

No gas source, no problem: pre-existing embolism may affect non-pressure driven embolism spreading in angiosperm xylem by gas diffusion

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Abstract

Embolism spreading in dehydrating angiosperm xylem is driven by gas movement between embolised and sap-filled conduits. Here, we examine how proximity to pre-existing embolism and hydraulic segmentation affect embolism propagation. Based on the optical method, we compared xylem embolism resistance between detached leaves and leaves attached to branches, and between intact leaves and leaves with minor veins cut ($n = 6$ species). Moreover, we directly compared the optical and pneumatic method on detached leaves. Embolism resistance of detached leaves was significantly lower than leaves attached to stems, except for two species with all vessels ending in their petioles. Cutting of minor veins showed embolism spreading in narrow vessels near the cuts prior to wide vessels in major veins. Moreover, embolism spreading between open and intact vessels occurred at largely similar xylem water potentials than embolism spreading between intact vessels, resulting in strong similarity between the optical and pneumatic method. We conclude that embolism spreading may depend on a direct connection to pre-existing embolism as gas source, is not exclusively pressure-driven, and indirectly related to conduit size. Hydraulic segmentation, however, can minimise embolism spreading due to confined and/or poorly interconnected conduits, which may increase hydraulic safety by slowing down gas diffusion.

Key-words

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

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Running title: Pre-existing embolism may affect embolism spreading

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Abstract

Embolism spreading in dehydrating angiosperm xylem is driven by gas movement between embolised and sap-filled conduits. Here, we examine how proximity to pre-existing embolism and hydraulic segmentation affect embolism propagation. Based on the optical method, we compared xylem embolism resistance between detached leaves and leaves attached to branches, and between intact leaves and leaves with minor veins cut ($n = 6$ species). Moreover, we directly compared the optical and pneumatic method on detached leaves. Embolism resistance of detached leaves was significantly lower than leaves attached to stems, except for two species with all vessels ending in their petioles. Cutting of minor veins showed embolism spreading in narrow vessels near the cuts prior to wide vessels in major veins. Moreover, embolism spreading between open and intact vessels occurred at largely similar xylem water potentials than embolism spreading between intact vessels, resulting in strong similarity between the optical and pneumatic method. We conclude that embolism spreading may depend on a direct connection to pre-existing embolism as gas source, is not exclusively pressure-driven, and indirectly related to conduit size. Hydraulic segmentation, however, can minimise embolism spreading due to confined and/or poorly interconnected conduits, which may increase hydraulic safety by slowing down gas diffusion.

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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 Jolly, 1896; 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 efficiency of water, 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 is based on two processes, namely mass flow and diffusion, we prefer the general term embolism spreading 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, which may occur in conduits from a previous growth ring or protoxylem (Kitinet *et al.* 2004; Sano *et al.* , 2011; Hochberg *et al.* , 2016). If this would be correct, the mechanism behind embolism spreading may not dependent on xylem pressure only (Avila *et al.* , submitted). Embolised conduits could also occur when a herbivore or xylem feeding insect damages conduits, or when a plant organ experiences die-back, which may result in local embolism spreading. 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 the 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 (Ψ_{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 approach (Oliveira *et al.* , 2019; Pereira *et al.* , 2016, 2019). Both experimental approaches rely on cut plant organs either due to the requirements to measure hydraulic conductivity, or the 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 (Brodrribb *et al.* , 2016a, b; Choat *et al.* , 2016; Lamarque *et al.* , 2018).

The amount of embolism propagation could be limited by hydraulic segmentation of the conduit network, which represents a hydraulic constriction or bottleneck (Zimmermann, 1983; Tyree *et al.* , 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).

