



RESEARCH PAPER

Xylem embolism in leaves does not occur with open stomata: evidence from direct observations using the optical visualization technique

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Abstract

Drought represents a major abiotic constraint to plant growth and survival. On the one hand, plants keep stomata open for efficient carbon assimilation while, on the other hand, they close them to prevent permanent hydraulic impairment from xylem embolism. The order of occurrence of these two processes (stomatal closure and the onset of leaf embolism) during plant dehydration has remained controversial, largely due to methodological limitations. However, the newly developed optical visualization method now allows concurrent monitoring of stomatal behaviour and leaf embolism formation in intact plants. We used this new approach directly by dehydrating intact saplings of three contrasting tree species and indirectly by conducting a literature survey across a greater range of plant taxa. Our results indicate that increasing water stress generates the onset of leaf embolism consistently after stomatal closure, and that the lag time between these processes (i.e. the safety margin) rises with increasing embolism resistance. This suggests that during water stress, embolism-mediated declines in leaf hydraulic conductivity are unlikely to act as a signal for stomatal down-regulation. Instead, these species converge towards a strategy of closing stomata early to prevent water loss and delay catastrophic xylem dysfunction.

Keywords: Drought, embolism, hydraulics, optical visualization, stomatal closure, water stress, xylem.

Introduction

Water deficit is one of the most significant abiotic constraints to growth and survival in terrestrial plants. More frequent and severe water shortages associated with current climate warming have the potential to overcome the ability of long-lived plant species to adapt to, and survive, these rapidly changing conditions (Choat *et al.*, 2018).

For plants, a major trade-off in the acquisition of CO₂ for photosynthesis is the loss of water by transpiration, which under

well-watered conditions is routinely compensated via water uptake by the roots from the soil. However, under periods of soil-water deficit and high atmospheric evaporative demand, water transport through the plant vascular network is exposed to increasing tension (Dixon and Joly, 1895). Under severe drought, tension within the water column can exceed the species-specific air-seeding threshold (P_c), leading to cavitation and embolism propagation through the xylem vessel network,

preventing water transport (Tyree and Sperry, 1989). This can result in organ desiccation and even plant death (Tyree and Sperry, 1989; Choat *et al.*, 2012; Urli *et al.*, 2013; Anderegg *et al.*, 2014), with global incidents of drought-induced plant mortality increasingly linked to xylem embolism (Brodrribb and Cochard, 2009; Brodrribb *et al.* 2010; Choat *et al.*, 2012; Urli *et al.* 2015; Anderegg *et al.*, 2016; Adams *et al.*, 2017). Plant resistance to xylem embolism is a key fitness-related trait that is generally estimated with the xylem water potential generating 50% loss of hydraulic conductance (P_{50}), and is critical for modellers to better predict plant responses and future distributions under environmental changes (Brodrribb and Hill, 1999; Blackman *et al.*, 2012; Bourne *et al.*, 2017).

Stomatal regulation is the primary means for a plant to manage efficient carbon assimilation and transpiration while preventing the risk of xylem embolism. Stomatal closure and embolism resistance have often been considered functionally coordinated (Jones, 1998; Buckley, 2005), with several studies reporting that down-regulation of stomatal conductance is related to hydraulic decline in leaves (Brodrribb and Holbrook, 2003; Torres-Ruiz *et al.*, 2015a; Scoffoni *et al.*, 2017b) and that leaf xylem embolism may provide an important hydraulic signal for stomatal closure (Nardini and Salleo, 2000; Sperry, 2000). However, the sequence of water potential thresholds for these drought tolerance traits (leaf xylem embolism occurring before, at, or after stomatal closure) in response to desiccation remains unresolved despite the importance of this interaction in determining overall plant function during drought (Brodrribb and Holbrook, 2003; Bartlett *et al.*, 2016). Some studies have suggested that plants routinely undergo diurnal cycles of embolism accumulation and repair while stomata are open and gas exchange is maintained (Zufferey *et al.*, 2011; Trifilò *et al.*, 2015), whilst others have emphasized that plants may consistently close their stomata early to avoid xylem embolism (Salleo *et al.*, 2000; Cochard and Delzon, 2013; Hochberg *et al.*, 2017; Martin-StPaul *et al.*, 2017). Additionally, methodological limitations of measuring xylem embolism resistance in long-vessel angiosperms under tension, due to the open-vessel artefact (Cochard *et al.*, 2005, 2010), can result in significant underestimations of embolism resistance (Cochard and Delzon, 2013; Cochard *et al.*, 2013; Martin-StPaul *et al.*, 2014), further obscuring this relationship. Such methodological concerns, as well as the lack of studies coupling real-time monitoring of stomatal conductance and xylem embolism formation during desiccation across a large range of plant taxa, have impaired our ability to resolve this debate and is urgently needed to better predict plant functioning under climate change (McDowell *et al.*, 2013).

