Detection of *Pythium ultimum* with a Species-Specific Monoclonal Antibody

G. Y. YUEN, M. L. CRAIG, and F. AVILA, Department of Plant Pathology, University of Nebraska, Lincoln 68583-0722

ABSTRACT

Yuen, G. Y., Craig, M. L., and Avila, F. 1993. Detection of *Pythium ultimum* with a species-specific monoclonal antibody. Plant Dis. 77:692-698.

We obtained four monoclonal antibodies to *Pythium ultimum* using a crude preparation of *P. ultimum* cell wall material as the immunogen. Initial selection was based on positive reactions with a culture of *P. ultimum* and negative reactions with one isolate each of *P. irregulare* and *Phytophthora cinnamomi* in indirect enzyme-linked immunosorbent assay (ELISA). The antibodies were screened further against 21 isolates of *P. ultimum* (including 11 asexual strains), 39 isolates of 16 other *Pythium* spp., and 24 isolates of 12 other genera of soil fungi. Antibody E5, the most specific to *P. ultimum*, was highly reactive to all *P. ultimum* isolates and had low or no reactivity to other *Pythium* spp. The other three antibodies exhibited cross-reactivity to various other species of *Pythium*. Two serogroups were distinguished within *P. ultimum* on the basis of reactivity to these three antibodies. None of the four antibodies reacted with other genera of fungi. A protocol was developed for the rapid identification of *P. ultimum* using E5, in which raw extracts from 2- to 3-day-old fungal cultures grown in liquid media are tested by ELISA. This system identified *P. ultimum* isolates among 246 cultures of pythiums isolated from sugar beet roots and soil with greater than 99% accuracy. An antigen competition assay, also using E5, was developed for detecting *P. ultimum* in roots. It was effective in detecting the fungus in sugar beet seedling roots that contained more than two infections per 10 cm of root.

Pythium ultimum Trow infects a wide range of host species. It causes preemergence and postemergence damping-off and also can reduce growth in mature plants through chronic root tip attack. Infection of mature plants by *P. ultimum* frequently is not manifested in aboveground symptoms other than reduced plant vigor (27). For this reason, measurements of disease severity or disease incidence do not adequately express the pathogenic capacity of the fungus.

Incidence of root infection by *P. ultimum* and soil inoculum levels commonly are determined by plating of root tissues and soil on agar media (2,7,8). These methods, however, do not provide accurate information on the extent of individual infections or the amount of pathogen biomass in infected tissues. The value of the information gained from the plating approach also is limited by the effort and time required to identify the pythiums cultured from roots and soil.

Accurate identification to the species level is necessary because other *Pythium* pathogens, such as *P. irregulare,* are commonly isolated from roots in conjunction with *P. ultimum* (2,8,27). In addition, antagonistic *Pythium* spp., such as *P. oligandrum* (16), may compete with *P. ultimum* for organic matter and root niches. Although gross colony morphology and rapid growth on media are useful in differentiating *Pythium* from other genera of fungi, *P. ultimum* cannot be readily distinguished from other *Pythium* spp. solely on the basis of these characteristics. Positive identification of *P. ultimum* requires culturing for as long as 10 days to produce diagnostic sexual structures. Furthermore, some isolates of *P. ultimum* do not produce sexual structures in culture. These asexual isolates produce only hyphal swellings and are difficult to distinguish from heterothallic species, such as *P. sylvaticum,* and from isolates in the HS group (24) that may be associated with other *Pythium* spp. (20).

Restriction analysis of mitochondrial DNA has been reported to be effective in identifying *P. ultimum* (15,17). Analysis of soluble proteins by isoelectric focusing (1) or electrophoresis (5) and isozyme variation (5) also have been used to distinguish *P. ultimum* from other *Pythium* spp. Although these techniques are extremely useful in systematics, serology-based methods are more efficient for ecological and epidemiological studies. Enzyme-linked immunosorbent assay (ELISA) has the advantages of speed and simplicity, requires little equipment, and thus can be modified for field research. The practicality of antibody-based methods has been demonstrated by the efficiency of commercial genus-specific immunoassay kits for pythiaceous fungi. These assays have been used to detect zoospores in runoff water (3) and to monitor pathogens in nursery stock (4) and turfgrass (21). Antibodies used in immunostaining procedures also have made possible direct observation of propagules in soil and hyphae associated with infected plant tissues (13,14).

Polyclonal antisera have been used to detect *P. insidiosum,* a human and animal pathogen (18), and *P. graminicola* (25) and to investigate relationships among *Pythium* spp. (11). However, the high degree of cross-reactivity of polyclonal antisera seriously limits their use for detecting single *Pythium* spp. Monoclonal antibodies (MAbs), in contrast, have been shown to provide the specificity required for species-level identification. Species-specific MAbs recently developed to *P. aphanidermatum* (6) and *Phytophthora cinnamomi* (9) not only enabled detection of the target species, but also permitted staining of specific cell wall features.

We set out to develop MAbs specific to *P. ultimum* to augment conventional plating methods by providing a method for the rapid identification of the pathogen in culture and in root tissues. We also hoped to use the MAbs eventually for immunostaining of *P. ultimum* in soil and roots. We report here on the specificity of four MAbs and the application of one antibody in separate ELISA formats for differentiating *P. ultimum* from other pythiums isolated from soil and roots and for directly detecting the fungus in roots. An abstract of this study has been published (26).

