

Characterization of a *Pythium ultimum*-Specific Antigen and Factors That Affect Its Detection Using a Monoclonal Antibody

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University of Nebraska Agricultural Research Division Journal Series 10975.

We thank L. Lane for technical and scientific advice, M. Craig for technical assistance, and J. Martinez for assistance in photography.

F. Avila was supported in part by the Fundación Gran Mariscal de Ayacucho, Venezuela, and the Latin American Scholarship Program of American Universities.

Accepted for publication 21 July 1995.

ABSTRACT

Avila, F. J., Yuen, G. Y., and Klopfenstein, N. B. 1995. Characterization of a *Pythium ultimum*-specific antigen and factors that affect its detection using a monoclonal antibody. *Phytopathology* 85:1378-1387.

A *Pythium ultimum*-specific monoclonal antibody (MAB E5) recognized a 46-kDa protein band from *P. ultimum* in Western blots, but did not react with any protein from five other *Pythium* spp. Loss of MAB E5 reactivity to the antigen following treatment of the antigen with trifluoromethane sulfonic acid, periodate, or laminarinase indicated that the antigen was a glycoprotein with a carbohydrate epitope containing β -1,3 linkages. The antigen was thermostable. MAB E5 reactivity to mycelia and specific structures was affected by fungal age. The E5 antigen content in *P. ultimum* mycelia cultured in Czapek-Dox broth increased during the first 3 days, but subsequently declined despite continuing

fungal growth. The antigen was detected in all young *P. ultimum* structures by immunofluorescence, but fluorescence decreased or disappeared as the structures matured or became dormant. Fluorescence reappeared upon transferring aged structures to fresh nutrient solution. Reactivity of MAB E5 to the fungus also was influenced by culture nutrients. *P. ultimum* cultured in media containing different seed exudates or different sugars varied in E5 antigen content. *P. ultimum* could not be detected in infected sugar beet and common bean hypocotyls using enzyme-linked immunosorbent assay (ELISA). The fungus was detected in infected sugar beet hypocotyls by Western blot, but not in infected bean tissue.

Additional keywords: cell wall, immunoassay, oomycete, *Pythiaceae*, serology.

Pythium ultimum Trow typically coexists with other *Pythium* spp. in soil and roots (16,20,39), confounding its detection in these materials. Yuen et al. (38) investigated a *P. ultimum*-specific monoclonal antibody, MAB E5, as a method for identification, detection, and quantification of *P. ultimum*. The antigen recognized by MAB E5 was detected in mycelia and also in culture fluid, which allowed rapid and easy identification of *P. ultimum* in pure cultures by enzyme-linked immunosorbent assay (ELISA). MAB E5 did react with some isolates of other *Pythium* spp. in ELISA at very low levels. These reactions raised questions as to whether the antigen recognized by MAB E5 occurred exclusively in *P. ultimum* or occurred in *P. ultimum* at higher levels than in other *Pythium* spp. Use of MAB E5 for detection of *P. ultimum* in plant tissues was more problematic; the pathogen in highly infected root tissues was detected with MAB E5 using an antigen competition ELISA, but not when using standard ELISA.

Little is known about the properties of the E5 antigen that would serve in designing better in situ assays involving MAB E5. Such methods require that the fungal structures be detectable in different substrates at all stages of development. *P. ultimum* occurs in nature as sporangia, oospores, and mycelia of diverse age (34). The fungus grows in a wide variety of nutrient substrates, such as plant tissues (20) and exudates from seed and roots (1,22,24,25,26,28), and the composition of nutrients from these sources can vary depending on the plant species (28). *P. ultimum*

grown in Czapek-Dox broth was more reactive to MAB E5 than when it was grown in a corn meal-based medium (38), suggesting that the composition of the natural substrate may affect the detection of *P. ultimum* using MAB E5. Evidence that substrate composition affects serological quantification has been reported for *Trichoderma harzianum* (7).

We conducted this study to characterize the E5 antigen and to determine the factors that may affect the detection of *P. ultimum* by MAB E5. One objective was to determine the chemical composition and thermostability of the E5 antigen. Another objective was to use Western blot analysis in determining the presence of the E5 antigen in non-*P. ultimum* isolates with low ELISA reactivity and to test Western blot analysis as an alternative detection system for *P. ultimum* in plant tissue. A third objective was to determine if reactivity of *P. ultimum* to MAB E5 was influenced by fungal age, developmental stage, and nutrient composition of the substrate. Preliminary results have been published in abstract form (3,4).

MATERIALS AND METHODS

Fungal isolates. Isolates of *Pythium* spp. (Table 1) were obtained from the collection of G. Y. Yuen. P201 served as the immunogen to produce MAB E5 (38) and was the primary *P. ultimum* isolate used in the characterization experiments. P225, an asexual isolate of *P. ultimum* (identified on the basis of the diameter of sporangia, or hyphal swellings, and no mating with *P. sylvaticum* in dual culture), was used to produce cultures with sporangia only for immunofluorescence experiments. *P. irregulare* Buisman, isolate P205, served as the negative control in all ex-

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periments. Other *Pythium* spp. were tested for reactivity to MAb E5 by Western blot analysis because they exhibited low-level reactions with MAb E5 in ELISA (38). Unless otherwise specified, each isolate was cultured in 50 ml of Czapek-Dox broth for 3 days at 25°C to yield mycelia.

ELISA methods. E5 antigen in mycelia and culture fluids was quantified using a modification of the indirect ELISA system reported by Yuen et al. (38). Mycelial extracts were prepared as described (38) and aliquots (0.2 to 0.6 ml) were dried at 80°C for 12 h on aluminum foil sheets to determine biomass density (mg of dry weight/ml). Total mycelial mass within aliquots of mycelial extract was estimated by multiplying the density by the aliquot volume. Protein concentrations of mycelial extracts were determined using a commercial Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA). In most ELISA experiments, mycelial extracts were diluted to 2 µg/ml using phosphate-buffered saline (PBS) (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂KPO₄, and 1.7 mM KH₂PO₄ [pH 7.5]) prior to testing. Culture fluids were tested directly. A 70-µl aliquot of sample was coated on each of three wells in two microtiter plates. MAb E5 was used at a 1:30,000 dilution, and goat anti-mouse IgG alkaline phosphatase conjugate (Cappel, Durham, NC) was used at a dilution of 1:5,000.

Optical density (OD) measurements from the three wells in a plate were averaged before statistical analysis. In some experiments in which mycelial extract samples of different mass were tested, ELISA results for mycelia were expressed as the ratio of OD to milligrams of mycelial dry weight in the 70-µl aliquot. ELISA results for culture fluids were expressed as unadjusted OD values. Dunnett's test was used to compare specific treatments with the control. Otherwise, Tukey's test was used for mean separation. Experiments were conducted twice with six replications per treatment. All statistical procedures were performed using Statistical Analysis Software (SAS Institute, Inc., Cary, NC). Results from repeated experiments were pooled when heterogeneity of variance was not indicated.

