

Xylem Embolism Resistance Determines Leaf Mortality during Drought in *Persea americana*^{1[OPEN]}

Amanda A. Cardoso, Timothy A. Batz, and Scott A. M. McAdam^{2,3}

Purdue Center for Plant Biology, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

ORCID IDs: 0000-0001-7078-6246 (A.A.C.); 0000-0002-0601-4361 (T.A.B.); 0000-0002-9625-6750 (S.A.M.M.).

The driver of leaf mortality during drought stress is a critical unknown. We used the commercially important tree *Persea americana*, in which there is a large variation in the degree of drought-induced leaf death across the canopy, to test whether embolism formation in the xylem during drought drives this leaf mortality. A large range in the number of embolized vessels in the petioles of leaves was observed across the canopy of plants that had experienced drought. Despite considerable variation between leaves, the amount of embolized vessels in the xylem of the petiole strongly correlated with area of drought-induced tissue death in individual leaves. Consistent with this finding was a large interleaf variability in xylem resistance to embolism, with a 1.45 MPa variation in the water potential at which 50% of the xylem in the leaf midrib embolized across leaves. Our results implicate xylem embolism as a driver of leaf mortality during drought. Moreover, we propose that heterogeneity in drought-induced leaf mortality across a canopy is caused by high interleaf variability in xylem resistance to embolism, which may act as a buffer against complete canopy death during prolonged drought in *P. americana*.

Extreme drought events have been intimately associated with widespread episodes of tree mortality across the globe especially in the past few decades (Allen et al., 2010; Anderegg et al., 2016; Choat et al., 2018). In addition to extreme drought, periods of moderate soil water limitation are regularly associated with episodes of canopy area reduction through the shedding of branches and leaves (Rood et al., 2000; Hochberg et al., 2017). This mortality is often heterogeneous across the canopy of drought-stressed trees, with live and dead branches and leaves coexisting side by side (Rood et al., 2000; Davis et al., 2002). The cause of heterogeneity in drought-induced branch and leaf mortality remains unclear.

Multiple mechanisms have been hypothesized to drive canopy mortality and ultimately plant death during drought (McDowell et al., 2008; Brodribb and Cochard 2009; Sala et al., 2010; Choat et al., 2018). These explanations range from carbon starvation through to failure of the hydraulic system. Recent evidence

suggests that hydraulic failure is likely to be the primary determinant of plant mortality during drought stress (Choat et al., 2018). This is because plants replace the water lost by transpiration through a network of xylem conduits responsible for lifting water from the soil under negative tension. The integrity of a water column under negative tension is under constant risk of failure from the invasion of air (Tyree and Sperry 1989). Whenever tensions in the xylem exceed a critical threshold, air is pulled into the conduit lumen, where it rapidly expands forming an air cavity that blocks the flow of water through the conduit (i.e. embolism). Increasing periods of water deficit result in higher tensions placed on the water column and an increased probability that embolism will spread throughout the xylem network causing systemic failure of the hydraulic system. The formation of embolism in the xylem is not reversible when the water column is under tension (Charrier et al., 2016), and consequently, major declines in hydraulic conductance occur once embolism forms during drought. This decline in hydraulic function leads to extensive canopy death and tree mortality during drought (Brodribb and Cochard 2009; Barigah et al., 2013).

Considerable variation in the vulnerability to embolism across species has been documented (Choat et al., 2012). The large differences in vulnerability between species appear to be driven by intrinsic features of the xylem, especially conduit dimensions and the porosity of pit membranes (Pittermann et al., 2010; Lens et al., 2011). The lethal risk of xylem embolism formation during water deficit places a strong selective pressure on the evolution of traits that either increase the resistance of xylem to embolism formation or that prevent

¹This work was supported by the U.S. Department of Agriculture National Institute of Food and Agriculture (Hatch Project 1014908 to S.A.M.M.).

²Author for contact: smcadam@purdue.edu.

³Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Scott A. M. McAdam (smcadam@purdue.edu).

A.A.C. and S.A.M.M. conceived the experiment, collected data, and wrote the manuscript with help from T.A.B.

^[OPEN]Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.19.00585

plants experiencing negative water potentials (Ψ_l) during drought (i.e. highly efficient stomatal closure and minimal cuticular conductance to water vapor; Martin-StPaul et al., 2017). Many studies have found associations between the distributional limits of plant species across aridity gradients and xylem resistance to embolism, with species native to dry environments often having highly resistant xylem (Pockman and Sperry 2000; Choat et al., 2012).

Whereas studies on the variability in hydraulic vulnerability among plant species are common, there are limited data demonstrating within-species variation (Hacke and Sauter 1996; Mencuccini and Comstock 1997; Kolb 1999; Jacobsen et al., 2014; Lamy et al., 2014; Schuldt et al., 2016), and even fewer studies documenting the variation in vulnerability between similar organs within a plant (Cochard et al., 1999; Wortemann et al., 2011; Bouche et al., 2016). Furthermore, due to the technical limitations of measuring embolism in leaves, the vast majority of these studies have been performed on stems. The few studies performed so far using noninvasive imaging techniques capable of generating a vulnerability curve from a single leaf suggest that a large, and as yet unexplained, interleaf variability in embolism resistance exists in many species (Johnson et al., 2018; Lamarque et al., 2018; Rodriguez-Dominguez et al., 2018). This high variability in leaf vulnerability within an individual may be a major driver of within-canopy or population responses to prolonged drought.