MATERIALS AND METHODS

Antigen preparation. *P. ultimum* P201 isolated from strawberry in California (27) was used to immunize mice for antibody production and served as the standard culture for the species in all procedures throughout the study. The fungus was cultured for 7 days in Czapek-Dox broth (CDB), amended with yeast extract (0.5 g/L) and with glucose substituted for sucrose, to produce the mycelial preparations used as antigen. Mycelial mats were collected by suction filtration, washed with three volumes of sterile 0.01 M phosphate-buffered saline (PBS), pH 7.0, and then chopped in PBS, which had been chilled to 4 C, in a blender at high speed for four 30-sec intervals. Chopped mycelia were washed three times by slow-speed centrifugation and resuspension in PBS. After the final centrifugation, the pellet was resuspended in a minimum amount of PBS, divided into 5-ml fractions, and stored frozen at −80 C. These crude prepara-

Journal Series Paper 10107 of the Agricultural Research Division, University of Nebraska.

Accepted for publication 11 February 1993.

© 1993 The American Phytopathological Society

tions consisted primarily of cell wall and membrane fragments. Before the antigen preparations were used in experiments, aliquots were thawed, ground further in a Potter-Elvehjem tissue homogenizer (Fisher Scientific, Springfield, NJ), and diluted with PBS to desired protein concentrations. Protein content of antigen preparations was estimated by measuring absorbance at 260 and 280 nm (12).

Antibody production. Hybridomas and MAbs were produced at the Monoclonal Antibody Core Facility of the Center for Biotechnology, University of Nebraska-Lincoln. BALB/c mice were immunized with three injections of P201 mycelial preparation (50 μg of protein) given at 2-wk intervals. Spleen cells from immunized mice were fused with SP2/O myeloma cells and hybridomas were cultured as described by Oi and Herzenberg (19).

Nine days after fusion, hybridomas were screened for production of antibodies with differential reactivity to *P. ultimum* P201, *P. irregulare* P205, and *Phytophthora cinnamomi.* The indirect ELISA procedure described below was used for this test, except that hybridoma culture fluid was applied in undiluted form to antigen-coated microtiter plates, and this was followed by reactions with goat antimouse antibody to all subclasses of mouse immunoglobulin (Ig) (Cappell Research Products, Durham, NC) and with rabbit antigoat IgG (whole-molecule) antibody conjugated to alkaline phosphatase. Reactions with absorbance readings of at least half of the highest reading in a given assay plate were considered to be positive.

Hybridoma clones that produced antibodies that reacted only with *P. ultimum* antigens were selected for further subcloning. Each clone was subcloned four times before being injected into mice for the production of ascites.

Indirect ELISA for specificity testing. MAbs in the form of ascites were tested for reactivity to isolates of *P. ultimum,* other *Pythium* spp., and other genera of fungi (Table 1). *Pythium* isolates were obtained primarily from culture collections of J. Hancock, F. Martin, and G. Yuen. All isolates were maintained on potato-dextrose agar or cornmeal agar (CMA).

Pythium cultures were identified to species with the help of a key by van der Plaats-Niterink (24). Cultures that did not produce oogonia in axenic culture were crossed with *P. sylvaticum* isolates ATCC18195 and ATCC18196 on CMA. Those that did not mate with *P. sylvaticum* were separated further into asexual *P. ultimum* and "other HS" isolates. Asexual isolates were distinguished by globose hyphal swellings 20–25 μm in diameter and included some isolates that produced very few oogonia in culture. Hyphal swellings of other HS isolates were smaller than those of asexual isolates or were not globose.

Mycelium was prepared as described for *P. ultimum.* Protein content of antigen preparations was adjusted to 100–300 μg/ml. Diluted antigen in 70-μl volumes was applied to wells of microtiter plates (MaxiSorb F96 Immunoplate, Nunc, Denmark), incubated for 2 hr at room temperature, and rinsed with tap water. The wells were filled with a blocking solution of 5% nonfat dry milk and 0.2% sodium azide in PBS for 90 min at 36 C. The next two reagents were applied sequentially in 70-μl volumes and incubated for 90 min at 36 C: first, mouse ascites diluted

Table 1. Species, host, location of isolation, and source of fungi tested

Species	Isolate numbers[a]	Host/Location[b]	Source[c]
Pythium ultimum	P10	Carnation/CA	1
	P32, P179, P201	Strawberry/CA	1
	P160	Soil/CA	1
	P161	Sugar beet/CA	1
	527	Sugar beet/MT	4
	67-1	Cotton/CA	3
	84-7	Alfalfa/CA	3
	110-2	Soil/FL	2
P. ultimum asexual isolates	P5	Carnation/CA	1
	P156	Celery/CA	1
	P164, P183	Strawberry/CA	1
	P202	Bean/NE	1
	78-11	Alfalfa/CA	3
	P221	Corn/NE	1
	P222	Sugar beet/NE	1
	P147	Soil/CA	1
	P225, P226	Soil/NE	1
P. acanthicum	A6	Soil/NC	2
P. aphanidermatum	P330	Bluegrass/NE	1
P. catenulatum	CBS 461.75	Soil/India	2
P. graminicola	1986-1	?/FL	2
P. intermedium	P170	Strawberry/CA	1
P. irregulare	P2	Carnation/CA	1
	P6	Celery/CA	1
	P8	Soil/CA	1
	P36, P81, P97, P99	Strawberry/CA	1
	P125	Bean/NE	1
	P126	Corn/NE	1
	P162, P166, P205	Soil/NE	1
	83-6, 89-6	Alfalfa/CA	3
	P215	Alfalfa/NE	1
P. mamillatum	P12	Soil/CA	1
P. myriotylum	1987-63	Bean/MD	2
P. oligandrum	17-4	Soil/CA	2
P. paroecandrum	79-2	Bean/CA	3
P. spinosum	P240	Soil/NE	1
	79-4	Soil/FL	2
P. sylvaticum	ATCC18195, ATCC18196	Soil/TN	5
	P216, P217	Corn/NE	1
	P227	Soil/NE	1
	1987-14	Corn/IA	2
P. torulosum	FTCC808	Bluegrass/OH	2
P. vanterpoolii	FTCC809	Bluegrass/OH	2
Pythium HS	P98	Strawberry/CA	1
	P140, P223	Soil/CA, NE	1
	P218, P220	Corn/NE	1
Alternaria spp.	1	Milkweed/NE	1
Bipolaris sorokiniana	1	Wheat/NE	1
Curvularia intermedia	1	Turfgrass/NE	1
Cylindrocarpon destructans	3	Strawberry/CA	1
Fusarium oxysporum	1	Soil/NE	1
Laetisaria arvalis	1	Soil/NE	1
Macrophomina phaseolina	1	Soybean/NE	1
Phytophthora cinnamomi	1	African violet/NE	1
Rhizoctonia cerealis	2	Wheat/NE	1
R. solani	9	Various/N. America	1
Sclerotinia sclerotiorum	1	Bean/NE	1
Trichoderma harzianum	1	Soil/NE	1
Verticillium dahliae	1	Tomato/NE	1