Western blot analysis. Mycelial or plant extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). Extracts for Western blots were prepared by freezing mycelia or plant tissues in liquid nitrogen and grinding them with a mortar and pestle. The ground material was then suspended in 0.5 ml of grinding buffer (30 mM Tris [pH 7.7], 5 mM CaCl₂, 5 mM MgCl₂, 15 mM β-mercaptoethanol, 1 mM phenyl-methyl sulfonyl fluoride, and 1% Tween 20). Protein concentrations of the extracts were adjusted to 3 mg/ml by dilution with the grinding buffer. Thirty-microliter aliquots of the extracts were mixed with equal volumes of 2× loading buffer (1 M Tris [pH 7.7], 10% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromophenol blue) (27). The mixtures were boiled for 5 min and centrifuged at 2,500 × g for 5 min at 25°C to remove undissolved debris, and then 20 µl of the supernatant (30 µg of total protein) was used in electrophoresis. Electrophoresis was performed in 1-mm-thick minigels using glycine SDS buffer (5 mM Tris, 38.6 mM glycine, and 0.02% SDS at pH 7.8 to 8.4) for 45 to 60 min at a constant current of 35 mA. Two protein gels were run in parallel for mycelial extracts. One gel was stained with silver for total protein and the other used in Western blot analysis. Proteins were electrotransferred from the unstained gel to a nitrocellulose membrane using 12 mM Tris (pH 8.3) with 96 mM glycine and 20% methanol at a constant voltage of 25 mV for 1.75 h. After blotting, the membranes were rinsed with deionized water, dried at 25°C for 12 to 24 h, and blocked for 12 to 24 h with a fresh blocking solution consisting of 5% nonfat dry milk in PBS. Blocked membranes were then incubated in a sealed plastic bag with 10 ml of MAb E5 solution, containing 10 µg/ml of total protein in the blocking solution, for 2 to 12 h at 25°C with constant agitation. The membranes were subjected to three 5-min washes with PBS and two 5-min washes with Tris-buffered

saline (TBS) (136.9 mM NaCl and 24.8 mM Tris [pH 8.5]). After washing, membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel) diluted in TBS with 3% bovine serum albumin (BSA) at 25°C for 2 to 12 h. Unbound antibody conjugate was removed from the membranes by three 5-min washes with TBS and one wash of 5 min with alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris [pH 9.5]). Binding of MAb E5 was detected using bromochloroindolyl phosphate and nitro blue tetrazolium (BCIP/NBT) as described in Harlow and Lane (19).

Immunofluorescence microscopy. Immunofluorescence microscopy was used to determine the reactivity of specific fungal structures with MAb E5. Mycelia collected from liquid cultures were washed gently with distilled water and then incubated in a fixing solution for 90 min at 25°C or overnight at 4°C. The fixing solution consisted of 1.5 ml of 4% paraformaldehyde in 0.15 M K₂HPO₄, adjusted to pH 6.5 with citric acid crystals. The fixed mycelia were washed three times with 0.1 M potassium phosphate (pH 6.5) and incubated for 90 min in a 1:1,000 dilution of MAb E5 in PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂KPO₄, and 1.7 mM KH₂PO₄ [pH 7.5]) containing 3% BSA. Subsequent procedures were conducted in the dark. Following three washes with PBS, the mycelia were incubated for 1 h with fluorescein-conjugated, affinity-purified antimouse-antibody from goat (whole molecule) (Cappel) diluted 1:200 in PBS with 3% BSA. The conjugate was removed by three washes with PBS, and a small sample of the stained mycelia was suspended in a drop of Gelvatol (19) on a clean microscope slide and covered with a cover slip. After the Gelvatol dried (3 to 12 h), the preparation was observed under epifluorescence illumination at 450 to 490 nm using a Nikon Optiphot microscope (Nikon, Inc., Melville, NY). Detection of E5 antigen was based on the presence of bright green fluorescence from fluorescein as opposed to yellow from autofluorescence. Photographs were taken with exposure times kept the same across different samples within an experiment to permit comparison of fluorescence intensity. Controls in the experiments consisted of *P. irregulare* treated with MAb E5, the two isolates of *P. ultimum* treated with a non-*P. ultimum* mouse monoclonal antibody, and all isolates treated with MAb E5 but without the fluorescein-conjugated antibody.

Antigen characterization. To characterize the E5 antigen, three types of degradation treatments were applied to mycelial extracts: chemical, enzymatic, and heat. Chemical and enzymatic treatments were used to determine the involvement of carbohydrate groups in the epitope. The chemical treatments were with trifluoromethane sulfonic acid (TFMSA) and sodium periodate. TFMSA hydrolyzes glycosidic bonds without hydrolyzing the

TABLE 1. Isolates of *Pythium* spp. tested in this study

<i>Pythium</i> species	Isolate ^a	Host/location ^b
<i>P. aphanidermatum</i> (Edson) Fitzp.	P330	Kentucky bluegrass/NE
<i>P. irregulare</i> Buisman	P36	Strawberry/CA
	P126	Corn/NE
	P205	Soil/NE
<i>P. mamillatum</i> Meurs	P12	Soil/CA
	P318	Rhododendron/CA
<i>P. spinosum</i> Sawada	P247	Soil/FL
<i>P. sylvaticum</i> Campbell & Hendrix	P204	Soil/TN
<i>P. ultimum</i> Trow	P161, P222	Sugar beet/CA
	P201 ^c	Strawberry/CA
	P202	Common bean/NE
	P221	Corn/NE
	P225, P226	Soil/NE

^a *P. mamillatum* P318 = isolate 72-14 from J. G. Hancock, University of California, Berkeley. *P. spinosum* P247 = isolate 79-4 from F. N. Martin, University of Florida. *P. sylvaticum* P204 = American Type Culture Collection isolate 18196.

^b CA = California; FL = Florida; NE = Nebraska; TN = Tennessee.

^c *P. ultimum* isolate used as the immunogen to produce MAb E5 (38).

peptide backbone (31). The TFMSA treatment used was a modification of the method described by Sojar and Bahl (31). Mycelial extracts (four samples) containing 100 μ g of total protein were dispensed into glass tubes, vacuum dried for 15 to 30 min, and placed on ice. One sample served as the untreated control. The other three samples were treated with 50 μ l of TFMSA along with 0, 10, or 25 μ l of anisole, and incubated on ice for 3 h. TFMSA was neutralized with 125 μ l of *n*-ethylmorpholine, after which proteins were precipitated by adding 5 to 10 volumes of acetone to the tubes, covering with Parafilm, and mixing. Tubes were stored for 12 h at -20°C and then centrifuged for 10 min at $3,500 \times g$. After the supernatant was discarded, the pellet was dried under vacuum and suspended in 30 μ l of grinding buffer. The protein solution was prepared for SDS-PAGE as described above.

