# Immunofluorescence Microscopy for the Detection and Identification of Propagules of *Phaeolus schweinitzii* in Infested Soil

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## ABSTRACT

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In liquid culture, the fungus *Phaeolus schweinitzii*, which causes a root- and butt-rot of conifers, secretes a number of species-specific and strain-specific polypeptides which are detectable by dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focussing. Indirect immunofluorescence microscopy was used to detect the binding of species-specific antisera to these antigens to extracellular macromolecules secreted by the fungus, to the cell surface of basidiospores and chlamydospores, and to the cell surface and cross walls of mycelia. Common antigenic determinants were present in extracellular culture filtrate material and walls of mycelia, chlamydospores, and basidiospores. Indirect immunofluorescence, performed by using antisera to culture filtrate molecules has been used to demonstrate the presence of mycelium, and on occasions chlamydospores, in naturally and artificially infested soil samples. This makes possible identification of the kind of propagule most likely to be the source of field isolates of the organisms; this information, which cannot be obtained by using selective media, strongly suggests that the pathogen can survive saprophytically in the soil. In contrast, isolated mycelial cell wall preparations did not prove to be a suitable source of immunogenic material for these studies.

*Additional key words*: extracellular glycoprotein, *Polyporus schweinitzii*.

*Phaeolus schweinitzii* (Fr.) Pat. (=*Polyporus schweinitzii* Fr.) causes a root- and butt-rot in a wide range of conifers in both Europe and North America (3,12). Common forest species such as *Picea sitchensis* (Bong.) Carr., *Pseudotsugae menziesii* (Mirb.) Franco, *Larix* spp., and *Pinus* spp. are among the most susceptible. Damage is sporadic both within and between forests, but loss of timber within an infected tree may be considerable (6). Although infections may be initiated (presumably by hyphae developing from basidiospores) through wounds in aerial parts of trees, by far the most common mode of penetration in plantation forestry in Britain is through roots (5,6). The results of studies of the "strain" (genotype) distribution of this organism between trees has suggested that basidiospores are also involved in the infestation of the soil, and the subsequent colonization of roots (2). The presence of *P. schweinitzii* in soil samples can be determined by using a selective medium (1), but information on the form of the fungus propagule in the soil samples is necessarily lost.

The purpose of the studies reported here was to use serological techniques (10,11) to generate species-specific antisera against cell surface and extracellular immunogens of *P. schweinitzii*, and to test the utility of using these antisera in indirect immunofluorescence microscopy studies to determine the presence and form of propagules of the fungus in soil samples.

## MATERIALS AND METHODS

**Culture of *Phaeolus schweinitzii*.** Ten isolates of *P. schweinitzii* were obtained from the diseased stumps of 10 recently felled *P. sitchensis* in the Forest of Dean, U.K. While their gross morphological characteristics in plate cultures were distinct, they were indistinguishable microscopically. When paired with each other, sensu Barrett and Uscuplic (2), they produced a zone of antagonism suggesting that each isolate was a different genotype. Subcultures were maintained by culture on 5% malt extract agar plates. When required, the fungus was grown in liquid plate culture in a 5% malt extract (Oxoid; Basingstoke, U.K.) at 23 C for 28 days in darkness.

**Preparation of cell surface and extracellular antigens.** Mycelial mats were collected from liquid plate culture by filtration, washed three times with distilled water, weighed, chopped into small fragments, and frozen in liquid $N_2$. Culture filtrates and washings were dialyzed for 72 hr against five changes of distilled deionized water at 4 C, lyophilized, and stored at −20 C. Cell walls were prepared from mycelial mats by grinding the frozen fragments to a paste with sand in a mortar with a pestle. The cell walls were washed by suspension and centrifugation (20,000 $g_{avg}$ for 5 min) twice in 0.1 M potassium phosphate buffer (pH 7.2) and three times in distilled, deionized water. The washed walls were resuspended in water, kept at 4 C, and sonicated in 1.5-ml batches for 3 × 5 min with 5-min intervals in a Dawe type 6441A Soni-Cleaver. The walls were separated from the sonication-released material by centrifugation and the walls were resuspended in water and centrifuged. The supernatant from this wash was added to the initial sonicate extract, lyophilized and stored at −20 C. The remaining wall material was resuspended in water, autoclaved for 1 hr at 121 C, 103.4 kPa (15 psi) and centrifuged. The supernatant heat-released material was decanted, lyophilized, and stored at −20 C.

Typically a batch culture of fungus yielded: 20 g (wet weight) of mycelial mat, the cell walls of which provided 6 mg of sonication-released material and 15 mg of heat-released material; and 60 ml of culture filtrate containing 70 mg dry weight of material of which about 40% was protein as determined by a modification of the Lowry method (9).