There is evidence for variation in the threshold of mortality across the canopy of an individual during drought (Rood et al., 2000). This temporal and spatial variation in mortality occurs as whole branches, individual leaves, and even within an individual leaf – referred to as partial leaf area mortality. Partial leaf death associated with negative Ψ_l is commonly observed in evergreen species (which lack the capacity to shed leaves in response to drought; Tyree et al., 2002). Hoffmann et al. (2011) found that the percentage of leaf death in a branch from a plant exposed to drought correlates with the amount of native embolism in the branch bearing these leaves, and similarly, Davis et al. (2002) found that shoot dieback during drought was likely linked to stems reaching threshold water potentials known to induce embolism. Given that during drought, stomatal closure precedes the formation of embolism (Hochberg et al., 2017; Martin-StPaul et al., 2017), a uniform Ψ_l is often measured across the canopy. Thus, we would hypothesize that variation in partial leaf mortality across a canopy exposed to a uniform Ψ_l during drought may be driven by differences in the embolism resistance between leaves.

We selected *Persea americana*, a species in which partial leaf death during drought is well described (Neuhaus et al., 2007), to test whether leaf embolism drives partial leaf area mortality and whether heterogeneous leaf mortality is associated with a large variation in embolism resistance between individual leaves. We exposed *P. americana* plants to a controlled drought

until incipient signs of leaf mortality were observed, after which plants were rewatered and allowed to fully rehydrate. Emboli in the xylem of the petioles of individual leaves were imaged after drought using cryo-SEM and the degree of variation in embolism resistance across the canopy was determined by the optical method.

RESULTS

The withholding of water from *P. americana* plants leads to a gradual decline in Ψ_l , such that 18 d after the withholding of water, Ψ_l had declined to -2.5 ± 0.3 MPa (mean \pm SE; Supplemental Fig. S1). The exposure of plants to the drought stress resulted in a range of visible leaf necrosis across the canopy (Fig. 1A; Supplemental Fig. S2). On average, the drought resulted in a $41 \pm 8\%$ (mean \pm SE) reduction in viable leaf tissue area across the three individuals exposed to the stress (Fig. 1A). The variation across the canopy in the distribution of this area of necrosis was considerable (Fig. 1A; Supplemental Fig. S2). A small percentage of leaves (4%) in all individuals showed no sign of leaf necrosis, surviving the drought stress with no visible damage (Fig. 1A). In contrast, damage to neighboring leaves in the same canopy was highly variable, ranging from a small percentage of total leaf area through to 12% of leaves in the canopy exhibiting 80% to 100% leaf area mortality as a result of the drought stress. The majority of leaves across the canopy displayed 40% to 59% leaf area mortality. There was a degree of heterogeneity in the location of necrotic regions across leaves within the same canopy (Supplemental Fig. S2). Whereas most regions of leaf death appeared to have an origin at the distal leaf tip with varying degrees of expansion toward the base of the leaf, some leaves displayed patterns of leaf death associated with particular sides of the midrib or extended from the leaf margin into laminal regions between major veins.

Drought stress also led to the formation of embolism in the xylem of leaf petioles (Fig. 1, B and C). The observed embolism in the xylem of the petiole occurred in vessels randomly distributed across the xylem, regardless of the degree of embolism (Fig. 1C). A large variation in the area of embolized vessels was observed in the petiole of leaves taken from across the canopy. All plants had leaves with a percentage of embolized vessels in the petiole xylem ranging from 3% through to nearly 100%. The area of embolized vessels in the xylem of the petiole strongly correlated with the percentage of leaf area mortality caused by drought ($r = 0.80$, $P < 0.001$; Fig. 1B).

A considerable variation in xylem resistance to embolism formation was observed using the noninvasive optical vulnerability technique across leaves of *P. americana* (Fig. 2A). This variation among leaves was observed in the Ψ_l at which (1) embolism was first observed, (2) at which 50% of the accumulated embolized area of the xylem had occurred (P_{50}), (3) at which 88% of

