

# No gas source, no problem: proximity to pre-existing embolism and segmentation affect embolism spreading in angiosperm xylem by gas diffusion

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## Abstract

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## Key-words

Bordered pits, embolism, vessel network, angiosperm xylem, drought stress, optical method, pneumatic method, gas diffusion

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**Running title:** Pre-exist embolism affects embolism spreading

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## Author contributions

XG performed the experiments, with help from LP and SAMM. SJ and XG conceived the original ideas. SJ with KC supervised the project. XG wrote the manuscript with support from SJ, and revisions from all authors.

## Introduction

Xylem sap in plants is frequently transported under negative pressure (Dixon and Joly, 1895; Jansen and Schenk 2015). Under conditions of low soil water content and/or high transpiration rates, the tensile force of xylem sap may increase considerably, which could lead to interruption of water transport in tracheary elements by large gas bubbles (embolism). Understanding the frequency and mechanism behind embolism formation in plant species is important because the amount of embolised conduits may affect the transport of xylem sap, and therefore photosynthesis (Zhu *et al.* , 2013; Martin-StPaul *et al.* , 2017). There is strong and convincing evidence that drought-induced embolism formation occurs via bordered pits in cell walls of adjacent conduits (Zimmermann, 1983; Sperry & Tyree, 1988; Jansen *et al.* , 2018; Kaack *et al.* , 2019). It has frequently been assumed that once the pressure difference between sap-filled conduits (under negative pressure) and embolised ones (under atmospheric pressure) exceeds a certain threshold, embolism spreads from an embolised conduit to a neighbouring one via the mesoporous pit membranes of bordered pits (Choat *et al.* , 2008; Tixier *et al.* , 2014; Wason *et al.* , 2018; Avila *et al.* , submitted). Although embolism spreading from previously embolised conduits has been well presented in many textbooks and papers (Zimmermann, 1983; Crombie *et al.* , 1985; Choat *et al.* , 2016; Lamarque *et al.* , 2018), various basic questions about this process remain unclear (Kaack *et al.* , 2019). Since gas movement across pit membranes may involve

two different processes, namely mass flow and diffusion, we prefer the general term embolism spreading or propagation instead of air-seeding, which includes mass flow of gas across a pit membrane only.

An important question is whether spreading of embolism in xylem tissue is facilitated by the presence of pre-existing embolised conduits, because this would raise questions about the absolute value of embolism resistance, and whether or not the mechanism behind embolism spreading depends on a certain pressure difference. Pre-existing embolism could be embolised conduits from a previous growth ring or protoxylem (Kitin *et al.* 2004; Sano *et al.* , 2011; Hochberg *et al.* , 2016). Embolised conduits and local spreading of embolism could also occur when herbivores or xylem feeding insects damage conduits, or when a plant organ experiences other types of damage or die-back. Artificial embolism spreading may occur when xylem tissue has been cut open to take embolism resistance measurements, because when a transpiring plant is cut in air, the air-water meniscus is quickly pulled back into the conduit lumina until it stops at an interconduit pit membrane (Zimmermann, 1983). A widely used approach to evaluate embolism resistance is to measure the xylem water potential that corresponds to 50% loss of hydraulic conductance ( $\Psi_{50}$ , MPa), while the xylem water potential corresponding to 50% of the total amount of gas that can be extracted from a dehydrated xylem tissue has been suggested as an alternative, direct approach of embolism quantification (Pereira *et al.* , 2016, 2020a; Zhang *et al.* , 2018; Oliveira *et al.* , 2019). Both experimental approaches rely on cut plant organs either due to the requirements to measure hydraulic conductivity, or gas diffusion kinetics of dehydrating samples. Moreover, dehydration of a cut branch or leaf can proceed much faster than dehydration of an intact plant (Cochard *et al.* , 2013; Hochberg *et al.* , 2017). Other methods, however, such as microCT observations and the optical method can be used to quantify embolism in a non-destructive way in intact plants (Brodribb *et al.* , 2016a, b, 2017; Choat *et al.* , 2016; Lamarque *et al.* , 2018).

The amount of embolism propagation could be limited by hydraulic segmentation, which represents a hydraulic constriction or bottleneck of the conduit network (Zimmermann, 1983; Tyree & Ewer, 1991; Levionnois *et al.* , 2020). In a broad sense, hydraulic segmentation has also been described as compartmentalisation, connectivity, sectoriality, or modularity, and may include narrow conduit dimensions and/or poorly interconnected conduits, which increase the resistance of the hydraulic pathway (Ellmore *et al.* , 2006; Loepfe *et al.* , 2007; Espino & Schenk, 2009). It has frequently been suggested that these constrictions of the hydraulic pathway may cause a difference in embolism resistance, which is defined as vulnerability segmentation (Tyree and Ewers, 1991; Levionnois *et al.* , 2020). Leaves, for instance, are said to be less embolism resistant than stem xylem based on the vulnerability segmentation hypothesis, although results across a broad range of species are mixed and could partly be explained by the different methods used (Zhu *et al.* , 2016; Klepsch *et al.* , 2018; Skelton *et al.* , 2018; Levionnois *et al.* , 2020). Also, it has not been tested yet whether vulnerability segmentation is affected by pre-existing embolism.

In a few studies, considerable differences in embolism resistance have been reported between intact plants and xylem tissue. Cut-open stem xylem of *Vitis vinifera* and *Laurus nobilis* , for instance, were suggested to underestimate embolism resistance (Choat *et al.* , 2010, Torres-Ruiz *et al.* , 2015; Lamarque *et al.* , 2018). Removal of leaves in seedlings of the ring-porous species *Quercus robur* was found to result in artificial embolism formation in stem xylem based on microCT (Choat *et al.* , 2016). In a few species, however, the bench dehydration method, which is a widely applied method for hydraulic estimations of embolism resistance, was found to show no difference in embolism resistance between cut, dehydrating branches and dehydration of intact plants of *Quercus* and *Populus* (Bréda *et al.* , 1993; Tyree *et al.* , 1992; Skelton *et al.* , 2018). While more species need to be studied to understand a possible artefact associated with embolism spreading from cut-open xylem, two explanations could be suggested for this observed discrepancy, i.e. why cut-open xylem may reduce embolism resistance and facilitate embolism propagation. First, it is possible that the cutting of conduits with sap under negative pressure introduces a cutting artefact, with embolism formation due to a sudden pressure drop (Wheeler *et al.* , 2013; Torres-Ruiz *et al.* , 2015). Second, embolism spreading could be prevented by hydraulic segmentation, which may occur at growth rings, nodes, and the transition between organs, such as leaf petioles or side branches (Sano *et al.* , 2011; Levionnois *et al.* , 2020). Indeed, vessels are known not to run completely randomly, but may end near nodes, side branches, stem-petiole transitions, and between the vascular bundles of the petiole and major veins (Salleo *et al.* , 1984;

André *et al.* , 1999, André, 2005, Wolfe *et al.* , 2016).

