Cavitation and Its Discontents: Opportunities for Resolving Current Controversies[1][C]

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Cavitation has long been recognized as a key constraint on the structure and functional integrity of the xylem. Yet, recent results call into question how well we understand cavitation in plants. Here, we consider embolism formation in angiosperms at two scales. The first focuses on how air-seeding occurs at the level of pit membranes, raising the question of whether capillary failure is an appropriate physical model. The second addresses methodological uncertainties that affect our ability to infer the formation of embolism and its reversal in plant stems. Overall, our goal is to open up fresh perspectives on the structure-function relationships of xylem.

A central question in the biology of vascular plants is under what conditions the continuity of the liquid phase, essential for the transport of water from soil to leaves, is lost (Tyree and Zimmerman, 2002). Without the high-conductance pathway for water movement through the xylem, vascular plants could not sustain the water loss associated with the diffusional uptake of CO_2 from a subsaturated atmosphere. As a result, substantial effort in the field of xylem transport focuses on quantifying vulnerability to cavitation and its impact. Yet, a number of recent studies raise questions regarding how well we understand cavitation in plant stems, pointing to apparently anomalous or inconsistent experimental results that suggest methodological artifacts (Choat et al., 2010; Ennajeh et al., 2011; Wheeler et al., 2013). Such results provide the motivation for this Update. We recognize that there is currently no consensus on the extent to which any particular experimental approach is subject to a problem; in addition, we note that the implications of such potential artifacts for the estimation of leaf xylem vulnerability involves further methodological considerations beyond our current scope. Our intention with this Update is to clarify the physical basis for a number of potential experimental artifacts relating to angiosperm stem xylem, in the hope that this will be useful for designing experiments that can resolve these issues.

In this spirit, we begin with a discussion of how cavitation occurs, sketch an alternative model to meniscal failure for how air seeding across homogenous pit membranes could occur in the absence of discrete pores, and discuss the implications of these two models for the relative importance of probabilistic versus deterministic constraints on air-seeding resistance. We then turn to recent evidence that xylem vulnerability to cavitation in some species may have been overestimated and consider possible physical effects that could lead to biases sensitive to conduit size in the three principal methods of vulnerability estimation (dehydration, air injection, and centrifugation); to the extent that such biases are quantitatively important, these methods cannot be considered independent for long-vesseled species. Experimental approaches that may prove helpful in reconciling some of the divergent results between methods or protocols are proposed. The potential for measurement artifacts to impact our understanding of embolism repair is also discussed.

THE MODE OF CAVITATION IN PLANT XYLEM

The metastability of liquid water in plant xylem is maintained by the activation barrier of the nucleation process that leads to a stable vapor state: although thermodynamically unstable, a water column under tension is kinetically stable, remaining intact until the activation barrier is overcome (Scholander et al., 1961; Caupin et al., 2012). Theoretical limits for the existence of negative pressure in water are below −120 MPa at room temperature, at which point cavitation occurs by homogenous nucleation. Negative pressures approaching this magnitude have been observed for water-filled inclusions in quartz crystals, while other experimental approaches (centrifuge, acoustic, and inclusions in gels) demonstrate minimum pressures of −20 to −30 MPa in the 20°C to 30°C range. These less negative cavitation thresholds likely represent heterogenous nucleation events arising on the walls of whatever encloses the liquid, or on impurities in the liquid (Caupin et al., 2012), rather than the inherent homogenous limit for water.

In plant xylem, cavitation occurs at tensions that can be reconciled with heterogenous nucleation on imperfectly wetted conduit surfaces (e.g. hydrophobic surfaces with a contact angle greater than 90°), air seeding by vapor nuclei stabilized in hydrophobic cracks, as well as the introduction of an air bubble through a pit membrane (hereafter "air seeding"; Pickard, 1981; Wheeler and Stroock, 2009). However, preexisting vapor nuclei would not be expected to exist in current-year xylem not previously stressed, and nucleation on imperfectly wetted surfaces requires very large contact angles difficult to reconcile with observed values (Zwieniecki and Holbrook, 2000). Hydrophobic patches as large as the critical radius for bubble expansion to occur could exist, yet these would have to be small enough to not affect macroscopic observations of xylem contact angles (Wheeler and Stroock, 2009). Early studies comparing levels of embolism induced by bench drying with those produced by air injection supported the original air-seeding hypothesis (Zimmerman, 1983; Cochard et al., 1992), and subsequently, most investigations of the cavitation limits of plant xylem have focused on air seeding and the properties of pit membranes.

Because pits develop between cells (i.e. not to air-space voids), a prerequisite for air seeding are processes that bring pit membranes into contact with a gas phase. Air entry into xylem lumens can occur through damage to leaves or stems, and at least in natural populations, such forms of damage are ubiquitous. Freezing can lead to the formation of gas bubbles as air is excluded during ice formation (Davis et al., 1999; Sevanto et al., 2012), providing a mechanism by which xylem conduits become air filled during winter. Air entry into the xylem may also simply arise as an inevitable consequence of growth: the destruction by stretching of protoxylem forms air-filled lacunae in mature tissues. Shared pits between metaxylem and protoxylem conduits may then represent the Achilles heel of the vascular system, necessary to maintain a continuous source of water to expanding tissues yet providing the point of entry of gas into the metaxylem of mature tissues. An important aspect of cavitation by air seeding, then, is that at any particular xylem tension, whether a vessel becomes cavitated depends on more than its inherent vulnerability (which is all that matters for heterogenous nucleation); an adjacent conduit must have previously cavitated. Thus, the spread of embolism by air seeding in stems may be strongly influenced by network effects and patterns of vessel-to-vessel connectivity (Loepfe et al., 2007; Cai et al., 2010; Brodersen et al., 2013a; Cochard et al., 2013).

Advances in imaging have recently provided information on the spatial propagation of embolism in vivo that appears to support the protoxylem as Achilles heel hypothesis for current-year shoots. Brodersen at al. (2013b) used high-resolution x-ray computed tomography (HRCT) to repeatedly image 5-mm-long volumes of young grape (Vitis vinifera) stem during an imposed drought, observing a pattern of propagation of embolism along radial files of xylem that extended outward from a ring of initially embolized conduits surrounding the pith. In contrast,

lower resolution NMR scans of older stems show no spatial pattern of embolism formation (Holbrook et al., 2001). As both technologies allow only a narrow slice of total vessel length to be imaged, whether differences in axial propagation contributed to the different patterns cannot be evaluated; in addition, the removal of leaves for water potential measurements between some of the HRCT scans could have influenced the observed pattern of empty vessels. Alternatively, in older stems, the xylem walls may harbor stable vapor nuclei in hydrophobic cracks as a result of winter embolism and refilling via root pressure, and spatially random heterogenous nucleation (at lower tensions than air seeding) might then become the dominant mode of cavitation. Yet, while older vessels have been found to be more vulnerable relative to younger vessels in Acer species (Choat et al., 2005), this appears to be due to changing pit properties (Sperry et al., 1991; Hacke et al., 2001) rather than to heterogenous nucleation. Recent work correlating acoustic emission and the appearance of cavitation events in conifer wood embedded in hydrogel supports the idea that heterogeneous nucleation is rare relative to the propagation of embolism by air seeding (Ponomarenko et al., 2013). Thus, there is currently little reason to question the current consensus that the dominant cause of fracture in xylem water columns, whether newly grown or previously embolized and refilled, is the failure of a pit membrane to exclude air.

