SELECTIVE DEGRADATION OF LIGNIN AND CONDENSED TANNINS OF DOUGLAS-FIR BARK SCLEREIDS BY FUNGI[1]

William D. Ross[2] and *Malcolm E. Corden*
Departments of Forest Products and Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331

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ABSTRACT

Chemical analysis of decayed bark confirms visual observations that colored materials rich in tannins and cell-wall lignin are removed separately from bark of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) by different species of fungi colonizing the bark. Ball-milled sclereid lignin is similar in many ways to lignin from Douglas-fir sapwood. In one instance, lignin is removed from sclereid walls by fungal action that leads to wall "bleaching," and tannins are only darkened, but not removed. In a second instance, tannins are removed from primary walls and lumina of sclereids, but wall lignin is not removed. Two fungi suspected as causal agents for the two white rots, *Bispora betulina* (Cda.) Hughes and a fungus resembling *Isaria*, failed to produce typical white rots in axenic culture, but evidence strongly suggests that they are involved in the "bleaching" process.

Additional keywords: Pseudotsuga menziesii, decay, extractives.

INTRODUCTION

Degradation of woody tissues by fungi, as evidenced by "bleaching" (e.g. white rot), involves removal of colored substances (e.g. tannins) from cell lumina (Kirk and Lundquist 1970; Scheffer 1936) and lignin and carbohydrates from cell walls (Liese 1970). Visual evidence indicates that in Douglas-fir bark, lignin and reddish-brown materials (e.g. tannins) are removed individually by different fungi to produce two different types of cell "bleaching" (Ross and Corden 1973). Thus, the more common view that white rot is associated with simultaneous removal of lignin and tannins (along with other colored substances), does not apply to these two types of "bleaching."

In the forest, *Bispora betulina* (Cda.) Hughes infests bark tissue in which condensed tannins have been darkened and sclereid walls are "bleached" white. Similarly a fungus resembling *Isaria* infests tissue in which the parenchyma cells are "bleached" white and colored materials are removed from the primary walls and lumina of sclereids. Numerous attempts were made to produce "bleaching" by growing these fungi on bark or bark components, but these attempts were unsuccessful. Therefore, the chemical changes that result in loss of color in the bark tissues were studied in tissues "bleached" by fungi in the forest.

This study was limited to examination of chemical changes occurring in the sclereid fraction of Douglas-fir bark as a result of fungal attack that lead to "bleaching" of sclereid walls or removal of colored materials from the primary walls and lumina of sclereids. Sclereids are the only cells in Douglas-fir bark that contain both wall lignin and condensed tannins, and they were isolated from other bark components to simplify chemical analysis. Visual observations suggested that lignin and condensed tannins are removed separately by different fungi even though both occur together in the same cell.

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[2] Present address: Plant Science Division, University Station, Box 3354, Laramie, WY 82071.

The two fungi commonly associated with "bleaching" of sclereid cell walls (*Bispora betulina*) or "bleaching" of parenchyma and removal of tannins and other colored materials from sclereids (a fungus resembling *Isaria*) were tested for their abilities to alter sclereid lignin and extracts containing condensed tannins and other colored substances.

MATERIALS AND METHODS

Fungal isolates

Bispora betulina and the fungus resembling *Isaria* were isolated from the outer bark of living trees growing at several different locations in western Oregon. Bark for isolation was selected for the presence of either "bleached" sclereids in the presence of darkened parenchyma or "emptied" sclereids in the presence of "bleached" parenchyma (Ross and Corden 1973). All isolates were maintained on slants of potato-dextrose-agar.

Source of normal and decayed bark

Inner-bark, outer-bark, normal- and decayed-bark samples were all collected from living trees growing in the Siuslaw National Forest in western Oregon. Outer bark described as "normal" in this paper was carefully selected to avoid any tissue that was discolored or damaged. Inner bark was collected in early June when it could be separated easily from both the vascular cambium and the innermost periderm layer of the outer bark. Bark that was collected for isolation of "bleached" or "emptied" sclereids was uniformly decayed to insure a high percentage of sclereids representative of the two decay types.

Culture medium

The medium used to generate mycelial mats of *B. betulina* and the fungus resembling *Isaria* for subsequent incubation with tannin-extract or sclereid-lignin medium was modified Bf-2 medium (Fåhraeus and Tullander 1956). Tannin extracts or lignin were also incorporated into half-strength, modified Bf-2 medium when fungi were tested for their abilities to degrade tannins and lignin. The Bf-2 medium was modified by replacing asparagin with NH_4NO_3 (1 g/liter), omitting phenylalanine, reducing iron to one-tenth strength and using sucrose (2 g/liter). The medium was sterilized by autoclaving and it had a pH near 5.6 (without tannin or lignin incorporated). The flasks of medium were inoculated with small plugs of fungus cut from cultures on potato-dextrose-agar, or mycelial mats previously grown in other Bf-2 medium were aseptically washed with sterile water and transferred to fresh medium.

