RESIN-CASTING: A METHOD FOR INVESTIGATING APOPLASTIC SPACES

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A method has been developed in which liquid resin can be injected or infiltrated into spaces within a plant body, for example the lumens of vessels, fibers, and intercellular spaces. After polymerization of the resin, plant tissues are digested away with two solutions used in sequence: first, equal parts concentrated hydrogen peroxide and glacial acetic acid; second, concentrated sulfuric acid. Complete digestion renders three-dimensional casts of the spaces in the original tissue. These can be examined with scanning electron microscopy, light microscopy, or a dissecting microscope. Casts have such high fidelity and high resolution that details of pit canals, pit chambers, and perforation plates can be studied. Vessel casts over 15 cm long and revealing the details of several thousand constituent vessel elements have been obtained easily. Casts of the lumens of all cell types and of narrow intercellular spaces are obtained by prolonged infiltration with a low-viscosity resin solution. Alternatively, rapid, brief injection of the resin by vacuum produces casts predominantly of just those spaces that were open to the resin at the cut ends of the sample, for example the lumens of vessels and secretory ducts or the intercellular space network of an aerenchymatous parenchyma.

A plant body contains a variety of nonprotoplasmic spaces whose size, shape, interconnections, and organization are important. Examples are the water-conducting lumens of the xylem vessels or the mesophyll’s intercellular spaces that permit the diffusion of carbon dioxide away from stomata. The study of the three-dimensional organization of such spaces has been greatly aided by scanning electron microscopy (SEM). But SEM has the limitation of being able to image only internal parts of a tissue or cell that are facing a cut surface; cells in the foreground obscure the cells and spaces deeper in the sample. Long vessels and secretory cavities are rarely observed in their entirety because it is difficult to cut them open uniformly over a long distance.

In animals, studies of the three-dimensional organization of the vascular/lymphatic system have been enhanced by resin-casting (Murakami, 1971). Unpolymerized resin monomers are injected into the vascular system; after polymerization, the animal’s tissues are digested away, rendering a cast of the entire lumen interior. Casts of complex spaces have been produced.

A similar technique can be used to study plant tissues (Saiki, Goto, and Sakuno, 1975; Okumura, Harada, and Saiki, 1976; Taneda et al., 1979). An initial study of the wood of oak and beech showed that high-resolution, high-fidelity casts could be made of lumens of tracheary elements, fibers, and even of intercellular spaces in rays (Fujii, in press). We are currently using this method to study the three-dimensional arrangement of vessels and vessel clusters in the wood of cacti. Our initial results indicate that the method should be widely applicable, and we wish to alert the botanical community to its potential.

MATERIALS AND METHODS

Preparation of the sample—Dry wood or herbarium material—Dry wood samples taken from the herbarium at the Forestry and Forest Products Research Institute could be used directly with no preparation other than to cut a piece to an appropriate size (see also Fujii, in press).

Living wood samples—Wood of living cacti was fixed and stored in 50% ethanol, then dried in one of two ways: 1) freeze-drying (Figs. 1–4). The sample was rehydrated through a graded series of ethanol/water solutions, then the wet samples, immersed in water, were frozen and placed in a freeze-drying machine. Pith parenchyma was well preserved. 2) Solvent evaporation (Figs. 5, 6, 9). Ethanol was replaced with n-pentane through a graded series, then the pentane was allowed to evaporate. Wooden structures were preserved very well. We did not use crit-

