Selective Delignification of Eastern Hemlock by *Ganoderma tsugae*

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ABSTRACT

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Ganoderma tsugae caused two distinct types of degradation in naturally decayed eastern hemlock, *Tsuga canadensis*. Large areas of wood were selectively delignified and a white rot, characterized by simultaneous removal of lignin and carbohydrates from the cell walls, occurred in localized areas. Scanning and transmission electron micrographs of delignified wood demonstrated a diffusible ligninolytic system that caused degradation of the compound middle lamella without substantial alteration of the secondary wall. The S_2 layer of the secondary wall remained least affected. Chemical analyses of delignified wood indicated hemicelluloses were removed in preference to cellulose when lignin was degraded. Pseudosclerotia, or zone lines, present in the outer edges of decayed logs and stumps were morphologically different from the black areas that occurred only in delignified wood. Micromorphological and ultrastructural studies made possible a schematic illustration of decay patterns caused by *G. tsugae* in eastern hemlock wood.

Ganoderma tsugae Murr. causes a soft white spongy rot in stumps and wood of fallen eastern hemlock (5,11). The fungus apparently is not responsible for decay in living trees, and few investigations have been carried out describing the patterns of timber degradation. West (28) recognized the decay as a peculiar type of rot containing horizontal and vertical cracks completely filled with white mycelium as well as large areas of soft white spongy wood with numerous black spots scattered throughout. Two closely related species, which cause similar decay patterns, are *G. oregonense* Murr., primarily on dead but occasionally on living Douglas-fir, spruce, hemlock, and pines in western United States (2), and *G. lucidum* (Fr.) Karst., which causes a widely distributed root rot and decay of deciduous trees (5,11).

Many wood-destroying fungi can degrade lignin, but only a relatively small number can selectively remove lignin without concomitant removal of appreciable amounts of cellulose and other wood carbohydrates (3,9,21). Fungi that selectively degrade lignin have great potential application in bioligninolytic systems for conversion of lignocellulosics into livestock feed, production of cellulosic products (eg, pulp) and treatment of lignin-derived wastes (7,16). Wood decayed by *G. tsugae* was selected for study because of the large white zones that macroscopically resembled delignified wood caused by white-pocket rot fungi (3,21). Unlike white-pocket rot that occurs in localized areas of living trees, decay by *G. tsugae* results in extensive areas of white, apparently delignified wood in dead trees.

The purpose of this investigation was to examine wood decayed by *G. tsugae* for selective delignification, identify the patterns of cell wall degradation, and to obtain a better understanding of the decomposition process by *G. tsugae* in eastern hemlock.

MATERIALS AND METHODS

Decayed wood from 18 eastern hemlock logs and stumps, bearing sporophores of *G. tsugae*, was obtained from the Chequamegon National Forest, Bayfield County, WI, USA, for chemical and morphological studies. Cubes of wood were cut with a razor blade and infiltrated with water under low vacuum using a hand-operated vacuum pump. Radial, tangential, and transverse sections 15- to 20-μm thick were cut with a cryostat microtome at −20 C. Sections were stained with zinc-chlor-iodide or phloroglucinol-HCl as described by Jensen (12). Sections were also prepared for scanning electron microscopy as described previously (3). Additional blocks were prepared for transmission electron microscopy by fixation in 3.3% glutaraldehyde with 500 μg of ruthenium red per milliliter in 0.1 M sodium cacodylate buffer for 48 hr followed by 1% osmium tetroxide with 500 μg of ruthenium red per milliliter in 0.1 M sodium cacodylate buffer for 15 hr. Samples were placed under low vacuum for 30 min while in the glutaraldehyde-ruthenium red fixative. Tissues were rinsed in buffer, dehydrated in an acetone series over a 4-hr period, embedded in Spurr's (26) epoxy resin formulation B containing 33% less D.E.R. 736 than the standard mixture and sectioned with a diamond microtome knife. Thin sections were stained with uranyl acetate and subsequently with lead citrate. Thick sections, approximately 0.5 μm, were also cut from the plastic-embedded samples with a glass microtome knife. The plastic embedding medium was removed by using techniques described by Mayor et al (20). Sections were stained with rhodamine 6G (0.25% aqueous) and observed with incident illumination from a mercury vapor lamp. An Aus Jena Fluoval fluorescence microscope with a BG12 exciter filter and OG1 barrier filter was used.

The specific gravity based on green volume was determined for sound and decayed wood (13,23). Ten replications were used for each sample. Sulfuric acid lignin was determined as previously described (8) and high pressure liquid chromatography was used to quantify the amount of wood sugars (22,25,27). An average was obtained from three replications for each analysis.

RESULTS

Wood decayed by *G. tsugae* typically showed a mottled rot consisting of two distinct types of decay (Fig. 1a): large uniformly white areas of delignified wood (labeled dl in Fig. 1a) containing black, spindle-shaped zones (Fig. 1a, arrowheads), and yellow or tan areas that were white-rotted (labeled wr in Fig. 1a) with numerous small holes filled with fungal mycelium. These two types of decay were easily differentiated when phloroglucinol-HCl was applied to the surface of the wood block. A bright carmine red

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coloration (dark areas in Fig. 1b) indicated the presence of lignin. The yellowish or tan wood around the holes containing masses of mycelium always gave a positive reaction to the stain. No color reaction was observed in the white areas, indicating that lignin had been altered or removed. Black zones within these white areas lost color intensity and gradually disappeared after phloroglucinol-HCl treatment.