In this paper, we aim to test to what extent cut-open angiosperm xylem has an effect on embolism spreading in leaves across a diverse selection of six temperate species. In the first and the second experiment we investigate if embolism resistance of leaf xylem was affected by the proximity to cut-open conduits. We hypothesise that leaf xylem would be more vulnerable to embolism for detached leaves with a cut petiole compared to leaves attached to stem segments. However, not only the proximity to cut-open vessels, but also hydraulic segmentation at the stem-leaf, or the petiole-leaf blade transition could affect embolism spreading, and may prevent a potential artefact in measurements of embolism resistance near cut xylem tissue. We included species with both deciduous and marcescent leaves (i.e. species that retain dead leaves on the plant), and diffuse porous and ring-porous wood, because hydraulic segmentation can be associated with leaf phenology and vessel dimensions. If pit membranes in bordered pits of vessels and tracheids would function as safety valves that avoid the spreading of embolism from embolised to functional conduits, it is possible that embolism spreading is reduced by the number of interconduit endwalls and/or the connectivity between conduits (Kaack *et al.* , 2019; Johnson *et al.* , 2020). Species that show hydraulic segmentation, may have safety valves composed of many tracheids and/or narrow, fibriform vessels.

Since drought-induced embolism is frequently reported to initiate in large vessels, while narrow and short vessels or tracheids embolise typically later at lower xylem water potentials (Scoffoni *et al.* , 2017; Klepsch *et al.* , 2018), we tested if embolism spreading in minor veins with narrow and short conduits would also be affected by the proximity to a cut-open vein. In this second experiment, we expect that narrow and short vessels near cut minor veins would embolise before embolism occurs in the large vessels of major veins, which would make narrow vessels seemingly more vulnerable than wide ones. We also predict that embolism spreading in minor veins near artificially induced cuts has a rather limited, local distribution due to the short dimensions of minor veins.

Finally, we aimed to test whether or not embolism spreading differs between intact vessels that are close to cut-open vessels in a petiole, and intact vessels in leaf veins that are located further away from cut-open xylem. For this reason, the Pneumatron and the optical vulnerability method were applied to the same detached leaf. If the pneumatic method would be subject to a potential artefact due to gas extraction from intact vessels that are neighbouring embolised, cut conduits, this method could systematically underestimate embolism resistance compared to the optical method. The pneumatic method, which estimates the changing gas volume in intact vessels during dehydration, showed a good agreement with hydraulic methods applied to stem segments (Pereira *et al.* , 2016, Zhang *et al.* , 2018). Direct comparison of the pneumatic and optical method to detached leaves of *Eucalyptus camaldulensis* suggested no significant difference for this species (Pereira *et al.* , 2020a), although a larger number of species should be tested to confirm this finding.

The three complementary sets of experiments will help us to address the question of whether embolism spreading in angiosperm xylem relies on a certain pressure difference threshold between embolised and functional conduits.

## Materials and methods

### Study site and plant material

The six angiosperm species studied included *Betula pendula* , *Carpinus betulus* , *Fagus sylvatica* , *Liriodendron tulipifera* , *Prunus avium* , and *Quercus petraea* . Samples were collected at Ulm University, Germany (48°25'20.3" N, 9°57'20.2" E). Except for *L. tulipifera* , all species are common, native angiosperm trees to Ulm. Various individuals of *L. tulipifera* were grown at the Botanical garden of Ulm University. Most species studied were diffuse porous, except for *Q. petraea* , which was ring-porous. Also, *C. betulus* and *Q. petraea* were considered as species with marcescent leaves during winter, with partial marcescent leaves in *F. sylvatica* . The remaining three species are deciduous. We selected four to five mature trees for each species. Healthy, mature and sun-exposed branches were sampled between June and September 2019 for experiment 1 and 3, and in May 2020 for experiment 2.

## Vessel length measurements

Maximum vessel length of stems and leaf petioles was determined by applying the air injection method (Greenidge, 1952). After connecting the basipetal part of a stem or petiole to a syringe, a ca. 150 kPa pressure was applied, while the acropetal part was kept under water. Successive cuts at the proximal stem/petiole end were made under water with a razor blade at intervals of 10 mm for stems and 2 mm for leaves until the first continuous stream of air bubbles could be seen emerging from the cut end. The corresponding length was then measured and recorded as the maximum vessel length of a stem or leaf petiole ( $MVL_{\text{stem}}$  and  $MVL_{\text{petiole}}$ , respectively). At least six stems or leaves were taken for these measurements (Figure S1).

We measured the maximum vessel length at the stem-leaf transition for leaves attached to 0.5 cm long stem segments. This approach was applied to six samples per species based on the air injection method. The syringe was connected to the short stem sample, and the leaf was shortened until bubbles could be seen emerging from the cut end.

The vessel length distribution of leaf xylem was measured with a Pneumatron device (Pereira *et al.*, 2020b). Instead of injecting air (Cohen *et al.*, 2003; Wang *et al.*, 2014; Pan *et al.*, 2015), the amount of gas that could be sucked up via cut-open conduits allowed us to measure the air conductivity of open vessels while shortening leaf petioles. We then plotted the air conductivity of the cut-open vessels against the petiole length. The average vessel length was obtained by fitting the vessel length equation from Sperry *et al.* (2005) to our data.

We defined a segmentation index as the maximum vessel length at the petiole end divided by the petiole length. This index indicated to what extent the longest vessels from the petiole end run into the leaf blade. Values  $< 1$  indicated that vessels ended before the leaf blade started, while values  $> 1$  suggested that at least some vessels starting at the petiole end run directly into the midrib of the leaf blade.

