MICROSCOPIC AND HISTOCHEMICAL CHANGES IN DOUGLAS-FIR BARK ACCOMPANYING FUNGAL INVASION[1]

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

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ABSTRACT

Douglas-fir bark in the process of biological deterioration contained two types of tissue "bleaching." Sclereid walls were "bleached" as wall lignin was removed in tissues infested with *Bispora betulina*, and parenchyma and sclereids were "bleached" as a result of the selective removal of condensed tannins from wall surfaces and lumina in tissues consistently infested with an unidentified fungus resembling *Isaria*. Bleached tissue symptoms were not produced in bark tissues inoculated with *B. betulina* or the fungus resembling *Isaria*, but both fungi significantly altered specific bark components.

Additional keywords: *Pseudotsuga menziesii*, lignin, condensed tannin, *Bispora betulina*, decay.

INTRODUCTION

Macroscopic examination of cuts through the bark of Douglas-fir frequently reveals biologically degraded tissues of two distinct types. In one case, tan-colored sclereids with normally lignified walls stand out against a background of "bleached," white parenchyma, and in the second case, "bleached," white sclereids appear on a background of dark brown parenchyma. It was suspected from these observations that two major classes of phenolic components in Douglas-fir bark, lignin and condensed tannins, were differentially degraded.

The present study was initiated to confirm the location of lignin and condensed tannins in bark tissues, to describe "bleaching" of specific cell types through degradation of colored materials, and to identify the associated fungi.

PREVIOUS WORK

General descriptions of outer bark and of ontogeny and structure of the inner bark and periderm layers of Douglas-fir are available (Chang 1954; Grillos 1956; Grillos and Smith 1959; Ross and Krahmer 1971).

The reddish-brown appearance of the cut surface of Douglas-fir outer bark is due largely to polymeric polyphenols in the phloem tissue (Hergert 1960). Specific fractions of these polyphenols have been named (e.g. phenolic acids, phlobaphenes, and tannins) depending primarily on their solubility in various solvents (Kurth et al. 1949). All these fractions are chemically similar and are polymers of leucocyanidins and catechins (Fujii and Kurth 1966). In the present study, all reddish-brown materials encrusting cell walls and residual protoplasts of bark cells are called condensed tannins.

Although lignin fractions from Douglas-fir bark have been described (Kurth and Smith 1954; Kiefer and Kurth 1953; Holmes and Kurth 1961; Fujii and Kurth 1966), a substance that fits the criteria for lignin suggested by Brauns and Brauns (1960)

