Antiserum to the Fungus *Phialophora mutabilis* and Its Use in Enzyme-Linked Immunosorbent Assays for Detection of Soft Rot in Preservative-Treated and Untreated Wood

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ABSTRACT

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Polyclonal antiserum was raised against mycelial extracts from the wood-degrading soft rot fungus *Phialophora mutabilis*. In enzyme-linked immunosorbent assays (ELISA), the antiserum reacted strongly with its homologous antigen and cross-reacted strongly to moderately with six other soft rot *Phialophora* species. With the exception of *Ceratocystis albida*, the serum reacted weakly or not at all with 11 other mold, blue stain, and rot fungi occurring frequently in or on wood. The antiserum also cross-reacted strongly with antigens in extracellular filtrates from *P. mutabilis* cultures that contained about 40 ng/ml of protein. Ultrastructural and immunocytochemical studies on wood degraded by *P. mutabilis* showed specific localization of the antibody on the fungal cell wall and certain intracellular structures. Extracellular labeling within soft rot cavities and sites of erosion decay of wood also were noted. The antiserum was assessed by ELISA for detecting the presence of the fungus and soft rot in untreated and preservative-treated wood blocks of pine and birch degraded for periods of 1–12 mo. *P. mutabilis* was detected in samples from all wood blocks degraded to low or high weight loss. Highest ELISA readings were recorded for wood blocks with highest substrate losses and vice versa.

Additional keywords: immunogold labeling, transmission electron microscopy.

In many parts of the world the principal groups of organisms attacking wood in ground contact and causing soft rot are the Ascomycetes and Fungi imperfecti. Within this group, the genus *Phialophora* contains some of the most destructive and frequently isolated fungi from preservative-treated timber in-service (4,7,8). *Phialophora* species occur widely and are destructive pathogens of wood under a wide range of environments. In Sweden, *P. mutabilis* is one of the principal biodegraders of preservative-treated utility poles in-service (18). Its success may be due to its considerable preservative tolerance (7,8).

Soft rot is a term originally proposed by Savory (21) to describe a type of wood decay where characteristic cavities of various shapes are produced within the secondary (S_2) walls of wood cells (type I attack, Fig. 1A). The cavities are produced through enzymatic activity of hyphae growing parallel to the cellulose microfibrils within the wood cell walls. In addition to cavity formation, some soft rot fungi also cause characteristic erosion of wood cell walls through the activity of lumenal based hyphae (type II attack, not shown). Soft rot decay, even at low substrate losses, can have a considerably negative affect on the strength of wood (17,23) and lead to serious practical problems with in-service timber situations.

Currently, the occurrence of wood decay in preservative-treated timber (e.g., utility poles) is evaluated under field conditions by use of one or more of the following methods: visual examination, subjective or measured impaction (i.e., probing with a knife and/or use of the Pilodyn instrument, Proceq SA, Zurich, Switzerland), or measurement of electrical conductivity (22). Core samples also may be taken for later examination by microscopy. Except for the Pilodyn, which has been found as the most reliable method for predicting impact failure for in-service utility poles (11), these methods may be subjective and time-consuming. Thus, there is a considerable need to develop a more rapid diagnostic test for routine application.

In recent years biological methods, including both enzymatic (4) and a number of immunological assays, have been evaluated for their ability to detect incipient (early stages) decay and the presence of rot fungi in wood (1,9). Immunological studies have so far concentrated on the use of either the enzyme-linked immunosorbent assay (ELISA) (10,12,13) or immunoblotting (20) for detection of basidiomycetes and decay in wood and poles, or the presence of blue stain fungi in stored lumber (5).

The purpose of the present work was to produce and characterize an antiserum to the important soft rot fungus *P. mutabilis* and to assess its use in ELISA as a practical means of detecting soft rot decay in situ.

MATERIALS AND METHODS

Antigen production. The isolate *P. mutabilis* (van Beyma) Schol-Schwarz (strain 24-E-1-1) (sensu *Lecythophora mutabilis*) was obtained from the collection maintained at the Department of Forest Products, Swedish University of Agricultural Sciences. This isolate, previously obtained from transmission poles treated with chromated-copper-arsenate (CCA) in Sweden (18), possesses considerable copper and arsenic tolerance and is aggressive on preservative-treated wood (8). The fungus is routinely maintained on 2.5% malt agar (Difco Labs., Detroit, MI). For antigen production, the fungus was cultivated in liquid salt medium according to Berg (2): $NH_4H_2PO_4$, 2 g; KH_2PO_4, 0.6 g; K_2HPO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; ferric citrate, 10.0 mg; $ZnSO_4 \cdot 7H_2O$, 4.4 mg; $CaCl_2$, 55.0 mg; $CoCl_2 \cdot 6H_2O$, 0.1 mg; $MnSO_4 \cdot 4H_2O$, 5.0 mg; yeast extract, 0.1 g; distilled water to 1,000 ml, pH 5.8. Cultures were grown in triplicate in 100 ml of medium for 4 wk at 25 C in 250-ml Erlenmeyer flasks under stationary conditions. Flasks were inoculated with five disks from actively growing cultures maintained on malt agar. Flask contents were filtered using a Büchner funnel (Munktell filter paper, Stora Kopparberg, Grycksbo, Sweden) and washed in several changes of phosphate-buffered saline (PBS), pH 7.4. The collected mycelia were resuspended in 1.0 ml of PBS and ground using a mortar and pestle. Samples were subsequently centrifuged at 5,000 *g* for 15 min, and the resulting pellets frozen and then freeze-dried until further use.

Production of antiserum. The antiserum to *P. mutabilis* (whole fungus) was produced by intramuscular injections of 0.5 ml of antigen suspension (2.0 mg/ml) emulsified with 0.5 ml of Freund's complete adjuvant into a New Zealand white rabbit. Booster injections 3 and 5 wk after initial immunization were given using 0.5 ml of antigen in Freund's incomplete adjuvant. The rabbit was bled 2 wk after the second booster injection and thereafter weekly.

