The functional morphology of interconduit pit membranes in fresh and dehydrated xylem tissues of angiosperm species

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Introduction

Xylem anatomy of angiosperms in relation to water transport

Composition of the secondary xylem

The xylem is the main pathway for water transport in most plants. A highly efficient transport system of the xylem has been proposed to contribute to the dominance of angiosperms (Hudson et al. 2010; Feild & Wilson 2012). A typical secondary xylem in angiosperms consists of four types of cells: vessel elements, tracheids, fibres and parenchyma cells (Jane 1956; Esau 1977).

Vessel elements are specialised water conducting cells in most angiosperms. They become dead cells after maturation when hydrolases dissolve their protoplast and the primary wall in perforation plates. With a perforation between adjacent cell wall, various vessel elements form a long hollow multicellular vessel. A vessel can be a few centimeters to meters long. For example, a long vessel of 7.73 m was reported in a woody vine of Pithecoctenium crucigerum (Ewers et al. 1990).

Tracheids are unicellular conduits, which dominate the wood of gymnosperms and also are present in the xylem of angiosperms. Similar to vessel elements, their lateral secondary cell walls become thick and lignified during development. However, no perforation is formed between two tracheids. Tracheids are normally shorter and narrower than vessels, and less than 7 mm in length (Sperry et al. 2006; Klepsch et al. 2016a). Angiosperm tracheids have a lower efficiency of water transport than vessels.

Fibres are elongated, narrow cells with a sharp end. During maturation, the secondary cell walls of tracheids become thick and lignified, which contributes to their mechanical support capacity, but reduces their lumina considerably. Therefore, fibres may contribute to hydraulic capacitance by capillary water (Jupa et al. 2016), but this cell type typically does not transport water under negative pressure. The lack of intact, functional pit membranes between fibres may also indicate that their cell do not contribute to the hydraulic pathway (Sano et al. 2011).
Introduction

Parenchyma cells are living cells and divided into two types, i.e., ray parenchyma and axial parenchyma. Both type of parenchyma form a highly interconnected system (Zimmermann & Tomlinson 1966). The functions of ray and axial parenchyma (Morris & Jansen 2016) include storage of water, minerals, and non-structural carbohydrates (e.g., Holbrook 1995; Trockenbrodt 1995; Plavcová & Jansen 2015), defence against pathogens (e.g., Shigo 1984), the transition of sapwood into heartwood (e.g., Pinto et al. 2004), and biomechanical contributions (e.g., Reiterer et al. 2002).

Interconduit bordered pits

Bordered pits represent openings in the secondary cell walls of tracheids and vessels, which provide a direct pathway for water transport between neighbouring conduits (Jane 1956; Sano et al. 2011). In angiosperms, a bordered pit (Fig. 1a) is composed of an elongated to circular pit border forming a pit cavity and a homogeneous pit membrane, which is unlike torus-margo pit membranes in conifers (Carlquist 1988). The pit border is assumed to provide mechanical support to the pit membrane, and the latter prevents the spreading of air and pathogens between conduits (Zimmermann 1983; Choat et al. 2008; Morris et al. 2016). The pit membrane, with the nanoscale pores formed by the cellulose fibrils, functions as a safety valve, but at the same time, accounts for 58 % of the total xylem hydraulic resistivity (Sperry et al. 2006; Choat et al. 2008).
Fig. 1 A bordered pit in *Drimys winteri* based on transmission electron microscopy (TEM) and air-water menisci at the surface of a pit membrane. (a) The pit borders (PB) overarching a homogeneous pit membrane (PM) form two pit cavities (PC). (b) The pressure required to force an air-water meniscus through a pit membrane is related to the the contact angle ($\phi$), the radius of the pore size (R) between cellulose fibrils (CF), the pore shape correction factor, and the surface tension.

How is water transported through angiosperm xylem?

*The cohesion-tension theory*

Water transport in plants is explained by the prevailing “cohesion-tension” theory (Dixon & Joly 1895; Pickard 1981), which states that water is pulled up from roots to leaves under a negative hydrostatic pressure generated by transpiration. When water molecules evaporate at the leaf surface, the capillary force arises at an air-water meniscus and pulls water upwards to replace the former meniscus. This pressure gradient is transferred to the bulk liquid and results in a further drop in water pressure, which drives water up (Sperry 2011).

According to the cohesion-tension theory, a continuous water column is needed to pull water from roots to the canopy. However, air bubbles dissolved in water could expand under a highly negative pressure, and when this happen, embolism forms in
conduits, which leads to a rupture of the continuous water column. If such embolism in xylem affects many conduits, the hydraulic failure of the xylem may eventually cause desiccation moristermotic tissue and lead to plant die-back (Sperry 2011).

The air-seeding hypothesis

The capillary force of an air-water meniscus (Fig. 1b) in a pit membrane between an embolised and functional conduit may stop the spreading of air bubbles from the embolised to the functional conduit. According to the Young-Laplace equation, the pressure difference ($\Delta P$, MPa) required to force an air bubble through a pit membrane is a function of the radius of the pore size (R, m) (Schenk et al. 2015):

$$\Delta P = 2\kappa\gamma\cos\phi/R$$  \hspace{1cm} (1)

where $\kappa$ is the pore shape correction factor, ranging between 0 and 1 (Emory 1989), $\gamma$ is the surface tension (mJ m$^{-2}$), and $\phi$ is the contact angle between liquid and the surface of the capillary. Assuming a pore shape correction factor of 0.5, a contact angle of zero, and pure water with a surface tension of 72 mJ m$^{-2}$ (Meyra et al. 2007; Caupin et al. 2008), a meniscus could spread through a pit membranes with a pore size of 20 nm at -7.2 MPa. However, the surface tension of xylem sap can be much lower by the presence of surfactants. Therefore, air-seeding would occur at a lower pressure, for example at -2.4 MPa in the xylem sap containing amphiphilic lipids with a surface tension of 24 mJ m$^{-2}$ (Lee et al. 2001). Once the pressure exceeds the capillary force of an air-water meniscus, air-seeding would occur, and embolism would spread through one air-filled conduit to the neighbouring water-filled conduit (Zimmermann 1983). This air-seeding hypothesis explains embolism formation caused by drought stress (Sperry et al. 1988; Choat et al. 2008). The optical method showing the spreading of embolism in the entire leaf network gives the clear evidence for air-seeding (Brodribb et al. 2016a).
The ultrastructure of pit membranes in angiosperms

Composition of pit membranes

The ultrastructure of pit membranes in bordered pits is important for embolism formation since the pore size of pit membranes plays a crucial role in the air-seeding process (Meyra et al. 2007). In angiosperms, the homogeneous interconduit pit membrane consists of layers of non-woven cellulose fibrils (Choat et al. 2008; Jansen et al. 2009). These cellulose fibrils have a diameter of 10-25 nm (Esau 1977; Wang & Zhang 2012), and remain present after hydrolysis, while non-cellulosic substances such as hemicellulose and pectins are enzymatically removed (O’Brien 1970; Klepsch et al. 2016b). Moreover, some compounds such as amphiphilic lipids are also found on and/or within interconduit pit membranes (Schenk et al. 2017; 2018), which would affect the contact angle of the air-water mensicus in pit membranes (McCully et al. 2014; Jansen et al. 2018). The spatial arrangement of cellulose fibrils in pit membranes determines the porosity and thickness of pit membranes (Choat et al. 2008; Jansen et al. 2009).

Porosity of pit membranes

The porosity of pit membranes, which represents the pore volume fraction, has been investigated in many angiosperm species based on different methods, and shows a large variation (5-800 nm) in the pore size of pit membranes (Shane et al. 2000). These methods include scanning electron microscopy (SEM) observation (Sano 2005; Hacke et al. 2007; Hillabrard et al. 2016), air injection (Crombie et al. 1985), dextran perfusion (Van Alfen et al. 1983), and colloidal gold perfusion (Choat et al. 2003; 2004). Large pores (up to 700 nm in diameter) in pit membranes, especially shown under SEM obtained from hydrated samples (Sano 2005; Hacke et al. 2007; Hillabrard et al. 2016) should be interpreted with care because dehydration during preparation could cause an irreversible shrinkage of pit membranes (Li et al. 2016). A pore size of 5-20 nm based on fresh samples may give a more accurate range for the porosity of pit membranes (Choat et al. 2003; 2004).
Introduction

Thickness of pit membranes

The thickness of pit membranes shows a large variation, from 60-1184 nm based on records of 131 angiosperm species (Li et al. 2016), although some data come from dehydrated or samples that were frozen for a long time. The highest value was found in fresh pit membranes of *Acacia pataczekii* with 1184 nm, and the lowest value came from dried pit membranes of *Tetracentron sinense* with 60 nm (Li et al. 2016). Fresh pit membranes of 67 species showed a mean value of 315 ± 221 nm, while non-fresh pit membranes of 70 species had a mean value of 227 ± 108 nm (Li et al. 2016).

An interesting correlation between the thickness of pit membranes and embolism resistance has been found for many species (Jansen et al. 2009; Scholz et al. 2013; Li et al. 2016), which indicates that a thick pit membrane shows a higher embolism resistance than a thin pit membrane. The difference in porosity could be an explanation (Jansen et al. 2009; Li et al. 2016). Moreover, an increase of a porous medium may show a higher tortuosity, which is the ratio of the actual flow pathway length to the length of the medium (Vallabh et al. 2011). However, we know little about the porosity and tortuosity of pit membranes, which represent a porous medium between conduits.

Pit membranes upon dehydration

Differences in the porosity and thickness of pit membranes between fresh and dried samples have been reported in some angiosperms (Pesacreta et al. 2005; Jansen et al. 2008; Li et al. 2016). A simple model of pit membranes (Fig. 2) may show these differences of pit membranes under wet and dried conditions. Assuming a 20 nm diameter of one cellulose fibril, and a 20 nm distance between cellulose fibrils under a wet condition, a 700 nm thick fresh pit membrane would consists of 18 layers of cellulose fibrils. In the simplified model of Fig. 2, it is assumed that cellulose fibrils in each layer are aligned parallel to each other, and that each cellulose fibril layer has a 45 degree orientation to the next neighbouring layer. The porosity of a fresh pit membrane can then be calculated and is 73.63 %. When the pit membrane has dried
and shrunken, the distance between the layers of cellulose fibrils is expected to be zero, and cellulose fibrils within the same layer randomly in pairs of 2 or 3 fibrils. Under this condition, the thickness of a dried pit membrane in the model of Fig. 2 is 360 nm, which means that the membrane has shrunken by ca. 50 %, and the porosity has reduced to 47.94 %. However, more studies are needed to understand the spatial arrangement of cellulose fibrils in pit membranes of angiosperms.

**Fig. 2** A simple model of interconduit pit membranes of angiosperms showing how the cellulose fibril arrangement differs between a fresh and a dried pit membrane

**Xylem embolism resistance measurements**

*The vulnerability curve*

Xylem embolism resistance is an important functional trait for woody species to define their limits of drought tolerance (Choat et al. 2012). Xylem embolism resistance of angiosperm species has been assessed in many ways (Cochard et al. 2013). All the methods construct a vulnerability curve to evaluate xylem embolism
resistance, although disagreements among the difference methods are common (Torres-Ruiz et al. 2014; Jansen et al. 2015) because most method are destructive and invasive and face the artifacts of “open vessel”. The vulnerability curve exhibits the relationship between the xylem water potential ($\Psi$, MPa) and embolism (Tyree & Sperry 1989), from which $\Psi_{50}$ is calculated to show the embolism resistance when there is a 50 % loss of xylem hydraulic conductivity.

Techniques for building the vulnerability curve

Methods constructing the vulnerability curve differ in how to induce water stress in xylem and how to assess embolism (Cochard et al. 2013). The water stress in the xylem can be induced by natural dehydrating (e.g., Sperry 1986, Sperry et al. 1988), centrifugal force (e.g., Li et al. 2008; Wang et al. 2014), and air-injection (e.g., Cochard et al. 1992; Salleo et al. 1992). Quantifying the degree of embolism is based on hydraulic measurements (e.g., Tyree & Dixon 1986; Sperry et al. 1988), detection of acoustic emissions (e.g., Mayr & Rosner 2011; Wolkerstorfer et al. 2012), X-ray microtomography (e.g., Torres-Ruiz et al. 2015; Choat et al. 2016), optical visualisation (e.g., Brodribb et al. 2016b; 2017), and the recently proposed pneumatic method (Pereira et al. 2016). Since each technique has its pros and cons, it is important and necessary to compare and evaluate different techniques (Jansen et al. 2015) in order to accurately determine xylem embolism resistance.
Aims of this thesis

The contents of this thesis can be divided into two parts. The first part (chapter 1 and chapter 2) focuses on the ultrastructure of interconduit pit membranes of angiosperm species. The second part (chapter 3) pays special attention to measuring xylem embolism resistance of angiosperms.

Chapter 1 focuses on bordered pits in vesselless angiosperm species. The ultrastructure of bordered pit membranes in vesselless angiosperms is poorly studied. Although the occurrence of vessels or cryptic vessels was reported in vesselless angiosperms before (Hacke et al. 2007; Ren et al. 2007), this chapter aims to test whether earlier controversial findings of perforation plates in vesselless angiosperms are affected by preparation artefacts, and how dehydration leads to structural changes of intertracheid pit membranes. The potential preparation artefacts during dehydration are hypothesised to account for the misinterpretation of tracheids as vessels or cryptic vessels.

Chapter 2 focuses on the porosity of intervessel pit membranes in angiosperms that differ in embolism resistance. Important questions are:

1) What is the pore size of intervessel pit membranes in angiosperm species?
2) Is the porosity of pit membranes different between species that differ in xylem embolism resistance?
3) How may dehydration change pit membrane porosity?
4) Are effects of dehydration on pit membranes reversible or not?

These are important questions that are highly relevant to understand the hydraulic resistance affected by pit membranes, the mechanism behind the spreading of air from an embolised to a water-filled conduit (i.e., air-seeding), and to understand potential consequences of hydraulic failure for water transport.

Chapter 3 aims to validate the pneumatic method, which estimates xylem embolism
resistance. Since this technique has only been applied to tropical and subtropical species (Pereira et al. 2016), this chapter aims to test whether the pneumatic method is suitable to temperate species, including both diffuse-porous and ring-porous species. The long vessels in earlywood of ring-porous species provide a potential problem for vulnerability curves based on the centrifuge method due to an open-vessel artefact (Torres-Ruiz et al. 2014; 2015). The pneumatic method has not been applied yet to ring-porous species. An important question is also whether or not this technique can be applied to conifers, which have a different pit membrane structure than angiosperms. By applying the pneumatic method to angiosperms and conifers, the importance of extracting gas from conduits is assumingly related to the pit membrane structure and its behaviour under dehydration, which links this chapter with the two first chapters.
Research topics and copyright permissions

Chapter 1 - Bordered pits in xylem of vesselless angiosperms and their possible misinterpretation as perforation plates

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Chapter 3 - Testing the plant pneumatic method to estimate xylem embolism resistance in stems of temperate trees

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Summary of chapters

Chapter 1

Vesselless wood represents a rare phenomenon within the angiosperms, characterizing Amborellaceae, Trochodendraceae and Winteraceae. Anatomical observations of bordered pits and their pit membranes based on light, scanning and transmission electron microscopy (SEM and TEM) are required to understand functional questions surrounding vesselless angiosperms and the potential occurrence of cryptic vessels. Interconduit pit membranes in 11 vesselless species showed a similar ultrastructure as mesophytic vessel-bearing angiosperms, with a mean thickness of 245 nm (± 53, SD; n = six species). Shrunken, damaged and aspirated pit membranes, which were 52 % thinner than pit membranes in fresh samples (n = four species), occurred in all dried-and-rehydrated samples, and in fresh latewood of Tetracentron sinense and Trochodendron aralioides. SEM demonstrated that shrunken pit membranes showed artificially enlarged, > 100 nm wide pores. Moreover, perfusion experiments with stem segments of Drimys winteri showed that 20 and 50 nm colloidal gold particles only passed through 2 cm long dried-and-rehydrated segments, but not through similar sized fresh ones. These results indicate that pit membrane shrinkage is irreversible and associated with a considerable increase in pore size. Moreover, our findings suggest that pit membrane damage, which may occur in planta, could explain earlier records of vessels in vesselless angiosperms.
Chapter 2

Intervessel pit membranes represent porous media for water transport between neighbouring vessels. The cellulose fibrils form a three dimensional network similar to a fibrillar, non-woven porous media that shrinks by dehydration. The size of pore spaces is largely unknown because of the difficulty in measuring its dimensions at the nanoscale without preparation artefact. The ultrastructure of fresh and dried-rehydrated pit membranes in petioles of three angiosperm species was observed with transmission electron microscopy (TEM). Perfusion with colloidal gold was performed to compare the relative porosity between fresh and dried-rehydrated membranes in three angiosperm species. Fresh pit membranes showed a considerable variation in thickness, with 268 nm in Acer pseudoplatanus, 686 nm in Cinnamomum camphora, and 504 nm in Persea americana. Dehydration caused a 46-50 % shrinkage in the three species. While 20 nm gold particles penetrated fresh pit membranes of A. pseudoplatanus, 5 and 10 nm colloidal gold particles could be seen within fresh pit membranes of C. camphora and P. americana. However, no gold particles penetrated the shrunken, dried-rehydrated pit membranes of the three species tested. Shrinkage of pit membranes after dehydration appears to be irreversible and is associated with a considerable reduction of the pore spaces in the pit membranes. The cellulose fibrils are suggested to form a tightly packed structure with a “Velcro effect”, which might be functionally similar to aspiration of a torus-bearing pit membrane in gymnosperms.
Chapter 3

