In Situ Visualization of the Dynamics in Xylem Embolism Formation and Removal in the Absence of Root Pressure: A Study on Excised Grapevine Stems\textsuperscript{1}[OPEN]

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Gas embolisms formed during drought can disrupt long-distance water transport through plant xylem vessels, but some species have the ability to remove these blockages. Despite evidence suggesting that embolism removal is linked to the presence of vessel-associated parenchyma, the underlying mechanism remains controversial and is thought to involve positive pressure generated by roots. Here, we used in situ x-ray microtomography on excised grapevine stems to determine if embolism removal is possible without root pressure, and if the embolism formation/removal affects vessel functional status after sample excision. Our data show that embolism removal in excised stems was driven by water droplet growth and was qualitatively identical to refilling in intact plants. When stem segments were rehydrated with \( H_2O \) after excision, vessel refilling occurred rapidly (<1 h). The refilling process was substantially slower when polyethylene glycol was added to the \( H_2O \) source, thereby providing new support for an osmotically driven refilling mechanism. In contrast, segments not supplied with \( H_2O \) showed no refilling and increased embolism formation. Dynamic changes in liquid/wall contact angles indicated that the processes of embolism removal (i.e. vessel refilling) by water influx and embolism formation by water efflux were directly linked to the activity of vessel-associated living tissue. Overall, our results emphasize that root pressure is not required as a driving force for vessel refilling, and care should be taken when performing hydraulics measurements on excised plant organs containing living vessel-associated tissue, because the vessel behavior may not be static.

"If the air moved high up in the vine,... then a transport system depending upon cohesion in the vessels would surely get jammed by the expanding gas...", and "...one may rightly wonder how recurring breaks in water columns could possibly be repaired" (Scholander et al., 1955).

Water transport through xylem conduits is disrupted when water columns break and the lumen becomes blocked by air embolisms. It has long been speculated that xylem embolism removal is dependent on a plant's ability to generate positive root pressure, which either expels trapped embolisms from the xylem network or forces the gas back into solution (Tyree et al., 1986; Sperry et al., 1987; Cochard et al., 1994; Tibbett and Ewers, 2000; Cobb et al., 2007; Isnard and Silik, 2009). Many plant species have the ability to generate root pressure, but definitive and widespread documentation of embolism removal and the underlying processes across species and organ types remain elusive (for review, see Brodersen and McElrone, 2013).

Grapevines have long been used as a model system for studying processes associated with long-distance water transport including root pressure generation, xylem vulnerability to cavitation, and embolism repair (e.g. Scholander et al., 1955; Sperry et al., 1987; Choat et al., 2010; Brodersen et al., 2010, 2013; Knipfer et al., 2015a). Since the recognition of their ability to generate root pressure (Hales, 1727), it has been speculated that grapevines utilize root pressure to refill xylem vessels that have become air-filled during recovery from winter freeze-thaw events (Sperry et al., 1987) and drought (Holbrook et al., 2001). Recently, two grapevine species (\textit{Vitis arizonica} and \textit{V. riparia}) were identified for their ability to remove embolism after generation of high root pressure following rewatering after drought (Knipfer..."
et al., 2015b). However, this trait is not universal within the genus, because another species (V. champinii) examined by Knipfer et al. (2015b) failed to remove embolism while always exhibiting high root pressure. This raises the question: does the force generated by root pressure drive embolism removal? Brodersen et al. (2010) and Knipfer et al. (2015b) provided direct, visual evidence that embolism removal in stems of living grapevine plants is initiated by water droplets emerging through vessel sidewalls into the gas-filled vessel lumen. These results are in support of the idea that embolism removal depends on the activity of living cells located adjacent to embolized vessels (Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Hacke and Sperry, 2003; Salleo et al., 2004, 2006; Secchi and Zwieniecki, 2010), but the apparent role of root pressure in this process remains unclear.

Embolism removal in grapevine stems requires several hours (10–20 h) after rewatering (Holbrook et al., 2001; Brodersen et al., 2010; Knipfer et al., 2015b). Preceding embolism removal, plant water status recovers in the first few hours after rewatering. The embolism removal process can be distinguished into four phases (Brodersen et al., 2010): (1) water droplet growth, (2) droplet coalescence, (3) expansion of water columns, and (4) completion of vessel refilling. During this process, liquid/wall contact angles (θ) of emerging water droplets with the inner vessel wall were found to be variable, suggesting heterogeneous wettability of the wall surface (Brodersen et al., 2010). On the other hand, Brodersen et al. (2010) also observed refilling failures, where water columns retracted into neighboring, filled vessels presumably under tension. Vessel-associated cells may also have contributed to this efflux from the vessel lumen, but the involvement of living tissue in this process has not been considered yet.

Whether the processes described above for intact plants also occur in excised stem samples disconnected from the roots and foliage is unknown. Temporal changes in vessel functional status in excised plant segments would have important implications for methodologies designed to accurately estimate plant hydraulic function using destructive sampling. It has been reported that some hydraulic methods induce substantial measuring artifacts, but the source of these artifacts are still under debate (e.g. Cochard et al., 2014; 2015; Torres-Ruiz et al., 2015; Knipfer et al., 2015b). To minimize potential artifacts when using invasive hydraulic methods, Wheeler et al. (2013) suggested that stem segments be rehydrated after excision to allow tissue equilibration before analysis. However, as speculated by Trífiló et al. (2014) and Venturas et al. (2015), this may facilitate vessel refilling, which would lead to an overestimation in xylem hydraulic transport capacity. To date, direct visualization of embolism formation and removal in excised plant segments has been limited to a single study (Rolland et al., 2015), and the corresponding biological mechanism that may induce these phenomena is still unknown.

