X-ray microtomography (micro-CT): a reference technology for high-resolution quantification of xylem embolism in trees

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Abstract

As current methods for measuring xylem embolism in trees are indirect and prone to artifacts, there is ongoing controversy over the capacity of trees to resist or recover from embolism. The debate will not end until we get direct visualization of vessel content. Here we propose desktop X-ray microtomography (micro-CT) as a reference direct technique to quantify xylem embolism and thus validate more widespread measurements based on either hydraulic or acoustic methods. We used desktop micro-CT to measure embolism levels in dehydrated or centrifuged shoots of Laurel—a long-vesseled species thought to display daily cycles of embolism formation and refilling. Our direct observations demonstrate that this Mediterranean species is highly resistant to embolism and not vulnerable to drought-induced embolism in a normal range of xylem tensions. We therefore recommend that embolism studies in long-vesseled species should be validated by direct methods such as micro-CT to clear up any misunderstandings on their physiology.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.12391

Keywords
Xylem physiology; Cavitation; Embolism; Methods; X-ray microtomography; Imaging.

Highlights

There is ongoing debate over the validity of the techniques used to measure xylem vulnerability. X-ray microtomography (micro-CT) is proposed here as a reference technique as it enables direct visualizations of xylem embolism. These direct visualizations clearly show that xylem conduits are highly resistant to cavitation, which means embolism is unlikely to form and refill on a daily basis.

Introduction

In any experimental discipline, scientific progress is often tied to the development of breakthrough technologies. The field of tree hydraulics made decisive strides forward with the emergence of operational techniques for measuring leaf water potentials (Scholander *et al.* 1965), sap flow (Granier 1985) and xylem embolism (Milburn and Johnson 1966; Sperry *et al.* 1988; Cochard 2005). A crucial phase for validating these techniques was to confront them with reference methods. Pressure chamber data have been validated by psychrometer readings (Boyer 1967), sap flow in tree trunks has been validated by lysimetric recordings (Steinberg *et al.* 1990), but reference methods for xylem embolism data remain a glaring omission. The upshot is persistent unanswered questions over the validity of current indirect measurement-based techniques like loss of hydraulic conductance or ultrasound acoustic emissions.

The lack of a reference method for xylem embolism is closely related to the nature of sap transport in trees, which occurs under large negative pressures in microscopic conduits. These conduits have opaque walls that rule out direct optical observations of xylem content, and any intrusion into the xylem can immediately drag air into the conduits. All the current methods are indirect (Cochard *et al.* 2013) and therefore potentially prone to artifacts that may skew our understanding of plant physiology and ecology (Delzon and Cochard 2014). The recent literature is rife with reports of methodological biases (Cochard *et al.* 2010; Christman *et al.* 2012; Sperry *et al*. 2012; Tobin *et al*. 2013; Wheeler *et al.* 2013; Torres-Ruiz *et al.* 2014; Trifilò *et al.* 2014a; Wang *et al.* 2014). In short, there is no consensus on whether current techniques are valid as none of studies performed can make reference to a standard technique.

The recent publications by Wheeler *et al.* (2013) and Trifilò *et al.* (2014a) in this journal are illustrative of the situation. Wheeler *et al.* (2013) used indirect techniques to demonstrate that even cutting stressed xylem segments under water still causes air to enter the xylem conduits. The implication is that a sampling artifact could explain why other teams found high levels of embolism in vessels at midday when xylem is under large tension that were apparently repaired through the afternoon as xylem tension drops. In this issue, the study by Trifilò *et al*. (2014a) contradicts Wheeler's findings. Trifilò's team repeated Wheeler's experiments using similar techniques but concluded that cutting stressed segments under water caused a rapid entry of water into the xylem conduits, presumably by rapid refilling. Again, a sampling artifact could explain the low level of embolism observed by Wheeler *et al.* (2013) when xylem tensions were relaxed prior to sample excision. The discrepancy in the interpretation of similar experiments is patent, and the confusion will remain until the content of the intact xylem conduits is known for a normal range of operating xylem tensions.

