Interrelationships of Seedling Age, Inoculum, Soil Moisture Level, Temperature, and Host and Pathogen Genotype in Phytophthora Root Rot of Alfalfa

R. G. Pratt and J. E. Mitchell

Former Research Assistant and Professor, Department of Plant Pathology, University of Wisconsin, Madison 53706 Present address of senior author: Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331.

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ABSTRACT

Resistance of two susceptible cultivars and of two resistant lines of alfalfa was compared by transplanting 2-, 4-, 6-, and 8-week-old seedlings into soil infested with cornmeal-sand inoculum of *Phytophthora megasperma*. Taproot disease was usually most severe in seedlings transplanted at 2 weeks of age and least severe in those transplanted at 8 weeks of age. Lines and cultivars differed in resistance at all ages tested. Disease severity in seedlings transplanted when 6 weeks old was greater at low dilutions of inoculum (1/8 or 1/32, v/v) than at the highest dilution (1/2,048), but four-fold or greater dilutions did not always result in less disease. Resistance of lines and cultivars did not differ when disease was severe at the lowest inoculum dilution (1/8, v/v). With few exceptions, disease was more severe at 20 and 24 C than at 16 or 28 C. Periodic flooding of soil caused greater disease in all lines and cultivars at one or more temperatures. Differences in resistance were evident at all temperatures. Growth of roots of cuttings from field-resistant plants was generally greater than growth of roots of susceptible plants in the presence of *P. megasperma*. Differences in virulence of *P. megasperma* isolates were not related to geographic areas of origin and do not suggest the existence of different pathogenic races.

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Phytophthora root rot, caused by *Phytophthora megasperma* Drechsler, is a destructive disease of alfalfa in low-lying fields throughout North America. The development and use of resistant cultivars currently offers the only practical means of disease control.

Resistance to *P. megasperma* in individual plants and cultivars was first reported by Erwin in 1966 (5). Marks and Mitchell (14) observed differences in susceptibility of cultivars and related these to differences in responses of alfalfa roots to *P. megasperma*. Hypersensitive reactions occurred in infected cortical cells at tips of roots of tolerant plants, and diameters of steles in surviving roots of plants grown in the presence of the pathogen were larger than in noninoculated roots. Erwin (5) and Marks and Mitchell (14) both reported that resistance to *P. megasperma* was not expressed at the young seedling stage, but Gray et al. (10) observed enhanced resistance in progeny of plants which survived pre- and postemergence damping-off. Pratt et al. (16) described resistant and susceptible reactions in alfalfa cotyledons inoculated with zoospores of *P. megasperma* and reported significant correlations between resistance in cotyledons and in roots. Lu et al. (13) studied the inheritance of resistance and concluded that susceptibility was conditioned by one tetrasomic gene with incomplete dominance.

Lehman et al. in California in 1967 released two *Phytophthora*-tolerant germplasms to be used as sources of resistance in breeding programs (11, 12). Frosheiser (9), Barnes (1), and others later released from Minnesota two germplasm sources (9) and two cultivars, Agate (1) and Ramsey (3), with resistance to *P. megasperma*. They also described in detail their greenhouse and field selection techniques (8).

Although several new alfalfa cultivars have now been developed with resistance to *P. megasperma,* no reports have yet described the stability of this resistance under changing environmental conditions. The purpose of work reported here was to compare reactions of resistant and susceptible cultivars at different ages to the pathogen at various inoculum levels and under a range of temperature and soil moisture conditions. The reaction of roots of isogenic rooted cuttings from resistant and susceptible clones of alfalfa to the presence of *P. megasperma* isolates from diverse areas of the United States was also studied.

MATERIALS AND METHODS.—Alfalfa plants of the susceptible cultivars Vernal and Saranac, and of the resistant lines MnP-B1 and MnP-D1 (9), were used in all experiments with seedlings. Scarified seeds were germinated on moist filter paper for 2 days, and seedlings were planted into a steamed sand:loam (1:1, v/v) mixture in 474 cm^3 wax-treated paper cups and grown in the greenhouse at 20 C with daily watering. After 10 days, a thin layer of unsteamed soil was added to the surface of each cup. After 2, 4, 6, or 8 weeks of growth, seedlings were transplanted into infested soil.

