# Sieve tube longevity in white ash (*Fraxinus americana*) studied with a new histochemical test for the identification of sugar

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A histochemical test for functioning sieve tubes, based on the 5-diazouracil sugar reaction, has been used to identify functioning sieve tubes in *Fraxinus americana* L. The plant material was lyophilized, treated with the reagent, and sectioned in a cryostat. Before the resumption of cambial activity, when buds are still closed (late April – early May), starch grains of the storage tissues in bark and wood disappear and rays, cambrium, and phloem are loaded with sugars. In early spring, sugars begin to accumulate in last year's sieve tubes, especially in those which had not been fully differentiated in the previous active season and those which were functioning but had formed toward the end of the active season. Girdling experiments, carried out during May, showed that translocation in the phloem does occur, to some extent, during the period when the buds develop and the young leaves grow. The reactivated sieve tubes provide the channel of translocation to developing buds for sugars, formed of the dissolution of starch grains. The first early-phloem sieve tubes are formed at the end of May and loaded with sugars during June. There seems to be a short period of simultaneous import into young leaves via reactivated phloem and export via new phloem. After this, last year's sieve tubes are crushed. A noticeable pressure flow does not appear in the main stem until mid-June.

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Les auteurs ont utilisé un test histochimique pour identifier les tubes criblés fonctionnels du *Fraxinus americana* L.; ce test est basé sur la réaction des sucres avec le 5-diazouracil. Le matériel végétal est d'abord lyophilisé, puis traité au réactif avant d'être sectionné dans un cryostat. Avant la reprise de l'activité cambiale, lorsque les bourgeons sont encore fermés (fin avril – début mai), les grains d'amidon dans les tissus de réserve disparaissent de l'écorce et du bois et les rayons, le cambium et le phloème sont bourrés de sucres. Au début du printemps, les sucres commencent à s'accumuler dans les vaisseaux criblés de l'année précédente, surtout dans ceux qui ne se sont pas pleinement différenciés au cours de la saison active précédente ainsi que ceux qui étaient fonctionnels mais qui se sont formés vers la fin de la saison active. Des expériences d'étranglement, conduites en mai, montrent que la translocation dans le pholème se fait, dans une certaine mesure, pendant la période où les bourgeons se développent les feuilles poussent. Les vaisseaux criblés réactivés permettent l'acheminement, vers les bourgeons en développement, des sucres provenant de la dissolution des grains d'amidon. Les premiers vaisseaux de phloème se forment à la fin de mai et se chargent de sucres en juin. Il semble y avoir une courte période pendant laquelle l'importation dans les jeunes feuilles via le phloème réactivé est simultané avec l'exportation via le nouveau phloème. Par après, les vaisseaux criblés de l'année précédente sont écrasés. Il n'y a pas d'écoulement sous perssion significatif dans la tige principale avant la mi-juin.

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## Introduction

Every study of phloem function must overcome two major problems. First, phloem sieve tubes are known as very sensitive cells. *P*-protein tends to aggregate upon any kind of injury or improper fixation. Second, translocating sugars dissolve quite easily in water, alcohol, and certain organic solvents that may be used while handling plant tissue.

Although many kinds of sugars, sugar alcohols, and other substances were found to be translocated in sieve tubes of different plants (Ziegler 1975; Eschrich and Heyser 1975), the predominant one is sucrose. As a nonreducing sugar, sucrose is very difficult to identify by means of histochemical reaction. The common phloem stains (aniline blue and resorcin blue (lacmoid) (Esau 1948); lacmoid (Aloni and Sachs 1973)) are specific to callose. Recently, Fisher (1978) attempted to estimate sugar concentration in sieve tubes by negative staining.

The aphid feeding technique, which was first

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employed by Mittler (1957, 1958) and used successfully by Weatherly *et al.* (1959), Hill (1962), Peel (1974), Zimmermann (1961, 1964), and others, can be introduced only in relatively young branches where the aphid's stylet can reach the functioning sieve tubes. It cannot be employed in studies of internal phloem, in branches with thick bark, or in stems of perennial monocotyledons.

