PHYTOPATHOLOGICAL NOTES

A Quantitative Method for the Isolation of Pythium ultimum from Soil

M. E. Stanghellini and J. G. Hancock

Research Assistant and Assistant Professor, respectively, Department of Plant Pathology, University of California, Berkeley 94720.

Three sphaerosporangiate *Pythium* spp., *P. ultimum* Trow, *P. debaryanum* Hesse (4), and *P. irregulare* Buis., are primarily associated with pre- and post-emergence damping-off of seedlings. *P. ultimum,* however, is the most common pathogenic species encountered. Although several quantitative methods have been reported for the direct isolation of *Pythium* spp. from soil (3, 5, 6, 7), those methods exhibit little or no selectivity with regard to specific species within the genus *Pythium,* require special antibiotic media, and are time-consuming. The purpose of this investigation was to develop a rapid and accurate method for the direct isolation of *P. ultimum* from soil. Two biological characteristics of this species, rapid propagule germination and high growth rate, were utilized in attaining this purpose (8).

A naturally infested Salinas Valley sandy loam agricultural soil (pH 5.9) was used throughout the study unless otherwise specified. Soil dilutions (g soil/ml sterile distilled water) of 1:50, 1:100, and 1:200 were thoroughly mixed for 2 min on a Vortex tube mixer. One-ml aliquots of the mixture were removed immediately and dispensed as small drops on the surface of 3-day-old 2%-water agar in petri plates. Three-day-old agar plates enhanced the rapid absorption of water from the drops, and facilitated the growth of *Pythium* into the agar. In this study, each ml was dispensed in 40 drops, 10 drops/plate. One drop was placed in the center and nine drops around the periphery of the agar plate. The size and number of drops used per plate is governed by the *Pythium* population. Fewer and larger drops are satisfactory when dealing with low populations, whereas numerous small drops are better for high populations. Plates were incubated at 24 C and read after 18-24 hr. Readings, made under low power (×10) on a dissecting microscope with fluorescent illumination, consisted of counting the number of hyphal strands emerging from the perimeter of each drop. Hyphae of *P. ultimum* were readily distinguished from other fungal hyphae by their rapid growth and tendency to grow in a straight line away from the drop (Fig. 1). To minimize errors in counting, soils containing high populations of *Pythium* should be diluted so that not more than four hyphal strands occur per drop.

Our studies showed a linear relationship between the number of *Pythium* hyphae emerging from the drops and the soil dilution (Fig. 2). Microscopic examination showed that 96% of the emerging hyphal strands originated from single spores, and branching within the drop accounted for the remainder (Table 1). Hyphal tip isolations consistently yielded pure cultures of *Pythium* spp. Bacteria and other contaminants were eliminated by the subsurface hyphal growth of *Pythium* through the agar. Identifications were made on rolled oat agar (2). Of 192 isolates, 190 were identified (4) as *P. ultimum* and two as *P. aphanidermatum* (Edson) Fitzp.

The term "spore" is used as the exact nature of the *Pythium* propagule (oospore or sporangium) cannot be determined after germination (1, 9). Microscopic examination of soil smears prior to spore germination revealed the presence of thin-walled sporangiallike bodies which, upon recovery and subsequent culturing, were identified as *P. ultimum*. Although no thick-walled oospores were observed, the possibility that they were present in low numbers in the soil and contributed to plate counts cannot be eliminated.

To determine the percentage recovery from soil, known numbers of sporangia of *P. ultimum* were added to a sterilized and to a naturally infested field soil. Recovery of *P. ultimum* from the sterilized and from the naturally infested soil, after correcting for the background population of *P. ultimum,* ranged from 87-100%.

Pimaricin was added to water agar (10 μg/ml) to determine if a selective antibiotic would increase the efficiency of *Pythium* recovery from field soil. Recovery was no more efficient. Comparison of *Pythium* spp. isolated by both methods showed no selective species retrieval by the addition of pimaricin. It was also observed that pimaricin tended to enhance the rate of growth and the degree of hyphal branching within the drop, making counting more difficult. Addition of nutrients to water agar supported extensive mycelial growth of *Pythium* and made it impossible to count single hyphal strands.

P. debaryanum Hesse (ATCC 9998), *P. mamillatum* Meurs (ATCC 1121), *P. irregulare* Buis., *P. splendens* Braun, and four isolates of *P. ultimum* were separately incorporated into moist field soil to determine if these sphaerosporangiate species could be quantitatively recovered. Five-mm discs were removed from 2-day-old potato-dextrose agar cultures of each species and placed in petri dishes containing 20 ml sterile distilled water and two rolled oat seeds. Plates were incubated for 2 weeks at 24 C; the mycelial mats were then removed, washed, placed in nonsterile, moist field soil, and incubated for 2 weeks. The mycelium was lysed, and only oospores and/or sporangia were observed in the soil after 2 weeks. Samples from each artificially infested soil were processed as described above.

Results showed that only *P. ultimum* and *P. splendens* exhibited the characteristic rapid hyphal growth and tendency to grow in a straight line away from the drop. Hyphal strands of both species originated from sporangia. A linear relationship between the number of hyphae emerging from the drops and the soil dilution was shown for both species. All other *Pythium* spp. exhibited a slower growth rate and required an incubation period up to 40 hr. Extensive hyphal branching within the drop prevented the quantitative aspect of counting single hyphal strands.