Figs. 1–4. Styrene casts from freeze-dried, vacuum-infiltrated samples. 1. A cast of vessel elements of Rhipsalis wood. Resin was drawn primarily into vessels, not into adjacent paratracheal cells. Broad pit-canal casts cover the surface. ×232; Bar = 100 μm. 2. Hylocereus. Casts of several paratracheal parenchyma cells are present. ×136; Bar = 100 μm. 3. Function of two vessel elements and one paratracheal parenchyma cell from Cleistocactus. The lower, right element has a long tail, and all elements are interconnected by pit-pairs. Remember that the cast represents the lumen, and spaces where there is no resin correspond to where cell walls had been located. Pit casts are narrow in the pit canal region (where they attach to lumen casts) and are broad in the pit chamber region. The flat, free surface of most pit chamber casts is a result of the presence of a smooth pit membrane. Several intact pit-pairs are present (arrows; enlarged in Fig. 4). ×1,400; Bar = 10 μm. 4. Magnification of part of Fig. 3. In the two casts of bordered pit-pairs, notice the extremely narrow space between the two pit chamber casts (arrow). The space results because the pit membrane excluded resin. The hole and irregularity in the pit chamber cast in face view may be an imperfection in the cast, may represent an irregularity in the pit membrane, or may have resulted from a deposit on the pit membrane. ×3,994; Bar = 10 μm.
Figs. 5–9. Solvent evaporation (Figs. 5, 6, 9) and ethanol/styrene infiltration series (Figs. 7, 8). 5. Rhipsalis. Mercox cast of two vessels, one with perforation visible. Pitting is scalariform, and many pit membrane casts have small bumps as if pit membranes had indentations or small holes. ×1,042; Bar = 10 µm. 6. Mercox cast of cells of inner cortex of Rhipsalis. Narrow spaces represent primary walls, resin represents cell lumen. Thin areas in wall represent primary pit fields (arrowheads). Surface of the cast replicates the inner face of cell walls that had been parallel to the
ical-point drying, but that might give the best preservation of structure in parenchymatous samples such as leaf mesophyll or ovary locules.

Infiltration — We used four methods for infiltrating the unpolymerized resin into wood samples. First, to study vessel size, shape, and distribution, a vacuum line was attached to one end of a wood sample and liquid resin was applied by pipet to the other end. Long vessels that had been cut open at both ends of the sample quickly filled with resin. The vacuum also was able to pull resin into vessels that were cut open only on the resin-application end and which terminated within the sample, not being cut open on the vacuum end. Of course, the vessels had to be free of tyloses or gums, so sap wood was more suitable than heartwood. The aerenchymatous pith also quickly filled with resin.

Second, to obtain casts of all types of cells present, small samples were completely immersed in unpolymerized resin for prolonged periods. This is basically the same method as embedding tissue for transmission electron microscopy (Figs. 10-12).

Third, some samples in 50% ethanol were passed through a graded ethanol series, transferred to resin (styrene, see below) monomer solution, then allowed to polymerize (Figs. 7, 8, 13, 14). Tissue shapes were well preserved, not being noticeably shrunken or collapsed. All spaces were infiltrated by resin, but possibly due to imperfect exchange from ethanol to resin, gaps and cavities occurred in some resin casts (Fig. 13).

Finally, partially polymerized styrene resin was forced into samples with pressure from a syringe. Excessive pressure might cause distortion, but high pressure may actually be advantageous in forcing resin to penetrate through pit membranes. Such penetration would be useful for studying intervessel pitting and three-dimensional organization of vessels within wood.

After infiltration, resin was heated to 60 C until fully polymerized, from overnight to several days.

Resins — We have had excellent results with both Mercox (produced by Dai-Nihon Co., Ltd., delivered by Oken Ltd., Japan; supplied in the United States by Ted Pella, Inc., Tustin, CA) and polystyrene. Both have low viscosity, but Mercox has a higher viscosity and polymerizes rapidly — within as little as 10 minutes after the accelerator is added, even if held at room temperature. Mercox could be vacuum-infiltrated into vessels where it remained, rather than leaking into paratracheal fibers and creating a solid three-dimensional matrix trapping everything. During digestion, most fibers were empty and so dissolved completely, leaving mostly just casts of vessels and some occasional casts of paratracheal parenchyma cells. Despite its higher viscosity, it penetrated pit canals and pit chambers, giving high-resolution casts. Mercox’s rapid polymerization may be a problem if extremely long casts are desired from long samples. However, a piece of Celastrus stem over 15 cm long and 2 cm diameter was easily infiltrated and digested, producing 15-cm-long vessel casts.

