

Detection, Isolation, and Pathogenicity of *Phytophthora megasperma* from Soils and Estimation of Inoculum Levels

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ABSTRACT

Phytophthora megasperma was isolated and identified using 3-day-old alfalfa seedlings floated over flooded soil as bait. The seedlings were injured prior to use by pinching the hypocotyl with a pair of fine tweezers. The fungus sporulated vigorously on the seedling 72-96 hr after immersion, and could be identified directly.

Soil isolates were as virulent to vernal alfalfa as an isolate from a tap root lesion. They caused rapid and extensive rot in the unthickened roots and

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lesions on the tap roots, followed by foliar discoloration, wilting, reduced top growth, small leaves, and frequently plant death.

The baiting method was used to study the distribution of the pathogen in alfalfa fields. The highest population density of the fungus occurred in a poorly drained heavy silt loam with a history of severe alfalfa root rot. The fungus could not be detected on contiguous well-drained hill slopes and crests. *Phytopathology* 60:1687-1690.

Phytophthora megasperma Drechs. has been associated with root rot of alfalfa (*Medicago sativa* L.) and is considered to be an important pathogen (14). Damage is greatest on heavy, poorly drained soils in low-lying areas after prolonged periods of rain. The fungus has been isolated in many parts of the United States from diseased alfalfa roots (3, 8, 10, 11, 14).

Direct isolation of the alfalfa pathogen from soil, in the absence of the diseased host, has not been reported. In order to study the distribution of this pathogen independent of alfalfa, a method was needed to isolate the fungus consistently from the soil. This report deals with a technique developed for detecting and isolating *P. megasperma* from soil, its use in estimating the distribution of the fungus, and the results of estimating the inoculum levels in infested soils.

MATERIALS, METHODS, AND RESULTS.—*Isolation attempts using existing procedures.*—Blue lupine (5) (*Lupinus angustifolium* L.) and apple (*Pyrus malus* L. 'Delicious') (4) have been used as baits in general surveys for *Phytophthora* spp. When pieces of these baits, exposed to soil inoculum, were plated on an antibiotic medium (7) containing penicillin (50 mg/liter), pimarinin (100 mg/liter), and polymyxin (50 mg/liter) incorporated in 20% Campbell's V-8 juice agar, it is reported (13) that cultures of the *P. megasperma* were sometimes obtained. We tried both baits without success for *P. megasperma* with alfalfa soils. P. T. Jenkins (*personal communication*) successfully trapped *P. cinnamomi* in Australia, using partly ripened Pakham Triumph and Sweet William pears placed directly on soil flooded to a depth of 1 inch. This method was tried with Bartlett pears, with no success. Simultaneous colonization of the tissue by *Pythium* spp. made identification of the *Phytophthora* virtually impossible. Flowers & Hendrix's technique (9) for direct soil isolation using a medium including gallic acid also proved unsuccessful, due to the large numbers of *Pythium* spp. present in the alfalfa soil samples.

Isolation methods using alfalfa seedlings.—Alfalfa seedlings and stem pieces have been used to induce

sporulation in *Phytophthora* spp. (8). This suggested that they might be of use as selective bait. Initial attempts using vigorous seedlings failed because seedlings grew rapidly when floated in soil water and did not become infected. They may have acquired a degree of resistance to the pathogen in the short time they were exposed, as they do to *Pythium* spp. (6), or they may not have been in close enough contact with the soil. Successful attempts were made with seedlings whose growth had been retarded by heat (60 C for 2 min), and a technique was finally standardized as follows. Vernal alfalfa seed was surface-sterilized with 3% sodium hypochlorite for 30 sec, thoroughly washed in sterile water, and germinated on moist filter paper in petri dishes. Germination occurred between 24-36 hr at 22-24 C, and the seedlings were ready for use after 72 hr, when the radicle was 6-10 mm long and the ruptured testa could be slipped off the young cotyledons. About 30 cc of soil were placed in a 90-mm petri dish and covered to a depth of 2-3 mm with 35-40 ml of distilled water. We floated six seedlings in water in each dish, taking care not to bury them. Hypocotyl and radicle tissues were crushed at two or three points with a pair of fine tweezers. This treatment slowed the growth and maturation in the seedlings. They did not die unless they became infected, but appeared to be more susceptible to infection. Moreover, it was easier to keep the whole seedling submerged at all times. The dishes were incubated at 20-22 C for 6 days.

