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Immunocytochemical Localization of Laccase L1 in Wood Decayed by *Rigidoporus lignosus*

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The cellular distribution of laccase L1 during degradation of wood chips by *Rigidoporus lignosus*, a tropical white rot fungus, was investigated by using anti-laccase L1 polyclonal antisera in conjunction with immunolabeling techniques. The enzyme was localized in the fungal cytoplasm and was associated with the plasmalemma and the fungal cell wall. An extracellular sheath, often observed around fungal cells, often contained laccase molecules. Diffusion of laccase within apparently unaltered wood was seldom observed. The enzyme penetrated all degraded cell walls, from the secondary wall toward the primary wall, including the middle lamella. Xylem cells showing advanced stages of decay were sometimes devoid of significant labeling. These data suggest that the initial attack on wood was not performed by laccase L1 of *R. lignosus*. Previous alteration of the lignocellulose complex may facilitate the movement of laccase within the wood cell walls. This immunogold study revealed that laccase localization during wood degradation seems limited not in space but in time.

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are blue copper-containing glycoproteins widely distributed in some higher plants and fungi (42). Among the fungi, several genera belonging to the subdivisions Ascomycotina, Deuteromycotina, and Basiodiomycotina are known to produce laccase. *Rigidoporus lignosus* (Klotsch) Imazeki is a basidiomycete causing a white rot of roots in more than 100 species of trees. *Hevea brasiliensis* (the rubber tree), a tropical tree cultivated for its latex production, is particularly affected by this pathogen in West African plantations (36). During root colonization, this fungus excretes several enzymes (18) that probably contribute to the rotting of roots (20, 37, 38). Among these enzymes, laccases, isolated and purified from culture filtrates, have been demonstrated to degrade a lignin isolated from *H. brasiliensis* by releasing low-molecular-weight material (13, 16).

The first interest in these enzymes was related to their production as a fungal taxonomic tool (2). However, the function and properties of fungal laccases have been extensively studied (33), with particular attention having been paid to their possible role in lignin biodegradation, cell detoxification, or both (23, 27, 30, 33). To improve our understanding of wood alteration by white rot root pathogens, investigations have been conducted ultrastructurally to localize wood-degrading enzymes (5). A great deal of information has been obtained in vitro on the localization of such enzymes by immunocytochemical methods and electron microscopy. Polyclonal and monoclonal antibodies raised against purified enzymes from wood decay fungi have been used in conjunction with colloidal gold to label antigens in hyphae and in infected wood or wood fragments. The distribution of xylanases (6), cellulases (45), Mn(II)-dependent peroxidases (MnP) (9), and lignin peroxidases (LiP) (6, 8, 9, 15, 48) in xylem cell walls has thus been studied with wood decayed by various fungi, including *Phanerochaete*, *Coriolus*, *Pleurotus*, *Lentinula*, *Phellinus*, and *Fomitopsis* species.

Surprisingly, ultrastructural localization of laccases with respect to the fungus during wood degradation is poorly documented. Previous work on cytochemical distribution of these enzymes has been carried out by using DOPA (3,4-dihydroxyphenylalanine) (47) and syringaldazine (26) as substrates. However, such techniques may lead to results that are unspecific or otherwise difficult to interpret. To our knowledge, the only direct visualization of laccases within decayed wood was performed on beech wood infected by *Coriolus versicolor* (12). Although this work was of interest, it did not focus on the distribution of laccases over xylem cell wall elements with respect to the decay process, including wall penetration by fungal hyphae and localization with a time course study.

We have addressed this question by studying the localization of laccase L1 of *R. lignosus* in infected wood fragments. In this article, we report results from an immunocytochemical investigation aimed at gaining insight into the role of laccases in wood biodegradation.

MATERIALS AND METHODS

Fungal strains. *R. lignosus* (strain 1) was collected from infected rubber tree roots in the Ivory Coast. Inoculation studies have shown this isolate to be virulent (40). It was routinely maintained on 2.5% malt agar (Difco, Detroit, Mich.) plates. For laccase production, the mycelium was grown in 250-ml Erlenmeyer flasks containing 50 ml of a mineral medium supplemented with glucose (1 g/liter), copper sulfate (0.5 mg/liter) and *p*-hydroxybenzoic acid (1 mM) according to the method of Galliano et al. (13). After being incubated for 2 weeks at 28°C, the cultures were filtered through a sintered-glass filter with a porosity of 1, and the filtrates showing laccase activity were pooled (19).

Purification of laccase L1. Enzyme was purified from culture filtrates in a two-step procedure (batch DEAE-

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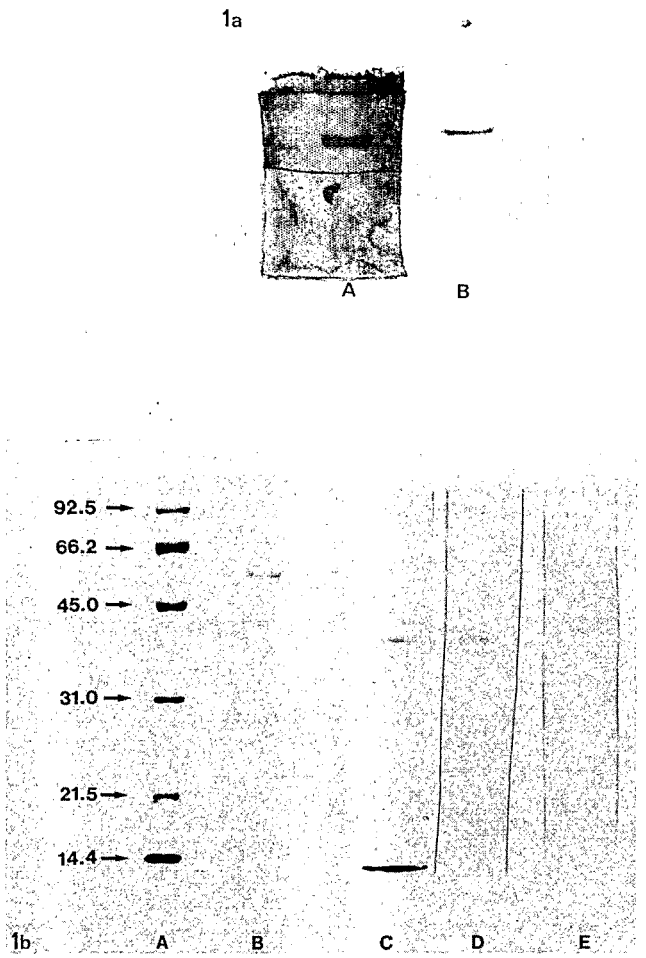


FIG. 1. PAGE and Western immunoblotting of purified laccase L1 from *R. lignosus*. (a) Native gels showing one protein band stained with silver nitrate (lane A) and one band after the gel was stained with guaiacol for laccase activity (lane B). (b) SDS-PAGE of purified laccase stained with Coomassie blue (lane B) and compared with various molecules of known molecular weight (lane A; molecular weights indicated in thousands at left). Antiserum protein was assessed by Western blotting after the protein (lane C, stained with silver nitrate) was transferred to nitrocellulose and incubated with the laccase protein antiserum (lane D). No band is visible after incubation of the protein with the rabbit preimmune antiserum (lane E).

cellulose and DEAE-cellulose column chromatography) according to the method of Geiger et al. (19). Enzyme purification control was carried out by both native and denaturing polyacrylamide gel electrophoresis (PAGE). Under native conditions, PAGE was performed with 10% (wt/vol) polyacrylamide slab gels at pH 8.3. The laccase activity was detected by immersing the gels in 0.2% guaiacol–0.10 M sodium phosphate buffer (pH 6.0). They were then transferred to 7% acetic acid as soon as color appeared (19). Denaturing PAGE was performed with 15% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Samples were boiled for 4 min in a buffer consisting of 10% (vol/vol) glycerol and 2% (wt/vol) SDS in a mixture of 63 mM Tris-HCl (pH 6.8) and 5% (vol/vol) 2-β-mercaptoethanol. For both types of PAGE, bromophenol blue (0.01%, wt/vol) was the tracking dye and the gels were run under constant current (25 mA). Proteins were stained either with a solution of 0.1% Coomassie blue in 5% acetic acid or with a solution of silver nitrate (Fig. 1) (19, 50).

