Conditions Affecting the Detection of Phytophthora megasperma in Soils of Wisconsin Alfalfa Fields

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ABSTRACT

Phytophthora megasperma was detected by a seedling baiting technique in freshly collected soil samples from 18 of 100 alfalfa fields in Dane County, Wisconsin, in 1970. All samples were collected from low areas of fields which showed possible evidence of past or present root rot. When 52 samples initially rated negative were retested after planting to alfalfa for 8 weeks, *P. megasperma* was detected in 12. The pathogen was also detected in 48 of 109 samples collected throughout Wisconsin in 1971. Samples from 35 of the 54 counties sampled were positive. Soils commonly infested were clay loams and silty clay loams; sandy loams were less frequently infested.

The baiting technique was most sensitive when 25 cc of soil was covered with 3-4 mm of water and the seedlings were added without any preflooding period. Infection of seedlings was greater at 15 C and 20 C than at 25 C; no infection occurred at 30 C. Planting naturally infested soils containing low levels of inoculum to alfalfa for 8 weeks, greatly increased the probability of detecting *P. megasperma*. Diluting samples with a steamed sand-sandy loam mixture, frequently increased seedling infection by *P. megasperma*, and also reduced the probability that seedlings would be infected by *Pythium*. It is concluded that *P. megasperma* is generally distributed throughout Wisconsin and may be an important pathogen of alfalfa in low-lying fields.

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Phytophthora root rot of alfalfa, discovered in California in 1952 (5), is considered a serious disease in many parts of North America. The causal agent was originally considered to be *P. cryptogea* Pethybridge & Lafferty (1, 6, 13, 17), but in 1965 Erwin reclassified it to *P. megasperma* Drechsler following a study of isolates from California, Illinois, Ohio, and Australia (8). *P. megasperma* has also been reported as a causal agent of alfalfa root rot in Arizona (12), Mississippi (13), the upper Midwest (10, 11, 14), Washington (4), and Canada (2). In all instances, the disease has been associated with poorly drained or heavily irrigated soils, or with periods of excessive rainfall.

Prior to 1970, the presence of *Phytophthora* in alfalfa fields could be confirmed only by isolating it from or observing it sporulating on diseased roots. Several investigators reported difficulty in obtaining isolations. Erwin (6) was able to isolate *Phytophthora* consistently only from young root lesions showing red-brown necrosis. Bushong & Gerdemann (1) and Frosheiser (10) were unable to isolate it from diseased roots in midsummer. Schmitthenner (17) reported difficulty in isolating *Phytophthora* from diseased seedlings due to contamination with *Pythium*.

In 1970, Marks & Mitchell (14) described an alfalfa-seedling baiting technique by which *P. megasperma* could be detected in soil and isolated in the absence of naturally infected plants. Inoculum levels in soils of alfalfa fields were estimated with the baiting technique by assays at a series of dilutions with uninfested soil. In each of two fields, the fungus could be detected only in samples from low-lying areas. Isolates obtained from soil by the baiting technique were as virulent to 'Vernal' alfalfa as isolates from root lesions.

The baiting technique of Marks & Mitchell appeared to offer a promising means for making comprehensive surveys of the presence of *P. megasperma* in soils. The purpose of this study was to determine: (i) conditions affecting the detection of *P. megasperma* in soils, (ii) the distribution of *P. megasperma* in alfalfa fields in Wisconsin, and (iii) the extent of morphological variation of *Phytophthora* isolates from alfalfa fields in Wisconsin.

MATERIALS AND METHODS.—Soil samples were collected from low areas of alfalfa fields with sparsely spaced, stunted, or chlorotic plants. Individual sampling sites usually comprised areas less than 100 square yards, although in some fields similar stand conditions existed over much larger areas. Three to five 3-liter samples of soil from the upper 6 inches at random points adjacent to plants within each site were thoroughly mixed and stored in sealed plastic bags at 23-25 C for up to 3 weeks, prior to testing or planting to alfalfa.

Dilutions of infested soils giving ratios of 1:0, 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32, were prepared by mixing them on a roller mill with appropriate quantities of a steamed and cooled mixture of sand and sandy loam (1:1).