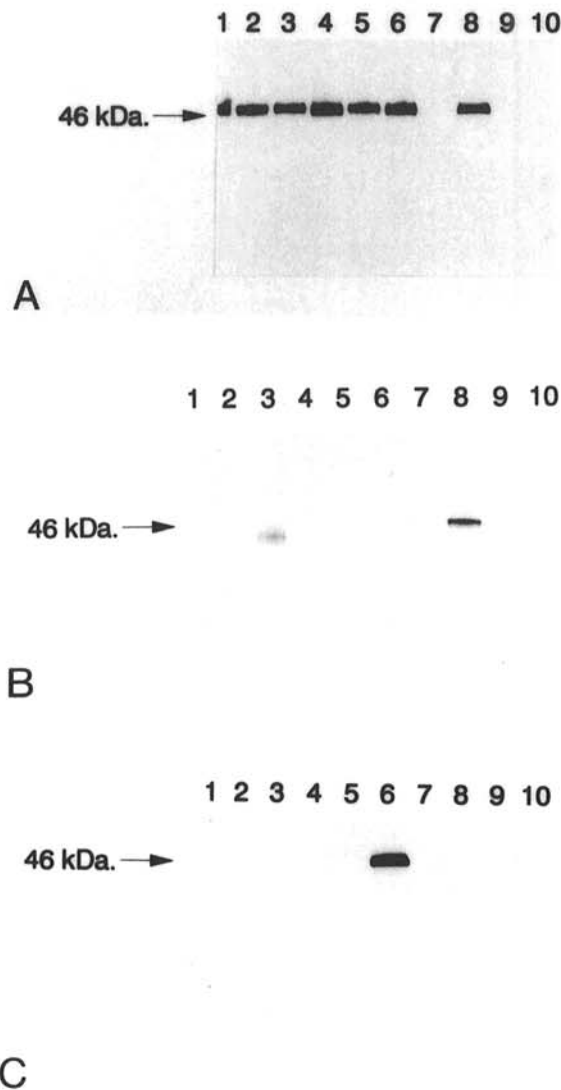


Fig. 1. MAb E5-probed Western blots of mycelial extracts of *Pythium ultimum* and other *Pythium* spp. **A**, Seven isolates of *P. ultimum*: P161 (lane 1), P226 (lane 2), P225 (lane 3), P202 (lane 4), P222 (lane 5), P221 (lane 6), P201 (lane 8); two isolates of *P. irregulare*: P36 (lane 7) and P205 (lane 9); and molecular weight standards (lane 10). **B**, Different *Pythium* spp.: *P. irregulare* P205 (lane 1), *P. aphanidermatum* P330 (lane 2), *P. ultimum* P201 (lane 3), *P. spinosum* P247 (lane 4), *P. mamillatum* P12 and P318 (lanes 5 and 6), *P. sylvaticum* P204 (lane 7), *P. ultimum* P201 (lane 8), *P. irregulare* P126 (lane 9), and molecular weight standards (lane 10). **C**, Effects of protein deglycosylation with trifluoromethane sulfonic acid (TFMSA): *P. ultimum* P201 with no treatment (lane 6); P201 treated with 50 μ l of TFMSA (lane 7); P201 treated with 50 μ l of TFMSA + 10 μ l of anisole (lane 8); P201 treated with 50 μ l of TFMSA + 25 μ l of anisole (lane 9); *P. irregulare* P205 treated with TFMSA and anisole in the same concentrations applied to *P. ultimum* (lanes 2, 3, 4, and 5); molecular weight standards (lanes 1 and 10). Molecular weight standards are not visible in these photographs.

Periodate oxidation cleaves α -glycol groups (15). For the periodate treatment, polystyrene microtiter plates were coated with mycelial extracts from *P. ultimum* (P201) or *P. irregulare* (P205), and blocked as described for ELISA (38). Bound antigens were treated for 2 h with 100 μ l of sodium meta-periodate solution at 20 and 40 mg/ml in 50 mM sodium acetate (pH 4.5) at 4°C (36). To stop the reaction, plates were drained, rinsed three times with washing solution (0.1% Triton 100 in PBS), and rinsed 10 times with tap water. Antigen reactivity was evaluated as described for ELISA.

Enzymatic treatments were performed with laminarinase and cellulase from *Trichoderma* (Sigma Chemical Co., St. Louis). Laminarinase liberates glucose monomers from β -1,3-linked glucose residues (23) and cellulase primarily breaks down β -1,4-linked glucose residues (30). Antigen-coated microtiter plates were incubated with 100 μ l of laminarinase or cellulase solution per well. Treatments with laminarinase were at 37°C for 24 h with 1.25 to 10 enzyme units/ml suspended in 50 mM sodium acetate (pH 5). Cellulase treatments were at 37°C for 4 h with 1.25 to 10 enzyme units/ml suspended in 0.2 mM sodium acetate (pH 5). To stop enzyme reactions, plates were drained, washed three times with washing solution, and rinsed 10 times with tap water. Reactivities of the treated antigens were then determined by ELISA.

Reduction of reactivity in ELISA after laminarinase treatment suggested that the epitope could be a carbohydrate composed of β -1,3-linked glucose residues. Therefore, a competition assay was performed with laminarin, a carbohydrate with a high content of β -1,3-linked glucose residues, to determine if β -1,3-linked glucose residues compete with the *P. ultimum* antigen for binding with MAb E5. MAb E5 at 1:30,000 dilution was mixed with laminarin at 50, 100, 500, and 1,000 μ g/ml and incubated for 2 h at 37°C . Seventy-microliter volumes of MAb E5 and laminarin mixture were applied to the wells of antigen-coated microtiter plates and further incubated at 37°C for 90 min. Treatments with MAb E5 solution without laminarin and with a solution containing only laminarin were used as controls. The effect of competition by laminarin on MAb E5 binding was determined following previously described ELISA methods.

Heat treatments were performed to determine thermostability of the E5 antigen. Samples of *P. ultimum* and *P. irregulare* mycelial extracts containing 20 μ g of protein/ml were placed in microcentrifuge tubes, boiled in a steam bath for periods of 10 to 120 min, and cooled to 25°C . Boiled extracts were coated in microtiter plates and reactivity with MAb E5 was determined by ELISA.

Age and fungal development. The effects of culture age on antigen production were determined on *P. ultimum* and *P. irregulare* isolates grown in Czapek-Dox broth. Growth was stopped after 1, 3, 5, and 7 days by adding 0.5 ml of 10% sodium azide. ELISA was then performed on mycelial extracts and culture fluids.

The reactivity of different developmental stages of *P. ultimum* with MAb E5 was determined by immunofluorescence. Isolates of *P. ultimum* (P201 and P225) and *P. irregulare* P205 were grown in flooded lima bean agar media (8). MAb E5 reactivity with sporangia, oogonia, antheridia, and oospores was studied with isolate P201; whereas, MAb E5 reactivity during sporangial maturation and germination was assessed with isolate P225, which produces only sporangia. At different developmental stages (3 to 5 days for sporangial formation, 5 to 10 days for oogonia and oospore formation, and 60 or more days for aged mycelia with dormant reproductive structures), mycelia growing in the liquid were collected and prepared for immunofluorescence. Aged mycelia with dormant structures also were exposed to fresh Czapek-Dox broth to stimulate hyphal growth and germination of sporangia and oospores. Mycelial samples were collected 1, 2, and 3 h after treatments with fresh broth and examined by immunostaining for reactivity to MAb E5.

Nutrient composition. Seed exudates and carbohydrate composition were investigated separately as factors that may affect E5

antigen production. The effects of seed exudates were investigated by growing fungi in variations of Czapek-Dox broth in which exudates from seeds of soybean (*Glycine max* (L.) Merr.), sugar beet (*Beta vulgaris* L.), common bean (*Phaseolus vulgaris* L.), and pea (*Pisum sativa* L.) were substituted for sucrose. Exudates were obtained from 100-g seed samples, which had been surface-disinfested by immersion in 10% commercial bleach (5.25% sodium hypochlorite) for 2 min and rinsed with sterile water. Seeds were then soaked in 100 ml of sterile, deionized water at 25°C for 24 h with occasional shaking. The liquid was aseptically filtered through Whatman No. 1 paper, sterilized by filtration through 0.2- μ m membranes, and tested for carbohydrate concentrations with the anthrone reagent (21). Mineral components of Czapek-Dox broth were prepared separately as concentrated (10 \times) stock solutions and then filter sterilized. Seed exudates and mineral solutions were mixed with sterile, deionized water to form media containing 0.1 g of carbohydrate per liter. Czapek-Dox broth containing sucrose at 0.1 g/liter was used for comparison, and a broth containing no carbohydrate served as a negative control.

The effect of carbon source on antigen production was determined by culturing the isolates in Czapek-Dox broth containing sucrose or in the same medium but in which fructose, glucose, maltose, mannose, galactose, ribose, or xylose was substituted for sucrose. Sugar solutions were prepared as 20 g/liter stock solutions and then filter sterilized. Broths containing 2 g of carbohydrates per liter were prepared as described for the seed exudate.

