INTRACELLULAR SUBERIN: OCCURRENCE AND DETECTION IN TREE BARK

by

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Summary

A protocol is outlined for histochemical detection of intracellular suberin linings on the inner surface of the cell walls in impervious tissues of wounded and infected bark, and in bark forming rhytidome. Thin intracellular suberin linings (circa 0.5 μm) were detected in all 15 woody angiosperms examined. Intracellular suberisation was strongly associated with individual cells or cell layers (boundary zone) that displayed imperviousness with fluid diffusion tests. Tests include use of phloroglucinol + HCl and Sudan black B to selectively quench autofluorescence of lignin and suberin, respectively. Blue-violet excitation is used to enhance the Sudan IV test for suberin, cutin, and waxes.

Key words: Suberin, histochemistry, fluorescence, bark, rhytidome, wound tissue.

Introduction

Suberin determination in inner bark tissues of trees has received renewed interest stimulated by the work of Mullick (1977) who demonstrated the apparent nonsuberised nature of an impervious tissue formed prior to phellogen regeneration at wounds and infections, and during rhytidome formation. Formation of nonsuberised impervious tissue (NIT) has been demonstrated in all four families of the Coniferales and from five angiosperms (Mullick, 1977; Soo, 1977).

Work by Biggs et al. (1983) demonstrated the presence of a similar nonsuberised impervious tissue in *Populus*. However, recently Biggs et al. (1984) re-evaluated the nonsuberised nature of cells in the impervious zone of *Populus* and other woody angiosperms. Intracellular suberisation is difficult to detect, and techniques for its histochemical detection are few (Scott, 1950; Mullick, 1975). This paper outlines a series of conventional histochemical tests which can be used in association with fluorescence microscopy for more precise determination of intracellular suberin in impervious tissues in the bark of several hardwoods.

Materials and Methods

The series of tests outlined below were performed on bark tissues collected in the field from *Acer saccharum* Marsh., *Amelanchier arborea* (Michx. f.) Fern., *Betula papyrifera* Marsh., *Castanea dentata* (Marsh.) Barkh., *Fraxinus americana* L., *Ostrya virginiana* (Mill.) K. Koch, *Populus maximowiczii* Henry × *trichocarpa* Torr. & Gray (hybrid NE-388), *Prunus avium* (L.) L., *P. domestica* L., *P. persica* (L.) Batsch, *P. serotina* Ehrh., *Quercus alba* L., *Q. rubra* L., *Sassafras albidum* (Nutt.) Nees., and *Tilia americana* L. During July and August, wounds deep enough to penetrate the living phellogen were made with a sharpened 4 mm diameter cork borer. The wounded area and adjacent bark tissues were removed with a 2.5 cm diameter arc punch at 4, 5, 9, 10, 17, 18, 24 and 25 days post-wounding. Also included were bark samples of *Populus* hybrid NE-388 and *P. persica* infected in the laboratory with *Cytospora chrysosperma* (Pers.) Fr. and *C. leucostoma* (Pers.) Fr., respectively. Samples were taken from the transition zone between healthy and diseased tissue (Biggs et al., 1983). In addition, rhytidome was examined from field collections of *Ostrya virginiana, Castanea dentata, Quercus rubra, Sassafras albidum, Prunus serotina* and *P. avium*. All samples were brought to the laboratory in a Dewar flask and immediately subjected to the ferric chloride-potassium ferricyanide (F-F) test (Mullick, 1975) for determination of impervious tissue. A minimum of three replicate trees were utilised at each sampling time except for *Betula papyrifera* and *Castanea dentata* where only one tree was available for examination.

At the end of the F-F test period, usually six days, the bark disks were halved longitudinally with a razor blade and one half was fresh-frozen and the other half fixed in formalin-acetic acid-alcohol (FAA). Bark disks not subjected to the F-F test were examined, also. Fresh tissues were frozen on a freezing stage (Bailey Instruments, Inc., Saddle Brook, NJ) using O.C.T. (optimum cutting temperature) compound and sectioned at 20 μm. Fixed tis-

sues were dehydrated, infiltrated, embedded in paraffin (Biggs et al., 1983), softened (10% glycerol; v/v), and sectioned at 10 μm on a rotary microtome.

