Pectin degradation during root decay of rubber trees by *Rigidoporus lignosus*

MICHEL R. NICOLE[1]

ORSTOM – Forestry Canada, Quebec Region, 1055 rue du PEPS, Sainte-Foy (Québec), Canada G1V 4C7

AND

NICOLE BENHAMOU

Département de phytologie, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Sainte-Foy (Québec), Canada G1K 7P4

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Aplysia gonad lectin, which binds polygalacturonic acid, was complexed to colloidal gold and used for localizing molecules that contain polygalacturonic acid in rubber tree roots infected with the white-rot root fungus *Rigidoporus lignosus*. Colonization of root tissues was associated with strong wall alteration of phloem cells and with degradation of the compound middle lamella in both the phloem (10 weeks after inoculation) and the xylem (15 weeks after inoculation). Our data suggest that pectin breakdown during root decay likely occurs after cellulose and lignin breakdown and may result from the fungal pectinase activities that were detected *in vitro*. Released pectin oligomers may act as inducers of both fungal laccase and of the tree defense system during root invasion.

Key words: root rotting fungi, rubber, pectin, cellulose, cytochemistry.

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La lectine de gonade d'Aplysia reconnaît spécifiquement les acides polygalacturoniques, monomères constitutifs de la pectine. Conjuguée à l'or colloïdal, elle a été utilisée pour localiser ce polymère à l'échelle ultrastructurale dans les racines d'Hévéa infectées par le champignon *Rigidoporus lignosus*, agent du pourridié blanc. L'analyse cytochimique des coupes ultra-fines a révélé que l'altération des lamelles moyennes du phloème (10 semaines après les infections artificielles) et du xylème (15 semaines après les infections artificielles) est associée à de profondes modifications des lignocelluloses des parois cellulaires. Nos résultats suggèrent que la dégradation de la pectine résulterait de l'activité d'enzymes pectinolytiques, détectée *in vitro*. Les oligomères de pectine ainsi libérés dans les cellules-hôtes pourraient agir en tant qu'inducteurs à la fois des laccases fongiques et de certaines réponses de l'arbre à l'infection racinaire.

Mots clés : pourridés, Hévéa, pectine, cellulose, cytochimie.

Introduction

Decay of tree roots by white-rot fungi results in the alteration of wall-bound polymers such as suberin, lignin, and polysacharrides, including cellulose, hemicellulose, and pectin (7). Pectin polysaccharides, defined as galactosyluronic acid-rich polymers (24), are mainly localized in the true middle lamella and the primary wall of plant cells. In xylem elements of trees, the compound middle lamella of young cells is largely pectin in nature but becomes highly lignified with cell aging (25). A number of ultrastructural and biochemical studies were devoted to the degradation of lignocellulose complexes (7), but little attention has been paid to the alteration of pectin in wood infected by root-rot fungi (13).

Rigidoporus lignosus (Kl.) Imazeki is a tropical white-rot fungus that causes severe losses in *Hevea brasiliensis* (Muell.) Arg. (rubber tree) plantations in West Africa (18). Microscopic observations of artificially infected roots revealed that infection occurred via wounds or after direct penetration of walls of dead surface cells (22). The fungus was shown to severely degrade host cell walls with respect to root tissue colonization (19). The marked decomposition of phellem suberized cell walls and of phloem cellulosic walls facilitated *R. lignosus* invasion into lignified xylem elements, the main target for the pathogen. Standard electron microscope investigations of fungal ingress into the root tissues showed unambiguously that the fungus was capable of growing within middle lamellae, thus causing marked changes in intercellular areas (19, 22). Although previous biochemical studies demonstrated the production of a wide range of extracellular enzymes by *R. lignosus* (11, 15, 19), to our knowledge there has been no report on the occurrence of fungal pectin enzymes during wood alteration of rubber tree roots.

In considering the broad spectrum of research devoted to the alteration of wall-bound polymers during host–pathogen interactions, it is apparent that visualizing the location and spatial distribution of these polymers could improve our understanding of the specific action of fungal enzymes. In this context probes, including anti-pectin antibodies (8, 14), pectinases (2), or the *Aplysia depilans* gonad lectin (AGL) (3, 4, 5), were successfully applied in conjunction with colloidal gold for localizing molecules containing polygalacturonic acid in various plant tissues. In this study, we used AGL for studying the distribution of pectic polymers in rubber tree roots infected with *R. lignosus*. In parallel, an exoglucanase–gold complex was used for localizing β-(1,4)-D-glucans (1, 19) to examine cellulose alteration.

Materials and methods

Fungal culture and growth conditions

Isolates of *R. lignosus* were collected in rubber tree plantations in the Ivory Coast. The mycelium was obtained by growing *R. lignosus*, strain 1, on a 2% malt agar medium (Difco Lab. Detroit, Mich.) at 28°C.

[1]Author to whom all correspondence should be addressed at ORSTOM, Laboratoire de Phytopathologie, BP 5045, 34 000 Montpellier, France.

