2, Celia M. Rodriguez-Dominguez2,3, Ximeng Li1, Sylvain Delzon4, Andrew King5, Rosana López1,6,7, Belinda E. Medlyn1, David T. Tissue1, Tim J. Brodribb2 and Brendan Choat1

1Hawkesbury Institute for the Environment, Western Sydney University, Richmond, NSW 2753, Australia; 2School of Biological Sciences, University of Tasmania, Private Bag 55, Hobart, Tas 7001, Australia; 3Irrigation and Crop Ecophysiology Group, Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS, CSIC), Avenida Reina Mercedes, 10, Sevilla 41012, Spain; 4UMR BIOGECO, INRA, Univ Bordeaux, Talence 33450, France; 5L’Orme de Merisiers, Synchrotron SOLEIL, 91190 Saint-Aubin-BP48, Gif-sur-Yvette Cedex, France; 6Departamento de Sistemas y Recursos Naturales, Universidad Politècnica de Madrid, Madrid, Spain; 7PIAF, INRA, University of Clermont-Auvergne, 63100 Clermont-Ferrand, France

Summary

- Hydraulic failure of the plant vascular system is a principal cause of forest die-off under drought. Accurate quantification of this process is essential to our understanding of the physiological mechanisms underpinning plant mortality. Imaging techniques increasingly are applied to estimate xylem cavitation resistance. These techniques allow for in situ measurement of embolism formation in real time, although the benefits and trade-offs associated with different techniques have not been evaluated in detail.
- Here we compare two imaging methods, microcomputed tomography (microCT) and optical vulnerability (OV), to standard hydraulic methods for measurement of cavitation resistance in seven woody species representing a diversity of major phylogenetic and xylem anatomical groups.
- Across the seven species, there was strong agreement between cavitation resistance values (P50) estimated from visualization techniques (microCT and OV) and between visual techniques and hydraulic techniques.
- The results indicate that visual techniques provide accurate estimates of cavitation resistance and the degree to which xylem hydraulic function is impacted by embolism. Results are discussed in the context of trade-offs associated with each technique and possible causes of discrepancy between estimates of cavitation resistance provided by visual and hydraulic techniques.

Introduction

Drought has a defining influence on the structure and productivity of terrestrial ecosystems (Ledger et al., 2011). Extreme drought events combined with heat waves have the potential to reduce primary productivity and trigger mass forest die-off (Ciais et al., 2005; Michaelian et al., 2011; Duke et al., 2017). Increasing temperatures associated with climate change may lead to droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011). Extreme droughts of greater intensity and duration (Trenberth et al., 2011).

According to the cohesion–tension theory proposed by Dixon & Joly (1894), liquid water is transported through the xylem under tension. Although the cohesion–tension mechanism allows large volumes of water to be transported from the roots to the leaves at little direct energetic cost to the plant, it relies on a system that is inherently unstable. Limited soil moisture and high evaporative demand cause increasing tension on the xylem water column. When tension reaches critical values, the water column ruptures (cavitation) which leads to the formation of gas bubbles or ‘emboli’ in xylem vessels and tracheids. Although the resultant air emboli are isolated within individual vessels or tracheids, embolism spreads rapidly through the network of xylem conduits via air seeding when critical xylem tensions are reached. Gas emboli block the transport of water and cause a progressive loss of xylem hydraulic conductivity until complete hydraulic failure occurs (Tyree & Sperry, 1989).

Xylem cavitation resistance, also referred to as vulnerability to embolism, is described by the relationship between the xylem water potential (Ψx in MPa) and the loss of hydraulic

Key words: cavitation, hydraulic, methods, optical, stem.
conductivity relative to a maximum reference state (PLC in %). This trait defines limits on the magnitude of water stress that a plant can withstand before complete loss of functionality occurs in the xylem. Cavitation resistance varies dramatically across species in accordance with site aridity (Brodersen & Hill, 1999; Pockman & Sperry, 2000; Choat et al., 2012; Li et al., 2018) and has been quantitatively linked to lethal (minimum recoverable) water potential (Brodersen & Cochard, 2009; Urli et al., 2013; Hammond & Adams, 2019). Although drought tolerance and mortality processes are complex, hydraulic failure via cavitation is considered to be a principal cause of drought-induced tree mortality (Adams et al., 2017; Choat et al., 2018), and a key trait for understanding the evolution and ecology of plant species in relation to aridity (Pittermann, 2010; Larter et al., 2017). It is therefore imperative that we have confidence in the accuracy of measurements and threshold values determined by standard measurement techniques.

Cavitation resistance is typically assessed by generating vulnerability curves for a given species or population of plants. Species hydraulic thresholds are often compared using the water potential at which 50% of conductivity is lost (P50), with more negative P50 denoting greater cavitation resistance. Techniques used to quantify cavitation resistance traditionally have relied on measurements of water flow rates through detached plant organs (stems, roots or leaves). Although these techniques have provided the foundation for the field of plant hydraulics, a number of experimental artefacts associated with flow-based methods have been identified in the last decade (Rockwell et al., 2014). These artefacts are related primarily to cutting the xylem while it is under tension (Wheeler et al., 2013) or the generation of embolism by centrifugal force (Cochard et al., 2010; Choat et al., 2010) and air injection (Ennajeh et al., 2011). It is important to note that although these artefacts may cause significant errors in the estimation of cavitation resistance, they can be largely eliminated by use of the appropriate precautions and selection of appropriate techniques for a given xylem anatomy (Torres-Ruiz et al., 2017; López et al., 2019). Additionally, in the last two decades, advances in imaging technologies have facilitated the development of new techniques to measure cavitation resistance (Choat et al., 2015; Cochard et al., 2015; Brodersen et al., 2016). These methods are noninvasive or in situ and allow us to visually detect embolism in intact plants, while providing high spatial and temporal resolution of embolism spread through the conductive tissue.