Because specific stains for suberin are unknown, a variety of stains were employed in the initial investigations (Mullick, 1975). The Feulgen reaction (Margolena, 1932), Nile blue sulfate (Jensen, 1962), IKI-H_2SO_4 (Scott, 1950) and Sudan black B (Jensen, 1962) were used in addition to the procedures outlined by Mullick.

Casselman (1964) indicates that although the oil-soluble colourants (the Sudan dyes) are generally regarded as specific reagents for lipids, actually, they are only highly selective reagents. Because they are not completely inert chemically, they can act as weakly basic dyes staining some tissue elements that probably contain no lipid. Nonspecific staining can be distinguished from true 'sydanophilia' by decolouration and recolouration. This procedure was utilised with Sudan IV and Sudan black B with 95% ethanol as the decolourising solvent. Material that gives a true sudanophilic reaction can be decoloured and recoloured repeatedly provided it is not extracted by the decolourising reagent.

In addition to the tests for suberin, other tests for lignin or lignin-like compounds were employed for fluorescence examination because of their quenching effect. This procedure is essential in the light of recent evidence indicating the importance of phenolics in the molecular composition of suberin (Kolattukudy, 1980). Phloroglucinol + HCl, toluidine blue O, and the Mäule reaction (Jensen, 1962) were employed in this regard. Sudan black B was used to quench autofluorescence of suberin and its use confirmed the chemical nature of the intracellular linings.

Fluorescence microscopy was used with methylene blue and phosphine, for autofluorescence examinations before and after application of phloroglucinol + HCl or Sudan black B, and to increase the sensitivity of the Sudan IV reaction. Primary and induced fluorescence was examined with a Leitz 50W HBO mercury burner with filter combinations A (340–380 nm excitation and 415 nm barrier filters) and H2 (390–490 nm excitation and 510–515 nm barrier filters).

Tissues were extracted with 0.5 M NaOH at room temperature for 18 hours to remove wall-bound phenolic acids (Ride & Pearce, 1979) or with 80% H_2SO_4 which is reported to digest wall components other than suberin or cutin (Johansen, 1940). Extracted tissues were treated separately with Sudan IV and phloroglucinol + HCl and examined for fluorescence as described above.

Observations

Although the F-F test was useful in determining the presence of an impervious zone, it was an extremely difficult test to evaluate because imperviousness did not develop evenly around the perimeter of a wound or across the margin of an infection site. In addition, it was a very time consuming test and required considerable skill. Simpler methods such as the permeation of water soluble fluorescent brighteners may offer a less laborious approach to detection of impervious zones.

Results of tests described below allowed for rapid detection of the suberised or nonsuberised nature of impervious tissues. Chemical fixation of tissues did not appear to affect determination of intracellular suberin. In all species and samples examined, imperviousness was associated with a thin (0.5 μm) intracellular suberin lining observed in all species 18 days after wounding. On occasion, F-F test colouration could be detected within the cell wall at the boundary of tissue perviousness. Restriction of movement of the F-F test solutions appeared directly associated with the thin intracellular lining. Use of these tests should allow for detection of individual impervious cells formed in the earliest stages of impervious tissue development.

The tests listed in Table 1 were found to be the simplest and least difficult to interpret under these experimental conditions. It is recommended that all the tests be performed before a conclusion is reached regarding the nature of impervious tissues. Examination of tissue at × 250 followed by examination of individual cells at × 1000 magnification is suggested.

Autofluorescence — Fluorescence filter combination of 340–380 nm excitation and 410 nm barrier impart a violet to purple autofluorescence to the suberin intracellular linings. *Tilia americana, Castanea dentata, Quercus rubra, Q. alba, Fraxinus americana, Ostrya virginiana* and members of the Rosaceae possessed violet to purple autofluorescent inner linings that were easily observed prior to any other chemical treatment. Observation of the linings is often obscured by the bright blue autofluorescence of the lignified walls of cells having suberised linings. Although autofluorescence alone sometimes allowed visualisation of the linings, presence of other autofluorescent compounds limited the ability to establish the exclusive association of suberised linings with imperviousness or the extent of imperviousness. Filter combination H2 alone was not effective in observations regarding intracellular suberisation except in members of the genera *Prunus* and *Quercus*.