AIR SEEDING THROUGH HOMOGENOUS PIT MEMBRANES: IS CAPILLARY FAILURE THE RIGHT MODEL?

For the homogenous pit membranes of angiosperms, the ability to exclude a gas phase despite lower pressures in the liquid phase has been interpreted as a capillary phenomenon, with air seeding the result of meniscal failure within a membrane pore (Zimmerman, 1983; Sperry and Tyree, 1988). In this view, the wettability of the membrane, maximum pore size, and surface tension of water together determine the maximum pressure difference between the gas and liquid phases that can be supported (Sperry and Tyree, 1988; Stroock et al., 2014). The relevant pores are generally conceptualized as determined by the cellulose microfibril network, with the matrix of polysaccharides playing only an indirect role in air-seeding resistance (Sperry and Tyree, 1988), as discussed further below. Nevertheless, recent work on hydrated pit membranes suggests that the cellulose network is completely embedded within a dense hydrogel phase (Pesacreta et al., 2005; Lee et al., 2012), raising the question of whether capillary sealing is the appropriate model if the spaces between cellulose microfibrils are filled with a gel phase rather than a liquid phase.

As pit membranes are derived from primary cell walls in which the cellulose network and cross-linking glycans are embedded in a more soluble matrix of pectic polysaccharides (Willats et al., 2001), we can assume that pit membranes initially have a similar construction. However, the matrix may be modified during apoptosis and vessel

maturation, as noted by Plavcová and Hacke (2011). The exact chemical composition of the pit membrane matrix remains controversial, particularly with regard to the presence or absence of the homogalacturonan (HG) pectin species (Choat et al., 2003; Pesacreta et al., 2005). Yet, the presence or absence of HG should not be confused with the presence or absence of a pectic gel phase in the matrix. For example, another species of pectin, rhamnogalacturonan II, is present in primary cell walls, is not degraded by known plant pectinases, can form calcium cross links, and, with respect to detection by immunolabeling, binds different antibodies than HG (Willats et al., 2001).

Evidence that calcium removal from pit membranes can lower air-seeding thresholds was observed in some of the earliest work on vulnerability to cavitation and has recently found renewed interest; the effect is attributed to a disruption of calcium cross links in a pectin-based gel phase stabilizing the relative positions of cellulose microfibrils and, thereby, the pore sizes of the cellulose network that are thought to form the capillary seal (Sperry and Tyree, 1988; Herbette and Cochard, 2010). Indeed, treatment of stems with pectolyase has been shown to shift the pressure at which 50% of stem conductance has been lost (P_{50}) from –2.8 to –0.3 MPa and from –2.5 to –0.5 MPa in *Fagus* and *Populus* species, respectively (Dusotoit-Coucaud et al., 2014). Such a stabilizing role for the matrix in pit membranes is consistent with current views on primary cell wall mechanics, in which cellulose is thought to be the main load-bearing component even as the extensibility of the cellulose network is strongly influenced by the gel matrix (Willats et al., 2001; Chanliaud et al., 2002). However, pore diameters, as estimated from scanning electron microscopy (SEM) imaging of the cellulose network of pit membranes, are often far smaller than the diameter predicted for air-seeding pores based on the Young-Laplace (capillary) equation and whole-stem vulnerability estimates (Choat et al., 2003; Jansen et al., 2009; Lens et al., 2011). Choat et al. (2003) suggested that the discrepancy might be due to deformation of the membrane as the pressure difference supported by the membrane increases, a rare large pore occurring either naturally or due to damage, or heterogenous nucleation.

This idea of a rare pore has been expanded into the "pit-area" hypothesis, according to which the distribution of pore sizes produced by vascular development has a long tail of large diameters such that the vulnerability of a particular vessel in a particular species is most strongly influenced by its total pit area (Wheeler et al., 2005; Hacke et al., 2006). More recently, however, a study of hybrid poplar (*Populus trichocarpa* × *deltoides*) found pore sizes estimated from SEM to be dramatically larger than expected based on a rare-pit model and whole-stem vulnerability (Plavcová et al., 2011). The observed pore sizes could only be reconciled with a rare-pit model of capillary air seeding if the average of the top 7.5% of observed pore diameters in SEM was taken as the maximum pore diameter in vivo. The authors suggest that the gelatinous matrix, shrunken or absent in SEM, may in its fully hydrated state reduce the effective pore diameter to that expected by the rare-pit model.

Here, then, a difficulty arises in determining the pore diameters relevant to air seeding: differences in preparation methods for SEM result in pit membranes that range in appearance from porous microfibril meshes to smooth homogenous surfaces (Jansen et al., 2008; Plavcová et al., 2011). Sano (2005) found localized extraporous regions with one or more large pores (up to 300 nm) in 5% to 20% of pit membranes in species of *Salix*, *Acer*, and *Betula*, with a subset of membranes showing very large pores (300–700 nm). Yet, these large pores are still too common to be considered "rare" pits that might contribute to between-vessel variation in air-seeding pressure. Also in that study, *Fraxinus mandshurica* had no discernible pores extending through the thickness of the membrane, consistent with a report for *Fraxinus americana* that found resolvable pores (up to a maximum of 30 nm) in only a few membranes. Thicker pit membranes appear more likely to have a homogenous surface in SEM (Jansen et al., 2009), and atomic force microscopy (AFM) has shown a change from an apparently smooth to rough surface between hydrated and air-dried membranes (Pesacreta et al., 2005).

