Isolation and Characterization of a Metalaxyl-Insensitive Isolate of *Phytophthora megasperma* f. sp. *medicaginis*

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ABSTRACT

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Investigations on the survival and activity of *Phytophthora megasperma* f. sp. *medicaginis* in a naturally infested, nonsterile field soil were hampered by lack of specificity of the alfalfa seedling bait bioassay, interference by fast-growing *Pythium* spp., and inability to distinguish between added and indigenous propagules of *P. m.* f. sp. *medicaginis*. An isolate (Pm20) of the pathogen with insensitivity to the fungicide metalaxyl was obtained without mutagenesis by screening encysted zoospores on an agar medium amended with metalaxyl. Except for insensitivity to metalaxyl, Pm20 did not differ from the wild type in total growth, growth rate, sporulation, propagule germination, or pathogenicity to alfalfa. Pm20 was unique in its insensitivity to metalaxyl from 41 isolates of *P. megasperma* from nine hosts in six states. A relationship was observed between colony morphology on cornmeal agar, sensitivity to metalaxyl, and pathogenicity to alfalfa. Pm20 was easily recovered after 6 wk in a nonsterile field soil on an antibiotic medium amended with metalaxyl. There was no growth of *P. m.* f. sp. *medicaginis* on this medium from soil to which Pm20 had not been added. By using Pm20 and incorporating metalaxyl into an antibiotic recovery medium selective for *Phytophthora*, the obstacles of assay nonspecifity, interference by *Pythium* spp., and lack of distinction between added and indigenous *P. m.* f. sp. *medicaginis* were overcome.

Additional key words: ecology, Phytophthora root rot, survival

The life history of a soilborne pathogen can be determined most accurately by using a soil in which the pathogen exists naturally. In such a soil the pathogen will have evolved strategies to ensure its persistence. Ecological studies frequently involve adding propagules of an organism to soil and assessing its behavior over time. If an unsterilized field soil is used, it is often difficult to distinguish the added propagules from propagules of that same organism or of closely related and morphologically similar organisms naturally present in that soil (27,28). This problem arose in studies on the survival of the alfalfa root pathogen *Phytophthora megasperma* f. sp. *medicaginis*.

P. megasperma is a member of the oomycete family Pythiaceae (30). Other pythiaceous fungi, especially *Pythium* species, commonly inhabit the same soil environment as *P. megasperma* (9,17,22,24,27). Some of these pythiaceous fungi have much greater growth rates on media than does *P. megasperma* and can seriously interfere with its recovery from soil.

The most frequently used assay for studying the ecology and survival of *P. m.* f. sp. *medicaginis* has been the alfalfa seedling bait bioassay (17,22,32). However when this assay was used to study survival of *P. m.* f. sp. *medicaginis* in alfalfa field soils in New York, it lacked specificity for the alfalfa pathogen; isolates of *P. megasperma* not pathogenic to alfalfa, as well as other *Phytophthora* species, could not be distinguished from *P. m.* f. sp. *medicaginis*.

To facilitate the study of *P. m.* f. sp. *medicaginis* within its natural soil environment, a marker was sought that would allow distinction of experimental propagules of the pathogen from the indigenous population and would improve the selectivity of its recovery from soils in which the faster growing pythiaceous fungi were present. The acylalanine fungicide metalaxyl is toxic to many pythiaceous fungi (29) including *P. m.* f. sp. *medicaginis* (6). Since native tolerance to metalaxyl has been reported for some *Phytophthora* species (14,21), we investigated the possibility of using metalaxyl insensitivity as a marker for *P. m.* f. sp. *medicaginis*.

For a marked isolate to be useful for survival studies in field soil, it should be identical to the wild type in all respects except sensitivity to metalaxyl. It also should be unique in its insensitivity to metalaxyl from other *Phytophthora* isolates that might be present in these soils. Accordingly, the objectives of this study were to obtain an isolate of *P. m.* f. sp. *medicaginis* with insensitivity to metalaxyl, to characterize this isolate with respect to sensitive *P. megasperma* and other isolates of *Phytophthora*, and to determine its suitability for studying survival of *P. megasperma* in a soil naturally infested with the pathogen. A preliminary report of this work has been published (26).

For the purpose of communication, the "forma specialis *medicaginis*" designation for isolates of *P. megasperma* Drechs. pathogenic to alfalfa has been adopted (7,16), despite problems that may be associated with this concept for *P. megasperma* (1,11).