Methods to estimate xylem embolism resistance generally rely on hydraulic measurements, which can be far from straightforward. Recently, a pneumatic method based on air flow measurements of terminal branch ends was proposed to construct vulnerability curves by linking the amount of air extracted from a branch with the degree of embolism. We applied this novel technique for 10 temperate tree species, including six diffuse, two ring-porous and two gymnosperm species, and compared the pneumatic curves with hydraulic ones obtained from either the flow-centrifuge or the hydraulic-bench dehydration method. We found that the pneumatic method provides a good estimate of the degree of xylem embolism for all angiosperm species. The xylem pressure at 50% and 88% loss of hydraulic conductivity (i.e., $\Psi_{50}$ and $\Psi_{88}$) based on the methods applied showed a strongly significant correlation for all eight angiosperms. However, the pneumatic method showed significantly reduced $\Psi_{50}$ values for the two conifers. Our findings suggest that the pneumatic method could provide a fast and accurate approach for angiosperms due to its convenience and feasibility, at least within the range of embolism resistances covered by our samples.
References


References


References


Chapter 1

Bordered pits in xylem of vesselless angiosperms and their possible misinterpretation as perforation plates

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ABSTRACT
Vesselless wood represents a rare phenomenon within the angiosperms, characterizing Amborellaceae, Trochodendraceae and Winteraceae. Anatomical observations of bordered pits and their pit membranes based on light, scanning and transmission electron microscopy (SEM and TEM) are required to understand functional questions surrounding vesselless angiosperms and the potential occurrence of cryptic vessels. Interconduit pit membranes in 11 vesselless species showed a similar ultrastructure as mesophytic vessel-bearing angiosperms, with a mean thickness of 245 nm (±53, SD; n = six species). Shrunk, damaged and aspirated pit membranes, which were 52% thinner than pit membranes in fresh samples (n = four species), occurred in all dried-and-rehydrated samples, and in fresh latewood of Tetracentron sinense and Trochodendron aralioides. SEM demonstrated that shrunk pit membranes showed artificially enlarged, >100 nm wide pores. Moreover, perfusion experiments with stem segments of Drimys winteri showed that 20 and 50 nm colloidal gold particles only passed through 2 cm long dried-and-rehydrated segments, but not through similar sized fresh ones. These results indicate that pit membrane shrinkage is irreversible and associated with a considerable increase in pore size. Moreover, our findings suggest that pit membrane damage, which may occur in planta, could explain earlier records of vessels in vesselless angiosperms.

Key-words: bordered pit; perforation plate; pit membrane; tracheed.

INTRODUCTION
The evolution of a water transport system from tracheids to vessels has been proposed to contribute to the success of angiosperms over the last 132 million years (Bailey 1944; Bond 1989; Solis et al. 2006; Hudson et al. 2010; Feld & Wilson 2012). The shift from tracheids to vessels greatly increases the xylem water supply efficiency, which may enable the adaptive radiation of plant growth form and habitat (Sperry 2003; Hudson et al. 2010). In almost all gymnosperms and ferns, unicyclic tracheids serve as water conducting units with bordered pits on their lateral lignified walls (June 1956; Sperry 2003). Tracheids are limited to about 7 mm in length (Hucke et al. 2004; Klepsch et al. 2016a). Vessels, however, which appear in almost all angiosperms, are multicellular and consist of many vessel elements. These are axially connected to each other and are characterized by bordered pits on joint vessel walls as well as perforation plates near their cell tips (June 1956; Carlquist & Schneider 2002; Jansen & Nardini 2014). Unlike bordered pits, perforations represent a complete opening in the cell wall. Vessels can attain a greater maximum diameter (up to 500 μm) than tracheed (≤80 μm) and can be several meters long (Zimmermann 1983; Sperry 2003).

Bordered pits on lateral walls provide the only direct pathway for water transport between neighbouring conduits (Sano et al. 2011). One of the most important components of bordered pits include the pit membrane, which are traditionally assumed to prevent the spread of air and pathogens between conduits (Zimmermann & Brown 1971; Sperry & Tyree 1988; Choat et al. 2008; Morris et al. 2016), but at the same time may increase the hydraulic resistance (Sperry et al. 2005; Hucke et al. 2006; Choat et al. 2008). In conifer tracheed, the characteristic torus-margo pit membrane imposes much less hydraulic resistance than the homogeneous pit membrane in angiosperm vessels (Pittermann et al. 2005; Choat et al. 2008), because the pores in the margo can be >100 nm (Bouche et al. 2014). The size of pit membrane pores in angiosperms is poorly understood and thought to be below 100 nm, with most pores in the range of 5 to 20 nm (Choat et al. 2003; 2004). Hence, angiosperm pit membranes generate an ~60 times higher area-specific flow resistance (Hucke et al. 2007; Choat et al. 2008).

The water conducting system in vesselless angiosperms (Amborellaceae, Trochodendraceae and Winteraceae) has attracted attention for more than 100 years (Van Tieghem 1900; Bailey & Thompson 1918; Bailey & Swamy 1948; Bailey 1957; Carlquist & Schneider 2001). The hypothesis that vessellessness represents a primitive character state in angiosperms has frequently been suggested and is supported by the vesselless wood of Amborella trichopoda, which takes a sister position to all other angiosperm lineages (Bailey & Tupper 1918; Carlquist 1998; Baas et al. 2003). The vesselless Winteraceae and Trochodendraceae, however, are placed among vessel-bearing clades (Solis et al. 1999; Doyle & Endress 2000; Bremer et al. 2009). These two families could be secondarily vesselless, returning to a tracheid-based vascular system due to frost adaptation (Feld & Brodribb 2001; Feld et al. 2002). Frost-induced embolism formation is less
likely in narrow conduits than in wide ones (Davis et al. 1999; Pittermann & Sperry 2006). However, not all vesselless angiosperm species experience frost in their natural environment. Alternatively, the irreversible vessel evolution hypothesis would require nine independent vessel origins within angiosperms (Doye & Endress 2000; Feldl et al. 2002).

The wood of vesselless angiosperms is composed of tracheids, ray parenchyma cells and axial parenchyma cells, with distinct growth rings in *Tetracentron* and *Tetracentron* (Carlquist 1988, 1989; Suzuki et al. 1991; Carlquist & Schneider 2001). Unlike conifers, tracheids in vesselless angiosperms lack a torus-margo structure (Meylan & Butterfield 1982). Hydraulic data from 20 vesselless angiosperm species suggested a low resistance of bordered pits between tracheids, which was similar to conifers, and much lower than eudicot intersubcellular pits (Hakse et al. 2007). This low pit resistance could be caused by a porous pit membrane (Hakse et al. 2007; Chout et al. 2008), but little information is available about the ultrastructure of bordered pits in vesselless angiosperms. The fine structure of the pit membrane in intertracheid pits of *Pseudowintera* *dandy* (Winteraceae) was described as ‘a porous, open-textured microfibril structure typical of a hydrolysed wall’ (Meylan & Butterfield 1982).

Bordered pits not only occur in walls of tracheids but can also be found in vessel-parenchyma and parenchyma-parenchyma cells in numerous species of woody angiosperms (Schmid & Machado 1968; Carlquist 2007). The pits between vessels and parenchyma cells are typically half-bordered on the vessel side, and an ‘amorphous layer’ (also called protective layer) is most pronounced close to the pit membrane on the parenchyma side (Machado & Schmid 1964; Schmid & Machado 1968; Czaninski 1977; Fujii et al. 1980; Barnett et al. 1993; Plavcová & Jansen 2015). Besides the amorphous layer, vessel-ray pit membranes may also differ in the chemical composition of their pit membranes, such as the characteristic presence of a ‘black cap’ (Plavcová & Hakse 2011; Kim & Daniel 2013, 2014). It is unknown if an amorphous layer and black cap occur in vesselless angiosperms in tracheid-parenchyma pits.

Angiosperm pit membranes consist of various layers of cellulose microfibrils (Jansen et al. 2009; Schenk et al. 2015; Klepsh et al. 2016b). The density and arrangement of microfibrils determine the porosity of a pit membrane (Chout et al. 2008), which strongly influence the hydraulic resistance and vulnerability to embolism via air-seeding (Jansen et al. 2009).

A thin pit membrane has been assumed to be related to lower hydraulic resistance and high porosity (Chout et al. 2008; Jansen et al. 2009; Lens et al. 2011; Scholz et al. 2013b). A positive correlation between intersubcellular pit membrane thickness (Tsub) and P50 (i.e. the stem water potential at 50% loss of hydraulic conductivity) indicates that pit membrane thickness determines hydraulic safety (Li et al. 2016). Since P50 values for vesselless angiosperms were found to vary from ~1.5 MPa in *Tetracentron sinense* to ~5.5 MPa in *Pseudowintera* *travertici* (Hakse et al. 2007; Perciara et al. 2016; Trueba et al. 2016), considerable variation can be expected in intertracheid pit membrane thickness of vesselless angiosperms.

In this study, we aim to investigate the structure of intertracheid pits, tracheid-parenchyma pits and parenchyma-parenchyma pits by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) in 11 vesselless angiosperm species. We expect that intertracheid pits have a thin pit membrane to provide a low pit resistance because vesselless angiosperms retain a similar sapwood-specific resistance as conifers (Sperry et al. 2007). We also hypothesize that earlier observations of perforation plates in vesselless angiosperms are unlikely, assuming that the occurrence of vessels or ‘cryptic’ vessels represents preparation artefacts (Feldl et al. 2000; Hakse et al. 2007; Rcn et al. 2007). Inappropriate preparation methods for SEM and TEM can lead to misinterpretation of the pit membrane as a perforation plate, with potentially pit membrane remnants (Carlquist & Schneider 2007; Feldl & Wilson 2012). Chemical treatments (e.g. acetone, ethanol and hydrogen peroxide) and dehydration of wood can result in shrinkage, mechanical damage or tearing of pit membranes, causing an artificial increase in porosity and the apparent occurrence of a perforation plate instead of a bordered pit (Thorsen 2000; Paezera et al. 2005; Jansen et al. 2008, 2009; Li et al. 2016).

Additional attention is given to the potential occurrence of lipids in intertracheid pit membranes as reported in vessel-bearing angiosperms, which would have important consequences for air-seeding (Schneider et al. 1999; Meylan et al. 2007; Westhoff et al. 2008; Jansen & Schent 2015; Schent et al. 2017). We will also examine the presence of an amorphous layer, potential differences in intertracheid pit membrane structure between earlywood and latewood and the occurrence of tracheid dimorphism, which was reported in *Tetracentron sinense* (Suzuki et al. 1991).

An additional side goal was to link various wood anatomical features with embolism resistance. Based on earlier work, it can be expect that P50 values of vesselless angiosperm species correlate with intertracheid pit membrane thickness, intertracheid wall thickness, tracheid diameter, pit membrane pore size and the intertracheid pit membrane surface area (Wheeler et al. 2005; Hakse et al. 2007; Lens et al. 2011; Scholz et al. 2013b; Li et al. 2016). The latter has been given considerable attention in support of the rare pit hypothesis (Wheeler et al. 2005; Hakse et al. 2007). Testing these functional correlations was based on six species for which fresh wood material was available.

**MATERIAL AND METHODS**

**Plant material**

Eleven species from a total of three plant families were studied (Table 1). For six species, fresh samples were collected in November and December 2015 from twigs that were ~30 cm long and 5–10 mm thick, including samples from one or two individuals in each species for analysis, and from two or three specimens were studied for five species (Table 1). After wrapping the material in wet tissue and plastic bags, samples were hand-carried or express mailed to the lab for sample preparation. Fixation of the samples for...
Table 1. List of the vesselless angiosperm species studied with their family classification based on APG III (Bremer et al. 2009), collecting sites, condition (fresh or dried) and the electron microscopy method applied

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Number of samples</th>
<th>Collecting site</th>
<th>Condition</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amborella trichopoda Baill.</td>
<td>Amborellaceae</td>
<td>2</td>
<td>Bonn University, Botanical Garden, Germany; Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Bellisum gracile A.C.Sm.</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Dried</td>
<td>SEM</td>
</tr>
<tr>
<td>Bellisum hupleos (B.L.Burtt) A.C.Sm.</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Dried</td>
<td>SEM</td>
</tr>
<tr>
<td>Bubbia oligocarpa (Sebhr.) B.L. Burtt</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Dried</td>
<td>SEM</td>
</tr>
<tr>
<td>Bubbia senevraioides (F.Muell.) B.L.Burtt</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Dried</td>
<td>SEM</td>
</tr>
<tr>
<td>Drimys winteri J.R.Forst. and G. Forst.</td>
<td>Winteraceae</td>
<td>2</td>
<td>Ulm University, Botanical Garden, Germany; Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Drimys winteri J.R.Forst. and G. Forst.</td>
<td>Winteraceae</td>
<td>2</td>
<td>Ulm University, Botanical Garden, Germany; Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Pseudowintera colorata (Raoul) Dandy</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Dried</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Tasmannia lanceolata Baill.</td>
<td>Winteraceae</td>
<td>1</td>
<td>Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Tetracentron sinense Oliv.</td>
<td>Trochodendraceae</td>
<td>2</td>
<td>Rosberg, Westport, County Mayo, Ireland; Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
<tr>
<td>Trochodendron aralioides Siebold and Zucc.</td>
<td>Trochodendraceae</td>
<td>3</td>
<td>Bonn University, Botanical Garden, Germany; University of Greifswald, Botanical Garden, Germany; Royal Botanic Gardens, Kew</td>
<td>Fresh</td>
<td>SEM, TEM</td>
</tr>
</tbody>
</table>

TEM observation was conducted within 5 d. During storage, stems were kept in water and put in a refrigerator to avoid dehydration and fungal growth. Dried samples were removed from twig material for a total of six species at the herbarium of the Royal Botanic Gardens, Kew. The taxonomic identity of all species was verified by using the International Plant Names Index database.

Transmission electron microscopy

Transmission electron microscopy techniques on fresh samples were applied at the Electron Microscope Centre of Ulm University according to a standard preparation protocol (Jansen et al. 2012; Scholz et al. 2013b; Li et al. 2016). In general, fresh wood samples of six species were cut into ~1 mm³ and fixed in a standard fixative (2.5% glutaraldehyde, 0.1 mol phosphate, 1% cacochlar and pH 7.3) overnight at room temperature. Then, samples were washed in a 0.2 M phosphate buffer and postfixed with 2% buffered osmium tetroxide for 2 h at room temperature. After washing with a buffer solution, the samples were dehydrated with a gradual ethanol series (30%, 50%, 70%, 90% and 100%). Then, samples were block stained with a saturated solution of uranyl acetate in ethanol for 30 min at 37°C, except for fresh samples of Tasmannia lanceolata, which was prepared at the Royal Botanic Gardens, Kew. The ethanol was gradually replaced with Epon resin (Sigma-Aldrich, Steinheim, Germany) at room temperature, and the resin was polymerized at 60°C for 48 h. After trimming, embedded samples were sectioned on an ultra-microtome (Ultracut E, Reichert-Jung, Austria). Transverse, semi-thin (~500 mm thick) sections were cut from the embedded samples with a glass knife, heat-fixed to glass slides, stained with 0.5% toluidine blue in 0.1 M phosphate buffer and mounted on slides with DPX (Agar Scientific, Stansted, UK). Transverse, ultra-thin (60 to 90 nm) sections were cut with a diamond knife and attached to 300 mesh hexagonal copper grids (Agar Scientific, Stansted, UK). Observations were conducted with a JEOL 1400 TEM (JEOL, Tokyo, Japan) at 120 kV accelerating voltage, and TEM images were taken with a digital camera (Soft Imaging System, Münster, Germany).

Dried wood samples of nine species (Table 1) were cut into 1 mm³ blocks and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%). The ethanol was gradually replaced with LR White Medium Grade Acryl resin (London
Resin Co., Reading, UK) over several days, with the resin changed approximately every 12 h. The resin was polymerized in a Weiss Gallenkamp (Loughborough, U.K.) vacuum oven at 60°C and 1000 mm Hg for 24 h. Embedded samples were trimmed with a Leica EM specimen trimmer (Leica Microsystems, Vienna, Austria). Semi-thin and ultra-thin sections were prepared as described earlier. The ultra-thin sections were attached to Formvar grids (Agar Scientific) and stained with uranyl acetate and lead citrate by using a Leica EM Stainer (Leica Microsystems). Similar post-staining was also applied to fresh sections of *Tetraminum lanceolatum*. Observations were carried out by using a JEM-1210 TEM (Jsool, Tokyo, Japan) at 80 kV accelerating voltage, and digital images were taken by using a MegaView III camera (Soft Imaging System, Münster, Germany).

**Scanning electron microscopy**

Wood samples (5–10 mm long) were split in a tangential or radial plane, air-dried at room temperature, fixed to aluminium stubs and coated with gold by using a sputter coater (FL-0946 Balzers, Fürstenfeld, Switzerland) for 2 min. Observations were carried out by using a SEM (Phenom-XL-0067-L, Netherlands) at an accelerating voltage of 5 kV.