X-ray computed microtomography (microCT) is an imaging technique that can provide noninvasive measurements of xylem functional status and cavitation resistance in intact plants (Choat et al., 2016; Losso et al., 2019). The very high spatial resolution (down to 1 μm per pixel) attainable by microCT (Cochard et al., 2015) also allows the location and spread of embolism in the xylem to be observed at a level of detail that was not previously possible (Brodersen, 2013). Although microCT can potentially be applied to any species or plant organ, its use is constrained by access to synchrotron facilities or high-cost laboratory instruments. Plants also are exposed to high doses of X-ray radiation, which may cause injury to living cells within the xylem (Kim & Lee, 2010; Choat et al., 2016; Nardini et al., 2017; Petruzzellis et al., 2018). However, it is unlikely that X-ray exposure reduces the efficacy of microCT to measure cavitation resistance because the tracheary elements are dead at maturity and there is no evidence that X-rays generate cavitation events during scanning (Choat et al., 2016; Venturas et al., 2019).

The optical vulnerability (OV) technique (Brodersib et al., 2016, 2017) is an in situ imaging method initially developed to study cavitation in the leaf mid-rib and vein network. It allows the dynamics of embolism formation to be followed during propagation in the venation system (Brodrrib et al., 2016). This method was subsequently adapted and used to visualize cavitation in flowers (Zhang & Brodrrib, 2017), stems (Brodrrib et al., 2017) and roots (Rodriguez-Dominguez et al., 2018). Stem P50 values estimated by the OV technique closely agree with values produced by a centrifuge-based hydraulic method in 13 conifers species (Brodrrib et al., 2017), although disagreement between hydraulic and OV estimates of stem P50 have been reported for some angiosperm species (Venturas et al., 2019; Pratt et al., 2020). The OV technique is inexpensive and portable (i.e. can be used in the field or in the lab) with flexible configurations enabling increased automation and throughput. Potential limitations of the OV technique include the small region of the xylem surface observed, that only emboli in the outer layers of the xylem are captured, and that removal of the bark may potentially generate artificial drying of tissue or embolism (Venturas et al., 2019). Although the OV technique provides unparalleled temporal resolution when successful, incorrect implementation of this method, particularly by incomplete or interrupted image capture, can produce errors and vulnerability curves that are artificially vulnerable.

Imaging methods have been criticized on the grounds that they do not directly measure the impact of drought stress on xylem flow rates, instead relying on detection of embolism in xylem conduits at discrete locations (Jacobsen et al., 2015). Errors may occur in the translation of conduit diameter measurements into theoretical flow rates (Venturas et al., 2019), or if immature conduits that are fluid-filled but not yet functional are counted as functional conduits (Pratt & Jacobsen, 2018; Bouda et al., 2019). Discrepancies between visual and hydraulic techniques could also occur if the area of embolized conduits observed is unrepresentative of decreases in xylem hydraulic conductivity caused by drought. Thus, a number of unresolved issues cloud our interpretation of cavitation resistance data and the application of various methods to the study of plant hydraulic function. In this study, we compared estimates of cavitation resistance generated by two in situ imaging techniques (microCT and OV) with those obtained by standard hydraulic methods (bench dehydration or centrifuge). The principal objective of the study was to determine whether imaging and hydraulic techniques provided significantly different estimates of cavitation resistance.

Measurements were made on seven woody species encompassing a broad range of xylem anatomies, allowing us to evaluate trade-offs associated with each technique in relation to xylem anatomical grouping. Specifically, we tested the hypothesis that visual and hydraulic techniques would provide a similar estimate.
of cavitation resistance despite differences in the way the impact of embolism is quantified – that is, whether vulnerability curves based on percentage of embolized conduits, embolized area of xylem and loss of hydraulic conductivity all produce similar estimates of \( P_{50} \). The results are discussed in the context of advantages and disadvantages of techniques used.

**Materials and Methods**

Vulnerability curves were constructed for each of the target species using three different methods: two *in situ* imaging methods, microcomputed tomography (CT) and the optical vulnerability method (OV); and one destructive method, either benchtop dehydration (BD) or the cavitron flow-centrifuge method (CA). The latter two are common hydraulic methods (HM) used to measure xylem cavitation resistance.