[a]Individual *Pythium* isolates are identified; for other genera, only the total number of isolates is given.
[b]CA = California, FL = Florida, IA = Iowa, MD = Maryland, MT = Montana, NC = North Carolina, NE = Nebraska, OH = Ohio, TN = Tennessee.
[c]1, G. Y. Yuen; 2, F. N. Martin, University of Florida; 3, J. G. Hancock, University of California; 4, D. Mathre, Montana State University; 5, American Type Culture Collection (ATCC).

1:500 to 1:3,000 in PBS, depending on the antibody; and second, goat anti-mouse IgG (whole-molecule)-alkaline phosphatase conjugate (Cappell) diluted 1:5,000 in 50 mM TrisHCl (pH 8.0) with 1 mM $MgCl_2 \cdot 6H_2O$, 2% bovine serum albumin, and 0.04% sodium azide. Between each reagent step, the wells were washed three times with PBS containing 1 ml of Triton X-100 per liter and rinsed with tap water. Finally, 150 µl of substrate solution (6 mg of *p*-nitrophenyl phosphate in 15 ml of 10 mM diethanolamine [pH 9.5] with 0.02% levamisole) was added to each well and incubated for 12–18 hr at room temperature. Absorbance was read at 405 nm with an ELISA plate reader. The average absorbance reading from four replicate wells for each fungal isolate was compared with a positive-negative threshold determined as the mean plus three times the standard deviation of P205. *P. ultimum* P201 and *P. irregulare* P205, as the positive and negative controls, respectively, were included in each microtiter plate, in addition to reagent controls. Preimmune mouse serum and heterologous mouse MAb also were included as negative controls in early experiments but were later excluded because they gave absorbance readings similar to that of the reagent controls.

Identification of *Pythium ultimum* isolated from soil and roots. We developed an immunoassay system for the rapid identification of *P. ultimum* in culture using MAb E5 and tested the system for its accuracy in differentiating *P. ultimum* from other *Pythium* spp. isolated from field soil and sugar beet roots. Sugar beet seedlings and soils were collected from western Nebraska as part of a separate study on the prevalence of fungal pathogens on sugar beet. Seedling roots were washed free of soil with tap water and placed on water agar (WA) to isolate *Pythium* spp. Over a 48-hr period, we transferred hyphal tips growing from the roots to CMA amended with streptomycin and penicillin at 100 mg/L. To obtain isolates from soil samples, we plated drops of soil suspensions on WA, as described for the *P. ultimum* soil assay of Stanghellini and Hancock (22), and after 24 hr transferred from hyphae growing from soil drops to antibiotic-amended CMA. All cultures collected were stored at 4 C. We used 195 soil isolates and 51 root isolates selected at random from this collection to test the identification system.

In preliminary experiments, supernatants from broth cultures of *P. ultimum* were found to react with MAb E5 to nearly the same extent as *P. ultimum* mycelia. Therefore, we tested two types of liquid cultures for their effectiveness in producing the E5 antigen in soluble form for use in the identification system. In one approach, *Pythium* isolates were cultured for 2 days in 1-ml volumes of CDB in microcentrifuge tubes. CDB was selected because of the minimal medium volume and incubation period required to produce detectable E5 antigen from *P. ultimum* in preliminary experiments. For the other method, 2- to 3-day-old cultures of the isolates on antibiotic-amended CMA in petri dishes 5 cm in diameter were flooded with 8 ml of sterile distilled water for an additional 3 days. Flooded CMA was tested because of its usefulness in producing colonies of the fungi for visual identification, in the hope that this one medium might serve both purposes.

All isolates were cultured in both media. Culture fluids were tested for reactivity to E5 in ELISA without further purification or standardization of protein concentration; 70-µl aliquots of undiluted culture fluid were applied as the coating antigen to duplicate wells. All other steps in the indirect ELISA were as described above, except that E5 ascites was used at a dilution of 1:30,000. Fluids from CDB and flooded CMA cultures of P201 and P205 were used in each assay plate as the positive and negative controls, respectively.