Table 1. Appearance of intracellular suberin lamellae under brightfield and fluorescence microscopy in combination with various histochemical reagents.

Species	Brightfield Sudan IV	Fluorescence (H2) Sudan IV	Autofluorescence (A)*	Autofluorescence (A)* Phloroglucinol quenching
Acer saccharum	pink	bright red	violet	violet
Amelanchiet arborea	nd**	bright red	violet	nd/violet
Betula papyrifera	nd	nd	nd/violet	violet
Castanea dentata	nd	nd/red-brown	violet	nd/violet
Fraxinus americana	nd	nd	violet	violet
Ostrya virginiana	nd	nd	violet	violet
Populus NE-388	nd	bright red	violet	violet
Prunus avium	pink	nd/red	violet	violet
Prunus domestica	pink	bright red	violet	violet
Prunus persica	pink	bright red	violet	violet
Prunus serotina	nd/pink	nd/red	violet	violet
Quercus alba	pink	bright red	violet	violet
Quercus rubra	pink	bright red	violet	violet
Sassafras albidum	nd	nd	nd/violet	violet
Tilia americana	nd	red	violet	violet

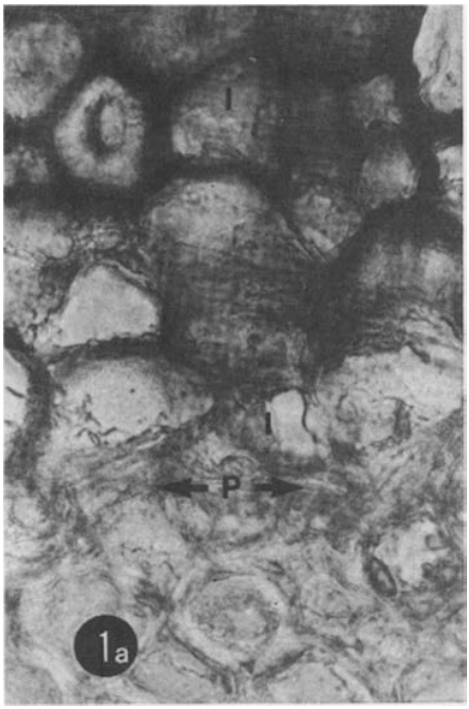
* Fluorescence filter combinations A (340–380 nm excitation and 415 nm barrier filters) and H2 (390–490 nm excitation and 510–515 nm barrier filters).

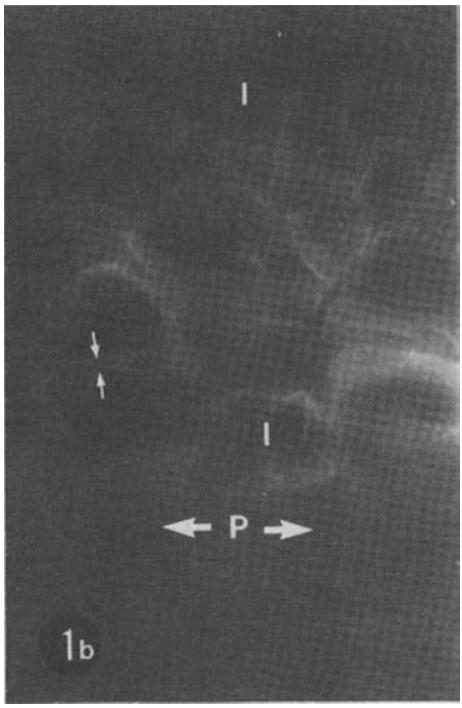
** nd: difficult to detect or not detected.

