Short title: Hydraulic failure and repair in grapevine

Correspondence to: Dr. Guillaume Charrier

e-mail: guillaume.charrier@bordeaux.inra.fr

Phone: +33 5 40 00 36 64

Address: UMR 1202 Biodiversité Gènes & Communautés INRA/Université Bordeaux, Bâtiment B2 - allée G. St Hilaire, CS 50023, 33615 Pessac Cedex – France
Evidence for hydraulic vulnerability segmentation and lack of xylem refilling under tension

Charrier G1,2*, Torres-Ruiz JM2, Badel E3, Burlett R2, Choat B4, Cochard H3, Delmas CEL5, Domec JC6,7, Jansen S8, King A9, Lenoir N10, Martin-StPaul N11, Gambetta GA1, Delzon S2

1 Bordeaux Science Agro, Institut des Sciences de la Vigne et du Vin, Ecophysiologie et Génomique Fonctionnelle de la Vigne, UMR 1287, F−33140 Villenave d’Ornon, France
2 BIOGECO, INRA, Univ. Bordeaux, 33610 Cestas, France
3 PIAF, INRA, UCA, 63000 Clermont-Ferrand, France
4 Hawkesbury Institute for the Environment, University of Western Sydney, Richmond, NSW 2753, Australia
5 UMR SAVE, INRA, BSA, Univ. Bordeaux, 33882, Villenave d’Ornon, France
6 Bordeaux Science Agro, UMR 1391 ISPA, F-33882 Villenave d’Ornon, France
7 Nicholas School of the Environment, Duke University, Durham, North Carolina 27708, USA
8 Institute for Systematic Botany and Ecology, Ulm University, Ulm D-89081, Germany
9 Synchrotron SOLEIL, L’Orme de Merisiers, Saint Aubin-BP48, Gif-sur-Yvette CEDEX, France.
10 CNRS, University of Bordeaux, UMS 3626 Placamat F-33608 Pessac, France
11 INRA, UR629 Ecologie des Forêts Méditerranéennes (URFM), Avignon, France

*Corresponding author

Authors’ contributions: S.D. and C.E.L.D. conceived the original screening and research plans (tomography); E.B., A.K., N.L., R.B., J.M.T.R., H.C., N.M-P, S.J., B.C. and S.D. performed the HRCT scans; G.C. and J.M.T.R. performed leaf hydraulics experiments; G.C. and J.C.D. performed gas exchange experiments. C.E.L.D. provided plant materials; G.C., G.A.G. and S.D. analyzed the data and wrote the article with contributions of all the authors.

One-sentence summary

Direct, non-invasive observations of embolism formation and repair reveal a lack of refilling under negative pressure and a xylem hydraulic vulnerability segmentation in grapevine.

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Present address: UMR 1202 Biodiversité Gènes & Communautés INRA/Université Bordeaux, Bâtiment B2 - allée G. St Hilaire, CS 50023, 33615 Pessac Cedex – France

Phone: +33 5 40 00 36 64
e-mail: guillaume.charrier@bordeaux.inra.fr

Abstract

The vascular system of grapevine has been reported as being highly vulnerable, even though grapevine regularly experiences seasonal drought. Stomata would consequently remain open below water potentials that would generate a high loss of stem hydraulic conductivity via xylem embolism. This situation would necessitate daily cycles of embolism repair to restore hydraulic function. However, a more parsimonious explanation is that some hydraulic techniques are prone to artifacts in species with long vessels, leading to overestimation of vulnerability. The aim of this study was to provide an unbiased assessment of (i) the vulnerability to drought-induced embolism in perennial and annual organs, and (ii) the ability to refill embolized vessels in two Vitis species.

X-ray micro-CT observations on intact plants indicated that both V. vinifera and V. riparia were relatively vulnerable, with the pressure inducing 50% loss of stem hydraulic conductivity ($\Psi_{50Stem}$) = -1.7 and -1.3 MPa, respectively. In V. vinifera, both the stem and petiole had similar sigmoidal vulnerability curves, but differed in $\Psi_{50}$ (-1.7 and -1.0 MPa for stem and petiole, respectively). Refilling was not observed as long as bulk xylem pressure
remained negative (e.g. at the apical part of the plants): $P = -0.11 \pm 0.02\text{MPa}; \Delta PLC = 0.02 \pm 0.01\%$. However, positive xylem pressure was observed at the basal part of the plant ($P = 0.04 \pm 0.01\text{MPa}$), leading to recovered conductance ($\Delta PLC = -0.24 \pm 0.12\%$).

Our findings provide evidence that grapevine is unable to repair embolized xylem vessels under negative pressure, but its hydraulic vulnerability segmentation provides a significant protection of the perennial stem.

**Keywords:** drought stress, stem, petiole, leaf, embolism resistance, hydraulic conductance, 3D imaging, *Vitis vinifera.*
Introduction

The plant hydraulic system is located at the interface between soil water and the atmosphere. Evaporative demand from the atmosphere generates a tension within a continuous xylem water column, pulling water from the soil, through roots, stems, petioles, and leaves (Dixon, 1896). Under drought conditions, the overall resistance to water flow through the soil-plant continuum increases. Increased resistance to water flow results from changes in the resistance at multiple specific locations along the flow pathway: in the soil, at the soil-root interface, in the roots, the main plant axis (i.e., stems, branches), the petioles, and the leaves. Two primary mechanisms controlling the resistance are stomatal closure (leaf-to-air water flow) and the loss of xylem hydraulic conductivity (soil-to-leaf water flow; Cochard et al., 2002). Stomatal closure is closely related to decreasing plant water status (Brodribb & Holbrook, 2003) and is often considered to be a protective mechanism against the loss of xylem hydraulic conductivity (Tyree & Sperry, 1988; Jones & Sutherland, 1991). Loss of xylem hydraulic conductivity occurs when the water potential of xylem sap reaches levels negative enough to disrupt the metastability of the water column, potentially resulting in embolism.