The goal of this study was to investigate if vessel refilling exists in excised grapevine stems, and in turn reveal whether (1) embolism removal can occur in the absence of root pressure, (2) requires a long-distance signaling mechanism delivered from distal organs, and/or (3) is driven predominantly by vessel-associated tissue. Temporal and spatial dynamics of embolism formation and removal were visualized using in situ computed microtomography (microCT) imaging, and the presence of living xylem tissue surrounding vessels was determined using fluorescence light microscopy.

RESULTS

Temporal dynamics of vessel refilling and embolism formation in excised stems differed considerably among the three treatments (i.e. H2O only; H2O+PEG; and no-H2O; see Fig. 1). When stem segment ends were supplied with H2O only (Fig. 1A), embolized vessels began refilling in less than 1 h, and the percentage of water-filled vessels increased rapidly over time. After 10 h, more than 90% of the entire population of initially embolized vessels was refilled (Fig. 1A, left panel). Simultaneously, no additional vessels embolized over time (Fig. 1A, right panel). Refilling rate and success was similar between stems obtained from plants at 2.5 MPa, 4.4% to 7% (Ψstem of ~1.3 MPa), and 89% to 68% (Ψstem of ~3.0 MPa). No differences in temporal dynamics of vessel refilling and embolism formation were observed in excised stems coated with petroleum jelly or those coated with petroleum jelly and sealed in a plastic bag (Fig. 1A, gray diamond symbol; Fig. 1C, black and white circle symbols). In addition, no differences in temporal refilling or embolism formation dynamics were observed in stems subjected to H2O at 0.2 kPa or 6 kPa (Fig. 1A, gray square symbol) hydrostatic pressure.
The process of vessel refilling is shown for a representative excised stem supplied with only H$_2$O in Figure 2 (black circle symbols in Fig. 1A). Transverse microCT images revealed that all embolized vessels refilled (indicated in blue) after 8 h (Fig. 2A). Vessels close to the pith refilled first. Corresponding longitudinal microCT images showed that vessel refilling was initiated by water droplets emerging into the gas-filled vessel lumen from vessel-associated tissue (i.e., difference between top and bottom $\theta$ of same droplet; see enlarged image of Figure 2C at $t = 2.5$ h). Over time, water droplets increased in size, coalesced, and formed expanding water columns ($t = 0.8$ h, 2.5 h and 4.3 h). In water columns, longitudinal images showed that $\theta$ of the meniscus increased during column expansion (Fig. 2C); meniscus-$\theta$ measured on several water columns was initially much less than 90° (mean = 47° ± 6° SE, $n = 6$) and greater (mean = 115° ± 3° SE, $n = 12$) during column expansion. This process resulted in complete refilling of the imaged vessel lumen by $t = 7.6$ h. In excised stem segments supplied with H$_2$O+PEG, microCT data showed that the process of vessel refilling was qualitatively similar (data not shown). MicroCT images provided no indication of a temporal refilling of vessels by drainage of liquid driven by gravitational or capillary forces, or by gels/tyloses (i.e., of irregular shape compared to water droplets and largely detached from the vessel surface; Czemmel et al., 2015).
Embolisms proliferated over time in excised stems in the no-H2O treatment, where the cut ends and the entire stem surface were sealed with petroleum jelly (Figs. 3 and 4). Time series analysis of transverse microCT images showed that in this treatment, vessels embolized in a random pattern, and typically in isolation from other embolized vessels (Supplemental Fig. S1). Simultaneously, some embolized vessels refilled over time within close proximity to the cambium within the functional xylem (Fig. 3).

Longitudinal microCT images of representative excised stems from the no-H2O treatment showed that remaining liquid in vessels retracted from both ends, and disappeared after 2.8 h (vessel V1; Fig. 4A). Both observed vessels (labeled V1 and V2) were spatially isolated from other embolized vessels (Fig. 4A, bottom panels) and surrounded by only parenchyma cells and fibers at the site of water efflux (Fig. 4, B and C). The mean contact angle of retracting water columns to the wall was much less than 90° (48° ± 3° SE, n = 10; Fig. 4B). Simultaneously, other vessels in the same stem showed water droplets emerging into the vessel lumen, and expansion of water columns (Fig. 5). In contrast to retracting water columns (see above), the measured contact angle of the meniscus to the vessel wall of expanding water columns was much greater (mean = 106° ± 4° SE, n = 10). These data were qualitatively similar to excised stems supplied with H2O (see Fig. 2).