Formation of callose on sieve plates has been used by many investigators as a criterion to determine when conduction ceases. Similarly, others considered callose disappearance as an indication of reactivation. However, there is no direct evidence that callose dissolution enables sieve tubes to function again, although it is certainly a prerequisite.

Evert *et al.* (1968) identified conducting sieve tubes by two criteria: the presence of turgid protoplast and the lack of secreted sheath around the stylet tip of feeding aphids.

Radioactive tracers or fluorescent dyes seem to be useful for detecting conducting sieve tubes (see Canny 1973, pp. 53 and 127). But one must consider that in many experiments the concentrations of externally applied substances are rather high and might cause unnatural situations within the tissue. Tracing radioactive materials requires sophisticated methods and is time consuming. Furthermore, these techniques are not very useful when transport is minimal or absent as during the dormant period.

The method used in this study combines the Raybin test for sucrose identification (Raybin 1933, 1937) and an industrial process used for precipitating sucrose from the "cossettes' (slices of sugar beet) by means of lime treatment (Hassid and Ballou 1957). The lime provides an alkaline pH which is essential for the reagent reaction. The new technique can serve as a quick and reliable method for investigation of the presence of sugar *in situ* even under conditions of no transport.

## Materials and Methods

Three- to nine-year-old twigs and stems of white ash (*Fraxinus americana* L.) were sampled every 2 weeks from trees during the dormant season (October–April) and during the beginning of the active season (April–June). The cut branch portions were immediately cooled in dry ice, carried to the laboratory and, while still frozen, dissected as follows. One centimetre of the branch at the cut end was discarded and the next 2-cm piece was cut into blocks 1 cm long, 0.5 cm wide, and 0.3 cm thick. The blocks included the bark, the cambial zone, and a thin layer of wood. These samples were freeze-dried in a Labcenco freeze dryer 3 for 5–7 h. They were then embedded in paraffin or directly used for the histochemical test for sugars. The dried tissue was left for 3–5 days in the staining solution which had been freshly prepared as follows: lime (CaO) crystals were pulverized, dissolved in distilled water, and filtered to make a 3% (w/v) solution. 5-Diazouracil (Raybin 1933, 1937) powder was added to the lime solution to produce a 0.5% dye solution. The solution was stirred, filtered, transferred to an International–Harris cryostat, model CTD, and cooled at −7°C until it approached the freezing point. The freeze-dried samples of tissue were then immersed in the dye solution, and the temperature in the cryostat was lowered to −15°C. Samples were left in the frozen solution for 3–5 days. Transverse and longitudinal sections were prepared in the cryostat at −15°C. Section thickness was maintained at 30–50 μm to avoid loss of sieve tube content. The sections were then transferred to room temperature for observation and photographed through a microscope with a fiber optics lamp. Solutions of glucose, fructose, and sucrose and raffinose ($2 \times 10^{-2}$ and $2 \times 10^{-3} M$) were treated with the reagent solution and photographed at different periods of time in order to establish the color reaction for the sugars.