Generally, high resistance to embolism is observed in species distributed in dry environments, whereas highly vulnerable species are distributed in wet environments (Maherali et al., 2004; Choat et al., 2012). Although grapevine (Vitis vinifera) is widely cultivated, including in regions where it is frequently exposed to water deficit during the growing season (Lovisolo et al., 2010), recent studies have produced contrasting estimates of its resistance to embolism. Grapevine has been described as either vulnerable (Zufferey et al., 2011; Jacobsen & Pratt, 2012), or relatively resistant (Choat et al., 2010; Brodersen et al., 2013). In Vitis species, and V. vinifera especially, stomatal closure is typically observed for midday leaf water potentials ($\Psi_{\text{leaf}}$) < -1.5MPa (Schultz, 2003). Thus, according to some studies, significant losses in xylem hydraulic conductivity should be observed before stomatal closure ($\Psi_{50}$ >-1.0MPa; Jacobsen & Pratt, 2012; Jacobsen et al., 2015), implying that embolism would be commonplace.

Risk of hydraulic dysfunction is mitigated along the hydraulic pathway by hydraulic segmentation, i.e. more distal organs such as leaves and petioles will be at greater risk to embolism than more basal organs such as the trunk (Tyree and Zimmermann 2002; Choat et al., 2005). This could promote hydraulic safety in larger, perennial organs, which represent a
greater investment of resources for the plant. Hydraulic segmentation may occur in two ways. During transpiration, the xylem pressure will always be greater in more distal parts of the pathway (leaves and petioles). All else being equal, this translates to a greater probability of embolism in distal organs. However, organs may also differ in their vulnerability to embolism, compensating or exacerbating the effects of differences in xylem pressure along the pathway. If leaves or petioles were more vulnerable to embolism than branches and the trunk, then they would be far more likely to suffer embolism during periods of water-stress. This would allow petioles, leaves (Nolf et al., 2015), or even young branches (Rood et al., 2000), to become embolized without significant impact on the trunk and larger branches. In grapevine, petioles have been described as extremely sensitive to cavitation (Ψ50 ca. -1.0 MPa; Zufferey et al., 2011). However, the hydraulic methods employed in these previous studies have been shown to be prone to artifacts (Wheeler et al., 2013; Torres-Ruiz et al., 2015), necessitating the use of a non-invasive assessment of drought-induced embolism.

High-Resolution Computed Tomography (HRCT) produces three dimensional images of xylem tissue in situ, allowing for a non-invasive assessment of embolism resistance. This technique has provided robust results in various plant species with contrasting xylem anatomy (Charra-Vaskou et al., 2012; 2016; Torres-Ruiz et al., 2014; Dalla-Salda et al., 2014; Bouche et al., 2016; Cochard et al., 2015; Knipfer et al., 2015). Synchrotron-based tomography facilities allow the visualization of intact plants, offering a non-invasive, in vivo estimation of the loss of hydraulic conductivity within the xylem (Choat et al., 2016). Moreover, the quality of the X-ray beam in the synchrotron facilities provides high resolution and signal to noise ratio, making image analysis simple and accurate.

If grapevine were as vulnerable to xylem embolism as suggested in some studies, refilling of embolized vessels would be expected to occur on a frequent (daily) basis in order to maintain hydraulic continuity (Sperry et al., 1994; Cochard et al., 2001; Charrier et al., 2013). Various refilling mechanisms have been proposed to date, including positive root/stem pressure, and refilling while the xylem is under negative pressure via water droplet growth (Salleo et al., 1996; Brodersen et al., 2010; Knipfer et al., 2016). Positive pressure in xylem sap can be related to mineral nutrition and soil temperature in autumn or spring (Ewers et al., 2001), and to soluble carbohydrate transport into the vessel lumen during winter (Améglio et al., 2001; Charrier et al., 2013). Refilling under negative pressure is based on the hypothesis that embolized vessels are isolated from surrounding functional vessels, permitting positive pressures to develop and the embolism to dissolve (Salleo et al., 1996; Tyree et al., 1999).
This process has been related to the chemistry of conduit walls (Holbrook & Zwieniecki, 1999), the geometry of interconduit bordered pits (Zwieniecki & Holbrook, 2000), and phloem unloading (Nardini et al., 2011). While refilling via positive pressure has been described frequently (Sperry et al. 1987; 1994; Hacke & Sauter 1996; Cochard et al., 2001; Améglio et al., 2004; Cobb et al., 2007), refilling under negative pressure remains controversial (Cochard et al., 2013; 2015). In grapevine particularly, imaging techniques have provided evidence of refilling in embolized vessels (Brodersen et al., 2010), but uncertainties remain regarding the xylem water potential measurement at the position of the scan.

The goal of the current study was to provide a non-invasive assessment of (i) the vulnerability to drought-induced embolism in two widespread grapevine species in perennial (Vitis vinifera and V. riparia) and annual (V. vinifera) organs, and (ii) the ability to refill embolized vessels under positive or negative pressure (V. vinifera). This approach would indicate whether embolism formation and repair are likely to occur on a daily basis, and/or if hydraulic segmentation could protect perennial organs from drought stress. Stems and petioles from intact V. vinifera cv. Cabernet Sauvignon, and V. riparia plants were scanned using Synchrotron-based HRCT, characterizing their vulnerability to embolism and quantifying their ability to refill at different positions along the plant axis (base and apex) in relation with bulk xylem pressure. These data were integrated with other non-invasive techniques assessing leaf hydraulics and transpiration.

**Results**

**HRCT imaging, and embolism vulnerability in V. vinifera and V. riparia**

Embolism in stems (V. vinifera and V. riparia) and petioles (V. vinifera) was characterized by direct observation provided by HRCT images. Two dimensional, transverse slices of xylem were extracted from a 3D volume for image analysis. Typical cross sections were presented in Figure 1 for V. vinifera. Embolized (i.e. air-filled) vessels appear as black spots (highlighted red in insets). Well-hydrated plants ($\Psi_{stem} > -0.5\text{MPa}$) exhibited none or very few air-filled vessels in stems and petioles (Figure 1A and D). For both organs, the percent loss of conductivity (PLC) measured was lower than 5%. At further dehydration (ca. -1.1\text{MPa}), only a few vessels became air-filled in stems generating 9% loss of hydraulic conductance (Figure 1B), whereas half of the vessels were already embolized in petioles (PLC = 46.2%; Figure 1E). A more negative water potential ($\Psi_{stem} = -1.7\text{MPa}$) induced a
considerable increase in the number of air-filled vessels in both stems, and petioles, PLC reaching 50.5%, and 96.5%, respectively (Figure 1C and F).