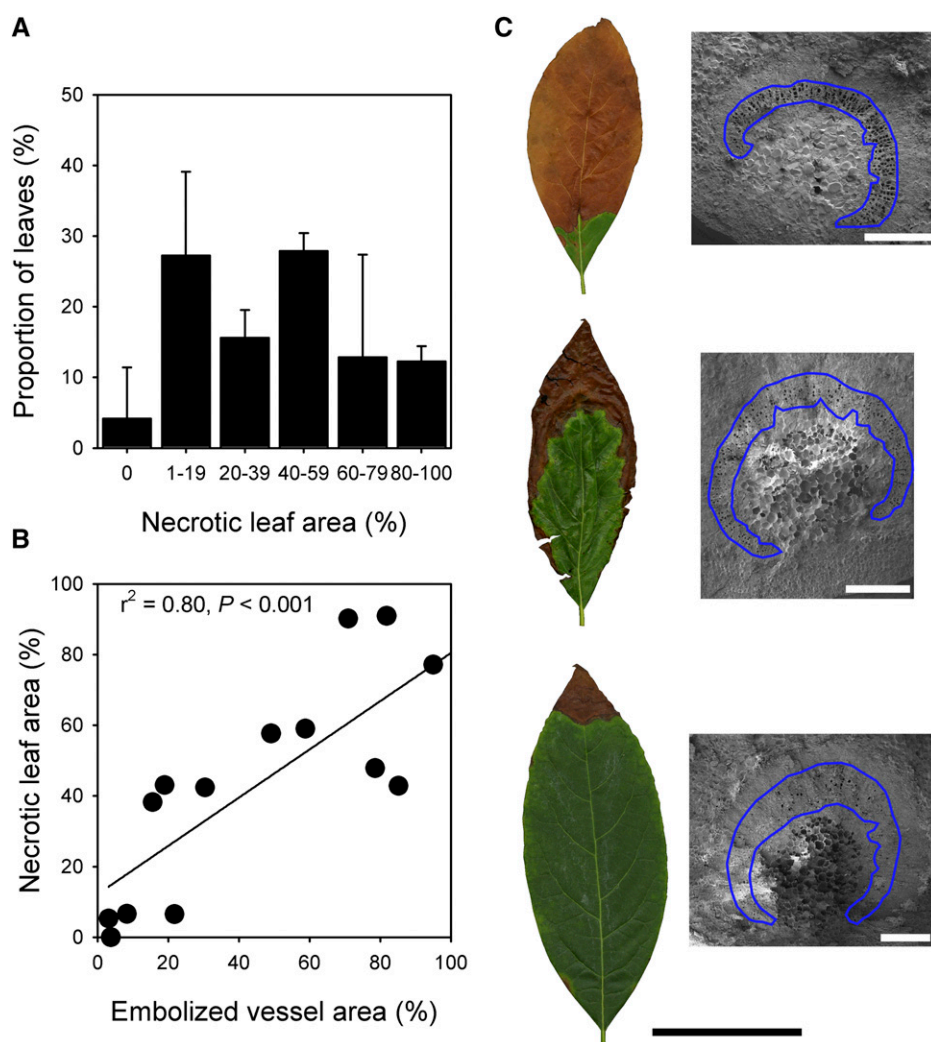


Figure 1. Drought-induced embolism in the petiole correlates with the percentage of necrotic leaf area caused by drought. **A**, Frequency distribution (\pm SE) of leaves across percentage classes of drought-induced necrotic leaf area from $n = 3$ drought-stressed plants. **B**, Correlation between the drought-induced percentage of necrotic leaf area and the percentage of embolized vessel area in the petiole. **C**, Representative leaves (black scale bar = 10 cm) displaying partial drought induced leaf mortality and the corresponding cryo-SEM cross section of the petioles (white scale bars = 500 μ m) showing the degree of xylem embolism. Blue lines delineate the xylem area of the petiole. Data were obtained from leaves of *P. americana* plants 2 months after recovery from drought stress.

the accumulated embolized area of the xylem had occurred (P_{88}), and (4) the Ψ_l at which the last embolism was observed. The Ψ_l at the onset of embolism ranged from -0.90 to -2.32 MPa. P_{50} was similarly variable across leaves, ranging from -1.51 to -2.96 MPa (Supplemental Table S1). P_{88} ranged from -2.30 to -3.49 . The Ψ_l at which the last event of embolism occurred was the most variable across leaves, ranging from -2.41 to -4.50 MPa. Furthermore, the window of Ψ_l between the first and last embolism varied among individual leaves and was not associated with any key threshold Ψ_l . In some leaves, embolism events started and finished within less than 1.0 MPa, whereas in one particular leaf, total embolism occurred over a range of 3.6 MPa. In most leaves, however, embolism occurred over a range of 1.30 MPa. Leaf embolism was consistently first observed in the midrib (Fig. 2B). Usually, several events occurred in the midrib before emboli were observed in the secondary and tertiary veins. Minor veins were consistently the last order of vascular tissue in the leaf to embolize.

To test whether the surviving, nonnecrotic portion of the leaves remained functional following plant

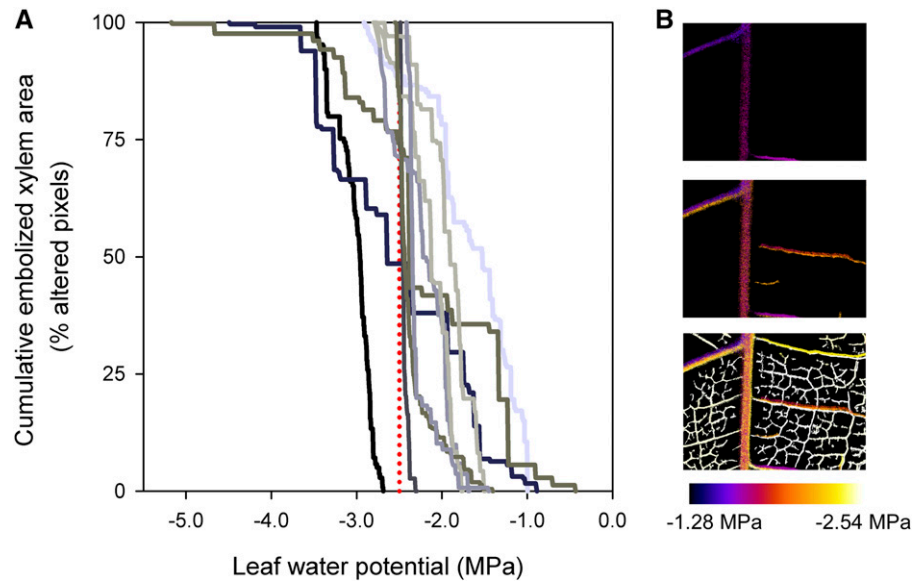
rehydration, we quantified leaf gas exchange in the surviving tissue of leaves that had experienced the drought stress. Rates of gas exchange were highly variable among the different surviving leaves in the canopy (Fig. 3). Strong negative correlations were observed in both net CO_2 assimilation rates and the percentage of necrotic leaf area (Fig. 3A), with the lowest rates of gas exchange observed in leaves with the largest areas of necrosis. Declines in stomatal conductance correlated with declines in net CO_2 assimilation rates ($r = 0.95$, $P < 0.0001$; Fig. 3B), but not with declines in Ψ_l nor increases in foliage levels of abscisic acid (ABA; Fig. 3C), which remained unchanged across leaves. Lower midday Ψ_l was measured in leaves that had higher stomatal conductance (Fig. 3D).

