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Research paper

Short-time xylem relaxation results in reliable quantification of embolism in grapevine petioles and sheds new light on their hydraulic strategy

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In recent years, the validity of embolism quantification methods has been questioned, especially for long-vesseled plants. Some studies have suggested that cutting xylem while under tension, even under water, might generate artificial cavitation. Accordingly, a rehydration procedure prior to hydraulic measurements has been recommended to avoid this artefact. On the other hand, concerns have been raised that xylem refilling might occur when samples are rehydrated. Here, we explore the potential biases affecting embolism quantification for grapevine (*Vitis vinifera* L.) petioles harvested under tension or after xylem relaxation. We employ direct visualization of embolism through X-ray micro-computed tomography (microCT) to test for the occurrence of fast refilling (artifactually low per cent loss of conductivity (PLC) due to rehydration prior to sample harvest) as well as excision-induced embolism (artifactually high embolism due to air introduction during harvest). Additionally, we compared the response functions of both stomatal regulation and xylem embolism to xylem pressure (Ψ_x). Short-time (20 min) xylem tension relaxation prior to the hydraulic measurement resulted in a lower degree of embolism than found in samples harvested under native tensions, and yielded xylem vulnerability curves similar to the ones obtained using direct microCT visualization. Much longer periods of hydration (overnight) were required before xylem refilling was observed to occur. In field-grown vines, over 85% of stomatal closure occurred at less negative Ψ_x than that required to induce 12% PLC. Our results demonstrate that relaxation of xylem tension prior to hydraulic measurement allows for the reliable quantification of native embolism in grapevine petioles. Furthermore, we find that stomatal regulation is sufficiently conservative to avoid transpiration-induced cavitation. These results suggest that grapevines have evolved a strategy of cavitation resistance, rather than one of cavitation tolerance (diurnal cycles of embolism and repair).

Keywords: cavitation, hydraulics, microCT, refilling, *Vitis vinifera*, vulnerability curves, X-ray micro-computed tomography.

Introduction

Plants transport water in a metastable state, and consequently, the water column in the xylem is at risk of cavitation: given the presence of a nucleating void, xylem sap will rapidly change from the liquid to the gas phase, ultimately resulting in the formation of an embolism as the conduit becomes completely filled with air. Embolism formation can cause a substantial reduction in the xylem conductivity and is therefore of critical importance for the understanding of plant physiology (Tyree and Sperry 1989). Several methods have been developed for quantifying the degree of embolism (Milburn and Johnson 1966, Sperry et al. 1988, Cochard et al. 1992, Pockman et al. 1995) and numerous publications have used these methods in a wide range of species (summarized by Choat et al. 2012).

In recent years, the validity of such embolism measurements has been questioned, calling into question our understanding of the hydraulic strategies of long-vesseled plants (reviewed by Cochard et al. 2013). Xylem vulnerability curves conducted

using several techniques on the same genotypes have been found to be very different (Choat et al. 2010, Torres-Ruiz et al. 2014), suggesting a methodological bias. Since the xylem sap is normally under tension, the cutting of a xylem vessel in the open air immediately results in an embolism. For that reason, common practice has been to prepare samples for hydraulic measurements by excising xylem segments under water. However, even when cut under water, vessels may cavitate, probably due to the metastable or supersaturated state of the xylem sap (Wheeler et al. 2013, Torres-Ruiz et al. 2015). Wheeler et al. (2013) suggested that air bubbles, released from the apoplast upon cutting or arising from imperfectly wetted defects on the cutting surface, may be sucked into opened conduits, biasing estimates of native embolism with a magnitude dependent on the tension level. However, such biases have not been found in all species for reasons that are not well understood, further complicating our ability to assess reliable methods for estimating vulnerability to cavitation (Wheeler et al. 2013, Venturas et al. 2015).

Methods allowing the direct observation of the xylem embolism in intact plants such as magnetic resonance imaging and X-ray micro-computed tomography (microCT) represent reliable options to visualize the phenomenon (Dalla-Salda et al. 2014, Cochard et al. 2015). However, these methods are expensive and the technical facilities are not widely available, facts that have motivated efforts to eliminate artefacts from hydraulic methods. For example, Wheeler et al. (2013) recommended xylem 'relaxation' (i.e., the increase of water potential close to the atmospheric level) prior to the excision of a xylem segments under water. Yet, Trifilò et al. (2014) presented evidence that such relaxation steps could refill native emboli within a short period (<1 h), suggesting that xylem relaxation might overcome one artefact only to result in another. These controversies call for in-depth investigation of the xylem relaxation–refilling interaction in order to establish unbiased methods for embolism quantification.