MATERIALS AND METHODS

Isolates and media. Forty-one isolates of *P. megasperma* from nine hosts in six states and four other *Phytophthora* species were used. These included 12 isolates from alfalfa, *Medicago sativa* L. (eight obtained by R. Millar, J. Stack, H. Wilkinson, Department of Plant Pathology, Cornell University, Ithaca, NY; two from A. Frosheiser, ARS, USDA, St. Paul, MN; one from D. Erwin, Department of Plant Pathology, University of California, Riverside; one from D. Maxfield, Department of Plant Pathology, University of Wisconsin, Madison); three isolates from arrowleaf clover, *Trifolium vesiculosoum* Savi. (from R. Pratt, ARS, USDA, Department of Plant Pathology and Weed Science, Mississippi State University, Mississippi State); three from soybean, *Glycine max* (L.) Merr. (from S. Cohen, Department of Botany and Plant Pathology, Michigan State University, East Lansing); 10 isolates from apple, *Malus domestica* Borkh. (from S. Jeffers, Department

Because of an error during the electronic processing of this manuscript, the pathogen trinomial was incorrectly printed as *P. m.* f. sp. *megasperma* instead of *P. m.* f. sp. *medicaginis* when this paper originally appeared in the September issue of *Phytopathology* (pp. 1014-1019). The article is reprinted here in entirety with correct pathogen nomenclature.

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of Plant Pathology, New York State Agricultural Experiment Station, Geneva); five isolates from cherry, *Prunus avium* L. (four from S. Mircetich, ARS, USDA, Department of Plant Pathology, University of California, Davis, and one from J. Hayes, Department of Plant Pathology, New York State Agricultural Experiment Station, Geneva); eight weed isolates (three from white clover, *Trifolium repens* L.; two from wild carrot, *Daucus carota* L.; one from black medic, *Medicago lupulina* L.; two from white cockle, *Lycnis alba* Mill., from R. Millar); and one isolate of *P. cactorum* (Lebert & Cohn) Schroeter, two isolates of *P. cinnamomi* Rands (from S. Jeffers) and one isolate each of *P. cryptogea* Pethybridge & Lafferty and *P. drechsleri* Tucker (from S. Mircetich). All isolates were maintained on V-8 juice agar (200 ml of V-8 juice, 3 g of $CaCO_3$, 15 g of agar, and 800 ml of distilled, demineralized water. Zoospores were produced by flooding 10- to 14-day-old V-8 agar cultures with sterile, distilled, demineralized water and placing them at 15 C for 12–15 hr. Comparisons of growth rates and sensitivities to metalaxyl were made on cornmeal agar (Difco Laboratories, Detroit, MI) amended with 0 or 20 μg/ml of filter-sterilized (Millipore Filter Corp., Bedford, MA) metalaxyl (Ciba-Geigy Corp., Greensboro, NC).

The medium of Masago et al (19) was modified to include potato-dextrose agar (Difco) amended with benomyl at 20 μg/ml (E. I. duPont de Nemours and Co. Inc., Wilmington, DE), pentachloronitrobenzene at 133 μg/ml (Olin Mathieson Chemical Corp., Little Rock, AR), nystatin at 25 μg/ml (Sigma Chemical Co., St. Louis, MO), ampicillin at 500 μg/ml (Sigma), rifampicin at 10 μg/ml (Sigma), and hymexazol at 50 μg/ml (Sankyo Co., Tokyo, Japan). When appropriate, it was supplemented with metalaxyl at 20 μg/ml.

Soil. Field soil was collected from the Mt. Pleasant Research Farm (Agronomy Department, Cornell University), Tompkins County, New York. The soil was a silt loam (18.4% sand, 56.0% silt, and 25.6% clay) with 6.0% organic matter and pH 7.4 (determined with water). It was from a field that had been under an alfalfa-corn-oats rotation. During the last rotation sequence, alfalfa had been planted without tillage into corn stubble. The soil was collected 1 yr later from an area in which severe Phytophthora root rot had occurred in the seeding year. Soil from the top 15 cm was sieved (6-mm openings) and stored in galvanized trash cans at 4 C. The soil moisture content at the time of storage was about 25%.

Specificity and reliability of seedling bioassay. To determine the suitability of the seedling bioassay for distinguishing *P. m.* f. sp. *medicaginis* Kuan and Erwin from other isolates of *P. megasperma* and other *Phytophthora* species, the following isolates were tested at least three times: *P. m.* f. sp. *medicaginis* (AFI, Pm20, GL4), *P. megasperma* from apple (088) and white clover (WCL1 and WCL2), *P. cinnamoni* (R-105, P12), *P. cactorum* (P13), and *P. drechsleri*. Alfalfa seedlings, 3–5 days old, grown from surface-sterilized seed (0.54% NaClO for 15 min) on moist filter paper in sterile petri plates were placed on a V-8 agar culture of each isolate (six seedlings per plate). These plates were held at 24 C for 12–15 hr. Three of the seedlings were removed from each dish, surface-sterilized for 1 min in NaClO, washed in sterile, distilled, demineralized water, and placed in 15 ml of sterile water for 24–48 hr. The other three seedlings were transferred directly from the culture to sterile water and held for 24–48 hr. All seedlings were observed over time for the production of sporangia. After observations were completed, the seedlings were blotted dry and placed on the selective medium. These plates were held at room temperature for 24–48 hr and observed for hyphal growth from the seedlings.

