

# X-ray microtomography observations of xylem embolism in stems of *Laurus nobilis* are consistent with hydraulic measurements of percentage loss of conductance

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## Summary

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- Drought-induced xylem embolism is a serious threat to plant survival under future climate scenarios. Hence, accurate quantification of species-specific vulnerability to xylem embolism is a key to predict the impact of climate change on vegetation. Low-cost hydraulic measurements of embolism rate have been suggested to be prone to artefacts, thus requiring validation by direct visualization of the functional status of xylem conduits using nondestructive imaging techniques, such as X-ray microtomography (microCT).
- We measured the percentage loss of conductance (PLC) of excised stems of *Laurus nobilis* (laurel) dehydrated to different xylem pressures, and compared results with direct observation of gas-filled vs water-filled conduits at a synchrotron-based microCT facility using a phase contrast imaging modality.
- Theoretical PLC calculated on the basis of microCT observations in stems of laurel dehydrated to different xylem pressures overall were in agreement with hydraulic measurements, revealing that this species suffers a 50% loss of xylem hydraulic conductance at xylem pressures averaging  $-3.5$  MPa.
- Our data support the validity of estimates of xylem vulnerability to embolism based on classical hydraulic techniques. We discuss possible causes of discrepancies between data gathered in this study and those of recent independent reports on laurel hydraulics.

## Introduction

Ongoing climate changes and the related increase in the severity of drought events (Trenberth *et al.*, 2014; Diffenbaugh *et al.*, 2015) are posing serious challenges to survival and productivity of forests and crops (Allen *et al.*, 2015; Lesk *et al.*, 2016). Accurate prediction and reliable modelling of future impacts of aridity on natural and cultivated lands, as well as breeding and selection of more drought-tolerant forest trees and crops, require a detailed understanding of the key functional traits assuring plant survival under severe water shortage (Mencuccini *et al.*, 2015). Several recent studies have highlighted the fundamental role played by vulnerability to xylem embolism in setting the functional limits to plant survival under extreme drought (Davis *et al.*, 2002; Brodribb & Cochard, 2009; Nardini *et al.*, 2013; Urii *et al.*, 2013; Anderegg *et al.*, 2015; Rowland *et al.*, 2015). In particular, the safety margin in relation to catastrophic hydraulic failure, that is, the difference between critical species-specific xylem tension triggering massive embolism and minimum xylem pressure actually

experienced by plants during extreme droughts (Choat *et al.*, 2012), has recently been shown to be a major predictor of mortality risk across different woody species around the globe (Anderegg *et al.*, 2016). Hence, future efforts to predict and mitigate the impact of climate change on vegetation should incorporate reliable estimates of species-specific and organ-specific vulnerabilities to drought-induced xylem dysfunction.

Drought-induced xylem embolism is caused by air bubbles being aspirated into conduits filled with water under tension from nearby gas-filled compartments (Sperry & Tyree, 1988). This process of 'air seeding' occurs at the level of interconduit pits, and its output is the loss of hydraulic conductance of embolized conduits, with consequent impacts on whole-plant water transport and leaf gas exchange (Savi *et al.*, 2015). If xylem embolism is severe enough to disrupt root-to-leaf water supply, plant desiccation and death might occur as a consequence of hydraulic failure (Barigah *et al.*, 2013). The vulnerability to xylem embolism of different species/organs is generally quantified by measuring 'vulnerability curves' (VCs), that is, plots of

progressive percentage loss of hydraulic conductance (PLC) vs xylem pressure. In most cases, these are generated by progressively dehydrating whole plants or detached branches, and sampling stem segments at different degrees of dehydration. Stem segments are cut from larger branches under water and connected to a hydraulic apparatus to measure their hydraulic conductance under low water pressure, before displacing embolism by a high-pressure 'flush' and repeating measurements under low pressure to quantify maximum hydraulic conductance, and finally calculating PLC. Several alternative methods to generate VCs have been proposed and tested, including monitoring of acoustic emissions rate by dehydrating stems (Milburn, 1973; Tyree & Dixon, 1983; Jackson & Grace, 1996; Nolf *et al.*, 2015), real-time measurements of hydraulic conductance of stems spinning in a centrifuge (Cochard, 2002; Li *et al.*, 2008), induction of embolism by positive air pressures applied to stems (Cochard *et al.*, 1992; Salleo *et al.*, 1992), single-vessel air injection (Venturas *et al.*, 2016) and quantification of the air volume extracted from progressively dehydrated branches (Pereira *et al.*, 2016). All these methods can be potentially affected by pitfalls and artefacts (Cochard *et al.*, 2013), and the recent literature is rich in controversies regarding their validity (Jansen *et al.*, 2015).