Determination of noncarbohydrate components of sclereids

Total extractive content of sclereid preparations was estimated by weight loss due to chlorite bleaching (Wise et al. 1946). Sclereids were isolated from both inner and outer bark tissue as described previously (Ross and Krahmer 1971). Outer-bark phloem was separated by hand from cork tissue before pulverizing to avoid contamination of sclereids with broken cork tissue. Three batches (0.2 g) of each unextracted sclereid type were placed in 30-ml, tared Gooch crucibles, stoppered from the bottom and covered with small, inverted crucible lids. The sclereids were incubated with 15 ml of acidic $NaClO_2$ solution at 75 to 80 C, and fresh chlorite and acetic acid were added every 30 minutes until a wet brightness of 76 or better was obtained with a Photo Volt brightness meter according to Tappi Standard Method T452 m-58 (1958). The bleaching solution was drawn from the crucibles and the resulting holocellulose was washed with cold water and finally with acetone. Weight loss due to extraction of the sclereids was determined after drying the crucibles containing the sclereid holocellulose.

Sclereids were bleached to a minimum brightness of 76 because staining with $K_3Fe(CN)_6$-$FeCl_3$ (Barton et al. 1952) demonstrated that almost all the phenolic materials had been removed from the sclereid walls at this point, and microscopic examination showed no signs of colored

substances in lumina of any chlorite-bleached sclereids.

Hydrolysis of sclereid walls and determination of sugar ratios

The ratios of five major structural sugars found in sclereid walls were determined for normal and decayed sclereids. Extractive-free sclereids were treated with acidic $NaClO_2$ (Wise et al. 1946) for removal of noncarbohydrate material. All holocellulose preparations were dried under vacuum, and 0.2 g of each was placed in test tubes with 10 ml of 72% H_2SO_4 and stirred continuously for 2 h at 18 to 20 C. The hydrolysate was diluted to 373 ml with distilled water and refluxed for 3 h. After cooling, the hydrolysate was filtered through a sintered glass crucible and adjusted to pH 3.8 with Amberite IR-4B (hydroxide form) ion exchange resin. The resulting sugar solution was reduced to about 5 ml in a rotary evaporator at 30 C and then diluted to 10 ml with water.

The sugars were applied to water-prewashed Whatman No. 1 chromatography paper and separated by descending development with ethyl acetate-acetic acid-water (9:2:2) or n-butanol-pyridine-water (10:3:3) (Mian and Timell 1960). The sugars were located with CD-1 spray reagent (Gorden et al. 1956), and after elution from the paper (Saeman et al. 1954) relative amounts of glucose, galactose, mannose, xylose and arabinose were determined (Timell et al. 1956). The values were corrected for sugar degradation during hydrolysis using correction factors obtained when known amounts of corresponding sugars were passed through the entire process.

Preparation of ball-milled lignin from inner-bark sclereids

When large quantities of sclereids were needed to prepare ball-milled lignin, only inner bark was used. This avoided contamination of the final lignin preparation by extractives from broken cork and the condensed tannins from the outer-bark sclereids. After bark sclereids were separated from other cell types, only washed sclereids that had passed a 100-mesh Tyler screen were used. Inner-bark sclereids that passed 100-mesh Tyler screens were ground in a Wiley Mill using a 60-mesh screen. Even though the long, thin sclereids could pass a 60-mesh screen if oriented properly, most were cut one or more times and this increased access of solvents to lumina during subsequent extractions.

Ground sclereids were extracted in a Soxhlet apparatus 48 h with ethanol-benzene (1:2), then 48 h with 95% ethanol and finally were stirred in water at 50 C for 2 h. The meal was desiccated under vacuum over anhydrous $CaSO_4$ and placed in a milling jar containing glass marbles. The jar was purged with helium, sealed, and rotated at about 20 rpm for five weeks.[3]

Lignin was prepared from ball-milled sclereid meal by the methods of Björkman (1956) except that the lignin was freeze-dried after precipitating in water. Ball-milled sapwood meal was kindly supplied by Dr. K. V. Sarkanen, and wood lignin was prepared from it by the Björkman method.