To estimate if vessel wall properties remained the same during the H2O, H2O+PEG, and no-H2O treatments, θ of water droplets were compared (Fig. 6). In all treatments, values of θ ranged from 36° to 91°, while higher θ-values were measured for droplets with smaller droplet base diameter. Regression analysis predicted a linear relationship of droplet base diameter and θ (P < 0.01) while regressions were not different between treatments (P > 0.05). In general, mean liquid/wall contact angles (θ) of water droplets to the vessel wall were comparable among the H2O (64° ± 2° SE, n = 43), H2O+PEG (64° ± 2° SE, n = 41), and no-H2O treatments (66° ± 2° SE, n = 43; P > 0.05; Fig. 6). However, among treatments, the expansion rate of water droplets was fastest when excised stems were supplied with H2O only (average growth constant, k, of 0.67 h−1), and slower when supplied with H2O+PEG (k of 0.35 h−1) or no-H2O (k of 0.19 h−1; see Supplemental Fig. S2).

A three-dimensional (3D) volume rendering of a dehydrated stem segment showed that xylem tissue surrounding a vessel was composed of fibers and axial parenchyma cells (Fig. 7). The interior wall of the vessel (labeled V1 in Fig. 7A) did not share a perforated wall with other vessels within the scanned area (Fig. 7, B–D), and the high abundance of pit connections along its length were exclusively to vessel-associated fibers and axial parenchyma cells. Xylem...
tissue adjacent to vessel-associated tissue was largely composed of fibers. This is illustrated in more detail in the enlarged image (Fig. 7E). Vessel-associated fibers and parenchyma cells were in direct contact to the vessel lumen. The 3D image shows files of three to 10 axial parenchyma cells and vessel-associated fibers with many pit connections to the vessel lumen (Fig. 7E).

The xylem of *V. riparia* was composed of an extensive matrix of living tissue surrounding the vessels (Fig. 8). Transverse stem sections stained with FDA showed that xylem tissue remained metabolically active (green

Figure 3. Time series of transverse microCT images from representative stem (*V. riparia*) segments that were entirely sealed with petroleum jelly after excision ($t = 0$ h; i.e. no-$H_2O$ treatment). Stems were obtained at A, $\Psi_{\text{stem}}$ of $-1.7$ MPa (gray diamond symbols in Fig. 1C); B, $\Psi_{\text{stem}}$ of $-2.5$ MPa (black triangle symbols in Fig. 1C); and C, $\Psi_{\text{stem}}$ of $-3.0$ MPa (open square symbols in Fig. 1C). Water- and air-filled tissue appears in light and dark gray, respectively. Vessels that additionally embolized (yellow, brown, red; different color for each time step) and refilled (blue) over time are highlighted in color.

Figure 4. A, Time series of longitudinal microCT images (top panels) obtained from a representative excised stem (*V. riparia*) segment that was entirely sealed with petroleum jelly (i.e. no-$H_2O$ treatment, open square symbols in Fig. 1C). After excision ($t = 0$ h), images show the retraction of water columns (white arrows indicate direction of movement) from the vessel lumen (labeled V1 and V2). (Bottom panels) Transverse microCT images (position indicated by dashed line in top panels) show that vessels V1 and V2 were not adjacent to other embolized vessels. B and C, Enlarged microCT images (position indicated by white box in A) showing the presence of xylem parenchyma cells and fibers adjacent to vessels V1 and V2. The position of the enlarged longitudinal image is indicated in the transverse image by a dashed line. The contact angle of vessel wall with the meniscus of the retracting water column was $<90^\circ$. pf, perforation plate; p, parenchyma cells; f, fibers.
color) in well-watered (Fig. 8A) and drought-stressed (Fig. 8, B and C) plants. The metabolically active and heavily lignified tissue in proximity to xylem vessels is highlighted in an enlarged image of Figure 8D. The intensity of green fluorescence signal was strongest in xylem ray parenchyma and pith, but substantial activity was also detected in the xylem tissue that was found to be composed of fibers (see Fig. 7). No green fluorescence signal was detected in an unstained stem section (Fig. 8E); also, our control experiment showed that green fluorescence signal emitted from stems that were incubated at 2°C before staining was very weak, but increased in stems incubated at 4°C and 23°C (Fig. 8, F–H). These data illustrate that much of a grapevine’s xylem is living at maturity.

**DISCUSSION**

This study on excised grapevine stems provides visual evidence that localized embolism removal can occur in the absence of root pressure and is driven by vessel-associated tissue (i.e., parenchyma cells and fibers), and that a long-distance signal is not required to trigger this process. After excision, embolism removal by water droplets emerging into the vessel lumen was initiated shortly after stems were rehydrated with H2O (1 h). The average time required to refill 50% of embolized vessels was only 1.6 h. However, when excised stems were supplied with an H2O solution of lower water potential (by adding PEG), the speed with which vessels refilled was reduced by approximately 4-fold. In stems that were sealed after excision and not rehydrated, approximately 50% of additional vessels embolized in less than 2 h and vessel refilling was negligible. Based on microCT and fluorescence light microscopy data, this study shows that both vessel-associated fibers and axial parenchyma cells directly connected to the vessel lumen via pits could induce liquid movement in and out of the vessel lumen. Given the observed dynamics in embolism formation/removal on excised grapevine stems, great care should be taken when interpreting measurements of xylem vulnerability using destructive hydraulic methods on detached plant organs that contain living vessel-associated tissue, regardless of their purported refilling ability.

