The cohesion theory debate continues

Response by Hervé Cochard, Thierry Améglio and Pierre Cruiziat

In two recent publications[1,2], we have reported that cryo-scanning electron microscopy (CSEM) observations of vessel content during transpiration in petioles of well-watered walnut trees were artifacts of the freezing procedure. We concluded that xylem vessels were all filled with sap during tree transpiration. Some of our experiments have been repeated recently[3]. Although the experiments yielded the same results, Martin Canny and colleagues came to the different conclusion that many vessels were filled with gas (embolized) during transpiration. We will summarize our experiment in this letter and explain where our views diverge.

Canny and colleagues quantified vessel embolism by the proportion of vessels seen filled with gas on a cross section in a CSEM (Ref. 4). In our work, we have also measured the proportion of loss of hydraulic conductance[5] (PLC) as a result of air embolism. The CSEM data suggest that many vessels are filled with gas in samples frozen intact on the tree but none in samples frozen shortly after excision under water. The PLC value of samples excised under water is also zero. Both techniques being invasive and, therefore, giving indirect information about the actual vessel content *in planta*, the current debate is about the interpretation of these data. We have argued that the number of gas-filled vessels is abnormally high on samples frozen intact on the tree because cavitations (artifacts) occur during the freezing procedure. By contrast, Canny *et al.* have argued that the number of gas-filled vessels and PLC values are abnormally low on samples excised under water because of rapid embolism refilling (artifacts). We know from past experiments that walnut petioles exposed to low xylem pressures (less than –1.2 MPa) become filled with gas at atmospheric pressure and that rapid vessel refilling does not occur (because the PLC values are not zero). However, in theory, walnut petioles exposed to the pressure value naturally occurring in a well-watered tree (about – 0.7 MPa) might contain cavitated vessels filled with gas at near-vacuum pressure. In this scenario, samples cut under water would rapidly refill and the PLC technique would underestimate the actual degree of embolism. In our opinion, the dye coloration experiment reported by Canny *et al.* is not a thorough test of this hypothesis because dye absorption could also have been caused by tissue rehydration[6] or capillary rise instead of embolism refilling.

To test whether the PLC or the CSEM technique causes artifacts, we did the following decisive experiment. We measured the PLC value of excised samples cut under water and exposed to the negative xylem pressure prevailing *in planta* (about –0.7 MPa). We used centrifugal force to expose samples to negative pressures and to force water through them at the same time. In doing so, we were able to record the PLC value while the pressure was lowered from 0 MPa to –2 MPa. If the refilling artifact was valid, then the PLC value at –0.7 MPa should have been significant because emptying vessel lumens must lower xylem conductance. Our data dismissed this hypothesis (Fig. 1). PLC values increased significantly only for pressures lower than –1.2 MPa. Therefore, our experiment shows that no cavitation occurs at the xylem pressure prevailing in well-watered trees and further establishes the relevance of the standard hydraulic technique[5].

Considering that there where no air-filled vessels, the PLC value of samples excised under water was zero and the PLC value of samples excised under water and exposed to the prevailing xylem pressure before excision was also zero, we conclude that the many vessels filled with gas in samples frozen intact on the tree were artifacts of the CSEM technique. Therefore, we strongly recommend that people involved in hydraulic measurements freeze xylem segments and measure PLC values on samples cut under water.

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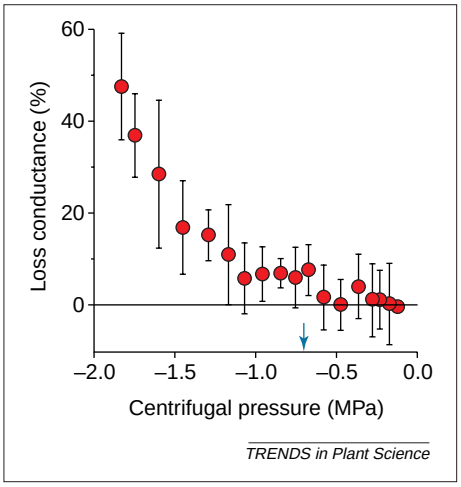

Fig. 1. Changes in the percent loss of hydraulic conductance (PLC) of a walnut petiole segment exposed to increasingly lower negative pressures from a rotational pull. The conductance was measured while the sap in the segment was under negative pressure. The PLC value was still insignificant at the xylem pressure prevailing *in planta* (arrow) and increased only for pressures less than –1.2 MPa. The error bars represent ±1 SD. Adapted from Ref. 1.

2 Cochard, H. *et al.* (2001) Vessel content debate revisited. *Trends Plant Sci.* 6, 13
3 Canny, M.J. *et al.* Cryo-scanning electron microscopy observations of vessel content during transpiration in walnut petioles. Facts or artifacts? *Plant Physiol. Biochem.* (in press)
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The cohesion theory debate continues: the pitfalls of cryobiology

Readers of *Trends in Plant Science* will by now be familiar with the opposing views of Martin Canny's group[1] and their colleagues, Hervé Cochard *et al.*[2,3]. The good thing about this debate is that the basic facts are more or less agreed upon. Petioles of *Juglans regia* (walnut) when frozen *in situ* by means of liquid nitrogen show a rather puzzling daycourse pattern in the cryo-scanning electron microscope (CSEM). All the vessels are filled with water early in the morning and late in the afternoon, whereas they are mostly empty during the noon hours. Cochard *et al.* [4] were not the first to observe this: Canny[5,6] and his

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co-workers[7,8] repeatedly found such daycourses in roots as well as leaves, and they used them as an argument against the cohesion–tension theory of water transport.