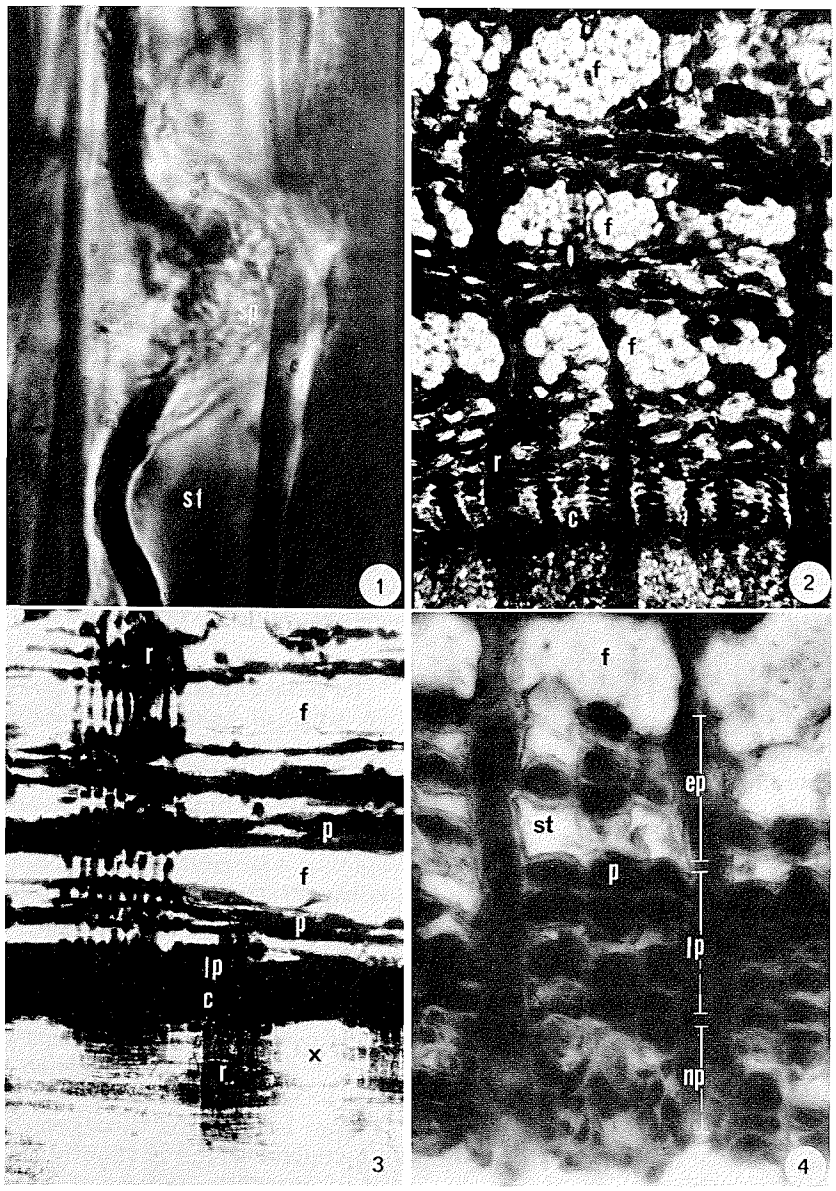
Three young trees were completely disbudded on May 4 at a time when the buds were swollen and about to burst. Newly developing buds were removed every other day. Some trees were girdled (ca. 1 cm wide) with a razor blade at breast height at the beginning of the experiment, others a month later, in order to see whether translocating materials would accumulate in the phloem just above the girdle. The control (intact) trees were also girdled on May 4 and examined once a week until the end of May while they had only very young leaves.

## Results

The 5-diazouracil reagent gives a fairly distinctive range of colored precipitates when reacting with pure sugar solutions. The reaction with glucose is brown, with fructose deep red, and with sucrose and raffinose blue. Sieve tube exudate of white ash (normally containing ca. 0.1 *M* sucrose, raffinose, and D-mannitol each, and about 0.2 *M* stachyose) gave a brownish reaction. A brownish color was also observed in sugar-containing cells of tissue sections. Some variations in color intensity did occur among the cells, but it was not possible to identify individual sugars by the color reaction *in situ*. However, the chemical test provides a useful tool for distinguishing between functioning and nonfunctioning sieve tubes.

Figure 1 shows the shrunken (lyophilized) sieve tube content which is slightly stained. It appears that when callose is formed, a certain amount of sugar solution and cytoplasm remain within the sieve tubes.