TABLE 1. Intensity of labeling over *R. lignosus* mycelium obtained with anti-laccase L1 antiserum and gold-labeled goat anti-rabbit antiserum in infected wood samples[a]

No. of days after inoculation	Intensity of labeling of mycelium[b]	
	Fungal cytoplasm	Fungal wall
3	46.83 (12)	159.73 (13)
6	67.32 (11)	484.75 (8)
11	16.21 (13)	131.29 (10)

[a] Anti-laccase L1 antiserum was used at a 1:200 dilution.
[b] Intensity of labeling is expressed as the number of gold particles per square micrometer. The number of samples is indicated in parentheses. The standard error of the mean on a transformed scale ($\log_{10}$) may be obatined by dividing the estimated standard deviation (0.388) by the square root of the number of samples within a given category.

Production of polyclonal antibodies to laccase L1. The anti-laccase L1 antiserum was raised in New Zealand White rabbits periodically immunized over a 3-month period after four intracutaneous injections (an initial injection and later injections 15, 30, and 70 days after the first injection) of the purified nondeglycosylated enzyme (250 μg of protein) emulsified in Freund's incomplete adjuvant (Difco). After each injection, 5 ml of serum was collected from each rabbit and tested for production of antibodies by enzyme-linked immunosorbent assay. The immunoglobulin fraction (immunoglobulin G) containing laccase L1 antibodies was subsequently prepared by ammonium sulfate precipitation using standard methods (49).

Immunoblotting. Unstained proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by the procedure of Towbin et al. (50). Western blotting (immunoblotting) was performed at 0.6 A for 1 h with a buffer composed of 25 mM Tris base, 190 mM glycine, and 20% methanol. Immunological labeling was performed by the method of Benhamou et al. (3). The primary antibody and the peroxidase-labeled goat antiserum to rabbit immunoglobulins were diluted 1:500 (Fig. 1).

Tissue processing. Wood slices (0.5 by 0.2 by 0.1 cm) were taken from fresh birch (*Betula papyrifera*), autoclaved for 1 h at 110°C, and placed at the edge of 2-day-old *R. lignosus* malt agar cultures. Autoclaved slices used as controls were placed on noninoculated malt agar cultures. All dishes were incubated in the dark at 28°C and approximately 90% relative

TABLE 2. Intensity of labeling over infected and healthy wood samples obtained with different controls

Infection status of wood	Sample	Intensity of labeling with indicated control[a]	
		Antibody plus antigen	Preimmune serum
Infected	Background	0.63 ± 0.39	0.45 ± 0.53
	Host cell wall	0.76 ± 0.59	0.32 ± 0.26
	Mycelium	0.54 ± 0.63	2.04 ± 1.73
Healthy	Background	0.31 ± 0.10	0.35 ± 0.41
	Host cell wall	0.27 ± 0.23	0.31 ± 0.22

[a] Intensity of labeling is expressed by the number of gold particles per square micrometer ± standard deviation. No labeling occurred with goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles. Because of the low level of labeling intensities in each sample compared with intensities reported in Table 1, statistical analysis was not performed on control data.

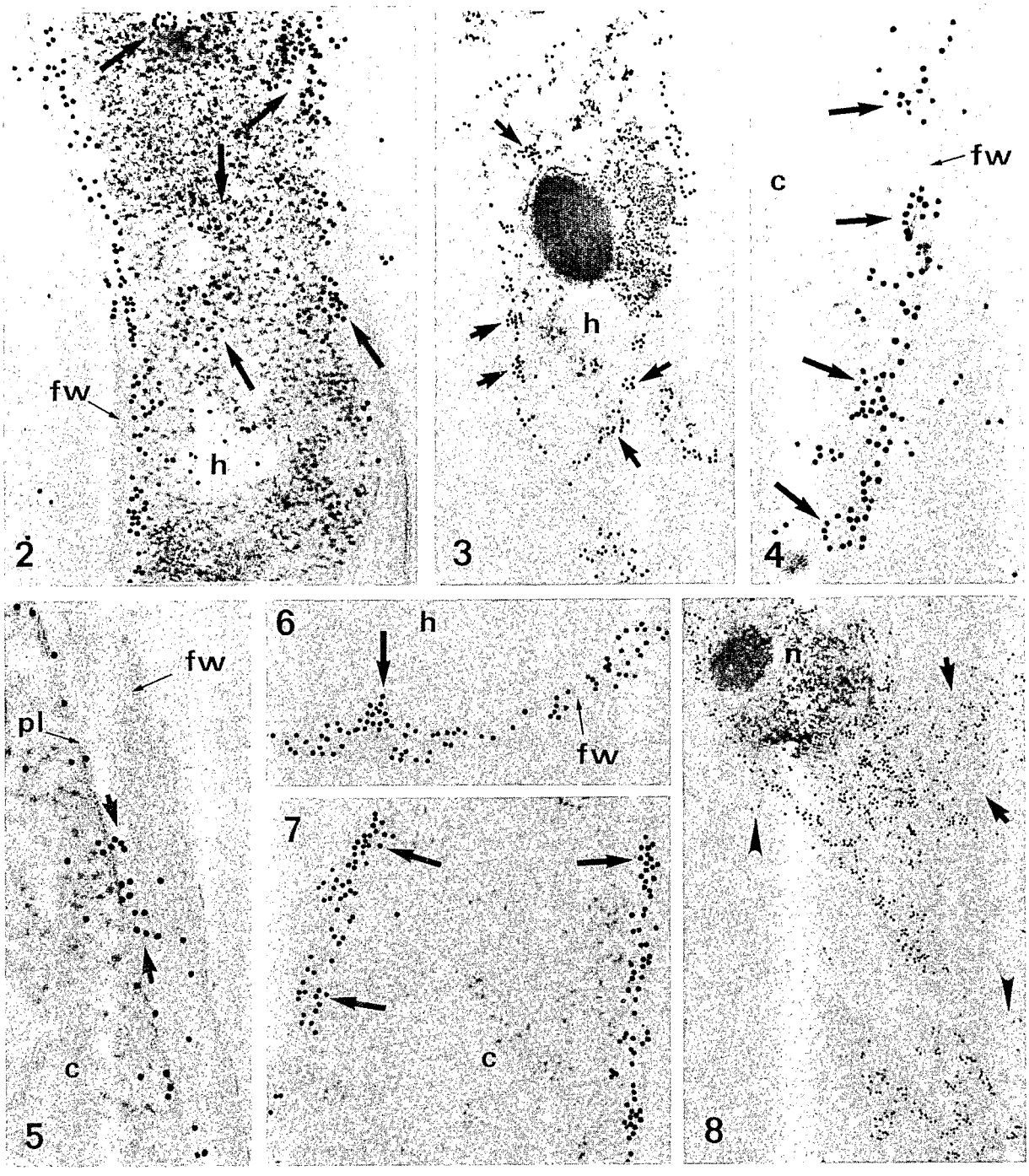


FIG. 2–8. Localization of laccase in *R. lignosus*. fw, fungal wall; h, hypha; c, cytoplasm; pl, plasma membrane; n, nucleus.