In a few studies, considerable differences in embolism resistance have been reported between intact plants and xylem tissue in cut organs of *Vitis vinifera* and *Laurus nobilis* , with cut-open xylem potentially underestimating stem embolism resistance (Choat *et al.* , 2010; Torres-Ruiz *et al.* , 2015; Lamarque *et al.* , 2018). 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* (Breda *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, three explanations could be suggested for the observed discrepancy. First, it is possible that the cutting of conduits with sap under negative pressure introduces a cutting artefact, although artificially embolised conduits near stem ends can be removed before hydraulic measurements are made (Wheeler *et al.* , 2013; Torres-Ruiz *et al.* , 2015). A second explanation is that embolism spreading could be avoided by hydraulic segmentation, which may occur at the transition

between organs, growth rings, and nodes (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). Thirdly, embolism may also occur in conduits that are not connected to embolised conduits, although the frequency of such de novo process is unclear (Brodersen *et al.* , 2013; Choat *et al.* , 2015, 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 experiment we aimed to 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 transition could affect embolism spreading, and may prevent a potential artefact in measurements of embolism resistance near cut xylem tissue. We therefore 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). Xylem tissue that shows hydraulic segmentation could include many tracheids and/or narrow, fibriform vessels (Rančić *et al.* , 2010). Species that show little or no hydraulic segmentation, may not have these safety valves. Removal of leaves in seedlings of the ring-porous species *Quercus robur* , for instance, was found to result in potential embolism formation in the stem based on microCT observations (Choat *et al.* , 2016).

Whether or not embolism spreading depends directly on vessel dimensions was tested in a second experiment by cutting minor leaf veins. 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.* , 2016; Klepsch *et al.* , 2018). These observations may give the impression that wide conduits are more vulnerable to embolism, although any functional explanation for such differential embolism resistance remains unclear. Indeed, pit membrane thickness, which is a major determinant of vulnerability to embolism (Li *et al.* , 2016; Kaack *et al.* , 2019), is not related to conduit dimensions (Klepsch *et al.* , 2018; Wu *et al.* , 2020; Kotowska *et al.* , 2020). If the proximity of a gas source would determine embolism spreading, 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.

Finally, we applied a methodological comparison of embolism resistance in leaf xylem between the optical method and the pneumatic method. If the pneumatic method would be subject to a potential artefact due to gas extraction from intact vessels that are neighbouring or connected to 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). While direct comparison of the pneumatic and optical method to detached leaves of *Eucalyptus camaldulensis* suggested no significant difference for this species (Pereira *et al.* , 2019), a larger number of species should be tested to generalise this finding.

The three complementary sets of experiments will contribute to a better understanding of the driving forces behind embolism spreading in xylem tissue.

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

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_{stem} and MVL_{petiole} , respectively). At least six stems or leaves were taken for these measurements (Figure S4).

Hydraulic segmentation between short (0.5cm in length) stem segments and leaf petioles was also tested for 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.*, submitted). Instead of injecting air (Cohen *et al.*, 2003; Wang *et al.*, 2014; Panet *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_{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 cm². 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 PEP₅₀ 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.* , 2019, 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 (Δn) 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⁻¹ K⁻¹), 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.* , 2019, 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.* , 2019). The volume of air discharged (AD, μL) from vessels could then be calculated based on the ideal gas law, with P_{atm} being the atmospheric pressure: AD = 10⁶ × Δn R T / P_{atm} (Eqn 3),

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

$$\text{PAD} = 100 \times (\text{AD} - \text{AD}_{\min}) / (\text{AD}_{\max} - \text{AD}_{\min}) \quad (\text{Eqn 4})$$

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 (Ψ), with a fitting by the following equation (Pammenter and Vander Willigen, 1998):

$$\text{PAD or PEP} = 100 / (1 + \exp(S / 25) (\Psi - P_{50})) \quad (\text{Eqn 5})$$

S represented the slope of the fitted curve, and P₅₀ represented the water potential at 50% of air discharged, or 50% of the total embolised pixels of the leaf area scanned. Values of P₁₂ (water potential at 12% of air discharged or embolised pixels) and P₈₈ (water potential at 88% of air discharged or embolised pixels) were calculated following the equations by Domec and Gartner (2001):

$$P_{12} = 2 / (S / 25) + P_{50} \quad (\text{Eqn 6})$$

$$P_{88} = -2 / (S / 25) + P_{50} \quad (\text{Eqn 7})$$

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 mm²) 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. S1). 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. S2).