Two distinct technologies have been employed to visualize xylem embolism in intact samples: Magnetic Resonance Imaging (MRI) and X-ray microtomography (micro-CT) (see Cochard *et al.* 2013 for a review). MRI systems suffer from poor spatial resolution (20 μm at best) and limited access to the technology (Holbrook *et al.* 2001). Micro-CT, in contrast, offers a spatial resolution typically around 1 μm, which is more than enough to visualize the lumen content of xylem tracheids or vessels. However, until recently, micro-CT has remained a fairly niche technology limited to synchrotron-based micro-CT systems (Brodersen *et al.* 2013). The recent emergence of powerful and affordable desktop-based micro-CT systems offers the first opportunity to propose this technology as a reference for xylem embolism studies (Charra-Vaskou *et al.* 2012; Suuronen *et al*. 2013, Torres *et al*. 2014).

Here, we illustrate how X-ray microtomography can serve as a reference method to visualize the exact content of intact xylem conduits. We will recommend this technique to be included by default in studies dealing with 'novel' xylem refilling (embolism repair under tension) or addressing the validity of more indirect methods in order to consolidate their conclusions. Reports suggest some tree species are able to reverse embolism (*Laurus, Sambucus, Olea, Populus*) and others not (*Acer, Sorbus*) (see Brodersen & McElrone 2013 for a review), and it was generally concluded that species that are highly vulnerable to drought-induced embolism rely on refilling embolized vessels as a survival strategy. We thus focused here on *Laurus nobilis* L.—a species known to having long xylem conduits and considered to be highly vulnerable to cavitation.

Materials and Methods

Experiments were conducted on 1 m-tall potted Laurel saplings grown outside in the INRA lab nursery at Clermont-Ferrand in fall 2013, i.e. when cambial activity had stopped.

Current-year leafy shoots were cut in air from well-watered *Laurus* saplings in the morning after sunrise, and brought to the lab where the shoots were rehydrated by immersing their cut end under tap water until experiments started. Two independent procedures were used to induce cavitation. First, four excised shoots (30 cm long) were allowed to dehydrate on a lab bench and their xylem pressure was measured with a Scholander bomb (PMS Instruments, Corvallis, USA) on covered leaves selected in the lower half of the shoots. An imaging cross-section was selected on an internode located 10 cm below the apex of each sample. All leaves below this internode were removed, but leaves above the internode were preserved in order to prevent air entry into the xylem vessels. The leaves were gently taped around the stem and wrapped in plastic paraffin film. A micro-CT scan was then recorded at 10 cm below the apex of each shoot, following the procedure detailed below.

For the second procedure, we used centrifugal force to induce cavitation. We selected two shoots similar to the previous ones, removed the leaf blades, cut (in air) their distal end just below the terminal leaf, and recut the proximal end to adjust the length to 28 cm. The imaging section was selected exactly at the middle of the segment, i.e. where xylem tension is highest during centrifugation (Cochard *et al.* 2005). A first micro-CT scan was acquired as described below to reveal the native state of embolism in each shoot. The shoots were then placed in a dedicated rotor (Cochard *et al.* 2005) with both ends inserted in two identical cuvettes containing 1 cm of distilled water to prevent any flow through the samples during centrifugation. The segments were centrifuged for two minutes in order to lower the xylem pressure at the imaging section to -1 MPa. The samples were removed from the centrifuge and a second micro-CT scan was acquired at the exact same imaging section. The procedure was repeated in 1 MPa steps until a minimum pressure of -6 MPa was reached.

To visualize xylem embolism, samples were first dropped in liquid paraffin wax in order to prevent their drying during the 38-minute scan, then placed in an X-ray microtomograph (Nanotom 180 XS, GE, Wunstorf, Germany) at the PIAF laboratory (INRA, Clermont-Ferrand, France). The field-of-view was a 4.6 × 4.6 × 4.6 mm^3 volume and covered the full cross-section of the samples. X-ray source settings were 50 kV and 275 μA, and 1000

images were acquired during the 360° rotation of the sample. At the end of the experiment, the sample was cut 3 mm above the scanned cross-section, injected with air (0.1 MPa), and re-scanned to visualize the complete map of the emptied vessels. It is important to note that if immature vessels were present, their lumen would have remained filled with cytoplasm after air-injection and they would not have been included in our embolism measurements.