DISCUSSION

Partial Leaf Area Mortality Is Associated with Xylem Embolism

A moderate drought stress, during which plants experienced a Ψ_l that approached P_{50} , resulted in a highly

Figure 2. A large variation in the resistance to embolism among leaves. A, Percentage of cumulative embolism recorded in the leaves of *P. americana* ($n = 9$). The vertical, red dotted line indicates the mean Ψ_l plants were exposed to during drought. Curves are colored according to degree of resistance to embolism. B, Images depicting the sequence of embolism events observed in the leaf of *P. americana*; each embolism event is marked by a different color corresponding to the Ψ_l at which the embolism event occurred. Large embolism events that spanned much of the width of the major veins were observed first in all leaves, followed by embolism events that at times covered large areas of the network of minor veins.

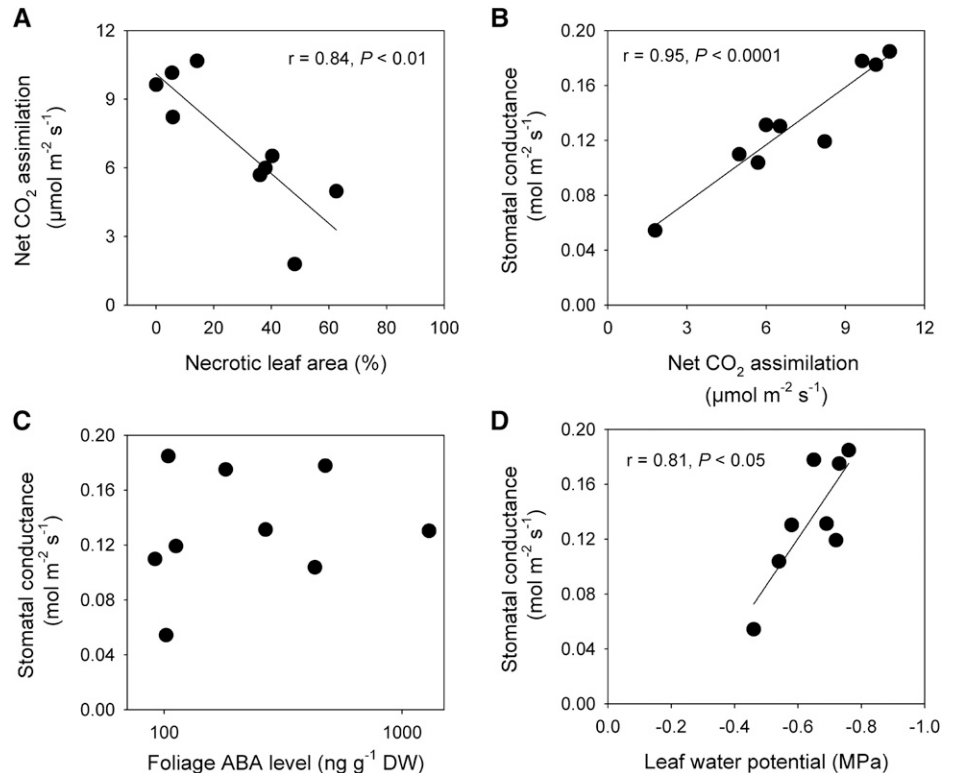


heterogeneous range of partial leaf mortality among leaves in the *P. americana* canopy. Our findings of a strong correlation between leaf area mortality, as represented by the area of necrosis, and the amount of embolism in the leaf petiole (Fig. 1B) indicate that drought-induced leaf embolism may play a causal role in leaf death during drought.

A number of studies have found considerable declines in leaf hydraulic conductance and gas exchange, particularly at the tips of leaves, caused by midrib

disruption (Hüve et al., 2002; Nardini and Salleo 2003; Sack et al., 2003). These observations suggest that emboli formed in the xylem closer to the base of the midrib or in the petiole are highly threatening to the continual supply of water to distal portions of the leaf (Sack et al., 2003). We do not know what the direct cause of cell death was in the necrotic portions of the leaf that formed during water deficit. However, the link between leaf area mortality and the degree of embolism in the xylem suggests that the supply of water to expired

Figure 3. Leaf gas exchange in surviving tissue is compromised in leaves displaying higher degrees of drought-induced leaf death. A, Correlation between percentage necrotic leaf area and net CO_2 assimilation. B, Correlation between net CO_2 assimilation and stomatal conductance. C, Lack of correlation between stomatal conductance and foliage levels of ABA expressed in terms of dry weight. D, The negative correlation between leaf water potential and stomatal conductance. Data were collected from the surviving, nonnecrotic portion of leaves from *P. americana* plants ($n = 3$) 2 months after recovery from drought stress.



tissue may have ceased or declined to rates below that necessary to maintain metabolic function in mesophyll cells. This decline in hydraulic supply to laminal tissue may have been exacerbated by both the collapse of xylem cells in the minor veins (Zhang et al., 2016) and dramatic declines in hydraulic conductivity outside the xylem as mesophyll cells lose turgor (Scoffoni et al., 2017b). An alternative explanation for this observed pattern of death across the lamina may have been an extreme loss of turgor leading to cytorrhysis in the mesophyll cells furthest from the vein, or midrib (Scoffoni et al., 2017a).

Heterogeneous Leaf Mortality during Drought and Embolism Resistance across the Canopy

The embolism resistance of leaves of *P. americana* was highly variable, with P_{50} ranging from -1.51 to -2.96 MPa (Fig. 2A). If we consider the mean minimal Ψ_l reached during drought (-2.5 MPa), a canopy is likely to have leaves with a percentage of embolism ranging from 0% to 100%, with most leaves presenting c. 70% to 80% embolism, if osmotic adjustment has not occurred (Cardoso et al., 2018). Such variation in the embolism resistance of individual leaves across a canopy likely explains the very large range of percentage of embolized vessels in the petiole and partial leaf area mortality observed across the canopy of drought-stressed plants (Fig. 1A; Supplemental Fig. S2). We assume that there is connectivity between the petiole and xylem in the midrib, which accounts for the majority of the hydraulic conductivity of the leaf. Considerable variation in the degree of embolism observed in the petiole of even-aged, drought-exposed leaves, and the link between this embolism area and the area of leaf necrosis, suggests that there is a strong coordination between hydraulic supply through the petiole and leaf photosynthetic function. Our data suggest that, unlike the stem, there is very little hydraulic oversupply in the petiole. Thus the petiole may represent a critical bottleneck for plant gas exchange and leaf drought tolerance.