**SDS-polyacrylamide gel electrophoresis.** Samples for electrophoresis were dissolved in dissociation buffer containing 0.01 M sodium phosphate buffer pH 7.0, 1% (w/v) sodium dodecyl

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sulphate (SDS) and 1% (v/v) 2-mercaptoethanol and heated at 100 C for 2 min. Slab (140 × 80 × 2.7-mm) polyacrylamide gel electrophoresis was performed with a 5% stacking gel and a 10 or 12% separation gel (8). Routinely, 1 mg of protein in 40 μl of sample was loaded per track and electrophoresed at 70V for 20 min and then at 150V for 5 hr. The electrode buffer was tris (0.05 M)-glycine (0.38 M) pH 8.4 containing 0.1% SDS. Gels were fixed overnight in methanol/glacial acetic acid/water (50:7:43, v/v) and then stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 in fixative and destained in methanol/glacial acetic acid/water (30:10:70, v/v). Glycoproteins in gels were detected by the periodic acid-Schiff's reagent technique of Zacharius et al (14). Molecular weight markers were obtained from British Drug Houses, Ltd., Poole, Dorset, England.

**Isoelectric focussing.** Isoelectric focussing was performed on LKB (Croydon, U.K.) PAG plates, pH interval 3.5–9.5 (5% gel, Ampholine concentration 2.4%) (13). Samples (0.25–0.5 mg dissolved in 10 μl $H_2O$) were applied directly onto the gel surface and focussed at constant power (15 W) for 1.5 hr at 4 C. Focussed (glyco)proteins were fixed for 1 hr in 30% (v/v) aqueous methanol containing 3.46% (w/v) sulfosalicylic acid and 11.5% (w/v) trichloracetic acid and stained with Coomassie Brilliant Blue. Determinations of pI gradient were by Pharmacia (Uppsala, Sweden) isoelectric focussing marker kit.

**Preparation of antisera.** One half milligram of antigen or washed mycelial fragments were suspended in 1.5 ml of phosphate-buffered saline (PBS) and thoroughly mixed with 1.5 ml of Freund's complete adjuvant (Calbiochem, La Jolla, CA). Rabbits were injected intramuscularly with 1 ml of antigen-adjuvant mixture in each hind leg. After 4 wk, booster injections using similar materials and quantities were given, and, after an additional 4 wk, each rabbit was bled by cardiac puncture. Serum was stored at −20 C or further fractionated by ammonium sulfate precipitation (7). The antisera were characterized by Ouchterlony diffusion and precipitin tests. Immunoelectrophoresis was by the method of Grabar and Williams (4). After electrophoresis of antigen (2 mg in 20 μl of tris-barbiturate buffer, pH 8.6) the immunoelectrophoretogram was developed by addition of 100 μl of antiserum to longitudinal troughs and incubation for 7 days at 100% relative humidity and 25 C.

**Indirect immunofluorescence microscopy.** Blocks (~1 $cm^3$) from the growing edge of mycelium cultured on 5% malt extract agar plates were removed and incubated under sterile conditions at 25 C on microscope slides for 7 days during which the mycelium grew across the slide from the agar block. The agar plug was then removed and the mycelium air-dried in situ for 60 min. Slides were either heat fixed or fixed in ethanol/chloroform/formalin (6:3:1, v/v) for 3 min, then placed in 95% ethanol for 4 min, distilled $H_2O$ for 0.5 min, air-dried, and used directly or stored at −20 C. The fixed mycelium was covered with a droplet of rabbit immunoglobulin G (IgG) culture filtrate or sonication-released material (usually diluted 1:20 with PBS), and incubated at 25 C in a moist chamber for 20 min. Excess test IgG was removed by two washes (5 min) in PBS before the mycelium area was covered with goat fluorescein isothiocyanate-labeled anti-rabbit IgG serum (diluted 1:10 with PBS) and incubated in a moist chamber in the dark for 20 min. Excess fluorescent antibody was removed by two washes in PBS, surface moisture was removed by gentle suction, and mycelium was mounted in 0.01 M sodium phosphate buffered glycerol pH 8.0.

For indirect immunofluorescence studies of soil samples, approximately 50 mg of soil was suspended in either a droplet of water or a droplet of Haupt's adhesive plus a droplet of 4% aqueous formalin, mixed, spread over the entire slide, and air-dried. Samples suspended in water were heat fixed. Both types of slides were either stored at −20 C or used directly for indirect immunofluorescence microscopy as described above. Samples from liquid grown cultures were prepared in a similar manner.