Sections through several annual rings of decayed wood demonstrated that earlywood tracheids were preferentially delignified (Fig. 2a). Sections treated with phloroglucinol-HCl and observed with a stereoscope using transmitted light showed intense staining of latewood tracheids (Fig. 2a, arrowheads), but tracheids in the earlywood portion of the annual rings were not stained. Positive staining with phloroglucinol-HCl suggested substantial amounts of lignin remained in latewood cells; however, the adjacent earlywood tracheids were free of lignin.

Sections cut from non-delignified areas (wr in Fig. 2b) and examined by scanning electron microscopy showed tracheid cell walls with intact middle lamellae and exposed lumens. In contrast, tracheids from delignified wood (labeled dl in Fig. 2b) readily separated and could not be cut to expose the cell lumen. The cells of the delignified areas appeared to lack middle lamellae (Fig. 2c) and individual tracheids could be pulled apart easily (Fig. 2d). Ray parenchyma cells were completely destroyed. Latewood that stained a deep carmine color with phloroglucinol-HCl had decay that resembled a typical white rot; lignin and carbohydrates were removed simultaneously from isolated areas. Holes were present in the tracheid cell walls, and large voids resulted from the gradual coalition of degraded areas; these were filled with fungal mycelium (Fig. 2e and f). Ray parenchyma cells also contained holes similar to the white rot degradation of the tracheids.

Transverse thin sections, examined with a transmission electron microscope, demonstrated the cell wall layers in sound eastern hemlock (Fig. 3a and b). Cell wall ultrastructure consisted of compound middle lamella, composed of a true middle lamella and primary wall, secondary wall with S_1, S_2, S_3 regions, and warty layer. Thin sections of wood from white, delignified areas ($59.5 \pm 3.9\%$ weight loss) decayed by *G. tsugae* exhibited various degrees of degradation in the compound middle lamella (Fig. 3c–f). The S_2 layer of the secondary wall remained intact and alterations of the warty and S_3 layer were evident (Fig. 3c–e). Cells separated easily from one another demonstrating a defibration of the wood (Fig. 3f). Although the middle lamella was completely removed, the S_2 layer remained relatively unaltered. The selective decomposition by *G. tsugae* of the compound middle lamella was not limited to a few specific areas of a cell, but occurred throughout a large portion of the wood. Photomicrographs, obtained by using fluorescence microscopy of sound (Fig. 4a) and decayed wood (Fig. 4b) demonstrated that selective delignification occurred in many tracheids. The decayed tracheids appeared distorted and collapsed due to the removal of compound middle lamella between cells (Fig. 4b).

Chemical analyses showed that sound eastern hemlock wood contained 32.4% lignin, 48.0% glucose, 4.2% xylose, and 13.8% mannose. Decayed wood had a $65.4 \pm 2.9\%$ weight loss, and the white delignified wood, when separated from the white-rotted wood, had a $59.5 \pm 3.9\%$ weight loss (Table 1). 98.3% of the lignin was removed from the white, delignified wood as well as various amounts of hemicellulose (Table 1). There was a 26.6% loss of glucose from the delignified wood when compared to sound wood.