Wood samples from the Royal Botanic Gardens, Kew (Table 1), were observed by using a Hitachi S-4700 field-emission SEM (Hitachi High Technologies Corp., Tokyo, Japan) at an accelerating voltage of 2 kV.

**Anatomical measurements**

Anatomical measurements were conducted by using IMAGEJ software (version 1.48, National Institutes of Health, Bethesda, MD, USA). Measurements were conducted separately for earlywood and latewood of *Trichodenotus* and *Tetraecenotus*. An overview of the definitions and abbreviations of pit characteristics is provided in Table S1 in the Supporting Information. Based on TEM images, the pit membrane thickness (\(T_{\text{pm}}\), nm) was based on three measurements: at opposite sides near the pit membrane annulus (i.e., close to the pit border) and in the centre. The thickness of the amorphous layer (\(T_{\text{a}}\), nm) was measured in a similar way for fresh material. In general, pit measurements were taken from at least 20 pits per species.

SEM images were used for measuring the intertracheid pit membrane diameter (\(D_{\text{pm}}\), \(\mu m\)), outer pit aperture diameter (\(D_{\text{oa}}\), \(\mu m\), i.e. the pit aperture as viewed from the outermost cell wall) and intertracheid pitfield fraction (\(F_{\text{pf}}\), the ratio of intertracheid surface area occupied by intertracheid pits to the total intertracheid wall area), with mean values based on at least 50 pits per species. The pit membrane diameter (\(D_{\text{pm}}\)) was calculated as the diameter of the circle having the same area as the pit membrane measured. The largest (\(D_{\text{pm}}\)) and shortest diameter (\(D_{\text{pm}}\)) of the outer pit aperture were measured separately. The ratio of \(D_{\text{pm}}\) and \(D_{\text{pm}}\) was also calculated. Pit density (\(P_{\text{d}}\), number 100 \(\mu m^{-2}\)) was defined as the number of pits on a tangential wall per 100 \(\mu m\) tracheid length, which was randomly selected, including tracheids tips and central areas. At least 10 tracheids for each species were examined. Field-emission SEM images of dried samples were used for measuring the pore size (\(D_{\text{ps}}\), nm) at its broadest point and the microfibril thickness (\(T_{\text{mf}}\), nm) on pit membranes with at least 25 counts.

Semi-thin sections were observed under a light microscope (LM) (Zeiss Axios Zoom V16, Göttingen, Germany) for measuring the intertracheid wall thickness (\(T_{\text{ww}}\), \(\mu m\)), tracheid diameter (\(D_{\text{t}}\), \(\mu m\)) and intertracheid contact fraction (\(F_{\text{c}}\)). \(T_{\text{ww}}\) was measured as the double intertracheid wall thickness in the middle of adjacent tracheids. \(F_{\text{c}}\) was calculated as the diameter of the circle having the same area as the measured tracheid and was limited to five species for which fresh samples were available. \(F_{\text{c}}\) was calculated as the ratio of the sum of the intertracheid contact perimeter to the total tracheid perimeter. More than 100 measurements were taken to calculate mean values per species.

Tracheid length (\(T_{\text{l}}\), \(\mu m\)) was measured on macerated samples of the stem xylem according to standard procedures (Jansen et al. 1998; Scholz et al. 2013a). Dried earlywood slivers were immersed in a mixture with one part glacial acetic acid and two parts hydrogen peroxide at 60°C for 2 d. Samples were washed three times with water, stained with 0.05% safranin and observed under a LM (Zeiss Axios Zoom.V16, Göttingen, Germany). Measurements of the tracheid length were based on at least 50 tracheids per species.

The total intertracheid pit membrane surface area per tracheid (\(A_{\text{pm}}\), \(m^2\)) was calculated as \(A_{\text{pm}} = F_{\text{pf}} \times T_{\text{l}}\) (Wheeler et al. 2005), and \(A_{\text{pm}}\) (the tracheid surface area, \(m^2\)) and \(F_{\text{pf}}\) (pit fraction) were calculated as follows:

\[
A_{\text{pm}} = \pi \times D_{\text{pm}} \times T_{\text{l}}  \tag{1}
\]

\[
F_{\text{pf}} = F_{\text{c}} \times T_{\text{l}}  \tag{2}
\]

where \(T_{\text{l}}\) represented tracheid length, \(F_{\text{c}}\) the intertracheid contact fraction and \(F_{\text{pf}}\) the intertracheid pitfield fraction. \(D_{\text{pm}}\) (tracheid lumen resistivity diameter, \(\mu m\)) was based on the tracheid lumen resistivity (\(R_{\text{L}}\), \(\text{MPa} \cdot \text{s} \cdot \text{m}^{-1}\)), which was calculated based on the Hagen-Poiseuille law:

\[
R_{\text{L}} = \frac{128 \times \eta}{(\pi \times D_{\text{pm}}^4)}  \tag{3}
\]

with \(\eta\) as the water viscosity index (1.002 × 10^{-5} MPa s at 20°C). The total tracheid lumen resistivity was obtained by measuring several sectors of a stem, and the average \(R_{\text{L}}\) was then put back to the Hagen-Poiseuille law to obtain \(D_{\text{pm}}\).

**Perfusion experiments with colloidal gold particles**

Branch segments of *Pinus pinaster* (15 to 25 cm) were collected from the greenhouse of the botanical garden at Ulm University between August 2016 and April 2017. Samples wrapped in wet tissue were transferred to the laboratory, submerged in water, and 3 to 5 cm at both ends was removed with a sharp razor blade. The branches were then cut into 2 cm long segments, which were much longer than the maximum tracheid length of 2943 \(\mu m\), and connected to a perfusion system (Fig. S1 in the Supporting Information).
Samples were perfused with phosphate buffer (pH 6) to which we added 1 mL of 5 or 20 nm colloidal gold solution (Sigma, Saint Louis, USA) with a concentration of $1 \times 10^{-9}$ g mL$^{-1}$. During the perfusion experiment, low pressure was applied by a 50 cm water column and the gold was introduced to the system via a three-way stopcock (Fig. S1).

In an initial experiment with a saturated acid fuchsin solution, we noticed that the dye appeared in the outflow after 10 to 20 s. In the perfusion experiments with colloidal gold, we therefore ignored the outflow during the first 30 s and collected the outflow solution for 10 min until we had at least 0.5 mL. Perfusion with colloidal gold particles was applied to four fresh samples of *D. winteri* for each particle size (i.e. 5 and 20 nm particles).

We also conducted gold perfusion experiments with dried- and rehydrated branches of *D. winteri*. Branches were dried for more than 48 h at room temperature and rehydrated with a vacuum pump overnight to remove xylem embolism. Drying of 2 cm long stem segments over 2 d at room temperature resulted in 96% desiccation of the samples. In addition, these samples were flooded with distilled water at 0.2 MPa until no air bubbles could be seen escaping from the immersed stem segments. Due to potential shrinkage of the pith tissue, we applied superglue on the pith to avoid potential leakage of colloidal gold via this tissue. Perfusion of dried and rehydrated branches with a saturated acid fuchsin solution showed that the dye appeared in the outflow solution after >10 min, indicating that xylem conductivity was much lower than in the fresh samples. Therefore, we started collecting the flow output 15 min after injection of colloidal gold, sampling 0.2-0.3 mL over a total of 10 min.

We used a silver enhancer kit (Sigma, St Louis, USA) to quantify the amount of colloidal gold in the output solution. The initial extinction factor of a mixture of 0.5 mL outflow solution and 2.4 mL demineralized water was measured at an absorption of 600 nm with a Modell A550 UV/VIS-Spectrometer (Perkin-Elmer, Waltham, USA). A sample of 2.9 mL demineralized water was measured as reference solution. Then, we added 0.1 mL of a freshly made 1:1 mixture of silver solutions A and B from a silver enhancer kit (Sigma, St Louis, USA) to the 2.9 mL solution. The final extinction factor was measured after putting the entire solution in a dark box for 30 min due to light sensitivity of the silver enhancer. The concentration of gold particles in the outflow solution was calculated based on the extinction factor values that were obtained from a calibration curve of known gold concentrations (Fig. S2).

To visualize gold particles under TEM, we perfused a 2 cm long fresh stem segment of *Drimys winteri* with an equal mixture of 5 and 20 nm colloidal gold particles following the previously mentioned method. A similar perfusion was applied to a 2 cm long branch segment that was dried for 2 d at a room temperature and rehydrated in a similar way as described earlier. After perfusion for 10 min, 1 mm$^3$ wood blocks were cut at a proximal distance of 5 mm from the injection side, corresponding to 1.5 times the maximum tracheid length of *D. winteri*, and prepared for TEM. In an additional experiment, we mixed equal amounts of a solution containing 20 and 50 nm gold particles to perfuse fresh and dried-and-rehydrated branches of *D. winteri*, but this time, the 1 mm$^3$ wood blocks that were prepared for TEM were taken at a distance between 17 and 20 mm from the injection, that is the final distal part of the stem segment. TEM preparation of colloidal gold samples was similar to the methodology described earlier, except that OsO$_4$ postfixation was emitted to enhance the detection of gold particles under TEM, which could be difficult to distinguish from the OsO$_4$ stained lipids in pit membranes (Schlenk et al. 2017). About 100 tracheids were observed with TEM, including 20 to 30 intertracheid pit membranes for detection of gold particles.

### Xylem embolism resistance

$P_{50}$ values of six species for which fresh material was available were based on data from Hacke et al. (2007), Pereira et al. (2016) and Baret et al. (2016).

### Statistics

SPSS software (version 21, IBM Corp, Armonk, New York) was used for statistical analyses. A Mann-Whitney U-test was applied to compare perfusion with 5 and 20 nm colloidal gold particles as well as pit membrane thickness between vesselless and vessel-bearing angiosperms. Pearson correlation analyses were performed to determine the relationships between $P_{50}$ and pit characteristics. Correlations were considered significant at $P \leq 0.05$.

## RESULTS

### Tracheid anatomy and intertracheid pits

No perforation plates could be found in any species studied based on light and electron microscopy of sections and macerations. Uniseriate intertracheid pits were bordered in all species studied (Fig. 1a). Biseriate, circular pits were obvious in species of *Belliotium*, *Bubbia*, *Drimys*, *Pseudowintera* (Fig. 1b) and *Tetracentron*. Multiseriate, alternate pits were found in *Belliotium gracile* (Fig. 1c), *Bubbia oligocarpa*, *Drimys* and *Tetracentron*. Scalariform pits were notable in *B. oligocarpa* and earlywood of *Tetracentron* (Fig. 1d) and *Trophodenodon*. A warty layer was observed on inner tracheid walls of *Drimys*, *Pseudowintera* (Fig. 1b) and *Trophodenodon* (earlywood and latewood).

The mean pit membrane diameter ($D_{\text{PM}}$) was 6.73 ± 1.42 µm ($n = 11$ species, mean ± SD), varying between 4.29 (Tasmanian lanceolata) and 9.36 µm (Bubbia venenapora). The outer pit aperture was typically oval, with a mean longest diameter of 4.87 ± 1.45 µm and a mean shortest diameter of 1.72 ± 0.41 µm. The mean ratio of $D_{\text{PM}}$ to $D_{\text{A}}$ was 0.37 ± 0.05 ($n = 11$ species). The total intertracheid pit membrane surface area per tracheid area ($A_{\text{P}}$) varied between 0.010 (*Anobolea trichopoda*) and 0.023 mm$^2$ (*Drimys winteri*) with a mean value of 0.017 ± 0.006 mm$^2$ ($n = 5$ species).

The mean pit density ($P_{50}$) was 17.49 ± 7.44 per 100 µm of tracheid length ($n = 11$ species).
The tracheid length varied from 1116 ± 168 (Tassaninia lanceolata) to 3524 ± 880 μm (Belliholom gracile) with a mean value of 2116 ± 705 μm (n = 11 species). The mean tracheid diameter (Dₙ) was 16.4 ± 2.3 μm (n = five species). Trochodendron aralioides had the smallest tracheid diameter of 13.5 ± 3.9 μm (excluding latwood) and Drimys brasiliensis the widest tracheid diameter of 19.4 ± 4.9 μm. The mean intertracheid wall thickness was 4.6 ± 1.3 μm (n = nine species).

No significant correlations were found between Pₜ₀ values and pit characteristics in six species (Table S2). There was no significant correlation between Pₜ₀ and the total intertracheid pit membrane surface area per tracheid area (Aₚ) (r = 0.28, P = 0.648, n = 5). Pₚ₀ and pit fraction (Pₚ) (r = 0.302, P = 0.621, n = 5) and Pₜ₀ and pit density (P₁₀) (r = 0.32, P = 0.536, n = 5). In addition, outer pit aperture diameter at the longest and shortest axes (Dᵣₚ₀ₐ and Dᵣₚ₀ₑ) was not related with Pₜ₀ (r = 0.493, P = 0.32, n = 6; r = 0.401, P = 0.431, n = 6).

**Intertracheid pit membranes**

All tracheids observed showed bordered pits with a pit membrane. In dried samples, SEM images showed visible pores in the intertracheid pit membranes (Fig. 1e–g), with a mean pore size (Dₚₚ₀) of 79 ± 54 nm (n = nine species). A single pore with a diameter of 319 nm was observed in Belliholom haplopus, while the smallest Dₚₚ₀ value was 13 nm in Antherostemma tychopoda. The microfibril thickness (Tₐ) is based on SEM images ranging from 23 to 53 nm with a mean value of 43 ± 12 nm (n = five species).

Intertracheid pit membranes of fresh samples under TEM showed a transparent and loosely arranged texture with small, dark (i.e. electron dense) particles (Fig. 2a,c,e,g) and a pronounced, dark pit membrane annulus. Larger pit membrane particles (61 ± 19 nm) were most common on the outermost layers of the pit membranes, while smaller ones (20 ± 7 nm) were associated with the inner layers (Fig. 3a,b). Higher magnification with TEM showed the irregular, clumped shape of most particles, which were sometimes grouped together. These particles showed a random to slightly linear orientation, probably reflecting the arrangement of cellulose microfibrils.

A second type of mainly circular particles could be seen on the pit membrane, near pit borders and on inner tracheid walls (Fig. 3b). These showed a dark outline and grey or empty inner part. The size of these particles was highly variable, from 55 to
Chapter 1

Bordered pits in xylem of vesselless angiosperms

Figure 2. TEM images of intrachalcal pit membranes in wood samples of vesselless angiosperms that were fresh (a, c, and e–h) when prepared for TEM or dried-and-rehydrated (b and d). (a) Fresh pit membrane of Drimys winteri. (b) Dried-and-rehydrated pit membrane of D. winteri. (c) Fresh pit membrane of Amborella trichopoda. (d) Dried-and-rehydrated pit membrane of A. trichopoda. (e) Fresh pit membrane in earlywood (EW) of Tetracentron sinense. (f) Fresh pit membrane in latewood (LW) of T. sinense. (g) Fresh pit membrane in earlywood of Trochodendron aralioides. (h) Fresh pit membrane in latewood of T. aralioides. The fresh pit membranes show a transparent and loose texture with many small dark spots due to \( \text{NaOCl} \) staining (a, c, e, and g). Unlike earlywood (e and g), pit membranes in latewood (f and h) show a shrunken pit membrane with a more homogeneous appearance, similar to intrachalcal pit membranes in a dried-and-rehydrated sample (b and d). All scale bars = 1 \( \mu \text{m} \).

Colloidal gold perfusion experiment

After injecting 5 nm colloidal gold particles in stem segments of Drimys winteri, the amount of gold detected in the outflow solution was on average \( 0.270 \pm 0.114 \times 10^{-7} \text{ g mL}^{-1} \) (\( n = 4 \)) (Fig. 4). Injection of 20 nm colloidal gold particles in fresh stem segments of this species resulted in reduced gold levels in the outflow solution, with a mean concentration of \( 0.034 \pm 0.0005 \times 10^{-7} \text{ g mL}^{-1} \) (\( n = 4 \)), suggesting that most gold particles did not pass intrachalcal pit membranes. Injection of 20 nm colloidal gold particles into dried-and-rehydrated stem segments, however, resulted in a mean concentration of \( 0.274 \pm 0.090 \times 10^{-7} \text{ g mL}^{-1} \) (\( n = 4 \)) gold particles, which was not significantly different from the colloidal gold concentration in the perfusion with 5 nm particles (Mann–Whitney \( U = 11 \), \( P = 0.343 \)). Compared with the initial concentration of the colloidal gold solution (\( 1 \times 10^{-4} \text{ g mL}^{-1} \)), a low gold concentration was measured in the perfusion solution of all samples (i.e. fresh – 20 nm, fresh – 5 nm and dried-and-rehydrated – 20 nm). However, the concentration of gold particles was eight times lower in the outflow solution after perfusion of fresh samples with 20 nm particles than in the two other conditions.

