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Modelling the mechanical behaviour of pit membranes in bordered pits with respect to cavitation resistance in angiosperms

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- **Background and Aims** Various correlations have been identified between anatomical features of bordered pits in angiosperm xylem and vulnerability to cavitation, suggesting that the mechanical behaviour of the pits may play a role. Theoretical modelling of the membrane behaviour has been undertaken, but it requires input of parameters at the nanoscale level. However, to date, no experimental data have indicated clearly that pit membranes experience strain at high levels during cavitation events.

- **Methods** Transmission electron microscopy (TEM) was used in order to quantify the pit micromorphology of four tree species that show contrasting differences in vulnerability to cavitation, namely *Sorbus aria*, *Carpinus betulus*, *Fagus sylvatica* and *Populus tremula*. This allowed anatomical characters to be included in a mechanical model that was based on the Kirchoff–Love thin plate theory. A mechanistic model was developed that included the geometric features of the pits that could be measured, with the purpose of evaluating the pit membrane strain that results from a pressure difference being applied across the membrane. This approach allowed an assessment to be made of the impact of the geometry of a pit on its mechanical behaviour, and provided an estimate of the impact on air-seeding resistance.

- **Key Results** The TEM observations showed evidence of residual strains on the pit membranes, thus demonstrating that this membrane may experience a large degree of strain during cavitation. The mechanical modelling revealed the interspecific variability of the strains experienced by the pit membrane, which varied according to the pit geometry and the pressure experienced. The modelling output combined with the TEM observations suggests that cavitation occurs after the pit membrane has been deflected against the pit border. Interspecific variability of the strains experienced was correlated with vulnerability to cavitation. Assuming that air-seeding occurs at a given pit membrane strain, the pressure predicted by the model to achieve this mechanical state corresponds to experimental values of cavitation sensitivity ($P_{90}$).

- **Conclusions** The results provide a functional understanding of the importance of pit geometry and pit membrane structure in air-seeding, and thus in vulnerability to cavitation.


**INTRODUCTION**

In angiosperm trees, xylem sap is transported under negative pressure by way of interconnected vessels according to the accepted cohesion–tension theory (Angeles et al., 2004). During drought events, large negative sap pressure increases between vessel ends, and cavitation events could occur. As a result, air could be aspirated from an air-filled vessel to the adjacent functional vessel by way of pores located in the double cell wall: the intervessel pits (Cochard, 2006; Lens et al., 2013). Cavitation may result in an air embolism that leads to a loss of hydraulic conductance (Cochard, 2006). Xylem embolism represents an important constraint on plant survival and productivity (Brodribb and Cochard, 2009; Choat et al., 2012). Thus, xylem functional traits have to manage both water flow efficiency and protection against air entry and propagation in the hydraulic conduit network (Tyree and Zimmermann, 2002).

Intervessel bordered pits are microscopic openings in the double cell wall. They show overarching walls that form a bowl-shaped chamber, which contains in its centre a thin membrane formed from the middle lamella and primary walls (Fig. 1). This airtight membrane is the physical boundary between the two vessels and prevents the spread of air embolisms between vessels. Since the ascent of water between angiosperm vessels involves the crossing of many bordered intervessel pits, the hydraulic behaviour of the pits is a key factor for controlling water movement (Shane et al., 2000; Tyree and Zimmerman, 2002). On the one hand, the flow resistance through intervessel pits accounts for $>50\%$ of the total resistance of the vessel network in angiosperms (Wheeler et al., 2005; Hacke et al., 2006). Their number and their hydraulic
conductivity, which involves the pit area and its water permeability, are positive factors that improve the intervessel water flow. On the other hand, the pits are the main sites for the air pathway from an air-filled vessel into a functional vessel (Tyree and Sperry, 1989a). Altogether, their structure has been reported to reflect a compromise between efficiency and safety of the water flow (Tyree and Sperry, 1989a; Chou et al., 2009; Jansen et al., 2009). Cavitation probably occurs in a functional conduit as air bubbles seed from an embolized neighbouring conduit through pores of intervessel pits (Tyree and Zimmermann, 2002; Cochard, 2006). When an embolized vessel is connected by bordered pits to a functional vessel under negative pressure, a pressure difference develops between both sides of the pit membranes. Drought-induced embolism resistance is determined by both qualitative and quantitative pit characteristics (Lent et al., 2013; Scholz et al., 2013). The probability of air entry into a vessel would increase in a stochastic fashion with its pit area (pit quantity). Considering variability in the porosity of pit membranes, the largest pore of the intervessel pit area will determine air-seeding. The larger the pore, the higher is the chance for the air to spread and to cavitate the vessel (Tyree and Sperry, 1989b; Cochard, 2006). The importance of the pit quantity to cavitation has been proposed as the rare pit hypothesis (Jarbeau et al., 1995; Wheeler et al., 2005; Christman et al., 2009). A strong correlation between cavitation resistance and the average area of pit overlap between vessels was found for >80 species (Wheeler et al., 2005; Hacke et al., 2006; Scholz et al., 2013). However, this correlation may not be valid when considering a relatively narrow range of cavitation resistance, suggesting also the importance of qualitative pit features such as pit membrane thickness (Lent et al., 2011; Scholz et al., 2013).