Mean absorbance values of isolates cultured in one type of medium were compared with positive-negative thresholds based on absorbance of P205 cultured in the same medium. An isolate was tested only once by ELISA if both types of culture fluid reacted with E5. Isolates that gave negative or low positive reactions with E5 for one or both culture fluids were purified on WA amended with streptomycin (100 mg/L), subcultured in both media, and retested at least once. Isolates were maintained for 10–14 days on flooded CMA and also were cultured on boiled grass blades (24). Both types of cultures were used for visual identification of isolates.

Detecting *P. ultimum* in roots. We tested two variations of the indirect ELISA using MAb E5 for their efficacy in detecting *P. ultimum* associated with root tissues. In the first variation, test samples were applied directly to wells of microtiter plates as the adsorbed antigen. The sample solutions (70-µl volumes) were incubated in each well for 2 hr at room temperature. All other ELISA procedures, beginning with the blocking step, were as described above.

The second variation was an antigen competition assay in which the test samples were first incubated in mixture with an equal volume of E5 ascites at a $1:10^4$ dilution for 90 min at 36 C. Aliquots (70 µl) of each mixture were then applied to four microtiter plate wells that had been precoated with 70 µl of *P. ultimum* P201 mycelial preparation diluted to a protein concentration of 300 µg/ml. After the sample-ascites mixtures had been incubated in the precoated wells for 90 min at 36 C and then washed out, the conjugate and the substrate were applied as previously described. In the competition assay, *P. ultimum* antigen in a sample should bind with the antibody in the sample-ascites mixture, thereby reducing the amount of E5 available to react with P201 antigen adsorbed on the ELISA plates. Thus the presence of *P. ultimum* in a test sample would be indicated by absorbance readings lower than those of control samples containing no *P. ultimum*. Student's *t* test was used to analyze differences between the absorbance means.

Various types of samples were used to test the two assays. One type consisted of *P. ultimum* P201 mycelial preparation diluted with PBS, sugar beet root extract, or soybean root extract to protein concentrations ranging from 10 to 400 µg/ml. The root extracts were prepared by grinding 150 cm of sugar beet roots

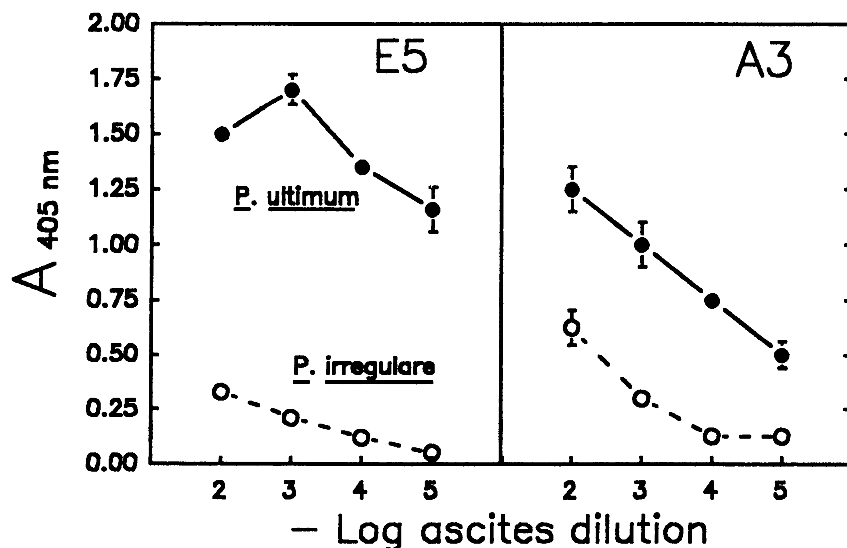

Fig. 1. Reactivity of *Pythium ultimum* isolate P201 and *P. irregulare* P205 to *P. ultimum* antibodies E5 and A3 as measured by indirect enzyme-linked immunosorbent assay. Error bars indicate standard error.

or 50 cm of soybean roots from 2-wk-old seedlings in 2 ml of PBS. Negative controls in this group included PBS and root extracts with no antigen added and the diluents amended with *P. irregulare* P205 mycelial preparation. Another type of sample consisted of 100 cm of *P. ultimum*-infected roots ground in 2 ml of PBS. The infected roots were obtained from 2-wk-old sugar beet seedlings planted in pots of artificially infested soil. To determine levels of infection, subsamples of 100–200 cm of root were aligned on WA, and numbers of *P. ultimum* colonies growing from the roots were counted after 24 hr. Extracts made from roots of seedlings grown in noninfested soils served as negative controls.

RESULTS

Reactivity of MAbs. Four of over 900 hybridoma clones screened produced antibodies (designated E5, A3, A4, and X4) that reacted with *P. ultimum* P201 but not with *P. irregulare* P205 or *Phytophthora cinnamomi*. All of the antibodies were IgG1 with κ light chains.

Ascites produced from the four hybridomas differed markedly in reactivity to *P. ultimum* P201. In titration experiments, MAb E5 had the highest level of reactivity, with maximum activity at a 10^{-3} dilution of ascites and absorbance readings exceeding 1.0 even at a 10^{-5} dilution (Fig. 1). E5 also gave the greatest separation between P201 and *P. irregulare* P205. Absorbance of E5 with P205 was less than 0.4 at all dilutions of ascites tested, whereas absorbance of A3 with P205 exceeded 0.5 at dilutions below 10^{-4} (Fig. 1). The reactivity of MAb A4 to P201 and P205 was similar to that of A3, while absorbance levels of X4, the least reactive antibody, did not exceed 1.0 with either fungus at any dilution (*data not shown*). All four MAbs gave absorbance values below 0.1 with *Phytophthora cinnamomi* (*data not shown*).