A significant step forward in our understanding of plant hydraulics may be achieved using direct visualization techniques. Direct imaging enables reliable and minimally invasive measurements of xylem vulnerability to embolism in intact plants (McElrone *et al.*, 2013; Choat *et al.*, 2015; Charrier *et al.*, 2016; Nolf *et al.*, 2017; Skelton *et al.*, 2017a; Klepsch *et al.*, 2018), while overcoming possible issues associated with standard hydraulic methods (e.g. during sample preparation and the use of short segments; see Wheeler *et al.*, 2013; Torres-Ruiz *et al.*, 2015b). Among these techniques,

the most recently developed has been the optical vulnerability (OV) technique (Brodrribb *et al.*, 2016). While most direct imaging techniques have been primarily used on stems, the OV method captures both timing and real-time propagation of embolism in the leaf xylem network of a desiccating plant. This technique has been cross-validated with a range of other hydraulic techniques (Brodrribb *et al.*, 2016; Skelton *et al.*, 2017a, 2018) and, as the OV method is relatively non-destructive, it also provides the opportunity to make concurrent measurements of other traits such as gas exchange on the same plant, thus providing a cohesive picture of whole-plant water transport.

This study aimed to resolve the sequence of water potential thresholds for both stomatal closure and embolism formation in leaves of dehydrating plants by coupling the OV technique with gas exchange measurements concurrently on the same intact plants. We tested the hypothesis that trees optimize their stomatal behaviour to close stomata at the onset of embolism formation (Klein, 2014; Skelton *et al.* 2015; Anderegg *et al.* 2016), and plants that are more resistant to xylem embolism will allow stomata to remain open longer (i.e. at lower water potentials) during drought. Our approach was 2-fold: (i) simultaneously monitoring stomatal conductance and leaf embolism during intact plant desiccation in three woody angiosperm species with contrasting embolism resistance; and (ii) performing a literature survey based on studies that used the OV method to assess the timing of stomatal closure and incipient leaf embolism across a greater range of taxa.

Materials and methods

Species and growing conditions

The timing of embolism formation and stomatal closure was monitored over the course of whole-plant desiccation in three woody angiosperm species, representing different families, that were expected to differ in their resistance to xylem embolism due to contrasting stem xylem hydraulic vulnerability (data not shown): (i) *Arbutus unedo* L. (family: Ericaceae, order: Ericales), a small evergreen tree native to the Mediterranean basin; (ii) *Ligustrum japonicum* Thunb. (family: Oleaceae, order: Lamiales), an evergreen shrub native to eastern Asia and widely used as ornamental plants in Europe; and (iii) *Prunus persica* L. Batsch (family: Rosaceae, order: Rosales), a deciduous tree native to Northwest China and widely cultivated in Southern Europe.

In May 2017, 3- to 4-year-old plants of each species were sourced from local nurseries (PlanFor nursery, Uchacq-et-Parentis, France and Le Lann nursery, Gradignan, France) and placed into climate-controlled chambers (Microclima Arabidopsis system, Snijders Labs, Tilburg, The Netherlands). Chamber CO₂ concentration, relative humidity, and temperature were set at 400 ppm, 60%, and 22 °C (day)/18 °C (night), respectively, while the photosynthetically active radiation (PAR) was 1200 μmol m⁻² s⁻¹ with a daily 12 h day/12 h night cycle. Prior to the initiation of the experiment, all pots were hand-watered daily to saturation (water was added to each plant until it leaked from the bottom drainage holes) to ensure that plants were under non-limiting soil-water conditions. Three replicate plants per species were used for all measurements. Just prior to the initiation of the optical measurements, and immediately following the installation of the stem psychrometer, the intact sapling was carefully removed from its pot to avoid damaging the root system, which was loosely wrapped in a plastic bag to ensure steady dehydration, but avoid artefacts in hydraulic measurements due to cut open vessels.

Visualization of leaf vulnerability to embolism

The accumulation of leaf vein embolism during desiccation was monitored using the OV method (Brodribb *et al.*, 2016) to construct leaf xylem vulnerability curves. One healthy, fully expanded leaf per plant, from three replicate plants per species, was selected for continuous embolism visualization while still attached to the parent plant. Immediately following the plant removal from the pot, an individual leaf was placed on the scanner (Perfection V800 Photo, EPSON, Suwa, Japan) and fixed with a microscope slide and clear adhesive tape to minimize leaf movement due to shrinkage during dehydration. Scan resolution was set at 2400 dpi which ensured sufficient visualization of midrib and major veins. Each leaf was scanned in transmission mode every 4 min until the leaf was observed to turn from green to brown, indicating tissue death. The scanner was placed within the climate chamber to ensure the entire plant was exposed to a homogeneous light and temperature environment.

