

Seeing is believing: what visualising bubbles in the xylem has revealed about plant hydraulic function

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ABSTRACT

Maintaining water transport in the xylem is critical for vascular plants to grow and survive. The drought-induced accumulation of embolism, when gas enters xylem conduits, causes declines in hydraulic conductance (K) and is ultimately lethal. Several methods can be used to estimate the degree of embolism in xylem, from measuring K in tissues to directly visualising embolism in conduits. One method allowing a direct quantification of embolised xylem area is the optical vulnerability (OV) technique. This method has been used across different organs and has a high spatial and temporal resolution. Here, we review studies using the OV technique, discuss the main advantages and disadvantages of this method, and summarise key advances arising from its use. Vulnerability curves generated by the OV method are regularly comparable to other methods, including the centrifuge and X-ray microtomography. A major advantage of the OV technique over other methods is that it can be simultaneously used to determine *in situ* embolism formation in leaves, stems and roots, in species spanning the phylogeny of land plants. The OV method has been used to experimentally investigate the spreading of embolism through xylem networks, associate embolism with downstream tissue death, and observe embolism formation in the field.

Keywords: cavitation, drought, embolism, imaging methods, optical vulnerability, plant hydraulics, vascular plants, xylem network.

Introduction

The transport of water within specialised conduits in the xylem is essential for vascular plant function, supplying water to photosynthetic tissues to enable growth and reproduction (Brodribb *et al.* 2007; Skelton *et al.* 2017b; Xiong and Nadal 2020). When stomata on leaves open during the day to facilitate CO_2 entry for photosynthesis, water vapour is lost from the leaf interior to the atmosphere via transpiration (Raven 2002; Brodribb *et al.* 2020b). This evaporation lowers water potential (Ψ) creating negative pressure on the water column and driving a flux of water from hydrated soils to the leaves.

During extended periods of soil drought, especially when associated with high evaporative demand, negative pressure increases within the xylem conduits, increasing the likelihood of air bubbles being pulled into the conduit lumen, expanding and forming an air cavity that blocks water flow through that conduit (i.e. embolism) (Tyree and Sperry 1989a; Kaack *et al.* 2021). As drought progresses, xylem function is threatened by hydraulic failure, which occurs when embolism spreads throughout the xylem network and causes a progressive decline in hydraulic conductance (K) that can ultimately result in plant death (Sperry and Tyree 1988; Torres-ruiz *et al.* 2016; Choat *et al.* 2018; Johnson *et al.* 2020; Guan *et al.* 2021).

Xylem vulnerability (or resistance) to hydraulic failure can be quantified by vulnerability curves (VCs) that describe the loss of K (or the accumulation of embolism) as a function of Ψ . Many of the earliest VCs were determined by quantifying the effect of Ψ on K (Sperry *et al.* 1988; Tyree and Sperry 1989a), with progressive declines in K during drought being attributed to increased embolism. Hydraulic VCs can be

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constructed in a number of different plant organs (Sperry *et al.* 1988), gravimetrically (Pockman *et al.* 1995), through measurements of rehydration kinetics (Brodribb and Cochard 2009) and evaporative flux from leaves (Brodribb and Holbrook 2006), or by measuring flow through xylem driven by centrifugation (i.e. Cavitron) (Cochard *et al.* 2013).

VCs can also be constructed by methods that associate physical phenomena with a quantification of xylem embolism. These include recordings of acoustic emissions (Tyree and Sperry 1989b) or the volume of air expelled from tissue under vacuum (i.e. the pneumatic method) (Pereira *et al.* 2016), both being associated with the amount of embolised conduits. There are also methods that directly distinguish between air-filled and fluid-filled conduits; e.g. dye colouration of functional xylem conduits (Hietz *et al.* 2008), cryo-scanning electron microscopy (cryo-SEM) (Cochard *et al.* 2000), magnetic resonance imaging (MRI) (Holbrook *et al.* 2001), X-ray microtomography (micro-CT) (Brodersen *et al.* 2010), and more recently, the optical vulnerability (OV) technique (Brodribb *et al.* 2016a).

The OV technique allows for the direct visualisation of embolism-induced changes in light transmission through or reflectance of the xylem while plants desiccate (Brodribb *et al.* 2016a). This technique can be conducted using relatively common and affordable equipment, which contrasts with other embolism visualisation techniques such as cryo-SEM, MRI and micro-CT, which are limited to researchers that have access to these specialised technologies. Thus, the OV method provides a highly accessible and affordable method for investigating xylem hydraulic function to a global scientific workforce (Cardoso *et al.* 2020b; Levionnois *et al.* 2020).

In this review, we conducted a meta-analysis of studies using the OV method that have been published following the development of this method (Brodribb *et al.* 2016a). First, we provide a detailed description of how the method works and how VCs are constructed using this approach, compare methods for determining cardinal Ψ from an optical VC, and discuss the limitations of this technique. Then, we summarise key insights related to xylem function that have been gained from the use of this method, including: (1) the association between xylem embolism and declines in K ; (2) embolism formation and spread; and (3) xylem resistance to embolism across species and organs. Finally, we provide future perspectives on the use of this method in the plant hydraulic field.

Visualising xylem embolism and constructing vulnerability curves using the OV technique

The development of the OV method was the first, simple, and affordable method that could provide a clear visualisation of embolism formation and spread through the xylem network

through rapid changes in light transmission associated with embolised, gas-filled xylem (Brodribb *et al.* 2016a). To date, this method has been applied to visualising drought-induced embolism events in stems (Brodribb *et al.* 2017; Rodriguez-Dominguez *et al.* 2018; Gauthey *et al.* 2020), roots (Rodriguez-Dominguez *et al.* 2018; Li *et al.* 2020b), leaves (Brodribb *et al.* 2016b; Cardoso *et al.* 2018; Rodriguez-Dominguez *et al.* 2018; Cardoso *et al.* 2020a; Chen *et al.* 2021; Guan *et al.* 2021; Jacob *et al.* 2022), and floral parts (Zhang and Brodribb 2017; Bourbia *et al.* 2020) (see Supplementary Table S1). A key benefit of using the OV method to construct VC is that in most cases, it does not require the use of excised segments, which places it amongst a small number of purely non-invasive techniques for quantifying hydraulic function, making it useful for tracking dynamic embolism formation in intact plants in the field (Wagner *et al.* 2022). Numerous studies have utilised intact plants when constructing OV method VCs (Skelton *et al.* 2017a; Cardoso *et al.* 2018, 2020a; Johnson *et al.* 2018; Corso *et al.* 2020; Gauthey *et al.* 2020; Li *et al.* 2020b). To date, VCs of a dozen economically important herbaceous species as well as early-diverging species (i.e. ferns, lycophytes, and Polytrichalean bryophytes) have been constructed using intact plants (Skelton *et al.* 2017a; Cardoso *et al.* 2018, 2019; Johnson *et al.* 2018; Brodribb *et al.* 2020a; Corso *et al.* 2020; Suissa and Friedman 2021).