Isolate selection from zoospores. Isolate AFI of *P. megasperma* obtained in 1978 from an alfalfa field in Aurora, NY, was used as the parent for selection of metalaxyl-insensitive progeny. AFI was typical of *P. m.* f. sp. *medicaginis* isolates from alfalfa roots or soils in New York. Cultures of AFI grown 14 days on V-8 agar at 24 C were flooded with sterile, distilled, demineralized water and placed at 15 C. Large populations of zoospores were produced in 12–15 hr. Zoospores were induced to encyst by agitation (Vortex-Genie, Scientific Industries Inc., Springfield, MA) for three 20-sec intervals. Suspensions, each 0.1 ml containing approximately 10,000 encysted zoospores, were dispersed evenly over the surface of cornmeal agar plates amended with metalaxyl at 0–500 μg of active ingredient per milliliter. The plates were placed at 24 C and observed daily for zoospore germination, hyphal growth, and colony development. Isolates insensitive to metalaxyl were selected and compared with the parent isolate for growth rate and stability of insensitivity on cornmeal agar amended with metalaxyl at 0 or 200 μg/ml. One isolate (Pm20) that was stable and equal to the parent in growth rate was compared with the parent for characteristics of zoospore production, encystment, and germination and for pathogenicity.

Survey of isolates for insensitivity to metalaxyl. Several isolates of *P. megasperma* and different *Phytophthora* species from different geographic locations and hosts were tested for sensitivity to metalaxyl at 20 μg of active ingredient per milliliter. Agar plugs (5-mm diameter) from actively growing colonies of each isolate were transferred to cornmeal agar amended with metalaxyl at 0 (CMA0) or 20 (CMA20) μg/ml, and the plates were held at 24 C. Radial growth (largest diameter) was determined at 24-hr intervals for at least 7 days. Three plates per metalaxyl concentration were used per isolate. The experiment was repeated twice.

Several isolates of *Phytophthora* tentatively identified as *P. megasperma* were isolated from roots of weeds growing in alfalfa fields. These isolates differed in colony morphology and pathogenicity to alfalfa (R. L. Millar, *unpublished data*). They were compared with Pm20 for insensitivity to metalaxyl as described above.

Specificity of selective medium and recovery of Pm20 from soil. To determine the suitability of Pm20 for studies in field soil, nonsterile soil from an alfalfa field with an indigenous *P. m.* f. sp. *medicaginis* population was used. *Pythium* species in this soil had repeatedly impaired the detection of *P. m.* f. sp. *medicaginis* via plating. In addition, this alfalfa soil harbored the weed isolates of *P. megasperma*, some of which were nonpathogenic to alfalfa and insensitive to metalaxyl. This soil was assayed to determine whether these insensitive isolates would yield false positive results in the assays. Alfalfa seedling tissue colonized with either Pm20 or AFI was mixed into the alfalfa field soil and the soil kept at room temperature for 6 wk. The soil then was subjected to an extended seedling bait bioassay. Soil (30 ml) was placed in each of three glass petri plates (9.0-cm diameter), allowed to dry gradually to approximately 5% moisture (7 days), then remoistened with 10 ml of distilled, demineralized water. After 3 days, the soil was flooded with 40 ml of sterile, distilled, demineralized water, and six 3- to 5-day-old alfalfa seedlings were floated in the water above the soil for 3 days. The seedlings were removed from the soil-water dishes and observed microscopically for sporangia resembling those of *P. megasperma*. The seedlings then were blotted dry and plated on the selective medium amended with metalaxyl at 0 or 20 μg/ml. The plates were observed daily for *P. megasperma* colonies. Isolates obtained from colonies on these plates were identified. The same alfalfa field soil, to which Pm20 was not added, was assayed in the same way to determine the response of the indigenous *P. m.* f. sp. *medicaginis* and to test whether the indigenous metalaxyl-insensitive weed isolates would yield false positive results.

Direct plating of Pm20-infested alfalfa field soil also was done. Suspensions of Pm20 zoospores (300 zoospores per milliliter) were added to field soil (10-ml suspension per 30 ml of soil) containing indigenous *P. m.* f. sp. *medicaginis* and weed isolates, and this soil was subjected to an abbreviated seedling bait bioassay. The soil (30 ml) was added to glass petri plates, flooded with 40 ml of distilled, demineralized water, and six 3- to 5-day-old alfalfa seedlings were floated in the water above the soil. After 3 days, the seedlings were removed, blotted dry, and plated on the selective medium amended with metalaxyl at 20 μg/ml. The water then was drained from the soil plates, and the soil was infiltrated with 15 ml of water agar containing the antibiotics of the selective medium plus metalaxyl at 20 μg/ml, stirred, and allowed to solidify. Soil-agar plugs taken with a no. 2 cork borer were transferred (10 plugs per plate) to the selective medium plus metalaxyl at 20 μg/ml. From each soil-agar

plate, 10 random plugs were transferred to each of three selective medium plates. The plates were held at room temperature (about 24 C) and observed daily for growth of Pm20. Soil not infested with Pm20 was treated similarly as a control and to test whether the indigenous metalaxyl-insensitive weed isolates would yield false positive results. There were 10 replicate plates per treatment.