## Xylem embolism resistance of leaves

### Sample preparation

All samples were collected before 9:00 in the morning. After cutting samples from a tree in air, large branches were immediately put in a water-filled bucket and a ca. 20 cm stem segment was cut from the stem base under water to avoid air entry as much as possible. The length of the remaining branch samples was between 80 and 160 cm, which was much longer than the maximum vessel length ( $MVL_{\text{stem}}$ ). Samples were covered up with a dark plastic bag, transferred to the lab within 10 min, and then rehydrated for more than two hours.

### The optical method

The optical method following Brodribb *et al.* (2016a, b) was applied to quantify the amount of leaf xylem embolism. A healthy, mature and undamaged leaf from each branch was fixed under a stereo microscope (Axio Zoom.V16, Zeiss, Jena, Germany) or in optical clamps (for more details, see <http://www.opensourceov.org/>). The surface area of the leaf, which was scanned for both the stereo microscope and clamps, was about 1 cm<sup>2</sup>. In general, images were taken every 5 min, and the water potential was simultaneously monitored with a stem psychrometer at 10 min intervals (see below). Then, images were processed using the Fiji version of ImageJ (Schindelin *et al.*, 2012) and the “OpenSourceOV ImageJ Toolbox” was used to analyse the images. Image stacks were made to determine changes in the brightness of leaf veins, which was due to embolism formation. The Percentage of Embolised Pixels (PEP) was quantified over time at decreasing xylem water potentials, with PEP<sub>50</sub> representing the xylem water potential corresponding to 50% of total embolised pixels (Brodribb *et al.*, 2016b).

### Pneumatic measurements

An automatic Pneumatron apparatus was applied to measure gas diffusion kinetics of desiccating leaves (Pereira *et al.*, 2020a, Jansen *et al.*, In press). The principle of this apparatus was similar to the manual approach of Pereira *et al.* (2016) and Zhang *et al.* (2018), but with a much higher accuracy and temporal

resolution. When samples for pneumatic measurements were prepared, the cut-open conduits embolised intentionally, which happened quickly by shaving the sample ends with a fresh razor blade in air.

Pneumatic measurements were taken every 15 min. For this a 40 kPa of absolute pressure was created with a vacuum pump, which extracted gas from a petiole end via a solenoid valve. The amount of gas extracted in a discharge tube with known volume could be measured with a pressure sensor. The vacuum pump reached 40 kPa (i.e., the initial pressure  $P_i$ ) within less than a second. Pressure data were recorded in a SD card at a time interval of 500ms. The final pressure ( $P_f$ ) was taken after 30s. According to the ideal gas law, the moles of air extracted from vessels ( $\Delta n$ ) could be calculated following the equation below:

$$\Delta n = n_f - n_i = \frac{(P_f - P_i) \times V}{RT} \quad (\text{Eqn 1})$$

where  $n_i$  and  $n_f$  represented the moles of air molecules at the initial and final pressure,  $V$  was the fixed volume of the discharge tube (1.1 mL),  $R$  was the gas constant (8.134 kPa L mol<sup>-1</sup> K<sup>-1</sup>), and  $T$  was the room temperature in the lab (around 25°C). Since a small  $V$  is needed to increase the measurement precision when a tiny amount of air is sucked from plant tissue, which is the case for detached leaves (Pereira *et al.*, 2020a, Jansen *et al.*, In press),  $V$  was estimated as the maximum gas volume that could be extracted when leaves were fully dehydrated ( $AD_{\max}$ , see below) divided by 510.2 (Pereira *et al.*, 2020a). The volume of air discharged ( $AD$ ,  $\mu\text{L}$ ) from vessels could then be calculated based on the ideal gas law, with  $P_{\text{atm}}$  being the atmospheric pressure:  $AD = 10^6 \times \Delta n R T / P_{\text{atm}}$  (Eqn 2),

Finally, the Percentage of Air Discharged (PAD, %) was calculated:

$$\text{PAD} = 100 \times (AD - AD_{\min}) / (AD_{\max} - AD_{\min}) \quad (\text{Eqn 3})$$

where  $AD_{\min}$  was the minimum volume of air discharged when the leaf was well hydrated, and  $AD_{\max}$  was the maximum volume of air discharged when the leaf was strongly dehydrated.

Vulnerability curves were generated by plotting PAD or PEP against the corresponding leaf water potential ( $\Psi$ ), with a fitting by the following equation (Pammenter and Vander Willigen, 1998):

$$\text{PAD or PEP} = 100 / (1 + \exp(S / 25) (\Psi - P_{50})) \quad (\text{Eqn 4})$$

$S$  represented the slope of the fitted curve, and  $P_{50}$  represented the water potential at 50% of air discharged, or 50% of the total embolised pixels of the leaf area scanned. Values of  $P_{12}$  (water potential at 12% of air discharged or embolised pixels) and  $P_{88}$  (water potential at 88% of air discharged or embolised pixels) were calculated following the equations by Domec and Gartner (2001):

$$P_{12} = 2 / (S / 25) + P_{50} \quad (\text{Eqn 5})$$

$$P_{88} = -2 / (S / 25) + P_{50} \quad (\text{Eqn 6})$$

### Water potential measurements

Psychrometers (PSY1, ICT International, Armidale, NSW, Australia) were applied to obtain water potential values from the leaves that were simultaneously measured with the optical and pneumatic method. Sand paper with a grit size of 400 was used to carefully remove a small area (10 mm<sup>2</sup>) of leaf cuticle under a stereomicroscope, while paying special attention not to abrade the vascular bundles and introduce air-entry into the xylem. Vaseline was used to seal the psychrometer chamber to the abraded surface of the leaf. Water potential measurements were taken every 10 min, and the measurements were stopped when leaves were completely desiccated, or the water potential showed no further decrease over a long period. The duration of measurements was between one and two days.

At the beginning of each dehydration experiment, the leaf water potential dropped rapidly within one hour. After that, the water potential decreased slowly and steadily. To test the accuracy of the xylem water potential values taken with psychrometers, we applied two different approaches. Firstly, for leaves that were attached to long branches, we measured not only leaf xylem water potential, but also stem xylem water potential with another stem psychrometer (Fig. S2). This comparison provided a reasonably good match

between both organs, although differences became more pronounced at high levels of dehydration for some species, with xylem water potential decreasing more quickly for leaves than stems. An exception was *F. sylvatica*, which had more negative xylem water potentials for leaves than stems when water potentials in leaves were less negative than -5 MPa. Secondly, water potential values of detached leaves that were measured with the Pneumatron and optical method were compared to a second set of leaves. Therefore, two leaves with a similar size were excised from the same branch at the same time. One leaf was attached to a psychrometer, while the other one was left to dry under similar conditions (same light intensity, temperature, and humidity). The water potential of the second leaf was measured with a pressure chamber (PMS Instrument Company, Albany, OR, USA) at an interval of 20 to 60 min to validate the accuracy of the stem psychrometer measurements during the first hours of dehydration. These tests revealed an overall good agreement between both methods for three species tested (*C. betulus*, *F. sylvatica*, and *Q. petraea*; Fig. S3).

