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Functional xylem characteristics associated withdrought-induced embolism in angiosperms

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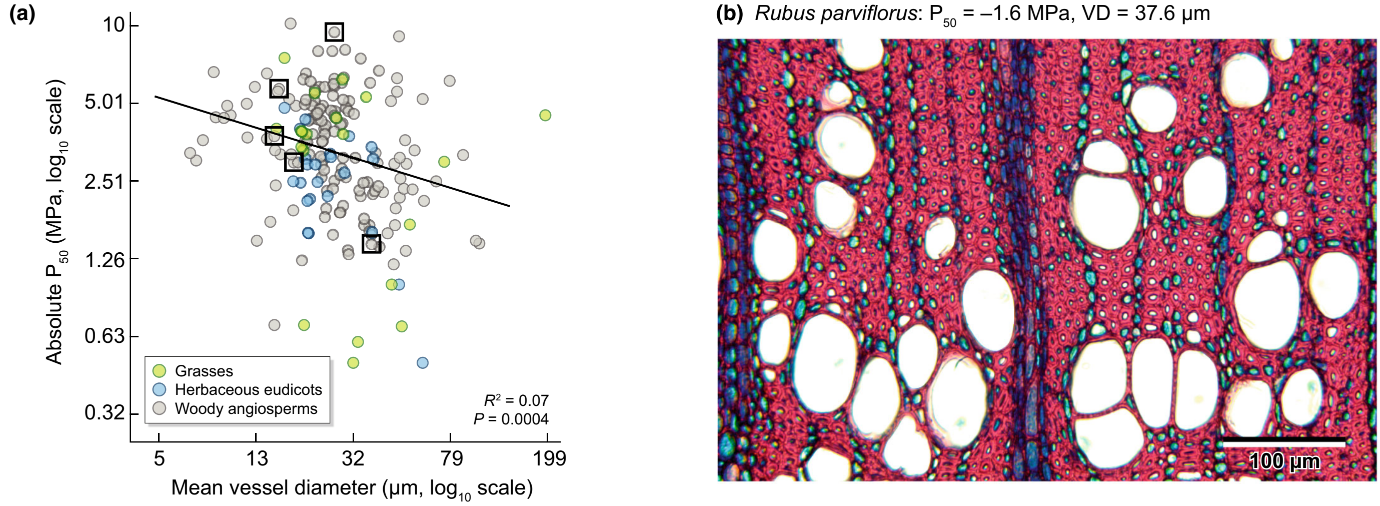
I. Introduction

Photosynthesis in vascular plants requires the long-distance transport of water from the soil to the sites of gas exchange in leaves. This water is pulled up through narrow conduits (e.g. xylem vessels in angiosperms) under negative pressure using energy provided by the sun and atmosphere (Tyree & Sperry, 1989; Angeles *et al*., 2004; Choat *et al*., 2012). During drought, embolism is avoided through stomatal closure (Martin-StPaul *et al*., 2017). However, stomatal closure may result in reduced photosynthetic productivity, growth and reproduction (Brodribb *et al*., 2021). If drought persists and xylem pressure continues to decline, embolism may eventually form in the xylem conduits and obstruct root-to-leaf water transport. Under conditions of intense and prolonged drought, the accumulation of embolism in xylem conduits results in the loss of hydraulic conductance (McDowell *et al*., 2008; Allen *et al*., 2015; Anderegg *et al*., 2016; Adams *et al*., 2017; Choat *et al*., 2018; Brodribb *et al*., 2020), possibly resulting in the desiccation and dieback of tissues, organs and entire plants (Davis *et al*., 2002; Venturas *et al*., 2016; Pratt *et al*., 2020). A lethal level of embolism that represents a physiological point of no return is thought to be reached in angiosperms when the maximum water transport capacity is reduced by *c*. 88% (P_{88}; Urli *et al*., 2013; Li *et al*., 2015). Despite the importance of sap flow to plant functioning, drought-induced embolism remains poorly understood, with most efforts so far mainly focusing on direct or indirect relations between xylem anatomical characteristics and P_{50}, that is the xylem sap pressure inducing 50% loss of hydraulic conductance (Lens *et al*., 2011; Kaack *et al*., 2019, 2021). Understanding anatomical and physico-chemical traits associated with drought-induced embolism will help us to understand (1) how plants will respond to a warming climate and how to manage ecosystems under these novel environmental conditions (Choat *et al*., 2018; Brodribb *et al*., 2020), and (2) how crop breeding programmes can safeguard food production in a future climate that is less favourable for plant growth (Brodribb *et al*., 2015; Gleason, 2015; Meza *et al*., 2020).

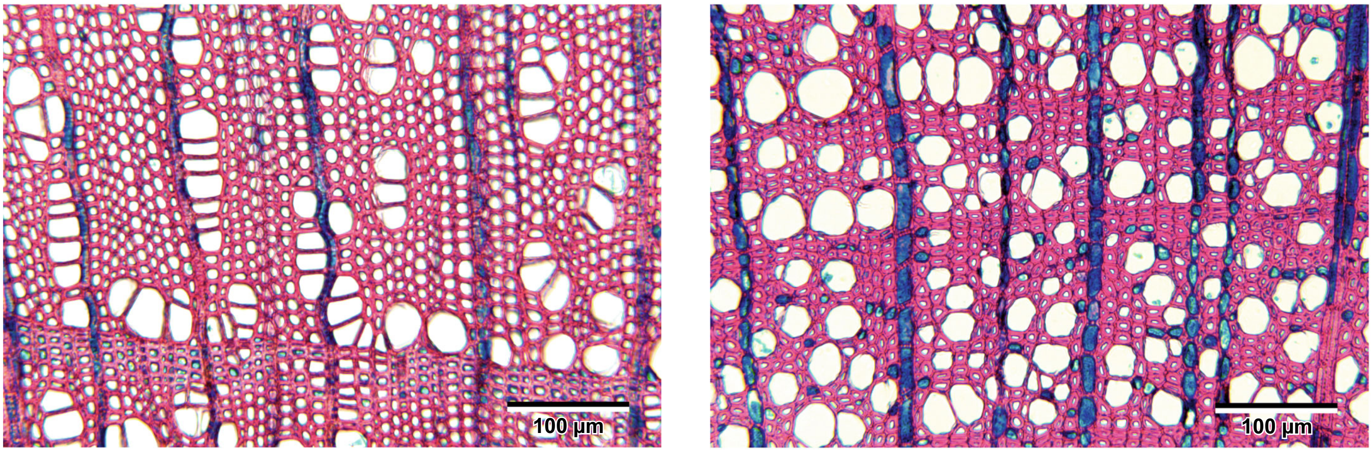
One of the most frequently measured anatomical traits associated with drought-induced embolism in angiosperms is the diameter of vessels, which varies over 70-fold across species and even within species (e.g. many lianas exhibit vessel dimorphism; 10–700 μm; Hacke *et al*., 2017; Olson, 2020). Wider vessels are generally longer within species (Ewers & Fisher, 1989; Cai *et al*., 2010; Pan *et al*., 2015; Liu *et al*., 2018), but more data based on accurate vessel length measurements are needed to quantify this relationship across species (Ewers *et al*., 1990; Hacke *et al*., 2006; Jacobsen *et al*., 2012). There is general agreement that wider and longer vessels conduct water more efficiently because of the reduced number of vessel endwalls, presenting less points of hydraulic resistance along the transport pathway (Gleason *et al*., 2016; Bouda *et al*., 2019). Species with wide, long vessels have also been shown to be more susceptible to freeze–thaw-induced embolism (Sperry & Sullivan, 1992; Davis *et al*., 1999b; Sevanto *et al*., 2012; Lintunen *et al*., 2013; Charrier *et al*., 2014). However, there is controversy regarding the impact of vessel diameter on *drought-induced* embolism, which is the focus of this review paper (Choat *et al*., 2005; Sperry *et al*., 2006; Lens *et al*., 2011; Gleason *et al*., 2018; Olson *et al*., 2018; Barigah *et al*., 2021). A pervasive and longstanding assumption is that species with wide vessels are more vulnerable to drought-induced embolism, which is based on both wood anatomical–ecological inference (Carlquist, 1966, 2001; Baas *et al*., 1983; Anfodillo & Olson, 2021; Koçillari *et al*., 2021) and some experimental work (Hargrave *et al*., 1994; Cai & Tyree, 2010; Nardini *et al*., 2017; Jacobsen *et al*., 2019). Indeed, a weak but statistically significant inverse correlation is shown across a broad range of angiosperm species (186 spp. from 51 families) when P_{50} and mean vessel diameter are measured on the same branch (Fig. 1). However, counter to these reports, there is also convincing evidence that indicates no diameter–vulnerability link, which we address in this review. Likewise, there is no consensus on the mechanism that might underlie a possible correlation between vessel diameter and vulnerability to embolism. This uncertainty has many contributing factors. For instance, drought-induced embolism formation and spread in xylem is poorly understood due to complex nanoscale interactions occurring among the multiple interfaces between liquids, gases and solids, as highlighted in this review. These interactions are further complicated by the presence of solutes and insoluble xylem sap compounds that change the surface tension. In addition, several different methods have been used to quantify P_{50} and vessel diameter, sometimes with contrasting results, thus further complicating the interpretation of meta-analyses that have addressed this topic (Brodersen *et al*., 2013; Cochard & Delzon, 2013; Choat *et al*., 2016; Martin-StPaul *et al*., 2017; Creek *et al*., 2018; Emilio *et al*., 2019; Smith-Martin *et al*., 2020). In an attempt to reconcile this discrepancy, there is an urgent need for a critical and well balanced assessment of the methods applied and the data provided as evidence for/against a diameter–vulnerability link, as well as a general evaluation of mechanisms behind drought-induced embolism. Therefore, this review will summarise our current knowledge of the anatomical and physicochemical drivers that may be involved in drought-induced embolism (Box 1), and highlight key knowledge gaps that are essential to better understand drought-induced embolism in future plant research.