Vulnerability curves based on the OV method are often constructed using fully hydrated plants or organs, given that this method can only detect new embolism events, with a limited capacity to determine the extent of pre-existing embolism in the xylem. Intact plants can be taken to the laboratory and the soil washed from the roots to accelerate plant dehydration (Cardoso *et al.* 2018). Whenever intact whole plants are not available, such as plants grown in natural settings, long branches exceeding the length of the longest vessel can also be excised under water and transported to the laboratory so that the OV method can be performed in stems (Skelton *et al.* 2021), leaves (Guan *et al.* 2021), or in both simultaneously. Excessive transpiration can lead to Ψ decline and embolism events occurring before imaging commences, and to avoid this, transpiration from excised material can be reduced by bagging and maintaining under low light levels ($<10 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$). To capture images, a range of devices have been used, providing a considerable advantage in resource poor scientific communities. They include stereo microscopes with cameras (Cardoso *et al.* 2018), custom-made clamps (<http://www.opensourceov.org>) (Johnson *et al.* 2020; Avila *et al.* 2021), flatbed scanners (Hochberg *et al.* 2017; Lucani *et al.* 2019), or mobile phone cameras (Petruzzellis *et al.* 2020), all of which can provide sufficient spatial resolution to capture changes in light transmittance or reflectance on the formation of an embolism (Brodribb *et al.* 2016a; Cardoso *et al.* 2018; Rodriguez-Dominguez *et al.* 2018; Johnson *et al.* 2020).

Reflected light is generally ideal for imaging stems, while transmitted light is often ideal for leaves (Brodribb *et al.* 2016b, 2017; Avila *et al.* 2021). Changes in visual light transmission caused by embolism can be readily observed in intact heterobaric leaves, roots and flower parts. Homobaric leaves can also be successfully imaged when imaged close to the midrib (Brodribb *et al.* 2016b). When leaves are imaged on excised branches that exceed maximum vessel length, there appears to be little impact of the excised end on the determination of leaf vulnerability (Brodribb *et al.* 2016b; Zhang and Brodribb 2017; Skelton *et al.* 2018; Levionnois *et al.* 2020; Guan *et al.* 2021). Care should be taken not to harvest individual leaves or leaves into which vessels extend from the excision, as this results in the rapid propagation of embolism through the leaf at very modest Ψ (Guan *et al.* 2021). In stems, a small area of periderm must be carefully removed so the xylem can be observed (Brodribb *et al.* 2017; Johnson *et al.* 2020). Touching the xylem when removing the periderm may induce localised embolism and must be avoided. A layer of adhesive hydrogel applied to the exposed xylem is required to improve light transmission and reduce evaporative water loss (Johnson *et al.* 2020). Even though debarking the stem to expose the xylem has been questioned, with Venturas *et al.* (2019) claiming it might damage the xylem and alter the way this tissue will respond to dehydration, the amount of artificial embolism induced by careful bark removal and the application of hydrogel is negligible (Johnson *et al.* 2020). For species where the periderm cannot be easily removed without damage to the xylem, or in species with a vascular stele such as lycophytes and ferns, thin paradermal sections can be made to expose the xylem before the hydrogel is applied (Cardoso *et al.* 2020c; Suissa and Friedman 2021).

Materials dehydrating slowly (*c.* a few days) under dark conditions (to allow whole plant Ψ equilibrium) are used to image the xylem to construct OV curves, and imaging frequency is generally in the range of 1–3 mHz. The Ψ can be assessed using two primary methods, although others are potentially available (Dainese *et al.* 2022). A psychrometer (ICT PSY Armidale, Australia) is typically attached to the stem and readings are taken at a frequency of 0.5–1.6 mHz. At the same time, a regular pressure chamber can be used to periodically cross-validate these stem Ψ measurements using leaves (Brodribb *et al.* 2016b; Cardoso *et al.* 2018; Gauthey *et al.* 2020; Johnson *et al.* 2020). Although both measurements of Ψ often match (Gauthey *et al.* 2020), the reliability of xylem Ψ measured in leaves using the pressure chamber once extensive embolism has accumulated is low (Ritchie and Hinckley 1971). Thus, the use of a stem psychrometer ensures an accurate estimation of Ψ , especially in species in which vulnerability segmentation occurs, whereby leaves are less resistant to embolism than stems (Charrier *et al.* 2016; Levionnois *et al.* 2020; Avila *et al.* 2021). Upon completion of desiccation and the

cessation of embolism formation, a regression (mostly but not always linear) between the drying time and Ψ measurements is yielded and used to describe the change in water status as plants dehydrate (Brodribb *et al.* 2016b; Cardoso *et al.* 2018).

Image sequences are terminated when all conduits are embolised (Brodribb *et al.* 2016a). To ensure that all embolism events are captured, images can be taken for several hours after the last embolism event is registered (Cardoso *et al.* 2018) and finally by severing the organ within 2 cm of the imaged area while still imaging can be used to ensure no additional embolism events were missed. Following data collection, embolism events are identified from the image sequence using a simple image subtraction method described by Brodribb *et al.* (2016a) and Brodribb *et al.* (2016b). The most common approach is the use of open-source ImageJ (NIH) software where successive pairs of images are subtracted, yielding the embolism-induced changes in the light transmission between the two images, whenever embolism is present. Noise from image subtraction needs to be next removed through a series of tools provided by ImageJ, such as smothering filters to increase the difference between noise and embolism events and thresholding the image sequence by choosing a threshold that maintains the maximum extent of events and minimisation of background noise. Any potential residual noise and small artefacts must then be manually removed, which is the most time-consuming aspect of this method (see below for common examples of noise and artifacts in image collection in the limitations section). A more detailed description on how to capture, process and analyse image stacks, can be found at <http://www.opensourceov.org/>. This website also provides users with the OpenSourceOV ImageJ Toolbox; i.e. a special macro for ImageJ (Fiji) containing several functions to facilitate data analysis. The manual image processing of vulnerability curves using ImageJ software is labour intensive, but there are reports of the potential development and utilisation of AI technology which has the potential to greatly enhance the speed of image processing (Y. Sorek, pers. comm.; <https://twitter.com/YonatanSorek/status/1417164616044589061>). This would be a widely adopted advance as image analysis remains the primary disadvantage of the OV method in terms of human hours and computer processing power for the rapid generation of VC.