By continuously scanning the leaf, changes in light transmission through the xylem were observed that corresponded to air entry into the xylem conduits (Brodribb *et al.*, 2016). Image analysis was carried out using ImageJ (NIH), with embolism events easily identified in image difference subtractions. The 'Analyse Particles' function was used to measure the area of embolized pixels in each image; threshold analyses were used to remove noise related to leaf movement from drying. An optical vulnerability curve was obtained for each individual by converting the cumulative embolized area to a percentage of total embolized pixels (by dividing the cumulative embolized area by the total cumulative area), incorporating both major and minor vein orders, and combining this with the water potential (as described below) to obtain percentage increase in embolism as a function of water potential (P , -MPa) (see <http://www.opensourceov.org> for the detailed image analysis protocol). Each replicate per species was fitted with a Weibull curve using the 'fitcond' function in the 'fitplc' package in R (Duursma and Choat, 2017). From these curves, the water potentials inducing 12% (P_{12}), 50% (P_{50}), and 88% (P_{88}) of embolized pixels were obtained per individual before average values were calculated per species ($n=3$ per species). P_{50} indicates the xylem tension inducing a 50% cumulative embolized area. The P_{50} of a species is widely considered to represent xylem vulnerability to hydraulic dysfunction and is used for comparisons across species. P_{12} is considered to represent the water potential threshold for the initial air entry causing embolisms, and P_{88} to represent the tension associated with a high level of functional impairment or mortality risk (Urli *et al.*, 2013; Hammond *et al.*, 2019).

Stem water potential

Stem water potentials (P , -MPa) were monitored every 30 min in each individual plant throughout desiccation via a psychrometer (ICT Armidale, NSW, Australia) that was fitted on the stem, as close as possible to the leaf that was placed into the scanner (between 10 cm and 20 cm). Reference leaf water potentials and stem water potentials (individual leaves that were dark-adapted for 30 min prior to measurement) were taken 3–4 times a day during the drying period using a Scholander pressure chamber (DG-Meca, Gradignan, France). At least two leaves were sampled during each time point, one from the branch that was undergoing optical imaging, and the other from the branch being measured for stomatal conductance (g_s), in order to capture spatial variation. The water potentials among leaf replicates at each time point were consistently similar ($\pm 10\%$), irrespective of sampling location, indicating that water potential was equilibrated throughout the plant. We also found good agreement of ± 0.5 MPa between the psychrometer values and those obtained with the pressure chamber, indicating that the psychrometers accurately captured water potentials of leaves undergoing imaging and stomatal conductance measurements (see Supplementary Fig. S3 at JXB online).

Stomatal conductance measurements and safety margin calculation

To determine the water potential at stomatal closure, we measured g_s simultaneously on plants that were being scanned for embolism visualization.

One fully expanded leaf of each replicate plant (three plants per species), on a branch adjacent to the leaf being imaged, was enclosed in an open gas exchange system (GFS-3000, Heinz Walz GmbH, Effeltrich, Germany) and exposed to saturating ambient light ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$), ambient atmospheric CO_2 of $400 \mu\text{mol mol}^{-1}$, relative humidity $\sim 60\%$, leaf vapour pressure deficit (VPD) maintained between 0.9 kPa and 1.4 kPa, and leaf temperature of 22°C . To ensure that we captured maximum stomatal conductance ($g_{s\text{max}}$) for the species, g_s measurements were commenced prior to plant desiccation to ensure that each individual replicate was measured at full turgor (i.e. under well-watered conditions). Measurements of g_s were conducted on each replicate plant in the morning, throughout plant dehydration, until stomatal closure was observed in the individual plant.

For each species, the response of stomatal conductance to decreasing water potential was parameterized using local polynomial regression and fitting a 'loess' model in conjunction with the 'fitcond' function in the 'fitplc' R-package. The resulting fit was used to determine the water potential corresponding to a 90% reduction in stomatal conductance (herein referred to as stomatal closure; P_{close}). The P_{50} safety margin was calculated as the difference in water potentials between P_{close} and P_{50} , whereas the P_{12} safety margin was calculated as the difference between P_{close} and P_{12} as proposed by Martin-StPaul *et al.* (2017).

Literature review

In order to minimize the uncertainty associated with different techniques used to measure vulnerability to embolism in leaves (Scoffoni *et al.*, 2017b), the literature review included only species measured via direct observations using the OV method on intact plants. The conservative approach utilized in the bibliographic search limited the possible methodological issues associated with more destructive methods that require detached leaves; thus, we report P_{50} values associated with leaf xylem only, and not P_{50} values of the whole leaf (as obtained with the standard techniques). Additionally, the literature synthesis has enabled the examination of the relationship between stomatal closure and embolism formation across a broader range of angiosperm species, in addition to the empirical data collected in the experimental study.

We extracted leaf xylem traits and the water potential associated with stomatal closure from recent published sources (see Supplementary Table S1 for species details). When values of P_{12} and P_{50} were not reported in numerical form, they were extracted from published graphs of vulnerability curves using grab graph data on linux (g3data 1.5.2, Github.com). If stomatal closure data were not available, we used the leaf water potential at turgor loss (P_{tip}) as a surrogate for P_{close} (Brodribb and Holbrook, 2003; Martin-StPaul *et al.*, 2017). Using a similar approach to Martin-StPaul *et al.* (2017), we analysed the association between P_{close} and P_{12} and P_{50} , and fitted a segmented regression to the data using the Segmented package in R. The model was used to identify the break points in the x -axis (i.e. the embolism resistance value at which there is a change in the covariation between P_{close} and leaf embolism resistance).