Nitrobenzene oxidation of lignin

Lignin was oxidized with alkaline nitrobenzene (Stone and Blundell 1951; Bland 1960) in stainless steel bombs (2-ml capacity) containing 10 mg lignin, 0.06 ml nitrobenzene, and 1.0 ml 2N NaOH. The bombs were rotated endwise in an oil bath at 160 C for 3 h, and after cooling the contents were transferred to small bottles, adjusted to pH 3.0 with HCl, and allowed to settle overnight. After centrifugation, the supernatant was combined with two washings (10-ml volumes of water) of the solid residue and partitioned three times with 10-ml portions of chloroform. The chloroform extract was dried under vacuum (Bland 1960), and the residue containing the aldehydes was dissolved in 2 ml of 95% ethanol. Vanillin was located under ultra-

[3] Procedures for milling wood meal for preparation of lignin were generously described by Dr. K. V. Sarkanen.

violet light, removed from the plates, and extracted from the gel with 95% ethanol in a micro-Soxhlet extractor for 1 h. Vanillin concentration was determined spectrophotometrically (Stone and Blundell 1951).

RESULTS

Determination of sodium chlorite extractives in normal and decayed sclereids

In the presence of condensed tannins (in this paper the term "condensed tannins" refers to all reddish-brown material including phenolic acids, phlobaphenes, and condensed tannins), the quantitative isolation of cell-wall lignin is problematical. However, if condensed tannins and other substances extractable by acidic chlorite bleaching (Wise et al. 1946) could be selectively removed without removing cell-wall lignin, then weight loss due to chlorite extraction would estimate the lignin content of sclereid walls. Sclereids from which condensed tannins and lumen contents have been selectively removed by fungal action in tissue infested with the fungus resembling *Isaria* provide this kind of material for analysis.

Lignin content of sclereids was determined indirectly by comparing amounts of sodium chlorite extractives present in three of the four sclereid types described below. Sclereids from normal and decayed bark were analyzed for total extractives removed by acidic sodium chlorite (Table 1).

As described by Ross and Corden (1973), condensed tannins encrust primary walls of bark sclereids (outer surfaces of isolated sclereids) at the time live inner bark is converted to dead outer bark. Thus, normal outer-bark sclereids (Fig. 1-B) contain primary-wall tannins, wall lignin, and lumen contents rich in tannins; but normal inner-bark sclereids (Fig. 1-A and 1-C) contain only wall lignin and lumen contents.

Fifty-two percent (see footnote c of Table 1) of the sclereids from bark naturally infested with the fungus resembling *Isaria* had empty lumina (called "emptied" sclereids in this paper) and were surrounded in the bark by parenchyma tissue that was "bleached" white (Fig. 1-E). Outer-bark sclereids normally appear reddish-brown because of colored primary-wall tannins, but "emptied" sclereids are light tan, similar to inner-bark sclereids, because their primary-wall tannins have been degraded. Thus, the sample of "emptied" sclereids analyzed had no primary-wall tannins, 48% possessed lumen contents (52% were empty), and 100% of the wall lignin was present.

Sclereids from bark infested with *B. betulina* had walls that were "bleached" white (called "bleached" sclereids in this paper). "Bleached" sclereids (Fig. 1-D) were assumed to contain 100% of their lumen contents, an unknown amount of wall lignin and 96% of the primary-wall tannins were missing as a result of fungal activity (see footnote b of Table 1).

By solving the simultaneous Eqs. 1, 2, and 4 (Table 1) it was estimated that primary-wall, total-wall, and lumen extractives were 10.0%, 21.2%, and 10.6% respectively, of the dry weight of normal outer-bark sclereids. If primary-wall extractives are condensed tannins and total-wall extractives are lignin, then normal outer-bark sclereids contain about 10% condensed tannin encrusting the

TABLE 1. *Total chlorite extractive content of sclereids determined by weight loss resulting from acidic sodium chlorite extraction*

Type of sclereid		Equation for source of extractive materials		Weight loss in % (extractive content)
Normal outer bark	1	1.00 S[a] + W + 1.00 L =		41.8
Normal inner bark	2	0.00 S + W + 1.00 L =		31.8
Walls "bleached" (96%)[b]	3	0.04 S + W[d] + 1.00 L =		18.8
Lumina emptied (52%)[c]	4	0.00 S + W + 0.48 L =		26.3

[a] S = Extractive materials on the outer surfaces (encrusted primary walls) of isolated sclereids
W = Extractive materials in the total wall of sclereids.
L = Extractive materials in the lumina of sclereids.

