

# Toward an improved model of maple sap exudation: the location and role of osmotic barriers in sugar maple, butternut and white birch

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Received February 20, 2007; accepted February 26, 2008; published online June 2, 2008

**Summary** Two theories have been proposed to explain how high positive pressures are developed in sugar maple stems when temperatures fluctuate around freezing. The Milburn–O’Malley theory proposes that pressure development is purely physical and does not require living cells or sucrose. The osmotic theory invokes the involvement of living cells and sucrose to generate an osmotic pressure difference between fibers and vessels, which are assumed to be separated by an osmotic barrier. We analyzed wood of *Acer saccharum* Marsh., *Juglans cinerea* L. and *Betula papyrifera* Marsh. (all generate positive pressures) examining three critical components of the osmotic model: pits in cell walls, selectivity of the osmotic barrier and stability of air bubbles under positive xylem pressure. We examined the distribution and type of pits directly by light and scanning electron microscopy (SEM), and indirectly by perfusion of branch segments with fluorescent dyes with molecular masses similar to sucrose. The latter approach allowed us to use osmotic surrogates for sucrose that could be tracked by epifluorescence. Infusion experiments were used to assess the compartmentalization of sucrose and to determine the behavior of gas bubbles as predicted by Fick’s and Henry’s laws. The SEM images of sugar maple revealed a lack of pitting between fibers and vessels but connections between fiber-tracheids and vessels were present. Fluorescein-perfusion experiments demonstrated that large molecules do not diffuse into libriform fibers but are confined within the domain of vessels, parenchyma and fiber-tracheids. Results of the infusion experiments were in agreement with those of the fluorescein perfusions and further indicated the necessity of a compartmentalized osmolyte to drive stem pressure, as well as the inability of air bubbles to maintain such pressure because of instability. These results support the osmotic model and demonstrate that the secondary cell wall is an effective osmotic barrier for molecules larger than  $300 \text{ g mol}^{-1}$ .

*Keywords:* bubbles, pressure, sap flow, sucrose, sugar maple.

## Introduction

Some tree species exhibit a phenomenon known as sap exuda-

tion, which is typically the result of either an increase in root pressure or an increase in stem pressure, although other forms of exudation are also possible, such as wound or oleo-resin gland exudation or guttation (Wieler 1893, Kozłowski and Pallardy 1996). Root pressure is not as widespread a mechanism among seasonal temperate species as it is in wet tropical environments where 61 out of 109 species surveyed by Fisher et al. (1997) developed at least some root pressure of which 15 species developed a root pressure of  $> 0.05 \text{ MPa}$ , which is enough to push water to a height of 5 m or more when transpiration is zero. However, some temperate species like grape (*Vitis vinifera* L.) and birch (*Betula* spp.) are known to generate considerable root pressures (Milburn and Kallarackal 1991).

Maple trees (*Acer* spp.) and a few other species (see Wiegand 1906) have a unique way of developing stem pressure that has for decades received special attention (Sachs 1860, Clark 1875, Wiegand 1906, Marvin 1958, Sauter et al. 1973, Milburn and O’Malley 1984, Johnson et al. 1987). Unlike root pressure in birch, stem pressure in maple is highly dependent on temperature; several freeze-thaw cycles are needed to initiate significant positive pressures (Tyree and Zimmermann 2002). Walnut and butternut trees (*Juglans regia* L. and *J. cinerea* L.) can also generate positive stem pressures in much the same way as maples, and contain comparable sugar concentrations in the sap (Améglio et al. 2001, Ewers et al. 2001). Maple species differ in the magnitude of the pressure they can generate, which is positively correlated with the concentration of sucrose in the sap (Johnson et al. 1987).

Sachs (1860) first presented a model based on the physical properties of expanding gases to explain maple sap flow. O’Malley and Milburn (1983) expanded on this model by proposing a system composed of gas-filled fibers and liquid-filled vessels in which the gas in the fibers would contract during the cooling phase drawing water through the fiber wall. This water would freeze on the lumen side creating a layer of ice that increased in thickness due to vapor distillation, reducing the lumen space and compressing the gas (Milburn and O’Malley 1984). During the warming phase, the compressed gas would expand pushing water out of the fibers and into the vessels as it

thawed. A potential flaw in this model became evident later when Yang and Tyree (1992) and Tyree and Yang (1992) showed that bubbles under pressure can dissolve, reducing the pressure to atmospheric within a few hours, yet stem pressures persist in maples for many days.

An alternative theory proposes that long-term pressurization of maple sap can be explained as an osmotic process (Tyree 1995, Tyree and Zimmermann 2002). Sucrose concentration in the vessels of sugar maple (*Acer saccharum* Marsh.) becomes progressively higher as the sap season unfolds (Sauter et al. 1973); and it has been established that pressure and volume of exudation following a freeze-thaw cycle are positively correlated with sucrose concentration (Marvin et al. 1967, Johnson et al. 1987). The observation that sucrose appears in the vessels when the sap season starts suggests that living cells must be involved in this phase of the process (Wiegand 1906, Marvin et al. 1967, Sauter 1974, Johnson et al. 1987). The presence of sucrose in the vessels was not considered in the Milburn–O'Malley model, but a possible role for it had been suggested much earlier by Wiegand (1906) and later by Marvin (1958), and further investigated in maple by Tyree (1983), Cortes and Sinclair (1985) and Johnson et al. (1987) and in European walnut (*Juglans regia*) by Améglio et al. (2001, 2004).

Tyree (1995) presented a quantitative model to explain how air bubbles can remain stable and pressures sustained for many hours based on the osmotic model. Johnson et al. (1987) observed that maple twigs, unlike other species they tested, absorbed water (sap uptake) during the exotherm while being frozen. Although gas pressurized during the freezing process may contribute to sap flow, pressures recorded for sugar maple are so elevated (up to 200 kPa) that compressed air bubbles in the system would become highly unstable and would likely dissolve quickly as predicted by Henry's Law and Fick's Law (Yang and Tyree 1992). This is particularly true if bubbles are in a cell of small diameter like a fiber (Ewers 1985, Tyree 1995).

Sucrose is the primary osmolyte lowering water potential of the sap in maple vessels. Derived from starch catabolism, it is actively transported out of ray parenchyma cells and into vessels across connecting pit membranes (Sauter 1972, Sauter et al. 1973, Decourteix et al. 2006). A key assumption of the osmotic model is that once sucrose is in the vessels it does not diffuse into surrounding cells; and this requires the existence of a semi-permeable barrier to confine sucrose molecules to the vessels while allowing water to move freely in and out of a reservoir of surrounding cells. Tyree (1995) proposed that lignified cellulose might provide an effective osmotic barrier preventing sucrose from moving from vessels into surrounding sclerenchyma. But such a barrier would work only if pits are absent in the common walls, because pit membranes (primary wall) would be permeable to sucrose. If pits are present, they may become plugged, as reported for *Fraxinus* sp. (Wheeler 1981) and *Ulmus americana* L., where plugging of wall pits isolates bacterial wetwood (Thompson and Jagels 1983).