Results

Stomatal closure

Mean maximum stomatal conductance (g_s), measured under well-watered conditions, was $114.3 \pm 15.5 \text{ mmol m}^{-2} \text{ s}^{-1}$ for *A. unedo*, $77.0 \pm 7.5 \text{ mmol m}^{-2} \text{ s}^{-1}$ for *L. japonicum*, and $125.5 \pm 3.3 \text{ mmol m}^{-2} \text{ s}^{-1}$ for *P. persica* (Fig. 2). As expected, g_s declined in response to increasing desiccation in all three species (Fig. 2) with stomatal closure (P_{close} ; $g_s < 10\%$ of maximum) occurring at water potentials ranging from -1.83 MPa in *A. unedo* to -2.42 MPa in *P. persica* (Table 1).

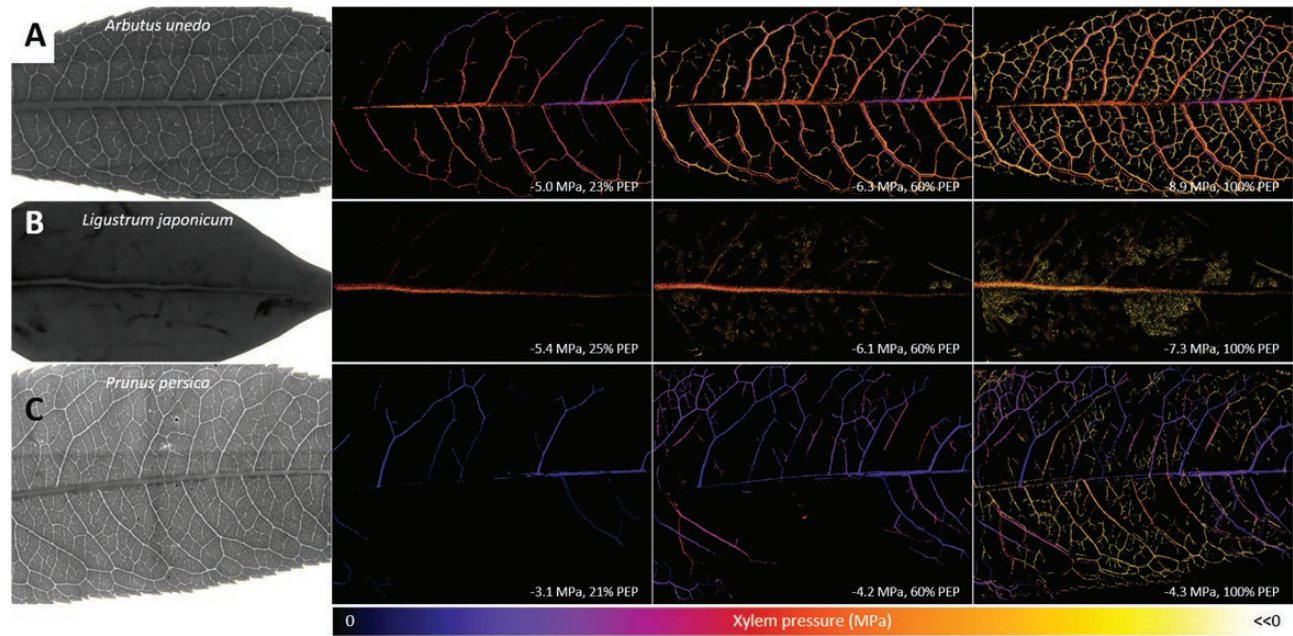


Fig. 1. From the series of leaf images taken during whole-plant desiccation of (A) *Arbutus unedo*, (B) *Ligustrum japonicum*, and (C) *Prunus persica*. Each row corresponds to the same individual leaf of the same species. From left to right for each species: the transmitted light image of the leaf highlighting the majority of leaf veins. The additional three colour map images for each species show the spatial progression of embolism at decreasing water potentials of the same leaf corresponding to ~25, 60, and 100% accumulated area of embolism events of the leaf vein network, respectively. The blue colours indicate embolism events occurring at water potentials higher than the events shown in orange and yellow.

Leaf vulnerability to embolism

Leaf xylem embolism was clearly visualized in the three woody angiosperm species using the optical technique (Fig. 1). The onset of leaf embolism was visualized between 31 h (in *P. persica*) and 166 h (in *A. unedo*) following the installation of the stem psychrometers and removal of the plant from the pot and excess soil. No major embolism events (i.e. the percentage area embolized remained below 12%) in the leaf were detected while the stomata were open (i.e. from the beginning of the dehydration until reaching P_{close}). Midribs and major veins in the leaves of the three species appeared to embolize before (i.e. at higher water potentials) minor veins (Fig. 1).

The safety margin between stomatal closure (P_{close}) and embolism formation (P_{12}) was greatest in *L. japonicum* with a margin of 2.88 MPa followed by *A. unedo* with a margin of 2.79 MPa. In contrast, the stomata of *P. persica* remained open at water potential values closer to incipient embolism formation in the leaf, with a reported safety margin of 0.43 MPa. The shortest time between stomatal closure and P_{12} was 21 h (in *P. persica*) and the longest time was 123 h (in *A. unedo*) (Supplementary Fig. S2). However, there was large variation in the time that individual plant replicates took to dry after cessation of watering, which was most probably due to variation in root mass and leaf area (data not shown).