Natural forest soil, which had been artificially "seeded" with basidiospores of *P. schweinitzii* and incubated in a covered pot at room temperature for 2 mo was also examined by a combination of wet sieving and immunofluorescence microscopy (10). Samples (5 g) were washed through a series of wire screens of decreasing mesh sizes, and particles that passed through the 40-μm screen were collected on a 0.45 μm Millipore filter before transfer with a drop of water to a glass slide for immunofluorescence staining.

Slides were examined using either a Nikon or Leitz epi-fluorescence microscope with halogen or mercury vapor UV light sources, respectively. Photographs were taken using Ektachrome 200 ASA or 400 ASA films with exposure times of between 0.5 min and 5.0 min. Magnification was ×400.

## RESULTS AND DISCUSSION

**Selection of source of antigenic material.** No discrete polypeptides were observed on SDS polyacrylamide gel electrophoretograms of sonication-released or heat-released cell wall material stained with Coomassie Blue. However, electrophoresis revealed the presence of a number of polypeptides in the filtrates from liquid cultures of *P. schweinitzii* and comparison of strains 4, 6, and 9 demonstrated (glyco)polypeptides that were common to all strains examined and others of more restricted distribution (Fig. 1). This was confirmed by comparison of the distribution of extracellular (glyco)peptides after isoelectric focussing (Fig. 2).

Significant but minor changes in the pattern of extracellular (glyco)proteins on SDS-polyacrylamide gel electrophoretograms were observed during culture development. Despite clear strain differences there were, using both techniques, discernible, common patterns associated with extracellular polypeptides of *P. schweinitzii*. These patterns were different from those associated with the extracellular polypeptides of other fungal plant pathogens under study in this laboratory, eg, *Colletotrichum lindemuthianum* (Fig. 2) or more closely related species such as *Piptoporus* (= *Polyporus*) *betulinus* (Fr.) Karst., *Laetiporus* (= *Polyporus*) sulphureus (Fr.) Murri and *Meripilus* (= *Polyporus*) *giganteus* (Fr.) Pers. (Fig. 3). Hence in this system culture filtrates were biochemically an appropriate source of antigens for the generation of species- and strain-specific antisera.

**Characterization of antisera.** Antisera raised against the macromolecules present in culture filtrates of strains 4, 6, and 9 gave reactions of identity when tested against the respective culture filtrate antigens of these strains using the method of Ouchterlony. There were no spurs or other suggestions of less than complete antigenic identity. When antisera against culture filtrates from strains 6 and 9 were reacted with the culture filtrates from the other eight strains of *P. schweinitzii*, extensive serological identity was observed. In a number of cases there was evidence of more than one antigen in the culture filtrates. No reaction was observed in this test between these antisera and either sonication-released or heat-released cell wall antigen preparations.

Although antisera to culture filtrates were not strain-specific they appeared to be species-specific, giving no reaction with culture filtrate antigens from either unrelated phytopathogenic fungi such as *C. lindemuthianum* and *Phytophthora megasperma* f. sp. *glycinea* or more closely related species such as *L. sulphureus, M. giganteus*, and *Polyporus squamosus*. Similar results were obtained using precipitin tests.

Immunoelectrophoretic analysis of the interaction between culture filtrate material and the antisera raised against these antigens clearly showed the presence of at least four antigens common to the culture filtrates of strains 6 and 9 (Fig. 4). Furthermore, whereas antiserum raised against material from strain 6 was active against four antigens in culture filtrates of both strain 6 and strain 9, the antisera raised against material from strain 9 was active against only two of these antigens in the culture filtrates of both strains. This indicated the presence of more than one antigenic determinant in the culture filtrates of both strains.

Material released from cell walls by sonication gave antisera which were only weakly active in Ouchterlony and precipitin tests against sonication-released and culture filtrate material and inactive against heat-released material. The antisera were not strain-specific. Similarly heat-released wall material gave non-strain-specific antisera that were weakly active against heat-