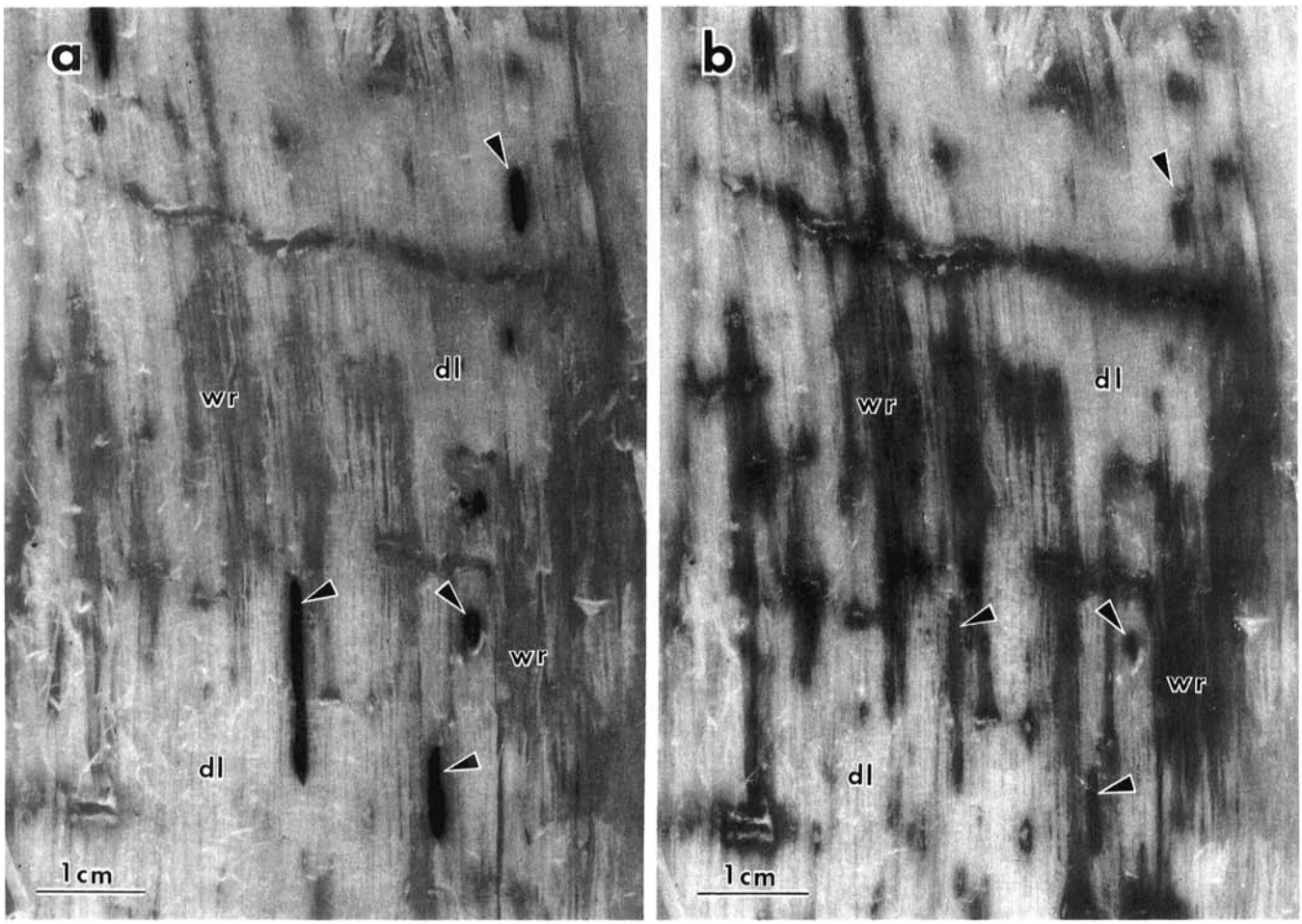

Fig. 1. a, Eastern hemlock wood decayed by *Ganoderma tsugae* with delignified wood (dl) containing several black areas (arrowheads) and white-rotted wood (wr). **b,** The same section of decayed wood shown in (a) after treatment with phloroglucinol-HCl. The white-rotted areas (wr) that appear dark are actually a bright carmine color, indicating a positive reaction for the presence of lignin. White, delignified wood (dl) remained white and black areas (arrowheads) were partially decolorized.