Inoculum consisted of *P. megasperma* growing on a mixture of cornmeal and sand. It was prepared by mixing 374 g silica sand and 9.0 g cornmeal in 500-ml flasks, adding 100 ml of deionized water, and autoclaving. Flasks inoculated with *P. megasperma* were incubated at 25 C and shaken every 3 days to insure colonization of the cornmeal-sand mixture.

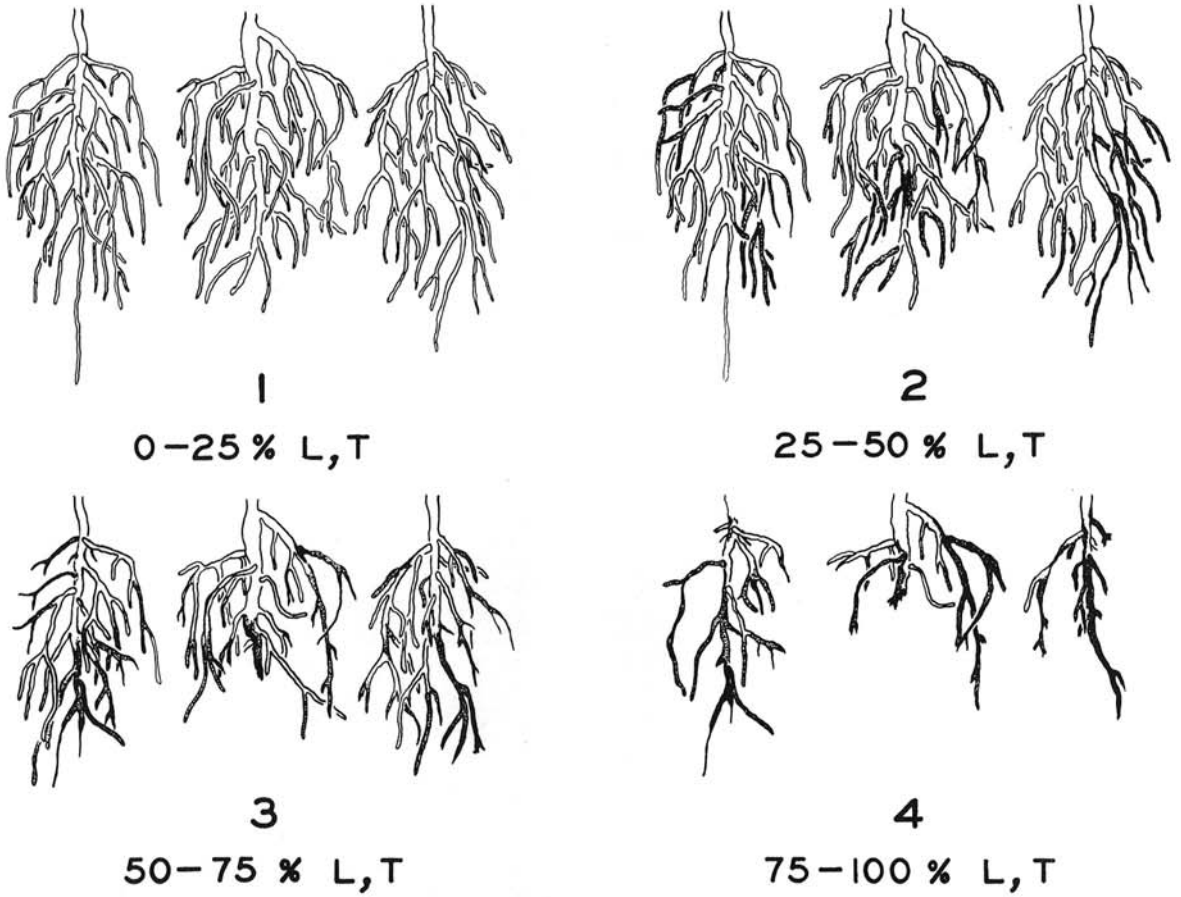

Fig. 1. Diagrammatic representation of scores of lateral root and taproot disease in alfalfa plants differing in severity of Phytophthora root rot. 1 = 0-25% of root(s) browned, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%. Lateral root and taproot disease was scored independently.