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.

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 (3rd or 4th 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

To estimate leaf vulnerability to embolism, both the optical method and the Pneumatron were applied to the same detached leaves. 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 8.19 ± 1.47 and 8.02 ± 1.33 cm, 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 ± 0.9 cm, which was followed by *L. tulipifera* (3.52 ± 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 dehydration time for each leaf or branch usually took 10 to 48 hours. Linear regressions were fitted based on the water potential in the period that corresponded to 200 to 600 minutes after dehydration was started. The slope of this fitting characterised the speed of the dehydration process (Table S3). *L. tulipifera* and *B. pendula* were the slowest dehydrating species, with a xylem water potential drop of 0.0012 and 0.0009 MPa min^{-1} for detached leaves, respectively, and a similar slope of the xylem water potential in leaves attached to a short branch for these species. *Q. petraea* showed a relatively high speed of dehydration of -0.0038 MPa min^{-1} for detached leaves and leaves attached to a short branch. Moreover, 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 S3).

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 S3). 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 S1). 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 S1).

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 P_{12} , P_{50} and P_{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 of 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 S1).

Experiment 2: Does embolism spreading also depend on the conduit size?

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 3rd or 4th 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 S3). 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 the artificially induced embolism events near the cuts, embolism spreading occurred 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 1.1mL.

Vulnerability curves based on the two methods were obtained for five species (Figure 4). The PEP_{12} , PEP_{50} and PEP_{88} values of detached leaves obtained with the optical method in experiment 1 did not differ significantly from those obtained in experiment 3. A difference in embolism spreading from cut conduits to intact ones versus spreading between intact conduits would especially be reflected in PAD_{12} and PEP_{12} values. Indeed, average PAD_{12} values based on the Pneumatron were lower than the average PEP_{12} values based on the optical method for four species, except for *P. avium*. The difference between PAD_{12} and PEP_{12} 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_{12} value than PEP_{12} for *P. avium* and *L. tulipifera*.

Despite minor differences between PEP_{12} and PAD_{12} , 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_{50} and PEP_{50} , and between PAD_{88} and PEP_{88} (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_{50} and PEP_{50} , and between PAD_{88} and PEP_{88} . Nevertheless, *P. avium* showed a 0.67 MPa difference ($P = 0.287$) between PEP_{50} and PAD_{50} , and a 0.61MPa difference (P

= 0.454) between PEP_{88} and PAD_{88} (Table S2).

Discussion

Experiment 1 and 2 show that spreading of drought-induced embolism can be strongly dependent on the proximity of the xylem area studied to the cut xylem tissue. Hydraulic segmentation, however, may limit the potential artefact caused by cut conduits due to differences in conduit dimensions, conduit connectivity, and/or the non-random arrangement of vessel ends. Yet, the proximity of pre-existing embolism was found to be more important for embolism spreading than conduit dimensions per se, with narrow vessels in minor veins embolising due to the presence of gas in neighbouring vessels prior to embolism formation in major veins. Moreover, no major differences were found between the optical and pneumatic method for five species, despite minor differences in the P_{12} values of both methods. Our findings throw novel light on the driving mechanisms behind drought-induced embolism, and may have consequences for estimations of embolism resistance based on cut xylem tissue (Wheeler *et al.*, 2013; De Baerdemaeker *et al.*, 2019).

Embolism spreading depends on pre-existing embolism as gas source

As shown in experiment 1 and 2, embolism spreading happens largely from one embolised conduit to a neighbouring one, which is in line with the air-seeding hypothesis (Zimmermann, 1983; Sperry and Tyree, 1988). Embolism formation appears to be unlikely if a conduit is not connected to a pre-existing embolism. Novel embolism formation has been observed 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 and its limited resolution to accurately detect the narrow vessel ends (Oskolski and Jansen, 2009), did not allow us to confirm that these conduits were completely disconnected from neighbouring gas sources.

