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Title: DEGRADATION OF PHENOLIC COMPONENTS OF DOUGLAS-FIR BARK BY FUNGI

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Deposition of condensed tannins in phloem tissue at the time of periderm formation, as well as the location and general nature of cell wall lignin are described for Douglas-fir bark. Fungal degradation of condensed tannins and lignified sclereids was studied.

An Isaria-like fungus was associated with outer-bark parenchyma cells and sclereids from which condensed tannins were removed (parenchyma "bleaching"). This fungus alters ortho- and meta-hydroxylated phenols and guaiacyl compounds related to lignin. It rapidly precipitated condensed tannin extracts in culture, but lignin in tissue from which the fungus was isolated was not degraded.

Cell wall lignin located with the Wiesner color reaction was restricted to secondary walls of sclereids. This bark lignin gave elemental analyses, vanillin yield following nitrobenzene oxidation, a methoxyl content and ultraviolet absorption spectrum slightly different,

but within a reasonable range of the same measurements for conifer wood lignins. The infrared absorption spectrum for the bark lignin was similar to those published for conifer wood lignins; however, the bark lignin has a much lower molecular weight than wood lignins, probably because sclereids lack compound middle lamellae, which in wood contain the lignin fractions having the highest molecular weights.

Bispora betulina is associated with sclereids which have had their lignified walls "bleached" white, but this fungus failed to substantially degrade ball-milled sclereid lignin in liquid culture. B. betulina caused darkening of condensed tannins in cultured bark, and used guaiacyl compounds related to lignin as a sole carbon source, but failed to cause sclereid "bleaching" in culture.

Degradation of Phenolic Components of Douglas-fir Bark by Fungi

by

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INTRODUCTION

About 500 million cubic feet of tree barks are generated annually as byproducts of forest industries of Oregon (Lehmann, 1968). Research has been conducted by private and public laboratories for over 30 years in an effort to develop useful products from tree barks, but about half of the bark presently removed at Oregon mills is still burned as waste. The largest source is from Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco.

Douglas-fir bark is so rich in phenolic components that the quality of almost all products developed from the bark (e.g., tannin, flavanoids for various uses, molded products, pressed boards, bast fibers for reinforcing agents in plastics, etc.) are probably affected by modifications which change the phenolic substances. Much of the unexplained variation in bark products could be accounted for if well-described batches of bark containing documented modifications in phenolic content were used during product development. Besides variation which results from diverse growth patterns, modifications by microorganisms contribute to variation of properties. Although many types of bark degradation are revealed by color changes in phloem and cork, these changes are not obvious unless looked for, and the effects of bark modifications on products will not be known until

products are made from batches of bark containing known modifications.

Examination of bark following fungal attack revealed two distinct types of tissue modification. In one case, cells of tissues normally impregnated with reddish-brown condensed tannins were "bleached" white, but lignified walls of sclereids surrounded by these "bleached" tissues remained normal. In the other case, lignified sclereid walls were "bleached" white while surrounding condensed tannins were darkened, but not removed. Thus, it appeared that highly specific attacks on two major classes of phenolic substances of Douglas-fir bark occurred in living trees.

The purposes of this study were to describe chemical changes that occurred in "bleached" tissue, to identify the fungi associated with these changes, and to study the capabilities of these fungi for altering condensed tannins, sclereid lignin and phenolic compounds related to lignin and condensed tannins.

Fungi which penetrate and degrade bark of living Douglas-fir have not been described, and their effects on bark should be considered when planning research designed to develop products from bark. Enzymes capable of substantially "depolymerizing" condensed tannins under mild conditions could be valuable tools in studying the nature of the chemical bonds of this group of substances. An enzyme system which specifically attacks a limited number of phenolic

materials might be valuable for purifying one type of phenolic substance in the presence of another. Better understanding of phenolic components of bark and wood could lead to useful products from bark, less offensive pulping and bleaching procedures for wood and a reduction in air and water pollution resulting from burning of bark and "disposal" of pulping wastes.

REVIEW OF LITERATURE

Structure of Douglas-fir Bark

The ontogeny and structure of the inner bark and periderm layers of Douglas-fir have been described, and general descriptions of the outer bark have been made (Chang, 1954; Grillos, 1956; Grillos and Smith, 1959).

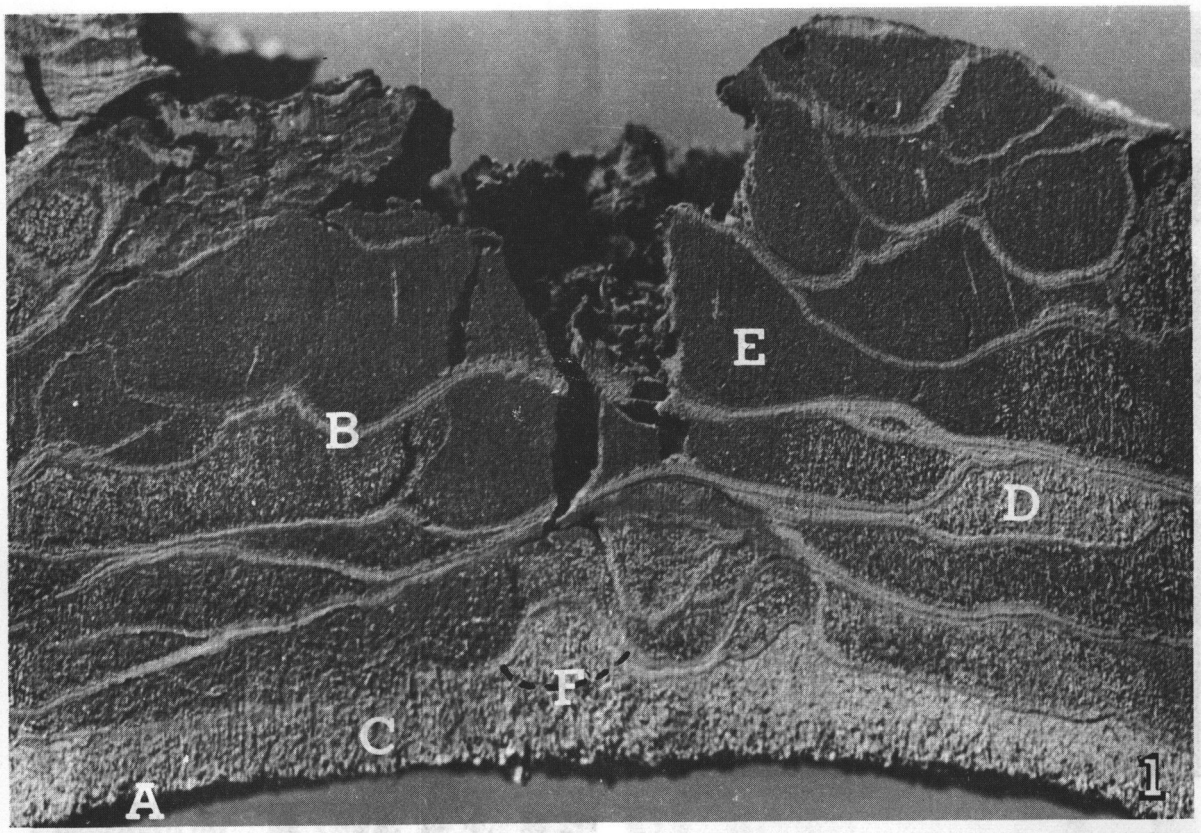
In cross-section, old growth Douglas-fir bark consists largely of reddish-brown phloem tissue which is traversed by many buff-colored, anastomosing periderm (cork) layers (Figure 1). All tissue lying outside of the innermost periderm layer is dead and is termed outer bark. Light tan-colored tissue lying between the vascular cambium and the innermost periderm layer is called inner bark. Many cells of the inner bark are living. Outer-bark phloem is encrusted with large amounts of condensed tannins and appears darker than inner-bark phloem where massive deposition of tannins has not yet occurred (Hergert, 1960). The vascular cambium adds cells to the inner bark, but the inner bark fails to increase in thickness because new periderm layers periodically form in the outer part of the inner bark (Figure 1-F) and living inner-bark cells are converted to dead outer-bark phloem (Grillos, 1956).

In cross-section there is a distinct difference in tissues lying on opposite sides of the vascular cambium. To the inside, the xylem

Figure 1. A cross-section of Douglas-fir bark from a 235-year-old tree. (A) Vascular cambium. (B) A periderm layer. (C) Inner-bark phloem. (D) Outer-bark phloem with the ends of many sclereids visible. (E) Outer-bark phloem in which most of the sclereids have been decayed. (F) A dotted line to illustrate a possible location for future periderm differentiation in the inner bark. (magn. 1.5:1).

Figure 2. A cross-section of inner bark. (A) Sieve cells. (B) Parenchyma cells containing stained starch grains. (C) A sclereid with condensed tannins in the lumen. (magn. approx. 430X).

Figure 3. A cross-section of outer-bark phloem. (A) Expanded phloem parenchyma cells. (B) Crushed sieve cells. (C) An outer-bark sclereid with condensed tannin encrusting the outer cell wall layer. (magn. approx. 550X).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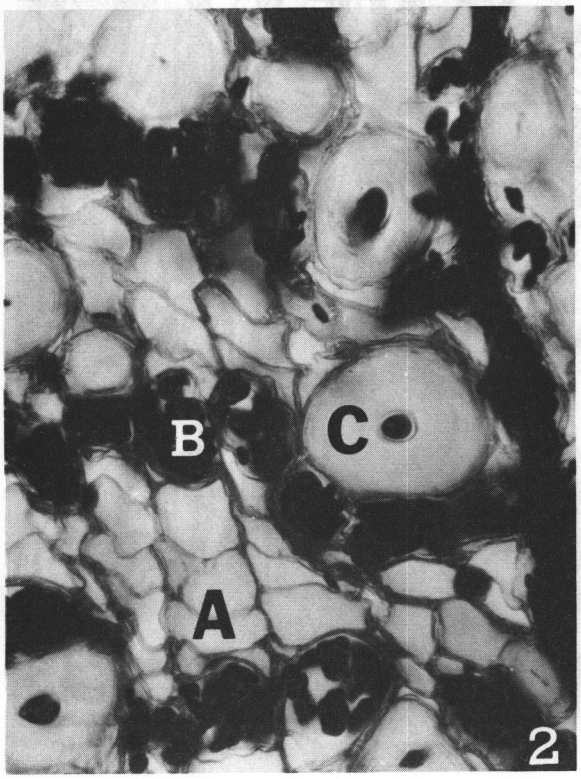
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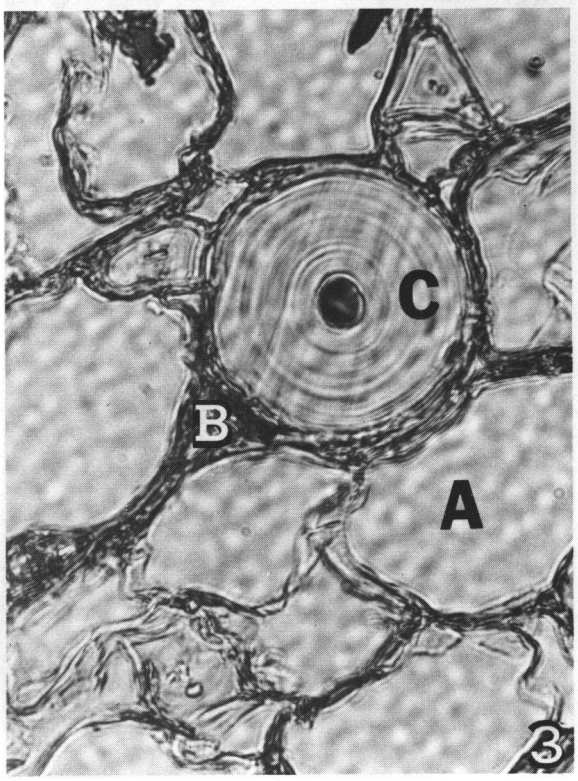
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(wood) is composed of radial bands of relatively homogeneous, thick-walled, lignified tracheids bordered by thin, inconspicuous rays. The tracheids are all dead and empty a short distance from the cambium and only ray parenchyma cells remain alive and store food materials (Brown, Panshin and Forsaith, 1949). To the outside of the vascular cambium, the most predominant cells of the phloem are thin-walled, non-lignified sieve cells and axial parenchyma cells. These two cell types alternate in discontinuous tangential bands of two to six rectangular sieve cells bordered by tangential bands, usually one cell wide, of circular parenchyma cells (Chang, 1954 (Figure 2). Axial and ray parenchyma cells of inner-bark phloem remain alive and store food materials (Figure 2) until they become part of the outer bark. Thick-walled sclereids (bast fibers) differentiate from short, thin-walled axial parenchyma cells at a distance of about 15 cells from the vascular cambium (Chang, 1954). At maturity, sclereids contain dark reddish-brown materials in their lumina (Grillos, 1956) (Figure 2). Thus, the sclereids occur individually and are surrounded by relatively thin-walled, non-lignified parenchyma or sieve cells (Figure 2). Periderm layers are formed from living axial and ray parenchyma cells of the inner bark (Grillos, 1956). In the outer bark the parenchyma cells are much expanded and crushed sieve cells are hardly discernible (Chang, 1954) (Figure 3).

Condensed Tannins

Disappearance of reddish-brown materials from bark tissues occurs where fungal mycelium is present. Based on previous anatomical and chemical descriptions of similar reddish-brown inclusions in parenchyma cells of living wood and bark and from all cell types in heartwoods of certain trees, such materials are certainly polyphenolic compounds (Hillis, 1962; Pew, 1949).

Polymeric polyphenols of Douglas-fir bark are classed as condensed tannins (phlobatannins) rather than hydrolyzable tannins (gallo- or ellagitannins) (Haslem, 1966; Hubbard and Kurth, 1949).

Specific fractions of the polymeric polyphenols of Douglas-fir have been variously named largely depending on methods of isolation. Hot-water-soluble polymeric polyphenols of Douglas-fir bark capable of fixing hide powder are generally referred to as tannins (Kurth, Hubbard and Gekeler, 1949). High-molecular-weight polyphenols insoluble in hot water, but extractable in hot, 95 percent ethanol are termed phlobaphenes (Hubbard and Kurth, 1949; Kurth et al., 1949). Phlobaphene is also a term used to describe reddish-brown polymers formed when leucocyanidins or catechins are condensed in the presence of mineral acids (Robinson, 1967).

Kiefer and Kurth (1953) found that a "wall or matrix or reddish-brown lignified material" surrounding sclereids of Douglas-fir bark can be removed by dilute alkali, but is insoluble in mineral acids or

organic solvents normally used to remove extractives from bark. This latter fraction contains carboxyl groups and is termed "phenolic acids" by Kiefer and Kurth (1953).

There is rather broad agreement that the condensed tannins arise from the polymerization of flavan-3-ol or flavan-3, 4-diol precursors (Haslam, 1966; Robinson, 1967; Swain, 1965). The polyphenols of Douglas-fir appear to form from leucocyanidins and catechins (Hergert, 1960). Recent work indicates that the "phenolic acids" of Douglas-fir bark, like the phlobaphenes and tannins, are polymers of leucocyanidins and catechins (Fujii and Kurth, 1966).

The tannins, phlobaphenes and "phenolic acids" of Douglas-fir bark are reddish-brown and have similar chemical compositions. Thus, in the present study, all reddish-brown materials encrusting cell walls or residual protoplasts of bark tissues are referred to as condensed tannins.