II. What is known about drought-induced embolism formation?

According to the well supported cohesion–tension theory, capillary wicking in leaf mesophyll cell walls exposed to intercellular air spaces is the driving force that creates a gradient of negative xylem pressure, pulling up sap towards transpiring leaves (Dixon & Joly, 1895; Angeles *et al*., 2004). A longstanding question is how a continuous column of liquid xylem sap may become interrupted by gas embolism (Steudle, 2001). Based on estimated water potential values that are known to cause embolism in xylem sap (below *c*. −0.5 MPa; Choat *et al*., 2012), embolism does not result from homogeneous nucleation (Briggs, 1950; Chen *et al*., 2016; Kanduč *et al*., 2020), is unlikely to occur via heterogeneous nucleation by small solutes, but may perhaps be more likely due to pre-existing gas bubbles, including tiny nanobubbles or surface bubbles associated with hydrophobic vessel surfaces (Tyree *et al*., 1994; Hölttä *et al*., 2002; Lohse & Zhang, 2015; Schenk *et al*., 2017). Indeed, there is

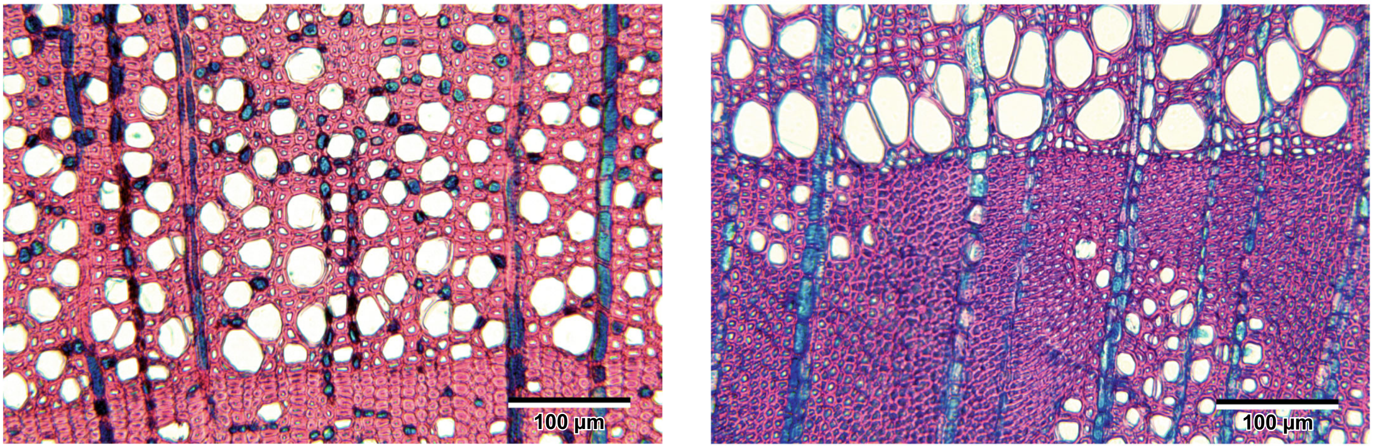


(b) *Rubus parviflorus*: P_{50} = –1.6 MPa, VD = 37.6 μm



(c) *Acer glabrum*: P_{50} = –3.0 MPa, VD = 23.0 μm

(d) *Amelanchier alnifolia*: P_{50} = –4.4 MPa, VD = 21.1 μm



(e) *Amelanchier utahensis*: P_{50} = –6.5 MPa, VD = 20.2 μm

(f) *Ceanothus crassifolius*: P_{50} = –9.4 MPa, VD = 26.5 μm

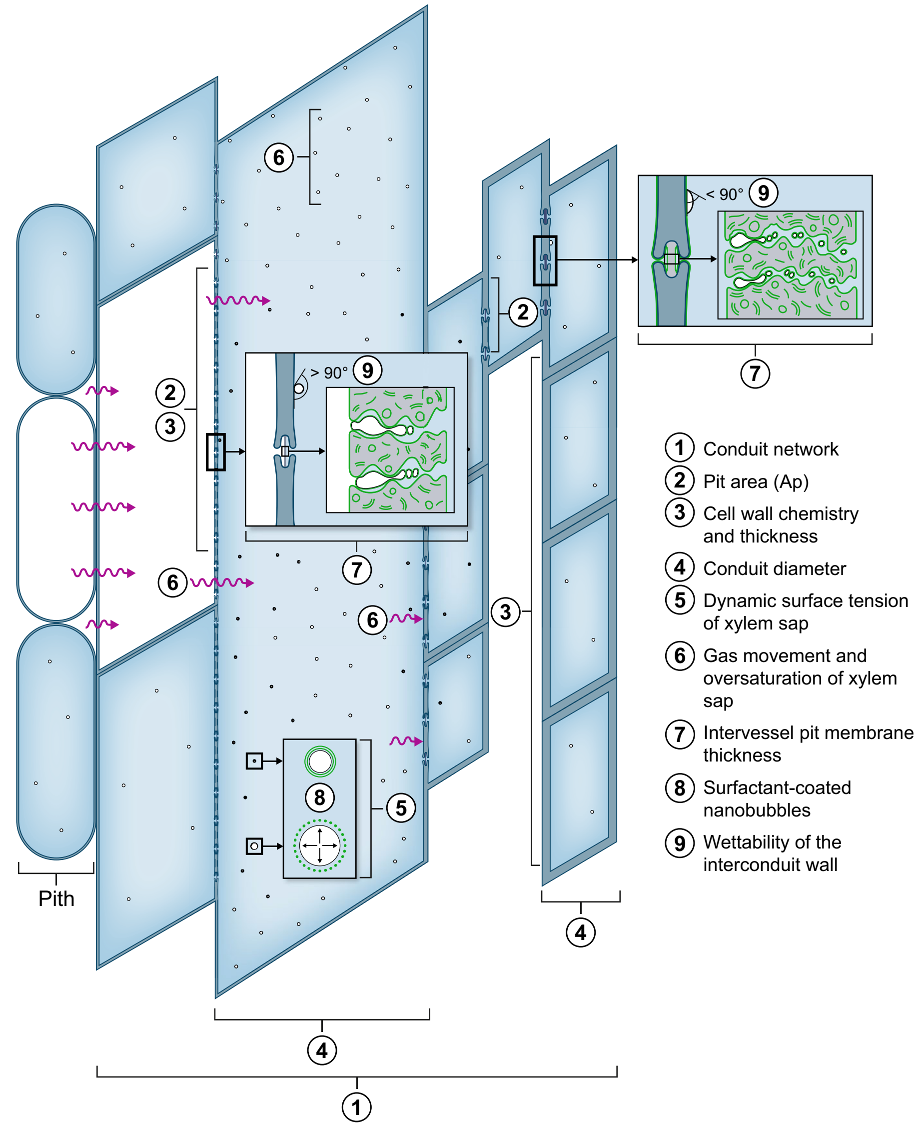
Fig. 1 Weak correlation between the xylem pressure inducing 50% loss of hydraulic conductivity (P_{50}) and mean vessel diameter (VD) across angiosperm branches from 186 species belonging to 51 families based on linear regression analysis (a), illustrated with light microscope images from transverse sections (b–f) of species represented by rectangles in (a). Species with narrow vessels (< 20 μm) have a higher chance to be embolism resistant, but there is large scatter in P_{50} for a given mean vessel diameter. Microscopic illustrations are taken from samples of the same individuals/populations measured for embolism resistance. P_{50} and vessel diameter (based on equivalent circle diameter) are taken from the revised dataset of Lens *et al*. (2016), which is based on the original dataset of Choat *et al*. (2012) with exponential vulnerability curves removed (see Fig. S1 for a graph with exponential vulnerability curves included). Dataset is included in Table S1.

compelling evidence that xylem sap contains not only water and dissolved gases, but also tiny (undissolved) gas bubbles, and a wide array of (in)soluble molecules, including ions, proteins, lipids and sugars (Herbette & Cochard, 2010; Nardini *et al.*, 2011; Schenk *et al.*, 2017, 2020). Of particular interest are the polar lipids due to their insoluble nature and potent surface activity, resulting in a wide range of dynamic surface tension of xylem sap. As will be explained further in this review, these surface-active molecules may play a major role in the stability of tiny gas bubbles in xylem sap under negative pressure, which secures the water transport system and makes it more resilient to drought-induced embolism than traditionally thought (Schenk *et al.*, 2017, 2020; Yang *et al.*, 2020; Ingram *et al.*, 2021; Jansen *et al.*, 2022).