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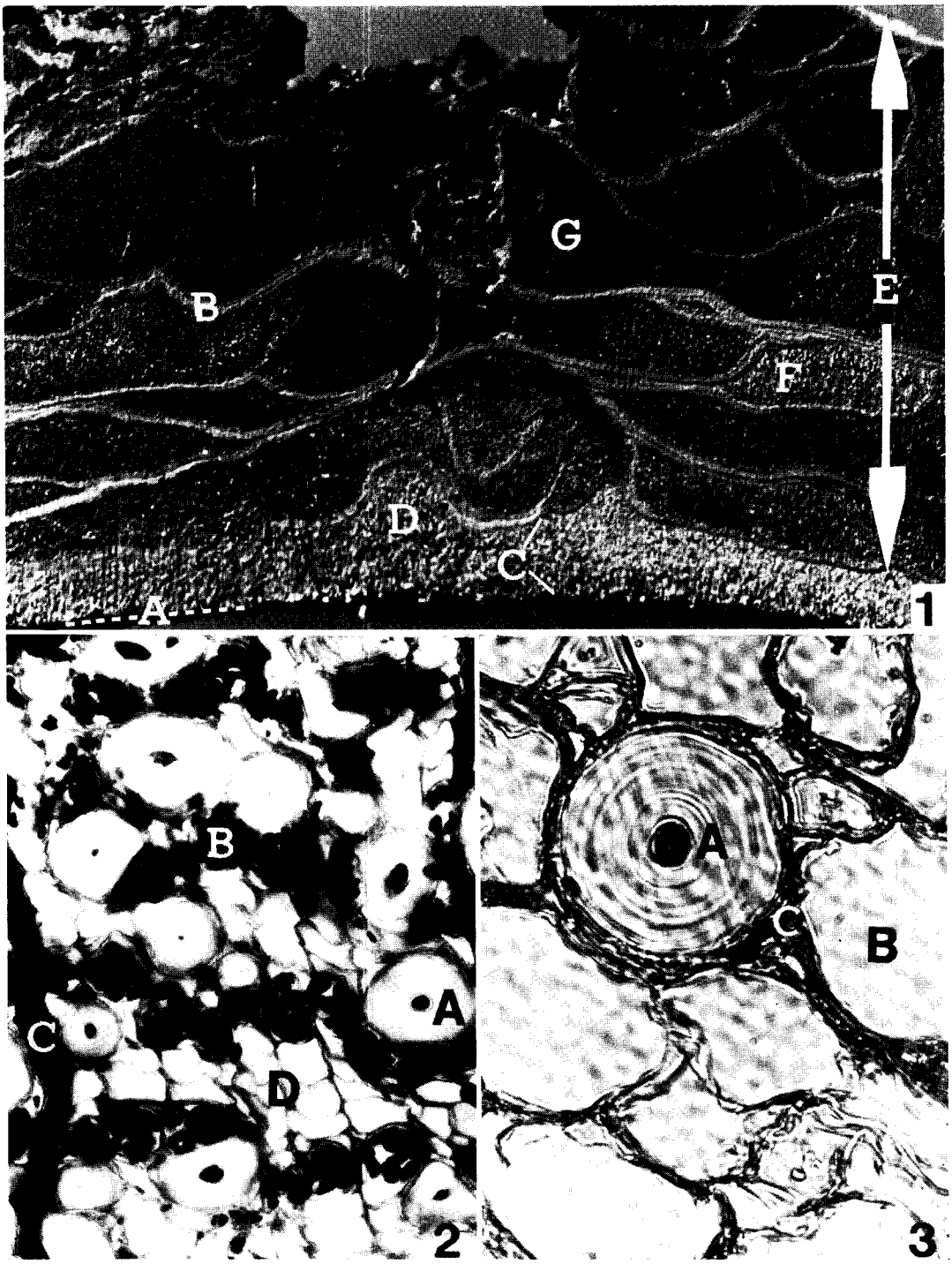
[2] Present address: Box 3354, University Station, Plant Science Division, University of Wyoming, Laramie, Wyoming 82070.



FIGS. 1–3) 1) Cross-section of Douglas-fir bark from a 235-year-old tree, with (A) vascular cambium, (B) periderm layer, (C) inner-bark phloem, (D) a dotted line illustrating a possible location for future periderm differentiation in the inner bark, (E) outer bark with, (F) the ends of sclereids visible, and (G) sclerieds decayed.

and Kratzl (1965) has not been isolated. Thus, the presence in sclereid walls of lignin meeting several criteria of the latter authors was sought in the present study.

Early lignin preparations from Douglas-fir bark failed to give a positive red to violet reaction when treated with Wiesner reagent (phloroglucinol-HCl) (Kurth and Smith 1954); however, Fujii and Kurth (1966) later reported that a Douglas-fir bark preparation "which contained some bast fibers and cork fragments" did give a positive Wiesner test. Not all cell types in tree barks give a uniform Wiesner reaction, but fibers and sclereids of coniferous barks generally do (Srivastava 1966).

The chemical composition of isolated sclereid fractions from Douglas-fir bark has been studied, and three "lignin" fractions have been described (Kiefer and Kurth 1953). The total lignin content of the sclereids was 44.8% as determined by the Klason procedure; however, one fraction, constituting 49% of the Klason lignin, was isolated by extracting the sclereids with 1% NaOH. This fraction, termed "phenolic acids," contained a relatively low methoxyl value (4.3%), a high carboxyl content (4.9–5.3%), gave a relatively low yield of vanillin (1.63%) by nitrobenzene oxidation, but failed to give a positive Wiesner reaction (Kiefer and Kurth 1953).

A second lignin preparation was obtained by extraction with a dioxane-HCl reagent following the removal of "phenolic acids" with 1% NaOH. This fraction was probably most similar to lignins found in the wood of gymnosperms and had a methoxyl content of 13.5–14.3%. The results of a Wiesner test and vanillin yield following nitrobenzene oxidation were not given for this preparation (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.

MATERIALS AND METHODS

Isolation and selection of fungi associated with decay of bark

To isolate fungi associated with decay symptoms in bark, individual bark cells or small groups of cells with discrete evidence of fungal attack were plated in a range of solid media (Ross 1971). Following incubation at 20–24 C, individual fungi were transferred to potato-dextrose agar slants for storage.