Both 5 and 20 nm gold particles could be seen within intrachalcal pit membranes of fresh and dried-and-rehydrated TEM samples taken beyond 5 mm from the injection point (Fig. 5a, b). This observation suggests that 20 nm gold particles

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Figure 3. TEM images of intertracheid pit membranes of fresh wood samples of vesselless angiosperms. (a) Pit membrane of Drimys winteri showing a thin (~40 nm) layer of dark and electron dense material on the pit border (PB) walls. The inset shows a higher magnification of the pit membrane (PM). Larger, dark particles occur on the outermost layers of the pit membranes and smaller ones on the inner layers. (b) Pit membrane of Anaborella trichopoda. Some circular particles with a dark outline and grey inner part could be seen on the pit membrane (arrows) and are different from the smaller, electron dense particles that are more or less homogeneously distributed across the pit membrane. (c) Fairly thick (~364 nm) pit membrane in latwood of Tetradendron winteri. Unlike the shrunken pit membranes in latewood as shown in Fig. 2f, the pit membrane here is loose and transparent with only few dark particles. This picture was taken opposite the earlywood of the next growth ring. AN = pit membrane annulus, PA = pit aperture, PB = pit border, PM = pit membrane, PW = primary cell wall and middle lamella, SW = secondary wall. Scale bar = 1 μm in a and 200 nm in the insert, b and c.

can pass through fresh pit membranes and are not completely filtered out. Interestingly, however, when examining the most distal wood samples with TEM, 20 nm gold particles were not found in the fresh samples (Fig. 5c), which is in line with the very low concentration of 20 nm gold particles shown in Fig. 4. Moreover, in the distal TEM sections of the dried-and-rehydrated sample, we found 20 nm gold particles within aspirated pit membranes, and few 50 nm gold particles (Fig. 5d). While most pit membranes were unaspirated in fresh samples, most pit membranes in the dried-and-rehydrated samples were fully or partially aspirated, but the drying treatment over 2 d did not result in fully shrunken pit membranes (Fig. 5b).

Tracheid-parenchyma pits

Tracheid-parenchyma pits, which were half-bordered on the tracheid side, occurred between tracheids and ray parenchyma cells as well as tracheids and axial parenchyma cells. A black cap with a dark and granular structure was observed on the tracheid side of the pit membrane in Drimys, Tetradendron and Trochodendron (Fig. 6a) but absent or indistinctly present in Anaborella (Fig. 6b). The dark particles were sometimes grouped in larger clusters and occurred both inside the pit membrane and on the outermost layer. Generally, there was a clear border between the actual pit membrane and the amorphous layer on the parenchyma side (Fig. 6b). The amorphous
layer typically showed a more homogeneous structure than the pit membrane and showed a less granular appearance with smaller or no dark particles (Fig. 6a,b). The amorphous layer was present along the entire inner parenchyma cell wall but was most pronounced near the pit membrane. The thickness of the amorphous layer (T_A) showed a large variation across species and within a sample, which was most likely due to its irregular thickness. Moreover, T_A varied depending on where the section was cut through the pit and where the amorphous layer was measured. The mean value of T_A was 350 ± 93 nm (n = five species), with a maximum value in _Trichosandra arenicolous_ (T_A = 1095 nm) and a minimum in _Dracyns brasiliensis_ (T_A = 95 nm). Lipid droplets were frequently observed in the protoplasm close to the tracheid-parenchyma pit, as well as vesicles and a well-developed endoplasmic reticulum.

**Parenchyma-parenchyma pits**

Parenchyma-parenchyma pits between ray parenchyma cells, axial parenchyma cells and ray-axial parenchyma cells were slightly bordered or simple. The pit membrane appeared to be a continuation of the middle lamella and primary walls.

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**Figure 4.** Gold concentration in the outflow solution after perfusing 2 cm long segments of _Dracyns watsoni_ with 5 and 20 nm colloidal gold particles. A high concentration of 5 nm gold particles was detected in the outflow solution of fresh branches, while 20 nm gold particles did not pass. However, a high concentration of 20 nm gold particles was detected in the outflow solution after injection in dried samples that were rehydrated prior to perfusion. All mean values were based on four measurements. The bars show mean values of gold particle concentration, and the error bars show the standard errors. Different letters indicate statistical significance.

**Figure 5.** TEM images of intertracheid pit membranes of _Dracyns watsoni_ after injection of 2 cm long segments with colloidal gold particles of known size (5 and 20 nm in a and b; 20 and 50 nm in c and d). Samples were fresh (a and c) or dried-and-rehydrated (b and d). TEM sections were made at a proximal side (i.e., >5 mm from the injection point, a and b) or distal side (i.e., >17 mm from the injection point, c and d). Information about the sample and particle sizes is provided in the upper right corner. Both 5 (white arrow) and 20 nm gold particles (black arrows) occur within the pit membrane and pit border of fresh (a) and dried-and-rehydrated (b) samples at the proximal end. While no gold particles occur in the fresh samples at the distal end (c), both 20 (black arrow) and 50 nm particles (double white arrow) can be seen at the distal end in the dried-and-rehydrated sample (d). The black double ended arrows show intertracheid pit membranes, which are highly transparent under TEM without OsO₄ treatment. AN = pit membrane annulus, PM = pit membrane, SW = secondary wall. Scale bar = 100 nm in a and b and 500 nm in c and d.

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Figure 6. TEM images of tracheid-parenchyma pits (a and b) and parenchyma-parenchyma pits (c and d) in vesselless angiosperms. (a) *Tectoduodendron aralioides* showing a tracheid-parenchyma pit with the amorphous layer (AL), pit membrane (PM), and a black cap (BC, arrow) on the tracheid side of the pit membrane, (b) A tracheid-parenchyma pit in *Amborella trichopoda* with a clear boundary between the pit membrane (PM) and the amorphous layer (AL), (c) *Tectoduodendron aralioides* showing a parenchyma-parenchyma pit membrane with various plasmodesmata (arrow), (d) A parenchyma-parenchyma pit in *A. trichopoda* with plasmodesmata (arrow). AL = amorphous layer, BC = black cap, P = parenchyma cell, PM = pit membrane, T = tracheid. All scale bars = 500 nm.

Numerous plasmodesmata traversed the pit membrane between the adjacent parenchyma cells (Fig. 6cd). The average diameter of individual plasmodesmata was 28 ± 2 nm (n = 24 counts).

Tracheid dimorphism

Tracheid dimorphism was found in secondary xylem of *Tetracentron sinense* (Fig. 7). In addition to normal tracheids, a second type of vessel-like tracheids showed multiserrate, alternate bordered pits in tangential walls and almost no pits in radial walls. Normal tracheids in *T. sinense* had scalariform or circular bordered pits on radial walls, but almost no pits on tangential walls (Fig. 7b). The vessel-like tracheids were adjacent to ray parenchyma cells and arranged in radial series (Fig. 7a). They had a significantly shorter length (422 ± 105 μm) and wider diameter (48 ± 6 μm) than normal tracheids, which showed a mean length of 1.886 (± 377) μm and a mean diameter of 16 (± 3.6) μm. No perforation plates could be detected in the vessel-like tracheids of *T. sinense* based on LM sections and maceration samples.

**DISCUSSION**

Intertracheid pit membranes in freshly embedded TEM samples were found to be similar in their ultrastructure compared with intervessel pit membranes in vessel-bearing angiosperms. The pit membranes showed loosely arranged microbribs and a homogenous thickness over the entire membrane, confirming the absence of any central, torus-like thickening. The consistent presence of a pit membrane in bordered pits of all species studied also indicates that these pits cannot be interpreted as perforation plates. Although bordered pits and perforation plates represent different structures, the latter can be interpreted as modified pits without a pit membrane or with pit membrane remnants (Jansen & Nardini 2014). These findings are in line with earlier LM and TEM observations (Bailey & Thompson 1918; Meylan & Butterfield 1982; Carlquist 1989; Carlquist & Schneider 2003) and raise questions about earlier records of perforation plates and 'cryptic' vessels in vesselless angiosperms (Feld et al. 2000; Hacke et al. 2007; Ren et al. 2007).

A potential explanation for earlier records of perforation plates in vesselless angiosperms is that dried samples undergo considerable pit membrane shrinkage and damage, resulting in a condensed and compressed arrangement of microbribs. This shrinking effect is likely irreversible because dried samples that were rehydrated prior to perfusion with 20 nm
colloidal gold particles showed an eightfold reduced filtering capacity compared with fresh (i.e. non-dried) samples. Comparison of distal and proximal samples from fresh material (Fig. 5a,c) also indicates that 20 nm gold particles may pass several pit membrane tracheids but are gradually filtered out from the perfusion solution over a <2 cm distance from the injection point. This finding suggests that most pores in fresh pit membranes are below 20 nm in diameter. Dried-and-rehydrated samples, however, allowed both 20 and 50 nm particles to pass intertracheid pit membranes in distal TEM samples, which provides clear evidence for increasing porosity in even partially dried pit membranes. We assume that the 2-d drought treatment at room temperature resulted in many embolized tracheids but not in complete desiccation of the sample. Indeed, TEM observation of ray parenchyma suggested that at least some ray cells retained their cytoplasm. Although pit membrane thickness could not be measured and accurately seen in the TEM samples prepared without OsO₄, the distribution of the gold particles in rehydrated samples suggested that these membranes were not fully shrunk as could be seen for TEM samples based on herbarium material (Fig. 2b,d,Lh). Although special care was taken to refill embolized tracheids in the dried-and-rehydrated samples, the reduced conductance suggested that not all tracheids were refilled (Espino & Schenk 2011). Moreover, the increased porosity in pit membranes of dried-and-rehydrated samples seemed not to compensate for the different rate of perfusion between fresh and dried-and-rehydrated samples.

Differences in the thickness and porosity of intertracheary pit membranes between dried and wet samples have been found in various vessel-bearing angiosperms (Peschera et al. 2005; Jansen et al. 2008; Scholze et al. 2013b; Li et al. 2016). Based on cryo-SEM observations of maize root xylem, even short-term, partial drying during preparation was found to cause large pores in pit membranes of metaxylem vessels (Shane et al. 2000). Observations using atomic force microscopy on samples that were first scanned wet and then dried also demonstrated that the overall arrangement of hydrated microfibrils in intervessel pit membranes was much less compact than those seen in dried material (Peschera et al. 2005). Thus, estimations of pit membrane porosity based on SEM are likely affected by this shrinkage, which indicates that SEM images cannot be used to determine the exact pit membrane pore sizes within a plant and that preparation of fresh samples is essential for TEM observation of intertracheid pit membrane thickness. Enlarged pores in SEM samples are especially found in the central area of a pit membrane, where an aspirated pit membrane is mechanically unsupported by the pit border. Re-arrangement of cellulose fibrils during pit membrane dehydration may also result in large pores near the pit membrane annulus (Sano 2005; Hacke et al. 2007; Hillbräund et al. 2016), although this could not be confirmed in our observations of mainly aspirated pit membranes.

The observation of dried, shrunk and frequently aspirated pit membranes in freshly embedded lateward of *Tetradendron* and *Tetracentron* suggests that shrinkage of pit membranes may also occur in plants. It can be suggested that these lateward tracheids are no longer involved in xylem water transport but only function in mechanical support. However, it is unclear whether the lateward tracheids in these genera contribute to water transport, and when these pit membranes in lateward tracheids shrink. It is possible that the pit membranes shrink after embolism has occurred, which may subject the pit membranes to large pressure differences across a pit pair and/or to desiccation. While an embolized conduit would initially be fully saturated with water vapour, it is likely that this conduit will gradually dry up over time, which would create pit membrane shrinkage. Embolism occurrence is known to cause air-seeding in *fatigued* (Hillbrand et al. 2016; Li et al. 2016), which provides additional evidence that pit membrane shrinkage is irreversible. The irreversibility of pit membrane damage deserves more attention with respect to temporal changes, seasonality, the potential occurrence of refilling and the application of standard lab protocols such as flushing of samples for conductivity measurements and air injection for air-seeding or maximum vessel length measurements. Similar observations of shrunk pit membranes were reported in non-conductive imperforate tracheary elements of vessel-bearing angiosperms, with ‘pierced’ openings in their thin and strongly reduced pit membranes, while water conductive imperforate tracheary elements that remained conductive showed an intact pit membrane (Sano et al. 2011).

Major similarities in the ultrastructure between intertracheid pit membranes of vesselless angiosperms and intervessel pit membranes in vessel-bearing species include the average pit membrane thickness and staining reaction with OsO₄. The average thickness (Tₚₚₚ = 245.55 ± 53.92 nm) in fresh sample of vesselless angiosperms showed no significant difference (Mann-Whitney U = 192, P = 1.00) with Tₚₚₚ values for 64 vessel-bearing angiosperms (average Tₚₚₚ = 321.50 ± 223.45 nm; Li et al. 2016). Therefore, our data do not support the hypothesis that intertracheid pits in vesselless angiosperms have a thin pit membrane as could be expected based on their relatively low pit resistance (Hacke et al. 2007). Tₚₚₚ can be affected by two factors: (1) the number of microfibril layers that are stack onto each other within a pit membrane and (2) the spatial distance between cellulose fibrils, which varies from loosely arranged to highly compact and condensed (Scholze et al. 2013b; Li et al. 2016). Although pit membrane thickness was found to be a good predictor of embolism resistance (Li et al. 2016), this correlation was not supported for the six vesselless angiosperm species studied.

The OsO₄ staining of intertracheid pit membranes and the occurrence of a black cap on tracheid-parenchyma pit membranes provide evidence that lipids accumulate at these places in a similar way as vessel-bearing angiosperms (Schaffer & Wünsierski 1989; Wünsierski et al. 1991; Schenk et al. 2017). In fact, the cellulose composition of pit membranes (Kim & Daniel 2013; Herbette et al. 2015; Klepeis et al. 2011b) is hardly visible under TEM due to its high transparency (Fig. 5), while post-fixation with OsO₄ makes the pit membranes visible due to the binding of osmium to double carbon bonds in unsaturated fatty acid chains of phospholipids (Riemersma 1968; Fig. 2 & 3). Therefore, the electron dense particles represent osmium bound to surfactants that accumulate in pit membranes pores (Schenk et al. 2017), and which are hypothesized
to affect water transport under tension (Christensen-Dalsgaard et al. 2011; Jansen & Schenk 2015; Schenk et al. 2015, 2017; Vera et al. 2016). A similar appearance of the black cap and surfactants accumulating in intertracheal pit membranes suggests that the lipids are produced by the parenchyma cells that are neighbouring tracheids, although this needs further research. It is also unclear whether or not there is seasonal variation in the amount and chemical nature of xylem sap surfactants, as could be suggested by the variation in electron density of pit membranes (Schmidt & Machado 1998; Schmitz et al. 2012). The fairly thick and electron transparent pit membranes as observed in the latewood adjacent to earlywood in *Tetracerton sinense* (Fig. 3c) may play a limited role in water transport, which could explain the low accumulation of xylem sap surfactants in its pit membranes. More research is also needed to investigate if the various types of vessels on inner tracheid walls, pit borders and pit membranes may represent liquid filled vesicles or (surface) nanobubbles (Schenk et al. 2017).

Contrary to our expectation, $P_{tr}$ values of five vesselless angiosperm species based on literature show no correlations with any of pit characteristics measured, including the pit membrane thickness, the total pit membrane area per tracheid and the shape of pit aperture. Therefore, these data do not support the rare pit hypothesis, which suggests that greater embolism resistance is associated with a reduction of the average interconduit pit membrane area per vessel ($A_{P}$) (Wheeler et al. 2005; Christman et al. 2012). However, $P_{tr}$ and $A_{P}$ were found to show a significant correlation ($r = 0.731$, $P = 0.001$) for 17 vesselless angiosperm species (Hacke et al. 2007). This discrepancy could be caused by the limited number of species investigated in this study, and the fact that the $P_{tr}$ values from literature were based on three different methods (i.e. the static centrifuge, cavitation and pneumatic method) and on different plant samples than those used here (Jansen et al. 2015).

Similar to vessel-parenchyma pits (Schmid 1965; Chafe 1974; Wisniewski & Davis 1989; Barnett et al. 1993; Muralakshmi et al. 1999), the amorphous layer in tracheid-parenchyma pits shows a loose and fibrous configuration and may surround the entire parenchyma protoplast. The multiple functions of the amorphous layer in vessel-bearing angiosperms include (Speier 2014) tylosis formation (Foster 1967; Chafe 1974; De Misco et al. 2016), protection of parenchyma cells against oscillations in hydrostatic-pressure from neighbouring vessels (Van Bel & Van der Schoot 1988), regulating the ability of exhibiting deep saperecooling (Wisniewski & Davis 1989) and preserving apoplastic continuity around the protoplast of a lignified cell (Barnett et al. 1993). The function of the amorphous layer in tracheid-parenchyma cells of vesselless angiosperms may serve similar functions due to their structural similarity with vessel-parenchyma pits.

Finally, the vessel-like tracheids in *Tetracerton sinense*, which were described as ‘unusual tracheids’ by Suzuki et al. (1991), appear to be characteristic of this species, while this feature is missing in *Tetragonolobus australiensis*. These vessel-like tracheids might play a role in efficient water movement in a radial direction because of their dense, alternate pits on tangential walls.