Specificity of MAbs. When the antibodies were tested further against isolates of *P. ultimum*, other *Pythium* spp., and other genera of soil fungi, E5 proved to be the most specific to *P. ultimum*. E5 diluted 1:3,000 reacted with all isolates of typical and asexual *P. ultimum*, with absorbance values ranging from 0.60 to 1.90 (Table 2). No reaction was detected with most of the isolates from 14 other *Pythium* spp. or with any HS isolates. Four of 15 isolates of *P. irregulare*, one of six of *P. sylvaticum*, and one of *P. intermedium* gave reactions above the positive-negative threshold. Absorbance values for these isolates (<0.3) were significantly lower than that of the least reactive *P. ultimum* isolates according to Student's *t* test.

Reactions with *P. ultimum* isolates varied between E5 and the other three MAbs (Table 2). Asexual and typical *P. ultimum* could not be distinguished from each other on the basis of any antibody reactivity pattern. However, serogroups could be distinguished within both *P. ultimum* categories: isolates in serogroup I reacted with all four MAbs, whereas isolates in serogroup II reacted only with E5. The serogroup of an isolate did not appear to be related to its host or geographic origin.

MAbs A3, A4, and X4 were not specific to *P. ultimum;* each cross-reacted with isolates of other *Pythium* spp., including all six isolates of *P. sylvaticum* (Table 2). A3 reacted with the most species. Two serogroups also could be distinguished within *P. irregulare* on the basis of reactivity with A3, A4, and X4.

All four antibodies proved to be specific to the genus *Pythium;* none reacted with 24 isolates of soil- and root-inhabiting fungi representing 12 other genera, including *Phytophthora* (*data not shown*). Absorbance values from tests with genera other than *Pythium* did not exceed 0.1. The only exception was a low positive reaction (absorbance 0.4) of MAb A3 with one isolate of *Cylindrocarpon destructans.*

Rapid identification of *P. ultimum* with E5. *P. ultimum* isolates among 246 isolates of *Pythium* from sugar beet roots and soil were readily identified on the basis of reactivity to antibody E5 in ELISA. Agreement between ELISA and microscopic visual identification in differentiating *P. ultimum* from other *Pythium* spp. was greater than 99%, regardless of the medium used to culture the fungi. All 188 isolates identified as *P. ultimum* or asexual *P. ultimum* on the basis of morphology reacted positively to E5 in ELISA.

However, reaction intensity varied among *P. ultimum* isolates and between culturing methods. In initial ELISA tests of CDB cultures, absorbance values for all but a few isolates of *P. ultimum* exceeded 0.4, and absorbance readings of *P. ultimum* isolates overlapped very little with those of other isolates (Fig. 2). The few *P. ultimum* isolates that were negative or low positive (absorbance readings of 0.2–0.4) were strongly positive when retested after further purification. More *P. ultimum* isolates gave low positive readings when the fungi were cultured in flooded CMA, and reactivity of the *P. ultimum* isolates in flooded CMA generally was lower than for those in CDB (*data not shown*).

Fifty-eight isolates tested were determined by morphology to be other *Pythium* spp., with *P. irregulare, P. spinosum,* and *P. intermedium* accounting for more than half of the isolates. All but one of these isolates were negative in their reactions to E5, and nearly all gave absorbance values less than 0.1 (Fig.

Table 2. Reactivity of isolates of *Pythium ultimum* and other *Pythium* spp. to monoclonal antibodies (E5, A3, A4, and X4) produced to *P. ultimum* P201

Species and serogroup[a]	Number of isolates	ELISA reaction[b]			
		E5	A3	A4	X4
P. ultimum					
Serogroup I	7	+++	++	++	+
Serogroup II	3	+++	0	0	0
P. ultimum asexual isolates					
Serogroup I	7	+++	++	++	+
Serogroup II	4	+++	0	0	0
P. acanthicum	1	0	0	0	0
P. aphanidermatum	1	0	0	0	0
P. catenulatum	1	0	++	0	0
P. graminicola	1	0	++	0	0
P. intermedium	1	+	++	0	+
P. irregulare					
Serogroup I	13	0[c]	0	0	0
Serogroup II	2	+	++	++	+
P. mamillatum	1	0	++	++	+
P. myriotylum	1	0	0	0	0
P. oligandrum	1	0	0	0	0
P. paroecandrum	1	0	0	0	0
P. spinosum	2	0	0	++	+
P. sylvaticum	6	0[c]	++	++	+
P. torulosum	1	0	++	0	0
P. vanterpoolii	1	0	++	0	+
Pythium HS	5	0	0	0	0

[a]Serogroups were identified in three species: *P. ultimum* serogroup I included isolates P160, P161, P179, P201, 527, 67-1, and 84-7; *P. ultimum* serogroup II included isolates P10, P32, and 110-2; asexual *P. ultimum* serogroup I comprised P147, P164, P183, P202, P221, P226, and 78-11; asexual *P. ultimum* serogroup II comprised P5, P156, P222, and P225; *P. irregulare* serogroup I included P2, P6, P8, P81, P97, P99, P125, P126, P162, P166, P205, 83-6, and 89-3; and *P. irregulare* serogroup II included P36 and P215.

[b]Absorbance >0.8 (+++); 0.3–0.8 (++); <0.3 but above the positive-negative threshold value (mean of *P. irregulare* P205 plus three standard deviations) (+); or less than the positive-negative threshold value (0). Values are averages across isolates and experiments.

[c]Isolates P6 and P81 of *P. irregulare* serogroup I and isolate ATCC18195 of *P. sylvaticum* had low-positive (+) reactivity with E5.