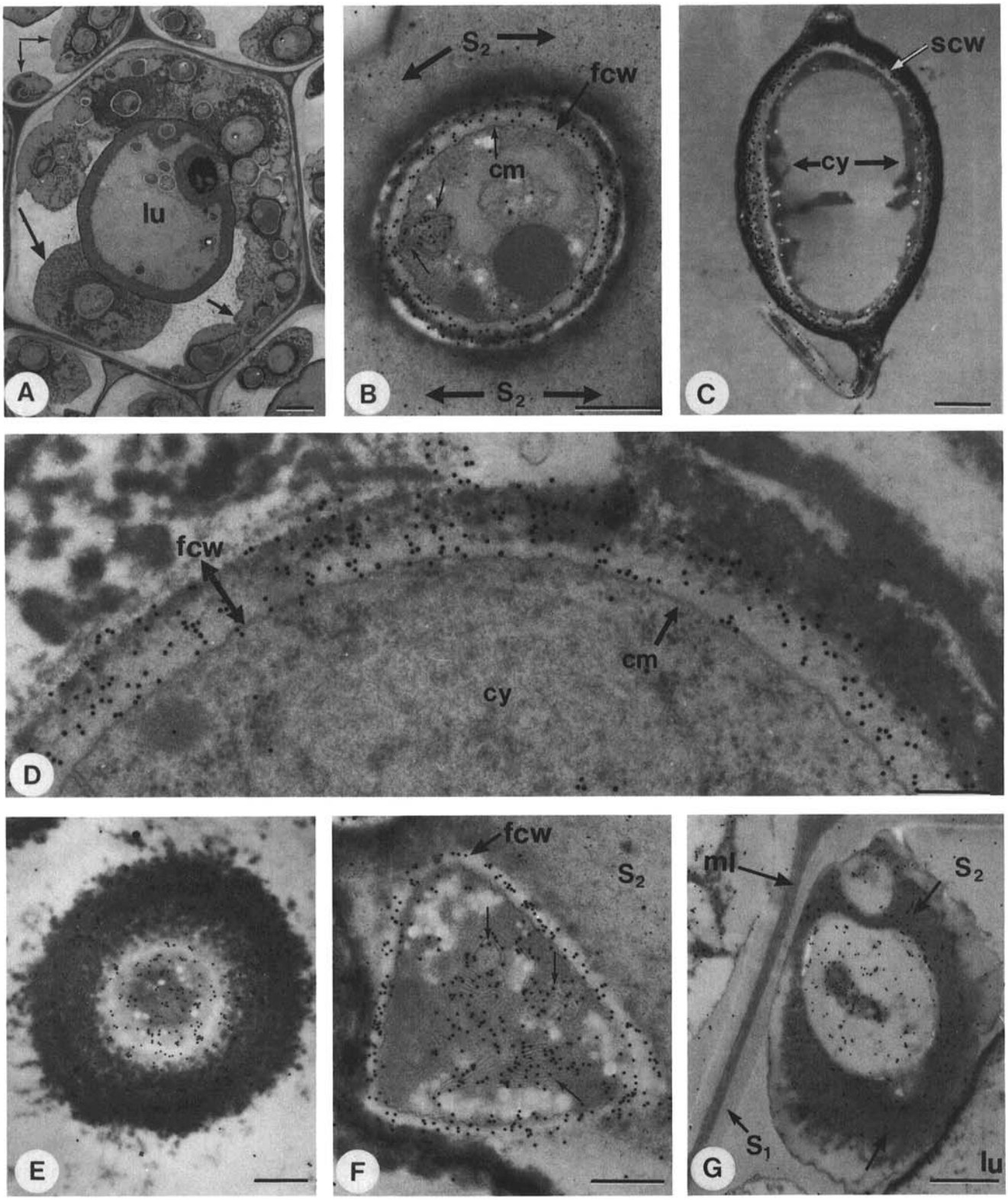

Fig. 1. *Phialophora mutabilis* decay of birch wood as revealed by immunocytochemical labeling with its homologous antiserum (1:100 dilution). **A,** Transverse section of birch fiber showing typical appearance of soft rot cavities (arrows) and of hyphae within the S_2 layer. **B–D,** Transverse sections showing immunogold labeling of hyphal (**B, D**) and spore (**C**) cell wall components. Membrane invagination (arrows, **B**) of the cell membrane also were labeled. **E,** Labeling of a heavily melanized hypha from the cell lumen. **F,** Labeling of membranes (arrows) and vesicular structures within the hyphal cytoplasm. **G,** Typical labeling of the outer attacked region of a soft rot cavity (arrows) within S_2. Bars = 2.0, 0.5, 1.0, 0.25, 0.5, 0.5, and 1.0 μm for A–G, respectively. Abbreviations: cm, cell membrane; cy, cytoplasm; fcw, fungal cell wall; lu, fiber lumen; ml, middle lamella; scw, spore cell wall; S_1 and S_2, secondary fiber wall layers.

Results reported here are for serum from the third bleeding about 3 wk after the second booster. Serum isolation was as described by Johnstone and Thorpe (15). Reactivity of the antiserum was initially tested using gel immunodiffusion assays (19). Preimmune serum was removed from the rabbits before immunization with antigen.