HRCT imaging was used to establish stem vulnerability curves (i.e. variation in PLC as a function of xylem pressure). In *V. vinifera*, vulnerability curves of both organs exhibited a similar sigmoid shape with the air-entry point (Ψe) observed at -1.22, and -0.26MPa in stems and petioles, respectively (Figure 2; Table I). Water potential inducing 50% loss of hydraulic conductance differed between stems (Ψ50Stem = -1.73MPa) and petioles (Ψ50Petiole = -0.98MPa). Thus, when the water potential reached stem Ψe, petioles had already lost 66% of their conductivity. Significant differences were observed between *Vitis* species (*P* = 0.002; Figure 3): *V. riparia* being more vulnerable than *V. vinifera* (Ψe: -0.70 vs -1.22MPa, and Ψ50Stem: -1.29 vs -1.73MPa, for *V. riparia* and *V. vinifera*, respectively).

Integration with leaf hydraulic conductance and gas exchange in *V. vinifera*

Changes in leaf hydraulic conductance (noted *K*Leaf, but including a part of the petiole) and transpiration were assessed and the data were integrated with those obtained from the HRCT analyses above. Loss of *K*Leaf exhibited a similar pattern to loss of hydraulic conductance in petioles: Ψ50Petiole = -0.98MPa; Ψ50Leaf = -1.08MPa (Table I), however with differences in the sensitivity (69 < slp < 129 %MPa⁻¹). Apparent *K*leaf (*K*Leaf_Ap) was shifted compared to *K*Leaf (similar sensitivity: 134 %MPa⁻¹, higher Ψ50Leaf_Ap: -0.46MPa). Parameters of all vulnerability curves were significantly different from 0 (*P*< 0.001; Table I).

Considering the stem to leaf gradient in water potential measured during the gas exchange experiment (i.e. when stomata remained open, and water potential gradient maintained; ΨStem = 0.866 * ΨLeaf + 0.083; R² = 0.870), loss of hydraulic function across stems, petioles and leaves was calculated depending on ΨLeaf (Figure 4). The petiole and leaf were closely coordinated, with 50% loss of function at ca. -1.0MPa, whereas the stem remained almost non-embolized (PLC = 2.5%) at this water potential and transpiration was reduced (5.4%). At lower water potentials, almost complete hydraulic dysfunction in petioles (PLC_Petiole = 88% at Ψ = -1.70MPa) was observed and the stem exhibited significant embolism (PLC_Stem = 32.2%). The margin between Ψ50Stem and either Ψ50Petiole or Ψ50Leaf was relatively narrow (0.65 to 0.75MPa). However, taking the gradient in Ψ from stem to leaf into account, the ‘effective’ safety margin was slightly greater (0.80 to 0.90MPa). Under well-watered conditions, with high VPD (approx. 2500Pa), leaf and stem water potentials reached -
0.62 +/- 0.03MPa and -0.39 +/- 0.03MPa (mean +/- SE, n = 36), for leaves and stems, respectively. Under the normal operating range of water potential, the amount of PLC in the stem and petiole would therefore be low (0 and 17%, respectively), while transpiration would be limited (Kap = 42%).

**Xylem refilling in V. vinifera**

Re-watered plants were scanned either in the basal (1 cm above the grafting), or in the distal part (ca. 1 m above soil). In the basal part, significant changes in the amount of air-filled vessels were observed over a 24 hours period, after the plant was re-watered. Most vessels were dark gray (i.e. air-filled) before re-watering (PLC = 86.8%; Figure 5D). After 7.5 hours, evidence of xylem refilling and an increase in the number of functional vessels was observed (Figure 5E), even though PLC was barely affected (PLC = 81.2%). After 15.5 hours, many additional vessels had refilled, decreasing the PLC to 57.4% (Figure 5F). In contrast, in the upper part of re-watered plants, even after more than 48 hours of re-watering, there was no significant change in PLC (Figure 5A-C), even though most living cells remained alive (Fig. S1). Refilling was not observed at the apex (∆PLC = 0.02 ± 0.01%), regardless of the initial levels of embolism (13.7% < PLC < 92.4%).

Figure 6 thus depicts the changes in basal and apical portions of the same plant, where xylem refilling was observed at the base (∆PLC = -15.5%), and, at the same moment, no significant change in PLC was observed in the upper part (∆PLC = +5.7%). Pressure transducers indicated that bulk xylem pressure was positive at the base (ΨStem = +0.023 MPa) and negative at the apex (ΨStem = - 0.015 MPa). Although stem water potential quickly increased after re-watering, it does not completely equilibrate along the whole stem even after more than 80 hours (Fig. S2). Negative pressure was indeed measured at the apex (Ψ = - 0.013MPa), whereas it was positive at the base of the same plant (ΨStem = +0.033 MPa). Although not all plants exhibited individual vessels being refilled with sap or positive pressure, significant changes in theoretical hydraulic conductance were only observed when xylem pressures were positive (Fig. 7A). Differences in water potential (P = 0.011) and PLC (P = 0.006) were thus observed depending on the distance from the soil, among the 5 replicates (Fig. 7B).

**Discussion**
Despite the fact that *Vitis vinifera* can be adapted to environments experiencing seasonal drought, studies differ in estimates of its hydraulic vulnerability and its classification as drought sensitive (Wheeler *et al*., 2005; Jacobsen & Pratt, 2012), or drought resistant (Choat *et al*., 2010; Brodersen *et al*., 2013). Discrepancies among studies most probably lie in methodological issues, especially considering that *Vitis vinifera* is a long-vesselled species (Cochard *et al*., 2013; Rockwell *et al*., 2014; Zhang *et al*., 2014). Here, for the first time, a non-invasive estimation of complete vulnerability curves was obtained using direct observations on intact *Vitis* plants by HRCT. Our results demonstrate that *V. vinifera* stems are more resistant to xylem embolism than previously estimated by centrifugation technique, and can sustain water potential lower < -1MPa (Ψ_{50Stem} = -1.7MPa). Contrastingly, *V. riparia* originates from riparian habitats and exhibited higher drought sensitivity – (Ψ_{50Stem} = 1.3MPa). Our findings also show that petioles are more vulnerable to embolism than stems, providing evidence for hydraulic vulnerability segmentation in grapevine. Xylem conduits refilling was observed in the basal part of the plant, where positive bulk pressure was recorded (Figure 5D-F; Fig. 6), but not in the apical part, where bulk pressure remained negative under experimental conditions (Figure 5A-C; Fig. 6).

