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Methods paper

Identifying which conduits are moving water in woody plants: a new HRCT-based method

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In vivo imaging methods are useful for examination of plant vascular tissues, particularly in the identification of fluid vs gas-filled conduits; however, these methods may not allow for the simple identification of conductive conduits. Our aim in the present study was to develop a method that would allow for the in vivo identification of conductive conduits. Intact plants and segments of grapevine (*Vitis vinifera* L.) and intact American chestnut (*Castanea dentata* (Marshall) Borkh.) saplings were examined. We found that iohexol, a water soluble iodine-rich molecule, was a useful contrast agent. We also stained the xylem of segments and gas-dried samples to compare between intact scans and excised segments. Iohexol could be readily fed through cut roots or stems into the transpiration stream, was successfully transported through the xylem and marked conductive vessels within high-resolution computed tomography (HRCT) scans. Iohexol results were comparable to those obtained by staining cut segments, with iohexol detecting greater numbers of smaller conduits in some samples. Samples contained gas-filled conduits, as well as both conductive (containing iohexol tracer) and non-conductive (no iohexol tracer) fluid-filled vessels. Fluid-filled non-conductive vessels were likely still developing or were not connected to the sap stream by a low resistance pathway. We found minimal differences between intact and excised segments other than excision-related dilution of iohexol. Both vessels and vasicentric tracheids were filled with iohexol in chestnut, providing a new tool to study the functions of these different cell types. The use of iohexol as a tracer to identify conductive vessels may greatly improve the utility of HRCT as a tool in the study of plant hydraulic function. Future studies using HRCT will likely need to incorporate conductive vessel markers or controls into experiments due to the presence of non-conductive fluid-filled vessels within the xylem.

Keywords: active xylem staining, cavitation, conductivity, dye ascent, HRCT, microCT, tracheids, vessels, X-rays, xylem.

Introduction

Terrestrial plants lose large amounts of water in exchange for CO_2. This water moves between the roots and the leaves through vessels and tracheids via bulk flow according to the cohesion-tension theory of plant water transport (Venturas et al. 2017). Studying plant water transport in the xylem has been a topic of keen interest as it has yielded interesting insights into the evolution of plants and it is linked to photosynthesis (Brodribb 2009), productivity and drought resistance (Pratt et al. 2014).

One challenge to studying plant vascular function is that measurements can disturb the behavior of the vascular system such that it differs from an undisturbed system, leading to artifacts. There are a number of reasons why artifacts may arise for various methods that are beyond the scope of this paper; nevertheless, some efforts to develop new methods have aimed to avoid or minimize the possibility of artifacts. One set of methods that has been used are in vivo imaging methods. Two imaging methods that have been used are magnetic resonance imaging (MRI; Windt et al. 2006) and high-resolution computed tomography (HRCT; Brodersen 2013). High-resolution computed tomography, in particular, has seen a substantial increase in use in the last decade.

In vivo imaging methods readily distinguish fluid- from gas-filled regions in conduits within vascular tissues. For HRCT this is accomplished because fluid absorbs X-rays much more strongly

than gas. Beyond fluid and gas, HRCT can distinguish other structural details in many species such as distinguishing cell walls from fluid-filled regions or conduit lumens. One critique that has been raised is that fluid-filled conduits (vessels or tracheids) may not be functional in transporting water (Jacobsen and Pratt 2012, Hacke et al. 2015), which becomes important in studies aimed at studying the flow of sap and comparing HRCT data to other methods that account for the presence of non-functional vessels, such as direct hydraulic measurements (Sperry et al. 1988).

Fluid-filled conduit lumens that are not conductive may occur for several reasons (Jacobsen et al. 2018). The first is that conduits may be immature. During xylogenesis, conduits expand and develop as living cells. During the final stages of this process, the plasma membrane breaks down, the end walls open, and the cells become functional in transporting water. Up until that time, the cell is not substantially contributing to bulk axial transport. Another reason why a conduit might be fluid-filled and not transporting water is because it is occluded by gels, gums or tyloses (Kitin et al. 2010, Jacobsen and Pratt 2012, De Micco et al. 2016). Types and amounts of occlusions produced are species specific. If a conduit is occluded, it will not function in transporting water, but it may contain substantial amounts of water and thus appear fluid-filled in HRCT images as has been shown for gels in grapevine (Jacobsen et al. 2018). Spatially, developing conduits will be most common in the vicinity of the vascular cambium and occluded vessels, while they could be anywhere, will be more common among the older portions of healthy tissue (De Micco et al. 2016). Finally, flow patterns of sap are determined by the connections between tracheary elements and the structure of the 3D vessel network (Waisel et al. 1972, Sperry et al. 1988, Utsumi et al. 2003, Gebauer et al. 2008, Schenk et al. 2008). A vessel may not be axially conductive because it is hydraulically isolated from the sap stream by non-functional conduits (Loepfe et al. 2007). This is possible if adjacent vessels connected to such a vessel become embolized and leave the vessel without connections to other portions of the transpiring tissue. This may be especially common in older growth rings and for species that are deciduous or for evergreens that lack direct leaf connections to older xylem (Maton and Gartner 2005).

Methods available to study flow patterns have been limited to dye ascents (MacDougal 1925, Kozlowski and Winget 1963, Newbanks et al. 1983, Sano et al. 2005, Umebayashi et al. 2007) or dye perfusions through excised segments (Sperry et al. 1988, Hargrave et al. 1994). Cryogenic scanning electron microscopy (Cryo-SEM) has also been used (Utsumi et al. 2003, Sano et al. 2005). Patterns of xylem staining are often attributed to gas-filled conduits (ones filled with emboli) that are not functional (Hargrave et al. 1994) or to the presence of blockages, such as gels and gums, within conduits (Ewers et al. 1989, McManus et al. 1989, Jacobsen and Pratt 2012). In stems that are hydrated, non-staining conduits have been observed abundantly near the vascular cambium and interpreted as immature conduits (Halis et al. 2012, Jacobsen et al. 2015, 2018).

Staining methods have yielded important insights into flow patterns of water in plants, but have some limitations. One challenge that can arise is when dye in one conduit bleeds into another that it is in contact with leading to 'contact staining' (Hargrave et al. 1994). This may make it difficult to assess fine scale patterns in some species. This has been addressed by freezing samples in liquid nitrogen immediately after staining (Kitin et al. 2010), careful preparation of samples (Sano et al. 2005) and the rapid processing of samples (Jacobsen et al. 2018). Dye selection is also important (MacDougal 1925, Sano et al. 2005).

The ability to identify conductive conduits from non-conductive ones using in vivo methods would be a powerful tool and this motivated the present study. Some nuclear MRI-based in vivo methods can yield information on flow rates of xylem and, in some cases, phloem (Köckenberger et al. 1997, Windt et al. 2006, Hochberg et al. 2017), thus being able to distinguish transporting vs non-transporting regions of tissue; however, there are disadvantages to these methods. Commercial MRI equipment is not well configured to plant studies and plants have to be laid on their sides, and it can be difficult to mount them in the equipment (Windt et al. 2006). Also, these systems are not widely available so they have not been used extensively. Finally, the resolution of these systems is limited at present, limiting the identification of small individual conduits, which also limits their utility.