2). One isolate of *P. mamillatum* gave reactions of approximately 0.4 and 0.6 when cultured in CDB and flooded CMA, respectively. Absorbance values for the negative control, P205, were typically below 0.1 in both media, and positive-negative thresholds were around 0.2.

Detecting *P. ultimum* in root tissues. ELISA could not detect *P. ultimum* in root extracts adsorbed onto wells of microtiter plates (*data not shown*). Presumably, root constituents blocked the adsorption of *P. ultimum* antigen onto the wells. Absorbance readings of root extracts containing *P. ultimum* were not significantly higher than those of the negative controls.

The antigen competition assay proved to be more effective in detecting *P. ultimum* associated with root tissues. When known quantities of mycelial preparations of *P. ultimum* were added to PBS or to soybean root extract, absorbance readings in the competition assay decreased with increasing *P. ultimum* concentration (Fig. 3). The dilution matrix affected the ELISA reactions, as indicated by different absorbance values for PBS and soybean root extract at the same concentration of *P. ultimum* (Fig. 3). Results were similar when sugar beet root extract and PBS were compared as diluents (*data not shown*). *P. irregulare* P205 added to the three diluents yielded absorbance readings similar to those of the diluents alone, regardless of the concentration of P205 protein (*data not shown*). The lowest concentration of *P. ultimum* antigen detected varied between experiments from 10 to 20 μg/ml.

The antigen competition assay could also detect *P. ultimum* in infected sugar beet roots. In one typical experiment (Table 3), *P. ultimum* was detected in two samples that contained more than 20 colonies of the fungus per 100 cm of root as determined by root plating. The fungus was not detected in two samples infected at lower levels (fewer than 10 colonies per 100 cm). Similar results were obtained when the experiment was repeated.

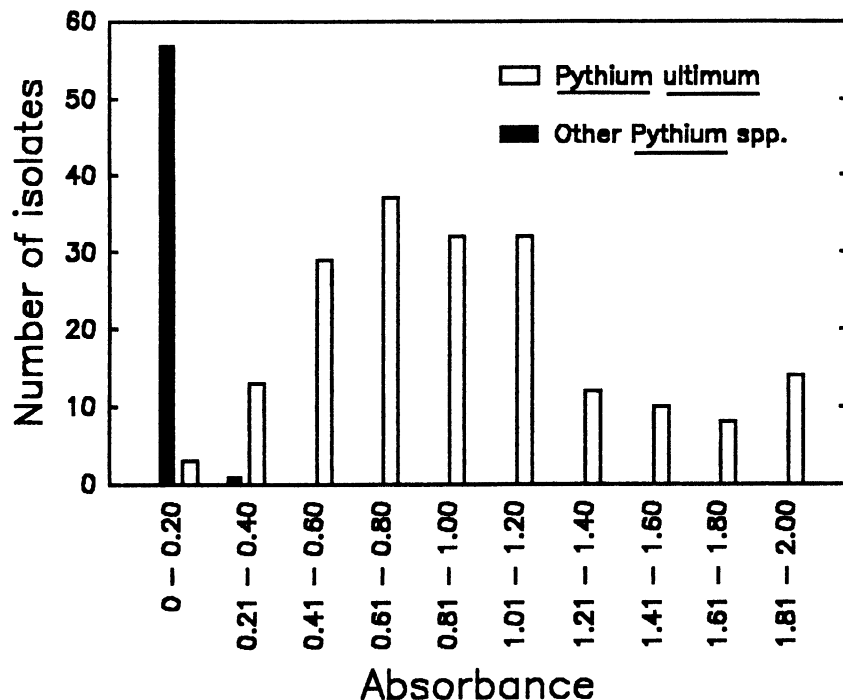

Fig. 2. Distribution of absorbance readings from enzyme-linked immunosorbent assays with antibody E5 among 246 isolates of *Pythium* spp. from sugar beet roots and soil. The isolates were identified on the basis of morphology observed in 10- to 14-day-old cultures. Culture fluids from 2-day-old cultures in Czapek broth were used for the assay.

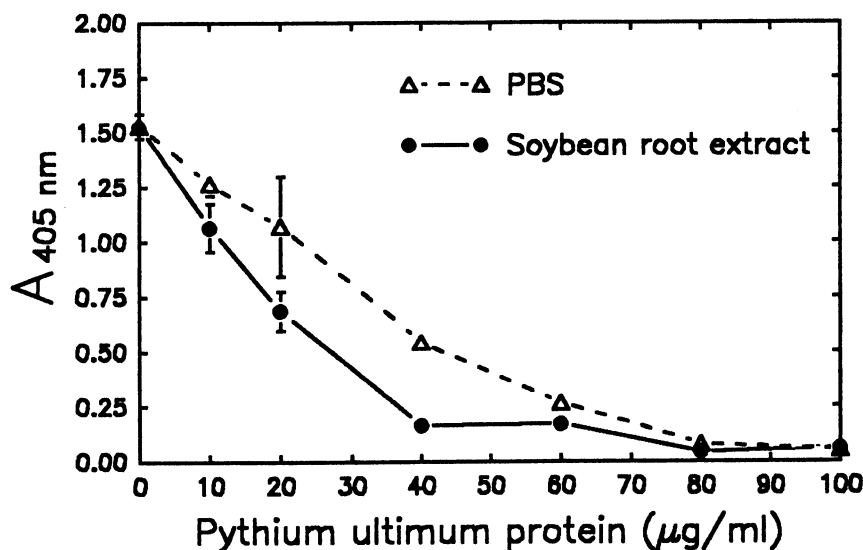

Fig. 3. Reactivity of antibody E5 to mycelial preparations of *Pythium ultimum* (isolate P201) diluted in phosphate-buffered saline (PBS) and in soybean root extract. Reactivity was measured by an antigen competition enzyme-linked immunosorbent assay. Error bars indicate standard error. Mycelia and root extracts were ground in PBS.

