The Preparation and Use of a Fluorescent Antibody Reagent for the Detection of Pythium graminicola

D. G. White

Former Lecturer, Department of Plant Pathology, The Ohio State University, Columbus 43210. Presently: Assistant Professor, Department of Plant Pathology, University of Illinois, Urbana 61801.

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ABSTRACT

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A fluorescent antibody reagent was prepared with *Pythium graminicola* and tested for specificity by staining *Pythium* and *Phytophthora* species on glass slides. The intensity of fluorescence could be used to separate *P. graminicola* from 17 other *Pythium* spp. and from two *Phytophthora* spp. No difference in fluorescence was noted among 15 different isolates of *P. graminicola*. Evidence of specificity of staining reactions, antigenic relationships of *Pythium* spp., and descriptions of methods used are presented.

Studies of specific *Pythium* spp. in soil would be enhanced if these species could be easily detected and identified by direct microscopic observation of soil preparations.

The fluorescent antibody technique has been used for the detection and identification of specific fungi and other microorganisms among large amounts of debris (2). The technique offers the possibility that *P. graminicola* could be identified by mycelium, sporangia, or oospores; thus, eliminating the necessity of having all these structures present.

Immunofluorescent staining has been used to identify species of *Phytophthora* (1, 5), but previously has not been used for identification of *Pythium* species. This paper reports the use of the fluorescent antibody technique for rapid identification of *Pythium graminicola*.

MATERIALS AND METHODS

The Fungi.—*Pythium aristosporum* (ATCC 11101), and *P. oedochilum* (ATCC 16016) were from the American Type Culture Collection, Rockville, MD. All others were from stock cultures of A. F. Schmitthenner, Ohio Agricultural Research and Development Center, Wooster.

Preparation of the antigen.—An isolate of *Pythium graminicola* was cultured at 28 C for nine days in 50 ml Center Mold Medium No. 1 (6). Mycelial mats were removed from the medium and washed with distilled water. The mats were then suspended in 0.05 M sodium phosphate-buffered saline (pH 7.0) and minced in an Omni-Mixer at high speed for 10 minutes. Further disintegration was achieved in a pyrex Ten Broeck homogenizer followed by sonication for 10 minutes with a Bronwill Biosonic III Sonifer (Bronwill Scientific, Inc., Rochester, NY) at an intensity of 63.2 watts/cm^2. The resulting preparation was centrifuged for 10 minutes at 12,000 *g* to remove larger mycelial particles and the supernatant was used as the antigen source.

Antigen preparations contained approximately 5.5 mg/ml protein based on the Lowry test (4), and approximately 10 mg/ml total fungal material. Preparations were divided into amounts to be used for injection of rabbits and stored at −20 C.

Immunization of animals.—New Zealand white doe rabbits (2.5-3.5 kg) were immunized with *P. graminicola* antigen preparations. Normal sera were obtained before injections were begun. Intramuscular injections with 1 ml antigen mixed with 1 ml Freund's Incomplete Adjuvant (Difco) were given weekly for 3 weeks. During the fourth week an intravenous injection of 0.5 ml antigen was given on day 1, followed with daily injections, increasing by 0.25 ml each day until the final injection on day 10 was 2.75 ml of antigen. Intraperitoneal injections of 1 ml of antigen were given on days 5 through 10. On day 6, intramuscular injections of 1 ml antigen mixed with 1 ml of the adjuvant were given. Trial bleedings were taken from each animal and the antibody content was checked by gel diffusion. Rabbits were bled when the highest titer was obtained. Blood was refrigerated overnight to allow it to clot and express serum. Sera were collected, clarified by centrifugation, and stored at −20 C.

Conjugation procedure.—The protein fraction of the serum was precipitated by mixing equal volumes of cold ammonium sulfate (3.9 M) and serum. The preparation was held at 4 C for 20-24 hours, and the resulting precipitate was then centrifuged and redissolved in a volume of distilled water equal to the original volume of the serum.

The solution was dialyzed (in 1.3 cm diameter tubing) against 0.85% sodium chloride at 4 C for 4 hours (3) and the protein concentration determined (4). One milliliter of cold 0.5 M carbonate buffer in 0.85% sodium chloride (pH 9.5) was added per 10 ml of solution. Fluorescein isothiocyanate (Nutritional Biochemicals Corp.,

Cleveland, OH) was added (50 μg of dye per mg protein) and the mixture was continuously stirred at 4 C for 20 hours (2). The conjugated sample was then passed through a Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column (25 × 110 mm), equilibrated with 0.05 M sodium phosphate-buffered saline (pH 7.2) with 0.001 M sodium azide (4), to remove unreacted fluorescein isothiocyanate. The conjugate was then stored at −20 C.