There are some known aspects of drought-induced embolism that are gaining convincing support. Firstly, there is increasing evidence that widespread embolism formation in conductive conduits is uncommon over relatively long periods in the field, and that embolism may be limited to periods of severe drought (Cochard & Delzon, 2013), after stomata have closed (Martin-StPaul *et al.*, 2017; Creek *et al.*, 2020). This contrasts with previous reports – discussed in the following paragraphs – that have found embolism formation (and reversal) occurs on a daily or seasonal basis (Tyree *et al.*, 1999; Salleo *et al.*, 2004; Nardini *et al.*, 2011). Assuming that embolism is strongly determined by the water potential of xylem sap, the case for drought-induced embolism being a rare event is supported by the most negative water potential measurements within a growing season ($P_{\min}$). Indeed, $P_{\min}$ was found to account for < 20% loss of stem conductivity among grasses (Lens *et al.*, 2016) and < 12% in various temperate tree species (Wason *et al.*, 2018; Guan *et al.*, 2022). Furthermore, it has been shown that the hydraulic safety margin (HSM), which can be defined as the difference between $P_{\min}$ and P_{88}, is overall positive (+2 MPa) across angiosperm species and biomes (Choat *et al.*, 2012), suggesting that lethal levels of embolism are only reached after an exceptionally intense episode of drought (Delzon & Cochard, 2014). Earlier reports of recurrent transitions between drought-induced embolism events and embolism repair under tension (summarised by Brodersen & McElrone, 2013; Delzon & Cochard, 2014) are likely to have arisen from methodological artefacts. Examples of artificial embolism formation result from freezing the xylem during cryo-SEM observations (Umebayashi *et al.*, 2016; Ogasa *et al.*, 2016), from centrifuging short xylem segments with many cut-open vessels (open vessel artefact; Cochard, 2002; Cochard *et al.*, 2013; please refer to subsequent paragraphs), and from cutting plant organs in water when the xylem sap is under negative pressure and oversaturated with gas (cutting artefact; Sperry, 2013; Wheeler *et al.*, 2013; Torres-Ruiz *et al.*, 2015; Schenk *et al.*, 2016). The open vessel artefact occurs when embolism is artificially induced with the centrifuge method in xylem segments that are shorter than the maximum vessel length, therefore resulting in at least some vessels being cut at both ends. These longer (and often wider) cut-open vessels embolise quickly when they are subjected to negative pressure, therefore resulting in a rapid substantial (exponential) decline in hydraulic conductivity, and therefore an overestimation of vulnerability (Cochard, 2002; Choat *et al.*, 2010; Cochard *et al.*, 2010, 2013; Fig. S1). However, it has also been suggested that the centrifuge method does not overestimate vulnerability to embolism in stem segments with cut-

Box 1 Shortlist of potential anatomical and physicochemical drivers of drought-induced embolism

(1) *3D-conduit network*: since embolism spreads from an embolised to a neighbouring, sap-filled conduit, 3D-vessel connectivity affects embolism propagation (Loepfe *et al.*, 2007; Mrad *et al.*, 2018, 2021; Wason *et al.*, 2021).
(2) *Ap*: average intervessel pit area per mean vessel affects conductivity, but has only a minor effect on embolism resistance for a given pit membrane thickness range (Wheeler *et al.*, 2005; Kaack *et al.*, 2021).
(3) *Cell wall chemistry and thickness*: ultrastructure and chemistry of cell walls – including conduit walls – influence transport efficiency of water and gas, and can serve as barriers allowing local pressure nonequilibrium and reducing embolism spread between adjacent cells (Lens *et al.*, 2016; Dória *et al.*, 2018; Pereira *et al.*, 2018; Thonglim *et al.*, 2020).
(4) *Conduit diameter*: wide vessels are traditionally assumed to be more vulnerable than narrow ones, although experimental data show a weak, indirect correlation (Fig. 1a; many papers cited in this review).
(5) *Dynamic surface tension of xylem sap*: surface tension has a strong effect on a gas–liquid–surfactant interface, and is highly variable depending on the local concentration of lipids per surface area over time (Schenk *et al.*, 2017, 2020; Yang *et al.*, 2020).
(6) *Gas movement and xylem sap oversaturation*: movement of gas molecules by diffusion or advection along a concentration gradient determines the amount of gas in conduits filled with sap, and gas under atmospheric pressure in intercellular spaces. The proximity to atmospheric gas volumes influences gas flow into xylem and may increase the likelihood of embolism propagation, for example by changing gas solubility in xylem sap potentially leading to oversaturation (Schenk *et al.*, 2016; Pereira *et al.*, 2020; Guan *et al.*, 2021).
(7) *Intervessel pit membrane thickness*: affects the number and therefore the size of pore constrictions in pit membranes, which determine flow of water and gas between adjacent vessels, and therefore embolism resistance (Fig. 2a; Lens *et al.*, 2011; Li *et al.*, 2016; Kaack *et al.*, 2021). Highly variable geometrical shapes of pit membrane pore shapes lead to snap-off events that generate undissolved tiny gas bubbles (nanobubbles) that are stabilised by surface active lipids (green).
(8) *Surfactant-coated nanobubbles*: tiny gas bubbles can be stable in xylem sap as long as they are coated with surfactants (green) and have a diameter below a critical threshold for a given pressure. The fate of these bubbles, their stability in plant xylem and potential link to embolism are largely unknown (Schenk *et al.*, 2015, 2017; Ingram *et al.*, 2021).
(9) *Wettability of the inner conduit wall*: inner conduit walls must be hydrophilic for an optimal solid–liquid interface (contact angle between water and conduit wall < 90°), while hydrophobic walls (contact angle > 90°) attract surface nanobubbles that may trigger embolism (Zwieniecki & Holbrook, 2000; McCully *et al.*, 2014; Schenk *et al.*, 2017; Brodersen *et al.*, 2018

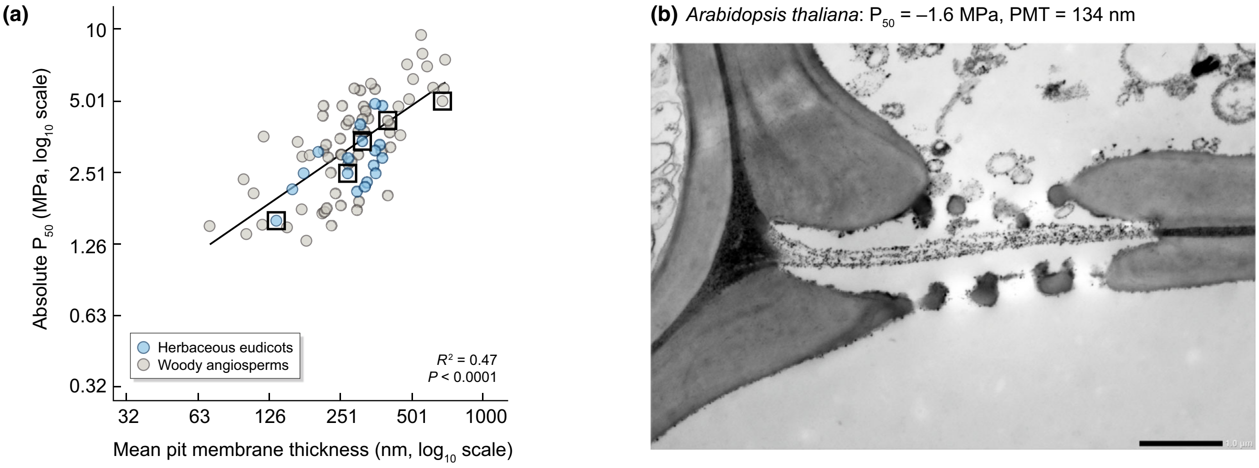


Box Fig 1. Summarising figure illustrating nine potential anatomical and physico-chemical drivers of drought-induced embolism inside a hypothetical stem. Large atmospheric gas sources in the pith (oval cells at the left) may induce embolism within the vessel network. Embolism formation and spread in this network is further affected by a complex interaction of the 3D-vessel connectivity (1), intervessel pit area per mean vessel (2), cell wall chemistry and thickness (3), conduit diameter (4), dynamic surface tension in xylem sap (5), gas movement among cells and intercellular spaces potentially leading to oversaturation of xylem sap (6), intervessel pit membrane thickness (7), surfactant-coated nanobubbles (8), and wettability of the conduit walls (9). Details for all these potential drivers and the extent to which they affect vulnerability to drought-induced embolism are discussed in Sections II, III and IV of the main text.