**Plant material and experimental design**

Plant material was selected to cover a range of xylem anatomies (tracheid-bearing conifers, diffuse-porous and ring-porous angiosperms) and drought vulnerability. Plant material from Australia included the following: 18 *Angophora costata* (OV = 4, CT = 4, BD = 10) and 12 *Eucalyptus crebra* (OV = -3, CT = 4, BD = 5) saplings from PlantsPlus Nursery (West Pennant Hills, NSW, Australia); 18 *Acacia aneura* (OV = 3, CT = 5, BD = 10), 19 *Wisteria braschytogryts* (OV = 3, CT = 5, BD = 11), 13 *Fraxinus oxyacarpa* (OV = 3, CT = 4, BD = 6), and 16 *Cedrus deodara* (OV = 3, CT = 5, BD = 8) saplings from Plantmark nursery (Vineyard, NSW, Australia). All plants except *A. aneura* were grown in 20-cm deep, 4-l plastic pots, using potting mix from the original nursery. At the point of measurement, plants were c. 1–1.50 m tall and between 1 and 3 yr old. Saplings of *A. aneura* were raised from seed by Greening Australia (Richmond, NSW, Australia) and then replanted and grown in 25-l woven bags filled with native loamy sand soil, local to dry sclerophyll forest in Menangle (Menangle Sand & Soil, Menangle, NSW, Australia). At the time of the experiment, the *Acacia* were 18 months old. All plants were grown in a sunlit polytunnel under ambient environmental conditions on the Hawkesbury campus of Western Sydney University (Richmond, NSW, Australia). In France, measurements were made on 20 individuals of *Pinus pinaster* (CA = 12, CT = 5, OV = 3) from Plantfor nursery (Uchacq, France) and grown on the INRA campus (Bordeaux, France). Individuals from the same species were purchased together and, therefore, age and height differed between but not within species. Note that some techniques produce curves that are composites of individuals points (BD and CT), whereas others produce full curves for each replicate individual (OV and CA).

All plants were maintained in well-watered conditions until experiments commenced in order to minimize native embolism formation in our samples. All experiments were carried out within two months of purchasing the plants. The OV, BD and CA measurements were made either directly before or after CT measurements were undertaken at synchrotron facilities. Owing to the low sample size for the CA technique applied to *P. pinaster*, data for an additional 10 individuals were added. These measurements were made on the same plants material and did not differ from \( P_{50} \) values obtained from the initial measurements.

**Water potential**

Stem water potential (\( \Psi_x \)) was assessed with a pressure chamber or stem psychrometer depending on experimental requirements. First a leaf was sealed in plastic film covered in aluminium foil for a minimum of 30 min before excision and measurement (McCutchan & Shackel, 1992); three leaves per plant were selected and measured at each time point. Then, leaves were slowly pressurized in a Scholander pressure chamber (PMS Instrument Co., Albany, OR, USA) until water was visible on the cut end of the petiole (Scholander et al., 1965). The \( \Psi_x \) also was recorded using a PSY1 Stem Psychrometer sensor coupled with a microvolt data logger used to store data (ICT International, Armidale, NSW, Australia) every 10 min. The psychrometer was installed mid-plant, c. 30–50 cm from the point where PLC was measured. The bark was removed gently, and the xylem washed with Milli-Q (deionized and filtered water, Merck KGaA, Darmstadt, Germany). Then the sensor was installed on the bare xylem and parafilm was wrapped around it to effectively seal the chamber. As the \( \Psi_x \) decreased, the waiting and cooling times were increased, to ensure a sufficient volume of water condensed onto the thermocouples during measurement cycles. For the benchtop dehydration measurements, water potential was measured on nontranspiring leaves using the Scholander pressure chamber. For OV and microCT measurements, a psychrometer was installed on the stem of the sample, recording water potential every 10 min. Stem psychrometer and pressure chamber measurements were compared regularly and showed close agreement (Supporting Information Fig. S1).

**Benchtop dehydration**

The benchtop dehydration method was applied following Sperry & Tyree (1988) and Choat et al. (2010). This method was selected for six of the seven target species because it is generally considered to be a reliable method (Cochard et al., 2013). In this case plants were dehydrated intact, rather than as cut branches. Plants were transported to the laboratory where their roots were washed gently with tap water to remove soil and facilitate dehydration. Plants were allowed to dehydrate freely for varying lengths of time and then sealed in a plastic bag for 30 min, so the water potential of the leaves would equilibrate with the stem and thus \( \Psi_x \) was measured. When the desired water potential was reached, plants were cut at the base of the stem under water. The cut end of the sample was then submerged in water for c. 30 min. This allowed the xylem tension in the branch segment to relax, significantly minimizing potential artefacts associated with cutting xylem under tension (Wheeler et al., 2013). The first cut was made at c. 30 cm upstream (i.e. at the base) from the intended final cut. The sample was slowly cut back from either end until
only a final segment of c. 10 cm in length with a diameter between 0.4 and 1.2 cm remains. If the sapling had three or more developed branches, then two to three samples per plant were used. The segment was then connected to silicone rubber tubing filled with filtered perfusing solution (deionized water and KCl, 2 mM) and attached to the flow meter (Liquiflow L13-AAD-11-K-10S; Bronkhorst High-Tech BV, Ruurlo, the Netherlands). This first measurement yielded the initial hydraulic conductivity ($K_{\text{ini}}$, kg s$^{-1}$ m$^{-1}$ MPa$^{-1}$) of the sample which was calculated as:

$$K_{\text{ini}} = \frac{Q \cdot l}{A \cdot \Delta P},$$

where $Q$, sap flow rate (kg s$^{-1}$); $l$, segment length (m); $A$, xylem cross-sectional area (m$^2$); and $\Delta P$, pressure drops across a segment of conducting tissue (MPa).