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

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

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

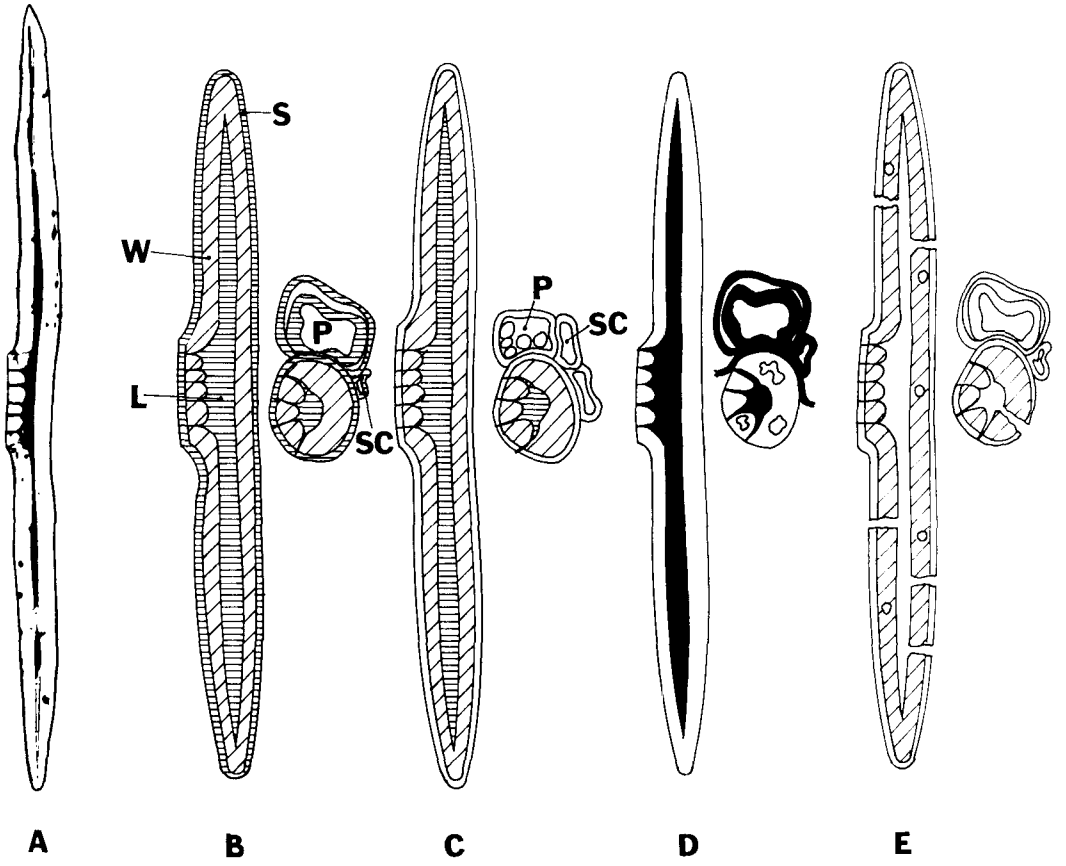

FIG. 1. Representations of cells from Douglas-fir bark showing chlorite extractive contents of normal and decayed sclereids and colored materials encrusting parenchyma cells. A) Photograph of an isolated sclereid from normal inner-bark phloem. B–E) Diagrams representing longitudinal and cross-sectional views of four sclereid types and accompanying parenchyma and sieve cells. S = Surface extractives in the outer layer (primary wall) of sclereids (condensed tannin). W = Wall extractives in thick total walls (lignin). L = Lumen extractives (rich in condensed tannins). B) Normal outer-bark sclereid with light-colored condensed tannins, dilated parenchyma cells (P) and crushed sieve cell (SC). Sclereid contains S + W + L extractives. C) Normal inner-bark sclereid lacking S tannins on sclereids and colored material encrusting parenchyma. Parenchyma cells are small, living, and contain starch. Sieve cells not yet crushed. Sclereid contains W + L extractives. D) "Bleached" outer-bark sclereid with darkened L extractives and lack of primary-wall layer. Sclereid has L extractives plus an unknown amount of W lignin. Parenchyma darkened. E) "Emptied" sclereid lacking S and L extractives, but containing W lignin and bore holes in the walls. Parenchyma cell with no colored encrustations.

primary walls and 21% wall lignin. Lumen contents, which are rich in condensed tannins, amount to 10% of normal outer-bark sclereids. These values compare favorably with the 44.8% Klason lignin content (condensed tannins and lignin) of bark sclereids before extraction with 1% NaOH (removes mainly phenolic acids and other tannin-like substances) and 22.8% after NaOH extraction found by Kiefer and Kurth (1953).