In view of the current debate on drought resistance of long-vesselled species (Sperry *et al*., 2012; Sperry, 2013; Cochard & Delzon, 2013; Hacke *et al*., 2015; Cochard *et al*., 2015), vulnerability curves imply that either embolism occurs under almost immediately negative water potentials of the xylem sap (‘exponential’ vulnerability curves), or that embolism does not take place until a threshold at a more negative water potential is reached (‘sigmoidal vulnerability curves). According to Figure 1, no embolism was observed at high xylem water potentials (Ψ>-1.0MPa) in stems of intact *V. vinifera* plants, suggesting that all vessels can support some level of negative pressure. In stems, the number of embolized vessels only increased once the pressure reached values lower than -1.5 MPa, which is consistent with results observed using Magnetic Resonance Imaging (MRI, Choat *et al*., 2010), and HRCT (Knipfer *et al*., 2015). Non-functional vessels (i.e. those that remained full of sap on our final cut images), represented ca. 5% of the theoretical conductance and were not included in our vulnerability curve analyses.

The high image resolution (ca. 3µm per voxel) provided by HRCT allowed the computation of a theoretical conductivity according to the diameters of individual vessels via the Hagen-Poiseuille equation (Figure 2; 3). Therefore, the theoretical loss of conductance
could be quantified at various xylem water potentials (as in Brodersen et al., 2013), whereas previous studies qualitatively assessed PLC from the number of air- vs sap-filled vessels. Combined with a high number of specimens at a wide range of water potentials, these results provide, for the first time, a complete vulnerability curve on intact stems ($\Psi_{50Stem} = -1.73\text{MPa}$) and petioles ($\Psi_{50Petiole} = -0.98\text{MPa}$) of *V. vinifera*. The vulnerability curves obtained are in agreement with the level of drought-induced embolism resistance observed for grapevine in studies using non-invasive techniques: synchrotron-based HRCT (Brodersen et al., 2013), Acoustic Emission analysis (AE; Vergeynst et al., 2015), and MRI (Choat et al., 2010). Although the source and signal interpretation qualitatively differ across non-invasive techniques, numerous studies combining these techniques on various species measured similar levels of embolism resistance (Choat et al., 2010; 2015; Charra-Vaskou et al., 2012; 2016; Charrier et al., 2014; Ponomarenko et al., 2014; Torres-Ruiz et al., 2014; Vergeynst et al., 2015). However, the $\Psi_{50}$ values observed in the current study are slightly less negative than those reported previously, with non-invasive methods (-1.7 vs ca. -2.0 MPa). This may have been due to differences in plant material. Ontogenic developmental stages of the plant might explain this discrepancy, where the development of secondary xylem along the course of the season would increase embolism resistance in grapevine (Choat et al., 2010). Our results demonstrate genotypic differences on stem vulnerability curves between *Vitis* species (*V. vinifera* vs. *V. riparia*; Figure 3) and are consistent with the higher drought-sensitivity of *V. riparia* compared to *V. arizonica* and *V. champinii* (Knipfer et al., 2015).

Petioles were more vulnerable to embolism than stems in *V. vinifera* cv Cabernet Sauvignon (Figure 1; 2). Only a few studies have reported petiole vulnerability curves for grapevine. Similar behavior is reported in other *Vitis vinifera* cultivars using flowmeter (Zufferey et al., 2011), pressure sleeve (Tombesi et al., 2014), or MRI (Hochberg et al., 2016). Loss of conductance in petioles (HRCT-based) and leaves (rehydration kinetic method) as measured with different techniques are remarkably similar (Figure 4) even though computations of hydraulic conductance from HRCT image data are only theoretical. Considering an inaccuracy of 2 voxels per vessel, average vessel diameters exhibited ca. 11 and 19% deviation in stem and petiole, respectively. However, PLC were only slightly affected ($\pm$ 0.9% in stem and petiole). HRCT-based images evidenced that xylem embolism limits conductance in petioles. However, the minimum water potential experienced by the petiole might have been lower than measured despite bagging the petiole for three hours.
before scanning it. This would have led to slightly over-estimated vulnerability curves, and would require additional observations using, for example, a small-sized psychrometer to monitor the petiole water potential during dehydration. In leaves, xylem embolism and extra-xylary (e.g. symplasmic) pathways both seem to contribute to the reduction of leaf hydraulic conductance (Kim & Steudle, 2007; Scoffoni et al., 2014; Bouche et al., 2016). These results question the validity of stem water potential measurement using bagged leaves for high level of stress (e.g. as presented on Fig. 6) i.e. when the leaf is hydraulically disconnected from the stem. Although embolism in petioles could represent a “hydraulic fuse” at the leaf level, under well-watered conditions, reduced transpiration (ca. 40%) substantially limits petiole embolism to less than 20%. In addition, the relatively young plant material used in this study (1 to 2 months old) is relatively vulnerable (Choat et al., 2010), but typically would not experience substantial drought in springtime.

A gradient in water potential along the entire plant might prevent embolism from propagating from distal to proximal parts without considerable difference in an organs’ embolism vulnerability per se (Fig. 6; Bouche et al., 2016). However, major anatomical differences in secondary growth, pit anatomy, and cell wall composition could also explain the higher embolism resistance of lignified organs, presenting fewer nucleation points, and lower primary xylem/secondary xylem ratio (Choat et al., 2005). Resistance to embolism is indeed tightly linked to xylem anatomy at the interspecific level (Lens et al., 2011), air bubbles nucleating onto cell walls, and propagating through pores of pit membrane (Jansen et al., 2009; Schenk et al., 2015). Through the gradient in water potential and hydraulic vulnerability segmentation, leaves and petioles isolate perennial parts of the plant from more negative water potentials and hydraulic failure under water deficit in grapevine (as demonstrated in this study) and some tropical tree species (Nolf et al., 2015).