The segment was then connected to a pressurized tank containing a degassed and filtered perfusing solution under 100–150 kPa for 30 min. This step flushed the sample, dissolving emboli inside the conduits and replacing it with the solution to regain maximum conductivity. Thus, when the segment subsequently was connected to the flowmeter (Liqui-Flow L10; Bronkhorst High-Tech BV), the hydraulic conductivity was assumed to be at its maximum. The percentage loss of conductivity was determined by:

$$\text{PLC} = \frac{K_{\text{max}} - K_{\text{ini}}}{K_{\text{max}}} \times 100,$$

where PLC, percentage loss of conductivity; and $K_{\text{max}}$, maximum hydraulic conductivity attained after flushing.

Cavitron

The cavitron technique (Cochard, 2002) was used to measure cavitation resistance in $P$. pinaster. This technique uses centrifugal force to create tension (negative pressure) in the xylem by spinning stem samples at their centre. The loss of hydraulic conductance is measured while the sample is spinning (under tension) in the centrifuge by generating a positive hydrostatic pressure difference between the two ends of the sample. Although centrifuge-based techniques are prone to an ‘open vessel’ artefact when applied to some angiosperm species (López et al., 2019), previous validation experiments have shown that the cavitron technique is reliable for tracheid-bearing species such as conifers (Cochard et al., 2005, 2010).

Measurements were performed at the University of Bordeaux (Talence, France) using a custom-built honeycomb rotor (SamPrecis 2000, Bordeaux, France) mounted on a Sorval RC5 superspeed centrifuge (Thermo Fisher Scientific, Munich, Germany). Samples were cut under water with all of the bark carefully removed. The stem was then recut under water to obtain a 22-cm sample in order to fit the stem into the cavitron. Both ends of the sample were sealed in plastic cuvettes filled with water. A solution of ultrapure and degassed water including 10 mM KCl and 1 mM CaCl$_2$ was used as the reference solution for hydraulic measurements. Flow rates through samples were measured by following the progress of a meniscus with a CCD camera. CAVI_SOFT software (v.5.0, University of Bordeaux) was used to control centrifuge speed and each sample was stepped through a set of increasing tensions. Meniscus position was logged for 1 min at each step after the desired rotor speed was reached. Sample hydraulic conductivity and percentage of loss of conductivity was calculated by CAVI_SOFT software at each step. At least two measures of PLC were taken for each pressure step until the sample reached 100% of PLC.

MicroCT

X-ray microCT enables noninvasive generation of xylem vulnerability curves from intact plants by means of visualization (McElrone et al., 2013; Choat et al., 2016). Data were acquired at two synchrotron facilities, the Australian Synchrotron (Clayton, VIC, Australia) and Synchrotron SOLEIL (Gif-sur-Yvette, France). All species except $P$. pinaster were scanned at the Australian Synchrotron, using the Imaging and Medical Beamline (IMBL). Samples were positioned in the beam using a robotic arm (Kuka, KR1000 Titan; KukaRoboter GmbH, Augsburg, Germany) and scanned on the main stem axis with a field of view of 28 mm × 20 mm. Scans were conducted at an X-ray energy of 30 keV, while the sample was rotated through 180 degrees using continuous rotation with images recorded at 0.1° angle increments. This yielded 1800 projections with additional flat field and dark field images recorded before and after each scan. Exposure time at each angle was 0.45–0.60 s giving a total scan time of 18–23 min. Scan volumes were reconstructed using XLICT workflow 2015 (CSIRO, Canberra, Australia) using either the Gridrec or FBP (Paganin et al., 2002) reconstruction algorithm, which resulted in better image contrast based on X-ray phase retrieval. The final resolution of images was 9.7 μm per voxel. Imaging of $P$. pinaster was undertaken at SOLEIL using the PSICHE (SOLEIL synchrotron, Gif-Sur-Yvette, France) beamline. Scans were carried out using a 25 keV monochromatic X-ray beam, while samples were rotated from 0° to 180° using a continuous rotation mode. The scan time was 75 s for each sample and yielded a stack of 1500 TIFF image slices and a field of view of 6 mm × 1.5 mm. Tomographic reconstructions were conducted using in PVHSt2 software (Miron et al., 2014) and resulted in images with a resolution of 2.9 μm per voxel. Cross-sections were compiled to generate 3D rendering using the open source software DRISHTI (https://github.com/nci/drishti) (Fig. S2).

Dehydration of plant material followed a similar protocol to that described for bench dehydration hydraulic measurements. Potted plants were transported to the facility where their roots were gently washed to facilitate dehydration. During dehydration, Ψ$_s$ was monitored primarily by stem psychrometers with occasional measurements using the pressure chamber as described above. After installation of psychrometers, plants were initially scanned while they were still fully hydrated and then placed outside to dry. When plants reached the desired Ψ$_s$ (to generate a vulnerability curve for each species), they were rescanned at the same site. The stem scan location was marked on each plant with
correction fluid, which provided a marker that was easily identified in X-ray images. This procedure continued until > 80% of the vessels were embolized (approx. four scans per plant). A final scan was made of each plant after the stem was cut just above the scan location to ensure all vessels were filled with air, providing us with the number of total vessels embolized which was assumed to be equivalent to a PLC of 100%. Dehydration of plants occurred over a period of 5 d.