One of the approaches to using HRCT to examine a wide range of tissues is to employ contrast agents that strongly absorb X-rays in order to enhance contrast in regions where contrast is naturally lacking (Blonder et al. 2012, Staedler et al. 2013, Teixeira-Costa and Ceccantini 2016). The present study fed a contrast agent into the xylem stream to visualize flow paths within the xylem tissue and to identify conductive tracheary elements. To do this, we chose a contrast agent with some particular characteristics. All useful contrast agents have high atomic mass numbers because greater mass is associated with greater X-ray absorption. Another consideration for a contrast agent was solubility and it was important that any chemical was water soluble to feed into the transpiration stream. An additional consideration was molecule size; molecules need to be small enough to pass through pit membrane pores, but also large enough so they do not readily diffuse across lipid membranes and broadly infuse tissues. Pit membrane pores are difficult to measure without artifact but estimates are that they range from 5 to 420 nm in diameter (Choat et al. 2008). Molecules that have topological surface areas >1.40 nm^2 are generally poor at lipid membrane penetration. Thus, a molecule useful for a wide range of species would have a size between 1.4 and 5 nm^2. Osmolality and toxicity is important to consider as well, since a solution with a highly negative water potential or one that is

toxic could lead to rapid stomatal closure and inadequate uptake.

Here we describe a simple HRCT-based method that allows for the determination of which conduits are actively transporting water in intact plants. We chose to sample two species: grapevine (*Vitis vinifera* 'Chardonnay' and 'Glenora') and American chestnut (*Castanea dentata*) that have rather different xylem anatomies. Grapevine was of keen interest because some studies have suggested that there are non-functional vessels that may appear functional in HRCT images (Jacobsen and Pratt 2012, Jacobsen et al. 2015), whereas other have suggested otherwise (McElrone et al. 2012, Hochberg et al. 2017). American chestnut was chosen because it is ring porous and has vasicentric tracheids. This broad range in conduit sizes was a good test for this method because being able to use a tracer that could mark both large and small conduits without broad diffusion of the tracer throughout the tissue would be maximally useful.

Materials and methods

Plants studied

Plants used in this study were well-watered and both greenhouse and field grown. Plants of *V. vinifera* L. 'Chardonnay' (Willis Orchards, Cartersville, GA, USA) and American chestnut *C. dentata* (Marshall) Borkh. (Burnt Ridge Nursery, Onalaska, WA, USA) were purchased from nurseries and grown in a climate-controlled greenhouse on campus at California State University, Bakersfield (CSUB) in small containers in April 2017. They were watered twice a day from overhead misters, once at dawn and a second time at solar noon, which served to keep the soil moist at all times. The soil used was a standard potting soil mixture with ample drainage (abundant perlite). For measurements, plants were selected for study that were relatively uniform in height, because the maximum height that could fit in our HRCT system was 0.5 m. In total, five grapevine plants and four chestnut plants were scanned. Another, field-grown, grapevine variety was also used (*V. vinifera* L. 'Glenora') from a common garden 0.5 km from the laboratory at CSUB. This grapevine was watered deeply about every other day and was kept well hydrated. This specimen produced an abundant fruit crop and actively grew all summer and fall, both of which indicated a lack water limitation. Experiments were conducted in late summer to early fall (July–September 2017). A pressure chamber was used to measure transpiring leaf water potential during experiments to verify that plants were hydrated (PMS, Albany, OR, USA).

High-resolution computed tomography (HRCT) and chemical contrast agents

Plants were scanned using an HRCT system (Bruker Corporation, Skyscan 2211, Billerica, MA, USA) at the CSUB Biology 3D Imaging Center. The objective was to determine which vessels were actively transporting water in our sampled plants by feeding them a chemical compound that would be visible in the intact plant when scanned via HRCT. High-resolution computed tomography systems use X-rays to examine tissues. Differences in X-ray absorbance between air, water, cells and chemicals create contrast that can be observed in HRCT images. It is common with this method, particularly in medical applications, to use chemicals that strongly absorb X-rays to create contrast that allows visualization of tissues of interest. Chemicals with relatively high atomic mass units will absorb X-rays more strongly than native tissues and the water that is abundant in live specimens.

In this study, various chemicals were considered, but the one that was the best candidate was an iodine-rich chemical, iohexol (TCI America, Portland, OR, USA). Iohexol met all of the requirements that we examined for a compound that would be a promising xylem sap contrast agent. It has a relatively large molecular size (topological polar surface area = 2 nm^2) so as to minimize diffusion across lipid membranes and unwanted bleeding into xylem parenchyma cells. It can also be used at relatively high (less negative) osmotic potentials so cells do not get exposed to a large osmotic gradient, which could be harmful or cause stomata to close and limit uptake. Indeed, iohexol is one of the chemicals that is injected into human vascular systems to enhance contrast in medical imaging applications, which gives some indication of its low toxicity to eukaryotic cells (Lusic and Grinstaff 2012). Iohexol is water soluble and is readily fed through cut organs into the transpiration stream. We dissolved iohexol in deionized water (150 mM) and filtered it with a 100 nm membrane filter (Osmonics, Inc. MAGNA Nylon Supported Plain 0.1 µm, 90 mm GE, New York City, USA). The contrast of various iohexol solutions was evaluated by placing different concentrations in centrifuge tubes into the HRCT and visually assessing which ones were different enough from pure using the real-time output of the HRCT camera. The 150 mM solution was chosen as the lowest concentration that provided sufficient contrast from water to unambiguously highlight vessels containing the solution. A 150 mM solution of iohexol was measured to have an osmotic potential of −0.29 MPa (WP4, Decagon Devices, Pullman, WA, USA).

Treatments and measurements

Plants were fed iohexol through their root systems or cut stems (Figure 1). To feed root systems, the soil of hydrated greenhouse grown plants was carefully removed from the roots under water. Roots tips were then cut under water with a fresh razor blade (GEM Single edge stainless steel PTFE-coated blades, Electron Microscopy Sciences, Hatfield, PA, USA) so that they were all open. For field-grown grapevine plants, long branches about 1.5 m long were cut underwater from plants. After transport to the lab (about 10 min) with the cut end under water during transport, we made successive cuts underwater to cut the branch to length, while maintaining the intact shoot tip and leaves. The whole root system of intact plants was placed into a centrifuge tube filled with 150 mM iohexol solution (Figure 1)

Figure 1. Grapevine (chardonnay) with root system immersed in iohexol tracer solution and placed outdoors to allow transpiration-induced uptake of the tracer.

or, for cut segments, the stem base was placed into a centrifuge tube filled with 150 mM iohexol solution. Plants or shoot segments were placed outside or in a greenhouse for 2–3.5 h, between 09:00 and 14:00 h, to allow them to take-up the solution via transpiration. The time of uptake was determined volumetrically and when the plants had taken up at least 5 ml they were then scanned. All plants readily took up the solution as it was found throughout the branches we examined.

