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

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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

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plants experiencing negative water potentials ($\Psi_t$) 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; Chat 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; Schulte 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 $\Psi_t$ 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 $\Psi_t$ is often measured across the canopy. Thus, we would hypothesize that variation in partial leaf mortality across a canopy exposed to a uniform $\Psi_t$ 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 $\Psi_t$, such that 18 d after the withholding of water, $\Psi_t$ had declined to $-2.5 \pm 0.3$ 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 $\Psi_t$ 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...
the accumulated embolized area of the xylem had occurred \( (P_{88}) \), and (4) the \( \Psi_t \) at which the last embolism was observed. The \( \Psi_t \) 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 \( \Psi_t \) 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 \( \Psi_t \) between the first and last embolism varied among individual leaves and was not associated with any key threshold \( \Psi_t \). 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 \( (r = 0.95, P < 0.0001; \text{Fig. 3B}) \), but not with declines in \( \Psi_t \) nor increases in foliage levels of abscisic acid (ABA; Fig. 3C), which remained unchanged across leaves. Lower midday \( \Psi_t \) 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 \( \Psi_t \) that approached \( P_{50} \), resulted in a highly
A 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
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 loose 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 $\Psi_i$ 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 $\Psi_i$ in leaves matches declines in leaf hydraulic conductance (Brodrrib 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; Rodriguez-Dominguez 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 (Brodrrib 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 (Brodrrib 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 (Tyrere 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 a natural photoperiod of ~12 h with a maximum light intensity of ~1500 μmol m⁻² s⁻¹.

Drought stress was imposed in three individuals by withholding water, and Ψₛ 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. 5–6 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 nondonutrient-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 ± 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:

\[ \text{Total vessel area} = \text{Total xylem area} \times 0.1252 \]  

(1)

Then, the percentage embolized vessel area was calculated as:

\[ \text{Embolized vessel area} \times 100 \]  

(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 (Brodribb 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 μmol m⁻² s⁻¹) to allow the xylem to embolize. Embolized leaves were placed under a stereo microscope (SZMT2, OPTIKA) for image capture every 600 s, whereas Ψₛ was measured using a psychrometer and verified by measuring Ψₛ using a Scholander Pressure Chamber. Image sequences were manually analyzed using ImageJ. 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 \( \Psi_r \) and embolism formation, a linear regression was fitted between time and \( \Psi_r \); and the equation used to determine the exact \( \Psi_r \) 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 1300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), temperature to 25°C, \( \text{CO}_2 \) concentration at 400 ppm \( \mu \text{mol} \), 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 6460 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.

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### LITERATURE CITED


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