MicroCT images provided good contrast between water-filled (grey) and air-filled (black) vessels. Image analysis was performed on a median Z-projection image (11 2D cross sectional images) by counting embolized vessels (darker than water-filled ones) using the ‘Threshold’ and ‘Analyse Particles’ functions in IMAGEJ, or by counting vessels manually using the ‘multi-point’ action (Nolf et al., 2017). For all angiosperm species and P. pinaster, vulnerability curves were based on the percentage of conduits embolized at a given $\Psi$. Percentage embolism was calculated for each scan image following:

$$\%\text{ embolism} = \frac{\text{Number of vessels embolised}}{\text{Total number of vessels}} \times 100.$$ 

Vulnerability curves for C. deodara were based on measurement of embolized xylem cross-sectional area, because the spatial resolution of images attained at IMBL was not sufficient to count individual tracheids in this species. The embolized area of tracheids was transformed to an estimate of percentage embolized tracheids with the aid of microscope images, which allowed tracheid number per area to be accurately assessed (Fig. S3). In selected species, we examined the relationship between the percentage of embolized conduits and the percentage of loss of theoretical conductivity (PLC). The theoretical hydraulic conductivity ($K_h$) for a given stem was calculated from conduit diameters measured in microCT images following the Poiseuille–Hagen law (Tyree & Ewers, 1991; Nolf et al., 2017):

$$K_h = \frac{\pi D^4}{128 \eta n} \sum_{i=1}^{n} (d_i^4),$$

$\eta$, coefficient of viscosity of water ($= 1.002 \times 10^{-3}$ MPa s at 20°C); $D$, conduit diameter (m); and $\rho$, density of water ($= 998.2$ kg m$^{-3}$ at 20°C).

Optical vulnerability technique

The optical vulnerability (OV) method was initially described in Brodribb et al. (2017). Briefly, a Raspberry Pi single board computer (Raspberry Pi Foundation, http://www.raspberrypi.org) was used to control and store images produced by a 8-Mp camera, which was contained within a 3D-printed clamp used to fix the sample in place. The sample field of view was magnified by a ×20 lens and illuminated by six bright light-emitting diodes (LEDs) that provided reflected light from the sample surface. Details of the clamp construction and data analysis are provided at https://github.com/OpenSourceOV.

Intact plants were installed on the benchtop and a small section (rectangle of 2.5 × 1 cm) of the bark was removed from the stem 40–90 cm from the root collar to expose the xylem. This distance varied between plants with differing stem diameters and lengths such that the target segment was of similar diameter between measurements. Stems ranged from 0.5 to 1.2 cm in diameter. Exposed xylem was covered with a conductive adhesive gel (Tensive®, Parker Laboratories Inc., Fairfield, NJ, USA) in order to reduce heterogeneity in the speed of desiccation across different xylem layers. The camera clamp assembly was positioned to best view the prepared xylem and fixed in place. Stem water potential was measured concurrently using a stem psychrometer installed at the base of the stem between 30 and 50 cm from the camera. Images were recorded every 5 min until the sample was completely dry or until no more cavitation events were observed for ≥ 10 h. Image sequences were downloaded from the Raspberry Pi and processed using IMAGEJ (Schneider et al., 2012) to calculate the area of embolism, which was coupled with a concomitant measurement of water potential. Cavitation events were identified with the aid of image subtraction, which clearly reveals areas of embolism in the field of view (Brodribb et al., 2016). These data were used to determine the percentage of embolism as:

$$\%\text{ embolism} = \frac{A_{\text{cav}}}{A_{\text{max}}} \times 100,$$

$A_{\text{cav}}$, cumulative area of cavitated xylem at time $t$; and $A_{\text{max}}$, maximum area of cavitated xylem at the end of each sample dehydration (Fig. S4).

Data analysis

All statistical analyses were conducted with R 3.1.2 (R Core Team, 2017) using RSTUDIO (RStudio Team, 2015). We compared $P_{50}$ for techniques measuring loss of hydraulic conductivity (BD and CA) with techniques that allow for visual measurement of the percentage embolism (CT and OV). Vulnerability curves were fitted to a Weibull function with the fitPLC package (v.1.3) following methods developed by Duursma & Choat (2017). For two of the methods (CA and OV), a full vulnerability curve was generated for each replicate individual. For these methods, sample was incorporated as a random effect to account for repeated measures on a single individual. A 95% confidence interval (CI) for $P_{50}$ was obtained using a standard profiling method. The CT and BD methods produce ensemble curves, in which measurements from multiple individuals are used to produce a curve. For these methods, a 95% CI for the estimate of $P_{50}$ was generated using a bootstrapping approach with 2000 resamples.

Previously, estimates of $P_{50}$ from different techniques have been compared by testing whether 95% CIs overlap; $P_{50}$ values generated from different methods have been considered to be statistically different if there is no overlap in the confidence intervals (Bourne et al., 2017; Nolf et al., 2017). However, it is possible for estimates to be statistically different even where CIs overlap (Cumming et al., 2007). Here, we compared estimates from different techniques by generating bootstrapped 95% CIs for the difference in estimates between each pair of methods. Bootstrap
distributions generated as part of curve fitting in FITPLC were extracted for CT and BD methods. For OV and CA methods, bootstrap distributions were generated to represent the confidence intervals obtained. We then resampled 2000 values from each of these distributions and used them to generate a bootstrap CIs for the difference between each pair of techniques. Where these CIs do not overlap zero, there is no significant difference between these two methods.