As a further test of the selective medium's specificity, nonalfalfa soils with *P. megasperma* populations were subjected to the same assays. Soil was collected from two apple orchards and a nursery in Wayne County, New York. The soil samples were taken from nine sites previously determined to be positive for *Phytophthora* species (S. N. Jeffers, *personal communication*). Some of the fruit isolates shown to have insensitivity to metalaxyl were originally isolated from these soils. Soil from each site was tested by the alfalfa seedling bait bioassays (abbreviated and extended) and the soil-agar plug assay. Three plates per soil sample were used in each assay.

RESULTS

Specificity and reliability of assays. Only *P. cactorum* could be distinguished consistently from *P. m.* f. sp. *medicaginis* by the morphological characteristics of sporangia produced from alfalfa seedlings. Isolates of *P. cinnamomi* and *P. drechsleri* and the weed and fruit isolates of *P. megasperma* varied sufficiently in sporangium morphology that they could not be distinguished (Fig. 1). When infected seedlings were plated on Masago's medium, only *P. cactorum* could be distinguished, due to its inability to grow on this medium; all other isolates grew and were indistinguishable macroscopically (Fig. 1). These results were observed in several trials.

Selection of metalaxyl-insensitive isolate from zoospores. Six zoospores provided cultures that were insensitive to metalaxyl; the occurrence of insensitivity was approximately one in 12 million zoospores. Because growth of five of the six isolates was significantly reduced (50–75%) in the presence of metalaxyl (10–50 μg/ml) and their responses to metalaxyl were inconsistent in several trials, they were not used. One zoospore yielded an isolate designated Pm20, which was obtained from cornmeal agar with metalaxyl at 10 μg/ml. This isolate was stable and showed no growth reduction in the presence of metalaxyl at 20 μg/ml. Pm20 did not differ from the parent isolate AFI in amount of growth or in growth rate in the absence of metalaxyl. Amount of growth and growth rate of Pm20 was unaffected by 20 μg/ml, was significantly less at 500 and 1,000 μg/ml, and was completely inhibited by metalaxyl at 5,000 μg/ml.

AFI-Pm20 comparisons. The parent isolate and Pm20 did not differ significantly in zoospore production, germination of encysted zoospores, morphology or numbers of oospores produced on V-8 agar or within infected alfalfa seedlings, or morphology, number, or germination (direct or indirect) of sporangia produced from infected alfalfa seedlings. In addition, AFI and Pm20 were equally pathogenic in three tests; each rotted off taproots and caused death of plants in the same time period, and both were easily recovered from diseased tissues on the selective medium without metalaxyl.

Metalaxyl sensitivity among isolates of *Phytophthora* spp. Pm20 was compared for sensitivity to metalaxyl at 20 μg/ml with 12 isolates of *P. megasperma* from alfalfa, 15 isolates from fruit trees, three isolates each from clover and soybean, and four other soil-inhabiting *Phytophthora* spp. All of the alfalfa isolates were extremely sensitive to metalaxyl and 11 did not grow on CMA20. After 5 days, one alfalfa isolate (GL4) grew very sparsely. Unlike the alfalfa isolates, the fruit isolates were much less sensitive to metalaxyl; their growth was inhibited 30–75% on CMA20 relative to that on CMA0.