Grapevines (*Vitis vinifera* L.) are long-vesseled plants known for their open vessel artefact (Choat et al. 2010). It was previously suggested that grapevine petioles are highly susceptible to cavitation and even that petiole cavitation could take part in stomatal regulation (Zufferey et al. 2011). Recently, in vivo visualization showed that petioles are significantly more vulnerable to cavitation than shoots, meaning that petiole cavitation is of greater physiological importance under field conditions (Hochberg et al. 2015*b*). In the current research, we have studied the artefact of cutting xylem under tension in grapevine petioles. The speed of xylem refilling in relaxed vessels was investigated using X-ray microCT imaging, and the vulnerability curves of relaxed (but not refilled) petioles were compared with vulnerability curves that were built using microCT images. Finally, to understand the hydraulic strategy of grapes, per cent loss of conductivity (PLC) and xylem pressure (Ψ_x) were evaluated and compared in field-grown grapevines subjected to water deficit.

Materials and methods

We undertook a research programme divided into three phases. The first phase was conducted in October 2014 with three specific objectives: (i) to evaluate the length of the vessels that pass through the petiole and continue into the stem in order to estimate the minimal sample length that is required to avoid any open vessels, (ii) to determine the hydraulic head sufficient to measure the native conductivity of petiole segments without displacing native embolism and (iii) to repeat the Wheeler et al. (2013) experiment in grapevines in order to test whether cutting vessels under tension results in artifactual embolism.

The second phase of the research was conducted in March 2015. We compared bench drying and hydraulic estimates of PLC (Sperry et al. 1988) versus microCT visualization of embolism to: (i) investigate the time required for refilling vessels when they are placed under slight positive pressure and (ii) test whether short-time xylem relaxation results in a reliable measurement of embolism.

The third phase of the research was conducted between July and August 2015. The objective was to study the relationship between PLC and stomatal conductance (g_s), while avoiding the artefact described by Wheeler et al. (2013). For this purpose, field-grown Merlot grapevines were subjected to deficit irrigation (30% of the crop evapotranspiration (ET_C)); periodical measurements of g_s and Ψ_x were performed, and the xylem vulnerability curves of petioles were built.

Plant material and growth conditions

The first phase of research was conducted on mature *V. vinifera* (cultivar unknown), growing in the orchard of INRA Clermont-Ferrand (France). In October 2014, before any autumn frost, the vines appeared very healthy with extremely long shoots (3–4 m). The vines were growing in semi-shaded conditions and were not artificially irrigated.

For the second phase of the experiment, 1-year-old *V. vinifera* (Syrah × Grenache progeny; Coupel-Ledru et al. 2014) grafted on R110 rootstock were grown in a controlled environment during the winter of 2015, in INRA, Clermont-Ferrand (France). The vines were potted in 10 l pots as described in Coupel-Ledru et al. (2014) and irrigated daily in order to maintain the water content close to field capacity. Starting on 20 December 2014, the vines were subjected to a 10/25 °C night/day cycle with 2 h of supplemental light. Bud break occurred on 15 January and the experiments took place between 1 and 15 March, when the vines had five mature leaves.

The third phase of the research took place in a 5-year-old Merlot vineyard (grafted on SO_4 rootstock) located in the University of Udine experimental farm (North-eastern Italy, 46° 02′ N, 13° 13′ E; 88 m a.s.l.). Before the experiment started, the vines were irrigated at 120% ET_C. The petiole xylem vulnerability curve was measured (as described below) a month before veraison.

At veraison, the irrigation of 12 vines was switched to 30% ET_C, while the others were maintained at 120% ET_C. Subsequently, g_s and Ψ_x were periodically measured (for a total of nine time points, four plants from each treatment) in adjacent leaves, using a portable gas exchange system (LiCor6400, LI-COR, Lincoln, NE, USA) and pressure chamber (Soil Moisture Co., Santa Barbara, CA, USA), respectively.

Evaluation of the length of vessels that travel from the petiole to the stem

Some proportion of xylem conduits are open (i.e., without an intervening end-wall bearing intervessel pit membranes) all the way from the stem through the petiole and into the leaf blade (Chatelet et al. 2006). We evaluated the length of such vessels as follows: following perfusion of five 2- to 3-m-long shoot samples with water at 150 kPa for 60 min to remove all native embolism, samples were perfused with air at 200 kPa from the cut basal stem end to embolize all the open vessels. For each sample, a 3 cm sample from the centre of the petioles originating from the fourth internodes above the cut end was excised under water and its PLC was determined as described below.

