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Cutting-edge research or cutting-edge artefact? An overdue control experiment complicates the xylem refilling story

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Arguably, the biggest current mystery in water relations research is how embolized xylem vessels refill despite negative pressures in the surrounding transpiration stream (Nardini, Lo Gullo & Salleo 2011). In this issue, Wheeler *et al*. (2013) set out to investigate the phenomenon in two known refillers, *Acer rubrum* and *Fraxinus americana* (Zwieniecki & Holbrook 1998; Zwieniecki *et al*. 2000). Instead, they made the sobering discovery that the 'refilling' in these species was an experimental artefact. And the artefact was seen in other species too. They report what amounts to a series of control experiments that should have been done decades ago. The results demand attention from anyone interested in xylem function and plant water relations.

Xylem studies are complicated by the remarkable fact that the liquid sap is transported under negative pressure. Negative gas pressures are impossible, but negative liquid pressures can exist as long as intermolecular forces hold. The strong bonding between water molecules combined with exclusion of large gas bubbles or other destabilizing surfaces allows liquid sap to be pulled up to the leaves by pressures far below vapour pressure. By analogy with a solid that can be placed under tension, negative sap pressures can also be referred to as (positive) tensions. The xylem water column is consequently metastable, and any gas bubble over a critical size nucleates 'cavitation': the phase change to vapour. The resulting vapour void can grow to occupy the entire conduit, and this gas 'embolism' blocks transport. The phenomena of cavitation and its reversal have grown over the years from a minor footnote to a significant chapter in plant physiology.

The metastability of xylem sap creates experimental difficulties. Any observation of xylem conduit functioning, whether by direct imaging or measurement of a bulk property like hydraulic conductivity, is only accurate if the observation itself does not nucleate cavitation and embolism. The need to validate observations with the appropriate control experiment is probably the single most limiting factor to progress in the field. It can be hard to know when a control is even necessary. There have been many interesting setbacks over the years when observations have subsequently been shown by belated controls to be experimental artefacts (Tyree 1997; Cochard *et al*. 2000).

The artefact exposed by Wheeler *et al*. has to do with how plant organs are excised prior to standard measurements of xylem hydraulic conductivity (see Fig. 1). As any florist knows, the excision must be made under water. If a stem is cut in air, negative pressure pulls the sap away from the surface and aspirates air into the open conduits, reducing future water uptake. But what Wheeler *et al*. show very convincingly, is that in 3 out of 4 species, significant blockage can be created even if the cut is made under water (see Fig. 1a,b schematics). Only if the negative pressures are relaxed before making the final underwater cut is the additional embolism avoided (Fig. 1c,d). The potential for the 'excision artefact' was shown to increase with more negative sap pressures, but to decrease with the amount of embolism present before cutting.

The mechanism of the excision artefact is unknown. The likely explanation is that gas gets drawn into the conduits during the cut by the sap negative pressure, blocking the openings to the conduits (Fig. 1a,b). The gas could either be aspirated from existing pockets in the tissue exposed by the cutting edge, or from cavitation nucleated as the blade slices open the conduit. Greater negative pressure would pull more gas and create more blockage. The blockage may be localized close to the exposed surface (e.g. Fig. 1b) because sap pressures should moderate quickly as water and gas are taken up. Interestingly, the only species showing no sign of the artefact was paper birch (*Betula papyrifera*), which has scalariform perforation plates. The narrow gaps in these perforation plates may be sufficient to trap gas near the cut surface, preventing measurable blockage.

Of historical interest is that the excision artefact was predicted long ago by Dixon and Joly (Dixon 1914, quoted at the beginning of the Wheeler *et al*. Discussion). Precautionary protocols designed to avoid the artefact were detailed in the Materials and Methods of M.H. Zimmermann's pioneering 1978 article on hydraulic architecture (Zimmermann 1978). Early practitioners of these methods continued these practices (Sperry, Donnelly & Tyree 1988), but the precautions were gradually dropped from the Materials and Methods sections. Why? Because no one . . . neither Dixon, nor Zimmermann, nor anyone else . . . had ever bothered to demonstrate whether the excision artefact actually existed. If you do not know whether it matters, it is human nature to forget over time about taking precautions.

Wheeler *et al*. show that the excision artefact does indeed matter. In some species, like red maple (*A. rubrum*), it matters a lot: cutting negatively pressurized xylem under water can decrease the hydraulic conductivity by 2–3-fold below what is arguably the correct value. From now on, researchers know that they really do have to take the precaution of relaxing xylem pressure before the final excision of a segment for assaying xylem conduit functioning.