Staining of fungal isolates.—Four-day-old slide cultures of *Pythium* species were stained with conjugated antiserum. Slide cultures were prepared by placing a drop of solid basal medium on a sterile microscope slide supported by a bent glass rod on moist filter paper in a sterile petri dish. The medium was inoculated and the culture was incubated at 28 C. Mycelium that grew from the medium onto the slide surface was fixed in acetone for 20 minutes. Fixed mycelium was flooded with conjugated antiserum for 20 minutes and rinsed with distilled water, followed by two soakings (10 minutes each) in phosphate buffered saline (pH 7.2). The slide was then rinsed with distilled water, allowed to dry and mounted in buffered glycerol (pH 7.2). Slide cultures treated with conjugated normal serum were used as a control. Slides were examined for fluorescence with a Leitz Ortholux microscope with a HBO-200 mercury vapor light source, a 3-mm blue excitation filter (BG-12), and a K-510 barrier filter.

Mycelia and sporangia were visually rated on the basis of the amount of fluorescence. Slides were coded during observation in order not to bias the ratings. Overall fluorescence ranged from little or no fluorescence (rating 0) to the most intense (rating 4). Intermediate fluorescence was given values of 1, 2, or 3.

RESULTS

Preliminary experiments indicated common antigens among *P. graminicola* and other *Pythium* spp. Conjugated antiserum was diluted 1:2 and 1:10 with 0.05 M sodium phosphate-buffered saline (pH 7.2) in an attempt to improve specificity of the stain.

Mycelium and sporangia of several *Pythium* spp. fluoresced the same as *P. graminicola* isolates when stained with 1:2 diluted conjugated antiserum (Table 1). All 15 isolates of *P. graminicola* were rated 4. *Phytophthora cactorum* Lebert & Cohn and *Phytophthora citricola* Swada were also stained, but did not fluoresce. When slide cultures were stained with conjugated antiserum diluted 1:10, mycelial fluorescence distinguished *P. graminicola* from all *Pythium* spp. except *P. aristosporum* (Table 1). Unstained slides were examined for autofluorescence. Oospores of all *Pythium* spp. autofluoresced a bright yellow. Autofluorescence of sporangia and mycelium was a barely detectable dull green.

Slide cultures inoculated with *P. graminicola* and one other *Pythium* sp. isolate were stained with conjugated antiserum diluted 1:10. In mixed cultures, *P. graminicola* could be easily separated from those *Pythium* species that had been rated 1 to 2 in previous experiments. Mycelium of *P. aristosporum* and *P. graminicola* could not be separated nor could any difference be detected among *P.*

TABLE 1. Fluorescence ratings[a] of *Pythium* spp. isolates stained with conjugated antiserum diluted 1:2 and 1:10, and cross-adsorbed conjugated antiserum diluted 1:3.

	Conjugated antiserum diluted 1:2		Conjugated antiserum diluted 1:10		Cross-adsorbed, conjugated antiserum[b] diluted 1:3	
Pythium spp.	Mycelium	Sporangia	Mycelium	Sporangia	Mycelium	Sporangia
P. acanthicum Drechsler	4	4	3	3	2	2
P. aphanidermatum (Edson) Fitzp.	4	4	3	4	3	3
P. aristosporum Vanterpool	4	4	4	4	3	3
P. arrhenomanes Drechsler	4	4	3	4	1	1
P. dissotocum Drechsler	4	4	3	3	1	1
P. inflatum Matthews	4	4	3	4	0[c]	0
P. irregulare Buisman	2	3	1	1	0	0
P. mamillatum Meurs	2	2	1	1	0	0
P. oedochilum Drechsler	2	...[d]	1	...	0	...
P. oligandrum Drechsler	4	4	3	3	3	3
P. periplocum Drechsler	4	4	3	3	3	3
P. pulchrum Minden	2	2	1	2	0	0
P. rostratum Butler	2	2	1	1	0	0
P. spinosum Swade	2	2	1	1	0	0
P. torulosum Coker & Patterson	4	4	3	4	0	0
P. ultimum Trow	3	3	2	2	0	0
P. vanterpoolii V. & H. Kouyeas	4	4	3	3	0	0
P. vexans de Bary	2	2	1	2	0	0
P. graminicola Subram.	4	4	4	4	3	3

[a]All fluorescence ratings were based on at least six separate preparations. Little or no differences were obtained among preparations. Fluorescence ratings between minimal (rating 1) and intense (rating 4).