A key benefit of the OV method is that it requires a lower number of biological samples to construct VCs when compared with other methods that directly quantify xylem embolism, particularly those that do so in leaves. By using the OV method, a complete vulnerability curve can be obtained in a single leaf, petal, or stem. Most other methods utilise several organs (often exceeding 20) to construct each VC (Table S1). This is because samples can only be measured once (Savi *et al.* 2017). Additionally, the OV method can be performed in almost any laboratory (Petrizzellis *et al.* 2020), contrasting with the cryo-SEM, MRI and micro-CT methods that are geographically restricted to a few locations, or are

costly. The combination of both simplicity and reliability contrasts with these other techniques (Cochard *et al.* 2019; Cardoso *et al.* 2020b). To date, VCs have been performed in 126 plant species since its development in 2016. When compared with the micro-CT, for instance, which was developed in 2010, the OV method has been utilised in less studies (36 compared to 47) but in considerably more species (126 compared to 43) and to construct VCs of several soft organs not often visualised by micro-CT (e.g. leaves, roots, floral parts in angiosperms as well as stipes and rhizomes in ferns and lycophytes) (Table 1; Table S1).

The use of micro-CT to validate embolism resistance quantified through the OV method

Several studies have compared the P_{50} (water potential inducing 50% embolism in the xylem or 50% loss in K , depending on the method) obtained from VCs using the OV method with other hydraulic or imaging-based techniques for determining xylem embolism resistance. There are many papers that report similar P_{50} values across a diversity of species when embolism is determined using micro-CT and the OV method in stems (Cardoso *et al.* 2020c; Gauthey *et al.* 2020; Pratt *et al.* 2020) and leaves (Emilio *et al.* 2019; Corso *et al.* 2020) (closed symbols; Fig. 1a; Supplementary Table 2). In comparing the P_{50} values obtained using micro-CT and the OV methods for stems and leaves when published in separate studies but in the same species, we also found strong congruence between values (open symbols; Fig. 1a; Table S2). Across leaves and stems of 20 species and 20 studies, the relationship between the micro-CT determined P_{50} and the OV determined P_{50} yielded a regression that closely agreed with the 1:1 relationship (Fig. 1a) ($r = 0.96$, $P < 0.0001$).

Table 1. The number of studies that have used the optical vulnerability (OV) technique or the micro-CT method (either synchrotron or desktop-based) to construct vulnerability curves (VCs), the number of plant species in which embolism resistance was assessed by each of the two methods in stems, or in organs other than stems (e.g. leaves, roots, flower parts, fern stipes), and the number of angiosperm and non-angiosperm species assessed by each of the two methods.

| Parameters | OV technique | Micro-CT |
|--|--------------|----------|
| Number of studies using the technique | 36 | 47 |
| Number of species for VCs in stems | 58 | 37 |
| Number of species for VCs in organs other than stems | 89 | 26 |
| Number of angiosperm species used for VCs | 103 | 37 |
| Number of non-angiosperm species used for VCs | 23 | 6 |

Data were obtained from all papers using at least one of the aforementioned techniques and that were published up to August 2021. See Table S1 for complete dataset and references.

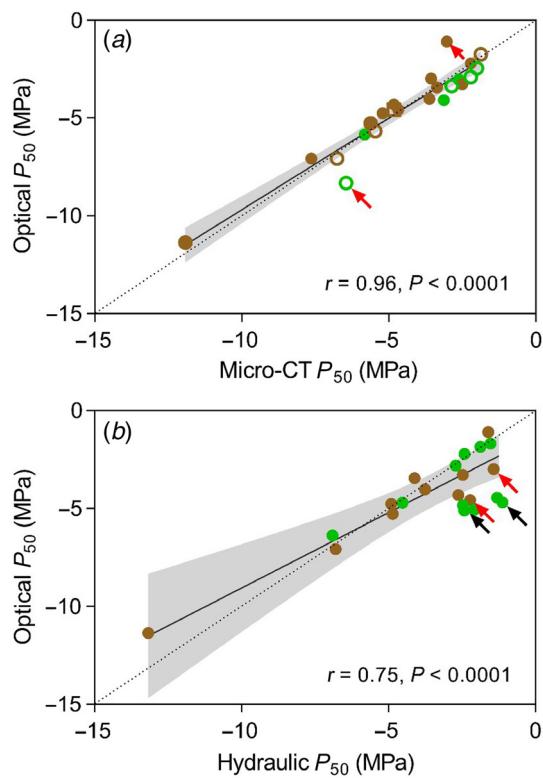


Fig. 1. (a) Correlation between mean P_{50} measured with the OV method and the micro-CT [either synchrotron-based (circle symbols) or desktop-based (square symbol)] ($n = 25$). (b) Correlation between mean P_{50} measured with the OV method and the standard hydraulic method ($n = 23$). Data are from leaves (green symbols) and stems (brown symbols) of a wide range of plant species obtained from studies that performed more than one method in the same species (closed symbols) or from different studies performed on the same species (open symbols). Arrows highlight data that are discussed in more detail within the main text. All linear regressions closely agree with the 1:1 line (dotted line). Grey area represents the 95% confidence interval. See Table S2 for complete dataset and references. Optical and micro-CT P_{50} (water potential inducing 50% embolism in the xylem). Hydraulic P_{50} (water potential inducing 50% loss in hydraulic conductance).

The strong agreement between the micro-CT P_{50} and OV P_{50} (Fig. 1a), supports the conclusions of Johnson *et al.* (2020) who performed micro-CT and the OV method in the same stems and found that the OV method reliably captured xylem embolism events to a depth of 0.3 mm into the stem and that this range of analysis is representative of embolism across the entire stem, when small stems are measured. Minimal congruence between these two methods is reported in one study examining the branches *Populus trichocarpa* Torr. & A. Gray (Venturas *et al.* 2019) (brown symbol pointed by an arrow in Fig. 1a). However, a number of unconventional approaches were taken in the generation of the OV data in this study. These authors utilised silicone vacuum grease on the exposed and imaged stem surface rather than a transparent hydrogel, which might have resulted in air

entry to the xylem or obscured minor embolism events. Secondly, when determining the VC from the image stack, the authors considered all embolism events as equal in value despite observing differences in the amount of pixels per embolism event, which is known to represent differences in the embolised xylem area. This procedure for embolism determination renders the curve an accumulation of embolism event numbers (not sizes) across Ψ , and this curve has no interpretive value for hydraulic function whatsoever. There is also some disagreement between the OV method determined P_{50} of -8.32 MPa and the micro-CT determined P_{50} of -6.46 MPa for leaves of *Laurus nobilis* L. (green symbol pointed by an arrow in Fig. 1a). It is possible, however, that this variation occurred due to a high intra-specific variability in leaf embolism resistance within this species, given that leaves were collected from different individuals adapted to different regions in Europe (Klepsch *et al.* 2018; Lamarque *et al.* 2018; Rodriguez-Dominguez *et al.* 2018). A ~ 1.50 MPa variation in embolism resistance between leaves in the same canopy has been observed in other Lauraceae species and has been attributed to being the cause of partial leaf and canopy death during drought (Cardoso *et al.* 2020a). There are also reports of considerable variation across the canopy in branchlet embolism resistance in the highly drought resistant conifer *Callitris rhomboidea* R. Br. (Johnson *et al.* 2022), and these differences have also been associated with differences in the lethal Ψ threshold for branch survival under drought.