Antisera specificity. The specificity of the antiserum to other *Phialophora* species and isolates was determined by ELISA. Test species included: *P. mutabilis* (van Beyma) Schol-Schwarz (= *Lecythophora mutabilis* (van Beyma) W. Gams) 202-E-15y-5; *P. malorum* M. N. Kidd & A. Beaumont 211-c-15-1; *P. malorum* (Kidd & Beaumont) CBS-245-60; *P. cyclaminis* (van Beyma) CBS-245-69; *P. fastigiata* (Lagerberg & Melin) Conant 731-1-31; *P. alba* (van Beyma) 154-D-16-2; *P. hoffmannii* (van Beyma) Schol-Schwarz 24-E-1-2; *P. richardsiae* (Nannf. apud Melin & Nannf.) Conant kx 2140, and *Phialophora* sp. A, isolates 831005, 35-1, and TS 340-M2. In addition, specificity also was determined for other soft rot ascomycetes: *Chaetomium globosum* (Fr. var. *globosum*) F171-1; *Ceratocystis albida* (13-23) and several basidiomycetes causing either white (*Phanerochaete chrysosporium* Burdsall P127-1; *Trametes [Coriolus] versicolor* [L.:Fr.] Lloyd A361; *Schizophyllum commune* [Fr.:Fr.] 12) or brown rot (*Gloephyllum sepiarium* [Wulfen:Fr] Karst 10 BS-2-Ar; *Fomitopsis pinicola* [Swartz:Fr.] Karst. 71349) in wood. Cross-reactivity with the blue stain fungi *Scytalidium lignicola* Pesante 415-BS-3, *Alternaria alternata* (Fr.:Fr.) IV-1-4-4-BM, *Aureobasidium pullulans* (de Bary) (Arnaud var. *pullulans*) 377-MB, and the mold fungus *Aspergillus fumigatus* Fres. var. *fumigatus* also were examined. All fungal isolates were obtained from the collection maintained at the Department of Forest Products, Swedish University of Agricultural Sciences or from the Centraalbureau voor Schimmelcultures, Baarn. For the soft rot-causing species, the fungi were grown in liquid culture and mycelia harvested and processed by freeze-drying as described above. All other fungi were obtained by scraping mycelia from cultures actively growing on malt agar. Liquid culture samples were ground with a mortar and pestle after freeze-drying, while fresh samples were frozen and then ground under liquid nitrogen.

Cross-reactivity of the antiserum against extracellular filtrates produced by *P. mutabilis* 24-E-1-1 and two other soft rot species also was studied using ELISA. The fungi were grown under the same liquid culture conditions as described above but with media supplemented with 1% cellstoff (Swedish Tissue AB, Kista, Sweden) containing equal amounts of bleached pine sulphite and bleached sulphate pulp as a carbon source. After removal of mycelia by filtration, the extracellular filtrates from 1-L cultures were concentrated with a MINITAN-S ultrafiltration device (Millipore Corp., Bedford, MA) fitted with a polysulfone (PTGC) 10,000 molecular weight (MW) cutoff filter. Filtrate concentrations were adjusted for ELISA in coating buffer (sodium carbonate buffer, pH 9.6). Total protein in the filtrates was estimated using the methods of Bradford (3).

IgG purification. Partial purification of the immunoglobulin (IgG) involved adding 9 ml of distilled water to 1 ml of antiserum and then adding 10 ml of saturated ammonium sulfate solution to precipitate the IgG. The IgG was pelleted via centrifugation (3,000 *g*), diluted with 1 ml of distilled water, and thoroughly dialyzed against half-strength PBS. The IgG was eluted through a DEAE cellulose column and the unadsorbed fractions collected. The absorbance at 280 nm was adjusted to 1 mg/ml. This partially purified IgG fraction was used for all assays.

Wood samples and decay tests. Small wood blocks (30 × 15 × 5 mm) of birch (*Betula verrucosa* Ehr.) and pine (*Pinus sylvestris* L.) were partially buried in 125-ml Erlenmeyer flasks containing moist loamy garden soil. After autoclaving, the flasks were inoculated with 5 ml of a mycelium and spore suspension (in PBS) of *P. mutabilis* 24-E-1-1 previously grown on 2.5% malt agar plates. The flasks were maintained in the dark at 25 C and 70% relative humidity. After periods ranging from 1 wk to 1 yr, triplicate blocks were removed for weight loss determination and subsequent ELISA analysis. Weight loss was determined by drying blocks over 3 days at 40 C. Some extra wood blocks also were processed for transmission electron microscopy and immunocytochemical studies.

Additional ELISA analyses were performed on wood blocks preservative-treated with CCA (i.e., a 2% commercial K33 CCA formulation [Cu 14.8, Cr 26.6, As 34.0%, w/w]) or an ammoniacal copper sulphate solution (1.2% NH_3, v/v, and 1.8% $CuSO_4$, w/w) and decayed for 5 mo with *P. mutabilis* 202-E-15y-5 under similar conditions as described above.

ELISA: Antiserum sensitivity and cross-reactivity. The indirect ELISA procedure was modified from that described by Clark and Adams (6). Initially freeze-dried antigen as prepared for immunization was used to determine the sensitivity of the *P. mutabilis* 24-E-1-1 antiserum. For this 10 mg of the freeze-dried and ground mycelium was mixed with 1 ml of 0.1 M sodium carbonate buffer (pH 9.6) (coating buffer) in Eppendorf tubes and shaken at 4 C for 1 h. After centrifugation at 1,000 *g* for 5 min to remove mycelial debris, the antigen was diluted in coating buffer and 200 μl pipetted into adjacent wells (triplicate wells) of an ELISA plate (A/S Nunc, Kamstrup, Denmark). After incubation overnight at room temperature, the plates were washed (2× in PBS, pH 7.2) followed by blocking in PBS containing 0.2% bovine serum albumin (BSA), 2% polyvinylpyrrolidone (PVP) (MW 10,000) and 0.05% Tween 20 (2 × 15 min). The *P. mutabilis* antiserum diluted 1:1,000 in PBS-0.2% BSA-Tween 20 was added (200 μl per well) and the plates incubated for 1 h at 37 C. After washing in PBS (2 × 15 min) and PBS-BSA-PVP-Tween 20 (2 × 15 min), 200 μl of an enzyme-conjugated IgG (goat-antirabbit IgG alkaline phosphatase, Sigma A-8025) diluted 1:1,000 in PBS-BSA-Tween 20 was added to the wells. After incubation for 1 h at 37 C the plates were washed as above and the enzyme substrate, *p*-nitrophenyl phosphate (1.0 mg/ml, 100 μl per well) diluted in diethanolamine buffer (pH 9.8), was added. The enzyme reaction was monitored at 405 nm with a Bio-Tek EL 311 SL microplate recorder (Bio-Tek Instruments, Inc., Burlington, VT) after 20 min without termination.

