Hydrolase treatments help unravel the function of intervessel pits in xylem hydraulics

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Intervessel pits are structures that play a key role in the efficiency and safety functions of xylem hydraulics. However, little is known about the components of the pit membrane (PM) and their role in hydraulic functions, especially in resistance to cavitation. We tested the effect of commercial chemicals including a cellulase, a hemicellulase, a pectolyase, a proteinase and DTT on xylem hydraulic properties: vulnerability to cavitation (VC) and conductance. The effects were tested on branch segments from Fagus sylvatica (where the effects on pit structure were analyzed using TEM) and Populus tremula. Cellulose hydrolysis resulted in a sharp increase in VC and a significant increase in conductance, related to complete breakdown of the PM. Pectin hydrolysis also induced a sharp increase in VC but with no effect on conductance or pit structure observable by TEM. The other treatments with hemicellulase, proteinase or DTT showed no effect. This study brings evidence that cellulose and pectins are critical components underpinning VC, and that PM components may play distinct roles in the xylem hydraulic safety and efficiency.

Introduction

In plants, long-distance sap transport occurs under negative pressures in xylem conduits. Sap flows between adjoining conduits through pits that form pores in the walls and play a key role in the safety and efficiency of the hydraulic system through the xylem (Choat et al. 2008). These pits facilitate the flow of water while preventing the passage of air bubbles. Under water stress conditions, xylem tensions increase and the conduits become vulnerable to cavitation. Cavitation provokes air embolism, leading to a loss of hydraulic conductance that can potentially result in organ or whole-plant death.

Strong correlations have been found between the drought tolerance of a species and its xylem vulnerability to cavitation (VC) (Maherali et al. 2004, Tissier et al. 2004, Choat et al. 2012). Substantial variations have also been found within species, between genotypes or depending on environmental conditions (Cochard et al. 2007, Dalla-Salda et al. 2009, Awad et al. 2010, Herbette et al. 2010, Wortemann et al. 2011). Xylem resistance to cavitation is thus considered a major adaptive trait for tree drought tolerance, and is thought to be one of the most promising criteria for screening for this feature. However, the literature provides only limited insights on the molecular and genetic factors involved.

The most likely mechanism of cavitation would be located on vessel pit (Sperry and Tyree 1988, Cochard 2006). According to the ‘air seeding’ hypothesis, water stress-induced cavitation would occur when an air bubble passes through a pit membrane (PM) (Cochard et al. 1992, 2009). Hence, VC would be strongly influenced by the porosity of the PM (Sperry and Tyree 1988, Cochard

Abbreviations – DTT, dithiothreitol; P50, xylem pressure inducing a 50% loss of conductance; PM, pit membrane; TEM, transmission electronic microscopy; VC, vulnerability to cavitation.
2006) and its mechanical properties during the cavitation process (Choat et al. 2004, Sperry and Hacke 2004). Clearly, the structural, physical and chemical properties of the PM are central determinants of cavitation. Pit structure has been the most intensively investigated property to date. Within angiosperms, there is a strong correlation between PM thickness and resistance to cavitation (Jansen et al. 2009, Lens et al. 2011). Pits with thicker PMs have smaller pores and are thought to be mechanically stronger, allowing them to resist air seeding. In conifers, which have pits with a thick torus surrounded by a thin margo, a major character for embolism resistance would be the size ratio of the torus-to-pit aperture diameter (Hacke and Jansen 2009, Pittermann et al. 2010). In contrast with pit structure, our knowledge on PM biochemistry is limited, with contradictory findings in the literature. Insights on PM composition are urgently needed, since it can strongly influence the porosity, permeability and mechanical properties of the PM and the air-water interface in this cavitation process.