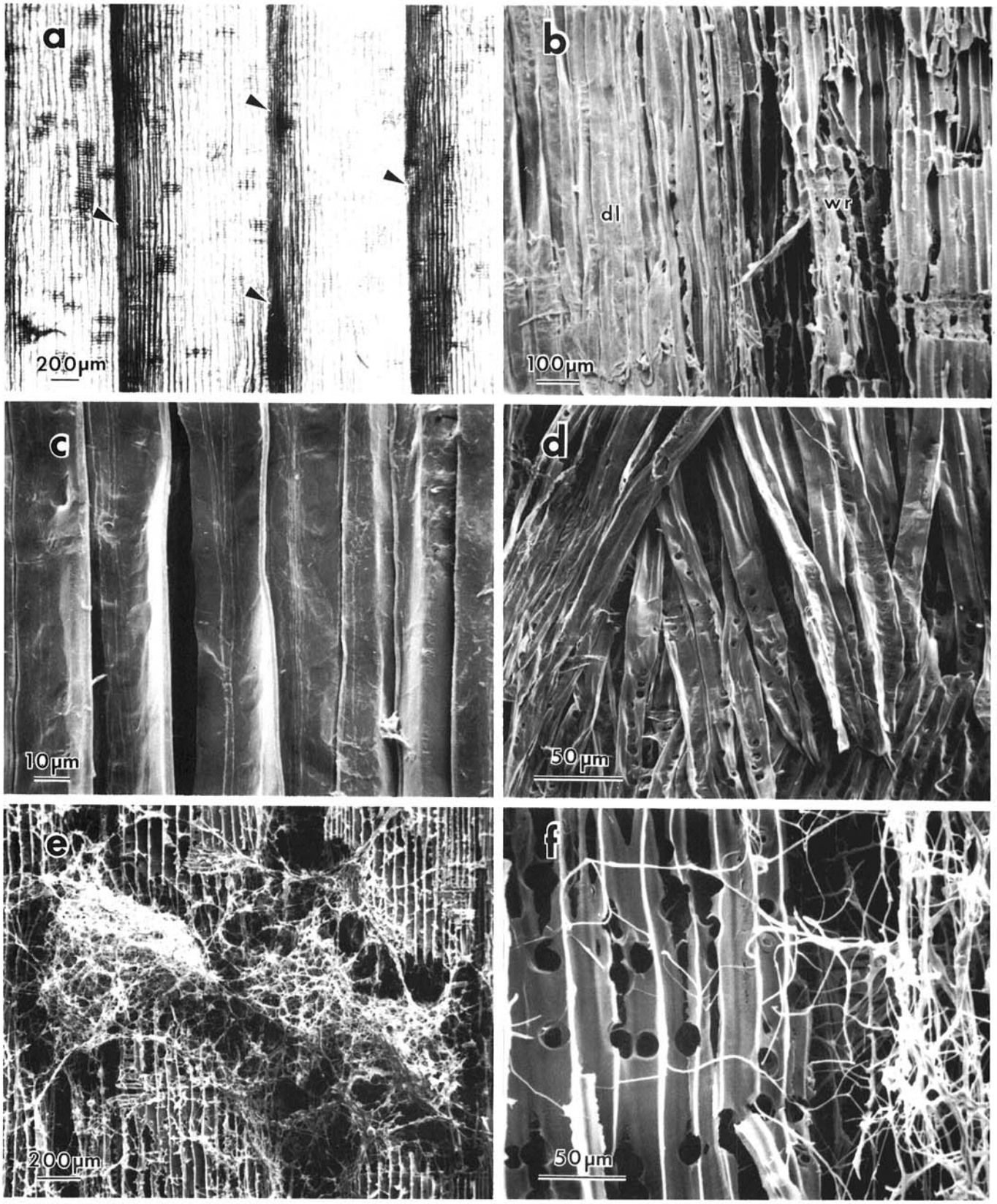

Fig. 2. Light **(a)** and scanning electron micrographs **(b to f)** of decayed eastern hemlock. **a,** Latewood tracheids stained a bright carmine color (arrowheads) when treated with phloroglucinol-HCl while earlywood cells remained colorless. **b,** Tracheids from white, delignified areas (dl) separated between cells and adjacent white-rotted wood (wr). **c,** Tracheids from delignified wood showed smooth outer surfaces without middle lamellae. **d,** Delignified tracheids separated easily, resulting in a defibration of the wood. **e,** White-rotted wood that stained positively with phloroglucinol-HCl contained large voids that were filled with fungal mycelium. **f,** Tracheids from white-rotted wood contained holes caused by a progressive thinning of the cell wall. Large voids resulted from the gradual coalescence of degraded areas.