FIG. 2 AND 3. Gold particles are grouped in clusters in the fungal cytoplasm as well as close to the plasma membrane (thick arrows and short arrows). Magnifications: Fig. 2, ×64,000; Fig. 3, ×40,000.

FIG. 4. Gold particles are observed in peripheral areas of the fungal cytoplasm. They seem to be associated with the plasma membrane (thick arrows). The fungal wall is evenly labeled. Magnification, ×80,000.

FIG. 5. Numerous gold particles (thick arrows) are localized in the paramural area. Few gold particles are bound to the plasma membrane. Magnification, ×80,000.

FIG. 6. Gold particles are concentrated in a vesiclelike structure bound to the plasmalemma (thick arrow). The fungal wall is consistently labeled. Magnification, ×64,000.

FIG. 7. The fungal cell wall is unevenly labeled. Some areas show numerous gold particles (arrows), while others are devoid of labeling. Magnification, ×64,000.

FIG. 8. Localization of laccase around a fungal hypha. Some extracellular regions are strongly labeled (arrows), while others exhibit only a few gold particles (arrowheads). The nucleus does not show any significant labeling. Magnification, ×32,000.

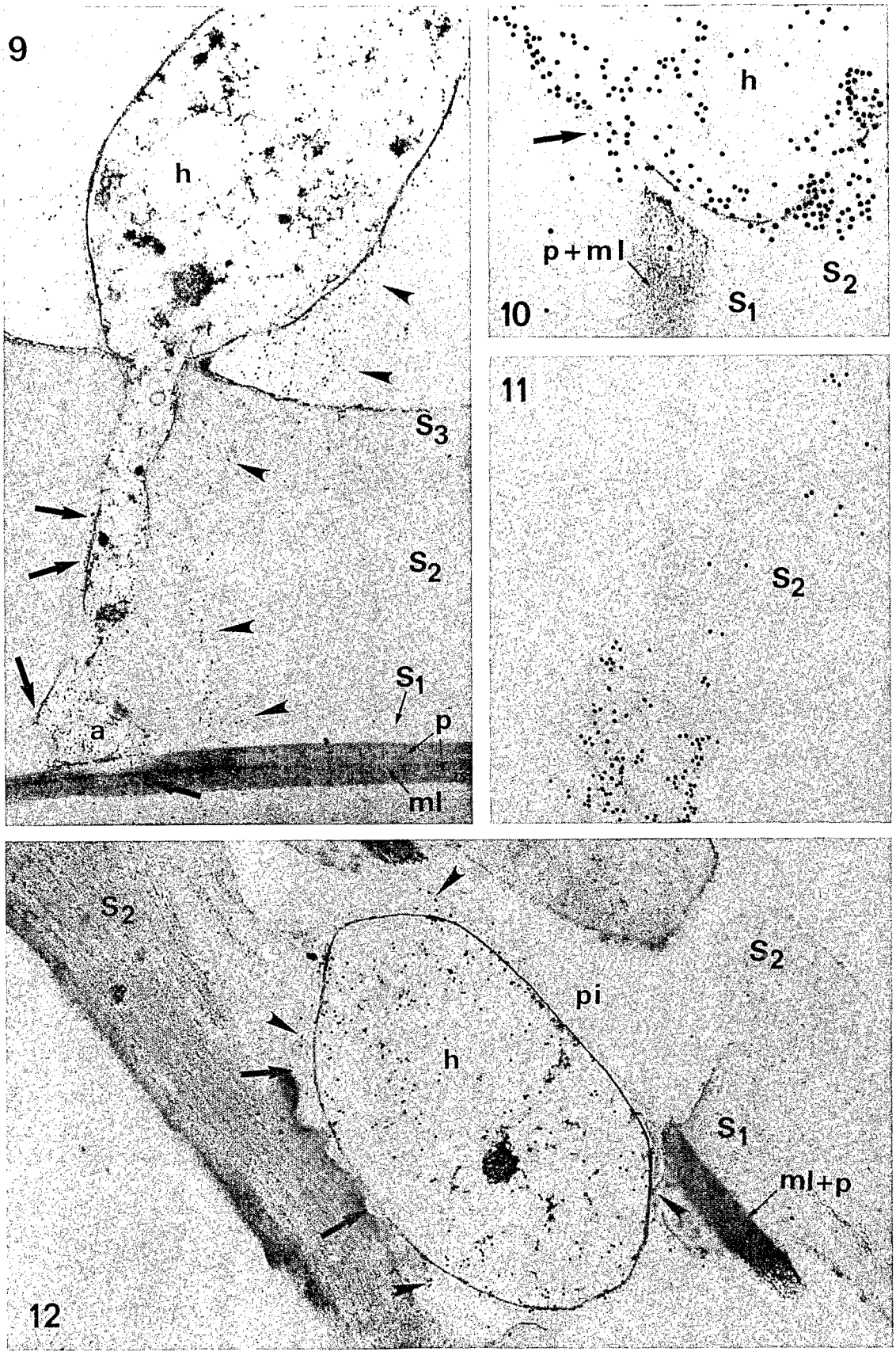


FIG. 9–12. Various patterns of laccase localization over xylem cell walls infected with *R. lignosus*. h, hypha; a, apical region; p, primary wall; ml, middle lamella; pi, pit area.

humidity. Three, six, and eleven days later, healthy and decayed wood slices were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h. Samples were rinsed three times for 20 min each time in buffer, dehydrated, and embedded in Quetol 651 (J.B.EM Inc., Dorval, Québec, Canada) by procedures previously described by Abad et al. (1). Samples in resin were cured at 74°C for 8 h. Also, samples of artificially infected roots of young rubber trees (35) were doubly fixed and embedded by a procedure described by Nicole et al. (39). Selected material was sectioned with a diamond knife on a Reichert Ultracut E microtome, and sections were collected on nickel grids and processed for immunolabeling studies.

Immunogold labeling. Ultrathin sections of material not treated with osmium tetroxide were pretreated with 0.1 M glycine (pH 7.1) in phosphate-buffered saline (PBS) to quench aldehyde groups induced during fixation. Sections from osmium tetroxide-treated wood were treated with saturated sodium metaperiodate. They were then incubated for 10 min at room temperature on a drop of normal goat serum (1:20 dilution) in 0.01 M PBS (pH 7.1). Next, sections were incubated on the primary antibody in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. Various antiserum dilutions and periods of incubation were tested to determine optimal labeling conditions. In the present study, sections were incubated for 3 h at 25°C with a crude rabbit antiserum (1:800 dilution) or with a partially purified antiserum (1:200). After primary antiserum incubation, the sections were washed in PBS-BSA-Tween 20 (pH 7.2) six times for 5 min each time and placed on a drop of Tris-HCl–BSA–Tween 20 (pH 8.2) for 10 min before incubation for 1 h at room temperature with a gold (15 nm)-labeled anti-rabbit immunoglobulin G conjugate (goat anti-rabbit GAR15; BioCell Research Laboratories, Cardiff, United Kingdom) diluted 1:20 in Tris-HCl–BSA–Tween 20. The samples were then washed thoroughly in Tris-HCl buffer and finally in distilled water. After being stained with uranyl acetate and lead citrate, the grids were examined under a JEOL 1200EX electron microscope operating at 80 kV (Département de Phytologie, Université Laval).