In this study, we examined the relationship between anatomical features and sap isolation in sugar maple, butternut

and white birch (*Betula papyrifera* Marsh.) xylem. We focused on (1) determining microscopically how the cells in the xylem of sugar maple, butternut and white birch are interfaced and whether this anatomical evidence supports the osmotic model, and (2) testing vessels for semi-permeability with perfusion experiments, using fluorescent dyes as sucrose surrogates. We also conducted experiments to test whether (1) bubble pressure dissipates in the absence of sucrose, and (2) movement of water between vessels and fiber-tracheids is regulated by an osmotic membrane.

## Materials and methods

### Plant material

Three sugar maple trees of about 15 cm DBH (diameter at breast height) were felled in June 2003 at the Proctor Maple Research Center, Department of Botany and Agricultural Biochemistry, University of Vermont. Main stem segments were selected from the base, breast height and crown base. Segments from small branches were also obtained. Branch and main stem segments were stored in 50% ethanol at room temperature. Additional branches were collected at the Proctor Center 1 week before the sap season (February 27), during the sap season (March 2) and 4 weeks later (March 30, 2004). Another set of branch segments was collected at the University of Maine and quickly frozen at  $-20^{\circ}\text{C}$ . Air-dried portions of each stem segment were separately stored without further processing to provide material for macerates. Butternut and white birch branches were collected from trees on the University of Maine campus and stored in the same way as described for sugar maple.

### Light microscopy

Cross (X), radial (R) and tangential (T) sections 10- $\mu\text{m}$  thick were obtained with a sliding microtome (Model 860, American Optical, Buffalo, NY) and stained with safranin-fast-green, acid fuchsin or Bismark brown. The sections were mounted in Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI) and observed by light microscopy. Photomicrographs were captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop microscope.

### SEM: wood blocks and branches

Rectangular blocks were sawn from main stem segments taking care that one side of each block coincided with ray orientation so as to ensure a proper radial surface. The X, R and T surfaces were smoothed on different but contiguous blocks with a sliding microtome. Smooth X surfaces were also obtained from small branch segments. Smoothed samples were further dehydrated through a series of alcohols to acetone and allowed to air dry. In addition, R surfaces from blocks and branches were prepared by first air-drying the wood and then splitting along the radial face. To take advantage of the SEM's 3-D capabilities, X, R and T faces were smoothed on the same block and the intersecting corner of smoothed surfaces was mounted

upward on an angled metal base. The base was affixed to an SEM stub and a coating of silver paint was applied between the wood and the base, and between the base and the stub. The remainder of the specimens were mounted with double-sided carbon tape on SEM stubs and further grounded with silver paint. Branches of 1 to 2 cm in diameter from white birch and butternut were processed in the same way as the sugar maple branches, and X and R split surfaces mounted for SEM observation.

#### SEM: freeze-fractured twigs

Young frozen sugar maple twigs with a maximum diameter of 5 mm were cut into 2-cm-long segments and stripped of their bark. Some of the thicker twigs were lightly scored in the middle with a sharp blade to create an initiation point for subsequent fracture before nitrogen submersion. While still frozen, twig segments were quickly immersed in liquid nitrogen and kept submerged until boiling stopped. Needle-nose pliers were used to manipulate and snap the twig segments in half while immersed in liquid nitrogen. Once broken, the specimens were transferred to 50% ethanol and dehydrated in an alcohol series to acetone. Specimens were air dried and mounted on SEM stubs with a hot-glue gun, with the fractured end pointing upward. Grounding was achieved with silver paint.

#### SEM: wood macerates

Macerates were prepared by chipping off small splinters from dry sugar maple wood, and incubating in Franklin's solution at 60 °C (Franklin 1945) for 12, 24 and 48 h to obtain different degrees of maceration. Macerates were thoroughly washed in several changes of distilled water and dehydrated to 100% acetone. The test tubes were agitated to create a suspension. Successive drops of macerate suspension were placed on a bare SEM stub and allowed to air dry before adding the next drop to avoid excessive migration of cells toward the edges of the stub.

All SEM specimens were coated with a 30 to 40 nm gold layer and observed with an Amray AMR-1000 scanning electron microscope (AMR, Bedford, MA) with a 5 KeV potential and a 200 µm aperture. Images were captured on Polaroid film with an analog camera attached to the electron microscope. Negatives were digitized with a back-lit high resolution flat-bed HP Scanjet G4010 Photo Scanner (Hewlett-Packard, Palo-Alto, CA).

#### Perfusion experiments

Sugar maple, white birch and butternut branches from mature (50+ years) trees were collected in the field at the University Forest and from the main campus at the University of Maine on June 9, 2003. Branches were submerged in water and cut back about 30 cm from the cut end to prevent embolism formation. The samples were kept in water and immediately transported to the laboratory where they were blotted with paper towels and stored at -20 °C for a maximum of 4 months.

During the sap-flow season other sugar maple stem segments 10 mm in diameter and 50 cm in length were cut and immediately mounted in a refrigerated box kept at 2 °C. The ends of each segment were connected to tubing, by compression fit-

tings (Tyree 1983), to a custom-fabricated flow meter that could measure low flow rates with a pressure drop of just a few kPa across a capillary tube. The principle of operation of the flow meter is similar to the ultra-low flow meter described by Tyree et al. (2002). A system of tubing and valves permitted three types of measurement: axial flow rate; infusion flow rate; and pressure with no flow as explained in the caption of Figure 1. This system could be used to replace the xylem vessel contents with any desired solution (see "Perfusion with sucrose" section) and to pressurize air in fibers by infusion flow without the need to freeze the segments to drive water in by vapor distillation.