Recent studies suggested the importance of the structure of pits in xylem functional traits, illustrating interspecific and intraspecific variation of pit membrane porosity (Chou et al., 2003, 2008; Sano, 2005; Jansen et al., 2009). Pit membrane thickness has been shown to be correlated with pit membrane porosity and thus with the pressure threshold required to force gas across the intervessel pit field (Jansen et al., 2009). Intriguingly, the pore size calculated to allow air-seeding was always greater than the pore size measured using perfusion with colloids or scanning electron microscopy (SEM; Choat and Pitterman, 2009; Jansen et al., 2009). The discrepancy between observed and calculated pore size can be explained by many factors related to microscopy techniques and preparation artefacts. First, microscope methods show limitations for measuring pore sizes in thick membranes (Jansen et al., 2009). Secondly, it is difficult to find the largest pore responsible for air-seeding. Thirdly, pits observed under SEM or transmission electron microscopy (TEM) are typically in a relaxed (i.e. non-aspirated) state, whereas pit membrane pores may enlarge by stretching when they undergo a pressure difference across two neighbouring conduits (Tyree and Zimmermann, 2002; Chou et al., 2004; Sperry and Hacke, 2004). Silicon injection under pressure allowed the observation of the deflection of pit membranes against the outer pit aperture (Chou et al., 2008), illustrating the stretching properties of pit membranes (Thomas, 1972). In such a case, porosity could increase by rupture or reversible stretching of the pit membrane (Sperry and Hacke, 2004). This stretching would then be a function of (1) geometrical characteristics of the membrane and the pit chamber and (2) the intrinsic mechanical properties of the membrane. Experiments based on colloidal gold perfusion across a pit field showed that increasing pressure allowed the perfusion of larger particles through intervessel pit fields (Chou et al., 2004). Moreover, since no colloidal gold was found to penetrate pit fields when the pressure was decreased, this study suggested that the increasing pressure difference generated the stretching of the pit membrane.

These recent experimental results strengthen the theoretical hypothesis of Sperry and Hacke (2004) that the mechanical behaviour of the pit structure could be a key point in cavitation resistance. Lens et al. (2011) showed a correlation between the pressure required to reduce hydraulic conductance by 50 % ($P_{50}$) and various pit anatomical features in Acer species. Correlations were found for the pit chamber depth, the pit membrane thickness, the pit chamber diameter and the aperture fraction. A mechanistic explanation integrating bordered pit parameters is needed to understand how pit morphological features may control vulnerability to cavitation. The effects of pit geometry on the degree of stretching of the pit membrane have been investigated. Sperry and Hacke (2004) proposed a model of the membrane deflection in response to a pressure difference. They considered the structure of the membrane as a composite material made of the superposition of cellular microfibril spokes. Starting from this nanoscale level, this very complete modelling was able to evaluate the elastic rigidity of the pit membrane. These data allowed investigation of the theoretical mechanical behaviour of the membrane according to the dimensions of the pit chamber such as its diameter and its depth. Finally, the impact of the membrane deflection on the cavitation process was discussed.

Using a similar mechanical approach to that of Sperry and Hacke (2004), we proposed a mechanical model of the pit membrane under a pressure difference between neighbouring conduits. In our case, the modelling was only based on the geometrical structure of the components of the pit we were able to measure on TEM images, i.e. the chamber depth, the diameter of the membrane and of the aperture, and the thickness of the membrane.

![Diagram of a bordered pit as seen in transverse section. Grey lines show a relaxed and aspirated pit membrane.](image)
We also obtained proof that pit membranes experience important strains during cavitation events, and our model allowed these strains to be quantified. We investigated the variability of the geometry of the pit structure for several species that show varying cavitation resistance and contrasted bordered pit anatomy in order to test how mechanical properties based on our model may scale with vulnerability to cavitation.

MATERIALS AND METHODS

Plant material

Four species that show a wide range of cavitation sensitivity were used. Sorbus aria, Carpinus betulus, Fagus sylvatica and Populus tremula branches were harvested in September 2011 from the Allagnat forest in the centre of France (45°45’23”N, 2°56’26”E, 1000 m a.s.l.). To avoid light effects (Herbette et al., 2010), we harvested only fully sun-exposed branches. Samples of 50 cm long were cut from the plants, wrapped in moistened paper and kept at 5°C in a sealed black plastic bag for a maximum of 6 d. Three samples per species were collected from 1–3 individus. Vulnerability to cavitation measurements and TEM analyses of pits were performed on samples from the same branches.

Vulnerability to cavitation

Xylem vulnerability to cavitation was assessed using the Cavitron (Cochard, 2002; Cochard et al., 2005) on 0-28 m long stem samples. The centrifugal force increases xylem water tension in branch segments and allows at the same time measurement of the loss of hydraulic conductance. A vulnerability curve was built by plotting the percentage loss of xylem conductance (PLC) vs. xylem water tension in three branches per species. A sigmoidal function was fitted for each curve using the following equation (Pammeter and Vander Willigen, 1998):

\[
PLC = \frac{100}{1 + e^{b(P - P_{50})/a}}
\]

where \(P_{50}\) is the pressure causing 50% loss of conductance, and \(S\) the slope of the curve at this point. In order to investigate the effect of pressure difference on pit membranes, branches of F. sylvatica and P. tremula were submitted to a –4 MPa pressure with the centrifuge. Then, the central parts of the branches, which correspond to the area with the highest centrifugal forces, were prepared for TEM analysis of pits.