Detection of *Phialophora* spp. in degraded wood blocks. For degraded wood samples the ELISA procedure was as described above, but with slight modification of antigen preparation. Decayed wood blocks were ground with a Wiley mill fitted with a 40-mesh screen. Samples then were added to coating buffer in Eppendorf tubes and agitated at 4 C for 1 h. Thereafter samples were centrifuged at 2,000 *g* for 5 min to remove larger lignocellulose debris. Preliminary studies in which the time of the initial premixing of antigen and coating buffer was varied (i.e., 1, 4, and 24 h at 4 C) showed 1 h to be adequate.

To reduce inhibitory cross-reactions between wood extracts and the antiserum, wood samples were used at 0.1 and 0.01 mg/ml. Further reduction of nonspecific reactions between the polyclonal antiserum and wood components solubilized during the prebuffer incubation stage was obtained with antiserum cross absorbed (shaken at 4 C for 1 h using a vortex apparatus) with undegraded birch or pine sawdust (diluted 1:1,000 in PBS; 40 mesh, 1 mg/1 ml). The sawdust then was removed from the antiserum via centrifugation at 2,000 *g* for 5 min. Controls included undegraded wood samples, separate omissions of the primary and secondary antibodies, and buffer only. Incubation with *P. mutabilis* was used as a positive control.

Light microscopy. To assess soft rot decay of wood blocks, initial observations were made of razor blade sections cut from blocks and stained with either 1% (w/v) safranin or lactophenol sky blue.

Transmission electron microscopy and immunocytochemistry. Small matchstick samples (about 1.0 × 1.0 × 5.0 mm) were removed from degraded wood blocks from a parallel experiment and fixed for 3 h in either 3% (v/v) glutaraldehyde containing 1% (v/v) paraformaldehyde in 0.1 m of sodium cacodylate buffer (pH 7.2), or with 4% (v/v) paraformaldehyde in buffer for 3 h at room temperature. Thereafter glutaraldehyde-fixed material was washed in buffer (2 × 30 min) and postfixed for 2 h at room temperature in 1% (w/v) osmium tetroxide also in buffer. After further washes in buffer and distilled water, samples were dehydrated in an ethanol series and embedded in London resin

(London Resin Co., Basingstoke, UK). Paraformaldehyde-fixed samples were dehydrated directly without postfixation and embedded in resin. Selected material was sectioned with a Reichert FCD4 ultramicrotome (Buffalo, NY) and sections collected on nickel grids.

For immunocytochemistry, ultrathin sections were pretreated with either 10% H_2O_2 (unosmicated samples) or saturated metaperiodate (osmicated samples) and washed in PBS (pH 7.2). Unosmicated samples were further incubated with 0.1 glycine (30 min) to quench aldehyde groups induced during fixation. Sections then were incubated in drops of normal goat serum in PBS (1:30) and then subsequently with the *P. mutabilis* antiserum diluted either 1:100 or 1:1,000 in PBS and containing 1% BSA and 0.05% Tween 20. Incubation was overnight (12–14 h) at 4 C. After primary antiserum incubation, the sections were washed in PBS-BSA-Tween 20 (pH 7.4) and in Tris-hydrochloride-BSA-Tween 20 (pH 8.2) and then incubated with a gold-labeled antirabbit IgG conjugate (goat-antirabbit 615) (B-2340: Janssen Life Science Products, Beerse, Belgium) in Tris-BSA-Tween 20 (pH 8.4) for 1 h. Sections then were washed thoroughly in Tris (pH 8.4) and finally in distilled water. Poststaining was in 4% aqueous uranyl acetate (15 min). Controls included omission of the primary antiserum and use of the *P. mutabilis* antiserum previously preadsorbed with *P. mutabilis* mycelia.

RESULTS

Sensitivity. Using ELISA and freeze-dried preparations of the homologous antigen, concentrations as low as 10 ng/ml and 100 ng/ml were detected for *P. mutabilis* 202-E-15y-5 and *P. mutabilis* 24-E-1-1, respectively (Table 1). Apart from the slightly greater sensitivity of the antibody for *P. mutabilis* 202-E-15y-5 at low concentrations, both fungal antigens reacted similarly with the dilutions used and both produced a reduced response at 1.0 mg/ml (Table 1).

Specificity. Results of specificity of the antiserum against *Phialophora* and non-*Phialophora* species are shown in Table 2. The antiserum showed a range of specificity with strongest reactions recorded for its own antigen and isolates of *Phialophora* sp. A. Slightly reduced response was recorded for *P. malorum* isolates followed by moderate reactions for both *P. hoffmannii* and *P. fastigiata* (Table 2). A slightly reduced reaction (A_{405nm} = 0.35) was obtained for *P. alba* and only weak to negative cross-reactions for *P. cyclaminis* and *P. richardsiae* (Table 2). Apart from a moderate (A_{405nm} = 0.644) cross-reaction with *C. albida* and a weak response from *P. placenta* (A_{405nm} = 0.19), all other non-*Phialophora* species tested (i.e., mold and blue stain, and basidiomycetes), including representatives frequently isolated from wood, gave very weak or negative reactions (Table 2).