In conclusion, our anatomical observations indicate that no perforation plates occur in the 11 species of vesselless angiosperms studied. Intertracheal pit membranes were similar in structure to vessel-bearing angiosperms. Moreover, the aspirated and damaged intertracheal pit membranes in dried-and-rehydrated wood samples showed a 52% shrinkage compared with pit membranes in fresh samples. The perfusion experiments with colloidal gold particles revealed that 20 and 50 nm particles could pass pit membranes in dried-and-rehydrated samples, but not in fresh ones, which suggests that drying causes irreversible pit membrane damage by increasing pit membranes pore size. This drying artefact, which may also occur in planta, may explain previous records of vessels in vesselless angiosperms.

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**CONFLICT OF INTEREST**

We declare that we have no conflict of interest.

**REFERENCES**


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Chapter 1

Bordered pits in xylem of vesselless angiosperms


Chapter 2

Intervessel pit membranes in angiosperms show reduced porosity upon dehydration

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Abstract

Intervessel pit membranes represent porous media for water transport between neighbouring vessels. The cellulose fibrils form a three dimensional network similar to a fibrillar, non-woven porous media that shrinks by dehydration. The size of pore spaces is largely unknown because of the difficulty in measuring its dimensions at the nanoscale without preparation artefact. The ultrastructure of fresh and dried-rehydrated pit membranes in petioles of three angiosperm species was observed with transmission electron microscopy (TEM). Perfusion with colloidal gold was performed to compare the relative porosity between fresh and dried-rehydrated membranes in three angiosperm species. Fresh pit membranes showed a considerable variation in thickness, with 268 nm in Acer pseudoplatanus, 686 nm in Cinnamomum camphora, and 504 nm in Persea americana. Dehydration caused a 46-50 % shrinkage in the three species. While 20 nm gold particles penetrated fresh pit membranes of A. pseudoplatanus, 5 and 10 nm colloidal gold particles could be seen within fresh pit membranes of C. camphora and P. americana. However, no gold particles penetrated the shrunken, dried-rehydrated pit membranes of the three species tested. Shrinkage of pit membranes after dehydration appears to be irreversible and is associated with a considerable reduction of the pore spaces in the pit membranes. The cellulose fibrils are suggested to form a tightly packed structure with a “Velcro effect”, which might be functionally similar to aspiration of a torus-bearing pit membrane in gymnosperms.

Key words: angiosperm xylem, bordered pit membranes, cellulose fibrils, dehydration, porosity
Introduction

Plant water transport through neighbouring conduits occurs via bordered pit pairs in conduit cell walls (Jane 1956; Sano et al. 2011). In angiosperms, pits represent openings in the secondary cell wall of vessels and tracheids with an elongated to circular border overarch ing the pit membrane (Carlquist 1988). The border is assumed to provide mechanical support to the pit membrane, while the aperture is a hydraulic bottle neck (Carlquist 1988; Sperry & Hacke 2004). The pit membrane, which consists of the primary cell wall and an intervening middle lamella of adjacent vessels, is found to contribute to 58 % of the total xylem hydraulic resistivity (Sperry et al. 2006; Choat et al. 2008). A crucial role of the porous pit membrane is to serve as a capillary safety valve (Zimmermann 1983) because the nanoscale pores formed by the cellulose fibrils in pit membranes prevent the spreading of air and pathogens between vessels (Zimmermann & Brown 1971; Choat et al. 2008; Morris et al. 2016).

Intervessel pit membranes consist of layers of non-woven cellulose fibrils (Choat et al. 2008; Jansen et al. 2009; Schenk et al. 2015). The diameter of cellulose fibrils in pit membranes is estimated to be between 20 and 50 nm based on atomic force microscopy of Sapium sebiferum (Pesacreta et al. 2005). The density and arrangement of cellulose fibrils, which determine the porosity and thickness of pit membranes, accounts for the hydraulic resistance of the pit membrane, and also the embolism resistance (Choat et al. 2008; Jansen et al. 2009; Lens et al. 2011; Scholz et al. 2013). A positive correlation is found between the intervessel pit membrane thickness and \( \Psi_{50} \) (the xylem water potential at 50 % loss of hydraulic conductivity), suggesting that a thick pit membrane typically shows a high embolism resistance (Li et al. 2016).

Under drought stress conditions, embolism could happen in the xylem when water-filled vessels become air-filled. The air-seeding hypothesis suggests that embolism is triggered when air bubbles are sucked from an air-filled vessel into an adjoining water-filled vessel. Air-seeding may happen when the pressure difference between neighbouring vessels exceeds the capillary force of the air-water meniscus in the pore pathway of a pit membrane (Zimmermann 1983; Crombie et al. 1985; Sperry
& Tyree 1988; Choat et al. 2008). The chemical composition and ultrastructure of intervessel pit membranes plays a major role in the air-seeding process (Meyra et al. 2007; Schenk et al. 2015). While non-cellulosic substances such as hemicellulose and pectins are enzymatically broken down during the vessel development, intervessel pit membranes are mainly composed of cellulose fibrils (O’ Brien 1970; Herbette et al. 2015; Klepsch et al. 2016). Amphiphilic lipids are also found on and/or within intervessel pit membrane (Schenk et al. 2017). These chemicals would affect the contact angle of the air-water mensicus in pit membranes (McCully et al. 2014; Jansen et al. 2018).

Dehydration could change the density and arrangement of cellulose fibrils and cause irreversible shrinkage of pit membranes (Hillabrand et al. 2016; Li et al. 2016; Zhang et al. 2017). Pit membranes are reported to shrink by 28-52 %, while enlarged pores in dried-rehydrated samples suggest that dehydration changes the arrangement of cellulose fibrils (Li et al. 2016; Zhang et al. 2017). The rupture and stretching of cellulose fibrils during the drying process may explain the weakened xylem showing an air-seeding fatigue with less embolism resistance and increased air permeability (Hacke et al. 2001; Stiller & Sperry 2002). Large pores (up to 700 nm) based on SEM images are likely formed because of the rearrangement of cellulose fibrils during sample preparation (Shane et al. 2000; Sano 2005). This dehydration artefact may explain the very large variation in pit membrane pores as observed under SEM (Harvey & van den Driessche 1997; Sano 2005; Hacke et al. 2007; Hillabrand et al. 2016). A large variation in pore sizes (82-200 nm) based on air injection is also suggested for Rhododendron ponticum (Crombie et al. 1985).

Pore sizes examined in wet samples of some angiosperms also show a large variation. Perfusion by polystyrene spheres in hydrated stems indicated a pore size of 20 nm in Heteromeles arbutifolia and 82 nm in Malosima laurina (Jarbeau et al. 1995). Pore sizes of Medicago sativa based on reduction of hydraulic conductance by dextran perfusion was estimated to be 800 nm in stems and 100 nm in petiole junctions (Van Alfen et al. 1983). Colloidal gold perfusion experiments show that the pores in fresh
pit membranes are between 5 and 20 nm in *Alphitonia excelsa*, *Austromyrtus bidwillii*, *Brachychiton australis*, and *Cochlospermum gillivraei* (Choat et al. 2003), *Fraxinus americana* and *Sophora japonica* (Choat et al. 2004), and around 20 nm in *Drimys winteri* (Zhang et al. 2017).

Pit membrane pores should be viewed in a three-dimensional way, with highly variable and geometrically complex pore volumes, which are connected by a throat (i.e., a restriction between two adjoining pore spaces). Pore sizes in pit membranes determine the porosity of pit membranes. Pit membrane porosity, i.e., the pore volume fraction in a pit membrane, is important to understand hydraulic resistance and air-seeding. The behaviour of an air-water meniscus is a function of the pore geometry, wettability and pressure difference according to the Young-Laplace equation (Schenk et al. 2015). The tortuosity can be used to quantify the geometric complexity of pores in porous media such as pit membranes (Jansen et al. 2018), and can be defined as the ratio of the actual flow path length to the thickness of the porous medium (Koponen et al. 1998; Vallabh et al. 2011). However, we know little about the porosity and tortuosity of pit membranes under natural conditions within the plant. Besides, it is unclear whether the porosity of pit membranes differs between fresh and dried-rehydrated samples and whether porosity is associated with pit membrane thickness.

This study aims to investigate pit membrane porosity of species that differ in embolism resistance and pit membrane thickness. Colloidal gold with different sizes of gold particles was perfused through fresh and dried-rehydrated petioles to test a potential difference in intervessel pit membrane porosity. We hypothesise that thick pit membranes indicate high embolism resistance, but show a similar porosity to species with thin pit membranes. Moreover, gold particles of 5, 10 and 20 nm are expect to penetrate the fresh pit membrane, while 50 nm gold particles are filtered out. Based on earlier observations of *Drimys winteri* (Zhang et al. 2017), we also speculate that large pores are formed in shrunken and damaged dried-rehydrated pit membranes, which may show an increased porosity.
Material and methods

Plant material

Three species with long petioles, i.e., Acer pseudoplatanus L., Cinnamomum camphora (L.) J.Presl and Persea americana Mill. were selected in this study. Petioles were chosen for our perfusion experiments to obtain narrow, undamaged xylem segments, which reduced the amount of colloidal gold that was needed. A. pseudoplatanus petioles were collected between October 2017 and early December 2017 at the campus of Ulm University. Leaf petioles of C. camphora and P. americana were collected between October 2017 and March 2018 from plants growing in the greenhouse of the botanical garden of Ulm University.

Vessel length distribution

To know the mean vessel length in petioles and whether there are open vessels in petioles chosen for the colloidal gold perfusion, the vessel length distribution in petioles was determined in the three species according to Sperry et al. (2005) and Wheeler et al. (2005). Four adult leaves from each species were collected in the morning, kept under water, and then brought to the laboratory. After cutting the leaf blade, the intact petiole was put in water under vacuum overnight to remove any potential embolism. Meanwhile, a freshly made 10:1 mixture of Rhodorsil ESA 7250 A and B (Bodo Müller Chemie GmbH, Offenbach/M, Germany) was prepared, to which 2-3 drops of 1% UNITEX solution (Ciba Specialty Chemicals Inc., Basel, Switzerland) was added. The mixed silicone was then put under vacuum to remove all bubbles, and injected into the petiole under a constant pressure of 200 kPa overnight with a Scholander pressure chamber (Model 1000 Pressure Chamber Instrument, PMS Instrument Company, Albany OR, USA). Cross sections at different lengths of the petiole were made with a microtome (Schenkung Dapples, Zürich, Schweiz) and observed under a Leica DMRBE fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). The maximum vessel length was determined at which the section showed only one silicone-filled vessel. The percentage of silicone-filled
vessels per xylem area was calculated for each section. Vessel length distributions were plotted with an exponential decay function and the mean vessel length was calculated based on Sperry et al. (2005) and Wheeler et al. (2005).

Colloidal gold perfusion

Colloidal gold perfusion experiments were applied to petioles of three species according to Zhang et al. (2017). Healthy, adult leaves were cut in the morning, kept in distilled water and brought to the laboratory within 10 min. Then, petioles were re-cut under distilled water to an arbitrary length of 10 cm for A. pseudoplatanus, 3 cm for C. camphora, and 4 cm for P. americana. For the fresh samples, six petioles from each species were put into a vacuum pump overnight to remove any potential xylem embolism. For the dried-rehydrated samples, six petioles from each species were dried at room temperature until a minimum of 90 % water loss was reached and then put in water under vacuum for 24 h to rehydrate.

Both fresh and dried-rehydrated samples were connected to a 60 cm column of distilled water via a three-way stopcock, with an acropetal direction of water flow. Petioles were flushed with distilled water for 2-3 min, then 1 ml 1:1 mixture of 20 and 50 nm colloidal gold solution (Sigma-Aldrich, St. Louis, USA) was injected to the system via the three-way stopcock. The perfusion was stopped when the red colour of the colloidal gold solution was shown at the terminal end of the petiole. Moreover, 1 ml of a 1:1:1:1 mixture of 5, 10, 20 and 50 nm colloidal gold solution was perfused to samples of C. camphora and P. americana. Gold particles of 5 and 10 nm provided additional information about the pit membrane porosity, while 50 nm particles were generally assumed not to pass pit membranes (Choat et al. 2003, 2004; Zhang et al. 2017). Therefore, the combination of smaller colloidal sizes with 50 nm particles was useful to determine conduits that were cut open at the injection point.

We also perfused 1 ml of a 1:1:1:1 mixture of 5, 10, 20 and 50 nm colloidal gold solution into the system under 200 kPa pressure with a Scholander pressure chamber (Model 1000 Pressure Chamber Instrument, PMS Instrument Company, Albany OR,
USA) in both fresh and dried-rehydrated samples of *C. camphora* and *P. americana*. The 200 kPa pressure was chosen because the flow rate was very slow at 6 kPa, and 200 kPa was unlikely to cause mechanical deformation and compression of pit membranes (Tixer et al. 2014). For each treatment, five to six petioles were used.

*Visualisation of colloidal gold*

Gold particles were studied with TEM. Several 1 x 2 x 2 mm xylem cubes close to the middle of petioles for each treatment were cut under water for TEM observation. The sample preparation for TEM was performed according to a standard preparation protocol. The cubes were first fixed in a standard fixative solution (2.5 % glutaraldehyde, 0.1 mol phosphate buffer, 1 % saccharose and pH 7.3) overnight in the fridge, and washed with phosphate buffered saline three to four times. Samples were then dehydrated in a rising propanol series (30 %, 50 %, 70 % and 90 %) for 3 min each and put in a 20 mg/ml uranyl acetate solution for 25 min at 37 °C to improve contrast. Samples were then embedded in propylene oxide with a rising amount of Epon resin (2:1, 1:1, 1:2) for 60 min and then with pure Epon resin overnight at room temperature. Semi-thin transverse sections with a ca. 500 nm thickness were cut with an ultra-microtome (Leica Ultracut UCT, Leica Microsystems GmbH, Wetzlar, Germany). Then, sections were dyed with 0.5 % toluidine blue and mounted for observation under a light microscopy (Zeiss Axio Lab.A1, Carl Zeiss Microscopy GmbH, Jena, Germany). Ultra-thin sections with a 60-90 nm thickness were prepared and put on 300 mesh copper grids or slit grids. Intervessel pit membranes and gold particles in different sizes were observed under a JEOL JEM-1400 TEM (Jeol Germany GmbH, Freising, Germany). Since no OsO₄ was used as post-fixative, pit membranes were electron transparent (Schenk et al. 2017, 2018), and individual gold particles of all sizes could easily be observed as circular, electron dense structures. Pictures were taken with a digital camera (Soft Imaging System, Münster, Germany).
Chapter 2

Pit membrane thickness measurements

Intervessel pit membrane thickness \((T_{PM}, \text{ nm})\) in petioles of three species was measured based on TEM pictures via ImageJ software (version 1.50i, National Institutes of Health, Bethesda, MD, USA). Intervessel pit membrane thickness was calculated as the mean value of three measurements at opposite sides near the pit membrane annulus and at the centre of the pit membrane. At least 16 different intervessel pits were measured in both fresh and dried-rehydrated petioles of each species.

Xylem embolism resistance determination

Xylem embolism resistance of \(C.\ camphora\) and \(P.\ americana\) was determined for stem xylem based on the pneumatic method (Pereira et al. 2016; Zhang et al. 2018). While xylem embolism resistance may be different between leaf petioles and stems, there is considerable evidence that xylem embolism may not be different between there organs at least in some species (Bouche et al. 2016a, b; Wason et al. 2018; Klepsch et al. 2018).

Briefly, a fresh branch that was 50-80 cm long and 7-10 mm thick was enclosed in a black bag and connected to a pneumatic apparatus via a three-way stopcock, which was linked to a syringe and a vacuum reservoir. By pulling the syringe, a pressure of ca. 40 kPa was created in the vacuum reservoir and the initial pressure \(P_i\) (kPa) was recorded immediately. Then, the branch-vacuum reservoir pathway was opened and the final pressure \(P_f\) (kPa) in the vacuum reservoir was recorded after 2 min. The amount of air discharged from the branch was calculated according to the ideal gas law and transformed to the volume of air discharged at atmospheric pressure. The branch xylem water potential \((\Psi_x, \text{ MPa})\) was taken as the average of two water potential measurements on leaves, assuming that a water potential equilibrium was reached between the branch and leaves. Superglue (Loctite 431) was applied on the leaf cutting surface to avoid any air infiltration. Then, the branch was taken out of the bag and dried on the bench at room temperature for 15-30 min for the first two
measurements and 1 h or long for additional measurements. After drying, the branch was kept in the black bag for 1 h to obtain a xylem water potential equilibrium. Pneumatic measurements and water potential measurements were repeated several times until the branch showed intense dehydration, or the water potential reached -10 MPa. The percentage of air discharged (PAD), which was assumed to be equivalent to the percentage loss of conductivity (PLC), was calculated as:

\[ \text{PAD} = 100 \times \frac{(\text{AD}_i - \text{AD}_{\text{min}})}{(\text{AD}_{\text{max}} - \text{AD}_{\text{min}})} \]  

(1)

where \( \text{AD}_i \) was the volume of air discharged at each measurement, \( \text{AD}_{\text{min}} \) the minimum volume of air discharged when the branch was hydrated, and \( \text{AD}_{\text{max}} \) the maximum volume of air discharged at the lowest xylem water potential. The vulnerability curve was plotted by the PAD and \( \Psi_x \) values in the following function:

\[ \text{PAD} = 100 \times \frac{1}{1 + \exp\left(\frac{S}{25} \times (\Psi_x - b)\right)} \]  

(2)

with \( S \) representing the slope of the curve, and \( b \) the \( \Psi_{50} \) value, which was the xylem water potential at 50 % air discharged in the branch. The \( \Psi_{50} \) value was obtained from the vulnerability curve based on five branches per species.

