Challenges in understanding air-seeding in angiosperm xylem

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Abstract

Bordered pit membranes play a crucial role in drought-induced embolism formation via the process of air-seeding, which remains one of most important challenges in our understanding of water transport under negative pressure. Recent progress in the structural and chemical composition of pit membranes provides a much desired, novel view on the mechanisms behind air-seeding. In this brief review, we discuss the functional importance of pit membrane thickness in relation to embolism resistance, the potential occurrence of shrinkage and increased porosity of pit membranes during dehydration, and the role of surface active compounds in air-seeding.

Keywords: cellulose fibril, embolism, intervessel pit membrane, pit border, porosity, nanobubble, surfactants

INTRODUCTION

Water transported through angiosperm xylem passes through thousands of interconnected conduits, including unicellular tracheids and multicellular vessels. Water molecules are dragged from one conduit to another via paired openings in two neighbouring secondary cell walls, which form together a bordered pit pair (Schmid and Machado, 1968). This opening, which is characterised by an inner aperture on the conduit lumen side and a wider aperture on the outermost side, varies in size from minute to large, with a horizontal pit diameter from <4 to >10 µm. Much smaller (<50 nm) openings occur in the primary wall and middle lamella, which together form the pit membrane and undergo some developmental modification during cell death of the conduit (Figure 1A, B). The nanoscale pores between the cellulose fibrils in interconduit pit membranes contribute to ca. 50% of the entire xylem hydraulic resistance (Sperry et al., 2006), but at the same time provide hydraulic safety by preventing to some extent the entry of gas and pathogens into the hydraulic pathway (Tyree and Zimmermann, 2002; Choat et al., 2008). Therefore, interconduit pit membranes are generally viewed as safety valves.

There is clear evidence for drought-induced embolism at the pit membrane level via air-seeding, but how exactly the underlying mechanisms work is poorly understood (Sperry and Tyree, 1988; Cochard et al., 1992; Tyree and Zimmermann, 2002; Brodribb et al., 2016). According to Tyree and Zimmermann (2002), “air-seeding occurs when an air bubbles is sucked into a water-filled lumen via a pore from an adjacent air space”. An important factor determining the likelihood of air-seeding is the pressure difference between the xylem sap in a functional conduit (typically under negative pressure) and the atmospheric pressure of the gas in an embolised, neighbouring conduit. In addition, the tortuosity of a pit membrane (defined as the ratio of the length of a flow path through a pit membrane to the width of the pit membrane) plays a critical role in air-seeding and is determined by the geometry of pore volumes, the porosity (i.e., the pore volume fraction), and the pore connectivity. Thus, the term tortuosity quantifies the convoluted flow of water through pit membranes and is a measure of their geometric complexity, but has hardly been used for flow through pit

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membranes. Interestingly, the air-seeding pressure is reduced by ca. 50% of what one would expect if all pores were perfectly circular and straight rather than complex shapes in the cellulose network of pit membranes (Schenk et al., 2015).