An important criticism of classical hydraulic measurements of stem embolism rate has been raised by Wheeler *et al.* (2013). In their study, Wheeler *et al.* showed that recorded PLC values were substantially higher when measured in stems cut while the xylem was under considerable tension, even if stems were cut under water, than values obtained after partial stem rehydration to relax xylem tension before cutting the sample for hydraulic measurements. Other studies have later suggested that the artefact might arise from rehydration itself, as relaxation of xylem tension and stem rehydration might favour refilling of embolized conduits (Salleo *et al.*, 1996; Nardini *et al.*, 2011; Savi *et al.*, 2016), thus leading to underestimation of PLC (Trifilò *et al.*, 2014; Knipfer *et al.*, 2016). Some studies have also shown that in several species, cutting stems or petioles under water while under tension does not introduce artefactual xylem embolism (Scoffoni & Sack, 2015; Venturas *et al.*, 2015).

Validation of indirect hydraulic measurements of embolism rate in plant organs by multiple techniques (see earlier) is a recommended procedure, including direct visualization of the functional status of xylem conduits by nondestructive imaging techniques like magnetic resonance imaging (Holbrook *et al.*, 2001; Zwieniecki *et al.*, 2013; Ogasawara *et al.*, 2016), or X-ray microtomography (microCT) (Brodersen *et al.*, 2010; Choat *et al.*, 2015; Ryu *et al.*, 2016). Direct microCT imaging of xylem conduits revealed patterns of embolism formation and recovery during dehydration and rehydration of stems and leaves (Kim & Lee, 2010; Knipfer *et al.*, 2015), and also allowed centrifuge techniques for PLC determination to be tested (Choat *et al.*, 2016). However, examples of experimental validation of hydraulic techniques are still scarce, mostly because of limitations in terms of access to microCT facilities.

In some cases, microCT analyses of embolism rates in stems sampled from plants at different water potentials cast doubts on previous studies based on hydraulic techniques. This is the case in a recent study by Cochard *et al.* (2015), reporting lack of

significant embolism in stem xylem of *Laurus nobilis* dehydrated to xylem pressures as low as  $-6$  MPa. Such a high resistance to xylem embolism apparently contrasts with published VCs measured for the same species on the basis of traditional hydraulic techniques, suggesting that this species suffers  $> 50\%$  loss of stem hydraulic conductance at xylem pressures of between  $-2$  and  $-3$  MPa. Notably, *L. nobilis* is a woody species that has been reported to perform significant refilling of embolized conduits (Salleo *et al.*, 1996; Tyree *et al.*, 1999), even at moderately negative xylem pressures, and several components of the possible osmotic mechanism leading to embolism repair have been initially described and elucidated in this species (Salleo *et al.*, 2004, 2006, 2009). Hence, novel evidence about artefacts leading to important errors in hydraulic PLC determination in this species would cast doubts on the validity of previous studies and would call for a significant revision of current theories regarding the occurrence and possible mechanisms of embolism repair processes in woody plants (Mayr *et al.*, 2014). However, it has to be noted that the study by Cochard *et al.* (2015) did not report a hydraulic VC measured on the same plant material used for microCT, so that a direct comparison between results obtained with the two techniques in this species is still lacking. In this study, we report on a direct comparison of vulnerability curves obtained with hydraulic and microCT techniques on stems sampled from the same individual of *L. nobilis*.

## Materials and Methods

All experiments were performed using 4-yr-old branches of a single *Laurus nobilis* L. (laurel) tree,  $> 25$  yr old, growing in the Botanical Garden of the University of Trieste. At the beginning of May 2015, that is, 1 month before hydraulic experiments and microCT observations, *c.* 80 straight branches of similar length and thickness were randomly selected. The terminal portion of each branch, *c.* 30 cm long and 2 yr of age, was carefully defoliated with scissors. Only four to five leaves in the terminal 5 cm were not removed. Only occasional and small rainfall events occurred during the study period in the area, and temperatures were above normal, with daily maxima averaging  $26.3^{\circ}\text{C}$ .