#### Perfusion with fluorescent probes

Because sucrose cannot be traced directly in stem xylem, we used two fluorescent dyes to test whether these substances could move across lignified cell walls. The fluorescein dyes were fluorescein disodium salt (Na-fluo, FLUKA brand) and fluorescein isothiocyanate (commonly called FITC, SCN-fluo hereafter; FLUKA brand). The dyes were chosen based on molecular mass which, in both cases, is similar to that of sucrose (376, 389 and 342 g mol<sup>-1</sup> for Na-fluo, SCN-fluo and sucrose, respectively). SCN-fluo was also chosen for its non-polarity, because ionic molecules like Na-fluo could theoretically react with polyuronic acids in the cell wall. Na-fluo was dissolved in double distilled water to 0.1% (w/v), and 0.1% (w/v) SCN-fluo was prepared in 0.05 M phosphate solution. We added 1.9% (w/v) sucrose to both fluorescein solutions to bring the perfusate to a similar osmotic activity as the solution present in the vessels during the sap-flow season. To prepare samples for perfusion, branch portions of about 1-cm diameter were selected from each species and subdivided with a band-saw into 4–5-cm-long segments while still frozen. These samples were quickly cleaned to remove saw dust and thawed

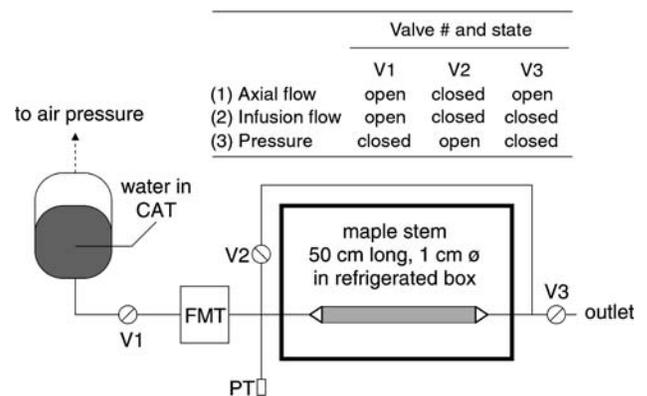


Figure 1. Experimental setup for measuring (1) axial flow through a sugar maple stem segment from left to right in the diagram, (2) infusion flow into both ends of the segment where flow is presumed to go from vessels into air-filled fibers and (3) pressure with no flow. Valve settings for each measurement type are shown. Abbreviations: CAT = captive air tank used to provide water under pressure from a pressure regulator; V1, V2 and V3 are valves; and FMT = a flow meter that includes PT = one of two pressure transducers in the FMT, i.e., the one on the down-stream side of the FMT.

while fully submerged in distilled water at room temperature.

Twigs were inserted into one end of a tubing system equipped with a low-flow peristaltic pump that flushed the samples for 30 min with distilled water. The water source was kept above the twigs to aid the pump and help keep the flow as stable as possible. A 60-ml syringe containing the fluorescent dye-sucrose mixture plus 10–20 ml of air between solution and plunger was attached to the tubing system. Two valves were used to prevent backflow of water into the fluorescein solution while flushing, and the backflow of fluorescein into the tubing while perfusing.

After flushing, water flow was stopped and the fluorescein-sucrose was perfused through the twig. The air in the syringe served as a pressure buffer to avoid transferring excessive pressure to the tubing and connectors. Sufficient positive pressure was applied while perfusing to allow the fluorescein to move laterally in the stem segment if an open pathway existed. Perfusion was carried out for about 5 min, until the fluorescein concentration of the perfusate was equal to the concentration of the solution in the syringe, as determined by spectrophotometric analysis. Once perfused, low pressure air was passed through the segments to eliminate excess liquid in the vessels, and twigs were blotted with paper towels. Samples were partially air dried for 2 h to avoid smearing when sectioned.

Thin sections (20  $\mu\text{m}$ ) of perfused twigs were obtained with a sliding microtome. A piece of Magic Scotch tape (3M Corporation) was gently pressed onto the sample before sectioning and the tape plus section were adhered to a cover-slip which in turn was taped to a microscope slide with the back of the tape that contained the section facing down. Sections were observed in epifluorescence UV light from a mercury-halide lamp source. A set of overlapping images was captured for each specimen from the pith outward and a digital composition was created to form a single image, encompassing the tissue from pith to bark.

In addition to the above described perfusion, twigs from both sugar maple and white birch were subjected to a perfusion-diffusion experiment. After perfusion, the twigs were wrapped in Parafilm and stored in an upright position at 4 °C for 2 h to allow the fluorescein to diffuse. Sections were obtained as previously described. Digital image processing was used to obtain yellow channels from the images and identify diffusion patterns (see “Imaging” section).

#### *Perfusion with sucrose*

Sugar maple stem segments were first perfused with either 10 mM NaCl or 10 mM NaCl + sucrose to give an osmotic pressure of either 0.1 MPa or 0.25 MPa. The perfusion was deemed complete when the refractive index of the solution flowing from the outlet equaled the refractive index of the input solution (see Figure 1).

To examine the stability of air bubbles under pressure, the segments were perfused with 10 mM NaCl then infused with the NaCl solution under a pressure of 100 kPa until the infusion flow rate fell to about zero (after 2 h) at which point the air bubbles were presumed to be in pressure equilibrium

with the perfusate. Pressure was then measured with no flow for 145 h.

To examine the osmotic properties of the putative osmotic barrier, the stem segments were perfused with a known solution to replace the vessel contents with the perfusate and then infusion rates were measured while increasing the applied pressure from 0 to 350 kPa at a rate of 30 kPa  $\text{min}^{-1}$ . A putative osmotic barrier should retard the rate of infusion when sucrose is present until the applied pressure exceeds the osmotic pressure of the sucrose. Johnson et al. (1987) have previously reported that NaCl has no osmotic effect in maple stem segments.

#### *Imaging*

All image composing and editing was done with GIMP (GNU Image Manipulation Program; <http://www.gimp.org/>). Spatially sequential images were overlapped to give a clear view of the entire stem section from pith to bark. GIMP was used to separate CMYK (Cyan-Magenta-Yellow-Black) channels, isolate the Y channel and invert the image to identify semi-quantitatively the diffusion patterns for sugar maple and white birch. In an RGB (Red-Green-Blue) image, the green channel is used for both yellow and cyan shades, thus making it difficult to separate signals of fluorescence coming from lignin and fluorescein. Because CMYK has a separate cyan component, the Y channel carries little information on lignin fluorescence while providing most of the intensity values of the fluorescein signal. Cell measurements were taken with ImageJ (Image Processing and Analysis in Java; <http://rsb.info.nih.gov/ij/>).

## **Results**

#### *Light and scanning electron microscopy*

Based on our wood anatomy analysis, we define fiber-tracheids as dead elongated cells with bordered pits, and libriform-fibers (or simply fibers) as dead elongated cells usually smaller in diameter than fiber-tracheids and with simple pits only. Both types of cells are part of the sclerenchyma, but fiber-tracheids may also take part in water transport, whereas fibers provide only mechanical support. The relevance of this distinction and the roles of each cell type are considered in the discussion.