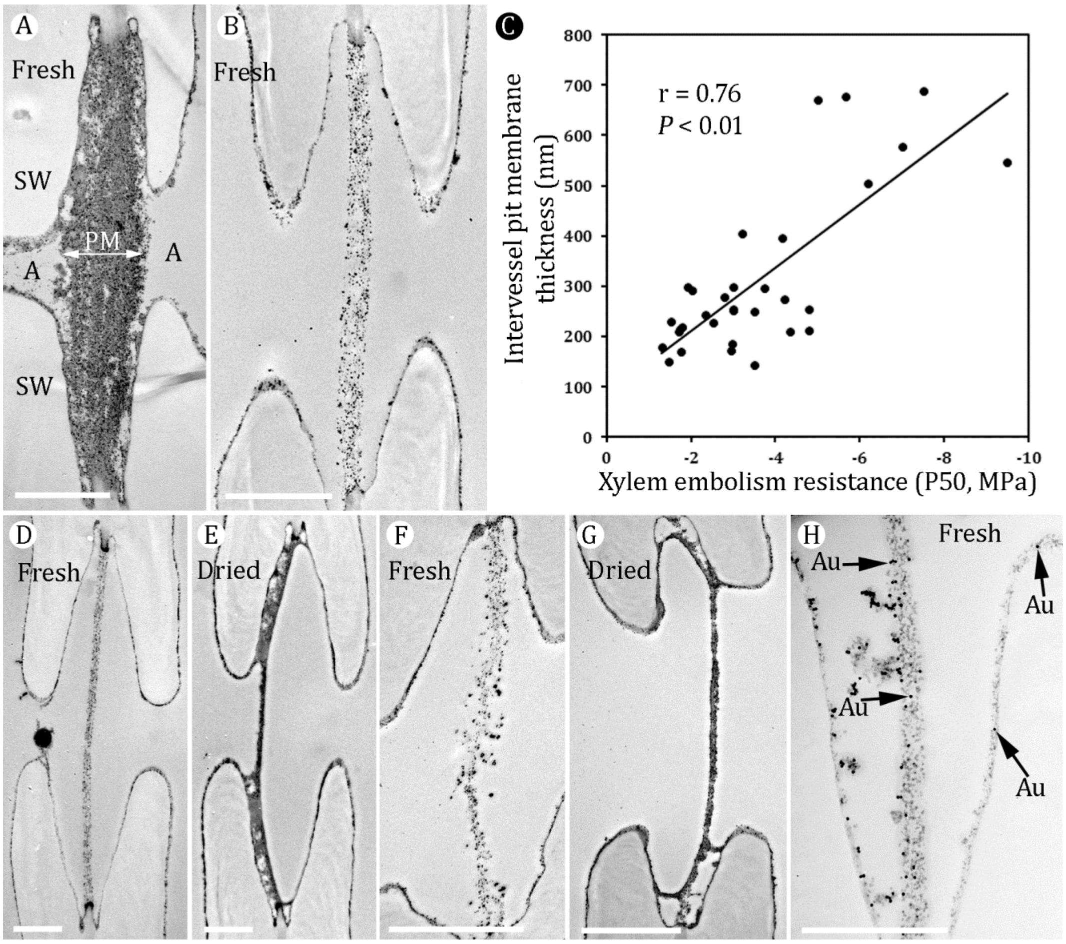

Figure 1. Transmission electron microscopy images of bordered pits and pit membranes between neighbouring vessels (A, B, D-H), and the relationship between xylem embolism resistance (P50, MPa) and pit membrane thickness (nm) for 32 angiosperm species (C; modified from Li et al., 2016). Freshly embedded pit membranes (A, B, D, F, H) differ in their ultrastructure from dried-rehydrated pit membranes (E, G). A. *Quercus ilex*; B. *Corylus avellana*; D & E. *Carpinus betulus*; F & G. *Alnus glutinosa*; H. Pit membrane of *Carpinus betulus* after injection with 20 nm colloidal gold particles, which were injected in the vessel element on the left; various gold (Au) particles penetrated the pit membrane (left arrows); two crossed the pit membrane completely and are found on the pit border (right arrows). SW = secondary wall, A = pit aperture; PM = pit membrane. All scale bars = 1 μm.

Two additional challenges in understanding the mechanisms behind air-seeding include the surface tension of xylem sap at the air-water-pit membrane interface and the wettability of cellulose fibrils in pit membranes (Meyra et al., 2007; Jansen and Schenk, 2015; Schenk et al., 2017). Considering recent studies on the ultrastructure of pit membranes (Jansen et al., 2009; Li et al., 2016; Zhang et al., 2017) and the potential significance of xylem sap surfactants for water transport under negative pressure (Schenk et al., 2017, 2018), this paper aims to provide an updated view on the mechanisms and future challenges in understanding air-seeding. In particular, we will briefly discuss the following questions: 1) Why is pit membrane thickness related to embolism resistance? 2) What are the consequences of pit membrane shrinkage for hydraulic safety? 3) Which role do surface active agents play in air-seeding? Addressing these questions could provide important steps forward in our understanding of air-seeding.

RESULTS AND DISCUSSION

Why is pit membrane thickness related to embolism resistance?

Pit membranes represent considerable variation in their thickness across angiosperm species, with a more than five-fold variation from ca. 150 nm to 1,000 nm based on transmission electron microscopy (TEM) images of freshly embedded samples (Jansen et al., 2009; Li et al., 2016) (Figure 1A-C). Transmission electron microscopy enables measurements of the pit membrane thickness at the nanoscale resolution, while super resolution confocal microscopes provide a promising, state of the art method. Dried-rehydrated samples were found to show 30 to 50% thinner pit membranes (Figure 1E, G) than fresh samples from the same plant species (Zhang et al., 2017) (Figure 1D, F). Sample storage (e.g., in ethanol or frozen) and preparation (including dehydration during TEM preparation) could cause additional shrinkage of pit membranes, which represent apoplastic structures not protected by a cell membrane. It is therefore recommended not to mix up dried, alcohol stored, or fresh material when comparing pit membrane thickness.