## Experimental design

We applied three different experiments to investigate the potential effect of cut-open xylem on embolism spreading.

Experiment 1: Comparison of detached leaves with leaves attached to a short and long stem segment

Embolism resistance of leaf xylem was measured using the optical method. For each of the six species studied, we considered three different types of samples: (1) a detached leaf with a cut petiole, (2) a single leaf and petiole connected to a short (0.5 cm) stem segment, regardless of the vessel length in stem xylem, and (3) a leaf attached to a long branch, which was at least two times the maximum vessel length as measured for stem xylem. Depending on the species, the branch length of the latter samples had a length of ca. 80 to 160 cm. Four to five replicates were tested for each sample type. The dehydration time for each leaf or branch usually took 10 to 48 hours. *L. tulipifera* and *B. pendula* were the slowest dehydrating species. Also, the dehydration speed of detached leaves was much faster than leaves attached to a long branch for *F. sylvatica*, *P. avium*, and *C. betulus* (Table S1).

Experiment 2: Comparison of intact leaves vs leaves with cut-open minor veins

To compare the potential impact of cut-open narrow vessels or tracheids in leaf xylem on embolism spreading, the optical method was applied on two adjacent leaves attached to a long branch, which was more than twice the maximum vessel length in stem xylem. This approach was applied to all six species. Two to three leaf pairs were tested for each species. Two mature, healthy and adjacent leaves were selected and placed under the stereomicroscope. In one leaf, we cut a few minor veins (3<sup>rd</sup> or 4<sup>th</sup> vein order) with a razor blade. Four to six cuts were made and the length of each cut was about 1 to 2 mm. The other leaf selected was kept intact. Cuts on leaves were made at the beginning of the dehydration procedure, and images were taken every 10 seconds to obtain a high temporal resolution of embolism formation within the first 10 minutes after making the cuts. Moreover, a transparent tape was applied to both leaf areas observed to avoid any potential difference in dehydration between the cut and intact leaf. After the first ten minutes of scanning the leaf, images were taken every five minutes.

Experiment 3: Comparison of the optical method with the Pneumatron

Both the optical method and the Pneumatron were applied to the same detached leaves to estimate embolism resistance of xylem tissue. The Pneumatron was connected to the cut leaf petiole, while the optical method was applied to the upper part of the leaf blades, as far away from the cut petiole as possible. In this way, we obtained the highest hydraulic distance between both methods, with the optical method focussing on the intact vessels in the upper leaf veins, and the Pneumatron measuring gas diffusion between the cut-open and first series of intact conduits of the petiole, and probably the lower part of the leaf blade. For each species, four replicates were tested.

## Data analysis

Vulnerability curves were plotted and fitted using SigmaPlot 14 (Systat Software Inc., Erkrath, Germany). After testing data for normal distribution and homogeneity of variance, a one-way ANOVA was applied to test for significant difference between xylem embolism resistance of a detached leaf, and a leaf attached to a short or long branch. An independent t-test was applied to determine whether the optical and pneumatic method differed. Statistics were performed in SPSS 22 (IBM, Armonk, New York, USA), and all graphs were drawn in SigmaPlot.

## Results

### Petiole vessel length measurements

*L. tulipifera* and *Q. petraea* had the longest petiole vessels of the six species studied, with an average length of  $8.19 \pm 1.47$  and  $8.02 \pm 1.33$  cm (mean  $\pm$  standard deviation), respectively. Despite having the longest petioles and the largest leaf surface area, vessels of *L. tulipifera* were always shorter than the petiole length, indicating that vessels at the base of the petiole ended well before the base of the lamina. Cut-open vessels in the petiole of *B. pendula* also ended before the base of the lamina. Petiole vessels of *Q. petraea*, however, were found to run from the petiole base up to the middle of the midrib. A similar observation was found for cut-open vessels in *F. sylvatica* and *P. avium*, which reached to half the length of the midrib. *C. betulus* had petiole xylem with vessels that were only slightly longer than the petiole (Table 1).

*Q. petraea* had the longest average vessel length in petiole xylem, with a value of  $5.1 \pm 0.9$  cm, which was followed by *L. tulipifera* ( $3.52 \pm 1.17$  cm). The mean vessel length in petioles of *B. pendula*, *F. sylvatica*, and *C. betulus* was shorter than 1 cm. Since the petiole of *P. avium* had a pronounced notch, we were unable to obtain accurate vessel length measurements for this species with the Pneumatron, even when using glue or parafilm to avoid any leakage (Table 1).

### Experiment 1: Does embolism spreading depend on the proximity to cut conduits?

The shape of the vulnerability curves obtained was consistently sigmoidal for the three sample types, i.e. detached leaves, leaves attached to a short stem segment, and leaves attached to a long stem segment (Figure 1). Embolism expansion in leaf veins started typically in major veins and proceeded to minor veins (Figure S4). We did not see different patterns in the progression of embolism formation among detached leaves and leaves attached to a short or long stem segment.

There was considerable variation in the  $PEP_{12}$ ,  $PEP_{50}$  and  $PEP_{88}$  values among the three types of samples for several species. Comparison of detached leaves with leaves attached to short stem segments showed a significant difference ( $P < 0.05$ ) in  $PEP_{50}$  for *C. betulus*, *F. sylvatica*, *P. avium* and *Q. petraea* (Figure 1, Table S2). Detached leaves of these species showed a ca. 1.5 MPa less negative  $PEP_{50}$  value compared to  $PEP_{50}$  values of leaves on short stem segments. A minor difference in  $PEP_{50}$  with no significant difference was obtained for *L. tulipifera* and *B. pendula* (Figure 1, Table S2).