Artificial inoculations of plant material

Rubber tree seeds, clone GT 1, were collected in a plantation of the Institut de Recherche du Caoutchouc in the Ivory Coast (IRCA). After germinating in sand, young seedlings were transferred to tubs (1 × 1 × 1 m) filled with forest soil. A high moisture level was maintained in the soil by daily watering to saturation and was monitored with a neutronic moisture gauge (Solo 20, Nardeux, France). Seedling inoculation was performed as described previously (17). Briefly, five preinfected rubber wood fragments were applied against the tap root of each 1-month-old seedling, 20 cm deep in the soil. Root samples were collected 4, 10, and 15 weeks following inoculation. Uninfected plants were used as controls.

Preparation of samples for electron microscopy

Portions of infected roots were fixed in toto for 1 h in 3% glutaraldehyde (v/v) (EM grade, Sigma Chemical Co., St. Louis, Mo.) in 0.1 M sodium cacodylate buffer, pH 7.2. Small root fragments were then excised and fixed again for 1 h with glutaraldehyde, postfixed with 1% aqueous osmium tetroxide (EM grade, Sigma) for 1 h at 4°C, washed in sodium cacodylate buffer before dehydration in a graded ethanol series, and embedded in Epon 812 or in Araldite. Ultrathin sections, collected on Formvar-coated nickel grids (200 mesh) (JBEM Chemical Co., Pointe Claire, Que.), were processed for cytochemical labeling. Observations were carried out with a JEOL 1200 EX electron microscope operating at 80 kV.

For each step of the infection process, five samples per infected plant were treated for TEM. Samples from healthy rubber tree roots were processed as mentioned above and used as controls.

Cytochemical labeling of polygalacturonic acids

Colloidal gold (Sigma) was prepared according to Frens (9) to get particles averaging 15 nm in diameter. The AGL (a gift from Dr. Gilboa-Barber) was conjugated to colloidal gold as described previously (3). Briefly, 200 μg of lectin was dissolved in 100 μL of distilled water and mixed with 10 mL of colloidal gold at pH 9.5. After centrifugation at 28 000 × *g* for 60 min at 4°C, the dark red sediment was resuspended in 0.5 mL of phosphate-buffered saline (PbS) (pH 8.0) containing 0.02% (w/v) of PEG 20 000. Sections of infected and healthy root samples were first floated on a drop of PBS–PEG (pH 8.0) for 5 min, then transferred to a drop of AGL–gold complex diluted 1:4 in the same buffer for 30 min at room temperature in a moist chamber. They were then washed with PBS, pH 7.2, rinsed with distilled water, and finally contrasted with uranyl acetate and lead citrate before examination.

Specificity of the labeling was assessed by means of the following control tests: (*i*) incubation of sections with the AGL–gold probe to which an excess of D-galacturonic acids was previously added; (*ii*) incubation of sections with the uncomplexed protein followed by incubation with the gold complexed protein; (*iii*) incubation of sections with the gold suspension alone.

Cytochemical labeling of β-(1-4)-D-glucans

An exoglucanase, a β-(1-4)-D-glucan cellobiohydrolase (EC 3.2.1.91) was complexed to colloidal gold as previously described (1). Sections of infected and healthy root samples were first floated on a drop of PBS–PEG, pH 6.0, then incubated on a drop of the enzyme–gold complex for 30 min at room temperature in a moist chamber. They were then thoroughly washed with PBS, pH 7.4, rinsed with distilled water, and finally contrasted with uranyl acetate and lead citrate before examination.

Specificity of the labeling was assessed by means of the following control tests: (*i*) incubation of sections with the exoglucanase–gold complex to which 5 mg of β-(1-4)-D-glucans was previously added; (*ii*) incubation with a bovine serum albumin (BSA) – gold complex; (*iii*) incubation of a nonenzymic protein–gold complex.

Quantitation of labeling

The density of labeling (D) over cell walls of healthy and decayed wood, as well as over fungal cell walls, was compared by determining the number of gold particles per square micrometre. Determination of areas (Sa) and number of gold particles (Ns) were made using Image 1.41, a picture processing shareware loaded on a Macintosh II computer (Apple). For each estimation 8 to 15 micrographs taken at a magnification of 12 000 – 40 000 were scanned. Density of labeling was calculated as follows: D = Ns/Sa. Average gold particle densities for healthy and infected root tissues were compared through the Kruskal–Wallis rank test that is based on a statistic distributed as χ^2 with 1 df. The value of the statistic and the corresponding *P*-value are given in Tables 1 and 2.