Polystyrene has lower viscosity and remains fluid for weeks at room temperature even with the initiator benzoyl peroxide present. It is possible to infiltrate large blocks and obtain casts of all cell types present. The solid matrix that results with polystyrene is more difficult to digest; however, this is not a serious problem, it merely requires digesting for 1 or 2 weeks rather than for 2 or 3 days. Polystyrene should be effective for casting closed spaces such as the chambers of secretory cavities or intercellular air spaces in compact tissues.

Digestion of organic material — After the resins had polymerized completely, samples were prepared for digestion. If large regions of organic material were free of resin (as when Mercox was sucked into a wood sample, leaving the sides of the sample uninfiltred), they could be placed directly into the digestion solution. If all surfaces were encased in resin, then some resin had to be cut away to expose organic material.

Plant material was digested by soaking samples alternately in two solutions. The first consisted of approximately equal parts concentrated hydrogen peroxide and glacial acetic acid. This was kept at 60 C. The second solution was concentrated sulfuric acid. Because sulfuric acid caused samples to become hot, sample bottles were placed in a cold water bath while sulfuric acid was being added. After the initial exothermic reaction was completed, samples sat at room temperature for the remainder of sulfuric acid digestion. The length of time in each solution was not critical; each was at least overnight for the smallest samples (about 1 ml) and up to several days for the largest (about 100 ml). After sulfuric acid treatment, debris was washed away with water. If digestion was not complete, all steps could be repeated as necessary.

Sulfuric acid caused some swelling of cell walls during digestion, and tended to push casts of neighboring cells apart somewhat. Hydrofluoric acid causes less swelling than sulfuric acid and is advantageous if precise cell-cell orientations are required, but the dangers of hydrofluoric acid must be considered. An acidic sodium chlorite solution may also be used for digestion of styrene-infiltrated materials, but it too may be more toxic than a mixture of hydrogen peroxide and acetic acid. We did not test digestion with sodium hydroxide or other bases.

Merox resin becomes soft and flexible in acidic so-
Figs. 10–14. 10-12. Slow, complete infiltration of all spaces. 10. Mercox casts of fiber lumens in Cleistocactus. $\times 990$; Bar $= 10$ $\mu$m. 11. Pith of Hylocereus is compact, but there are intercellular spaces that filled with resin (arrows). Such casts show that intercellular spaces connect into a three-dimensional network that would permit gaseous diffusion of oxygen and carbon dioxide. Freeze-dried sample. $\times 500$; Bar $= 100$ $\mu$m. 12. Styrene cast of vessel cluster of Hylocereus. Digestion of walls has not caused paratracheal cells to separate from vessels in this sample. In Hylocereus
Neutralization by soaking in a solution of pH 7 or higher may be useful in the final wash stage. Removal of the final wash water is important. If casts of individual cells and vessels are desired, wash water can simply be allowed to evaporate. If three-dimensional relationships are desired, a much more gentle procedure is necessary. When wash water was removed by freeze-drying, complex vessel clusters and associated paratracheal cells remained together. Critical-point drying may be even more beneficial, but certain types of polymers may swell or melt in the process.

Polystyrene must be polymerized in a closed container such as gelatin capsules or test tubes; this resulted in total immersion of the sample, and the sides or ends had to be cut away (in some cases, chiseled away) to permit digestion solutions to reach organic material. However, this problem is outweighed by polystyrene's benefits: it gives an extremely fine-grained, precise replica that is stable during digestion.

Retention of three-dimensional organization—During infiltration, liquid resin moved through primary cell walls to at least some degree, filling adjacent cells. But as walls were digested, casts of individual cells separated from casts of adjacent cells. Casts of the various vessel elements of an individual vessel remained together as a single cast due to the continuity of resin through the perforations (Figs. 1, 2). Also, paratracheal parenchyma cells and the several vessels of a vessel cluster remained together in one sample; this may indicate either that there were small holes in the pit membranes of this sample or that digestion of the pit membranes, even though prolonged, was not complete (Figs. 7, 12, 15).