Transmission electron microscopy

Three stem samples of S. aria, C. betulus, F. sylvatica and P. tremula were embedded for TEM analysis of pits. Samples were fixed overnight at room temperature using Karnovsky’s solution (Karnovsky, 1965). They were then washed in 0-1 m phosphate buffer (pH 7-4) and post-fixed in buffered 1% osmium tetroxide for 4 h at room temperature. Six washes of 5 min each using phosphate buffer were performed, and samples were gradually dehydrated in ethanol at 4°C. Samples were embedded in LR white resin (London Resin Company, Reading, UK), which gradually replaced the ethanol. When 100% LR white resin was reached, the resin was polymerized for 2 d at 55°C in air-free tubes. Transverse sections with a thickness of 60–90 nm were stained with uranyl acetate for 10 min and lead citrate for 1 min. The TEM observations were carried out using a JEOL JEM1210 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV accelerating voltage and with a Hitachi H7650 transmission electron microscope. Image analysis was performed using ImageJ software (Schneider et al., 2012) with at least 24 intervessel pits studied for each species. Measurements of pit features (Fig. 1, Table 1) were performed on images with pits showing two apertures. We measured pit membrane thickness (\(T_m\)), pit chamber depth (\(L_p\)), which is defined as the distance from the unaspirated pit membrane to the inner pit aperture, pit chamber diameter (\(D_p\)) and pit aperture diameter (\(D_a\)). The \(T_m\) data were the mean value of three measurements per pit membrane. The \(L_p\) and \(D_a\) data were the mean values of two measurements per pit. For P. tremula and F. sylvatica, pit membrane length (\(L_m\)) and \(D_p\) were measured.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit</th>
<th>Definition</th>
<th>Equation</th>
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<tbody>
<tr>
<td>(P_{50})</td>
<td>MPa</td>
<td>Pressure required to deflect the membrane against the inner aperture</td>
<td>(P_{50} = (64 \times D \times L_p)/(D_a^2) - (D_j/2)^2)</td>
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<tr>
<td>(\varepsilon_{res})</td>
<td></td>
<td>Residual plastic strain</td>
<td>(\varepsilon_{res} = (L_m - D_p)/D_p)</td>
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<tr>
<td>(D)</td>
<td>MPa</td>
<td>Pit membrane flexural rigidity</td>
<td>(D = (E \times T_m)/[12(1 - \nu^2)])</td>
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<tr>
<td>(\nu)</td>
<td>–</td>
<td>Poisson’s ratio</td>
<td>(\nu = 0.3)</td>
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<tr>
<td>(L_m)</td>
<td>(\mu m)</td>
<td>Pit membrane length</td>
<td>(L_m = \frac{\pi}{4}D_p^2)</td>
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<tr>
<td>(D_p)</td>
<td>(\mu m)</td>
<td>Pit chamber diameter</td>
<td>(D_p = D_a - 2\nu)</td>
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<tr>
<td>(L_p)</td>
<td>(\mu m)</td>
<td>Pit chamber depth, i.e. the distance from the pit membrane surface to the inner pit aperture</td>
<td>(L_p = \frac{D_a}{2})</td>
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<tr>
<td>(D_a)</td>
<td>(\mu m)</td>
<td>Pit aperture diameter</td>
<td>(D_a = \frac{D_m}{2})</td>
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<tr>
<td>(T_m)</td>
<td>(\mu m)</td>
<td>Pit membrane thickness</td>
<td>(T_m = \frac{D_a}{2})</td>
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<tr>
<td>(\varepsilon)</td>
<td></td>
<td>Maximum pit membrane strain</td>
<td>(\varepsilon = 3 \times (D_j/2)^2 \times (1 - \nu^2)/[8 \times E \times T_m]) if (P &lt; P_b)</td>
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<td></td>
<td></td>
<td></td>
<td>(\varepsilon = 3 \times (D_j/2)^2 \times (1 - \nu^2)/[8 \times E \times T_m] + \varepsilon_1) if (P &gt; P_b)</td>
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<tr>
<td>(W_{Fig})</td>
<td>(\mu m)</td>
<td>Pit membrane deflection index</td>
<td>(W_{Fig} = D_p^2/[(64 \times D) \times D_j^2])</td>
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<td>(e_1)</td>
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**Table 1. List of parameters of the pit model with symbols, units, definitions and equations.**
and compared between samples submitted to –4 MPa and samples not subject to centrifugal forces. Residual strains ($\varepsilon_{res}$) were calculated as:

$$\varepsilon_{res} = \frac{(D_p - L_m)}{D_p}$$

Data from the literature

Anatomical and $P_{50}$ data were compiled from previous studies (Sperry and Hacke, 2004; Jansen et al., 2009; Plavcova et al., 2011; Lens et al., 2011; Plavcova and Hacke, 2012). We considered studies for which at least $T_{in}$, $D_p$, and $P_{SO}$ were measured. Lens et al. (2011) compiled $T_{in}$, $D_p$, $L_p$, $D_a$ and $P_{50}$ data for six Acer species: A. negundo, A. glabrum (including var. glabrum and var. diffusum), A. grandidentatum, A. saccharinum, A. platanoïdes and A. pseudoplatanus. Jansen et al., (2009) compiled $T_{in}$ and $D_p$ data for 12 angiosperm species: Corylus avellana, Fraxinus americana, Ilex aquifolium, Olea europaea, Populus fremontii, Quercus robur, Salix alba, Sambucus nigra, Sophora japonica, Larix nobilis, Betula nigra and Betula pendula. Corresponding $P_{50}$ values were extracted from Chat et al. (2012). $D_a$ measurements were added for Olea europaea and Larus nobilis. $T_{in}$, $D_p$, $D_a$ and $P_{50}$ from Nerium oleander L. and Populus trichocarpa x deltoides (clone H11-11) were reported by Sperry and Hacke (2004) and by Plavcova et al. (2011) and Plavcova and Hacke (2012), respectively.

Model description

We modelled the mechanical behaviour of the pit membrane in order to quantify (1) the effect of the pressure difference on the strain; (2) the pressure that is required to deflect the membrane beyond the aspiration pressure. The model assumed circular membranes and was based on the Kirchhoff–Love thin plates theory (Bauchau and Craig, 2009). Basic axioms of this theory consider a continuous, elastic and homogeneous circular plate that (1) can bend in two directions and twist; (2) is initially flat; and (3) shows a transverse dimension (thickness) that is small compared with the diameter. This latter axiom allowed shear effects to be neglected. As a boundary condition, the pit membrane was presumed to be clamped to the pit border. Finally, the pressure was assumed to be uniform on the whole membrane area. All the mechanical parameters were computed for each pit using their intrinsic measured dimensions.

Flexural rigidity ($D$) of the membrane was calculated as the force couple required to bend the membrane to a unit curvature.

$$D = \frac{(E \ T_{in}^2)}{12 \ (1 - \nu^2)}$$

where $E$ is the Young’s modulus of pit membrane and $\nu$ is its Poisson’s ratio. The Young’s modulus is the ratio of stress ($\sigma$ (Pa)) to strain ($\varepsilon$), and measures the elasticity of a material. Using atomic force microscopy (AFM) on fresh samples, the Young’s modulus for intervessel pit membranes in Populus deltoides was found to be around 400 MPa (Capron et al., 2014). As the available evidence suggests that intervessel pit membranes in angiosperms may show a larger variation in their ultrastructure and chemical composition than previously thought, we used this Young’s modulus value by default as a constant value across angiosperm species. Moreover, the Poisson coefficient $\nu$, which refers to the transversal contraction of the material, was estimated to be 0.3 for all our computations because most polymers exhibit values of about 0.3. Moreover, this value is in line with the $\nu$ value for cellulose (Nakamura et al., 2004), which represents the main component of intervessel pit membranes. Considering these elasticity parameters as constants permitted us to focus on how the pit geometry is involved in the interspecific variability of vulnerability to cavitation.

Following the above axioms, the deflection ($W$) of a membrane that experiences a uniform pressure ($P$) fulfils the following equation:

$$\nabla^4 [W(r)] = \frac{P}{D}$$

(2)

In the case of a circular membrane, the Nabla operator ($\nabla$) can be written in circular co-ordinates as follows:

$$\frac{d}{dr} \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dW(r)}{dr} \right) \right] = \frac{P}{D}$$

(3)

where $r$ is the radial position from the centre of the membrane. Solving this equation using the above-written boundary conditions, leads us to the relationship

$$W(r) = \frac{(R^2 - r^2)^2}{64D} - \frac{P}{D}$$

(4)

where $R$ is the radius of the membrane ($R = D_p/2$). Solving eqn (3) for $r$ being equal to the radius of the aperture ($r = D_p/2$) and $W$ being equal to $L_p$ enabled us to evaluate the pressure required to deflect the pit membrane against the aperture ($P_b$).

$$P_b = \frac{1}{3} \left( 1 - \nu^2 \right) \frac{T_{in}^3}{(D_p^2 - D_a^2)^2} \ L_p$$

(5)

The strain is the degree of the pit membrane deformation. When $P$ increases, the strain increases as the pit membrane is stretched. According to the mechanical model, the maximum strain level ($\varepsilon_{max}$) occurs at the centre of the pit membrane and increases linearly with $P$:

$$\varepsilon_{max} = \bar{\varepsilon} (r = 0) = -\frac{3}{8} \left( 1 - \nu^2 \right) \frac{R^2}{E} \frac{P}{T_{in}}$$

(6)

When $P < P_b$, $R = D_p/2$. However, when $P > P_b$, the peripheral area of the membrane is supported by the pit border and only the membrane region that is not supported by the pit border should be considered for deformation. In this case, assuming that no further displacement occurs at the aperture boundary, we can consider the previous eqns (4) and (6) with $R = D_p/2$.