Excised xylem segments of long-vesseled species should have a higher number of open vessels (compared with common short-vesseled species). Since the embolism in open conduits is much easier to remove than the embolism in conduits bearing an end-wall, the maximal pressure for accurate conductivity measurements of embolized vessel (k_{init}) was evaluated. The conductivity of plant segments of different lengths (1 cm petiole, 4 cm petiole and 4 cm petiole attached to a 4 cm stem), containing a different number of open vessels, was measured over a series of pressures (increasing from 5 up to 200 kPa). Prior to the measurements, all segments were flushed with air (200 kPa) from both ends to ensure 100% embolism. All conductivity values were normalized by the maximal conductivity that was measured when water was perfused at 200 kPa in the absence of any embolism.

Xylem vulnerability curves

To evaluate the effect of cutting xylem under tension, we compared the PLC both in relaxed petioles and those cut under tension (Wheeler et al. 2013). Long shoot segments (1.5 m) were bench dehydrated to a range of pressures between –0.5 and –2 MPa. Shoots were covered with black plastic bags for 20 min to equilibrate the leaf and stem water potentials before measuring Ψ_x. Ψ_x was estimated by measuring leaf water potential with a pressure chamber (PMS, Albany, OR, USA). For the hydraulic conductance measurements, three petioles were excised under water and connected to the Xyl'em apparatus (Xylem Embolism meter, Bronkhorst, Montigny-les-Cormeilles, France) (i.e., cut under tension treatment). The cut surface of the remaining shoot was held under water for 10 min and then three additional petioles were cut under water and connected to the Xyl'em apparatus (relaxed treatment). For all treatments, the initial hydraulic conductance (K_i) was determined with a hydrostatic pressure head of 4–5 kPa, and distilled and degassed water supplemented with 15 mM KCl was used as the perfusion solution. The petioles were then flushed twice for a period of 2 min with water pressurized at 0.18 MPa and remeasured to determine the maximum conductance (K_{max}). Finally, PLC was calculated as:

$$PLC = 100 \times \left(1 - \frac{K_i}{K_{max}}\right) \quad (1)$$

In the second phase of the research, the per cent embolized vessels ($\%_{emb}$) of bench-dried shoots from two genotypes hypothesized to differ in terms of their xylem vulnerability to cavitation (043 and 064, Grenache × Syrah progeny; Coupel-Ledru et al. 2014) were measured using the same hydraulic method. The shoots were submerged under 4 cm of water for 20–40 min (depending on the initial tension) to promote xylem tension relaxation. The difference in relaxation time (compared with 10 min in the first phase of the research) resulted from the understanding that a refilling artefact is not probable within an hour time frame (described in Results).

Quantification of embolized vessels with X-ray microCT

Vines (043 and 064, Grenache × Syrah progeny; Coupel-Ledru et al. 2014) were transferred to the INRA laboratory, and Ψ_x was measured. The petiole of the third or fourth leaf from the bottom was fastened to the stem so that the blade lay parallel to the shoot axis: all other leaves were removed. A plant segment, composed of 20 cm of stem two to three internodes beneath the remaining petiole insertion, was cut from the vine in air, and the leaf inserted into a plastic sample holder. The shoot was quickly submerged in wax (60 °C) and immediately cooled in water to prevent dehydration during the scan. The sample was placed in an X-ray microtomograph (Nanotom 180 XS, GE, Wunstorf, Germany) at the PIAF laboratory (INRA, Clermont-Ferrand, France) and scanned. X-ray settings were chosen in order to minimize the scan time and the X-ray dose the samples experienced (60 kV, 240 µA, 21 min). The spatial resolution was adjusted according to the sample dimensions and varied from 3 to 4 µm per voxel. After 3D reconstruction, one transverse 2D cross section was extracted, always from the same 10 µm depth along the petiole. Images were then analysed with ImageJ software (Abràmoff et al. 2004) in order to quantify the number of embolized conduits.

Between two consecutive images, Ψ_x was decreased using the single end air injection technique (Cochard et al. 1992). The segment was placed in a pressure chamber (PMS) with only the stem protruding from the lid. The wax was removed from the tip of the shoot, and the chamber was pressurized to the desired pressure for a period of 5–20 min, until equilibrium was reached (Cochard et al. 1992). Ψ_x was gradually decreased from –0.5 to –0.75 to –1 to –1.25 to –1.5 MPa, while the petiole was

scanned at each Ψ_x. Finally, to assess the total number of mature vessels, the petiole was cut 1 mm below the scanned area. The $\%_{emb}$ was calculated as the per cent of embolized vessels out of the total vessel number.