All species showed a sigmoidal response in percentage embolized vein area to decreasing water potential, with an initial plateau phase where no embolism was detected until a critical water potential threshold was crossed. This initial

plateau was shortest for *P. persica* (<12% embolized area) at -2.85 MPa, whereas *A. unedo* and *L. japonicum* were similarly resistant, with the onset of embolism occurring at around -4.62 MPa and -4.78 MPa, respectively (Fig. 2; Table 1). Individual leaves of each species showed little variation in patterns of embolism accumulation; P_{50} varied between individual leaves by 0.21 MPa in *P. persica* and 0.6 MPa in *A. unedo* (Supplementary Fig. S1).

Literature survey

To date, five other studies have included simultaneous measurements of leaf xylem embolism via the OV and stomatal closure (P_{close}) or turgor loss point (P_{tip}) on intact plants, and thus were included in the literature synthesis (Supplementary Table S1 for details).

Leaf embolism resistance (as described by P_{50}) of the 15 species included in the literature survey ranged from -1.15 MPa for *Helianthus annuus* to -6.25 MPa for *Bursaria spinosa* (Fig. 3b). The onset of leaf embolism (P_{12}), which is a more conservative descriptor of embolism resistance, varied from -1.06 MPa for *H. annuus* to -5.8 MPa for *B. spinosa* (Fig. 3a). The range of water potentials over which the same species closed their stomata (P_{close}) was narrower than embolism resistance and varied from -0.7 MPa for *V. vinifera* to -3.2 MPa for *Quercus douglasii* (Fig. 3a, b).

Fitting a segmented regression (as per Martin-StPaul et al. (2017) between P_{close} and embolism resistance (either P_{12} or P_{50}) provided a better fit of the data than a linear model (segmented model $R^2=0.84$ contrasting with linear model $R^2=0.4$). This model was then used to determine the breakpoints in the x -axis,

which correspond to the water potential at which there is a change in the covariation between P_{close} and leaf embolism resistance, P_{12} and P_{50} . Thus, the breakpoint for the segmented relationship between P_{close} and P_{12} is -2.6 ± 0.2 MPa and that between P_{close} and

P_{50} is -3.47 ± 0.3 MPa. The y -axis intercept for this breakpoint corresponds to the upper limit of P_{close} , which was approximately -2.65 MPa, with P_{close} reaching a plateau by -3 MPa.

For the 12 species with P_{12} values higher than the breakpoint, the relationship between P_{12} and P_{close} was close to the 1:1 line, indicating that stomatal closure occurred very close to (or at) the point of incipient leaf embolism (i.e. the safety margin is ~ 0 MPa), with only three species, *Quercus kelloggii*, *Q. lobata*, and *Q. douglasii*, having a P_{12} higher than P_{close} (Fig. 3a). All species included in the literature synthesis closed their stomata prior to reaching their corresponding P_{50} values; that is, the P_{50} safety margin was always positive (Fig. 3b). Additionally, the most resistant species to xylem embolism had the widest safety margin between P_{close} and their corresponding P_{12} (Fig. 3c) or P_{50} (Fig. 3d).

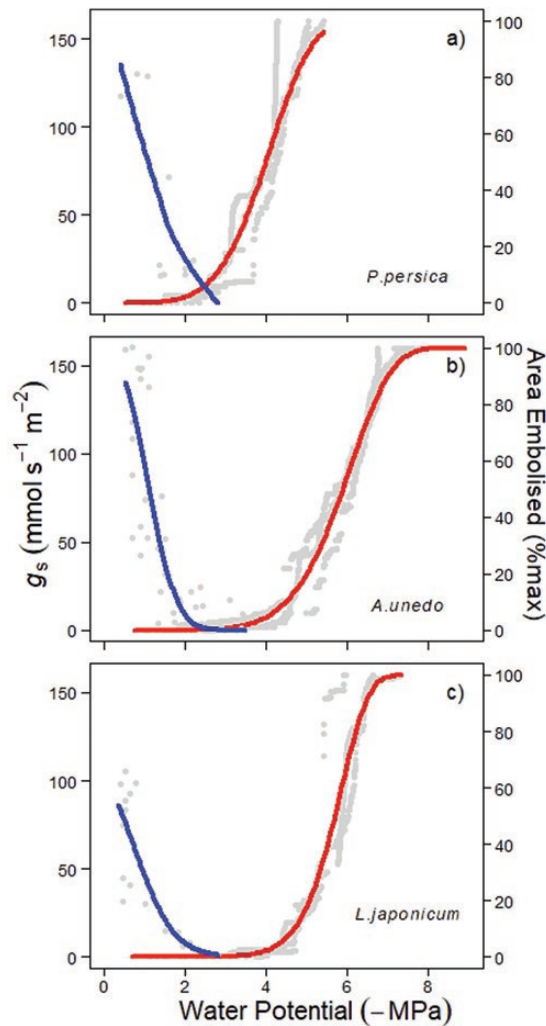


Fig. 2. Response of stomatal conductance (g_s ; blue lines) during plant dehydration and optical vulnerability curves (as represented by percentage area embolized; red lines) to decreasing water potential (MPa) for the three species measured in this study: (a) *P. persica*, (b) *A. unedo*, and (c) *L. japonicum*. The grey points represent all individual data points from three individuals per species.