Natural populations of *P. ultimum* from 64-3,800 propagules/g of soil were recorded from agricultural clay loam and sandy loam soils. A population of 13,000

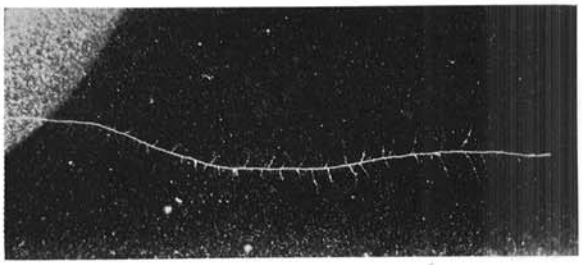

Fig. 1. Hyphal strand of *Pythium ultimum* emerging from a portion (upper left corner) of a drop of an aqueous soil suspension placed on water agar. Hypha originated from a sporangium. This photograph was taken after 18-hr incubation at 24 C. (×30)

propagules of *P. splendens*/g of soil was recorded from the rhizosphere of a diseased Easter lily plant.

Several selective methods for the isolation of *Pythium* and/or *Phytophthora* spp. from soil have been reported (3, 5, 6, 7). Only two of those techniques yielded a quantitative estimation of the *Pythium* spp. population (3, 7) as high as obtained in the present study, using the same naturally infested field soil. However, the toxicity of photoreactants originating from the rose bengal in those media rendered isolation and species identification unfeasible.

The technique described in this study has several desirable features in the isolation of certain *Pythium* spp.: (i) it is simple, rapid, and requires only water agar; (ii) quantitative population estimations are possible for *P. ultimum, P. splendens,* and possibly *P. aphanidermatum;* (iii) pure cultures are directly obtained by hyphal tipping; and (iv) the linear relationship between the *Pythium* population and the range of soil dilutions indicates that competitive effects on the plate (a persistent problem with conventional soil dilution plates) do not significantly interfere with the accuracy of this method. Although the method detected *P. splendens* and *P. aphanidermatum* as well as *P. ultimum,* it is possible to distinguish among these species directly on the plate. With experience, characteristic patterns of hyphal growth can be used as a means of identifying those *Pythium* spp. that exhibit a rapid growth.

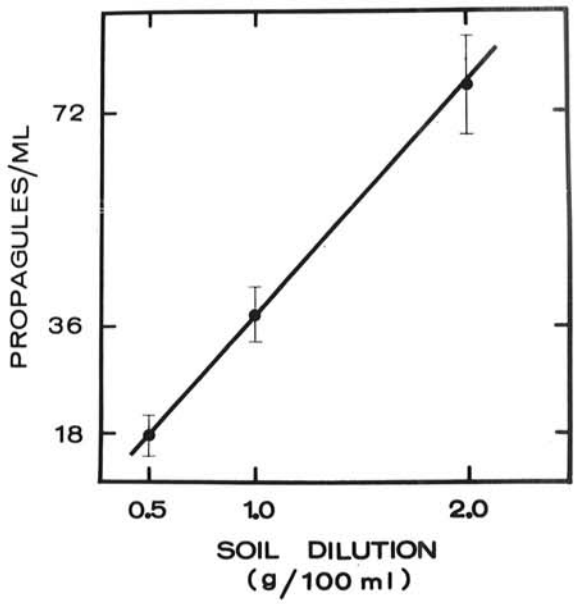

Fig. 2. Relationship between *Pythium ultimum* population and the soil dilution. The mean and standard error of five separate soil samples, each replicated three times, are plotted.

TABLE 1. Origin of hyphal strands of *Pythium ultimum* emerging from drops of an aqueous soil suspension placed on water agar in petri dishes after 18-hr incubation at 24 C

Experiment	Drops	Hyphae	Hyphae traced to single spores[a]	% Association of single hyphae with single spores[a]
1[b]	69	110	105	95.4
2	62	91	89	97.8
3	23	43	39	90.7
4	62	91	90	98.9
Total	216	335	323	Avg 95.7
5[c]	150	243	234	96.3

[a] The term "spore" is used as the nature of the propagule (oospore or sporangium) cannot be determined after germination.

[b] Experiments 1-4 were made using a naturally infested Salinas Valley sandy loam agricultural soil.

[c] Experiment 5 was made using a nonsterile field soil artificially infested with sporangia of *P. ultimum*.

LITERATURE CITED

1. DRECHSLER, C. 1946. Zoospore development from oospores of *P. ultimum* and *P. debaryanum* and its relation to rootlet-tip discoloration. Plant Dis. Reptr. 30:226-227.
2. GOODING, G. V., & G. B. LUCAS. 1959. Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. Phytopathology 49:277-281.
3. KERR, A. 1963. The root rot-*Fusarium* wilt complex of peas. Australian J. Biol. Sci. 16:55-69.
4. MIDDLETON, J. T. 1943. The taxonomy, host range and geographical distribution of the genus *Pythium*. Mem. Torrey Botan. Club 20:1-171.
5. OCANA, G., & P. H. TSAO. 1966. A selective medium for the direct isolation and enumeration of *Phytophthora* in soil. Phytopathology 56:893 (Abstr.).
6. SCHMITTHENNER, A. F. 1962. Isolation of *Pythium* from soil particles. Phytopathology 52:1133-1138.
7. SINGH, R. S., & J. E. MITCHELL. 1961. A selective method for isolating and measuring the population of *Pythium* in soil. Phytopathology 51:440-444.
8. STANGHELLINI, M. E., & J. G. HANCOCK. 1969. Role of sporangia in the survival of *Pythium ultimum*. Phytopathology 59:1051 (Abstr.).
9. TROW, A. H. 1901. Observations on the biology and cytology of *Pythium ultimum* n. sp. Ann. Bot. 15: 269-312.