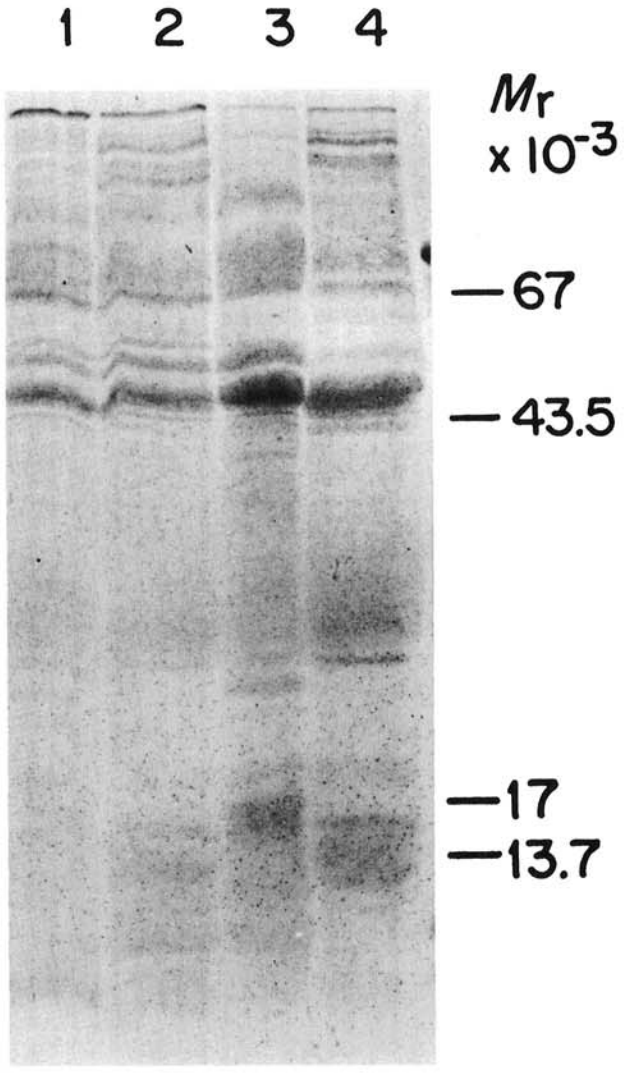


**Fig. 1.** Sodium dodecyl sulphate 17%-polyacrylamide gel electrophoresis of culture filtrate polypeptides of *Phaeolus schweinitzii*: lane 1—strain 4, 2 wk culture; lane 2—strain 4, 4 wk culture; lane 3—strain 6, 4 wk culture; and lane 4—strain 9, 4 wk culture.

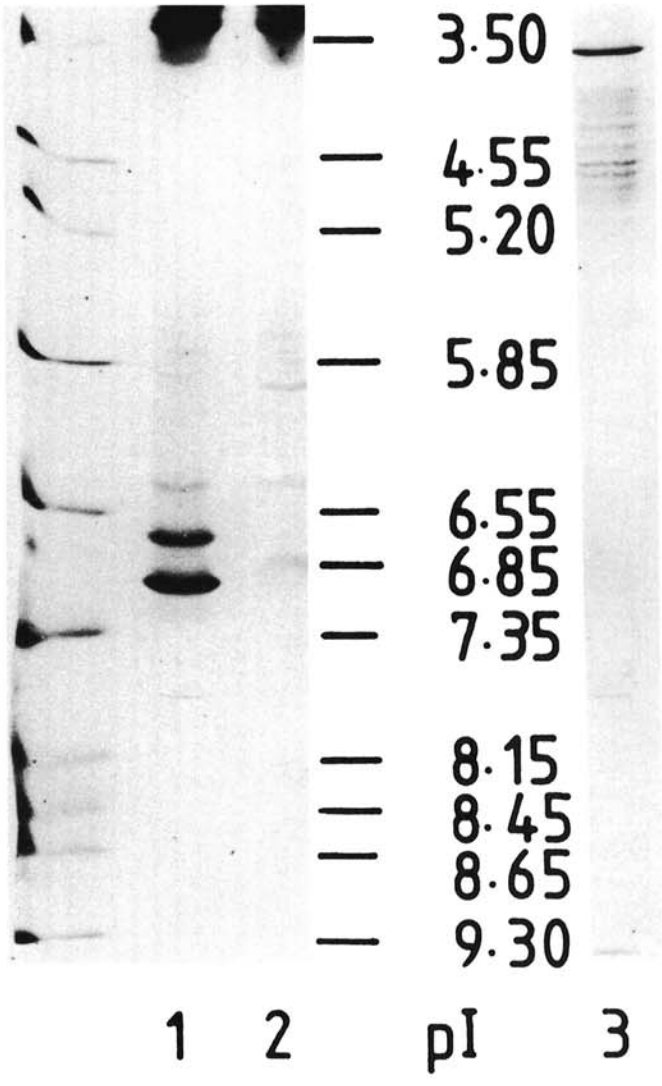


**Fig. 2.** Flat bed isoelectric focussing of culture filtrate polypeptides of *Phaeolus schweinitzii*: lane 1—strain 6; lane 2—strain 9; and lane 3—*Colletotrichum lindemuthianum* race $\beta$.