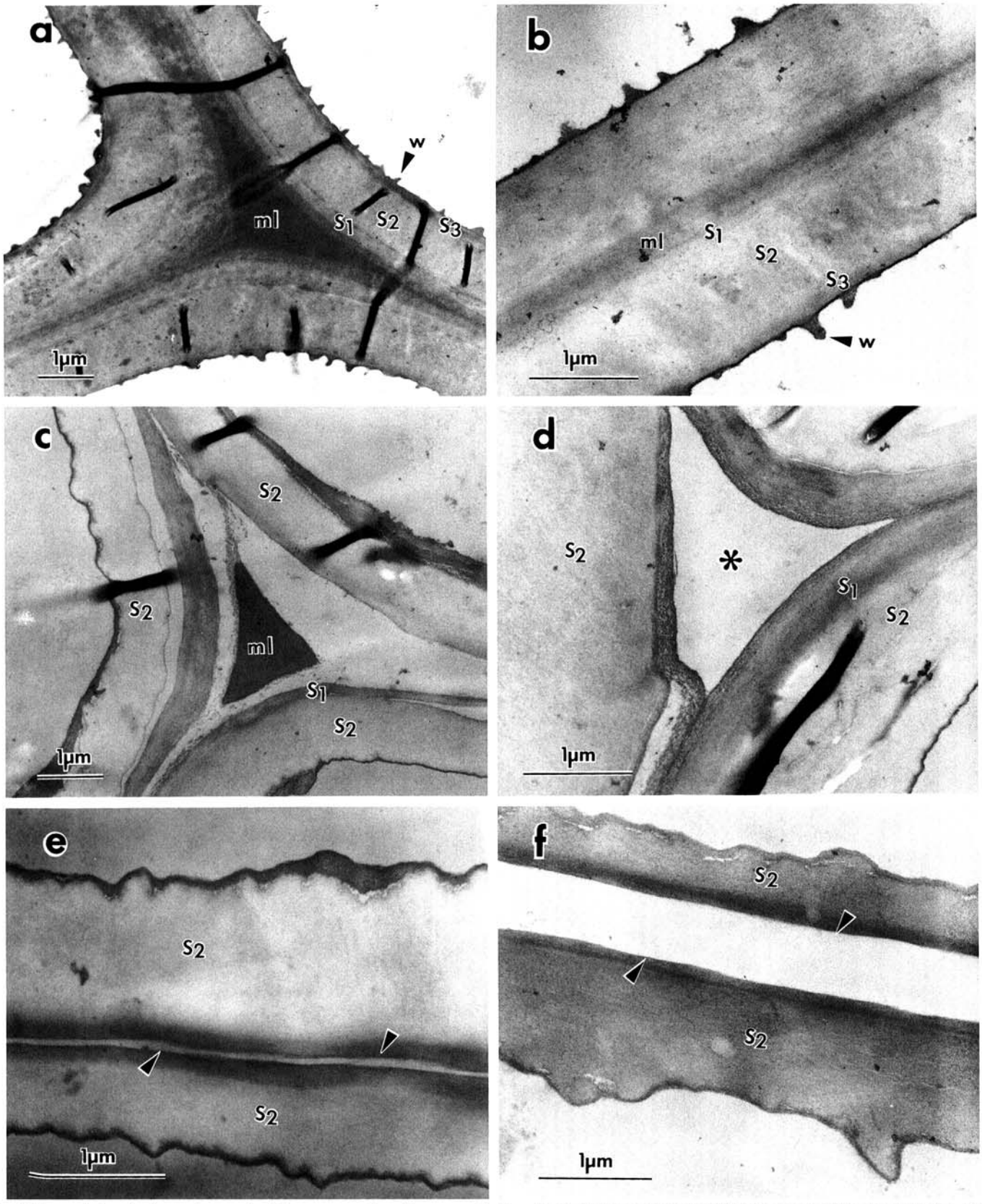

Fig. 3. Transmission electron micrographs of transverse sections from sound **(a,b)** and delignified wood **(c to f)** exhibiting a group of three tracheids **(a,c, and d)** and two adjacent tracheids **(b,e, and f). a and b,** Tracheids with unaltered compound middle lamella (ml), secondary wall (S_1, S_2, and S_3 regions) and warty layer (w). **c,** Partially degraded compound middle lamella with S_2 regions of the secondary wall remaining relatively unaltered. **d,** The compound middle lamella was extensively decomposed (*) without alteration of the secondary wall. **e,** Tracheids with completely degraded middle lamella (arrowheads) contained secondary walls with rough surfaces in the cell lumens. **f,** Delignified tracheids separated due to a lack of middle lamella (arrowheads). The warty layer and S_3 region of the secondary wall also was removed, but a large portion of the S_2 region remained.

The proportion of carbohydrate to lignin lost was 0.27, 0.77, and 0.88 for glucose, xylose, and mannose, respectively.

Light microscopy of the black spots in delignified wood showed tracheids containing thick-walled swollen hyphae with transparent, yellowish deposits around them (Fig. 5a). Scanning electron micrographs of these black areas did not reveal any micromorphological differences of the tracheid cell walls when compared to nonpigmented areas. This was due to the lack of compound middle lamellae resulting in tracheids that separated from one another, so that only the outer surface of delignified tracheids could be observed.

Black pseudosclerotia or zone lines frequently occurred at the outer edge of wood decayed by *G. tsugae*. The wood around the pseudosclerotia was not delignified and reacted when phloroglucinol-HCl was applied. The black coloration of the pseudosclerotia was not altered by the dilute HCl solution. The pseudosclerotia contained copious amounts of deposits that completely filled the tracheids and appeared to be morphologically different from the occlusions in black areas of delignified wood (Fig. 5b). Masses of hyphae were observed in and around the occlusions.

A schematic illustration obtained from transverse, tangential, and radial sections of wood decayed by *G. tsugae* is presented to summarize the results. A comparison of a sound wood block (Fig. 6) and decayed block (Fig. 7) demonstrates the patterns of white rot and selective delignification by *G. tsugae*.

DISCUSSION

Selective lignin degradation by *G. tsugae* occurred without concomitant loss of appreciable amounts of cellulose. The micromorphological and ultrastructural evidence indicated the cellulose-rich S_2 layer remained relatively unaffected while the

TABLE 1. Percent weight loss and percent loss of lignin and wood sugars in eastern hemlock decayed by *Ganoderma tsugae* under field conditions

	Weight loss[a]	Loss of original components (%)[b]			
		Lignin	Glucose	Xylose	Mannose
Entire decay	65.4 ± 2.9	80.8	56.2	64.4	83.8
White tissues	59.5 ± 3.9	98.3	26.6	76.0	86.1

[a] Average of 10 replications with standard deviation.
[b] Average of three replications.