Limitations to OV method

A primary limitation to performing the OV method is the considerable human hours associated with analysing images to generate the VCs. Although algorithms can be customised to identify and quantify embolism events during imaging analyses (Hochberg *et al.* 2017, 2019), all images need to be further manually analysed to remove any potential remaining noise originated from movements, insects, tissue drying fronts, bubbles in the hydrogel, and minor changes to light environment. In this regard, automating the analysis of the VCs yielded with the OV method would be a considerable improvement to the utility of this method for the screening of phenotypes across large populations. This, however, might be improbable in the near future given the diverse nature of interference to an image stack.

Another intrinsic limitation to the OV method is that it cannot detect native or pre-existing embolism (Chen *et al.* 2021), which can impact quantification of relative xylem embolism resistance (Avila *et al.* 2022), and the translation of embolised xylem area to a percent loss of K . As such, only plants cultivated under well-water conditions, plants that have been previously exposed to drought but are known to refill their xylem (Cardoso *et al.* 2018), and

branches and leaves formed during wet seasons (which are expected to have very low levels of pre-existing embolism) are ideal to construct VCs with the OV method. Recently, it has been suggested that significant native embolism produced by drought is restricted to very extreme events in field grown trees, so the issue of pre-existing embolism on accurate VC determination in field grown plants may be minimal (Guan *et al.* 2022; Wagner *et al.* 2022). It is important to maintain the plant material in plastic bags with damp paper towels until the beginning of experiments whenever collecting branches in the field to construct VCs in order to prevent excessive water loss and any potential embolism prior to analysis (Skelton *et al.* 2021). Although the OV method cannot directly quantify the native embolism level, it has been very recently demonstrated that it is possible to indirectly assess the level of native embolism with high confidence by utilising the VCs obtained using this method and measurements of the lowest Ψ experienced by the plant in the field (Chen *et al.* 2021).

Similar to other methods that directly assess embolism resistance, the OV method is liable to error due to open vessel artifacts if the branch or leaf being measured is shorter than the longest vessel (Guan *et al.* 2021). Hence, knowledge of maximum vessel length is required prior to harvesting branches for image analysis, so that images can be captured beyond the length of the longest open vessel (Martin-StPaul *et al.* 2014; Torres-Ruiz *et al.* 2014). During analysis, there is a number of major classes of artefacts that appear in image subtractions that could be confused for embolism events to an untrained eye (e.g. movement, drying from post embolism, and insects), although embolism events are readily apparent in image subtractions (Fig. 2).

Movement

During the process of capturing images, stems and leaves might move if not firmly secured. In some species with large volume changes in stems or leaves during dehydration, movement will occur during imaging regardless of how secure the sample is, particularly prior to the formation of embolism (Johnson *et al.* 2018). If there is a sudden movement of the sample, it could appear as a widespread change, or localised changes, during image subtraction. This can be avoided, when possible, by making sure the sample is secured during imaging. An alternative is to increase the imaging rate, although this results in trade-off with processing time. Imaging at a frequency of 1.0 Hz could eliminate most movement noise while still capturing embolism events, at the expense of considerable processing power and time to analyse the larger image stacks. If movements do occur while taking imaging at a lower frequency, they are often easy to recognise as distinct from embolism events, as the movement will affect large or localised regions of the sample area in one image rather than events in individual xylem elements

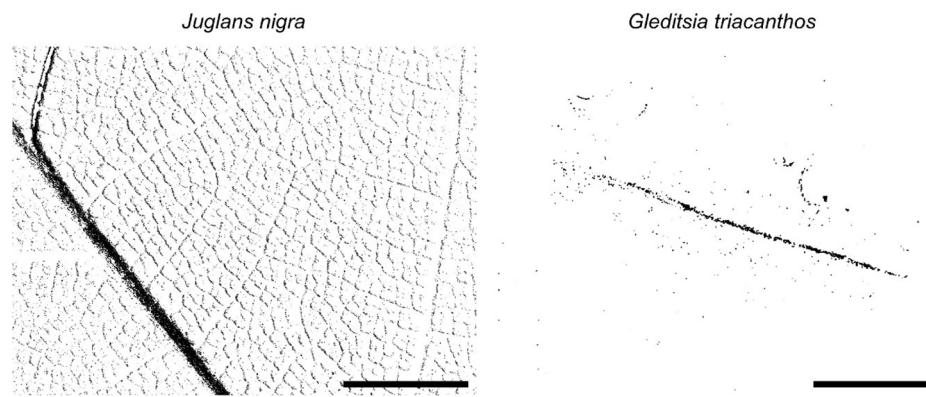


Fig. 2. Embolism events in the leaf veins of *Juglans nigra* L. and the stem xylem of *Gleditsia triacanthos* L. Images depict image subtractions before the removal of noise. Scale bars = 250 µm.

(Fig. 3). The hydrogel, or bubbles in the hydrogel, may also move with time in the imaged area, but the movement is very slow and can easily be recognised in the raw images.

Drying front post embolism

The image subtractions may also identify a sequential series of colour changes even if the sample is firmly secured during the final stages of drying after most, if not all embolism events have occurred. This results from a rapidly moving drying front, often observed in leaves as the mesophyll desiccates. A drying front has a tendency to spread from a location and radiates across the imaged area in each sequential subtraction (Fig. 4).

Insects

If the plant material is collected from plants grown in field settings, the presence of insects may be recognisable during image subtractions and it needs to be deleted from image sequences. Smaller bugs can also move onto a leaf or stem, attracted by the light used for imaging. Insects are easily distinguishing from embolism events during image subtraction because they appear as small circles that occur in a random nature in single frames. Additionally, the organisms are visible and easy to be recognised in the raw images (Fig. 5). It is also possible that larger insects may appear and fully obscure the field of view. Phloem feeding insects that can move onto the leaf during imaging may be observed feeding, and there are reports that such feeding can cause an embolism events (Kim 2013). To reduce the impact of insects on OV curve collection, damp paper towels can gently clean both leaf surfaces, or insecticide can be used to greatly reduce insect load.

Light intensity

The visualisation of embolism using the OV method is based on changes in light, either transmitted or reflected, meaning

that any change in ambient or applied light intensity could cause an artefact across the whole sample or in one region of the imaged sample. This could happen, for example, if using a microscope to image, and the light levels in the room fluctuate. To avoid this error, a cylindrical or conical shield can be placed over the sample to reduce the effect of any sudden changes in ambient light intensity. Light must also be constant and consistent, which can be challenging to maintain when using a flatbed scanner. It is also important to appropriately set the light intensity or camera exposure time at the beginning of an experiment, to avoid over saturation of the vessels at high light levels, thus rendering embolism events undetectable in the images, or not visible at low light intensities. Also, specific requirements for intensity need to be evaluated for each species individually.