Discussion

For the three tree species in our experimental study, the onset of leaf hydraulic dysfunction always occurred after closure of the stomata. For the most hydraulically vulnerable species, *P. persica*, which exhibited the smallest safety margin of 0.43 MPa between P_{close} and P_{12} , stomatal closure and incipient leaf xylem embolism were temporally separated by at least 21 h (under conditions of rapid desiccation in the growth chamber). For the most drought-resistant species, *A. unedo*, with a safety margin of 2.79 MPa, embolism was not visualized in leaf xylem for at least 2 d after stomatal closure. After stomatal closure, subsequent water loss from the leaf (via cuticular transpiration and stomatal leakiness) is generally minimal (Brodrribb *et al.*, 2014; Duursma *et al.*, 2019). Hence, under natural conditions, stomatal regulation would largely prevent these species from reaching water potentials likely to result in significant xylem embolism, except under cases of severe and/or prolonged drought.

Our finding that stomatal closure and subsequent incipient leaf embolism is temporally separated, in some cases by >2 MPa and as many as 5 d (Fig. 2; Supplementary Fig. S2), does not support the idea that leaf embolism is a driver of stomatal closure in these species, but instead indicates that stomatal down-regulation occurs to avoid the leaf embolism threshold altogether (Tyree and Sperry, 1988). In agreement, Hochberg *et al.* (2017) and Cardoso *et al.* (2018) showed via direct imaging in grapevine and sunflower, respectively, that complete stomatal closure preceded the appearance of

Table 1. Mean (95% confidence interval in parenthesis) water potentials at which 12, 50, and 88% loss of embolized pixels and a reduction of 10% and 50% stomatal conductance and stomatal closure (corresponding to 90% reduction in g_s) occur in leaves of *Arbutus unedo*, *Ligustrum japonicum*, and *Prunus persica* saplings ($n=3$)

Species	Stomatal response			Leaf vulnerability to embolism		
	P_{gs10} (MPa)	P_{gs50} (MPa)	P_{close} (MPa)	P_{12} (MPa)	P_{50} (MPa)	P_{88} (MPa)
<i>Arbutus unedo</i>	-0.54 (NA, -0.83)	-1.14 (-0.95, -1.59)	-1.83 (-1.53, -2.17)	-4.62 (-4.60, -4.64)	-5.90 (-5.89, -5.91)	-6.93 (-6.92, -6.94)
<i>Ligustrum japonicum</i>	-0.34 (NA, -0.64)	-0.96 (-0.72, -1.21)	-1.90 (-1.30, -2.30)	-4.78 (4.76, -4.81)	-5.70 (-5.68, -5.72)	-6.40 (-6.38, -6.42)
<i>Prunus persica</i>	-0.75 (NA, -1.09)	-1.28 (-0.99, -1.59)	-2.42 (1.45, NA)	-2.85 (-2.84, -2.86)	-3.99 (-3.99, -4.00)	-4.99 (-4.97, -5.00)