Mechanisms by which monomers polymerize to form condensed tannins have been reviewed (Brown, 1964; Haslam, 1966; Swain, 1965), but the nature of the bonds between the monomers is still uncertain. Two mechanisms by which flavan-3-ols and flavan-3, 4-diols polymerize are acid-catalyzed condensation and oxidative quinone polymerization. The first mechanism involves an acid catalyzed condensation such as that between the C-2 of one flavanoid monomer and the C-6 of a second molecule to form a carbon-carbon

bond (Freudenburg, 1960). The second mode, as examplified by catechin polymerization, is by enzyme-catalyzed quinone formation (or autoxidation) at the B-ring followed by polymerization between the C-6' of the oxidized B-ring and the C-8 of the phloroglucinol ring of the second monomer (Hathway and Seakens, 1957).

The oxidative mechanism is thought to be more important than the acid catalyzed mechanism in the formation of condensed tannins Brown, 1964; Swain, 1965), and a possible scheme is shown in Figure 4. Spectral characteristics of condensed tannins of conifers suggest that some ether linkages may also occur between monomers (Hergert, 1960). Also, optically-active tannin from heartwood of Acacia mollisima was hydrolyzed by dilute acid to give a flavan-3, 4-diol, and this was best explained by the presence of acid-labile ether linkages between some of the flavan-3, 4-diol monomers (Roux and Paulus, 1961).

Flavan-3-ols and flavan-3, 4-diols have two and three asymmetric carbon atoms respectively. Presence of asymmetric carbon atoms within the monomer units of condensed tannins could account for two types of asymmetric bonds requiring isomer-specific enzymes for cleavage. Should carbons 2, 3, or 4 of the flavanoid monomers be linked to other monomers of a tannin molecule, cleavage of these bonds might require enzymes capable of binding a specific configuration about each of these bonds between monomer units. Should intact

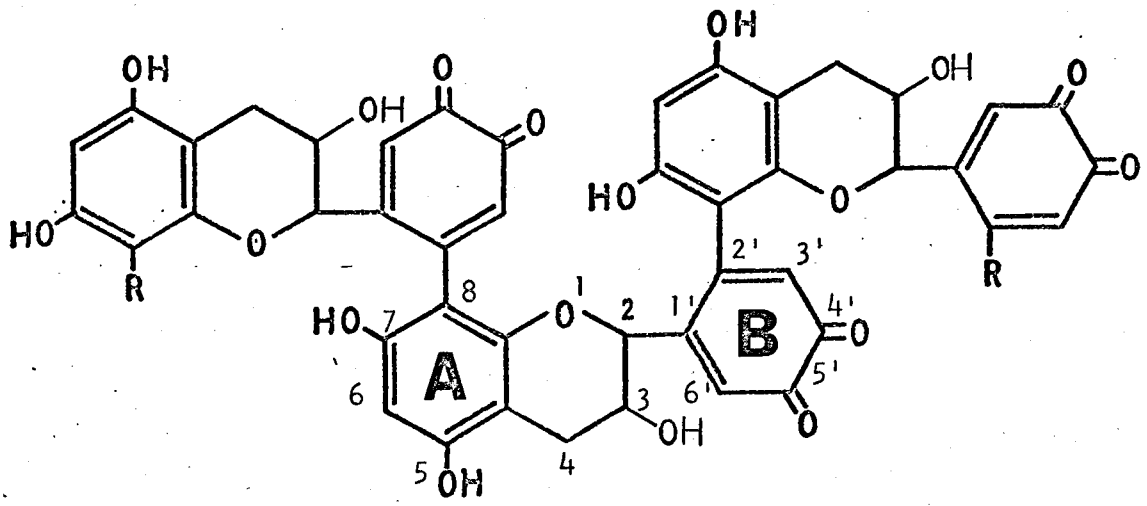

Figure 4. A schematic representation of a condensed tannin formed by quinone polymerization of catechin (Hathway and Seakins, 1957; Robinson, 1967).

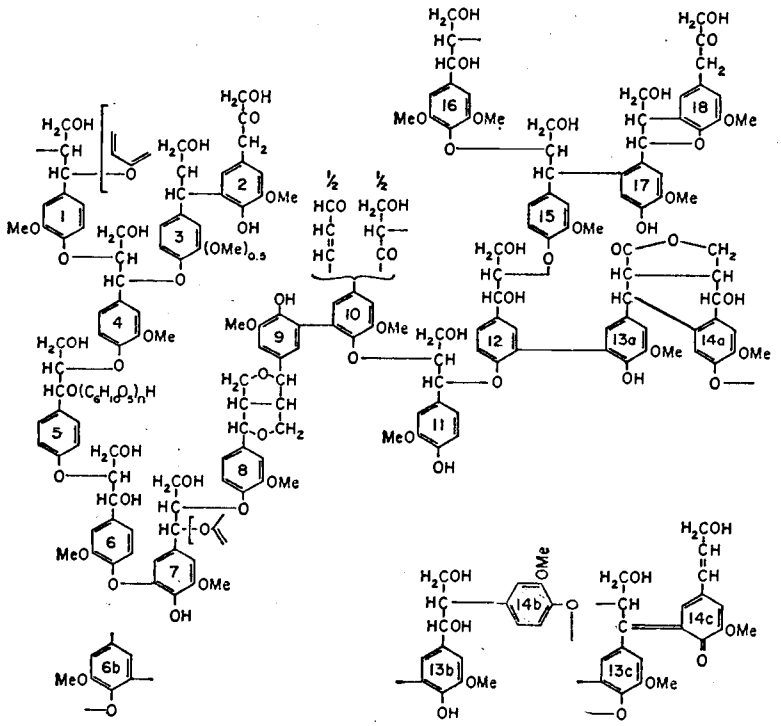

Figure 5. A schematic model for spruce lignin (Freudenburg, 1965, 1966).

monomers be released, further utilization of the monomers again would involve asymmetric bonds within monomer units. Fungi have been shown to selectively deplete only naturally-occurring stereo-isomers of catechin from liquid culture (Brown and Bocks, 1963).

Lignin of Douglas-fir Bark

The second type of bark decomposition studied appears microscopically and histochemically to involve degradation of cell wall lignin of bark sclereids.

An early definition described lignin as

> that incrusting material of the plant which is built up mainly, if not entirely, of phenylpropane building stones; it carries the major part of the methoxyl content of the wood; it is unhydrolyzable by acids, readily oxidizable, soluble in hot alkali and bisulfite, and readily condenses with phenols and thio compounds (Brauns, 1952, p. 15).

More recently, Brauns and Brauns (1960) included, among other qualifications, that from coniferous woods, high yields of vanillin are produced by alkaline nitrobenzene oxidation of lignin. A list of chemical criteria for determining the lignin character of plant substances has been presented (Kratzl, 1965).

While a number of earlier papers present quantitative data and chemical descriptions of "lignin" fractions from Douglas-fir bark (Fujii and Kurth, 1966; Holmes and Kurth, 1961; Kiefer and Kurth, 1953; Kurth and Smith, 1954), a substance which would fit the criteria suggested by Brauns and Brauns or Kratzl has not been described for

the bark.

Early tests with lignin preparations from Douglas-fir bark failed to demonstrate a positive color reaction when treated with phloroglucinol-HCl (Kurth and Smith, 1954). Later, Fujii and Kurth (1966) mentioned that a Douglas-fir bark sample "which contained some bast fibers and cork fragments" gave a positive phloroglucinol-HCl test for lignin. All cell types in tree barks generally do not give a uniform Wiesner reaction (a red to violet color given by lignin when treated with a phloroglucinol-HCl reagent), although fibers and sclereids of coniferous barks generally give a positive reaction (Srivastava, 1966).

The chemical composition of isolated sclereid fractions from Douglas-fir bark has been studied, and three "lignin" fractions were described (Kiefer and Kurth, 1953). A total "lignin" content of 44.6 percent was determined for the sclereids by the Klason procedure. This procedure involves treatment of samples with 72 percent sulfuric acid to remove all non-"lignin" materials, and then residual acid-insoluble material is weighed and termed Klason, or acid-insoluble lignin (TAPPI, 1954). One fraction, constituting 49 percent of the Klason lignin, was obtained by extracting the sclereids with one percent sodium hydroxide. This fraction was termed "phenolic acids" and contained a relatively low methoxyl content 4.3 percent), a high carboxyl content (4.9-5.3 percent), gave a low yield of vanillin (1.63

percent) by nitrobenzene oxidation and failed to give a positive Wiesner reaction (Kiefer and Kurth, 1953).

A second preparation was obtained by extraction with a dioxane-HCl reagent following the removal of "phenolic acids" with the sodium hydroxide. This second fraction is probably most similar to that for softwood lignins and had a methoxyl content of 13.5-14.3 percent. Reaction of this fraction with phloroglucinol-HCl and vanillin yield from nitrobenzene oxidation were not given (Kiefer and Kurth, 1953).

About one-fourth of the Klason lignin remained after the first two extractions and this residue was considered the third "lignin" fraction.

Much information has been compiled on the biosynthesis and structure of wood lignins in recent years, and there are a number of reviews of the subject (Brauns, 1952; Brauns and Brauns, 1960; Freudenburg, 1965, 1966; Harkin, 1969; Pearl, 1967; Schubert, 1965).

Wood lignin is a three-dimensional polymer of optically-inactive phenylpropane monomers. Lignin bonds such as β-aryl ether linkages form by free radical condensation of phenylpropane units, and asymmetric carbon atoms are formed. This asymmetry produces two possible configurations about the β carbon atoms of phenylpropane monomers, but randomization of these bonds leads to racemization (Freudenburg, 1964). Freudenburg's model for spruce lignin

(Freudenburg, 1965, 1966) is presented in Figure 5 for comparison with the general scheme for a condensed catechin tannin presented in Figure 4.

MATERIALS AND METHODS

Field Sampling

Bark samples with signs of phenolic degradation were collected east and west of the Cascade Mountains from northern California to central Washington.

Culture Media

Fåhraeus' Bf-2 medium and modifications thereof were used as the defined media in this study (Fåhraeus and Tullander, 1956). The modified Bf-2 medium was prepared by replacing asparagin with ammonium nitrate, omitting phenylalanine, reducing the iron to one-tenth strength and either omitting sugar or using 0.2 percent sucrose.

An inner-bark extract medium was used to screen fungi isolated from bark for their ability to modify polyphenols from Douglas-fir bark. The medium was prepared by leaching 20 g of dry inner-bark fines (to be described below) in 500 ml of water for 0.5 hours. Mineral salts (one-tenth strength iron) and vitamins of Bf-2 medium were added along with 20 g of sucrose. The mixture was sterilized by passing it through a Millipore HA filter and was then diluted to one liter with either sterile water of a liquid agar solution (1.5 percent wt./vol.) to obtain a liquid or solid medium.

Cultures were incubated in the dark at 20 to 24° C, either still

or on a rotary shaker.

Production of Inoculum

Two to three ml of a spore and mycelial suspension of the Isaria-like fungus obtained from colonies growing on potato-dextrose-agar (PDA) in test tubes was added to 50 ml of modified Bf-2 medium with 2 percent sucrose. The Isaria-like fungus was grown one to two months in still culture in 125-ml Erlenmeyer flasks to obtain sufficient mycelium for inoculum.

B. betulina failed to grow sufficiently in less than two to three months when started from spores in the above medium, but its growth was stimulated by methanol extracts of outer-bark phloem; therefore inoculum was grown on a rotary shaker in 500-ml Erlenmeyer flasks with 100 ml of modified Bf-2 medium to which 40 mg of methanol extract was added in 2 ml of ethanol.

Mycelia of both fungi were collected by aseptically filtering the cultures through Whatman no. 1 filter paper in a Buchner funnel. The mycelia were washed with sterile water and transferred with a sterile spatula to modified Bf-2 media containing phenolic substances.

Incubation of Bark Samples with Fungi

Bark samples were prepared for incubation with fungal isolates in an attempt to reproduce "bleaching" of parenchyma tissue and

sclereids typical of that found in growing trees. Various bark samples were cut to include whole bark, entirely outer bark or entirely inner bark. Isolated sclereid and cork fractions (see section on isolation of sclereid fractions) were also incubated with fungal cultures. The bark samples were sterilized by autoclaving or with an ethylene oxide-carbon dioxide mixture, and were adjusted to various moisture levels before inoculation.

Incubation of Fungi with Phenolic Substances

Fungal cultures were incubated in still culture with 50 to 100 ml of modified Bf-2 medium in 250-ml Erlenmeyer flasks. Phenolic compounds were added to the medium as ethanol solutions (1-2 ml) to give a final concentration of approximately 400 ppm. Methanol extracts of outer bark phloem were similarly added at 1600 ppm to 100 ml of modified Bf-2 medium in 500-ml DeLong culture flasks.

Preparation of Isolated Sclereid Fractions

It is possible to separate whole bark into fairly homogeneous batches of cork, sclereids and parenchyma cells (Hergert and Kurth, 1952; Kiefer and Kurth, 1953) by first cutting the bark into small pieces and air drying. After pulverizing the dry pieces in a Waring Blender, the mixture is placed on a stack of Tyler standard screens of meshes 28, 35, 60, 80, 100, 150 and 170 and shaken with a Syntron

shaker for 15 minutes. The material retained on the 28-mesh screen was fairly clean cork, screens 80-150 mesh were rich in sclereids, and material passing through 170 mesh (referred to as bark fines) was mainly parenchyma and sieve cells with small amounts of broken cork and sclereids. Material retained on screens 80-170 mesh was washed several times to float off contaminating fines and was then air dried. Only that washed material that passed through a 100-mesh screen was considered as a relatively homogeneous sclereid sample. When large amounts of sclereids were desired for the preparation of ball-milled lignin, only inner bark was used. This avoided contamination by broken cork and high concentrations of condensed tannins.

To obtain reasonably clean sclereids from the outer bark, phloem tissue was carefully scraped from the cork tissue before the phloem was pulverized. Isolated sclereids were prepared from uninfested outer bark, outer bark with "bleached" sclereid walls and outer bark with "bleached" parenchyma and "emptied" sclereids.

Preparation of Ball-milled Lignin from Inner-bark Sclereids

The inner-bark sclereids retained on 150- and 170-mesh Tyler screens were ground in a Wiley Mill using a 60-mesh screen. Although all the long, thin sclereids could pass 60 mesh if oriented properly, most were cut transversely one or more times by the Wiley Mill. This allowed better penetration of extracting solvents into the narrow

lumina than would have been afforded by the few simple pits in the sclereid walls.

Ground sclereids were extracted 48 hours in both ethanol-benzene (1:2) and ethanol (95 percent), and were stirred in water at 50° C for two hours. The meal was thoroughly dried in a desiccator under vacuum over anhydrous calcium sulfate. The dry sclereid meal was placed in a milling jar containing glass marbles, the jar was purged with helium, sealed tight and rotated at about 20 rpm for five weeks. [1]

A lignin preparation designated sample "A" was prepared from ball-milled sclereid meal by the methods of Björkman (1956). Lignin sample "B" was extracted from sclereid meal with dioxane-water (96:4) and freeze-dried. The fluffy light tan powder was redissolved in 90 percent aqueous acetic acid, dropped into water, washed twice with water and finally freeze-dried.

No attempt was made to quantitatively extract lignin from sclereid meal, and in general yields were much less than from sapwood meal. They ranged between 1.5 and 2.0 percent of the dry, ball-milled bark meal. The most exhaustive extraction was carried out with three batches of 400 to 600 ml of 96 percent aqueous dioxane over 10 days using 93.4 g of sclereid meal.