Following iohexol uptake, water potential of plant leaves was measured with a pressure chamber, and then plant leaves were covered in plastic wrap (to halt transpiration), and plants or shoots were placed in an HRCT and imaged intact. Water potential leaves were taken as far away as possible from the point of scanning to avoid any air-entry into the region to be measured by HRCT. The intact scan was conducted on the main stem of the plant, which was about 7.5 cm above the stem–root junction for the main stems. For some samples, we excised the main stem under water and reimaged to see what effect the excision had on the distribution of stain or if the cutting lead to any changes in the distribution of water and air-filled conduits. This was important because excised segments are generally more practical to scan, thus being able to use them would broaden the utility of this method. After scanning the main stem, for some grapevine intact plants, we also excised, underwater, distal 2-year-old and 1-year-old stem segments and scanned them. This sampling was chiefly done to assess if the iohexol had traveled the full length of the vascular system. These samples were not transpiring because their foliage was plastic-wrapped after having been already scanned in the HRCT. Scanned locations on samples were marked with a strip of metal tape so that this area of the sample could be matched in subsequent sampling. Chestnut seedlings did not have older branches so only their main stems were scanned.

Following HRCT imaging, and for a subset of samples, a 0.14 m sample that included the scanned portion of the stem was excised underwater. This shorter segment was stained using a 0.1% (m/v) dye solution of crystal violet that was pulled up through the stems at approximately 2 kPa pressure for 30 min. This was done by attaching stems to a tube connected to a closed reservoir of ultrapure water on one end and the stem tip was immersed in dye on the other end. The reservoir was then lowered about 20 cm below the tip of the stem immersed in dye. This created a pressure gradient of about −2 kPa to drive dye uptake in the stem xylem via siphoning. Thin cross sections were prepared from near the scanned area by hand using a razor blade (GEM Single edge stainless steel PTFE-coated blades, Electron Microscopy Sciences) and mounted on slides in glycerol. Photos of the entire cross section were taken using a microscope (Leica DM750, Leica Microsystems, Switzerland) with an attached camera (Leica MC120 HD, Leica Microsystems, Wetzlar, Germany).

Following scanning, all stems were dehydrated. To do this, a 2-cm section of stem, which included a portion of the scanned volume, was excised from each sample. This section was attached to a tubing system and gas was pushed through the sample at 50 kPa for 5 min following the method of McElrone et al. (2013) for the identification of potentially conductive vessels. This method forces gas into conduits and dehydrates the tissue, thereby increasing the contrast between walls and lumens for easier identification of conduits that are not permanently occluded.

To compare iohexol to crystal violet staining of excised segments, we counted the number of conduits in cases where we had both scanned stems and stems that we stained with crystal violet ($n = 6$). For the HRCT iohexol samples, software was used to selectively analyze xylem area and threshold, and automatically count the iohexol-filled conduits that were >12 μm in diameter (CTAn Software, Bruker microCT, Bruker Biospin, Billerica, MA, USA). Doing this removed fiber lumens and conduits with very small lumen diameters that minimally contribute to sap flow. In cases where thresholding data were ambiguous, both dehydrated sections and micrographs of the same samples were used to ensure accurate counts. Crystal violet stained conduits were counted manually. The number of conduits was compared using standardized major axis regression to test if the slopes were equal to 1.

To evaluate the potential for iohexol to assist in the automatic analysis of conductive vessels within scans, we conducted an analysis on grapevine scans. Using CTAn software (Bruker Corporation, Billerica, MA, USA) we analyzed a volume of interest that included the xylem portions of the sample (excluding the bark and pith) and analyzed all of the objects (vessels) above a

minimum size established through thresholding. The same procedure was repeated on the dehydrated sample. This allowed us to assess the number and diameter of iohexol-containing vessels and to compare them to the number and size of vessels in dehydrated samples.

Results

None of the plants had water potentials in a range that would indicate water shortage (transpiring leaf water potentials ranged from −0.28 to −0.85 MPa). This is consistent with all of the plants being well hydrated.

Iohexol was readily taken up into the transpiration stream of plants and provided strong contrast between conductive conduit lumens and surrounding tissues. For intact grapevine plants that were fed iohexol solution through their roots, scans of segments from the basal oldest portions of plants (3-year-old main stems; Figure 2a) and distal positions of plants (1-year-old stems; Figure 2b) showed that iohexol solution was transported along the entire stem length of the plants. This indicates that the