Cross sections of sugar maple observed by light microscopy revealed no pits connecting fibers to vessels. In a few cases, blind simple pits were observed in fiber walls adjacent to vessels (Figure 2a). Fiber to fiber simple pit pairs were observed (Figure 2b). Vessel area occupied  $10 \pm 1.5\%$  of total area of analyzed sections. Vessels were solitary or arranged in clusters of 2–3, occasionally 4–5, with a linear radial arrangement. Inter-vessel pits were common.

In longitudinal sections, both fiber-tracheids interconnected with bordered pit pairs and libriform fibers with or without sparse simple pitting were observed. Fiber-tracheids were usually associated with vessels and were connected to them through bordered pit pairs (Figures 2c and 2d). No connections were found between libriform fibers and fiber-tracheids.

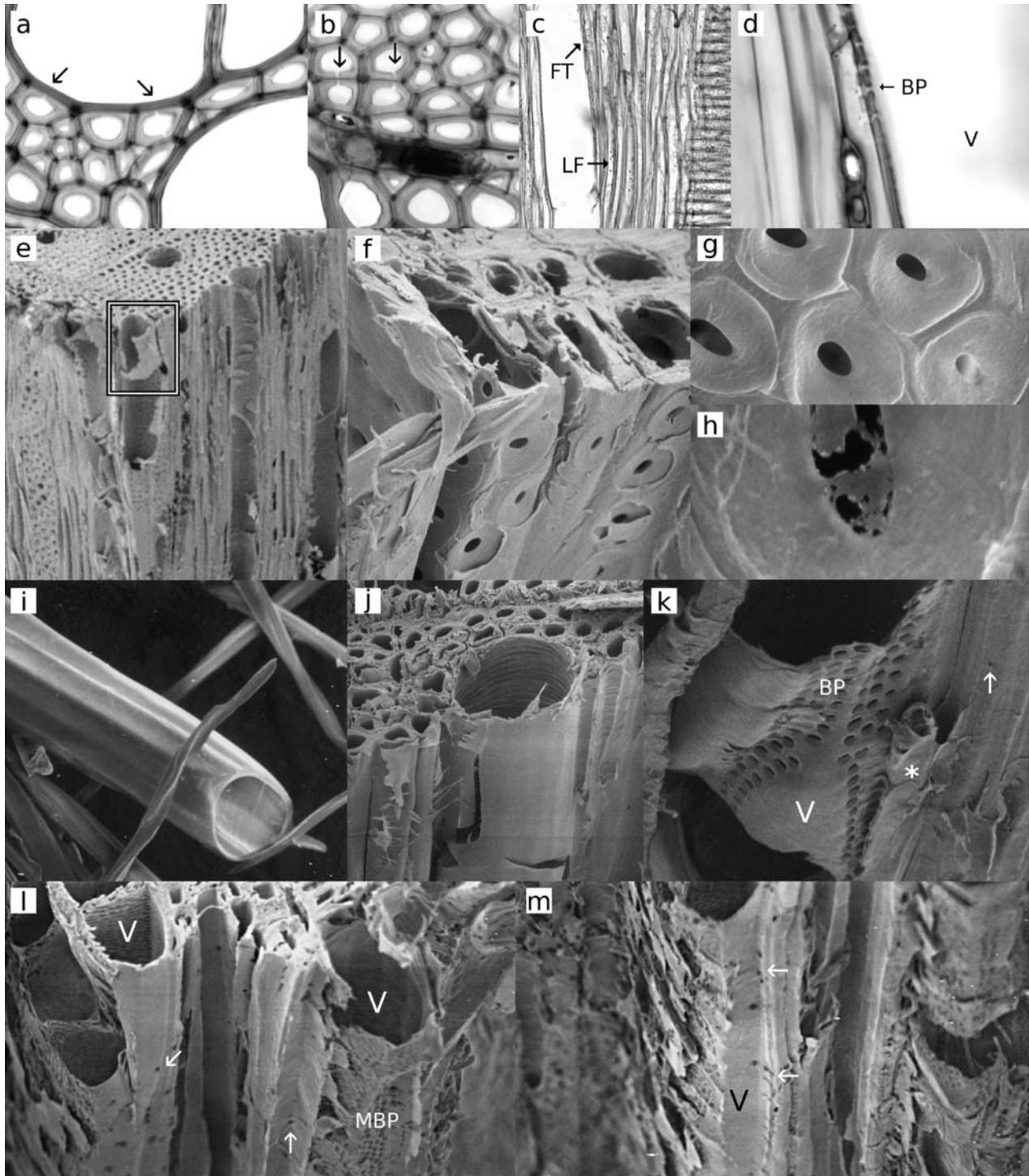


Figure 2. Light and electron microscopy images of xylem anatomy of (a–k) sugar maple and (l and m) white birch. (a) Blind libriform fiber-to-vessel pits (arrows). (b) Fiber-to-fiber simple pits (arrows). (c) Fiber tracheids (FT) with bordered pits associated with vessels and libriform fibers (LF) with simple pits. (d) Fiber tracheid connected to a vessel (V) through bordered pit pairs (BP). (e–k) SEM images of sugar maple wood tissue. (e) A vessel has been ruptured and exposed at the corner, the rectangle indicates area enlarged in (f). (f) Detail of fiber tracheids with profuse bordered pits and connection to a vessel element. (g) Bordered pits on a vessel element. (h) Remains of a ruptured pit membrane in a bordered pit. (i) Sugar maple macerate; a vessel element with impressions of surrounding libriform fibers showing a complete lack of pits. (j) Cross section of radial split edge of a sugar maple twig. A vessel surrounded by libriform fibers with no pits. Fibers also show a general scarcity of pits. (k) A vessel element (V) shows the bordered pit (BP) patterns that interface with two fiber-tracheids, one still visible (\*). Nearby libriform fibers show few simple pits (arrow). (l and m) SEM images of white birch wood. Vessel elements (V) show minute bordered pits (MBP, inter-vessel pits) and simple pits that are in contact with fibers (arrows).

Vessels were pitted where they came in contact with ray parenchyma cells through half-bordered pit pairs. Cross sections showed that this pitting occurred only between vessels and uniseriate rays or uniseriate portions of multiseriate rays (not shown). Similar pit pairs were found between vessels and sparse paratracheal parenchyma.