After 3D reconstruction, the spatial resolution of the image was 2.0 × 2.0 × 2.0 μm per voxel. One transverse 2D cross-section was virtually extracted from the middle of the volume using VGStudioMax software (Volume Graphics, Heidelberg, Germany) then analyzed using ImageJ software (Rasband 2014) to determine percent embolism of each sample. Briefly, the diameters of the air-filled vessels were determined and their hydraulic conductivity K was calculated using the Hagen-Poiseuille equation. Only the conduits with a diameter >10 μm were considered, thus removing all non-conductive air-filled fibers from the analysis. The same procedure was applied to the cut samples, which enabled us to calculate the total sample conductivity K_{max}. Percent embolism in the sample was then computed as K-to-K_{max} ratio. Following Salleo *et al.* (2004) and Salleo *et al.* (2006), the percentages of embolism reported here for centrifuged samples are net of native levels measured at 0 MPa.

Maximum vessel length was determined using the air-injection method (Ewers and Fisher 1989). Eleven shoots similar to the ones used in the experiments described above were cut from the trees. The proximal cut end was connected to a compressed air tank (0.1 MPa) and the distal end was immersed in tap water. The shoots were then shortened by cutting 0.5 cm segments from the distal end until bubbles were seen coming out of the cross-section. This indicated that one vessel was cut open at both ends and the remaining sample length was taken as a measure of maximum vessel length.

Results

The micro-CT images revealed that level of embolism measured in the central position of the two segments used for these experiments was 17% and 22% (Fig. 1A). This level of 'native' embolism may be mostly induced during sample preparation. Indeed, maximum vessel length averaged 23.6 cm (SD=3.6, n=11) in the current-year Laurel shoots. Therefore, we expected to find a low proportion of vessels cut open and air-filled at center in the 28 cm-long samples used for centrifugation. Assuming that vessel end-walls at both sample-ends decrease according to an exponential law (Cohen *et al.* 2003), an estimated 12% of vessels were cut open at the micro-CT imaging cross-section, and we cannot exclude that several vessels may have become air-filled at the imaging cross-section when distal leaf blades were removed.

The number of air-filled vessels at the imaging section increased post-centrifugation only when xylem pressure dropped below -3 MPa (Fig. 1B, Fig. 2, open circles), and only 30% embolism was induced by exposing samples to -6 MPa (Fig. 1C). These values were consistent with the levels of embolism we measured using micro-CT images of *Laurus* branches exposed to xylem pressures in the range of -4.5 to -6 MPa by bench dehydration (Fig. 2, open squares).

Discussion

Brodersen *et al* (2013) were the first to publish a xylem embolism vulnerability curve (VC) using a synchrotron-based micro-CT system on *Vitis*. Torres-Ruiz *et al.* (2014) then constructed a VC for *Olea* trees using a desktop-based micro-CT. Both teams visualized the spread of embolism in xylem vessels during dehydration. Strikingly, they found that vessels remained full of sap until xylem pressures reached a threshold value as low as -1.5 MPa for *Vitis* and -3 MPa for *Olea*, and reached 50% embolism at -2.5 MPa for *Vitis* and -4 MPa for *Olea*. Furthermore, they found that most VCs obtained with indirect methods greatly overestimated xylem vulnerability in these long-vessel species, therefore suggesting artifacts due to the procedure for measuring embolism (cutting stems under water while the xylem was under large tensions or constructing VCs on samples containing cut open vessels). A similar artifact can be hypothesized for the work of Trifilò *et al.* (2014a) on *Olea* that reported a high level of xylem embolism above -2 MPa, i.e. within a range of pressures where micro-CT was unable to find any embolism events. The discrepancy is so high between the direct micro-CT observations of Torres-Ruiz *et al.* (2014) and the indirect estimates of xylem embolism by Trifilò *et al.* (2014) that it cannot be explained solely by differences in plant material. Therefore, direct micro-CT observations challenge the existence of daily cycles of embolism and refilling in Olive proposed by Trifilò *et al.* (2014a) and others before (Secchi *et al.* 2007).