This study provides new lines of evidence regarding the potential artefacts that lead to vulnerability curves with an ‘exponential’ shape. The ratio between vessel and sample length impairs hydraulic measurements in long-vesselled species (Ennajeh et al., 2011; Martin-StPaul et al., 2014; Torres-Ruiz et al., 2014; Choat et al., 2016), although this is disputed by other studies (Sperry et al., 2012; Pratt et al., 2015). Furthermore, the ‘exponential’ shaped vulnerability curves imply that a grapevine stem would be 50% embolized before its leaf and stomatal conductance decrease, which seems unlikely (Nardini & Salleo, 2000). Moreover, investing carbon into structures (i.e. conduit walls) that would lose their function so readily
seems unlikely, especially considering the functional importance of carbon in plant physiology (Mencuccini, 2003; McDowell, 2011; Sala et al., 2012; Hartmann et al., 2013; Charrier et al., 2015; Hartmann, 2015). Finally, the minimal water potential experienced by a plant on a seasonal basis ($\Psi_{\text{min}}$) is generally less negative than its $\Psi_{50}$ value (Choat et al., 2012).

The current study does not support high vulnerability of grapevine stems (Jacobsen et al., 2015). In the present study, drought-stressed $V. \text{vinifera}$ plants (10% to 90% stem PLC) were able to refill embolized vessels at the stem bases, but not the upper, distal stem portions (Figure 5-6). When observed, embolism refilling was always associated with positive root pressure (Fig. 7), consistent with the results of Knipfer et al. (2015). In the upper part the xylem sap remained at negative pressure (Fig. S2) and showed no refilling, even though vessel associated cells remained alive (Fig. S1). Root pressure has been credited as a strategy to recover from winter embolism (Ewers et al., 2001) and has been observed in various angiosperm dicot species, such as $Alnus \text{sp}$ (Sperry et al., 1994), $Betula \text{sp}$ (Sperry, 1988), $Juglans \text{sp}$ (Améglio et al., 2002; Charrier et al., 2013), $Vitis \text{sp}$ (Hales, 1727; Sperry et al., 1987), and some tropical and temperate vines and lianas (Ewers et al., 1997; Cobb et al., 2007). These studies suggest that particular species are able to actively refill their vessels by generation of positive pressure in the early Spring. In both this paper and in previous studies, HRCT-based observations of xylem refilling in grapevine reveal water droplets clinging on vessel walls, which then increase in volume towards the center of the conduit lumen (Brodersen et al., 2013; Knipfer et al., 2015; Fig. 5). This may suggest that apoplastic sap is pressurized before invading conduits’ lumen. Recently, Knipfer et al. (2016) reported xylem refilling in the absence of a root system $i.e.$ in 3-5 cm long excised stem segments connected to a 2-cm tube, filled with a solution at 0.2 kPa (corresponding to 2 cm column height). However, excised segments no longer exhibited tension nor pressure and slight hydrostatic pressure, when connecting the sample at both ends, which, combined with capillary forces, might have been sufficient to observe xylem refilling. In the present study, even xylem positive pressure may not successfully lead to xylem refilling in all cases. Xylem pressures of 0.02 to 0.05 MPa magnitude were observed, which should correspond to a 2 to 5 m high water column, while apical portion remained at a slightly negative potential (-0.02 to -0.1 MPa), without refilling observed at the apex (Fig. 7). Xylem pressure may have been dissipated along the plant stems, and/or gas did not dissolve into xylem sap, delaying the occurrence of
positive pressure at higher parts. Although xylem refilling was not observed at the apex during our experiment, it may have been occurred after a longer period. However, the occurrence of negative water potential after more than 3 days without active transpiration, suggests that this phenomenon is not routine for *Vitis vinifera*. It is important to consider that only bulk xylem pressures were assessed in the current study. There is a possibility that pressure gradients are not homogeneous across a portion of the stem, or even between vessels that lie in close proximity to each other. Currently, experimental approaches do not exist for assessing *in situ* pressures at this scale, but this difficulty needs to be acknowledged. Given that refilling is a phenomenon occurring at the level of an individual vessel, one would expect that it would be the local pressure gradient environment that would dictate whether or not refilling would occur, and not necessarily the bulk level property, nor living cells’ activity.

Previous observations of refilling under negative pressure may have resulted from artifacts such as those documented by Wheeler *et al.* (2013). Cutting stems under water when sap is under negative pressure may induce the artificial formation of air bubbles, leading to an overestimation of embolism vulnerability (Torres-Ruiz *et al.*, 2015; Ogasa *et al.*, 2016; Umebayashi *et al.*, 2016). Therefore, normal diurnal fluctuation in xylem tension could produce artefactual PLC fluctuations in stems (Torres-Ruiz *et al.*, 2015) or petioles (Zufferey *et al.*, 2011). Equally, variation in tension along the plant axis could cause misleading interpretations of refilling under negative pressure if the leaves sampled for measuring stem water potential are not directly adjacent to the part of the stem being scanned and/or if leaves experienced levels of stress great enough to result in their hydraulic disconnection from the parent plant. We thus observed negative leaf water potential, although bulk xylem pressure was positive at the base (*e.g.* on Fig. 6). This point should be of particular concern in light of the high vulnerability of grapevine petioles characterized in this and other studies. Water potential measurements would therefore have to be performed on downward leaves located as close as possible to the position of the HRCT area scanned (but only for a moderate level of stress). Alternative methods could include cutting stem segments after equilibration to atmospheric pressure, or the use of stem psychrometers.

**Conclusion**

Stems of *V. vinifera* are more resistant to drought stress than those of *V. riparia*, and are not able to refill under negative bulk xylem pressure. The hydraulic segmentation
generated from stem to leaf is reinforced by vulnerability segmentation between perennial and annual parts, which prevents perennial parts from experiencing more severe losses in hydraulic function. The insights obtained here about the drought response of *Vitis* highlighted the limitations of current methods to assess *in situ* xylem sap water potential. These results will help to assess drought resistance of different grapevine genotypes, and to manage irrigation in the field, and should also be of significant interest for other economically important long-vasseled plants (*e.g.*, *Quercus* sp, *Olea* sp, *Eucalyptus* sp).