open vessels (Jacobsen & Pratt, 2012; Sperry *et al.*, 2012; Tobin *et al.*, 2012; Hacke *et al.*, 2014), and that there is no accidental formation of gas-filled vessels in xylem segments that are cut in water whilst under negative pressure (no cutting artefact observed in Venturas *et al.*, 2015, 2019; Nardini *et al.*, 2017). These alternative viewpoints suggest that exponential vulnerability curves may be valid (Sperry *et al.*, 2012), and consequently support previous reports of high levels of native embolism (up to 60–90%) in functional sapwood, even under mild drought (Pockman & Sperry, 2000; Domec *et al.*, 2006; Jacobsen *et al.*, 2007; Chacon *et al.*, 2020; Percolla *et al.*, 2021). This discrepancy in xylem vulnerability continues to cause confusion about the frequency of drought-induced embolism events *in planta*. Indeed, disagreement within the literature generally centres around just a few long-vesseled genera, *Quercus* and *Vitis*. These are systematically prone to measurement errors, which appears to be the most parsimonious answer when confronted with multiple modes of measurement (Choat *et al.*, 2010; Wason *et al.*, 2019). For example, stem P_{50} in grapevine species is estimated to be *c.* −2.5 MPa based on sigmoidal (s-shaped) vulnerability curves using bench dehydration, microCT, and validated by nuclear magnetic resonance (Choat *et al.*, 2010; McElrone *et al.*, 2012; Charrier *et al.*, 2018) vs −0.5 MPa using exponential curves based on short segments (0.14 and 0.27 m) spun with a centrifuge (Jacobsen & Pratt, 2012). Similarly, stem P_{50} across oak species ranges from −3 to −6 MPa based on s-shaped curves using bench dehydration and the optical vulnerability technique (Martin-StPaul *et al.*, 2014; Skelton *et al.*, 2018, 2021) vs much less negative P_{50} values (often around -0.5 MPa) based on exponential curves generated mainly by centrifuge methods spinning short stem segments (Sperry *et al.*, 2012; Percolla *et al.*, 2021). Altogether, there is increasing evidence that the artefacts mentioned above overestimate vulnerability, suggesting that plants do not suffer from major drought-induced embolism events in the field under daily, natural growing conditions. Therefore, natural selection appears to have shaped the hydraulic systems of plants, including the nonxylem pathways in roots and leaves, in such a way that the impact of drought-induced embolism is minimised. This can be done in three ways: (1) by building resistant xylem (Davis *et al.*, 1999a), (2) by preventing xylem sap pressures from exceeding critical thresholds (Martin-StPaul *et al.*, 2017), and/or (3) by developing new wood tissue that leads to hydraulic recovery (Gauthey *et al.*, 2022).

Secondly, it is generally accepted that embolism spread between adjacent conduits predominantly happens via the weakest link in the interconduit walls, where pore diameters are typically between 5 and 50 nm. Such pores in angiosperms are typically found within the pit membranes located inside the bordered pits between adjacent vessels, whereas the nonpitted areas of the intervessel walls are much denser (with pore diameters < 2 nm; Donaldson *et al.*, 2018). Traditionally, the movement of an air–water interface across a pit membrane has been described as 'air seeding' (Zimmermann, 1983; Sperry & Tyree, 1988). However, considering that the air-seeding theory was developed decades before major advancements in anatomical and physiological methods, the original authors did not fully understand the influence of three-dimensional pit membrane structure, nor the complex interactions of gas–liquid–solid interfaces, such as snap-off events (see below; Box Fig. 1; Kaack *et al.*, 2021; Park *et al.*, 2021). Modern techniques and recent studies largely confirmed that interconduit pit membranes represent the main pathway for gas movement as suggested by these authors, but also identified more insights into the anatomical and mechanistic details underpinning embolism spread. For example, there is solid evidence for the spreading of embolisms from an already embolised vessel to a sap-filled neighbouring one; this has been observed *in vivo* in twigs and small branches of intact plants using microcomputed tomography (microCT; Brodersen *et al.*, 2013; Choat *et al.*, 2016), and with the 'optical method' in leaf veins (Brodribb *et al.*, 2016a,b; Skelton *et al.*, 2021), stems (Gauthey *et al.*, 2020; Johnson *et al.*, 2020), roots (Rodriguez-Dominguez *et al.*, 2018) and inflorescences (Bourbia *et al.*, 2020). There is also convincing evidence that intervessel pit membrane thickness is one of the major drivers of drought-induced embolism vulnerability in angiosperms (Box 1), both between species (Jansen *et al.*, 2009; Lens *et al.*, 2011, 2013; Li *et al.*, 2016; Dória *et al.*, 2018; Levionnois *et al.*, 2020a) and within species (Thonglim *et al.*, 2020; but questioned by Lemaire *et al.* (2021) who did not find ultrastructural intervessel pit differences within a poplar hybrid), with thick intervessel pit membranes providing higher embolism resistance than thin ones (Fig. 2; Box Fig. 1). The functional explanation for this relationship between P_{50} and intervessel pit membrane thickness is that membrane thickness has a strong effect on the size and number of pit membrane pore constrictions, which represent bottlenecks or pore throats between interconnected pore voids across an entire pit membrane pathway (Box Fig. 1). As the size of the narrowest pore constriction – which is most crucial for water and gas transport – strongly depends on the number of consecutive pore constrictions, the rate of fluid transport and embolism spread relates to pit membrane thickness (Kaack *et al.*, 2019, 2021; Yang *et al.*, 2020; Zhang *et al.*, 2020). Unlike the traditional view that air seeding occurs via a single bubble penetrating through the largest pore of the entire pit membrane, the multilayered nature of pit membranes with multiple pore constrictions makes this process extremely unlikely. Instead, air–water interfaces within the highly variable geometrical shapes of pore voids are very likely to show snap-off events, which are driven by the local increase in liquid pressure by entry of gas–water menisci, and thermodynamically by surface-area minimisation to keep tensile energy low. These snap-off events are well known in porous media, occur when the radius of a pore constriction is less than half the radii of the pore void on either side of the constriction (Roof, 1970; Kovscek & Radke, 1996; Berg *et al.*, 2013; Schenk *et al.*, 2015), and are likely to lead to tiny, undissolved gas bubbles that are surrounded by surface active lipids (i.e., surfactant-coated nanobubbles; Box Fig. 1). These novel insights improve the traditional 2D-view of air-seeding, and suggest that a truly mechanistic understanding of embolism spread will require the careful consideration of a 3D-nanoscale pit membrane structure. Therefore, pit membrane thickness, along with the geometry of the pore constrictions and the nanoscale behaviour of gas–liquid–surfactant–solid interfaces within 3D-pit membranes, are important components contributing to a mechanistic understanding and predictive power of embolism resistance.

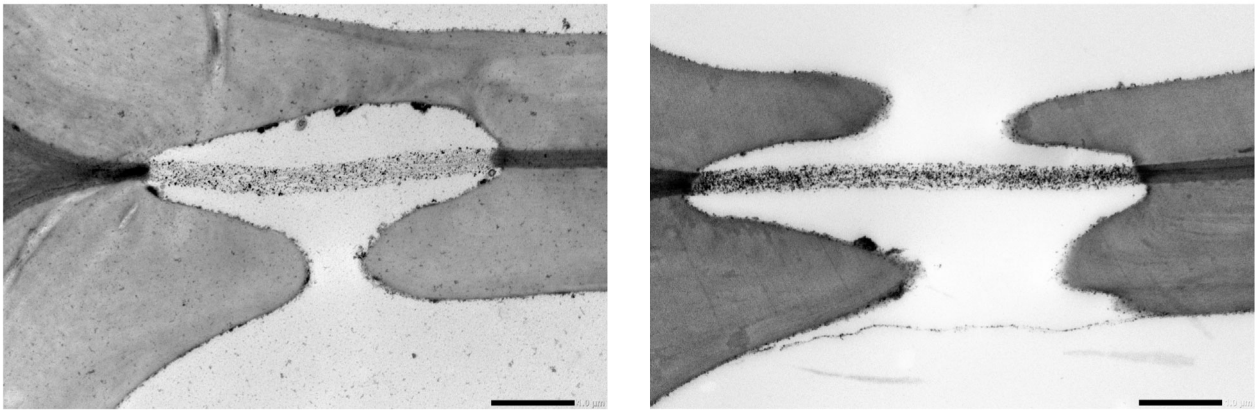
A third point that we are fairly confident about relates to the location and temporal distribution of the first drought-induced embolism events in plant xylem. MicroCT observations indicate that the xylem vessels close to the pith in twigs and branches generally embolise first (Box Fig. 1; Brodersen *et al.*, 2013; Cochard *et al.*, 2015; Choat *et al.*, 2016; Venturas *et al.*, 2019), and that the widest conduits in the xylem of the main leaf veins usually embolise first (Brodribb *et al.*, 2016a; Scoffoni *et al.*, 2017; Guan *et al.*, 2021; but please refer to Ocheltree *et al.*, 2020). The reason for these patterns in embolism spread in stems and leaves is not well understood, but could be related to differences in the chemical composition and thickness of conduit walls in primary xylem (conduit walls partly or entirely lignified) vs secondary xylem (conduit walls entirely lignified; Choat *et al.*, 2005). Interestingly, the amount of lignification in cell walls may determine gas diffusion kinetics across conduit walls, and therefore could reduce the speed of embolism propagation in stems with increased levels of lignification or woodiness (Box Fig. 1; Lens *et al.*, 2016; Dória *et al.*, 2018; Pereira *et al.*, 2018; Thonglim *et al.*, 2020; Box 1) and/or wood density (Greenwood *et al.*, 2017; Liang *et al.*, 2021). Alternatively, the first-formed vessels in woody stems may be subjected to cumulatively more stress events resulting in a reduced functional life span as embolism accumulates over time (Hacke *et al.*, 2001; Umebayashi *et al.*, 2019), or may be more closely connected with large atmospheric gas sources, such as intercellular gas in pith tissue or dysfunctional primary xylem (Guan *et al.*,