The two above-hypothesized roles for how the matrix may influence the air-seeding properties of the cellulose mesh, stabilizing pore size and reducing the effective pore size, are consistent with the physical model of membrane structure proposed to explain ion-mediated effects on pit membrane hydraulic conductance (Zwieniecki et al., 2001). In this model, Poiseuille flow occurs through pores whose diameter varies with the swelling of a hydrogel "coating" on the cellulose microfibrils (Fig. 1A). An alternative hypothesis that explains ion-mediated flow changes as the result of electroviscous effects adopts a similar view of the pore structure (van Doorn et al., 2011; Santiago et al., 2013). Yet, recent work using AFM on hydrated pit membranes of tobacco (*Nicotiana tabacum*; Lee et al., 2012) found no evidence of discrete pores through the membrane (Fig. 1B), consistent with Pesacreta et al. (2005). In the study by Lee et al. (2012), pit membranes were imaged first in deionized water, followed by reimaging in a 50 mM KCl solution. In deionized water, the membrane surface was relatively smooth and soft; in the KCl solution, the height of the surface dropped and became harder, with the rougher topography attributed to the underlying microfibril network. In this view, KCl-mediated increases in membrane hydraulic conductance could result from a reduction in membrane thickness (Lee et al., 2012), although electroviscous effects could also occur (A.D. Stroock, personal communication). These observations beg the question: if the gel phase is continuous, how does air seeding occur?

TOWARD A MODEL OF AIR SEEDING ACROSS MEMBRANES EMBEDDED IN HYDROGEL

In contrast to a porous solid, the polymer network in a gel is thermally active. As explained by Wheeler and
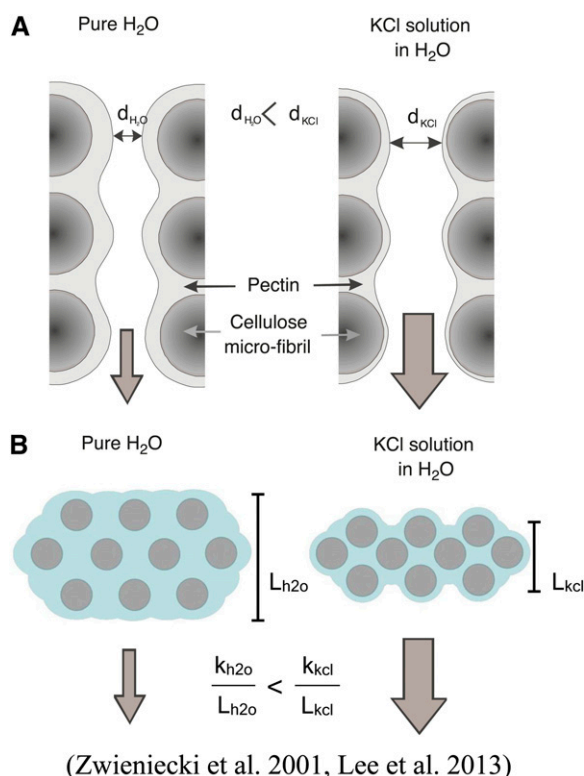
Figure 1. Two models for flow-through pit membranes. A, Discrete pores filled with a discrete phase of liquid water and solutes provide a path for bulk Poiseuille-type flow, with an effective radius d. In this conception, water in the gel phase (Pectin) is stationary: Shrinking and swelling of the hydrogel in response to ionic concentrations in the liquid phase control the effective pore diameter. B, The gel phase occupies the entire noncellulosic matrix of the pit membrane through which water permeates. In this conception, explaining the response of membrane hydraulic conductance to ions requires that the effect of shortening the length of permeation dominates the effect of increasing polymer density on hydraulic conductivity, or $k_{h2o}/L_{h2o} < k_{kcl}/L_{kcl}$. [See online article for color version of this figure.]

Stroock (2009), molecular-scale mixing of water and polymer leads to thermodynamic stabilization of the solvent, such that the chemical potential of water in a gel has osmosis-like contributions from interacting with the polymer (Wheeler and Stroock 2009). Indeed, the Flory-Rehner model cited by Wheeler and Stroock (2009) for the chemical potential of water inhabiting a gel can be written in a form analogous to plant cell water relations as follows:

$$\mu_{w,gel} = \mu_o(T) + \underbrace{\overline{V}_{w,liq}G\left(\phi_p^{1/3} - \phi_p/2\right)}_{P\overline{V}_{w,liq}}$$

$$- \underbrace{RT\left[-\ln\left(1-\phi_p\right) - \phi_p - \chi\phi_p^2\right]}_{-RTc\overline{V}_{w,liq}}$$

where $\overline{V}_{w,liq}$ is the molar volume of water, G is the poroelastic shear modulus of the gel, ϕ_p is the polymer

volume fraction in the gel, χ is the Flory interaction parameter that describes the energy of interaction between the polymer and water, R is the ideal gas constant, and T is the temperature (in K); with respect to the analogous plant water relations terms, P is the turgor pressure and c is the concentration of solutes, and the relationship between water potential and chemical potential is given by $\mu_w = \psi_w\overline{V}_{w,liq}$ (Slatyer and Taylor, 1960). In a freely swollen gel at equilibrium, the contractile stress of the network is experienced as a compressive stress in the solvent (Hong et al., 2008), such that the "pore pressure" in a hydrogel is more positive than it would appear from the perspective of a rigid porous solid. The thermodynamic state of water in a hydrogel can then be analogized to that of water in a plant cell (Rockwell et al., 2014), in which swelling due to osmotic effects and the restoring force of the wall leads to a pressure difference between the external and internal solvents (i.e. turgor). While the concept of a pore pressure in gels remains controversial (e.g. not physically well defined in all instances; Hong et al., 2010), the important point is that the water inhabiting the gel phase is in a thermodynamically (not just kinetically) stable state. For example, in the hydrogel experiments of Wheeler and Stroock (2009), the pore pressure of the gel phase (equivalent to P in the equation) remained at approximately +0.6 MPa even as the pressure in the pure fluid inclusions fell to −20 MPa.

One approach to modeling air seeding in such a material is to apply the capillary failure model to the polymer network itself. In this mode of failure, phase separation of the gel into discrete domains of water and polymer must occur for the tension in the bulk to be transmitted to the polymer pore space, followed by meniscal failure (Wheeler and Stroock, 2009). However, the pore size of the membrane matrix appears to be too small to explain air seeding in plant xylem. For example, based on macromolecular exclusion, the effective pore size of the pectin network for the primary cell wall of soybean (*Glycine max*) root cells grown in culture was found to be 6.6 to 8.8 nm (Baron-Epel et al., 1988), about 1 order of magnitude too small to explain air seeding as a result of meniscal failure. Nor is it clear what would cause the initial phase separation to occur. In the manufactured hydrogel of Wheeler and Stroock (2009), cavitation events in water-filled inclusions showed a highly uniform distribution of tensions (clustered around 22 MPa). This is not consistent with a failure mode dependent on the largest pore of the polymer network, making it more likely that the gas phase was seeded in this system by heterogenous nucleation (on hydrophobic patches of the gel) or impurities in the liquid. Thus, application of the capillary model at the scale of the porosity of the polymer network may not be physically realistic.