Statistics

Statistical analyses were conducted in SPSS Statistics (Version 21, IBM Corporation, Armonk, USA). The Shapiro-Wilk-Test was applied to test the normal distribution of the data compiled. Independent-samples T-test was applied with normally distributed data to compare the means of two samples. If data were not normally distributed in two independent samples, a nonparametric test, i.e., the Mann-Whitney-U-Test, was used. Figures showing comparison of means were made with SPSS (Version 21, IBM Corporation, Armonk, USA) and vulnerability curves were made with SigmaPlot 12.5 (Systat Software Inc., Erkrath, Germany).

Results

Pit membrane thickness and embolism resistance

Intervessel pit membrane thickness (\( T_{PM} \)) in both fresh and dried-rehydrated petioles
of the three study species showed considerable variation (Fig. 1). For the fresh petioles of the three species, the thickness of intervessel pit membranes ranked as *C. camphora* > *P. americana* > *A. pseudoplatanus*. In *A. pseudoplatanus*, the mean $T_{PM}$ was $268 \pm 12$ nm (mean $\pm$ SE, $n = 18$) in fresh petioles. In dried-rehydrated petioles, the mean $T_{PM}$ was $143 \pm 13$ nm ($n = 16$), showing a 46.5 % shrinkage. A significant difference ($t(32) = 7.022, p < 0.0005$) was shown when comparing means of $T_{PM}$ in fresh and dried-rehydrated petioles of *A. pseudoplatanus*. In *C. camphora*, means of $T_{PM}$ in fresh and dried-rehydrated petioles were $686 \pm 18$ nm ($n = 24$), and $369 \pm 22$ nm ($n = 30$) respectively, with a significant difference ($U = 23, p < 0.0005$) being tested. Intervessel pit membranes in dried-rehydrated petioles of *C. camphora* showed a 46.1 % shrinkage compared to fresh petioles. In *P. americana*, there was a significant difference ($t(44) = 9.733, p < 0.0005$) between the average $T_{PM}$ values in fresh and dried-rehydrated petioles, with $504 \pm 19$ nm ($n = 28$) and $247 \pm 16$ nm ($n = 18$). Intervessel pit membranes in dried-rehydrated petioles of *P. americana* shrank by 51.0 % compared to fresh petioles.

![Figure 1](image.png)

**Fig 1.** Comparison of intervessel pit membrane thickness between fresh and
dried-rehydrated petioles of three species. Significant difference ($p < 0.05$) is shown by different letters. The box plot shows quartiles, top and bottom 25% of the data. Circles in *Persea americana* indicate outliers.

The maximum vessel length of petioles was 8.0 cm in *A. pseudoplatanus*, 2.6 cm in *C. camphora*, and 6.8 cm in *P. americana*. The mean average vessel length of petioles was $1.52 \pm 0.77$ cm ($n = 4$) in *A. pseudoplatanus*, $1.04 \pm 0.14$ cm ($n = 4$) in *C. camphora*, and $3.23 \pm 0.35$ cm ($n = 4$) in *P. americana*. The $\Psi_{50}$ value of *C. camphora* was $-2.27 \pm 0.11$ MPa and $-1.62 \pm 0.11$ MPa for *P. americana*, indicating that branches of *C. camphora* have a higher embolism resistance than *P. americana* (Fig. 2).

![Vulnerability curves of Cinnamomum camphora and Persea americana based on the pneumatic method. Data are based on five branches for each species. Solid circle and solid line represent data for C. camphora, and blank circle and dashed line show data for P. americana.](image)

**Fig. 2** Vulnerability curves of *Cinnamomum camphora* and *Persea americana* based on the pneumatic method. Data are based on five branches for each species. Solid circle and solid line represent data for *C. camphora*, and blank circle and dashed line show data for *P. americana*. 

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Gold particles under TEM observation

Gold particles could be observed at the surface of pit membranes, on the pit border walls, or on inner conduit walls. Gold particles that were observed at a minimum distance of 50 nm from the pit membrane surface were considered to be able to penetrate the pit membrane. This criteria was easier and more reliable to determine the penetration capacity of colloidal gold than determining whether or not a particular conduit had an open end at the injection point. Open conduits could indeed be observed based on the presence of 50 nm particles, but the lower amount of 50 nm particles compared to smaller sizes made it difficult to distinguish non-open conduits from open conduits in a single cross section. Since the presence of 50 nm gold particles could be overlooked, the absence of 50 nm gold particles did not provide strong evidence that a particular conduit represented an open conduit.

A detailed summary of the occurrence of gold particles in different sizes under each treatment was given in Table 1. In *A. pseudoplatanus*, 20 nm gold particles could penetrate the fresh intervessel pit membrane under 6 kPa pressure (Fig. 3a, b), but could not cross membranes in dried-rehydrated petioles (Fig. 3c, d).

In *C. camphora*, no gold particles could penetrate intervessel pit membranes under 6 kPa pressure, irrespective of their fresh or dried-rehydrated condition (Fig. 4a, b, c and d). When 200 kPa pressure was applied, 5 and 10 nm gold particles were found within fresh intervessel pit membranes (Fig. 4e, f), but not in dried-rehydrated membranes (Fig. 4g, h). In Fig 3h, some 10 and 20 nm gold particles showing in the pit membrane were considered not penetrating the pit membrane because the distance between these gold particles and the pit membrane border was less than 50 nm.

In *P. americana* petioles perfused under 6 kPa pressure, 5 and 10 nm gold particles were shown within fresh intervessel pit membranes (Fig. 5a, b), but not in dried-rehydrated membranes (Fig. 5c, d). Gold particles of 5 and 10 nm could be seen inside fresh intervessel pit membranes under 200 kPa pressure (Fig. 5e, f), but not in dried-rehydrated membranes (Fig. 5g, h).
Table 1. Summary of the distribution of gold particles under TEM in fresh and dried-hydrated petioles of three species injected at 6 and 200 kPa. + = gold particles were found within intervessel pit membranes, indicating they could penetrate at least partly pit membranes. - = gold particles were not seen within intervessel pit membranes, suggesting they could not penetrate pit membranes. NA indicated data were not available. The 200 kPa pressure was not applied to petioles of Acer pseudoplatanus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Condition</th>
<th>Pressure</th>
<th>5 nm</th>
<th>10 nm</th>
<th>20 nm</th>
<th>50 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acer pseudoplatanus</em></td>
<td>Fresh</td>
<td>6 kPa</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dried-rehydrated</td>
<td>6 kPa</td>
<td>NA</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cinnamomum camphora</em></td>
<td>Fresh</td>
<td>6 kPa</td>
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<tr>
<td></td>
<td>200 kPa</td>
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<td></td>
<td>Dried-rehydrated</td>
<td>6 kPa</td>
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<tr>
<td><em>Persea americana</em></td>
<td>Fresh</td>
<td>6 kPa</td>
<td>+</td>
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<td>200 kPa</td>
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<td>Dried-rehydrated</td>
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</tr>
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</table>

Some grouping of colloidal gold particles could be found and showed an electron dense under TEM (Fig. 3a, b; Fig. 4d; Fig. 5d) because these hydrophobic gold
particles were trapped by some surfactants such as phospholipids. Besides, porosity may not be homogeneous and constant across pit membranes. Because a different electron density within one pit membrane could be distinguished (Fig. 3b, d; Fig. 4a, b; Fig. 5b), and gold particles penetrating the pit membrane could be seen at different part of the pit membrane and showed different depths (Fig. 3a; Fig. 4e, f; Fig. 5b).

**Fig 3.** The distribution of gold particles in TEM images of intervessel pits in xylem tissue of fresh (a, b) and dried-rehydrated (c, d) petioles of *Acer pseudoplatanus* injected with 20 and 50 nm gold particles under 6 kPa. Gold particles of 20 nm occur within the fresh pit membrane (b), and at the surface of the dried-rehydrated membrane (d). PM = intervessel pit membrane; PB = pit border. Black lines represent the pit membrane borders.
Fig 4. The distribution of gold particles in TEM images of intervessel pits of fresh (a, b, e and f) and dried-rehydrated (c, d, g and h) petioles of *Cinnamomum camphora* injected with 5, 10, 20 and 50 nm gold particles at 6 and 200 kPa. No gold particles are found within pit membranes under 6 kPa pressure (b and d). Gold particles of 5 and 10 nm penetrate fresh pit membranes at 200 kPa (e and f), but do not penetrate...
dried-rehydrated pit membranes (g and h). PM = intervessel pit membrane; PB = pit border. Black lines represent the pit membrane borders.

**Fig 5.** The distribution of gold particles in TEM images of intervessel pits of fresh (a, b, e and f) and dried-rehydrated (c, d, g and h) petioles of *Persea americana* injected with 5, 10, 20 and 50 nm gold particles at 6 and 200 kPa. Gold particles of 5 and 10
nm occur within fresh pit membranes under 6 and 200 kPa (b and f), but not within dried-rehydrated membranes (d and h). PM = intervessel pit membrane; PB = pit border. Black lines show the pit membrane borders.

**Discussion**

*Do pit membranes that differ in thickness show a different porosity?*

Species with thick pit membranes are expected to show high embolism resistance (Jansen et al. 2009; Lens et al. 2011; Scholz et al. 2013; Li et al. 2016). Our results based on two species are in line with this hypothesis because the thickest pit membranes and the most negative $P_{50}$ value was found in *C. camphora*. Although sufficient care is need based on the two species studied here, a strong correlation between the thickness of pit membranes and $P_{50}$ values was based on 37 species (Li et al. 2016).

A functional explanation behind the relationship between pit membrane thickness and embolism resistance could be a difference in porosity (Jansen et al. 2009; Li et al. 2016). The intervessel pit membranes show a different porosity in the three species studied (Table 1; Fig. 3, 4 and 5). Gold particles of 5 and 10 nm could pass through fresh pit membranes in *P. americana* (Fig. 5a, b), but not in *C. camphora* at 6 kPa (Fig. 4a, b). This may indicate that the lowest porosity occurs in *C. camphora*. Alternatively, the flow at 6 kPa pressure was simply too low in *C. camphora* because 5 and 10 nm gold particles penetrated at 200 kPa (Fig. 4e, f). However, pit membranes of *A. pseudoplatanus* showed the highest porosity with a pore size around 20 nm. Colloidal gold particles of 20 nm were also found to penetrate pit membranes of *Alnus glutinosa* ($T_{PM} = 174 \pm 7$ nm) and *Carpinus betulus* ($T_{PM} = 188 \pm 5$ nm), while few 10 nm particles could be seen in pit membranes of *Hibiscus schizopetalus* ($T_{PM} = 353 \pm 7$ nm) but not in *Nerium oleander* ($T_{PM} = 469 \pm 14$ nm) (unpublished data). Therefore, thin pit membranes appear to show slightly larger pores than thick pit membranes. However, gold particle size may not equal pore size, but is likely to be a relative indication of the pore throat, while electroviscosity (Santiago et al. 2013), a boundary
layer, and/or hydrophobic nature of the colloidal gold may affect its penetration capacity through nano-sized pores.

The 5-20 nm pore size in fresh pit membranes shown in the three species studied is in line with earlier reports based on colloidal gold experiments in *Alphitonia excelsa*, *Austromyrtus bidwillii*, *Brachychiton australis*, and *Cochlospermum gillivraei* (Choat et al. 2003), *Fraxinus americana* and *Sophora japonica* (Choat et al. 2004), and *Drimys winteri* (Zhang et al. 2017). However, this size range strongly disagrees with records of larger pore sizes based on other methods. Pores in pit membranes were estimated to be smaller than 64 nm in *Eucalyptus regnans* when suspensions of colloidal carbon or colloidal gold were perfused under a vacuum (Cronshaw 1960). Dextran perfusion indicated a pore size of 800 nm in stems and 100 nm in petiole junctions of *Medicago sativa* (Van Alfen et al. 1983). A pore size of 82-200 nm was shown in *Rhododendron ponticum* based on air-injection (Crombie et al. 1985). Perfusion of polystyrene spheres indicated a pore size of 82 nm in *Malosima laurina*, and 30 nm for *Heteromeles arbutifolia* (Jarbeau et al. 1995).

The pore size in pit membranes plays a crucial role in embolism formation via air-seeding. According to the Young-Laplace equation, the pressure difference forcing a bubble through a pit membrane is a function of the radius of the pore size (Schenk et al. 2015). Assuming a contact angle of zero, and a pore shape correction factor of 0.5 (Meyra et al. 2007; Caupin et al. 2008; Schenk et al. 2015), a meniscus could cross through pit membranes with a pore of 20 nm diameter at -7.2 MPa in pure water, and at -2.4 MPa in the xylem sap containing amphiphilic lipids with a surface tension of 24 mJ m$^{-2}$ (Lee et al. 2001). Flow through pit membranes with 20 nm pores would be 75-625 times more efficient than non-pitted conduit cell walls, which are suggest to have 4-6.8 nm pores (Carpita et al. 1979; Carpita 1982; Baron-Epel et al. 1988) according to the Hagen-Poiseuille equation. Pit membranes with 20 nm pores could provide an efficient and also safe pathway for water transport.

An alternative explanation for the $T_{PM}$-$\Psi_{50}$ relationship is the pathway length in pit membranes. Assuming that pit membranes possess cellulose fibrils with a similar
diameter and a similar porosity, thick pit membranes provide a longer pathway than thin pit membranes. It was also suggested that an increase of the porous medium may result in a higher tortuosity and a higher flow resistance for a given porosity and fibril diameter. However, this needs to be tested for pit membranes (Vallabhb et al. 2010, 2011). Based on atomic force microscopy of Sapium sebiferum, a cellulose fibril is expected to be 20-50 nm thick, and the distance between cellulose fibrils in fresh pit membranes ranges from 100 to 300 nm (Pesacreta et al. 2005). Assuming a similar cellulose fibril diameter and distance between cellulose fibrils across species, a low porosity pit membrane would have a high number of cellulose fibril layers, which indicates a thick pit membrane. Considering that the thickest pit membranes and the lowest porosity occur in C. camphora, we could expect that there are more layers of cellulose fibrils in C. camphora than in P. americana and A. pseudoplatanus.

*Dehydration leads to decreased porosity of intervessel pit membranes*

Shrunken intervessel pit membranes were observed in dried-rehydrated petioles of three species, which were 46-50 % thinner than fresh pit membranes (Fig. 1, 3, 4 and 5). This shrinking effect caused by dehydration is similar to previous studies (Pesacreta et al. 2005; Scholz et al. 2013; Li et al. 2016; Zhang et al. 2017) and mostly likely irreversible because rehydration did not seem to restore the pit membrane in its original thickness. This irreversible nature of pit membrane shrinkage calls embolism refilling into question. Refilling would make no sense if pit membranes would become highly shrunken and provide a hydraulic resistance that is similar to the tiny pores in non-pitted cell wall area. While embolism refilling remains controversial (Brodersen & McElrone 2013; Cochard & Delzon 2013), an unresolved question remains the speed of conduit dehydration after embolism and the time required for pit membrane shrinkage to occur *in planta.*

Although SEM images cannot be used to determine the exact pore sizes of pit membranes, many SEM images show large pores in the center of pit membranes and also near the pit membrane annulus (Sano 2005; Hacke et al. 2007; Hillabrand et al. 2016). Colloidal gold perfusion of Drimys winteri also suggests enlarged pores (> 50
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nm) in dried-rehydrated pit membranes (Zhang et al. 2017). Contrary to this finding, dried-rehydrated pit membranes in the three species studied showed no enlarged pores. Pore sizes of *A. pseudoplatanus* in dried-rehydrated pit membranes were smaller than 20 nm whereas 20 nm gold particles could penetrate fresh pit membranes (Fig. 3). In *C. camphora* and *P. americana*, pore sizes in dried-rehydrated pit membranes were below 5 nm and no gold particles could cross pit membranes even under 200 kPa (Fig. 4, 5). These results do not support the air-seeding fatigue hypothesis, which suggests an increased porosity in dried samples as reported in *Aesculus hippocastanum*, *Helianthus annuus*, *Populus angustifolia*, and *P. tremuloides* (Hacke et al. 2001; Stiller & Sperry 2002). However, no air-seeding fatigue was found in xylem of *Acer negundo*, *Alnus incana* and *Betula occidentalis* after a drying-refilling cycle (Alder et al. 1997; Hacke et al. 2001). The very thin and flimsy pit membranes of *Aesculus hippocastanum* (93 nm) and *Populus* (Jansen et al. 2009) may account for air-seeding fatigue. An alternative explanation could be the formation of large pores in dried pit membranes by pathogens using cellulose. In all cases, however, air-seeding fatigue may not occur in nature without embolism refilling. Moreover, air-seeding fatigue should be tested on a wider range of species to validate the hypothesis that this phenomenon is limited to species with thin and fragile pit membranes.