A strong cross-reaction of the antiserum also was recognized against extracellular filtrates from liquid cultures of *P. mutabilis* 24-E-1-1 and *P. malorum* 211-C-15-1 (Table 3). Filtrates containing 40 ng/ml protein gave A_{405nm} values of 0.843 and 0.067 for *P. mutabilis* and *P. malorum,* respectively. A corresponding A_{405nm} for the soft rot fungus *C. globosum* at about 40 ng/ml protein was 0.040.

Detection of *P. mutabilis* in degraded wood samples. All ELISA assays performed on extracts from untreated and preservative-treated birch and pine wood degraded by *P. mutabilis* yielded strong positive absorbance values at the two sample dilutions used (Tables 4 and 5). With untreated birch and pine samples, a strong positive ELISA reaction was obtained after only 1 wk of exposure to the fungus when very little weight loss had taken place (Table 4). With extracts from degraded birch, the ELISA absorbance levels increased progressively up to the 12-mo

TABLE 1. Sensitivity of the *Phialophora mutabilis* antiserum against two *P. mutabilis* isolates as determined by indirect enzyme-linked immunosorbent assay (ELISA)[a]

Mycelial dry weight[b] (μg/ml)	Absorbance at 405 nm[c] *P. mutabilis* 24-E-1-1	*P. mutabilis* 202-E-15y-5
1,000	1.266 ± 0.013	1.182 ± 0.100
100	1.490 ± 0.128	1.495 ± 0.079
10	1.677 ± 0.174	1.629 ± 0.117
0.1	0.492 ± 0.061	0.593 ± 0.025
0.01	0.000	0.029 ± 0.015
PBS control	0.000	0.000

[a] Ground mycelia diluted in coating buffer were added to microtiter plates and left overnight. Following washing in phosphate-buffered saline (PBS) and blocking in PBS-BSA-PVP-Tween, *P. mutabilis* 24-E-1-1 antiserum (1:1,000 in PBS-BSA-Tween) was added and plates were incubated. After washing in PBS-BSA-PVP-Tween, alkaline phosphatase conjugated to goat antirabbit IgG (1:1,000 in PBS-BSA-Tween) was added, and plates were incubated. Plates then were washed with buffer and the enzyme substrate *p*-nitrophenyl phosphate (1.0 mg/ml) in diethanolamine buffer was added. Enzyme reaction was monitored at 405 nm.

[b] *P. mutabilis* isolates were grown in liquid media, freeze-dried, ground using a mortar and pestle, and diluted in coating buffer.

[c] Absorbance values were means of three adjacent wells and are blanked against PBS-control.

TABLE 2. Results of indirect enzyme-linked immunosorbent assay (ELISA)[a] for cross-reactivity of *Phialophora mutabilis* 24-E-1-1 antiserum with other *Phialophora*[b] spp. and various rot and mold fungi[c]

Fungal species	Absorbance at 405 nm[d] Isolate	0.1 mg/ml
Ascomycetes		
Phialophora soft rotters		
P. mutabilis	24-E-1-1	1.49 ± 0.13
P. mutabilis	202-E-15y-5	1.49 ± 0.08
P. sp. A	35-1	1.54 ± 0.06
P. sp. A	TS-340-M2	1.52 ± 0.14
P. sp. A	831005	1.09 ± 0.01
P. malorum	211-C-15-1	1.03 ± 0.06
P. malorum	CBS-245-60	1.02 ± 0.06
P. hoffmannii	24-E-1-2	0.74 ± 0.06
P. fastigiata	731-1-31	0.68 ± 0.02
P. alba	154-D-16-2	0.35 ± 0.03
P. cyclaminis	CBS-245-69	0.09 ± 0.01
P. richardsiae	kx 21-10	0.06 ± 0.01
Non-*Phialophora* soft rotters		
Ceratocystis albida	13-23	0.644 ± 0.05
Chaetomium globosum	F171-1	0.090 ± 0.01
Ascomycetes - Mold fungi		
Aspergillus fumigatus		0.05
Ascomycetes - Staining fungi		
Aureobasidium pullulans	377-MB	0.02
Alternaria alternata	IV-1-4-4-BM	0.02
Scytalidium lignicola	415-BS-3	0.06
Basidiomycetes		
White rot species		
Phanerochaete chrysosporium	P127-1	0.04 ± 0.01
Trametes versicolor	A361	0.07 ± 0.01
Schizophyllum commune	Nr 12	0.01 ···
Brown rot species		
Postia placenta	QM 1010	0.19 ± 0.03
Fomitopsis pinicola	71349	0.00[e] ···
Gloeophyllum sepiarium	10-BS-2-AK	0.01 ···
PBS control	···	0.000

[a] Ground mycelia in coating buffer were added to microtiter plates. After washing in phosphate-buffered saline (PBS) and blocking in PBS-BSA-PVP-Tween, *P. mutabilis* 24-E-1-1 antiserum (1:1,000 in PBS-BSA-Tween) was added, and plates were incubated. After washing in PBS-BSA-PVP-Tween, alkaline phosphatase conjugated to goat antirabbit IgG (1:1,000 in PBS-BSA-Tween) was added, and plates were incubated. Plates then were washed with PBS-BSA-Tween, and the enzyme substrate *p*-nitrophenyl phosphate (1.0 mg/ml) in diethanolamine buffer was added. Enzyme reaction was monitored at 405 nm.

[b] Isolates were grown in liquid media, freeze-dried, and ground with mortar and pestle.

[c] Mycelium were scraped off agar plates, weighed, and ground with mortar and pestle.

[d] Absorbance values are means of triplicate wells plus standard deviation, or single results, and are blanked against PBS control.