The baiting technique of Marks & Mitchell (14) was used to test soil samples for *Phytophthora*. Three-day-old seedlings of alfalfa (*Medicago sativa* L. Vernal) with green cotyledons, germinated on moist filter paper in petri dishes, were each firmly pinched with fine tweezers at the tip of the radical, base of the hypocotyl, and below the cotyledons. Six injured seedlings were scattered over petri dishes containing a 25-cc aliquot of a soil sample which had been flooded with deionized water to a depth of 3-4 mm above the soil surface; the plates were incubated at room temperature (23-25 C) unless otherwise specified. After 80-96 hr, seedlings were gently washed, placed in a shallow layer of water in a petri dish, and examined for *Phytophthora* sporangia under a dissecting microscope at 30X.

Phytophthora was isolated by plating cotyledons that had been surface-sterilized for 30 sec in 1% NaOCl on potato-dextrose agar (PDA) or V-8 juice agar (V-8A, clarified by centrifugation after autoclaving with $CaCO_3$) containing 50 mg/liter pimaricin and 100 mg/liter vancomycin (3). Cultures from single hyphal branches were obtained from hyphae growing on 2% water agar. Sporangia were produced on injured alfalfa seedlings that had been infected when placed over agar cultures flooded with a shallow layer of water and then transferred after 48 hr to plates containing water extract of soil.

TABLE 1. Influence of temperature on detection of *Phytophthora megasperma* in naturally infested soils by baiting with alfalfa seedlings

Soil sample	Temperature (C)	Ratio, original soil:diluent soil[a] 1:0	1:1	1:2	1:4	1:8	1:16	1:32
11	30	0[b]	0	0	0	0	0	0
	25	12	1	3	2	2	0	0
	20	17	17	17	18	9	11	11
	15	18	13	18	17	15	8	3
13	30	0	0	0	0	0	0	0
	25	2	3	6	0	1	0	0
	20	3	14	15	13	16	12	1
	15	1	14	14	13	6	7	0
67	30	0	0	0	0	0	0	0
	25		1	0	2	2	2	0
	20		5	3	2	2	2	0
	15		10	9	6	1	4	0

[a] Diluent soil a 1:1 sand:sandy loam mixture, 25 cc soil per plate.

[b] Total number of seedlings on which *Phytophthora* sporangia were observed in three plates with six seedlings per plate after incubation for 4 days at 30 C and 25 C, 5 days at 20 C, and 6 days at 15 C.

TABLE 2. Influence of amount of soil in plates on detection of *Phytophthora megasperma* in naturally infested soils by baiting with alfalfa seedlings

Soil sample	Soil/plate (cc)	Ratio, original soil:diluent soil[a] 1:0	1:1	1:2	1:4	1:8	1:16	1:32
53	10	1[b]	0	1	1	0	0	0
	25	5	16	7	9	4	1	2
	40	13	13	8	5	11	1	0
67	10	1	2	1	0	0	0	0
	25	1	7	6	5	4	0	0
	40	3	10	6	5	1	2	0
81	10	0	0	0	3	0	0	0
	25	4	4	14	4	1	0	0
	40	5	4	7	4	1	1	2

[a] Diluent soil a 1:1 sand:sandy loam mixture.

[b] Total number of seedlings on which *Phytophthora* sporangia were observed in three plates with six seedlings per plate after incubation for 4 days at 25 C.

RESULTS.—*Primary testing of Dane County soils.*—Soil samples were collected from 100 fields in Dane County, Wisconsin, between 1 June 1970 and 30 September 1970. Three plates of soil with six seedlings per plate were used to assay each sample. *Phytophthora* was initially detected in samples from 18 fields. When large numbers of seedlings were infected, lush colonies with masses of full and emptied sporangia often developed on the cotyledons. When only a few seedlings were infected, colonies were smaller and sometimes a few sporangia were found on only one of the 18 seedlings. Pieces of plant debris and organic matter were occasionally attached to the seedlings by *Phytophthora* mycelium. *Pythium*, saprolegniaceous fungi, and protozoa also developed on seedlings, both in the presence and absence of *Phytophthora*.