$\bar{\varepsilon}$ representing the increase in length. In order to calculate $\bar{\varepsilon}$ for a given pressure, it was assumed that the membrane expands to form a spherical arc:

$$\bar{\varepsilon} = \frac{P}{R} a \sin \left( \frac{R}{P} \right) - 1$$

(7)
where \( r \) is the radius of the sphere in which the deformed membrane is inscribed.

\[
\rho = \frac{[R^2 + W(0)^2]}{2W(0)}
\]

(8)

with \( R = D_a/2 \) if \( P < P_b \), and \( R = D_a/2 \) if \( P > P_b \).

Finally, we computed a deflection index (\( W_{I_a} \)) on the aperture that was calculated on the basis of eqn (4). This index considered the membrane area that was unsupported by the pit border and was not dependent on pressure. It indicates the ability of the membrane to deflect for a given pressure difference between its two sides.

\[
W_{I_a} = \frac{(D_a/2)^4}{64D}
\]

(9)

The mechanical parameters derived from morphological pit features are reported in Table 1.

**RESULTS**

**Vulnerability to cavitation**

Vulnerability to cavitation showed significant differences for \( P_{50} \) according to a Student \( t \)-test. Mean \( P_{50} \) values (± s.e.) were \(-5.67 \pm 0.17 \) MPa for \( S. aria \), \(-2.42 \pm 0.03 \) MPa for \( P. tremula \), \(-3.18 \pm 0.16 \) MPa for \( F. sylvatica \) and \(-4.17 \pm 0.06 \) MPa for \( C. betulus \). PLC values for samples submitted to \(-4 \) MPa were 76.7 % (± 7.4) and 100 % (± 0.0) for \( F. sylvatica \) and \( P. tremula \), respectively.

**Quantitative pit characteristics and model inputs**

The four species studied showed considerable variation in their pit morphology (Fig. 2A–D; Supplementary Data Table S1). Intervessel pits differed in their pit membrane thickness, but also in diameter, chamber depth, and aperture diameter. In addition, differences in pit membrane position and length were observed between pits from control samples of \( F. sylvatica \) and \( P. tremula \) (Fig. 2C, D), and pits submitted to a pressure difference of \(-4 \) MPa (Fig. 2E, F). The frequency of aspirated pit membranes was clearly higher for \(-4 \) MPa samples (2 % for \( F. sylvatica \) and 17 % for \( P. tremula \)) compared with the control (0 MPa) samples (0 and 8 % for \( F. sylvatica \) and \( P. tremula \), respectively) (Fig. 2E). Samples of \( F. sylvatica \) showed a low number of aspirated pit membranes, but deformation of the pit membrane could be seen (Fig. 2F). In addition, the thickness of the pit membrane in aspirated and deformed pit membranes was found to decrease compared with the control conditions. Pit membranes that were aspirated or deformed also showed a higher electron density and were less transparent under TEM than the control samples.

The residual strain \( \varepsilon_{res} \) (Fig. 3) ranged from 0 to 0.035 for pit membranes of \( P. tremula \) and from 0 to 0.017 for \( F. sylvatica \). The residual strain is a typical pattern that reveals that the membranes experienced large non-recoverable plastic deformation. \( \varepsilon_{res} \) was significantly different between control samples and \(-4 \) MPa samples for both species. \( P. tremula \) showed significantly higher \( \varepsilon_{res} \) than \( F. sylvatica \), which is less sensitive to cavitation.

An overview of the pit features for the species studied is shown in Fig. 4. The \( T_{me}, D_p, L_p \) and \( D_a \) values varied considerably in

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**Fig. 2.** TEM images of bordered intervessel pits from four species with contrasting vulnerability to cavitation. *Sorbus aria* (A; \( P_{50} = -5.67 \pm 0.17 \) MPa, mean ± s.e.), *Carpinus betulus* (B; \( P_{50} = -4.17 \pm 0.06 \) MPa), *Fagus sylvatica* (C, E; \( P_{50} = -3.18 \pm 0.16 \) MPa) and *Populus tremula* (D, F; \( P_{50} = -2.42 \pm 0.03 \) MPa). TEM images are representative pictures of pits observed in control stems (A–D) or stems submitted to a xylem pressure of \(-4 \) MPa (E, F). TEM analysis and vulnerability curves were performed on similar branch samples. Scale bar = 1 \( \mu \)m.
magnitude. Values of \(D_p\) for \(P. tremula\) and \(C. betulus\) were similar, but were higher than \(D_p\) values of \(F. sylvatica\) and \(S. aria\). \(L_p\) and \(D_p\) showed limited interspecific variability. Nevertheless, \(P. tremula\) showed pits with significantly deeper chambers (\(L_p\)) than other species, and pits of \(S. aria\) pits had a significantly larger pit aperture diameter (\(D_p\)).

There were also differences in intraspecific variability. \(Fagus sylvatica\) had the highest variability for \(D_m\) (Fig. 4B) and \(D_s\) (Fig. 4D), while \(F. sylvatica\) and \(S. aria\) had high variability for \(T_m\) (Fig. 4A).