PMs are composed of the middle lamella plus the primary walls of adjacent cells that have undergone modifications. This means they would initially be made of tightly interwoven cellulose microfibrils in a matrix of hydrated hemicelluloses and pectins, including various proteins. However, the modifications that occur as the PM matures are unknown. Hydrolysis of the cell wall was observed in PM and this would remove most of the non-cellulosic polysaccharides unprotected by lignins (O’Brien and Thimann 1967, O’Brien 1970, Butterfield and Meylan 1982). Hence, there is ongoing debate on the final composition of a mature pit, especially the presence of pectins and/or hemicelluloses (Zwieniecki et al. 2001, Herbe et al. 2010, Plavcova and Hacke 2011), while lignin deposition and protein composition have retained little attention. Studies using staining techniques suggest that the PM contains lignin (Fromm et al. 2003) and pectin (Gortan et al. 2011), whereas others suggest that all but the highly methylated pectins get removed from pit as it matures (Czaninski 1972, 1979, Catesson 1983). Explanations for such discrepancies are the weak specificity and sensitivity of staining techniques and the diverse composition of PMs across species. The ion-mediated water flow variation in xylem observed for several species has been attributed to the hydrogel properties of PM pectins (Zwieniecki et al. 2001, Boyce et al. 2004, Cochard et al. 2010). Although an alternative hypothesis to pectin shrinking has been proposed, there is no experimental evidence for it (van Doorn et al. 2011). Immunolabeling studies in poplar did not detect any pectin in most of the PM but found clusters of pectins in the annulus (Plavcova et al. 2011, Plavcova and Hacke 2011). Immunolabeling has the advantage of bringing direct evidence for the presence of a compound but can be hindered by accessibility to the epitope, especially in cell walls. In other words, no labeling can be explained by either an absence of pectins or by other compounds masking a pectic epitope.

Beyond this debate on the composition of PM, the role of its respective components remains to be addressed, especially in terms of resistance to cavitation. As a first step, we tested the effect of commercial hydrolases including a cellulase, a hemicellulase, a pectolyase and a proteinase on xylem hydraulics properties: VC and conductance. The pit structure was then analyzed by TEM to explain the effect of the hydrolases and propose hypotheses on the role of the respective target components. These investigations were performed on beech, a species in which we had previously demonstrated a role of calcium in VC (Herbertte and Cochard 2010), but as the debate on pectin in PM has shifted toward poplar, we also tested the effect of hydrolases on VC for a poplar species.

Materials and methods

Plant material

The perfusion experiments were carried out on stems from an old beech tree (*Fagus sylvatica*) from Allagnat forest in central France (45°45′23″N, 2°56′26″E, 1000 m a.s.l.) and on stems from an adult poplar tree (*Populus tremula*) from Bort-l’Étang in central France (45°47′02″N, 3°25′43″E, 333 m a.s.l.). Stems were sampled on the same tree to avoid intraspecific variation. Branches were harvested from October to December, i.e. after the vegetative season and before the temperature decrease drastically below 0°C. We analyzed sunlit shoots of comparable age and growth. Selected stems were 0.5–1 cm in diameter and 0.5 m long. The freshly sampled stems were sealed in airtight black plastic bags to reduce water loss through transpiration and taken straight to the lab to measure xylem hydraulic conductance on the same day or the day after. Samples taken to study VC were wrapped in moist paper, bagged and stored at 4°C until analysis.

Chemical treatments

Chemical treatments were applied just prior to hydraulic analysis. Samples were infiltrated under vacuum by connecting their terminal part to a vacuum pump while their base was immersed in a flask containing an excess of solution until at least 5 ml had perfused through the samples. We tested six different chemical treatments: four solutions prepared in 5 mM MES buffer (pH 5), i.e. MES buffer at pH 5 as control; 1% w/v cellulase...
Xylem VC

Xylem VC was assessed on 0.28 m long stem samples using the centrifugal technique (Alder et al. 1997, Cochard 2002). The technique uses the centrifugal force to increase water tension in a xylem segment and a XYL’EM apparatus (Bronkhorst, Montigny-les-Cormeilles, France) to measure the decrease in hydraulic conductance. Before centrifugation steps, we first determined sample maximal conductance \( K_{\text{max}} \) under 6–9 kPa. The stem segments were perfused with a solution containing 10 mM KCl and 1 mM CaCl2. Samples subjected to water tension were then connected to the hydraulic apparatus, and a conductance \( K \) was measured. Xylem pressure was reset to a more negative pressure, and the new sample conductance \( K \) was determined. Percent loss of conductance \( \text{PLC} \) was then computed as:

\[
\text{PLC} = 100 \times \left(1 - \frac{K}{K_{\text{max}}} \right)
\]

where \( P_{50} \) is the pressure causing a 50% loss in hydraulic conductivity and \( s \) is the slope curve at this point.