The relationships between theoretical PLC values and percentage embolized vessels extracted from microCT images were examined using regression analysis. A Standardized Major Axis (SMA) approach was used to test the if the slope or elevation of the line differed from the 1 : 1 relationship using the SMATR package (v.3.4.8; Warton et al., 2012).

**Results**

Imaging techniques (CT and OV) for measuring cavitation resistance were compared with standard hydraulic methods using a range of species with diverse xylem anatomy: two conifers (C. deodara and P. pinaster), three diffuse porous angiosperms (An. costata, Ac. aneura and E. crebra), a ring porous angiosperm (F. oxycarpa) and a liana (W. brachybotry). The degree of embolism and its impact on xylem hydraulic function were evaluated differently in each technique. MicroCT produces visualization of embolism within a stem that can be viewed as 2D slices or 3D volume renderings (Fig. 1a,b). In this case, images clearly showed the size and location of embolized conduits, allowing for counts and morphometric analysis. It also was possible to track how embolism spread through the xylem with increasing water stress and to separate different regions of the stem for independent analysis. The OV technique detects the area of embolized conduits from one surface of the stem (Fig. 1c). Although the full cross-section of the xylem is not sampled, imaging occurs every 5 min allowing the temporal dynamics of embolism development to be explored in greater detail.

Previous studies utilizing microCT to measure cavitation resistance have based vulnerability curves on calculations of theoretical hydraulic conductance from measured vessel diameters. In this study, we compared metrics derived from analysis of microCT images to estimate the impact of embolism on xylem hydraulic function. For species in which vessel diameters could be measured, we observed strong agreement ($r^2 = 0.94$, $P < 3.4844e-16$) between theoretical PLC and percentage loss of

---

**Fig. 1** Visualization of embolism in stems of *Wisteria brachybotrys* during dehydration by microcomputed tomography (microCT) and the optical vulnerability (OV) technique. (a) Three-dimensional reconstruction of the stem volumes acquired by microCT. The accumulation of gas-filled vessels during dehydration is shown in orange. (b) Single transverse slice from within the microCT volume showing the location of embolized vessels (black) within the stem. The xylem water potential ($\Psi_x; \text{MPa}$) corresponding to each microCT image is indicated in grey. (c) Cavitation events recorded during dehydration by the OV technique. A colour map shows the spatial progression of cavitation with declining $\Psi_x$ over the course of dehydration.
Fig. 2 Relationship between the percentage of loss of vessels (PLV) and the percentage of loss of theoretical conductivity (PLC) calculated from conduit diameter following the Hagen–Poiseuille law. The grey line indicates the standard major axis (SMA) line fit between PLV and PLC, the black dotted line represents the 1 : 1 relationship and the grey shading represents the 95% confidence interval (CI). The SMA regression line was not significantly different in slope or elevation to the 1 : 1 line.

Discussion

We found that visual and hydraulic techniques produced similar estimates of xylem cavitation resistance (P50) across a range of woody plant species. Our results demonstrate that visual estimation of embolized conduits can accurately capture the impact of cavitation and embolism on xylem hydraulic function. Although some previous studies have compared visual and hydraulic techniques, this is the first study to compare microcomputed tomography (microCT) and optical vulnerability (OV) techniques directly with hydraulic methods across a diverse range of species representing major xylem anatomical groupings (tracheid bearing, diffuse porous, ring porous). We note that our results contrast with some recent studies reporting differences in estimates of P50 obtained from visual and hydraulic methods for two angiosperm species (Venturas et al., 2019; Pratt et al., 2020). Below, we discuss the advantages and disadvantages offered by each technique as well as causes of potential discrepancies between methods when estimating cavitation resistance.

Measurement of cavitation resistance with microCT

Trade-offs in sampling regime, image resolution, accessibility and expense were apparent between the techniques utilized in this study. MicroCT provides several advantages, including noninvasive assessment of plant hydraulic parameters and precise visualization of embolism spread through the xylem network. However, it also requires access to specialized facilities and instrumentation. This precludes the application of microCT in remote field-based studies, whereas laboratory-based studies are usually limited by relatively short periods of beamtime or the expense of using commercial instruments. Additionally, recent work has
identified potential sources of error and artefacts associated with microCT that bear further discussion. Potential errors and artefacts may be derived from damage to xylem tissue caused by high doses of X-ray radiation during scanning (Savi et al., 2017). With regards to measurements of cavitation resistance, there is no evidence that X-ray absorption during scanning causes cavitation; previous work has demonstrated that repeated scans do not alter the number of embolized vessels (Brodersen et al., 2010; Bouche et al., 2016; Choat et al., 2016; Venturas et al., 2019). In the current study, we observed no burning or damage to stems resulting from multiple scans. The strong agreement between hydraulic and microCT-based estimates of $P_{50}$ shown here and in previous studies provides further evidence that any damage caused by X-ray radiation has little impact on measurement of cavitation resistance.