(b) *Arabidopsis thaliana*: P_{50} = –1.6 MPa, PMT = 134 nm

(c) *Glebionis segetum*: P_{50} = –2.2 MPa, PMT = 318 nm

(d) *Cladanthus mixtus*: P_{50} = –2.9 MPa, PMT = 269 nm



(e) *Argyranthemum gracile*: P_{50} = –4.0 MPa, PMT = 406 nm

(f) *Olea europaea*: P_{50} = –5.0 MPa, PMT = 670 nm

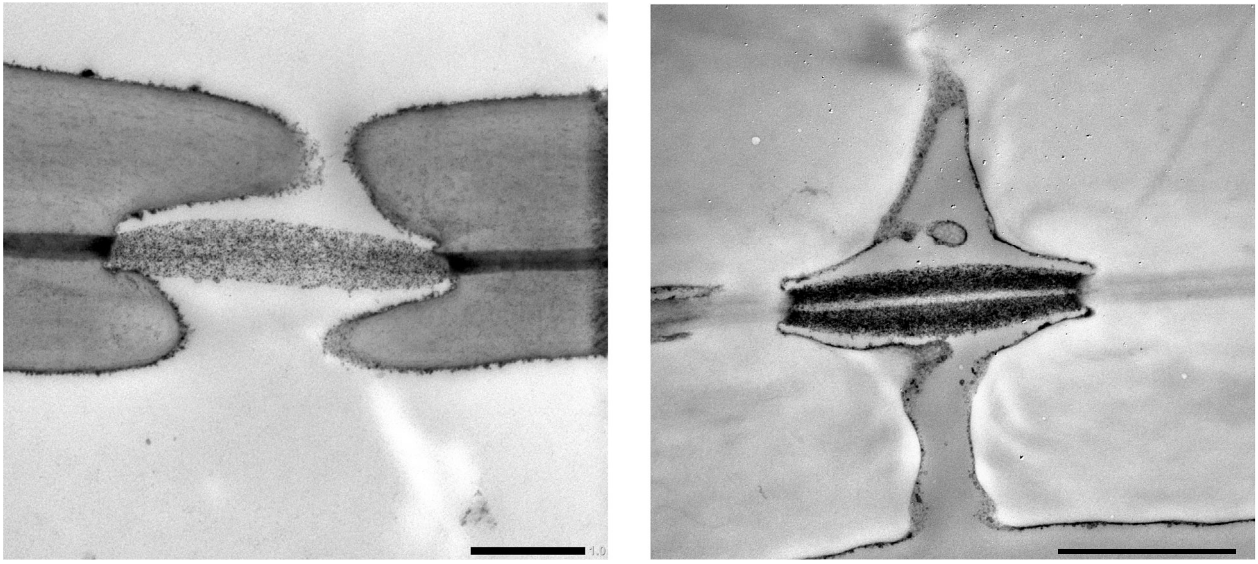


Fig. 2 Strong correlation between the pressure inducing 50% loss of hydraulic conductivity (P_{50}) and mean intervessel pit membrane thickness (PMT) across stems of angiosperm species based on linear regression analysis (a), illustrated with transmission electron microscope images from cross-sections (b–f) of species represented by rectangles in (a). Species that are more resistant to embolism formation have on average thicker intervessel pit membranes. Microscopic illustrations are taken from samples of the same individuals measured for embolism resistance (except for f). Bars: 1000 nm (b–e); 2000 nm (f). Dataset included in Table S1.

2021). Bearing this in mind, it is important to realise that angiosperm studies investigating drought-induced embolism at the whole-plant level have focused largely on small-sized individuals (woody seedlings or saplings) due to methodological constraints, thereby leaving out observations in the sapwood of trunks with vessels that are far removed from pith or primary xylem vessels.

Finally, vulnerability to drought-induced embolism is often assumed to be related to the mean intervessel pit area of a given vessel with average dimensions (Ap), as proposed by the pit area (aka rare pit) hypothesis (Wheeler *et al.*, 2005; Hacke *et al.*, 2006, 2009; Christman *et al.*, 2009, 2012). Based on this hypothesis, permeability to gas and embolism propagation increases with increasing Ap (Box 1). Although the pit area hypothesis correctly emphasises the functional importance of intervessel connectivity (Loepfe *et al.*, 2007), the idea that a rare pit membrane would be highly leaky, leading to embolism propagation, is not compatible with our more recent insights into the 3D-nature of pit membranes. In addition, although Ap may affect P_{50} for a certain range of intervessel pit membrane thickness (*c.* 150–300 nm), there is new evidence from both 3D-modelling and experiments that P_{50} has a stronger relationship with pit membrane thickness (Fig. 2a) than Ap (Kaack *et al.*, 2021). This is also in line with other studies demonstrating that Ap values do not consistently increase with increased vulnerability to embolism (Lens *et al.*, 2011; Wason *et al.*, 2018). Moreover, the pit area hypothesis was published before the open vessel artefact was understood, and was originally proposed based on P_{50} measurements taken from short, water-flushed stem segments (14.2 cm long) using centrifuge methods that are likely to have overestimated the vulnerability of species with longer – and often wider – vessels, which commonly have higher Ap values (e.g. Choat *et al.*, 2010; McElrone *et al.*, 2012; Cochard *et al.*, 2013; Torres-Ruiz *et al.*, 2014; Martin-StPaul *et al.*, 2014; Charrier *et al.*, 2018; Skelton *et al.*, 2018; however, please refer to the above studies that question the open vessel artefact). Similarly, estimations of Ap are challenging due to the requirement to obtain accurate vessel length distributions. Most vessel length estimations have been based on silicone injections, which may not always fully penetrate from the injection point to the vessel ends (Pan *et al.*, 2015; Gao *et al.*, 2019). For instance, the ratio of underestimation of vessel length via the silicone-injection method was found to be higher in medium-to-long-vesseled species compared with short-vesseled species (Gao *et al.*, 2019), and could be overcome by applying more recent pneumatic approaches based on gas injection or extraction (Cohen *et al.*, 2003; Pereira *et al.*, 2020). Last but not least, intervessel connectivity, which is important to estimate Ap values, is highly challenging to quantify. Indeed, the Ap model assumes that vessel endings are distributed randomly, whereas this may not be true in some cases, for example near nodes, side branches, stem–petiole transitions, etc. (Salleo *et al.*, 1984; André *et al.*, 1999; Wason *et al.*, 2021). Altogether, the available evidence suggests that Ap is probably not a main driver of drought-induced embolism resistance, and that there are likely to be various anatomical or physicochemical variables that play a more decisive role (Box 1). We therefore recommend to be careful when interpreting the pit area hypothesis as a mechanistic explanation for a possible correlation between P_{50} and vessel diameter.