[e] Reading less than control.

sampling time where the strongest A_{405nm} value of 2.052 was recorded for a dry substance weight loss of 46.2% (Table 4). Similarly with pine, the A_{405nm} values also increased progressively until 2 mo, but thereafter remained fairly stable for 4-, 6-, and 12-mo samples. Pine weight losses for the 4- to 6-mo period varied between 11.3 and 17.4% and were considerably lower than that recorded for birch (i.e., 39.3 and 46.2% for 6 and 12 mo, respectively).

Strong positive ELISA reactions also were obtained for the CCA (K33)- and ammoniacal copper-treated birch and pine samples attacked by *P. mutabilis,* indicating that the assay also was effective in detecting the fungus in preservative-treated wood. The preservative treatments decreased soft rot decay of birch by *P. mutabilis* 202-E-15y-5. With the 2% K33- and ammoniacal copper-treated samples, dry weight losses of only 10% were recorded. Equivalent weight losses for the untreated control and 1% K33-treated birch wood were 50.8 and 31.2%, respectively.

TABLE 3. Sensitivity of the *Phialophora mutabilis* 24-E-1-1 antiserum against extracellular filtrates from three soft rot fungi as determined by indirect ELISA[a]

Extracellular filtrate (protein concentration μg/ml)[b]	Absorbance at 405 nm[c]		
	P. mutabilis 24-E-1-1	*P. malorum* 211-C-15-1	*C. globosum* F171-1
40	2.327	1.038	0.332
20	1.894	ND[d]	0.298
4	1.749	0.553	0.281
0.4	1.200	0.309	0.160
0.04	0.843	0.067	0.040
0.004	0.170	0.015	0.032
0.0004	0.049	0.034	0.030
PBS control	0.000	0.000	0.000

[a]Extracellular filtrates in coating buffer were added to microtiter plates. After washing in phosphate-buffered saline (PBS) and blocking in PBS-BSA-PVP-Tween, *P. mutabilis* 24-E-1-1 antiserum (1:1,000 in PBS-BSA-Tween) was added and plates incubated. After washing in PBS-BSA-PVP-Tween, alkaline phosphatase conjugated to goat antirabbit IgG (1:1,000 in PBS-BSA-Tween) was added, and plates incubated. Plates were washed with PBS-BSA-Tween and the enzyme substrate *p*-nitrophenyl phosphate (1.0 mg/ml) in diethanolamine buffer added. Enzyme reaction was monitored at 405 nm.
[b]Extracellular filtrates concentrated from 3-wk-old flask cultures (400 ml of culture medium) of the various fungi.
[c]Absorbance values represent the mean of two adjacent wells and were blanked against PBS control.
[d]Not determined.

ELISA A_{405nm} readings also increased as the birch samples were more decayed and highest values were recorded for the untreated (1.804 at 0.1 mg/ml) and 1% K33 (1.744 at 0.1 mg/ml) treated samples. Results for 2% K33- and ammoniacal copper-treated birch samples were similar (1.223 and 1.447 at 0.1 mg/ml), as were their respective weight losses (Table 5). Unlike birch, preservative treatment of pine reduced decay to nonsignificant levels (i.e., <2% weight loss) (Table 5). Despite this, significant ELISA readings were obtained for the treated pine samples suggesting the presence and colonization by the fungus, a result confirmed by visual observations of wood blocks before grinding. Strongest ELISA readings were obtained for the untreated pine sample (1.067 at 0.1 mg/ml) where the greatest weight loss also was recorded. Control ELISA readings for undegraded birch and pine were low and nonsignificant in comparison with decayed samples from the two experiments (Tables 4 and 5). An increase in the quantity of wood sample (i.e., 0.01 to 0.10 mg/ml) used in the initial incubation procedure did cause, however, a slight increase in the ELISA absorbance (Table 4). Use of high wood concentrations (i.e., >1 mg/ml) caused a marked inhibitory cross-reaction between wood extracts and ELISA absorbance (data not shown).

Immunogold labeling. The purpose of the ultrastructural immunocytochemical studies was to determine sites of antibody binding on the fungus and also to determine whether extracellular binding of the antibody occurred in wood cell walls undergoing soft rot decay. Fixation had an important effect on the ultrastructural quality and antigenic response of fungal hyphae. While aldehyde-fixed samples produced the best antigenic response, samples postfixed in osmium tetroxide provided improved fungal ultrastructure. Photos shown here are from samples postfixed in osmium tetroxide and treated with a 1:100 antisera dilution.

Hyphae within soft rot cavities in the S_2 cell wall of fibers or within the cell lumen of fibers were heavily labeled by the antiserum (Figs. 1B, D–G and 2A). Spore and hyphal cell walls reacted intensely (Fig. 1B–D), as did hyphae covered in electron-opaque materials considered to be melanin (Fig. 1E). Labeling also occurred on hyphal intracellular membrane structures (Fig. 1B and F) including invaginations of the outer cell membrane (Fig. 1B). Hyphae lacking cellular components and apparently dead also reacted strongly. Extracellularly, the antisera reacted with wood cell wall regions undergoing degradation (Fig. 2A) and were apparent on both the outer regions of soft rot cavities (Fig. 1G) and on fiber lumen cell walls subjected to erosion decay (Fig. 2B). Labeling also occurred in lumena of fibers when filled with meshwork of electron-dense products originating from fungal

TABLE 4. Detection by enzyme-linked immunosorbent assay (ELISA)[a] of *Phialophora mutabilis* 24-E-1-1 antigens in birch and pine wood decayed for various periods of time[b]

	Degradation time (weeks)								Control (undegraded wood)
	1	2	3	8	12	16	26	52	
Birch wood									
0.1 mg/ml[c]	0.908[d]	1.405	1.227	ND[e]	1.760	ND	1.896	2.052	0.035
0.01 mg/ml	0.214	0.454	0.532	ND	1.003	ND	1.434	1.500	0.020
Percent weight loss	2.1	4.0	8.0	ND	28.7	ND	39.3	46.2	...
Pine wood									
0.1 mg/ml	0.902	1.030	1.374	1.447	ND	1.274	1.451	1.340	0.159
0.01 mg/ml	0.310	0.432	0.616	0.682	ND	0.434	0.822	1.131	0.055
Percent weight loss	1.7	1.4	4.4	7.4	ND	11.3	17.4	ND	...