**Material and methods**

**Plant material**

Two widespread grapevine species were measured: *Vitis vinifera*, which is cultivated for grape production, and *Vitis riparia*, which is commonly used as a rootstock. The domesticated grapevine species *V. vinifera* L originates from the Caucasian area (Zecca *et al.*, 2012), and has been cultivated worldwide. This species was compared with *V. riparia* Michx., a native American grape distributed in North America, which is known to be much more drought-sensitive than *V. vinifera* (Carbonneau, 1985). One-year old potted plants from *V. vinifera* cv Cabernet Sauvignon and *V. riparia* ‘Gloire de Montpellier’, both grafted on *V. riparia* ‘Gloire de Montpellier’ were grown in 7.5L pots filled with commercial potting soil for 2 months until they reach ca. 1m height and 1cm basal stem diameter (5 to 10 leaves). Different sets of plants (n = 5 to 10 plants per pool) were used for HRCT scans, leaf hydraulic conductance (*K*<sub>Leaf</sub>), and gas exchange measurements (see below).

In the HRCT pool, 10 *V. vinifera* and 10 *V. riparia* plants were exposed to different levels of water stress for one to three weeks to cover a wide range of water potentials. In 2015, the plants were scanned at ca. 1m height, two to three times during the four days HRCT observations (Mid-April 2015). Among this pool, 3 *V. vinifera* plants were re-watered after scanning until the soil was water-saturated to measure their ability to recover from different level of initial embolism (50% < PLC < 90%) in upper part. Re-watered plants were stored in shaded conditions to prevent active transpiration and scanned every 6 hours for up to 48 hours, while stem water potential was regularly measured (see details below). An additional rewatering experiment was performed in May 2016, on 5 additional plants of the same age and morphology as in 2015, focusing on the difference between apex and base (right above
the rootstock). The $K_{\text{Leaf}}$ measurements were carried out two months later (June 2015) on eight well-hydrated plants of *V. vinifera*, which were up-rooted prior to measurements to allow their progressive dehydration within a daily course. In the gas exchange pool, eight *V. vinifera* plants were exposed to different levels of water stress, but of lower intensity than the HRCT plants (pre-dawn water potentials > -1.2MPa).

**High Resolution X-ray Computed Tomography**

Synchrotron-based computed microtomography was used to visualize air- and sap-filled vessels in the main stem and petiole of *V. vinifera* cv. Cabernet Sauvignon, and the main stem of *V. riparia*. In April 2015, plants were brought to the HRCT beamline (PSICHE) at the SOLEIL synchrotron facility. This beamline has a large, empty rotary stage, which allowed us to scan plants at different heights (*e.g.* basal and upper portions). Three hours before each scan, one leaf, located 10mm above the scanned area, was wrapped in a plastic bag and covered with aluminium foil in order to provide accurate stem water potential values ($\Psi_{\text{Stem}}$). The water potential was then measured right before the scan with a Scholander pressure chamber (Precis 2000, Gradignan, France). At the height of the scan, one leaf was carefully attached to the stem using a piece of tape. The main stem and petiole were scanned simultaneously using a high flux ($3.10^{11}$ photons.mm$^{-2}$) 25 keV monochromatic x-ray beam. The projections were recorded with an Hamamatsu Orca Flash sCMOS camera equipped with a 250 µm thick LuAG scintillator. The complete tomographic scan included 1,500 projections, 50 ms seconds each, for a 180° rotation. Thus, samples were exposed for 75 s to the x-ray beam. Tomographic reconstructions were performed using PyHST2 software (Mirone *et al.*, 2014) using the Paganin method (Paganin, 2006), resulting in $1536^3$ 32-bit volumic images. The final spatial resolution was $3^3$ µm$^3$ per voxel. Complementary measurements to visualize embolized conduits in grapevine petioles and refilling at the stem base were undertaken at the Diamond Light Source (DLS) and Swiss Light Source (SLS) synchrotron facilities, where similar plant material and the same experimental setup were used. For details of the I12 beamline (DLS) and the TOMCAT X02DA beamline (SLS), please refer to Bouche *et al.* (2016) and Choat *et al.* (2016), respectively.

**Measurement of xylem pressure/tension**

During rewatering experiments, xylem water potential was measured using three different set-ups (Fig. S2). Two were dedicated to measure xylem negative pressure:
scholander pressure chamber (described above), and psychrometers (PSY-1, ICT international, Armidale, Australia). In 2015 experiment, xylem water potential was only measured using Scholander pressure chamber. In 2016, stem psychrometers were mounted on the stem of two different plants, 10 cm above grafting, before re-watering. A 5-cm long portion of the stem was wrapped in parafilm (Alcan, Montreal, Canada) to ensure psychrometer sealing, at 5 to 10 cm below the scanning area. About 2 cm² of bark (and parafilm) was removed and a psychrometer was attached with clamps. The third set-up was dedicated to measure positive xylem pressure. When a clear decrease in the amount of embolized conduits was observed at the base, the apex of the plant was cut and immediately connected to a pressure transducer probe (26PCFFA6D, Honeywell, Morristown, USA), using an adapter tube, filled with deionized and degassed water (Thitianakul et al., 2012). Data was recorded on a CR1000 logger (Campbell, Logan, USA) at a time interval of 30 seconds. Once the signal stabilized (ca. 15 min.), the base was cut and connected to the pressure transducer following the same procedure.

**Image analysis and vulnerability curves**

On transverse cross section taken from the center of the scanned volume, the diameter and area of each individual air- and sap-filled vessels (embolised and functional, respectively) were measured in stems and/or petioles of each species using ImageJ software (http://rsb.info.nih.gov/ij). Air-filled vessels were highly contrasted with surrounding tissues. Thus a binary image was generated and vessels were extracted according to their dimensions, discarding particles lower than 10µm² (ca. 4 pixels).

