A Nylon Fabric Technique for Studying the Ecology of *Pythium aphanidermatum* and Other Fungi in Soil

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ABSTRACT

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A nylon fabric was used to support and recover propagules of *Pythium aphanidermatum* after burial in clay loam and sandy loam soils. Oospores were trapped in the fabric mesh, buried in soil, incubated, retrieved, and examined microscopically. Destruction of the oospores was estimated from percentages that appeared to be disintegrating and their viability was determined by dislodging them from the fabric and plating them on a medium selective for *Pythium*. Fungistasis of soil to oospores could be assessed by burying nylon-mounted oospores in asparagine-amended soil and nonamended soil and comparing germination. Lysis of mycelia of *P. aphanidermatum* and production of sporangia from mycelia buried in soil were determined by assessing the number of mycelial strands and digitate sporangia present in unit areas of fabric. The nylon fabric also allowed convenient examination of fungal structures by scanning electron microscopy. Other structures examined by the nylon fabric method were zoospores of *P. aphanidermatum*; oospores of *P. ultimum, P. myriotylum,* and *Aphanomyces euteiches;* mycelia of *Rhizoctonia solani;* sclerotia of *Sclerotinia minor*; microconidia of *Fusarium oxysporum;* and microsclerotia and conidia of *Verticillium dahliae.*

Additional key words: antagonism, soil ecology, microparasitism.

The study of the ecology of soilborne plant pathogens is sometimes limited by the techniques available. Observations on the behavior of microscopic fungal structures in soil are hampered by their small size, the opacity of soil, and the difficulty of recovering the structures from a large volume of soil. For these reasons, studies of survival of fungal propagules within soil have relied heavily on indirect methods such as baiting, plating dilutions of soils on selective media, or assessing disease incidence in infested soil. These methods supply valuable information, but do not allow direct observations of the physical or physiological states of survival structures or the soil biota that affect survival.

Methods of direct observations may give more specific information than indirect methods, but may also be difficult to use, or unreliable. Several direct methods are available. Glass slides, often coated with nutrients or test fungi embedded in agar (6), have been buried and retrieved. Similar methods involve burial of cellulose filter paper (6), cellophane (6), or membrane filters (1). All of these methods involve applying fungal structures to a solid substrate which may be difficult to view microscopically, may not adequately hold the structures, and may require the addition of interfering nutrients. Other techniques require application of test organisms to soil surfaces and recovery with adhesive cellulose acetate tape (11), plastic films (5), or water agar (5,9). These techniques have the advantage of introducing no additives or solid supports to the soil during incubation, but investigations are confined to the soil surface.

Glass or nylon fabrics have certain advantages for the study of fungi in soil. These fabrics provide a discontinuous surface that permits free movement of gases and water and allows penetration by roots and soil animals. They are flexible and can be packed tightly against the soil. Glass fiber tape has been used to study mycelia of *Phytophthora* spp. in soil (4), but a disadvantage is that the fibers are irregular in arrangement. Nylon fabric also has been used to study fungal propagules added to soil (3,9) as well as indigenous soil fungi (10). Recently, nylon fabrics with a wide range of uniform pore sizes with mesh made of nylon monofilaments have become available. The possibility of using these materials for mounting, burying in soil and observing fungal propagules was investigated and techniques were devised to study *Pythium aphanidermatum* (Ed.) Fitz. and other fungi in soil. A brief account of this technique has been presented (7).

MATERIALS AND METHODS

The primary isolate used in this study was *P. aphanidermatum* (PaB4) from a diseased bean plant grown in a soil from Salisbury, MD. Other isolates were: *P. ultimum* Trow (PuB5); *P. myriotylum* Drechs. (PmP3); *Rhizoctonia solani* Kühn (R-35); *Fusarium solani* (Mart.) Appel & Wr. f. sp. *cucurbitae* Snyd. & Hans. (FC-1); *Sclerotinia minor* Jagger (SS-13); and *Aphanomyces euteiches* Drechs. (Ae-7), all from the Soilborne Diseases Laboratory collection. *Verticillium dahliae* Kleb. (V-57) was obtained from J. Krikun.