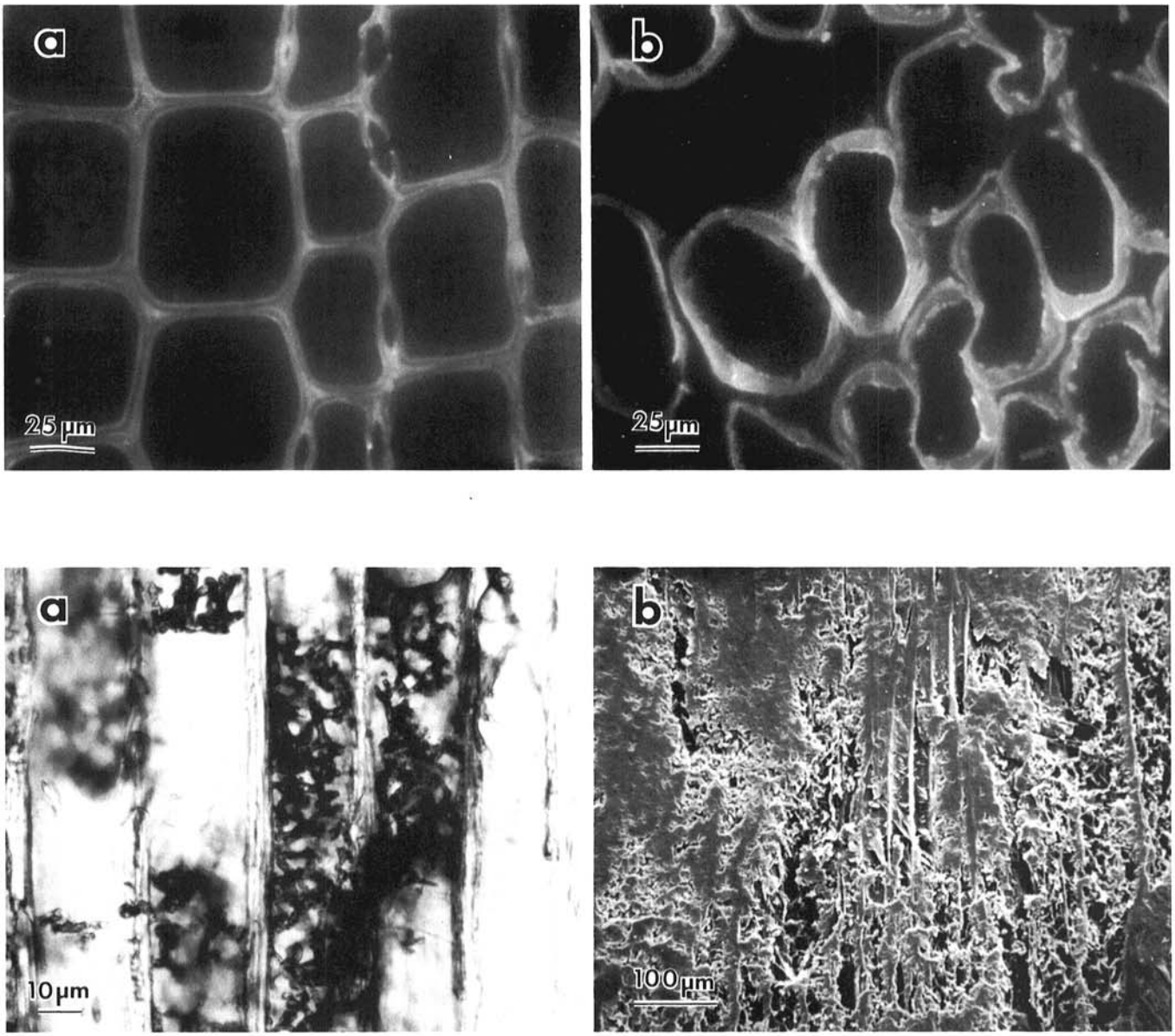

Fig. 5. a, Light micrograph of a black area within delignified wood. Tracheids contained thick-walled swollen hyphae and transparent occlusions. **b,** Scanning electron micrograph of a pseudosclerotium (zone line) showed profuse deposits that filled the tracheids and masses of fungal hyphae within, as well as surrounding, the occlusions.

compound middle lamella was removed. A highly diffusible ligninolytic system apparently is produced, since fungal hyphae located in the cell lumens caused degradation throughout compound middle lamellae. Cowling (6) postulated that extracellular enzymes of white-rot fungi have the ability to diffuse from the fungal cell wall. Unlike most white-rot fungi, however, *G. tsugae* produces a selective degradation system that apparently can diffuse and remove lignin at considerable distances. Recently, Eriksson et al (10) and Ruel et al (24) demonstrated that a mutant strain of *Phanerochaete chrysosporium* Burds., lacking cellulase enzymes, caused fiber separation in spruce wood. A progressive thinning of the cell wall from the lumen outward, commonly observed in wood decayed by white-rot fungi (4,17,29), was not apparent in wood delignified by the cellulase-less mutant (10) or by *G. tsugae*.

Kirk et al (15) and Ander and Eriksson (1) have shown that lignin cannot be degraded without a loss of some carbohydrate from the wood. White-rot fungi, such as *Coriolus versicolor* (L.:Fr.) Quél and the wild type *P. chrysosporium* require glucose or cellulose in order to metabolize lignin (15). The results reported here indicate that *G. tsugae* does not remove large amounts of cellulose when lignin is degraded, but 76–86% of hemicellulose is lost. Decay by the cellulase-less mutant also removed substantial amounts of hemicellulose as well as low-molecular-weight sugars in wood when