Enzyme assays

The production of pectinases by *R. lignosus* (strain 1) was assayed *in vitro* after the fungus was grown for 30 days (28°C) in 2 mL of the following medium: KH_2PO_4, 0.5 g; Na_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.25 g; asparagine (DL), 2.0 g; pectin, 1.0 g; $FeCl_3$, 2 mL (0.5% soln.); V8 juice, 100 mL of supernatant after centrifugation; H_2O, 989 mL. The pH was adjusted to 7.0 with NaOH. Prior to electrophoresis, the culture was filtered and the crude liquid medium was boiled for 4 min; aliquots of 25 μL of this medium were then collected and mixed with 25 μL of denaturating buffer as described by Ried and Collmer (25). Polyacrylamide gel electrophoresis (PAGE) under denaturating conditions was performed at 25 mA in 15% polyacrylamide gels containing 0.2% pectin from citrus fruits (Sigma). After the one-dimensional PAGE, gels were incubated overnight in 50 mM of renaturating buffer (Tris–HCl, pH 7.5) containing 1% (w/v) Triton X-100, at room temperature with gentle shaking. After incubation, gels were stained with 0.02% aqueous ruthenium red (2 h at room temperature) and destained with water.

Controls were tested in similar conditions using (*i*) *Phellinus noxius*, another tropical root-rot fungus known for excreting pectinases both *in vitro* and *in vivo* (11) and (*ii*) a commercial pectinase (100 μg/25 μL) (Sigma).

Results

Cytochemical localization of polygalacturonic acids

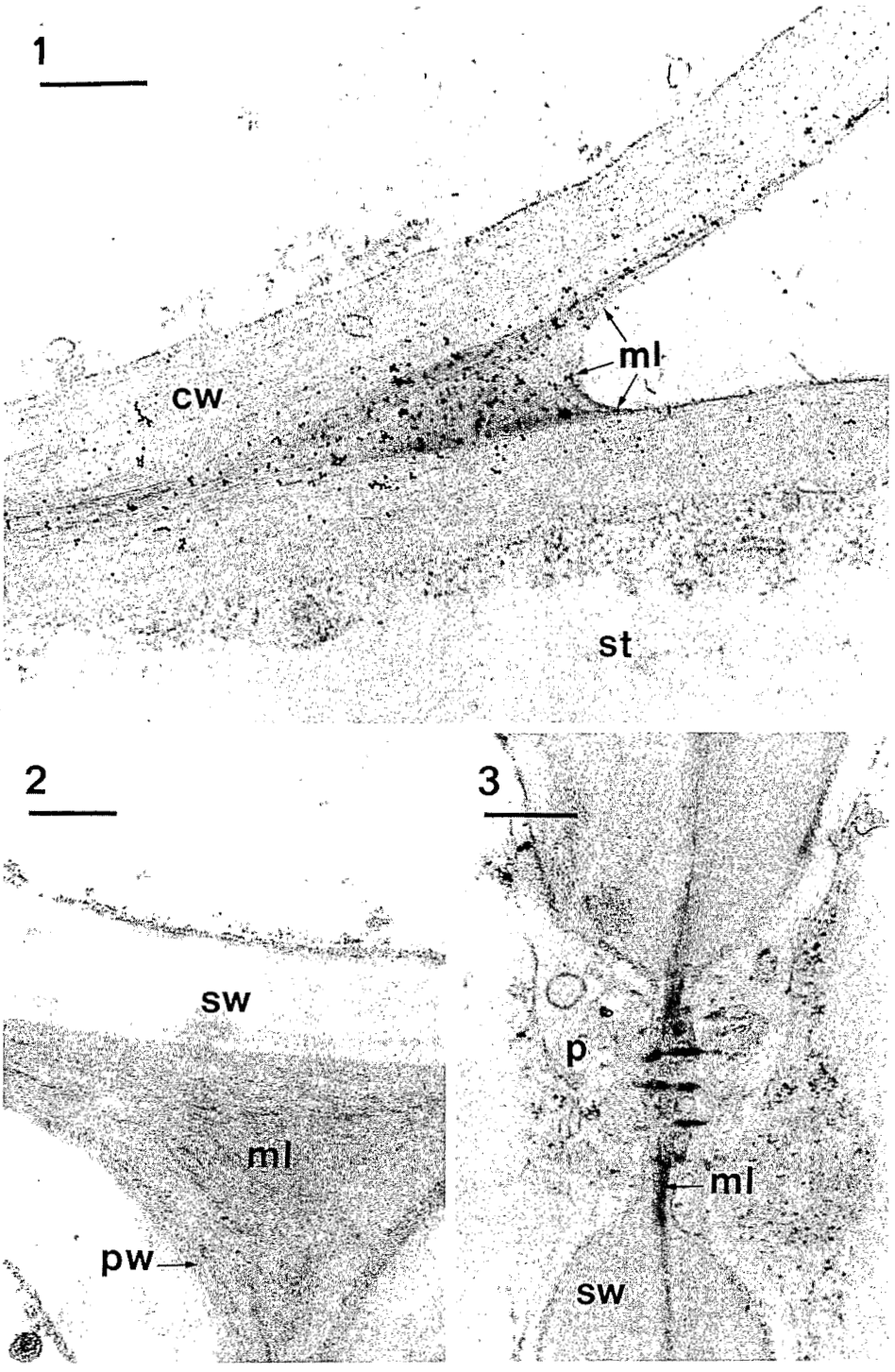
Labeling pattern observed over healthy root tissues

Following incubation of ultrathin sections of noninoculated young root tissues with the AGL–gold complex, various labeling patterns were observed over the root cell walls (Table 1). In the phellem, cell walls and middle lamelllae did not exhibit a significant labeling compared with background labeling. A few gold particles were scattered over phloem primary cell walls (Fig. 1), while the middle lamellae were heavily labeled (Table 1). In the xylem, very low labeling occurred over the lignified middle labellae (Fig. 2) and over pit areas (Fig. 3). No gold particles were found to be associated with primary and secondary cell walls (Table 1; Figs. 2 and 3).