III. Why are the mechanisms behind drought-induced embolism formation so complicated?

One of the many factors raising uncertainty about the mechanisms behind drought-induced embolism involves the gas bubble initiation, its potential stabilisation in a liquid under negative pressure, and physical processes at the gas–liquid–solid–surfactant interface that determine fluid flow and the likelihood of embolism. Gas oversaturation or oversolubilty is a common phenomenon in soil water and well understood to take place in nanoconfined spaces (Ho *et al.*, 2015; Coasne & Farrusseng, 2019), and therefore also likely in cell walls and pit membranes of xylem tissue. Although the origin of embolism initiation is largely unknown, one hypothesis is that an embolism arises from a pre-existing nanobubble that expands in the xylem sap (Box Fig. 1). Tiny gas bubbles can be stable in xylem sap under negative pressure if they remain below a critical size threshold, and only when they are coated by surfactants (surface-active agents), which are amphiphilic, polar lipids that lower the surface tension (Box 1; Schenk *et al.*, 2015, 2017). If embolism would be a largely stochastic process based on pre-existing nanobubbles, wide and long vessels with a large volume and high amount of nanobubbles would have a higher likelihood to encounter embolism than narrow, short vessels (Box Fig. 1). Yet, it is unclear how stable these surfactant-coated nanobubbles are over time, and under which fluctuating conditions (variation in temperature, pressure, gas solubility of xylem sap, gas concentrations in the surrounding xylem tissue) these tiny gas bubbles may expand to form a large embolism (Ingram *et al.*, 2021; Box Fig. 1). Similarly, we do not know the *in vivo* surface tension of surfactant-coated nanobubbles, and their size and concentration inside plant xylem, which are both critical aspects to estimate the stability of tiny gas bubbles in xylem sap (Schenk *et al.*, 2017, 2020; Yang *et al.*, 2020; Box 1). Consequently, it is becoming clear that multiphase interactions between cell walls, pit membranes, xylem sap, surfactants, nondissolved nanobubbles and dissolved gas may show dynamic changes over time, and are affected by local changes in temperature and pressure. Most of these parameters are likely to affect drought-induced embolism formation over time (Box 1), which may not be a function of xylem water potential only (Avila *et al.*, 2021; Guan *et al.*, 2021). Therefore, understanding these nanoscale interactions under negative pressure remains an important priority.

Given the potential for drought-induced embolism to be triggered at gas–liquid–solid–surfactant interfaces, a longstanding but unresolved question is how plants manage to transport water in a system in which negative pressure is common (McCully *et al.*, 2014; Schenk *et al.*, 2017, 2018). That they manage to do so without continuously creating large gas bubbles implies that the surface chemistry of inner vessel walls is important in keeping water columns largely intact (Box 1). For example, hydrophilic surfaces – with contact angles between water and conduit walls close to zero – enable water to distribute itself uniformly along the wall surface, leading to high wettability of the walls (Box 1). By contrast, hydrophobic vessel surfaces – with water–wall contact angles above 90° – are prone to surface bubbles that stick to the conduit walls (Lohse & Zhang, 2015). We might therefore expect embolism vulnerability to be reduced by hydrophilic surfaces and exacerbated by hydrophobic surfaces (Box Fig. 1). However, some studies have shown that inner vessel wall surfaces feature a microscale mosaic of hydrophilic and hydrophobic patches. Inner vessel walls are partly hydrophobic, especially at pit borders and pit aperture rims (Zwieniecki & Holbrook, 2000; McCully *et al.*, 2014; Schenk *et al.*, 2017; Brodersen *et al.*, 2018), whereas most of the remaining vessel surface mainly consists of a mixture of cellulose and lignin, making these neither completely hydrophilic nor completely hydrophobic (Schenk *et al.*, 2018). Interestingly, continuous layers of amphiphilic and polar lipids are observed on inner vessel walls, pit borders, and pit membranes. This results in these surfaces being more hydrophilic, and is likely to reduce the probability of surface bubbles on these surfaces (Box Fig. 1; Schenk *et al.*, 2017, 2018, 2020). In summary, there is growing evidence that conduit surface chemistry is relevant to keep the water transport pipeline intact.

Drought-induced embolism spread from embolised conduits to adjacent, sap-filled conduits prevails over de-novo drought-induced embolism events. This conclusion is based on microCT and/or optical technique observations in stems (Brodersen *et al.*, 2013; Knipfer *et al.*, 2015; Choat *et al.*, 2016; Skelton *et al.*, 2017; Torres-Ruiz *et al.*, 2017) and leaves (Brodribb *et al.*, 2016a,b; Skelton *et al.*, 2017, 2018; Klepsch *et al.*, 2018; Lamarque *et al.*, 2018) across a diverse range of species (see earlier). However, at this point, it is not clear how rare *de novo* embolism is, nor whether these embolism events are likely to have arisen from pre-existing nanobubbles in the xylem sap. *De novo* embolism events are difficult to identify in microCT images because 3D-reconstructions of wood are based on stem segments that are much shorter than the length of vessels (1 to > 200 cm), thereby obscuring whether or not a seemingly isolated vessel in a cross-section is connected to a neighbouring embolised vessel upstream or downstream of the field of view (Knipfer *et al.*, 2015; Choat *et al.*, 2016). In leaves, microCT observations revealed rare isolated embolism events in the veins of a minority of *Arabidopsis* samples studied (Scoffoni *et al.*, 2018). In addition to the uncertainty concerning *de novo* drought-induced embolism, it remains largely unknown what happens to xylem sap that is released when vessels become fully embolised. It is possible that the water displaced by the formation of water vapour may be released into the transpiration stream or taken up by cell walls or cytoplasm of living xylem cells (Vergeynst *et al.*, 2015; Yazaki *et al.*, 2020). At the same time, the process of embolisation itself may temporarily reduce the chances of air seeding in adjacent vessels, based on a modelling study that suggests that gas may be extracted from adjacent tissues via interconduit pit membranes during the initial stages of embolisation (Wang *et al.*, 2015).

A final potential source of variation in drought-induced embolism vulnerability is that plasticity of the water transport system may occur as plants progress across seasons, or through developmental stages from seedlings to mature life forms (Olson *et al.*, 2018). For example, the embolism resistance of grapevine stems and leaves increases towards the end of the growing season (Charrier *et al.*, 2018; Sorek *et al.*, 2020). The explanation for this seasonal change by Sorek *et al.* (2020) in leaves was shrinkage of intervessel pit membranes, which leads to reduced pit membrane pore constrictions, whereas the amount of lipids associated with pit membranes increased over time. Furthermore, the xylem network could be different within and between current-year stems and trunks, meaning that seasonal or interannual changes in vessel connectivity may impact embolism spread (McElrone *et al.*, 2021). Studying seasonal or temporal changes in the vulnerability of the same vessels over time is a promising experimental strategy. This is because it keeps anatomical variation to a minimum, and allows identifying factors other than vessel dimensions in generating vulnerability to drought-induced embolism.

IV. Why are wider vessels not necessarily more vulnerable to drought-induced embolism than narrower vessels?

A positive correlation between drought-induced embolism vulnerability and vessel diameter comes largely from comparative and ecological studies on wood anatomy, but this correlation is often not found in experimental studies (Box 1; please refer to subsequent paragraphs). A frequently cited argument supporting this link is that earlywood vessels, which are often formed during the spring in temperate regions or during the wet season in seasonally dry regions, are always wider than latewood vessels formed later in the season, often under drier conditions (Fig. 3a,b; Carlquist, 2001). This not only applies to the typical temperate ring-porous species, but also to (semi-)ring-porous species from (sub)tropical regions (Fig. 3a,b) and to the diffuse-porous species that have abundant unicellular latewood tracheids, which have been suggested to be more resistant to embolism compared with the longer, narrow latewood vessels (Fig. 3c; Baas *et al.*, 1983; Carlquist, 2001; Pratt *et al.*, 2015b; Percolla *et al.*, 2021). However, this does not necessarily provide evidence that earlywood and latewood vessels differ in vulnerability to drought-induced embolism per se, because the difference in vessel diameter may reflect a developmental difference in growth, and may depend on turgor-driven cell expansion and/or seasonal differences in hydraulic demands for transpiration (Lockhart, 1965; Ray *et al.*, 1972; Coussement *et al.*, 2018). Furthermore, whereas natural selection favours photosynthesis, the role of xylem hydraulic efficiency as potential determinant of photosynthetic productivity can be masked, for instance by apoplastic and symplastic control at the outer-xylem tissue level in leaves (Scoffoni *et al.*, 2017). In other words, linking conduit diameter with vulnerability to embolism can be misleading when taking only comparative and ecological wood anatomy studies into account.