DISCUSSION

The results of this study show that MAb E5 is a useful tool for detecting and identifying *P. ultimum*. E5 accurately differentiates asexual *P. ultimum* isolates from HS isolates affiliated with other *Pythium* spp. Because of its specificity, the antibody also will be useful in investigations of competition between *P. ultimum* and other *Pythium* spp.

MAb E5 when used in ELISA for direct detection of *P. ultimum* in sugar

Table 3. Detection of *Pythium ultimum* in sugar beet roots by an antigen competition assay with monoclonal antibody E5

Sample[a]	Estimated infection[b]	Absorbance reading[c]
Infected		
1	1	0.966
2	7	1.055
3	23	0.250*
4	40	0.396*
Noninfected controls		
1	0	1.023
2	0	0.763
Mean		0.893

[a] Infected root samples were taken from 2-wk-old sugar beet seedlings grown in soils artificially infested with *P. ultimum* isolate P201. Noninfected controls were from same-age plants grown in noninfested soil.

[b] Infection level was estimated by aligning 100–200 cm of root from a separate subsample on water agar and counting the number of colonies of *P. ultimum* per 100 cm of root after 24 hr.

[c] Values are means of four replicate wells. An asterisk denotes a significant ($P = 0.05$) difference between infected samples and the mean of the noninfected controls according to Student's *t* test.

beet roots is more effective at higher levels of infection. Low infection levels commonly found on seedling root systems in the field—for example, fewer than 10 colonies per 100 cm of root—may not be detectable with the antigen competition assay. Other immunoassay designs, such as double-antibody capture ELISA and immunoblots using nitrocellulose, may increase sensitivity.

Regardless of the type of assay, sensitivity may need to be examined specifically for each species of host plant. Production of the E5 antigen by P201 varied among culture media differing in nutrient composition (G. Y. Yuen and F. Avila, *unpublished*), and thus E5 antigen production by *P. ultimum* may also vary among hosts. The accuracy and precision of the antigen competition assay with E5 for estimating *P. ultimum* biomass have not been fully determined. Theoretically, the method could be used to quantify *P. ultimum* biomass by comparing absorbance readings of a sample to a set of calibration standards containing known amounts of *P. ultimum.* Care must be taken to prepare calibration standards using the same type of plant tissue or fluid as that of the samples, as we found that plant tissue components affect the assay.

The more immediate application of E5 in ELISA is for the rapid identification of *P. ultimum* in culture. The procedure is facilitated by the release of the antigen into liquid culture media. This discovery was surprising, because the immunization process was intended to yield antibodies to cell wall- or membrane-bound antigens. Preliminary application of E5 in immunofluorescence microscopy also indicated localization of the antigen in cell walls of *P. ultimum* (G. Y. Yuen, *unpublished*).

When ELISA with E5 is used for identification of *P. ultimum,* any isolate that reacts positively with E5 can be assumed to be *P. ultimum,* whereas isolates with reactivity below the positive-negative threshold should be retested, particularly when there is a possibility of contamination. We obtained false-positive readings in this study with some isolates of *P. irregulare, P. intermedium, P. mamillatum,* and *P. sylvaticum.* These false positives, however, were few in number (2% of field isolates), and the absorbance values were relatively low compared to those of *P. ultimum* isolates.

Absorbance data from the specificity experiments, in which pure cultures were used and protein content was standardized, indicated that *P. ultimum* and non-*P. ultimum* isolates were mutually exclusive populations. Use of Chebychev's equivalent (mean of the negative control plus three standard deviations) to establish the positive-negative threshold in this study did not provide complete separation of the populations. Alternative approaches for establishing thresholds for ELISA data, such as the use of histograms (23), may be more appropriate.

Our choice of *P. irregulare* P205 as the negative control isolate was arbitrary; its level of reaction to E5 in ELISA may not represent the range of true negatives. Using one of the low-positive non-*P. ultimum* isolates instead as a negative control would have raised the threshold for positive reactions and thus cut down on or eliminated false positives but could also have resulted in false negatives.

The three other MAbs to *P. ultimum* in this study (A3, A4, and X4) cross-reacted with *P. irregulare, P. mamillatum,* and *P. sylvaticum.* Krywienczyk and Dorworth (11) made similar observations for the same species using polyclonal antisera. Affinity between *P. ultimum* and other *Pythium* spp. also was suggested in a study by Chen et al (5) in which particular isolates of *P. irregulare* were found to cluster with *P. ultimum* in cluster analysis and principal component analysis of data relating to soluble proteins. In contrast, Hendrix and Papa (10) placed *P. ultimum* in a widely different subgroup from those of the other three species on the basis of morphology. This separation was supported by data from a study by Martin and Kistler (17), which revealed species-specific mitochondrial DNA restriction patterns for *Pythium* spp.: *P. ultimum* had less than 50% similarity to other *Pythium* spp., in contrast to the more than 80% similarity between *P. ultimum* isolates (15).