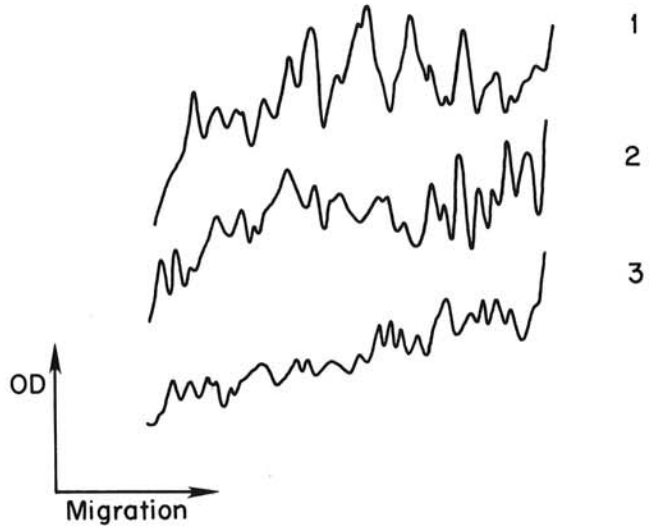


**Fig. 3.** Sodium dodecyl sulphate 12%-polyacrylamide gel electrophoresis of culture filtrate polypeptides of *Phaeolus schweinitzii* and closely related species. The patterns are represented by densitometer scans of culture filtrates from: curve 1—*Phaeolus schweinitzii* strain 9; curve 2—*Laetiporus sulphureus*; and curve 3—*Meripilus giganteus*.

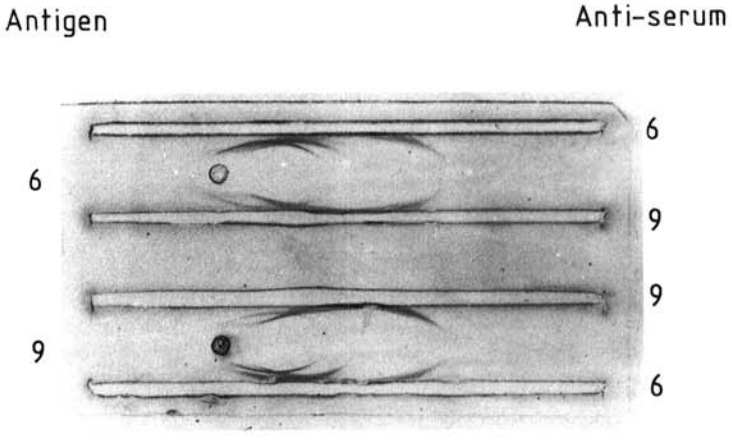


**Fig. 4.** Immunoelectrophoresis of culture filtrates of strains 6 and 9 of *Phaeolus schweinitzii*. The immunoelectrophoretograms were developed with anti-strain 6 and anti-strain 9 culture filtrate immunoglobulin G.

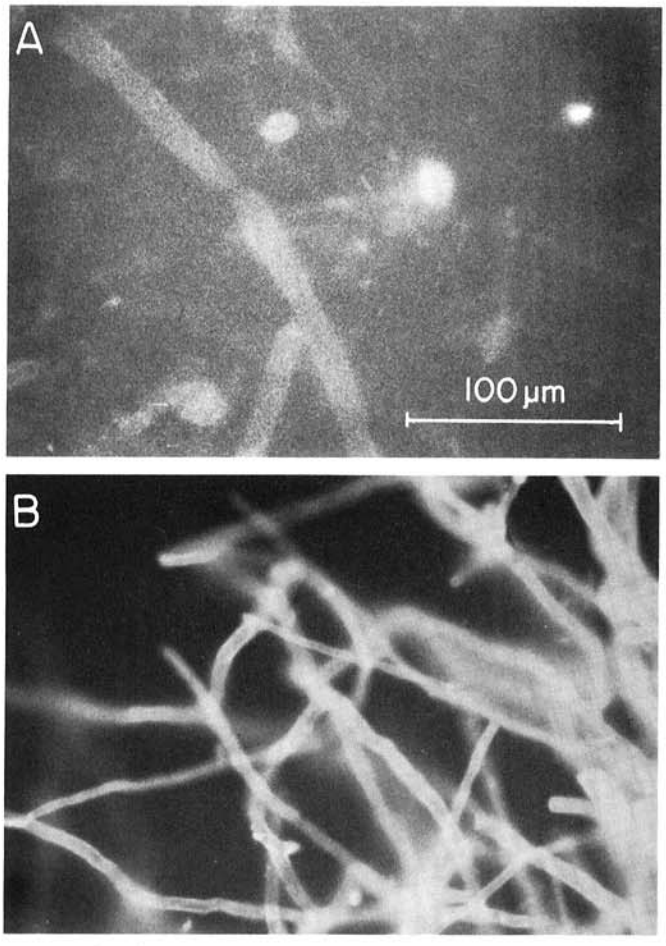


**Fig. 5.** Indirect immunofluorescence micrographs of mycelia of *Phaeolus schweinitzii* strain 1 grown in liquid culture. **A,** Pre-immune immunoglobulin G (IgG) and **B,** IgG against strain 6 culture filtrate. Both were diluted 1:20 with phosphate-buffered saline.