A positive, exponential correlation ( $R^2 = 0.52$ ,  $P < 0.05$ ) was found for the shift in embolism resistance between detached leaves and leaves attached to a short stem segment, and the segmentation index of leaf xylem (Figure 2). Leaves with a segmentation index  $> 1$  were strongly affected by the cut-open vessels at the petiole end, resulting in a shift in  $PEP_{12}$ ,  $PEP_{50}$  and  $PEP_{88}$  of 1MPa or more between detached leaves and leaves attached to stem segments. *L. tulipifera* and *B. pendula*, which had all vessels ending within their petiole, were clearly less affected by the proximity to cut xylem conduits compared to the four other species with vessels running from the petiole end into the midrib.

A significant difference in  $PEP_{12}$  and  $PEP_{50}$  was also found between leaves attached to a short branch, and those on a long branch for *F. sylvatica*. No significant difference in xylem embolism resistance was found between leaves on a short stem segment and leaves on a long stem piece for the other species studied, except for  $PEP_{88}$  values of *B. pendula* and *P. avium*. Any similarity or dissimilarity in  $PEP_{50}$  between the three types of samples was mostly reflected in  $PEP_{12}$  and  $PEP_{88}$  (Figure 1, Table S2).

### Experiment 2: Does the proximity to cut conduits also affect embolism spreading in minor leaf

## veins?

The percentage of cumulative embolised xylem area in leaves with cut-open minor veins and intact leaves was plotted against time for the six species studied (Figure S5). For leaves with several artificial cuts in the 3<sup>rd</sup> or 4<sup>th</sup> order veins, no immediate embolism formation was detected after the cutting, except for the vessels that were cut open. In all six species, however, we observed embolism events in minor veins adjacent to the cuts during the first hours of desiccation (Figure 3, Figure S4). These embolism events in minor veins, with relatively narrow and short vessels, occurred prior to embolism formation in the wide and long vessels of major veins. Since the amount of embolised pixels near the cuts had a local effect only and was relatively small compared to the total amount of embolised pixels that could be detected in the entire leaf area, there was no large overall difference in embolism spreading between intact and cut leaves. Therefore, the embolism spreading patterns did not differ when the percentage of embolised pixels of the entire scanned leaf area was plotted against time. After artificially induced embolism events occurred near the cuts, embolism spreading followed in the major veins, and then spread to the minor veins in both intact and cut leaves (Figure 3).

### Experiment 3: Is the Pneumatron underestimating embolism resistance compared to the optical method?

The optical method and Pneumatron were simultaneously applied to the same detached leaves for all six species. We were unable to obtain Pneumatron measurements for *B. pendula*, because the gas volume that could be extracted from the leaves was too small to meet the sensitivity requirements of the tube volume, even if we reduced the volume of the discharge tube to the minimum volume of 1.1 mL.

Vulnerability curves based on the two methods were obtained for five species (Figure 4). The PEP<sub>12</sub>, PEP<sub>50</sub> and PEP<sub>88</sub> values of detached leaves obtained with the optical method in experiment 1 (Fig.1, Table S2) did not differ significantly from those obtained in experiment 3 (Table S3). A difference in embolism spreading from cut conduits to intact ones versus spreading between intact conduits would especially be reflected in PAD<sub>12</sub> and PEP<sub>12</sub> values. Indeed, average PAD<sub>12</sub> values based on the Pneumatron were lower than the average PEP<sub>12</sub> values based on the optical method for four species, except for *P. avium*. The difference between PAD<sub>12</sub> and PEP<sub>12</sub> was more than 0.6 MPa for *Q. petraea*, *F. sylvatica*, and *C. betulus*, although these differences were not statistically significant. Also, there was considerable variation among the samples tested (Fig. 5a), with several samples showing a slightly more negative PAD<sub>12</sub> value than PEP<sub>12</sub> for *P. avium* and *L. tulipifera*.

Despite minor differences between PEP<sub>12</sub> and PAD<sub>12</sub>, the vulnerability curves based on the optical and pneumatic method showed strikingly similar patterns for the five species tested, with a strong correlation between PAD<sub>50</sub> and PEP<sub>50</sub>, and between PAD<sub>88</sub> and PEP<sub>88</sub> (Fig. 5b, c). When intraspecific differences in embolism resistance were found within a species, both methods matched each other very well (Figure 4, 5, Figure S6). Although curves based on the optical method had a relatively steeper slope compared to the pneumatic curves for *F. sylvatica*, *C. betulus* and *Q. petraea*, this difference was not significant. No significant difference was found between PAD<sub>50</sub> and PEP<sub>50</sub>, and between PAD<sub>88</sub> and PEP<sub>88</sub>. Nevertheless, *P. avium* showed a 0.67 MPa difference ( $P = 0.287$ ) between PEP<sub>50</sub> and PAD<sub>50</sub>, and a 0.61MPa difference ( $P = 0.454$ ) between PEP<sub>88</sub> and PAD<sub>88</sub> (Table S3).

## Discussion

Experiment 1 and 2 show that spreading of drought-induced embolism and thus embolism resistance can be strongly affected by the proximity of the xylem area studied to pre-existing embolism such as cut xylem tissue. The observation that embolism initiation (PEP<sub>12</sub>) occurs over a > 1MPa range of xylem water potential for four out of six species studied challenges the assumption that embolism spreads once a certain threshold difference has been reached. While cut-open conduits may facilitate embolism spreading, hydraulic segmentation may limit this potential artefact. As predicted, embolism spreading in minor veins was also affected by the proximity to cut conduits, but showed a rather limited, local distribution without affecting the entire leaf. Also, we found no difference between the optical and pneumatic method for five species, despite considerable variation in the P<sub>12</sub> values of both methods. The consequences and broader significance

of these findings present some old questions and assumptions in a new light, and provides a novel gas diffusion hypothesis as a possible mechanism for embolism spreading.

### Embolism spreading depends on pre-existing embolism

Since embolism spreading happens largely from one embolised conduit to a neighbouring one, as predicted by the air-seeding hypothesis (Zimmermann, 1983; Sperry and Tyree, 1988), the observation that embolism formation may depend on proximity to an existing gas source such as cut-open conduits or pre-existing embolised conduit is not surprising. Embolism formation appears to be unlikely if a conduit is not connected to a pre-existing embolism. Novel, de novo embolism formation has been observed in very few conduits that are not connected to embolised ones based on microCT (Brodersen *et al.*, 2013, Choat *et al.*, 2015, 2016), and embolism formation in seemingly isolated conduits could occasionally be observed in our experiments. However, the rather two-dimensional view associated with the optical method, its limited resolution to accurately detect narrow vessel ends (Oskolski and Jansen, 2009), and its shortcoming to detect pre-existing embolism, did not allow us to confirm that these conduits were completely disconnected from neighbouring gas sources.