Ninety-six percent of the sclereids isolated from bark naturally infested with *B. betulina* lacked colored primary walls and were "bleached" white. There are at least two possible ways to explain the lack of color on the outside surfaces of these isolated sclereids "bleached" by fungal action. Either the tannins were removed from the primary walls, or the entire primary walls are missing (Fig. 1-D). If only the tannins

were missing, the total chlorite-extractive content of "bleached" sclereids should be about 32% (Table 1), but since the extractive content was only 18.8% and the lumina of the "bleached" sclereids remained filled, the fungal "bleaching" must also involve loss of lignin from the sclereid walls.

Carbohydrate content of cell walls of normal and decayed sclereids

Cell walls of sclereids "bleached" by fungal action, which were isolated from outer bark naturally infested with *B. betulina*, were heavily degraded. However, except for numerous tiny bore holes, the walls of "emptied" sclereids isolated from bark naturally infested with the fungus resembling *Isaria* were normal and stained brilliantly with Wiesner reagent (Ross and Corden 1973). Thus, only the walls of "bleached" outer-bark and normal inner-bark sclereids were analyzed for the five major structural sugars to determine the effects of decay.

The hemicellulose sugars were removed from the sclereid walls more rapidly than cellulose during the course of natural fungal "bleaching" of walls as judged by glucose content compared with the other sugars (Table 2). Arabinose was not detected from sclereids "bleached" by fungal activity in bark naturally infested with *B. betulina* even when the amount of hydrolysate analyzed was increased to three times that used to detect the other sugars.

TABLE 2. *Relative amounts of sugars in sclereid cell walls*

Sugar	Normal inner-bark sclereids[a]	"Bleached" outer-bark sclereids[a]
Glucose	1.00	1.00
Galactose	0.06	0.05
Mannose	0.29	0.15
Arabinose	0.05	none detected
Xylose	0.15	0.10

[a] Relative sugar content expressed as the ratio of each sugar to the glucose content of the sclereid type being analyzed.

Fungal alteration of bark extracts containing condensed tannins

The ability of the bark fungi to alter condensed tannins from outer bark was studied. Bark fines (100 g) from unmodified outer-bark phloem tissue were shaken for 12 h on a rotary shaker in absolute methanol (400 ml). The solids were removed by filtration on Whatman No. 1 paper, the filtrate was centrifuged, and the supernatant taken to dryness in a rotary evaporator at 42 C. The dried extract (160 mg) was added to 100 ml of modified Bf-2 medium (one-half strength) with 2 g sucrose/liter in 500-ml Erlenmeyer flasks, the medium was inoculated with mycelial mats of the bark fungi, and the flasks were placed on a rotary shaker.

After 5 days in shake culture, colonies of the fungus resembling *Isaria* were caked with a brown precipitate, many slightly darkened particles of the extract remained in suspension, and the culture fluid was light brown. At this time, the *B. betulina* and uninoculated cultures contained a light rose-colored precipitate in a yellow-brown culture fluid. When mycelium of the fungus resembling *Isaria* was harvested and placed in fresh methanol-extract medium, the fungus caused complete clearing of the medium in less than 24 h, and the mycelial mats were heavily caked with a dull brown precipitate. After 1 month, much of the bark extract was converted to a dark brown precipitate by *B. betulina*, but the culture fluid remained colored.

Alteration of ball-milled sclereid lignin by B. BETULINA

B. betulina is associated with bark tissues in which "bleached" sclereids with substantial degradation of lignified cell walls appear. Thus, the ability of this fungus to degrade isolated sclereid lignin was studied. Ball-milled lignin from both sapwood and inner bark of Douglas-fir gives a similar dark violet color with Wiesner reagent, but chemical analysis (Table 3) indicated that the bark lignin was somewhat different from Douglas-fir sapwood lignin as reported by Sarkanen et al. (1967). The infrared spectrum of sclereid lignin (Fig. 2-A) was