Phloem activity began early in March with the beginning of callose dissolution. This continued for about a month. During this period the cambium initials were showing lower amounts of sugars compared with last year's sieve tubes and parenchyma and the previous year's phloem parenchyma. In April, when the buds were still closed and the cambium was dormant, starch grains began to disappear. It is assumed that starch was hydrolysed and the resulting sugars were translocated via the rays to the cambial zone and to last year's




FIGS. 1–4. Sections through bark, freeze-dried and stained with 5-diazouracil. The brownish color of sugar-containing cells appears dark grey or black in these photographs. Sections have been cut thick to avoid loss of cell content. *c*, cambial zone; *ep*, early phloem, *f*, fibers; *lp*, late phloem; *np*, new phloem; *p*, parenchyma; *r*, ray; *st*, sieve tubes; *sp*, sieve plate; *x*, xylem. Fig. 1. Sieve tube, sectioned longitudinally, in December. The content is stained for sugars and appears shrunken. × 960. Figs. 2, 3. Cross section (Fig. 2) and longitudinal section (Fig. 3) made in April when the trees were still dormant. The cambial zone, last year's late phloem, and xylem and phloem rays stained strongly. Fig. 2. × 190. Fig. 3. × 95. Fig. 4. Cross section made at the end of May when cell divisions resumed in the cambial zone. Most of the sieve tubes in last year's early phloem are empty, while last year's late-phloem sieve tubes contain sugars. Sugars have begun to accumulate in the newly formed phloem. × 380.

phloem (Figs. 2, 3). Buds and cambium cells began to swell at the end of April.

In early May, cell divisions were observed in the cambium zone. At this time, last year's late phloem showed a strong sugar reaction while the newly formed phloem just began to show an increased sugar content. Last year's early-phloem sieve tubes appeared empty (Fig. 4).

Stem girdles, applied on disbudded trees in early May, showed the typical swellings, indicating that phloem transport can take place even in the absence of leaves. The source of nutrient for this