Alternatively, we can take a continuum view of the gel and its mechanical properties. In this view, the gel phase in the matrix stabilizes the water while transferring the load imposed by the pressure difference between the liquid and gas phases to the cellulose network. The important quantity for air seeding may then be

not the polymer "pore" sizes but the macroscopic mechanical properties of the gel, specifically its ability to resist crack formation and propagation as the membrane is strained due to flexing of the cellulose microfibril network as the pressure difference increases. While many hydrogels are physically weak, a study of the material properties of pectin networks at concentrations typically found in plants (30%, w/w) found that the tensile modulus based on uniaxial extension ranged from 15 to 166 MPa depending on the ionic strength of the bathing solution, on the order of cell wall moduli (Zsivanovits et al., 2004).

In a composite pit membrane, crack formation likely involves the bonding interactions of all of the components. Hydrogels composed of interpenetrating ionically cross-linked and covalently cross-linked networks can be much more resistant than either network would be alone: the covalent network can bridge cracks, stabilizing them before they reach their critical length for spontaneous growth, while the ionic cross links can "unzip" over a broad area as the strain increases, dissipating stress (Sun et al., 2012). Cracks formed by breaking ionic cross links in such gels also heal when the stress is relaxed, with a time scale on the order of days, similar to that seen for recovery from cavitation fatigue (Stiller and Sperry, 2002). Interestingly, in the manufacture of pressure vessels, the walls are designed to leak by being made thinner than the critical length leading to spontaneous crack growth and (explosive) fracture (Hertzbert, 1996). In plant pit membranes, such "designed leaks" would offer an obvious selective advantage if they allow breaches of the membrane to be reversible by a "healing" process of ionic repair.

Finally, from an engineering perspective, the use of porous solids to generate negative pressures by capillarity has been marked by failure due to the difficulty in creating materials with a sharp cutoff in the distribution of pore sizes, as required to avoid a single large pore from allowing air entry. However, coating porous ceramics with a cross-linked hydrogel has been shown to stabilize the behavior of the system and increase the maximum tensions supported (Thut, 1928; Wheeler and Stroock, 2009). Given the robustness of a gel-based system for balancing negative pressures versus capillary seals subject to rare defects that may be as difficult for biological developmental processes to control as they are for manufactured systems, it would be surprising if evolution did not favor the usage of gels for sealing pit membranes against air as well.

PROBABALISTIC VERSUS DETERMINISTIC CONSTRAINTS ON VESSEL SIZE

The two models of pit membrane sealing discussed here, capillary versus polymer gel, lead to broadly different expectations for the constraints plants face in building resistant xylem. Sealing by capillarity may be expected to be sensitive to developmental defects in pit membranes and, therefore, associated with rare, highly vulnerable pits that lead to a broad distribution of vulnerabilities at the level of individual vessels. In this scenario, there is a cost imposed by such rare defects proportional to the number of vessels that are nonfunctional at normal midday operating xylem tensions. In addition, a constraint on large vessel diameter emerges, as the likelihood of a defect rendering the vessel physiologically nonfunctional is expected to increase with pit membrane area (expected to scale with diameter for the sake of hydraulic efficiency; Wheeler et al., 2005). In the extreme case, such behavior would be consistent at the level of stems with "r-shaped" vulnerability curves (Sperry et al., 2012), which show large losses of hydraulic conductivity at moderate levels of water stress (i.e. an exponential rise of percent loss of conductivity [PLC] with increasing xylem tension; Cochard et al., 2013). In the case of sealing with a gel rather than microporous interface, one might expect a narrower distribution of pit and, therefore, vessel vulnerabilities and more "s-shaped" stem PLC curves characteristic of high cavitation resistance at moderate water potentials (i.e. a sigmoidal rise in PLC with increasing xylem tension; Cochard et al., 2013). In this case, constraints on vessel size could emerge from deterministic tradeoffs between resistance to air seeding and hydraulic conductance at the individual pit level, as discussed further below, combined with resistance matching between end walls and lumens (Sperry et al., 2006).

While we currently lack experiments to test the air-seeding threshold or mechanical properties of individual pit membranes, axial air injection through glass microcapillaries has been used to estimate vulnerability at the scale of individual vessels. For *Quercus gambelli*, 40% of vessel files injected air seeded below 1 MPa, in general consistent with an r-shaped vulnerability curve for this species (Christman et al., 2012; Sperry et al., 2012), and thus an important role for rare defects consistent with capillary failure. On the other hand, a study of seven *Acer* species (typically with more s-shaped vulnerability curves) found that deterministic properties such as membrane thickness, pit aperture fraction (aperture area over membrane area), thickness-to-span ratio (intervessel wall thickness over vessel diameter), and wood density contributed more to a safety versus efficiency tradeoff for vessel size than pit area or pit number per vessel (Lens et al., 2011). These differences between two co-occurring *Quercus* and *Acer* species may mean that the nature of the safety-efficiency tradeoff differs between these taxa, with *Q. gambelli* overbuilt to accommodate a high rate of functionally defective vessels.

Standing in the way of progress on such questions are an increasing number of reports that the various methods for assaying xylem vulnerability to cavitation are subject to artifacts that may interact with wood structure and, potentially, vessel size. Clarifying these methodological issues so that we can be sure our experiments give us an unbiased measure of vulnerability then assumes a high priority.

ARE CURRENT METHODS TRULY INDEPENDENT OR SUBJECT TO A SHARED BIAS?

Over the past several years, the reliability of each of the three principal methods for assaying vulnerability to cavitation (dehydration or bench drying, air injection, and centrifugation) has been challenged. The biological question immediately at stake is whether an r-shaped vulnerability curve is indicative of artifacts, and most if not all xylem is strongly resistant to cavitation over the range of its normal operating potentials, with an s-shaped vulnerability curve (Sperry et al., 2012; Cochard et al., 2013). Because each of the three methods imposes stress through different physical mechanisms, the assumption has been that the sources of errors in the methods are independent; therefore, agreement between two or more methods offers assurance that the consensus vulnerability curve is free of artifacts. Most authors have assigned extra weight to curves derived from dehydration, as in that method the stress is applied uniformly along the stem, coming closest to the state of stress in intact plants (Choat et al., 2010; Ennajeh et al., 2011; Sperry et al., 2012). Thus, other methods are validated by comparison with dehydration. However, Wheeler at al. (2013) demonstrated that cutting stems under water while the xylem is under large tensions can inflate PLC in the measured segment. More work remains to be done to determine if such artifacts contribute to r-shaped vulnerability curves; the tests are nontrivial, as they require removing a maximum vessel length of material after the initial cut (under tension) in water in order to generate a sample for measurement with no conduits open to the initial cut. Nevertheless, dehydration methods can no longer be assumed to provide an unbiased reference curve.