Xylem hydraulic conductance and embolism rate

Xylem hydraulic conductance \( K \) was measured on 0.3 m long beech stems using the XYL’EM apparatus. Samples were cut under water to avoid air entry. To explore the effect of hydrolyases on xylem conductance, samples were first perfused with control solution (MES buffer at pH 5) with their distal part connected to the XYL’EM. Initial conductance was measured under 6 to 9 kPa using the MES buffer. The stems were then perfused with control solution or one of the four enzymatic solutions described above. After incubation, the new conductance was scored and expressed relative to initial conductance. Conductance variations were determined for six to nine stems per treatment. After analysis, samples were wrapped in moist paper, bagged and stored at 4°C for further tests on VC.

To check the effects of the enzymes on VC, centrifuge-induced embolism was measured at the sample center. After enzymatic treatments, 0.28 m long beech segments were submitted to a xylem pressure of −2.5 MPa using a centrifuge (cavitron). Then, the middle 5 cm long part of the stem was prepared by cutting underwater. Initial conductivity \( (K_i) \) was measured using the XYL’EM apparatus. A water flush of 0.15 MPa was then applied to the segments for 5 min to dissolve air bubbles. Hydraulic conductivity was determined again and the flushes were repeated until the maximum conductivity \( (K_{\text{m}}) \) was reached. The embolism rate \( E \) was calculated as follows:

\[
E = 100 \left( \frac{K_{\text{m}} - K_i}{K_{\text{m}}} \right)
\]

Transmission electronic microscopy

After enzymatic treatments, some branches were directly prepared for TEM analysis whereas others were subjected to a xylem pressure of −4 MPa before being prepared for TEM analysis. Half or quarter sections were taken in the stem center with a clean razor blade. Fragments were cut to a size of about 2 mm3. Fragments were then fixed and included in LR-White resin (Sigma-Aldrich) as described by Jansen et al. (2007). Transverse and 70 nm-thick sections were made with an ultramicrotome (UC6, Leica, France), mounted onto copper grids, stained with uranyl acetate and lead citrate, then observed under a Hitachi H-7650 transmission electron microscope (Hitachi, Elexience, France) and photographed with a CCD AMT HR camera (Hamamatsu, 1024 × 1024 pixels).

Cellulase activity assays

The cellulase activity of the commercial enzymes was tested by measuring reducing sugar release from soluble carboxymethyl cellulose (CMC) (Miller 1959). CMCase activity was determined in a 200 μl assay mixture that contained 1% (w/v) CMC (Sigma-Aldrich) dissolved in 10 mM MES buffer at pH 5. We added 50 μl of the enzyme [Pectolyase 0.1% (w/v) or hemicellulase 3.7% (w/v) or cellulase 0.01% (w/v)] dissolved in 10 mM MES buffer at pH 5 prior to incubation at 37°C for 2 h. Samples were examined for presence of reducing sugars by the dinitrosalicylic acid method.
Controls were run in an identical manner except that the enzyme samples were boiled for 5 min prior to incubation. The amount of reducing sugars was determined spectrophotometrically by measuring the absorbance of the solution at 530 nm and comparing the absorbance values against a glucose standard curve.

**Statistical analyses**

ANOVA was used to investigate the effects of treatments on VC and on hydraulic conductance. If the effects were significant, mean values were compared using Tukey’s Honestly Significant Difference (HSD) test ($P < 0.05$).