[a]Extracts from ground wood diluted in coating buffer were added to microtiter plates. After washing in phosphate-buffered saline (PBS) and blocking in PBS-BSA-PVP-Tween, cross-absorbed *P. mutabilis* 24-E-1-1 antiserum in PBS-BSA-Tween was added, and plates were incubated. After washing in PBS-BSA-PVP-Tween, alkaline phosphatase conjugated to goat antirabbit IgG (1:1,000 in PBS-BSA-Tween) was added. Plates were washed with PBS-BSA-Tween, and the enzyme substrate *p*-nitrophenyl phosphate (1.0 mg/ml) in diethanolamine buffer was added. Enzyme reaction was monitored at 405 nm. Nonspecific reactions between *P. mutabilis* 24-E-1-1 antiserum and wood components were reduced by cross-absorbing the serum with birch or pine sawdust (1:1,000 in PBS, 1 mg/ml).
[b]After decay, the wood blocks were dried at 40 C and weight losses determined (mean of triplicate blocks). Wood blocks were then ground in a Wiley mill and the sawdust incubated under agitation with coating buffer. After removal of lignocellulose debris and appropriate dilution the supernatant (antigen) was assayed using ELISA.
[c]Represents original amount of sawdust used for incubation.
[d]A_{405nm} values were means of two adjacent wells and are blanked against PBS control.
[e]Not determined.

activity. Controls using antiserum preadsorbed with *P. mutabilis* mycelia (Fig. 2C) or omission of the primary antibody stage produced negative results.

DISCUSSION

The present *P. mutabilis* antisera showed a high degree of sensitivity to their homologous antigen as well as a broad cross-reaction to six of the eight *Phialophora* species tested by ELISA. Two species, *P. cyclaminis* and *P. richardsiae,* gave insignificant ELISA results. *P. cylaminis* has not been reported so far to cause soft rot in wood and, therefore, may not be important in the present context. Apart from *P. alba,* these cross-reacting species are all recognized as important soft rotters of treated and untreated wood, suggesting that the antiserum could function in any broad spectrum assay system to detect the general presence of *Phialophora* in wood. The strong antigenic response of the antisera with extracellular filtrates from *P. mutabilis* and *P. malorum* and the immunogold labeling of wood regions undergoing decay also indicate a further cross-reaction with soluble secretory products likely to be found in soft rotted wood. Additional ELISA studies (G. Daniel, *unpublished*) have shown *P. mutabilis* culture filtrates to cross-react with polyclonal antibodies made to *Trichoderma reesei* cellobiohydrolases and endoglucanases, and to a lesser extent with those from *Phanerochaete chrysosporium,* suggesting that the filtrates likely possess cellulase activity.

Apart from the preservative-treated pine samples, all samples from *P. mutabilis*-inoculated birch and pine blocks showed significant ELISA reactions (compared with undegraded wood samples) and an absorbance at 405 nm that continued to increase with

TABLE 5. Detection by indirect enzyme-linked immunosorbent assay (ELISA)[a] of *Phialophora mutabilis* 202-E-15-y-5 in preservative-treated wood blocks of birch and pine decayed for 5 mo[b]

	Control (untreated)	Preservative treatment/Absorbance at 405 nm[c]			Control (untreated; undecayed)
		1% K33	2% K33	$NH_3 + CuSO_4$	
Birch wood					
0.1 mg/ml[d]	1.804	1.744	1.223	1.447	0.122
0.01 mg/ml	1.440	1.232	0.617	0.752	ND[e]
Percent weight loss	50.8	31.2	10.4	9.7	ND
Pine wood					
0.1 mg/ml	1.067	0.936	0.851	1.286	0.066
0.01 mg/ml	0.405	0.338	0.287	0.488	ND
Percent weight loss	18.5	< 2.0	< 2.0	< 2.0	ND

[a] Extracts from ground wood diluted in coating buffer were added to microtiter plates. After washing in phosphate-buffered saline (PBS) and blocking in PBS-BSA-PVP-Tween, cross-absorbed *P. mutabilis* 24-E-1-1 antiserum in PBS-BSA-Tween was added and plates incubated. After washing in PBS-BSA-PVP-Tween, alkaline phosphatase conjugated to goat antirabbit IgG (1:1,000 in PBS-BSA-Tween) was added and plates incubated. Plates were washed with PBS-BSA-Tween and the enzyme substrate *p*-nitrophenyl phosphate (1.0 mg/ml) in diethanolamine buffer added. Enzyme reaction was monitored at 405 nm. Nonspecific reactions between *P. mutabilis* 24-E-1-1 antiserum and wood components were reduced by cross-absorbing the serum with birch or pine sawdust (1:1,000 in PBS, 1 mg/ml).

[b] After decay, the wood blocks were dried at 40 C and weight losses determined (mean of duplicate blocks). Wood blocks were then ground in a Wiley mill and the sawdust incubated under agitation with coating buffer. After appropriate dilution the supernatant was assayed using ELISA.

[c] A_{405nm} values were means of two adjacent wells and are blanked against PBS control.

[d] Represents original amount of test sample sawdust used for incubation.

[e] Not determined.