In experiments with seedlings, equal quantities of inoculum of five isolates of *P. megasperma,* each from different counties in Wisconsin (15), were mixed. The composite inoculum was mixed with unsteamed sand:loam mixture (1:7, v/v), and further dilutions were made serially to give inoculum levels of 1/8, 1/32, 1/128, 1/512, and 1/2,048. Alfalfa seedlings were transplanted into the infested soil in 474-cm^3 cups and maintained in a 20 C greenhouse for 4 weeks for inoculum-level and seedling-age experiments. For temperature and soil-flooding experiments, transplanted seedlings were maintained in growth chambers at 16, 20, 24, and 28 C with a 12-hour photoperiod under combined cool-white fluorescent and incandescent lamps (total intensity= 19,400-21,500 lux). In all experiments with seedlings, twelve plants of each cultivar or line were used in each treatment (three seedlings per cup, four cups per treatment).

Disease severity in seedlings of all ages was rated according to percentages of the roots which were visibly browned (rotted) after 4 weeks of growth in infested soil. Browning of lateral roots and taproot were scored independently. Plants were scored 1 if 0-25% of the lateral

TABLE 1. Taproot disease severity in plants of resistant and susceptible alfalfa lines and cultivars of four ages grown in the presence of *Phytophthora megasperma*

Alfalfa line or cultivar[a]	Plant age and disease severity[b]			
	2 weeks	4 weeks	6 weeks	8 weeks
Saranac	3.8 A	3.6 A	4.0 A	3.4 A
Vernal	4.0 A	3.3 AB	3.7 A	2.7 AB
MnP-B1	3.1 B	2.8 B	2.5 B	1.8 BC
MnP-D1	2.6 C	2.0 C	1.6 C	1.4 C

[a]Saranac and Vernal = susceptible cultivars, MnP-B1 and MnP-D1 = resistant lines.

[b]Plants of all ages were transplanted into soil infested with *P. megasperma* at 1/32 (v/v) inoculum level and grown 4 weeks at 20 C. Disease severity score 1 = 0-25% of taproot browned, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%. Individual scores = means of scores of three plants in each of four replicate cups. Means within a column not followed by the same letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.

TABLE 2. Taproot disease severity in plants of resistant and susceptible alfalfa lines and cultivars grown in the presence of *Phytophthora megasperma* at five inoculum levels

Alfalfa line or cultivar[a]	Inoculum level (v/v) and disease severity[b]				
	1/8	1/32	1/128	1/512	1/2,048
Saranac	3.6 A	4.0 A	3.3 A	3.2 A	3.3 A
Vernal	3.6 A	3.7 A	2.7 A	3.0 A	1.4 B
MnP-B1	3.3 A	2.5 B	2.4 B	2.6 A	1.3 B
MnP-D1	3.4 A	1.6 C	1.3 B	1.3 B	1.7 B

[a]Saranac and Vernal = susceptible cultivars, MnP-B1 and MnP-D1 = resistant lines.

[b]Six-week-old plants were transplanted into soil infested with *P. megasperma* and grown 4 weeks at 20 C. Disease severity score 1 = 0-25% of taproot browned, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%. Individual scores = means of scores of three plants in each of four replicate cups. Means within a column not followed by the same letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.

TABLE 3. Taproot disease severity in plants of resistant and susceptible alfalfa lines and cultivars grown in the presence of *Phytophthora megasperma* at four temperatures with and without flooding of soil

Alfalfa line or cultivar[a]	Flooding[b]	Temperature and disease severity[c]			
		16	20	24	28
Saranac	+	2.5 AB	4.0 A	3.4 AB	3.3 A
	−	2.6 A	2.9 A	3.0 AB	2.3 ABC
Vernal	+	3.2 A	2.8 A	3.8 A	2.9 AB
	−	1.5 C	1.8 BCD	3.2 AB	2.3 ABC
MnP-B1	+	1.7 BC	2.6 AB	2.8 AB	2.2 BC
	−	1.4 C	1.3 D	2.5 B	1.5 CD
MnP-D1	+	1.7 BC	2.3 ABC	2.9 AB	2.3 BC
	−	1.0 C	1.6 CD	1.5 C	1.0 D

[a]Saranac and Vernal = susceptible cultivars, MnP-B1 and MnP-D1 = resistant lines.