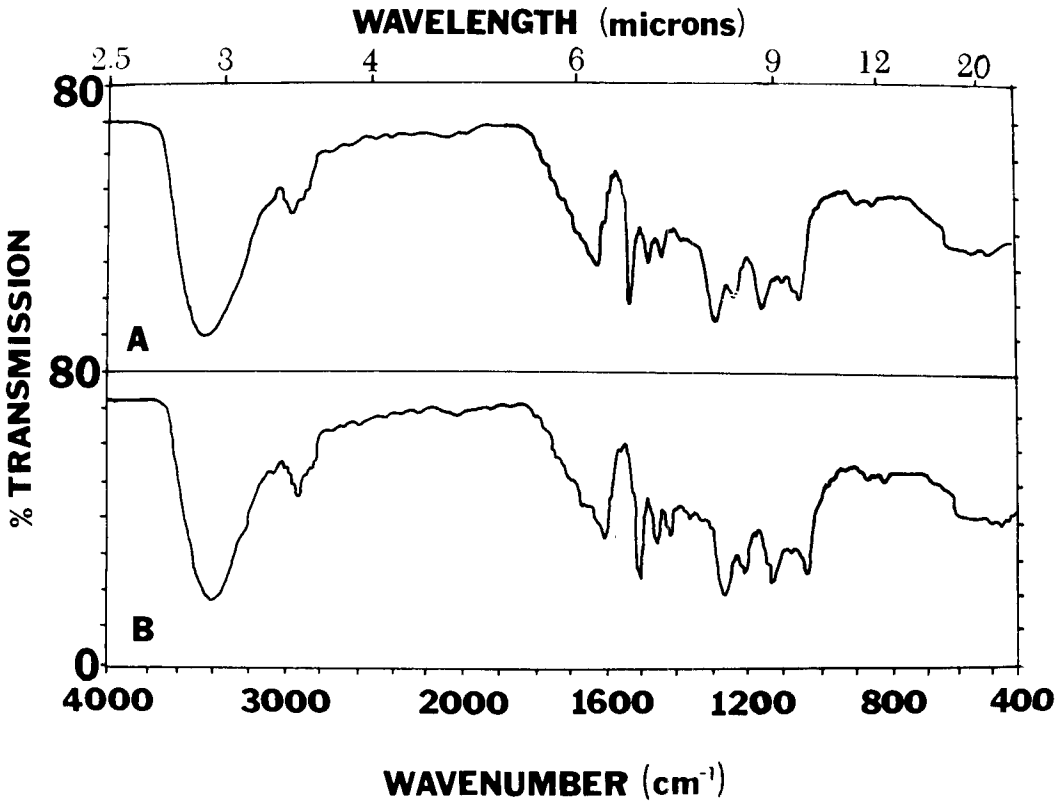

FIG. 2. Infrared absorption spectra of bark lignins taken from KBr pellets. A) Bark lignin cultured with sterile medium. B) Bark lignin cultured with *Bispora betulina*.

similar to those of spruce (Brown et al. 1968) and larch lignins (Sarkanen et al. 1967).

Sclereid lignin was sterilized by saturating 0.5-g batches with 95% ethanol and washing the saturated lignin into sterile 500-ml DeLong flasks with additional ethanol. The ethanol was removed under vacuum and the dried lignin was covered with 100 ml of ½ strength, modified Bf-2 medium. Mycelial mats of *B. betulina*, previously grown on modified Bf-2 medium, were placed in the medium and the cultures were incubated in the dark for 2 weeks on a shaker and then for 4 weeks in still culture. During this time *B. betulina* grew over the entire surface of the lignin and appeared to darken it.

The culture solids were collected by centrifugation and the lignin was extracted with 96% aqueous dioxane. The solubilized lignin was freeze-dried, taken up in 90% acetic acid, precipitated in water, washed three times with water, and finally freeze-dried again. This lignin preparation was then compared to a similar sclereid lignin preparation incubated with sterile medium

TABLE 3. *Comparative analysis of ball-milled lignin from Douglas-fir bark sclereids and from sapwood in percent dry weight*

Component	Sclereid[a] % dry wt.	Sapwood[b] % dry wt.
Carbon	59.4	63.4
Hydrogen	5.9	6.1
Oxygen	34.4	30.6
Methoxyl	13.8	15.1
Vanillin	17.8	--

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

[b] Data for sapwood lignin from Sarkanen et al. (1967).

TABLE 4. *Composition of ball-milled bark lignin components after incubation with* Bispora betulina *in liquid culture*

Component	*B. betulina* % dry weight	Sterile medium % dry weight
Carbon	59.4	60.4
Hydrogen	5.5	6.0
Oxygen	35.2	33.8
Methoxyl	13.4	13.8
Vanillin	17.3	17.8

to determine if *B. betulina* had degraded the lignin. Carbon, hydrogen, oxygen, and methoxyl content; vanillin yield from nitrobenzene oxidation; the infrared spectra; and molecular weight distribution using gel permeation chromatography were measured.