Limitations to analysis

There are some species in which generating VC using the OV method is not possible. Our research group have had issues measuring both leaves and stems of certain species, and we assume that these issues can be attributed to two broad anatomical causes. First, wood colour is one major limitation when assessing stem vulnerability. Once the periderm is removed most species have greenish or yellowish sap wood that changes in colour on embolism formation; i.e. a change that is apparent to the naked eye and after imaging. In contrast, other species including *Aesculus glabra* Willd. and *Magnolia salicifolia* Maxim. have very pale sapwood, so that embolism is very challenging to visualise even when using directed light and high-resolution microscopic imaging. Second, thick, homobaric leaves also pose a challenge for generating VCs using the OV method. Leaf veins require the transmission of light to capture embolism events and if a leaf has no bundle-sheath extensions (like in homobaric leaves) and is thick, very little light will transmit through the veins. We have found this to be an issue in some species such as *Ginkgo biloba* L.. A simple test to check

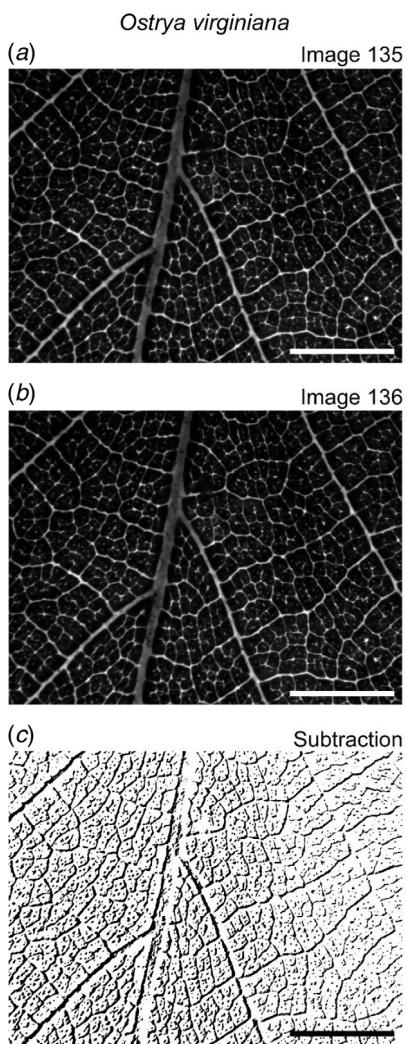


Fig. 3. Images of two sequential frames of a leaf of *Ostrya virginiana* (Mill.) K. Koch captured 300 s apart (a, b) that was shrinking on mesophyll desiccation. The image subtraction of these two frames is shown (c), with movement due to shrinking occurring across the entire leaf surface. Scale bars = 250 μ m.

whether a leaf will be appropriate for image analysis is to hold it up to the sun or high light source and see if midribs and minor order veins are visible. If so, then OV vulnerability curves should be accessible. In the case of homobaric leaves, one option is to generate OV curves for leaves from imaging the midrib solely (Lucani *et al.* 2019).

Another intrinsic limitation of the OV method remains in understanding how much the hydraulic vulnerability, especially in leaves or soft organs, is impacted by the percentage of cumulative embolised area of the xylem. Although, most data collected so far demonstrate a close association between the OV P_{50} and the hydraulic P_{50} (Fig. 1b; Supplementary Table 2), this may not occur in all species (see below).

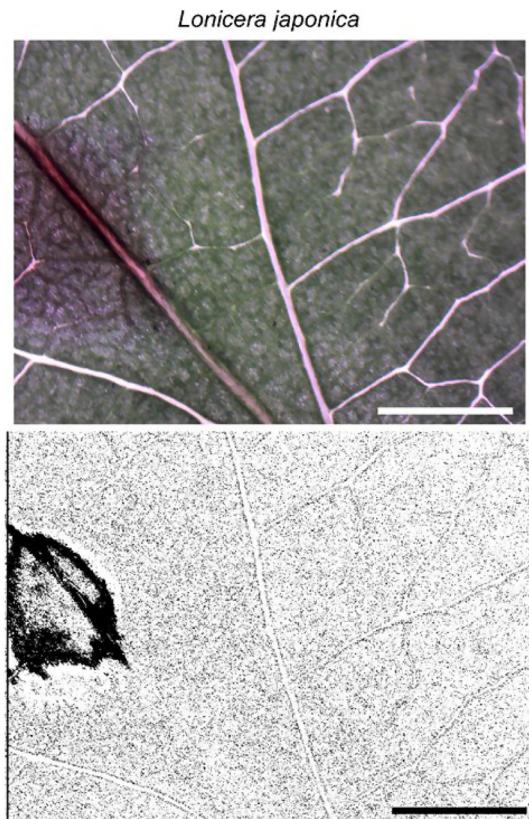


Fig. 4. The onset of a drying front spreading across a fully embolised leaf of *Lonicera japonica* Thunb. The upper image depicts the raw image, and the lower image depicts an image subtraction showing the progress of the drying front over 5 min. Scale bars = 250 μ m.

Issues with determining cardinal water potentials from OV curves

Most plant hydraulic physiologists are interested in comparing the embolism resistance of genotypes or species. A key metric for this comparison is P_{50} but other cardinal Ψ such as P_e (the Ψ of first embolism), P_{12} (the Ψ when 12% of xylem is embolised or conductivity is lost) and P_{88} (the Ψ when 88% of the xylem is embolised or conductivity is lost) are often used. P_{88} is often associated with leaf or canopy death in angiosperms (Brodrribb and Cochard 2009). A key advantage of the OV method is that these cardinal Ψ values can be taken directly from VCs. However, issues can arise when trying to extract Ψ values for a particular % embolism when individual embolism events span that threshold. Often values are taken from the Ψ when an embolism event spanned the given cardinal threshold. Curve fitting is commonly used to determine cardinal Ψ from invasively generated VCs but it is unnecessary and can be even problematic for determining cardinal Ψ from OV curves (Fig. 6). Using a dataset of vulnerability curves from six leaves of *Persea americana* Mill., we show that mean Ψ at P_{50} taken directly from the curves is -2.24 ± 0.08 MPa.