If availability of a pre-existing gas source or embolised conduit is important, then where does the gas come from to induce embolism in intact xylem? It is possible that there is almost always an embolised conduit available, perhaps in primary xylem or in older xylem from an older growth ring. This would be an obvious gas source if functional, sap-filled conduits show any direct connection with these embolised conduits via bordered pits. However, these pre-existing gas sources may be limited due to poor connectivity or compartmentalisation of the hydraulic network (Kitin *et al.*, 2004; Morris *et al.*, 2016). Since vessels and tracheids do not share pits with non-conductive fibres (Sano *et al.*, 2011), it is unlikely that air entry from these cells or intercellular spaces will contribute to embolism formation in conduits.

### Hydraulic segmentation reduces embolism spreading in xylem tissue

While the proximity of a studied xylem area to cut conduits seems to be important, the speed of embolism spreading over a certain distance also depends on the vessel dimensions. If each individual vessel would embolise separately (Johnson *et al.*, 2020), wide and long vessels would show a faster propagation of embolism over a given distance than narrow, short vessels. Spreading of embolism would especially be reduced in xylem with a high degree of hydraulic constrictions, making xylem patches at the distal side of embolised conduits seemingly more resistant to embolism. The four species that showed a reduced embolism resistance in detached leaves as compared to leaves attached to branches, have open vessels running directly from the base of the petiole into the midrib (Table 1, Figure 2). Since the maximum vessel length in petioles of *L. tulipifera* and *B. pendula* were shorter than the petiole length (Table 1), both species showed relatively small differences in embolism resistance between detached leaves and leaves attached to a stem segment (Fig. 1c, e).

Wide and long vessels in the midrib and secondary veins were found to embolise before the high 3<sup>rd</sup> to 5<sup>th</sup> vein orders. This pattern confirms various studies based on the optical method and microCT observations (Brodribb *et al.*, 2016a, b; Scoffoni *et al.*, 2017; Klepsch *et al.*, 2018). However, the observation of local spreading of embolism in minor veins near cut vessels in experiment 2 supports the hypothesis that embolism can spread from pre-existing gas sources, and in minor veins prior to embolism formation in the large vessels of major veins (Fig. 3). This observation suggests that proximity to a gas source is the main driver for embolism spreading, and not the conduit diameter per se. Nevertheless, the limited and short dimensions of minor veins (Chatelet *et al.*, 2006; Lechthaler *et al.*, 2019; Hua *et al.*, 2020) are a plausible explanation for why artificially induced embolism near cuts in minor veins propagate locally, rather than spread across the lamina. Short conduits in minor vein orders may have been selected for in order to maximise leaf hydraulic function over the life span of a leaf. Minor orders of leaf veins are believed to be prone to xylem embolism formation over the life of a leaf due to localized damage caused by insects, pathogens or mechanical damage to the lamina, and because they sustain the most negative pressures (Brodribb *et al.*, 2010). Considerable restriction to the extent of embolism spread through minor vein orders would ensure a limited impact on

the hydraulic capacity of a leaf that sustains localised damage to the lamina.

It is also possible that wide and long vessels are more likely connected to a pre-existing embolism than narrow, short conduits, and that large vessels show a higher amount of intervessel pit membrane area than narrow, short vessels. In other words, the reason why large and wide vessels are likely to embolise first, may reflect a difference in the rate of air entry, which is caused by their connectivity to a gas source, and not any inherent difference in embolism resistance per se. We are not aware of an alternative mechanism that would explain why wide conduits are more vulnerable to embolism than narrow ones. Pit membrane thickness, which is strongly associated with embolism resistance (Liet *et al.* , 2016; Kaack *et al.* , 2019), was found not to be related to conduit diameter (Kotowska *et al.* , 2020; Wu *et al.* , 2020).

### **Vulnerability segmentation may reflect hydraulic segmentation, but not intrinsic differences in embolism resistance per se**

The observation that embolism formation in leaves of an individual tree may occur under highly variable ( $> 1$  MPa) xylem water potentials, suggests that embolism resistance may represent a relative trait that does not capture the absolute, intrinsic embolism resistance of its xylem. It is likely that hydraulic segmentation includes highly reduced conduit dimensions, especially with respect to conduit length and width, with a high number of interconduit end walls over a short stretch of xylem tissue. Conduit end walls have been suggested to hold up embolism spreading at least temporarily, with pit membranes functioning as safety valves and preventing further spreading of embolism due to their tiny pores (Zhang *et al.* , 2017, 2020; Kaack *et al.* , 2019; Johnson *et al.* , 2020). Moreover, narrow and short tracheids or fibriform vessels may be more confined than long and wide vessels, with a small interconduit pit membrane area for air entry in narrow tracheids.

Quantifying embolism resistance across the entire xylem pathway could be complicated by measuring artefacts or the proximity to pre-existing embolism, such as cut xylem. In our study, comparison of xylem embolism resistance of leaves attached to long stem segments with embolism resistance of stem xylem based on previous papers (Klepsch *et al.* , 2018; Zhang *et al.* , 2018), indicates that leaf xylem of all six species was between 0.5 and  $> 1$  MPa more embolism resistant than stem xylem. Our result for *B. pendula* was consistent with Klepsch *et al.* (2018), with leaf xylem being more resistant than stem xylem. Most angiosperms species, however, showed that stem xylem was either more embolism resistant, or equally resistant than leaf xylem (Zhu *et al.* , 2016; Skelton *et al.* , 2018; Losso *et al.* , 2019). Therefore, caution is needed to directly compare absolute values of embolism resistance between organs, since measured values of embolism resistance could be relative estimations only, especially if destructive methods are used and cut-open xylem accelerates embolism spreading.