Two of these media were particularly successful for culturing bark fungi. An inner-bark extract medium was prepared by leaching 20 g of dry inner-bark fines (see below) in 500 ml of water for ½ hr. Mineral salts (iron reduced to ⅒ strength) and vitamins of Bf-2 medium (Fåhraeus and Tullander 1956) were added with 20 g/liter of sucrose. The medium was sterilized by filtration through a Millipore HA filter, and agar (15 g/liter) was added when a solid medium was desired. In addition, modifications of the Bf-2 medium were used in which NH_4NO_3 (1 g/liter) was substituted for asparagin, phenylalanine was omitted, the iron content was reduced to ⅒ strength, and various carbon sources replaced sucrose.

Fungi repeatedly isolated from bark were incubated in a liquid inner-bark extract medium to determine their ability to modify the bark polyphenols. The medium (100 ml) in 250-ml Erlenmeyer flasks was inoculated with mycelial fragments and the cultures were incubated 2–4 weeks in the dark at 20–24 C, either still or on a rotary shaker. Fungi causing precipitation of reddish phenolic materials in the medium were retained for further study.

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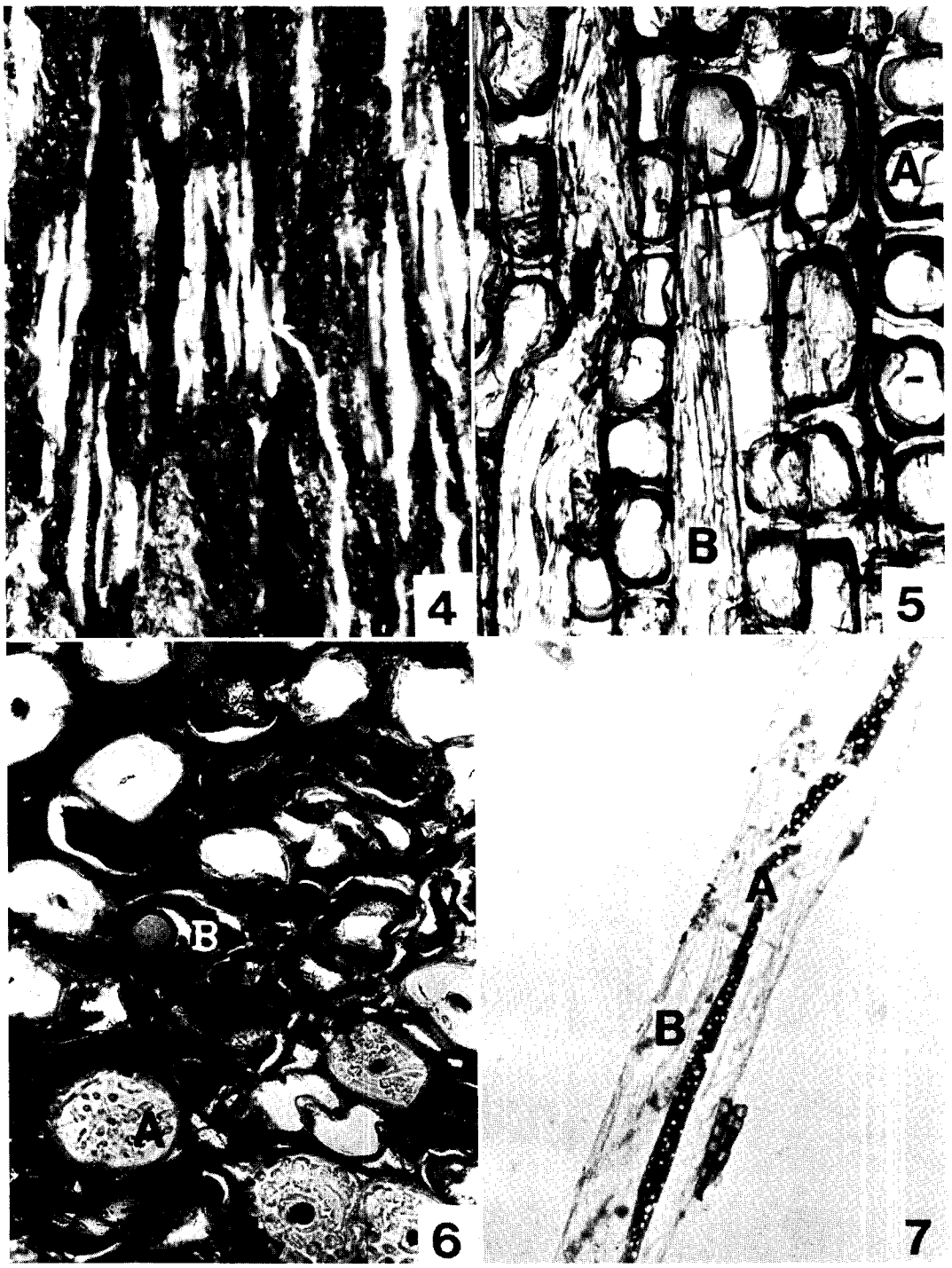
2) Cross-section of inner bark with (A) a sclereid with condensed tannins in the lumen, (B) an axial parenchyma cell containing starch grains, (C) ray parenchyma, and (D) sieve cells.