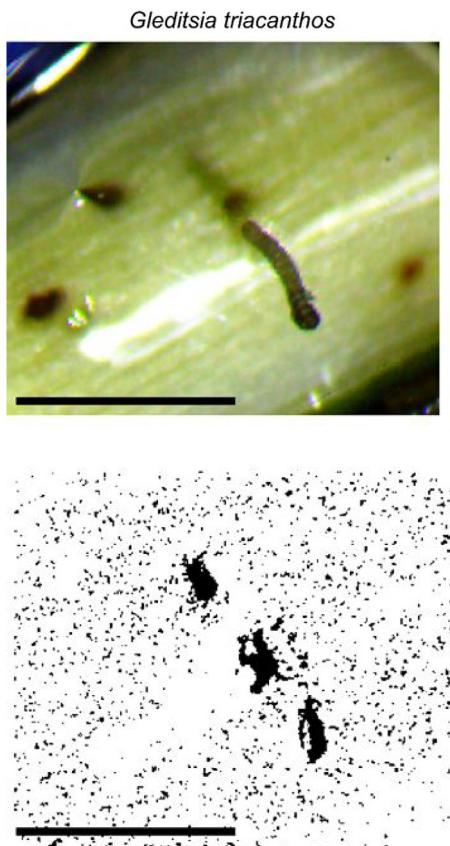


Fig. 5. Caterpillar imbedded in hydrogel and moving as images were taken of a stem of *Gleditsia triacanthos*. The upper panel depicts a raw image of the trapped caterpillar, and the lower panel depicts an image subtraction demonstrating the movement of this insect and shadow in 5 min. Scale bars = 250 μ m.

This value is similar to the P_{50} estimated from a fitted Weibull model -1.91 ± 0.01 MPa and to the mean of individually fitted Weibull functions to the data obtained on individual leaves (-2.24 ± 0.08 MPa) (Table 2). Differences, however, arose when comparing P_{12} and P_{88} values for fitted curves with the absolute values of embolism resistance (Table 2). Thus, we recommend that mean Ψ and standard errors are calculated and plotted rather than using fitted curves for OV curves as per Avila et al. (2021).

What we have learned about water transport in the xylem by utilising the OV method

Associating xylem embolism with declines in hydraulic conductance

Xylem embolism has long been known to reduce K (Sperry et al. 1988; Tyree and Sperry 1989a). Thus, methods that quantify declines in K were believed to measure embolism

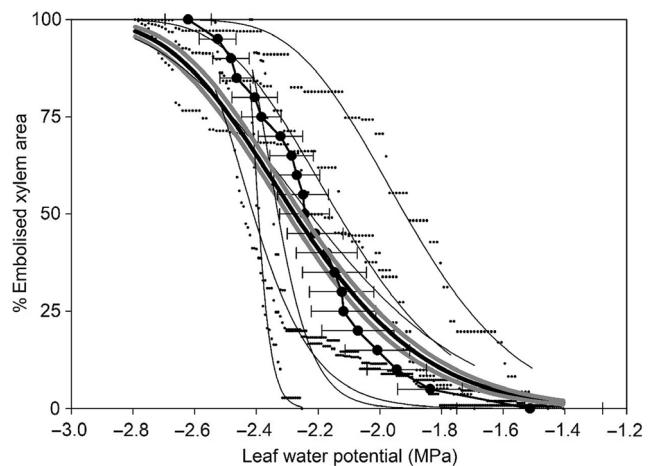


Fig. 6. Comparison of methods used to analyse optical vulnerability curves of the leaves of *Persea americana* (data taken from Cardoso et al. 2020a). Small dots represent optical vulnerability data from six individual leaves. Thin black curves represent individual best fits of a sigmoidal Weibull function to data obtained on single leaves [fitted using the fitplc package in R (Duursma and Choat 2017)]. The thick black curve represented the fitted Weibull sigmoidal curve through the entire dataset (grey lines represent the upper and lower confidence intervals). Large dots with standard errors represent the mean water potential at 5% increments of embolised xylem area.

formation (Sperry et al. 1988; Cochard et al. 1992, 1999, 2005, 2013; Stout and Sala 2003; Stiller 2009; Awad et al. 2010). The development of reliable methods directly quantifying xylem embolism, mainly the micro-CT and more recently, the OV method, have allowed researchers to test whether declines in K are always the result of xylem embolism formation, irrespective of the plant organ or species. The OV P_{50} for the stems of several species closely matches the P_{50} obtained with the Cavitron (*in situ* flow centrifuge), which assess declines in K (Brodribb et al. 2017). In leaves, the hydraulic P_{50} determined by rehydration kinetics also matches the OV determined P_{50} in several plant species (Brodribb et al. 2016b; Skelton et al. 2017a; Corso et al. 2020). A strong agreement between the OV method-determined P_{50} and the hydraulic method- P_{50} can be observed across the literature (Fig. 1b; Table S2).

The main progress attributed to methods directly quantifying xylem embolism (including the OV) relies in demonstrating that although xylem embolism will always result in decreased K , the latter might also occur prior to the formation of embolism as a result of extra-xylary events. Early studies confirmed these reductions to K outside of the xylem utilising cryo-SEM (Cochard et al. 2004; Zhang et al. 2014) and micro-CT (Bouche et al. 2016) in the leaves of conifers (*Taxus baccata* L., *Pinus* spp., and *Podocarpus grayi* de Laub.), which can experience considerable shrinkage and deformation of transfusion tracheids during drought, resulting in K declines in the absence of xylem embolism

Table 2. Mean cardinal water potentials (Ψ) from vulnerability curves of six *Persea americana* leaves (data taken from Cardoso et al. 2020a).

| | P_{12} | s.e. | P_{50} | s.e. | P_{88} | s.e. |
|--|-----------|----------|-----------|----------|-----------|----------|
| Means taken directly from curves | – 2.00787 | 0.104472 | – 2.2442 | 0.080448 | – 2.48253 | 0.059365 |
| Curve fitted through all data | – 1.8499 | 0.041634 | – 1.91886 | 0.010426 | – 2.62706 | 0.027756 |
| Mean from curves fitted to data from individual leaves | – 0.92 | 0.154179 | – 2.23573 | 0.083634 | – 2.45307 | 0.063634 |

For means taken directly from curves, cardinal Ψ were taken as the Ψ recorded when embolism events crossed the threshold percentage. Curves were fitted through the whole dataset using the `fitplc` package in R (Duursma and Choat 2017) or through the percentage embolism datasets for each leaf, which were then used to calculate mean and standard error values for each cardinal Ψ . $P_{12, 50, 88}$ (water potential inducing 12, 50, 88% embolism in the xylem).

(Brodribb and Holbrook 2005). In addition, recent studies have demonstrated with measurements of evaporative flux determined leaf K and micro-CT that leaves from a number of woody angiosperm species experience declines in leaf K decoupled from xylem embolism (Scoffoni et al. 2017), a result also demonstrated for leaves of *Caragana* spp. (green symbols pointed by black arrows in Fig. 1b) by comparing data from the OV method with evaporative flux determined VCs (Yao et al. 2021). Finally, by comparing VCs obtained hydraulically, by rehydration kinetics, and the OV method, the shoot K of two *Selaginella* spp. have been recently demonstrated to decline considerably prior to embolism formation in the stem (brown symbols pointed by red arrows in Fig. 1b) (Cardoso et al. 2020c). In the later study, the utilisation of micro-CT revealed that an air-filled lacuna formed in the stems when plants of *Selaginella* were dehydrated, disconnecting the central vascular stele from the cortex and likely explaining the relatively hydrated declines in the shoot K (Cardoso et al. 2020c).