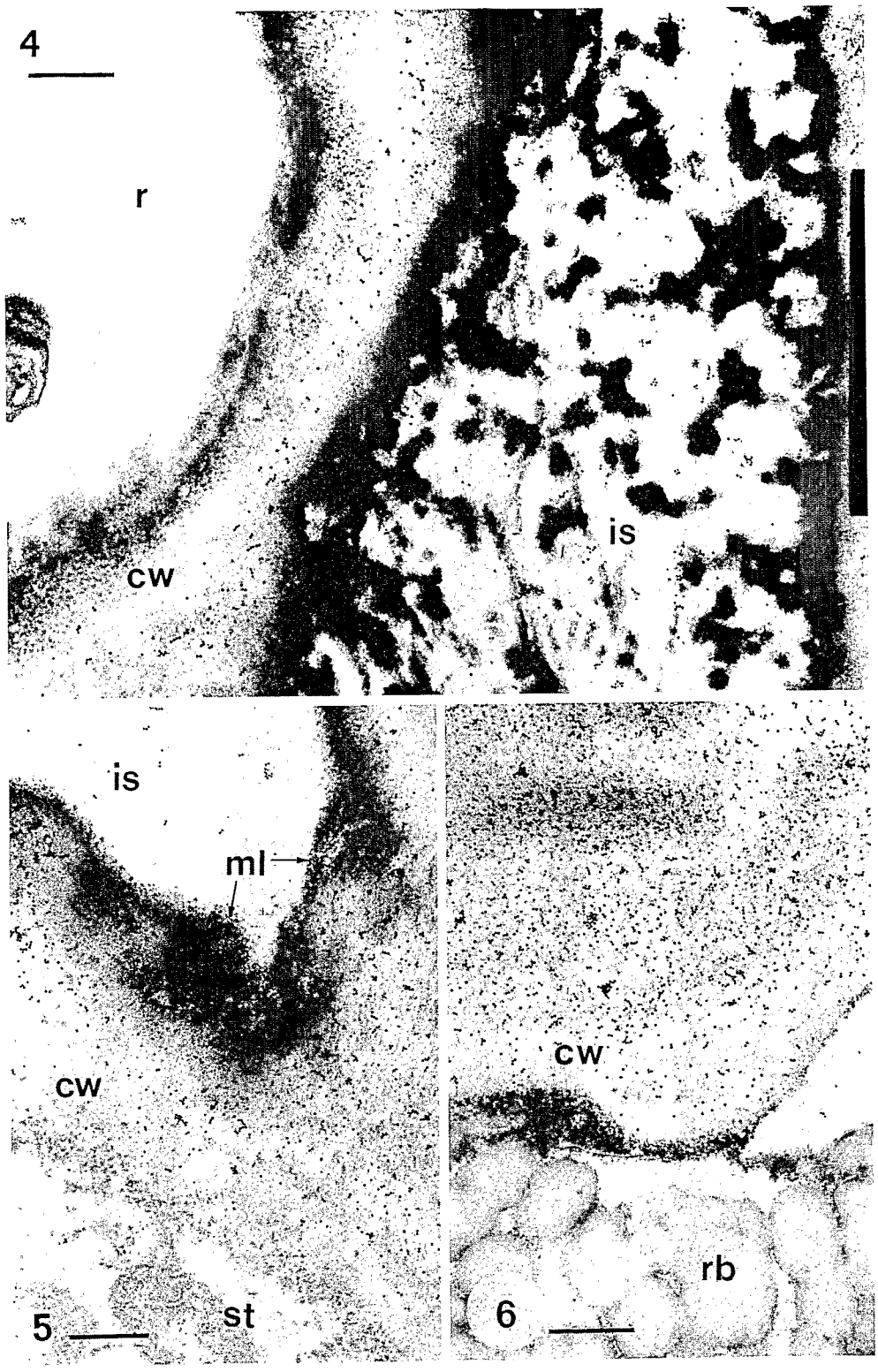
Labeling pattern observed over infected root tissues

Density of gold particles was not significant over middle lamellae in the phellem 4 weeks after inoculation; however, over cellulosic walls, a difference in labeling was observed compared with labeling found over healthy phellem (Table 1).