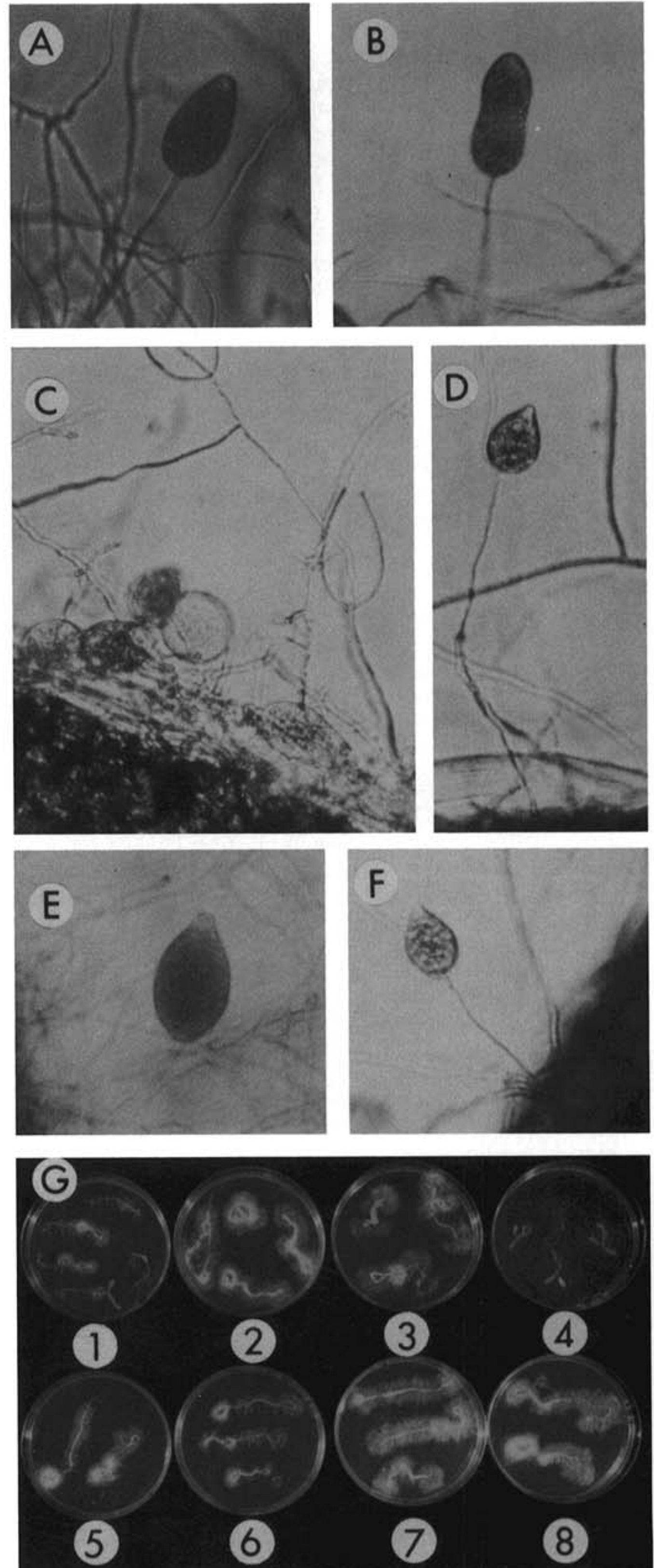

Fig. 1. Sporangium and colony morphology of *Phytophthora* isolates. **A, B, D, and E,** Variation in morphology for sporangia of *P. megasperma* f. sp. *medicaginis*. **C,** Sporangium of *P. drechsleri* produced from infected alfalfa seedling. **F,** Sporangium of *P. megasperma*, isolate WCL1 from white clover, which was not pathogenic to alfalfa. **G,** Alfalfa seedlings infected by various isolates of *P. megasperma* and *Phytophthora* spp. plated on selective medium without metalaxyl: plates 1 and 5, isolates of *P. m.* f. sp. *medicaginis* from alfalfa; plates 2 and 6, isolates of *P. megasperma* from white clover (plate 2 contains isolate WCL1, which is not pathogenic to alfalfa, and plate 6 contains isolate WCL2, which is pathogenic to alfalfa); plates 3 and 7, two isolates of *P. cinnamomi*; plate 4, an isolate of *P. cactorum* that did not grow on this medium; and plate 8, an isolate of *P. drechsleri*.

On CMA0, colonies of the fruit isolates had an appressed appearance, but those of the alfalfa isolates had a fluffy texture. In the presence of metalaxyl, however, the colony morphology of the fruit isolates was more fluffy than appressed. The weed isolates of *P. megasperma* could be separated into two distinct groups: those with a fluffy colony morphology that were pathogenic to alfalfa and extremely sensitive to metalaxyl, and those with an appressed morphology that were not pathogenic to alfalfa and much less sensitive to metalaxyl. The clover and soybean isolates were extremely sensitive to metalaxyl; their growth was inhibited 100 and 98%, respectively. Growth of *P. cryptogea*, *P. drechsleri*, and *P. cactorum* was inhibited 100%; growth of *P. cinnamomi* was inhibited 92%.

Specificity of selective medium and recovery of Pm20 from soil. The problem of distinguishing introduced from indigenous *P. m.* f. sp. *medicaginis* was well illustrated (treatments II and III, Table 1). For treatment III, 11 of 18 seedlings (in two of three plates) became colonized from soil to which *P. megasperma* was not added. Infection of these seedlings resulted from the indigenous population. For treatment II, 12 of 18 seedlings (in two of three plates) were positive for *P. megasperma*; based only on microscopic examination, it was not possible to determine whether the seedlings were colonized by the indigenous or introduced *P. megasperma*. Because only the Pm20-infected seedlings yielded colonies on the selective medium with metalaxyl (Fig. 2), plating the seedlings on the selective medium with metalaxyl distinguished introduced *P. m.* f. sp. *medicaginis* from indigenous *P. megasperma* (treatment I).