[b]Flooding = soil watered to saturation for 3 days followed by 3 days watering with drainage unimpeded. Nonflooded plants watered daily with drainage unimpeded.

[c]Seven-week-old plants were transplanted into soil infested with *P. megasperma* and grown 27 days. Disease severity score 1 = 0-25% of taproot browned, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%. Individual scores = means of scores of three plants in each of four replicate cups. Means within a column not followed by the same letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.

roots were brown, 2 if 25-50%, 3 if 50-75%, and 4 if 75-100% (Fig. 1). Similar scores were assigned to taproots. Whenever a taproot was completely girdled by rot, all of the root below the lesion was considered to be rotted. When a portion of a taproot was rotted off, disease was scored by presuming that the length of the taproot if completely intact would have been similar to roots of control plants.

Experiments with rooted cuttings were conducted with plants selected for field resistance or susceptibility from a Phytophthora root rot plot maintained by Paul Sun of Teweles Seed Co., at Clinton, Wisconsin, in 1972. Plants were selected after one season of growth with frequent overhead irrigation in infested or uninfested soil. Plants which were classified resistant were taken from infested areas of the plot and had a few restricted lesions on taproots and large lateral roots. Plants presumed to be susceptible were taken at random from uninfested areas of the plot from lines or cultivars in which few or no plants showed resistance in infested areas. Sections of stems 8-13 cm long with one aerial node were rooted in moist sand in 20-cm diameter pots in the greenhouse for 4 weeks at 28 C.

Single-zoospore cultures were obtained from two isolates of *P. megasperma* from Arizona (from R. B. Hine), two from California and one from Mississippi (D. C. Erwin), one from Washington (M. M. Kraft), and three from Wisconsin (15). Zoospores were obtained from colonies flooded with deionized water after 10 days of growth in light on V-8 juice agar (16). Single-zoospore cultures were obtained by transferring isolated spores germinating on cornmeal agar to individual cultures. All isolates of *P. megasperma* were maintained on cornmeal agar at 4-5 C with periodic transfers and growth at 25 C.

Statistical significance in all experiments was determined by analysis of variance with completely random or factorial designs and by use of Duncan's multiple range test (17).

RESULTS.—*Symptom development.*—Leaves and shoots of seedlings transplanted into infested soil often became wilted and chlorotic after 8-10 days. Necrosis appeared after 10 days, and foliage of some plants was completely necrotic after 4 weeks. Lateral roots and taproots became light-brown after 10 days, and were often severely rotted and dark-brown after 4 weeks. When control seedlings were transplanted into uninfested sand:loam mixture, portions of lateral roots sometimes became slightly discolored, but most lateral roots and all taproots remained white.

Disease severity at different plant ages and inoculum levels.—Seedlings of the susceptible cultivars Saranac and Vernal, and of the resistant lines MnP-B1 and MnP-D1, 2, 4, 6, and 8 weeks old, were transplanted into a sand:loam mixture infested with *P. megasperma* at inoculum levels of 1/8, 1/32, 1/128, 1/512 and 1/2,048. At the 1/32 inoculum level, taproot disease was usually most severe in 2-week-old seedlings and least severe in 8-week-old seedlings (Table 1). Differences in resistance among lines and cultivars were observed in seedlings of all ages, but these were most evident in seedlings transplanted into infested soil after 4 or 6 weeks. With seedlings 2 weeks old, nearly all plants of the two susceptible cultivars and many plants of the resistant lines were killed; with 8-week-old seedlings, few plants of any cultivar or line were killed after 4 weeks in infested soil. Significant differences between the cultivars Saranac and Vernal in disease severity were not observed in seedlings of any age. Plants of MnP-B1 differed significantly in disease severity from those of the two susceptible cultivars in seedlings 2 and 6 weeks old, and those of MnP-D1 did