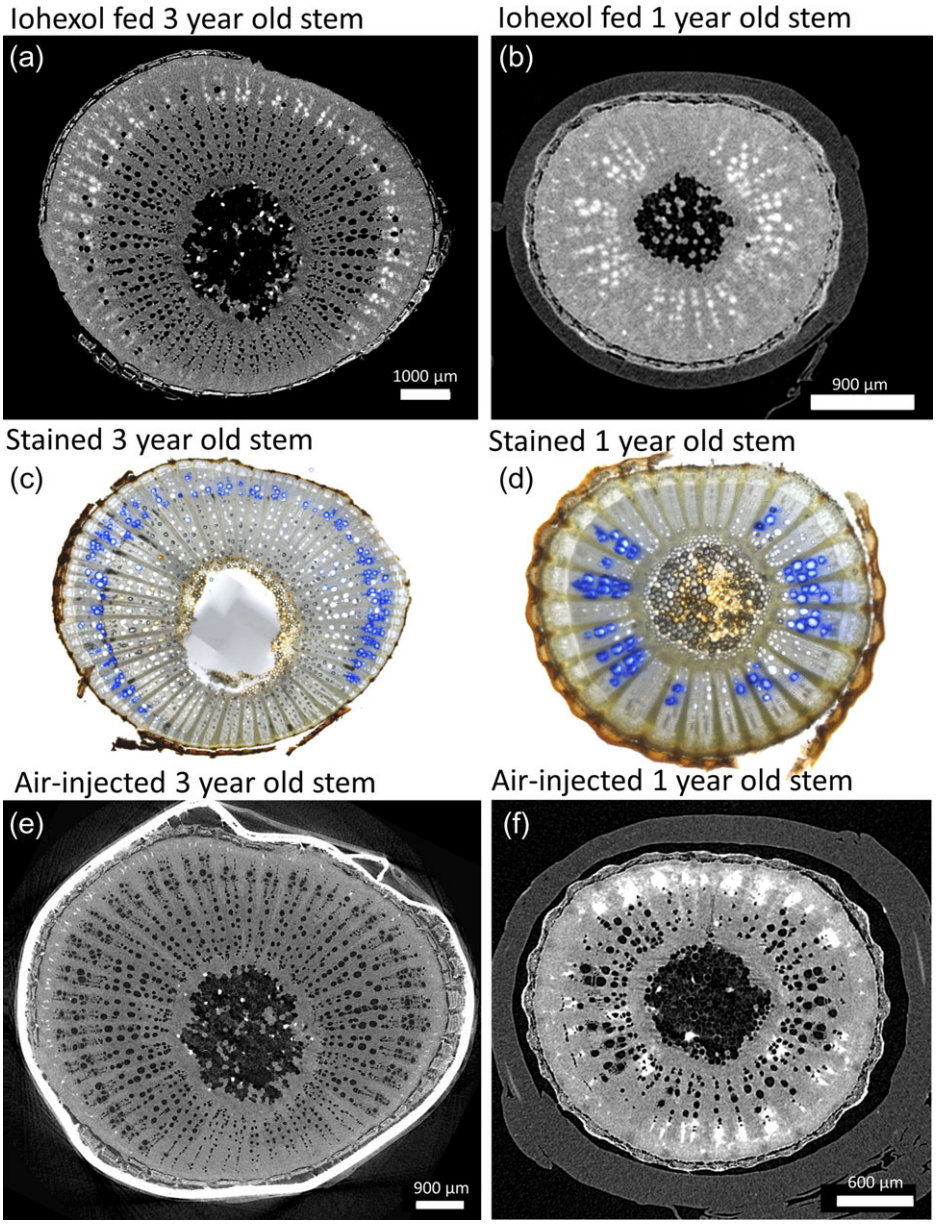

Figure 2. Iohexol-fed grapevine ('Chardonnay') images of a 3-year old-main stem from an HRCT scan of an intact plant (a), a light micrograph of the same stem stained with crystal violet (c) and an HRCT scan of the same stem following gas dehydration (e). Also shown is a 1-year old stem (b, d, f) from the same plant and with the same images and treatments. The 1-year-old stem was excised prior to imaging. Light (white) areas in the xylem in panels (a) and (b) show the iohexol-rich areas, indicative of conductive vessels, that strongly absorb X-rays, dark (black) areas are gas with little X-ray absorption, and gray areas absorb intermediately to iohexol and air. Crystals that strongly absorb X-rays can also be seen in the pith and bark in all HRCT images. In the gas-dehydrated branch, dried iohexol crystals are also visible as bright white regions in the xylem and bark (f).

volume of iohexol fed to the plants over 2–3.5 h (>5 ml) was sufficient to move throughout the plant vascular system. We did not examine leaves, but this could be easily done in future studies (Blonder et al. 2012).

The conductive vessels were primarily found in the current growth ring of xylem. In the main stems, the inner and older growth rings of grapevine had virtually no active vessels (Figure 2a), whereas there were some in chestnut (white spots in older ring in Figure 3a and stained vessels in 3d). Most inner growth ring vessels were gas-filled (Figures 2a and 3a). The current-year growth rings did not have a substantial amount of embolism (Figures 2a, b and 3a), which was expected because plants were hydrated. The one exception was that there was about 40 large vessels that were embolized in the current growth of grapevine (Figure 2a), which corresponds to about 20% of the largest vessels (smaller ones excluded) in the outer growth ring (the total number of large vessels are most easily observed in Figure 2e). For intact chestnut plants, which where both smaller and younger than the grapevine, only main stems were examined, which were 2 years old. Mature chestnut has ring porous-type xylem. Characteristically, for species with this xylem-type, the first year of growth is diffuse porous and ring porosity is not observed until year two or later. Diffuse porous first year xylem and the second year ring porous-type could be easily observed in our sampled plants, especially when they were air-dehydrated (Figure 3c).

Staining xylem by siphoning dye through excised segments generally showed agreement with iohexol treated samples with some notable exceptions (Figures 2a, c and b, d, and 3a, d). The number of conduits recorded containing iohexol was significantly and positively correlated to the number containing crystal violet (Figure 4). The number of conduits filled with iohexol was greater than the number for crystal violet (Figure 4), but the difference was not significant (slope = 1.56 and 95% CI = 0.86), i.e., the slope was not different from 1. There were some interesting differences observed for iohexol and crystal violet staining patterns. Iohexol was observed in current-year vasicentric tracheids that could be seen as white bands

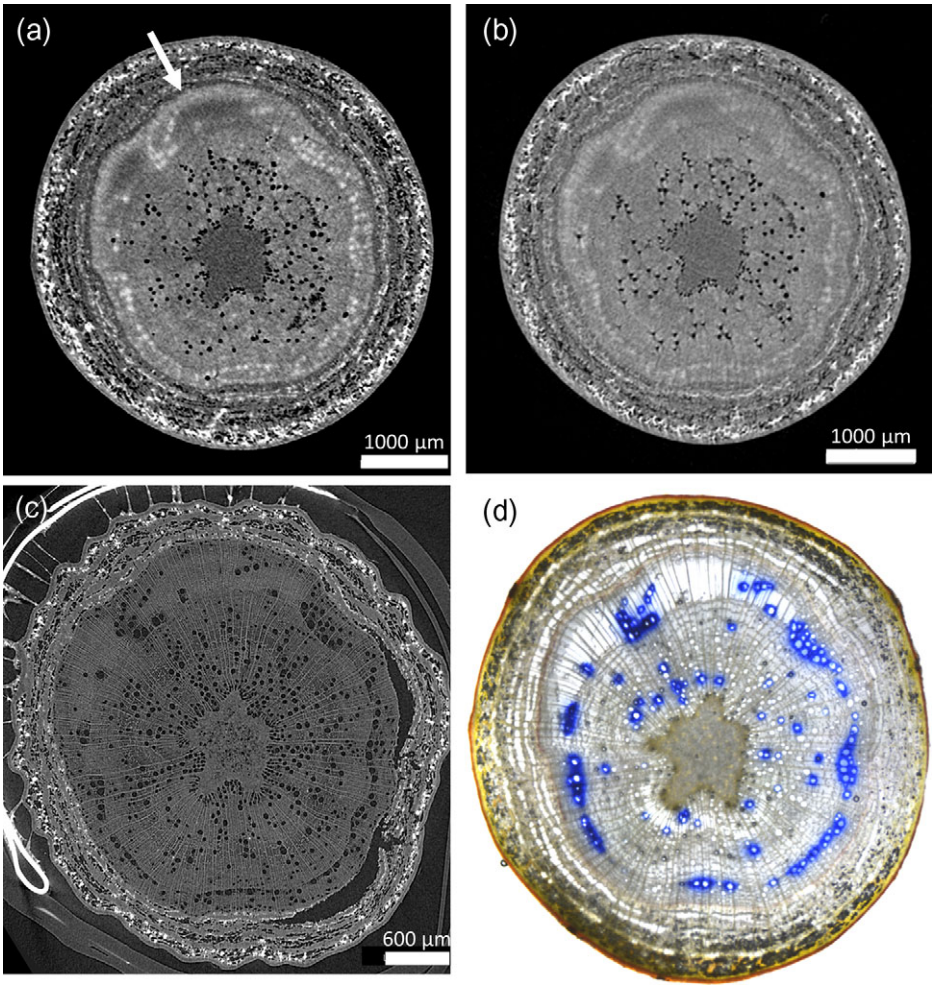

Figure 3. Iohexol-fed American chestnut 2-year-old main stem of an intact plant (a), the same stem after it had been excised under water (b) and the same sample after it has been dehydrated (c) to clearly see the vessels. Panels (a), (b) and (c) are images from HRCT scans. The stem was also stained with crystal violet (d) and photographed using light microscopy. The arrow in panel (a) points to a band of white iohexol marking an extensive region of conductive vasicentric tracheids.