Within 24 hr after plating, hyphae grew from the seedlings into the agar. When the wild type grew from an agar plug or an infected seedling onto an agar medium containing metalaxyl, the hyphae had a gnarled, aberrant morphology and extended at most a few millimeters. When aberrant hyphae were transferred from the metalaxyl medium to a medium minus metalaxyl, normal growth and development of *P. m.* f. sp. *medicaginis* resulted. Some of the seedlings from the Pm20-infested soil, when plated onto the selective medium with metalaxyl, gave rise to normal appearing hyphae and also gnarled, aberrant mycelium. When the gnarled hyphae were transferred to a medium minus metalaxyl, normal *P. megasperma* developed from it. When subcultured again onto medium plus or minus metalaxyl, it grew only on the medium minus metalaxyl. It presumably was the wild type isolate of *P. megasperma*. This demonstrated that a single bait seedling could be infected simultaneously by both the wild type and the marked isolate. Dual infections also were observed occasionally for plants used in pathogenicity tests done with field soil.

Direct plating of Pm20-infested and naturally infested (no Pm20 added) soils also was done. The soils were subjected to the seedling bait bioassay. All 10 plates for both the Pm20-infested and the naturally infested soils had seedlings with sporangia of *P. megasperma*. On these seedlings, Pm20 and wild type sporangia could not be distinguished. When these seedlings were plated on the selective medium with metalaxyl, the seedlings from all 10 plates of the Pm20-infested soil gave rise to colonies of *P. megasperma*. Upon subculturing, they were positiviely identified as Pm20. None of 60 seedlings from any of the 10 plates for the naturally infested soil gave rise to a colony of *P. megasperma* on the selective medium with metalaxyl. Results were similar when these soils were plated directly on the selective medium with metalaxyl. Soil-agar plugs from eight of the 10 plates of Pm20-infested soil gave rise to colonies of *P. megasperma*. No soil-agar plugs from any of the plates with naturally infested soil gave rise to colonies of *P. megasperma*. When soil-agar plugs from either soil were plated on the selective medium minus metalaxyl, the plates were overrun by *Pythium* species, making detection of *P. megasperma* impossible. Although most of the seedlings exposed to the naturally infested soil had *P. megasperma* sporangia, neither the seedlings nor the soil yielded colonies of *P. megasperma* on the selective medium containing metalaxyl.

When soils from two apple orchards and a nursery in Wayne County, New York, were subjected to the alfalfa seedling bait bioassay, only *P. cactorum* was isolated. When these same soils were plated on the selective medium with metalaxyl (20 μg/ml), no

TABLE 1. Production of sporangia and isolation of *Phytophthora megasperma* f. sp. *medicaginis* from alfalfa seedling baits used to bioassay naturally infested soils amended with isolates insensitive and sensitive to metalaxyl

		P. megasperma isolated from[a]	
Treatment[b]	Sporangia on seedlings[c]	Seedlings plated on M	Seedlings plated on M+R
I. Pm20-colonized tissue	2	+	+
II. AFI-colonized tissue	2	+	–
III. None[d]	2	+	–

[a] Mycelial growth of *P. megasperma* from seedlings used to bioassay soil. After microscopic observations, three seedlings were blotted dry and plated on a selective medium minus metalaxyl (M) and three were plated on the medium with metalaxyl (M+R).

[b] Alfalfa seedling tissue was colonized by isolate Pm20 (metalaxyl insensitive) or AFI (sensitive) mixed into an alfalfa field soil, and the soil was kept at room temperature for 6 wk. The soil then was subjected to the baiting bioassay (extended) described in the text. Soil that did not receive tissue also was baited.

[c] Six seedlings in each of three plates were removed from the soil water and observed microscopically for the presence of sporangia of *P. megasperma*. The value given equals the number of soil/plates which had seedlings with sporangia of *P. megasperma*.

[d] Data represent several experiments in which *P. megasperma* was isolated from seedlings plated on M but never from seedlings plated on M+R.