![Fig. 3. Residual strain in pit membranes induced during cavitation events in \(Populus tremula\) (A) and \(Fagus sylvatica\) (B). Stem samples were control stems (0 MPa) or stems subject to –4 MPa. Pit membrane length (\(L_m\)) was compared with pit chamber diameter (\(D_p\)) and the residual plastic strain was calculated as \(\varepsilon_{res} = (L_m - D_p)/D_p\). Significant differences (\(P < 0.01\), Wilcoxon rank sum test) are shown by asterisks.](image)

**Mechanical characteristics of the pit structure and model outputs**

Figure 5 shows the relationship between the mechanical properties of the pits (\(D\) and \(W_{f1}\)) and \(P_{50}\). The pit membrane flexural rigidity (\(D\)) was calculated for each pit based on its membrane thickness \(T_m\) for 25 angiosperm species including the four species studied. \(D\) showed an exponential relationship with \(P_{50}\) (\(R^2 = 0.72\)). Species showing higher cavitation resistance had higher \(D\). The pit membrane deflection index (\(W_{f1}\)) was calculated using \(T_m\) and \(D_s\) for 14 angiosperm species and showed a strong relationship with \(P_{50}\) (\(R^2 = 0.86\)). Species showing higher cavitation resistance had a lower deflection index.

The measurements of \(D_m, D_s, T_m\) and \(L_p\) for \(S. aria, C. betulus\), \(F. sylvatica\) and \(P. tremula\) (Fig. 4) allowed modelling the maximum (\(\varepsilon_{max}\)) and mean (\(\bar{\varepsilon}\)) pit membrane strain as a function of pressure (\(P\); Fig. 6). The maximum strain (Fig. 6A) occurs at the centre of the membrane, whereas the mean strain (Fig. 6B) represents the increase in the total membrane length. When the pit membrane reaches the aperture and is fully aspirated at the \(P_b\) pressure, there is a breakpoint in the strain function. Beyond this \(P_b\) value, only the pit membrane area that is not supported by the borders is deformed. The membrane strains ranged from 0 to 0.15. A different behaviour of pit membrane strain was observed for a low pressure. When \(P\) was > 2 MPa, species with higher vulnerability to cavitation tended to experience higher levels of strain. When \(P\) exceeded \(P_b\), the slope of the strain was higher for \(\varepsilon\) than for \(\bar{\varepsilon}\).

The relationship between the mechanical properties based on our model and the vulnerability to cavitation was tested in 11 angiosperm species (Fig. 7). \(P_b\) and \(P_{50}\) (Fig. 7A) were strongly correlated (\(R^2 = 0.73\)). In general, \(P_b\) was < 1 MPa, and less negative than the \(P_{50}\) value for all species studied, except for \(S. aria\).

**DISCUSSION**

Our observations that show residual strains provide experimental proof that the pit membrane may undergo strains during air-seeding events. This corroborates recent results of Capron.
et al. (2014) who measured the deflection of membranes after silicon injection for different pressures. We propose a model of a pit membrane that undergoes a pressure difference between neighbouring conduits. This integrative model considers various morphological features of the bordered pit. The mechanical output of the model showed that variability of the pit features leads to a great interspecific variability of the pit membrane strains for a given pressure. These strains were correlated with $P_{50}$ among the species studied and permitted us to present a functional understanding of the importance of the pit geometry for vulnerability to cavitation.

**Modelling the pit membrane behaviour under pressure**

Mechanical behaviour of the pit membrane in response to pressure is a function of its structure, which is determined by geometrical parameters (Fig. 1) and the intrinsic mechanical properties of the pit membrane. The pit membrane elasticity ($E$) or its Poisson’s coefficient ($\nu$) depends on the composition and microstructure of the membrane (Sperry et al., 2004; Herbette and Cochard, 2010; Lens et al., 2013). So far, interspecific variability of $E$ has not been investigated because of the technical difficulty in applying AFM measurements on wet (i.e. fresh) pit membranes (Pesacreta et al., 2005; Zwieniecki and Secchi, 2012). We used the only available value (Capron et al., 2014). If $E$ should depend on the thickness of the membrane because of the number of cellulose microfibrils sheets (Sperry and Hacke, 2004), it should also be influenced by the composition of the membrane and the cross-links between components. Equations (5) and (6) show that the pressure required for the membrane to reach the pit border ($P_b$) varies linearly with $E$, and the membrane deformation is inversely proportional to $E$. Considering $\nu$ and $E$ as constant parameters permitted us to focus on the role of the pit geometry and the involvement of simple and measurable anatomical parameters in the interspecific variability to cavitation resistance. Moreover, we should consider the point that we modelled the aspirated pit membrane as a clamped circular plate that does not slip against the pit border.

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**Fig. 5.** Relationships between mechanical properties of the pit membrane ($D$, $W_L$) and $P_{50}$. Pit membrane flexural rigidity ($D$) was calculated based on the pit membrane thickness ($T_m$) for 25 angiosperms species (A). The pit membrane deflection index ($W_L$) was calculated using mean pit values of $T_m$ and $D$ for 14 angiosperms species (B). Data are means values (+ s.e.). The $y$-axis is log scaled. Exponential regression lines are indicated in grey.