Immunocytochemical controls. Controls used for immunolabeling of healthy and infected wood included samples (i) incubated with anti-laccase L1 antiserum previously absorbed with the antigen, (ii) incubated with preimmune rabbit serum instead of the primary antiserum, and (iii) incubated without the primary antibody incubation step. The specificity of labeling for each observed section was systematically estimated.

Quantitation of labeling. The densities of labeling on *R. lignosus* hyphae and over healthy and decayed wood were compared by determining the number of gold particles per square micrometer. Areas and numbers of gold particles were determined with Image Analyst 7.22 (Automatix; license 1596) picture processing software and Image shareware loaded on a Macintosh II computer (Apple). For each estimation, 8 to 15 micrographs taken at a magnification of ×12,000 to ×40,000 were scanned. The density of labeling was calculated as the number of gold particles divided by the area. Gold particle densities were transformed to their base 10 logarithm to stabilize their variance and achieve normality. An analysis of variance was then performed according to a completely randomized 3×2 factorial design with time (3, 6, and 11 days) and cell localization (cytoplasm and cell wall) as the two factors.

RESULTS

PAGE of purified laccase L1 and immunoblotting. PAGE of laccase L1 purified from liquid culture filtrates resulted in the protein profile illustrated in Fig. 1. Silver nitrate staining of eluted laccase on native gels exhibited a single band of protein (Fig. 1a, lane A) whose activity was revealed by guaiacol assay (Fig. 1a, lane B); both bands migrated at the same level on gels. The specificity of the antiserum raised against the purified laccase L1 protein was confirmed by the single band observed after Western blotting (Fig. 1b, lane D) of the purified laccase preparation (Fig. 1b, lanes A [molecular weight marker] to C). The use of rabbit preimmune serum instead of laccase antiserum did not reveal any protein band on nitrocellulose (Fig. 1b, lane E).

Immunocytochemical labeling. In our experiments, sections were treated with various dilutions of both crude and purified antiserum. A 1:200 dilution of partially purified antiserum exhibited the most specific labeling. At this dilution, no significant differences between cell wall labeling (0.27 ± 0.22 gold particles per μm^2) and nonspecific background labeling (0.20 ± 0.19 gold particles per μm^2) in tissue sections from sound wood samples were observed. Wood samples fixed with glutaraldehyde and embedded in Quetol 651 retained a good degree of protein antigenicity (1), although the ultrastructural preservation of the fungal membranes suffered from the omission of osmium tetroxide. On the other hand, removal of osmium from sections of doubly fixed rubber roots with sodium metaperiodate did not yield significant labeling (data not shown).

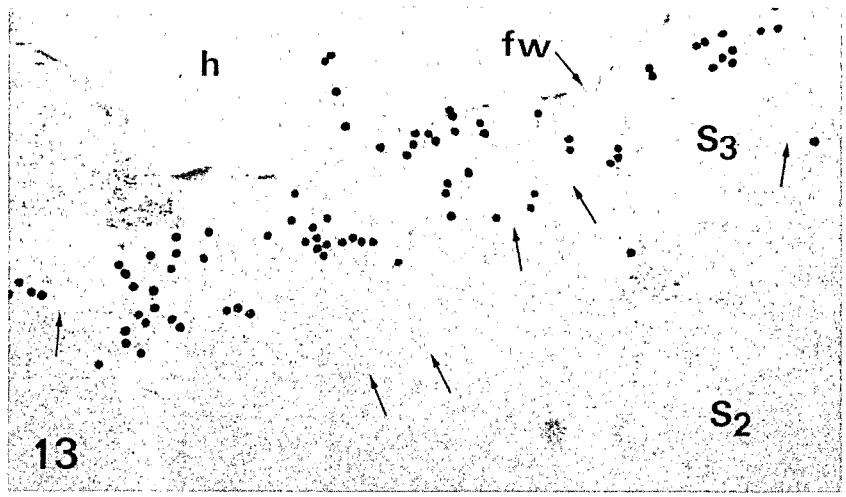
Localization of laccase L1 in *R. lignosus* cells. Observation of sections from wood samples colonized for 3, 6, and 11 days revealed gold particles localized over both the cytoplasm and the cell wall of *R. lignosus*. Statistical analysis of gold particle densities showed that labeling increased significantly over the fungal cytoplasm and cell wall 6 days after inoculation ($P = 0.0101$ [Table 1]). A significant decrease in gold particle density was noticeable in both the fungal cytoplasm and the cell wall ($P = 0.0001$) 11 days after wood inoculation. When gold particles were abundant in the fungal cytoplasm, they often appeared grouped in clusters (Fig. 2

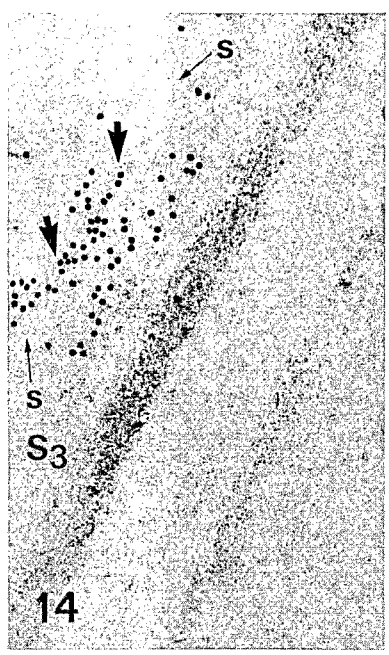
FIG. 9. Degradation of xylem cell walls in association with a microhypha penetrating the wall outward from the cell lumen. Numerous gold particles are present within the apical region of the microhypha, close to which the primary wall and the S_1 layer of the secondary wall show lightly altered zones (two-headed arrow). Labeling is also observed close to the fungal wall (thick arrows) or at a distance from the fungus (arrowheads), in the secondary wall (S_1, S_2, and S_3), or in the xylem cell lumen. A few gold particles are seen in the primary wall. Magnification, ×18,000.

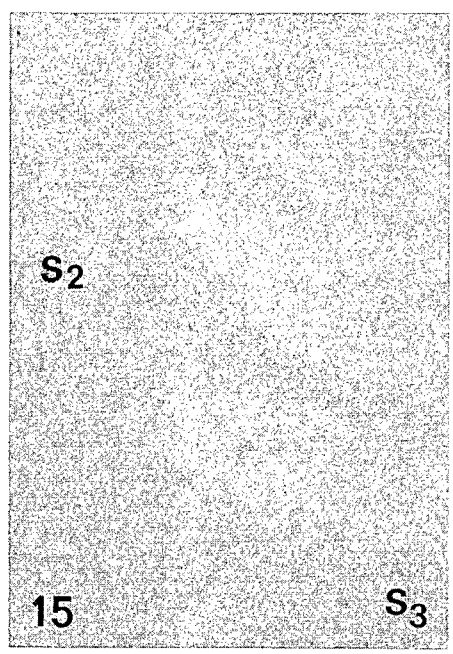
FIG. 10. Degradation of the xylem cell walls by a fungal hypha. The cell walls (S_1, S_2, and p) and the middle lamella are degraded by the fungus (h). Labeling is observed in the fungal cytoplasm and on the S_2 layer of the secondary wall. Gold particles are also seen over fibrillar material between the fungus and the wood cell (thick arrow); few are seen over the primary wall and the middle lamella. Magnification, ×64,000.