Evaluation of refilling under pressure

To ensure that Ψ_x relaxation did not result in the refilling of embolized vessels, we quantified the number of embolized vessels before and after submergence under water. For this, we scanned the vines petioles using microCT (exactly as described in the previous section). Three vines of two genotypes (two 006 and one 082) were detached from their roots and bench dried to a Ψ_x of −1.03, −1.1 and −1.5 MPa. A similar plant segment (one leaf attached to a 20-cm-long stem) to the one used to establish xylem vulnerability curves was harvested and scanned. Vine segments were subjected to a low positive pressure (4 cm H_2O) for different time intervals between two consecutive measurements. Each segment was scanned and fully submerged under water alternately for periods of 10, 30, 120 and 120 min (for a cumulative total of 280 min) and then left under water overnight (15 h).

Statistical analysis

In order to evaluate the Ψ_x that corresponds to the measured 50% PLC or 50% embolism (Ψ_{50}), vulnerability curve data were fitted with an exponential sigmoidal regression ($P < 0.05$) as follows:

$$PLC = \left(\frac{100}{1 + e^{\alpha(\Psi_x - \Psi_{50})}} \right) \quad (2)$$

To test for significant differences between the methods (with or without relaxation; microCT or hydraulic apparatus), coefficients α and Ψ_{50} were determined from the linearized form of Eq. (2), as follows:

$$\ln\left(\frac{100}{PLC - 1} \right) = \alpha(\Psi_x - \Psi_{50}) \quad (3)$$

Transformed values were analysed through a regression model ($P < 0.05$), with Ψ_x, the method and their interaction as model effects. To compare the vulnerability to cavitation of the different genotypes, PLC values of the same Ψ_x were averaged and compared.

Results

The results from the first part of the experiment showed that long vessels pass from the petiole to the stem. In the long shoot vines that were used in the first part of the experiment, conduits passed through the petiole and into the stem without the presence of a pit membrane, sometimes for over four internodes, equivalent to 37 cm (Table 1). Specifically, cutting the stem at 5 cm below the internode resulted in a loss of conductivity of 18.6% in the central part of the petiole. The addition of one, two or three internodes to this segment (5 cm shoot) reduced the conductivity loss to 9.8, 7.2 and 2.2%, respectively (Table 1). When using less developed plants, as in the second phase of the research, we noticed a lack of embolism in the scanned segments that were cut under tension (in the air) while they had only 20 cm of stem connected down from the petiole. This indicated that the vessels extended a shorter distance from the petiole into the stem (see Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online).

The embolism in short plant segments, which contained a higher percentage of open vessels, was shown to be removed at lower pressures of water compared with long segments (Figure 1). The embolism present in the 1-cm-long petiole segments was partly removed at 10 kPa and completely removed at 50 kPa. On the other hand, the 4-cm-long petioles connected to a 4-cm-long stems showed very few signs of embolism flushing, even at 50 kPa. A petiole segment of 4 cm long (an easy segment to use from a practical point of view) showed no sign of embolism removal at 20 kPa.

With the above results to guide our sampling and choice of pressure heads, we next tested the effects of cutting and relaxation on estimates of native embolism. Comparison of xylem vulnerability curves measured with 10 min relaxation or without prior

Table 1. Assessment of the conductivity (as a per cent total petiole conductivity) of long vessels passing through the petiole and into the shoot. Data are presented as a function of the distance they pass (in cm) and number of internodes down from the petiole junction. Data are means ± SE ($n = 5$).

No. of internodes	0.5	2	3	4
Distance (cm)	4.7 ± 0.66	16.3 ± 3.05	26.9 ± 5.22	36.9 ± 7.48
Conductivity (%)	18.58 ± 3.59	9.8 ± 5.51	7.22 ± 1.93	2.21 ± 1.45

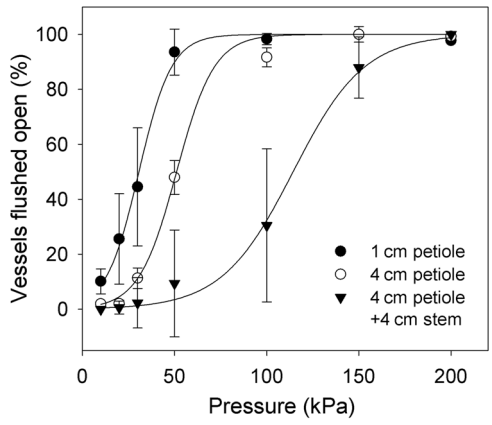

Figure 1. Assessment of the minimal pressure required to flush vessels in grapevine petiole (or petioles connected to stems) segments of different lengths. Data are means ± SE ($n = 5$). The sigmoidal regression showed that the inflection point was 30, 51 and 114 kPa for the 1, 4 and 4 + 4 cm long segments, respectively.