3) Cross-section of outer-bark phloem with (A) an outer bark sclereid with condensed tannin encrusting the outer cell-wall layer, (B) expanded phloem parenchyma cell and (C) crushed sieve cells.



FIGS. 4–7. 4) A split piece of infested outer-bark phloem containing white ("bleached") sclereids and darkened parenchyma tissue.

5) Longitudinal section of bark containing "bleached" sclereids, with (A) parenchyma cells containing darkened residual protoplasts and fungal hyphae, and (B) degraded sclereid walls.

Incubation of fungi on bark

Bark samples and isolated sclereids were incubated with fungal isolates in an attempt to reproduce specific decay symptoms.

Vial cultures. Outer bark was cut into 1-cm cubes and a 2-mm hole was bored through the center of each cube. Two bark cubes were enclosed in each vial (21 × 70 mm) by attaching a glass rod (2 × 60 mm) to the vial stoppers and passing the rod through the holes in the two bark cubes. Moisture content of the bark was maintained by submerging the lowest bark sample halfway in water in the bottom of the vial. The upper bark sample was above the water in a saturated atmosphere. These bark sample assemblies were sterilized with ethylene oxide, the bark cubes inoculated with mycelial fragments and the vial cultures incubated in the dark at 20–24 C.

Jar cultures. Bark samples of various thicknesses in radial measurement up to about 2.5 cm and about 2 cm square were cut parallel to the cambium. Thickness varied with total bark thickness or depended on whether only outer bark, inner bark, or whole bark was used. Sterilized bark samples were inoculated with fungal mycelium and placed inside small jars on top of plastic test tube caps covered with filter paper wicks that transported nutrient salts (Domsch and Corden 1970) from the bottom of the jars to the bark samples. The samples were observed microscopically at various times for evidence of fungal attack on the bark. Water was added periodically.

Slide cultures. Microscope slides were dipped in Silica Gel G, dried in an oven at 90 C for 1 hr, and placed in petri dishes with filter paper wicks covering each end of the slides. Sterile whole bark samples (about 2 × 2 × 1 cm), or isolated sclereids were inoculated with mycelial fragments while lying on the silica gel. Sterile Bf-2 medium was added to allow thorough wetting of the silica gel via the paper wicks. The slide cultures were incubated in the dark at 20–24 C.

Preparation of isolated sclereid fractions

Whole bark can be ground and then fractionated by screening into homogeneous batches of cork, sclereids, and parenchyma cells (Hergert and Kurth 1952; Kiefer and Kurth 1953). Methods used in this study and descriptions of the resulting bark fractions are given by Ross and Krahmer (1971). Washed sclereids that passed through a 100-mesh Tyler screen were considered free of other cell types. Material passing through a 170-mesh screen (bark fines) was rich in parenchymatous material and was used in making bark extract medium.

RESULTS AND DISCUSSION

Lignin and condensed tannins in bark

The inner bark of Douglas-fir has been described in detail (Chang 1954; Grillos 1956), but details of outer bark structure and development, such as the location of lignin in cell walls and the deposition of condensed tannins in the phloem when periderm layers form, are notably lacking. Therefore, observations and histochemical tests were run on bark tissues to provide necessary background information for studies on fungal degradation of outer bark.