FIG. 11. Labeling of a degraded secondary wall (S_2) which is apparently not in contact with the fungus. Magnification, ×48,000.

FIG. 12. Presence of a fungal hypha in the pit area of xylem cell walls. The cell walls (S_1, S_2, and p) and the middle lamella, showing slightly degraded areas (thick arrows) in close contact to the fungus, are weakly labeled (arrowheads). Magnification, ×32,000.







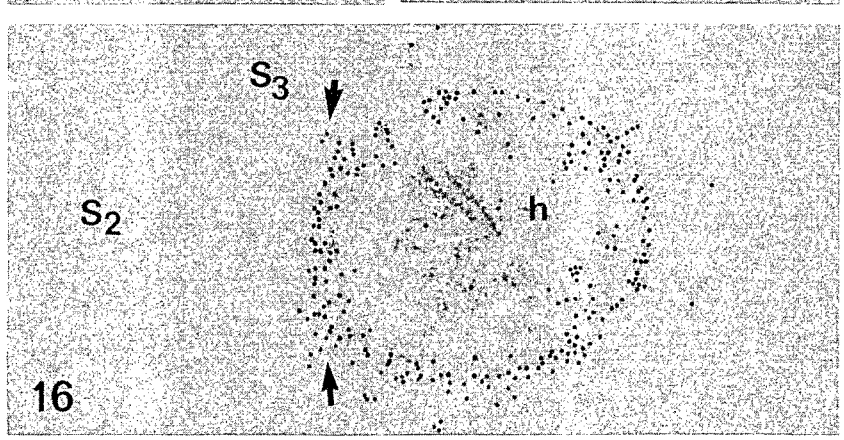


FIG. 13–16. Time course study of laccase localization in wood 3 and 6 days after inoculation.

FIG. 13. Time course study of laccase localization in wood 3 days after inoculation. Note the erosion of the inner portion of the S_3 secondary wall layer (arrows), over which gold particles are observed. Few gold particles are present over the S_2 layer. Magnification, ×91,000. fw, fungal wall; h, hypha.

FIG. 14–16. Time course study of laccase localization in wood 6 days after inoculation.

FIG. 14. Localization of labeling over the S_3 layer of the secondary wall. Gold particles are visible over the fungal wall (thick arrows) and the sheath (s). Magnification, ×64,000.

FIG. 15. Secondary cell wall layers (S_2 and S_3) showing advanced stages of decay without contact with the fungus. No labeling is present over these altered layers. Magnification, ×32,000.

and 3), even when localized close to the plasmalemma (Fig. 2, 4, and 6). Numerous gold particles were concentrated in the periplasmic area (Fig. 5) or in vesiclelike structures bound to the plasmalemma (Fig. 4 and 6). The fungal cell wall was consistently labeled, especially in samples collected 6 days after inoculation (Table 1; Fig. 4 and 6). However, gold particles were unevenly distributed, with some wall regions showing intense labeling (Fig. 7) while others were unlabeled (Fig. 2, 3, and 7). Similarly, hyphae were surrounded by areas with variable labeling (Fig. 8). Further examination at a higher magnification showed that gold particles were associated with small fibrils (see Fig. 26). No significant labeling over other fungal organelles, including mitochondria and the nucleus, was recognized (Fig. 8).

Localization of laccase L1 in wood cells. Wood samples colonized by *R. lignosus* displayed various patterns of degradation, ranging from slight erosion to advanced stages of degradation (Fig. 12, 13, and 15). Generally, in vitro cell wall degradation occurred outward from the cell lumina to the middle lamella (Fig. 9); cell wall degradation occurred inward from lumina only when hyphae were in close contact with the middle lamella (Fig. 10). Labeling was irregular over altered wall areas of xylem elements. A significant number of gold particles was seen over degraded wood both at a distance from and close to *R. lignosus* mycelium (Fig. 10 to 12). Apparently, intact walls of infected cells were generally not labeled (Fig. 16), although there were exceptions. Gold particles were observed over wall regions close to penetrating microhyphae as well as around the mycelium, which differentiated such microhyphae (Fig. 9).

A time course investigation of wood degradation revealed that no gold particles were observed over walls of apparently noninvaded cells of all inoculated wood samples. In the samples collected after 3 days, gold particles accumulated over the degraded innermost layer of the secondary wall of xylem cells (Fig. 13), although particles were occasionally seen in the other layers of secondary walls (Fig. 11). Labeling was absent from the primary wall and the middle lamella. Six days after wood colonization, labeling occurred over the secondary wall layers of invaded cells (Fig. 14 and 27). The middle lamella and the primary wall were also sometimes labeled, but only when the mycelium was in close contact with them. Wall regions exhibiting advanced decayed cell walls were generally devoid of gold particles (Fig. 15). Investigation of samples after 11 days revealed gold labeling over all cell walls from the innermost part of the secondary wall (Fig. 17 and 18) deep into the primary wall and in the middle lamella (Fig. 21 and 22), including the pit membranes (Fig. 19). However, some regions of cell walls that were penetrated by hyphae sometimes were not labeled (Fig. 20). Gold particles were found near penetrating microhyphae (Fig. 21 and 22) or scattered over large portions of wood cell walls adjacent to hyphae, but often with a few gold particles distributed over other adjacent cell walls (Fig. 21). Layers of the secondary wall showing advanced signs of fungal attack were found to be weakly labeled; the labeling was restricted to residual fragments (Fig. 23).

A slimelike sheath with an obvious fibrillar structure surrounded hyphae mainly 6 and 11 days after wood inoculation (Fig. 20 and 24 to 29). The sheath apparently connected hyphae and the wood (Fig. 20, 24, and 27). Sometimes slime appeared to be detached from the hyphae (Fig. 26 and 28). Association of labeling with loosened (Fig. 26 and 28) or dense (Fig. 27) slime layers was indicated by the presence of gold particles along some of their fibrils. However, this labeling was not seen related to that observed over adjacent altered cell walls. Heavy deposition of gold particles (Fig. 27) or a low level of labeling (Fig. 24 and 25) was seen over sheaths in contact with apparently undegraded wall regions. In other cases, slime was nearly devoid of significant labeling (Fig. 20 and 29).

Immunocytochemical controls. The specificity of the labeling was assessed with several controls. Preadsorption of the anti-laccase L1 antibodies with the purified laccase resulted in a very low level of nonspecific labeling over sections from both infected and uninfected wood (Table 2). Similarly, the use of preimmune rabbit serum rather than anti-laccase L1 antiserum did not yield significant labeling in either infected or healthy wood (Table 2). The intensity of labeling of fungal cells from infected wood obtained with the preimmune serum (Table 2) was not significant compared with the intensity of labeling (10 times higher) obtained with anti-laccase L1 antiserum over mycelium in infected wood (Table 1).

