

Seedling and Rootlet Diseases of Forage Alfalfa Caused by *Pythium irregulare*

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ABSTRACT

Hancock, J. G. 1991. Seedling and rootlet diseases of forage alfalfa caused by *Pythium irregulare*. Plant Dis. 75:691-694.

Isolates of *Pythium irregulare* caused seedling diseases of alfalfa (*Medicago sativa*) over a range of temperatures (16–32 C); however, postemergence damping-off and root-forking were most severe at 16 and 21 C. Alfalfa shoot growth was retarded when seedlings were grown in either pasteurized or raw soils amended with culture-grown inocula adjusted to give inoculum densities of propagules within ranges measured in field soils. A negative correlation was found between infection rates of feeder roots by *P. irregulare* and shoot dry matter during early harvest cycles with greenhouse-grown plants. However, degrees of reduction in shoot dry matter differed significantly between cultivars of alfalfa.

Additional keywords: *Pythium paroecandrum*, *Pythium ultimum*

Oomycetous fungi cause a variety of seedling and root diseases of alfalfa (*Medicago sativa* L.) in North America (20). Certain cosmopolitan species of *Pythium* cause preemergence damping-off and root-forking (10,12), whereas *Phytophthora megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin causes widespread and serious root diseases of both seedlings and mature plants (20). Species of *Aphanomyces* are reported to cause seedling and root diseases in the midwestern United States and Canada in poorly drained soils (6,22). All of these pathogens may cause root diseases of alfalfa where shoot symptoms are inconspicuous (13,20). A general growth retardation in forage alfalfa caused by small amounts of infection or weakly virulent pathogens can, however, easily go unrecognized in the field.

Species of *Pythium* are implicated as causing subclinical diseases of alfalfa and other crops (13,15,16,27–29). In the Central Valley of California, the main species or species complexes of *Pythium* associated with feeder roots of alfalfa are *P. ultimum* Trow, *P. irregulare* Buisman/*P. paroecandrum* Drechs., and *P. violae* Chesters & C. J. Hickman (13). Rootlet infection by species of *Pythium* is most intense in spring and fall (13,14). In previous greenhouse studies, rootlet infection reduced shoot growth of alfalfa in pasteurized soil reinfested with *P. irregulare*/*P. paroecandrum* and *P. ultimum* (13).

Because *P. irregulare*/*P. paroecandrum* was the most common *Pythium* complex isolated from rootlets of forage alfalfa in the Central Valley of California (13), investigations of the virulence of isolates of this group were continued. In preliminary studies, forage alfalfa crops maintained or enhanced soil inoculum density of *P. irregulare*, increasing its potential as a factor in replant diseases. Thus, in this investigation, emphasis was placed on morphologically distinctive isolates of *P. irregulare* as both seedling and rootlet pathogens of alfalfa (4,31). An isolate of *Pythium*, morphologically similar to the taxonomic description of *P. paroecandrum*, also was included in this study for purposes of comparison (31).

MATERIALS AND METHODS

Preparation of inoculum. Isolates of *Pythium* were recovered from feeder roots of forage alfalfa grown in the Central Valley. One isolate (79-2) identified as *P. paroecandrum* was recovered from a lesion in a hypocotyl of common bean (*Phaseolus vulgaris* L.). Stock cultures were maintained in a hemp seed/water medium (26). Short-term maintenance of cultures was on potato-dextrose agar slants.

For inoculum, isolates of *Pythium* were grown on oatmeal slants in petri dishes (9 cm diameter) filled with 10 ml of water at room temperature in a laboratory with regular fluorescent lighting (11). Mycelia, oospores, and sporangia were harvested from the water's surface after about 10 days' growth. Mycelial mats were rinsed with distilled water and added to small quantities of moistened (about -0.01 MPa) metham-sodium treated soil, which was prepared as described previously (13). This mixture was incubated in a closed container for 5–7 days at 12 C to promote mycelial

lysis. Then, infested soil was air-dried, ground in a mortar with a pestle, and stored at 12 C until used to infest larger quantities of soil for experimentation. Prior to dilution with noninfested soil, inoculum densities (ID) of *Pythium* spp. were measured in the stock soils with the soil-drop method (11) or on a selective medium (25).