The variation in fresh pit membrane thickness has been shown to scale directly with embolism resistance (quantified as P50 values, i.e., the xylem water potential corresponding to 50% loss of hydraulic conductivity) across a wide range of angiosperm taxa: species that are resistant to xylem embolism show typically thicker pit membranes than species that are more vulnerable (Figure 1C). Pit membrane thickness was also significantly different between species from a temperate and Mediterranean biome, with thicker pit membranes in Mediterranean than temperate species (Li et al., 2016). A significant relation between pit membrane thickness and embolism resistance was also found between closely related species within *Acer* and *Prunus* (Lens et al., 2011; Scholz et al., 2013), and between specimens of a hybrid poplar clone and *Fagus sylvatica* (Plavcová and Hacke, 2011; Schuldt et al., 2016). Pit membrane thickness was also found to be correlated with predawn water potential for eight fern species in a Mexican cloud forest (Klepsch et al., 2016a).

Initially, this finding was explained by the assumption that pore sizes depend on pit membrane thickness, with large pores being characteristic of thin pit membranes, while narrow pores would characterise thick pit membranes (Jansen et al., 2009). Although this hypothesis requires further testing, there is no evidence that the actual pit membrane thickness affects the distance or the spaces between cellulose fibrils, and therefore the pore volumes or diameters. Considering that both thin and thick pit membranes are composed of similar sized (ca. 5-25 nm thick) cellulose fibrils, with little or no difference in other chemical compounds, porosity should largely be determined by how much a pit membrane is swollen or shrunken (i.e., how far cellulose fibrils are spaced apart), and probably not by the thickness per se or the actual number of cellulose fibrils overlying each other. This hypothesis, however, currently lacks experimental evidence.

The images presented in Figure 1 show a pronounced collapse of pit membranes between the freshly embedded samples and the dried-rehydrated condition. Since pectins are absent in intervessel pit membranes (Herbette et al., 2015; Klepsch et al., 2016b), swelling and shrinkage of pit membranes cannot be explained by hydrogel behaviour of pectins. However, shrinkage is largely caused by a reduction of the pore volumes, which become much smaller, while the fibrils become more tightly packed and may increase in diameter. Based on atomic force microscopy (AFM), no clear difference was observed in the width of cellulose fibrils in wet (i.e., never-dried) versus dried pit membranes, with values between 22 and 50 nm for *Triadica sebifera* (Pesacreta et al., 2005). AFM measurements, however, are most likely overestimating the size of cellulose fibrils due to tip broadening, with dimensions between 3 and 5 nm for the principal unit of plant cellulose fibrils (Thimm et al., 2000; Ding et al., 2014). Interestingly, AFM observations of cellulose fibrils in parenchyma cell walls of celery showed that wet samples had thinner fibrils than air-dried samples, with mean diameters of 15.0±0.3 nm and 23.4±0.5 nm, respectively (Thimm et al., 2000). A similar difference in cellulose diameter was found between wet and dry fibrils of cotton based on AFM (Pesacreta et al., 1997). Therefore, it is likely that cellulose fibrils in pit membranes increase in diameter during dehydration by coating of proteins or other unknown substance. In addition, the microfibrils will aggregate during dehydration, with pre-aligned cellulose fibrils becoming glued together, resulting in a less uniform and more enmeshed network. Displacement of cellulose fibrils from their native position could be driven by very high capillary pressure of air-water interfaces with a curvature similar to cellulose fibrils (ca. 70 atm), and by attractive van der Waals force, which may increase by 10-fold during dehydration (Israelachvili, 1992; Thimm et al., 2000).

What are the consequences of pit membrane shrinkage for hydraulic safety?

Shrinkage of pit membranes during sample dehydration was frequently suggested to result in enlarged pore sizes, which can be pronounced when observing relatively thin pit membranes with a scanning electron microscope (SEM) (Shane et al., 2000; Sano, 2005; Jansen et al., 2008). Enlarged pores are especially common at the margins (i.e., near the pit membrane annulus) and in the center of the pit membrane, where the pit border does not provide mechanical support to an aspirated pit membrane (Sano, 2005; Jansen et al., 2009; Hillabrand et al., 2016). Since shrinkage of the pit membrane results in rearrangement of cellulose fibrils, enlarged pores near the annulus could be caused by the reduced mechanical flexibility of fibrils that are anchored in the primary wall.