Soils used were the loamy sand (designated soil A) and sandy loam (designated soil B) previously described (8) and were either used directly when collected from the field or steamed for 3 hr at 70 C. A silty clay loam and a sandy loam from Beltsville were used to assess the recovery of *P. aphanidermatum* oospores at −5 bars matric potential and at soil saturation (0 bars). Experiments were repeated at least once with at least four replicates.

Nylon fabric was obtained from Tetko, Inc. (420 Saw Mill River Road, Elmsford, NY 10523). Nitex® Swiss nylon monofilament fabric with a pore size of 25 μm was used for capturing oospores of *P. aphanidermatum* (oospore diameter 22–27 μm). Oospores were produced in V8-cholesterol medium as previously described (2). Cultures 2 wk-old were rinsed thoroughly in tap water, spread to dry on petri dishes, and blended in 25 ml of distilled water per culture mat in a Tekmar® blender. Most of the hyphae were removed by filtration through 100-μm pore-size nylon fabric. The resulting oospore preparation was diluted to 100 ml and contained about 10,000 oospores per milliliter. Nylon fabric was cut into 4.5-cm-diameter circles to fit a Millipore® (Millipore Corporation, Bedford, MA 01730) filter apparatus and was mounted on top of the filter support without a membrane filter. Oospores were drawn into the fabric with vacuum and the aid of a rubber policeman. Those oospores nearly the size of the fabric pores were caught, smaller ones passed through and larger ones were gently rinsed from the surface of the fabric. The nylon mesh disk containing oospores was cut into wedges about 1 cm at the widest portion by 2 cm long. The wedges were buried vertically and the soil pressed firmly around them. After incubation, the wedges were carefully retrieved, rinsed gently in water to remove soil particles, mounted on slides, stained with lacto-fuchsin, and examined. For germination tests, oospores were released from the fabric by vigorously pulling and stretching the fabric with forceps in 0.5 ml water. Drops containing oospores were plated on a *Pythium*-selective medium (2) and incubated at 30 C. Germinated oospores were counted after 18 hr.

Zoospores of *P. aphanidermatum* and structures of other fungi were trapped and buried in soil in fabrics having other pore sizes (Table 1), depending upon the propagule dimensions. Nitex® (Millipore Corporation, Bedford, MA 01730) fabric is available in 137 different pore sizes ranging from 1 μm to 5,000 μm. Thus, the size of the fungal structure to be held determined the pore size of fabric to be used.

The survival of mycelia in soil was studied by growing the fungi on media to produce sparse colonies intertwined among filaments of 100 μm pore-size fabric placed on the surface of the media. *P. aphanidermatum* was thus grown on the surface of cornmeal agar (CMA) and *R. solani* on the surface of one-half strength Difco potato dextrose agar (PDA). The cultures were incubated for 4 days at 30 C and the fabric containing mycelium was peeled off, cut into 1 × 3-cm wedges and buried in soil. The wedges were retrieved at 24-hr intervals, mounted, stained, and examined.

Oospores of *P. aphanidermatum* on 25-μm pore-size fabric were retrieved from soil and examined by scanning electron microscopy (SEM). The mesh containing oospores was rinsed gently in water, placed on moist filter paper in a petri dish and fixed with osmium tetroxide vapors (two drops 2% OsO_4 in a sealed dish for 24 hr). The oospore-nylon preparation was dehydrated in an ethyl alcohol series, critical-point dried, and mounted on SEM stubs. The samples were coated with 20–30 nm of gold-palladium in a Technics Hummer V sputter-coater device. The samples were viewed in a Hitachi HHS-2R scanning electron microscope operated at 10 or 15 kV.

RESULTS

Oospores of *P. aphanidermatum* were securely caught in the 25-μm pores of the nylon mesh (Fig. 1A). In addition, the frequency of retrieval of the oospores in the fabric from soil was consistently high (Table 1). Few oospores were lost from the nylon mesh when they were buried either in moderately dry (~−5 bars) silty clay loam or in sandy loam or in those soils saturated with water. The frequency of recovery was greater than 90% in all cases. The oospores recovered from saturated soils were less clearly viewed than those from the drier soils, because soil particles adhered to the oospores and the fabric in the moist soil. Many oospores recovered from moist soils could be clearly viewed, however. The weave of the nylon fabric afforded a convenient pattern for systematically viewing the microscope field and counting the oospores.