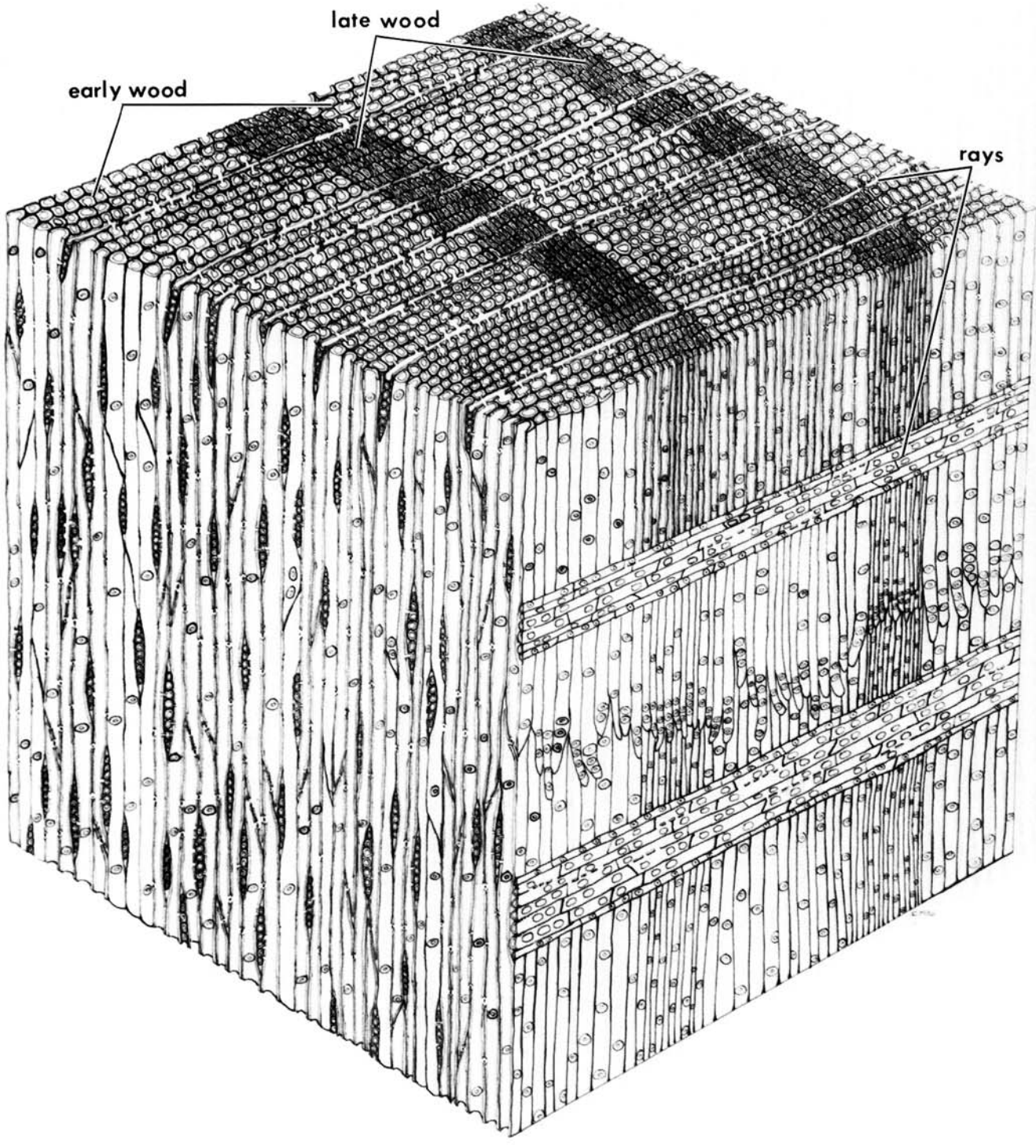

Fig. 6. Diagrammatic representation of a block of sound eastern hemlock wood showing two complete growth rings with earlywood and latewood cells. Tracheids and ray tracheids with bordered pits and uniseriate rays with simple pits are present. Normal resin canals are wanting.

lignin was removed (10). The arrangement of lignin and carbohydrate in the wood cell wall as proposed by Kerr and Goring (14) demonstrates the close spatial relationship of lignin and hemicellulose. This model provides an explanation of how the lignin and hemicellulose matrix could be selectively removed from wood without a substantial loss of cellulose.

Although selective delignification by *G. tsugae* occurred in large areas, it was not uniformly distributed throughout the wood (Figs. 1,7). The percent weight loss (Table 1) from samples of the entire decay were greater than loss from the delignified wood. The entire decay samples contained delignified wood, pseudosclerotia, and white-rotted wood. Chemical analyses of white-rotted wood (15) demonstrates the simultaneous removal of lignin and carbohydrates. A ratio of carbohydrate lost to lignin lost is usually