Cutting artifacts have a strong potential to interact with vessel size. The hypothesis put forward by Wheeler et al. (2013) is that the act of cutting seeds microbubbles/gas nuclei into the cut end and that the rapid acceleration of the fluid into the cut end expands and translates these bubbles into open vessels forming stable emboli (Fig. 2). In species with short vessels or tracheids, such embolism would be confined by end walls close enough to the cut end that they would generally be trimmed off the sample prior to measuring flow. Yet for species with long vessels, conduits opened by the initial harvest cut (under water) may extend into the sample used for flow measurements. In addition, it seems reasonable to expect that conduits with larger diameters are more likely to become seeded with a cutting artifact. If plant physiologists are so unfortunate that dehydration, air injection, and centrifugal techniques are all biased by artifacts sensitive to vessel size, then these methods are no longer strictly independent and agreement between methods offers a false sense of confidence.

AIR INJECTION AND THE POTENTIAL FOR OVERSATURATION ARTIFACTS

Ennajeh et al. (2011) investigated the effects of chamber and sample length on vulnerability curves produced using
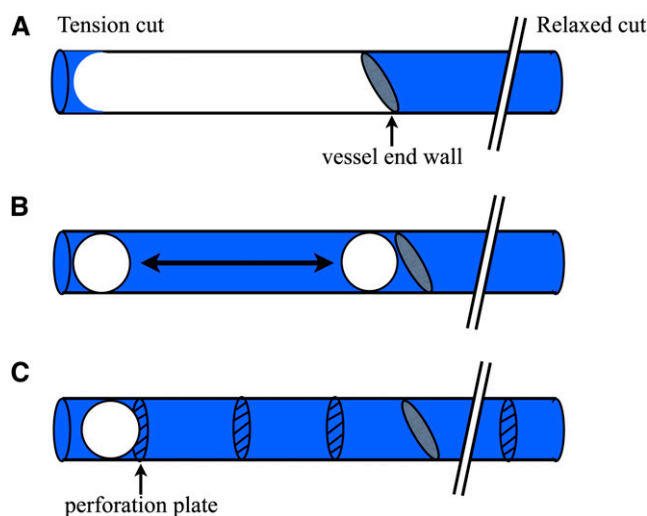
Figure 2. Hypotheses for the pattern of embolism formation due to a cutting artifact: Cutting a functional xylem conduit under tension may result in embolism even when the parent stem is severed under water. The suggestion is that the initially rapid acceleration of water into the severed conduits can expand emboli seeded from an imperfectly wetted cutting surface, nuclei in the reservoir water, or air spaces in the stem. A, The embolism may expand such that the vessel drains to its end wall, with a meniscus at the cut end as the reservoir water invades the conduit by capillarity. B, Alternatively, a bubble forms but either remains confined near the cut end or translates with the volume flow, as the downstream cells hydrate, until impeded by an end wall. C, Perforation plates present between vessel elements in some xylem may trap emboli at the cut end, where they would typically be trimmed off and not affect measurement. [See online article for color version of this figure.]

air injection, finding that the combination of sample lengths at least as long as maximum vessel length and short pressure chamber lengths gave more resistant curves than when samples approached the size of the chambers or were shorter than maximum vessel length. Their empirical rule specified that as the quantity (sample length − chamber length)/maximum vessel length approached 1, the air-injection curve could be expected to converge with a dehydration curve. Although the authors did not propose a mechanism, one hypothesis that might explain how chamber, sample, and vessel lengths could interact to make stems appear more vulnerable is that flow during conductivity measurements or flushing introduces bubble nuclei into upstream open vessels, such that if these vessels extend into the region of gas pressurization created by the chamber, emboli can form by diffusion of gas out of the supersaturated sap rather than by air seeding across a pit membrane (Fig. 3). If this hypothesis is correct, air injection of stems containing native sap may prove more resistant than stems perfused (e.g. during flow measurement) prior to pressurization.

It is worth noting too that single-vessel air injection is also potentially susceptible to oversaturation artifacts. The relevant length for the diffusion of dissolved gases to where they can come out of solution and form bubbles is not the sample length but the distance separating
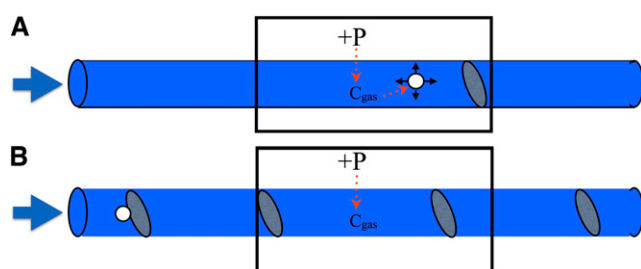
Figure 3. Hypothesis for the interactions of sample length, vessel length, and chamber length in the determination of PLC by air injection. A, Flushing or measurement flows sweep bubble nuclei through open vessels into the area subsequently injected radially at pressure *P*. As the dissolved gas will come into chemical equilibrium with the pressurized gas phase (although the pressure of the liquid phase remains nearly atmospheric), the water in the xylem will be oversaturated relative to atmospheric pressure and the nuclei will grow by diffusion as the excess gas comes out of solution. B, End walls filter the water such that no nuclei reach a region of sufficient oversaturation to form emboli. C_{gas}, Concentration of dissolved gas. [See online article for color version of this figure.]

the injected conduit from a conduit filled with water unfiltered by pit membranes, which may be as small as the thickness of a single pit membrane. A conduit in which oversaturated fluid comes into contact with nuclei for a gas phase will spontaneously produce a stream of bubbles. Using longer samples that contain multiple end walls, such that the injected conduit is not in contact with any open vessels, does seem to produce a tighter distribution of less vulnerable end walls than short segments chosen to try to measure a single end wall (Choat et al., 2005; Jansen et al., 2009). Yet, this may simply be due to the bias of sampling multiple end walls in series to make a single measurement of vulnerability. Which bias leads to greater error, measuring multiple end walls in series or diffusion to nuclei in open vessels, remains to be determined.