In both experiments, ELISA was performed on mycelial extracts and culture fluids after 3 days of fungal growth. Each experiment was performed twice with six replications per treatment. Analysis of variance was performed on data from each experiment separately; results from repeated experiments were pooled when heterogeneity of variance was not indicated. Tukey's test was used for mean separation.

Assays for *P. ultimum* in plant materials. Western blot analysis and ELISA using MAb E5 were compared for effectiveness in detecting *P. ultimum* in infected plant materials. Seeds of sugar beet and common bean were surface disinfested by immersion in 10% commercial bleach (5.25% sodium hypochlorite) for 2 min followed by several rinses with sterile, distilled water. The seeds were germinated for 2 to 3 days in sterile petri plates containing moist filter papers. Seedlings were transferred to the periphery of 2-day-old cultures of *P. ultimum* and *P. irregulare* on water agar. Seedlings with rotted hypocotyls were removed from the petri plates 2 to 3 days after inoculation, and the hypocotyls were excised from the seeds. Five bean hypocotyls and up to 50

sugar beet hypocotyls were pooled into individual samples. For each treatment there were three samples. Extracts of the hypocotyls were prepared as described for mycelia and tested by Western blot and ELISA procedures for the presence of *P. ultimum*, with *P. ultimum* and *P. irregulare* mycelial extracts as positive and negative controls. Seedlings transferred to water agar were used as another negative control. Heating was tested for its effects on reactivity of MAb E5 to *P. ultimum* in infected plant material in ELISA. Extracts of sugar beet and common bean hypocotyls infected with *P. ultimum* or *P. irregulare* were heated for 15 min. ELISA was then performed on heated and nonheated hypocotyls extracts. Heated and nonheated mycelial extracts of *P. ultimum* and *P. irregulare* were used as controls.

RESULTS

Antigen specificity and composition. MAb E5 recognized a 46-kDa band in all seven *P. ultimum* isolates tested in Western blot analysis (Fig. 1A). The antibody did not react with extracts of isolates of *P. aphanidermatum*, *P. irregulare*, *P. mamillatum*, *P. spinosum*, or *P. sylvaticum* (Figs. 1A and B) that previously exhibited low-level reactivity with MAb E5 in ELISA (38). A 46-kDa band was present in all *Pythium* spp. tested in parallel silver-stained SDS-PAGE gels (data not shown).

Deglycosylation of *P. ultimum* mycelial extracts by TFMSA treatment resulted in a loss of reactivity of MAb E5 with the 46-kDa band (Fig. 1C). Antigen reactivity with MAb E5 in ELISA was reduced significantly by all concentrations of periodate ($D = 0.26$) (Fig. 2) and laminarinase ($D = 0.47$) (Fig. 3) according to Dunnett's test. Laminarin applied in a competition assay at 1,000 μ g/ml did not affect the reactivity of MAb E5 in ELISA (data not

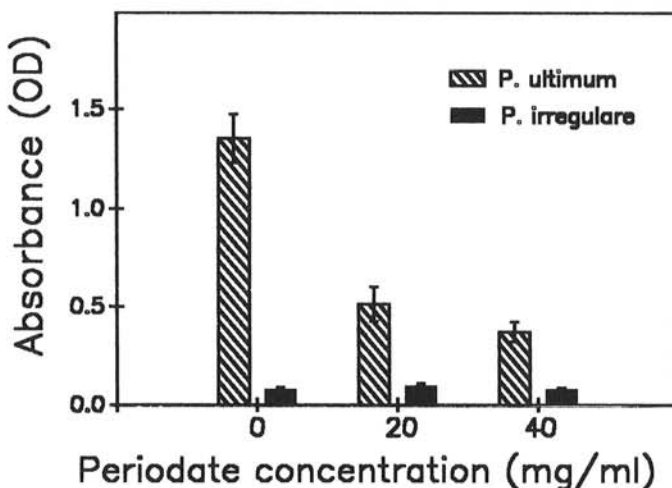


Fig. 2. Effects of periodate treatments on the reactivity of *Pythium ultimum* P201 and *P. irregulare* P205 mycelial extracts with MAb E5. Values are the means of six replications in two experiments. Bars represent the standard error.

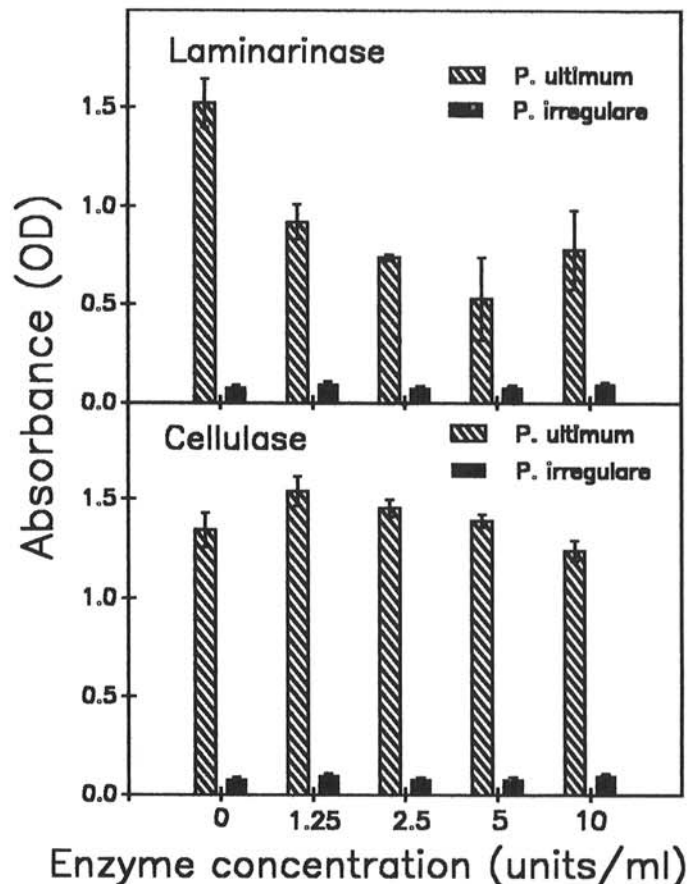


Fig. 3. Effects of laminarinase and cellulase treatments on the reactivity of *Pythium ultimum* P201 and *P. irregulare* P205 mycelial extracts with MAb E5. Values are the means of six replications in two experiments. Bars represent the standard error.

shown). The reactivity of the antigen to MAb E5 was undiminished after cellulase treatment (Fig. 3).

The antigen was found to be heat resistant. When *P. ultimum* extracts were boiled for 10 to 40 min, ELISA readings increased over that of nontreated *P. ultimum* extracts. The highest ELISA readings were observed in extracts boiled for 10 to 20 min; however, further boiling for 50 min to 2 h eliminated extract reactivity (Fig. 4).

Effects of culture age and developmental stages. Antigen content in *P. ultimum* changed with culture age, but was not directly proportional to biomass (Fig. 5). The ratio of absorbance to biomass (OD/mg of dry weight) increased in *P. ultimum* mycelia until the third day of fungal growth, after which the ratio decreased. The absorbance to biomass ratio in mycelia cultured for 5 days was half that of 3-day-old mycelia, and after 7 days it was similar to that of the first day. In the culture filtrates, the antigen concentration increased for 5 days and then no further change in absorbance was detected. Total fungal biomass increased throughout the 7 days of culture, following an exponential growth curve. ELISA readings of mycelial extracts and culture filtrates of *P. irregulare* were between 0.05 to 0.09 OD; whereas, biomass development of *P. irregulare* was similar to that of *P. ultimum* (data not shown).