Discrepancies between estimates of cavitation resistance also may arise from translation of microCT into percentage theoretical loss of hydraulic conductance (PLC) or of vessels (PLV) (Venturas et al., 2019). These effects could be derived from the presence of immature vessels, which appear water-filled but are not yet active (Pratt & Jacobsen, 2018; Bouda et al., 2019), or the influence of network level properties that are not taken into account when estimates of theoretical hydraulic conductivity ($K_h$), PLC or PLV are based on measurements of conduit dimensions in cross-section (Bouda et al., 2019). Our results show agreement between vulnerability curves constructed with microCT imaging and hydraulic methods, indicating that any discrepancies associated with translating visual data into loss of hydraulic function were small. This is consistent with previous studies that have shown good agreement between vulnerability
curves constructed with hydraulic methods and microCT (Torres-Ruiz et al., 2014; Choat et al., 2016; Losso et al., 2019). However, it is possible that small discrepancies observed between microCT and hydraulic measurements in our dataset are related to errors discussed above and these effects are important to consider when utilizing microCT for estimation of cavitation resistance. In some cases these effects can be easily accounted for; Nolf et al. (2017) demonstrated that $P_{50}$ derived from hydraulic measurements matched most closely to the $P_{50}$ of current year xylem estimated from microCT rather than the entire xylem cross-section. This provides an example of how our understanding of hydraulic dysfunction caused by water stress is enhanced by the application of noninvasive imaging techniques, which provide detailed information on the functional significance of embolism in different regions of the xylem.

The optical vulnerability technique

The OV technique provides a low cost and portable alternative to microCT that may be utilized in laboratory or field settings. When used in conjunction with psychrometers, the technique allows measurements of cavitation resistance to be fully automated, with water potential and cavitation monitored at short time intervals (Brodribb et al., 2017). This high temporal resolution reduces errors that may arise from less frequent sampling along the curve of one individual or construction of curves from multiple individuals. The technique also requires less plant material as a full curve is acquired for each individual and can be applied to multiple organs or sites on a given individual, for example simultaneous measurement of leaf, stem and root vulnerability (Rodriguez-Dominguez et al., 2018; Skelton et al., 2018).

In our dataset, $P_{50}$ values obtained with the OV technique were in close agreement with values obtained from hydraulic techniques and microCT (Fig. 6). Likewise, Brodribb et al. (2017) reported that estimates of $P_{50}$ provided by OV and hydraulic measurements were similar for 13 conifer species and one angiosperm (Brodribb et al., 2017). However, two recent studies have reported differences between vulnerability parameters derived from the OV method and hydraulic methods (Venturas et al., 2019; Pratt et al., 2020), and there are potential

![Fig. 4 Vulnerability curves for one ring porous and one liana angiosperm species produced using visual and hydraulic techniques (blue line, hydraulic method (a, d); pink line, microcomputed tomography (microCT) (b, e); and orange line, optical technique (c, f)). Coloured lines show the relationships between stem water potential ($\Psi_x$) and percentage of loss of conductivity (PLC, for hydraulic method), percentage of embolized conduits (for microCT) and percentage of embolized area (for optical technique). Each filled black dot is a measured datum. Vertical lines represent the $P_{50}$ (thick line) and the 95% confidence interval (CI, dotted lines). Grey area represents the CI of the curve.](image-url)
Considerations for hydraulic measurements

With reference to hydraulic techniques, we used the bench dehydration method for the majority of species in order to avoid issues associated with centrifuge and air injection techniques. Centrifuge-based techniques offer advantages in terms of time and plant material required to construct vulnerability curves, but can significantly underestimate cavitation resistance in angiosperm species with long vessels (Choat et al., 2010; Torres-Ruiz et al., 2014). However, centrifuge-based techniques are reliable for conifer species regardless of the variant of technique applied (Alder et al., 1997; Cochard et al., 2005, 2010; Li et al., 2007). Therefore, although the flow centrifuge (cavitron) method was used for measurement of P_{50} in our study, the estimate of P_{50} is directly comparable to those obtained from bench dehydration. Although the bench dehydration technique is labour-intensive, it is regarded as the most reliable hydraulic technique for measurement of cavitation resistance and provides a direct measure of impacts on hydraulic conductance. Even so, we note that a number of sources of experimental error have been identified with the bench dehydration method, including those associated

sources of experimental error that must be considered. Images are taken from a small region of the xylem on one side of a young stem and light only penetrates a short distance into the stem. This may create bias if the area of xylem visualized is not representative of the whole xylem cross-section, for example if recently formed outer conduits are more vulnerable to cavitation than conduits located deeper in the xylem. Additionally, if cavitation events within the xylem are patchy, as observed in some individuals with microCT imaging, then estimates of P_{50} will depend upon the timing of cavitation in the particular area of xylem being visualized. Such effects may explain the high level of variation in P_{50} observed in some cases (e.g. Fig. 4c, Fraxinus oxycarpa), although it also is possible that this variation represents plasticity between samples measured. In this case, the ability of the OV technique to capture entire replicate curves at high temporal resolution may be especially helpful in quantifying plasticity. Overall, the strong agreement between estimates of mean P_{50} for OV vs hydraulic and microCT techniques provides confidence that experimental error associated with the OV techniques can be overcome by sufficient sample size and care in preparation of plant material.
with excision of plant material (Wheeler et al., 2013) and measurement of xylem flow rates on the bench (Espino & Schenk, 2011; De Baerdemaeker et al., 2019). In this context, the close agreement between hydraulic methodology and in situ visual techniques suggests that all techniques tested here produce reliable estimates of cavitation resistance if the appropriate precautions are taken.