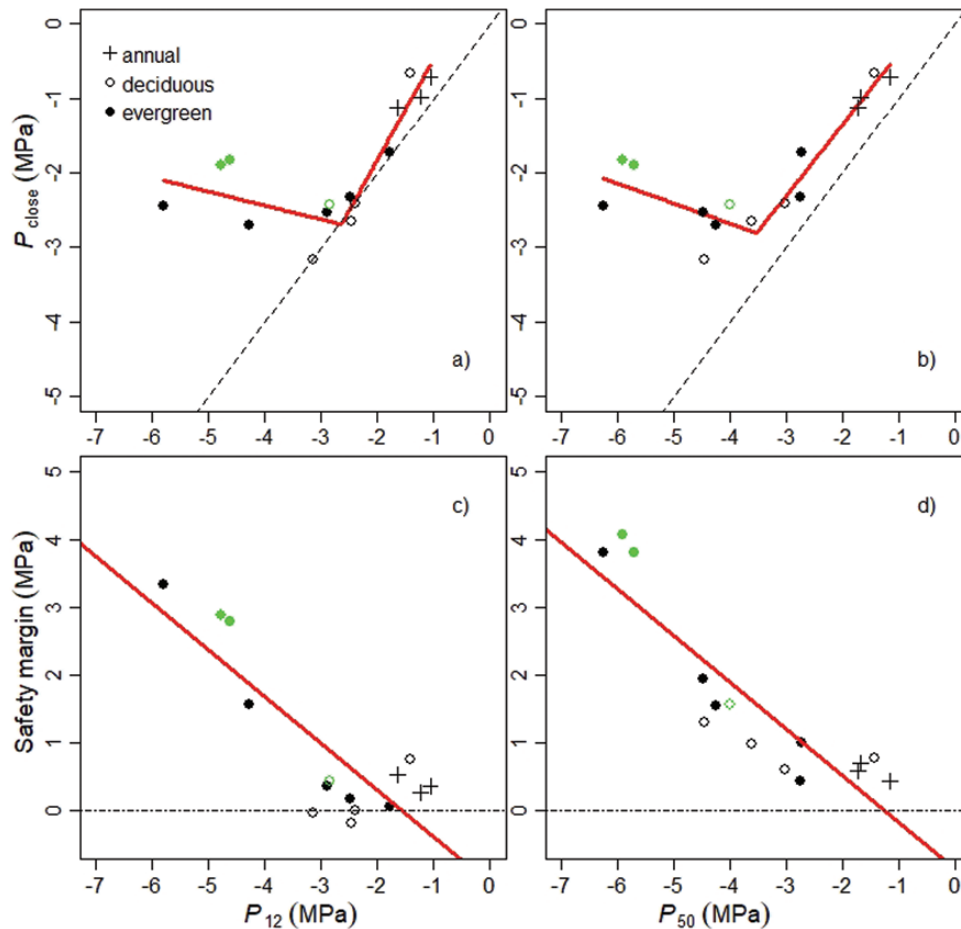


Fig. 3. Top row: relationship between stomatal closure (P_{close}) and (a) onset of leaf xylem embolism (P_{12}) and (b) P_{50} from a literature synthesis of 15 species that were measured via the OV technique. The dashed line is the 1:1 line. Any species below the line close their stomata after P_{12} . The red line is the best fit with a segmented regression, showing a significant breakpoint for P_{12} of -2.6 MPa or P_{50} of -3.47 MPa corresponding to an average P_{close} of about -2.6 MPa. Bottom row: the linear relationship between embolism resistance as represented by (c) leaf P_{12} and (d) leaf P_{50} and stomatal safety margin (the difference in water potential between stomatal closure and embolism formation; $P_{\text{close}} - P_{12}$) ($P < 0.001$). All points correspond to individual species; green points correspond to the three species measured by the authors. Different functional groups are indicated in the legend. Figure adapted from Martin-StPaul *et al.* (2017). Plant resistance to drought depends on timely stomatal closure. *Ecology Letters* 20, 1437–1447.

embolism in leaves by several days. However, such findings are inconsistent with previous studies that have suggested that embolism-mediated reductions in leaf hydraulic conductivity may act as a signal for stomatal down-regulation during water limitation (Nardini *et al.*, 2001; Brodribb and Holbrook, 2003; Lo Gullo *et al.*, 2003; Torres-Ruiz *et al.*, 2015a; Bartlett *et al.*, 2016; Savi *et al.*, 2016). Clearly, more work needs to be done to resolve the sequence of water potential thresholds associated with different drought tolerance traits within a plant, because this will inform our understanding of the mechanisms plants employ to avoid drought-induced hydraulic failure.

Furthermore, our literature synthesis showed that among the recently published studies incorporating direct observation of leaf vulnerability to embolism via the OV method with paired stomatal conductance, all species closed their stomata prior to leaf P_{50} , and 80% of them before P_{12} . Although our study provides a small representation of species due to the limited number of studies that performed direct observations of xylem embolism with concurrent gas exchange measurements, this synthesis has

been supported by the finding of a positive stomatal safety margin in stems in a recent meta-analysis by Martin-StPaul *et al.* (2017), where most P_{close} values were higher than their respective stem P_{50} values. Indeed, among the species in our synthesis, as well as in Martin-StPaul *et al.* (2017), there was a general pattern of increasing stomatal safety margins associated with increasing embolism resistance. The observation that the most drought-resistant species close their stomata at a water potential much higher than required to initiate embolism in both leaves and stems, does not support the hypothesis that plants maximize plant CO_2 assimilation under drought through tight coordination between P_{close} and embolism resistance (Klein, 2014; Skelton *et al.*, 2015; Anderegg *et al.*, 2016). Indeed, in a recent study of 20 broad-leaf tree species, larger safety margins between stomatal closure and embolism formation were found to be correlated with decreased tree mortality (Chen *et al.*, 2019). Taken together, this suggests that many woody angiosperm species employ a conservative stomatal strategy during water limitation, closing stomata early and sacrificing potential carbon gain in favour of protecting against hydraulic dysfunction. This is underpinned by our observation

that independent of the species resistance to embolism (P_{50}), no plant maintains stomatal conductance beyond -3 MPa, indicating that there is a threshold water potential beyond which stomatal conductance (and thus photosynthesis) does not occur. This was demonstrated by [Martin-StPaul et al. \(2017\)](#); importantly however, in our study, these traits were measured simultaneously on intact plants, rather than in separate individuals or on excised branches. It is important to note that safety margins increasing concurrently with decreasing leaf P_{50} does not necessarily lead to longer survival time during drought because plant survival capacity depends on the interaction of multiple traits that affect the rate of water loss after stomatal closure, including minimum leaf transpiration [water loss through the leaf cuticle and due to the stomatal leakiness (e.g. [Blackman et al., 2016](#))] and hormonal regulation [such as abscisic acid (ABA) ([Brodribb et al., 2014](#))]. Future work needs to integrate these mechanisms with our current understanding of leaf hydraulics.