### Estimating maximum vessel length

In order to avoid spurious air entry during sample preparation up to the apical point where hydraulic and microCT measurements had to be performed (see 'MicroCT observations'), the maximum vessel length was estimated using the air injection method (Jacobsen *et al.*, 2012). A total of 12 branches, 1 m in length, were excised in the field. Branches were re-cut at *c.* 8 cm from the apex and 1 cm of bark was carefully removed. The cut section was shaved with a razor blade, trimmed under water, and connected to a tubing system. Air was perfused from the apical (distal) cut section to the basal end, at a pressure of 30 kPa (Chatelet *et al.*, 2011). The basal end of the branch was progressively shortened by 1 cm cuts while immersed in water and observed with a magnifying lens. The maximum vessel length of each branch was recorded when at least one bubble stream could be seen emerging

from the cut section, indicating that at least one vessel was cut open at both ends.

## Hydraulic experiments

The vulnerability to xylem embolism of *L. nobilis* stems was initially measured using established hydraulic techniques. In particular, measurements were performed at the beginning of June 2015 using the bench dehydration technique. Partially defoliated (see earlier) 1-m-long branches were excised at dawn, immediately re-cut under water, and rehydrated for 24 h while covered with a black plastic bag to favour refilling of embolized conduits (Trifilò *et al.*, 2014). Branches were air-dehydrated in the laboratory at constant irradiance, temperature and relative humidity. At regular time intervals, three leaves per branch were wrapped in cling film and the branch was inserted into a black plastic bag. After 30–40 min of equilibration, the water potential of at least two leaves was measured with a pressure chamber (mod. 1505D; PMS Instruments, Albany, OR, USA) in order to estimate the xylem pressure ( $\Psi_{\text{xyl}}$ ). After  $\Psi_{\text{xyl}}$  determination, 4-cm-long stem samples were cut under water from the terminal portion of the branch (*c.* 8 cm from the apex). About 1 cm of bark was removed from the basal end of the sample, and additional 2–3 mm cuts were made with a sharp razor blade at both ends before connecting the sample to a hydraulic apparatus (Xyl'EM xylem embolism meter, Bronkhorst, Montigny-Les-Cormeilles, France). The samples were perfused with a reference solution (0.5 mM  $\text{Ca}^{2+}$ , 0.07 mM  $\text{Mg}^{2+}$ , 0.08 mM  $\text{Na}^+$ , 0.03 mM  $\text{NO}_3^-$ , 1 mM  $\text{HCO}_3^-$ , 0.15 mM  $\text{SO}_4^{2-}$ , 0.01 mM  $\text{F}^-$ , 10 mM KCl) (Nardini *et al.*, 2007) at a pressure of 8 kPa, and the initial hydraulic conductance ( $K_i$ ) was measured when the flow became stable (within 3–5 min). Samples were then flushed at high pressure (0.18 MPa) for 10 min and the maximum hydraulic conductance was measured ( $K_{\text{max}}$ ). Xylem embolism was quantified as PLC, calculated as  $[1 - (K_i/K_{\text{max}})] \times 100$ . PLC values obtained from stems dehydrated to different pressures were plotted against the corresponding  $\Psi_{\text{xyl}}$  values, thus obtaining a hydraulic VC.

## Control experiments

In order to check for eventual artefacts caused by sample preparation for microCT analysis, additional control experiments were performed, consisting of cutting stems under a sealing medium (Petroleum jelly; Cepsa Lubricantes, Madrid, Spain) preventing air entry and water loss before and during microCT observations (see later). Petroleum jelly was warmed to 60°C and stained with toluidine blue. Laurel branches sampled and rehydrated as described earlier were bench-dehydrated for at least 30 min. The terminal portion, 30 cm long, was cut while it was immersed in the 'stained' petroleum jelly. This procedure took <3 s, and once the cut section was exposed to air, the petroleum jelly apparently turned instantaneously to the solid state. Cross-sections of the basal cut region were obtained using razor blades and observed under a light microscope. These observations revealed that petroleum jelly had penetrated into xylem conduits for a maximum length of

5 mm, thus sealing the xylem and preventing air entry. In order to check that the cut under petroleum jelly did not affect the functional status of conduits in the terminal portion of the twigs, a control hydraulic vulnerability curve was obtained. Branches dehydrated to different  $\Psi_{\text{xyl}}$  values were cut under petroleum jelly at a length of 30 cm and wrapped in cling film. After *c.* 2 min, samples for hydraulic measurements were re-cut under water and the PLC of the terminal stem portion was measured using the same procedure as described earlier.

