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# Direct X-Ray Microtomography Observation Confirms the Induction of Embolism upon Xylem Cutting under Tension<sup>1</sup>

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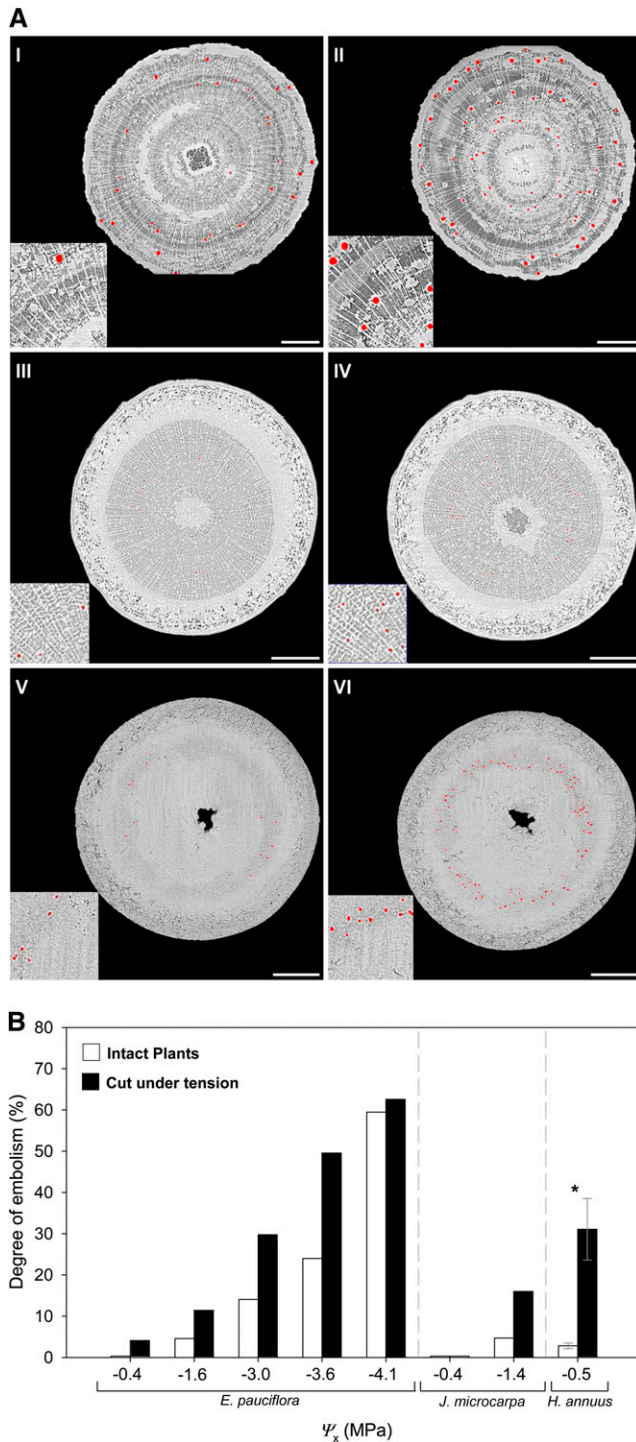
Embolism resistance is a critically important trait for evaluating the ability of plants to survive and recover from drought periods and predicting future drought-induced forest decline (Choat et al., 2012). However, recent publications have provided evidence that some measurement techniques used to evaluate the hydraulic function and vulnerability to cavitation of plant organs may be prone to artifacts (Sperry et al., 2012; Cochard et al., 2013; Torres-Ruiz et al., 2014; Trifilò et al., 2014). The discovery of these artifacts has raised questions regarding the reliability of some previously published plant hydraulics data, in particular data relating to the refilling of embolized xylem conduits while the xylem is under tension. In this context, Wheeler et al. (2013) reported that sampling plant organs by cutting while the xylem is under tension can induce artificial increases in the degree of embolism at the moment of sample excision, even when cuts are made under water. The methodology applied by Wheeler et al. (2013), however, did not allow the visualization of embolized or functional vessels, and native embolism levels could not be determined in intact plants before any cutting was done.

Whereas Scoffoni and Sack (2014) showed that the artifact described by Wheeler et al. (2013) has no impact on leaf xylem hydraulic conductance, there is

some uncertainty about its importance in stems or shoots (Trifilò et al., 2014; Venturas et al., 2014). The results of Wheeler et al. (2013) indicate that more embolism could be induced by cutting samples that are under midrange xylem tension (e.g. at midday or under conditions of water stress). Potential overestimation of embolism due to changes in the xylem tension during the day has important implications for our understanding of plant water relations, since they could erroneously suggest that daily patterns of embolism formation and repair are routine in many woody plant species. Debate continues regarding the implications of a cutting artifact for the existence of a mechanism that allows plants to repair embolism while the xylem is under tension, so-called novel refilling (Salleo et al., 1996; Cochard and Delzon, 2013; Sperry, 2013; Delzon and Cochard, 2014). To avoid the excision artifact, Wheeler et al. (2013) recommended the relaxation of the xylem tension prior to excision by rehydrating plant tissue for anywhere between 2 min and 2 h. However, recent results from Trifilò et al. (2014) indicated that the rehydration procedures used by Wheeler et al. (2013) for relaxing the samples might favor xylem refilling and embolism repair (rehydration artifact), suggesting that the artifact resides in the relaxing procedure rather than in the cutting procedure. In light of these data, the assessment of the artifact described by Wheeler et al. (2013) using noninvasive techniques on intact plants, such as direct observation using x-ray microtomography (micro-CT; McElrone et al., 2013; Cochard et al., 2014) or magnetic resonance imaging (Choat et al., 2010; Zwieniecki et al., 2013), is useful to visually assess changes in embolism after cutting stems.