The SEM analysis confirmed that bordered-pit pairs exist between fiber-tracheids and vessel elements (Figures 2f and 2k). Bordered pits measured an average of  $4.68 \mu\text{m}$  at the chamber and the openings were ellipsoidal with the semi-major and semi-minor axes measuring  $1.7$  and  $0.9 \mu\text{m}$ , respectively (Figure 2g), the semi-major axis being oriented perpendicular to the longitudinal axis of the vessel element. On rare occasions, remnants of the pit membranes were found. These were usually ruptured and had no apparent encrustations (Figure 2h). Wood macerates and radially split branch and main stem segments showed a complete absence of pit connections between libriform fibers and vessel elements (Figures 2i and 2j).

The SEM images confirmed a general scarcity of pits in libriform fibers. Simple pits in fibers were circular and measured  $0.78 \pm 0.35 \mu\text{m}$  in diameter. Macerates permitted a better view of the half-bordered pit pairs connecting vessel elements to ray parenchyma cells (not shown). These pits were large and ellipsoidal with the semi-major and semi-minor axes measuring  $3.8 \pm 0.6$  and  $2.5 \pm 0.5 \mu\text{m}$ , respectively.

Cross sections of butternut (*J. cinerea*) showed abundant banded confluent parenchyma. Because vessels were surrounded by axial paratracheal parenchyma, fiber to vessel contact was infrequent. Profuse pitting was observed between vessel elements and axial parenchyma. No connecting pits were found between fibers and axial parenchyma when observed in radial view (not shown). White birch (*B. papyrifera*), like sugar maple, showed a general scarcity of axial parenchyma. The SEM examination of white birch vessels showed the characteristic minute alternate inter-vessel bordered pitting. We occasionally observed simple pits connecting fibers to vessels (Figures 2l and 2m).

#### *Perfusion experiments with fluorescein*

In sugar maple, both the neutral and ionic forms of fluorescein could be seen primarily in the vessels after perfusion, whereas fibers revealed only the native blue lignin fluorescence (Rost 1995) at the UV wavelength used (Figure 3b). Fluorescein was also present in ray and longitudinal parenchyma (Figures 3b and 3e). Na-fluo seemed to be more readily absorbed by parenchyma cells than SCN-fluo.

Some cells surrounding vessels also contained a small amount of fluorescein. Usually the areas were not large and were typically associated with the presence of another nearby vessel or with ray parenchyma. These regions are presumed to be fiber-tracheids. In all cases, the pith failed to absorb any fluorescein and in some twigs the first one or two rings held only a limited amount of dye. Tangential sections confirmed the discrete disposition of the fluorescein that had been incorporated into the rays, whereas the surrounding fibers only showed the blue lignin fluorescence (Figure 3e).

Fluorescein (both Na-fluo and SCN-fluo) appeared to behave the same way in butternut as it did in sugar maple (Figure 3d). The occurrence of considerable banded and confluent parenchyma in butternut led to greater overall fluorescence of sections, but as in sugar maple, fluorescence was limited to vessels and parenchyma. In white birch, fluorescein staining occurred in non-parenchyma areas around vessels although at lower concentrations (Figure 3c). Also, longitudinal sections of fluorescein-perfused white birch twigs showed a clear diffusion of the dye outside the vessels (Figure 3f) which contrasted with the more restricted vessel and parenchyma distribution in sugar maple (Figure 3e).

#### *Perfusion-diffusion in sugar maple and white birch*

Perfusing with SCN-fluo and allowing it to diffuse for 2 h showed qualitative differences between sugar maple and white birch (Figure 4). Although sugar maple showed some diffusion around vessels, this is likely associated with fiber-tracheids or parenchyma cells, or both, and did not extend more than one or two cells outward (Figure 4a). In contrast, white birch showed large diffusion patches around some vessels, particularly those in the outermost rings (Figure 4b). This is in agreement with the previous perfusion experiments and with the observations on inter-cell pitting.

#### *Perfusion experiments with NaCl and sucrose solutions in sugar maple stem segments*

In preliminary experiments, sugar maple stem segments were perfused with 10 mM NaCl; then valves were adjusted to switch between measuring the flow rate of perfusion and infusion while holding the applied pressure constant at 100 kPa. The results showed that the flow rate declined by about 90% when switching between perfusion and infusion (Figure 5a). The perfusion rate is relatively independent of time, which is consistent with steady-state water flow along the axis of vessels. The infusion rate, in contrast, declines rapidly with time, which is consistent with water flowing into air spaces known to exist in fibers. The decline in flow rate is expected as air-bubbles compress and come into equilibrium with the applied infusion-pressure.

In another experiment, stems were perfused with 10 mM NaCl to displace all sucrose initially present in the vessels, then segments were infused with NaCl at a pressure of 110 kPa for 2 h. At time zero in Figure 5b, the valves were adjusted to measure pressure with no further water flux into or out of the stem segment. Pressure started at 105 kPa but rapidly fell to near  $-1$  kPa over a period of 4 h and remained low for another 36 h after which the pressure rose to a peak value of 71 kPa at 116 h then gradually fell. At 145 h, the vessel contents were sampled by perfusing a little more solution and were found to contain 1.5% solids by refractive index. The main solid component was determined to be sucrose.

As shown in Figure 5c, stem segments were perfused first with more NaCl solution; then the infusion rate was measured while increasing the pressure from 0 to 350 kPa at a constant rate of  $30 \text{ kPa min}^{-1}$ . Finally the pressure was returned to atmospheric for 1 h to allow water to flow out with expansion of