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[1] Thanks go to Dr. K. V. Sarkenen for describing to me the process for dry milling of wood meal for preparation of lignin.

Ball-milled meal of sapwood[2] was kindly supplied by Dr. K. V. Sarkanen, and wood lignin was prepared by the methods of Björkman (1956).

Thin-layer Chromatography

Silica Gel G (E. Merck Ag., Darmstadt, Germany) was used as layering material, and it was applied 0.25 mm thick to glass plates (20 x 20 cm) and dried at 90° C for about one hour.

Solvents used for the development of thin-layer chromatograms were:

1. Benzene-methanol-acetic acid (45:8:4) (Haslam, 1966).
2. n-butanol saturated with 3 percent aqueous ammonium hydroxide (Ishikawa, Schubert and Nord, 1963).
3. Chloroform-ethanol (50:1) (Kirk, 1968).

Spray reagents used to detect phenolic substances included:

1. Potassium ferricyanide-ferric chloride (1 percent each in water) (Barton et al., 1952).
2. Diazotized sulfanilic acid (Ibrahim and Towers, 1960).
3. Bis-diazotized benzidine (Roux and Haihs, 1960).

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[2] Ball-milled meal received was thought to be that of Douglas-fir sapwood, but low methoxyl (14.5 percent) and the infrared spectrum of the resulting lignin suggest otherwise. The infrared spectrum (Figure 17C) shows strong absorption at 1735 cm^{-1} and other features characteristic of the spectrum for juvenile pine milled-wood lignin given by Sarkanen, Chang and Allan (1967, Figure 4).

Phenolic compounds were also detected by their fluorescence or absorption under ultraviolet light.

Paper Chromatography

Sugars were separated by the methods of Mian and Timell (1960) using Whatman no. 1 filter paper and were developed in the descending direction with ethyl acetate-acetic acid water (9:2:2) or n-butanol-pyridine-water (10:3:3). All papers were prewashed with distilled water before spotting. Sugars were located with the CD-1 spray reagent of Gorden, Thornberg and Werum (1956).

Quantitative Determination of Phenolic Compounds in Fungal Culture Medium

Aliquots (150-200 µl) of phenolic medium were aseptically removed from cultures shortly after inoculation and again at the end of the experiments. The aliquots were streaked on silica gel plates (see thin-layer chromatography) and developed with either solvents 1 or 2. Phenolic compounds were located under ultraviolet light or by comparing positions on the plates with positions of authentic standards. The identities of compounds were confirmed by their ultraviolet absorption spectra. Compounds were removed from the plates by scraping bands of silica gel from them and extracting the gel three times with 2-ml volumes of 95 percent ethanol. The resulting phenolic

extracts were diluted to 6 ml with ethanol, filtered through Whatman no. 1 filter paper and their absorbances were determined at appropriate absorption maxima between 259 and 288 nm with a Beckman DB spectrophotometer. For each phenolic compound a standard curve of maximum ultraviolet absorption was prepared, and the concentration of each phenolic compound recovered from the medium was determined by comparing its ultraviolet absorption with the standard curve. Control experiments indicated a recovery of 90 to 95 percent of all phenols by this method.

Hydrolysis of Sclereid Walls and Determination of Sugar Ratios

Whole sclereids (both normal inner-bark and biologically-"bleached" outer-bark) were extracted in a Soxhlet apparatus with ethanol-benzene (1:2) for 10 hours followed by extraction with ethanol (95 percent) for 10 hours. Extracted sclereids were dried under vacuum in a desiccator over anhydrous calcium sulfate and then bleached using the chlorite method of Jayme and Wise (Wise, Murphy and D'Addieco, 1946). In the case of normal inner-bark sclereids, sodium chlorite and acetic acid were added to the bleaching solution every hour for four hours. The final holocellulose was very light yellow and the sclereid lumina appeared empty. Biologically-"bleached" sclereids had much more porous walls and were rendered white with empty lumina after one hour of extraction. Holocellulose

preparations were dried under vacuum.

Rather harsh methods were used to hydrolyze the holocellulose preparations to determine sugar ratios. Holocellulose (0.2 g) was placed in a test tube (2 x 20 cm) with 10 ml of 72 percent sulfuric acid and stirred continuously for two hours at 18 to 20° C. The hydrolysate was then diluted to 3 percent with distilled water and refluxed for three hours. After cooling, the hydrolysate was filtered through a sintered glass crucible and adjusted to pH 3.0 with Amberlite IR-4B (hydroxide form) ion exchange resin. The sugar solution was reduced to about 5 ml in a rotary evaporator at 30° C and finally diluted to 10 ml with water. The sugars were separated by paper chromatography using the methods of Mian and Timell (1960), eluted from the chromatograms (Saeman et al., 1954) and determined quantitatively by the method of Timell, Glaudemans and Currie (1956). Relative amounts of glucose, galactose, mannose, xylose and arabinose were determined and the values were corrected for sugar degradation during hydrolysis using correction factors obtained when known amounts of the five sugars were passed through the entire process. Losses were similar to those shown by Saeman et al. (1954) (Figure 7) for three hours of secondary hydrolysis.

Determination of Total Phenols in Sclereids

Total phenolic contents of sclereids with "bleached" walls,

sclereids with "emptied" lumina, normal inner-bark sclereids and normal outer bark sclereids were determined. Phenolic components of each sclereid preparation were removed using the chlorite holocellulose method (Wise et al., 1946) and weight loss due to chlorite bleaching was considered total phenolic content. Isolation of altered sclereids was tedious and only a small amount (about 2 g) of each type was recovered. Three batches (0.2 g each) of each type of sclereid were placed in separate 30-ml, tared Gooch crucibles (low form) which were stoppered from the bottom and covered with small, inverted crucible lids. The sclereids were incubated with 15 ml of acidic sodium chlorite solution (Wise et al., 1946) at 75 to 80°C. Fresh chlorite and acetic acid were added every 30 minutes until a wet brightness of 76 or better was measured with a Photo Volt brightness meter according to TAPPI Standard Method T452 m-58. Bleaching chemical was then drawn from the crucibles with a vacuum and the resulting holocellulose was washed with cold water and finally with acetone. Weight loss due to chlorite bleaching was determined by drying the crucibles containing the sclereid holocellulose and subtracting final dry weight from the dry weight of untreated sclereids of the same type.

Nitrobenzene Oxidation of Lignin

A modification of the micromethods for alkaline nitrobenzene

oxidation described by Stone and Blundell (1951) and Bland (1960) was used. Stainless steel bombs (2-ml capacity) containing 10 mg lignin, 0.06 ml nitrobenzene and 1.0 ml 2N sodium hydroxide were rotated endwise in an oil bath at 160° C for three hours. The reactions were stopped by submerging the bombs in cold water. The cooled reaction mixtures were then transferred to small bottles, adjusted to pH 3.0 with HCl and allowed to settle overnight. Soluble aldehydes were collected by centrifugation and combined with two subsequent washings (10-ml volumes of water) of solid materials. The aqueous solutions were partitioned three times with 10-ml portions of chloroform and the chloroform extract was taken to dryness under vacuum (Bland, 1960). Aldehydes were redissolved in 2 ml of ethanol, and 400-μl aliquots were streaked on silica gel plates. After developing the plates with solvent 2, the aldehydes streaks were scraped from the plates, extracted from the gel with ethanol (95 percent) in a micro-Soxhlet extractor for one hour, and concentrations of vanillin were determined spectrophotometrically by methods of Stone and Blundell (1951).

RESULTS

Normal, Unmodified Bark--Location of Cell Wall Lignin and Deposition of Condensed Tannins at the Time of Deep Periderm Formation

The inner bark of Douglas-fir has been described in some detail (Chang, 1954; Grillos, 1956), but outer bark has received only general description. The location of cell wall lignin and important changes which occur in phloem tissue at the time of periderm formation, especially with regard to deposition of condensed tannins, are two areas which have not been described for Douglas-fir. Thus, clarification of these matters was needed as background for studies on fungal degradation of the bark tissues.

Sections for microscopic examination were cut with razor blades from a large number of bark samples including many in which periderm layers were in early stages of differentiation in the inner bark. The Wiesner test (5 percent phloroglucinol in 95 percent ethanol acidified with hydrochloric acid) was used to stain lignified tissue, iodine-potassium iodide was used to stain starch, and Sudan IV and ferric chloride were used for suberized tissues and polyphenols, respectively (Johansen, 1940).

The thick-walled sclereids are the only cells in the inner and outer bark that stain brilliant reddish-violet with the Wiesner reagent, and the color is indistinguishable from that given by the wood.

Scattered periderm and parenchyma cells also stain, but when they do they generally have abnormally thick walls and are often near an injury At maturity sclereids are filled with reddish-brown material. Condensed tannins probably account for most of the color in lumina because the contents of sclereid lumina condense to phlobaphene-like material upon treatment with 72 percent sulfuric acid, and are released as intact units when the secondary walls are hydrolyzed by sulfuric acid.

Most parenchyma cells of the inner bark are living and many contain starch, tannins, crystals and/or other storage products. When a new periderm forms within the inner bark (Figure 1F), storage products, such as starch, disappear from cells that are to become either phellogen or outer bark phloem. As storage materials disappear, parenchyma cells become greatly dilated, and light reddish-brown materials encrust the expanded walls and residual protoplasts. A thin zone of periclinal divisions can be seen along an abrupt boundary between greatly-expanded parenchyma with dark encrusted walls, and smaller, undilated, light-colored parenchyma of the inner bark. The zones of cell divisions stain pink with Sudan IV suggesting the beginning of suberization of phellum cells.

The outer layer of sclereid cell walls (comparable to the original parenchyma cell from which each sclereid developed) also becomes impregnated with reddish-brown materials (Figure 3). The

impregnation of the outer walls of sclereids is particularly evident when light golden tan preparations of isolated sclereids from the inner bark are compared with the light reddish-brown sclereids from uninfested outer bark.

These microscopic observations fit well with previous chemical descriptions of Douglas-fir bark. The reddish-brown material in the outer layer of outer-bark sclereids is the same layer that Kiefer and Kurth (1953) described as "phenolic acids" soluble in dilute alkali. Holmes and Kurth (1961) found colorless d-catechin and l-epicatechin in inner bark of Douglas-fir, but not in the outer bark. Monomeric catechins and leucocyanidins have been found in highest concentration in the cambial zone of the bark and their concentration decreased in the centrifugal direction from the cambium (Hergert, 1960). Hergert also found that as monomers decreased in concentration, there was a corresponding increase in polymeric polyphenols. This implies that the colorless precursors to the reddish-brown, high molecular weight condensed tannins occur in parenchyma cells of the inner bark and polymerize on dilated parenchyma cell walls and residual protoplasts at the time of periderm formation. As condensed tannins increase in molecular weight, they show a parallel increase in their affinity for cellulose (Roux and Evelyn, 1958). Possibly the disappearance of starch and other storage products at the time of parenchyma expansion may contribute to synthesis of condensed tannins.

To summarize, at the time of periderm formation, small non-lignified, living parenchyma cells of the inner bark lose their storage materials, increase greatly in size and become impregnated with condensed tannins (Figure 2 vs 3). Inner bark sclereids, which contain a type of lignin in their thick secondary walls and condensed tannins in their lumina, change from a light tan to reddish-brown as their outer wall layer becomes impregnated with condensed tannin, just as surrounding parenchyma walls do (Figure 3). Thus, the sclereid is the only cell that contains both lignin and condensed tannin.

Modified Bark--Description of "Bleached" Sclereids

"Bleaching" of sclereid walls is quite common in the outer bark of Douglas-fir, especially in butt logs of older trees (over 100 years). Typically, most advanced degradation is centered in the outer bark midway between the outer surface and the vascular cambium. Less degradation occurs near the outer surface of the bark and in tissue contiguous with the inner bark. As trees age, decay appears to move inward toward the cambium more rapidly than it spreads outward. Infested phloem tissue is generally darker than uninfested outer bark phloem, and the periderm layers are dull gray rather than the normal light buff color. When the bark is broken longitudinally the sclereids, which are normally reddish-brown and difficult to distinguish from the

surrounding phloem tissue, appear as numerous white streaks (Figure 6). Microscopic examination reveals heavy accumulations of fungal mycelium and dark reddish-brown materials in the parenchyma tissue (Figure 8B). Cross-sections of lightly-"bleached" sclereids reveal bore holes running longitudinally within the thick walls of the sclereids (Figure 7). This type of growth in cell walls is characteristic of soft-rot fungi as described by Duncan (1960; Figure 3C). As decay progresses, sclereid walls become "bleached" white and are easily crushed under pressure as if the cementing lignin component of the walls had been degraded. "Bleached" sclereids fail to stain with Wiesner reagent. Thus, the final appearance of attacked sclereids more closely resembles white-rotted tissue (Figure 9), because typical soft-rotted wood usually resembles brown-rot decay in external appearance (Duncan, 1960; Figures 7 and 9). In advanced stages of decay, the secondary walls of sclereids were completely decomposed.

Condensed tannins in lumina of "bleached" sclereids and encrusting parenchyma cell walls and protoplasts are darker in color in infested tissue than in normal tissue (Figure 1D vs 1E; Figures 7 and 8 vs 3). Although these condensed tannins are darkened, they are not substantially removed from the tissue.

Thick green-black hyphae are often concentrated along periderm layers surrounding infested phloem tissue. As these hyphae penetrate

Figure 6. A split piece of infested outer-bark phloem containing white ("bleached") sclereids and darkened parenchyma tissue. (magn. approx. 45X).

Figure 7. Cross-section of bark containing "bleached" sclereids. (A) Condensed tannins in parenchyma tissue have been darkened. (B) Bore holes are oriented longitudinally within thick sclereid walls. (magn. approx. 330X).

Figure 8. Longitudinal section of bark containing "bleached" sclereids. (A) Degraded sclereid walls. (B) Parenchyma cells containing darkened residual protoplasts and fungal hyphae. (magn. approx. 200X).

Figure 9. A "bleached" sclereid. (A) The condensed tannins of sclereid lumina are darkened but not removed. (B) The nature of residual, "bleached" wall material. (magn. approx. 430X).