Damaged pit membranes were observed in dried samples of *Populus tremuloides* and *P. balsamifera* (Sperry et al. 1991; Hillabrand et al. 2016), and some vesselless angiosperms such as *Amborella trichopoda*, *Drimys brasiliensis*, *D. winteri*, *Tasmannia lanceolata*, and within a plant in latewood of *Tetracentron sinense* and *Trochodendron aralioides* (Zhang et al. 2017). Since the mean thickness of fresh pit membranes for these vesselless angiosperms is 245 nm (Zhang et al. 2017), thin pit membranes may be prone to damage during dehydration. No damaged pit membranes were shown in the three species studied, and no aspirated pit membranes occurred in dried petioles of *C. camphora* and *P. americana*. Although *A. pseudoplatanus* showed a similar pit membrane thickness to the vesselless angiosperms, dried pit membranes of *A. pseudoplatanus* were straight and intact under TEM (Fig. 3), except for some
aspirated ones. It is unclear whether cellulose chemistry differs in species.

Cellulose fibrils in the dried pit membrane in the three species studied group tightly together and show a “Velcro effect” with decreased porosity, which might be functionally similar to aspiration of a torus in bordered pits of gymnosperms. More compact cellulose fibrils in dried samples (Pesacreta et al. 2005) suggest that dehydration causes rearrangement of cellulose fibrils and strongly affects the porosity and thickness of pit membranes. Assuming that dehydration only causes a decreased distance between layers of cellulose fibrils, the dried and shrunken pit membrane would show an decreased porosity since the pore volume in pit membranes is decreased. Besides, a difference in diameter of cellulose fibrils between wet and dried samples was found in cotton (Pesacreta et al. 1997) and celery (Thimm et al. 2000). The cellulose fibrils may aggregate together during dehydration and form a less uniform and more enmeshed network (Jansen et al. 2018). However, it is unclear how the arrangement of cellulose fibrils changes and alters the porosity of pit membranes during dehydration.

In summary, colloidal gold perfusion applied in petioles of three angiosperm species showed a pore size of 5-20 nm in fresh pit membranes. Dried-rehydrated pit membranes, with an irreversible shrinkage of 46-50 %, showed a decreased porosity in the three species.

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References


Chapter 3

Testing the plant pneumatic method to estimate xylem embolism resistance in stems of temperate trees

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Methods paper

Testing the plant pneumatic method to estimate xylem embolism resistance in stems of temperate trees

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Methods to estimate xylem embolism resistance generally rely on hydraulic measurements, which can be far from straightforward. Recently, a pneumatic method based on air flow measurements of terminal branch ends was proposed to construct vulnerability curves by linking the amount of air extracted from a branch with the degree of embolism. We applied this novel technique for 10 temperate tree species, including six diffuse, two ring-porous and two gymnosperm species, and compared the pneumatic curves with hydraulic ones obtained from either the flow-centrifuge or the hydraulic-bench dehydration method. We found that the pneumatic method provides a good estimate of the degree of xylem embolism for all angiosperm species. The xylem pressure at 50% and 88% loss of hydraulic conductivity (i.e., $\Psi_{50}$ and $\Psi_{88}$) based on the methods applied showed a strongly significant correlation for all eight angiosperms. However, the pneumatic method showed significantly reduced $\Psi_{50}$ values for the two conifers. Our findings suggest that the pneumatic method could provide a fast and accurate approach for angiosperms due to its convenience and feasibility, at least within the range of embolism resistances covered by our samples.

Keywords: angiosperms, bench dehydration, conifers, embolism, flow-centrifuge, pneumatic method, secondary xylem, vulnerability curve.

Introduction

There is convincing and clear evidence for the occurrence of xylem embolism in plants, which implies that functional, water-filled conduits (vessels and tracheids) in xylem tissue become air-filled (e.g., Sperry and Tyree 1966, Tyree and Zimmermann 2002, Choat et al. 2016). There is less agreement, however, about the temporal aspects of drought-induced embolism formation in plants, i.e., whether embolism occurs on a daily basis or is limited to seasonal or extreme drought events in intact stems under natural conditions (Cochard and Delzon 2013, Wheeler et al. 2013, Jacobsen et al. 2014). There are even different interpretations about the frequency of embolism occurrence for the same species growing in a similar environment (e.g., grapevine, Jacobsen and Pratt 2012, Charrier et al. 2016, Hochberg et al. 2017). Discussions about xylem embolism resistance strongly rely on the method that is applied, which makes it important to compare and evaluate different techniques (e.g., Torres-Ruiz et al. 2014, Jansen et al. 2015). Vulnerability curves characterize the plant vulnerability to xylem embolism and express the relationship between xylem water potential ($\Psi$) and embolism (Tyree and Sperry 1989). Over the last decades, various techniques have been
used to construct vulnerability curves depending on how xylem embolism is measured, how water stress is induced and how the water potential is measured (Cochard et al. 2013). The xylem water potential can be measured directly with psychrometers, indirectly determined as the leaf water potential after equilibration, or considered as the centrifuge force applied. Quantifying the degree of embolism by hydraulic measurements is the most commonly used approach, which directly estimates the degree of embolism as the percentage loss of hydraulic conductivity (PLC; %; Crombie 1983, Tyree and Dixon 1986, Sperry et al. 1988). This method may seem to be straightforward, but is destructive and requires sufficient care to obtain stable flow measurements (Espino and Schenk 2011). Problems with hydraulic measurements could be pit membrane clogging, vessel occlusion (e.g. by resin or latex), wound response and an excision artefact (Wheeler et al. 2013, Torres-Ruiz et al. 2015). Alternative techniques such as acoustic emissions detection and X-ray microtomography have the main advantage of being non-destructive and non-invasive, but do not directly measure water transport (Mayr and Rosner 2011, Wolkenstorfer et al. 2012, Cochard et al. 2013, Chaoh et al. 2016).

Bench dehydration is one of the standard methods to induce xylem embolism at a wide range of xylem water potential values because it allows branches or intact plants to dehydrate naturally at an ambient environment (Sperry 1986, Sperry et al. 1988). However, intensive lab work and a large amount of plant material are required for this technique. Centrifugation techniques induce a centrifugal force and require less time and plant material, but an ‘open vessel’ artefact may limit the application of this technique on species with vessels that are longer than the branch segment (Li et al. 2008, Cochard et al. 2010, Wang et al. 2014, Torres-Ruiz et al. 2015, 2017). Air-injection uses a double-ended pressure chamber to maintain a target pressure on an inserted stem with two ends protruding out of the chamber (Cochard et al. 1992, 2013, Salleo et al. 1993). While this technique is fast, it is equally challenged by the ‘open vessel’ artefact (Chaoh et al. 2010, Einraej et al. 2011). The optical method to visualize embolism in leaves and stems provides a relatively fast and reliable method to assess xylem embolism resistance in a non-destructive way (Scotfion and Jansen 2016, Brodbibb et al. 2016, 2016b, 2017, OpenSourceOV 2018). Considering the importance of xylem embolism in discussions about large-scale tree mortality (Anderregg et al. 2016) and the potential limitations of various methods for constructing vulnerability curves, there is a need for an artefact-free, easy, field-friendly and fast method that would hasten the assembly of large and global datasets on xylem embolism resistance (Chaoh et al. 2012).

Recently, Pereira et al. (2016) proposed a pneumatic method, which is fundamentally different from the hydraulic methods. Vulnerability curves in this study were obtained for 15 species by plotting the xylem pressure vs the amount of air discharged from terminal branch ends, while drought stress was induced using the bench dehydration method. Assuming that the air volume discharged from branches that are subjected to different levels of drought stress is mainly affected by embolism, vulnerability curves could be made, and were found to be largely in agreement with hydraulic methods (Pereira et al. 2016). One major advantage of the pneumatic technique is minimal manipulation of the plant samples, while measuring the amount of gas that can be extracted from samples is less complicated and faster (i.e. <3 min) compared with hydraulic measurements.

Since the species tested by Pereira et al. (2016) were mainly tropical to subtropical and included species with diffuse- porous wood, this paper aims to validate the pneumatic method for both diffuse-porous and ring-porous species from temperate areas. Moreover, additional conifer species are required to test this method, given that the number of conifers in the study by Pereira et al. (2016) was limited to Cupressus sempervirens and Thuja plicata. Therefore, we selected 10 temperate tree species, covering eight angiosperm and two gymnosperm species. The pneumatic method was compared with the flow-centrifuge technique for diffuse-porous species and conifers, and with hydraulic measurements after bench dehydration for two ring-porous species (Fraxinus excelsior and Quercus robur). We expected that the pneumatic method would show similar embolism resistance as the flow-centrifuge method for diffuse-porous species and conifers. It was unclear if the pneumatic method would be suitable for ring-porous species due to the large, earlywood vessels, which are known to remain functional only for 1 year (Ellmore and Ewers 1985, 1986, Cochard et al. 1992, Umebayashi et al. 2010, Sano et al. 2011).

Materials and methods

Plant material

Plant material of eight angiosperm species and two gymnosperm species was collected at Ulm University (Germany) (48° 25’20.3”N, 9° 57’20.2”E) and the University of Bordeaux (Table 1) (44°47’55.4”N, 0°36’54.2”W). The species selected were all common trees in the forest surrounding Ulm University and the University of Bordeaux. Vulnerability curve measurements according to the pneumatic method and hydraulic-bench dehydration method were conducted at Ulm University. For the flow-centrifuge method, branches were express-shipped to the University of Göttingen, except for the two gymnosperm species, which were measured with a similar flow-centrifuge set-up at the University of Bordeaux.

The pneumatic method

Stem vulnerability curve measurements of 10 species following the pneumatic method were conducted according to Pereira et al. (2016). Briefly, five branches that were 50–100 cm long and with a 7–10 mm branch diameter were collected from five
Table 1. List of the 10 species studied with reference to their family classification, the technique applied, xylem pressure at 50% and 88% of the maximum air discharged ($\Psi_{50}$ and $\Psi_{88}$) or at 50% and 88% loss of hydraulic conductivity ($\Psi_{50}$ and $\Psi_{88}$) with standard deviation, vessel porosity and collecting site.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Technique</th>
<th>$\Psi_{50}$ (MPa)</th>
<th>$\Psi_{88}$ (MPa)</th>
<th>Vessel porosity</th>
<th>Collecting site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnus glutinosa (L.) Gaerth.</td>
<td>Betulaceae</td>
<td>Pneumatic</td>
<td>$-1.6 \pm 0.1$</td>
<td>$-2.3 \pm 0.3^*$</td>
<td>Diffuse-porous</td>
<td>Stöfflen, Ulm</td>
</tr>
<tr>
<td>Betula pendula Roth</td>
<td>Betulaceae</td>
<td>Pneumatic</td>
<td>$-1.8 \pm 0.1$</td>
<td>$-2.2 \pm 0.2$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Coriaria betulus L.</td>
<td>Betulaceae</td>
<td>Pneumatic</td>
<td>$-2.0 \pm 0.1$</td>
<td>$-2.3 \pm 0.1$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Corylus avellana L.</td>
<td>Betulaceae</td>
<td>Pneumatic</td>
<td>$-3.8 \pm 0.5$</td>
<td>$-5.6 \pm 0.6^*$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Fagus sylvatica L.</td>
<td>Fagaceae</td>
<td>Pneumatic</td>
<td>$-3.7 \pm 0.2$</td>
<td>$-4.5 \pm 0.1$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Fraxinus excelsior L.</td>
<td>Oleaceae</td>
<td>Pneumatic</td>
<td>$-2.8 \pm 0.4^*$</td>
<td>$-5.1 \pm 0.6^*$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Liriodendron tulipifera L.</td>
<td>Magnoliaceae</td>
<td>Hydraulic</td>
<td>$-2.4 \pm 0.4$</td>
<td>$-3.8 \pm 0.6$</td>
<td>Ring-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Pinus pinaster Atton</td>
<td>Pinaceae</td>
<td>Pneumatic</td>
<td>$-1.4 \pm 0.2$</td>
<td>$-1.9 \pm 0.3$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Pinus sylvestris L.</td>
<td>Pinaceae</td>
<td>Pneumatic</td>
<td>$-1.5 \pm 0.1$</td>
<td>$-1.8 \pm 0.1$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
<tr>
<td>Quercus robur L.</td>
<td>Fagaceae</td>
<td>Pneumatic</td>
<td>$-2.8 \pm 0.4^*$</td>
<td>$-4.0 \pm 0.8$</td>
<td>Diffuse-porous</td>
<td>Botanical Garden, Ulm University</td>
</tr>
</tbody>
</table>

Hydraulic = hydraulic-bench dehydration. Standard deviation values in the hydraulic-bench dehydration technique were not available for F. excelsior and Q. robur. Data with * showed a significant difference (P < 0.05) between the techniques applied (for details see Table 2).

Adult trees per species. Samples were collected during the early morning between June and September 2016 and 2017. After wrapping the branches in wet tissue and black plastic bags, samples were brought to the lab, re-cut under water and rehydrated for 1 h. The distal end of a branch was then connected to a pneumatic apparatus through a three-way stopcock, which included a syringe as a vacuum source and a pressure sensor (PX26-015GV, Omega). This pressure sensor is not an oil-filled transducer and is designed for working with liquids. For gases, however, the PX140 series was used by Pereira et al. (2016). Comparison of the PX140 and PX26 pressure sensors showed a perfect agreement for air pressure measurements ($R^2 = 1; y = -24.602x + 30.69$), indicating that both sensors are suitable for the pneumatic method.

Once the branch end was connected to the pneumatic tubing system, the tubing was not replaced and the branch ends were not shaved before a measurement was taken. For Pinus sylvestris, however, the surface was trimmed with a fresh razor blade to avoid obstruction of tracheids by resin before each measurement. Because resin did not appear to be a major problem for Pinus pinaster, we debarked the end of branches of this species to avoid an excessive resin exudation, but did not trim the surface cut each time a measurement was taken.

The rigid silicon tube between the branch end and the pressure sensor functioned as a vacuum reservoir. Firstly, the branch-vacuum reservoir pathway was closed, i.e., the branch end was open to the atmosphere. A pressure of ~40 kPa was obtained in the vacuum reservoir by pulling the syringe plunger.

Then, the vacuum reservoir-syringe pathway was closed and the branch-vacuum reservoir pathway opened. The initial pressure ($P_i$, kPa) was measured immediately after connecting the branch end to the vacuum reservoir. After extracting air from the branch to the vacuum reservoir for 2 min, the final pressure ($P_f$, kPa) was measured. Branches were bagged in a black plastic bag during the measurements. Two leaves from the branch were then cut and their water potentials were measured with a Scholander pressure chamber (PMS Instrument Company, Albany, OR, USA). Super glue (Locite 431) was applied to the branch where the leaf petiole was cut to avoid air-entry in the branch. The xylem water potential ($\Psi$, MPa) was the average of the two leaf water potentials measured. The branch was then detached from the apparatus and dried on the bench. The drying time was between 15 and 30 min for the first two measurements and 1 h or longer for measurements at more negative water potentials. After drying, the branch was bagged in a black plastic bag to equilibrate for 1 h and then connected to the apparatus to start the next measurement. Measurements were ended when the plants showed strong dehydration, such as dry leaves, which was around ~6 MPa for several species, and ca ~9 MPa for F. sylvatica.

According to the ideal gas law, the amount of moles of air ($\Delta n$, mol) discharged from the stem in the vacuum reservoir was calculated as follows:

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Table 2. List of the t-statistics (t), the degrees of freedom (df) and the significance values (P) of t-tests on $\Psi_{so}$ and $\Psi_{so}$ values in the 10 species studied. Xylem embolism resistance was measured using the pneumatic method ($\Psi_{so}$, $\Psi_{so}$) and flow-centrifuge/hydraulic-bench dehydration ($\Psi_{so}$, $\Psi_{so}$).

<table>
<thead>
<tr>
<th>Species</th>
<th>t</th>
<th>df</th>
<th>P</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnus glutinosa (L) Gaertn.</td>
<td>1.156</td>
<td>7</td>
<td>0.286</td>
<td>4.899</td>
<td>7</td>
<td>0.002</td>
</tr>
<tr>
<td>Betula pendula Roth</td>
<td>-1.360</td>
<td>9</td>
<td>0.207</td>
<td>-0.529</td>
<td>9</td>
<td>0.610</td>
</tr>
<tr>
<td>Corylus betulus L</td>
<td>0.169</td>
<td>4.855*</td>
<td>0.872</td>
<td>3.774</td>
<td>4.164*</td>
<td>0.018</td>
</tr>
<tr>
<td>Corylus ellipsoides L</td>
<td>0.425</td>
<td>9</td>
<td>0.681</td>
<td>1.769</td>
<td>9</td>
<td>0.111</td>
</tr>
<tr>
<td>Fagus silvatica L</td>
<td>-2.311</td>
<td>10</td>
<td>0.043</td>
<td>4.373</td>
<td>10</td>
<td>0.001</td>
</tr>
<tr>
<td>Fraxinus excelsior L</td>
<td>1.709</td>
<td>5</td>
<td>0.148</td>
<td>0.055</td>
<td>5</td>
<td>0.959</td>
</tr>
<tr>
<td>Liriodendron tulipifera L</td>
<td>-0.942</td>
<td>8</td>
<td>0.374</td>
<td>1.115</td>
<td>8</td>
<td>0.297</td>
</tr>
<tr>
<td>Pinus pinaster Aiton</td>
<td>-10.811</td>
<td>14</td>
<td>0.000</td>
<td>-2.618</td>
<td>14</td>
<td>0.020</td>
</tr>
<tr>
<td>Pinus sylvestris L</td>
<td>-6.628</td>
<td>8</td>
<td>0.000</td>
<td>0.672</td>
<td>3.757*</td>
<td>0.540</td>
</tr>
<tr>
<td>Quercus robur L</td>
<td>-2.826</td>
<td>6</td>
<td>0.030</td>
<td>4.299</td>
<td>6</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values of $\Psi_{so}$ with * suggested that the assumption of homogeneity of variance was broken and thus the degree of freedom was reduced. P < 0.05 indicated a significant difference.

\[
\Delta n = (P_1 - P_2) \times V / RT
\]

where $V$ represented the volume of the vacuum reservoir (0.0082 L), $R$ the gas constant, and $T$ the room temperature. Then, also according to the ideal gas law, the volume of the air discharged (AD, ml) was calculated by transforming $\Delta n$ to an equivalent volume of air at atmospheric pressure ($P_{atm}$, 94 kPa at 618 m, the altitude of Ulm University). Any leakage from the apparatus could be calculated from the pressure change over 2 min when the branch-vacuum reservoir pathway was closed. This minor error was then subtracted from AD (the air discharged).