Previous studies in which milled-wood lignins were incubated in liquid culture with various white-rot fungi indicated that methoxyl content generally decreased with a corresponding increase in hydroxyl, and vanillin yield following nitrobenzene oxidation decreased (Ishikawa et al. 1963). Carbon and hydrogen contents generally dropped as the amount of carbonyl and carboxyl groups increased. Infrared spectra of decayed wood lignin also revealed an increase in carbonyl and carboxyl absorption at about 6 μm (Ishikawa et al. 1963). On the basis of these changes in lignin described by Ishikawa et al., it appears that *B. betulina* failed to alter the bark lignin preparation significantly under the conditions used in the present study (Fig. 2-A vs. 2-B and Table 4).

Attempts were made to detect changes in molecular size distribution of sclereid lignin, which might have resulted from incubation with *B. betulina.* Lignin samples recovered from *B. betulina* and uninoculated control cultures were fractionated on columns (1.5 × 60 cm) or agarose beads (Bio-Gel A 15 m, 50–100 mesh, Bio-Rad Laboratories) similar to column A of Kirk et al. (1969). All solvents were mixed in a glove bag under nitrogen, and the reservoir containing dioxane (chromatoquality, 99+ mol percent)-water (1:1) was continuously flushed with prepurified nitrogen that had been passed through an alkaline pyrogallol oxygen trap (Vogel 1956). Five-ml fractions were collected, and the lignin concentration was assumed to be directly proportional to absorbance at 280 nm (Kirk et al. 1969).

The sclereid lignin as isolated in this study lacked a high-molecular weight peak that was found in wood lignin passed through the same column. While the procedures for isolating the wood and bark lignins were similar, differences in molecular weight distribution of the two lignins may not give a true picture of the relative molecular weights of the native lignins within cell walls. *B. betulina* did not substantially modify the molecular weight distribution of the isolated sclereid lignin under the culture conditions used in this study.

DISCUSSION

The two types of fungal decay of Douglas-fir bark described earlier (Ross and Corden 1973) and discussed in this paper result in "bleached" tissues that look like two different white rots. If white rots involve the removal of colored materials from cells (Scheffer 1936; Kirk and Lundquist 1970), then the condensed tannins (along with other colored materials) must be removed to produce "bleached" bark tissues. Also, white rots have been associated with removal of lignin (Liese 1970).

Microscopic study of outer-bark development has located the reddish-brown condensed tannins largely in parenchyma tissue and also as encrustations of primary walls and residual protoplasts of outer-bark sclereids (Ross and Corden 1973). The nature of the condensed tannins (including the reddish-brown phenolic acids, phlobaphenes, and condensed tannins in this paper) has been characterized (Hubbard and Kurth 1949; Kiefer and Kurth 1953; Hergert 1960; Fujii and Kurth 1966). However, the existence of cell-wall lignin in bark similar to that of wood was not established for Douglas-fir (Kiefer and Kurth 1953).

The present study located a Wiesner-positive substance in Douglas-fir bark which was limited primarily to the cell walls of sclereids. Ball-milled sclereid lignin was prepared from bark sclereids and results from elemental analysis, vanillin yield, percent methoxyl, infrared spectra, and Wiesner reaction all indicate that a substance similar in many ways to wood lignin occurs in the cell walls of bark sclereids. The methoxyl content (13.8%) and vanillin obtained following nitrobenzene oxidation (17.8%) were lower, but within a 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 low methoxyl content of sclereid lignin. Sarkanen et al. (1967) suggest that heartwood extractives are responsible for abnormally low methoxyl values in wood lignins, and the effect was related to the amount of polyphenols found in heartwoods of different species. They were unsuccessful in removing these contaminants which they felt were bound chemically to the lignin. From the present study it is apparent that fungal enzyme systems exist that selectively remove only condensed tannins (and possibly other nonlignin components) when the tannins and lignin occur together in the same cell.

The lignin preparations isolated from bark sclereids lacked a high-molecular-weight fraction that was present in the sapwood lignin. Similar high-molecular-weight fractions have been identified in enzymatically liberated wood lignin from Sitka spruce, and were located in the compound middle lamellae (Brown et al. 1968). It remains to be determined if the sclereid lignin, as it occurs natively in cell walls, also includes the higher-molecular-weight fraction found in wood lignins.

Fungal decay leading to each of the two white-rot symptoms found in Douglas-fir bark removes either condensed tannins (and other colored, nonlignin components) or wall lignin, but not both.