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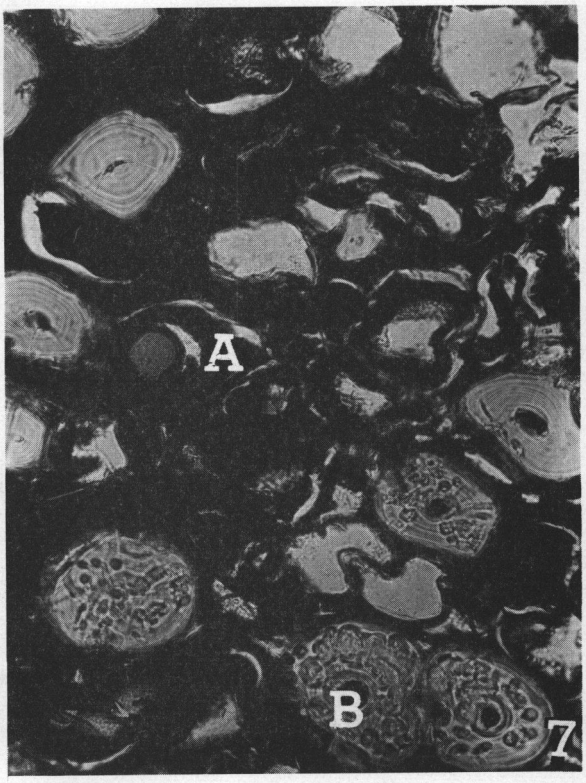

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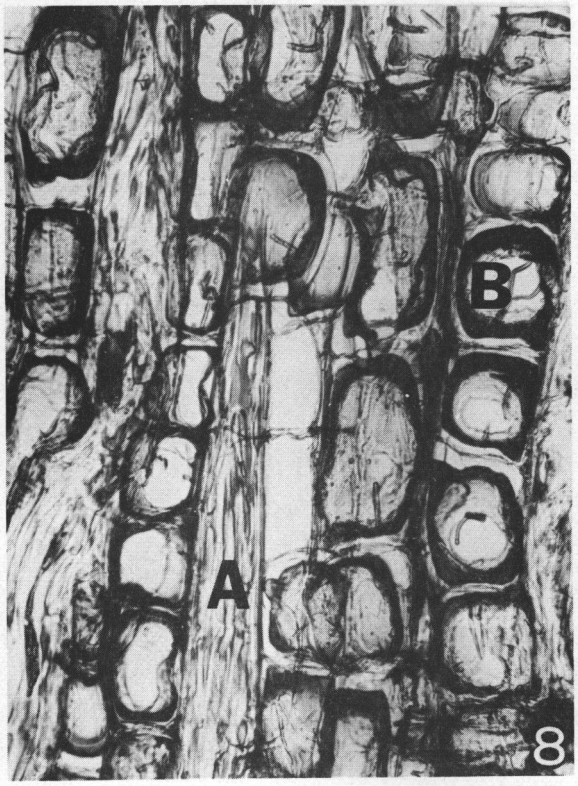

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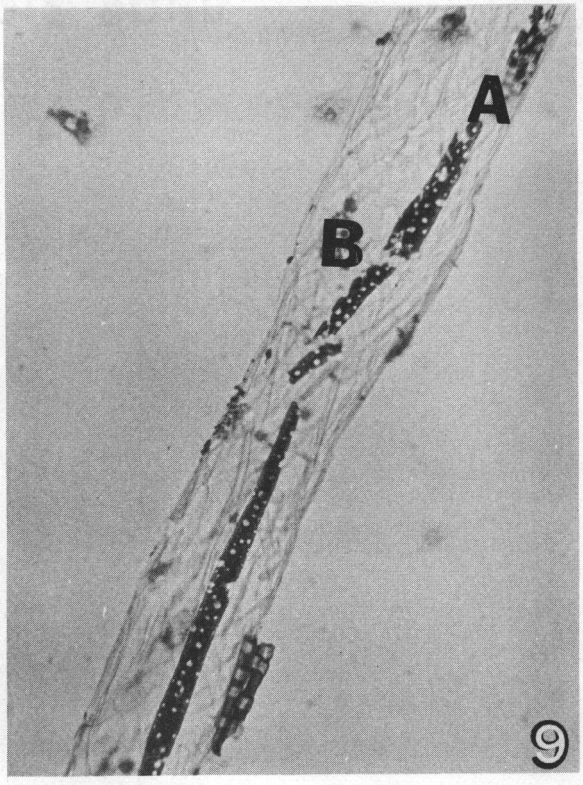

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the periderm layers, such layers change in color from light tan to dull gray.

Movement of the decay fungus (or fungi) is delayed by periderm layers, which are apparently only slowly penetrated. This is shown in Figure 1 where light is reflected from the ends of phloem sclereids at D, but not at E. Microscopic examination of areas such as E showed that the sclereids were nearly completely decayed. Where phloem tissue was exposed along the edges of the large central fissure shown in Figure 1, heavy sclereid degradation occurred up to the first intact periderms. However, beyond these periderms sclereids often were relatively undamaged (Figure 1).

In an attempt to identify an organism capable of producing the "bleached" effect, numerous isolations were made from infected bark. Individual "bleached" sclereids were picked out with fine-tipped forceps and planted on a variety of solid media. Greenish-black mycelium of Bispora betulina (Cda.) Hughes emerged from cultured sclereids much more frequently than any other microorganism[3] (Table 1). Not only did 62 percent of the isolation from infected bark give rise to B. betulina, but greenish-black mycelium similar to that of the fungus was observed within walls of "bleached" sclereids.

Cultures of B. betulina were used to inoculate numerous sterile,

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[3] The author is indebted to Dr. C. J. K. Wang for verifying the identity of the fungus as Bispora betulina.

Table 1. Isolation Frequencies of B. betulina and Other Fungi from Bark Containing "Bleached" Sclereids.

Culture Medium	Number of Isolates Which Produced B. betulina	Total Number of Isolates Counted
Inner-bark extr.	14	32
Water agar	5	5
Cornmeal-malt-yeast extr.	2	5
Tannic acid[1]	2	4
Gallic acid[1]	4	5
Inner-bark extr.	21	24
Fusarium medium[2]	17	33
Gottliebs' medium[3]	22	30
Fåraeus' Bf-2[4]		
Potato-dextrose-agar	5	6
Total	110	177

[1] Davidson, Bampbell and Blaisdell (1938).

[2] Domsch and Corden (1970).

[3] Gottlieb, Day and Pelczar (1950).

[4] Fåhraeus and Tullander (1956).

whole bark samples and isolated sclereid fractions. Although a number of different temperature and moisture conditions were used, typical symptoms of sclereid decay were not obtained in culture. B. betulina did invade bark tissue in culture and cause darkening of cork and parenchyma cells typical of tissues accompanying "bleached" sclereids.

Modified Bark--Description of Tissue Containing "Bleached" Parenchyma and "Emptied" Sclereids

A second modification of bark cells involves removal of

of condensed tannins from parenchyma cell walls and protoplasts and from outer wall layers and lumina of sclereids. While this type of bark degradation was observed in trees growing in a number of widely-scattered locations, it appears to be most prevalent in slow-growing trees on poor sites or in suppressed trees on better sites. It occurs in much younger trees than sclereid "bleaching," and generally extends over most of the length of infested boles. "Bleached" parenchyma cells are found immediately under the outside cortical tissues of the bark. Where advanced parenchyma degradation is exposed at the surface of the bark, loose sclereids are often seen lying free from the surrounding remains of parenchyma tissue.

Tissues with tannin degradation present a picture which is nearly the negative of that given by tissues containing "bleached" sclereids. In the former case the parenchyma cell walls are white and the sclereids are tan, while in the latter case the sclereids are "bleached" white and the parenchyma is dark reddish-brown (Figures 6 and 10). Microscopic examination shows that not only are tannins removed from parenchyma tissues and from outer walls of sclereids, but bore holes occur in the thick sclereid walls and tannins are removed from sclereid lumina as well (Figure 11).

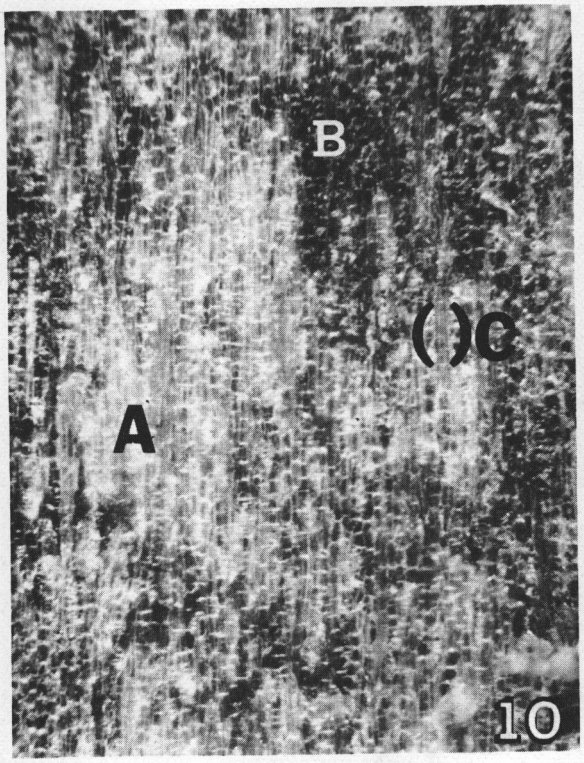
Condensed tannin is removed from the outer cell wall layers of sclereids found in infected tissue. Isolated sclereid preparations from this tissue are light tan in color, as is characteristic of inner

Figure 10. Outer-bark tissue containing "bleached" parenchyma cells. (A) Parenchyma cells appear to have had reddish-brown condensed tannins removed by fungal activity. (B) "Unbleached," but infested, tissue is slightly darker than uninfested tissue. (C) The sclereid within the parentheses appears light tan in color because tannins have been removed from the outer wall layer. (magn. approx. 45X).

Figure 11. Phloem tissue with "bleached" parenchyma cells. (A) A sclereid which has not yet had its lumen emptied by a fungus. (B) Sclereids with numerous bore holes in their walls and empty lumina. (C) Colorless, "bleached" parenchyma cells. (magn. 80X).

Figure 12. A typical colony of the Isaria-like fungus growing on PDA medium. (magn. approx. 5X).

Figure 13. Hyphae of the Isaria-like fungus grown on PDA. Note the characteristic spherical inclusions in the hyphae. (magn. 550X).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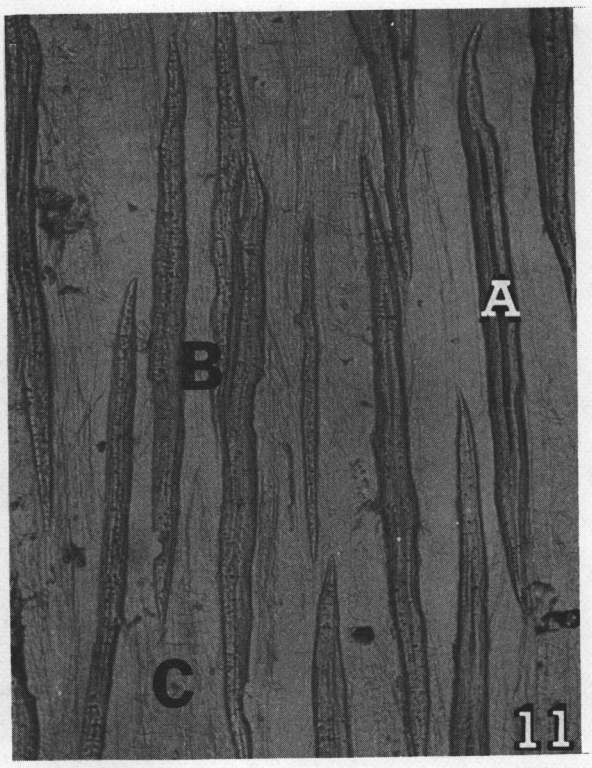
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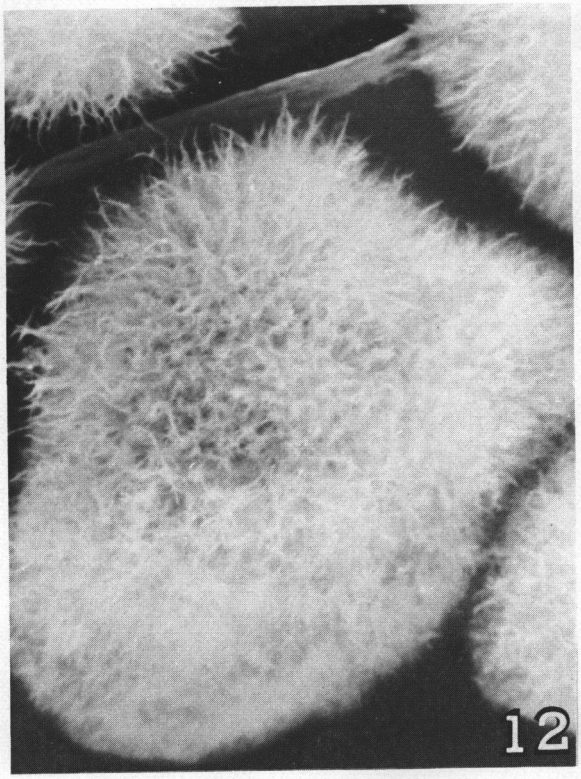
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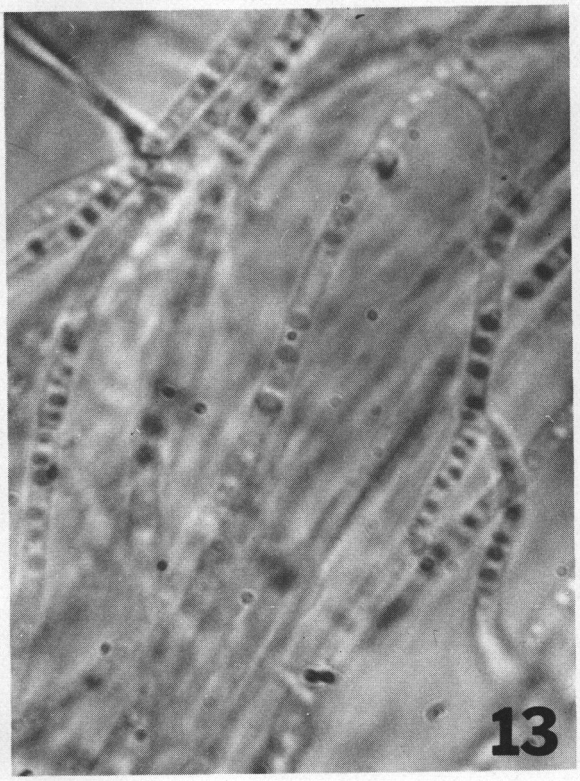
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bark sclereids, rather than the typical reddish-brown of normal outer bark sclereids.

Cell walls of "emptied" sclereids (Figure 11) stain brilliant red-violet with Wiesner reagent, while any portions of sclereid walls showing "bleaching" fail to stain with this reagent (Figures 6 and 7). This indicates that, where tannin degradation occurs, its removal is highly specific with little or no degradation of sclereid lignin.

Because tannin degradation occurs superficially in bark, a large number of microorganisms are present at the site of parenchyma "bleaching." Thus, no one organism dominantly outnumbers all others as is the case with B. betulina where sclereid "bleaching" occurs.

Either individual sclereids or very small groups of "bleached" parenchyma cells were aseptically planted on nutrient media, and frequencies with which different fungi grew from the tissues were determined.

A white, synnematous imperfect fungus was present in 13 of 45 isolations which produced fungi. The next most prevalent fungus appeared only six times. Isolations were from eight different trees growing in three different localities, and the white imperfect fungus was present at all three localities.

Fresh cultures grown on PDA formed fluffy tufts of white synnemata (Figure 12). Many seta-like, white hyphae protruded

laterally from the main axis of each synnema. The septate hyphae often contained numerous small hyaline, spherical inclusions (Figure 13). Older cultures grown on PDA in test tubes developed brown pigmentation and short, thick synnemata. Conidia were not seen attached to the hyphae, but on the basis of a limited number of detached, spherical spores and the general characteristics of the mycelium, the fungus best fit into the ill-defined form genus, Isaria (Fries) (Barnett, 1960; Barron, 1968; Morris, 1963). This fungus is referred to here as an Isaria-like fungus.

Sterile pieces of outer bark were placed into petri dishes containing silica gel dampened with water, and the bark was inoculated with the Isaria-like fungus. After six months, hyphae heavily colonized the bark and penetrated several sclereid cell walls. However, no parenchyma "bleaching" or removal of tannins from sclereid lumina was noted.

Both B. betulina and the Isaria-like fungus caused rapid precipitation of the inner-bark extract medium.