Those of us who have worked on quantifying water transport, for example, by means of heat balance methods or transpiration studies, tend to be rather perplexed by these findings. Diurnal patterns of water volume transport in plants are different. They show minima during the night, in the morning and in the evening, and a pronounced maximum around noon of a sunny day. How, then, can water for peak demand be conducted to the evaporating leaves, when we are told that conduits become empty just at the time their transport capacity is needed most?

Cochard *et al.*[4] presented several experiments in an attempt to prove that the voids in conduits observed in the CSEM images are artefacts. Martin Canny and co-workers repeated part of these original experiments. The latest stage of the controversy is about an experiment 'forgotten' by Canny *et al.*[1] in their discussion, that is, the measurements of conductivity on centrifuged petioles, which these authors neither repeated nor discussed. It is sometimes difficult to repeat each and every experiment when you do not have the equipment ready or have experience in using a particular method. In addition, we tend to overestimate the importance of experiments we did ourselves. A certain overestimation of an obviously cherished experiment is also visible in the dye-uptake study[1], which indeed does not prove that the colored water was sucked into cavitation voids.

The conductivity measurements[4] are of crucial importance, because they tell us that the minimum tension in the xylem water column necessary for reducing hydraulic conductivity is far higher than the one at which empty vessels show up after freezing. We are confronted with the following dilemma: At tensions of about –0.25 MPa, cavitations become visible in the CSEM. For a significant loss of hydraulic conductivity we need tensions of at least –1.2 MPa.

The interpretation by Cochard *et al.* is that freezing water will cause the water column in the xylem to snap under tensions not yet sufficient to cause cavitation by air-seeding. I think that this interpretation is supported by several independent facts. (1) Winter freezing is one of the two major mechanisms for embolization of xylem[9] in the field. We can therefore assume that the growth of ice crystals in conduits leads to cavitation and formation of emboli. The phenomenon is not well studied at the level of single conducting elements, therefore there is little information about the moment and the mechanism of rupture of the water column.

(2) The overall cooling rate achieved in the interior of petioles or roots by immersion in liquid nitrogen or by contact with cooled copper-jawed cryo-pliers must be completely insufficient for vitrification of the vessel fluid, which is almost pure water. All other methods imaginable, such as immersion into supercooled N_2 or precooled propane, would probably have no better success: the cooling rate of water in a trachea deep inside a petiole or a root depends on the Biot number of the sample, which is strongly influenced by its heat conduction properties as well as by its thickness[10]. (3) The heat conduction in plant tissues is low whereas the diameter of the plant organs under study was excessive in comparison with successfully vitrified specimens. Samples used in rapid cooling procedures[10] usually have a volume of $<0.25\ mm^3$, and even the most sophisticated approaches do not keep plant tissues ice-free to a depth of >0.6 mm from the surface of the sample. Therefore, it is no wonder that exotherms were observed during 'rapid' cooling of walnut petioles[4]. (4) Vitrification (immobilization of water molecules *in situ*) would require a cooling rate more rapid than $-100°C\ s^{-1}$, whereas the measured values correspond to about $-20°C\ s^{-1}$. However, only true vitrification could potentially immobilize the xylem sap in such a way that no gas bubbles are released and, thus, no free surfaces available for the explosive evaporation of water vapor.

The resolution of a scanning electron microscope is not high enough to show the microcrystalline structure of the solid vessel contents formed on immersion of a petiole in liquid nitrogen.

Many of the conduits appear to be partly filled with frozen water in the CSEM images; they show droplets or larger masses attached to the walls. Canny's group takes this ice as evidence for cycles of cavitation and refilling. I share the opinion[4] that we see remnants of the water column broken by freezing. Probably they consist of the microcrystalline ice first formed, which triggered a cavitation event and led to the rapid withdrawal of yet unfrozen water into neighboring conduits still under tension or to uptake by living cells. In various papers, the proportion of fully or partly empty conduits was estimated at up to 70% in a daycourse. However, let us not forget that CSEM images cannot give the true proportion of cavitated conduits because they show only cross-sections and not the whole length of the tracheae. A cross-section completely filled with ice is no evidence against voids above or below the plane of sectioning; the percentages estimated from direct observations are thus minimum values.

In summary, all evidence makes it plausible that the diurnal fluctuations in the filling state of frozen tracheae are artefacts. The counter-intuitive assumption that vessels become empty just when their transport capacity is needed most can thus be replaced by a far simpler sequence of events: water transport through hydraulic resistances leads to tensions, and freezing under tension leads to visible cavitations. Cochard *et al.*[4] have, in my opinion, once again shown that the cohesion–tension theory of water transport is rather tough and resilient. But so are its opponents.

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