Multiple end-wall bias could also form the basis for an alternative explanation of the chamber and sample length effects observed by Ennajeh et al. (2011). For short samples close to the chamber length, the assumption is that all vessels are tested at the imposed pressure and that all vessels have either radial vessel-to-vessel pitting or a region of end wall within the sample. For long samples of the same taxa and in the same length chamber, the injected air must cross more end walls axially to create the same degree of PLC as in a short sample; thus, it has more chances to encounter a sufficiently resistant end wall. Therefore, long samples would be artifactually resistant relative to a dehydration curve, in which the stress is applied radially along the whole length, and of course may also propagate axially as well.

The near convergence reported by Ennajeh et al. (2011) between the dehydration curve and the short chamber (3.5 cm) plus long sample (37.5 cm) air-injection curve for peach (*Prunus persica*; maximum vessel length of 42 cm), as opposed to the r-shaped curves from chambers approaching the sample length, does not support an

important end-wall bias effect. Nevertheless, the hypothesis suggests an experiment that may be useful in the absence of a reference curve. For taxa that show an r-shaped air-injection vulnerability curve for a given chamber and sample size but a more s-shaped curve when sample length is increased (i.e. the criteria of Ennajeh et al. [2011] equal about 1), it could be informative to excise samples of the short length (i.e. at the chamber ends) and measure their PLC. The rationale is that, in the absence of open vessel/oversaturation artifacts, these excised segments should be identical to the short samples and follow an r-shaped curve, indicating that a multiple end-wall bias affects the long samples. Conversely, if the excised segments were more similar to the curve of the parent (long) sample, this would be consistent with open vessels biasing the short samples toward hypervulnerability.

OPEN-VESSEL ARTIFACTS IN THE IN SITU FLOW CENTRIFUGE METHOD (CAVITRON): AN INDICATION OF A MORE GENERAL PROBLEM?

With respect to the centrifuge, a consensus has emerged that at least one variation of the method (Cavitron) suffers from a vessel length artifact (Cochard et al., 2010; Sperry et al., 2012). In Cavitron experiments, flow is measured in the centrifuge, whereas in the traditional centrifuge method, flow measurements are made outside the centrifuge once all tensions induced by spinning have collapsed. This difference motivated the hypothesis that, in the Cavitron, if open vessels extend from the upstream sample end close to the center of rotation, embolism nuclei (e.g. microbubbles or imperfectly wetted impurities) could be convected into a region of low pressure during spinning and form artifactual emboli at relatively low tensions (Cochard et al., 2005). Subsequent subsampling of spun stems showed increased embolism at the upstream end of stems in the Cavitron, supporting this view (Cochard et al., 2010).

There is no consensus, however, regarding whether centrifuge methods in which flow measurements are made outside the centrifuge also suffer from open-vessel artifacts (Jacobsen and Pratt, 2012; Sperry et al., 2012). A similar subsampling investigation of material spun in the absence of a flow imposed across the stem did not find a pattern of higher embolism at the ends than in the center, supporting the contention that this "traditional" method is free of open-vessel artifacts (Sperry et al., 2012). Yet, these stems (*Sorbus scopulina*) were also spun to relatively large tensions, at which point real cavitation may have masked any open-vessel artifacts. An intensive study on current-year shoots of olive (*Olea europaea*) found similar r-shaped curves for "short" stems (15 cm, with maximum vessel length of 82 cm) between centrifuge curves generated by measuring flow during or after spinning, with P_{50} of approximately -1 MPa, versus -5 MPa for benchtop dehydration using both hydraulic and imaging (HRCT) methods (Torres-Ruiz et al., 2014). Interestingly, in this study, longer samples (28 cm) with flow measurement after

spinning did produce a more resistant r-shaped curve than samples measured while spinning, although both were still more vulnerable relative to the benchtop data, with P_{50} on the order of -3 versus -1.5 MPa, respectively.

It seems unlikely that centrifuge reservoirs and flow measurement perfusing or flushing solutions are entirely free of bubble nuclei when artificial conduits are mechanically coupled to plant xylem; therefore, the liquid in any open vessel (unfiltered by a pit membrane) must be regarded as potentially contaminated. The larger the open volume of a vessel, the more likely it will be to contain such nuclei and the more likely the open volume will extend into a region of low pressure once spinning commences. Any microbubbles in an open vessel would be expected to move by buoyancy toward the axis of rotation of a spinning stem, until either the pressure decreases below their critical pressure, causing them to expand, or progress is blocked by an end wall (Fig. 4). In the case of unflushed and unmeasured material, for which open-vessel contents should still be clean native sap, nuclei from the reservoir could still potentially invade the xylem driven by buoyancy once centrifugal forces are induced by spinning. For example, in the case of grape (cv Chardonnay), centrifugation of stems with native sap produced more resistant curves than stems flushed prior to spinning in the traditional method (Choat et al., 2010), supporting the idea of a vessel length, bubble nuclei, and centrifugation interaction. The native sap grape curves showed higher PLC at moderate tensions than either dehydration or air injection on "long" samples (1 m versus an estimated maximum vessel length of 0.9 m), resulting in a curve with a long plateau. If the true vulnerability curve for grape is indeed s shaped, as is supported now by comparison of HRCT scanning of intact stems (versus centrifuged stem samples; McElrone et al., 2012; Brodersen et al., 2013b), then the plateau in PLC exhibited by stems spun with native sap might result from gas nuclei seeded by the reservoir. Interestingly, McElrone et al. (2012) also showed evidence that the distribution of cavitation induced by spinning grape stems to -0.5 MPa (29% of vessels cavitated) was skewed to large-diameter vessels compared with the in vivo cavitation produced by drying to between -1 and -1.5 MPa (8% of vessels cavitated), as might be expected if the probability of a vessel cavitating in the centrifuge is affected by the size of its opening to the reservoir.