The frequency of recovery of zoospores of *P. aphanidermatum* and oospores of other *Pythium* spp. and *A. euteiches* also was high (Table 1). Irregularly shaped propagules of other fungi were not as readily trapped in the nylon mesh as were oospores. Microsclerotia of *V. dahliae* were not caught in 100-μm pore-size nylon but small fragments were trapped in 30-μm pores. Conidia of this fungus filled the pores of 1-μm fabric. The conidia remained in the fabric after burial in soil, but recovery was not assessed. Microconidia of *F. solani* f. sp. *cucurbitae* also were recovered from soil with the 1-μm pore-size fabric. Sclerotia of *S. minor* were not easily trapped in the 1,000-μm nylon mesh by filtration procedures. Those caught in the pores became easily dislodged. Certain sclerotia with a slight constriction in the center of the propagule were selected from cultures and could be securely forced into the fabric opening. Although tedious, this procedure was used successfully to trap, bury, and recover sclerotia from soil for viewing with the SEM.

Sparse mycelia of *P. aphanidermatum* and *R. solani* on nylon mesh were successfully contained on the 100-μm pore-size fabric (Table 1). Dense mats of mycelium could be produced on the mesh and buried in soil, but the viewing of individual hyphal strands was hampered by the density of the mat.

Germination and destruction of oospores, lysis of mycelia, and formation of sporangia of *P. aphanidermatum* were observed following burial in a sandy loam (soil A) and in a loamy sand (soil B) (Table 2). Lysis of mycelia on 100-μm pore-size fabric was much greater in soil A than in soil B: after 3 days of burial at 30 C, 86% of the hyphae originally contained on the mesh had lysed in soil A, but

TABLE 1. Comparison of percentage of pores filled and frequency of recovery from soil of propagules of soilborne fungal pathogens trapped in nylon monofilament fabrics with different pore sizes

Fungus species and propagule type[a]	Pore size of fabric (μm)	Pores filled (%)	Recovery (% of control)
Pythium aphanidermatum			
oospores	25	76	99
zoospores	10	73	96
mycelium	100	100	96
P. ultimum			
oospores	20	83	98
P. myriotylum			
oospores	30	42	89
Aphanomyces euteiches			
oospores	30	50	85
Fusarium solani f. sp. *cucurbitae*			
microconidia	5	60	...[b]
Rhizoctonia solani			
mycelium	100	100	...[b]
Verticillium dahliae			
microsclerotia	30	10	...[b]
conidia	1	100	...[b]
Sclerotinia minor			
sclerotia	1000	few[c]	100

[a] Propagules were mounted in pore spaces by vacuum filtration and buried in soil for 1 day to 2 wk.

[b] Buried in soil, examined after recovery, but not counted.

[c] Only selected sclerotia with a constriction in middle could be lodged securely in the mesh.

only 7% had lysed in soil B. Also, more digitate sporangia formed from hyphae in soil B than formed in soil A. The sporangia were clearly visible even in areas where the mycelium had lysed (Fig. 1B).

Germination of oospores was assessed in these two soils by determination of the percentage of oospores of *P. aphanidermatum* that germinated in soil when buried on 25-μm pore-size nylon and incubated in soil amended or unamended with 0.01% asparagine. Little or no germination occurred in nonamended soil. In contrast, soil A supported considerable germination when amended, but soil B did not.

Destruction of oospores occurred equally in both soils. Microscopic examination of oospores with SEM revealed microorganisms both on and within the oospores and collapse of oospore walls. Frequently oogonial and oospore walls adhered to the nylon filaments after contents had disintegrated. Fungi frequently were associated with the oospores (Fig. 1C) as were

TABLE 2. Lysis of mycelia, formation of sporangia, germination of oospores, and destruction of oospores of *Pythium aphanidermatum* in soil

Soil type	Mycelial lysis[a] (% of control)	Formation of[b] sporangia/0.1 mm^2	Oospore germination[c] (%)		Oospore destruction[d] (%)
			With asparagine	Without asparagine	
Sandy loam (Soil A)	86	0.2	38	1	35
Loamy sand (Soil B)	7	6.7	8	0	44

[a] Mycelium grown on nylon mesh (100-μm pore-size) on cornmeal agar and buried in soil for 3 days at 30 C was recovered and hyphae in ten 400 μm^2 areas of nylon fabric were counted and averaged. Counts were based on the mycelia contained in the same unit areas on mesh not buried in soil.