relaxation shows a significantly lower PLC for the relaxed treatment ($P < 0.05$) for Ψ_x values higher than −1.4 MPa (Figure 2). The differences were clearer for plants with high Ψ_x. These results are similar to the findings of Wheeler et al. (2013) and are possibly linked to the number of non-conductive vessels (these are no longer prone to the artefact) in the intact petiole. The Ψ_{50} was −1.3 and −1.44 MPa in the relaxed and non-relaxed petioles, respectively.

The results from the second phase of the research showed that submerging plant segments under water for >4 h (the cumulative time reached 4 h 40 min) did not result in xylem vessels refilling (Figure 3; Figure S3 available as Supplementary Data at *Tree Physiology* Online). Refilling was observed only when the segments were left under water overnight (15 h). Interestingly, overnight refilling was very significant in all segments, with two out of three segments being completely refilled after overnight submergence in water.

The 043 genotype showed higher cavitation resistance compared with the 064 genotype, as measured by microCT imaging (Figure 4; Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online). Differences in $\%_{emb}$ were significant ($P < 0.05$) for most measured Ψ_x ($\Psi_x = -0.5$, −0.75, −1 and −1.5 MPa) but not for $\Psi_x = -1.25$ MPa. The vulnerability curves of the two genotypes were different, as evidenced by both methods of analysis (X-ray microCT or hydraulic apparatus; Figure 5). According to the direct X-ray microCT observations, Ψ_{50} of 064 was −1.01 MPa, while Ψ_{50} of 043 was −1.19 MPa. We tested

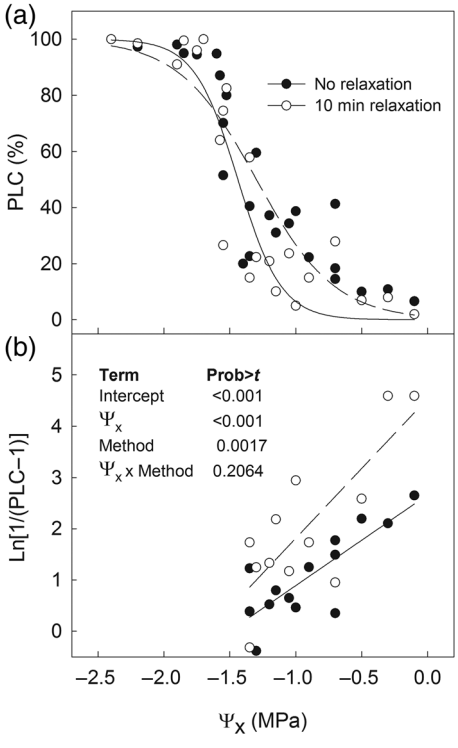

Figure 2. (a) The relation between PLC and Ψ_x in grapevine petioles under tension (filled circles) or after relaxation in which the shoots were submerged under water for 10 min (open circles). The sigmoidal regression suggest that the relaxation shifted the Ψ_{50} from −1.35 to −1.44 MPa. (b) Statistical comparison of the method (with or without relaxation) to generate xylem vulnerability curves in (a). The sigmoidal curves were linearized and compared using a regression model with Ψ_x, the method and their interaction as model effects.