**Embolism Removal and Root Pressure**

For intact grapevine plants, Brodersen et al. (2010) and Knipfer et al. (2015b) reported that the removal of drought-induced gas emboli after rewatering occurs via water droplets growing from surrounding tissue into the gas-filled vessel lumen. However, it remained
unknown whether this process is restricted to intact plants, and/or if a long-distance signal from distal tissues (e.g. roots or leaves) to sites of embolism in the stem is required for stimulation of this process (Lovisolo et al., 2008; Brodersen and McElrone, 2013). Various studies have suggested that signaling mechanisms are required to initiate this removal process (e.g. hormones, Bucci et al., 2003; Secchi et al., 2012; and sugars, Secchi and Zwieniecki, 2011), including a hydraulic signal generated by root pressure (McCully, 1999). Using in situ microCT imaging, these data show that the embolism removal process in excised grapevine stems is qualitatively similar to that in an intact plant (Brodersen et al., 2010), and does not require a long-distance signaling mechanism for initiation. Contrary to published hypotheses (Tibetts and Ewers, 2000; Isnard and Silk, 2009), our data also eliminate root pressure as a necessary driving force for embolism removal in V. riparia. Knipfer et al. (2015a,b) reported that in two grapevine species, the ability to generate higher root pressure after rewatering is positively correlated with the ability for embolism removal; however, a third species that exhibited negligible embolism removal always exhibited the highest root pressures. Together, these data strongly suggest that embolism removal in the stem is not dependent on the activity of roots and a high positive hydrostatic pressure force in the xylem network (i.e. water droplet growth and embolism removal was observed here at 0–6 kPa). The simultaneous processes of embolism removal and the generation of root pressure may be the result of the same cellular process of water movement into the vessel lumen in different tissues (i.e. roots and stems), but embolism removal does not appear to be directly driven by root pressure (Wegner, 2014).

We cannot discount that root pressure may play an indirect role in embolism removal by releasing xylem tensions in the non-transpiring plant, which would facilitate gas dissolution (Fisher et al., 1997), prevent a pull of liquid from refilling vessels via pits to functional vessels (Brodersen et al., 2010), or enable rehydration of vessel-associated tissue by reducing the liquid water potential in remaining functional vessels. In intact grapevines, plant water status always recovered before the initiation of embolism removal, and refilling was always delayed by more than 3 h after soil rewatering (Brodersen et al., 2010; Knipfer et al., 2015b). In this study, stem segments supplied with H2O to the cut ends showed embolism removal initiated in less than 1 h after excision, and several hours faster than intact plants. This emphasizes that localized rehydration of vessel-associated tissue in stems, or the relaxation of xylem tension in the functional vessels, may be a prerequisite for embolism removal. By applying water to the cut stem ends, our experimental approach facilitated the delivery of water to sites of vessel refilling by creating a shortcut, thereby bypassing much of the plant’s hydraulic resistance network that is present in an intact plant. Thus, the tissue rehydration process is expedited, allowing the initiation of embolism removal to occur earlier.

Xylem Parenchyma and Fibers

Embolism removal has often been documented in plant species that contain vessel-associated parenchyma cells, emphasizing a tight linkage of living tissue and vessel refilling (for review, Brodersen and McElrone, 2013).
Besides parenchyma cells, many shrub-like or vine woody plant species also contain living fibers that retain protoplasts (Fahn and Leshem, 1963), including grapevine (Pratt, 1974). Despite structural differences, Esau (1953) emphasized that there should be no metabolic functional differences between parenchyma cells and living fibers because both are able to fulfill the same cellular functions. Similarly, using microCT and fluorescence imaging, our data highlight that the tissue surrounding xylem vessel in *V. riparia* stems is living and composed of both vessel-associated cells and fibers (most abundant) with extensive pit connections to the vessel lumen (Fig. 7). This tissue remained metabolically active under well-watered and drought conditions. We speculate that the high efficiency of embolism removal in grapevine is related to the active role of both living fibers and parenchyma cells, and the absence of (to our knowledge) novel embolism removal in many woody tree species may be directly related to the lack of sufficient living xylem tissue to support the embolism removal process [e.g., Pine (*Pinus*), Borghetti et al., 1991; Martínez-Cabrera et al., 2009; Knipfer et al., 2015a; Giant Sequoia (*Sequoia*), Choat et al., 2015 and Morris et al., 2015]. Grapevine, like many other lianas, can be a structural parasite, and is therefore to some extent released from the structural constraints that are imposed on freestanding woody plants. Released from a structural role, grapevine may be able to utilize fibers and axial parenchyma in unique ways, allowing them a competitive advantage in certain habitats (Schnitzer and Bongers, 2011).