From microscopic and histochemical examination of these two types of phenolic degradation, it appears that within phloem tissue B. betulina is associated with degradation of lignified sclereid walls with some darkening, but no substantial removal of condensed tannins. The Isaria-like fungus is associated with removal of substantial amounts of condensed tannins from phloem tissue, but, besides tiny bore holes,

does not degrade lignified sclereid walls. This implies different and quite specific enzyme systems for the degradation of lignin versus condensed tannins.

Chemical Analysis of Normal and Infested Sclereids

Reason for Limiting the Analyses to Sclereids

Limited chemical analyses were made of the two types of "bleached" tissue to support the anatomical and histochemical evidence which suggested that fungi were removing phenolic components from phloem tissue.

Because of the complex and highly variable structure of the outer bark, a common cell type was used for comparative analyses rather than the whole bark. Chemical composition of isolated sclereid fractions from normal and decayed bark were compared because: 1) sclereids may be separated from bark in a form fairly free of other cell types, 2) sclereids are the only regularly-lignified cells in the bark, that contain condensed tannins in their lumina and outer wall layers, and 3) the two types of phenolic degradation to be studied occur in the sclereid fraction.

Determination of Total Phenolic Content of Sclereids

Normal inner-bark sclereids, normal outer-bark sclereids,

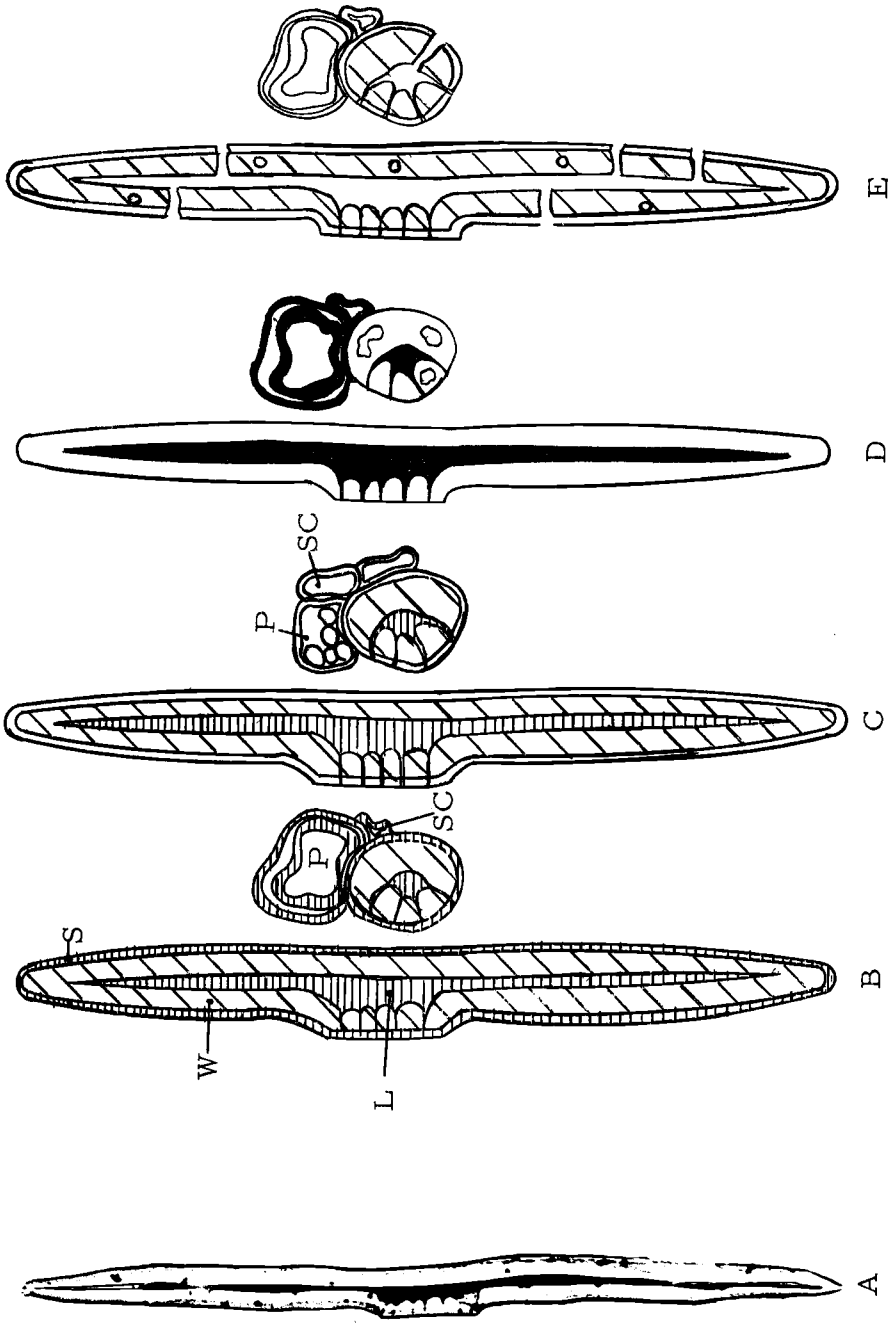
Figure 14. Diagrammatic representation of phenolic contents of normal and decayed sclereids and parenchyma cells. A. A photograph of an isolated sclereid from normal inner-bark phloem. B-E. Diagrams representing longitudinal and cross-sectional views of four sclereid types and accompanying parenchyma and sieve cells. S = Surface phenols in the outer layer of sclereid walls (condensed tannin). W = Wall phenols in thick secondary walls (lignin). L = Lumen phenols (condensed tannin).

B. Normal outer-bark showing light colored condensed tannins, dilated parenchyma (P) and crushed sieve cell (SC). Sclereid contains S + W + L phenols.

C. Normal inner-bark showing lack of S phenols on parenchyma and sclereid walls. Parenchyma are small, living, contain starch, and sieve cells not yet crushed. Sclereid contains W + L phenols.

D. "Bleached" outer-bark sclereid showing darkened L phenols and lack of outer-wall layer. Parenchyma darkened. Sclereid has L + an unknown amount of W phenols.

E. "Emptied" sclereid showing lack of S and L phenols and lack of tannin in parenchyma cells as well. Bore holes appear in cell walls. Sclereid contains only W phenols.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"bleached" sclereids (lignin degradation suspected), and "emptied" sclereids (condensed tannin removed from lumina and outer wall layer suspected (Figure 14) were analyzed for total phenolic content (Table 2).

Table 2. Total Phenolic Content of Sclereids Determined by Weight Loss Resulting from Acidic Sodium Chlorite Bleaching.

Type of Sclereid		Equation for Source of Phenolic Materials		Weight Loss in Percent
Normal outer bark	1	$S + W + L$[1]	=	41.8
Normal inner bark	2	$W + L$	=	31.8
Walls bleached (96%)[2]	3	$3.04S + W$[4] $+ L$	=	18.8
Lumina emptied (52%)[3]	4	$W + .48L$	=	26.3

[1] S = Phenolic materials on the surfaces of sclereids.
W = Phenolic materials in the secondary walls of sclereids.
L = Phenolic materials in the lumina of sclereids.

[2] By microscopic examination of 200 sclereids it was determined that 96% of this sample had "bleached" walls.

[3] By microscopic examination of 200 sclereids it was determined that 52% of this sample had "empty" lumina.

[4] The amount of W (wall) phenols in "bleached" sclereids is unknown.

Condensed tannins are lacking in outer surface layers of inner-bark sclereids but present in such layers of normal outer-bark sclereids, while sclereids from tissue showing "bleached" parenchyma lack condensed tannins both from their lumina and outer surface layers. All three types of sclereids contain lignin in their thick secondary walls. Thus, by determining differences in phenolic

contents of each sclereid type, it is possible to estimate the amounts of phenols in surface layers, secondary walls and lumina of these cells.

Sclereids were bleached to a minimum brightness of 76 (see methods) using the chlorite method because it was felt that phenolic content had been reduced to a very low level at this point. Although much longer treatment might have reduced phenolic levels even lower, it was feared that carbohydrate losses might occur. Bleaching to a brightness of 76 required 12 treatments over six hours for normal outer-bark sclereids (final brightness 76), but only four treatments over two hours for "bleached" sclereids (final brightness 97). Holocellulose from "bleached" sclereids gave no detectable stain with ferric chloride-potassium ferricyanide (Barton, Evans and Gardner, 1952), and the other three types of sclereids gave very faint green colors after 20 minutes in the stain. Microscopic examination showed no signs of tannin material in lumina of any chlorite-bleached sclereids.

To facilitate discussion, the phenolic components are referred to as the surface phenols (S), wall phenols (W) and the lumen phenols (L) (Figure 14).

Normal outer-bark sclereids contain surface, wall and lumen phenols while normal inner bark sclereids contain only wall and lumen phenols. The sclereid preparation from tissue infested with the Isaria-like fungus showed 52 percent empty lumina "emptied sclereids) and were surrounded in the bark by parenchyma tissue which was

"bleached" white. Outer bark sclereids normally appear reddish-brown because of reddish-brown surface phenols (Figure 14B), but "emptied" sclereids are light tan because their surface phenols have been degraded (Figure 14E). Thus, this fraction (Figure 14E) is considered to have no surface phenols, only 48 percent lumen phenols (52 percent of the preparation had "empty" lumina) and 100 percent wall phenols.

The sclereid preparation associated with B. betulina ("bleached" sclereids) had 100 percent lumen phenols which were considerably darkened but not removed, an unknown amount of wall phenols and 4 percent surface phenols (96 percent of the walls lacked surface phenols and they appeared white).

By solving simultaneous equations 1, 2 and 4 (Table 2) it was estimated that S = 10.0 percent, W = 21.2 percent, and L = 10.6 percent of the dry weight of normal outer-bark sclereids. If S (surface) and L (lumen) phenols are condensed tannins and W (wall) phenol is wall lignin, then normal outer bark sclereids contain about 20 percent condensed tannins and 21 percent wall lignin. An earlier report gave the Klason lignin content for an "extractive-free" sclereid fraction as 44.8 percent before extraction with 1 percent sodium hydroxide and 22.8 percent after sodium hydroxide extraction (Kiefer and Kurth, 1953).

There are at least two ways in which sclereids may become

"bleached" white. Either the surface phenols have been removed from the outer wall layer or the entire outer wall layer is missing. If only the surface phenols were missing, one would expect a total phenolic content of about 30 percent (Table 2). Since the lumina of "bleached" sclereids remain packed with darkened tannins (Figures 9 and 14D) and since only 18.8 percent of the phenols were present in this fraction, wall phenols (lignin) must have been removed from the cells. If a time course study on phenolic removal from sclereids had been possible, the value 18.8 percent would probably have been even lower since it will be shown that carbohydrates have also been removed from the walls of "bleached" sclereids.

Since cells encrusted with condensed tannin are not "bleached" in the presence of "bleached" sclereids, it is unlikely that surface phenols were removed from outer layers to produce white sclereids. Evidence presented below will support the proposal that as thick secondary walls of sclereids become decayed, the bonds between these walls and the outer, tannin-encrusted surface layers are weakened to the point that the outer colored wall layers easily break away from residual, white secondary walls when bark is milled or broken (Figure 14B vs 14D).

Determination of Carbohydrate Content of Sclereid Walls

Microscopic examination revealed that cell walls of "bleached"

sclereids were heavily degraded (Figures 7-9), but, except for numerous tiny bore holes, the walls of "emptied" sclereids appeared normal and stained brilliantly with Wiesner reagent. Thus, only "bleached" sclereids and normal inner-bark sclereids were analyzed for carbohydrate content.

Since fungal degradation of sclereids could not be produced in culture, time course studies of carbohydrate loss were not run. Thus, ratios of five major sugars in sclereid walls were determined for normal inner-bark sclereids and compared with those for "bleached" outer-bark sclereids (Table 3).

Table 3. Ratios of the Amounts of Five Major Sugars of Cell Walls of Normal Inner-bark Sclereids and Biologically-"bleached" Outer-bark Sclereids.

Sugar	Normal Inner-bark Sclereids	"Bleached" Outer-bark Sclereids	Sugar Ratios for Bark Sclereids[1]
Glucose	1.00	1.00	1.00
Galactose	.06	.05	.09
Mannose	.29	.15	.16
Arabinose	.05	None detected	.00
Xylose	.15	.10	.28

[1] Determined by Kiefer and Kurth (1953).

Preliminary experiments using relatively mild hydrolytic procedures designed to reduce plant cell walls to component sugars (Albersheim et al., 1967; Saeman et al., 1954) gave incomplete hydrolysis of sclereid holocellulose. Thus, the comparatively harsh

procedure presented in the methods section was used to obtain a more complete sample of the cell wall sugars.

Hemicellulose sugars were depleted from the "bleached" sclereids more rapidly than cellulose (glucose). Arabinose was not detected from "bleached" sclereids even when the amount of hydrolysate applied to chromatograms was increased to three times the amount used for the other sugars. The ratios for mannose and xylose were almost exactly opposite those obtained from data of Kiefer and Kurth (1953) (Table 3). The experiment was repeated and the same relative results were obtained both times.

Judging from literature dealing with relative amounts of mannose and xylose in coniferous wood cells, it is logical that a fiber-like cell, such as a sclereid, would contain more mannose than xylose. This is because the main hemicelluloses in conifers are mannans which are about double the amount of xylan (e. g., Abies balsamea and Pinus strobus) (Timell, 1965). Although whole bark of Picea engelmanni contains 3.8 percent xylose compared with 2.9 percent mannose (Ramalingam and Timell, 1964), it must be remembered that whole bark is rich in parenchyma cells. Pirela (1961) found that rays of pine and spruce contained more xylan than mannan, but tracheids contained much more mannose than xylose. Since rays are rich in parenchyma, just as bark is, this could account for whole bark having more xylose than mannose. For determination of sugar content

of sclereid cell walls, Kiefer and Kurth (1953) used all sclereids passing 40-mesh Tyler screens, thus they probably had a high percentage of sclereid bundles containing parenchyma contamination. In the present study, only those sclereids passing 100-mesh screens following a cold-water wash were used, and very little parenchyma contamination was present. Differences in amount of parenchyma could account for the disagreement in sugar ratios.

The disappearance of arabinose from "bleached" sclereids was unexpected. Amounts of arabinose have been determined in middle lamellae, primary walls, S_1, S_2 and S_3 wall layers of tracheids of spruce and pine (Meier, 1961). Arabinose accounted for 31.7 and 30.8 percent of the middle lamella and primary wall of spruce and pine respectively, but only 2.0 and 3.2 percent of entire tracheid walls were arabinose. Consistent with high arabinose levels in primary walls, is the fact that whole bark of P. engelmanni (rich in thin-walled parenchyma cells) contained 3.3 percent arabinose compared to only 1.0 percent for the wood of the same species (Ramalingham and Timell, 1964). As mentioned earlier, "bleached" sclereids could appear white only if surface phenols were removed or the entire outer wall was removed. If the fungus which produced "bleaching" could use wall carbohydrates and lignin, but not condensed tannins, the organism would be restricted to the secondary wall which would become loosened from the outer, tannin-encrusted layer as decay progressed

(Figure 7B). When the bark was broken or milled such degraded white centers of sclereids would break away from surrounding outer walls which were protected from fungal attack by encrusting tannins. With the outer primary wall missing, low amounts of arabinose in the remainder of the wall were probably not detected because of sugar loss during acid hydrolysis.