Laurel (*Laurus nobilis* L.) was the first species reported to possess a so-called 'novel' refilling capacity, i.e. a capacity to refill embolized xylem vessels while bulk xylem pressure is significantly negative (Salleo *et al.* 1996). Many authors have since reproduced these same observations in Laurel (Tyree *et al.* 1999; Hacke & Sperry 2003; Salleo *et al.* 2004; Salleo *et al.* 2006; Nardini *et al.* 2011; Oddo *et al.* 2014; Trifilò *et al.* 2014b)—but the evidence supporting this novel refilling in Laurel has always been based on indirect estimates of xylem embolism; and there is still that possibility that sampling or measuring artifacts could have biased these results. To test this hypothesis, we visualized, for the first time, xylem embolism in dehydrated shoots of this model species by direct micro-CT observations. Our results show

that xylem vessels in Laurel were outstandingly resistant to cavitation, as the threshold xylem pressure inducing cavitation was *below* -3 MPa (Fig.1 C-D, Fig. 2). This finding is in stark contrast to the cavitation data previously obtained with indirect techniques on Laurel (Fig. 2) that suggest a pressure threshold for cavitation *above* -1 MPa. Once again, this suggests that sampling or measuring procedures may have biased the conclusion of previous studies exploning rapid refilling in Laurel in a range where their xylem conduits are not prone to cavitation. The strong discrepancy between the VC obtained here by micro-CT visualization and the VC published by Cochard (2002) with the Cavitron technique can be explained by the now well-established 'open-vessel' artifact associated with centrifuge-based techniques (Torres-Ruiz *et al.* 2014). Vessels cut open at both sample ends cannot sustain large tensions and become air-filled before embolism forms in intact vessels. The Cavitron technique revolves around measuring entire-shoot conductance during centrifugation and is thus strongly impacted by embolism forming at sample ends. The micro-CT approach only detects embolism forming in the center of the sample. As laurel vessels are relatively long, a small proportion of cut open vessels emptied all the way up to the imaging point. These vessels were discarded from our analysis, and the VC was constructed solely with intact vessels.

These recent data on Vitis, Olive and Laurel illustrate how micro-CT observations can serve as a reference method for the study of xylem embolism and refilling and help swiftly resolve controversies that would otherwise remain unsolved with more traditional approaches. However, it is important to note that, contrary to synchrotron facilities, desktop-based micro-CT apparatuses are often closed systems with a small chamber, and sample size is usually limited to a few decimeters. It can also be speculated that the high radiation dose to the sample over long duration scans may promote embolism, kill living tissue and therefore prevent refilling. Under this hypothesis, xylem embolism in Olive and Laurel would occur at even more negative pressures than those reported here.

Conclusion

Our findings suggest that indirect techniques can no longer be assumed to provide an unbiased reference VC in long-vesseled species and that the experimental evidence for 'novel' refilling warrants revisit (Cochard & Delzon 2013). We therefore strongly encourage scientists working on this refilling mechanism to first strengthen their findings with direct micro-CT visualization in order to ground their conclusions on evidence provided by direct observations. There is an urgent need to consolidate the techniques for measuring xylem

embolism in long-vesseled species if we are to finally understand the physiological and ecological significance of this trait.