Traditional methods to quantify the embolism resistance of leaves are based on constructing vulnerability curves from an aggregate of many leaves. The ability to optically determine the embolism resistance of an individual leaf provides a means to observe the variation in embolism resistance across leaves in the canopy. We adopted the standard practice for quantifying embolism resistance using the optical method, whereby the area of embolism events was quantified. When assessed in such a way, cumulative embolism area curves plotted against Ψ_l in leaves matches declines in leaf hydraulic conductance (Brodrribb et al., 2016). Alternative methods for analyzing optical embolism data, like the assignment of a single value to an embolism event regardless of the size of that event, does not match declines in hydraulic conductance (Venturas et al., 2019), and so was not used here. Comparable with what we

observed here in *P. americana*, a similar or more pronounced variation in embolism resistance between leaves has been recently observed using the optical vulnerability method (Johnson et al., 2018; Lamarque et al., 2018; Rodríguez-Domínguez et al., 2018). Many of the species with pronounced variation in embolism resistance between leaves are adapted to dry environments and have an intrinsically high resistance to embolism formation in the xylem, such as the tree species *Olea europaea*, *Laurus nobilis*, and the cereal crop *Triticum aestivum*. In contrast, herbaceous and rainforest species (including *Helianthus annuus* and *Eucryphia moorei*) have been found to have minimal variability between leaves in embolism resistance observed using the same method (Brodrribb et al., 2016; Cardoso et al., 2018). Because classical methods for generating leaf vulnerability curves are reliant on a pooling of data sampled from many individual specimens, the data from these techniques cannot provide a quantification of embolism resistance in an individual leaf, and consequently there can be substantial spread in leaf hydraulic data collected using these techniques (Brodrribb et al., 2014; Lamarque et al., 2018). We suggest that this spread in hydraulic measurements in traditional leaf vulnerability curves may be indicative of high interleaf variability in embolism resistance that we observe using the optical method.

We do not yet know what mechanism(s) underlie the variation in embolism resistance observed between leaves across the canopy of the same species. Variation could be due to anatomical changes in intrinsic features of the xylem including conduit dimensions, and thickness and porosity of pit membranes of individual leaves may play an important role in defining their vulnerability to embolism (Blackman et al., 2010; Lens et al., 2011). The evolution of a high interleaf variability in embolism resistance across a canopy may have been selected for as a means of partially limiting whole-plant transpiration by eliminating more vulnerable leaves early in drought, prolonging survival. At the same time, the most resistant leaves would remain, although with some reduction in their function due to partial embolism. Leaf persistence in the canopy could provide the plant with an immediate source of photosynthates when drought ends, facilitating the rapid reestablishment of a new canopy postdrought. High interleaf variability in embolism resistance might also reflect a trade-off between hydraulic safety and efficiency, whereby a canopy is constructed of a mix of leaves containing some that are capable of high rates of gas exchange yet are susceptible to embolism and some that are less efficient yet more resistant to embolism. This might explain high variability between leaves in maximum hydraulic conductance and rates of gas exchange; however, these associations between hydraulic efficiency and safety have not yet been made at an individual leaf level. Alternatively, *P. americana* being native to the wet subtropics, and having quite vulnerable xylem, may, like many such species (Tyree et al., 2002), have never evolved (or has lost) the capacity to

shed leaves that experience incipient embolism during drought (Hochberg et al., 2017).

Leaf Gas Exchange Is Reduced by Persistent Embolism in Surviving Leaves

We also found that the amount of necrotic leaf area correlated with the rate of leaf gas exchange in the remaining viable tissue of the leaf, after drought stress (Fig. 3A). This suggests that embolism in the petiole, which persisted despite complete rehydration of the xylem, may have compromised the physiological capacity of the remaining viable tissue. We found a strong correlation between leaf photosynthetic rate and stomatal conductance in the surviving leaf tissue (Fig. 3B), but did not find any correlation between stomatal conductance and foliage ABA levels (Fig. 3C). This result suggests that photosynthetic damage that occurs during drought permanently reduces the rate of leaf gas exchange in surviving tissue after rewatering and that embolized vessels in leaves do not refill after rewatering in *P. americana*. Photosynthetic damage was likely exacerbated in leaves in which hydraulic conductivity was likely reduced by the formation of extensive embolism in the petiole. The lack of correlation with foliage ABA levels suggests that, once rewatered, gas exchanged was not limited by ABA-induced stomatal closure in the remaining viable leaf tissue.

CONCLUSION

We found that embolism in the petiole induced by drought correlates with the percentage leaf area mortality during drought in *P. americana*. The high variability between leaves in the degree of embolism that forms in the petiole during drought in *P. americana* is likely due to a large interleaf variability in the resistance to embolism formation in the xylem. Our results may have implications for the strategies used by plants to tolerate periods of prolonged water deficit, and suggest that embolism formation in the xylem may be critical for leaf survival during drought.