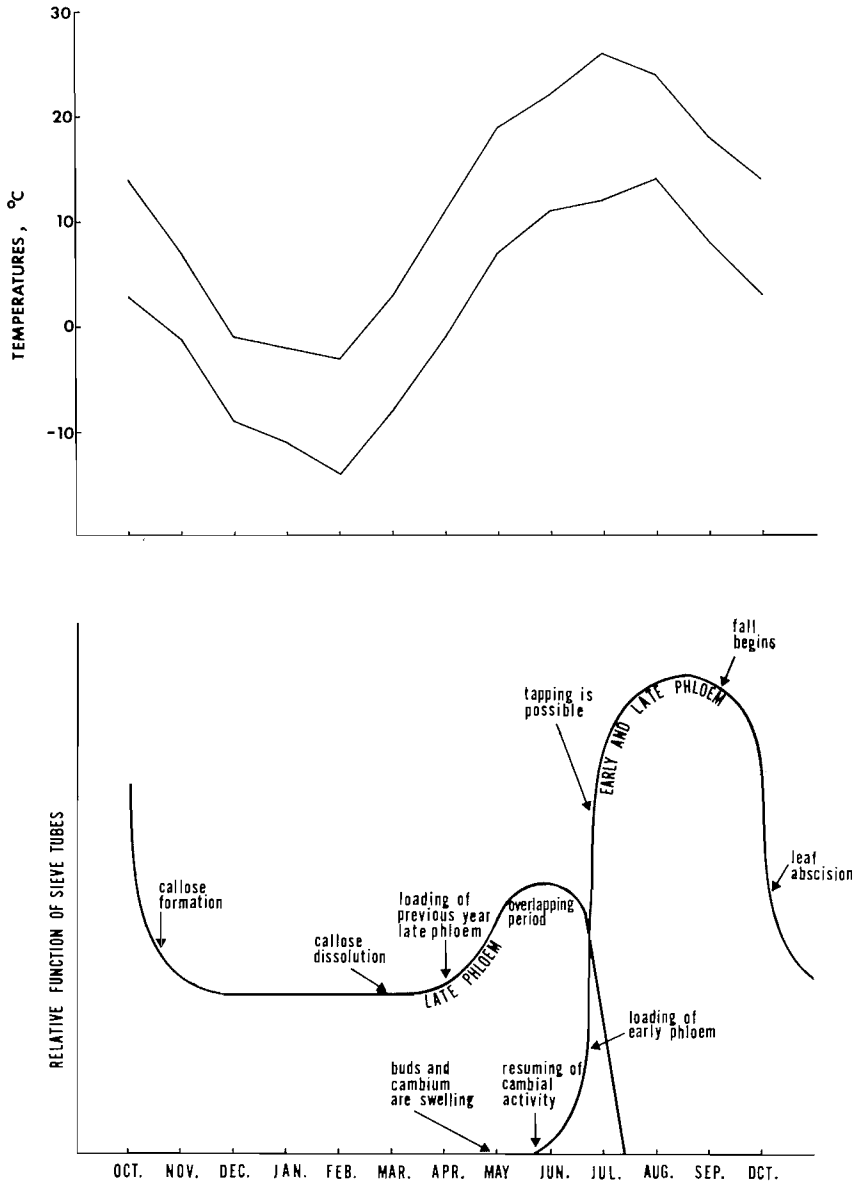



FIG. 5. A diagrammatic representation of sieve tube function throughout the year, summarized from anatomical observations and the intensity of the color reaction. The period mid-July through September is based upon earlier literature. The top graph shows monthly averages of daily minimum and maximum temperatures.

growth could only have been the stored reserve material. This transport must have taken place through reactivated phloem, because new phloem is not formed until the end of May. Disbudded trees yielded sieve tube exudate 2 weeks earlier (i.e., early June) than control trees (mid-June) when incision into phloem was made, and the volume of exudate in mid-June was about 20-fold greater in disbudded than in control trees. At the end of June, only small amounts could be obtained from the disbudded trees.

Figure 5 summarizes anatomical and histochemical observations in a graphical form. Reactivation of last year's sieve tubes and differentiation of sieve tubes, formed at the end of the previous season, serve the tree during the critical period of bud burst. The histochemical results indicate that last year's sieve tubes, especially those formed at the end of the season, may continue to function slightly beyond the time when the new sieve tubes have become functional at the beginning of the growing season. This overlapping functional life of

old and new sieve tubes assures that the meristematic zones (cambium, phellogen, and growing shoots) are provided with sufficient food.

Histochemical tests show that during the dormant period, parenchyma cells which are far away from the cambium, contained large amounts of sugars. Phloem ray cells did not become crushed as early as sieve tubes. They remained intact and active for a few years and seem to serve as a radial translocation path. Accumulation of sugars in the rays in early spring (April and May (Fig. 3)) provides the available sugars needed for the resumption of cell divisions in the cambium and phellogen (end of May) and for the loading of last year's late sieve tubes.

## Discussion

Mature functioning sieve tubes contain an aqueous solution and cytoplasmic remnants after the disintegration of cell organelles. The question of what can be seen in intact, well preserved, functioning sieve tubes is still open. Canny (1975, pp. 129–135) discussed this problem and summarized it by stating that "the fairest answer to the above question" (of what one can see) "seems on the evidence to be: Nothing."

Two major difficulties have to be faced when the role of sieve tubes is investigated: (1) the cells are very sensitive to any kind of injury, and (2) their contents, the translocate, consist of water-soluble compounds which are difficult to trace (Fisher 1976). We believe that the new technique used in this study overcomes the second problem and permits the identification of sugar-containing sieve tubes which can be regarded as functioning.

The freeze-drying procedure was found to be the most suitable method for preserving sieve tube constituents (see also Fisher 1976). Some reports state that freeze-drying caused either marked shrinkage (Fisher 1972) or partial collapse (Bieleski 1966; Trip and Gorham 1967), others showed that cells remain undamaged (Lawton and Biddulph 1964; Schmitz 1970). In our study only the sieve tube's content appeared shrunken. This proved to be an advantage because the sieve tubes' solution became thus more concentrated and more easily identified. Only the last 2 years' sieve tubes, especially those of the previous year, appeared to contain cytoplasmic remnants and solutes while the old sieve tubes appeared empty and crushed.