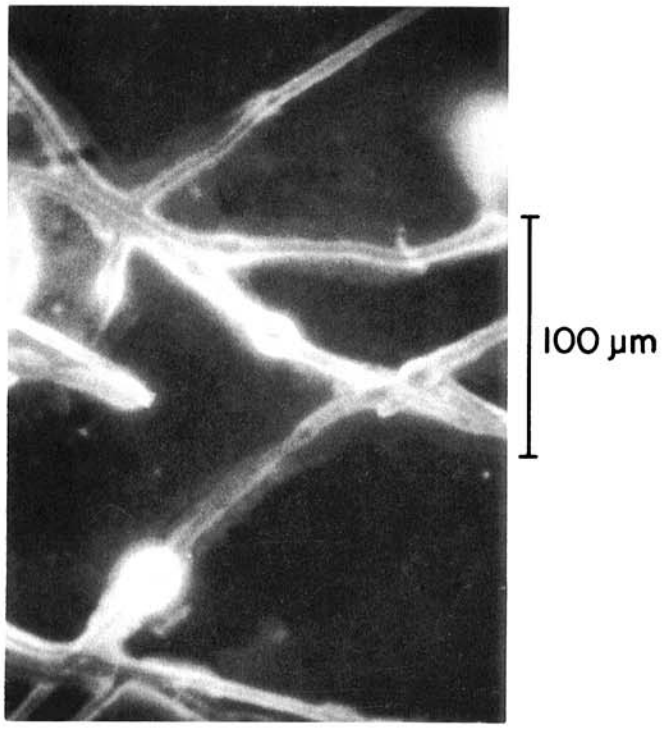


**Fig. 6.** Indirect immunofluorescence micrograph of mycelia of *Phaeolus schweinitzii* grown on a glass surface, using immunoglobulin G against strain 6 culture filtrate diluted 1:20 with phosphate-buffered saline.

released material and inactive against sonication-released and culture filtrate material in these tests.

**Indirect immunofluorescence microscopy of axenically cultured *P. schweinitzii*.** Rabbit anti-culture filtrate IgG bound extensively to mycelia of all 10 strains grown in liquid culture as judged by immunofluorescence microscopy using fluorescein isothiocyanate-labeled goat anti-rabbit IgG antiserum (Fig. 5). Routinely a 1:20 dilution of anti-culture filtrate IgG was used, since in dilution experiments this was found to be the lowest concentration giving extensive binding. An insignificant weak brown fluorescence was observed when control hyphae were examined under a UV light or when rabbit pre-immune serum was substituted for test IgG (Fig. 5). The anti-culture filtrate IgG also bound to the surfaces of basidiospores of *P. schweinitzii*, and to the surface of mycelium grown on a glass surface. In the latter case a halo of fluorescence was observed around the edge of the mycelium, indicating the presence of extracellular antigens secreted by the fungus (Fig. 6). The anti-culture filtrate IgG would bind only relatively weakly to the surface of mycelia obtained from liquid cultures of closely related species and when these species were grown on a glass surface there was no halo effect indicating absence of binding to extracellular molecules. These observations also demonstrated that the halo effect was not an artifact of fixation or a nonspecific binding of fluorescent-labeled antiserum. With unrelated species there was no binding either to extracellular macromolecules or the mycelial surface.

Mycelium of *P. schweinitzii* obtained from liquid culture was also stained by anti-sonication-released IgG, anti-heat-released IgG and antimycelial fragment IgG. Although there was binding to mycelium grown on a glass surface there was no halo effect.