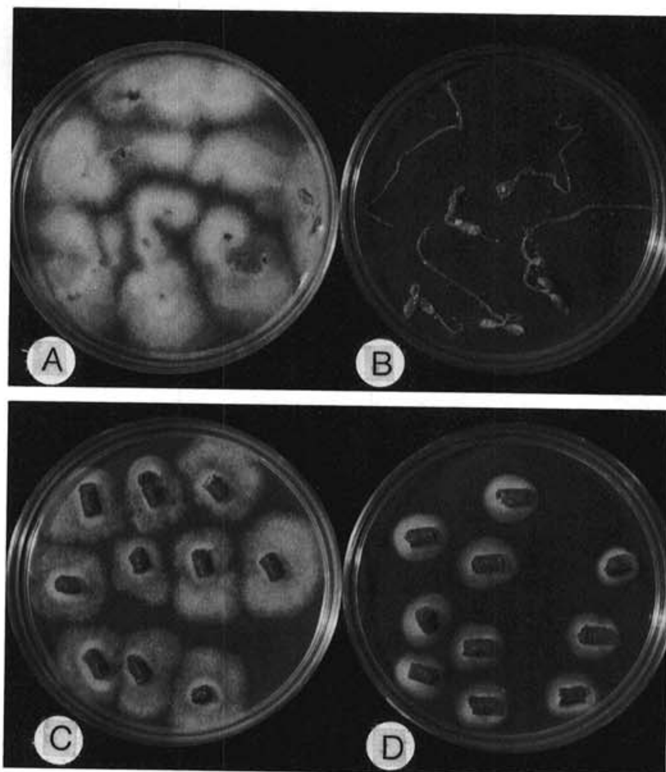

Fig. 2. Selective isolation of metalaxyl-insensitive isolate Pm20 of *Phytophthora megasperma* f. sp. *medicaginis* from seedlings and soil. Seedling baits from natural soil **A,** artificially infested with Pm20 or **B,** no Pm20 added, plated on selective medium with metalaxyl. Soil-agar plugs from field soil **C,** artificially infested with Pm20 or **D,** no Pm20 added, plated on selective medium with metalaxyl. In **A and B,** sporangia of *P. megasperma* were previously observed on all six seedlings on both plates. Since there was no growth on the selective medium with metalaxyl, the seedlings in **B** are presumably infected with the wild type *P. megasperma* indigenous to the field soil. In **D,** the growth is *Geotrichum candidum*, which can be easily distinguished from *P. megasperma*.

Phytophthora colonies developed. If fruit isolates of *P. megasperma* were present in these soils, they either were not detected by these assays or the population was below the detection limit.

DISCUSSION

Use of a physiological marker to facilitate detection and positive identification of *P. m.* f. sp. *medicaginis* in a natural soil environment permits investigations on the ecology of the pathogen that otherwise would not be possible. The alfalfa seedling bait bioassay has been used routinely to detect *P. m.* f. sp. *medicaginis* in soil (17). We found, however, by first observing the seedlings microscopically and then plating them on selective medium, that some seedlings that had not produced sporangia were infected. Therefore, if presence of sporangia were the only criterion used, false negative data could result. This could be a serious source of error especially in a soil environment where there are biotic and abiotic influences on sporangium production that cannot be stringently controlled (2,19,33).

In this investigation, sporangia of *P. megasperma* isolates not pathogenic to alfalfa, as well as the sporangia of different *Phytophthora* species, could not be distinguished microscopically from those of *P. m.* f. sp. *medicaginis* when the sporangia were produced on alfalfa seedlings. Others also have noted their inability to distinguish *Phytophthora* species by sporangium morphology (5,13). In New York's alfalfa fields, the population of *P. megasperma* includes both pathogenic and nonpathogenic isolates. Moreover, *P. cactorum* was baited from soil with alfalfa seedlings. Others have reported a diverse population of *P. megasperma* (10,22), as well as different *Phytophthora* species in alfalfa soils (3,15,20,23). These observations bring into question the reliability of the alfalfa seedling assay for the identification of *P. m.* f. sp. *medicaginis*.

P. m. f. sp. *medicaginis* was considered to be *P. cryptogea* for 12 yr before its reclassification (8). This attests to the difficulty of positively identifying *Phytophthora* species based on sporangium morphology, which is a variable trait (31). Thus, identification of a large number of specimens is difficult, especially in assays from soil where other organisms may influence both sporangium production and morphology (2,18,33). Isolation and identification of every specimen would be prohibitive in studies on population dynamics or other ecological aspects.

Working with naturally infested, nonsterile field soil presented the following obstacles: inability to distinguish experimental propagules of the pathogen from the indigenous population, inability to distinguish the pathogen from other *Phytophthora* species or nonpathogenic *P. megasperma* isolates, and inability to detect *P. m.* f. sp. *medicaginis* in the presence of faster growing soil fungi, especially *Pythium* spp. Using the metalaxyl-insensitive isolate Pm20 and incorporating metalaxyl into the selective recovery medium overcame these obstacles. The marked isolate Pm20 appears identical to the wild type (AFI) in all characters tested (total growth, growth rate, sporulation, propagule germination, morphology, and pathogenicity) except reaction to metalaxyl. Pm20 was derived from the wild type isolate AFI, which to our knowledge had never been exposed to metalaxyl before this study. The trait of insensitivity to metalaxyl therefore was present as a characteristic of the natural population of the pathogen. These results underscore the possibility of selection of metalaxyl-insensitive strains in nature, if metalaxyl is used to control Phytophthora root rot.