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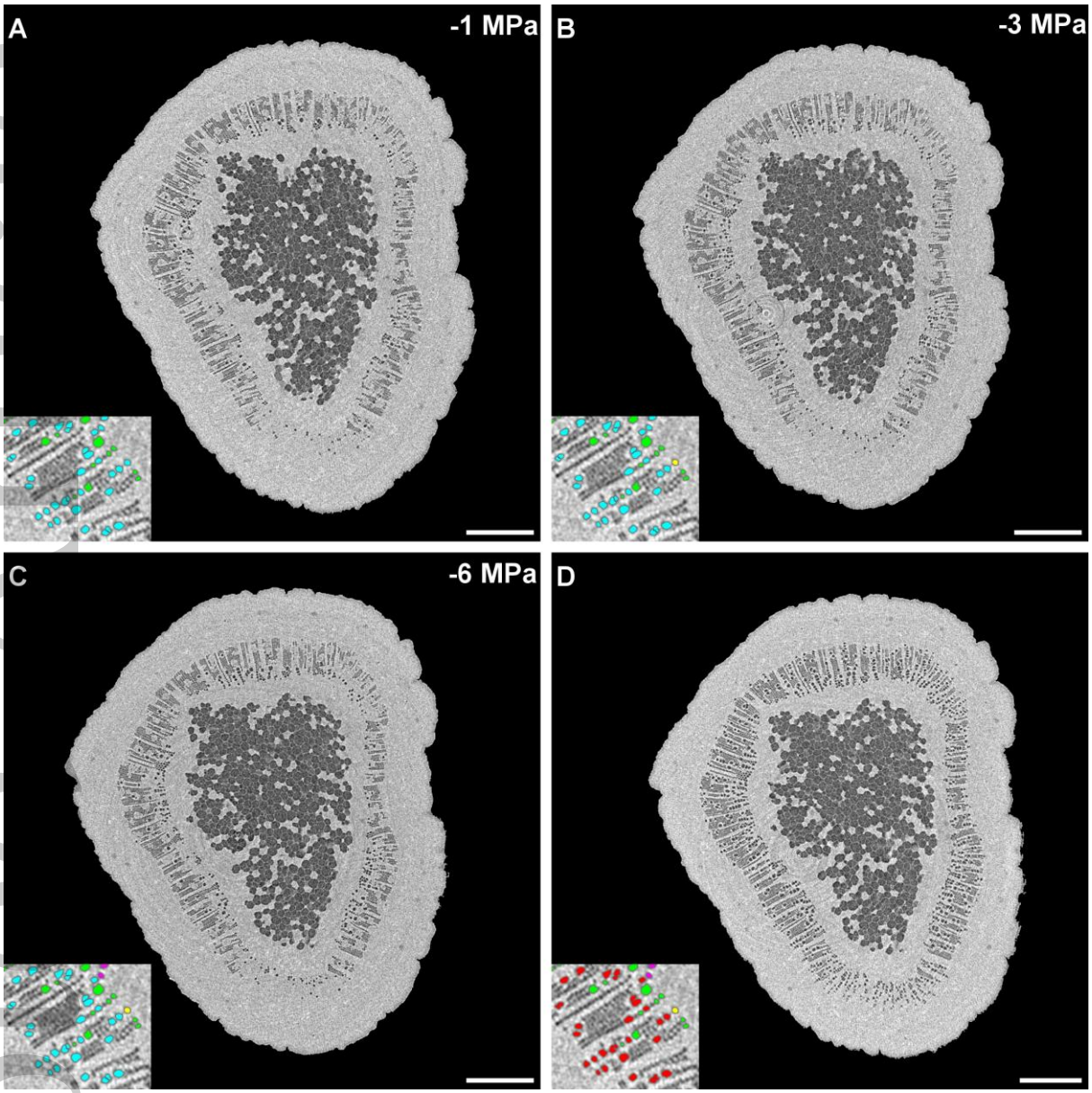
Figure 1: Direct visualization of xylem embolism by micro-CT technology. Laurel shoots were scanned in their central part and a 2D cross-section was reconstructed showing functional (gray) and air-filled (black) xylem conduits. Total number of xylem conduits was revealed by cutting the shoots a few mm above the initial scanned region (D). Newly-embolized vessels were observed above a native-state level only at pressures below -3 MPa. The magnified inserts show native embolism (green) and functional conduits (blue) becoming air-filled as xylem tensions increased (yellow then pink). Many vessels (red) have a cavitation pressure below -6 MPa. These direct observations demonstrate that Laurel is much more resistant to embolism that indirect methods had previously suggested. Scale bar = 1 mm.

Figure 2: Xylem embolism vulnerability curves for *Laurus nobilis* L. Xylem embolism formation and refilling have been intensively studied in Laurel by means of *indirect* methods (see references in the legend box). The results of these studies suggested that Laurel is highly vulnerable to embolism and that daily hydraulic failure and repair may therefore be routine. *Direct* observations of xylem embolism by X-ray microtomography paint a different picture (open symbols). Here, vessels remain functional until xylem pressure reaches a substantial level of tension that is far more negative than what indirect methods had suggested. Daily embolism is definitely not routine in Laurel and, therefore, which makes refilling irrelevant.



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