Intensity of green fluorescence upon immunostaining of *P. ultimum* with MAb E5 varied with the stage of development. Young mycelia were highly reactive with green fluorescence being concentrated in the cell walls (Fig. 6A). Fluorescence levels were also high in young sporangia (Fig. 6B). Intensity of green fluorescence in oogonia, antheridia, and young oospores was less than in young sporangia or actively growing hyphae (results not shown). Green fluorescence decreased in hyphae as the mycelia aged and was not detected in mature sporangia (Fig. 6C and D). Mature oospores exhibited yellow autofluorescence, but not the bright green coloration due to fluorescein (results not shown). Mature oospores in the negative control samples also were highly autofluorescent, but all other structures exhibited little or no autofluorescence.

The antigen became detectable again in hyphal tips within 1 h after aged mycelia were transferred to fresh Czapek-Dox broth (Fig. 6E). Green fluorescence also reappeared in sporangia within 40 min after exposure to fresh broth. Green fluorescence was apparent in the sporangial cell walls before germ tube emergence and intensified during germination (Fig. 6F). Following treatment of mature oospores with fresh broth, green fluorescence could be discerned in some oospores despite autofluorescence, but the timing of the reappearance of green fluorescence could not be

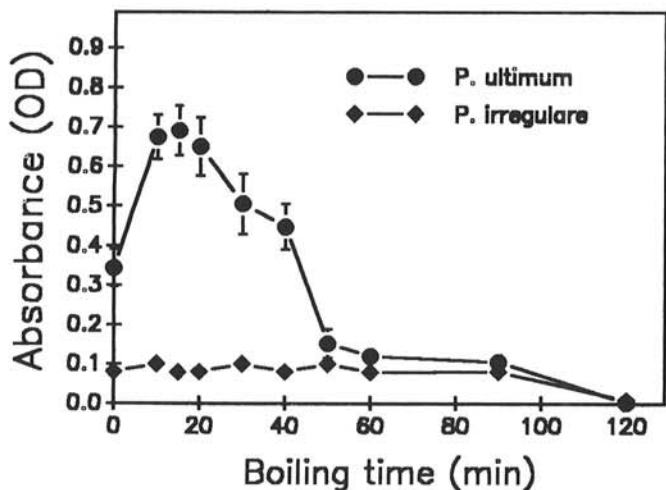


Fig. 4. Heat effects on the reactivity of *Pythium ultimum* P201 mycelial extracts with MAb E5. Values are the means of six replications in two experiments. Bars represent the standard error.

determined clearly (results not shown). The negative controls did not exhibit green fluorescence.

Effects of nutrient composition. Amounts of E5 antigen detected by ELISA varied among cultures of *P. ultimum* grown in exudates from different seeds (Fig. 7). There were statistical differences ($P = 0.05$) in the absorbance to biomass ratios of mycelial extracts among the different exudates. Media containing sugar beet seed exudates, pea seed exudates, or sucrose yielded higher amounts of antigen in mycelia than media with soybean or common bean exudates. The highest E5 antigen concentration in the culture fluid and the greatest amount of *P. ultimum* growth were measured in the soybean seed exudate medium. While *P. ultimum* grew to similar levels in exudates from sugar beet, pea, and common bean, as well as in sucrose, concentrations of E5 antigen in

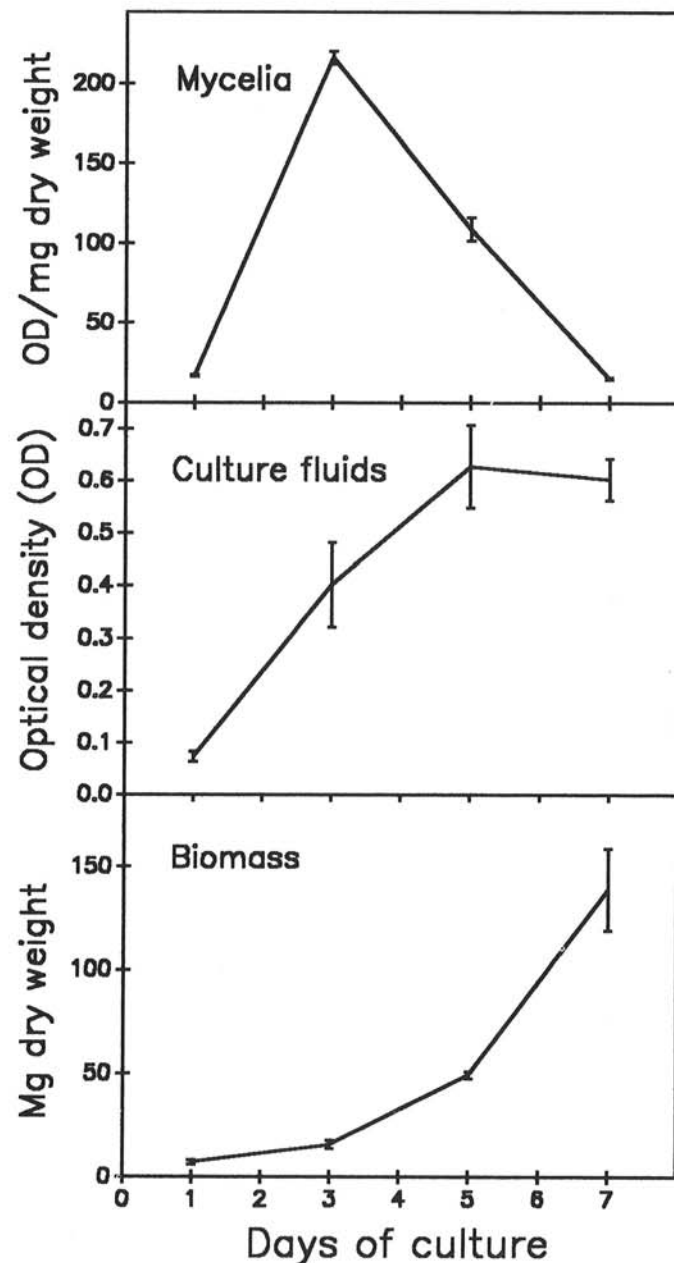


Fig. 5. Effects of culture age on E5 antigen content in *Pythium ultimum* P201 mycelial extracts and culture fluids, and on the growth of P201 in Czapek-Dox broth. The antigen content in mycelial extracts was expressed as the ratio of absorbance to mycelial mass (optical density [OD]/mg of dry weight of mycelia) to correct for differences in biomass among the samples. The antigen content in culture fluids was expressed as unadjusted absorbance (OD). Values are the means of two experiments with six replications each. Bars represent the standard error.

the culture fluids were significantly different among these cultures.

The type of sugar in the medium affected E5 antigen levels in mycelial extracts and culture fluids. The antigen content in mycelial extracts, expressed as absorbance per milligrams of biomass, was highest in the medium containing sucrose and lowest in media with galactose, xylose, or ribose (Fig. 8). Glucose was the best carbohydrate substrate for antigen accumulation in culture fluids, whereas, galactose, mannose, xylose, and ribose produced the lowest amounts of antigen. Antigen concentrations detected in culture fluids were related to growth of *P. ultimum*, except that moderate growth was exhibited in mannose, whereas, little E5 antigen was detected in the mannose culture fluid.