[b] Numbers of digitate sporangia formed on 100-μm pore-size nylon fabric during assay of the mycelial lysis described above in footnote a.

[c] Oospore germination in situ on 25-μm pore-size nylon mesh placed in soils amended or unamended with 0.01% asparagine, incubated for 18 hr at 30 C, retrieved, mounted, and examined microscopically.

[d] Percentage of total oospores enmeshed in 25-μm pore-size nylon fabric that were collapsed, disintegrated or otherwise deteriorated after 3-wk of incubation in soil at 25 C.

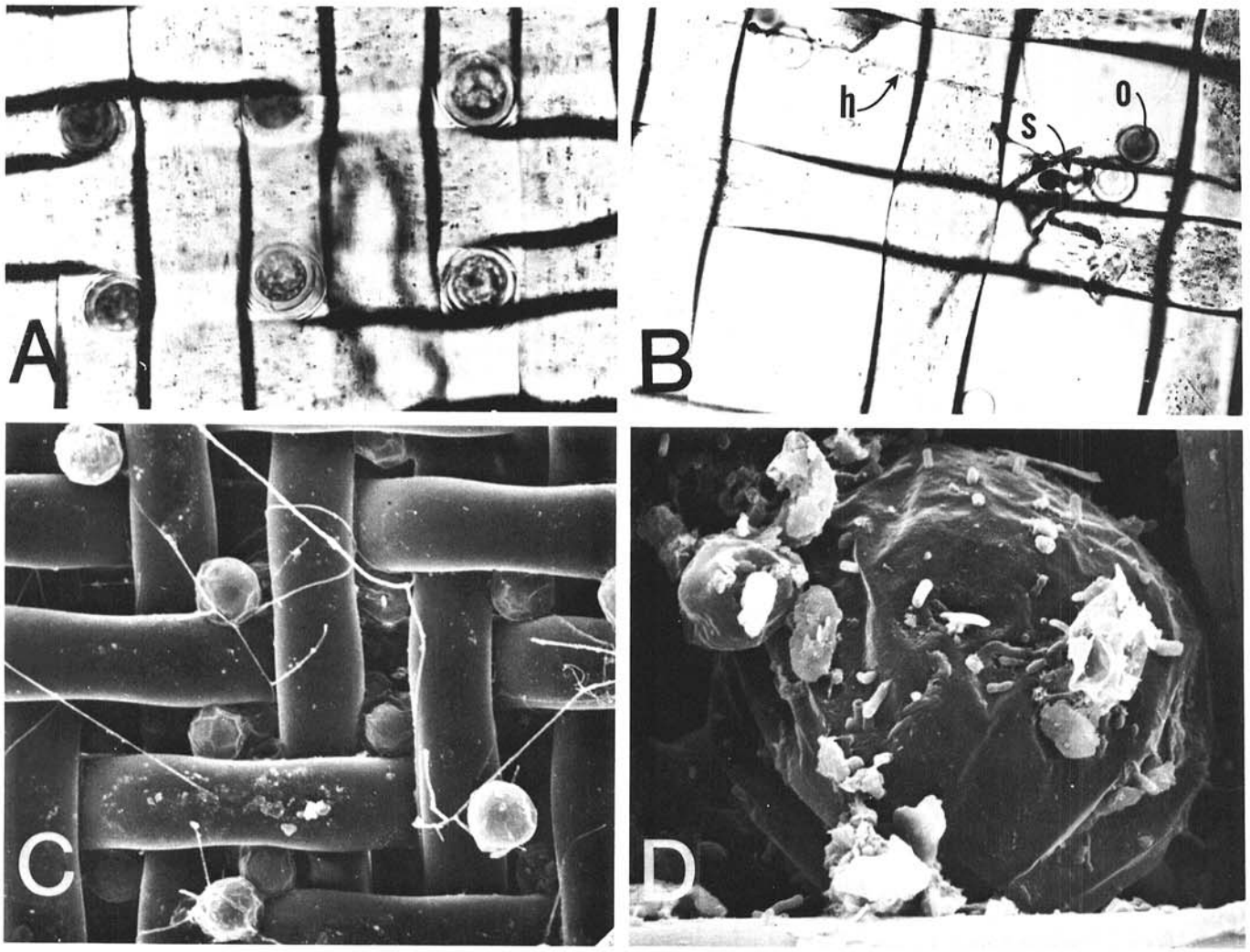

Fig. 1. Propagules of *Pythium aphanidermatum* on nylon fabric after burial and retrieval from soil. **A,** Oospores of *P. aphanidermatum* caught in the mesh openings of 25-μm pore-size fabric after burial in soil for 2 wk (×470). **B,** Mycelium of *P. aphanidermatum* undergoing lysis after burial for 3 days on 100-μm pore-size nylon, and the subsequent formation of sporangia and oospores (×280). Before burial each square contained 10–20 mycelial strands, O = oospore, S = sporangium, H = remaining hyphal strand. **C,** Scanning electron micrograph (SEM) of oospores of *P. aphanidermatum* and associated soil fungi after burial in soil for 2 wk on 25-μm pore-size nylon (×460). **D,** SEM of oospores of *P. aphanidermatum* and associated bacteria after burial in soil for 2 wk on 25-μm pore-size nylon (×3,800).

rod-shaped bacteria (Fig. 1D). Oospores buried in steamed soils were not destroyed during the incubation period.

The germinability of buried oospores was estimated by removing the oospores from the nylon and counting germinated oospores after incubation on a *Pythium*-selective medium. Percentages of germination agreed well with percentages of oospores that appeared to be viable prior to plating. Oospores removed from the nylon were also streaked on 2% water agar. Fungi and actinomycetes grew into the water agar after 24 hr of incubation at room temperature. The colonies appeared to originate from within oospores. When they grew into areas of the medium where the suspensions had not been streaked, hyphal strands were readily isolated, often in pure culture.

DISCUSSION