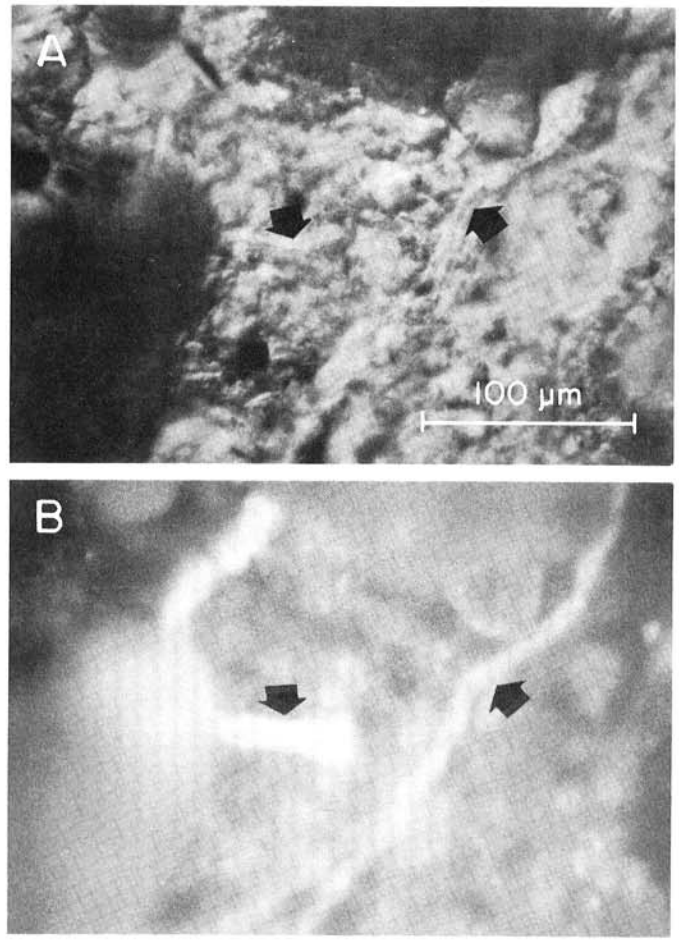


**Fig. 7.** Detection of mycelium of *Phaeolus schweinitzii* in a naturally infested soil sample: **A,** light micrography; **B,** indirect immunofluorescence micrograph obtained by using immunoglobulin G against strain 6 culture filtrate diluted 1:20 with phosphate-buffered saline. Arrows denote hyphae.

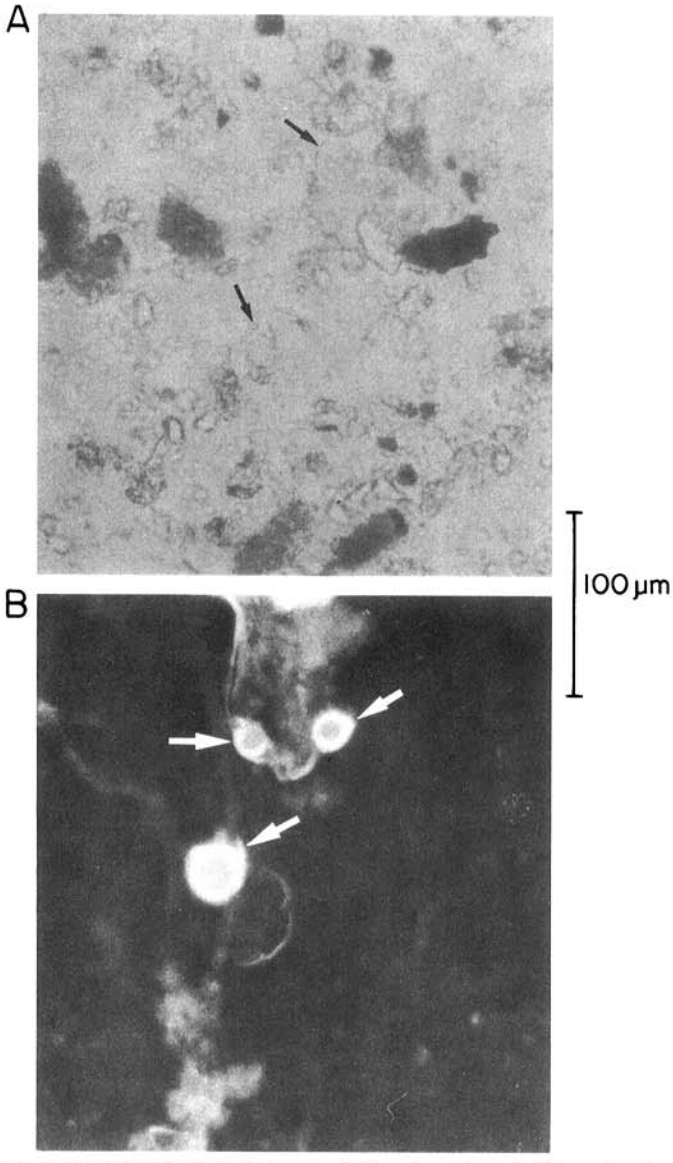


**Fig. 8.** Detection of chlamydospores of *Phaeolus schweinitzii* in a naturally infested soil sample: **A,** light micrograph; **B,** indirect immunofluorescence micrograph obtained by using immunoglobulin G against strain 6 culture filtrate diluted 1:20 with PBS. Arrows denote chlamydospores.