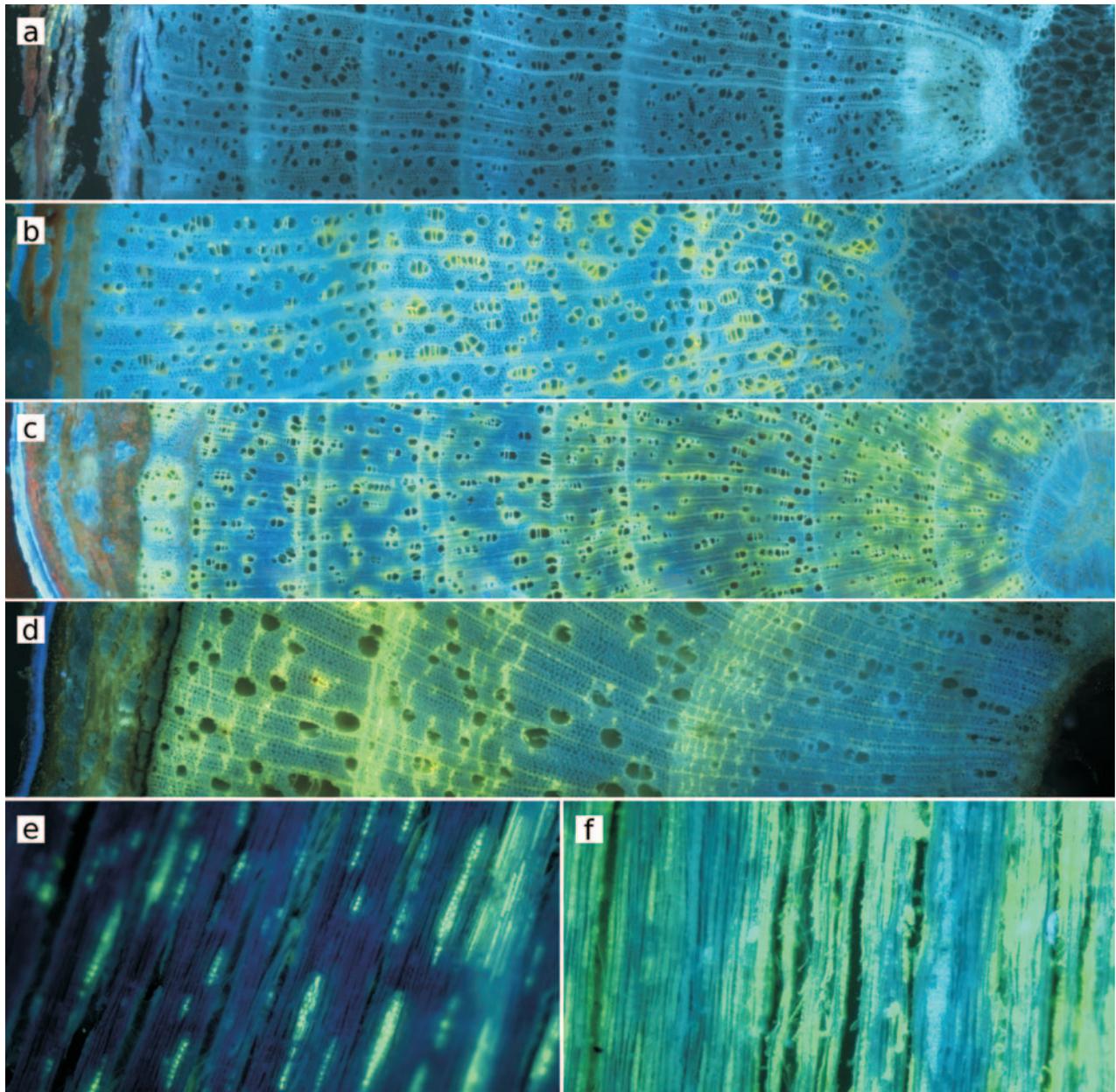


Figure 3. Epifluorescence images of sugar maple, white birch and butternut twigs. a–d are shown lined-up from bark to pith (left to right). (a) 24-bit color digital composition of an untreated sugar maple twig showing the natural fluorescence of lignin. Primary-wall-rich tissue such as ray and terminal parenchyma are shown in a lighter cyan. (b) Sugar maple twig after fluorescein perfusion, showing a discrete distribution of the fluorescein that is primarily confined to the vessels from pith to bark. (c) A white birch twig after perfusion with fluorescein. Considerable diffusion can be observed outside the vessels as a conspicuous green-yellow halo, particularly in the three innermost rings and the last ring (from pith to bark). (d) A butternut twig after perfusion with fluorescein. Banded confluent parenchyma (perpendicular to the rays) is abundant and gives a strong signal creating a halo effect. Ray parenchyma also took up a considerable amount of dye. Axial paratracheal parenchyma is less visible but also prone to absorb the dye and contribute to the glowing green signal. Considering all three types of parenchyma, distribution seems to be nonetheless discrete and fibers are shown in a lighter shade of blue. (e and f) Tangential sections of (e) fluorescein-perfused sugar maple and (f) white birch twigs. Dye distribution in sugar maple shows a clear localization to rays and is also visible in the edges of vessels; fibers appear deep blue and thus well isolated. The distribution in white birch is fuzzier and the dye is localized to large areas encompassing vessels as well as fibers.

the air bubbles compressed in the fibers. The segments were then perfused with 10 mM NaCl + sucrose at 0.1 MPa osmotic pressure and again pressurized at 30 kPa min<sup>-1</sup>. The rate of in-

filtration was delayed by the presence of sucrose. This experiment was performed three times, always with a 1-h resting period at atmospheric pressure. In the third experiment, the seg-

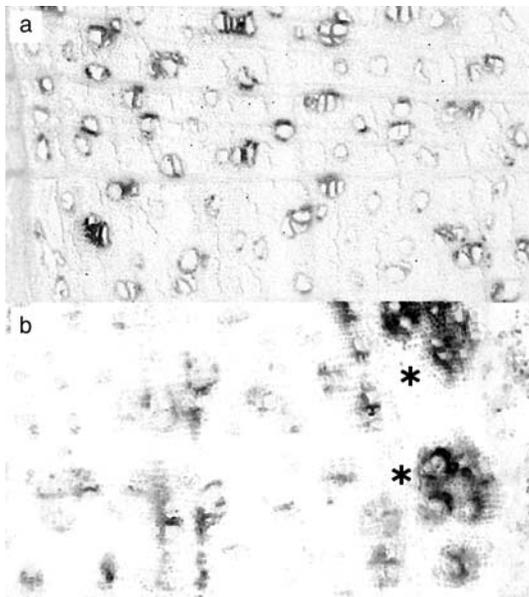


Figure 4. Inverted pixel-value images of isolated yellow-green channel combination extracted from CMYK and RGB originals. Darker areas indicate a higher concentration of fluorescein. (a) Sugar maple image showing a more detailed analysis of dye distribution which appears to be mostly associated with vessels. (b) White birch twig, showing areas with widespread diffusion of fluorescein outside vessels and into fibers (marked \*). Curving was applied to enhance the contrast of the signal but without manipulation of range of values, thus weaker signal from cyan shades may be visible in some areas.

ment was perfused with 10 mM NaCl + sucrose at 0.25 MPa osmotic pressure. The final experiment was done after perfusing with 10 mM NaCl. The flow rate of infusion was consistent with the predictions of Tyree (1995).

## Discussion

The involvement of sucrose in the generation of positive stem pressure has been previously discussed. While O'Malley and Milburn (1983) and Milburn and O'Malley (1984) have stated that some stem pressure can be generated without sucrose, their experiments often lasted for >100 h and they did not measure stem sucrose concentrations at the end of the experiment. In contrast, Johnson et al. (1987) and Johnson and Tyree (1992) have shown that sucrose is needed to reach and maintain the pressures measured in living stems. In Figure 5b, we show that sucrose reappears in sugar maple stem segments after incubation at 2 °C for more than 48 h and this may explain the disparity in claims between the earlier experiments of O'Malley and Milburn (1983) and Milburn and O'Malley (1984), and later experiments by Johnson et al. (1987) and Johnson and Tyree (1992).