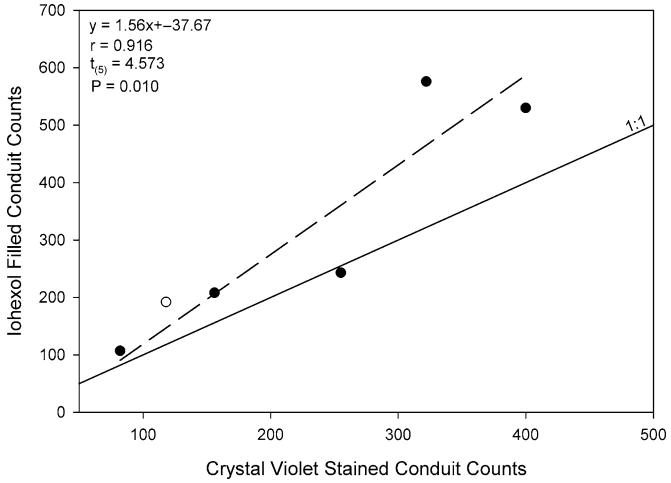

Figure 4. The number of iohexol-filled conduits (vessels and tracheids) related to the number crystal violet-stained conduits (closed symbols are grapevine and open symbol is American chestnut). There were a greater number of iohexol-filled conduits (slope >1), but not significantly so (lower 95% CL = 0.693 and upper 95% CL = 2.430). A 1:1 line is shown for reference.

of iohexol highlighted tissue near the outer edge of American chestnut samples (Figure 3a, arrow). Among these tracheids were some small latewood vessels that are most easily observed in the dried sample (Figure 3c). These outermost smaller vessels and tracheids were not stained by crystal violet (Figure 3d). Tracheids within the inner growth ring lacked iohexol and also did not stain with crystal violet, suggesting that they were not active in axial transport even though they were generally not air-filled (Figure 3a and d). In the 1-year-old grapevine sample there were also some conduits (almost all with relatively small diameter) that did not stain with crystal violet, but that did fill with iohexol (Figure 2b and d).

Comparative analysis of the iohexol and crystal violet methods suggests that some vessels were sap-filled, but not significantly contributing to transpiration (Figure 5). These vessels were fluid-filled in HRCT images of intact plants but did not contain iohexol (Figure 5c, right side arrows); however, crystal violet stained these same vessels in excised segments (Figure 5c, inset). This same pattern was observed in some samples in the current-year growth ring (Figure 5b, d and f, bracketed region). This was quite pronounced in one sample (Figure 5b), and upon close inspection of the plant, we noticed that there was a dead branch that had been previously pruned by the growers we obtained the plants from. Presumably, this sector of xylem was the pipeline to that branch and it was no longer connected to the axial transport pathway via vessel-to-vessel connections. This section of xylem did stain with dye when the segment was excised and stained with crystal violet, indicating that the vessels in this region were potentially functional (not occluded). In these samples, as in all others, all vessels became gas-filled with air-injection, regardless of their status prior to the injection as conductive or non-conductive (Figure 5e and f).

A cohort of water filled vessels were observed that contained neither iohexol nor crystal violet (Figure 6c and e), suggesting that such vessels were not contributing to transpiration and that they were occluded. These vessels were commonly observed in the outer growth ring, particularly in grapevine, suggesting that they were immature (Figure 6a and c). In some of the sections, when the samples were dehydrated with air these vessels showed some deformation suggesting that they were not fully lignified (Figure 6b, arrow; see Figure S1a and c available as Supplementary Data at *Tree Physiology* Online), but this was not always the case (Figure 5e and f). Many of these vessels also had thin walls seen in light micrographs (see Figure S1e and f available as Supplementary Data at *Tree Physiology* Online). It was also apparent in the light micrographs that the fiber matrix around these vessels was not fully developed (see Figure S1f available as Supplementary Data at *Tree Physiology* Online). The non-conductive vessels filled with air after being dehydrated by air-injection (Figure 6b) and this can been seen in longitudinal portions of scanned images (see Figure S1b and d available as Supplementary Data at *Tree Physiology* Online).

Intact and excised scanned segments were similar, with little to no change in the distribution of iohexol or emboli (Figure 3a vs b); however, cutting non-transpiring segments under water generally had the effect of diluting the iohexol leading to diminished contrast (fainter white in vessels, Figure 3b). Nevertheless, even though the contrast was diminished, the X-ray-dense iohexol was still visible in vessels. Being able to cut segments means that a wider range of samples and organs can be examined with this method because there is generally a limit to the dimensions that can be scanned within an HRCT.

It is common to use software to automate image analyses of the gas-filled conduits within HRCT-derived images. The X-ray-dense iohexol creates contrast that can be used to clearly identify vessels that are actively transporting water and provides enough contrast to separate these vessels from neighboring walls (this enables thresholding). However, there are important caveats to automatic analysis. We conducted an automatic analysis comparing the results of an iohexol-fed segment (Figure 6a and d) and the same segment after it had been air dried (Figure 6b and f). For iohexol, small vessels were difficult for the software to identify and large vessels were so bright in the image that the image analysis found them to be larger than air-filled vessels. The results reflected this in that the total number of vessels >20 μm identified by thresholding was 184 in the iohexol sample compared with 484 in the air-dried sample; moreover, the number of larger vessels (>60 μm) was 144 for the iohexol sample and 103 for the dried one. In gas-injected samples, the vessels were able to be more easily identified by the software in the thresholding step of the analysis.