Bark sections for microscopic examination were cut with razor blades from many bark samples, including some in which periderm layers in the inner bark were in the early stages of differentiation. The Wiesner reagent (5 g phloroglucinol/100 ml 95% ethanol acidified with HCl) was used to identify lignified tissue, iodine-potassium

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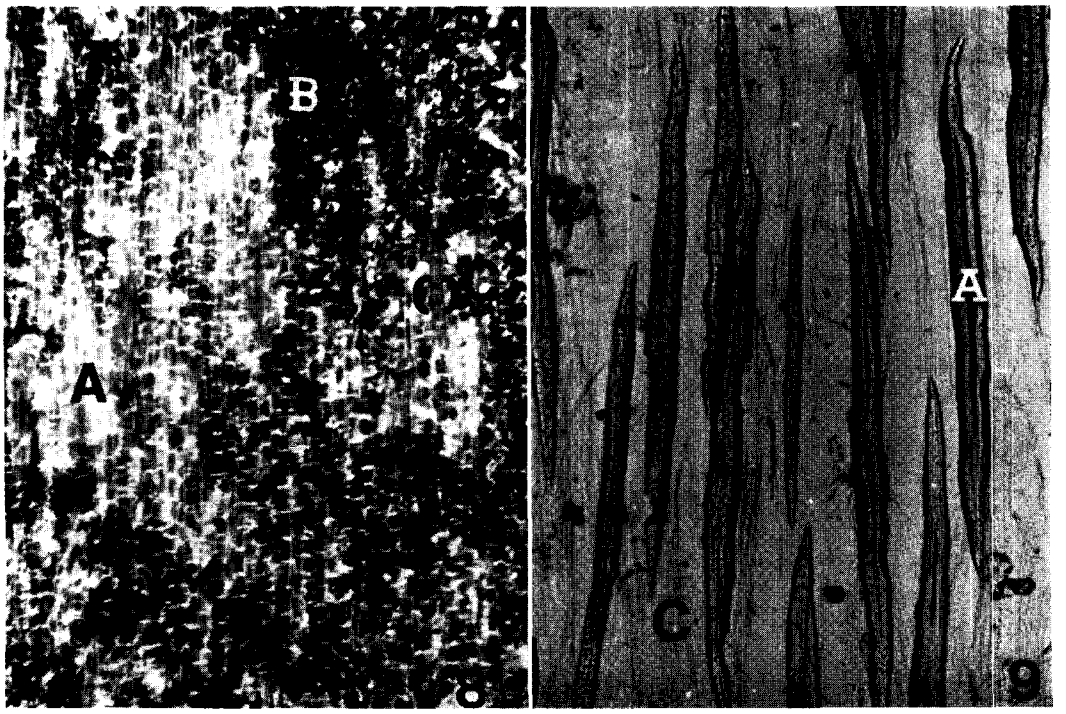
6) Cross-section of bark containing "bleached" sclereids, with (A) bore holes oriented longitudinally in thick sclereid walls, and (B) darkened condensed tannins in parenchyma tissue.

7) A "bleached" sclereid, with (A) darkened tannins in sclereid lumen, and (B) residual "bleached" wall material.



FIGS. 8–9. 8) Outer-bark tissue containing (A) "bleached" parenchyma cells that appear to have had condensed tannins removed by fungal activity, (B) "unbleached" but infested tissue slightly darkened, and (C) a light-colored sclereid with tannins removed from the outer wall layer.

9) Phloem tissue containing (A) sclereid with tannins in its lumen, (B) sclereids with numerous bore holes in their walls and empty lumina, and (C) "bleached" parenchyma cells.

iodide was used to stain starch, and Sudan IV and ferric chloride were used for suberized tissues and polyphenols, respectively (Johansen 1940).

In cross-section, old growth Douglas-fir bark consists largely of reddish-brown phloem traversed by many buff-colored, anastomosing periderm (cork) layers (Fig. 1). All tissue outside the innermost periderm layer is dead and is here termed outer bark (Fig. 1-E). The light tan-colored tissue between the vascular cambium and the innermost periderm layer is inner bark (Fig. 1-C). Many cells of the inner bark are living. Outer-bark phloem is encrusted with condensed tannins and appears darker than inner bark phloem where heavy tannin deposits have not yet occurred (Fig. 1). The vascular cambium adds cells to the inner bark, but the inner bark fails to increase greatly in thickness because new periderm layers periodically form in the outer part of the inner bark (Fig. 1-D) and living inner-bark cells are transformed to dead outer bark phloem.