Free Sugars in the Outer Bark

As mentioned above, starch and other cell inclusions disappear from parenchyma tissue at the time of periderm formation. Thus, few hot-water-soluble carbohydrates would be expected in the outer bark. Hot-water extracts from uninfested outer bark were concentrated and chromatographed on paper using the two solvents listed in the methods section. Arabinose and glucose were the only sugars detected and the intensity of the sugar spots with CD-1 spray reagent indicated that arabinose was considerably more concentrated than glucose. Manners (1965) detected arabinose and traces of glucose, rhamnose and xylose in hot-water extracts of Douglas-fir bark, but it was not clear if inner bark was included. Since arabinose is not a common storage sugar, possibly it was leached from hemicelluloses of the many expanded parenchyma cells of the outer bark and from primary walls of sclereids. B. betulina was incubated in Bf-2 medium containing 400 ppm tannin extract and various ratios of sucrose and

arabinose. Growth of the fungus was not stimulated in the presence of arabinose.

Alteration of Phenolic Substances by B. betulina and the Isaria-like Fungus

Any of several chemical differences between lignin and condensed tannins might account for the specificity with which parenchyma and sclereids are "bleached." Lignin is rich in methoxylated guaiacyl units (Figure 5), which, after demethoxylation, have exclusively an ortho hydroxylation pattern. Condensed tannins are composed mainly of non-methoxylated flavanoid compounds, and, while the B-rings of these flavanoids may have ortho hydroxylation patterns, the A-rings have meta hydroxylation patterns (Figure 6). Thus, organisms which utilize lignin should be able to alter guaiacyl compounds and ortho-hydroxylated products thereof, but organisms which utilize condensed tannins would be expected to alter both ortho- and meta-hydroxylated phenols, but not necessarily guaiacyl compounds.

Alteration of Lignin-related Compounds by the Fungi

Two types of experiments were run to test the ability of B. betulina to alter lignin-related compounds, but only one type of experiment was run with the Isaria-like fungus.

To determine if B. betulina could utilize lignin-related

compounds as sole carbon sources, this fungus was incubated in mineral salts[4] along with ferulic acid, vanillin or p-hydroxybenzaldehyde for five months. Ability to utilize these phenolic compounds was determined by gain in dry weight of mycelium (Table 4).

Table 4. Mycelium Dry Weights of Bispora betulina Grown Five Months in Liquid Medium Containing Mineral Salts Plus Vanillin, p-Hydroxybenzaldehyde or Ferulic Acid as the Sole Carbon Source.[1]

Carbon Source	Concentration of Carbon Source (ppm)	Mycelial Dry Weights (mg[2])
None		4.4
Vanillin	300	9.1
	100	12.6
	50	7.6
p-Hydroxybenzaldehyde	300	19.3
	100	12.0
	50	11.7
Ferulic Acid	300	18.0
	100	18.7
	50	24 2

[1]Salts were those used by Domsch and Corden (1970) except that NH_4NO_3 (5 g/l) was used in place of KNO_3.

[2]Each value is an average of two cultures.

Further experiments included both B. betulina and the Isaria-like fungus and ferulic acid, vanillin, vanillic acid and protocatechuic acid

[4]Mineral salts were those used by Domsch and Corden (1970) except that NH_4NO_3 (5 g/l) was used in place of KNO_3.

were used as substrates in modified Bf-2 medium, containing 0.2 percent sucrose. This series of phenols was chosen because it represents a logical pathway to opening of the benzene ring for lignin breakdown products (Haider, Lim and Flaig, 1964). Ability to alter these phenols was determined by spectrophotometrically measuring disappearance of the compounds from culture media with time (Table 5).

The Isaria-like fungus altered all of the lignin-related phenols. Cultures containing vanillin, vanillic acid and protocatechuic acid became brown and the ferulic acid culture produced a pink-white precipitate after two days with the Isaria-like fungus. No secondary products were noted when aliquots of the media (150-200 µl) were chromatographed on silica gel G using solvents 1, 2 and 3, but a reddish-brown material failed to move from the origins of chromatograms spotted with cultures of protocatechuic acid, vanillin and vanillic acid.

As expected, ferulic acid and vanillin were altered by B. betulina, but at much slower rates than when these phenols were cultured with the Isaria-like fungus (Table 5). However, growth was very slow in the presence of protocatechuic acid and vanillic acid at the concentrations used, and decrease in concentration of these phenols was not significantly different from controls. A long-term experiment using very low concentrations of the latter two phenols

Table 5. Summary of Changes in Concentrations of Lignin-related Phenols Cultured with B. betulina or the Isaria-like Fungus.

Compound	Fungus	First Time for Phenol Measurement	Concentration (ppm)	Last Time for Phenol Measurement	Concentration (ppm)
Vanillin	Isaria	3 days	264	17 days	96
	Bispora	3	320	17	189
	Control	3	368	17	301
Vanillic acid	Isaria	3 days	365	17 days	155
	Control	3	464	17	341
	Bispora	0	363	17	378
	Control	0	348	17	360
Ferulic acid	Isaria	3 days	152	17 days	67
	Control	3	333	17	277
	Bispora	0	300	17	177
	Control	0	360	17	321
Protocatechuic acid	Isaria	2 days	176	17 days	101
	Control	2	323	17	288
	Bispora	0	312	17	150
	Control	0	306	17	186

would be needed to determine if B. betulina could metabolize them. No secondary products were noted when aliquots of medium (150-200 µl) were chromatographed, but vanillic acid cultures turned light yellow.

Alteration of Tannin-Related Compounds by the Fungi

Both bark fungi were tested for their abilities to alter the tannin-related compounds, catechin and phloroglucinol. Ultraviolet absorbance for these compounds was low at the concentrations used so ability to alter the compounds was estimated from thin-layer chromatograms.

After two days incubation with the Isaria-like fungus, the catechin medium contained considerable brown precipitate, and after five days, the precipitate nearly covered the bottom of the flasks. Cultures of B. betulina with catechin showed no change after two days, were yellow after five days and were quite brown after 17 days. The controls were yellow after 17 days. After 17 days unsprayed chromatograms showed a dark gray streak for the control, a slightly lighter streak for B. betulina and no detectable gray for the Isaria-like fungus. Spraying with diazotized sulfanillic acid indicated yellow streaks for control and B. betulina cultures but barely-discernible streaks from cultures of the Isaria-like fungus.

The abilities of the two fungi to alter phloroglucinol were

strikingly different (Figure 15); the Isaria-like fungus removed it almost completely from the medium, but B. betulina showed no ability to modify the compound. A third fungus, an unidentified synnematous imperfect isolated from the same bark tissue as the Isaria-like fungus, was included in the experiment because it produced many products from phloroglucinol (Figure 15). In contrast, the Isaria-like fungus produced no spots detectable with ultraviolet light, ferric chloride-potassium ferricyanide or diazotized sulfanillic acid, other than a barely-detectable spot opposite phloroglucinol with the ferric chloride-potassium ferricyanide spray (Figure 15).

B. betulina grew better in phloroglucinol medium than in other phenolic media (media also contained 0.2 percent sucrose), but altered very little if any phloroglucinol as judged by the intensity of spots on the chromatograms after 35 days incubation (Figure 15). B. betulina also grew in the presence of resorcinol, but showed no significant reduction of the compound when compared with control cultures.

It would not be necessary for the Isaria-like fungus to alter guaiacyl compounds such as ferulic acid, vanillin and vanillic acid to utilize condensed tannins, but ability to alter the ortho-hydroxylated protocatechuic acid (similar to B-ring of flavanoids) as well as meta-hydroxylated phloroglucinol (similar to A-ring of flavanoids) would probably be required for this fungus to utilize condensed tannins. The

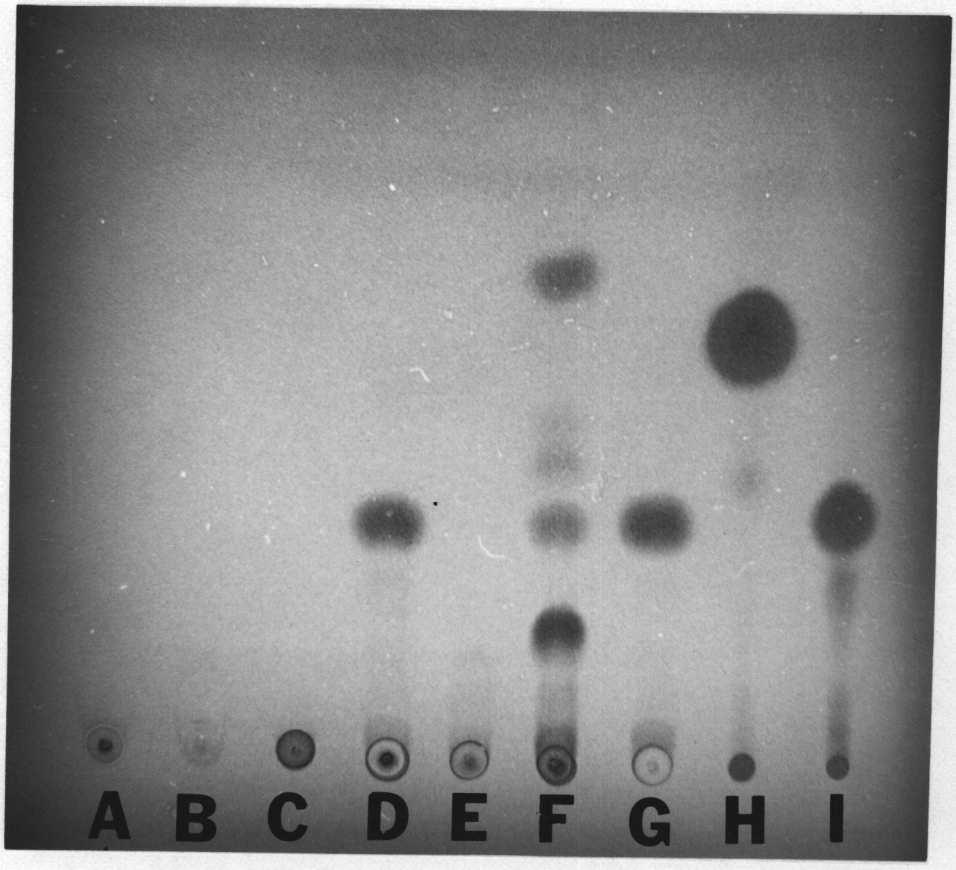

Figure 15. A thin-layer chromatogram of aliquots (50 µl) of media containing phloroglucinol in which B. betulina, the Isaria-like fungus and a second unidentified fungus from parenchyma-"bleached" tissue were growing for 35 days. A. B. betulina in phenolic-free medium. B. The Isaria-like fungus in phenolic-free medium. C. Unidentified fungus from parenchyma-"bleached" tissue in phenolic-free medium. D. B. betulina in phloroglucinol medium. E. The Isaria-like fungus in phloroglucinol medium. F. Unidentified fungus from parenchyma-"bleached" tissue in phloroglucinol medium. G. Sterile control culture containing phloroglucinol. H. Authentic resorcinol. I. Authentic phloroglucinol.

ability of the Isaria-like fungus similarly to use the guaiacyl compounds is not inconsistent with the contention that the Isaria-like fungus does not use lignin. Failure of this fungus to use lignin could lie in a limited ability to cope with such lignin bonds as β-aryl ether linkages, biphenyl linkages, etc.

On the other hand, B. betulina did not appear to significantly alter phloroglucinol or resorcinol, but could use ferulic acid, vanillin and p-hydroxybenzaldehyde as sole carbon sources. Inability to degrade meta-hydroxylated phenols would limit B. betulina in its use of tannins.

Although B. betulina did not appear to substantially alter protocatechuic acid (an ortho-hydroxylated phenol related to the B-ring of flavanoids), the concentration of this compound used was somewhat inhibitory making this result inconclusive (vanillin is also inhibitory at higher concentrations). Since the fungus could utilize vanillin as a sole carbon source, and protocatechuic acid is thought to be involved in the pathway to ring opening (Haider et al., 1964), B. betulina probably can alter this ortho-hydroxylated phenol at lower concentrations. It is possible that darkening of catechin by this fungus involves oxidative quinone polymerization (Hathway and Seakins, 1957) which could also account for darkening, but not removal, of condensed tannins in tissues containing "bleached" sclereids.

Incubation of Fungi with an Extract Containing Condensed Tannins

The capabilities of both fungi to alter a medium rich in condensed tannins of outer bark was studied. The object was to extract a fraction from the outer bark phloem which would be rich in the reddish-brown condensed tannins, but relatively free from the more highly methylated cell wall lignin found in the sclereids. Thus, 100 g of outer-bark fines from carefully-selected, unmodified outer-bark phloem tissue were shaken about 12 hours on a rotary shaker with 400 ml of absolute methanol. Solids were removed from the methanol extract by filtration with Whatman no. 1 filter paper followed by centrifugation, and the soluble extract was taken to dryness in a rotary evaporator at 42° C.

Modified Bf-2 medium (one-half strength with 2 percent sucrose) was used with 160 mg of the methanol extract in 100 ml of medium in 500-ml Erlenmeyer flasks. For still cultures, mats of the Isaria-like fungus that had grown 50 days in still culture with phenolic-free Bf-2 medium and B. betulina mycelium that had been shaken 36 days in Bf-2 medium containing 400 ppm outer-bark methanol extract were used as inoculum.

After five days of shaking, colonies of the Isaria-like fungus were caked with a brown precipitate, but many slightly-darkened particles of the extract remained in suspension, and the soluble

medium was light brown. B. betulina cultures looked nearly like the controls after five days. In control cultures the extract appeared as a light rose-colored precipitate in yellow-brown solution.

When mycelium of the Isaria-like fungus was harvested from the above cultures and recultured in fresh methanol-extract medium, the fungus caused complete clearing of the medium in less than 24 hours and the mycelial mats were heavily caked with a dull brown precipitate. The liquid portion of the cultures was clear and colorless. Sterile blocks of bark (five pieces approximately 0.5 x 0.5 x 0.25 inches) placed in the same medium were completely covered by the mycelium after seven days when the liquid portion of the medium turned clear and colorless. The hyphae eventually penetrated sclereid walls, but no "bleaching" of parenchyma or emptying of sclereid lumina was noted.

After two to three weeks, the white mycelium of the Isaria-like fungus penetrated the brown precipitate which covered the mycelium and grew out from it, leaving brown deposits of precipitate within the fungal colonies. Hurst and Burges (1967) indicate that caking of precipitate on fungal mycelium growing in humic acid media is quite common, and thus disappearance of humic acid (or tannins) from solution does not necessarily indicate that the tannins were utilized. They also found that prominant carboxyl absorption normally shown in infrared spectra of humic acids was reduced considerably in such

precipitated material. The precipitate was not analyzed in the present study.

After one month, much of the bark extract was converted to a dark brown precipitate by B. betulina, but considerable color remained in solution. The controls appeared unchanged.

The same types of changes occurred in still cultures; much slower in the case of the Isaria-like fungus, but at about the same rate for B. betulina. Thus, it appears that the Isaria-like fungus has the capability to rapidly and substantially alter condensed tannins, although actual utilization was not proven. Because the media were cleared so much more rapidly by cultures pregrown with methanol extract of outer-bark phloem, it appears that enzymes involved in the changes may have been induced to high levels during the initial incubation with the tannins.