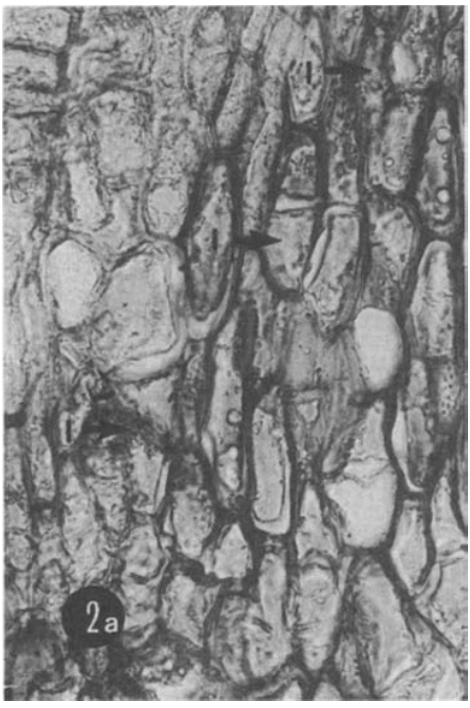
Tests for suberin — Although a specific histochemical test for suberin does not exist, the Sudan dyes are well known for their affinity for fatty substances. Mullick (1975) utilised Sudan III and observed thin linings in the NIT of *Abies amabilis* (Dougl.) Forbes. He attributed this result to the facile nature of the Sudan dyes and precipitation of the stain in the tissue. In the present study, Sudan IV was found to be extremely valuable when combined with fluorescence microscopy (Leitz combination filter H2). It was a very sensitive indicator for newly formed suberised phellem cells and intracellular linings of some species. Detection of linings with Sudan IV in brightfield was difficult because the refractive properties of the inner cell walls in wounded and nonwounded tissues sometimes gave similar pinkish-red colouration. Combined with fluorescence microscopy, Sudan IV colouration was often bright red against a yellow-green background. Destaining and restaining tissues with Sudan IV was necessary to rule out nonspecific colouration (Casselman, 1964). Not all species reacted well with Sudan IV, so other tests were necessary. Members of the genera *Prunus* and *Quercus*, and *Amelanchier arborea* exhibited Sudan IV colouration and red fluorescence most clearly.

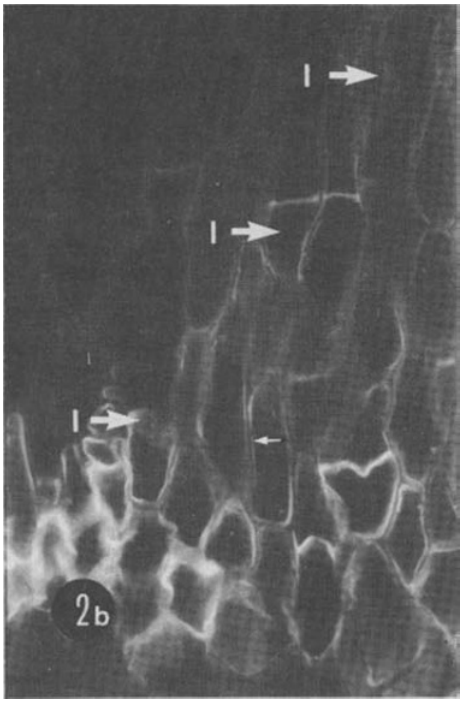
Additional tests for suberin should be utilised. Phosphine fluorescence was the least variable and imparted a silvery-white fluorescence to the suberin lining (Leitz filter block A). The lining will autofluoresce violet-blue (Leitz filter block A) but this was often difficult to detect because of bright autofluorescence of surrounding lignified tissues. Sudan black B was not effective in conjunction with the F-F test because it is similar in colour to F-F test boundaries and because its sensitivity was not increased with fluorescence. However, Sudan black B was used to selectively quench autofluorescence of intracellular linings thus providing additional evidence that they are suberised.

Tests for lignin and lignin-like compounds — Use of phloroglucinol + HCl was essential to this procedure because of its fluorescence quenching effect on tissues with which it reacts. When applied to impervious tissues and examined in bright field, phloroglucinol + HCl imparted a red colouration located in cell walls within and external to the impervious zone.