The first evidence of infection appeared within 2-4 days, when the cotyledons became water-soaked and sporangia of *P. megasperma* could be observed on the cotyledons and hypocotyls. Infected seedlings usually became quite flaccid. Sporangia frequently formed in floating masses on the water surface around the bait, and could be readily seen under a stereomicroscope (Fig. 1-A,B). In sterile soils, or soils taken from areas where *P. megasperma* was not found, the seedlings remained turgid and fresh for 7 to 10 days, even though growth had slowed down.

Isolation of Phytophthora megasperma.—*P. mega-*

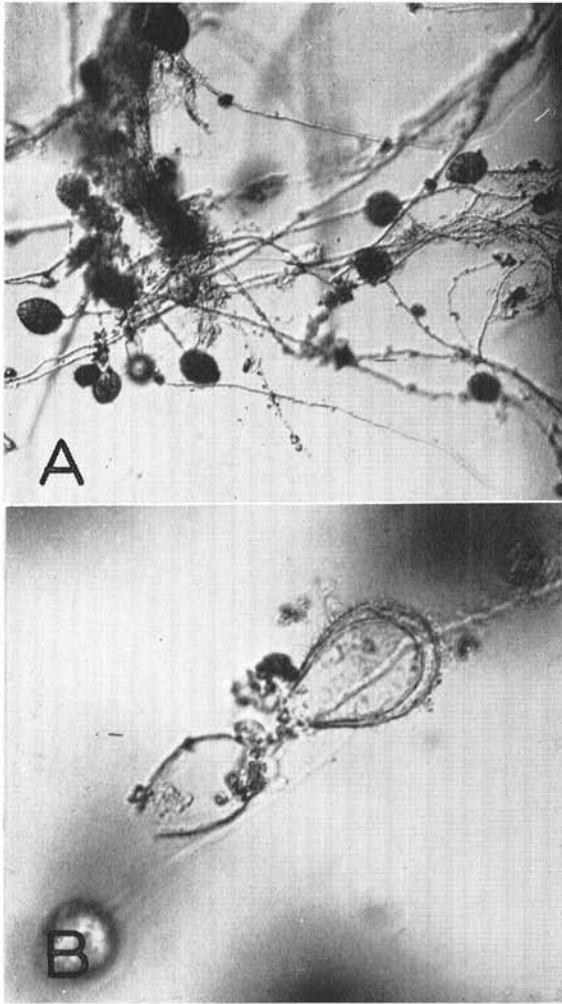


Fig. 1. A) Sporangia of *Phytophthora megasperma* on hypocotyls of alfalfa seedling bait ($\times 150$). B) Repetitive formation of sporangia ($\times 800$).

measperma was isolated by plating small pieces of freshly infected cotyledons on antibiotic V-8 juice agar. In situations where mixed infections of *P. megasperma* and *Pythium* spp. occurred, *P. megasperma* was isolated by carefully breaking off the sporangia with a pair of fine tweezers under a stereomicroscope, drying them by touching the lower side of the tweezer on blotting paper, and plating on antibiotic V-8 juice agar. The plates were incubated at 20-22 C and inspected after 16 to 24 hr. At this stage, it was possible to identify hyphae of *P. megasperma* and make hyphal tip isolations if required.

Fourteen isolates of the fungus were obtained from soils where alfalfa root rot was observed. They were similar to a culture from a diseased tap root of alfalfa (supplied by F. Frosheiser) that had been identified as *P. megasperma*. All cultures were identical to reported descriptions of *P. megasperma* (17). It is apparent that this fungus is endemic in Wisconsin.

Pathogenicity tests.—Since damaged seedlings were used as bait, it is possible that avirulent fungal strains were isolated from the soil. The virulence of a random selection of isolates was tested on 6-week-old vernal alfalfa maintained under a 16-hr light period at 20-26 C. The plants were raised from seed in steamed sand:sandy loam mixture (1:1) in 30-ml plastic cups. The inoculum was grown in 1-liter capacity bottles containing 60 ml of 20% V-8 juice, 2% CaCO_3 , and 0.001% thiamine. Tsao & Garber's method (16) was used to harvest the inoculum and infest the soils. The plants were repotted after 3 weeks in 250-cc capacity pots containing steamed and infested sand:sandy loam mixture. After inoculation, they were subjected to flooding for 2-day periods at intervals of 2 days.

No major differences were noted in the virulence of the soil isolates to vernal alfalfa. This species of alfalfa is apparently very susceptible to *P. megasperma*. The damage caused by the soil isolates was comparable to that caused by the Minnesota isolate which has been rated as highly virulent (10) and was used as a standard in these tests.