After about 4 h, pressure in the experiment illustrated in Figure 5b fell to -1 kPa and remained near zero for about another 24 h. This behavior is predicted by the osmotic model. The pressure at time zero is due to compressed air bubbles in fibers, but Henry's Law and Fick's Law would predict that the air

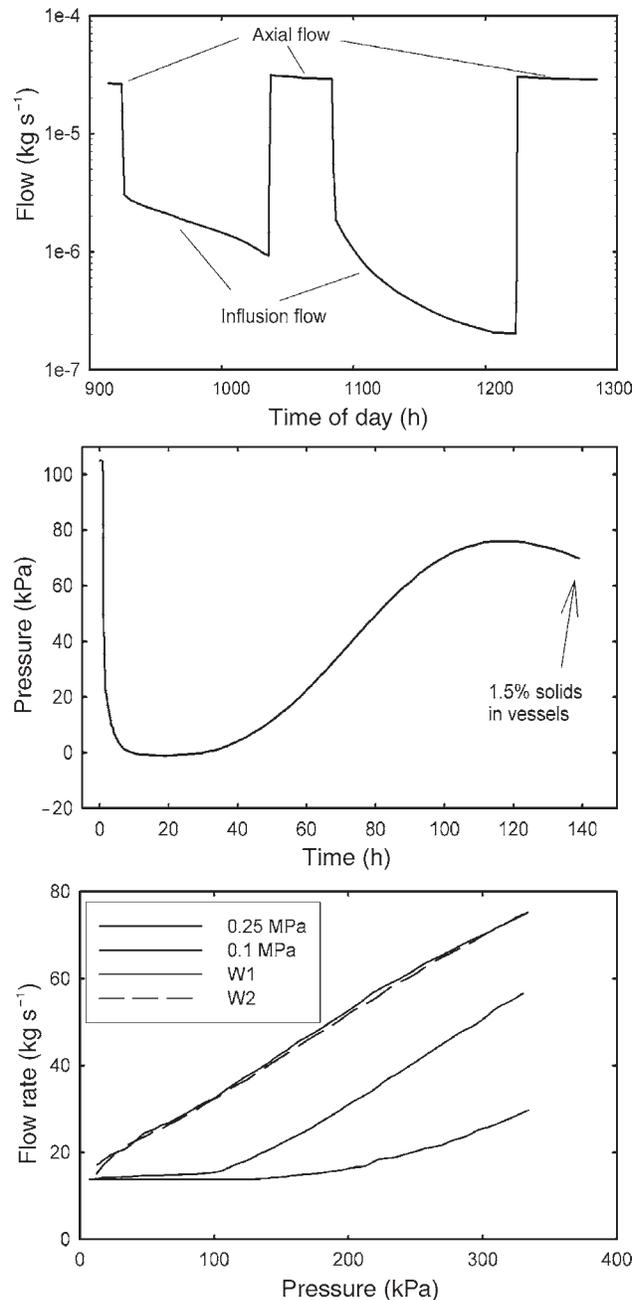


Figure 5. Representative examples from experiments repeated four times on four different sugar maple stem segments 1 cm in diameter and 50 cm in length. (a) Flow rate versus time of day. The flow-rate axis is on a logarithmic scale. Axial flow or infusion flow was measured while a constant pressure of 105 kPa was applied to the stem segment. Valves were opened or closed (see Figure 1) to direct flow either through the stem segment (axial flow) or into both ends of the stem segment (infusion flow). (b) A stem segment was first flushed with 10 mM NaCl to remove sucrose from vessels then infused with NaCl for 2 h at 100 kPa. At time zero, the valves in (a) were set to measure pressure versus time with no volume flow in or out of the stem segment. (c) Infusion flow rates versus infusion pressure when pressure increased at a constant rate of 30 kPa min<sup>-1</sup>. W1 and W2 treatments: infusion with 10 mM NaCl in vessels, W1 was done first and W2 last; 0.1 MPa treatment: infusion rate after filling vessels with 0.1 MPa sucrose + NaCl; 0.25 MPa treatment: similar to 0.1 MPa treatment except measured after filling vessels with 0.25 MPa sucrose + NaCl.

pressure will cause gas to dissolve and diffuse to the surface of the stem segment and come out of solution. Once equilibrium is established, the pressure in the air bubbles should approximately equal the pressure in the ambient air outside the stem. But capillarity at the air–water interface requires us to suppose that the pressure inside the bubble exceeds the pressure in the fluid; the pressure drop across the interface should equal  $4T/D$ , where  $D$  = the diameter of the fiber lumen (assuming the bubble fills the entire lumen) and  $T$  = the surface tension at the air–water interface. Hence, the capillary equation would predict a negative xylem fluid pressure under our measuring conditions in which pressure is measured with negligible water flow into the stem segment. For pressures to balance, we predict a slightly negative pressure in the fluid, which is the pressure measured by the flow meter. However, gradual appearance of sucrose in the vessels after the first few hours might prevent negative xylem pressure reaching the value predicted by  $4T/D$ .

The osmotic involvement of sucrose would require an absence of pits between fibers and vessels, because interruptions in the secondary wall would create openings mediated only by pit membranes allowing sucrose to diffuse out of the vessels, quickly equalizing the osmotic differential between vessels and fibers. Pit membranes, composed of middle lamella and primary wall, are porous enough to allow the passage of sucrose (Pérez 1995, Choat et al. 2003). Additionally, these membranes may be subjected to occasional rupture. Secondary walls must therefore be responsible for impeding the diffusion of larger saccharides (such as sucrose or maltose) outside the vessels.

Based on light and electron microscopy studies, we have identified pitting patterns in different cell types in sugar maple. The cells that we observed that connect to vessels by bordered pits have been defined as tracheids by Carlquist (1988). However, we use the term fiber-tracheid as defined by Baas (1986). This terminology is consistent with Panshin and deZeeuw (1980) who only recognize fiber-tracheids (either thin or thick-walled, and of varying diameters) in *Acer saccharum*. The vessel to fiber-tracheid connection in sugar maple has not previously been reported. This connection permits fiber-tracheids to be part of the water-transport vessel system as described in other hardwoods (Ilic 1997, Kitin et al. 2004).