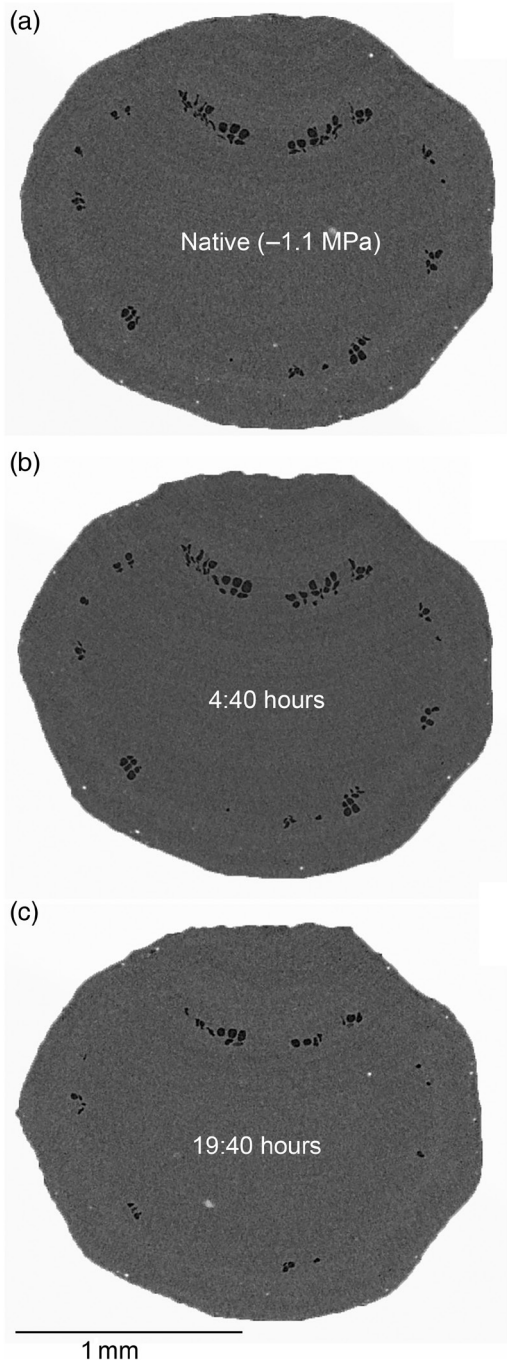

Figure 3. Assessment of vessel refilling in samples submerged under water (4 cm depth). X-ray microCT images revealed no difference in embolism between (a) the native state ($\Psi_x = -1.1$ MPa) and (b) after 4 h 40 min under water. Refilling was observed only when the samples were submerged under water overnight (c). Improved temporal resolution images are provided online (see Figure S3 available as Supplementary Data at *Tree Physiology* Online).

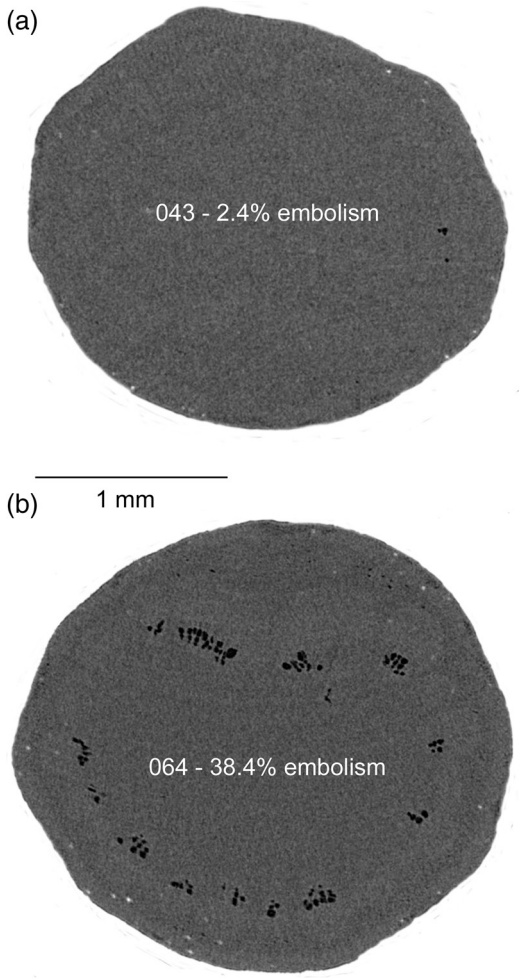

Figure 4. Comparison of embolism in petioles of two grapevine genotypes (a) 043 and (b) 064, from the Grenache × Syrah progeny. Plant segment of a leaf connected to a 20-cm-long stem was pressurized to 0.75 MPa before the X-ray scan. Images of the total vulnerability curve are provided online (see Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online).