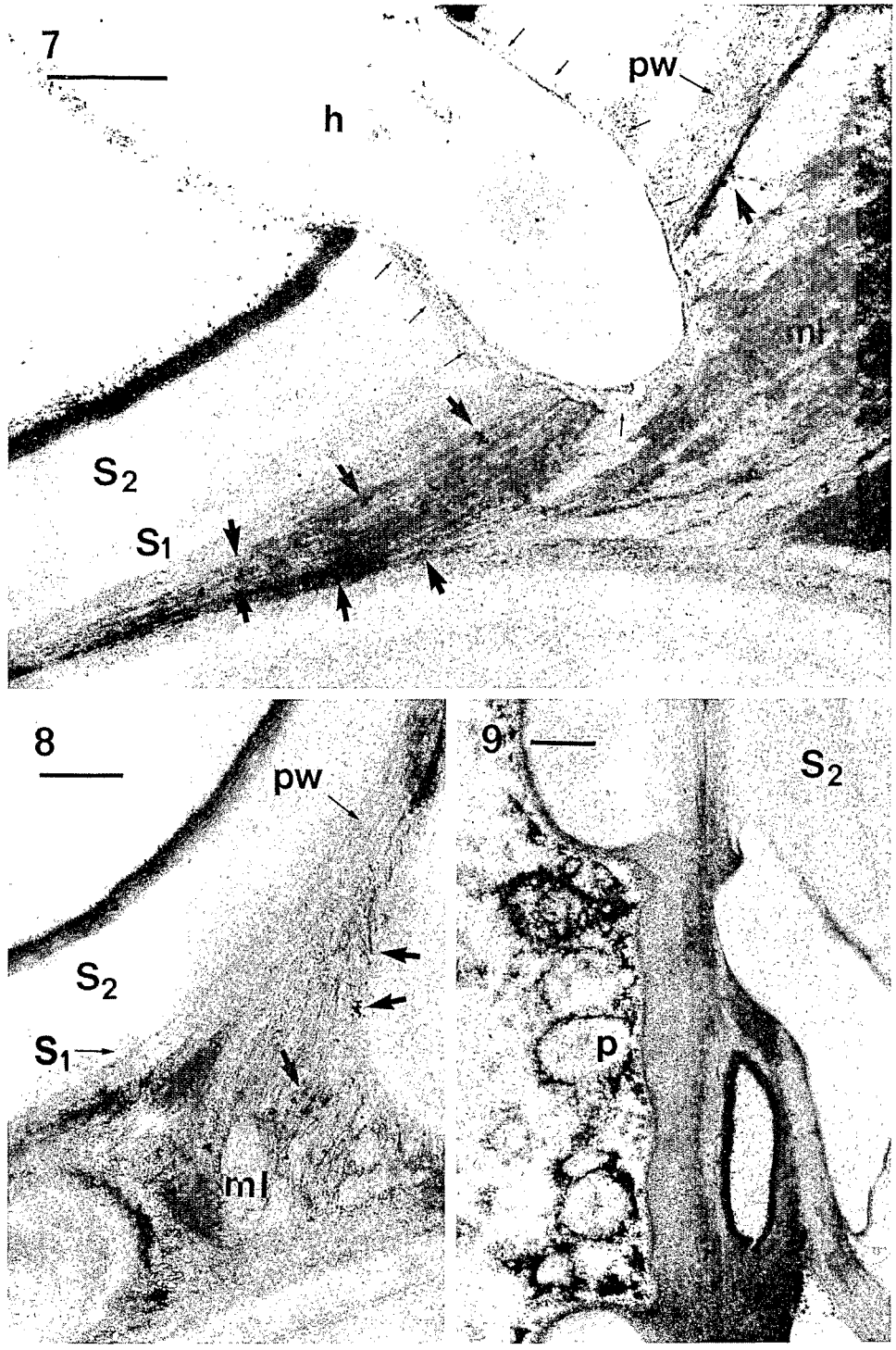
At 10 weeks after inoculation, phloem cells showed some damage; labeling was significantly different than that observed over healthy tissues (Table 1). A decrease in labeling of more than 50% was seen over the degraded middle lamellae (Table 1). Marked changes were observed in intercellular spaces of the infected phloem cells (Figs. 4 and 5) where electron-dense aggregates were apparently connected to the cell walls. Numerous gold particles accumulated in and around these degraded areas. Changes in the distribution of gold particles were also detected over the cellulosic cell walls. Labeling increased more than threefold compared with cell walls in healthy tissues (Table 1) and was generally intense except in the inner-