DISCUSSION

This article reports on the immunolocalization of laccase L1 of *R. lignosus* during the degradation of wood fragments. In this study, we used native laccase rather than a carbohydrate-depleted enzyme to raise anti-laccase L1 antibodies. Glycosylation of laccases (on average, 8% of the molecular weight in fungal laccase) (42) was demonstrated to maintain protein conformation and to protect the enzyme against protease (34). The carbohydrate moiety of fungal laccase mainly contains small amounts of sugars such as xylose, mannose, and fucose, which are rare components of both *R. lignosus* and wood cell walls. Although antiglycan immunoglobulins may induce a low degree of labeling, the use of highly diluted purified antibodies (1:200), the absence of significant labeling over sections of uninoculated wood and of roots fixed with osmium tetroxide (which does not alter glycan epitopes [24]) and in the various control experiments demonstrates the specificity of these antibodies for the protein moiety of the enzyme. In view of our results, sites of significant labeling over sections clearly indicate the localization of laccase in degraded wood. However, the high variation in laccase distribution in nearby hyphae and into wood cell walls, as well as the possible displacement of the enzyme during tissue processing, made the quantitation of gold particles according to the time course of wood degradation difficult.

Immunogold labeling showed laccase to be both intracellular and extracellular to fungal hyphae. Although fungal organelles were poorly preserved, the enzyme was found primarily in vesiclelike structures that were often located in peripheral cytoplasmic regions or bound to the plasmalemma. Laccase was also associated with the fungal cell wall and was seen in its paramural space. Such a feature of distribution, consistent with other immunocytochemical

FIG. 16. The layers of the secondary wall (S_2 and S_3) in close contact with a fungal hypha are devoid of gold particles. Note that the fungal wall is evenly labeled. Gold particles are also seen over the fungal cytoplasm and on fibrils of the extracellular slime sheath (arrows). Magnification, ×48,000.

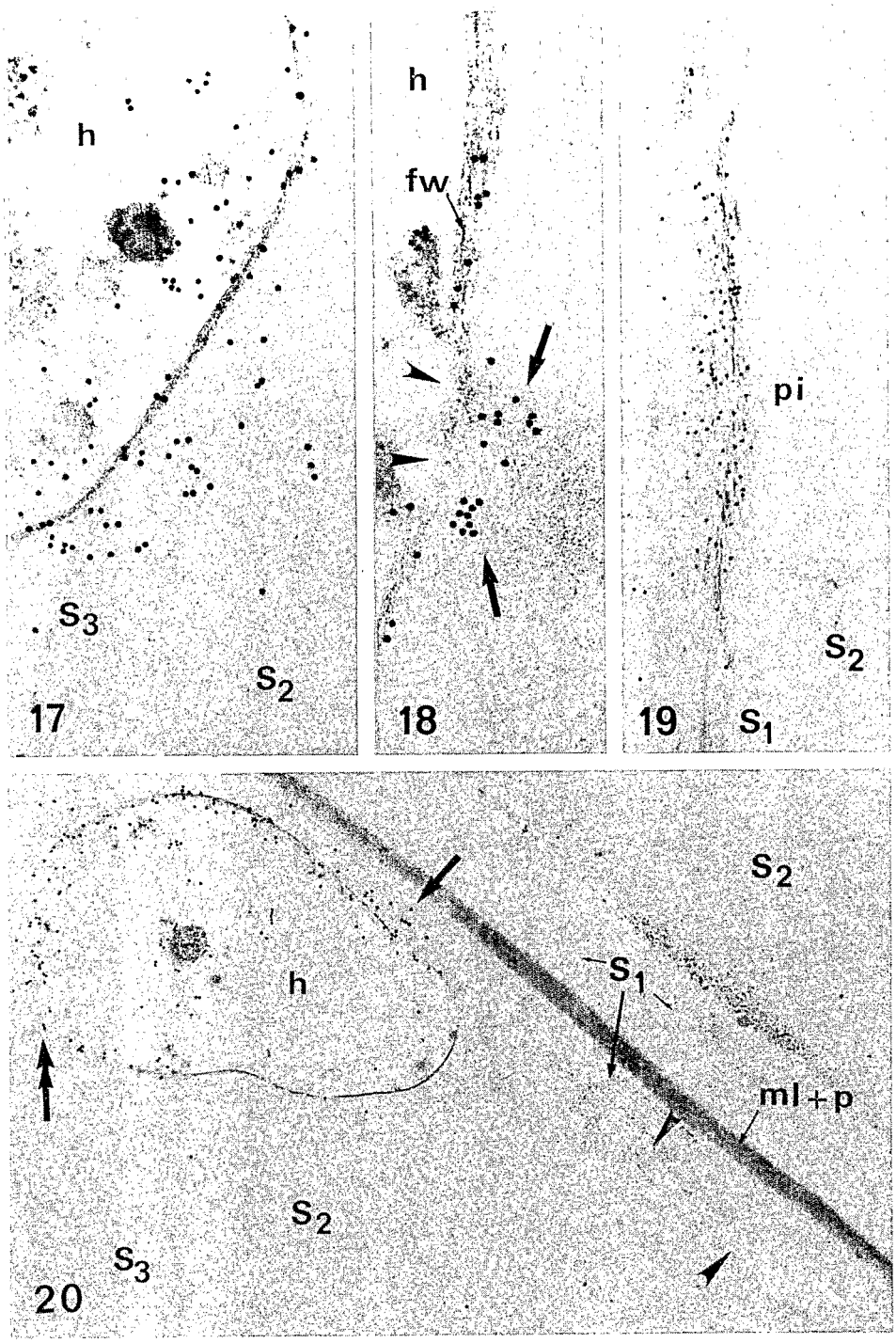


FIG. 17–20. Time course study of laccase localization in wood 11 days after inoculation. h, hypha; fw, fungal cell wall; pi, pit area; ml, middle lamella; p, primary cell wall.

FIG. 17. Gold particles are visible over apparently intact S_3 and S_2 layers of the secondary xylem cell wall. Magnification, ×60,000.

FIG. 18. The degraded S_3 layer of the secondary wall is labeled (thick arrows). The fungal cell wall in close contact with the S_3 layer seems disorganized (arrowheads). Magnification, ×60,000.

FIG. 19. Numerous gold particles are evenly distributed over the middle lamella of the pit area. Magnification, ×32,000. The S_1 and S_2 layers of the secondary wall are indicated.

FIG. 20. Gold particles, few in number, are seen only over the degraded S_1 layer of the secondary wall (thick arrow). The other wall layers (p, S_2, and S_3), including the middle lamella, do not exhibit significant labeling. Note the fungal slime in close contact with the degraded wall even at some distance (arrowheads) from the fungus (h). Labeling of slime occurs only close to the hypha (two-headed arrow). Magnification, ×32,000.