When phloroglucinol-treated tissues were examined under fluorescence (filter block A) only the intracellular linings were visible (Figs 1, 2). Under these conditions the linings appeared violet to purple. Toluidine Blue 0 provided the same effect as phloroglucinol. The Mäule reaction was always negative when applied to impervious tissues, however, positive red colouration was usually noted in phloem fibres, sclereids and phellem of some exophylactic and necrophylactic periderm (Srivastava, 1966). Cells in the impervious zone retained their autofluorescence under these conditions and intracellular linings were observable.

Extractions — Although extractions with 0.5 M NaOH yielded some interesting information regarding the nature of wall-bound phenolic acids in the impervious zone, no improvement on detection of suberin was provided. Extraction with H_2SO_4 provided no useful information.

Discussion

Integrated use of bright field and fluorescence microscopy in conjunction with conventional histochemical technique allowed for detection of thin suberin intracellular lamellae in cells comprising impervious zones in hardwood bark. Use of phloroglucinol + HCl and Sudan black B to selectively quench autofluorescence of lignin and suberin, respectively, is reported for the first time. Use of fluorescence to enhance the Sudan IV test has been mentioned (Mullick, 1975) but its use in these tests appears limited to recently derived suberin layers.

Impervious zones with intracellular suberin linings were a regular feature of the phellogen regeneration process and occurred during early stages of rhytidome formation, at the internal boundary of mechanical wounds, and external to sites of phellogen regeneration during fungal infection. The presence of intracellular suberin linings, as reported herein, is the first confirmation of Scott's (1950) results, although, intercellular linings as reported by Scott were not observed in any of the plant material examined and intracellular linings were observed only in boundary zone tissues.

Other optical techniques utilising polarised light have been used to examine the suberin-like linings of cells in wounded olive tissue (Hewitt, 1938). Although Hewitt indicated the anisotropic nature of the linings and concluded that they were not suberin but suberin-like, the birefringent nature of other cellular components limited the use of this technique in the present study.

In light of the present study and that of Scott (1950), it is essential that the work of Mullick (1975) and Soo (1977), regarding the non-suberised nature of impervious tissues, be re-evaluated. Because the series of tests described herein did not yield a series of identical results among species, the composition of suberin lamellae is probably slightly different in the different species. The similarity of results within species of *Prunus* and *Quercus* may reveal a chemotaxonomic relationship between the aliphatic components of the suberin complex within these genera. Extremely weak affinity for Sudan IV in suberised linings of *Fraxinus americana, Betula papyrifera*, and *Ostrya virginiana* suggests inclusion of other substances in the long chain fatty acid polymeric structure of suberin. Kolattukudy (1980) has discussed the importance of phenolic compounds in relation to suberin composition and has indicated that phenolic compounds may make up more than 50% of the total suberin complex. The relative amount of the phenolic component or the microstructure of the suberin complex may influence colouration by the Sudan dyes. The presence of suberin in bark, as described in this paper, and in wood (Scott, 1950; Pearce & Rutherford, 1981; Biggs, unpublished) suggests a crucial role for this compound in the establishment of impervious tissue and barrier zones.

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Fig. 1. Section through barrier zone in bark of *Betula papyrifera* 17 days after wounding. — 1a: Newly regenerated phellogen (P) and impervious tissue (I) treated with phloroglucinol + HCl; x 1000. — 1b: Same as Fig. 1 but viewed with fluorescence microscope after treatment with phloroglucinol. Note thin suberin linings on the inner surface of cells comprising the impervious zone; x 1000. — Fig. 2. Sections through barrier zone in bark of *Tilia americana* taken 17 days after wounding. — 2a: Impervious zone (I) just prior to phellogen regeneration treated with phloroglucinol + HCl; x 400. 2b: Same as Fig. 2 but viewed with fluorescence microscope after treatment with phloroglucinol. Note thin suberin linings on the inner surface of cells comprising the impervious zone; x 400.

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