Symptoms of *P. megasperma* root rot.—In 1943, Jones (12) gave detailed descriptions of the pathological anatomy of root rot of alfalfa caused by unidentified pathogens in Wisconsin. The type of root damage reported here is strikingly similar. Unthickened roots were invaded, and showed the greatest susceptibility to rot (Fig. 2). Roots that showed secondary growth were more resistant. Very little new root growth was observed in infested soil, and it is assumed that the lateral roots were killed as they emerged. Small, fusiform, black-colored streaks developed across the tap root at points where lateral roots emerged. These symptoms did not occur on controls subjected to the same flooding regime. Root and shoot growth remained vigorous in noninoculated plants, and numerous new rootlets were formed. In the absence of root rot pathogens, periodic flooding did not affect the viability or vitality of vernal alfalfa roots.

When 3-month-old plants growing in 2-liter capacity pots were inoculated by wounding using Frosheiser's method (11), large lesions were frequently formed on the tap root. These lesions were identical in form and color to those described by Erwin (8) and Frosheiser (10, 11).

Estimation of inoculum levels in soil.—A modification of Tsao's technique (15) was used to compare the level of infestation in different soils, using alfalfa seedlings as bait. The results are expressed as a population density index (PDI) which is defined as the reciprocal of the highest dilution at which infection occurs. A dilution level was considered to be infested if the sporangia of *P. megasperma* were observed on any one of the six seedlings, used as bait, in any of the three replicates. The dilution series was prepared by mixing 90 cc of soil with 90 cc of steamed, sand:sandy-loam mixture (1:1) on a roller mill. Three 30-cc aliquots of this mixture were tested at each dilution level, and the remainder was used to make the next dilution of the series. At the lower dilution, all seedlings usually

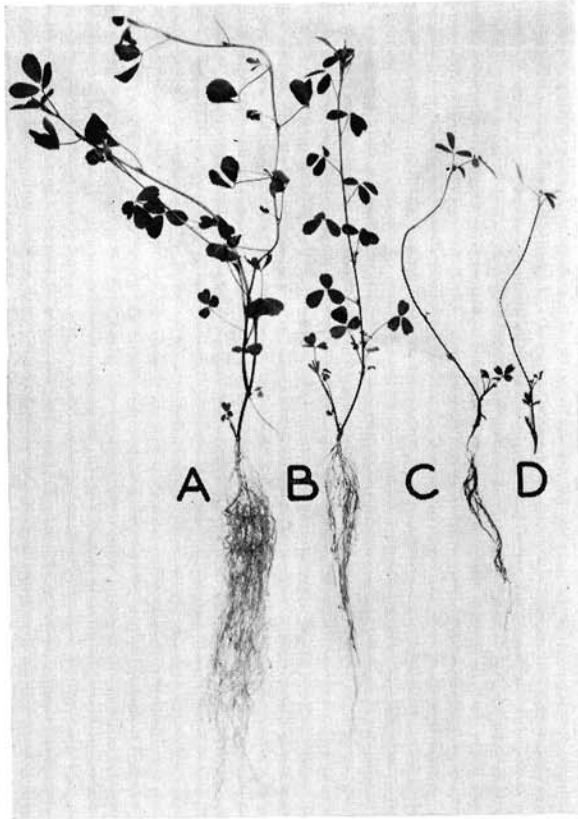


Fig. 2. Pathogenicity of *Phytophthora* spp. to 6-week-old vernal alfalfa. A) Control. B) Slight root rot caused by *P. parasitica* var. *nicotianae*. C), D) Extensive root rot caused by *P. megasperma*. In some cases the tap root was destroyed (D) ($\times \frac{1}{3}$).

were infected and, at the highest dilution at which infection occurred, only one or two seedlings were attacked.

The feasibility of this technique was examined, using soil samples from three areas in Wisconsin where alfalfa was grown. Thirty-two tests were carried out and the results were consistent (Table 1). Soil from an alfalfa field in central Wisconsin where a severe outbreak of root rot occurred in the summer of 1969 showed the highest PDI of 64. Those from two low-lying fields in southeastern Wisconsin had indices of 16 and 8.

Distribution of the fungus in relation to the topography of the field was examined. Two fields in southern Wisconsin were chosen because of their slope characteristics. Their history was not available, but it appeared that the alfalfa was 3-4 years old and had been cut shortly before samples were taken. The soils were podsolised, moderate to heavy silty loams. In the first field (Fig. 3-B), the fungus was detected only in the low-lying area at the foot of a steep slope where the stand was patchy and there was evidence of serious root rot. In the second field (Fig. 3-A) with a very gentle gradient, the fungus was detected for about one-third of the distance to the crest.