**Liquid Movement Inducing Embolism Removal/Formation**

In the past, only a few studies reported liquid/wall contact angles inside xylem vessels (Zwieniecki and Holbrook, 2000; Kohonen, 2006; Brodersen et al., 2010). Our data indicated that θ of water droplets with the inner vessel wall were comparable among treatments,
solution along the vessel wall during the time period of the experiment that would have affected \( \theta \) by changing liquid/wall interactive forces. Moreover, \( \theta \) of water droplets emerging into the embolized vessel lumen was initially high (around 90°) and declined when droplets became larger. We speculate that these dynamic changes in \( \theta \) point to a localized spread of liquid on the vessel surface during droplet expansion, which may have been facilitated by areas of relatively hydrophilic wall material (Brodersen et al., 2010) and surface roughness (Kohonen, 2006). A spread of liquid film on the vessel surface may positively contribute to the refilling process by increasing the surface area available for dissolution of gas emboli (Konrad and Roth-Nebelsick, 2009). After droplet coalescence, the observed changes in \( \theta \) of non-static water columns over time suggest changes in hydrostatic pressure of the refilling liquid (i.e. in addition to the pressure force generated by surface tension) as induced by efflux from vessel-associated tissue (Yuan and Lee, 2013). For example, meniscus-\( \theta \) of water columns changed from much less than 90° (concave-shaped) to greater values during expansion, pointing to an increase in liquid hydrostatic pressure by water flow into the vessel lumen driven by vessel-associated tissue; this applies when changes in temperature and gas pressure are negligible. But future research is needed to investigate in detail the spatial differences in vessel structure (e.g. lumen geometry, presence of perforation plates) and the temporal changes in vessel surface properties (e.g. variation in wall chemistry, hydrophobicity), which may contribute to these observed changes in \( \theta \) (see data on corn by McCully et al., 2014). In contrast, it has been reported that in another plant species where vessel refilling is purely driven by capillary forces (i.e. passive, water movement not linked to a cellular process), the meniscus-\( \theta \) of expanding water columns was much less than 90° (Rolland et al., 2015), and this refilling mechanism was observed in vessels of relatively small diameter (around 10 \( \mu m \)). For grapevine with relatively large diameter vessels, it can be calculated that capillary forces would only move a liquid column along a length of 12.4 cm (for \( d \) of 100 \( \mu m \), \( \theta \) of 65°, density of water (\( \rho \)) of 1000 Kg m\(^{-3}\), g of 9.8 m s\(^{-2}\), and surface tension of water (\( \gamma \)) of 0.072 N m\(^{-1}\) using the Young-Laplace equation in the form of \( 2 \cdot g \cdot \cos \theta / (\rho \cdot g \cdot (d/2)) \), seemingly insufficient for a grapevine vessel refilling where vessel lengths often exceed greater than 50 cm (Choat et al., 2010). Therefore, capillary forces alone are likely too weak to induce sufficient water movement to refill an embolized grapevine vessel, and these physical limitations may have contributed to the evolution of an additional active tissue-dependent embolism removal mechanism (i.e. water movement by osmosis linked to cellular solute transport).

Several conceptual models have been developed to explain tissue-dependent embolism removal by water droplet growth in intact plants (e.g. Holbrook and Zwieniecki, 1999; Brodersen et al., 2010; Wegner, 2015). It has been proposed that solute export from

![Figure 9](image-url). Cartoon illustrating the putative processes of embolism expansion and removal in an excised stem segment containing living vessel-associated tissue. (t = 0) At time of excision, xylem tensions are released and the water potential of vessel liquid (\( \Psi_v \)) is less negative as compared to that of vessel-associated tissue (\( \Psi_T \)). (i.e. osmotic adjustment under drought). (t = 1) The gradient in water potential induces a flow of liquid from the embolized vessel lumen via pits into vessel-associated tissue. The hydrostatic pressure (\( P_f \)) of the receding vessel liquid drops and the contact angle of meniscus with the inner vessel wall is \(<90°\). Xylem tissue surrounding vessels rehydrates and in turn turgor pressure increases. (t = 2) An export of solute into the apoplast is induced to aid the equilibration of xylem tissue water potential. This results in an osmotically driven growth of water droplets into the vessel lumen (and increases solute potential, \( \Psi_s \)). Water droplets form expanding water columns. The hydrostatic pressure inside expanding water columns increases by this flow and the meniscus contact angle is \( >90° \). During the expansion of water columns, gas pressure increases and gas embolism will be forced back into solution.

indicating that differences in liquid/wall interactive forces did not contribute to the observed dynamics of embolism removal, i.e. when excised stems were rehydrated (\( H_2O \) only, \( H_2O+PEG \)) or not (no-\( H_2O \)). This also excludes a possible drainage of externally supplied
vessel-associated parenchyma into the vessel lumen generates an osmotic gradient that induces liquid movement into the gas-filled vessel lumen (Tyrre et al., 1999; Holbrook and Zwieniecki, 1999; Hacke and Sperry, 2003; Salleo et al., 2006; Secchi and Zwieniecki, 2010, 2011; Brodersen et al., 2010; Brodersen and McElrone, 2013). This liquid movement may be facilitated by the existence of water-transporting proteins in xylem parenchyma that allow a cotransport of water and solutes against a gradient in free energy (Wegner, 2014). After liquid emergence on the inner vessel surface, liquid forms expanding droplets. This expansion increases the pressure inside gas embolisms that are subsequently forced back into solution. After an embolized vessel element is completely refilled, water enters the channel of bordered pits connecting the refilled element to the xylem network to functional vessels. Based on these assumptions and our experimental data, we propose a conceptual model of embolism formation/removal in excised stems as obtained from drought-stressed plants (Fig. 9):

\[ t = 0 \] At time of excision, the lumen of many vessels is gas-filled (i.e. drought-induced embolism), gas emboli may be trapped at distal ends of water-filled vessels (i.e. cutting-artifact; Torres-Ruiz, 2015), but xylem tension in water-filled vessels is released. Under the assumption that osmotic adjustment took place under drought to prevent tissue dehydration and maintenance of turgor (P; Hsiao, 1973; Patakas et al., 2002), the water potential of vessel-associated tissue \( \psi_{v, \text{t}} = P + \psi_{c} \) is much more negative than \( \psi_{v} \) of remaining liquid inside the vessel lumen.