TABLE 4. Dry weight of roots of cuttings of resistant and susceptible alfalfa clones grown in the presence of different isolates of *Phytophthora megasperma*

	Dry weight of roots, % of control[a]						
	Susceptible clones			Resistant clones			
Isolate	1	2	3	1	2	3	Mean[b]
Cal.–P844re	14	29	24	51	33	28	30 A
Wis.–Jefferson	19	20	30	33	26	53	30 AB
Wis.–Shawano	24	22	23	44	40	55	35 ABC
Ariz.–Snowflake	24	41	43	25	39	54	38 BCD
Wis.–Chippewa	33	22	37	36	48	56	39 BCD
Cal.–P192	19	31	20	45	61	57	39 CD
Washington	21	27	31	41	62	52	39 CD
Miss.–P339	34	53	49	42	44	47	45 DE
Ariz.–Parker	50	51	43	42	43	57	48 E
Mean[b]	26 A	33 A	33 A	40 B	44 BC	51 C	

[a]Values = mean dry weight of roots of plants grown in infested soil as percent of that of plants grown in noninfested soil. Three stem cuttings with roots trimmed to 1.5 cm were transplanted into each of three cups of sand:loam mixture infested with cornmeal-sand inoculum (1/32, v/v) of single-zoospore isolates of *P. megasperma*.

[b]Means not followed by the same letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.

so at all ages. Severity of disease in plants of MnP-D1 was less than in those of MnP-B1 in seedlings 2, 4, and 6 weeks old, but not in 8-week-old seedlings at the 1/32 inoculum level (Table 1).

Lateral roots of 6-week-old lines and cultivars differed in severity of disease only at one inoculum level, but disease in taproots differed at all inoculum levels except the highest (Table 2). At the 1/8 inoculum level, many plants of all lines and cultivars were killed, while few were killed at the 1/2,048 level. Differences in taproot disease severity between plants of Saranac and Vernal were significant at the 1/2,048 inoculum level, and differences between plants of MnP-B1 and MnP-D1 were significant at the 1/32 and 1/512 levels.

Disease severity under different temperature and soil moisture conditions.—Seven-week-old seedlings of the four cultivars and lines were transplanted into infested sand:loam (1/32 inoculum level) and maintained in growth chambers at 16, 20, 24, and 28 C for 27 days. Three cups (twelve seedlings) of each cultivar or line were watered daily with drainage unimpeded and three cups were flooded daily to soil saturation for periods of 3 days followed by watering for 3 days with drainage unimpeded.

Taproot disease was more severe at 24 C and usually also at 20 C than at 16 or 28 C, with or without soil flooding (Table 3). Flooding increased disease in all cultivars and lines, and this difference was significant in five of the sixteen comparisons. In the absence of flooding, disease in Saranac was significantly greater than that in all other lines and cultivars at 16 and 20 C, and was greater than in the two resistant lines at 24 and 28 C. Vernal differed significantly from MnP-B1 at 24 and 28 C only. With flooding, differences between lines and cultivars were significant only at 16 and 28 C.

Growth of alfalfa roots in the presence of different P. megasperma isolates.—Roots of cuttings of three plants from the *Phytophthora* nursery classified resistant and three plants considered susceptible were trimmed to a length of 1.5 cm. Three cuttings were transplanted into each of three cups of infested soil containing a 1/32 level of inoculum prepared from single-zoospore cultures of each of nine isolates of *P. megasperma*. Three control cups containing sand:loam mixture amended with uninfested cornmeal-sand were also planted with cuttings of each plant. Transplanted cuttings were maintained in a greenhouse at 24 C with daily watering for 7 weeks. Roots of cuttings were washed free of soil, excised from stems, and the total root mass of the three cuttings from each cup was weighed after drying for 24 hours in an oven at 100 C.

Roots of cuttings transplanted into uninfested soil grew to lengths of 15 cm and remained white. Roots of cuttings transplanted to infested soil were smaller and showed varying amounts of browning. Some cuttings from susceptible plants had no new root growth, and the transplanted roots were completely rotted. With few exceptions, cuttings from resistant plants had greater top and root growth and less root browning than cuttings from susceptible plants (Table 4).