**Fig. 6.** Maximum pit membrane strain ($\epsilon_{max}$) vs. pressure in four species with constraining vulnerability to cavitation. Mean values of pit features were used to calculate pit membrane maximum ($\epsilon$) and mean ($\bar{\epsilon}$) strain in *S. aria*, C. betulus, F. sylvatica and P. tremula (as indicated in the key). The maximum strain occurs at the centre of the membrane, whereas the mean strain represents the relative increase in membrane length. Strains increase as the pit membrane is aspirated against the pit border. When the pit membrane reaches the pit border for a given pressure $P_b$, there is a breakpoint in the strain function. The $P_b$ pressure differs between the species and can be deduced from the change in the slope of the strain function. Beyond this $P_b$ value, only the pit membrane area that is unsupported by the aperture is deformed. The circles in (A) indicate the $P_{50}$ values.
maximum membrane strain ($\varepsilon$, which is correlated with the pore size (Jansen character of a species. Pits differed in their pit membrane thickness measurements but probably leads to an optimal position. This also suggests that the measurements of a variable and depended on the species. Moreover, TEM observations showing the surface view of bordered pits would be required to quantify circularity.

**Relationship between pit geometry and vulnerability to cavitation**

Although *F. sylvatica* and *C. betulus* had similar values for membrane thickness, they had different pit chamber geometry, in particular the pit diameter ($D_p$). Modelling of the pit behaviour showed that high pressure generated contrasted interspecific strains that could explain their great difference in vulnerability to cavitation. The relationship found by Lens et al. (2011) between the chamber depth ($L_m$) and $P_{50}$ was not observed in the four species we studied. In order to understand the involvement of the pit geometry in vulnerability to cavitation, the anatomical features of the pit were integrated into the $\varepsilon$ values.

The residual strains in pit membranes after cavitation events in *P. tremula* and *F. sylvatica* (Fig. 3) supported the relevance of the modelling of pit membrane strains. The least cavitation-resistant species showed the highest residual strains. This observation also suggested that the cavitation process may be linked to large non-recoverable plastic strains that occur when high pressures are experienced (Choat et al., 2004) and that could also explain the cavitation fatigue phenomenon.

In order to investigate a relationship between pit geometry and vulnerability to cavitation, parameters derived from the model were computed and plotted against $P_{50}$ (Fig. 5). First the membrane rigidity $D$ showed a stronger correlation with $P_{50}$ ($R^2 = 0.72$) than the membrane thickness $T_m$ alone ($R^2 = 0.66$). Moreover, the pit membrane rigidity adds an important mechanical role to $T_m$ with respect to cavitation resistance. The deflection index ($W_l$) appears to be a relevant parameter to investigate pit membrane behaviour. $W_l$ integrates the size of the pit aperture $D_p$, which appears to be an important feature of the pit geometry, and affects the behaviour of an aspirated pit membrane. In general, the pit aperture $D_p$ represents a rather small and constant fraction of the pit border $D_b$, with a pit aperture of around 10% of the pit membrane area (Lens et al., 2011; Scholz et al., 2013). According to our modelling, the pressure required to deflect the pit membrane against its aperture is low ($< 1$ MPa). This corroborates the results obtained by the modelling of Sperry and Hacke (2004) who concluded that aspiration should probably occur early before air-seeding. Also, most of the strain occurs in the aperture. The high correlation between $P_{50}$ and the deflection index $W_l$ ($R^2 = 0.86$) argues that this parameter could be a key point for cavitation resistance.
Strains experienced by the membrane represent a suitable parameter to investigate the functional link between mechanical behaviour and resistance to cavitation. On the one hand, the strains experienced by the membrane may affect air-seeding via enlarging pores (Choat et al., 2003, 2004; Choat and Pittermann, 2009). On the other hand, the pit membrane may encounter some permanent damage, which could also lead to air-seeding (Sperry and Hacke, 2004). The computation of the strains showed contrasting results between the four species (Fig. 6). The pit membranes of P. tremula, which was the most vulnerable species, were rapidly deflected against the aperture for a pressure \( P_b \) value of 0-11 MPa and then underwent the highest strains in the aperture. A thin membrane with a large diameter explained this behaviour. Similarly, the pit membranes of C. betuloides were rapidly deflected against the aperture for a \( P_b \) of 0-35 MPa because of a large membrane area, but the strains increased slowly because of a small aperture diameter that made the structure more rigid. The pit membranes of F. sylvatica were aspirated for a higher \( P_b \) than for C. betuloides because of small pit membrane diameters. However, compared with C. betuloides, pits of F. sylvatica had a larger aperture \( D_p \), which resulted in a higher slope of the strain vs. pressure \( P \) function when \( P \) exceeded \( P_b \). The lower vulnerability to cavitation for C. betuloides can thus be attributed to the pit geometry that limits the membrane deformation rather than the intrinsic property of its pit membrane. Finally, the pit structure of S. aria required a much higher \( P_b \) (10-76 MPa) than the pit membranes of more vulnerable species. A thick membrane bearing a small diameter explained the high value for \( P_b \). The strains experienced by pit membranes in this species at 5 MPa were much lower than those of the three more vulnerable species. It is noteworthy that the strains experienced at high pressure difference were in accordance with the vulnerability to cavitation of the four species. However, considering the \( T_m/D_p \) ratio of pit membranes in S. aria (\( T_m/D_p = 0.12 \pm 0.007 \)), the use of the thin plate theory remains questionable. This theory does not involve shear deformations and should only be applicable for pit membranes having a thickness to pit diameter ratio of <0.05 (Zietlow et al., 2012). Thus the computations for S. aria are likely to underestimate the strains and deflection. This reservation could be applicable to other species, and the thick plate theory would be more relevant to model their membrane behaviour.