To some extent, artifacts of the type described above must occur whenever the cavitation resistance of conduits filled with water unfiltered by pit membranes is tested, simply based on physical principles; what is properly contentious is the magnitude of the problems such artifacts create in any given instance. Complicating evaluation of the magnitude of the problems is the potential for artifacts to be confounded with other sources of error. For example, in the centrifuge, the tension reported as inducing a particular level of PLC as measured by flow over the whole stem is only achieved at the center of rotation and falls off toward the ends. Only the air-seeding
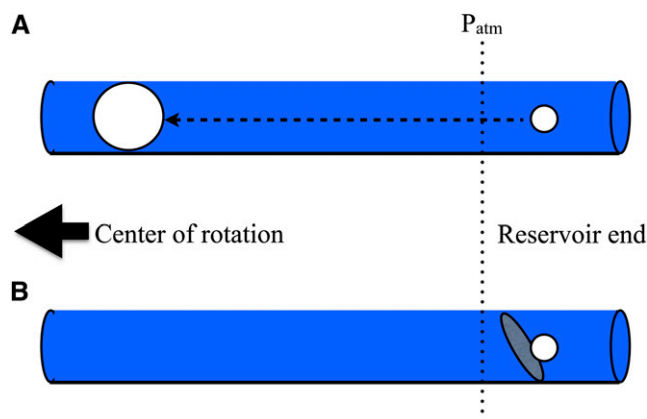

Figure 4. Hypothesis for a long-vessel artifact in centrifuge-induced PLC. In a spinning stem (A), bubble nuclei (microbubbles, dust motes with hydrophobic domains) less dense than the surrounding fluid may move toward the region of lowest pressure at the center of rotation by buoyancy, unless impeded by a vessel-end wall (B). Nuclei may also be introduced from the reservoir or seeded into open vessels by prior flushing or measurement flows. P_{atm}, Atmospheric pressure. [See online article for color version of this figure.]

threshold of pits falling within a restricted region near the center of rotation is actually tested at the reported tension. The operating assumption in relating peak tensions at the sample center to whole-sample PLC has been that if most vessels in a sample have some portion in the zone of maximum tension, the PLC of the whole sample and its central part will converge (Cochard et al., 2005; Cai and Tyree, 2010). Yet, where subsampling of stems has been done to check for the distribution of PLC in spun stems (*S. scopulina* [Sperry et al., 2012], peach [Cochard et al., 2010], and *Populus* spp. [Cai et al., 2010]), PLC in the central portion can exceed that at the ends by a factor of 2 to 3. Thus, artifactual nucleation of cavitation at the sample ends due to contamination from a reservoir or during initial flow measurement could actually improve the agreement between centrifuge whole-sample PLC and a dehydration reference curve. This might explain how PLC at one sample end could exceed midsample PLC in hybrid aspen (*Populus tremuloides*) stems spun and then subsampled for PLC determination along their length (Cai et al., 2010).

NAVIGATING METHODOLOGICAL LIMITS

A conservative protocol might be to simply avoid inducing experimental stresses on open vessels and not rely on the proposition that water unfiltered by pit membranes has the same tensile strength as native sap. In addition, because in air injection and centrifuge curves the length over which the stress is applied differs from the length over which flow is measured, rigorous comparison with dehydration curves (for which the stress is applied uniformly along the whole stem) requires knowledge of the vessel length distribution, network

topology, and connectivity (Cai and Tyree, 2010). This extra analytical complexity can be avoided simply by measuring flow in air-injection and centrifuge experiments only over the part of the sample subject to a uniform (or nearly uniform) stress. In the centrifuge, this would mean spinning stems long enough that no open vessels extend into the region of peak tension, which is then subsampled for the determination of PLC. This would obviously only be practical on a restricted set of species with sufficiently short vessels. For radial air injection, one would inject stems long enough that no open vessels extend into the pressure chamber and then subsample the portion of the stem within the chamber (after degassing to avoid oversaturation cutting artifacts; Wheeler et al., 2013) for estimation of PLC. Data from such protocols could then be compared with those obtained by dehydration, provided that the determination of dehydration-induced PLC is made on a sample containing no vessels cut open while under tension. The PLC of such samples is still subject to a network bias relative to the intact plant (i.e. flow in the plant may have significantly more opportunities to bypass embolized vessels than in short samples; Zimmerman, 1983; Cai and Tyree, 2010), but for comparing methods, it is sufficient to maintain a consistent sample length for flow measurement between methods if possible.

A further challenge for relating individual vessel air-seeding pressures to whole-plant behavior, or attempting to model axial versus radial propagation of embolism, is that air-seeding pressures may not be randomly distributed in the stem. That is, if radial air entry into the xylem is governed by protoxylem-metaxylem or primary-secondary pit connections, these may be more resistant to air-seeding than those of the secondary xylem vessel connections that predominate in axial flow and are tested in axial air injection, whether of whole stems or single vessels. In this view, the axial system may be "hardened" where it joins damaged or easily damaged xylem but less resistant internally, perhaps to optimize hydraulic conductance. Scaling up axial or single-vessel air-injection measurements to obtain the expected behavior of intact stems during dehydration becomes quite difficult if, in fact, many vessel-vessel connections are not tested during in vivo dehydration until other, more resistant pit membranes (interfacing with primary xylem or protoxylem lacunae) have air seeded. Again, where whole-stem behavior rather than xylem architecture is the primary interest, experiments in which a radial stress is applied uniformly along the length of the entire sample used for flow measurements sidestep these complications.

Looking forward, it is important to recognize that the above controversies regarding PLC determination are not merely technical in nature but have the potential to alter our understanding of the biological and ecological implications of xylem cavitation. As the magnitude of the proposed artifacts are predicted to scale with xylem tension, we need to understand the extent to which they contribute to apparent cycles of embolism and repair while the xylem remains under tension ("novel refilling"; Cochard and Delzon, 2013; Sperry, 2013; Wheeler et al., 2013).

REVISITING THE CURRENT PARADIGM OF DIURNAL CYCLES OF CAVITATION AND REPAIR

The idea that many plants undergo cavitation and repair on a routine or even daily basis (e.g. Zufferey et al. 2011) is, on many levels, unsatisfying. If tradeoffs between safety and efficiency are important in plants, why would natural selection favor xylem that is not functional when evaporative demand is strong and transpiration rates are near their peak? Other aspects of this paradigm are also perplexing. For example, evidence that embolism repair is reversible only up to a point: more severe levels of water stress result in cavitation that is not reversed even when xylem tensions are relieved (Secchi and Zwieniecki, 2010). One could hypothesize that embolism repair only occurs in some subset of conduits or that more severe levels of water stress impair the living cells that are thought to play a role in refilling. However, an alternative interpretation is that we have overestimated the extent to which plants are vulnerable to cavitation, with the distinction between reversible and irreversible levels of cavitation mapping directly onto measurements of artifactual versus real cavitation. A final concern is the absence of a physically consistent model for novel refilling. Despite nearly two decades of work on cavitation repair, how a refilled conduit could reconnect to functional conduits without recavitating remains as mysterious as ever (Zwieniecki and Holbrook, 2009).

IS NOVEL REFILLING AN ARTIFACT?