The percentage of air discharged (PAD, %), which was analogous to the percentage loss of conductivity (PLC, %), was calculated as follows:

\[
\text{PAD} = 100 \times \frac{(AD_{max} - AD_{min})}{AD_{max}}
\]

where $AD_{max}$ was the volume of air discharged at each measurement, $AD_{min}$ the minimum volume of air discharged when the branch was fully hydrated and $AD_{max}$, the maximum volume of air discharged at the lowest xylem water potential. Therefore, the initial air discharged from a fully hydrated stem is considered as a reference point, which accounts for the non-conduct air volume from the pith, intercellular spaces and outside air. If this non-conduct volume does not increase during branch dehydration, the air volume inside the branch would only increase if a new embolism is formed and connected to the cut base and apparatus.

The stem vulnerability curve was then constructed by plotting the PAD and $\Psi$ values with the following function (Famender and Vander Willigen 1998):

\[
\text{PAD} = 100 / (1 + \exp((S/25)(\Psi-b)))
\]

where $S$ represented the slope of the curve and $b$ was equal to $\Psi_{so}$, i.e., the xylem water potential at 50% air discharged from the stem. $\Psi_{so}$ (i.e., the xylem water potential at 88% air discharged in the stem) was calculated following Domec and Gartner (2001) as

\[
\Psi_{so} = -2 / (S/25) + b
\]

The hydraulic-bench dehydration method

Stem vulnerability curves of F. excelsior and Q. robur were constructed using the hydraulic-bench dehydration method since their maximum vessel length was longer than the flow-centrifuge rotor length (28 cm) (Chojnacki et al. 2005, Li et al. 2015). Data of F. excelsior were based on Li et al. (2015), who studied similar sized branches from the same trees as those used for this study.

For Q. robur, 15 branches that had a total length between 1.5 and 2 m were collected from six adult trees at the campus of Ulm University in the early morning of August 2017. The samples were wrapped in wet plastic bags and brought to the lab within 15 min. Branches were re-cut under water and rehydrated in water for 1 h to eliminate any potential cutting artefact (Torres-Ruiz et al. 2015). Then, branches were dehydrated in the lab with different time intervals from 1 to 24 h. After bagging the branches for 1 h to create equilibrium between the leaf and xylem water potential, three leaves from the current year stem were cut for water potential measurements using a pressure chamber. The average of the measurements was regarded as the xylem water potential ($\Psi$). Then, branches were re-cut under water into 3 cm long current year stem segments, which were connected to a modified Sperry apparatus (Sperry et al. 1988) to measure the percentage loss of hydraulic conductivity (PLC, %). Briefly, the distal end of the stem segment was connected to a 60 cm high water column and the proximal end to a pipette via
Chapter 3

Testing the pneumatic method in temperate trees

A silicon tube filled with water. We used deionized, filtered (0.2 μm) and degassed water with 10 mM KCl and 1 mM CaCl₂ for all hydraulic measurements. By measuring the time that was required to fill a volume of 0.01 ml in the pipette, the flow rate of the sample (F, μg s⁻¹) was calculated as the average of three continuous measurements. The hydraulic conductivity of the segment (Kₚ, kg m⁻¹ MPa⁻¹) was calculated as follows:

\[ Kₚ = F/P/L \]

(5)

where P represented the water pressure applied to the segment (0.006 MPa) and L (m) was the length of the segment.

Then segments were flushed with water at 1.20 kPa for 30 min to remove potential emboli, and connected to the Speroni apparatus to obtain the maximum conductivity (Kₚmax, kg m⁻¹ MPa⁻¹). The PLC was then calculated as follows:

\[ PLC = 100 \times (Kₚmax - Kₚb)/Kₚmax \]

(6)

The stem vulnerability curves were plotted with PLC and Ψ values as mentioned above.

The flow-centrifuge method

The flow-centrifuge technique (Cochard et al. 2006) was applied to the six diffuse-porous angiosperms and two conifer species (Table 1). Branches cut in the early morning were stored at 4°C in a MICROFUR solution (Kadyn, Wallisellen, Switzerland) and processed within a week. Stem segments of 28 cm length were excised under water with both ends debarked and trimmed, mounted in a custom-built rotor chamber, which uses a commercially available centrifuge as basis (Sorval RC-5C, Thermo Fisher Scientific, Waltham, MA, USA). The maximum sample conductivity (Kₚmax) was first determined at low speed and relatively high xylem pressure (~5 MPa). By increasing the rotational velocity, the xylem pressure (Ψ) was decreased stepwise and the hydraulic conductivity (Kₚ) was measured at each pressure level. The PLC was calculated as Eq. (6) and stem vulnerability curves were plotted as mentioned above.

Statistics

We first tested the Ψₛₒᵥ values determined by the pneumatic method (Ψₛₒᵥ) across species against those obtained by one of the hydraulic methods (Ψₒₓₒ, including the flow-centrifuge or hydraulic-bench dehydration method) using an independent-samples t-test. For the two ring-porous species, a one-sample t-test was used to determine the difference between Ψₛₒᵥ and Ψₒₓₒ values, since only a single vulnerability curve was obtained based on the hydraulic-bench method. In both tests, Ψₒₓₒ values were considered to be significantly different for a particular species when their 95% confidence intervals did not overlap. Then, we ran a linear regression analysis to test how Ψₒₓₒ could be predicted from Ψₛₒᵥ. Similar t-tests and a regression analysis were also applied to Ψₒₓₒ (Ψₒₓₒ based on the pneumatic method) and Ψₒₓₒ (Ψₒₓₒ based on the flow-centrifuge or hydraulic-bench dehydration method), and to Ψₒₓₒ (slope of the percentage of air discharge) and Ψₒₓₒ (slope of the percentage loss of hydraulic conductivity). All statistical analyses were done in SPSS 21 (IBM, Armonk, New York, USA) and all figures were made in SigmaPlot 12.5 (Systat Software Inc., Erklafl, Germany).

Results

Vulnerability curves based on the pneumatic method were obtained for the six diffuse-porous species (Figure 1). The 95% confidence bands of the pneumatic curves and hydraulic curves showed considerable overlap, which was especially the case for B. pendula. The Ψₒₓₒ values of these species showed no difference from the Ψₒₓₒ values (P > 0.05), except that a marginal difference was found for F. sylvatica (P = 0.04) (Tables 1 and 2). Ψₒₓₒ and Ψₒₓₒ values showed significant difference (P < 0.05) for A. glutinosa, C. betulus and F. sylvatica, but no difference for the other three diffuse-porous species (Tables 1 and 2).

A vulnerability curve based on the pneumatic method could also be obtained for 2- or 3-year-old branches of F. excelsior (Figure 2a). However, the method could not be successfully applied to 3- to 5-year-old branches of Q. robur because the amount of air discharged from fresh samples that were not subject to any drought stress was too high. Hence, the final pressure (P) reached atmospheric pressure even when the branch was fully hydrated. Since wide earlywood vessels in Q. robur are known to be functional for 1 year only, the xylem of previous growth rings and the pith tissue was glued off at the branch end, with only the xylem from the current growth ring directly exposed to the pneumatic apparatus. This minor modification resulted in vulnerability curves of this species as shown in Figure 2b. Comparison of Ψₒₓₒ and Ψₒₓₒ for the two ring-porous species showed a 0.3 MPa difference for F. excelsior and 0.4 MPa difference for Q. robur. A significant difference was found for Q. robur (P = 0.03), but not for F. excelsior (P = 0.15). Similarly, a significant difference between the Ψₒₓₒ and Ψₒₓₒ values was found for Q. robur (P < 0.01), but not for F. excelsior (P = 0.96) (Table 1).

The vulnerability curves for the two conifer species showed a large difference between the pneumatic and hydraulic method (Figure 2c and d). The Ψₒₓₒ and Ψₒₓₒ values differed significantly (P < 0.01) by 0.9 and 1.2 MPa for P. pinaster and P. sylvestris, respectively, while Ψₒₓₒ and Ψₒₓₒ values differed for P. pinaster (P = 0.02) and showed no difference for P. sylvestris (P = 0.54) (Table 1).

Ψₒₓₒ values from the pneumatic (Ψₒₓₒ) and hydraulic curves (Ψₒₓₒ) were strongly correlated (R² = 0.670, P < 0.01; Figure 3a). The fitted line was Ψₒₓₒ = 0.945Ψₒₓₒ - 0.383, which was close to the 1:1 line (Figure 3a). A stronger correlation was obtained when analysing the six diffuse-porous species...
species ($R^2 = 0.957, P < 0.01$), and the eight angiosperm species ($R^2 = 0.911, P < 0.01$). Two conifers (P. pinaster and P. sylvestris) did not fall within the 95% confidence band (Figure 3a).

$\Psi_{s}$ and $\Psi_{e}$ were also strongly correlated ($R^2 = 0.752, P < 0.01$; Figure 3b). The fitted line was $\Psi_{e} = 0.735 \Psi_{s} - 0.492$, which was close to the 1:1 line (Figure 3b). A higher correlation also occurred when analysing the six diffuse-porous species separately ($R^2 = 0.950, P < 0.01$), as well as the eight angiosperm species ($R^2 = 0.876, P < 0.01$). Only P. pinaster did not fall within the 95% confidence bands (Figure 3b).

The slope of the percentage of air discharge ($S_{a}$) was not significantly correlated with the slope of the percentage loss of hydraulic conductivity ($S_{h}$) ($R^2 = 0.198, P = 0.20$). When A. glutinoso was excluded as an outlier, however, a significant correlation was shown ($R^2 = 0.566, P = 0.02$) (Figure 3c).
Excluding A. glutinosa, the solid line showed the fitting: $S_H = 0.822S_T + 64.792$, while F. excelsior and Liriodendron tulipifera did not fall within the 95% confidence bands (Figure 3c).

**Discussion**

Vulnerability curves based on the pneumatic method were largely similar to those based on the hydraulic methods for most angiosperm species, with the exception of the two conifer species studied. Therefore, this finding validates the pneumatic method for temperate angiosperm species and is in agreement with a comparison of the pneumatic method and the hydraulic-bench dehydration method for mainly tropical and subtropical species (Pereira et al. 2016). However, significant differences between $\Psi_{50}$ and $\Psi_{90}$ for two out of eight angiosperm species studied (i.e., F. sylvatica and Q. robur) suggest that the pneumatic method may provide a relative approach to quantify embolism resistance, while there may not be an absolute, standard way to determine $\Psi_{50}$ values for a broad taxonomic range of species. Care should especially be taken for ring-porous species, which can show highly variable vulnerability curves depending on the method applied (Choat et al. 2010, Jacobsen and Pratt 2012, Hacke et al. 2015, Torres-Ruiz et al. 2017).

Agreement between the pneumatic method and flow-centrifuge method was highest for the six diffuse-porous angiosperms. The highest coefficients of correlation ($R^2$) were found for both $\Psi_{50}$ and $\Psi_{90}$ values. Hence, the pneumatic method appears to be reliable for constructing vulnerability curves of diffuse-porous angiosperms, which are known to show functional vessels over various growth rings (Unebayashi et al. 2010, Sano et al. 2011). This finding also suggests that most of the air discharged during dehydration corresponds to the amount of embolized conduits. While air can also be taken up through the bark and the leaves, the amount of external air was shown to be negligible within a time frame of 2 min (Pereira et al. 2016). Air diffusion via bark and leaf tissue is most likely a slow process, because the resistance of air flow was found to be considerably higher across the periderm, cambium and mesophyll cells than through lumen conduits and interconduit pit membranes (Comstock 1970, Sorz and Hietz 2006, Pereira et al. 2016). Air flow through bordered pit membranes may increase during dehydration and can be explained by pit
membrane shrinkage that is associated with increased porosity (Cohen et al. 2003, Pan et al. 2015, Zhang et al. 2017).

An interesting difference was found between the two ring-porous species studied, including 2-year-old branches of F. excelsior, and 3- to 5-year-old branches of Q. robur. Since it is known that wide, earlywood vessels in ring-porous species remain only functional over a single season and do not refill (Cochard and Tyree 1990, Cochard et al. 1997, Sano et al. 2011), the amount of gas that can be extracted from embolized earlywood vessels can be high. While working with current-year shoots might provide a solution to avoid this problem, the proportion of lignified xylem tissue to non-xylem tissue can be too small and may not provide sufficient mechanical support for applying the pneumatic method. In addition, the amount of leaves on current year shoots can be small, which would make repeated water potential measurements difficult. Given off previous growth rings, as done for Q. robur, could provide an alternative, easy solution, especially when dealing with multi-year branches.

Ψ50 values showed a significant difference between the pneumatic and flow-centrifuge method for the conifer species tested (Table 1, Figures 2c and d). Although the correlation between Ψ50 and Ψ50h (Figure 3a) and between Ψ50 and Ψ60h (Figure 3b) was significant in all species, it became weaker when the two conifer species were included. The finding of reduced embolism resistance for both conifers is largely in agreement with the two conifer species studied by Pereira et al. (2016). In this study, the difference in Ψ50 values between the pneumatic and hydraulic method was 2.4 MPa for C. sempervirens and 2.6 MPa for T. platanoides. Overall, these data suggest a reduced embolism resistance based on the pneumatic method for P. sylvestris, P. pinaster and C. sempervirens, but an increased embolism resistance for T. platanoides.

Resin in gymnosperm xylem may not be the main reason for the large discrepancy between Ψ50 and Ψ50h, because resin canals are absent in the xylem of C. sempervirens and T. platanoides (Wagenführ 2007, Cleary and Holmes 2011). Moreover, carefully trimming the cut surface of P. sylvestris did not seem to provide a shift towards more negative Ψ50 values. A possible explanation could be aspiration of the torus-mango pit membrane in conifers (Bouche et al. 2014). When a pit membrane is subject to a certain pressure difference between neighbouring tracheids, the torus could become aspirated and blocks off the pit aperture, preventing air flow from one tracheid to another (Cochard et al. 2009, Jansen et al. 2012). The pressure difference required to cause pit membrane aspiration in conifers was found to range from 0.01 MPa to 0.3 MPa (Bouche et al. 2014, 2015), which means that aspiration is likely when measuring AΔΨ50 values of conifer branches. Thus, pit membrane aspiration might underestimate the amount of air discharged during a pneumatic measurement, and the maximum PAD would be reached at a less negative water potential, which underestimates xylem embolism resistance. For the same reason, the application of the flow-centrifuge method may result in a shift towards less negative Ψ50 values if the flow difference across the two sample ends is too high, resulting in pit aspiration (Beinircher et al. 2010, Bouche et al. 2015). Nevertheless, this hypothesis does not explain the more negative Ψ50 value for T. platanoides as reported by Pereira et al. (2016). While further research is needed to test the pneumatic method on additional conifer species, the available evidence indicates that this method is not recommended for gymnosperms.

In conclusion, the pneumatic method may provide considerable advantages over other methods when studying xylem resistance to embolism of temperate angiosperm branches. Compared with hydraulic methods, extracting gas from terminal branch endings is fast and easy. Pneumatic vulnerability curves of several species can be constructed simultaneously within 2 to 3 days, depending on how fast branches dry at room temperature. This method may especially be useful for field measurements at remote locations, student projects, and when dealing with a large number of samples.
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Conflict of Interest

We declare that we have no conflict of interest.

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References


Testing the pneumatic method in temperate trees
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List of publications

Published papers:


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➢ The structure of intertracheid bordered pits in vesselless angiosperms
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➢ Estimation of xylem embolism resistance

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27/09/2017 The importance of bordered pit membranes for water transport in plants. 3rd Xylem International Meeting, University of Bordeaux, France.

05/09/2016 Bordered pits in xylem tracheids of vesselless angiosperms and their misinterpretation as vessels. The 46th annual meeting of the Ecological Society of Germany, Austria and Switzerland. Marburg. Germany.


PUBLICATIONS


Declaration

I declare that I prepared this doctoral thesis independently and did not use any sources or means other than those indicated by me and, furthermore, I cited the passages where I quote or referred to works by other people and their contents and I comply with the Statutes of Ulm University on Safeguarding Good Scientific Practice in the applicable version.

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Ya Zhang

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