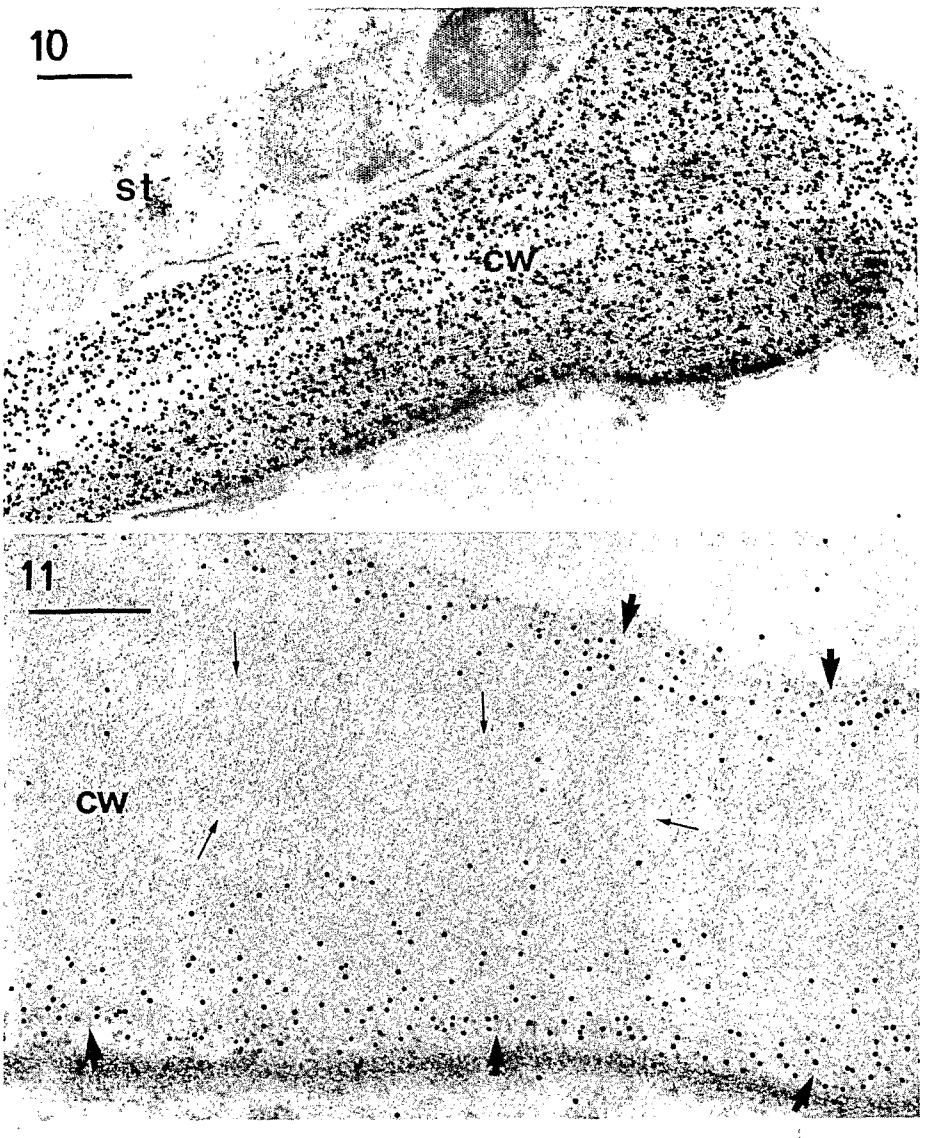
FIGS. 1–3. Transmission electron micrographs of healthy rubber tree roots double fixed with glutaraldehyde and osmium tetroxide. Sections were treated with the AGL–gold complex. Fig. 1. The fibrillar material of the middle lamella (*ml*) is intensely labelled whereas the cellulosic wall (*cw*) of sieve tubes (*st*) only exhibited some gold particles. Fig. 2. A few gold praticles occur over the middle lamella (*ml*) between xylem elements. Secondary walls of cells are nearly free of labeling. *pw*, primary wall; *sw*, secondary wall. Fig. 3. The membrane of the pit area (*p*) is devoid of significant labeling as well as the secondary walls (*sw*). *ml*, middle lamella. Scale bars = 0.4 μm.



FIGS. 4–6. Transmission electron micrographs of the infected phloem of rubber tree roots double fixed with glutaraldehyde and osmium tetroxide. Sections were treated with the AGL–gold complex. Fig. 4. Electron-dense material is localized in the intercellular space (*is*) between ray cells (*r*). Numerous gold particles are seen over and close to these aggregates. *cw*, cellulosic wall. Fig. 5. The degraded middle lamella (*ml*) appears highly electron dense. Intense labeling is observed over the sieve tube (*st*) cell wall. Gold particles occur over the intercellular space (*is*) whereas a few are seen over the innermost part of the wall. *cw*, cellulosic wall. Fig. 6. The cell wall (*cw*) of a laticiferous cell is intensely labeled, except in the innermost region. Unlabeled rubber particles (*rb*) are coagulated. Scale bars = 0.4 μm.



FIGS. 7–9. Transmission electron micrographs of the infected xylem of rubber tree roots double-fixed with glutaraldehyde and osmium tetroxide. Sections were treated with the AGL–gold complex. Fig. 7. A fungal cell (*h*) is penetrating xylem cell walls and the middle lamella (*ml*). Degraded areas are seen along the fungus pathway (small arrows). The middle lamella also shows eroded regions. Several gold particles are seen over the middle lamella (large arrows). The fungal cell as well as the secondary wall layers (S_1, S_2) and the primary wall (*pw*) are devoid of labeling. Fig. 8. Degradation of the middle lamella (*ml*) over which gold particles are unevenly distributed (arrows). The secondary wall layers (S_1, S_2) and the primary wall (*pw*) are devoid of labeling. Fig. 9. Gold particles are distributed over the highly electron-dense membrane of a pit (*p*). No labeling is observed over cell wall layers (S_2). Scale bars = 0.4 μm.