Pathogenicity studies. For studies on virulence of *Pythium*, soils were pasteurized with metham-sodium fumigation or were autoclaved. Once the IDs of stock soils were known, appropriate dilutions were made with pasteurized soil to yield desired inoculum densities. The two soils used in these studies were nonacid, Thermic Typic Xerorthents (Yolo silt loam [formerly designated Zamora loam] and Hanford sandy loam), both of which were used in previous studies (12,13). In experiments requiring 12 L of soil or less, soils were mixed in a liquid-solids blender with an internal rotating blade mechanism (Patterson-Kelly Co., Inc., East Stroudsburg, PA). In large-scale experiments, soils were initially mixed thoroughly with a shovel on a plastic tarp and, finally, in a cement mixer for 20 min. The IDs of diluted infested soils were measured prior to experimentation.

Tests of preemergence and postemergence damping-off were made in plastic pots (13 cm diameter) either in the greenhouse, where minimum and maximum temperatures averaged 18 and 24 C, respectively, or in temperature-controlled rooms at 16 ± 2 , 21 ± 2 , 27 ± 2 and 31 ± 2 C. U.C. mix (2) and soil pasteurized with metham-sodium were used in these tests, and five replicate pots were used for each isolate or control. Pots were arranged on the greenhouse bench in a randomized design. Thirty seeds per pot of Moapa 69, a nondormant cultivar, were planted and covered with about 2 mm of soil; the soil was initially moistened to saturation and remoistened daily. Experiments were evaluated 2 wk after seeding or when seedlings were at the first trifoliate leaf stage. All experiments were repeated.

In tests of pathogenicity toward roots, plants were either directly seeded into soil in pots and thinned or transplanted. Transplants were grown in the greenhouse in vermiculite (13). Seeds were treated with a commercial preparation of *Rhizobium meliloti* prior to planting. Uniformly sized seedlings were transplanted when they were at the third trifoliate leaf stage, and a small

This research was supported by a grant from the University of California Systemwide Integrated Pest Management Program.

Accepted for publication 14 December 1990.

quantity of vermiculite was transferred with the seedling and "root ball" (cone shaped, about 25 cm³) during transplanting to avoid damaging the feeder root system. Plants (one per pot) were grown in soil in the greenhouse in 15-cm-diameter plastic pots with six replicates per treatment. Plants were watered regularly, and shoots were harvested in successive cycles at the first evidence of flowering and dried to constant weight at 60 C.

In tests of cultivar susceptibility, plants were grown outdoors in Berkeley (July through October, 1988 and 1989) on raised benches in 20-cm-diameter white plastic pots filled with 3 L of soil (Hanford sandy loam) with five replicates per treatment. The soil had been autoclaved prior to reinfestation with *P. irregulare*. Plants were seeded directly with about 30 seeds per pot, and seedlings were thinned to five plants per pot 2 wk after emergence. A 2- to 3-cm layer of sterilized granite pebbles was laid in the bottom of each pot and overlaid with soil. Pots were watered daily and

fertilized with slow-nutrient-release pellets (Osmocote, Sierra Chemical Co., Milpitas, CA). Pots with infested and noninfested soil were maintained on adjacent benches to avoid cross-contamination. Pots were randomized within each grouping. Six and seven cultivars or germ plasm lines were tested in 1988 and 1989, respectively.

Infection of rootlets and root-length densities. In pathogenicity studies, rootlet infection was measured with a root plating technique where both the numbers of infection sites per unit of root length and the length of root colonized by *P. irregulare* were measured. Roots were sampled by taking soil cores from pots with a 2-cm-diameter tube sampler or by cutting 9-cm³ blocks of soil out of pots at least 0.5 cm from the edge or bottom of pots. Roots were washed from soil with tap water onto a screen with 0.833-mm² openings (20 mesh), suspended in 1% sodium hexametaphosphate for 30 min, and rewashed on the screen with tap water. Segments of the fine feeder roots were laid out on

1.5% water agar and infection was measured as described previously (13). Root-length densities were measured by the line-intersect method of Newman (24).