From a more xylem physiological point of view, there are three main explanations for why the role of vessel diameter on drought-induced embolism vulnerability has been debated. First, as highlighted in this review, there are various unknowns involved in the mechanisms behind drought-induced embolism, leaving room for multiple drivers that may change over time, such as xylem sap composition, chemistry and thickness of conduit walls, qualitative and quantitative intervessel pit traits, local differences

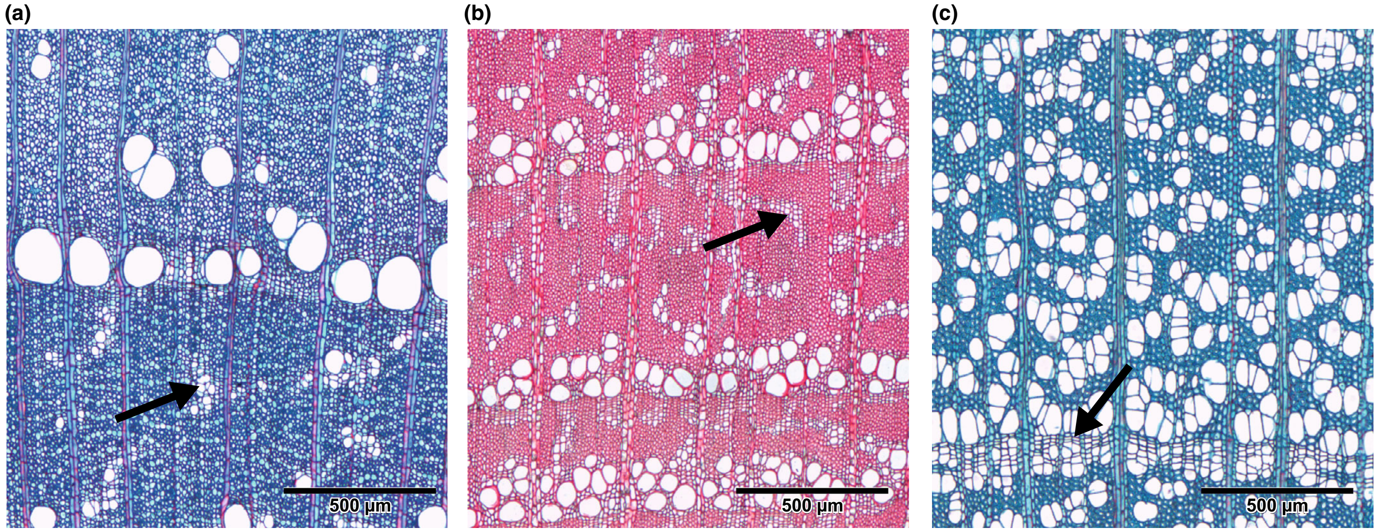


Fig. 3 Light microscope sections of angiosperm woods showing widespread patterns in earlywood vs latewood. (a, b) In nontemperate species experiencing considerable late season drought, such as *Ehretia matthewii* (native to India and Sri Lanka; Boraginaceae; a) and *Proustia cuneifolia* (native to Argentina, Chile, Bolivia and Peru; Asteraceae; b), vessels are always wider at the beginning of the growing season (earlywood) and narrower in the latewood (arrows). (c) In species such as *Sambucus racemosa* (native to western Europe and USA; Adoxaceae), the narrow latewood vessels are replaced by even narrower conductive imperforate tracheary elements called vascular tracheids (arrows).

in the pressure and temperature of xylem tissue, and the concentration and vicinity of dissolved and undissolved gas (Box 1). Also, the 3D-spatial positions and connections among vessels, including vessel compartmentalisation (also called segmentation or sectoriality), affect the number of potential pathways for embolism propagation to occur, and may be better predictors of embolism vulnerability than vessel diameter (Loepfe *et al*., 2007; Mrad *et al*., 2018, 2021; Guan *et al*., 2021; Wason *et al*., 2021).

Second, studies that compare vulnerability to drought-induced embolism in xylem across different organs such as roots, stems, and leaves show there is no direct, mechanistic link between conduit diameter and embolism resistance at the whole-plant level. For instance, various papers have suggested that leaves with narrow vessels have *more* vulnerable xylem than the xylem in perennial shoots including wider vessels (Pivovaroff *et al*., 2014; Charrier *et al*., 2016; Johnson *et al*., 2016; Creek *et al*., 2018; Skelton *et al*., 2019), or that roots are more vulnerable than stems (Alder *et al*., 1996; Hacke *et al*., 2000; Martínez-Vilalta *et al*., 2002; Maherali *et al*., 2006; Pratt *et al*., 2007, 2015a), although the generally longer vessels in roots may have led to an overestimation of root vulnerability in some angiosperm studies (as mentioned previously). Other studies found that the xylem tissue of leaves, stems, and roots is more or less equally resistant to embolism (Skelton *et al*., 2017; Creek *et al*., 2018; Wason *et al*., 2018; Losso *et al*., 2019; Smith-Martin *et al*., 2020; Wu *et al*., 2020; Levionnois *et al*., 2020b; Lübbe *et al*., 2022), or that leaves are more resistant to embolism than stems in some species (Zhu *et al*., 2016; Klepsch *et al*., 2018; Levionnois *et al*., 2020b; Guan *et al*., 2022). What clearly emerges from these studies is that (1) conduit diameter is not a good proxy for drought-induced embolism resistance at the whole-plant level, (2) distal tissues (leaves, twigs) are not necessarily more vulnerable than proximal tissues (main roots, stems) in the same individuals as predicted by the vulnerability segmentation hypothesis (Tyree & Ewers, 1991), (3) hydraulic measurements from only the most resistant organ may lead to a poor correlation between traits and climate, and (4) methodological differences should be carefully considered. Studies also show that embolism propagation depends on other variables such as the proximity to gas volumes under atmospheric pressure. Guan *et al*. (2021), for instance, showed that narrow vessels in minor veins can become *more* vulnerable to drought-induced embolism than wider vessels in major veins when the intact vessels in minor veins are directly exposed to atmospheric gas in cut-open vessels.

Third, some of the scatter in the diameter–vulnerability relationship (Fig. 1a) may be attributed to P_{50} being the most common measure of xylem vulnerability. This common practice ignores other determinants of embolism formation, such as xylem traits that may impact the shape of a vulnerability curve (Cai *et al*., 2014). More importantly, the 'mean' vessel diameter may not represent the most accurate parameter that reflects the variation in vessel width. For example, mean vessel diameter is sometimes calculated as tangential diameter and sometimes as an equivalent circle diameter, and is often hydraulically weighted based on either all vessels in a cross-section, or from a subset of these vessels (usually > 100) (Scholz *et al*., 2013). Furthermore, there are multiple ways for a plant to arrive at the same mean vessel diameter (i.e., different vessel diameter frequency distributions). It is therefore more important to know the vulnerability of each vessel separately, rather than the vulnerability of a 'mean' vessel that is assumed to represent all vessels combined within an organ, although this is technically challenging (e.g. via microCT). To further complicate matters, the pressure drop may not be uniform across all vessels in a cross-section (Bouda *et al*., 2019), which may overestimate the vulnerability of mid-sized vessels and underestimate the vulnerability of large diameter vessels (assuming that the variables in Box 1 and end wall resistivity across all vessels are similar). Additional

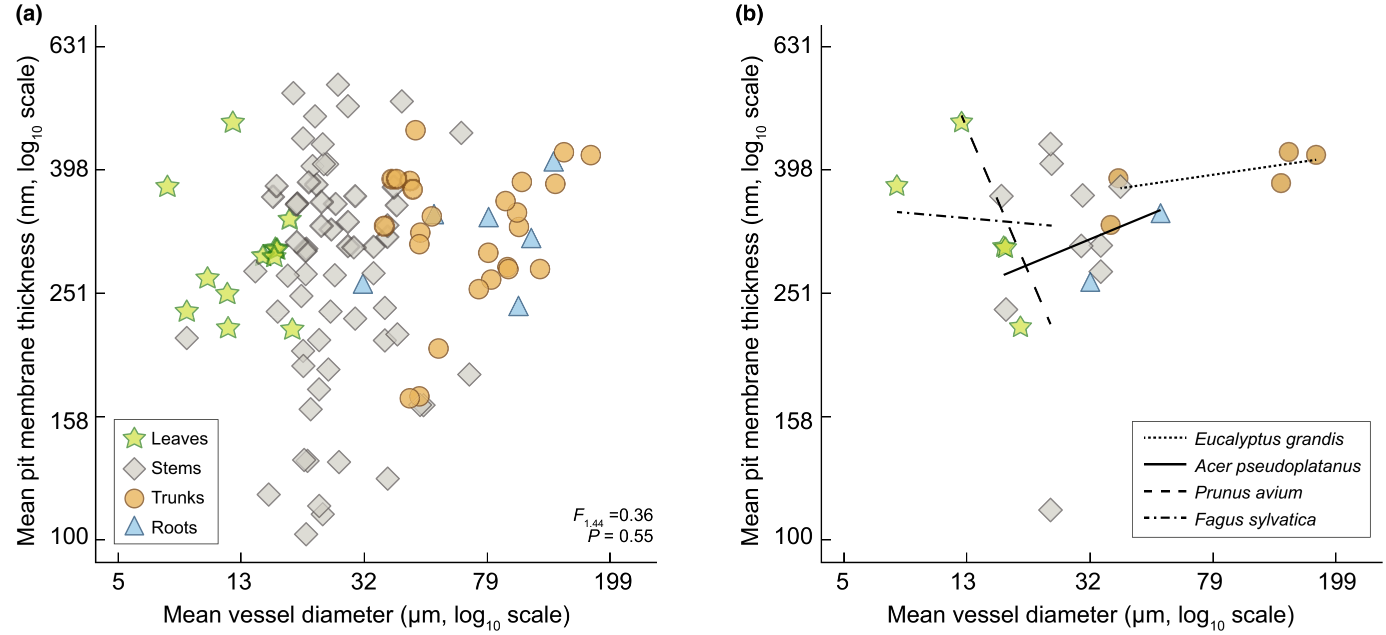


Fig. 4 Vessel diameter is not correlated with intervessel pit membrane thickness across xylem of leaves/midribs/petioles (stars), stems/branches (diamonds), trunks (circles), or roots (triangles) at the interspecies level (a), and variable at the intraspecies level for four species (b). At the interspecies level (a), data were fitted with a generalised linear mixed model, with organ and vessel diameter as fixed effects and species as a random effect; at the intraspecies level (b), different linear regression models were used for every species. Dataset is included in Table S1.