The question of sieve tube longevity has interested botanists for a long time. It is generally assumed that sieve tubes function only one season after which they are closed by definitive callose or crushed. There is more and more evidence of greater longevity in many arborescent gymnosperms and angiosperms and in perennial woody monocotyledons. Huber (1939), Grillos and Smith (1959), and Alfieri and Evert (1965, 1966) found that sieve cells in many gymnosperm trees function during parts of two growing seasons. Gill (1932) found that late-phloem sieve tubes of ash remain functional until new phloem is produced in the spring. Huber (1939) claimed that only sieve tubes of ring-porous trees last for two seasons while in diffuse-porous trees they cease to function at the end of every active season. On the other hand, Holdheide (1951) reported that the sieve elements function only one season in the majority of European dicotyledonous trees. Holdheide (1951) and later Evert *et al.* (1968) found 1- to 5-year-old functioning sieve tubes in *Tilia americana*. Parthasarathy and Tomlinson (1967) observed 50-year-old metaphloem sieve tubes in the palm *Sabal palmetto*.

All of the above-mentioned studies were based mainly on anatomical observations, whereby sieve tubes without *p*-protein or lacking the ability to produce callose were considered nonfunctioning. Our own conclusions are based on a histochemical reaction which stains sugars *in situ*, and on anatomical observations.

During the dormant period most sieve tubes are sealed with callose; the exception is those which are very close to the cambial zone and have not yet fully developed, i.e., they still contain organelles. The dormancy callose is produced and dissolved enzymatically by living cells. Callose-sealed cells must therefore contain cytoplasm as well as sugar solution.

The sugar reactions of phloem and xylem rays indicate that rays play an important role in the reactivation process by carrying storage sugars from the bark and xylem to the cambium. Reactivated sieve tubes from the previous year did not produce callose at the end of the second season; they lacked cytoplasm and did not contain sugar. Resumption of sieve tube activity was first described for the grapevine by Esau (1948). Later, Evert (1963) and Derr and Evert (1967) found that in *Pyrus malus* and *Robinia pseudoacacia* overwintering elements differentiate into sieve elements before new elements were formed by the reactivating cambium. Alfieri and Evert (1968) showed that the last-formed sieve cells of *Pinus resinosa* remain functioning during the winter.

Exudation from sieve tubes at the time of break of dormancy, known for some years, is also evidence of phloem reactivation. It has been found that the amino acid content of sieve tube exudates

from trees is relatively high during the period of senescence in the fall and during the break of dormancy in the spring, while it is very low during the major part of the vegetation period (Mittler 1958). Zimmermann (1964, p. 296) suggested that the sieve tube exudate at the break of dormancy contains "the very same substances that had been salvaged from aging leaves at the end of the previous season and remained in the phloem during the winter." Exudate, obtained at the time of break of dormancy (i.e., originating in reactivated sieve tubes), may also be different in sugar composition from summer exudate (Hill 1962). When trees were disbudded, exudation was obtained in early June, before the control trees showed summer exudation. This was probably still from reactivated phloem whose contents had not yet been used up because of disbudding. Preliminary analysis of this exudate indicated that constituents were different than the constituents of summer exudate, which was obtained at the end of June from control trees.

Two apparently conflicting pieces of evidence remain to be discussed. Histochemical evidence indicates the presence of sugar in sieve tubes from the time of break of dormancy, through the summer. The functional period of reactivated sieve tubes appears to last long enough to be replaced by the functional life of the new sieve tubes (Fig. 5). However, sieve tube exudation can be obtained only during a relatively brief period after break of dormancy. Exudation then stops until the new leaves are fully mature (about mid-June or somewhat later). This cessation of exudation seems to suggest a period of no transport (Zimmermann 1964). The answer to this question may be that we have to distinguish between the area of growing shoot tips, where local translocation processes continue from the time of break of dormancy throughout the summer, and the main stem, where transport may not be necessary before the time of long-distance export from leaves to roots.

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