The consequences of the excision artefact are significant. As emphasized by Wheeler *et al*., it casts doubt on numerous

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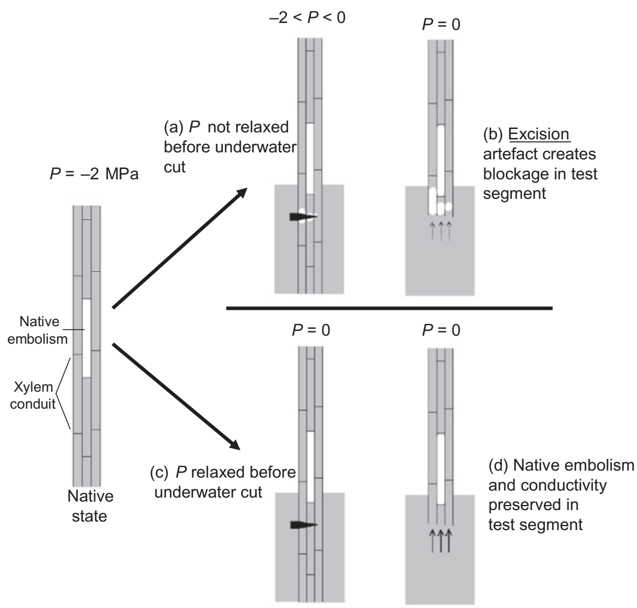

Figure 1. The 'excision artefact' exposed by Wheeler *et al.* (2013). Left-hand 'native state' schematic shows xylem conduits of the intact plant arranged in series and parallel. Xylem sap is under negative pressure (e.g. $P = -2$ MPa here). Some conduits are water filled (grey), others are gas filled or embolized (white). The upper arrow (a) shows what Wheeler *et al.* suggest that happens when test segments are cut underwater with no pressure relaxation. Gas bubbles initiated at the cutting blade (black wedge), or pulled from surrounding air spaces in the tissue (not shown), expand into the cut conduits by the negative (but quickly increasing) sap pressure. The result is artefactual blockage and reduced hydraulic conductivity in the test segment (b). The lower arrow (c) shows that the excision artefact can be avoided by relaxing the pressure to zero (or near to zero) before cutting. Additional blockage is thereby minimized, and the native conductivity and embolism is preserved in the test piece (d).

data sets showing diurnal changes in hydraulic conductivity from high at pre-dawn to low at mid-day and back up again at night. But the problem also is critical for any observation based on xylem conductivity measurements, including vulnerability-to-cavitation curves. Materials and Methods sections are often not explicit about the details of sample excision, so it is difficult to know how many previous studies are now questionable.

While sniffing out the excision artefact, Wheeler *et al.* tracked down yet another potential problem with refilling evidence. The original refilling study used a pressure sleeve to induce embolism by elevating the local air pressure around the stem high enough to push air into the xylem conduits (Salleo *et al.* 1996). The embolism thereby induced was seen to diminish with time, despite negative pressure in the transpiration stream. This protocol was repeated quasi-independently with the same result (Tyree *et al.* 1999), and has subsequently been adopted in a number of refilling studies (Secchi & Zwieniecki 2010, 2011). The potential problem exposed by Wheeler *et al.* is that the same pattern of embolism recovery is seen even when air pressures were too low to cause embolism in the intact sample. Their interpretation is that cutting the sample too soon after pressurizing causes the super-saturated sap to outgas and embolize the cut segment. When the sample was given time for gases to equilibrate prior to cutting, there was little excision-induced outgassing, a pattern that could create the illusion of embolism recovery. Similar to the excision artefact, this outgassing problem was noted previously in some of the first work using the air-pressurizing method, but it was not systematically documented (Sperry & Tyree 1990).

While the observations of Wheeler *et al.* must be taken very seriously, strictly speaking, they only repudiate the refilling report for *A. rubrum* and *F. americana* (Zwieniecki & Holbrook 1998; Zwieniecki *et al.* 2000). Since the report of refilling under negative pressure in 1996, there have been uncounted studies in numerous species, and not all can be tarred with the same brush. Some of the most compelling evidence comes from intact grapevines where there was no excision or air-pressurizing, yet refilling was non-invasively imaged (Brodersen *et al.* 2010). The deficiency of that otherwise excellent experiment was a paucity of convincing data on the xylem pressure at the time and site of the imaging. No one questions that refilling can occur at bulk xylem pressures near or above atmospheric (by root pressure, for example). The issue is how it can occur when the bulk xylem is truly under significant negative pressure adjacent to the refilling conduit. As shown by Wheeler *et al.* and others (Jacobson & Pratt 2012), xylem pressures can be spatially quite heterogeneous, even when pains are taken to promote their equilibration. Future refilling studies must not only take pains to avoid excision and air-pressure artefacts, but must also obtain the best possible resolution of local xylem pressures or tissue water potentials.

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