\[ t = 1 \] A flow of liquid is induced from vessel lumen into vessel-associated tissue, facilitated by vessel-to-parenchyma cell and fiber pitting, following this gradient in \( \Psi \). Consequently, the water column of liquid retracts and its hydrostatic pressure is reduced to sub-atmospheric (= tension) due to this outflow, as evidenced by a meniscus contact angle of liquid/wall \(<90^\circ\). This internal redistribution of water continues, and a flow of water propagates through the tissue matrix from vessel-associated tissue to bordering tissue layers tissue rehydration). This increases turgor, and in turn \( \psi_{c} \) of parenchyma cells and fibers after water uptake. This conceptual idea is in agreement with Scholander (1955), who mentioned that in an excised dehydrated grapevine stem a tension develops in liquid-filled vessels caused by strong osmotic forces that established themselves when the vine wilted.

\[ t = 2 \] To equilibrate \( \psi_{c} \) within the tissue matrix and with external \( \Psi \), which may be as high as atmospheric pressure, parenchyma cells and fibers are required to export solutes (i.e. increase \( \psi_{c} \) by an increase in \( \psi_{c} \)) into the apoplast until a new water flow equilibrium is reached; apoplast \( \psi_{a} \) and in turn external \( \psi_{c} \) decreases. This accumulation of solutes in the apoplast induces water droplet growth by osmosis (Holbrook and Zwieniecki, 1999) or water cotransport (Wegner, 2014), and ultimately embolism removal. A continuous flow of liquid from vessel-associated tissue into the gas-filled vessel lumen results in expansion of water-columns having a meniscus contact angle of liquid/wall of greater than \( 90^\circ \). The now convex-shaped meniscus is the result of an increase in liquid hydrostatic pressure caused by water inflow from vessel-adjacent tissue. In turn, gas emboli dissolve into solution driven by the increase in gas pressure between expanding water columns. The dissolution of gas is facilitated by the existence of a liquid film that spread from emerging droplets. Because of these internal processes, a previously embolized vessel may refill again while others embolize.

This conceptual model of Figure 9 explains the substantial increase in embolized vessels over time in stems not subjected to an external water source. Because vessels remained water-filled in stems supplied with \( \text{H}_{2}\text{O} \) only and all embolized vessels refilled, it can be concluded that the embolism removal process can be limited by the availability of free water for tissue rehydration and refilling. Moreover, rehydrating stems with \( \text{H}_{2}\text{O}+\text{PEG} \) solution of comparatively low \( \psi_{c} \) demonstrated that the rate of vessel refilling can be reduced artificially highlighting the importance of changes in tissue water relations during embolism removal (as suggested by Fig. 9). Because PEG 8000 was used at relatively low concentrations and the large PEG polymer is unlikely to permeate into cells, toxic effects caused by this osmoticum were considered negligible (Lawlor, 1970). However, it requires further investigations to resolve how tissue internal \( \Psi \) responds temporally and spatially to different external conditions. The observation of a random pattern in embolism formation in excised stems, as opposed to a radial embolism spread via vessel-to-vessel pit connections in vivo (Brodersen et al., 2010), further supports the importance of hydraulic properties and rehydration of vessel-adjacent tissue in this process.

**Implications for Hydraulic Measurements**

As mentioned in the introduction, native xylem hydraulic conductivity and susceptibility to embolisms have been evaluated extensively on excised plant segments using hydraulic methods, but some of these methods have been recently called into question (e.g. Choat et al., 2010; Sperry et al., 2012; Cochard et al., 2014; Torres-Ruiz et al., 2015). Using microCT imaging, it has been shown that cutting stem segments under xylem tension causes air to be pulled into the excised vessel ends (Torres-Ruiz et al., 2015), which may lead to a significant underestimation of hydraulic conductivity. To eliminate this artifact, it has been suggested that sample water potentials should be equilibrated for 0.5 to 2 h in sealed plastic bags and/or rehydrated at both cut ends after harvest and before excision of a stem segment (Wheeler et al., 2013). Finally, segment ends should be recut under water before hydraulic measurements (Torres-Ruiz et al., 2015). This method, however, is performed with the implicit assumption that no novel refilling (to our knowledge) is occurring. Nevertheless, more recent data indicate that the rehydration
procedure may facilitate vessel refilling (Trifilò et al., 2014; Venturás et al., 2015). Venturás et al. (2015) concluded that differences in hydraulic conductivity were mainly caused by a refilling artifact as opposed to a cutting-artifact. Here, the experimental design effectively simulated the methods many labs currently use for the preparation/maintenance of samples in xylem hydraulic conductivity measurements. Our data provide direct visual evidence that refilling of grapevine stems can induce vessel refilling in less than 1 h, which would lead to an overestimation of hydraulic conductivity. For example, Jacobsen and Pratt (2012) maintained excised grapevine shoots in sealed plastic bags for as long as greater than 12 h, with cut ends sealed with vacuum grease before hydraulic conductivity measurements. Our data suggest that when grapevine stem ends are sealed with no supplemental water (i.e., not rehydrated), many more vessels can embolize over time due to internal water-redistribution (see Fig. 9). Given our data, this sampling procedure would cause an overestimation of xylem embolism when using hydraulic methods, and potentially lead to r-shaped vulnerability curves. However, future work is needed to determine the extent and the impact of the dynamic process of embolism formation/removal in excised samples on measured hydraulic parameters in grapevine and other species.