Hence there were molecules in mycelia that were antigenically related to extracellular antigens secreted by mycelia, and furthermore these molecules were antigenically distinct from the antigens released from mycelial cell walls by sonication or heat treatment.

**Indirect immunofluorescence microscopy of soil samples.** Taken together, the immunochemical and immunofluorescence microscope studies indicated that the antisera against culture filtrate material were the most appropriate for the detection of *P. schweinitzii* in soil samples.

In test soil samples to which mycelium, chlamydospores, and basidiospores had been added, basidiospores and chlamydospores could only be discerned by careful observation with the light microscope. IgG against culture filtrate material strongly and specifically bound to such structures, and the presence and morphology of these propagules were more clearly revealed by indirect immunofluorescence microscopy than by light microscopy.

Examination, by immunofluorescence microscopy, of natural soils from locations where the disease was known to occur revealed the presence of mycelia of *P. schweinitzii* in 14 samples (Fig. 7). All 14 samples tested independently by selective isolation into culture, proved to be positive for the presence of *P. schweinitzii*. A few

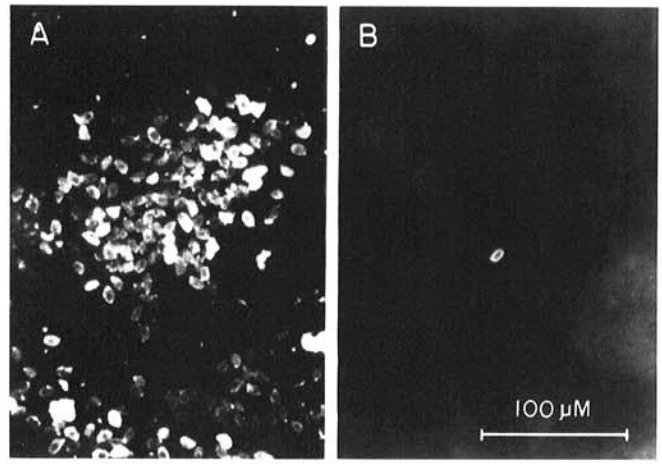


**Fig. 9.** Detection of basidiospores of *Phaeolus schweinitzii* by indirect immunofluorescence microscopy: **A,** preparation of purified basidiospores; **B,** soil artificially seeded with basidiospores.

chlamydospores were also observed in five of these samples (Fig. 8). No propagules were observed in several other samples, which later also proved to be negative when tested for the presence of *P. schweinitzii* by selective culture techniques, indicating an exact correlation between the two methods.

In soil artificially "seeded" with basidiospores and retained in the laboratory in a covered pot at room temperature for 2 mo, chlamydospores and mycelium, in addition to basidiospores, were found using indirect immunofluorescence microscopy coupled with both the direct slide and wet sieving techniques. Basidiospores were never seen in natural forest soils but only in laboratory and field soils that had been artificially "seeded" with basidiospores within the past 12 mo (Fig. 9). The wet sieving method proved inferior for examining this soil; while it had the advantages of removing some of the large particles, mycelia were necessarily fragmented and thus more difficult to recognize. Furthermore, it was impossible to separate out the clay particles that were the same size as chlamydospores (12–17 µm). Soils used in the present study contained a high proportion of such particles.

Chlamydospores, basidiospores, and mycelia of *P. schweinitzii* contain molecules antigenically related to species-specific antigens secreted by mycelia grown in liquid culture. The specificity of the antisera raised against these culture filtrate molecules has been used to determine the presence and form of the fungus in samples of naturally infested soil. Observations that mycelium and chlamydospores of *P. schweinitzii* are present in both natural forest soil and in soil artificially seeded with basidiospores of *P. schweinitzii* indicate that basidiospores can germinate in soil to produce a mycelium with chlamydospores. Mycelia and chlamydospores are, therefore, likely to be the propagules from which cultures are obtained via the selection medium. It is concluded that *P. schweinitzii* can exist saprophytically in the soil; thus, an opportunity for the generation of new strains (genotypes) sensu Barrett and Uscuplic (2) may occur prior to root infection. The longevity of the suggested saprophytic state in soil and its ability to extend from original sources of infection, are currently under investigation.

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