MATERIALS AND METHODS

Plant Material and Drought Stress

Individuals of *Persea americana* were grown from seed in 10-L pots containing a 2:1 mix of Indiana Miami topsoil and ground pine bark, reaching six months of age (~60–80 cm tall, with a canopy of ~20 even-aged leaves) in a glasshouse at Purdue University. During establishment, plants were watered daily and received liquid nutrients once per month. Conditions in the glasshouse were set at a night/day temperature of 22°C /28°C, respectively, and natural photoperiod of ~12 h with a maximum light intensity of ~1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Drought stress was imposed in three individuals by withholding water, and Ψ_l were monitored between 11:30 and 13:30 h using a Scholander pressure chamber (615D, PMS Instrument Company) in leaves collected every ~3 d as the drought treatment progressed (Supplemental Fig. S1). After 18 d under water-limited conditions, signs of leaf necrosis were visualized as areas of

brown necrotic leaf tissue. At this point plants were rewatered and maintained under well-watered conditions (soil water potential > -0.2 MPa) for 2 months. The rehydration period was applied to verify the long-term effects of the drought on leaves.

Leaf Area Mortality and cryo-SEM Observation of Embolism in the Petiole

Two months after rewatering, the remaining leaves from the three individuals were imaged and the partial leaf area mortality (i.e. brown necrotic regions [Supplemental Fig. S2]) was calculated as a percentage of total leaf area using the software ImageJ (National Institutes of Health).

Embolism in the petiole was quantified in *c.* five leaves from each individual by the direct visualization of embolized conduits in nontranspiring leaves using cryo-SEM as described by Cochard et al. (2000). This method of direct embolism visualization in xylem does not induce artifacts when the water column is not under negative tension (Cochard et al. (2000)). To ensure the xylem water column was not under negative tension, intact leaves were cut under water near the point of petiole insertion on the stem, and a 2-cm petiole segment from the cut end was immediately re-excised under water. Within approximately 15 s from re-excision a 10-mm segment, corresponding to the middle of the petiole, was excised under water and mounted onto a modified vise clamp holder with a 1:1 OCT cryo-gel and water. To prevent negative tension on the water column, a meniscus of water was maintained on the cut ends of the petiole segment during mounting. The petiole segments were immediately frozen in liquid nitrogen and placed into the Gatan Alto 2500 (Gatan Inc.) cryo-preparation chamber of the microscope (FEI Nova Nano 200), and maintained under vacuum at -170°C. The cryo-fracture was operated in the chamber, and all the analyses were performed in petiole segments that were fractured greater than 3 mm from the cut ends to avoid possible artifacts caused by petiole excision and manipulation. Specimens were briefly sublimated while viewing at -90°C to remove surface frost, transferred to the cryo-preparation chamber to cool and cease sublimation, and then subsequently sputter coated for 120 s at 8 mA using a platinum target. After coating, samples were returned into the SEM stage and imaged at -140°C. The efficiency of the sampling technique was independently validated using petiole samples prepared from a nondrought-stressed leaf and a fully embolized leaf prepared by out-of-water excision and bench drying, resulting in completely water or air-filled vessels, respectively.

For each cross-section image taken by the cryo-SEM, both the total xylem area (including conducting and parenchyma cells) and the embolized vessel area (air-filled conduits displayed in black) were directly measured using ImageJ. To identify the portion of the total xylem area corresponding to the vessel area, cross sections were analyzed using light microscopy in petioles. Because the vessel area in the xylem was very similar among samples ($12.52 \pm 0.11\%$), this number was used to calculate the total vessel area for each one of the cryo-SEM images. The total xylem area was calculated as follows:

$$\text{Total vessel area} = \text{Total xylem area} \times 0.1252 \quad (1)$$

Then, the percentage embolized vessel area was calculated as:

$$\text{Embolized vessel area}(\%) = \frac{\text{Embolized vessel area}}{\text{Total vessel area}} \times 100 \quad (2)$$

Optical Vulnerability of Single Leaves

Nine leaves from four well-watered individuals of *P. americana* grown as described above were used to determine the variation in leaf embolism resistance using the optical vulnerability method (Brodrribb et al., 2016). Soil was washed from the roots of each individual to enhance the rate of drying, and plants were kept bagged under very low light conditions (light intensity < 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to allow whole plant Ψ_l equilibration. Intact leaves were placed under a stereo microscope (SZMT2, OPTIKA) for image capture every 600 s, whereas Ψ_l was measured using a psychrometer and verified by measuring Ψ_l using a Scholander Pressure Chamber. Image sequences were manually analyzed using ImageJ⁶⁶. Briefly, embolism events were determined by obtaining the differences in pixels of individual images from the previous images, which were then transformed into masks for quantification. Embolism events were identified as changes in color associated with vascular tissue; background noise associated with changes in color outside the vascular tissue was manually removed from the image stack. The embolism area per image was calculated as the sum of nonzero pixels and expressed as the percentage of total embolism

area in the sequence. To analyze the relationship between Ψ_l and embolism formation, a linear regression was fitted between time and Ψ_l and the equation used to determine the exact Ψ_l at the time of each image capture. These values were then plotted against total embolism area for each image to produce the embolism resistance curves.

Leaf Gas Exchange and Foliage ABA Level

Leaf gas exchange and foliage ABA levels were measured in the remaining leaves from the same three drought-stressed and rewatered individuals used for leaf area mortality and cryo-SEM assessments. The experiments were performed on the surviving green portions of the leaves, being careful to avoid necrotic regions. Measurements were taken in the morning between 9 and 11: AM, in plants maintained under the same glasshouse conditions as previously described. Leaf gas exchange was measured using an infrared gas analyzer (LI-6800, Licor Biosciences). Conditions in the leaf cuvette were controlled for the duration of the experiment; light intensity was set at $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature to 25°C , CO_2 concentration at $400 \mu\text{mol mol}^{-1}$, and VPD at ~ 2.0 kPa. Following leaf gas exchange, the same portion of the leaves were harvested for foliar ABA analysis. Samples were weighted, covered in cold (-30°C) 80% (v/v) methanol in water, and immediately stored at -30°C . They were further purified, and the foliar ABA level was quantified by physicochemical methods with an added internal standard using an Agilent 6400 series triple quadrupole LC/MS according to McAdam (2015).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Decline in leaf water potential during drought.