Discussion

Iohexol root feeding is an effective tool for studying sap flow patterns in vivo

Feeding iohexol into the roots of plants coupled with HRCT of these intact plants produced images with abundant contrast that

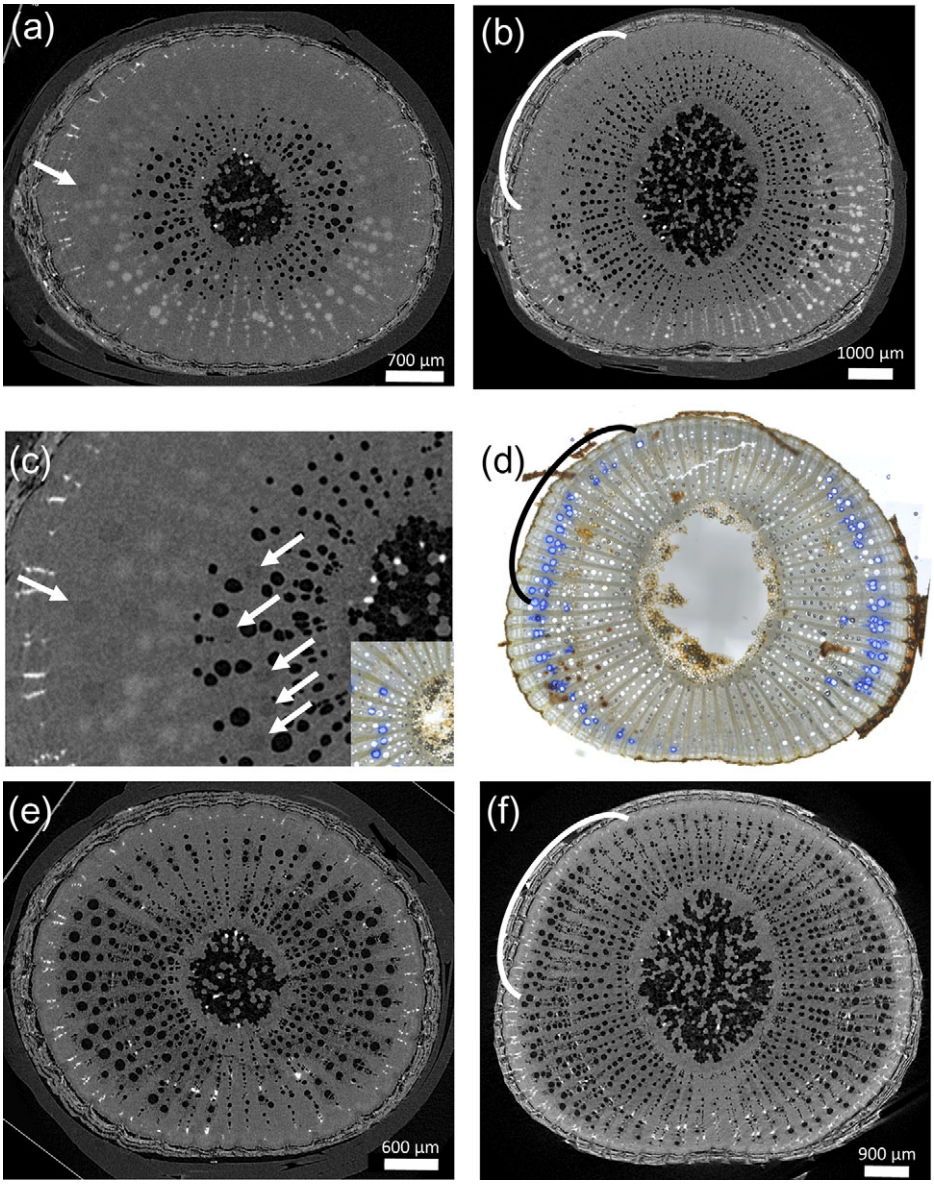

Figure 5. Iohexol-fed grapevine ('Chardonnay') images of a 2-year-old main stem (a), a magnified region of the same stem to highlight vessels that appeared fluid-filled that did not contain iohexol (c; arrows point to some of these vessels) and the same stem air-dehydrated (e). The inset in panel (c) shows the five vessels highlighted with arrows in the older xylem within panel (c) that were not conductive when using iohexol, but which later stained with crystal violet. Also shown are images from a different iohexol-fed grapevine ('Chardonnay') 3-year-old main stem (a), the same stem stained with crystal violet (d) and the same stem air-dehydrated (f). The bracketed areas in (b), (d) and (f) show a region of vessels that did not move iohexol (b), but that did stain with crystal violet (d) and that filled with air upon dehydration.

illuminated regions of xylem that were actively transporting sap. This worked for a woody liana (grapevine) and tree seedlings (American chestnut). The chestnut is ring porous and iohexol was observed abundantly in both the large earlywood vessels and small latewood ones. It also showed up abundantly in the vasicentric tracheids.

Siphoning dye through excised segments can be challenging for species that contain conduits with highly divergent diameters (e.g., ring porous species or species with both vessels and tracheids). This is the case because it takes a longer to stain the small diameter conduits, whereas the wide diameter vessels may commonly stain more quickly (Chiu and Ewers 1992, Umebayashi et al. 2007). If the staining time is too short, some of the smallest conduits may not even stain at all, and this may explain some of the discrepancies between small conduits observed between the iohexol method and the crystal violet staining used here. Species with a more even distribution of vessel diameters (diffuse porous) should be less affected. Depending on the objective of a particular study, failure to detect some small conduits may not be important because it is the large diameter conduits that contribute the most to hydraulic conductivity due to the Hagen–Poiseuille relationship. The iohexol