Data analysis. All experiments were repeated at least once. One-way or two-way completely randomized ANOVAs were applied to data in multiple comparisons of cultivar performance in tests of pathogenicity and virulence of isolates of *Pythium*. Linear regression was applied to data sets where disease or plant performance was measured as a function of ID.

RESULTS

Seedling stage disease. Isolates of *P. irregulare* and *P. paroecandrum* caused preemergence and postemergence damping-off of alfalfa but were somewhat less virulent than two isolates of *P. ultimum* in causing preemergence damping-off (Table 1). However, there were no consistent differences in the degrees of root-forking incited by the isolates of these species.

There was considerable variation between the virulence of the isolates of *Pythium* spp. when damping-off was measured as a function of temperature (Table 2). The isolates of *P. irregulare* and *P. paroecandrum* usually incited postemergence damping-off and root-forking most severely at the lower temperatures, but *P. paroecandrum* did not cause a significant reduction in emergence of alfalfa in any of the seedling-stage experiments (Tables 1 and 2). The same effects of temperature on seedling diseases caused by *P. irregulare* were found when this experiment was repeated. The isolates of *P. ultimum* were generally more virulent than the isolates of *P. irregulare* and *P. paroecandrum* in causing preemergence damping-off, and the degree of damping-off by *P. ultimum* was influenced less by temperature within the range of 16–31 C (Table 2).

Rootlet disease. Isolates of *P. irregulare* and *P. ultimum* infected roots and caused reductions in shoot dry matter during two or three harvests in two separate experiments. *P. paroecandrum* (79-2) affected shoot growth in one experiment but not the other. However, variation was high in both experiments, and observed differences were not statistically significant ($P > 0.05$).

Graded IDs of *P. irregulare* (83-6) were used to test the influence of infection intensity on several yield components of alfalfa. There was a significant positive correlation between ID and numbers of rootlet infections per root length or percent of root length colonized, and there was a significant negative correlation between ID and root-length densities in greenhouse experiments where temperatures fluctuated between 18 and 24 C (Table 3). Regression of shoot dry matter from the third harvest

Table 1. Effect of different isolates of three *Pythium* species on damping-off of alfalfa (cv. Moapa 69)

Treatment ^a	Mean no. of plants per pot				
	Experiment 1		Experiment 2		
	Emergence	Root-forking ^b	Emergence	PDO ^c	Root-forking
U. C. mix	21.5	0.0	17.2	0.0	0.0
Noninfested	22.7	0.0	24.7	0.0	0.0
<i>P. paroecandrum</i>					
79-2	20.8	0.8	19.7	1.0	1.0
<i>P. irregulare</i>					
83-6	13.2	3.3	16.7	4.8	1.8
84-12	19.0	1.2	25.8	0.5	0.3
<i>P. ultimum</i>					
82-28	6.8	2.2	7.5	2.2	1.7
84-7	9.3	5.2	9.2	3.0	1.7
LSD ($P = 0.05$)	3.2	1.5	4.7	3.1	1.1

^aSoil (Yolo silt loam) was fumigated with metham-sodium and reinfested with isolates of *Pythium*. *Pythium* was not detected in fumigated soil (noninfested). Initial inoculum densities were about 100 germinable propagules per gram of soil. Thirty seeds were planted per pot (five replicates per treatment). Soil temperatures fluctuated between 21 and 23 C.

^bRoot-forking refers to the number of seedlings with multiple adventitious primary roots.

^cPostemergence damping-off (PDO) refers to the number of seedlings with girdled hypocotyls.

Table 2. Influence of temperature on damping-off of alfalfa (cv. Moapa 69) by three *Pythium* species

Treatment ^b	Mean no. of plants per pot ^a											
	16 C			21 C			27 C			31 C		
	EM	PDO	FK	EM	PDO	FK	EM	PDO	FK	EM	PDO	FK
Noninfested	23.8	0.0	0.0	27.8	0.0	0.0	16.2	0.0	0.0	16.7	0.0	0.0
<i>P. paroecandrum</i> 79-2	20.7	4.0	3.0	28.0	0.2	6.5	21.2	0.2	0.8	17.8	0.0	0.0
<i>P. irregulare</i> 83-6	10.7	5.7	0.5	16.8	4.7	5.0	8.7	1.8	1.7	9.3	0.2	1.3
<i>P. ultimum</i> 84-7	6.3	5.0	0.5	6.0	1.2	1.7	6.8	1.7	3.8	6.2	0.8	1.3

^aThirty seeds were planted per pot (five replicates/treatment). EM = no. of seedlings that emerged, PDO = no. of seedlings with girdled hypocotyls, and FK = no. of seedlings with multiple primary roots. Using a two-way ANOVA, the LSDs ($P = 0.05$) for EM, PDO, and FK were 2.3, 0.8, and 0.8, respectively.