MATERIALS AND METHODS

Plant Growth

_Vitis riparia_ (‘Riparia Glorae’ plants were propagated from 5 to 10 cm herbaraceous cuttings obtained from established plants at the University of California, Davis, experimental vineyard (Knipfer et al., 2015b). Plants were grown in 0.7 L plastic pots filled with soil mix (40% washed sand, 20% sphagnum peat moss, 20% redwood compost, and 20% pumice rock) and maintained under greenhouse conditions (approximated day/night temperature of 8/25°C, photoperiod of 15/9 h, relative humidity of 35%) for 9 to 12 weeks. Plants were irrigated twice daily with water supplemented with macro- and micronutrients, and plants used in experiments were approximately 1 m tall. To induce drought stress, irrigation was stopped as obtained from drought-stressed plants (n = 15). Drought-stressed plants were transported from the greenhouse to the microCT facility (Beamline 8.3.2) at the Lawrence Berkeley National Laboratory Advanced Light Source (Berkeley, CA) on the day of scanning (Brodersen et al., 2010; McElrone et al., 2013; Knipfer et al., 2015a,b). _Ψ_ stem of the intact plant was measured <30 min before excising stem segments. Subsequently, the shoot of the plant was submerged in water and a basal stem segment of 7 to 9 cm in length (average diameter of 2.5–4.0 mm) was cut under water with pruning shears between two nodes (Supplemental Fig. S5). The stem segment was recut at both ends by 1 to 2 cm using a fresh razor blade to minimize the presence of air-filled tissue at distal ends and in turn maximize the connectivity of externally supplied solution with stem tissue (stems were 3–5 cm in length to facilitate tissue rehydration at sites of embolized vessels). Following this procedure, both ends were treated in the same way and the cut stem surfaces were supplied with either H2O (solute potential, $\psi_f$ of 0 MPa), H2O+PEG (Polyethylene glycol 8000; $\psi_f$ of −0.12 and −0.29 MPa), or covered with petroleum jelly (Vaseline Petroleum Jelly, Uni-lever, Englewood Cliffs, NJ; i.e., the no-H2O treatment). H2O+PEG solutions of relatively mild $\psi_f$ were used to minimize effects of osmotic shock on cellular activity during the experiment, and solutions of different $\psi_f$ were supplied to the stem to investigate if dynamics in embolism removal/formation can be manipulated externally and are dependent on the corresponding water potential gradient and in turn the rate of flow (rehydration) into the excised stem. The following procedures were used to supply different solutions: (1) an adapter piece (i.e., Connector-PVC tubing unit) was entirely filled with solution using a syringe; (2) the bottom end of the stem was tightly sealed to the PVC tubing of the adapter piece (approximately 2 cm in length) under water; and (3) the valve of the adapter was closed so that no solution was draining out of the stem bottom end. Using a valve instead of a screw cap had the advantage that the stem was not over-pressurized, which may have forced solution into embolized vessels. Subsequently, the top end of the stem was sealed to a 2-cm piece of PVC tubing that was filled with the corresponding solution using a syringe. The hydrostatic pressure of the supplied solution was 0.2 kPa (≈2 cm column height); to test if this hydrostatic pressure affected the dynamics of vessel refilling, some stems were also subjected to H2O at 6 kPa (≈60 cm column height). After sample installation, great care was taken that no air bubbles were present in the PVC tubing and adapter piece. For the no-H2O treatment, both stem ends were covered and sealed with petroleum jelly and a 2-cm piece of PVC tubing was slid over both stem ends to hold the petroleum jelly in place. After samples were prepared with the appropriate treatment (H2O, H2O+PEG, no-H2O), the entire surface of the stem was covered with petroleum jelly to prevent surface evaporation (<0.0007 mL at 8 h at 25°C; some segments were also inserted into a sealed plastic bag after they were coated with petroleum jelly to further ensure that water loss to the atmosphere was negligible. The entire procedure of sample preparation before microCT analysis was completed in approximately 5 min and is summarized in Supplemental Figure S3.