**Results**

**Effect of hydrolase treatments on xylem VC**

Beech or poplar segments infiltrated with different solutions showed highly-contrasted cavitation curves. Xylem vulnerability increased dramatically when segments were infiltrated with cellulase or pectolyase solutions whereas segments infiltrated with hemicellulase solution showed no difference compared to controls (Fig. 1). Mean $P_{50}$ ($±s.d.$) was $-2.81$ ($±0.42$) MPa and $-2.48$ ($±0.43$) MPa for beech and poplar control stems, respectively. These values are consistent with previous reports for both species (Herbette and Cochard 2010, Herbette et al. 2010). After hemicellulose treatments, branches had similar $P_{50}$ values to controls, at $-2.73$ ($±0.64$) MPa and $-2.10$ ($±0.30$) MPa for beech and poplar, respectively. After cellulase and pectolyase treatments, $P_{50}$ values were $-0.21$ ($±0.04$) MPa and $-0.32$ ($±0.01$) MPa for beech and $-0.27$ ($±0.02$) MPa and $-0.47$ ($±0.10$) MPa for poplar, respectively. To confirm that the loss of conductivity was related to cavitation and not to obstruction by enzymes or their degradation products, we measured embolism rate in the center of the treated samples. In beech stems subjected to a xylem pressure of $-2.5$ MPa, embolism rate was 89 and 100% in pectolyase- and cellulase-treated samples vs 33 and 38% in control and hemicellulase-treated samples (data not shown). To verify that the effect of pectolyase was not due to a contaminating cellulase activity, we measured the cellulase activity of the three enzyme solutions (Table 1). The cellulase activity levels found in the cellulase and hemicellulase solutions were consistent with the data given by the supplier, but no information was given for the pectolyase solution. The pectolyase solution showed some cellulase activity but at a significantly lower level than the hemicellulase solution. We can thus conclude that the effect of the pectolyase on the VC was not due to a cellulase activity.

**Effect of hydrolase treatments on xylem hydraulic conductance**

Beech branches (28 cm long) were infiltrated with control solution, and their hydraulic conductance $K$ was measured. The branches were then treated with control solution or a solution containing cellulase, hemicellulase or pectolyase, conductance was measured, and the variation was calculated relative to the initial
Fig. 2. Vulnerability curves of beech branches perfused with a protease or DTT. Branches were perfused with a control solution (dark circle) or a solution containing proteinase K (white square) or DTT (white triangle) and incubated for 2 h at 37°C. The vulnerability curves were then established. Data are means (±se) from three samples.

Fig. 3. Effect of hydrolases on xylem conductance. Beech branches were perfused with a control solution, and conductance was scored. The branches were then perfused with a control solution, or with a solution containing cellulase (Cell), hemicellulase (Hemi) or pectolyase (Pect), or a mixture of all three hydrolases (3 Enz), incubated for 2 h at 37°C, and perfused with a solution containing 1 mM CaCl₂ and 10 mM KCl. Then, conductance was scored, and the variation was expressed relative to initial conductance. Data are means (±se) from six to ten samples. Data highlighted with an asterisk are significantly different from controls according to a Tukey’s HSD test (P < 0.05).

conductance (Fig. 3). To test for a synergistic effect between the enzymes, we also infiltrated some branches with a solution containing a mix of the three enzymes. Cellulase treatment induced a significant mean 28% increase in conductance whereas the hemicellulase or pectolyase treatments had no significant effect. The treatment with all three enzymes gave similar results to treatment with cellulase alone.

Effect of hydrolase treatments on PM structure

To gain insights into the effect of hydrolases on xylem hydraulics, we performed TEM analyses of the xylem structure, focusing on PM structure. These analyses were performed both on branches infiltrated with hydrolases and on branches infiltrated with hydrolases and then submitted to full embolism. Xylem transverse sections showed no observable difference in vessel wall structure between the different treatments, suggesting that the treatments were soft enough to avoid damaging the vessel walls. When infiltrated branches were incubated with cellulase, the PM was either fully stripped or strongly destroyed (Fig. 4B and F), with no effect on pit borders. There was no observable effect on PM following hemicellulase or pectolyase treatment (Fig. 4C, D, G, and H), whether before or after cavitation.

Discussion

Against a background of ongoing debate over presence of pectins in PM, this study brings evidence that cellulose and pectins are critical components of VC, and that they likely have distinct roles in the efficiency and safety of xylem hydraulics.