After synchrotron experiments, all stems and petiole samples were wrapped up in moist paper and plastic bags and brought to the PIAF-INRA laboratory (Clermont Ferrand, France). Samples were cut 2mm above the previously scanned area, and scanned again using HRCT (Nanotom 180 XS; GE, Wunstorf, Germany) as described in Cochard et al. (2015). Vessels where sap was under negative pressure (i.e. functional vessels) immediately filled with air (as observed in Torres-Ruiz et al., 2015), whereas living vessels were not affected by cutting (i.e. cytoplasm was left intact in the individual vessel elements, see Jacobsen et al., 2015). Filled vessels in these images, were typically located in the outermost part of the xylem tissue, and discarded in the subsequent analyses.
For each species and organ, the theoretical specific hydraulic conductivity of a whole cross section \( K_{H} \) was calculated from the Hagen-Poiseuille equation using the individual diameter of sap- and air-filled vessels as:

\[
K_{H} = \sum_{i} \frac{\pi \phi_{i}^4}{128 \eta A_{Xyi}}
\]  

(1)

with \( K_{H} \): specific theoretical hydraulic conductivity \((\text{kg.m}^{-1}.\text{MPa}^{-1}.\text{s}^{-1})\); \( \phi \): mean feret diameter of vessels \((\text{m})\); \( \eta \): viscosity of water \((1.002 \text{ mPa.s at } 20^\circ\text{C})\), and \( A_{Xyi} \): xylem area of the cross section \((\text{m}^2)\).

The theoretical loss of hydraulic conductivity (PLC) was calculated as:

\[
PLC = 100 \cdot \frac{K_{HA}}{K_{HMax}}
\]  

(2)

with \( K_{HA} \) and \( K_{HMax} \) representing the theoretical hydraulic conductivities of air-filled vessels, in initial and cut cross sections, respectively.

Vulnerability curves (PLC as a function of water potential) were fitted using the nls function with R software (R Development Core Team, 2013), according to the following equation:

\[
PLC = \frac{1}{1 + e^{\frac{-25}{1} (\Psi - \Psi_{50})}}
\]  

(3)

with slp being the derivative at the inflexion point \( \Psi_{50Stem} \).

The air entry point \( (\Psi_e) \) was estimated from eq. 3 as \( 50/\text{slp} + \Psi_{50Stem} \) (Domec and Gartner 2001).

**Leaf hydraulic conductance**

Loss of \( K_{Leaf} \) was measured by using the rehydration kinetic method (Brodribb and Holbrook, 2003; Charra-Vaskou *et al.*, 2011) on eight *V. vinifera* cv Cabernet Sauvignon plants \(( N = 4-5 \text{ measurements per plant})\). Conductance measurements were performed using plants at different levels of water stress. Two contiguous fully-expanded leaves were bagged in plastic bags with wet paper towels for one hour before taking a measurement in order to cease transpiration and equilibrate water potential within the leaf. Leaf water potential \( (\Psi_{Leaf}) \) was measured on one leaf using a Scholander pressure chamber \((\text{Precis 2000, Gradignan}, 2000)\).
France), while $K_{Leaf}$ was measured on the second one. The second leaf was excised and immediately connected, under water, to a flow-meter to measure $K_{Leaf}$. The flow-meter was composed of a pressure transducer (Omega Engineering Ltd, Manchester, UK) connected to a datalogger (USB-TC-AI, MCC, USA), which measures the water pressure drop between a calibrated capillary PEEK tube and the leaf. This pressure drop was then converted into a flow rate to calculate the leaf conductance as the ratio between the maximum flow rate recorded during rehydration and the leaf water potential. Specific leaf conductance ($K_s$) was subsequently calculated dividing the leaf conductance by leaf area, which was measured using a leaf area meter (WinFolia 2007b, Regent Inst., Quebec, Canada). Leaf vulnerability curve (percent loss in $K_{Leaf}$ as a function of water potential) was fitted using the nls function with R software (R Development Core Team, 2013), according to the equation:

$$PLK_{Leaf} = \frac{1}{1 + e^{\frac{SP}{\Psi_{50Lea}}}$$

with slp being the derivative at the inflexion point $\Psi_{50\text{Leaf}}$.

**Gas exchange**

Pre-dawn water potential ($\Psi_{pd}$) was measured on one leaf per plant, close to the rootstock prior to any light exposure, on nine *V. vinifera* cv Cabernet Sauvignon plants exposed to different levels of water stress (-0.05 < $\Psi_{pd}$ < -2 MPa). Plants were then exposed to outside ambient conditions from 8:00 a.m. until 14:00 p.m., during a sunny day (PAR > 1500µmol.m$^{-2}$.s$^{-1}$; VPD > 2000Pa). Leaf gas exchange measurements were conducted on mature, well-exposed leaves using a portable open-system including an infrared gas analyzer (GFS 3000, Walz – Germany). Conditions in the cuvette (i.e. PAR, temperature, VPD, and CO$_2$) were set equal to environmental conditions. Leaf transpiration rate ($E$, mmol.m$^{-2}$.s$^{-1}$) was measured during the morning, from 8:00 until 14:00. Water potentials were measured on the leaf used for gas exchange ($\Psi_{Leaf}$), and on another one, wrapped for one hour in plastic bag covered with aluminium foil ($\Psi_{Stem}$), using a Scholander pressure chamber (Precis 2000, Gradignan, France). Apparent leaf hydraulic conductance ($K_{LeafAp}$) was calculated as the ratio between $E$ and $\Delta\Psi = \Psi_{Stem} - \Psi_{Leaf}$:

$$K_{LeafAp} = \frac{E}{\Delta\Psi}$$
A leaf vulnerability curve (percent loss in $K_{Leaf,Ap}$ as a function of water potential) was fitted using the nls function with R software (R Development Core Team, 2013), according to the equation:

$$PLK_{Leaf,Ap} = \frac{1}{slp \cdot e^{-\frac{\Psi_{50,Leaf,Ap}}{spp}}}(6)$$

with slp being the derivative at the inflexion point $\Psi_{50,Leaf,Ap}$.

**FDA staining**

Detection of viability of x-ray exposed xylem cells was performed using a 9.6-μm FDA (fluorescein-diacetate; Sigma-Aldrich, Milwaukee, WI) solution, in combination with fluorescence light microscopy. One plant was analysed ten days after first exposure to x rays. Stem slices were obtained from the exposed part and ten cm above this area. The stem was cut transversely, into 5mm thick slices, and immediately submerged into FDA solution for 30 minutes in the dark. Samples were rinsed with deionized water and placed onto a microscope glass slide. The sample surface was excited with green fluorescence light ($\lambda = 490$ nm) generated by a SOLA light engine SE 5-LCR-VB (Lumencor, Beaverton, USA), and observed for light $\lambda > 500$nm for detection of living and metabolically active tissue (green signal) using a macroscope Axiozoom V16 (Zeiss, Marly le Roy, France), connected to a camera Axiocam 105 (Zeiss, Marly le Roy, France).
Acknowledgments

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Supplemental material

Supplementary figure S1 shows cell vitality at a distal part of grapevine stems, ten days after x-Ray exposure by HRCT scans.