Analysis of variance was performed with root weights from all infested cups using a factorial design. The weight of roots from each of the three replicate cups for each host plant-isolate combination was expressed as a percentage of the mean weight of roots of the host plant grown in uninfested soil. Significant ($P = 0.05$) differences in root weights were attributed both to effects of host genotype and virulence of pathogen; no significant interaction between hosts and pathogens was found. Mean values of root weights of cuttings from the three susceptible plants were similar, and all were significantly less than those of

all resistant plants. *P. megasperma* isolate "P844re" was significantly more virulent than all others except two isolates from Wisconsin.

DISCUSSION.—Severity of Phytophthora root rot in alfalfa seedlings of resistant and susceptible lines and cultivars varied with plant age, inoculum level, temperature, and soil moisture content. The extent of growth of roots of cuttings varied with host genotype in the presence of *P. megasperma,* and with differences in virulence of isolates.

Two previous studies determined that severity of disease may vary with plant age and inoculum level. Frosheiser (7) reported less severe disease in 12-week-old seedlings of Vernal alfalfa than in 6-week-old seedlings at similar inoculum levels. Gray et al. (10) diluted inoculum 0-, 10-, and 100-fold and obtained progressively less disease in young seedlings. Both studies were conducted only with seedlings of predominantly susceptible cultivars. This study shows that severity of disease is affected by inoculum level and plant age in seedlings of both resistant and susceptible lines and cultivars. Resistance was expressed in taproots of seedlings from 2-8 weeks old and at all inoculum levels except the highest (1/8, v/v).

Four-fold or greater dilutions of inoculum did not always result in less disease in seedlings of either resistant or susceptible lines. It is possible that effects of diluting inoculum are sometimes obscured over 4 weeks by the ability of *P. megasperma* to rapidly increase in soils in the presence of alfalfa.

Reports of the extent to which temperature influences severity of Phytophthora root rot are conflicting. Erwin (4) reported that disease progressed less rapidly at 10-16 C than at 21-27 C, but that severity was about the same at temperatures of from 17 to 27 C (5). Bushong and Gerdemann (2) observed differences in severity of disease in young seedlings under different regimes of fluctuating temperatures. Similarly, in our study taproot disease in seedlings of resistant and susceptible germplasms was usually greater at 20 and 24 C than at 16 or 28 C in both flooded and unflooded treatments. However, resistance in taproots of seedlings was expressed at all temperatures with or without flooding of soil.

Although disease severity in most plants of the lines MnP-B1 and MnP-D1 was less than in plants of Vernal and Saranac, seedlings which could be classified "resistant" and "susceptible" were found in all lines and cultivars. Many plants also could not be clearly considered resistant or susceptible. These observations are compatible with the genetic model of Lu et al. (13), which explains phenotypic differences in disease reactions of plants by different dosages of recessive genes for resistance.

Several authors have recommended methods for screening alfalfa for resistance to Phytophthora root rot (6, 8, 10). For evaluation of disease in the greenhouse, we recommend transplanting seedlings 6 weeks old, with clearly differentiated taproots, into sand:loam infested with cornmeal-sand inoculum of *P. megasperma* at ratios of 1/32 to 1/128 (v/v). Plants should be maintained at 20-24 C for 4 weeks and watered daily. To enhance disease, soil may be periodically flooded for several days. Disease in plants may be easily scored using only taproots. Although several authors have scored plants in whole or part on the basis of kinds and sizes of lesions (5, 7, 8, 13), we suggest that scoring on the basis of total percentages of browning of taproots provides a convenient method for rating differences in resistance of plants.

Screening should be conducted using a composite of isolates of *P. megasperma*. Growth of roots of isogenic cuttings of six alfalfa clones varied significantly in the presence of different isolates, indicating that these differed in virulence. However, pathogenic races of this organism on alfalfa have not been described, and we found no evidence to suggest that differences in virulence of isolates may be explained by the existence of different pathogenic races. Differences in virulence were also not related to broad geographic areas of origin.

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