## MicroCT observations

X-ray microtomography observations were performed at the beginning of June 2015 at the SYRMEP beamline of the Elettra light source in Trieste (<http://www.elettra.trieste.it>). Partially defoliated (see earlier) 1-m-long branches were excised from the plant and rehydrated as already described. After *c.* 24 h, samples were removed from water at different time intervals and left to dehydrate on the laboratory bench in order to induce progressively lower  $\Psi_{\text{xyl}}$ . Samples were transported to the SYRMEP beamline while enclosed in black plastic bags. The water potential of at least two previously wrapped leaves was measured to estimate  $\Psi_{\text{xyl}}$  using a portable pressure chamber (3005 Plant Water Status Console; Soilmoisture Equipment Corp., Goleta, CA, USA). In order to observe the functional status of xylem conduits in whole shoots while avoiding spurious air entry in vessels during scans, microCT measurements were done on *c.* 30-cm-long shoots. In particular, samples observed at microCT had a total length of  $27.6 \pm 0.8$  cm, and the scanned region was, on average, at a distance of  $8.3 \pm 0.4$  cm from the shoot tip. Hence, the scanned region was at a distance of 19.3 cm from the base of the sample, corresponding to 1.6 times the maximum vessel length (see earlier). Moreover, samples for microCT analysis were cut with scissors while briefly immersed in petroleum jelly warmed to 60°C. The liquid petroleum jelly promptly turned to a solid state when the shoot was exposed to air after the cut, thus sealing the cut section. To prevent sample drying during the scan, shoots were tightly wrapped in Parafilm, and the remaining apical leaves were enclosed in cling film. Samples were secured to the sample holder, placed in the beamline and scanned at a position 8 cm away from the stem apex. MicroCT scans were performed in phase contrast modality applying the 'free propagation technique'. With its characteristic 'edge enhancement' effects, this imaging approach benefits from the spatial coherence of the synchrotron source and results in high image contrast and detailed visibility in weakly absorbing samples. Samples were acquired at a mean X-ray energy of 19 keV, using a detector with a 2.5  $\mu\text{m}$  pixel size. Each scan contained 1800 projections collected with 0.33° angular step over 180°. Exposure time per projection was 0.3 s. At the end of the 9 min scan, images were reconstructed and five slices per sample were selected for the quantitative analysis. A total of 15 samples at different initial  $\Psi_{\text{xyl}}$  were scanned. After each scan, total sample length and the distance from shoot tip to the scanned point were recorded.

## Anatomical analysis

In order to discriminate gas-filled from water-filled conduits in the slices, 15- $\mu\text{m}$ -thick cross-sections of all samples observed at the SYRMEP beamline were cut using a rotary microtome (Leica RM2245; Leica Biosystems, Wetzlar, Germany) from the same location (8 cm from the apex) at which the scan had been performed. Sections were stained with a solution of safranin and Astrablü (1% and 0.5% in distilled water, respectively) and permanently fixed with Eukitt (Bio-Optica, Milano, Italy). Overlapping images (*c.* 20%) of the whole cross-sectional area were acquired using a light microscope (Nikon Eclipse80i; Nikon, Tokyo, Japan) at  $\times 40$  magnification and then stitched together with PTGui (New House Internet Service BV, Rotterdam, the Netherlands). Anatomical analyses of reconstructed images of whole cross-sections were then performed with Roxas v 2.0 (von Arx & Dietz, 2005; von Arx & Carrer, 2014) and limited to the corresponding area of the microCT slices. The total xylem conductivity ( $Kb_{\text{MAX}}$ ) was assessed as the sum of the single vessel conductivities calculated from the Hagen–Poiseuille equation. A second Roxas analysis was run after deleting all the vessels not recognizable as gas-filled vessels in the corresponding microCT slice in order to calculate the total xylem conductance of nonembolized vessels ( $Kb_x$ ). The theoretical PLC of each sample was then calculated as  $[(Kb_{\text{MAX}} - Kb_x)/Kb_{\text{MAX}}] \times 100$ .