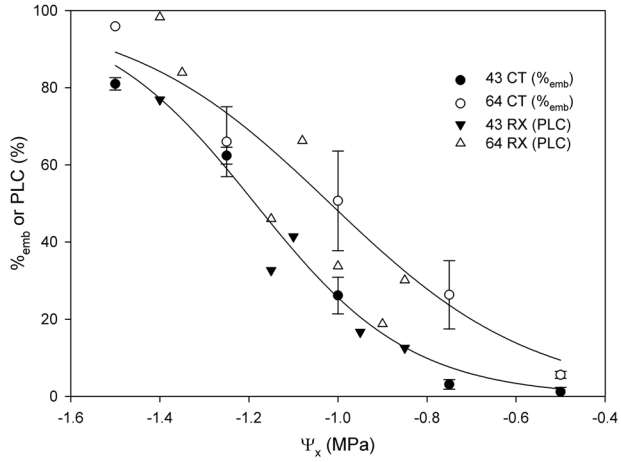

Figure 5. Comparison of xylem vulnerability curves built by two methods: imaging intact vessels with X-ray microCT (CT—circles) or by using the hydraulic apparatus with relaxed segments (RX—triangles). The relationship between the Ψ_x and the PLC or $\%_{emb}$ was assessed in grapevine petioles of two genotypes 043 (filled symbols) and 064 (open symbols). X-ray microCT data are averages of four different plants ±SE, while data points of the hydraulic apparatus represent averages of three petioles excised from the same shoot. The lines are the sigmoidal regressions of the X-ray microCT data.

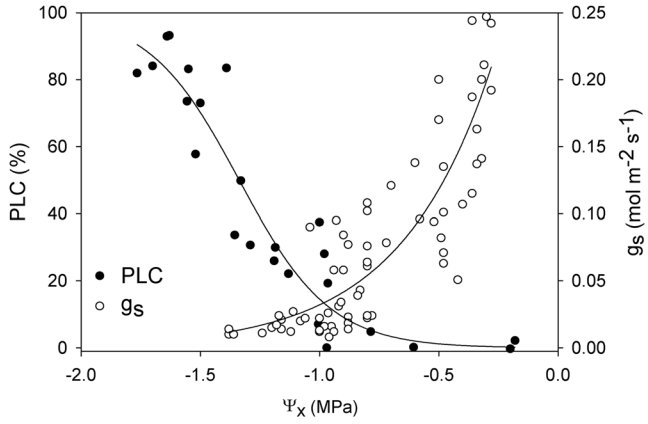

Figure 6. Comparison of g_s and petioles PLC under different Ψ_x in field-grown Merlot vines subjected to deficit irrigation. Ψ_x and g_s were measured simultaneously in adjacent leaves. The xylem vulnerability curve was recorded 1 month prior to the dehydration experiment using bench dehydration of shoots. Per cent loss of conductance data are averages of three leaves per shoot, while g_s represent a single leaf. The sigmoidal regression of both g_s and PLC in respect to Ψ_x is presented.

the similarity between the methods (comparing PLC with $\%_{emb}$). The regression analysis suggested that the genotypes and stem water potential had a significant effect ($P < 0.01$), while the method of analysis (X-ray microCT or hydraulic apparatus) did not ($P = 0.1666$).

Comparison of PLC and g_s regulation in dehydrating vines suggested that stomatal closure occurred at less negative values of Ψ_x than the ones leading to significant embolism (Figure 6). Analysis of the sigmoidal regression applied to both g_s and PLC data sets showed that 50% stomatal closure occurred at –0.51 MPa, whereas 50% PLC occurred at –1.33 MPa. At a Ψ_x equivalent to 12% PLC (–0.94 MPa), g_s was < 0.04 mol m^{-2} s^{-1} (equivalent to 15% of the maximal g_s). As water stress increased, vines also responded by abscising leaves. For the 30% ET_C treatment, the mean number of leaves decreased by 35%, from 214 to 141, after 22 days of treatment. During the same period of time, the vines maintained at 120% ET_C did not shed any leaves.

Discussion

Our results demonstrate that cutting the vessels of grapevine petioles under tension results in a biased assessment of native embolism, as suggested by Wheeler et al. (2013) for other species. A short xylem relaxation (20 min) enabled the accurate assessment of xylem embolism. Interestingly, the necessity for xylem relaxation prior to harvest for hydraulic measurements appears to have been broadly anticipated by recommendations nearly 40 years ago (Zimmermann 1978), recommendations

subsequently omitted from many hydraulic measurement protocols, perhaps due to uncertainty regarding its intended purpose or practicality.

While overnight relaxation resulted in xylem refilling, our results suggest that segments could be submerged under water for several hours without any risk of refilling. In contrast, Trifilò et al. (2014) suggested that fast refilling (<1 h) could account for the discrepancy between relaxed and native PLC results. These authors supported their hypothesis by showing that the difference was eliminated when key components in the hypothesized active refilling mechanism were inhibited (e.g., girdling, proton pumps). On the other hand, a proof that the girdling process itself does not inflict cavitation is still lacking (Rockwell et al. 2014). Current results and other X-ray microCT observations (Choat et al. 2015, Cochard et al. 2015) suggest that 'fast refilling' could be another artefact and is still under the burden of proof. It is important to mention that the potential damage to the refilling process that might result from the radiation emitted by the microCT should also be considered. The disagreements between recent studies that have evaluated the importance of excision-induced embolism (Trifilò et al. 2014, Ogasa et al. 2016, Scoffoni and Sack 2015, Torres-Ruiz et al. 2015, Venturas et al. 2015) highlight the need for a mechanistic understanding of the phenomenon.