We determined that neither vessels nor fiber-tracheids are connected through pits with libriform fibers. Therefore, on the basis of pit connections, vessels, associated fiber-tracheids, and axial and ray parenchyma collectively form an interconnected system that is isolated from axial sclerenchyma (Figure 6). Contrary to Wiegand's observation that "fibers are without markings" (Wiegand 1906), small simple pits in fibers were present but found to be blind when facing a vessel element. Butternut also displayed pitting isolation between vessels and fibers but contained a substantially higher volume of axial paratracheal parenchyma than sugar maple. White birch vessels have an inter-vessel pit pattern that differs from the pattern in sugar maple and butternut; white birch specimens (based on SEM evidence) showed simple pits on vessels that connected with surrounding fibers.

The hypothesis that secondary walls can restrict the movement of molecules with molecular masses similar to sucrose was evaluated with the fluorescent dyes Na-Fluo and SCN-

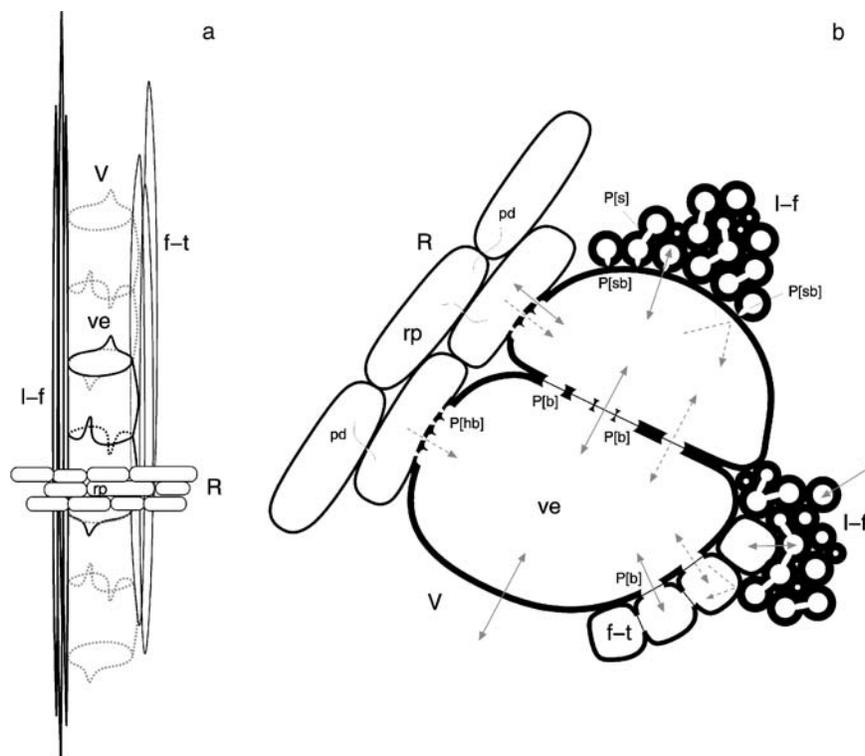


Figure 6. Schematic representation of the different cell types present in the xylem of sugar maple, and their spatial arrangement. Abbreviations: V, vessel; ve, vessel element; R, ray; rp, ray parenchyma (only living cells); l-f, libriform fibers; f-t, fiber-tracheids. (a) Radial view of a stack of vessel-elements surrounded by fibers (l-f) and f-t and in contact with a ray. (b) Cross section of an arrangement analogous to (a) showing pit connections (P[type]) (where [hb] = half-bordered; [b] = bordered; [s] simple; and [sb] = simple blind); plasmodesmata connections (pd) between living ray parenchyma; and potential pathways of H<sub>2</sub>O (solid line arrows) and sucrose (dashed line arrows).

Fluo. The fluorescent dye experiments revealed that molecules similar in size to sucrose can move between vessels, parenchyma cells and fiber-tracheids, whereas fibers are effectively isolated.

Our infusion experiments (Figure 5c) are consistent with the fluorescent dye experiments. When vessel contents are replaced with NaCl, the rate of infusion is a linear function of the applied pressure. But when sucrose is present in addition to NaCl, the rate of infusion is nearly zero until the infusion pressure exceeds the osmotic pressure of the sucrose. These observations are in agreement with those of Pérez (1995) and Choat et al. (2003) who demonstrated that pit membranes are porous to sucrose. Our results provide strong evidence that the lignified secondary wall can function as an effective barrier for molecules of similar or greater mass than sucrose, thus establishing the basis for developing an osmotic differential between the vessel system and fibers. We suggest that the secondary wall has pores of diameters that restrict the movement of molecules with a molecular mass cut-off above  $300 \text{ g mol}^{-1}$  as suggested by the experiments of Johnson et al. (1987) and Johnson and Tyree (1992) in which sucrose ( $\text{MW} \sim 342 \text{ g mol}^{-1}$ ) induced sap flow but hexoses ( $\text{MW} \sim 180 \text{ g mol}^{-1}$ ) did not.

Sugar maple and butternut behaved in the same way in our fluorescent dye experiments and these results are consistent with previous research showing similar sap pressurization behavior (Tyree 1995). Sauter et al. (1973) showed that enzymatic activity localized in the ray-vessel pits indicated that sucrose was being actively transported into vessels from ray cells. Our microscopic analysis confirmed their observation and, additionally, demonstrated that such specialized pits only occur in uniseriate rays or uniseriate tails of multiseriate rays. In contrast, white birch twigs showed some diffusion of fluorescein outside vessels into sclerenchyma, which is consistent with the observed occurrence of pits between fibers and vessels in this species. Physical barriers between fibers and vessels would not be essential in birch because it has been shown that root pressure instead of stem pressure is the responsible for sap exudation (Milburn and Kallarackal 1991).

In conclusion, we have presented anatomical evidence to support the osmotic model (Tyree 1995). We used fluorescent dye molecules as a surrogate for sucrose and showed that these dyes are excluded from fibers. We have demonstrated that air bubbles compressed by infusion experiments lose pressure in a few hours in the absence of sucrose (Figure 5b), but that sucrose and xylem pressure recover after prolonged incubation at  $2^\circ\text{C}$ . We have also shown that rate of infusion is influenced by the presence of sucrose in vessels (Figure 5c) in a way that is consistent with the osmotic model. Hence, we find support for the proposal that non-living tissue systems within the xylem can be an integral part of the osmosis-based model by restricting the passage of disaccharides. The precise molecular exclusion threshold of lignified secondary walls remains to be determined.

#### Acknowledgments

Funding for this research was provided by the USDA Forest Service,

Northeast Forest Experiment Station, Burlington, VT, and the Andrew W. Melon Foundation. The authors thank Dr. Alejandra Equiza for critical review of the manuscript. This is Maine Agriculture and Forest Experiment Station publication No. 2953.

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