In the case of soft organs, such as leaves, flower parts and fine roots, which are more likely prone to shrinkage (Scoffoni et al. 2014; Bouche et al. 2016) and observations that the K of leaves and roots relies on the conductance of several other tissues other than the xylem, including the activity of membrane aquaporins (Sade et al. 2014; Buckley et al. 2015; Moshelion et al. 2015), it is possible that outside xylary events taking place prior to xylem embolism will influence the hydraulic P_{50} of a number of other species. Therefore, we believe that the combination of hydraulic and observation xylem methods is a powerful approach to fully understand the sequence of physiological events taking place in plant organs during drought. Additionally, only by combining these two approaches, we will be able to thoroughly understand why agreement between the OV P_{50} and the hydraulic P_{50} is observed for some species, but not others.

Regarding potential artifacts associated with the OV method that could potentially result in contrasting hydraulic and OV P_{50} values, two recent studies assessing the stems of *P. trichocarpa* (Venturas et al. 2019) and *Quercus douglasii* Hook & Arn. (Pratt et al. 2020) found less negative P_{50} using hydraulic methods than the P_{50} obtained by the OV method (Pratt et al. 2020). The authors then suggested that the OV method overestimated the xylem resistance of *Q. douglasii* stems because this method was

believed to only sample a small portion of the outer xylem tissue, which can be more resistant to embolism than the xylem of more internal xylem regions. In this study the OV curves matched micro-CT curves, which instead points to issues with the determination of embolism resistance by hydraulic means in species with long vessels (Choat et al. 2010; Cochard et al. 2010, 2013; Jansen et al. 2015; Torres-Ruiz et al. 2017).

Embolism formation and spread

In addition to insights into the threshold Ψ for embolism formation, the OV method further provides a high spatial and temporal resolution to explore the mechanisms of embolism spread throughout the xylem network. In a study utilising the OV technique, Johnson et al. (2020) demonstrated that more than 80% of the stem embolism events occurred in single vessels in three dry forest angiosperm with low connectivity xylem networks, suggesting a close association between vessel arrangement and embolism spread. In another study utilising the OV method fully embolised, excised petioles, resulted in more vulnerable leaf xylem because of an enhanced spreading of embolism in six woody angiosperm species (Guan et al. 2021). Finally, the OV method can map the spread of embolism in leaves across spatial and temporal scales for the first time, revealing that larger vein orders present the first embolism events thus leading to a widespread embolism of minor veins (Brodribb et al. 2016a). This general sequence of xylem embolism throughout the leaf veins was further confirmed for several species with different venation patterns (Brodribb et al. 2016b; Skelton et al. 2017a; Johnson et al. 2018; Avila et al. 2021).

The widespread embolism formation in leaves has been associated with leaf mortality in a study that directly assessed the level of embolism in the petiole of drought-induced necrotic leaves using the cryo-SEM (Cardoso et al. 2020a). In this study, a positive strong correlation was obtained between petiole embolism level and foliar necrotic area in a set of leaves spanning different levels of necrosis and embolism and the variation in leaf mortality across canopy was linked to an intra-specific variability in the leaf P_{50} obtained through the OV method (Cardoso et al. 2020a). The OV method has been used to provide the first

foundational evidence confirming the connection between leaf hydraulic failure and tissue damage during water stress by exposing plants to drought, or leaves to severe evaporative stress, and associating the leaf area damaged by dehydration with the direct observation of embolism in the veins irrigating that region of the leaf using the OV method (Brodribb et al. 2021).

Xylem resistance to embolism across different species and organs

The OV method has been utilised to study more angiosperm species than any other plant group (Table 1; Fig. 7; Table S3), likely due to the larger number of species, economic importance of this lineage of land plants, and ease with which the OV method can obtain VCs in long-veined angiosperm species, which contrasts with prior methods that are restricted to short-veined and tracheid-bearing species (Choat et al. 2016; Scoffoni et al. 2017; Sergent et al. 2020). When comparing average data on the mean stem P_{50} of gymnosperms ($n = 16$) with those of angiosperms ($n = 60$), we obtain statistically similar values (-4.71 and -4.81 MPa, respectively) (Fig. 7a), with *Acacia aneura* F. Muell. (an angiosperm) being the most resistant species in the dataset (P_{50} of nearly -12 MPa) (Gauthey

et al. 2020). This dataset contrasts with the commonly held view that gymnosperms are more resistant to embolism than angiosperms due to the presence of torus-margo pit membranes (Maherali et al. 2004; Choat et al. 2012). It is possible that the conclusions from previous studies demonstrating gymnosperms outcompeting angiosperms in terms of embolism resistance have been drawn from meta-analyses including underestimated values of P_{50} for several angiosperm species, and an over-sampling of mesic temperate species, that were obtained with invasive-methods (Maherali et al. 2004; Choat et al. 2012). It should be noted that we only provide a partial dataset that does not include data from a wide diversity of gymnosperm species including *Callitris tuberculata* R. Br., the most embolism resistant tree species so far studied (Larter et al. 2015), and that additional studies and meta-analyses are necessary in order to more accurately compare the P_{50} of angiosperms and gymnosperms.

In addition to stem vulnerability, over 100 values ($n = 114$) of leaf P_{50} have been obtained with the OV in the last 5 years (Fig. 7; Table S3). Although xylem embolism in the stem is believed to be the main driver for plant mortality (Adams et al. 2017; Choat et al. 2018; Hammond et al. 2019), leaves are the key organs responsible for photosynthesis, with any embolism having major impacts on plant productivity (Brodribb et al. 2021). Leaves also represent one of the main resistors to water flow within plants (Sack and Holbrook 2006). The large amount of data collected for angiosperm leaves using the OV method allows us to confirm a general trend towards lower embolism resistance in leaves (P_{50} of -3.84 MPa) when compared with stems (P_{50} of -4.81 MPa) (Fig. 7b), as proposed by the hydraulic vulnerability segmentation hypothesis (Tyree and Ewers 1991). However, studies determining the P_{50} of different organs with the OV method within the same species (Table S4), yield contrasting results, suggesting instead that hydraulic vulnerability segmentation is species-specific. It has been observed that many species do present such segmentation across organs (Zhang and Brodribb 2017; Rodriguez-Dominguez et al. 2018; Skelton et al. 2018; Bourbia et al. 2020; Li et al. 2020b; Avila et al. 2021), while many other studies using either the micro-CT or the OV method do not report any vulnerability segmentation (Skelton et al. 2017a; Losso et al. 2019; Corso et al. 2020; Li et al. 2020a; Smith-Martin et al. 2020; Peters et al. 2020; Skelton et al. 2021).