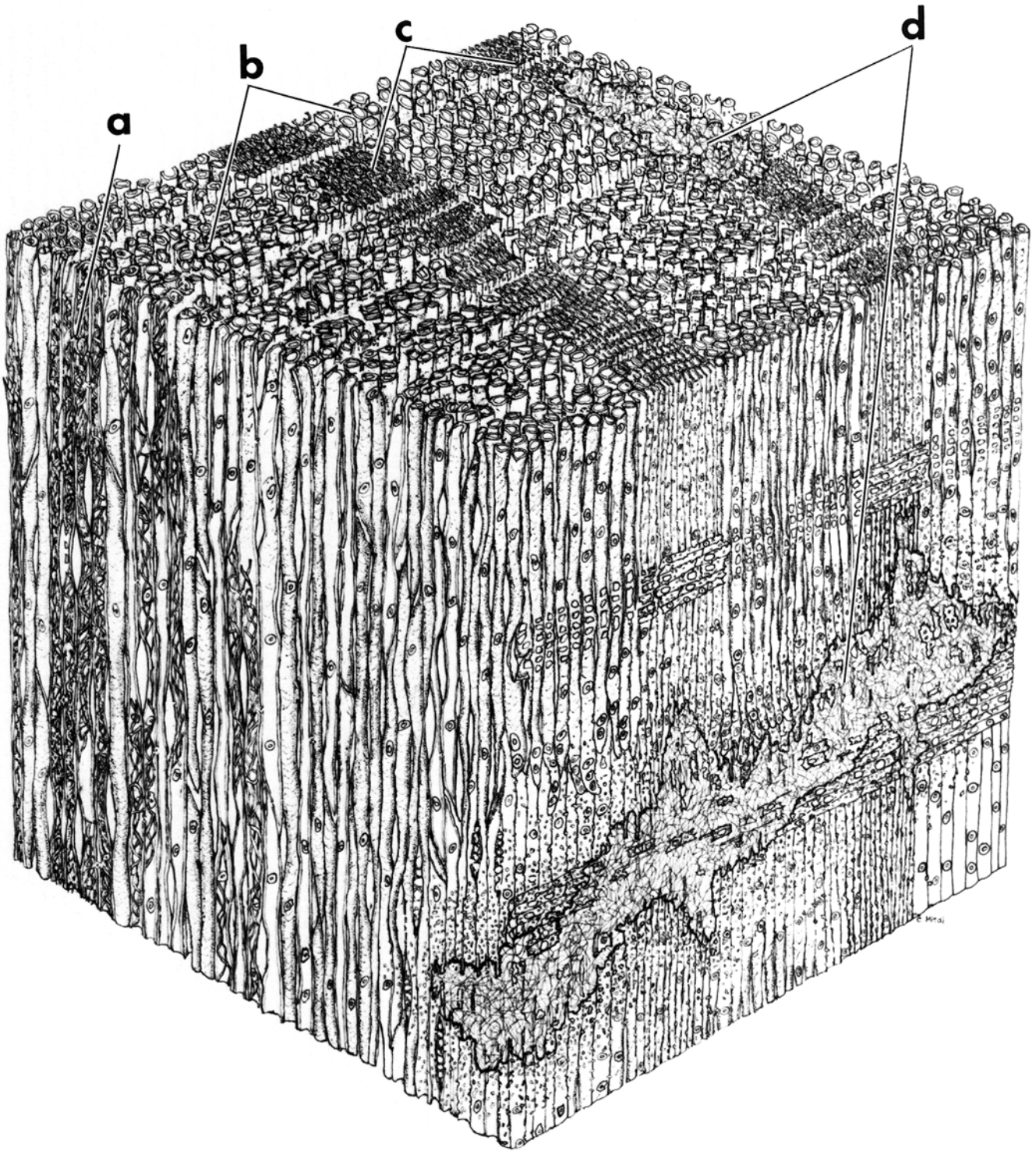

Fig. 7. Diagrammatic representation of a block of eastern hemlock wood decayed by *Ganoderma tsugae* showing two distinct types of decay. Dark areas within delignified wood (a) contained thick-walled hyphae with transparent deposits around them. Earlywood cells were delignified (b). The compound middle lamella was removed but the secondary wall remained relatively unaltered. Ray parenchyma cells were completely destroyed. Latewood tracheids were degraded by a typical white rot resulting in a simultaneous removal of cell wall components (c), but ray parenchyma cells were not removed. Lysed zones or erosion troughs produced around hyphae resulted in holes that formed within the cell walls. Gradual coalition of degraded areas caused large voids to form within the wood (d). These voids were filled with fungal hyphae. Tracheids surrounding the voids were white-rotted and exhibited a typical shot-hole appearance.

1:1 (6). The low ratio of glucose to lignin lost in the delignified wood demonstrates the selectivity for lignin removal. Although only 26.6% of the glucose is removed when 98% of the lignin is degraded, less cellulose degradation may occur at earlier stages of delignification.

The ability of basidiomycetes to produce two different types of decay has been previously demonstrated for *Phellinus pini* (Thore:Fr.) A. Ames (3) and *Inonotus dryophilus* (Berk.) Murr. (21). These white-pocket rot fungi cause delignification in localized areas in living trees, but can also cause a typical white rot in some areas of the wood. Anatomical and chemical obstructions limit the size of the delignified area. Resin ducts, earlywood tracheids, and occluded ray parenchyma cells in pine and larch (3) and medullary rays and occluded latewood cells in white oaks (21) restrict delignification by *P. pini* and *I. dryophilus*, respectively. In contrast, delignification of eastern hemlock by *G. tsugae* occurs in large undefined patches. Latewood tracheids appear to be the only restriction during the incipient stages of decay.

Pseudosclerotia, commonly referred to as zone lines, occurring at the edge of decaying logs and stumps appear to be formed in response to desiccation (19). These three-dimensional plates are similar to the dark pseudosclerotia previously described for many wood-destroying fungi (5,18). The black areas within wood delignified by *G. tsugae* are not pseudosclerotia. These localized areas of thick-walled hyphae with pigmented substances surrounding them are produced within extremely moist, decayed wood. The pigmented substances are decolorized with dilute HCl, whereas the pseudosclerotia are unaltered by this treatment. Since these black areas occurred only in delignified wood, they may consist of by-products of lignin degradation. Similar black spots occur in wood decayed by many species of basidiomycetes, such as *Heterobasidium annosum* (Fr.) Bref., and *Ganoderma applanatum* (Pers.:Wall.) Pat. (5). However, information concerning their function and chemical composition is not available.

One of the interesting features of *G. tsugae* is its ability to cause selective delignification under natural conditions. This decay has decomposition patterns similar to the cellulase-less mutants of *P. chrysosporium* (10,24). The ability of fast-growing (wild type) basidiomycetes to intermittently cause selective lignin degradation indicates that substrate and environmental factors may affect the degradation process, resulting in delignification identical to the decay by genetically altered cellulase-less mutants. The factors responsible for inducing delignification by *G. tsugae* instead of a simultaneous removal of lignin and cellulose are not well understood and should be further investigated. In vitro studies should also provide important information concerning the diffusibility of lignin-degrading enzymes and help elucidate the mechanism of action of the ligninolytic system.

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