Immediately after sample preparation, samples were fixed to a rotating stage and an approximately 3 mm portion located in the middle of the stem was scanned in the 21 keV synchrotron x-ray beam. Samples were scanned multiple times (4–8 times) over a time period of up to 18 h after stem excision (t = 0 h). During each scan (duration of approximately 10 min), the sample was scanned using the continuous tomography setting that yields 1025 two-dimensional (2D) images with a 3.1-μm pixel resolution captured on a CMOS camera (PCO.Eedge; PCO, Kelheim, Germany). From each scan, the acquired 2D projection images were reconstructed into a stack of transverse images using a custom software plugin for the Fiji image-processing software (www.fiji.sc, Image); National Institutes of Health, Bethesda, MD) that used the software Octopus (ver. 8.3; The Institute for Nuclear Sciences, Ghent University, Ghent, Belgium) in the background (Knipfer et al., 2013b). The process of refilling vessels formation and the status of water-filled and embolized vessels were determined from reconstructed transverse and longitudinal microCT images (Brodersen et al., 2010; Knipfer et al., 2015a,b). Longitudinal images were generated using the Slice Tool in the software AVIZO (ver. 6.2; Visualization Sciences Group/FEI, Hillsboro, OR). From the first scan, the total number of vessels (N_total) and the number of initially embolized vessels (N_initial/embol) was determined, and from the following scans, numbers of additional embolized (N_initial/embol refilled) and refilled (N_initial/embol refilled) vessels were determined. Refilled and embolized vessels were marked in color using the Brush Tool in Adobe Photoshop CS4 imaging-processing software (Adobe Systems, San Jose, CA). The percentages of refilled and embolized vessels were determined by (N_initial/embol refilled × 100% and (N_initial/embol refilled × 100%, respectively. The amount of functional vessels (N_functional = N_total – (N_initial/embol refilled + N_initial/embol)) was determined by (N_initial/embol refilled × 100%. Based on longitudinal microCT images acquired during the embolism removal process, the liquid/wall contact angle values ($\theta$) between the water droplet and inner vessel wall, and the meniscus of water columns to the inner vessel wall, were measured using the Angle Tool in the software Fiji. Droplet base diameter was measured using the Line Tool in the software Fiji. The expansion rate of water droplets until formation of water columns was estimated by measuring the 2D projected surface area of a droplet over time using the Polygon Tool in the software Fiji.

For detailed analysis of xylem structure and visualization of tissue connectivity, additional excised stem segments were obtained from plants at _Ψ_ stem > −0.5 MPa and dehydrated slowly for 4 d at relatively low temperature of 33°C (i.e. prevention of tissue cracking) before microCT imaging. After the
dehydration period, segments were scanned at 1.8-μm pixel resolution (as described above). After the 2D projections were reconstructed into a stack of transverse images, entire image stacks were uploaded into the software AVIZO (ver. 6.2) and a three-dimensional image was generated using AVIZO’s Volume Rendering Module (Knipfer et al., 2015a).

**FDA Staining**

For detection of cell viability and metabolic activity within the xylem tissue, a 9.6-μm FDA (fluorescein-diacetate; Sigma-Aldrich, Milwaukee, WI) solution was used in combination with fluorescence light microscopy (Krasnow et al., 2008). FDA-solution was prepared by adding 2 μL of a 4.8 μM stock FDA-solution (in acetone) to 1 mL of water. Plants were transported from the greenhouse to the laboratory <2 h before sample preparation. After measurement of the 95% an approximately 3-mm-long stem segment was cut transversely using a fresh razor blade, and was submerged immediately after for 30 min in FDA staining solution and incubated in the dark at room temperature (23°C). Before imaging, samples were rinsed with water, and placed on a microscope glass slide with the top end covered with a thin glass slide. The sample surface was viewed under violet fluorescence light (excitation filter 405 nm, dichromatic mirror 455 nm, barrier filter 455 nm) for detection of heavily lignified tissue (bright blue signal) and under green fluorescence light (excitation filter 490 nm, dichromatic mirror 500 nm, barrier filter 515 nm) for detection of living and metabolically active tissue (green signal) using a Olympus Vanox-AHBT (Olympus America, Melville, NY) compound microscope connected to a 600ES digital camera (Pixera, Santa Clara, CA). In principle, after absorption of nonfluorescent FDA an esterase-dependent enzymatic reaction is carried out inside the living cell that produces the green fluorescent metabolite fluorescein (i.e., indicator for cell viability). To test if changes in cell metabolism by temperature are reflected by changes in fluorescence intensity, a control experiment was carried out: stem samples (approximately 3 mm in length) were prepared from the same plant, submerged in water, incubated for 24 h at 23°C, 4°C, and −20°C, and transferred into a 4°C FDA solution for 30 min before preparation for imaging at the same camera settings.

**Data Analysis**

Linear regression and statistical analysis were performed with SAS (ver. 9.2; SAS Institute, Cary, NC) and SigmaPlot (ver. 8.0; Systat Software, San Jose, CA). The PROC REG procedure was used in SAS for linear regression analysis. Nonlinear regression analyses were performed in SigmaPlot. Mean values and ses were determined using the PROC MEANS procedure in SAS. Statistical differences between means and between slopes of linear regression lines were determined by ANOVA using the PROC GLM procedure at P < 0.05 in SAS.

**Supplemental Data**

The following supplemental materials are available.

- **Supplemental Figure S1.** Enlarged Transverse MicroCT Images at Three Different Locations along the Stem Showing Embolized Vessels in Isolation
- **Supplemental Figure S2.** Temporal Changes in Water Droplet Size during the Embolism Removal Process in Excised Stems
- **Supplemental Figure S3.** Cartoon Illustrating the Preparation Procedure of Excised Stem Samples for MicroCT Imaging

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