### **Embolism spreading may not rely on a fixed pressure difference threshold, but on pressure-driven diffusion**

The observation of embolism spreading under highly variable xylem water potentials in experiment 1 and 2 indicates that embolism formation may not always rely on a certain threshold of the pressure difference between a functional and embolised conduit, as frequently assumed based on the air-seeding hypothesis, and quantitatively estimated by the Young-Laplace equation (Sperry and Tyree, 1988; Choat *et al.* , 2008). Even if some of our xylem water potential measurements may not be fully accurate due to a pressure gradient heterogeneity (Bouda *et al.* , 2019), the  $> 1$  MPa difference in PEP<sub>50</sub> values between detached leaves and leaves connected to stems provides solid evidence that embolism formation in xylem tissue from the same organ of a species may occur under different xylem water potentials. This finding is not entirely new, and in line with earlier differences in embolism resistance between intact plants and cut plants (Choat *et al.* , 2010; Torres-Ruiz *et al.* , 2015; Lamarque *et al.* , 2018). For instance, a ca. 4 MPa difference in P<sub>50</sub> was found for *Laurus nobilis* based on microCT observation of cut branches and intact seedlings (Nardini *et al.* , 2017; Lamarque *et al.* , 2018). Similar to our results, Skelton *et al.* (2018) compared the vulnerability curves of cut branches and intact plants based on the optical method for *Quercus wislizenii* , and found a 1.5 MPa difference in P<sub>50</sub> between leaves attached to a long, cut branch, and leaves from an intact plant. The finding that cut plant material can be more vulnerable to embolism spreading than intact plants raises concerns

about embolism resistance measurements of plant material samples with pre-existing embolism, the possible induction of embolism due to cutting, and the application of the bench dehydration method on cut plant material (Sperry and Tyree, 1988).

If embolism spreading for given species may not rely on a certain pressure threshold, which mechanism does then trigger embolism? We believe that this is a highly important question in our understanding of water transport, even though this question cannot be fully answered based on the available evidence. We speculate that gas diffusion across interconduit pit membranes plays a role in determining the amount of gas dissolved in xylem sap, which may affect embolism nucleation. Although mass flow is theoretically  $10^5$  times faster than diffusion, gas diffusion in xylem is overall much faster and more common than mass flow. The main reason seems to be that gas diffusion takes place continuously over very large areas, while mass flow requires gas movement through multi-layered, tiny pore constrictions of mesoporous pit membranes (Kaack *et al.*, 2020). However, the continuous nature of gas diffusion and the high amounts of gas ( $\text{CO}_2$  and  $\text{O}_2$ ) surrounding conduits (Spicer and Holbrook, 2005; Teskey *et al.*, 2008) do not mean that gas concentration in xylem sap is always under equilibrium with gas in embolised conduits, as has frequently been assumed (Hammel 1967; Yang and Tyree, 1992; Wheeler *et al.*, 2013; Schenk *et al.*, 2016). Gas solubility of xylem sap is affected by pressure and temperature (Mercury *et al.*, 2003; Schenk *et al.*, 2016; Lidon *et al.*, 2018). Due to a nearly constant atmospheric pressure in a cut-open vessel, but a considerably variable liquid water pressure in sap-filled conduits, the driving force for gas diffusion is the unbalanced, anisobaric situation between embolised conduits and functional conduits. We ignore here the effect of temperature on gas solubility of xylem sap as all experiments were conducted in an air-conditioned lab under similar temperature (Schenk *et al.*, 2016). Moreover, oversolubility of gas may occur in nanoporous cell walls and mesoporous pit membranes, as is known for nanoconfined environments in general (Pera-Titus *et al.*, 2009; Hoet *et al.*, 2015; Coasne & Farrusseng, 2019), which could contribute to concentration gradients of gas dissolved in xylem sap.

A recently embolised intact vessel is not immediately filled with gas under atmospheric pressure, but eventually achieves equilibrium of its gas pressure with the atmosphere, depending on how fast gas is attracted from surrounding gas sources via diffusion. While cut-open vessels are immediately filled with air and reach atmospheric pressure immediately, intact vessels that embolise are initially filled with water vapour (ca. 2.4 kPa). It has been modelled that gas diffusion takes from 20 min to several hours to obtain atmospheric pressure (ca. 101.3 kPa) in embolised, intact vessels, which may depend on the distance to the nearest gas phase, and the interconduit pit membrane area for gas diffusion (Yang & Tyree, 1992; Wang *et al.*, 2015a, b). Although gas diffusion happens across conduit cell walls and pit membranes, the micropores ( $< 2$  nm) in hydrated walls are much smaller than the 5 to 50 nm dimensions of pit membrane pores (Donaldson *et al.*, 2019; Kaack *et al.*, 2019). Therefore, it is reasonable to assume that gas diffusion across hydrated, 200 to 600 nm thick pit membranes is much faster than across the much thicker layers of secondary cell walls (Yang *et al.*, submitted). Indeed, axial gas diffusion in wood is found to be about one to two orders of magnitude larger than radial diffusion (Sorz & Hietz, 2006). Moreover, end-wall resistivity of conduits has been suggested to be proportional to lumen resistivity (Sperry *et al.*, 2005; Hacke *et al.*, 2006), and conductance of gas increases to the 4<sup>th</sup> power with conduit diameter or pore diameter according to Hagen-Poiseuille's equation.

Further research is clearly needed to investigate how gas diffusion may contribute to the very high gas solubility of xylem sap (Schenk *et al.*, 2016), whether oversolubility occurs due to nanoconfined spaces such as pit membranes and cell walls (Pera-Titus *et al.*, 2009; Hoet *et al.*, 2015; Coasne & Farrusseng, 2019), how gas-xylem sap interfaces are affected by the dynamic surface tension of xylem sap lipids (Yang *et al.*, 2020), and how surfactant-coated nanobubbles may contribute to the gas concentration of xylem sap and embolism formation (Schenk *et al.*, 2015, 2017; Jansen *et al.*, 2018; Park *et al.*, 2019).

### **The optical method and Pneumatron show close similarity in measuring embolism resistance**

We found a strong agreement between the optical method and the Pneumatron, with no significant difference between either  $P_{12}$ ,  $P_{50}$  or  $P_{88}$  values obtained from the optical method and the Pneumatron in five deciduous species (Figure 4, Table S2). This finding is in line with the results from Pereira *et al.* (2020a) for *Eucalyptus camaldulensis*, and supports the hypothesis that the pneumatic method measures gas extracti-

on from embolism events in intact vessels (Jansen *et al.* , In press; Yang *et al.* , submitted). The fast and straightforward approach of taking pneumatic methods on small samples such as individual leaves makes the Pneumatron device also suitable for field observations and embolism resistance measurements at the intraspecific and intratree level.