## Results

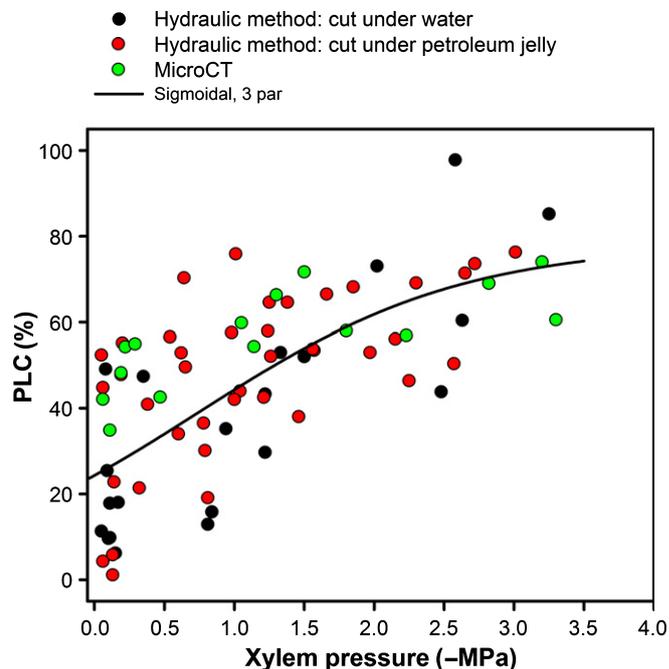
### Hydraulic measurements and vessel length

Branches of *L. nobilis* fully rehydrated overnight showed PLC values in the terminal twig sections ranging from 3% to 50%. Upon branch dehydration, PLC progressively increased, reaching values as high as 80% at  $\Psi_{\text{xyI}} = -3$  MPa (Fig. 1, black circles). When data were fitted to a three-parameter sigmoidal relationship, curve interpolation suggested an average baseline PLC value of *c.* 25% at  $\Psi_{\text{xyI}} = 0$  MPa, and a 50% loss of hydraulic conductance at  $\Psi_{\text{xyI}} = -1.3$  MPa. When subtracting the baseline PLC value to account for native irreversible embolism in terminal twigs, the  $\Psi_{\text{xyI}}$  inducing PLC = 50% ( $\Psi_{50}$ ) turned out to be  $-3.5$  MPa.

Mean ( $\pm$  SEM) maximum vessel length as estimated on the basis of the air injection method was  $11.9 \pm 2.5$  cm, consistent with previous estimates in the same species (Gascó *et al.*, 2008).

### MicroCT analysis

The preparation of samples for microCT analysis, as based on cutting terminal twig portions under liquid petroleum jelly, did not induce artefactual embolism, as PLC values recorded at different  $\Psi_{\text{xyI}}$  values for samples cut under petroleum jelly were not different from those recorded after cutting samples under water (Fig. 1, red circles). MicroCT observations of distal twigs sampled from branches at  $\Psi_{\text{xyI}}$  close to 0 showed that most vessels were water-filled, but with several gas-filled

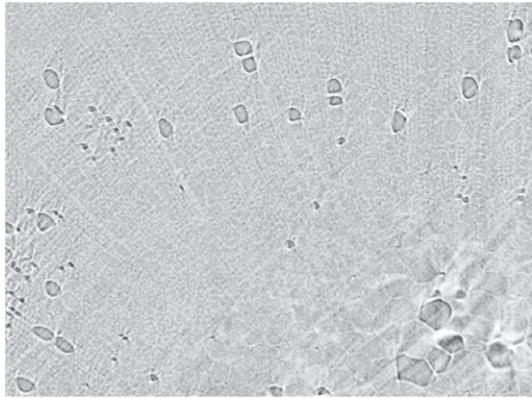


**Fig. 1** Changes in percentage loss of stem hydraulic conductance (PLC) as a function of xylem pressure, as measured during progressive dehydration of *Laurus nobilis* (laurel) branches using standard hydraulic techniques. Stem samples (4 cm long) for hydraulic measurements were obtained from the terminal part of 30 cm shoots cut either under water (black circles) or under warm liquid petroleum jelly (red circles; see text for details). Theoretical PLC values calculated on the basis of visual observations of the functional status of xylem conduits via X-ray microtomography (microCT) are also reported (green circles).