Our experiments suggest that even when sampling very long petioles (e.g., 11 cm long in Tombesi et al. 2014), the existence of open vessels is very likely. It should be noted that the vessel length evaluation was conducted on exceptionally long shoots (3–4 m), and smaller plants may have shorter vessels. These measured values could be used as an upper limit in future protocols, since vessel length variability among grapevine cultivars is known to be low (Chatelet et al. 2011). It should be noted that the presence of open vessels in the measured segment is not necessarily an issue. Once the tension is relaxed, open vessels might be an advantage in avoiding vessel clogging by particles present in the perfusion solution. However, for an accurate measurement of k_{init}, low pressure should be applied. The petioles of grapevines contain some vessels with diameter of 30 µm (Tombesi et al. 2014, Hochberg et al. 2015*a*) and accordingly (Young–Laplace equation), if a vessel is open at both ends, embolism might be displaced at pressures > 10 kPa. Our results showed that this is the case for the 1-cm-long samples, but longer samples (4 cm) could be safely measured with a higher (10–20 kPa) pressure level. It appears that the previous protocol, which was created specifically for grapevine petioles, used too high a pressure (40 kPa) for k_{init} measurement (Lovisolo and Tramontini 2010), resulting in partial displacement of air bubbles. This can explain why previous studies that used this protocol (Tramontini et al. 2014, Hochberg et al. 2015*a*) did not surpass 50% PLC despite very negative Ψ_x (below –2 MPa).

Therefore, three critical steps should be incorporated into future PLC protocols—in parenthesis are the specific indications for petioles of grapes: (i) the initial segment harvesting should not damage the vessels designated for PLC assessment (harvest a petiole connected to at least four internodes shoot), (ii) relax the tension by submerging the sample under water for a few minutes up to an hour and (iii) measure the initial conductance under low pressure (<20 kPa for a 4-cm-long petiole). These procedures should allow the accurate evaluation of PLC, avoiding the formation of excision-induced embolism. A short movie demonstrating the protocol is available at https://www.youtube.com/watch?v=YzpDN7laepo.

The artefact of excision-induced embolism may compromise previous measurements of PLC, and casts doubt on reports of in vivo xylem conduit refilling while the sap remains under tension. Specifically in grapevines, the measurements of diurnal patterns of cavitation and refilling (Lovisolo et al. 2008, Zufferey et al. 2011) were probably an artefact of the daily fluctuation in Ψ_x and the cutting of samples under native tension. Xylem refilling is a well-documented phenomenon (Brodersen et al. 2010), but there is no conclusive evidence for its occurrence under local tension. Excision-induced embolism probably resulted in apparent higher PLC values than the native state (Cochard et al. 2015). Grapevine stems, though not studied in the present research, may also be prone to excision-induced embolism. Similar to petiole observation, direct xylem imaging of grapevine stems (Choat et al. 2010, Brodersen et al. 2013, Hochberg et al. 2015*b*) suggested greater cavitation resistance than some hydraulic measurements (Lovisolo et al. 2008). Furthermore, our results suggest (Figure 6) that cavitation in grapevine petioles occurs only after significant g_s reduction, contrary to what was previously hypothesized (Zufferey et al. 2011, Tombesi et al. 2014). These findings indicate that grapevines regulate their stomata to prevent xylem embolism and would even shed a large proportion of their leaves in order to avoid cavitation tensions. This pattern of stomatal behaviour is more consistent with a strategy of cavitation resistance and avoidance, rather than a paradigm of tolerance featuring diurnal cycles of cavitation accumulation and repair.

Conclusion

Our results demonstrate the biased increase in embolism when cutting grapevine petioles under tension, confirming the findings of Wheeler et al. (2013). Direct X-ray microCT observations showed that a short xylem relaxation does not lead to refilling. Its incorporation into hydraulic measurements of grapevines would result in reliable evaluations of PLC. The new protocol showed that grapevines have evolved a strategy of cavitation resistance, rather than one of cavitation tolerance (diurnal cycles of embolism and repair).

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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