Davidse obtained metalaxyl-tolerant isolates of *P. m.* f. sp. *medicaginis* by hyphal adaptation and by mass selection of zoospores (6). These isolates, however, had an altered morphology, were less virulent and had a slower growth rate in the absence of metalaxyl. Davidse also obtained tolerant isolates characterized by high virulence, and a morphology and growth rate typical of the wild type, from zoospores that had been mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutagenesis was not used in the present investigation to obtain a marked isolate of *P. m.* f. sp. *medicaginis* to preclude inducing changes other than metalaxyl insensitivity. Because the Pm20 isolate was obtained by mass selection of zoospores without the use of a mutagen, this supports the view of Davidse that a stable, high level of tolerance can occur in *P. m.* f. sp. *medicaginis* naturally.

Because metalaxyl insensitivity was to be an important feature of ecological studies of Pm20 in natural soil, it was necessary to determine whether this trait was unique to Pm20. Forty-one isolates of *P. megasperma* from nine hosts as well as different *Phytophthora* species were tested for sensitivity to metalaxyl (26). *P. m.* f. sp. *medicaginis* was readily distinguished from *P. drechsleri, P. cryptogea, P. cinnamomi,* and *P. cactorum* and from the alfalfa and weed isolates of *P. megasperma* pathogenic to alfalfa, all of which were extremely sensitive to metalaxyl at 20 μg/ml. The fruit and weed isolates not pathogenic to alfalfa were less sensitive to metalaxyl.

Finding isolates of *P. megasperma* with some insensitivity to metalaxyl in New York soils required determining the specificity of the Pm20 selective medium and the possibility of recovering Pm20 from field soils. The selective medium with metalaxyl allowed growth only of Pm20 in tests with soils from alfalfa fields having metalaxyl-tolerant weed isolates or from apple orchards having metalaxyl-tolerant fruit isolates. Although baits from soils not infested with Pm20 had sporangia of *P. megasperma*, none of these baits yielded colonies on the selective medium with metalaxyl. It is therefore unlikely that any of the naturally occurring metalaxyl-tolerant isolates of *P. megasperma* will interfere in studies involving field soil.

The ability to recover Pm20 from soil was demonstrated by direct plating of soil or by plating baits from Pm20-infested field soil. Since single bait seedlings were simultaneously infected with Pm20 and the wild type *P. m.* f. sp. *medicaginis*, the population of *P. m.* f. sp. *medicaginis* indigenous to the field soil did not appear to interfere with studies of Pm20. By using the selective metalaxyl medium, Pm20 can be recovered in the presence of the wild-type *P. m.* f. sp. *medicaginis* from field soil, bait seedlings, and mature alfalfa roots. Within colonized tissue, Pm20 survived for at least 6 wk in field soil. Its ability to survive and give rise to inoculum in field soil appears the same as that of the wild type.

Association of host group (e.g., fruit isolates or alfalfa isolates) with sensitivity to metalaxyl has been observed with other isolates of *P. megasperma* (4,12,14). Coffey and Bower (4) obtained similar results with respect to the relative sensitivites of isolates of *P. megasperma* from different hosts. A direct comparison of their results to those presented here is not possible because the range of metalaxyl concentrations tested did not overlap. Isolates of *P. megasperma* from fruit trees or Douglas-fir were "weakly aggressive" on alfalfa and showed some insensitivity to metalaxyl. Isolates from alfalfa that were "aggressive" on alfalfa were extremely sensitive to metalaxyl. Isolates from alfalfa that were "weakly aggressive" on alfalfa were less sensitive to metalaxyl. In Oregon as in New York, the population of *P. megasperma* in alfalfa fields is diverse.

Brasier posed the questions ". . . do individuals of *Phytophthora* recognize and respond to each other (apart from sexual interaction)?" and "do . . . different genotypes compete or collaborate?" (1). Both appressed and fluffy types of *P. megasperma* have been isolated from the same plant species and in some cases the same plant (R. L. Millar, *unpublished data*). They appear to compete and survive equally well and they do not appear to affect the activity of each other. AFI and Pm20, however, have nearly identical genotypes, and both AFI and Pm20 were active in the same soils at the same time and could simultaneously infect the same alfalfa seedling or mature alfalfa root. Previous workers obtained from alfalfa roots isolates of *P. megasperma* that had fluffy, appressed, or intermediate colony types on V-8 agar (8,22). How those isolates compare (or relate) to those described here cannot be deduced. Although much more work is needed, based on the findings reported here, there was no indication that isolates of *P. megasperma* of either seemingly diverse genotype (appressed isolates from weeds vs. alfalfa isolates) or nearly identical genotypes (AFI vs. Pm20) interacted to the detriment or benefit of each other.

The use of Pm20 and the selective medium with metalaxyl provided the means for studying the ecology of *P. m.* f. sp. *medicaginis* in a field soil. Investigations on the movement through soil, competitive colonization of organic matter in soil, the effects of edaphic factors on propagule activity, and the role of alternate hosts in the life history of *P. m.* f. sp. *medicaginis*, which previously were either impossible or exceptionally difficult to undertake, were made possible (25).

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