Conditions affecting baiting efficiency.—Samples from Dane County rated positive for *Phytophthora*, and samples from a field at the Marshfield experiment station (14), were used to evaluate factors affecting the recovery of *Phytophthora* from soil by the baiting technique. In each experiment, three soil samples were tested separately in dilution series with three plates (six seedlings per plate) at each dilution level.

Temperature influenced infection of seedlings by *Phytophthora* (Table 1). Sporangia were never observed on seedlings at 30 C, but development of colonies with sporangia on seedlings appeared to reach a maximum after 4, 5, and 6 days incubation at 25, 20, and 15 C, respectively. With longer incubation, many sporangia emptied, and some seedlings became too decomposed to examine. Sporangia were not observed on seedlings in 4 days at 15 C, but they were often abundant after 6 days.

Infection was greater in two of three soil samples at 20 C than at 25 C, but no consistent differences were apparent between results at 20 C and 15 C. However, in the third soil sample (No. 67) which was less infectious, seedling infection appeared to be slightly greater at 15 C than at 20 C (Table 1).

Seedling infection in flooded plates appeared to be greater with 3-4 mm of water above the soil than with either 10 mm or only enough water to saturate the soil. Growth of other water molds greatly increased with 10 mm as compared to 3-4 mm water over the soil, rendering detection of *Phytophthora* much more difficult. Very few sporangia were observed on seedlings from plates flooded only to the soil surface, probably due to the absence of free water necessary for sporangium formation.

Increasing the total soil mixture from 25 to 40 cc per plate did not result in greater infection of seedlings, but infection was much less with 10 cc per plate (Table 2). Furthermore, infection of seedlings in plates containing 25 cc soil mixture at 1:4 and 1:8 dilutions was often greater than in plates containing 10 ml at 1:1 dilutions even though the former contained less infested field soil per plate. Similarly, in these and other experiments, more seedlings were often infected with 1:1 or 1:2 dilutions than with undiluted field soil (Tables 1, 2, 3, 4). No consistent differences in infection of seedlings were noted when steamed sand:sandy loam was compared with the unsteamed mixture or with sand alone as diluents of infested soil.

Preflooding naturally infested soils to activate inoculum did not consistently increase infection of seedlings by *P. megasperma* in the baiting technique. The organism was also more difficult to detect on seedlings added to plates which were preflooded for 1 and 3 days than with nonpreflooded plates, due to more numerous colonies of *Pythium* and saprolegniaceous fungi. Few seedlings were infected by *Phytophthora* in plates preflooded for 5 days (Table 3).

In all experiments, numbers of seedlings infected usually decreased from low to high dilutions with the sand:sandy loam mixture. However, results were often erratic between replicate plates at the same dilution, and higher dilutions (1:8, 1:16) did not always have fewer seedlings infected than lower dilutions (1:1, 1:2, 1:4) (Tables 1, 2, 3, 4). In an attempt to decrease variability among replicates and to determine whether inoculum was associated with a particular particle size in the soil, soil of two samples was fractionated mechanically by dry sieving and the individual samples assayed. No differences between infectivity of unsieved soil and fractions retained on 10-, 24-, and 50-mesh screens (openings of 2.00, .71, and .30 mm, respectively) were noted with either sample. In one sample, however, infection was much less with debris retained on a 100-mesh screen (openings .15 mm) than with unsieved soil, and no infection occurred for either sample with material which passed through a 100-mesh screen. Seedling infection was not more uniform with any size fraction than with unsieved soil.

It was possible that the inoculum levels in some soil samples were below what would normally be

TABLE 3. The effect of preflooding naturally infested soils on detection of *Phytophthora megasperma* by baiting with alfalfa seedlings

Soil sample	Number of days pre-flooding[b]	Ratio, original soil:diluent soil[a] 1:0	1:1	1:2	1:4	1:8	1:16	1:32
12	0	8[c]	9	4	6	3	2	0
	1	16	8	5	4	1	5	0
	3	10	10	0	1	0	0	0
	5	4	4	0	0	0	0	0
13	0	6	4	0	4	2	0	1
	1	1	5	7	6	0	3	0
	3	2	3	3	0	1	1	0
	5	0	4	0	0	0	0	0
14	0	5	4	4	2	2	1	2
	1	6	6	6	9	5	2	1
	3	1	2	6	8	10	0	0
	5	0	3	1	0	0	0	0

[a] Diluent soil a 1:1 sand:sandy loam mixture, 25 cc soil per plate.