[b]Cross-adsorbed with *P. torulosum*.

[c]No fluorescence was detected.

[d]Sporangia were not formed.

graminicola isolates. Classifications based on fluorescence were confirmed by morphological characters wherever possible.

Conjugated antiserum that had been cross-adsorbed with *P. torulosum* was used to stain *Pythium* isolates as in previous experiments. Cross-adsorbed antiserum was prepared by mixing equal volumes of antiserum and *P. torulosum* antigens. The precipitate was removed by centrifugation and the antiserum was conjugated as before. *Pythium graminicola* was rated 3 when stained with the resulting preparation. Conjugated cross-adsorbed antiserum could be diluted 1:3 without any loss of fluorescence when used to stain *P. graminicola*. Fluorescence of the *Pythium* isolates after staining with the *P. torulosum* cross-adsorbed antisera indicated elimination of antibodies which were common to many of the isolates (Table 1). *Pythium* species that had been difficult to separate from *P. graminicola* in earlier fluorescence tests either did not fluoresce or fluoresced with low intensity with the exception of four *Pythium* isolates which fluoresced intensely and could not be distinguished from *P. graminicola* on the basis of mycelial examination.

With all stains, fluorescence appeared to be located primarily on the hyphal wall, with very faint fluorescence in the cytoplasm. Many of the *Pythium* spp. that were rated + to ++ fluoresced in an alternating pattern along the hyphae. Fluorescence of *Pythium* spp. rated 3 and 4 was evenly distributed with younger mycelium and sporangia fluorescing more intensely. Some areas of the slides had mycelium with fluorescence halos which indicated diffusion of antigens outward from the hyphae. Stains with conjugated normal antiserum indicated little or no nonspecific adsorption onto the mycelium or the slide surface.

DISCUSSION

A species-specific reagent that reacted only with *P. graminicola* was not produced in these studies with either selective dilution or cross adsorption. After cross adsorption, cross reactions still occurred with certain other species; and the fluorescence of *P. graminicola* was greatly reduced. Best results were with conjugated antiserum diluted 1:10. On the basis of fluorescence, *P. graminicola* isolates could be distinguished from all other *Pythium* isolates except *P. aristosporum*. This may relate to the fact that *P. graminicola* isolates and *P. aristosporum* were pathogenic on corn seedlings in greenhouse tests (D. G. White, *unpublished*) and are morphologically similar.

The results indicated some relationships among the *Pythium* spp. tested. *Pythium graminicola* has inflated filamentous sporangia as do *P. aphanidermatum, P. aristosporum, P. arrhenomanes, P. inflatum, P. periplocum, P. torulosum,* and *P. vanterpoolii.* Mycelium of all *Pythium* isolates with inflated filamentous sporangia were rated 4 when stained with conjugated antiserum diluted 1:2 and 3 or 4 with conjugated antiserum diluted 1:10. *Pythium acanthicum* and *P. oligandrum* have contiguous sporangia and were rated 4 when stained with conjugated antiserum diluted 1:2 and 3 with conjugated antiserum diluted 1:10. *Pythium dissotocum* has sporangia which are similar to filamentous hyphae or slightly thicker and was rated 4 when stained with conjugated antiserum diluted 1:2 and 3 with conjugated antiserum diluted 1:10. The other *Pythium* isolates have spherical sporangia and were rated 2 to 3 when stained with conjugated antiserum diluted 1:2 and 1 to 2 with conjugated antiserum diluted 1:10.

The results suggest that *Pythium* spp. with inflated filamentous sporangia are antigenically similar to *Pythium* spp. with filamentous sporangia and those with contiguous sporangia. *Pythium* isolates with spherical sporangia have antigens found in *P. graminicola,* but the antibodies to these antigens were not found in as high a concentration in the antiserum.

Even though a species-specific reagent was not obtained, limited studies indicated that *P. graminicola* could be detected among *Pythium* spp. and other fungi growing on glass slides from root lesions of corn seedlings. The technique would be of greater value if corn seedlings. The technique would be of greater value if oospores of *Pythium graminicola* could be identified. However, this was not possible due to autofluorescence. The technique may be utilized by plant pathologists to a greater extent after the serological relationships of the fungi have been studied in more detail.

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