scatter in Fig. 1a could be explained by potential differences in methods that may (e.g. Venturas *et al.*, 2019) or may not (e.g. Gauthey *et al.*, 2020) impact vulnerability curves. Furthermore, temporal variation in 'native' embolism (daily or seasonal) or the high-pressure flushing of stems with water before taking an initial hydraulic measurement is known to affect the shape of a vulnerability curve (Domec *et al.*, 2006; Jacobsen *et al.*, 2007; Hacke *et al.*, 2014). Also branch age, growth rate and the number of growth rings may affect the vulnerability curve (Olson *et al.*, 2018; Pratt *et al.*, 2020; Weithmann *et al.*, 2022). In summary, mean vessel diameter does not seem to be a good proxy for drought-induced embolism, although vessel diameter could have an indirect effect via vessel length and interconnectivity. More fine-scale data at the level of individual vessels across time are desired to further clarify the role of vessel diameter on embolism resistance in more detail and to reconcile the available discrepancy in the literature.

Based on available data, there is no consistent evidence showing that mean vessel diameter is correlated with intervessel pit membrane thickness across woody angiosperms, at species (Fig. 4a) or organ level (Fig. 4b). For example, some authors have reported a positive correlation between these traits (*Eucalyptus grandis*; Pfautsch *et al.*, 2018), some studies found a negative correlation (*Prunus avium*; Guan *et al.*, 2022), and most papers reported no correlation across species (Lens *et al.*, 2011; Johnson *et al.*, 2014; Kotowska *et al.*, 2020; Guan *et al.*, 2022). Consequently, although information about intervessel pit membrane thickness within individual plants remains fairly limited, there does not seem to be a clear axial trend (Fig. 4b), which could explain why there is no consistent pattern in embolism resistance among organs as discussed before. Conversely, intervessel pit membrane thickness is strongly linked with P_{50} (Fig. 2a) and also with anatomical traits assumed to be involved in drought-induced embolism resistance (Box 1), such as vessel wall thickness (Jansen *et al.*, 2009; Li *et al.*, 2016), and the amount of stem lignification or woodiness in otherwise herbaceous lineages (Lens *et al.*, 2016; Dória *et al.*, 2018; Thonglim *et al.*, 2020). All this evidence, as well as the important role of intervessel pit membrane thickness in embolism spread (see above), suggests that this key trait serves as one of the main drivers of embolism resistance and explains why wider vessels are not necessarily more susceptible to drought-induced embolism than narrower vessels.

V. Research priorities: what do we need to focus on to understand the mechanisms behind drought-induced embolism?

Identifying the key drivers of drought-induced embolism vulnerability and predicting scaling relationships as well as the mechanistic processes behind these parameters (Box 1) remain important research priorities. A promising approach to improve our knowledge of the mechanisms behind drought-induced embolism is to build process-oriented models. Although there are very good water transport models at the plant level, these existing models do not extend down to the level of individual vessels and the three-dimensional pore network in pit membranes (Blackman *et al.*, 2016; Martin-StPaul *et al.*, 2017; Sperry *et al.*, 2017, 2019; Tai *et al.*, 2018; Venturas *et al.*, 2018, 2021). Therefore, we suggest that a new family of integrative xylem models is needed, allowing the linking of nanoscale interactions with processes at the microscale and macroscale. What is highly needed is a 3D-intervessel pit membrane model (Li *et al*., 2020; Zhang *et al*., 2020; Kaack *et al*., 2021), and especially a pore-network model (e.g. Weishaupt *et al*., 2022) of pit membranes to capture fluid transport and a large variety of physical processes and pore-scale mechanisms of interconduit pit membranes, taking also into account the possible deformation of pit membranes during their lifespan. Integrating these 3D-pit membrane models within a nanoscale to macroscale xylem model (Loepfe *et al*., 2007; Mrad *et al*., 2018, 2021; Wason *et al*., 2021) would allow the evaluation of pit membrane traits, vessel network traits (e.g. connectivity, vessel widening) and their interaction on whole-plant transpiration, construction costs and carbon income. Furthermore, accurate 3D models should include tissue capacitance (Gleason *et al*., 2014; Yazaki *et al*., 2020), which would clear the way towards evaluating the consequences of water storage in the short term (subsecond, seconds) and long term (minutes, hours, days). Finally, an integrative whole-plant model should be parameterised and validated with experimental results from methods that allow studying changes in the liquid and gas phase in xylem sap at a high temporal resolution (Pereira *et al*., 2020; Jansen *et al*., 2022), and should also account for variation in hydraulic demand during developmental plant stages across multiple species from different plant functional groups.

Advancing our understanding of drought-induced embolism will also rely on the finer details of what happens at the liquid–solid–gas–surfactant interfaces. We have made progress in understanding the 3D-nanostructure of pit membranes, and the hydraulic and ecological consequences of this mesoporous medium. For example, the effect of seasonality on pit membrane thickness and chemical composition appears to be important (Schmid & Machado, 1968; Sorek *et al*., 2020). However, details on how the 3D-construction of cellulose microfibrillar aggregates are interlinked with other pit membrane components, and how this microfibrillar network can mechanically deflect remains poorly understood (Capron *et al*., 2014; Tixier *et al*., 2014). This requires gene regulatory networks underpinning pit membrane ultrastructure, estimations of the elasticity and the strain to induce aspiration, as well as temporal changes that are likely to affect pit membrane function as plants develop (Herbette *et al*., 2015; Klepsch *et al*., 2016). The functional role of living tissues in water transport also needs more attention, including symplastic conductance between living cells and conduits, the role of amphiphilic, polar lipids such as galactolipids and phospholipids in the behaviour of nanobubbles in xylem sap during dynamic changes of pressure and temperature (Schenk *et al*., 2017, 2020; Yang *et al*., 2020; Jansen *et al*., 2022), as well as the role of ions in particle surface charge, with potential consequences to flow resistance or changes in fluid viscosity (Santiago *et al*., 2013).

In addition to the vessel-level and pit membrane-level traits discussed in the previous paragraph, whole-xylem and whole-plant structural characteristics are also thought to influence embolism vulnerability. Perhaps the most striking but least understood of these is the physiological role of hydraulic compartmentalisation (aka segmentation or sectoriality; McElrone *et al*., 2021) of xylem tissue, defined by a change in the nonrandom ending of vessels combined with reductions in conduit dimensions. For example, petioles, growth ring boundaries, nodes and side branches are places where the vessel network may exhibit an abrupt segmentation area, which may overlap with an abscission zone. Although there has been much speculation about the importance of these zones on hydraulic conductance, embolism spread and pathogen infection, there have been few data collected on the quantitative variation of the vessel network within plants and across species and their functional significance (André, 2005; Morris *et al*., 2020; Guan *et al*., 2021).

In summary, we suggest that a better understanding of drought-induced embolism could be obtained by focusing our research efforts on three broad objectives: (1) achieving a better understanding of how multiphase environments (including liquids, gases, solids, and surfactants) behave under negative pressures within the nano/microporous (< 2 nm pores), mesoporous (2–50 nm pores), and macroporous (> 50 nm pores) media of plant xylem, (2) identifying the mechanistic driver(s) of embolism initiation and spread under changing environmental conditions, and (3) evaluating the relative vulnerability of individual vessels against their surrounding xylem tissue, thereby linking local embolism to hydraulic failure of organs and whole-plants. This knowledge will be important to reconcile the current discrepancy in our discussion to which extent various traits may affect embolism vulnerability.

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Data availability

The data that support the findings of this study (Figs 1a, 2a, 4, S1) are available in the supplementary material of this article (Table S1).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Weak correlation between the xylem pressure inducing 50% loss of hydraulic conductivity (P_{50}) and mean vessel diameter (VD) across angiosperm branches, including s-shaped as well as exponential vulnerability curves.

Table S1 Datasets used for Figs 1a, 2a, 4 and Fig. S1.