The evidence for novel refilling based on hydraulic measurements is necessarily indirect, as the phenomenon is believed to occur only in intact plants; an experimental stress is imposed that, based on a vulnerability curve, is expected to result in substantial PLC, and then the stress is removed. After the water potential of the intact plant has recovered, the xylem is sampled for PLC determination, with novel refilling indicated by the finding of a degree of PLC closer to that expected for the water potential at the time of sampling rather than that predicted by the peak stress. That is, as long as the stress is not too great (as noted above), the hysteresis between water potential and PLC expected in the absence of embolism repair is not observed; instead, PLC seems to be a simple function of the water potential at the time of sampling. Wheeler et al. (2013) showed that for two species of *Acer*, this exact pattern could be explained by a previously underappreciated potential for the act of cutting xylem under tension (or when filled with oversaturated sap) to induce artifactual PLC. As noted by Sperry (2013), this does not allow us to conclude that all reports of novel refilling are necessarily artifactual. However, the fact that experimental artifacts can create the appearance of novel

refilling should shift the burden of proof for studies using similar methods.

For example, cryo-SEM has been used to document novel refilling in maize (*Zea mays*) roots (Canny, 1997; McCully et al., 1998). Subsequently, it was shown in walnut (*Juglans regia*) petioles that the in situ freezing required for sample preparation in cryo-SEM can create gas-filled conduits when the xylem is under tension (Cochard et al., 2000). Similarly, cutting artifacts taken together with the anomalies in the behavior of air injection (Choat et al., 2010; Ennajeh et al., 2011) and the centrifuge (Cai and Tyree, 2010; Choat et al., 2010; Cochard et al., 2010; Sperry et al., 2012) provide an alternative explanation for much of the hydraulic data currently interpreted as evidence for novel refilling. Experiments in which embolism repair is impaired or eliminated by phloem girdling would appear to be inexplicable by the sorts of sampling artifacts discussed here (Salleo et al., 1996; Zwieniecki and Holbrook, 1998). Yet, in the absence of controls showing that phloem girdling does not cause cavitation, we feel that these experiments must be treated with caution. Even a brief exposure of the vascular cambium to the air could locally increase xylem tensions to the extent that they cause cavitation. As a result, we are unaware of any PLC-based studies that unambiguously support the existence of a novel refilling mechanism in plants.

NEW TECHNOLOGIES: TOWARD RESOLUTION OF THE CONTROVERSY

While the methodological prescriptions of Wheeler et al. (2013) and Ennajeh et al. (2011), as well as those discussed above, may be sufficient to test for artifacts for many angiosperms, vessel lengths in some ring-porous taxa may be long enough to render these tests impractical. In vivo imaging such as HRCT provides an alternative approach whose power is elegantly illustrated in a recent study of embolism reversal in grape vines (Brodersen et al., 2010). As with an earlier magnetic resonance imaging-based study, demonstrating that such reversal is truly "novel" in the sense of occurring without pressures throughout the xylem being brought into the positive range will require more detailed monitoring of plant water status and studies of species in which root pressure does not develop (Holbrook et al., 2001; Sperry, 2013). HRCT imaging of plant xylem has been limited by access to synchrotron beamlines; however, the "desktop" version of the technology that uses an x-ray tube source may make this technique more widely available. Suuronen et al. (2013) used such a system to follow the spread of embolism in small (approximately 2 mm diameter) saplings of two species of *Betula* during drought. The resolutions achieved, 1 and 2.5 μm^3 voxel, were sufficient to distinguish between fibers and vessels. While the time course of stem water potentials during drying is not known, the authors found that, even in the well-watered state, up to 46% of the xylem fiber matrix was embolized, with the fraction increasing from the moment watering stopped. By contrast, no increase in vessel cavitation was observed over the first 5 d of the drought, underscoring the critical need to resolve vessel and fiber water content changes separately in order to infer changes in PLC.

Indeed, the inability to distinguish acoustic events derived from cavitation in vessel lumens versus fiber (xylem elements that contribute more to mechanical support than to axial flow) lumens has long been noted as a factor limiting the inferences that could be drawn from another noninvasive method, the monitoring of acoustic emissions triggered by bubble nucleation (Tyree and Sperry, 1989). However, recent progress in understanding the relation between emissions and PLC in conifer wood (Mayr and Rosner, 2011; Wolkerstorfer et al., 2012), and simultaneous visual and acoustic detection of cavitation and thin sections of conifer wood embedded in hydrogel, have confirmed that the energy of acoustic emissions correlates with xylem lumen dimensions (A. Ponomarenko, O. Vincent, A. Pietriga, H. Cochard, E. Badel, and P. Marmottant, unpublished data). This result may in the future lead to the ability to resolve the acoustic signature of cavitation in vessels versus fibers in angiosperms and, at present, probably represents the best hope for nondestructive PLC estimation in large woody plants.

CONCLUSION

The issues touched upon in this Update illustrate that our understanding of the stability of water under tension in the xylem remains a work in progress. The first order is to resolve methodological uncertainties in how we quantify the extent of embolism across species that differ markedly in the architecture of their hydraulic networks. Here, experimental approaches that are backed by physically well-defined models are needed. At stake are long-standing questions about the conditions under which the continuity of the water in the xylem is lost: Does cavitation occur as a routine or daily event, or is it limited to periods of low water availability and/or freezing temperatures? And then, under what conditions can embolism be reversed? A definitive answer to the latter is critical to assessing the impact of cavitation on future survival and productivity, as are the costs of replacing conduits lost permanently to cavitation.

Central to understanding structure-function relations are the constraints on conduit diameter. Are wide conduits inherently vulnerable to cavitation based on an increased probability of containing a weak pit, or do limits on conduit diameter emerge from the costs of mechanical reinforcement against implosion? Equally important is to understand the role of xylem architecture in determining cavitation resistance. To what extent do conduits remain water filled because their pit membranes have not come into contact with air versus being truly resistant to air seeding? Fibers, through their mechanical function, as sites of water storage (Hao et al., 2013), and as pathways

for water movement between vessels (Cai et al., 2014), affect xylem function and merit more attention.

Finally, understanding air seeding across pit membranes may require thinking outside the confines of the classical capillary model. Unfortunately, in doing so, the attractive simplicity of that model is lost and we are forced to confront what is likely to be a complex set of mechanical and chemical interactions between pit membrane components, the determinants of which even in well-studied primary cell wall systems remain incompletely understood (Willats et al., 2001). Taking a more phenomenological view of air seeding across pit membranes is surely a step backward from the goal of attaining a complete physical theory for cavitation in plants but one that may prove necessary for understanding the constraints imposed on plants by the necessity of containing the spread of embolism. Careful anatomical and functional investigations (Lens et al., 2011), as well as direct observation of pit-membrane behavior with AFM (Lee et al., 2012) and better understanding of the mechanical properties and roles of cell wall constituents (Zsivanovits et al., 2004), could provide a basis for future progress.

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