Conclusions
MicroCT and OV techniques produced similar estimates of P50 to hydraulic techniques when applied to the same plant material. This result held across seven species with contrasting xylem anatomy, demonstrating the general applicability of the techniques examined here. This is consistent with previous validation studies showing agreement between visual and hydraulic techniques for a further five angiosperm and 14 conifer species (Torres-Ruiz et al., 2014; Choat et al., 2016; Brodribb et al., 2017; Losso et al., 2019). We found that visualization of embolism produced similar vulnerability curves to those based on measurements of xylem hydraulic conductivity despite differences in the way embolism was quantified, that is, area of embolized xylem, counts of embolized vessels and theoretical hydraulic conductivity calculated from conduit diameter. Overall, these results provide confidence that a range of techniques can be employed to measure cavitation resistance, with the choice of technique dependent upon the particular questions posed, the location of experiments, and access to specialized facilities. The development of visual techniques presents numerous opportunities for improvements, including noninvasive observation of hydraulic

Fig. 6 Correlations between P50 values (means ± 95% confidence interval (CI)) obtained with microcomputed tomography (CT), optical vulnerability (OV) and hydraulic methods (HM) across seven species (a, b and c). The black line represents the 1 : 1 relationship. Confidence intervals (CI) for difference between the extracted bootstrap of two techniques (d, e and f). If this CI overlaps zero, then the two methods are not significantly different. Asterisks highlight the significant differences between methods.
function and full automation of measurements. Further work is required to resolve discrepancies between techniques observed by some authors (Venturas et al., 2019; Pratt et al., 2020), and to reduce potential sources of error that may arise from translation of visual data into loss of hydraulic capacity.

Acknowledgements

This work was supported by an ARC Discovery Project (DP170100761) to BC and TJB and an ARC Future Fellowship (FT130101115) to BC. We thank Daniel Hausermann, Chris Hall and Anton Maksimenko from the Australian Synchrotron Imaging and Medical Beamline in Melbourne for assisting with the micro-computed tomography methodology, as well as the technical staff at SOLEIL. Travel funding for AG to attend beamtime at SOLEIL was provided by the International Synchrotron Access Program (ISAP) managed by the Australian Synchrotron. CMR-D was supported by an Individual Fellowship from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no. 751918-AgroPHYS.

Author contributions

BC, TJB and AG conceived and designed the project; JMRP, MRCM, CMR-D, XL, RL, BC and AG collected the OV data and CT data from the Australian Synchrotron; SD contributed assistance with the cavitron technique and with AK, provided help with the CT technique in SOLEIL (France); and AG analyzed the data and wrote the first draft under the guidance of BC, DTT and BEM. All authors reviewed and assisted substantially with the manuscript development.

ORCID

Tim J. Brodribb https://orcid.org/0000-0002-4964-6107
Madeline R. Carins-Murphy https://orcid.org/0000-0003-4370-9485
Brendan Chat https://orcid.org/0000-0002-9105-640X
Sylvain Delzon https://orcid.org/0000-0003-3442-1711
Alice Gauthey https://orcid.org/0000-0002-4432-8249
Ximeng Li https://orcid.org/0000-0002-7816-5441
Rosana López https://orcid.org/0000-0003-3553-9148
Belinda E. Medlyn https://orcid.org/0000-0001-5728-9827
Jennifer M. R. Peters https://orcid.org/0000-0003-4627-7788
Celia M. Rodriguez-Dominguez https://orcid.org/0000-0003-2352-0829
David T. Tissue https://orcid.org/0000-0002-8497-2047

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

---

**Fig. S1** Relationship between the bagged leaf water potential measured using a Scholander chamber and the stem water potential measured using a psychrometer across three species with different xylem anatomy.

**Fig. S2** Example of stem reconstruction for four species with different xylem anatomy using the software DRISHTI.

**Fig. S3** Transverse surfaces of *Cedrus deodara* visualized by SEM and X-ray microCT.

**Fig. S4** Transverse slices produced with the OV technique for *Acacia aneura*.

**Fig. S5** Bootstrap CI for difference between extracted bootstrap of two techniques for *P*12 and *P*88.

**Table S1** *P*50 with CIs for the three different techniques and across seven species.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist Central Office*. 

---

**About New Phytologist**

- *New Phytologist* is an electronic (online-only) journal owned by the New Phytologist Trust, a **not-for-profit organization** dedicated to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews and Tansley insights.

- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication ‘as ready’ via *Early View* – our average time to decision is <26 days. There are **no page or colour charges** and a PDF version will be provided for each article.

- The journal is available online at Wiley Online Library. Visit [www.newphytologist.com](http://www.newphytologist.com) to search the articles and register for table of contents email alerts.

- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient, our USA Office (np-usaoffice@lancaster.ac.uk)

- For submission instructions, subscription and all the latest information visit [www.newphytologist.com](http://www.newphytologist.com)