Supplemental Figure S2. Representative leaves demonstrating the large range of necrotic area caused by drought stress.

ACKNOWLEDGMENTS

We acknowledge the use of the facilities of the Bindley Bioscience Center (National Institutes of Health-funded Indiana Clinical and Translational Sciences Institute) and the invaluable help of Robert Seiler at the Purdue Life Science Microscopy Facility.

Received May 13, 2019; accepted October 10, 2019; published October 17, 2019.

LITERATURE CITED

- Allen CD, Macalady AK, Chenchouni H, Bachelet D, McDowell N, Vennetier M, Kitzberger T, Rigling A, Breshears DD, Hogg EH, et al (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *For Ecol Manage* **259**: 660–684
- Anderegg WRL, Klein T, Bartlett M, Sack L, Pellegrini AFA, Choat B, Jansen S (2016) Meta-analysis reveals that hydraulic traits explain cross-species patterns of drought-induced tree mortality across the globe. *Proc Natl Acad Sci USA* **113**: 5024–5029
- Barigah TS, Charrier O, Douris M, Bonhomme M, Herbette S, Améglio T, Fichot R, Brignolas F, Cochard H (2013) Water stress-induced xylem hydraulic failure is a causal factor of tree mortality in beech and poplar. *Ann Bot* **112**: 1431–1437
- Blackman CJ, Brodrribb TJ, Jordan GJ (2010) Leaf hydraulic vulnerability is related to conduit dimensions and drought resistance across a diverse range of woody angiosperms. *New Phytol* **188**: 1113–1123
- Bouche PS, Jansen S, Sabalera JC, Cochard H, Burrett R, Delzon S (2016) Low intra-tree variability in resistance to embolism in four Pinaceae species. *Ann For Sci* **73**: 681–689
- Brodrribb TJ, Cochard H (2009) Hydraulic failure defines the recovery and point of death in water-stressed conifers. *Plant Physiol* **149**: 575–584
- Brodrribb TJ, McAdam SAM, Jordan GJ, Martins SCV (2014) Conifer species adapt to low-rainfall climates by following one of two divergent pathways. *Proc Natl Acad Sci USA* **111**: 14489–14493
- Brodrribb TJ, Skelton RP, McAdam SAM, Bienaimé D, Lucani CJ, Marmottant P (2016) Visual quantification of embolism reveals leaf vulnerability to hydraulic failure. *New Phytol* **209**: 1403–1409
- Cardoso AA, Brodrribb TJ, Lucani CJ, DaMatta FM, McAdam SAM (2018) Coordinated plasticity maintains hydraulic safety in sunflower leaves. *Plant Cell Environ* **41**: 2567–2576
- Charrier G, Torres-Ruiz JM, Badel E, Burrett R, Choat B, Cochard H, Delmas CEL, Domec J-C, Jansen S, King A, et al (2016) Evidence for hydraulic vulnerability segmentation and lack of xylem refilling under tension. *Plant Physiol* **172**: 1657–1668
- Choat B, Brodrribb TJ, Brodersen CR, Duursma RA, López R, Medlyn BE (2018) Triggers of tree mortality under drought. *Nature* **558**: 531–539
- Choat B, Jansen S, Brodrribb TJ, Cochard H, Delzon S, Bhaskar R, Bucci SJ, Feild TS, Gleason SM, Hacke UG, et al (2012) Global convergence in the vulnerability of forests to drought. *Nature* **491**: 752–755
- Cochard H, Bodet C, Améglio T, Cruiziat P (2000) Cryo-scanning electron microscopy observations of vessel content during transpiration in walnut petioles. Facts or artifacts? *Plant Physiol* **124**: 1191–1202
- Cochard H, Lemoine D, Dreyer E (1999) The effects of acclimation to sunlight on the xylem vulnerability to embolism in *Fagus sylvatica* L. *Plant Cell Environ* **22**: 101–108
- Davis SD, Ewers FW, Sperry JS, Portwood KA, Crocker MC, Adams GC (2002) Shoot dieback during prolonged drought in *Ceanothus* (Rhamnaceae) chaparral of California: A possible case of hydraulic failure. *Am J Bot* **89**: 820–828
- Hacke U, Sauter JJ (1996) Drought-induced xylem dysfunction in petioles, branches, and roots of *Populus balsamifera* L. and *Alnus glutinosa* (L.) Gaertn. *Plant Physiol* **111**: 413–417
- Hochberg U, Windt CW, Ponomarenko A, Zhang Y-J, Gersony J, Rockwell FE, Holbrook NM (2017) Stomatal closure, basal leaf embolism, and shedding protect the hydraulic integrity of grape stems. *Plant Physiol* **174**: 764–775
- Hoffmann WA, Marchin RM, Abit P, Lau OL (2011) Hydraulic failure and tree dieback are associated with high wood density in a temperate forest under extreme drought. *Global Change Biol* **17**: 2731–2742
- Hüve K, Remus R, Lüttschwager D, Merbach W (2002) Water transport in impaired leaf vein systems. *Plant Biol* **4**: 603–611
- Jacobsen AL, Pratt RB, Davis SD, Tobin MF (2014) Geographic and seasonal variation in chaparral vulnerability to cavitation. *Madroño* **61**: 317–327
- Johnson KM, Jordan GJ, Brodrribb TJ (2018) Wheat leaves embolized by water stress do not recover function upon rewatering. *Plant Cell Environ* **41**: 2704–2714
- Kolb S (1999) Transport constraints on water use by the Great Basin shrub, *Artemisia tridentata*. *Plant Cell Environ* **22**: 925–935
- Lamarque LJ, Corso D, Torres-Ruiz JM, Badel E, Brodrribb TJ, Burrett R, Charrier G, Choat B, Cochard H, Gambetta GA, et al (2018) An inconvenient truth about xylem resistance to embolism in the model species for refilling *Laurus nobilis* L. *Ann For Sci* **75**: 88
- Lamy J-B, Delzon S, Bouche PS, Alia R, Vendramin GG, Cochard H, Plomion C (2014) Limited genetic variability and phenotypic plasticity detected for cavitation resistance in a Mediterranean pine. *New Phytol* **201**: 874–886
- Lens F, Sperry JS, Christman MA, Choat B, Rabaey D, Jansen S (2011) Testing hypotheses that link wood anatomy to cavitation resistance and hydraulic conductivity in the genus *Acer*. *New Phytol* **190**: 709–723
- Martin-StPaul N, Delzon S, Cochard H (2017) Plant resistance to drought depends on timely stomatal closure. *Ecol Lett* **20**: 1437–1447
- McAdam SAM (2015) Physicochemical quantification of abscisic acid levels in plant tissues with an added internal standard by ultra-performance liquid chromatography. *Bio Protoc* **5**: e1599
- McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG, et al (2008) Mechanisms of plant survival and mortality during drought: Why do some plants survive while others succumb to drought? *New Phytol* **178**: 719–739
- Mencuccini M, Comstock J (1997) Vulnerability to cavitation in populations of two desert species, *Hymenoclea salsola* and *Ambrosia dumosa*, from different climatic regions. *J Exp Bot* **48**: 1323–1334
- Nardini A, Salleo S (2003) Effects of the experimental blockage of the major veins on hydraulics and gas exchange of *Prunus laurocerasus* L. leaves. *J Exp Bot* **54**: 1213–1219
- Neuhauser A, Turner DW, Colmer TD, Kuo J, Eastham J (2007) Drying half the root-zone of potted avocado (*Persea americana* Mill., cv. Hass) trees