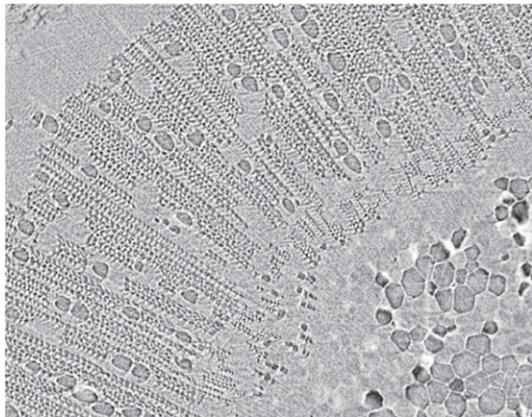
conduits interspersed in the reconstructed slices (Fig. 2a). Upon dehydration, the number of gas-filled conduits progressively increased, reaching a maximum for samples dehydrated down to  $\Psi_{\text{xyI}} \approx -3$  MPa (Fig. 2b). Theoretical PLC values calculated for samples scanned at microCT were plotted against the corresponding values of  $\Psi_{\text{xyI}}$  (Fig. 1, green circles), and the overall trend turned out to be similar to that recorded on the basis of hydraulic measurements. It should be noted that theoretical PLC values based on two-dimensional images might overestimate the loss of hydraulic conductance suffered by stem samples, because, in the three-dimensional xylem network, embolized vessels can be bypassed so that water can flow around gas-filled conduits.

Fig. 3 shows the fraction of embolized vessels for different diameter classes, as recorded on the basis of microCT analysis performed on three twig samples dehydrated to progressively lower  $\Psi_{\text{xyI}}$ . In fully hydrated samples ( $\Psi_{\text{xyI}} = -0.1$  MPa), low embolism rates ( $< 20\%$ ) were observed in conduits with diameter  $< 30$   $\mu\text{m}$ . Progressively higher embolism rates were detected for larger conduits, with a 50% peak for conduits with diameters close to 60  $\mu\text{m}$ . In samples dehydrated to  $\Psi_{\text{xyI}} = -1.0$  MPa,  $> 70\%$  of conduits with diameter  $> 40$   $\mu\text{m}$  were embolized, and the embolism rate increased to *c.* 40–60% even in smaller conduits ( $< 40$   $\mu\text{m}$ ). At  $\Psi_{\text{xyI}} = -3.2$  MPa, the fraction of embolized conduits was  $> 60\%$  for all diameter classes.

(a)  $\Psi_{xyl} = -0.11$  MPa



(b)  $\Psi_{xyl} = -3.3$  MPa

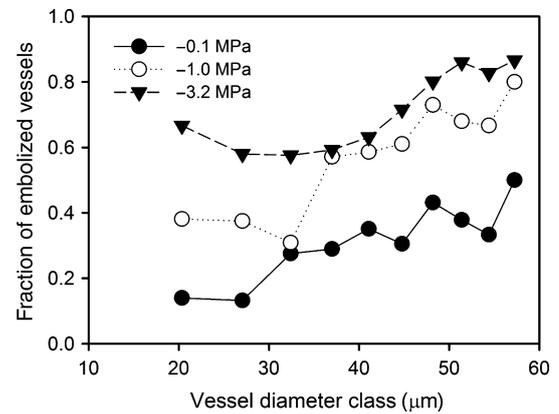


**Fig. 2** Direct visualization of xylem embolism by X-ray microtomography (microCT). *Laurus nobilis* (laurel) shoots 30 cm in length were scanned at 8 cm from the shoot apex, and a two-dimensional cross-section was reconstructed. Functional (grey) and air-filled (black) xylem conduits can be seen. The figure shows the image reconstruction for: (a) stem with a xylem pressure ( $\Psi_{xyl}$ ) of  $-0.11$  MPa; and (b) a second shoot dehydrated to  $\Psi_{xyl} = -3.3$  MPa.

## Discussion

X-ray microtomography observations of embolism rate in stems of laurel dehydrated to different xylem pressures overall were in agreement with hydraulic measurements. Theoretical PLC values derived on calculating the hydraulic conductance in water-filled vs gas-filled conduits largely overlapped with PLC values measured using standard hydraulic techniques. Samples used for hydraulic analysis were cut directly under water, and thus were potentially exposed to a ‘cutting artefact’ according to Wheeler *et al.* (2013). Indeed, the agreement between hydraulic measurements and direct observations of xylem functional status in 30-cm-long samples suggests that cutting laurel stems under tension does not introduce significant spurious embolism into samples, especially when re-cut several times, confirming previous findings by Trifilò *et al.* (2014) and Venturas *et al.* (2015).