[b] Water added to plates 0, 1, 3, and 5 days before addition of alfalfa seedlings.

[c] Total number of seedlings on which *Phytophthora* sporangia were observed in three plates with six seedlings per plate after incubation for 4 days at 25 C.

TABLE 4. The effect of preplanting naturally infested soils with alfalfa on detection of *Phytophthora megasperma* by baiting with alfalfa seedlings

Soil sample	Treatment	Ratio, original soil:diluent soil[a] 1:0	1:1	1:2	1:4	1:8	1:16	1:32
53	none	5[b]	16	7	9	4	1	2
	Alfalfa[c]	16	18	18	17	14	9	6
	Alfalfa[d] + flooding	17	16	18	15	16	10	13
67	none	1	7	6	5	4	0	0
	Alfalfa	5	17	16	16	16	10	9
	Alfalfa + flooding	12	16	17	9	9	8	4
81	none	4	4	14	4	1	0	0
	Alfalfa	16	18	16	18	18	16	13
	Alfalfa + flooding	10	18	16	17	10	14	1

[a] Diluent soil a 1:1 sand:sandy loam mixture, 25 cc soil per plate.

[b] Total number of seedlings on which *Phytophthora* sporangia were observed in three plates with six seedlings per plate after incubation for 4 days at 25 C.

[c] Three-week-old 'Vernal' alfalfa seedlings transplanted into soil (six seedlings per 6-inch pot) and watered normally every other day for 8 weeks.

[d] As in c, but soil watered normally every other day for 5 weeks, and then flooded and drained at 2-day intervals for 3 weeks.

Fig. 1. Distribution of *Phytophthora megasperma* in soils of alfalfa fields in 54 counties in Wisconsin in 1971. Dots = *P. megasperma* detected in soil by a seedling baiting technique following planting of soil samples to alfalfa; circles = not detected.

detected by the seedling baiting assay. To determine whether the ability to detect *Phytophthora* could be increased, seedling infection in three positive Dane County soils which received no treatment was compared to that which occurred after alfalfa had been planted in the same soils with both normal watering and with periodic flooding. The initial inoculum level in each soil was determined by the seedling assay at the beginning of the experiment. Vernal alfalfa seedlings with 6- to 8-inch-long root systems and 5- to 6-inch tops, grown 3 weeks in autoclaved sandy loam, were then transplanted into samples of the field soils in 6-inch pots. Six pots, with six seedlings per pot, were used for each soil sample; all pots were maintained in a greenhouse (ca. 25 C) and watered every other day for 5 weeks. Following this, three pots from each sample were alternately flooded to the soil surface for 2 days and then allowed to drain for 2 days over a period of 3 more weeks. The remaining three pots from each soil sample were watered every other day as needed to keep the soil moist with drainage unimpeded. After a total of 8 weeks, soils from the three flooded and the three unflooded pots of each sample were separately mixed and assayed with the seedling test after all larger root pieces were removed by hand.

Planting soils to alfalfa greatly increased the amount of *Phytophthora* inoculum in all three soils (Table 4). No consistent differences were evident between infection occurring in soil from flooded and unflooded pots.

The increase of inoculum observed in these soil samples following planting to alfalfa suggested that some "negative" samples might have low inoculum levels which could be raised to the point where detection with the seedling test was possible. Fifty-two of the 82 negative Dane County samples were planted to alfalfa as previously described, with normal greenhouse watering for 8 weeks. When the soil was then tested by the seedling baiting test, 12 of the 52 Dane County samples originally rated negative were found to contain *Phytophthora*. In all of these tests, the seedlings in the baiting test were heavily infected.