Direct comparison of fresh, non-dried pit membranes with dried ones showed much more loosely intermeshed fibrils in wet pit membranes than in dried ones based on AFM (Pesacreta et al., 2005). Since understanding pit membrane pore sizes in planta requires observation of wet, non-dried pit membranes, injection of fresh branches with colloidal gold particles with a known diameter could provide a more accurate method than SEM imaging (Figure 1H). Perfusion experiments with colloidal gold have been applied to ca. 10 angiosperm species, indicating that pore diameters are typically between 5 and 20 nm (Choat et al., 2003, 2004; Zhang et al., 2017) (Figure 1H). However, the accumulation of surfactants (see below) or other charged substances in pit membranes could trap colloidal gold particles, which are generally hydrophobic and charged. TEM images show indeed some coating of the pit membranes with colloidal gold particles after injection in xylem.

Moreover, TEM observations of dried-rehydrated samples show that pit membrane shrinkage and the associated changes in porosity are largely irreversible and may occur in the field under natural conditions (Zhang et al., 2017). This finding could provide a convincing explanation for the phenomenon of air-seeding fatigue (initially called “cavitation fatigue”), because once conduits have become gas filled and pit membranes have shrunken, these may become leaky and do not swell completely again after artificial refilling of the conduits, typically resulting in reduced air-seeding pressures (Hacke et al., 2001; Hillabrand et al., 2016). The temporal aspects of embolism-induced pit membrane shrinkage, and the associated changes in porosity, however, require further research. Increased porosity of pit membranes also has technological implications and explains why permeability of wood to gases gradually increases over time as wood dries (Siau, 1984; Cohen et al., 2003). Moreover, the largely irreversible nature of pit membrane shrinkage and its increased porosity raise questions about the occurrence of embolism refilling, including seasonal refilling as observed for instance in grapevine, and the potential artefacts associated with flushing stem segments for vulnerability curves (Cochard et al., 2013; Brodersen and McElrone, 2013; Charrier et al., 2016).

Which role do surface active agents play in air-seeding?

During conduit autolysis, non-cellulosic substances (especially pectins and hemicellulose) are enzymatically removed from the almost fully developed pit membrane (O’Brien, 1970; Herbette et al., 2015; Klepsch et al., 2016b). As such, cellulose fibrils form the major composition of the pit membrane, although additional compounds are likely present in interconduit pit membranes. There is convincing evidence for the occurrence of amphiphilic lipids associated with pit membranes, which make pit membranes visible in TEM sections after postfixation with OsO_4. Treatment with glutaraldehyde and poststaining with uranylacetate and lead citrate do not provide sufficient contrast to observe pit membranes in TEM sections of most species, which means that pit membranes in these samples are highly electron transparent and practically invisible (Schenk et al., 2017, 2018; Zhang et al., 2017). The reaction with OsO_4 is mainly due to osmium particles that bind to double carbon bounds in unsaturated fatty acid chains of lipids. Confocal microscopy using FM1-43 as a dye for amphiphilic lipids provide additional evidence for lipids on inner conduit walls and bordered pits (Schenk et al., 2018). Although the concentration of amphiphilic, insoluble lipids associated with pit membranes is unknown, their surface tension is dynamic and concentration dependent (Schenk et al., 2015, 2017). It is currently unclear if these surfactants only coat the outermost layer of a pit membrane, render hydrophobic surfaces more hydrophilic, or whether these lipids also penetrate into pit membranes and clog pore volumes (Schenk et al., 2018). The presence of surfactants makes modelling of pit membranes more complicated as four separate phases should be considered, namely liquid (water), gas (air), solid (cellulose fibrils), and surfactants (insoluble lipids).

The chemical composition of pit membranes has an important effect on the contact angle of an air-water meniscus spanning a pit membrane pore (McCully et al., 2014). While there are hydrophilic surfaces created by cellulose, even crystalline cellulose has some hydrophobic surfaces. Meyra et al. (2007) speculated that pit membrane surfaces would have to be partially hydrophobic, because the boundary layer of water molecules attached to entirely hydrophilic surfaces would cause a huge increase in viscosity if all surfaces within the nanopores of pit membranes would be completely hydrophilic.