A possible explanation for the comparably low resistance to drought-induced embolism in both hydraulic and microCT measurements is the occurrence of spurious embolism in both cases,



**Fig. 3** Fraction of embolized vessels for different classes of vessel diameter, as recorded on the basis of X-ray microtomography (microCT) observations in three *Laurus nobilis* (laurel) shoots dehydrated to xylem pressures ( $\Psi_{xyl}$ ) of  $-0.1$  MPa (closed circles),  $-1.0$  MPa (open circles) and  $-3.2$  MPa (triangles).

that is, even in the stem region scanned under microCT using 30-cm-long samples. In our study, as well as in Cochard *et al.* (2015), cut stems with a length of 30 cm were used for direct observations. It is possible that air was introduced into samples during preparation, resulting in an artefactual increase of embolism rates recorded using microCT, but we feel that this is unlikely for two reasons. First, control experiments revealed that cutting stems under petroleum jelly resulted in identical PLC values to those measured in samples cut under water. We can thus exclude this preparation procedure as a source of artefacts in microCT analysis. Second, the cut was done at a distance from the scanned point corresponding to 1.6 times the maximum vessel length, as estimated using the air injection technique. Hence, unless estimates of maximum vessel length were also biased by a different artefact and thus unreliable (Scholz *et al.*, 2013), any air bubble entering at the cut point should not travel up to the scan region but should be blocked at the vessel ends.

On the basis of the VCs based on both hydraulic and microCT data, the  $\Psi_{50}$  for laurel would average  $-3.5$  MPa when PLC is corrected for the baseline values predicted at  $\Psi_{xyl} = 0$  MPa. These data are in overall agreement with those based on VCs previously measured in this species in different studies (e.g. Salleo *et al.*, 1996; Cochard, 2002; Hacke & Sperry, 2003; Trifilò *et al.*, 2014). Our data, however, are in disagreement with Cochard *et al.* (2015) based on microCT analysis on the same species, reporting only 10% loss of conductance at  $\Psi_{xyl} = -3$  MPa, while  $\Psi_{50}$  was estimated to be  $< -6$  MPa. According to Cochard *et al.* (2015), such a negative  $\Psi_{50}$  would be in accordance with high drought resistance of this Mediterranean species, and would cast doubts on the validity of previous studies describing processes of embolism formation and repair in laurel (Tyree *et al.*, 1999; Salleo *et al.*, 2004). Indeed,  $\Psi_{50}$  values for plants living in Mediterranean biomes have been reported to range between  $-0.5$  and  $-14$  MPa, with mean values of  $-4.8$  and  $-4.0$  MPa in the Mediterranean basin and California Chaparral vegetation, respectively (Nardini *et al.*, 2014a). Hence, laurel does not appear as an unusually vulnerable species when compared with plants

co-occurring in the same geographical range. Moreover, the evolutionary and biogeographic traits of *L. nobilis* contrast with the common view of this species as ‘typically’ Mediterranean. In fact, laurel is a member of Lauraceae, a large family of woody plants comprising > 2500 species belonging to 50 different genera, all distributed from tropical to subtropical latitudes (Chanderbali *et al.*, 2001). In particular, the genus *Laurus* is considered as a typical relict of the Tethyan flora covering low and middle latitudes of the northern hemisphere during the Palaeogene, and represented by evergreen sclerophylls adapted to warm and humid habitats (Rodríguez-Sánchez & Arroyo, 2008). Still today, laurel distribution is restricted to warm climates with low seasonality and reduced water deficit, and water stress is considered as the main climatic factor constraining the southern distributional range of the species (Rodríguez-Sánchez & Arroyo, 2008). Accordingly, in Italy, natural populations of laurel occur only in humid and frequently shaded microhabitats (Filibeck, 2006; Pignatti *et al.*, 2015). Hence, the relatively high drought vulnerability of laurel emerging from our hydraulic and microCT analysis is consistent with the ecological requirements of the species.