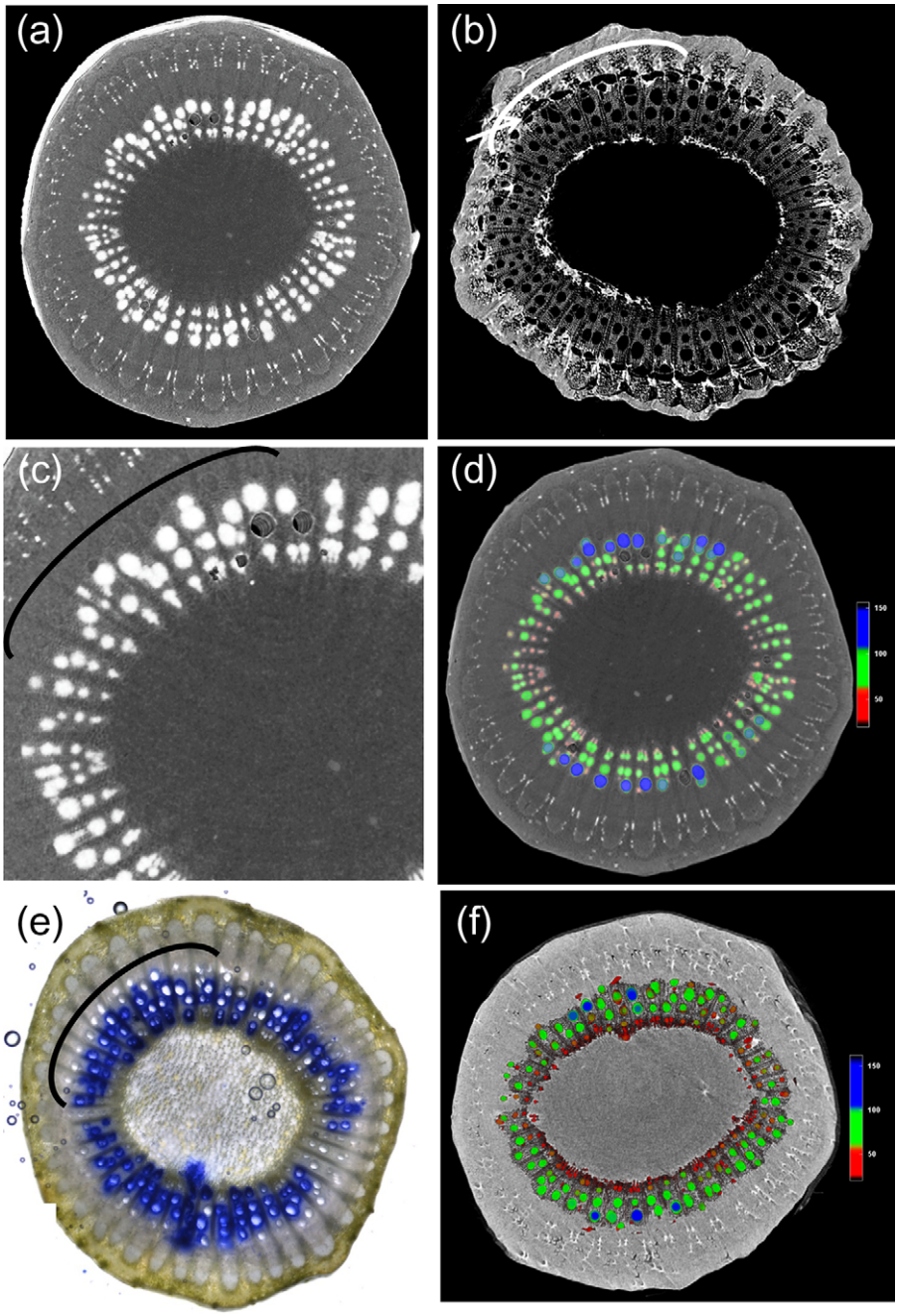

Figure 6. Iohexol-fed grapevine ('Glenora') images of a 1-year-old excised branch (a), a magnified region of the same stem to highlight vessels that appeared fluid-filled that did not contain iohexol (c; bracketed region shows some, but not all of these vessels), the same stem stained with crystal violet (e; bracketed area is the same as in c) and the same stem air-dehydrated (b, bracketed area is the same as in c and e). Arrow in panel (b) points to an example of a vessel that deformed after being dehydrated (see also Figure S1 available as Supplementary Data at *Tree Physiology* Online). Automated image analysis was conducted on this sample and colored circles in images (d) and (f) represents diameter size of conduits (μm) according to the scales shown.

method could be used in combination with the staining method to fine tune the time it takes to stain all of the functional tracheary elements in future studies.

Using iohexol may help with automatic analyses of the conductive vessels within HRCT-derived images, which is desirable when studies are seeking to estimate plant hydraulic function or conductivity. However, our first attempt at this did not yield satisfactory results. If this method is going to be automated, to get accurate vessel diameters, the iohexol solution will likely need to be adjusted to get the right level of contrast between it and the surrounding tissues.

Crucial to our ability to identify vessels with a limited role in bulk axial sap transport was the use of both iohexol feeding of roots and crystal violet staining of cut segments. The vessels that were

not moving sap but that were capable of moving sap were the ones that did not fill with iohexol but that did subsequently stain in cut segments with crystal violet. This combination of methods was important because the non-sap transporting vessels may have been occluded by cytosol (immature vessels, Utsumi et al. 2003), gels or gums and thus were not transporting because they were occluded (Kitin et al. 2010). Such vessels would likely have appeared sap-filled in HRCT, but would not have contained iohexol (Jacobsen et al. 2018). The crystal violet data allowed us to reject the possibility that some of these vessels were occluded. Cryogenic scanning electron microscopy (Cryo-SEM) has been shown to be effective in identifying maturing tracheids filled with cytoplasm (Utsumi et al. 2003); however, this may not work as easily to identify non-conductive vessels in the late stages of development (St Aubin et al. 1986, Kitin and Funada 2016).

Some have suggested that when vessels are injected with air and examined using HRCT, formerly occluded vessels should contain residual material that would enable them to be identified as non-functional in vivo. In the case of gels in grapevine vessels, this has been recently tested and shown to be false as gels were completely removed without leaving trace material (Jacobsen et al. 2018). In the present study, the non-functional vessels that were immature filled with air at the injection point. When examined in longitudinal section, these vessels were generally gas-filled along their length. It is possible that failure to fill with gas could occur if cytoplasmic contents get trapped in unopened perforation plates leaving the vessels partially evacuated, but our data suggest that this is not common. In the late stages of development, the cytoplasmic contents of vessels become very dilute and such vessels may fully drain upon air injection, which we commonly observed. These results indicate that using air-injected samples to determine the presence non-functional conduits in vivo is likely not a reliable method.

Patterns of sap flow illustrated by new iohexol method

Flow of sap through the xylem is determined by the connections between tracheary elements. We found that some potentially conductive vessels may not be directly connected to the transpiration stream by other tracheary elements (Utsumi et al. 2003). Most vessels were transporting sap via the low resistance vessel-to-vessel bulk-flow axial pathway (hereafter called axial transport) as evidenced by their filling with iohexol after feeding it through the roots. Other fluid-filled vessels did not fill with iohexol, suggesting that they were not directly connected to the transpiration stream via tracheary elements. Also possible is that these vessels were isolated from the transpiration stream because they were surrounded by embolized conduits. Such vessels are still likely to be connected to the sap stream via parenchyma (ray and axial) and intercellular spaces; however, these pathways will have high resistance to flow and will not contribute a large volume of water to sap flow. Nevertheless, these fluid-filled vessels may contribute to plant function in other important ways. For example, they may be important in releasing stored water (capacitance) and connecting radial pathways of water flow, and thereby buffering the pressure potential of the xylem sap in the vascular system on a daily basis (Meinzer et al. 2009). The intercellular pathways have been little studied in the context of their function (Kitin et al. 2009).

In some grapevine samples, vessels that were not axially transporting sap were numerous and had large diameters. This pattern may be common in species that have short-lived leaves yet maintain some functional xylem in the previous year's (or older) growth ring. Such conduits would likely not form pits with current year's xylem because pitting does not extensively occur across growth rings (however, see Utsumi et al. 2003, Sano 2004, Kitin et al. 2009). Both of the species we examined are winter deciduous, thus all leaves are produced in the current year and direct connections to older xylem would be very unlikely. Another possibility is that wounding or shedding of plant parts may lead to the presence of vessels not directly transporting sap (Choat et al. 2015). This appeared to be the case in some of our samples where we noticed that in a sample with non-active sections there was a branch that had been previously pruned. These patterns highlight the important role that connections between leafy branches and stem xylem play in determining xylem flow patterns (Maton and Gartner 2005). It also highlights the value of the method used here to further study this phenomenon.