Traditionally, air-seeding is assumed to occur when an air-water meniscus passes through the largest pit membrane pore between an embolised and functional vessel (Sperry and Tyree, 1988; Tyree and Zimmermann, 2002). This concept, however, is an oversimplification of the real situation, especially in wet pit membranes that never dried, because various aspects should be considered, such as the highly variable geometry of pore volumes, the dynamic nature of the surface tension of the air-water meniscus, and the behaviour of a surfactant-coated air-water meniscus under negative pressure. However, complete movement of the air-water meniscus through a large and short pore pathway with no constriction sites is likely to result in embolism spreading. This process is likely in a shrunken pit membrane with enlarged (>100 nm) pores, even under not very negative xylem water potential. The presence of surfactants will thereby reduce the surface tension of the air-water meniscus, which would considerably reduce the pressure difference for air-seeding.

In a never-dried pit membrane, however, cellulose fibrils will be more or less equally spaced apart from each other, which reduces the size of the pores but increases the pathway length and probably also the tortuosity. Due to contracted surfactant films with a surface tension far below 72 $mJ\ m^{-2}$ (i.e., the surface tension of pure water), an air-water meniscus will be pulled into a pit membrane pore. Making bubbles in water costs energy because of the energy of the bubble surface, which is referred to as surface tension. Therefore, bubble size is a direct and linear function of surface tension: bubbles will be formed more easily and will be much smaller when surface tension is strongly reduced due to the presence of insoluble, amphiphilic lipids at the air-water interface (Schenk et al., 2017). While the overall surface tension of xylem sap can be rather similar to pure water (Christensen-Dalsgaard et al., 2011; Losso et al., 2017), which is not surprising given the extremely low concentration of surfactants in xylem sap (between 0.5 and 1.36 $\mu M\ L^{-1}$; Schenk et al., 2017), dynamic, concentration dependent surface tension provides a different concept that could result in very low surface tension at the air-water interface in pit membranes.

Because matter tends to assume a geometry that minimizes energy, it is highly unlikely that an air-water meniscus will follow the entire, tortuous pathway with many narrowing, broadening, and meandering curves. Instead, bubble snap-off will occur when an air-water meniscus passes a pore volume that has <½ the diameter of the adjacent pore volume, which is a well-known foam-generating phenomenon (Roof, 1970). This snap-off event may lodge at some point in the porous medium, could break up into more bubbles, or could find a way through the pore pathway until it reaches the xylem sap in the pit border.

Since the speed of bubble generation and snap-off is currently unknown, it is possible that surfactants may not coat nucleating bubbles. Interestingly, however surfactant coated nanobubbles can be stable under negative pressure (Oertli, 1971), and can be observed in extracted xylem sap as nano-particles using a nanoparticle tracking device, with concentrations between 136 and 581 nL^{-1} and diameter modes between 85 and 218 nm (Schenk et al., 2017). Surfactant-coated nanobubbles could also be visualized based on freeze-fracture TEM (Schenk et al., 2017). Under rapidly declining liquid pressure, surfactant bubbles may expand, with the surfactant coating breaking up. Exposure of the bubble to xylem sap without any surfactant coat would then result in an increase of the surface tension and the LaPlace pressure, which leads to bubble compression and dissolution without embolism formation (Schenk et al., 2015).

According to this surfactant model, pit membranes cannot be considered as simple safety valves that prevent air-entry as much as possible. Instead, pit membranes would function as foam-producing structures, generating nanobubbles that are coated with surfactants. Further research is required to analyze the chemistry of amphiphilic lipids in xylem sap across all major groups of vascular plants, their dynamic surface tension, origin within the xylem tissue, exact location, and their hypothesized functions.

CONCLUSION

Challenges in understanding air-seeding will strongly rely on the functional importance of xylem sap surfactants combined with a more realistic, three-dimensional view of the pit membrane ultrastructure as a porous medium. Understanding air-seeding provides not only a key element to the long-standing question of how water transport in plants works under negative pressure without constant hydraulic failure, but also represents an important hurdle to be taken if man-made transport systems under negative pressure are desired. Pit membranes between water conducting cells in plant xylem require more attention with respect to their ultrastructure and potential artefacts due to shrinkage. More work is also needed to study the chemical composition of pit membranes, the nature of amphiphilic lipids, seasonal variation, and the three-dimensional network of the highly variable pore volumes.

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