Although A3, A4, and X4 are less specific than E5 for identifying *P. ultimum,* two subgroups can be distinguished within *P. ultimum* on the basis of these antibodies. Serological diversity within the species has not been observed previously. The reactivity of a given isolate of *P. ultimum* to the antibodies appeared to be consistent through repeated ELISA tests in this study. Therefore, these MAbs may be useful as markers to monitor populations of one serogroup against a background of the other, making studies of interaction between strains of *P. ultimum* feasible. Further research is needed to determine whether the antibody reactivity patterns in the species are related to any biological or ecological attribute and whether any additional serogroups exist.

ACKNOWLEDGMENTS

We thank Joseph Hancock and Frank Martin for providing isolates from their collections. We also thank Jole Williams for assistance in producing hybridomas.

LITERATURE CITED

1. Adaskaveg, J. E., Stanghellini, M. E., Gilbertson, R. L., and Egen, N. B. 1988. Comparative protein studies of several *Pythium* species using isoelectric focusing. Mycologia 80:665-672.
2. Ali-Shtayeh, M. S. 1986. Seasonal variation in population levels of *Pythium* species in irrigated and non-irrigated fields in the West Bank of Jordan and the Gaza Strip. Trans. Br. Mycol. Soc. 87:503-509.
3. Ali-Shtayeh, M. S., MacDonald, J. D., and Kabashima, J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Dis. 75:305-311.
4. Benson, D. M. 1991. Detection of *Phytophthora cinnamomi* in azalea with commercial serological assay kits. Plant Dis. 75:478-482.
5. Chen, W., Hoy, J. W., and Schneider, R. W. 1991. Comparisons of soluble proteins and isozymes for seven *Pythium* species and applications of the biochemical data to *Pythium* systematics. Mycol. Res. 95:548-555.
6. Estrada-Garcia, M. T., Green, J. R., Booth, J. M., White, J. G., and Callow, J. A. 1989. Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens. Exp. Mycol. 13:348-355.
7. Hancock, J. G. 1977. Factors affecting soil populations of *Pythium ultimum* in the San Joaquin Valley of California. Hilgardia 45:107-122.
8. Hancock, J. G., and Grimes, D. W. 1990. Colonization of rootlets of alfalfa by species of *Pythium* in relation to soil moisture. Phytopathology 80:1317-1322.
9. Hardham, A. R., Suzaki, E., and Perkin, J. L. 1986. Monoclonal antibodies to isolate-, species-, and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi.* Can. J. Bot. 64:311-321.
10. Hendrix, F. F., Jr., and Papa, K. E. 1974. Taxonomy and genetics of *Pythium.* Proc. Am. Phytopathol. Soc. 1:200-207.
11. Krywienczyk, J., and Dorworth, C. E. 1980. Serological relationships of some fungi of the genus *Pythium.* Can. J. Bot. 58:1412-1417.
12. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. 3:447-454.
13. MacDonald, J. D., and Duniway, J. M. 1979. Use of fluorescent antibodies to study the survival of *Phytophthora megasperma* and *P. cinnamomi* zoospores in soil. Phytopathology 69:436-441.
14. Malajczuk, N., McComb, A. J., and Parker, C. A. 1975. An immunofluorescence technique for detecting *Phytophthora cinnamomi* Rands. Aust. J. Bot. 23:289-309.
15. Martin, F. N. 1990. Taxonomic classification of asexual isolates of *Pythium ultimum* based on cultural characteristics and mitochondrial DNA restriction patterns. Exp. Mycol. 14:32-46.
16. Martin, F. N., and Hancock, J. G. 1986. Association of chemical and biological factors in soils suppressive to *Pythium ultimum.* Phytopathology 76:1221-1231.
17. Martin, F. N., and Kistler, H. C. 1990. Species-specific banding patterns of restriction endonuclease-digested mitochondrial DNA from the genus *Pythium.* Exp. Mycol. 14:32-46.
18. Mendoza, L., Kaufman, L., and Standard, P. 1987. Antigenic relationship between the animal and human pathogen *Pythium insidiosum* and nonpathogenic *Pythium* species. J. Clin. Microbiol. 25:2159-2162.
19. Oi, V., and Herzenberg, L. 1980. Immunoglobulin-producing hybrid cell lines. Pages 351-372 in: Selected Methods in Cellular Immunology. B. Mishell and S. Shiigi, eds. Freeman, San Francisco.
20. Schmitthenner, A. F. 1962. Isolation of *Pythium* from soil particles. Phytopathology 52:1133-1138.
21. Shane, W. W. 1991. Prospects for early detection of Pythium blight epidemics on turfgrass by antibody-aided monitoring. Plant Dis. 75:921-925.
22. Stanghellini, M. E., and Hancock, J. G. 1970. A quantitative method for the isolation of *Pythium ultimum* from soil. Phytopathology 60:551-552.
23. Sutula, C. L., Gillett, J. M., Morrissey, S. M., and Ramsdell, D. C. 1986. Interpreting ELISA data and establishing the positive-negative

threshold. Plant Dis. 70:722-726.
24. van der Plaats-Niterink, A. J. 1981. Monograph of the Genus *Pythium*. Stud. Mycol. 21.
25. White, D. G. 1976. The preparation and use of a fluorescent antibody reagent for the detection of *Pythium graminicola*. Phytopathology 66:523-525.
26. Yuen, G. Y., and Craig, M. L. 1990. Monoclonal antibodies specific to *Pythium ultimum* and *Pythium* spp. (Abstr.) Phytopathology 80:962.
27. Yuen, G. Y., Schroth, M. N., Weinhold, A. R., and Hancock, J. G. 1991. Effects of soil fumigation with methyl bromide and chloropicrin on root health and yield of strawberry. Plant Dis. 75:416-420.