Only the deciduous species in the literature synthesis had negative safety margins between P_{close} and the onset of leaf embolism formation ([Fig. 3](#)). These species had a more 'risky' hydraulic strategy and continued to fix carbon at water potentials close to those inducing hydraulic dysfunction. This could compensate for their shorter growing season than evergreen species, due to annual leaf shedding, by maximizing photosynthesis and subsequent carbon uptake at the expense of maintaining positive stomatal safety margins ([Markesteijn et al., 2011](#); [Fu et al., 2012](#)). Our finding is consistent with other studies that have found deciduous species tend to favour maximizing photosynthetic assimilation, whereas evergreen species favour increased leaf life span due to higher nutrient and carbon investment in leaf production ([Kröber et al., 2014](#); [Zhang et al., 2017](#)).

While many studies proposed that xylem embolism is primarily responsible for whole-leaf hydraulic decline during dehydration ([Brodribb et al., 2016](#); [Skelton et al., 2017a](#)), this was often based on indirect evidence ([Cochard et al., 2002](#); [Nardini et al., 2003](#); [Trifilo et al., 2003](#); [Johnson et al., 2009](#)). On the other hand, other studies have suggested that hydraulic conductance outside the xylem is the main driver of K_{leaf} decline, particularly prior to P_{close} or P_{tp} , and may help protect the xylem from incurring embolism ([Scoffoni et al., 2014, 2017a, b](#); [Trifilo et al., 2016](#)). It is important to be able to separate these two hydraulic pathways in a leaf when considering a species' vulnerability to drought-induced failure. Extra-xylary declines in K_{leaf} (prior to stomatal closure and/or turgor loss) are often rapidly recoverable ([Scoffoni et al., 2014](#); [Zhang et al., 2016](#)) when compared with declines due to embolism in the leaf xylem, which require longer recovery times and may cause long-lasting depressions of gas exchange ([Skelton et al., 2017b](#)). Indeed, rapid declines in K_{leaf} at high water potentials prior to leaf xylem embolism have been observed for some species ([Brodribb and Holbrook, 2006](#)). One important distinction between our study and those that found negative safety margins [i.e. embolism formation occurs while stomata are open (e.g. [Bartlett et al., 2016](#))], is that the OV method (used in this study and the additional studies included in the literature synthesis) provides a direct observation of leaf vulnerability to embolism. Many other common leaf hydraulic techniques, such as the rehydration kinetic method or the evaporative flux method, measure bulk K_{leaf} and incorporate both

xylary and non-xylary pathways when quantifying leaf hydraulic vulnerability to drought. By not disentangling the extra-xylary contribution to K_{leaf} decline during desiccation, we could be vastly overestimating the species' 'non-recoverable' vulnerability to hydraulic dysfunction, particularly during the initial vulnerability at mild water deficits ([Scoffoni et al., 2017b](#)). This lack of distinction could help explain some of the observations of negative stomatal safety margins (due to rapid extra-xylary reductions in K_{leaf}), as well as observations of diurnal cycles of hydraulic loss of conductivity and recovery in concert with midday depressions of stomatal conductance ([Brodribb and Holbrook, 2004](#); [Johnson et al., 2009](#); [Zufferey et al., 2011](#)), given the increasing evidence that embolism refilling under tension is rare ([Cochard and Delzon, 2013](#); [Charrier et al., 2016](#)). It is clear that our understanding of the mechanisms responsible for the desiccation-induced decline in K_{leaf} is incomplete. However, the capacity to easily measure leaf embolism accumulation in isolation from the extra-xylary pathway using the OV technique provides an important step in quantifying non-reversible leaf hydraulic decline under drought. In conclusion, embolism in the leaf xylem does not apparently form while the stomata are open, but only occurs after stomatal closure. It is therefore unlikely that xylem embolism-mediated reductions in leaf hydraulic conductivity act as a signal for stomatal down-regulation during water limitation. Instead, early stomatal closure provides an important pre-emptive safety mechanism limiting hydraulic dysfunction in leaves by reducing plant transpiration, and therefore the risk of reaching water potential values that induce the formation of embolism in the xylem. Thus, this study shows that the species we studied here have all converged towards a strategy of drought avoidance ([Delzon, 2015](#)) by minimizing water loss under drought conditions, at the expense of carbon fixation.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Species names, family, and functional group of the species included in the literature synthesis.

Fig. S1. Accumulation of leaf embolism with decreasing water potential ($-$ MPa) during desiccation measured using the optical vulnerability method in this study is shown for *A.unedo*, *L.japonicum*, and *P.persica*.

Fig. S2. Accumulation of leaf embolism over time during desiccation measured using the optical vulnerability method in this study for *A.unedo*, *L.japonicum*, and *P.persica*.

Fig. S3. Comparison of water potential measurements using two techniques for the three study species.

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