Effects of cellulose or pectin hydrolysis on xylem hydraulics

In a previous study, we infiltrated chemicals through beech branches to investigate the role of calcium in xylem hydraulics (Herbette and Cochard 2010). Here, we used the same methods on the same species to investigate the role of wall components in xylem hydraulics—only lignins could not be investigated as there is no treatment for them. Protein concentration remained unchanged after branch infiltration with solutions containing hydrolytic enzymes (data not shown), indicating that the hydrolases were correctly infiltrated throughout the samples. In support of this, pectolyase or cellulase infiltration strongly increased VC in the sample center (see section Results). We used commercial enzymes that were characterized and already used to investigate physiological functions (Schulte and Gibson 1988, Chappell et al. 1991, Vries and Visser 2001). We used supplier-recommended concentrations for preparing protoplasts, and so they were not limiting. We also checked that the pectolyase effect was not due to a contaminating cellulase activity.

The cellulase treatment induced a sharp increase in VC (Fig. 1). This effect has to be related to the PM breakdown whereas the vessel wall remained intact (Fig. 4). According to the “air seeding” hypothesis, xylem cavitation would occur by air aspiration through pores in the PM. VC would thus be a function of the diameter of the largest pore in the PM. In the cellulase-treated branches, the pore for air seeding is the pit aperture. The median aperture diameter of beech pits was 2.16 μm (data not shown). According to Laplace’s law, the deduced xylem pressure inducing
Fig. 4. TEM images of intervessel pit structure of hydrolase-treated branches. All images are from transverse and ultrathin (70 nm) sections prepared from three branches per treatment. Beech branches were perfused with a control solution (A, E) or with a solution containing cellulase (B, F), pectolyase (C, G) or hemicellulase (D, H) and incubated for 2 h at 37°C. Then, some branches were prepared for TEM analysis (A, D) of their intervessel pits while others were subjected to a xylem pressure of $-4 \text{ MPa}$ before being prepared for TEM analysis (E, H). PMs were absent in most of the pit from cellulase-treated branches (B, F). No differences were observed between pits from control branches (A, E) and pits from pectolyase- and hemicellulase-treated branches (C, D, G, H). Scale bar = 1 μm.

cavitation would thus be $-0.14 \text{ MPa}$, in agreement with $P_{50}$ values for these cellulase-treated beech branches. Cellulase-treatment also induced a 28% increase in xylem conductance in beech branches (Fig. 3) that can be attributed to the removal of PM resistance (Fig. 4). This resistance was previously evaluated on six tracheid-bearing species by dissolving PM with a cellulase (Schulte and Gibson 1988). For these species, PM resistance accounted for 14 up to 84% of xylem resistance. PM resistance was estimated to account for 80 and 87% of xylem hydraulic resistance in *Ulmus americana* and *Fraxinus americana*, respectively (Choat et al. 2006). This high contribution of PM to resistance is due to the lower lumen resistance of the earlywood vessels in these ring-porous species (Choat et al. 2006). Indeed, these species were selected because the large diameters of earlywood vessels (60–120 μm) facilitate their measurements. Values for PM resistance have also been provided by models (Sperry and Hacke 2004) and measurements on progressively shortened stems (Sperry et al. 2005, Wheeler et al. 2005, Hacke et al. 2006). According to these studies, end wall resistivity would average half of the total xylem resistivity, what agrees with the value found for the contribution of the PM alone.

TEM investigations have found cellulose microfibrils in PM in most species (Schmid and Machado 1968, Sperry and Tyree 1988, Sano 2005, Jansen et al. 2009). These studies suggest that cellulose is the PM framework, while our results on two tree species demonstrate that cellulose is critical for PM functions and structure.

The lack of effect on xylem hydraulics with hemicellulase, protease or DTT suggests wall proteins and hemicelluloses do not play important roles in pit hydraulic function. Besides, most hemicelluloses get hydrolyzed in PM as vessels mature (O’Brien 1970) and so were logically absent in mature PM of the few species investigated (Imamura et al. 1974, Dute et al. 2008, Alves et al. 2009). Although the presence of proteins in PM has still not been specifically addressed, a proline-, threonine- and glycine-rich protein has been localized in intervessel


PM in tomato (Harrak et al. 1999), suggesting a specific role in PM. Lack of effect with DTT or protease treatment suggested that this role would not related to hydraulics.