Detection of *P. ultimum* in inoculated hypocotyls. *P. ultimum* could not be detected in hypocotyls of inoculated sugar beet and bean by MAb E5 in ELISA. Absorbance values of hypocotyl extracts from seedlings infected with *P. ultimum* or *P. irregulare* and of extracts from noninoculated seedlings were all less than 0.2 (data not shown). Heating of hypocotyl extracts did not improve detection of *P. ultimum* over corresponding unheated samples (data not shown).

Using Western blot analysis, the 46-kDa band corresponding to E5 antigen was detected in the same extracts of inoculated sugar beet hypocotyls used for ELISA, but not in noninoculated hypocotyls or those inoculated with *P. irregulare* (Fig. 9). The 46-kDa band could not be detected in *P. ultimum*-inoculated bean hypo-

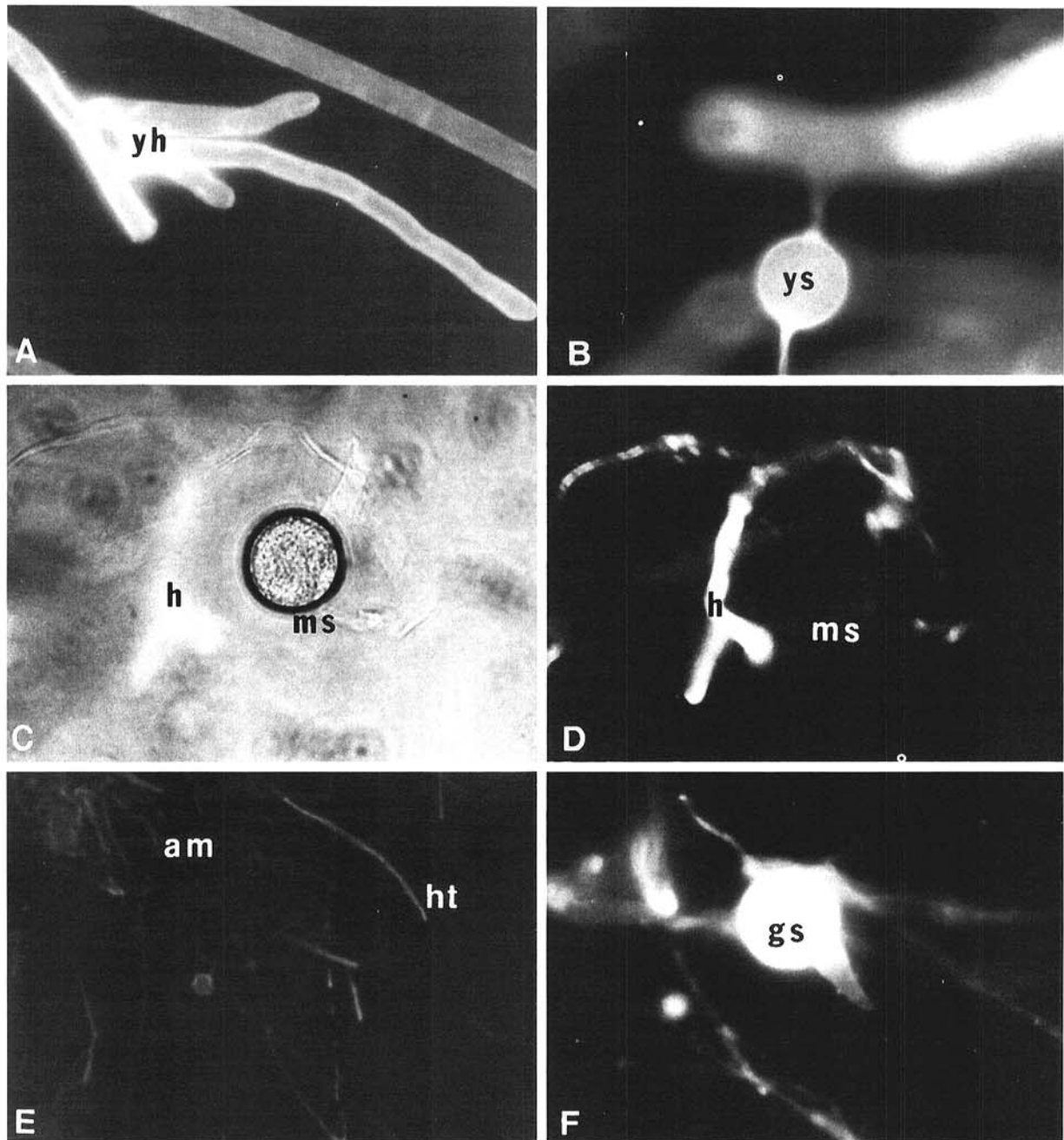


Fig. 6. Fluorescence in various developmental stages of *Pythium ultimum* P225 following immunostaining with antibody E5. **A**, Young hyphae (yh). **B**, Young sporangium (ys). **C**, Mature sporangium (ms) and hyphae (h) under epifluorescence and bright field illumination. **D**, Mature sporangium (ms) and hyphae (h) in the same field as Fig. 6C, but under epifluorescence illumination only. **E**, Aged mycelium (am), hyphal tips (ht), and mature sporangia (ms) following exposure to fresh Czapek-Dox broth for 2 h. **F**, Germinating mature sporangium (gs) with germ tube (gt) after exposure to fresh Czapek-Dox broth for 2 h. Figures 6A and 6E are at 1200X and 120X magnification, respectively; all other figures are at 500X magnification.

cotyls, although the same amount of total protein was assayed in all samples.

DISCUSSION

The exclusive reactivity of MAb E5 to the 46-kDa band from *P. ultimum* in Western blots confirmed the specificity of the MAb E5 to *P. ultimum* reported by Yuen et al. (38). It also indicated that low ELISA values previously observed with non-*P. ultimum* isolates could have been attributed to nonspecific background, rather than the presence of the E5 antigen. Protein bands of 46 kDa were found in all of the other *Pythium* spp. tested in this study by electrophoresis. The nonreactivity of MAb E5 to the heterologous

46-kDa proteins was perhaps attributable to differences in conformation from the E5 epitope or to variations in composition that were not discernible on the basis of size. Other *P. ultimum* MAbs (A3, A4, and X4) that were produced in conjunction with MAb E5 cross-reacted with other *Pythium* spp. in ELISA (38) and also recognized the 46-kDa *P. ultimum* band in Western blots (2), suggesting that MAbs A3, A4, and X4 recognized different epitopes on the same antigen as MAb E5. Although the relationship between the cross-reactivity of A3, A4, and X4 and 46-kDa proteins in other *Pythium* spp. has not been determined, it is conceivable that the species-specificity of MAb E5 is due to its recognition of a unique epitope on a protein that is otherwise common across a number of *Pythium* spp. MAbs specific to *Phytophthora cinnam-*