The thick-walled sclereids (Figs. 2-A and 3-A) are the only cells in the inner and outer bark that stain reddish-violet with the Wiesner reagent, and the color is very similar to that given by lignified wood elements. Scattered periderm and parenchyma cells may stain also but usually only when they are near an injury. At maturity, sclereids are filled with reddish-brown condensed tannins (Figs. 2-A and 3-A) that are converted to phlobaphene-like material following treatment with 72% H_2SO_4, and are released as intact units when the secondary walls are hydrolyzed by the acid.

Most parenchyma cells of the inner bark are living and contain starch, tannins, and other storage products (Fig. 2-B&C). When

a new periderm forms within the inner bark (Fig. 1-D), the storage products disappear from cells that are to become either phellogen or outer bark phloem. Cells destined to become outer bark phloem become greatly dilated (Fig. 2-B vs. Fig. 3-B), and then light reddish-brown materials encrust the expanded walls and residual protoplasts. The outer layer of outer-bark sclereid cell walls (i.e. the primary cell walls of the parenchyma cells from which the sclereids developed) also becomes impregnated with reddish-brown materials (Fig. 3-A). A thin zone of periclinal divisions forms along an abrupt boundary between expanded parenchyma cells and the smaller, undilated, light-colored parenchyma of the inner bark. This zone of cell divisions stains pink with Sudan IV, suggesting the beginning of suberization of the periderm.

These observations are consistent with previous chemical descriptions of Douglas-fir bark. For example, brown material in the outer wall layer of outer-bark sclereids was described by Kiefer and Kurth (1953) as "phenolic acids" soluble in dilute alkali. Hergert (1960) found high concentrations of monomeric catechins and leucocyanidins in the cambial zone of the bark and their concentration decreased centrifugally from the cambium where the polymeric polyphenols increased. This implies that the colorless precursors for 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 concomitant 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 periderm formation, small nonlignified, living parenchyma cells of the inner bark lose their storage materials, increase greatly in size, and become impregnated with condensed tannins. Inner-bark sclereids, which contain lignin in their thick secondary walls and condensed tannins in their lumina, change from light tan to reddish-brown as their outer wall layer becomes impregnated with condensed tannin, as do the surrounding parenchyma walls. Thus, the sclereid is the only bark cell that contains both lignin and condensed tannin.

Sclereid "bleaching"

"Bleaching" of sclereid walls is common in the outer bark of Douglas-fir, especially at the base of trees a hundred years or more in age. Generally, the most advanced degradation is in the outer bark midway between the outer surface and the vascular cambium. As the decay progresses, it moves inward toward the cambium more rapidly than it spreads outward. Infested outer bark phloem is darkened, and the periderm layers become dull gray rather than the normal light buff color. When the bark is broken longitudinally, the normally reddish-brown sclereids appear as white streaks and are easily distinguished from the surrounding phloem tissue (Fig. 4). Microscopic examination reveals heavy accumulations of fungal hyphae and dark reddish-brown materials in the parenchyma tissue (Fig. 5-A). Lightly-"bleached" sclereids have bore holes running longitudinally within their thick walls (Fig. 6-A); as do cells of wood attacked by soft-rot fungi (Fig. 3C *in* Duncan 1960). As decay progresses, the sclereid walls become "bleached," fail to stain with Wiesner reagent, and are easily crushed under pressure as though the lignin component of the walls has been degraded. In advanced stages of decay, the secondary walls of sclereids are completely decomposed. Thus, decayed sclereids (Figs. 4 and 7) more closely resemble elements of white-rotted wood than those of soft-rotted wood (Figs. 5 and 7 *in* Duncan 1960), which in external appearance generally is similar to brown-rot decay.

Condensed tannins in the lumina of "bleached" sclereids and on parenchyma cell walls and protoplasts are darker in decaying bark than in normal tissues (Fig.

1-G vs. F; Figs. 5 and 6 vs. 3). Although darkened, these condensed tannins are not substantially removed from the tissues.

Thick, greenish-black hyphae often proliferate along the periderm layers surrounding decayed phloem, suggesting that periderm layers slow the advance of bark fungi. When heavily infested, the periderms change from a light tan to a dull gray color. Where periderm tissue was broken along the edges of fissures in the bark (Fig. 1), severe sclereid degradation occurred up to the next intact periderm.