Because of the low sample number of species in which roots (P_{50} of -6.34 MPa, $n = 2$) and petals (P_{50} of -2.72 MPa, $n = 4$) in which embolism resistance using the OV method has been observed (Fig. 7b), it is hard to draw conclusions about the general vulnerability of these organs relative to other organs. In fact, roots have been observed to be more (Rodriguez-Dominguez et al. 2018), less (Peters et al. 2020) or equally resistant (Li et al. 2020b) compared to stems, depending on the species. Likewise, petals and flowers (in which embolism resistance was first assessed using the OV) have been demonstrated to display higher or similar P_{50}

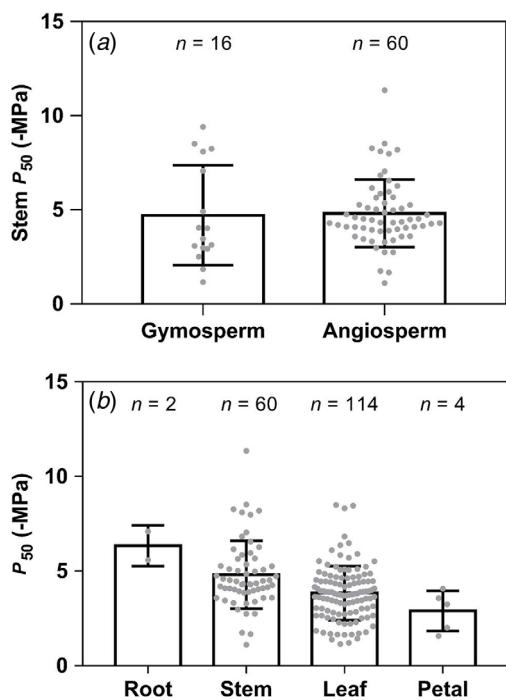


Fig. 7. (a) Stem P_{50} obtained from studies utilising the OV method for a set of gymnosperm and angiosperm species. No difference between the two groups was found according to Student's t -test ($P = 0.923$). (b) P_{50} of roots, stems, leaves and petals of angiosperms obtained from studies utilising the OV method. Data are means \pm s.d. Gray dots are original data. See Table S3 for complete dataset and references. P_{50} (water potential inducing 50% embolism in the xylem).

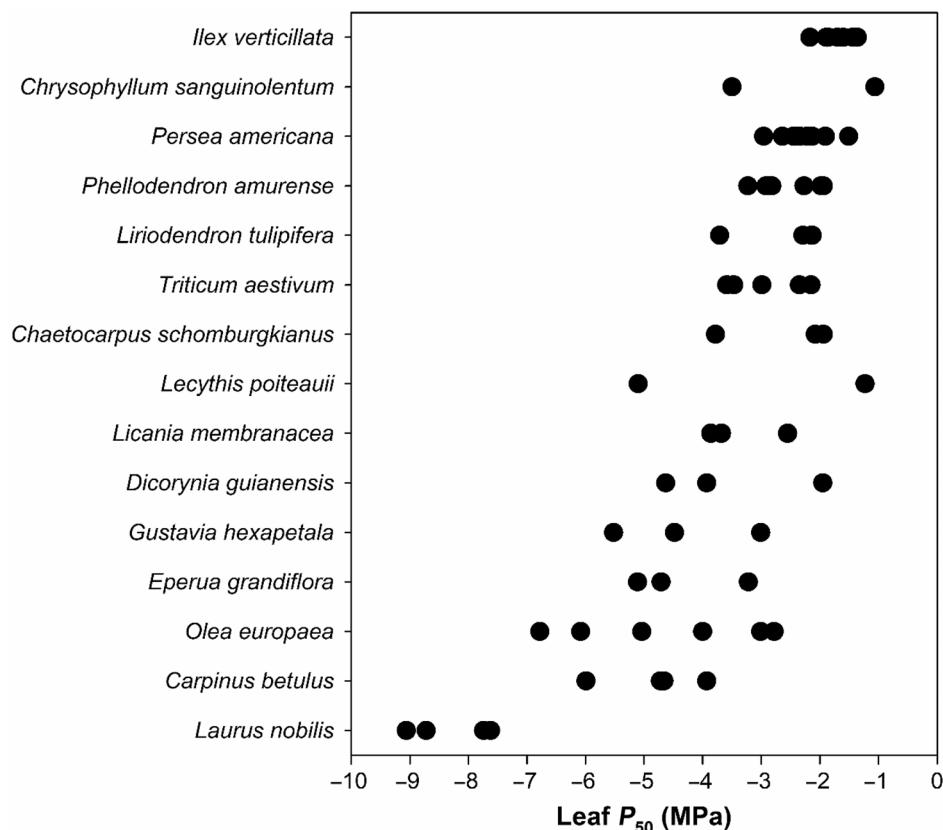


Fig. 8. Water potential inducing 50% embolism in the xylem (P_{50}) of individual leaves obtained by the OV method in several angiosperm species. See Table S5 for complete dataset and references.

than leaves across different species (Zhang and Brodribb 2017; Bourbia *et al.* 2020). Conversely, a recent study utilised the OV method to assess the xylem vulnerability to embolism in peduncles of tomato and found that they are considerably more resistant than leaves, allowing this annual species to maintain fruit development during drought (Harrison Day *et al.* 2022).

Finally, the newly developed OV method has allowed researchers to determine variation in embolism resistance across different leaves (Fig. 8; Table S5). Until the OV was developed, no other method could determine the P_{50} of a single leaf, so the variation of this trait across leaves could not be assessed. Recent studies using the OV method have found that there can be a considerable variability in leaf P_{50} between leaves in the same species (Fig. 8). Such large intra-specific variation in the leaf P_{50} can result in heterogenous leaf damage across canopies (Cardoso *et al.* 2020a).

Concluding remarks

Because of its reliability associated with simplicity and accessibility, the OV method has been extensively utilised by the plant hydraulic community since its development in 2016. Through this method, researchers have confirmed

several hydraulic mechanisms such as a threshold Ψ for embolism formation that is both species- and organ-specific and that embolism spread depends on the xylem conductivity and the presence of adjacent gas source. This method has also allowed researchers to demonstrate other hydraulic events for the first time such as: (1) mapping embolism spread throughout the leaf vein network; (2) intraspecific variability of leaf P_{50} (and even across the canopy); and (3) the direct association between leaf xylem embolism and leaf mortality. This method also provided researchers with the first measurements of embolism in floral parts and it is likely to continue being utilised to understand how embolism forms, spreads, and impacts all the plant organs (including soft ones) as well as early-diverging species such as ferns and lycophytes that have been neglected in the past.

Supplementary material

Supplementary material is available online.

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