Testing of statewide samples.—During June, July, and August of 1971, 109 soil samples were collected from low-lying areas of alfalfa fields showing evidence suggestive of Phytophthora root rot in 54 other counties in Wisconsin. One to three samples were collected in each county. Samples were stored in tightly sealed plastic bags at 23-25 C for up to 2 months. Portions of all samples were planted to 3-week-old Vernal alfalfa seedlings, and pots were watered every other day for 6 weeks. In many of the pots, alfalfa seedlings showed symptoms of *Phytophthora* infection, becoming chlorotic soon after transplanting and dead after 2 to 3 weeks. In others, chlorosis did not appear until 4 to 5 weeks. Plants grown in 39 samples showed top symptoms of chlorosis, necrosis, and stunting typical of Phytophthora root rot after 6 weeks. Soil of each sample was then assayed by the method described above for *P. megasperma* using three plates of seedlings per soil sample.

Thirty-three of the 39 samples in which plants had shown top symptoms assayed positive for *P. megasperma*, and the organism was also detected in 15 of the 70 samples in which top symptoms in the greenhouse were not noticed. The 48 positive samples originated from fields throughout Wisconsin (Fig. 1). In most cases, large numbers of seedlings were heavily infected, indicating high levels of inoculum. Statewide sampling sites were located on Soil Conservation Service maps of individual counties in Wisconsin and the soil textures characteristic of the sites were noted. There was some correlation between recorded soil type and presence of *Phytophthora*. Of samples from silt loam areas, the most common type sampled, 26 were positive and 28 were negative. Of 11 samples from sandy loam areas, one was positive and 10 were negative; of eight samples from clay loam or silty clay loam areas, five were positive and three were negative.

Isolation and characterization of Phytophthora from soils of alfalfa fields in Wisconsin.—Schmitthenner (17) and Marks & Mitchell (14) reported that *Phytophthora* was difficult to isolate from sporulating colonies on infected seedlings due to contamination with *Pythium*. We also found that even when *Phytophthora* colonies developed on all six seedlings in a plate of undiluted soil, isolations from bits of cotyledons on selective media most frequently yielded *Pythium* spp.

Since many seedlings were infected by *Phytophthora* at high dilutions of soils that had been planted to alfalfa (Table 4), it appeared possible that isolations might be more successful with such

seedlings than with those from undiluted soil plates because *Pythium* inoculum might be diluted out. Whole cotyledons of seedlings floated for 48 hr over soils previously planted to alfalfa and diluted 1:8 and 1:16 with steamed sand:sandy loam were surface-sterilized and plated on V-8A + pimaricin and vancomycin. Pure cultures of *Phytophthora* were easily obtained from at least half of the cotyledons plated from most soil samples, and few *Pythium* colonies developed.

Although *P. megasperma* was easily isolated from most soil samples assayed positive after planting to alfalfa, in some cases few or no cultures developed from plated cotyledons. All of the samples rated positive with the baiting test after planting to alfalfa, had been stored in plastic bags at 23-25 C for 2 to 3 months before isolations were attempted. When these samples were reexamined by the baiting technique after 3 to 4 months storage, most yielded seedlings heavily infected with *Phytophthora*. With some samples, however, few or no colonies developed. It appeared that the high inoculum levels which developed in the presence of alfalfa were stable over several months in some soil samples but not in others, and that this was a factor that would have to be considered in assaying soils for this pathogen.

A comparison was made of 155 *Phytophthora* isolates obtained from soils of Wisconsin alfalfa fields. These included 39 isolates from the Marshfield Experimental Farm and Dane County, and 116 from other areas of the state. A culture derived from a single hyphal branch was obtained for each isolate.

All isolates produced numerous nonpapillate, proliferating sporangia characteristic of *P. megasperma* when infected alfalfa seedlings were floated in aqueous soil extract, but sporangia never developed on agar media. Oospores developed on V-8A, and both paragynous and amphigynous antheridia were observed in all cultures examined intensely. Diameters of 10 oogonia and oospores were measured in each of 111 isolates. Mean values of oogonia ranged from 30.8 to 39.4 μ with the mode interval 35.5 to 37.4 μ. Means of oospore diameters ranged from 27.4 to 36.5 μ with the mode interval 33.5 to 35.4 μ. All of 110 isolates grew from 2 mm to several cm on PDA at 31-32 C in 2 weeks.