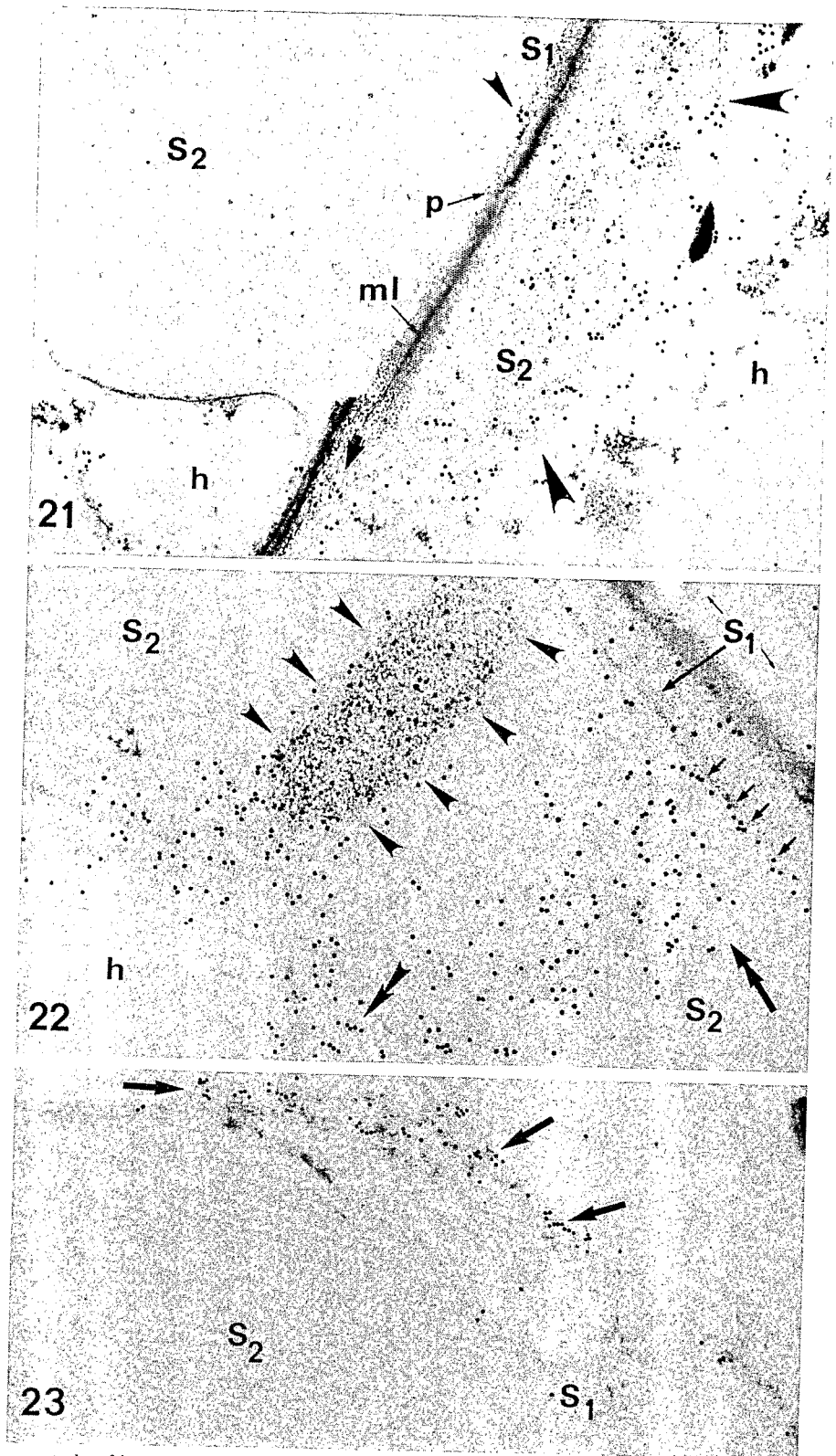


FIG. 21–23. Time course study of laccase localization in wood 11 days after inoculation. p, primary wall; h, hypha; ml, middle lamella; S_1 and S_2, layers of the secondary wall.

FIG. 21. The secondary wall of an invaded cell is evenly labeled (large arrowheads). In the adjacent cell, only a few gold particles are visible over the primary wall (small arrowhead). The pit area also is weakly labeled (thick arrow). Magnification, ×37,500.

FIG. 22. Labeling is observed deep in the secondary wall (two-headed arrow). The S_1 layer of the secondary wall and the middle lamella are also labeled. Gold particles are regularly distributed at the juncture of the S_1 and S_2 layers of the secondary wall (small arrows). Intense labeling is seen over the area demarcated by arrowheads and showing a longitudinal section through the wall of a microhypha. Note the labeling (double arrowhead) of the fungal sheath close to the fungus (h). Magnification, ×45,000.

FIG. 23. The S_1 layer of the secondary wall shows an advanced stage of degradation. Only a few gold particles are present over the remaining S_1 layer, while labeling is intense over residual wall fragments (arrows). Magnification, ×39,000.

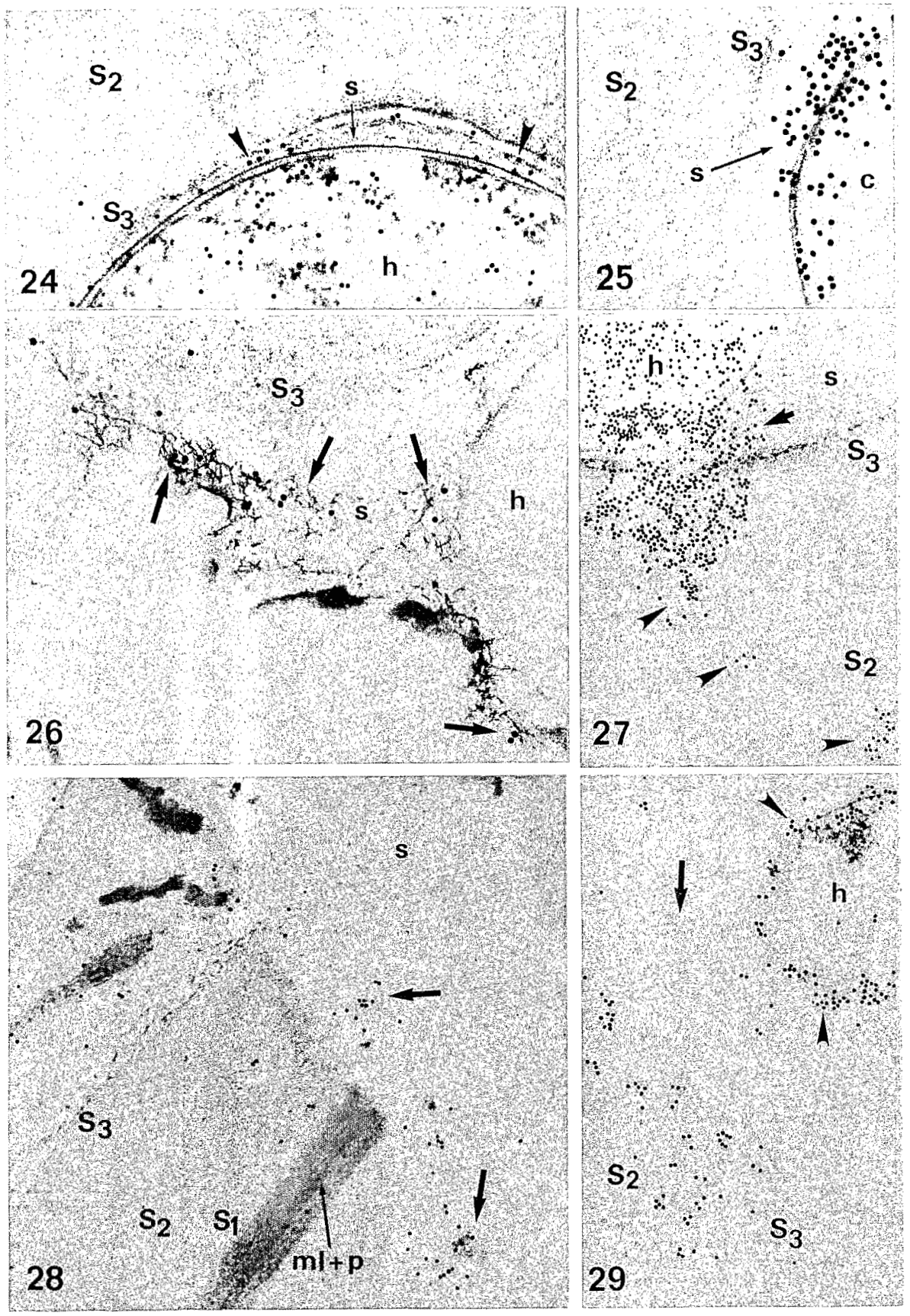


FIG. 24–29. Labeling pattern on fungal slime layers.