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**Figure 1.** A, Transverse sections based on consecutive micro-CT scans for *E. pauciflora* (I and II), *J. microcarpa* (III and IV), and *H. annuus* (V and VI) at  $\Psi_x$  of  $-3.6$ ,  $-1.4$ , and  $-0.4$  MPa, respectively. Images show the amount of cavitated or air-filled conduits before (intact seedling; left) and after (right) cutting the sample under water without releasing the xylem tension. Both images were scanned at the same height to allow before and after comparison. Cavitated or air-filled vessels are observed as red. Insets show the same magnified areas (2 $\times$ ) of each section. Bars = 500  $\mu$ m. B, Degree of embolism in *E. pauciflora*, *J. microcarpa*, and *H. annuus* at different  $\Psi_x$  in intact plants (white

## DISENTANGLING CURRENT CONTROVERSIES BY DIRECT VISUALIZATION USING MICRO-CT

Micro-CT is a noninvasive technique that allows researchers to directly visualize xylem conduits at high resolution and determine the degree of embolism and embolism spreading patterns (Brodersen et al., 2013). By using this technique, Cochard et al. (2014) recently found a lower amount of embolism in droughted *Laurus nobilis* stems than was reported previously for this species using indirect techniques (Cochard, 2002; Salleo et al., 2006).

We used two different Synchrotron-based micro-CT facilities (the Swiss Light Source [SLS] in Villigen, Switzerland, and the Advanced Light Source [ALS] in Berkeley, CA) to test the excision artifact described by Wheeler et al. (2013). Specifically, we examined the impact of cutting xylem under tension and under water in three woody and one herbaceous species: *Acer campestre*, *Eucalyptus pauciflora*, *Juglans microcarpa*, and *Helianthus annuus*. By direct visualizations, we tested whether (1) the degree of embolism of the samples varied before and after cutting them (underwater) for all four species and (2) how much xylem tension affected the degree of embolism observed in *E. pauciflora*, *A. campestre*, and *J. microcarpa* after cutting under water. All experiments were carried out on intact plants except for *A. campestre*, in which cut branches were used. Intact seedlings were scanned at approximately 10 cm above the base of the stem. For *A. campestre* branches, the scans before cuttings were done at a minimum distance corresponding to three internodes from the cut end (enough to avoid any open vessels at the basal end). All postcut scans were done immediately after cutting under water at the same location as the first scan. All scans were completed in approximately 5 min using the continuous tomography setting at 17 and 20 keV at the ALS and SLS facilities, respectively. The degree of embolism was calculated by comparing the amount of xylem area embolized before and after cutting with the total xylem area. Cutting under water was done a few centimeters away from the scanned area.

For all the species studied, images showed an increase in the degree of embolism after cutting while the xylem was under tension, whether the measurements were done on intact plants (Fig. 1A) or cut branches (*A. campestre*). For *A. campestre*, the degree of artificially increased embolism was 3.8% and 26.3% at a xylem tension (i.e. xylem water potential [ $\Psi_x$ ]) of  $-3$  and  $-3.5$  MPa, respectively. This is consistent with the excision artifact suggested by Wheeler et al. (2013). The percentages of embolism introduced by cutting are lower than those reported by Wheeler et al. (2013), who

columns) and after cutting them under tension and under water (black columns). Due to limited access to Synchrotron micro-CT facilities, only one replication per  $\Psi_x$  was carried out except for *H. annuus* ( $n = 3$ ). Columns indicate averaged values, error bars represent SE, and the asterisk indicates a significant difference ( $P < 0.05$ ).