Most isolates of *P. megasperma* collected from the Dane County and statewide surveys could be placed in one of three groups on the basis of colony morphology on V-8A: (i) "dense-fluffy" with thick, cottony aerial mycelium and few oospores formed on the agar surface; (ii) "scanty-fluffy" with sparse aerial mycelium and masses of oospores on the agar surface; and (iii) "appressed" with no aerial mycelium and with masses of oospores. Some isolates showed characteristics of more than one of these three groups in serial transfers on V-8A. All cultures produced dense aerial mycelium on PDA.

DISCUSSION.–Previous to this study, *P. megasperma* was only known from a few sites in Wisconsin (11, 14). It is now apparent that the organism must be regarded as an actual or potential problem for alfalfa production throughout the state. *P. megasperma* is common in silt loam, the soil type encountered most frequently in our survey. It is also more likely to be present in clay loam or silty clay loam soils than in sandy loams.

The seedling test is not sensitive enough to determine the presence of *P. megasperma* in many untreated field soils. Detection may be easy in soils with high inoculum levels or under moist conditions where alfalfa is present and infection and disease development are in progress. However, in some soils with low inoculum levels or in dry periods, the organism may only be detected following growth of alfalfa seedlings in the soil. This treatment greatly increases inoculum levels in all naturally infested soils. *P. megasperma* could easily be isolated from infected seedlings at 1:8 or 1:16 dilutions of soils planted to alfalfa but not from the same soils without dilution due to contamination by *Pythium*. This suggests that inoculum levels of *P. megasperma* are selectively raised by planting to alfalfa, while those of *Pythium* spp. are not. Thus, *Pythium* inoculum is apparently diluted out at high soil dilutions while sufficient *Phytophthora* inoculum is still present to cause infection of many seedlings.

Seedling infection in the baiting technique is affected more by temperature than is the occurrence and development of root rot in older plants. In two soil samples, seedling infection was greater at 20 C than at 25 C, and in a third sample infection was greatest at 15 C. However, Erwin (9) reported no differences in root rot severity at temperatures from 17 to 27 C. It is possible that a temperature affect occurs with the baiting technique because other fungi such as *Pythium* spp. may also invade injured seedlings, and *P. megasperma* might be at a greater competitive advantage at lower temperatures because growth at 15 C is only slightly less than at 25 C (9).

Several reports have indicated that more than one species of *Phytophthora* may be associated with alfalfa root rot (1, 13, 15). There is no question that all of the *Phytophthora* isolates from Wisconsin alfalfa fields are the same species, in spite of differences in colony morphology and oospore production. By the key of Waterhouse (19), the Wisconsin isolates would be referred to *P. megasperma* Drechs. var. *sojae* Hildebrand because oogonia average less than 40 μ diam and maximum growth occurs at temperatures greater than 30 C. By the concept of Savage et al. (16) they would be classified as *P. sojae* Kaufmann & Gerdemann. We agree with Erwin (8) that these and other *Phytophthora* isolates from alfalfa are best described as *P. megasperma* sensu Tompkins et al. (18). This concept is the most realistic because it allows for variation in diameters of oogonia and oospores within one species unit, as we observed in our isolates. Erwin (7, 8) previously described variation in colony morphology and oospore production in *P. megasperma* similar to that which we observed; this variation should not be considered important for species determination.

No correlations were noted between the nature or extent of field symptoms and the presence of *P.*

megasperma in either the Dane County or statewide surveys. Appearances of stands in Dane County fields in which the organism was detected by assay of untreated soil samples were not different from some others in which *Phytophthora* was not found even after planting to alfalfa. However, in both 1970 and 1971, sampling was done in dry periods, and very few sampling sites showed evidence of active disease development or spread. It is, therefore, possible that inoculum levels were so low in some cases that *P. megasperma* was not collected in samples even though it was present in the fields. It is also possible that very low inoculum levels in some soil samples might have been revealed by assay after repeated planting to alfalfa. It is, of course, also possible that *P. megasperma* was not present in many fields, and that sparse stands in some low areas were the result of damping-off, bacterial wilt, Aphanomyces root rot, flooding, or other yet unexplained causes. Nevertheless, because *P. megasperma* is generally distributed in alfalfa-growing areas in Wisconsin, it must be considered a potential limiting factor for alfalfa production in low-lying areas throughout the state.

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