Nylon monofilament fabric provides a convenient, versatile, and reliable method for study of the ecology and survival of *P. aphanidermatum* propagules in soil. This technique offers several advantages over other methods: it can be used to assess lysis, fungistasis (as measured by germination in soil), microparasitism, and production of secondary survival structures, such as sporangia; various pore sizes can be selected to accomodate different sizes of propagules, including those of soilborne fungi other than *P. aphanidermatum*; the mesh is easily filled with propagules, can be cut into conveniently sized pieces, is flexible for a tight fit against soil particles, and provides a discontinuous inert surface which is considered an advantage over solid support structures (6); propagules are usually firmly held by the mesh, are readily retrieved from soil, and the mesh provides a convenient grid for making counts in the microscopic field; propagules can be freed from the mesh after recovery from soil and their viability can be determined by plating on germination and growth media; and antagonistic microorganisms associated with deteriorating propagules can be viewed microscopically and can be isolated in pure culture.

Certain disadvantages or limitations also are apparent with this method: some propagules, such as oospores that have germinated on the mesh in soil, may be lost, especially if hyphae have grown into the soil and dislodge the oospores when the mesh is removed from the soil; visibility is sometimes obstructed, especially when the soil moisture content is high; the technique selects similar sized propagules which may be a disadvantage in fungal species with a wide range of propagule dimensions; irregularly-shaped propagules such as sclerotia of *S. minor* are not easily loaded into the mesh and are not always held firmly in place by the filaments. Limited application of the technique to the study of sclerotia is possible, however, by inserting selected sclerotia into the pores.

This method provides a convenient, versatile, and reliable means for studying the ecology of *P. aphanidermatum* and other soilborne pathogens in soil. A detailed account of the contrasting behavior of *P. aphanidermatum* in the two soils designated soil A and soil B using nylon fabric is in preparation.

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