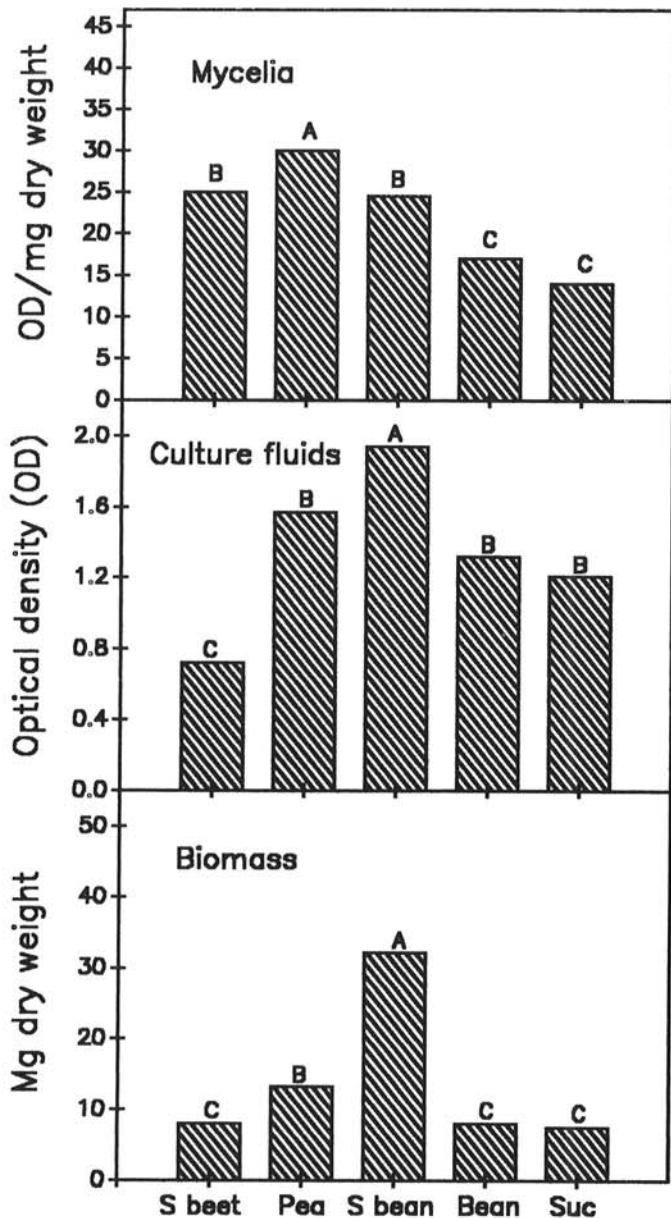


Fig. 7. Effects of seed exudates on E5 antigen content in *Pythium ultimum* P201 mycelia and culture fluids, and on the growth of P201. Media containing the mineral salts of Czapek-Dox broth were amended with seed exudates from sugar beet (S beet), pea, soybean (S bean), and common bean (bean). A medium containing sucrose (Suc) was used to compare with exudate treatments. The carbohydrate concentration in each medium was standardized to 0.1 g/liter. The antigen content in mycelia was expressed as the ratio of absorbance to mycelial mass (optical density/mg of dry weight of mycelia) to correct for differences in biomass among samples. Values are the means of two experiments with six replications each. Treatments in each graph with different letters are significantly different ($P = 0.05$) according to Tukey's test.

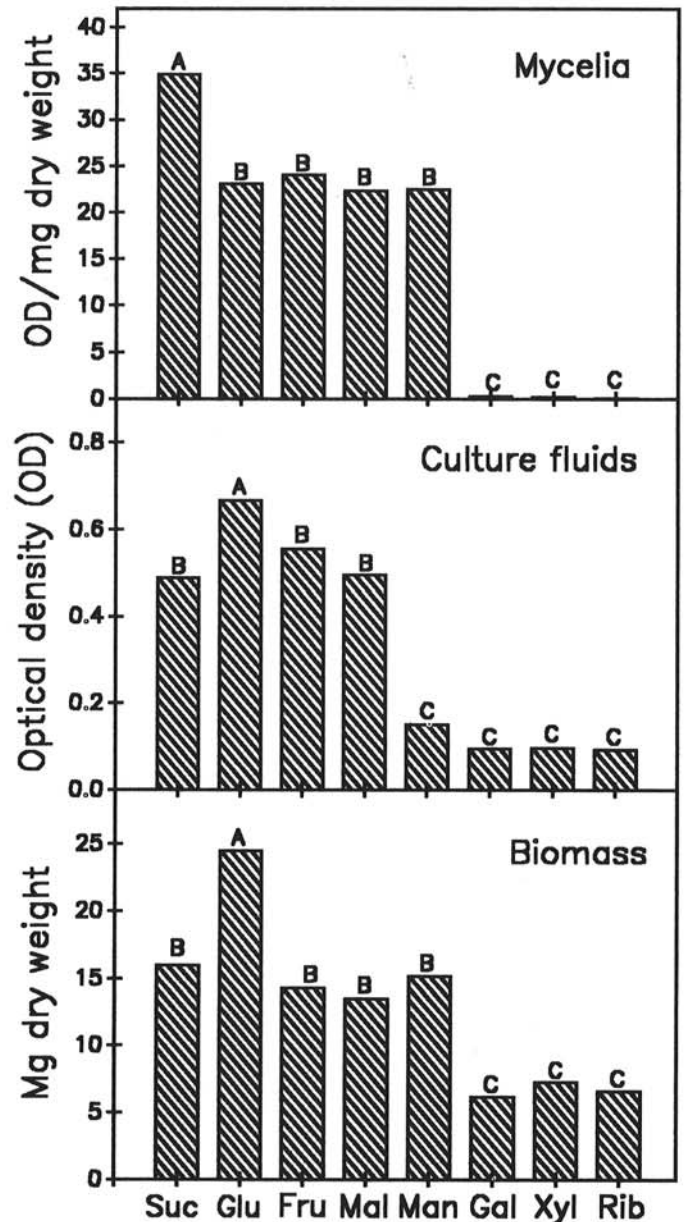


Fig. 8. Effects of different carbohydrates on E5 antigen content in *Pythium ultimum* P201 mycelia and culture fluids, and on the growth of P201. Media containing the mineral salts of Czapek-Dox broth were amended with sucrose (Suc), glucose (Glu), fructose (Fru), maltose (Mal), mannose (Man), galactose (Gal), xylose (Xyl), or ribose (Rib) to a carbohydrate concentration of 0.1 g/liter. The antigen content in mycelia was expressed as the ratio of absorbance to mycelial mass (optical density/mg of dry weight of mycelia) to correct for differences in biomass among samples. Values are the means of two experiments with six replications each. Treatments in each graph with different letters are significantly different ($P = 0.05$) according to Tukey's test.

moni (18) and *P. aphanidermatum* (10) were produced using zoospores as immunogens, but MAbs produced to mycelia of oomycete species other than *P. ultimum* have not proven to be species-specific (13,35).

The loss of the antigen band in the Western blot after TFMSA treatment and the decrease of MAb E5 reactivity in ELISA after periodate treatment indicated that the E5 antigen was a glycoprotein and that a carbohydrate was involved in the epitope. This conclusion was supported by the thermostability of the E5 antigen. The increase in reactivity after brief boiling may have been due to the facilitated binding of MAb E5 with the epitope after partial denaturation of the glycoprotein. The reduction in MAb E5 reactivity to the antigen by laminarinase treatment, but not by competition with laminarin, suggested that although the epitope contained β -1,3 linkages, it was not composed of simple β -1,3-linked glucose residues. The possibilities existed that more complex β -1,3-linked glucose residues were involved in the epitope or that the epitope was attached to the antigen molecule by β -1,3 linkages. Because reactivity did not change after cellulase treatment, it was unlikely that β -1,4 linkages were involved in the epitope.