The hydrolysis of pectins induced a sharp increase in xylem VC for both beech and poplar, with no effect on conductance (Figs 1 and 3). This is consistent with two previous studies demonstrating that calcium is important for resistance to cavitation but not for conductance in beech and poplar (Herbette and Cochard 2010, Plavcova and Hacke 2011). We did not find any clear effect of pectin hydrolysis on the pit or PM structure (Fig. 4), and cellulase-treated samples were more vulnerable to cavitation than pectolyase-treated samples in both species. Pectin hydrolysis and cellulose hydrolysis probably have distinct effects. Furthermore, the presence of pectin in PM is questioned. According to literature reviews (Choat et al. 2008, Nardini et al. 2011, van Doorn et al. 2011), there is no clear evidence for the presence of pectins in PM for most angiosperms. Exceptions would be for gymnosperm and a few angiosperm species having pits with torus and pseudo-tori, respectively. However, studies were mostly performed using dyes that lack sensitivity and accuracy, yet were not sufficient to exclude a role of pectins in PM swelling and shrinking properties (van Doorn et al. 2011). Accurate, specific and sensitive immunogold labeling showed that pectins are absent from most of the PM but that clustered in the PM annulus in poplar and other angiosperm species having homogeneous pits (Plavcova et al. 2011, Plavcova and Hacke 2011). This annulus is a conspicuous feature of the PM, and for the investigated species, the pectins were found to be more concentrated in this annulus than in the other part of the cell wall. Taken together, these studies and our findings suggested that pectin concentrated in the PM annulus would be involved in VC, at least for poplar. This restricted localization of pectins fits well with the lack of effect of pectin hydrolysis on hydraulic conductance.

**Hydraulic functions of PM**

Our results point to the conclusion that the safety and efficiency functions of PM, i.e. VC and hydraulic conductance, are uncoupled. The uncoupling of these two PM functions was also evidenced by an effect of sap ionic composition on conductance but not on VC (Cochard et al. 2010). Intriguingly, pectin hydrolysis did not seem to change PM structure or porosity, despite fairly high $P_{50}$ values in beech and poplar. According to Laplace’s law, beech PM would need a pore diameter of $0.91 \mu m$ to allow air seeding under $-0.32$ MPa. Our TEM observations were unable to find any hole in the pectolyase-treated PM. Using TEM, smaller holes with a mean diameter of $0.051 \mu m$ were observed in PM from different conifer species and fitted well with the pressure needed to explain their VC (Jansen et al. 2012). We can thus rule out that the TEM was unsuitable for investigating large pores in PM. When a pit allows water flow from one sap-filled vessel to another, the pressure differential across the PM is very small. In this situation, the PM is in a relaxed state, and cellulose offers the main pit resistance to sap flow as it is the main component. Here, only cellulose hydrolysis led to an increase in conductance, related to a complete breakdown of the PM. When the pit has to block air seeding from an air-filled vessel to a sap-filled vessel, there is a much bigger pressure differential (several MPa) across the PM. The PM thus gets stretched and deformed, and so VC would be dependent on the mechanical resistance of the pit (Choat et al. 2004, Sperry and Hacke 2004, 2008). The resulting VC would be a function of pit geometry and intrinsic PM properties. Pit geometry includes the PM thickness involved in both hydraulic efficiency and safety (Sperry and Hacke 2004, Lens et al. 2011). Pectin properties were critical for the mechanical properties of the cell wall, especially for wall extensibility (Chanelaud et al. 2002, Parre and Geitmann 2005, Pelloux et al. 2007). Pectins could thus contribute to the intrinsic mechanical properties of the PM.

Pectin hydrolysis, like calcium removal, had an effect on VC but not on conductance, whereas sap ionic concentration had an effect on conductance but not on VC (Cochard et al. 2010). The ionic effect on conductance cannot be due to the hydrogel properties of pectins, as was initially proposed by Zwieniecki et al. (2001), at least for beech and poplar and maybe most of angiosperms species. Our results are in agreement with the alternative hypothesis proposed by van Doorn et al. (2011) that the ionic effect can be attributed to any other polyelectrolyte of the PM. Hemicelluloses can be ruled out, since their hydrolysis had no effect on conductance. Two microspectrometry studies showed that there is lignin in PM for various species (Boyce et al. 2004, Schmitz et al. 2008). Hence, cellulose remains the best candidate for regulating the xylem conductance, while a role for lignins has to be addressed.

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