Supplementary figure S2 illustrates the recovery in water potential measured via different methods i.e. stem psychrometer, pressure chamber and bagged leaf, and pressure transducer.
Table I. Details of the fit of different experimental data with a sigmoid function in *V. vinifera*.

Different techniques were used according to the studied organ: HRCT image analysis in stems and petioles, measurement of rehydration kinetics at the leaf level and measurement of transpiration loss depending on the water potential gradient from leaf to root. Degree of freedom, residual sum of square and pseudo-R² are given. Values and significance of the two parameters (Slope and Ψ₅₀) are indicated (***, P<0.0001), and Ψₑ calculated from these latter.

<table>
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<tr>
<th>Organ</th>
<th>Technique</th>
<th>Df</th>
<th>SSres</th>
<th>Pseudo R²</th>
<th>Slope (%.MPa⁻¹)</th>
<th>Ψ₅₀ (MPa)</th>
<th>Ψₑ (MPa)</th>
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<td>0.948</td>
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<tr>
<td>Leaf</td>
<td>Transpiration</td>
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<td>2.171</td>
<td>0.596</td>
<td>133.6***</td>
<td>-0.456***</td>
<td>-0.830</td>
</tr>
</tbody>
</table>
**Figure caption**

**Figure 1.** Transverse HRCT images of intact *Vitis vinifera* cv Cabernet Sauvignon plants at different water potentials: stems (A-C) and petioles (D-F). Insets represent 0.25 mm² area. Functional (grey) and air-filled (black) xylem vessels are represented in blue and red in the insets, respectively. Theoretical loss of hydraulic conductivity for each image is indicated as PLC (%). White bars = 1 mm.

**Figure 2.** Percentage loss of hydraulic conductivity (%) versus xylem water potential (MPa) calculated from HRCT images in *Vitis vinifera* stems (black dots) and petioles (grey dots). Dashed lines represent the sigmoid fit of the data. Symbols and bars represent the mean and standard errors from 0.2MPa classes (n= 1 to 7 replicates per dot).

**Figure 3.** Percentage loss of hydraulic conductivity (%) versus xylem water potential (MPa) calculated from HRCT images in stems of *Vitis vinifera* (black dots) and *Vitis riparia* (white dots). The dashed lines represent the sigmoid fit of the data (solid and dashed lines, for *V. vinifera* and *V. riparia*, respectively). Symbols and bars represent the mean and standard errors from 0.2MPa classes in *Vitis vinifera* (n= 1 to 7 replicates per dot), and *V. riparia* (1 replicate per dot).

**Figure 4.** Percentage loss of hydraulic conductivity (%) in *Vitis vinifera* stems (solid line; HRCT images), petioles (long-dashed line; HRCT images), leaves (short-dashed line; rehydration kinetic method), and apparent leaf conductance (dotted-line; calculated from gas exchange measurements) depending on leaf water potential (MPa).

**Figure 5.** Cross sections of *Vitis vinifera* stems at two different height levels i.e. the upper, distal part (A-C) and bottom, proximal part above the graft (D-F) after re-watering drought-stressed plants. Time relative to rewatering (t = 0 h, i.e. the rewatering time) and the theoretical losses of hydraulic conductance (PLC, %) are indicated. White bar = 1mm scale.

**Figure 6.** Cross section at two different height levels i.e. the upper, distal part (A-B) and bottom, proximal part above the graft (C-D) of the same *Vitis vinifera* plant, before and after re-watering. Time relative to rewatering (t = 0 h, i.e. the rewatering time), theoretical losses of hydraulic conductance (PLC), and water potential (MPa) measured using pressure chamber on bagged leaf (Ψ\text{leaf}), stem psychrometer (Ψ\text{stem}) and pressure probes (Ψ\text{pp}) are indicated. White bar = 1mm scale. Discrepancy between Ψ\text{leaf} and Ψ\text{stem} probably originate from disconnection
from stem to leaf hydraulic pathway (according to Fig.4, when PLC_{apex} = 63\%, \Psi_{leaf} = \text{ca.} -2\text{MPa and PLC}_{petiole} = \text{ca.} 100\%).

**Figure 7.** Mean change in theoretical hydraulic conductance (-\Delta PLC, \%) and xylem water potential (MPa) for basal and apical scan positions in re-watered stems of *Vitis vinifera*. PLC and xylem water potential were significantly different across the apical and basal positions based on a Kruskall-Wallis test (n = 5; P = 0.006 and 0.011, for PLC and water potential, respectively).

**Figure S1.** Cross section of the upper stem part of a grapevine plant that was scanned with HRCT 10 days before treatment with fluorescein-diacetate. The fluorescent, green colored cells represent living cells, which were slightly more pronounced in the control image (B) compared to the area that was exposed to the x-ray beam (A). White bar = 1mm scale.

**Figure S2.** A. Water potential (MPa) versus time relative to rewatering (t = 0 h, i.e. the rewatering time) in re-watered *Vitis vinifera* plant. B. After significant change in theoretical hydraulic conductance was observed (as shown Fig. 5), water potential was successively measured on bagged leaf using pressure chamber (\Psi_{leaf}), then one pressure transducer was connected to the apex (black line), and ultimately to the base (dotted line).

**References**


Dixon HH (1896) Transpiration into a saturated atmosphere. Proceedings of the Royal Irish Academy.


Figure 1. Transverse HRCT images of intact *Vitis vinifera* cv Cabernet-Sauvignon plants at different water potentials: stems (A-C) and petioles (D-F). Insets represent 0.25 mm² area. Functional (grey) and air-filled (black) xylem vessels are represented in blue and red spots in the insets, respectively. Theoretical loss of conductivity for each image is indicated as PLC (%). White bars = 1 mm.
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Figure 7. Mean change in theoretical hydraulic conductance (-ΔPLC, %) and xylem water potential (MPa) for basal and apical scan positions in re-watered stems of *Vitis vinifera*. PLC and xylem water potential were significantly different across the apical and basal positions based on a Kruskall-Wallis test (n = 5; P = 0.006, and 0.011, for PLC and water potential, respectively).


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