While a high coefficient of determination ( $R^2=0.91$ ) indicated good agreement between  $PEP_{50}$  and  $PAD_{50}$  values (Fig. 5b), the vulnerability curves with the pneumatic method were less steep than with the optical method for four species studied, except for *L. tulipifera* . A somewhat weaker, but still significant correlation was found between the  $PEP_{12}$  and  $PAD_{12}$  values based on both methods (Fig. 5a). It is possible that the presence of gas in cut-open vessels affects the process of embolism spreading as discussed above, and this may explain why the percentage of gas discharged by the Pneumatron was slightly higher than the percentage of the cumulative embolised pixels during early stages of dehydration in four out of five species studied (Figure 3, Figure 5A). A similar finding was reported in Pereira *et al.* (2020a). However, the opposite was found for *P. avium* , which may have slower gas diffusion due to its thick pit membranes as compared to the other species (Kaack *et al.* , 2020). If intact vessels become embolised, but gas diffusion across pit membranes is slow, atmospheric pressure will not be quickly reached in a recently embolised vessel (Wang *et al.* , 2015a, b).

The xylem area selected to apply the optical method was deliberately chosen in the upper part of the leaf blade, where vessels in the leaf veins are separated from cut vessels at the petiole end by at least one and most likely various intervessel walls, as shown based on the maximum vessel length in petioles. Since the Pneumatron extracts gas from the petiole end, the tight similarity in embolism resistance between both methods suggests that the gas extracted with the Pneumatron comes from intact, embolised conduits. It is possible that there could be some overlap between vessels from which gas is extracted with the Pneumatron and those that are visualised with the optical method (Fig. 6). Yet, it remains unclear over how many end walls the Pneumatron is able to extract gas. We speculate that this number of end walls depends at least partly on the pit membrane thickness, the complete or partial hydration of the pit membrane, and whether or not porous medium characteristics of interconduit pit membranes change during dehydration, since these would determine gas diffusion considerably (Crombie *et al.* , 1985; Kaack *et al.* , 2019; Zhang *et al.* , 2020).

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## Tables

**Table 1.** Overview of the petiole length and vessel length dimensions for six temperate tree species studied. Maximum vessel length measurements were based on air injection, while the average length of vessels starting at the basipetal end of the petiole was estimated with a Pneumatron. Values indicate mean  $\pm$  standard deviation ( $n = 5$  or  $6$ ).

	Petiole length (cm)	Max. vessel length at the petiole end (cm)	Average vessel length at the base of the p
<i>B. pendula</i>	2.03 $\pm$ 0.48	1.07 $\pm$ 0.26	0.83 $\pm$ 0.09
<i>C. betulus</i>	1.37 $\pm$ 0.13	1.44 $\pm$ 0.30	0.96 $\pm$ 0.12
<i>F. sylvatica</i>	1.23 $\pm$ 0.06	3.41 $\pm$ 0.27	0.66 $\pm$ 0.43
<i>L. tulipifera</i>	8.68 $\pm$ 1.39	8.19 $\pm$ 1.47	3.52 $\pm$ 1.17
<i>P. avium</i>	3.53 $\pm$ 1.00	5.47 $\pm$ 0.83	/
<i>Q. petraea</i>	1.17 $\pm$ 0.21	8.02 $\pm$ 1.33	5.1 $\pm$ 0.90

## Figure legends

**Figure 1** . Leaf xylem vulnerability curves of six deciduous species using the optical method applied to different samples: a detached leaf (red), leaf attached to a short stem segment (blue), and to a long branch (green). For each sample type, four to five replicates were measured (pale colours). Bright lines are regression lines for each type of sample, and grey lines indicate 95% confidential intervals. PEP<sub>50</sub> values (MPa) of the three types of samples were presented with mean  $\pm$  standard deviation ( $n = 4$  or  $5$ ), with different small letters showing significant difference between each sample type ( $P < 0.05$ ). Note that the curve fitting may give the impression that embolism occurs at positive xylem pressures, while the raw data make clear that embolism spreading never occurs at a xylem water potential that is less negative than  $-0.1$  MPa.

**Figure 2** . Relationship between the segmentation index and the difference in PEP<sub>12</sub> (red), PEP<sub>50</sub>(yellow) and PEP<sub>88</sub> (green) values between detached leaves and leaves attached to a short stem segment. A segmentation index was defined as the maximum vessel length divided by the petiole length, with vessels running into the leaf blade when the value is  $> 1$ , and the longest vessels ending before the leaf blade when the value is  $< 1$ .

**Figure 3** . Maps of embolism events between an intact leaf (A-F) and a leaf with cuts in minor veins (G-L) of *Quercus petraea* . The cut and intact leaf pair were attached to the same long branch, with images taken for both leaves after a certain desiccation time (shown on the left). White solid lines represent artificial cuts of the 3<sup>rd</sup> or 4<sup>th</sup> veins, and white arrows point at embolism in minor veins near the cuts, which happened much earlier than embolism in major veins.

**Figure 4** . Xylem vulnerability curves of leaves of five deciduous species based on the optical and pneumatic method. Both methods were applied to the same detached leaf. Pale and bright red lines represent raw data and regression lines of the optical method, respectively. Blue lines represent raw data (pale blue) and regression lines (bright blue) based on the pneumatic method. For each species, four leaves were tested, and P<sub>50</sub> values (MPa) of both methods were presented.

**Figure 5** . Correlation between paired values of P<sub>12</sub> (a), P<sub>50</sub> (b), and P<sub>88</sub> (c) as measured with the optical and pneumatic method. Each dot represents a single leaf, solid black lines indicate the regression line, blue dashed lines indicate the 95% confidence intervals, and black dashed lines indicate the 1:1 line.

**Figure 6** . Illustration of the optical method and pneumatic measurements on detached leaves and possible embolism spreading during dehydration after 5 and 10 hours. The Pneumatron was connected at the basipetal end of the petiole, while the leaf area scanned with the optical method (pale yellow square) is separated from the cut-open vessels (white) by at least several end walls. (a) The initial status when leaves were fresh, and all intact vessels were water-filled (blue). (b, c) Embolism spreads to various intact vessels (green), from

which gas is extracted with the Pneumatron, and which are observed with the optical method when these are within the scanned leaf area. It is assumed here that the Pneumatron extracts gas across two end walls. Arrows indicate gas diffusion through intervessel walls towards the Pneumatron. Embolism formation in vessels only captured with the optical method are coloured yellow. Adapted from Jansen *et al.* (In press).

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