FIGS. 10 and 11. Transmission electron micrographs of the phloem of healthy and infected rubber tree roots double-fixed with glutaraldehyde and osmium tetroxide. Sections were treated with the exoglucanase–gold complex. Fig. 10. Healthy phloem. Gold particles are evenly distributed over cell walls (*cw*) of sieve tube (*st*) elements. No perturbation of cell wall organization is observed. Fig. 11. Infected phloem. A few gold particles are seen over the degraded cell wall (*cw*) except in innermost parts close to the cell cytoplasm (arrowheads). The cell wall appears loosened without compact arrangement of cellulose fibrils (small arrows). Scale bars = 0.4 μm.

most areas close to the cytoplasm (Figs. 5 and 6).

In the xylem, which coincides with approximately 15 weeks after inoculation, ultrastructural observations indicated that the middle lamella was degraded both in areas adjacent to penetrating hyphae (Fig. 7) or at a distance from them (Fig. 8). Labeling was found to occur over the middle lamella (Figs. 7 and 8) and in pit membranes (Fig. 9). Quantitation of gold particles demonstrated an increase of labeling at these sites compared with cells of healthy xylem (Table 1). In contrast, primary and secondary walls were unlabeled.

No significant labeling was observed over the fungal cell wall and host cytoplasm.

Several controls were performed to assess the specificity of labeling obtained with the AGL–gold complex. A significant reduction was observed over sections of both healthy and infected tissues incubated with the gold complex to which D-galacturonic acids were previously added in excess (Table 2) compared with Table 1.

Cytochemical localization of β-(1,4)-D-glucans

Labeling pattern observed over healthy phloem

In the phloem of healthy plants, gold particles were regularly distributed over cell walls of all cell types, including sieve tubes (Fig. 10) and laticiferous cells.

Labeling pattern observed over infected phloem

Examination of the colonized phloem cells showed that pronounced changes occurred in cell wall organization. Fibrils of cellulose appeared loosened without compact arrangement (Fig. 11). A decrease in the electron density of walls was also observed. A strong decrease of labeling (more than 90%) occurred over these walls, although innermost areas appeared

TABLE 1. Density of gold particles over cell walls in healthy and infected root tissues after incubation with the AGL−gold complex for localizing polygalacturonic acids

Host tissue	Healthy tissue	Infected tissue	χ^2	P
Phellem				
Middle lamella	0.99±0.83	0.51±0.41	ns	—
Suberized layer of cell wall	0.16±0.46	0.12±0.21	ns	—
Cellulose layer of cell wall	1.44±0.46	2.95±0.97	5.00	0.0253
Background	0.49±0.45	0.26±0.11	ns	—
Phloem				
Middle lamella	103.41±31.82	35.29±26.03	7.71	0.0055
Cell wall	22.47±11.58	72.58±24.89	15.41	0.0001
Background	0.25±0.34	0.65±0.48	ns	—
Xylem				
Middle lamella	1.57±2.05	24.24±15.64	11.86	0.0006
Cell wall	0.45±0.81	0.20±0.50	ns	—
Pit membrane	2.11±1.80	161.20±133.19	7.5	0.0062
Background	0.04±0.09	0.25±0.37	ns	—

NOTE: Data are expressed as means of 8−15 micrographs ± SE. χ^2 with 1 df.

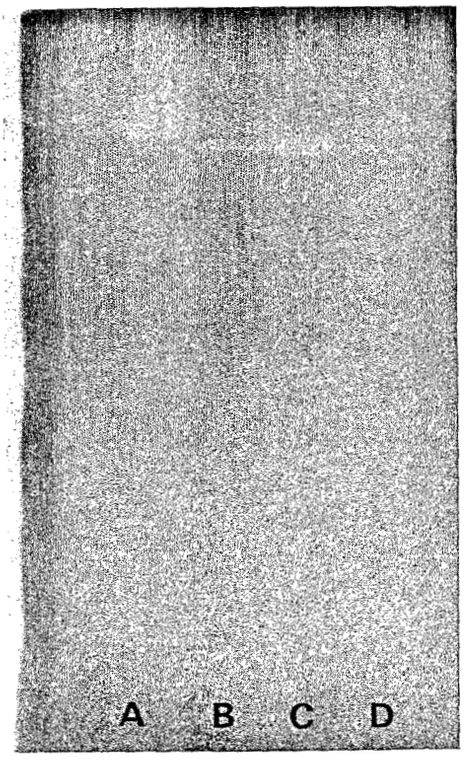


FIG. 12. Detection in SDS−PAGE of pectinases activities produced by *Rigidiporus lignosus* (strain 1, lane B; strain 68, lane D), *Phellinus noxius* (lane C), and of the activity of a commercial pectinase (lane A). Pectic enzymes were assayed on a pectate-containing gel stained with 0.02% aqueous ruthenium red and destained with water.

more intensely labeled (Table 3; Figs. 10 and 11).