Figure 7 shows the involvement of the pit geometry in interspecific variability of vulnerability to cavitation. If the pressure required to deflect the pit membrane against its aperture (\( P_{50} \)) has no direct mechanistic involvement in air-seeding, its relationship to \( P_{50} \) is interesting because the \( P_b \) parameter (1) integrates all pit features, including membrane, chamber and aperture dimensions; (2) has a strong impact on the strain experienced by the membrane; and (3) reveals different pit strategies among species. A strong correlation with \( P_{50} \) was found, and \( P_b \) always seemed to be lower than \( P_{50} \), except for S. aria, confirming the importance of the strains experienced by the membrane when it is deflected against the pit border. Comparison of computed \( P_b \) values and \( P_{50} \) values suggested that the air-seeding events occur when the pit membrane has already been deflected against the pit border (Fig. 6A).

Moreover, we computed the maximum pit membrane strain at a pressure \( P = P_{50} \), and we observed a low variability. Thus, assuming the \( P_{50} \) values correspond to a constant critical pit membrane strain (\( e = 0.06 \)), the model allowed evaluation of the corresponding pressure that is required to reach this mechanical state [eqn (6)]. This predicted value was then compared with experimental values for a set of 11 species (Fig. 7B). The correlation found suggests that the structure of the pit has a large impact on the cavitation resistance and that the air-seeding occurs when the pit membrane strain exceeds a threshold value that probably corresponds to mechanical damage.

Broadening our model to a larger number of species would provide insight into various evolutionary strategies of angiosperm pit membranes. According to our model, decreasing the diameter of the pit \( D_p \) showed the largest effect on the strains compared with other features of the pit. However, the correlation between pit diameter only and cavitation resistance tends to be low (Lens et al., 2011; Scholz et al., 2013). This corroborates the idea that not a single feature but rather various dimensions of the bordered pit can be adjusted and influence cavitation resistance. The gain in decreasing vulnerability to cavitation could be at the cost of a less efficient water flow in the pit chamber. This ‘trade-off’ hypothesis has been investigated by Sperry and Hacke (2004) who modelled the pit hydraulic efficiency in relation to the geometry of the bordered pits. However, their investigations finally showed no relationship between pit flow resistance and resistance to cavitation. Moreover, sensitivity analysis and covariance analysis of pit features would be of interest on a broader data set of species.

Insights into the mechanism of cavitation

The putative role of the deflection of the pit membrane has been investigated previously (Sperry and Hacke, 2004; Choat et al., 2008). Here, residual strains in pit membranes induced during cavitation events in P. tremula and F. sylvatica (Fig. 3) provided experimental proof that the pit membrane undergoes mechanical strains during cavitation. The advantage of TEM observations is that residual strains were quantified and compared between species.

Calculation of strains provides insights into the mechanisms of cavitation through air-seeding. First, the low pressure \( P_b \) calculated in this study corroborates the hypothesis that air-seeding should occur when the membrane is aspirated against the aperture. Thus, air-seeding probably occurs in the central region of the membrane that is not supported by the pit borders and subjected to the maximum strain. The strains experienced on the whole membrane inside the chamber alone cannot explain interspecific variability, because strains occur at low pressures that do not correspond to air-seeding pressure. The strains estimated by our model (Fig. 6) appear realistic, because they were higher than the residual plastic strains (Fig. 3). The membrane deformations would range from 5 to 15 % for such high pressures. The resulting enlargement of pores in pit membranes or their formation of micro cracks could be involved in air-seeding. Pore diameters calculated from the Laplace law are always larger than maximum pore diameters measured from SEM images (Jansen et al., 2009). Pit membrane strains would increase the pore diameter and could partially explain this difference. Considering the low range of deformation the membrane could experience, and according to the Laplace law, a 10 % increase in the pore diameter would be negligible for air-seeding pressure and too weak to explain the discrepancy between the observed and expected pore size (Jansen et al., 2009). Thus, our results reinforce the
hypothesis that air-seeding is probably not due to pit membrane pore enlargement. Alternatively, the residual plastic strains observed suggest that the pit membrane may experience some structural damage when air-seeding occurs. The observation of cavitation fatigue also supports the pit membrane rupture hypothesis (Hacke et al., 2001). The comparison between ε and ē opens the discussion on the point where the membrane air-seeding occurs. If pores have a more or less similar diameter across the pit membrane area, which would be expected based on the equal thickness of angiosperm pit membranes, then air-seeding should occur mostly in the middle of the membrane where maximum strain is experienced. SEM analyses of pits tend to support this hypothesis (Choat et al., 2004). In contrast, if we consider some variability in pore diameter and the idea that the largest pore is randomly distributed on the pit membrane surface (Sano, 2005), the mean membrane strain should be considered as a relevant parameter to evaluate air-seeding resistance. Both hypotheses are relevant and the debate will continue until the mechanisms of air-seeding are understood.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: interspecific variability of the pit structure, with measurements performed using TEM images.

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LITERATURE CITED