Cell-free Enzyme Reactions from the Isaria-like Fungus

Because the Isaria-like fungus so rapidly precipitated methanol extracts, it was suspected that high levels of extracellular enzymes were present in the medium. Cell-free culture filtrates were prepared from tannin cultures in hopes of detecting breakdown products of phenolic compounds which had been undetectable in the presence of the fungus. Mycelium of the Isaria-like fungus was filtered from seven-day-old cultures in Bf-2 medium containing

condensed tannins, and the filtrate was sterilized using a Millipore HA filter (0.45 μ). To six ml of catechin, ferulic acid, phloroglucinol, vanillin or vanillic acid in 0.2 M acetate buffer (pH 5.0) were added 18.0 ml of culture filtrate to give a final substrate concentration of 5×10^{-3} M. Reaction mixtures were incubated at room temperature in the dark for 24 hours. Controls using boiled culture filtrates were also incubated. After 24 hours all reaction mixtures were adjusted to pH 2.0 with hydrochloric acid and were partitioned two times with diethyl ether. Ether fractions were taken to dryness under vacuum, resuspended in small volumes of ethanol (95 percent), chromatographed on silica gel and developed with solvents 1, 2 and 3.

Unboiled reaction mixtures of ferulic acid turned a milky pink color while all other mixtures turned brown. Boiled reaction mixtures did not change. The only reaction mixture which produced detectable amounts of breakdown products was that containing ferulic acid. Ether extracts of this mixture produced five spots, including the origin and the ferulic acid spot with solvent 2, and six spots in solvents 1 and 3. The extract was streaked onto silica gel plates, and after development with solvents 1 and 2 all streaks were removed from the plates, extracted in ethanol (95 percent) and ultraviolet spectra were determined.

The spot opposite vanillin on chromatograms gave the same color reactions as vanillin with sprays 1 and 2. Ultraviolet spectra

were identical with those of vanillin giving well-defined peaks at 278 and 308 nm in ethanol (95 percent) and shifting to 352 nm in dilute potassium hydroxide in ethanol. Co-chromatography of vanillin with the extracted compound in solvents 1, 2 and 3 gave single spots on silica gel. No such spot was given with the boiled filtrates. Thus, the Isaria-like fungus probably cleaved the side chain of ferulic acid to give vanillin.

Incubation of B. betulina with Ball-milled Sclereid Lignin

Because B. betulina was so frequently associated with sclereid "bleaching" which seemed to involve a substantial degradation of lignified sclereid walls, the ability of this fungus to degrade isolated sclereid lignin was studied.

Ball-milled lignin was prepared from inner-bark sclereids and sapwood as described in the methods section. The ball-milled wood lignin was light tan, but the bark lignin was slightly darker with a faint rose-colored hue, suggesting slight contamination with tannin-like phenols. Both types of lignin gave similar dark violet colors with Wiesner reagent. Analytical data indicated that the bark lignin preparation was somewhat different than lignin from the sapwood (Table 6). Ultraviolet spectra of wood and bark lignins were slightly different (Figure 16). Infrared spectra were considerably different, especially in the region 1600 to 1700 cm^{-1} (Figure 17), but the spectra for

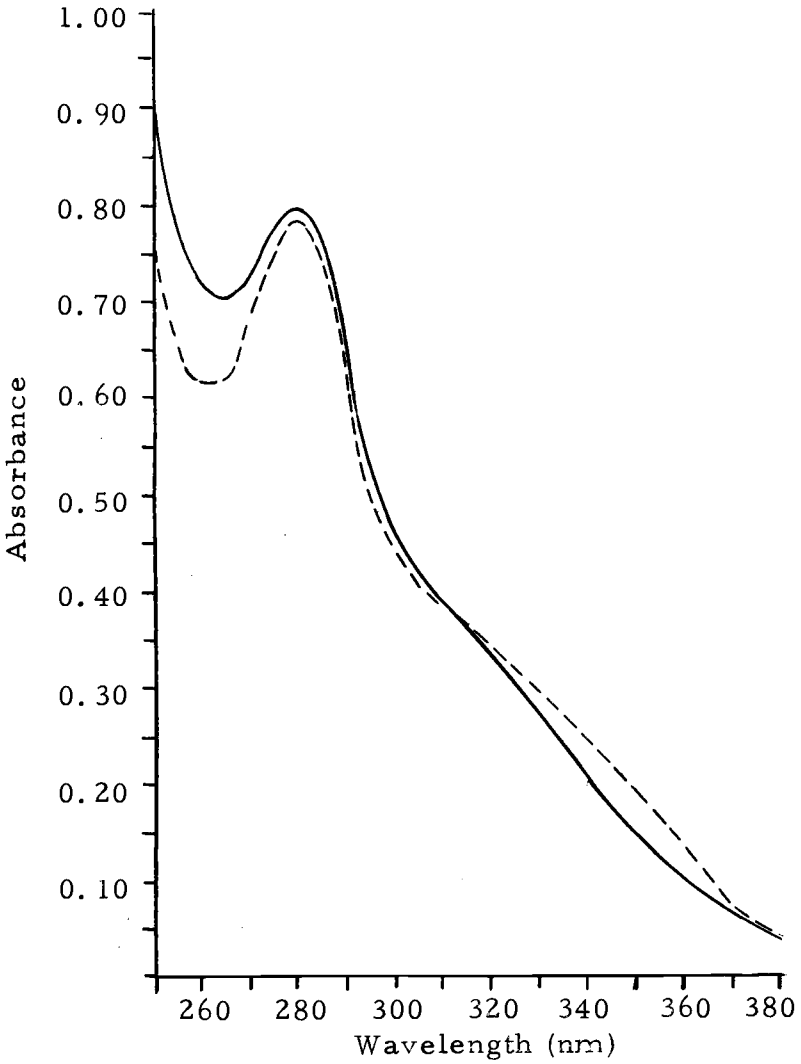

Figure 16. Absorption spectra for wood and bark lignin dissolved in dioxane-water (1:1). The solid line is for wood and the dashed line for bark lignin.

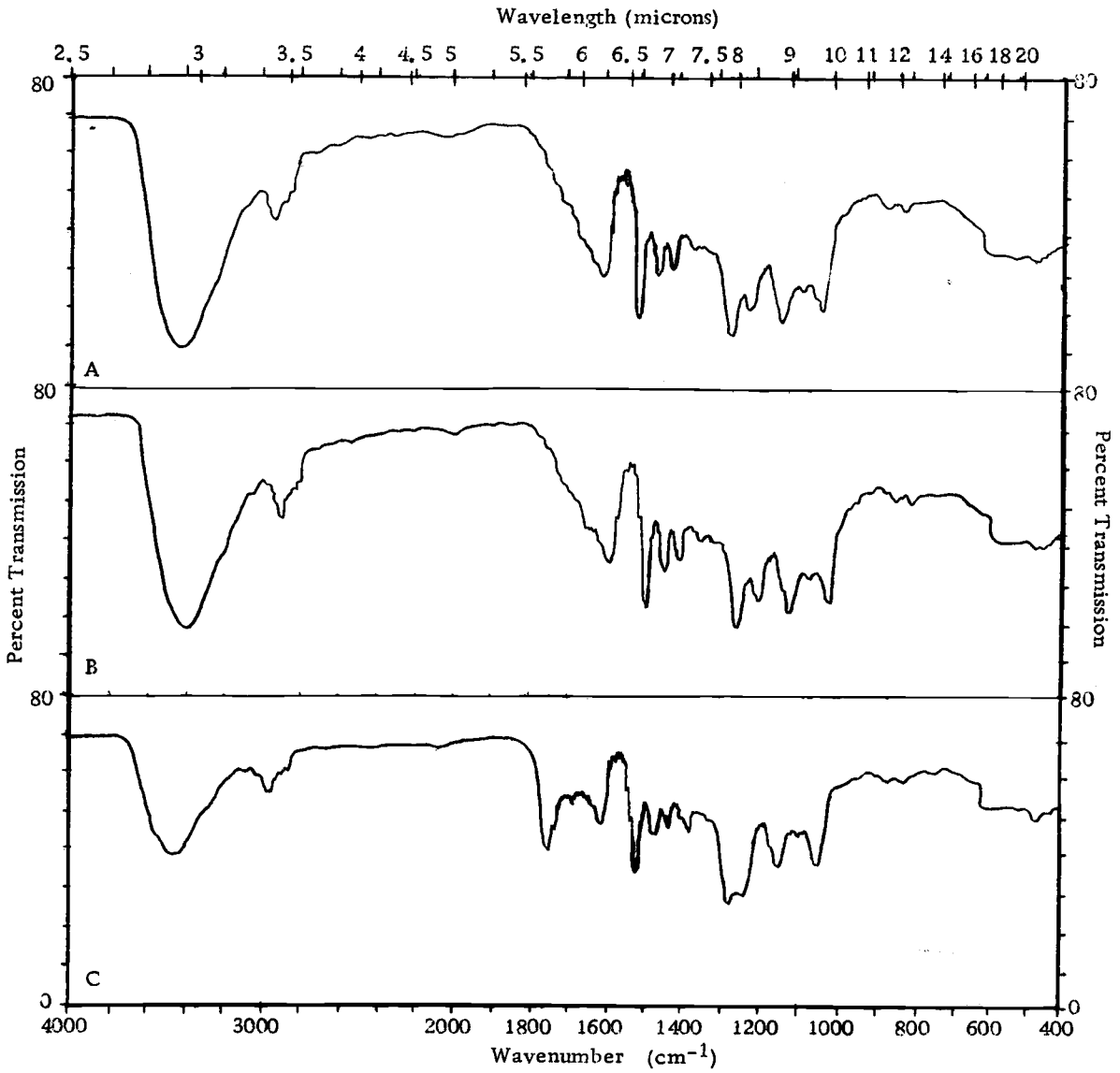

Figure 17. Infrared absorption spectra of bark and wood lignin taken from KBr pellets. A. Bark lignin sample A cultured with sterile medium. B. Bark lignin sample A cultured with B. betulina. C. Uncultured wood lignin.

(Thanks go to Joe Webber for producing the spectra.)

sclereid lignin (Figure 17A and B) were quite similar to infrared spectra shown for spruce lignins (Brown, Cowling and Falkehag, 1968) and for larch (wholewood) lignin (Sarkanen, Chang and Allan, 1967). As mentioned earlier, the infrared spectrum of the wood lignin control, which was thought to be from Douglas-fir, looked suspiciously like that of juvenile pinewood (see footnote page 19).

Table 6. Analytical Data for Ball-milled Lignins Prepared from Sapwood and Bark Sclereids.[1]

Type of Analysis[2]	Sapwood Lignin	Bar, Sclereid Lignin (Sa. B)
Carbon	61.41	59.40
Hydrogen	5.35	5.95
Oxygen	33.24[3]	34.41[4]
Methoxyl	14.55	13.79
Vanillin	-	17.85

[1]For description of sapwood see footnote page 19.

[2]Carbon, hydrogen, oxygen and methoxyl determined by Pascher and Pascher Mikroanalytisches Laboratorium, Bonn, West Germany.

[3]By difference.

[4]By analysis.

Sclereid lignin to be incubated with B. betulina was sterilized by saturating 0.5-g batches with ethanol (95 percent) and washing the saturated lignin into sterile 500-ml DeLong culture flasks with additional ethanol (about 30 ml). The flasks were placed under vacuum to remove most of the ethanol, and the partially-dried lignin

was covered with 100 ml of 0.5 strength Bf-2 medium containing 0.2 percent sucrose and 0.1 percent ammonium nitrate. B. betulina was added to the flasks as mycelial mats. Sterile lignin was incubated as controls. Inoculated cultures and sterile controls were incubated in the dark for two weeks on a shaker followed by four additional weeks as still cultures. During this time, B. betulina covered the entire surface of the lignin precipitate and appeared to darken it somewhat. After the incubation period, the solids were spun down by centrifugation and the lignin was extracted from them using 96 percent aqueous dioxane. The solubilized lignin was freeze-dried, taken up in 90 percent acetic acid, precipitated in water, washed three times with water and refreeze-dried. Lignin incubated with B. betulina was then compared to lignin incubated in sterile medium to see if B. betulina had degraded the lignin. The lignins were compared by determining carbon, hydrogen, oxygen, percent methoxyl, percent vanillin yield from nitrobenzene oxidation, infrared spectra and molecular weight distribution using gel permeation chromatography.

Previous work in which milled wood lignins were incubated in liquid culture with various white-rot fungi indicated that a number of changes in the lignin took place as a result of fungal activity (Ishikawa, Schubert and Nord, 1963). In this earlier work, percent methoxyl generally decreased with a corresponding increase in hydroxyl and decrease in vanillin yield following nitrobenzene oxidation. Carbon

and hydrogen contents generally dropped as the amount of carbonyl and carboxyl groups increased. Infrared spectra also revealed an increase in carbonyl and carboxyl absorption at about six μ (Ishikawa et al., 1963).

Based on types of changes in lignin composition described by Ishikawa et al., it appears that B. betulina failed to alter the bark lignin preparation significantly under the culture conditions used (Figure 17 and Table 7).

Table 7. Analysis of Bark Lignin (Sample A) Incubated with B. betulina for Six Weeks in Liquid Culture.

Types of Analysis	Lignin plus B. betulina	Lignin plus Sterile Medium
Carbon	59.36	60.41
Hydrogen	5.46	6.04
Oxygen[1]	35.18	33.82
Methoxyl	13.43	13.82
Vanillin	17.35	17.85

[1]By difference.

Although it appears from chemical analysis that B. betulina failed to alter the bark lignin significantly, efforts to detect changes in molecular size distribution of sclereid lignin which might have resulted from incubation with the fungus revealed some properties of sclereid lignin.

A column for determining molecular size distribution of lignin

samples was prepared with agarose beads of Bio-Gel A-15m, 50-100 mesh (Bio-Rad Laboratories) after the analytical column A of Kirk, Brown and Cowling (1969). Column dimensions were 1.5 x 60 cm and the dioxane was chromatoquality, 99^{+} mol percent (Matheson, Coleman and Bell). All solvents were mixed in a glove bag under nitrogen. The reservoir containing dioxane-water (1:1) was continuously flushed with prepurified nitrogen which had previously passed through an alkaline pyrogallol oxygen trap (Vogel, 1956). Glass tubing with polyethylene fittings carried the solvent from the reservoir to a Milton-Roy minipump which metered solvent to the column at a rate of 0.5 ml per minute. Five-ml fractions were collected, and lignin concentration was assumed to be directly proportional to absorbance at 280 nm (Kirk et al., 1969). Lignin recovered from control and fungal cultures was passed through the column described above.

There is some question as to whether distribution curves of molecular size for ball-milled lignin represent the true distribution of molecular size for native lignin as it occurs in unmilled cell walls (Brown et al., 1968). This is because ball-milling may fracture lignin molecules, and furthermore, all the lignin in cell walls was not extracted quantitatively by the methods used in this study (Björkman, 1956). However, such distribution curves should accurately represent the distribution of molecular size of the lignin samples used, and if B. betulina was capable of substantially altering the molecular size

of the sclereid lignin under the culture conditions used, it should have been detected by a shift in the molecular size distribution curve.