FIG. 24. A weakly labeled sheath (s; arrowheads) is in contact with an unlabeled xylem secondary wall (S_2 and S_3). Gold particles are present in the fungal cytoplasm (h). Magnification, ×60,000.

studies of lignin-degrading peroxidases (8, 15), shows that antigenic sites of laccase within the fungal cell are available during the early stages of wood colonization. The high density of gold particles over the fungal wall 3 to 6 days after inoculation suggests that laccase may be induced by the proximity of the lignocellulose substrate. The decrease in labeling over fungal cells 11 days after invasion of wood may indicate (i) a loss of excretion of the enzyme, (ii) a decrease in enzyme synthesis, or (iii) a modification (e.g., degradation) of the protein. Regulation of lignolytic enzymes is known to depend upon several parameters such as protease activity, MnO_2, and released products of wood degradation (10).

The present ultrastructural study also revealed the occurrence of fibrillar extracellular sheaths surrounding *R. lignosus* hyphae which closely adhered to degraded wood cell walls, suggesting their involvement in penetration and/or digestion of the lignocellulose complex. As previously stressed by Palmer et al. (41) and recently confirmed by Ruel and Joseleau (44), extracellular slimes appear to have an active role in the support and transport of wood-degrading enzymes to their substrates. Localization of laccase on the fungal slime matrix suggests that an interaction between the enzyme and the slime matrix may occur, in agreement with other immunocytochemical studies of extracellular fungal oxidases (5, 9, 12, 46). However, the irregular observation of laccase within the fungal matrix may indicate that release of the enzyme from hyphae depends on the accessibility of its substrates. Moreover, a direct contact between hyphae and the cell wall does not seem necessary to induce enzyme secretion during the attack of the lignin-polysaccharide complex. Thus, hyphal slimes may be involved in recognition of enzyme substrates in wood.

The localization of laccase L1 within the walls of xylem cells, including the middle lamella, shows that this enzyme can diffuse into decayed wood. Thus, laccase operates in both lignin-rich regions (middle lamella and primary walls) and polysaccharide-rich areas (layers of secondary walls) of wood cell walls. The enzyme distribution within wood cell walls was not always associated with a high level of wall degradation. Adjacent wood cells displaying advanced stages of decay sometimes have different amounts of gold labeling. Previous biochemical studies of laccase distribution in infected roots of living rubber trees have shown that wood in an advanced state of decay contained little or no laccase activity (17). Moreover, diffusion of laccase into intact cell wall layers seemed hindered. The enzyme localization in apparently intact cell walls suggests that the lignocellulose complex had been previously modified, providing laccase accessibility to active sites. For example, the observed restriction of laccase to the walls of, or close to, microhyphae moving through wood cell walls might result in the lack of such active sites. The observation that laccase L1 of *R. lignosus* might not be able to reach its substrate in undegraded lignocellulose is in agreement with observations made with LiP and MnP (5, 8, 15). It has also been pointed out that steric hindrance may prevent lignin-degrading enzymes from penetrating xylem cell walls (48). However, this is not consistent with the fact that the laccase enzyme itself, conjugated to colloidal gold, did not label lignin cell walls of sound wood (37a). As reported by Daniel et al. (8), the degree of enzyme diffusion into wood cell walls may vary with wood porosity, the level of lignification, and the degree to which fungi have degraded the wood.

It has been established that laccases play a role in lignin breakdown (4, 16, 23, 29, 30, 32, 34). Although biochemical mechanisms of lignin degradation have been extensively studied (25, 27, 28), the inherent role of laccases in phenylpropanoid alteration is far from being clearly determined (11, 30, 31). Nevertheless, they are known to catalyze removal of one electron from phenolic hydroxyl groups in lignin and lignin-related compounds, producing phenoxy radicals that undergo a variety of reactions (28). In view of our data, it can be assumed that laccase L1 from *R. lignosus* does not initiate lignin degradation. As shown for LiP and MnP, the initial attack on lignin may result from other enzyme activities or may be performed by mediator reactions (5, 31, 46). Hemicellulases are known to hydrolyze polysaccharides early in lignocellulose degradation. They may open channels in the secondary wall, allowing access to lignin oxidases (6). Cellulases secreted by *R. lignosus* during root decay of rubber trees (18) degrade wood in the early stage of infection (37). It is likely that in our experiment, cellulases initiated wood cell wall degradation, allowing laccases to move progressively into walls. The secretion of laccases from *R. lignosus* mycelium occurs with other oxidases involved in lignin degradation (13, 20). A recent study convincingly documented that laccases and MnP of *R. lignosus* (14) act synergistically in the solubilization of a radioactive lignin from *H. brasiliensis*, confirming the importance of these enzymes in lignolysis in the absence of LiP. Additional immunocytolocalization of both *R. lignosus* laccase and MnP may provide new information about the time course of production of these enzymes during wood degradation. The localization of laccase in wood cell walls or in degraded wall residues is also consistent with the involvement of this enzyme in the detoxification of host cell degradation products. The ability of laccase, as well as LiP (22, 46), to polymerize endogenous plant phenolic moieties and/or oxidative products released from degraded lignins (7, 16, 21, 43) protects the fungus against a toxic environment.

In conclusion, the present study confirms that laccase in degraded wood cell walls can be visualized by immunocytochemical methods. It appears that its distribution during wood decay occurs in all wall types, including the middle lamella. Since laccase accumulates in xylem regions displaying various stages of alteration, it is likely that the enzyme is associated with fungal modification of the wood. Whether

FIG. 25. Numerous gold particles are observed over some of the slime's fibers (s), in contact with the apparently undegraded S_3 layer of the secondary wall. Labeling also occurs in the fungal wall and cytoplasm (c). Magnification, ×80,000.

FIG. 26. Few gold particles are associated with the fibrils (arrows) of a loosened layer of slime (s). Magnification, ×80,000. h, hypha.

FIG. 27. In samples collected 6 days after inoculation, a dense layer of fungal slime (s) between the hypha and the secondary xylem cell wall is heavily labeled (arrow). Also, labeling is intense over the S_3 layer and the fungal hypha; gold particles are seen over the S_2 layer (arrowheads). Magnification, ×40,000.

FIG. 28. No significant labeling occurs in the loosened slime (s). Gold particles are seen over residual xylem cell wall fragments (thick arrows). Magnification, ×48,000. ml, middle lamella; p, primary wall.

FIG. 29. Three days after wood inoculation, fibers of the sheath are devoid of significant labeling (arrow), while gold particles (arrowheads) are seen close to the fungus (h). Labeling is also visible over the S_3 and the S_2 layers of the secondary wall.

laccase localized in degraded wood corresponds to a step in lignin degradation and/or to a detoxifying mechanism remains to be elucidated.

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