The E5 antigen shares characteristics that are common to other oomycete MAb systems (9,11,14,35,36,37) in that it is a glycoprotein with the epitope in the carbohydrate portion. Antigens have been characterized for nine MAbs produced to zoospore cell-surface components of *P. aphanidermatum* (9,11) and *P. cinnamomi* (14) and to cell wall/membrane preparations of *P. sulcatum* and *P. violae* (35). Of these, seven recognized glycoproteins; the remaining antibodies recognized a protein (9) and a polysaccharide (35). Carbohydrate epitopes also were found for most of these MAbs and for most of the MAbs in a library of 44 produced against extracellular glycoproteins and mycelial walls of *P. megasperma* f. sp. *glycinea* (36,37). Two antibodies from the library are highly specific to different polysaccharide epitopes comprised of β -1,3-linked glucans. It would appear from these collective studies that carbohydrate epitopes associated with glycoproteins in the oomycetes are highly immunogenic. Wycoff et al. (37) suggested that MAbs to *P. megasperma* f. sp. *glycinea* are predominantly carbohydrate-specific because of the abundance of carbohydrates. While cell walls of oomycetes consist of 80 to 90% carbohydrate composed primarily of glucose monomers (5), glycoproteins are a minor component of the cell wall as compared to structural carbohydrates and, therefore, the glycosidic portions that potentially could be epitopes are even less abundant. The immunogenicity of the carbohydrate epitopes is perhaps more related to the physiological role of glycoprotein antigens. Glycoproteins are recognized as important antigenic components for fungi in general (29). Glycoprotein antigens to oomycete antibodies have been associated with host recognition by zoospores (11,17) and attachment of cysts to root surfaces (9,14). Analysis of glycoproteins from several *Pythium* spp. by Takenaka and Kawasaki (33) revealed a similar composition between the fungal compounds and arabinogalactan proteins in higher plants, and the authors suggested a role homologous to cell-cell recognition and plant defense for the fungal glycoproteins.

Another similarity that MAb E5 shares with some of the other oomycete antibodies is the mobility of its antigen. Although MAb E5 was produced to a mycelial cell wall preparation, the antigen was present in the mycelium only temporarily before it was excreted or released into the surrounding medium. While in the mycelium, it was localized in the cell wall, as indicated by a loss of reactivity to MAb E5 in immunofluorescence following treatment of *P. ultimum* hyphae with driselase (3). MAbs produced to purified cell wall preparations or extracellular glycoproteins from *P. megasperma* f. sp. *glycinea* react with both extracellular and cell wall-bound antigens (36,37).

From the collective information from this and other studies on antibodies to oomycetes, several generalizations regarding the

production of species-specific antibodies are apparent. First, cell wall or cell surface components are likely the most immunogenic and may yield the desired specificity. Even if antibodies to soluble antigens are desired, cell wall antigens probably will be detectable in extracellular fluids also. Second, because specificity is likely to be found in cell wall glycoproteins, it would be advantageous to extract and purify these compounds for use as an immunogen. Finally, polyclonal antibodies should exhibit species cross-reactivity even if the antibodies are to be made to purified species-specific glycoprotein antigens, as was reported for a polyclonal antibody produced to a *P. iwayamai*-specific glycoprotein (33). Carbohydrate epitopes are commonly shared and can contribute to cross-reactivity (12). While the specificity of polyclonal antibodies can be increased by cross-absorption, MAbs are the best solution for producing antibodies that recognize only species-specific epitopes.

Although the production of species-specific MAbs for oomycetes appears to be facile, development of the antibodies into tools for detecting and quantifying fungi in vivo is a challenge. Difficulties in ELISA detection of *P. ultimum* in association with plant tissues were encountered previously in tests of infected sugar beet roots (38) and in this study. The detection problems may be related to plant compounds that interfere with the absorption of E5 antigen on the wells of the ELISA plates or with the antigen-antibody reaction. It also is conceivable that, because the E5 antigen is a glycoprotein, plant glycoproteins in natural substrates affect antigen-antibody reactions by binding to or competing with the E5 antigen. Further optimization of ELISA parameters to minimize the effects of competition from plant compounds could make the method more useful, but such modifications could also result in reduced sensitivity (6). The positive detection of *P. ultimum* in inoculated sugar beet seedlings by Western blot analysis suggested that interference from plant-derived compounds could have been minimized by electrophoresis and transblotting. The 46-kDa glycoprotein recognized by MAb E5 was an unambiguous marker for *P. ultimum* in Western blot analysis; whereas, positive detection of the fungus by ELISA was based on reactivity relative to negative controls.

Regardless of the assay method, use of MAb E5 for quantifying *P. ultimum* in vivo is complicated because E5 antigen production is nutrient dependent. Quantification using MAb E5 may be limited to particular hosts or substrates, and requires determining the relationship between reactivity and biomass for each host/substrate system. The detection of *P. ultimum* by Western blot in sugar beet, but not in bean, could have reflected lower production of E5 antigen in the mycelium within bean tissues. This conclusion was supported by the differential ELISA detection of E5 antigen in culture media containing various seed exudates. The effects of seed exudates on E5 antigen content were

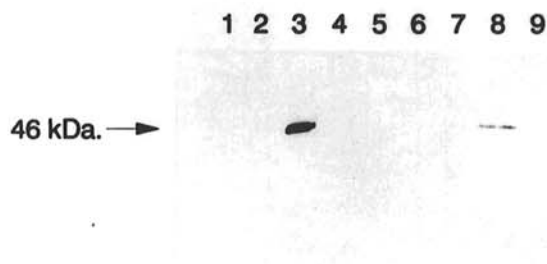


Fig. 9. MAb E5-probed Western blot of extracts of sugar beet and common bean hypocotyls infected with *Pythium ultimum* P201 or *P. irregulare* P205: molecular weight standards (lane 1); *P. irregulare* mycelial extract (lane 2); *P. ultimum* mycelial extract (lane 3); uninfected bean (lane 4); *P. ultimum*-infected bean (lane 5); *P. irregulare*-infected bean (lane 6); uninfected sugar beet (lane 7); *P. ultimum*-infected sugar beet (lane 8); *P. irregulare*-infected sugar beet (lane 9). Molecular weight standards are not visible in this photograph.

perhaps attributable to the differential stimulation of E5 antigen production by seed exudate components such as carbohydrates. Seed and root exudates of different plant species vary in composition and concentrations of nutritive (carbohydrates and amino acids) and nonnutritive compounds (1,24,26,28,32). The effects of specific plant exudates on sporangial germination of *P. ultimum* has been reported (24,26,28,32), but the role of exudates on the production of antigenic molecules has not been examined previously. This study demonstrated that sugar composition strongly influences E5 antigen production. Sugar concentration also is a critical factor (2).

E5 antigen levels in *P. ultimum* mycelia were not constant during development and, therefore, did not reflect total fungal biomass. The E5 antigen was associated with active metabolism as it was detected in young mycelia and sporangia and in aged mycelia and sporangia after exposure to fresh nutrients. The antigen was not detected in dormant structures, presumably because synthesis of antigen ceased while intracellular antigen was liberated into the surrounding substrate. Antigens associated with developmental stages have been found in other oomycetous fungi. Monoclonal antibodies to *P. aphanidermatum* (10) and *Phytophthora cinnamomi* (18) recognize antigens specific to components of zoospores and cysts. Several of the antigens are associated with the encystment process (9,11,14).

The metabolism-dependent characteristic of the E5 antigen could be an advantage when using MAb E5 to quantify *P. ultimum* because assays would be sensitive to metabolically active propagules or portions of the mycelium rather than to the total fungal biomass, which would include dormant and dead structures. This characteristic also could be exploited by immunostaining with MAb E5 to distinguish actively growing structures from dormant ones. Treatment of aged mycelia with a nutrient solution for 1 h appeared to stimulate metabolism and, thus, activated antigen production. This procedure might provide the means to assay the total potentially active biomass in a sample or to render *P. ultimum* detectable in a sample in which the antigen would not otherwise be produced. The reappearance of the E5 antigen in dormant structures after activation with nutrients also provides a means to study the influence of specific compounds on hyphal growth and propagule germination.

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