Some unexplained difficulties arose concerning the reproducibility of molecular size distribution curves. The first four sclereid lignin samples passed through the column (three samples are given in Figure 18A) all indicated a major peak between fractions 16 and 20 as compared to wood lignin, most of which passed through at the void volume. Control lignin (cultured with sterile medium) showed a peak at tube 8 which was not shown by either uncultured sclereid lignin or that cultured with B. betulina (Figure 18A). Control lignin was more slowly soluble in dioxane-water (1:1) than that cultured with the fungus, and possibly underwent some condensation under the culture conditions. Purified lignin cultured with the fungus was a light fluffy tan material slightly lighter than the control. However, in solution (dioxane-water, 1:1) the lignin was much darker than the control, and three distinct dark bands moved behind the main volume of the lignin on the column and emerged in tubes 22 and 25 (Figure 18A). This suggests that low molecular weight compounds were released from the lignin during incubation with the fungus. Unfortunately, all attempts to reproduce the patterns shown in Figure 18A failed. Instead, molecular size patterns for the sclereid lignin samples all shifted to the left (Figure 18B). Distributions in Figure 18B were reproducible as if all lignin samples had undergone some sort of condensation during storage in a

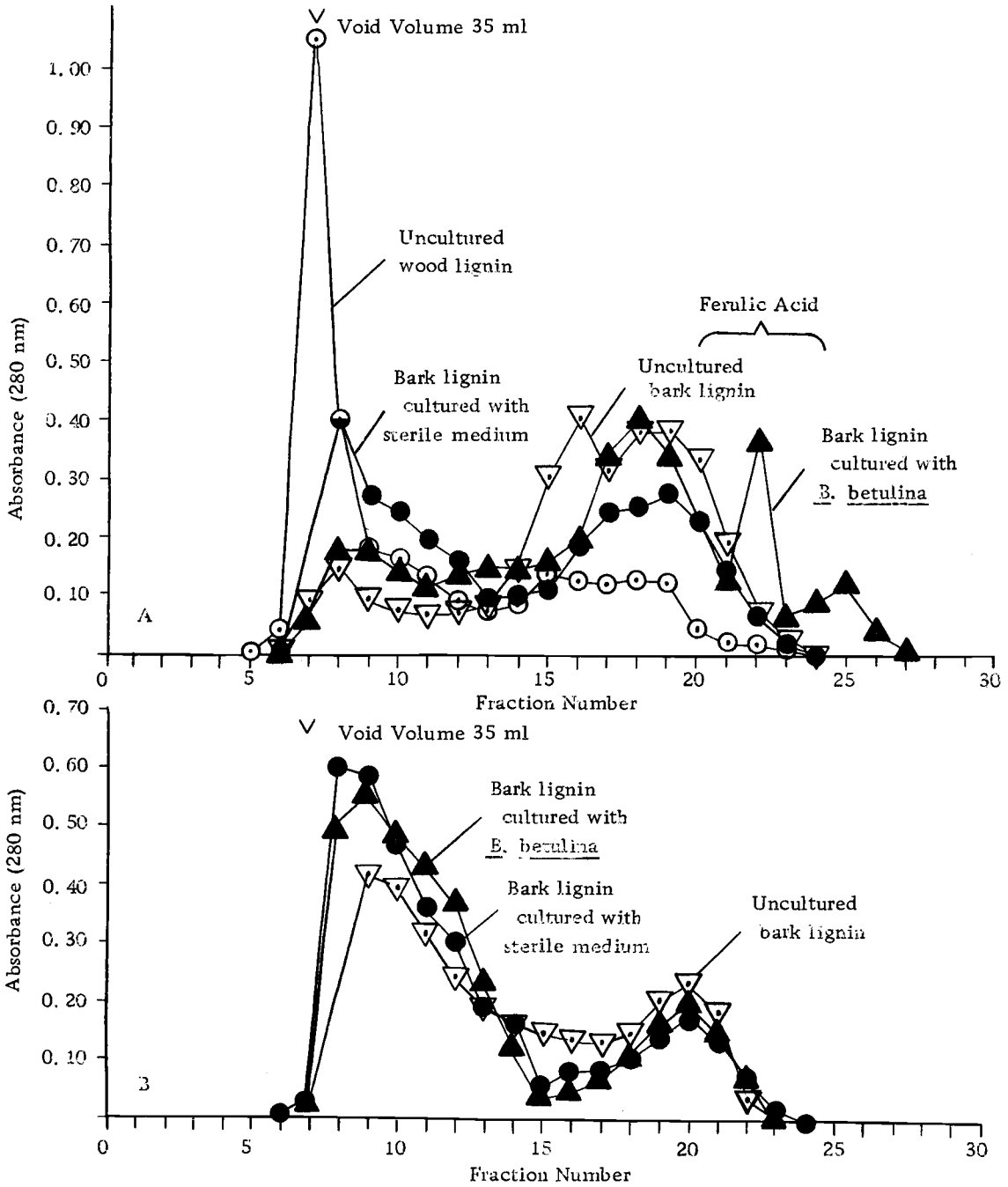

Figure 18. Molecular size distribution patterns for lignin samples determined by gel permeation chromatography. A. First series of molecular size distribution curves determined. Ferulic acid was passed through the column as a control representing a lignin monomer. B. Second series of molecular size distribution curves showing the large and reproducible shift to the left for molecular size of all bark lignin samples.

deep freeze between experiments. Resolving power of the column was not destroyed because ferulic acid (used as a model for a lignin monomer) peaked at tube 22 which was the same position that a late peak emerged from the "decayed" lignin (Figure 18A).

All molecular size distribution curves for sclereid lignin showed that the bark lignin was generally of a much lower molecular size than wood lignin (Figure 18A and B). Although ball-milled lignin may not represent the true molecular size distribution found in native cell walls, the molecular size distribution curve produced for wood lignin (Figure 18A) was quite similar to that shown for enzymatically-liberated sweetgum lignin which was obtained using a similar column (Kirk et al., 1969). Since ball-milled wood and bark lignins were prepared by similar techniques (see methods), the molecular size patterns shown in this study are strong evidence favoring the conclusion that bark lignin is of lower molecular weight than wood lignin.

Brown et al. (1968) isolated lignin from sweetgum and sitka spruce by subjecting wood blocks to decay by Lenzites trabea or Poria monticola respectively. These fungi remove almost solely carbohydrates thus releasing cell wall lignin to be extracted in a relatively unmodified form without ball milling. These two fungi decay wood cells from the lumina progressively toward the compound middle lamellae. Thus, by stopping decay at various levels of carbohydrate removal and subjecting released lignin to gel permeation

chromatography, they followed changes in molecular size distribution from the lumina to the middle lamellae. They found that the molecular size of the lignin increased as the middle lamellae were approached, and highest molecular weight lignin (approximately 14, 000) was released last, probably from the compound middle lamellae (Brown et al., 1968).

The ontogeny of Douglas-fir sclereids is quite different from that of wood tracheids or vessels, because sclereids arise secondarily by enlargement of parenchyma cells at some distance from the vascular cambium (Grillos, 1956). Sclereids are usually surrounded by parenchyma and sieve cells and are not contiguous with other lignified cells. Two types of middle lamellae associated with bast fibers have been described; one type holding contiguous bast fibers together is lignified and termed an "inner middle lamella, " while the second type attaching bast fibers to parenchyma cells is termed an "outer middle lamella" (Lewin, 1958). Only the "outer middle lamellae" are susceptible to retting which involves pectic enzymes, while "inner middle lamellae" are heavily lignified and not susceptible to retting (Lewin, 1959). When thin sections of inner bark of Doualas-fir were incubated two days in buffered pectic enzymes (Pectinol-100) they were more easily teased apart than sections incubated in buffer alone.

DISCUSSION

Lignin of Douglas-fir Sclereids

Microscopic study of "bleached" sclereids suggested that a lignin-like material was removed from cell walls by a fungus. Three "lignin" fractions have been described from sclereids (Kiefer and Kurth, 1953), and it has been suggested that in general the sclereid "lignins" are quite different from wood lignin. However, ball-milled sclereid lignin prepared in this study showed several similarities to conifer wood lignins. Sclereid lignin gave a color test with Wiesner reagent that was indistinguishable from that of wood lignin, and methoxyl content (13.79 percent) and vanillin obtained following nitrobenzene oxidation (17.85 percent) were lower, but nevertheless within reasonable range of values reported for wood lignins (Sarkanen et al., 1967; Schubert, 1965). Sclereid lignin was slightly darker than wood lignin with a faint rose color that suggested contamination by tannin-like polyphenols. Such contamination could be responsible for the lower methoxyl content of sclereid lignin. Sarkanen et al. (1967) suggest that heartwood extractives are responsible for abnormally low methoxyl values in wood lignins, and such an effect was related to the amount of polyphenols found in heartwoods of different species. They were unsuccessful in removing these contaminents which they felt were bound chemically to the lignin. Ideal sclereid material for the

isolation of lignin would lack polyphenols previously removed by an organism such as the Isaria-like fungus. Infrared spectra of bark lignin were also very similar to those for other coniferous wood lignins (Brown et al, 1968).

A significant difference between sclereid and wood lignins was the lower molecular weight of sclereid lignin which was possibly equivalent to a low molecular weight fraction of sitka spruce lignin thought by Brown et al. (1968) to be present in secondary walls of tracheids rather than compound middle lamellae.

Thus, it appears that Douglas-fir bark sclereids contain in their cell walls lignin that may be similar to that found in secondary walls of wood tracheids, but lack the high molecular weight lignin found in the compound middle lamellae of tracheids.

Sclereid "Bleaching"

Considerable circumstantial evidence indicates that B. betulina is involved in "bleaching" of sclereids. This fungus was present in 62 percent of the isolations made from tissue containing "bleached" sclereids. Longitudinal bore holes, typical of soft rot of wood, are present in "bleached" sclereids, and B. betulina has previously been described as a soft-rot fungus (Duncan and Eslyn, 1966). B. betulina slowly darkens condensed tannins and is capable of utilizing ferulic acid, vanillin and p-hydroxybenzaldehyde as sole carbon sources.

There is, however, some doubt about the involvement of this fungus in "bleaching" of sclereids, because it failed to attack sclereids in culture and did not substantially alter isolated sclereid lignin. If low molecular weight material was released from the lignin by B. betulina as suggested (Figure 18A), it was not accompanied by detectable changes in methoxyl, vanillin yield, elemental analysis or infrared spectra. Since milled-wood lignin is known to contain two percent or more carbohydrate (Björkman, 1956), it is possible that low molecular weight fractions of lignin could be released by breaking lignin-carbohydrate bonds, as has been shown by a mixture of polysaccharidases acting on lignin-carbohydrate complexes from spruce-wood (Kringstad and Cheng, 1969). Preliminary experiments showed that B. betulina had strong cellulose enzymes when it rapidly grew through cellophane disks placed over the surface of nutrient medium. Cleavage of lignin-carbohydrate bonds could conceivably occur without demethoxylations, etc. which normally accompany degradation of milled-wood lignins in liquid culture (Ishikawa et al., 1963). A second explanation for lignin degradation in the absence of detectable chemical change might be given by the type of lignin decay recently described by Kirk and Lundquist (1970) for Polyporus versicolor in sweetgum sapwood. Residual lignin isolated from decayed wood by the methods of Björkman (1956) was indistinguishable from lignin isolated from undecayed wood. It was felt that the fungus attacked limited amounts

of lignin and completely removed them before moving on to new lignin. Thus, residual lignin remained normal. However, such a mechanism is less likely with ball-milled lignin because access to fungal enzymes should be much greater than with intact wood.

Assuming that B. betulina does cause "bleaching" of sclereids and darkening of condensed tannins, the following events appear to take place. As seen from whole bark cultures and from bark collected from forest trees, B. betulina has an unusual ability to penetrate periderm (cork) layers, which is necessary to gain entry to phloem tissue not directly exposed by cracks or other openings. Upon entering the phloem tissue, the main source of carbohydrates is lignified walls of sclereids since all parenchyma and crushed sieve cells are impregnated with condensed tannins. B. betulina cannot utilize condensed tannins to a great extent, but can probably oxidize and possibly metabolize free B-rings of tannin flavanoid units. Thus, tannins are darkened as the fungus moves through parenchyma tissue to sclereids. Apparently a certain stage of fungal development or tannin oxidation must be reached before sclereid walls are entered. Possibly entry is slow because of a very slow penetration by the fungus (maybe mechanical) through condensed tannins which coat outer surfaces of sclereid walls. B. betulina is not known to cause white rot of wood, although it has been shown to cause soft rot which involves growth within cell walls (Duncan, 1960; Duncan and Eslyn,

1966) (Figure 3), and probably has a limited ability to use wood lignin. Possibly the absence of a white-rot symptom in soft-rot in wood is linked with the inability of the fungus to remove tannins as well as lignin. In wood, high molecular weight lignin of compound middle lamellae and cell corners (Brown et al., 1968) is more resistant to fungal attack by white-rot fungi than secondary wall lignin (Schmid and Liese, 1964; Scheffer, 1936; Wilcox, 1965). Since high molecular weight lignin of the middle lamellae and cell corners appears to be absent from sclereids (Figure 18A and B), possibly B. betulina is capable of slowly degrading the less-resistant, low molecular weight lignin of secondary walls of sclereids. Because the lumina and outer wall layers of sclereids are encrusted with resistant tannins, B. betulina would be restricted to growth within secondary walls causing degradations of the walls to the point where tannins were encountered in sclereid surfaces and lumina. It has been emphasized that "bleaching" of wood tissue by white-rot organisms, to a large extent, involves removal of colored substances (probably tannins) from cell lumina (Cowling, 1961; Kirk and Lundquist, 1970; Scheffer, 1936). When inner bark sclereids are sectioned, the thick secondary walls appear very pale yellowish-white. Tannins are not substantially removed from lumina of "bleached" sclereids, but phenolic loss from "bleached" sclereids is greater than can be accounted for by loss of surface phenols alone. Thus, wall lignin is probably removed along with

some carbohydrate to render the secondary walls white and to weaken the bonds between secondary walls and outer wall layers. When bark is broken or milled the outer, tannin-encrusted wall layers of sclereids easily break away from the residual, white secondary walls which remain as "bleached" sclereids.

B. betulina is occasionally associated with darkening of phloem and degradation of cork tissue amounting to substantial percentages of bark volume on old-growth butt logs. B. betulina was by far the most common fungus associated with internal tissues of the Douglas-fir bark samples collected in this study.

Parenchyma "Bleaching"

There is no doubt that the Isaria-like fungus is capable of altering a number of different phenolic substances including ortho- and meta-hydroxylated phenols and guaiacyl compounds. The rapid condensation of condensed tannins and subsequent growth through the condensation products suggests that this fungus is involved in removing condensed tannins from parenchyma cells and sclereids.

Although this fungus colonized whole bark in culture and penetrated some sclereid walls, actual "bleaching" of parenchyma and "emptying" of sclereids was not noted. However, assuming that the Isaria-like fungus does cause removal of condensed tannins from Douglas-fir bark, the following are events which appear to take place.

It appears from the removal of condensed tannins from both parenchyma cells and sclereids (Figures 10 and 11) that the fungus obtains a substantial portion of its energy from metabolizing these tannins. Secondary walls of sclereids were not degraded except for numerous tiny bore holes (Figure 11). Failure to use lignin may possibly be attributed to inability of the fungus to break bonds between monomer units, because the fungus did cleave the side chain from free ferulic acid. After tannins are removed, the parenchyma tissue is completely degraded leaving free sclereids, but many organisms are undoubtedly capable of metabolizing exposed, non-lignified parenchyma tissue from which tannins have been removed.

Thus, it appears that much of the resistance of Douglas-fir bark to microbial degradation lies in the fact that chemical components of the bark are far too diverse for any single organism to substantially degrade. It appears that certain fungi have become adapted to highly specific use of a limited number of complex substrates in the bark, and, because they are specialists, perhaps further study of such organisms would prove fruitful where research tools are needed to understand specific components of complex natural materials.

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