Controls performed to assess the specificity of the enzyme−gold complex did not yield significant labeling (not shown).

Detection of fungal pectinolytic activity after SDS−PAGE

The activity of pectic enzymes produced *in vitro* by *R. lignosus* (strain 1) was assayed after SDS−PAGE. Electrophoregrams revealed a single thin band for *R. lignosus* strain 1 (Fig. 12, lane B) and strain 68 (Fig. 12, lane D). Bands resulting from activities of a commercial pectinase (Fig. 12, lane A) and from that of pectinases secreted by *P. noxius* (Fig. 12, lane C) appeared thicker.

TABLE 2. Density of gold particles over cell walls in healthy and infected root tissues after incubation with the AGL−gold complex to which polygalacturonic acids were previously added in excess

Host tissue	Healthy tissue	Infected tissue
Phloem		
Middle lamella	0.38±0.60	0.00±0.00
Cell wall	0.30±0.42	0.16±0.35
Background	0.05±0.08	0.22±0.31
Xylem		
Middle lamella	0.11±0.25	0.18±0.40
Cell wall	0.00±0.00	0.03±0.08
Background	0.07±0.16	0.04±0.09

NOTE: Data are expressed as means of 8−15 micrographs ± SE.

TABLE 3. Density of gold particles over cell walls in the healthy and infected phloem after incubation with exoglucanase gold complex for localizing nonreducing ends of cellulose

	Healthy tissue	Infected tissue	χ^2	P
Cell wall	480.13±24.90	5.84±6.02	6.88	0.0006
Cell wall borders	496.27±21.04	68.51±46.33	6.72	0.0008
Background	0.71±0.31	2.25±1.49	ns	—

NOTE: Data are expressed by means of 8−15 micrographs ± SE. χ^2 with 1 df.

Discussion

Until now, ultrastructural studies of the wall degradation process in woody tissues by rotting fungi focused mainly on lignin degradation (6, 22). Although previous work on rubber tree roots infected with *R. lignosus* mentioned that structural changes occur in the middle lamella during fungal colonization (19, 22), no biochemical evidence has confirmed the implication of fungal pectinases in root decay (12, 15). The present

cytochemical investigation was undertaken to gain better insight into the degradation of pectin-containing molecules during rubber tree – *R. lignosus* interaction. Our results confirm that the fungus is capable of degrading pectin compounds.

In the infected phloem, large amounts of galacturonic acid molecules were found to be associated with degraded cell walls. This probably derives from an enzymatic degradation of cellulose, as revealed by the use of the exoglucanase–gold probe, and may result in an increased accessibility of the AGL probe to pectic compounds. Such compounds are assumed to be cross-linked to other cell wall polymers (i.e., cellulose and hydroxyproline-rich glycoproteins) (24). This indicates that pectin degradation likely occurs after, or coincides with, cellulose alteration. In our pathosystem, cellulose degradation occurs early during root colonization (19). Cellulases may open new channels for other wall-degrading enzymes, such as pectinases and lignin-oxidases (7, 21). Similarly, galacturonic acids were also found to be more accessible to the gold probe over degraded middle lamellae in the xylem. Early degradation of lignin, which protects pectin in middle lamella areas, may unmask pectic components that could be degraded later. Involvement of pectic enzymes in root decay by white-rot fungi was recently reported by Johansson (13) with *Heterobasidion annosum*. However, the role of such enzymes in the pathogenesis of *Armillaria* remains unknown, since root penetration is accomplished by mechanical forces (27). Although pectin-splitting enzymes of *R. lignosus* were only characterized *in vitro*, our data indirectly suggest that these enzymes may contribute to the root decaying process of rubber trees.

Damage caused by the pathogen is accompanied by the release of pectic fragments, mostly in nonlignified tissues. The dissolution of middle lamella matrices was frequently shown to be associated with the accumulation of pectic oligomers in intercellular spaces of neighboring degraded cells (3, 5). During the interaction of rubber tree roots with *R. lignosus*, these fragments may play several roles. First, pectin was shown to act in conjunction with phenolic substances as an inducer of laccases (16), which are polyphenoloxidases involved in lignin breakdown (10, 21). *Rigidoporus lignosus* laccases induced during early colonization of roots, and enhanced by released pectic fragments, may diffuse toward the xylem prior to fungal invasion via cellular flows in ray cells. Second, it has been demonstrated that cellular location of pectic oligosaccharides provided additional evidence that these oligomers can act as signaling molecules in plant defense (3, 26). Thus, one may speculate that oligouronide fragments could have similar functions to those exhibited by *R. lignosus* chitin oligomers in infected roots (i.e., cork cambium and defense lignin stimulation, callose deposition) (23).

In conclusion, the present cytochemical study demonstrates that pectin is degraded during root colonization of rubber trees by *R. lignosus*. Our results indicate indirectly that alteration of pectin polysaccharides results from fungal pectinases and suggests that degradation of this polymer mostly occurs during phloem and xylem colonization in decayed roots. Whether released pectic fragments in infected cells act as signals involved in tree responses to the fungal attack remains to be demonstrated.

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