Another class of vessels we observed were those that were not transporting sap and that were occluded. The chief type of occlusion that we observed was in the form of vessels that had not fully matured, thus their pits and end walls were obstructed and they did not contribute to bulk movement of water (Utsumi et al. 2003, Jacobsen and Pratt 2012). The evidence for such vessels was that they did not fill with iohexol during root feeding and they did not stain with crystal violet; moreover, such vessels were abundant in many samples in the outer region of the growth ring near the vascular cambium where one would expect immature vessels to be located. One other piece of evidence was that when we blew air into these samples after staining, some of them became deformed (partially collapsed) indicating that some of these vessels, but not all, were also not fully lignified (Kitin and Funada 2016). Finally, many of these vessels and the fibers surrounding them also had relatively thin secondary walls when viewed in light micrographs. Nevertheless, these vessels appeared to be fluid-filled and would likely be interpreted as functional if based on HRCT images alone. Such vessels will be more or less abundant depending on the timing and rate of maturation of vessels in a particular species (Jacobsen et al. 2018). Early in a growing season, when woody plants are actively growing, there will be many immature vessels and these vessels will eventually mature so that at the end of a growing season all the vessels will be mature out to the cambium as has been shown in grapevine (Jacobsen et al. 2015). This study was

conducted near the end of the growing season in our region so it was expected that some samples would have vessels mature out to the cambium as was found for some grapevines and was generally found for chestnuts, which had set bud and had not been growing for about a month before we conducted our experiments. One application of this new method is to examine the timing of when tracheary elements become functional to gain insight into their developmental patterns.

The finding that not all the vessels are active in bulk movement of sap in grapevine stands in contrast to the conclusions of a recent study. Hochberg et al. (2017) used a flow MRI method to examine bulk flow patterns in grapevine stems. They concluded that there were only very few non-conductive vessels near the edge of their stems that would represent immature vessels. Nevertheless, their data show substantial numbers of vessels that are minimally conductive and that flow is quite heterogeneous across the stem (their Figure 8B). These patterns in their data are consistent with our data and conclusions.

Chestnut xylem anatomy is different than grapevine, including chestnut being strongly ring porous and including abundant vasicentric tracheids. Grapevine xylem contains vascular tracheids, formed in the late wood (Carlquist 1985), but they are not as abundant as in chestnut nor as connected to the vessel network. In chestnut, there were abundant tracheids throughout the growth ring. In the latewood, small vessels filled with iohexol as well as bands of iohexol-filled tracheids. The role of vasicentric tracheids, as found in chestnut, is not well-established beyond arid and semi-arid taxa (Carlquist 1984, Pratt et al. 2015). Our results are novel in showing both the abundance and conductive function of tracheids in the wood, particularly the late wood, which, to our knowledge, has not been described. These tracheids create extensive connections throughout the current year of xylem growth. These connections should increase efficiency of hydraulic transport (Loepfe et al. 2007); however, they may also make chestnut more vulnerable to the spread of embolism or the spread of pathogens (Chatelet et al. 2006). Carlquist (1984) has argued that these tracheids promote safety by creating alternative pathways to circumvent embolized vessels (Pratt et al. 2015). The iohexol method has considerable promise to test competing hypotheses about the role of vasicentric tracheids in transport safety and efficiency.

Tracheids in the oldest portions of chestnut xylem did not fill with iohexol even though they appeared fluid filled. These tracheids did not stain with crystal violet either, thus we cannot say for certain that they were not occluded. Nevertheless, because they were in the older growth ring, and chestnut is deciduous (current leaves lack direct vascular connections to the inner growth rings), the older tracheids would not significantly contribute to axial transport of sap even if they were not occluded. In grapevine, latewood vascular tracheids filled with iohexol and also stained with crystal violet.

The results of this study have implications for HRCT methodology aimed at understanding plant hydraulics. There have been many studies using HRCT to examine topics such as vascular networks, refilling of vessels and vulnerability to cavitation, and such studies are likely to continue because of the value of this method (Blonder et al. 2012, Brodersen 2013, Vergeynst et al. 2014, Choat et al. 2015). For cavitation resistance studies, plants are commonly imaged across a range of water potentials and the number of air-filled conduits are simply counted or, better still, converted to conductivity (via Hagen–Poiseuille flow equations) and compared relative to the number of fluid-filled conduits or the flow estimated from those conduits. What our data show is that there are a number of conduits that are not moving sap. If one does not account for these non-conductive fluid-filled conduits then the estimated percentage loss of conductivity will be an underestimate, i.e., plants will appear more resistant to cavitation than they actually are. This error becomes especially important to consider when estimates of vulnerability are made across different methods. For example, some have compared HRCT estimates of loss in conductivity to values calculated from directly measuring hydraulic conductivity of cut stems (e.g., Choat et al. 2010). The hydraulic methods will not include flow through any occluded vessels in a sample, whereas HRCT estimates will. Some HRCT-based studies have reported that HRCT methods find species to be more resistant to cavitation and embolism formation when compared with hydraulic methods (e.g., Choat et al. 2010, Cochard et al. 2015), which is in the direction one would predict based on our findings. A challenge for future HRCT studies is to account for non-axially transporting vessels in HRCT estimates of conductivity. It appears that one cannot simply assume that all vessels that appear fluid-filled in an HRCT image are functional in axial sap transport. At least one study has attempted to account for this (Choat et al. 2015), but they did so in cut segments that would not have allowed them to account for non-occluded but non-axially transporting conduits in intact stems. These conduits could be identified with the iohexol approach reported here.

Our results also point to a potential artifact associated with hydraulic methods applied to cut stems. Hydraulic methods may include vessels that are not moving sap in the intact plant, potentially due to their being isolated, but that are not occluded. This creates an artifact because such vessels would not be functional in vivo. This means that when samples have a significant number of these vessels, hydraulic methods on cut segments will overestimate native hydraulic transport. The potential for this artifact to affect hydraulic measurements of cut segments, and the analyses that have been conducted with such data (e.g., safety and efficiency tradeoffs) require further study (Gleason et al. 2016).

Summary

The use of tracers to identify conductive vessels may greatly improve the utility of HRCT as a tool in the study of plant hydraulic

function. Iohexol appears to be a good tracer that is readily transported up the xylem is able to mark conductive vessels in images, and has limited bleeding into adjacent tissues. This tracer was useful in identifying conductive vessels and tracheids in both intact plants and cut segments and shows similar patterns in xylem vessel activity to more traditional active xylem staining techniques with some interesting exceptions. The iohexol method on intact plants appears to have advantages in identifying small vessels and tracheids than staining of cut segments, particularly in ring porous xylem. Future studies using HRCT will likely need to incorporate conductive vessel markers or controls into experiments due to the presence of non-conductive fluid-filled vessels within the xylem, particularly when comparisons are made to other methods that account for non-conductive vessels such as direct hydraulic methods. While at present this method does not allow for the estimate of flow rates it may be possible to modify it to yield such information.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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