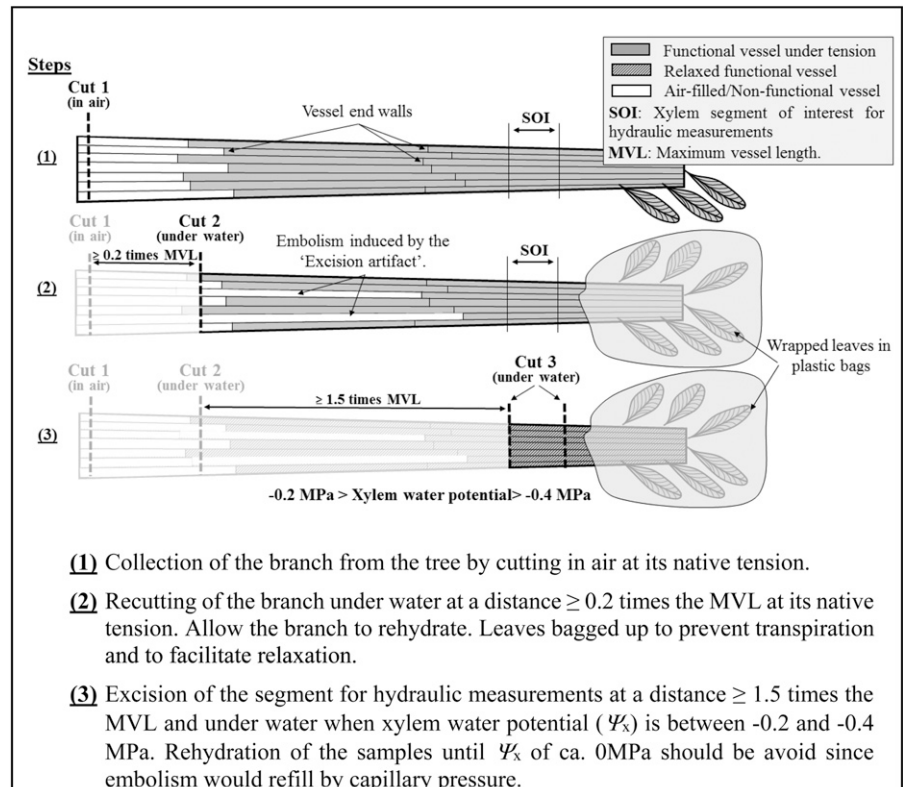
quantified the degree of embolism in longer xylem segments using a hydraulic method. The effect of vessel size was not taken into account in our percentage embolism estimates so, if the widest and longest vessels were the ones to embolize first, this would result in a higher loss of conductance (Cochard et al., 2013). Although these increases were relatively low, they clearly support the suggestions that the standard practice of cutting branches underwater to preserve the endogenous state of embolism can provoke the formation of bubbles into the vascular system, providing implications for accurate measurements of embolism. Although more access to micro-CT facilities is required for confirmation, the variability in our results suggests that the intensity of the cutting artifact is not constant within or between species even at similar xylem tensions, probably being affected by other factors (e.g. vessel diameter or amount of air in the pith). In *E. pauciflora*, *J. microcarpa*, and *H. annuus*, the possibility of working with intact seedlings allowed us to measure the occurrence of this excision artifact (i.e. increases in the degree of embolism compared with the intact stem) not only in woody species but also in herbaceous plants (Fig. 1). In addition, results from *E. pauciflora* and *J. microcarpa* showed how this artifact is influenced by the xylem tension: none or negligible increases were observed at low xylem tensions (i.e.  $\Psi_x$  of approximately  $-0.4$  MPa) and when the degree of native embolism was relatively high (Fig. 1B). The embolism induced by cutting, therefore, was a function of the species, the xylem tension, and the

degree of native embolism at the moment of the sample excision.

## IMPLICATIONS FOR PLANT PHYSIOLOGY AND ECOLOGY

Our results confirm that the excision artifact could have a significant impact on estimates of embolism at the stem level. This indicates that we must carefully reassess the accuracy of some previous results, especially those obtained from samples collected under tension (e.g. at midday, from dehydrating branches, and from water-stressed plants) and from long-vesseled species in which vessels could be open in the measurement segment. Artfactual increases in the degree of embolism can be misleading for two reasons: (1) they may suggest an erroneously high vulnerability to cavitation for some species; and (2) the variation of the xylem tension throughout the day would induce artifactual increases in the degree of embolism that could be interpreted as the occurrence of diurnal cycles of embolism formation and repair. Therefore, protocols of invasive techniques should be improved and validated by direct visualization for accurate evaluation of the hydraulic function or vulnerability curves. To minimize the excision artifact, the following protocol to collect and prepare samples should be followed (Fig. 2): if branches are initially harvested from a tree by cutting in air (cut 1), a second cut (cut 2) should be done under

**Figure 2.** Experimental protocol to avoid the excision artifact suggested by Wheeler et al. (2013) at the time of collecting xylem segments for hydraulic measurements.



water at a minimum distance of 0.2 times the maximum vessel length (MVL; Ewers and Fisher, 1989) of the species to hydraulically reconnect 50% of the vessels and then release the tension of the xylem by immersing the cut end in water and allowing the branch to rehydrate. Once the  $\Psi_x$  is between  $-0.2$  and  $-0.4$  MPa, the xylem segment of interest for hydraulic measurements should be excised under water (cut 3) at a minimum distance of 1.5 times the MVL from the initial cut to remove any artificial embolism generated during cut 1 and cut 2. Alternatively, one can determine the hydraulically weighted vessel length instead of the MVL (Cochard et al., 1994). After cut 3, the measurement on the segment of interest should be carried out immediately to avoid any possible effect of capillary pressure that will eventually passively refill embolized vessels.

Our results highlight the importance of both validating xylem embolism data and invasive hydraulic techniques by direct observation in intact plants for an improved understanding of water transport physiology in plants.

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