"Bleaching" of sclereid walls is accompanied by darkening, but not noticeable removal, of condensed tannins from sclereids or surrounding parenchyma. The relative amounts of chlorite extractives of normal and decayed sclereids (Table 1) indicate that lignin was removed from the cell walls of sclereids "bleached" by fungal action. These "bleached" sclereids remain filled with reddish-brown materials that condense to a single, intact unit of phlobaphene when the cell walls are hydrolyzed with mineral acid (72% H_2SO_4).

If condensed tannins are not removed enzymatically from the primary walls of sclereids "bleached" by fungal action, how are they removed? The arabinose content of primary walls of coniferous wood cells is relatively high, but negligible in the secondary walls (Meier 1961). Thus, the disappearance of arabinose from sclereid walls "bleached" by fungal action strongly suggests that the entire primary wall is missing. The arabinose in the remaining secondary wall was either utilized by the invading fungus or was below the detection limits of the assays used. Fungal bore holes largely run longitudinally within the secondary walls (typical soft rot decay), but failure of the fungus to remove condensed tannins forbids the decay of the primary wall, which is encrusted with condensed tannins. When bark is broken or milled the outer, tannin-encrusted primary walls, which have been loosened from the degraded secondary walls during decay, break away (like peelings from bananas), and "bleached" secondary walls appear as white streaks in the bark.

The second type of decay results in removal of condensed tannin (and other colored substances) from bark parenchyma (microscopic examination only) and from primary walls and lumina of sclereids. The evidence that lignin was not removed from the same sclereids is that the sclereid walls stained brilliantly with Wiesner reagent, the walls did not crush under mild pressure as those from "bleached" sclereids did, and the amounts of chlorite extractives obtained from "emptied" sclereids were very near to

the value that would be predicted if 52% of the lumina were empty (Table 2) but lignin was not removed.

B. betulina was associated in bark with "bleached" sclereid walls, but all efforts to reproduce sclereid "bleaching" by inoculating bark with *B. betulina* in axenic culture failed (Ross and Corden 1973). *B. betulina* was suspected as the causal organism for several reasons. Longitudinal bore holes, typical of soft rot of wood are present in "bleached" sclereids, and *B. betulina* previously has been described as a soft-rot fungus in wood (Duncan and Eslyn 1966). *B. betulina* slowly darkened condensed tannins in culture and the tannins in bark infested with the fungus are darkened, but not removed. This fungus was present in 62% of the isolations made from bark containing "bleached" sclereids. However, failure of *B. betulina* to produce "bleached" sclereids in axenic culture or to substantially alter ball-milled sclereid lignin in culture, leaves determination of the causal organism of sclereid "bleaching" uncertain.

Wood decayed by a soft-rot fungus, such as *B. betulina*, typically turns brown and is similar in gross appearance to a brown rot (Duncan 1960). However, it could be that soft-rot fungi degrade wood lignin, similarly to the way lignin is decayed in sclereid walls, but brown-rot symptoms result mainly because condensed tannin (or other colored substances) are not removed. Chemical analysis of wood that has undergone soft-rot is needed.

The fungus resembling *Isaria* was associated with "bleached" parenchyma and "emptied" sclereids in naturally infested bark. The fungus was isolated from bark containing "emptied" sclereids more frequently than any other fungus, and it rapidly cleared culture medium containing reddish-brown condensed tannins.

Hurst and Burges (1967) suggest that caking of a precipitate on fungal colonies growing in humic acid media, similarly to the way tannins caked to the fungus resembling *Isaria*, is common, and disappearance of humic acid (or possibly tannins) from solution does not necessarily indicate that they were utilized. The fungus resembling *Isaria* rapidly and substantially alters condensed tannins, although actual utilization was not proven. Because the medium was cleared more rapidly by the fungus pregrown on bark-extract medium, it appears that the enzymes involved may have been induced during the initial incubation with the tannins.

Again, failure to produce sclereid "emptying" or parenchyma "bleaching" in bark cultured axenically with the fungus resembling *Isaria* leaves the involvement of this fungus in producing these symptoms uncertain.

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 all of them. Besides the two resistant components, lignin and condensed tannins, Douglas-fir bark also contains resistant structures such as periderm layers and other resistant substances such as resins and the fungitoxic flavanone, dihydroquercetin (Hergert 1962). Apparently certain fungi have have become adapted to highly specific use of a limited number of complex substrates in the bark. Because they are specialists, perhaps further study of such organisms would prove fruitful where research tools are needed to describe specific components of complex natural materials.

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