The discrepancy between  $\Psi_{50}$  values reported by Cochard *et al.* (2015) and those in the present study, obtained on the same species, using very similar samples and the same visual technique, raises interesting questions about possible sources of variation in  $\Psi_{50}$  in laurel and/or possible artefacts arising from microCT observations. Laurel is a widely cultivated species and most individuals available in botanical gardens or nurseries, and frequently used for hydraulic research, are likely to belong to one of the 65 known cultivars available on the market (Hatch, 2015). Interestingly, these cultivars differ widely in terms of leaf size and shape, a morphological trait that has recently been found to correlate with cultivar-specific vulnerability to xylem embolism (Nardini *et al.*, 2014b; Schreiber *et al.*, 2016). Intraspecific variability in vulnerability to xylem embolism is generally considered to be relatively low (Lamy *et al.*, 2014), but in some cases  $\Psi_{50}$  was found to differ even by > 1 MPa between different cultivars/provenances (Pratt *et al.*, 2012; Jacobsen *et al.*, 2014; David-Schwartz *et al.*, 2016; Schreiber *et al.*, 2016). Another possible explanation for substantial discrepancies in  $\Psi_{50}$  records might reside in the phenomenon known as ‘cavitation fatigue’, that is, the progressive increase of vulnerability to xylem embolism suffered by plants exposed to cycles of drought stress and recovery (Hacke *et al.*, 2001). Our laurel plants were field-growing, and hence substantial ‘weakening’ of xylem hydraulic resistance over several summer seasons should not be ruled out. Indeed, several samples showed quite high PLC values even when fully rehydrated, indicating the presence of substantial and irreversible embolism as a possible legacy of ‘cavitation fatigue’.

While different plant material or drought history might be parsimonious explanations, yet to be tested, for discrepancies in terms of xylem vulnerability assessed by microCT in different laboratories, other sources of error should be considered as a warning for future efforts to validate hydraulic measurements by direct observation of xylem functional status. A first critical point relates to accurate measurements of xylem water potential, as eventual embolism in leaf veins might cause stomatal closure and lead to hydraulic isolation of stem xylem from leaves. In this case,

measurements of leaf water potential might cause values of stem xylem pressure to be underestimated, leading to incorrect calculation of  $\Psi_{50}$ . It is also possible that preliminary sample preparation based on partial stem defoliation, as done in our study 1 month before measurements, increased the vulnerability to xylem embolism, by creating scars by which air could have entered more easily into stems. However, at the time of measurements, the scars appeared as suberized and probably sealed. Finally, the X-ray dose supplied during the microCT scan might itself produce artefacts by damaging xylem conduits and inducing spurious embolism appearance. The energy and scan times used in the present study and by Cochard *et al.* (2015) are very similar, thus possibly ruling out the possibility that the observed discrepancies arise as a result of X-ray damage.

In conclusion, our data provide validation of data gathered on laurel using common hydraulic techniques, while confirming that it is highly recommended to test and validate hydraulic techniques aimed at assessing species- and organ-specific vulnerability to xylem embolism by as many independent techniques as possible, including direct *in vivo* observations of the functional status of xylem conduits, as suggested by Cochard *et al.* (2015). However, the discrepancies between observations performed on the same species suggest that the replication of such observations in different facilities and by different laboratories, as well as on different plant materials, should be increased to solve some of the possible methodological problems associated with X-ray-based microCT. Direct visualization of xylem conduits in intact plants at different water stresses (Choat *et al.*, 2016), followed by classical hydraulic measurements to compare the output and performance of the different experimental techniques appears to be a promising direction. However, it should be noted that microCT itself is still a relatively untested technique when used to assess the functional status of xylem conduits, and several concerns about the correct interpretation of microCT images of stems have been raised in previous studies (Jacobsen & Pratt, 2012; Hacke *et al.*, 2015). In any case, it is unlikely that synchrotron-based microCT will become a routine technique for processing large number of samples, at least in the near future. Hence, efforts to test the validity of more affordable hydraulic techniques should be increased, to finally converge on commonly accepted procedures to collect samples and measure embolism rates in different plant organs, with the minimum possible risk of introducing artefacts (Jansen *et al.*, 2015). However, tests of different methods should preferably be done on the same plant material, at the same time, and using the same handling protocol in order to obtain accurate and reliable comparisons.

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## Author contributions

A.N., S.S. and T.S. planned and designed the research. A.N. and T.S. performed hydraulic experiments. A.N., T.S., A.L., S.P. and G.T. performed microCT observations and image reconstruction. G.P. performed anatomical analysis and elaboration of microCT images. P.T. and M.L. contributed to the analysis and discussion of data. A.N., S.S. and S.M. wrote the manuscript, with contributions from all authors.

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