Prolonged infiltration with polystyrene resulted in casts of all cells and spaces; with careful, gentle washing, many individual casts remained together. Adhesion of individual casts may have been the result of several causes: casts of pits and pit chambers may have become tangled, and possibly polystyrene had infiltrated the pit membrane. In some species, the pit membrane becomes degraded and more permeable during differentiation of vessel elements (Vian et al., 1992). Whatever the cause, this adhesion permitted some retention of the three-dimensional organization of fibers, vessels, tracheids, and paratracheal parenchyma. With vigorous washing or with sonication, clusters of two or three vessels and associated paratracheal parenchyma remained together over long distances. Perhaps pit membranes have fine holes that permit resin to interconnect the casts of all associated cells; alternatively, pit membranes of Hylocereus may be particularly resistant to digestion. Note lack of pits on the facing surface of the central vessel element. x 225; Bar = 100 μm.

Fig. 15. Styrene cast of Hylocereus vessel cluster and associated cells. All cells, even paratracheal parenchyma, had thick walls; thus there are rather broad spaces between cells and peglike casts of simple pits. These samples were washed gently, and final wash water was removed by freeze-drying. Sonication probably would have disrupted this structure. x 362; Bar = 100 μm.

These casts have holes and gaps that are artifacts. x 1,000; Bar = 10 μm. This cast from the same sample as Fig. 13 has fewer artifacts. Five paratracheal parenchyma cells surround a vessel where it makes an abrupt turn. This is from a cortical bundle, not from wood. x 500; Bar = 20 μm.
all three-dimensional organization was lost. If extra resin
was allowed to remain outside the ends of the tissue after
infiltration, then this resin formed a base that held in
place all the casts of cells that had been cut open at the
ends of the sample (Fujii, in press).

A continuing objective of our research is to obtain casts
that retain the three-dimensional organization of vessels
within wood. That is, casts that will show if vessels or
vessel clusters form a branching and anastomosing three-
dimensional network. Such casts could show whether a
particular vessel or cluster follows a path such that at
certain levels it is part of the earlywood and at other levels
it is a latewood component. Because vessel casts can be
obtained for large, long pieces of wood, study of the three-
dimensional hydraulic architecture should be possible on
a large scale. At present, our casts indicate sites where
vessel-vessel pitting occurs between adjacent vessels, but
when pit membranes are digested, individual casts sep-
ate and further three-dimensional information is lost.
We are currently investigating methods that could remove
or at least partially degrade pit membranes by cellulase
such that casts of intervessel pit-pairs would be strong
enough to hold casts of vessels together, completely re-
taining the three-dimensional organization.

**Potential uses**—Our preliminary work has focused only
on vessel organization in wood, but numerous other stud-
ies are possible. SEM studies of casts of fibers and fiber-
tracheids could be used to measure the water-storage and
water-releasing capacity of wood. The vascular connec-
tion between parasitic plants and their hosts could be
studied in three dimensions with resin casts. It should be
possible to produce casts of numerous types of tissue
spaces such as those of secretory ducts and glands, or
within ovaries, styles, or anthers. Casts of internal spaces
of fruits, seeds, and ovules, especially during develop-
ment, would be of value. Fruiting bodies of many fungi
have complex cavities that could be studied with three-
dimensional casts, and certain algae, especially the large
and complex brown algae, could be investigated this way.
Domatia occur in many leaves and stems, and casts of
their lumens may aid in understanding how insects use
domatia. Casts of the burrows produced by boring insects
would also be useful in studying insect damage.

We would like to reemphasize the simplicity of this
 technique; it requires no special equipment or unusual
chemicals, casts can be examined by simple observation,
light microscopy or SEM; and most casts can be completed
in less than a week, sometimes only a few days.

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