While proximity between a studied xylem area and cut conduits seems to be important, the speed of embolism spreading over a certain distance also depends on the vessel dimensions. Wide and long vessels would indeed show a faster propagation of embolism over a given distance than narrow, short vessels if embolism spreading are mainly single-vessel events (Johnson *et al.*, 2020). Spreading of embolism would especially be reduced by xylem areas with hydraulic segmentation, making these xylem patches seemingly more embolism resistant. 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).

Why does hydraulic segmentation reduce embolism spreading? Firstly, it is likely that hydraulic bottlenecks show locally 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. A recently embolised intact vessel is not immediately filled with gas under atmospheric pressure, but eventually achieves Henry's law equilibrium based on the speed of gas diffusion. While cut-open vessels are immediately filled with air and reach atmospheric pressure immediately, intact vessels that embolise become filled with a mixture of water vapour and air. It has been modelled that it takes from 20 min to several hours to obtain atmospheric pressure in embolised, intact vessels, which depends on the distance to the nearest gas phase, and the interconduit pit membrane area for gas diffusion (Wang *et al.*, 2015a, b). Although gas diffusion happens also across conduit cell walls, 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 pit membranes is much faster than across the various layers of cell walls. Moreover, end-wall resistivity of conduits has been suggested to be proportional

to lumen resistance (Hacke *et al.* , 2006; Sperry *et al.* , 2005), while conductance of gas increases to the 4th power with conduit diameter or pore diameter according to Hagen-Poiseuille's equation.

Where does the gas come from to induce embolism in the first conduits? Since vessels and tracheids do not show pits with non-conductive fibres (Sano *et al.* , 2011), it is unlikely that gas diffusion from these cells or intercellular spaces will contribute to embolism formation of conduits. 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 not be available, or may not be connected to the hydraulic network of the current year's sapwood due to segmentation. When comparing 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), all six species studied showed that leaf xylem was between 0.5 and > 1 MPa more embolism resistant than stem xylem. Our result of *B. pendula* was consistent with Klepsch *et al.* (2018), with leaf xylem being more resistant than stem xylem. Other angiosperms species, however, showed that stem xylem was either more embolism resistant, or equally resistant than leaf xylem (Chen *et al.* , 2009; Zhu *et al.* , 2016; Skelton *et al.* , 2018; Losso *et al.* , 2019). This discrepancy seems to suggest that sufficient caution is needed to directly compare absolute values of embolism resistance between different methods and organs.

Embolism spreading may not be only pressure-driven

The more than 1 MPa difference in PEP₅₀ values between detached leaves and leaves connected to stems provided strong evidence that embolism formation may not always be pressure driven, as traditionally assumed based on the air-seeding hypothesis and the Young-Laplace equation (Sperry and Tyree, 1988; Choat *et al.* , 2008). The finding that embolism formation in xylem tissue from the same organ of a species may occur under different xylem water potentials 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 P₅₀ was found for *Laurus nobilis* based on microCT observation of cut branches and intact seedlings (Lamarque *et al.* , 2018; Nardini *et al.* , 2017). Similar to our findings, 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 P₅₀ 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).

An important question concerns the exact triggering mechanism behind embolism, which cannot be fully addressed based on the available evidence. However, since embolism spreading may not be pressure-driven, we speculate that gas diffusion across pit membranes will take place well before embolism formation has started. Although mass flow is theoretically 10⁵ times faster than diffusion, gas diffusion in xylem is 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 according to the air-seeding hypothesis relies on gas movement through multi-layered, tiny pore constrictions of mesoporous pit membranes. Further research is clearly needed to investigate whether or not gas diffusion may contribute to (super)saturation of xylem sap (Schenk *et al.* , 2016), how gas-water interfaces are affected by the dynamic surface tension of xylem sap lipids (Yang *et al.* , 2020), and how surfactant-coated nanobubbles may affect the gas concentration of xylem sap (Schenk *et al.* , 2015, 2017; Jansen *et al.* , 2018; Park *et al.* , 2019).