TABLE 1. Determination of population density indices of *Phytophthora megasperma* in soil in some alfalfa fields in Wisconsin

| Locality | Replicate no. | Reciprocal of soil dilution | | | | | | | |
|--------------------------|---------------|-----------------------------|---|---|---|----|----|----|-----|
| | | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| Marshfield ^a | 1 | + | + | + | + | + | + | + | - |
| | 2 | + | + | + | + | + | + | + | - |
| | 3 | + | + | + | + | + | + | + | - |
| Dane County ^b | 1 | + | + | + | + | + | - | - | - |
| | 2 | + | + | + | + | + | - | - | - |
| | 3 | + | + | + | + | + | - | - | - |
| Dane County ^c | 1 | + | + | + | + | - | - | - | - |
| | 2 | + | + | + | - | - | - | - | - |
| | 3 | + | + | + | + | - | - | - | - |

^a Heavy soil, severe root rot incidence. One-year-old plants. Repeated 3 times.

^b Heavy soil, evidence of previous damage at foot of steep slope. Repeated 2 times.

^c Heavy soil. Apparently healthy base of gentle slope. Repeated 2 times.

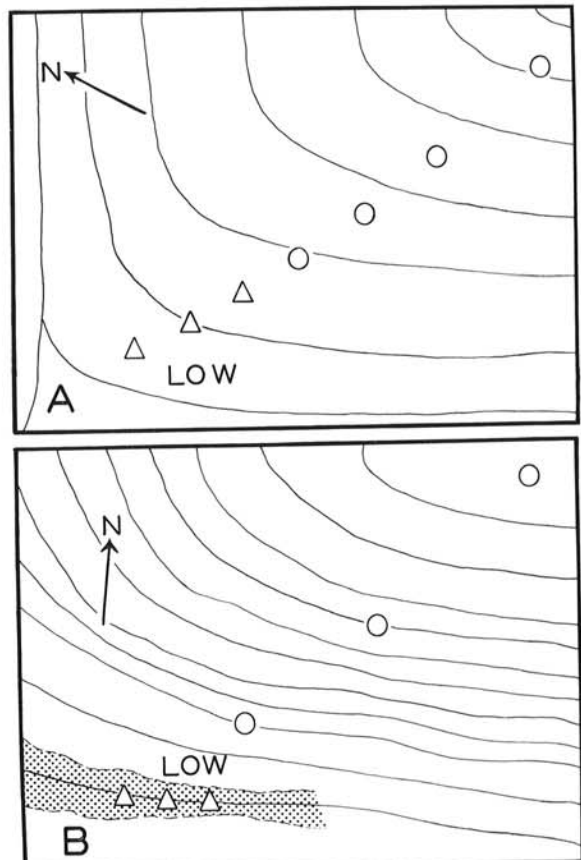


Fig. 3. Distribution of *Phytophthora megasperma* (Δ) in soil in relation to the topography of alfalfa fields. Arrow indicates north. The crosshatch shows area of severe root rot. Loci where no *P. megasperma* was detected are shown thus (O). A) A gently sloping field. The contour lines indicate 1-ft change in elevation. All samples were taken at 20-yard intervals. B) Distribution in a steeply sloping field. Contours represent 2-ft change in elevation with side hill samples taken at 30-ft intervals.

DISCUSSION.—Difficulty due to multiple colonization is frequently encountered in attempting to identify the fungi trapped on baits. Our initial attempts at identifying *P. megasperma* by culturing tissue pieces failed because of the large numbers of propagules of *Pythium* spp. present in the alfalfa soils. This may explain why the fungus has not been reported before on alfalfa in Wisconsin, even though evidence given above suggested that it has been present for many years in this state.

Our results show that alfalfa seedlings can be used as bait, and the fungus may be readily identified without isolation techniques. The effect of injuring seedlings is unknown, but it may prolong the period during which they are susceptible to infection (6).

The mechanism of infection of the seedlings is uncertain. Even though seedlings were in contact with the soil surface, it is possible that infection was initiated by zoospores. This mechanism is believed to occur in the case of lupine (5), avocado (18), pineapple (1), and sweet pear baits.

In wet, heavy soils, the fine root system of alfalfa shows extensive rot (13), and this is reflected in poor yields. The ability of alfalfa to survive under these conditions has been used as an indicator of slope and drainage efficiency in fields (2). Our results indicate that drainage per se cannot account for this situation because, over a short term period, intermittent waterlogging has little effect on root viability when fungal pathogens are not present in the soil. It is apparent that *P. megasperma* is present in a number of areas in Wisconsin. Consequently, its significance as a factor limiting growth of alfalfa in wet, heavy soils and on low-lying spots needs further assessment.

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