- avoids the symptoms of water deficit that occur under complete root-zone drying. *J Hortic Sci Biotechnol* **82**: 679–689
- Pittermann J, Choat B, Jansen S, Stuart SA, Lynn L, Dawson TE** (2010) The relationships between xylem safety and hydraulic efficiency in the Cupressaceae: The evolution of pit membrane form and function. *Plant Physiol* **153**: 1919–1931
- Pockman WT, Sperry JS** (2000) Vulnerability to xylem cavitation and the distribution of Sonoran Desert vegetation. *Am J Bot* **87**: 1287–1299
- Rodriguez-Dominguez CM, Carins Murphy MR, Lucani C, Brodribb TJ** (2018) Mapping xylem failure in disparate organs of whole plants reveals extreme resistance in olive roots. *New Phytol* **218**: 1025–1035
- Rood SB, Patiño S, Coombs K, Tyree MT** (2000) Branch sacrifice: Cavitation-associated drought adaptation of riparian cottonwoods. *Trees (Berl)* **14**: 248–257
- Sack L, Cowan PD, Holbrook NM** (2003) The major veins of mesomorphic leaves revisited: Tests for conductive overload in *Acer saccharum* (Aceraceae) and *Quercus rubra* (Fagaceae). *Am J Bot* **90**: 32–39
- Sala A, Piper F, Hoch G** (2010) Physiological mechanisms of drought-induced tree mortality are far from being resolved. *New Phytol* **186**: 274–281
- Schuldt B, Knutzen F, Delzon S, Jansen S, Müller-Haubold H, Burlett R, Clough Y, Leuschner C** (2016) How adaptable is the hydraulic system of European beech in the face of climate change-related precipitation reduction? *New Phytol* **210**: 443–458
- Scoffoni C, Albuquerque C, Brodersen CR, Townes SV, John GP, Bartlett MK, Buckley TN, McElrone AJ, Sack L** (2017a) Outside-xylem vulnerability, not xylem embolism, controls leaf hydraulic decline during dehydration. *Plant Physiol* **173**: 1197–1210
- Scoffoni C, Albuquerque C, Brodersen CR, Townes SV, John GP, Cochard H, Buckley TN, McElrone AJ, Sack L** (2017b) Leaf vein xylem conduit diameter influences susceptibility to embolism and hydraulic decline. *New Phytol* **213**: 1076–1092
- Tyree MT, Sperry JS** (1989) Vulnerability of xylem to cavitation and embolism. *Annu Rev Plant Physiol Plant Mol Biol* **40**: 19–36
- Tyree MT, Vargas G, Engelbrecht BMJ, Kursar TA** (2002) Drought until death do us part: A case study of the desiccation-tolerance of a tropical moist forest seedling-tree, *Licania platypus* (Hemsl.) Fritsch. *J Exp Bot* **53**: 2239–2247
- Venturas MD, Pratt RB, Jacobsen AL, Castro V, Fickle JC, Hacke UG** (2019) Direct comparison of four methods to construct xylem vulnerability curves: Differences among techniques are linked to vessel network characteristics. *Plant Cell Environ* **42**: 2422–2436
- Wortemann R, Herbette S, Barigah TS, Fumanal B, Alia R, Ducousso A, Gomory D, Roeckel-Drevet P, Cochard H** (2011) Genotypic variability and phenotypic plasticity of cavitation resistance in *Fagus sylvatica* L. across Europe. *Tree Physiol* **31**: 1175–1182
- Zhang Y-J, Rockwell FE, Graham AC, Alexander T, Holbrook NM** (2016) Reversible leaf xylem collapse: A potential “circuit breaker” against cavitation. *Plant Physiol* **172**: 2261–2274