Large conduits typically embolise first, but are not more vulnerable to embolism than narrow ones

Wide and long vessels in the midrib and secondary veins were found to embolise before the high vein orders (3rd – 5th vein orders) in the three experiments conducted. This pattern confirms various studies based on

the optical method and microCT observations (Klepsch *et al.* , 2018; Scoffoni *et al.* , 2017; Brodribb *et al.* , 2016a). However, the observation of local spreading of embolism in minor veins near cut vessels in experiment 2, supports the hypothesis that embolism spreads from pre-existing gas sources, which happened in minor veins prior to embolism formation in large vessels of major veins (Fig. 3). This also suggests that proximity to a gas source is the main driver for embolism spreading, and not the conduit diameter per se.

Nevertheless, wide and long vessels are more likely to be connected to a pre-existing embolism than narrow, short conduits, as discussed above. It is also possible that large vessels may show a higher amount of intervessel pit membrane area than narrow, short vessels. Although this relationship has been examined with respect to the rare pit hypothesis (Wheeler *et al.* , 2005; Hacke *et al.* , 2006), no support for this idea was found in *Acer* (Lens *et al.* , 2011). Yet, large and wide vessels are likely to embolise first, and this temporal difference could be caused by their connectivity to a gas source, and may not reflect any inherent difference in embolism resistance per se. No alternative mechanism is known why wide conduits would be vulnerable to embolism, since pit membrane thickness, which is strongly associated with embolism resistance (Li *et al.* , 2016), was not related to conduit diameter (Kotowska *et al.* , 2020; Wu *et al.* , 2020).

The optical method and Pneumatron showed strong 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.* (2019) for *Eucalyptus camaldulensis* , and supports the hypothesis that the pneumatic method relies largely on gas extraction from embolism events in intact vessels (Jansen *et al.* , In press). The fast and straightforward approach of taking pneumatic methods on small samples such as individual leaves make this method also suitable for field observations and further work at the intraspecific and intratree level.

While a high coefficient of determination ($R^2=0.91$) indicated good agreement between P_{50} values obtained from two methods (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 P_{12} values based on both methods (Fig. 5a). 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 the intact vessels that are equally affected by the presence of gas from the cut-open vessels, or close to those that are observed with the optical method (Fig. 6). It is possible that there could be an overlapping area with embolism measured in intact vessels based on both methods (Fig. 6). Yet, it is 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; Zhanget *et al.* , 2020; Kaack *et al.* , 2019).

It is possible that the pressure and humidity inside an embolised vessel affects the process of embolism spreading, with a more efficient propagation of embolism spreading from a cut vessel to an intact one than between two intact ones. 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), and a similar trend was found in Pereira *et al.* (2019). 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. 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). Interestingly, the amount of gas extracted with the Pneumatron is considerably lower than the amount of gas that would be available in intact vessels with average dimensions under atmospheric

pressure.

In conclusion, our work revealed the potential impact of cut-open conduits on embolism resistance in leaf xylem, and we found that embolism spreading during dehydration may happen at a less negative water potential when there is a direct connection to a gas source. This process could be minimised by hydraulic segmentation, with narrow and short conduits slowing down gas diffusion due to their confined and/or poorly interconnected nature.

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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

	Petiole length (cm)	Max. vessel length at the petiole end (cm)	Average vessel length at the base of the p
<i>C. betulus</i>	1.37±0.13	1.44±0.30	0.96±0.12
<i>F. sylvatica</i>	1.23±0.06	3.41±0.27	0.66±0.43
<i>L. tulipifera</i>	8.68±1.39	8.19±1.47	3.52±1.17
<i>P. avium</i>	3.53±1.00	5.47±0.83	/
<i>Q. petraea</i>	1.17±0.21	8.02±1.33	5.1±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 replicates were measured (pale colours). Bright lines are regression lines for each type of sample, and grey lines indicate 95% confidential intervals. P_{50} 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 ($P < 0.05$).

Figure 2 . Relationship between the segmentation index and the difference in P_{12} (red), P_{50} (yellow) and P_{88} (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 3rd or 4th 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_{50} values (MPa) of both methods were presented.

Figure 5 . Correlation between paired values of P_{12} (a), P_{50} (b), and P_{88} (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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