In an attempt to identify microorganisms capable of attacking bark, "bleached" sclereids were isolated from the outer bark of each of eight trees and planted in nine different media (Ross 1971). Of 177 fungal colonies obtained, 110 were *Bispora betulina* (cda.) Hughes. This fungus has greenish-black hyphae similar to hyphae often observed within walls of "bleached" sclereids.

Bispora betulina caused a heavy precipitation of reddish-brown materials after two weeks' growth in inner bark extract medium, and thus is capable of modifying bark components. However, when bark samples were inoculated with this fungus and incubated 12 and 16 months, respectively, in vial and jar cultures, the sclereids were not "bleached," although the condensed tannins of cork and parenchyma cells were darkened as in cells typically associated with "bleached" sclereids. Similarly, in slide cultures the fungus did not attack isolated sclereids.

Parenchyma "bleaching" and sclereid "emptying"

A second modification of bark cells involves removal of condensed tannins from parenchyma cell walls and protoplasts and from the outer walls and lumina of sclereids. This type of bark degradation is most prevalent in slow-growing trees on poor sites and in suppressed trees on better sites. It occurs in younger trees than sclereid "bleaching," and generally extends the length of the bole. "Bleached" parenchyma cells occur immediately under the outer cortical tissues of the bark, and when "bleaching" is severe, the sclereids become detached from the surrounding decayed tissues.

Degradation of condensed tannins in bark presents a picture that is the inverse 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 parenchyma remains a dark reddish-brown (Fig. 8 vs. 4). Microscopic examination of bark with "bleached" parenchyma reveals that not only are tannins removed from parenchyma cells and the outer walls of sclereids, but bore holes penetrate the thick sclereid walls and the tannins are removed from the sclereid lumina as well (Fig. 9).

When condensed tannins are removed from the outer cell wall layers of sclereids from outer bark (sclereids that are also "emptied"), they become tan-colored like inner-bark sclereids. Unlike cell walls of "bleached" sclereids, "emptied" sclereids (Fig. 9) stain brilliant red-violet with the Wiesner reagent. Thus, tannin degradation appears highly specific with little or no degradation of sclereid lignin.

Individual "emptied" sclereids and small groups of "bleached" parenchyma cells were isolated from the outer bark of each of eight trees from three sites. The bark cells were planted on nutrient media (Ross 1971) to select microorganisms capable of modifying the condensed tannins. Because these symptoms of degradation occur predominantly at or just below the bark surface, there is a complex microflora present. Nevertheless, 13 of 45 isolates were an imperfect fungus that, on the basis of mycelial characteristics best fits the ill-defined form genus, *Isaria* (Fries) (Barnett 1960; Morris 1963; Barron 1968). When grown on PDA, the *Isaria*-like fungus formed fluffy tufts of white synnemata with many seta-like white hyphae protruding laterally from the main axis of each synnema. In older cul-

tures short, thick brown synnemata formed. No conidia were produced. The next most prevalent fungus was isolated only six times.

The *Isaria*-like fungus caused a heavy precipitation of phenolic components of bark extract medium within 24 hr. When incubated with whole or outer bark samples in slide culture for six months, the fungal hyphae colonized the bark uniformly and penetrated some sclereid walls, but failed to "bleach" parenchyma or "empty" sclereid lumina. The fungus did not noticeably alter isolated sclereids in slide cultures.

CONCLUSIONS

From microscopic and histochemical examination of the degradation of bark by fungi, it appears that within phloem tissue, *B. betulina* is associated with alterations of lignified sclereid walls, and with darkening, but not removal of condensed tannins. A fungus resembling *Isaria* is associated with removal of substantial amounts of the condensed tannins from phloem tissue, but except for production of small holes, lignified sclereid walls are not extensively degraded by this fungus. Although these different patterns of degradation were not produced in bark tissues inoculated with *B. betulina* or the fungus resembling *Isaria*, significant alterations of bark components by them suggests decay capabilities. Unique enzyme systems are required for degradation of lignin versus condensed tannin.

Chemical changes that occur in "bleached" tissue and the capabilities of the suspected causal fungi for altering condensed tannins and sclereid lignin are described in a later paper. Discussion of the phenolic compounds related to lignin and tannins has been omitted from the later paper.

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