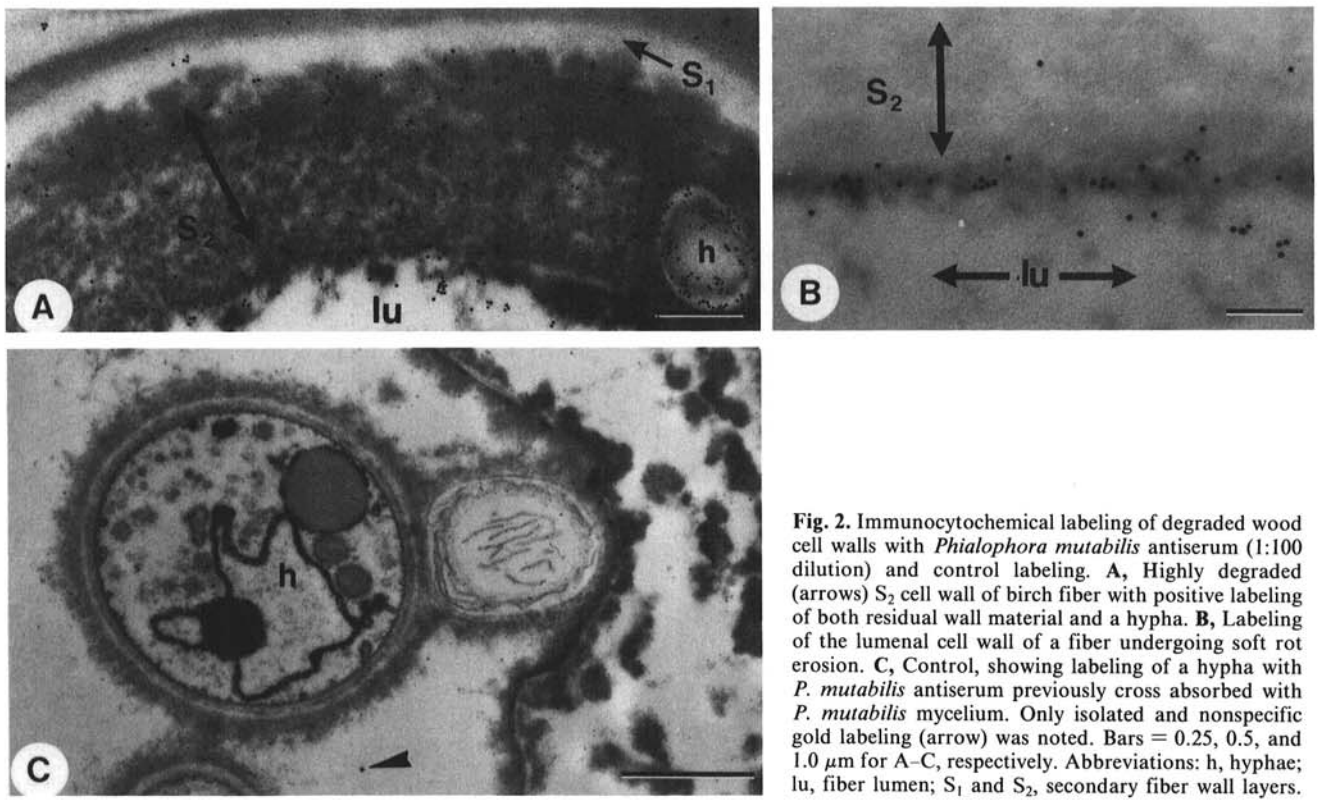

Fig. 2. Immunocytochemical labeling of degraded wood cell walls with *Phialophora mutabilis* antiserum (1:100 dilution) and control labeling. **A,** Highly degraded (arrows) S_2 cell wall of birch fiber with positive labeling of both residual wall material and a hypha. **B,** Labeling of the lumenal cell wall of a fiber undergoing soft rot erosion. **C,** Control, showing labeling of a hypha with *P. mutabilis* antiserum previously cross absorbed with *P. mutabilis* mycelium. Only isolated and nonspecific gold labeling (arrow) was noted. Bars = 0.25, 0.5, and 1.0 μm for A–C, respectively. Abbreviations: h, hyphae; lu, fiber lumen; S_1 and S_2, secondary fiber wall layers.

time and relative level of decay (expressed as percent dry weight loss). This trend was not observed with preservative-treated pine samples that gave significant ELISA readings despite having insignificant weight loss. Lack of degradation of the treated pine blocks was expected and is consistent with its known greater resistance compared with birch after preservative treatment. However, light microscopy and visual examination of these blocks decayed for 5 mo did show some colonization by hyphae and presence of spores that presumably were responsible for the strong ELISA reaction. This indicates that while the ELISA technique may be applicable for the detection of the fungus in wood, it may not give a true impression of the extent of decay.

ELISA performed on extracts from undegraded preservative-treated and nontreated wood samples gave weak but nonsignificant responses when 0.1 and 0.01 mg/ml of wood samples were used, although inhibitory responses were noted for 1 mg/1 ml samples. Jellison and Goodell (14) report similar observations for wood extracts and considered phenolic residues to be responsible for inhibitory cross-reactions. The effect of the preservative treatment, however, did not seem to cause a recognized increase in background readings (data not shown). This indicates that with appropriate sampling, the ELISA should prove a suitable bioassay method for detection of fungi in preservative-treated wood. A possible limitation of the ELISA technique with respect to quantification of decay is that the assay is primarily dependent on the amount of fungal biomass present in the degraded tissue. The immunogold cytochemical studies showed that both spores and dead hyphae cross-reacted with the antisera. Wood tissue colonized but not degraded (e.g., through heavy sporulation of the fungus), or in which the fungus has been active and then died (e.g., through toxic action of preservatives), also would be expected to give strong and positive ELISA results. The greatest possible use of the present immunoassay may therefore be in detecting the early occurrence of *Phialophora* species before, or at incipient stages of, decay in wood. In this respect, the method may have certain advantages because of its specificity and sensitivity over existing physical methods of decay analysis of treated wood that give no indication of the fungal flora present.

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