^bSoil (Yolo silt loam) was fumigated with metham-sodium and reinfested with *Pythium* spp. The control was not infested. Initial inoculum densities were 260, 160, and 90 germinable propagules per gram of soil of isolates 79-2, 83-6, and 84-7, respectively.

as a function of the rate of infection yielded a negative correlation coefficient ($r = -0.80, n = 6$). A similar relationship was observed in the third harvest of a second experiment ($r = -0.74, n = 6$). The coefficients of determination (r^2) in these experiments ranged between 0.5 and 0.65. Linear regression of combined data from the two experiments for harvest cycle 3 is shown in Figure 1.

Other effects of the intensity of rootlet infection on plant development include reductions in the number of stems per plant and a delay in flowering (Fig. 2).

Cultivar susceptibility. Effects of rootlet infection by *P. irregulare* (83-6) on shoot growth were studied in separate outdoor experiments in successive years with alfalfa cultivars and a synthetic germ plasm line in either the semidormant or nondormant classes of alfalfa (Table 4). In the 1988 experiment, there were sharp differences in shoot growth between plants grown in noninfested soil and soil where initial IDs were adjusted to about 200 propagules per gram. Growth differences were less obvious in 1989, when the IDs of infested soil at seeding were adjusted to about 50 propagules per gram.

In 1988 it was found that shoot growth of the cultivars Pierce and WL 605 were least affected by root infection by *P. irregulare* and that Moapa 69, Condor, and WL 516 were the most affected. No obvious differences were evident in the degrees of root colonization by *P. irregulare* between cultivars (Table 4). The variation in growth responses of different cultivars to root infection was obvious visually. In the 1989 experiment, the ID of *P. irregulare* was lower than in 1988, and the effects of rootlet infection on shoot growth were less distinctive; however, the shoot growth of germ plasm line A77-10B was reduced considerably (Table 4).

Symptomatology. Symptoms of rootlet infection by *P. irregulare* were usually inconspicuous when infection was moderate (15–20 infections/100 cm of root), but when it was high (>50 infections/100 cm of root), necrotic regions along the lengths of roots were readily observed. The color of infection sites ranged from very light yellow to brown. Water-soaking was associated with the brown lesions. If soils were waterlogged for several days prior to root sampling, feeder root tips were often dark brown and colonized by *Pythium*.

Root-forking was apparently caused by the development of adventitious roots behind (basipetal to) infected root apices at seedling stages. This symptom was found also on the feeder root systems of mature plants.

DISCUSSION

Pythium irregulare caused premergence and postmergence damping-off of alfalfa at both warm and cool soil

temperatures and at IDs similar to those occurring naturally in the field. On the whole, the isolates examined were not as virulent as the isolate of *P. ultimum*, especially at the warm temperatures. *P. ultimum* was reported previously as a uniformly virulent, seedling pathogen at the temperatures examined in this study (12). These differences in temperature responses between the two species may partially account for the lack of recognition of *P. irregulare* in the etiology of alfalfa seedling-stage diseases. In California, soil temperatures of exposed soil during the principal planting period from September to early October (13) should favor pathogenesis by *P. ultimum*, whereas the cooler soil temperatures during the March–April planting period could favor both species.

P. irregulare can cause significant reductions in shoot growth of alfalfa during early stages of plant development when rootlet infection exceeds 5–10 infections/100 cm of root. While such projections are undoubtedly influenced by other factors, including rootlet infection by other pathogens, they allow a basis for assessing potential disease

impact and, consequently, disease management tactics.

Selection of resistance to chronic, nonspecialized root pathogens was advocated strongly by Bruehl (5). Supportive findings for such an approach with *Pythium* spp. are described for root rot resistance of common bean and pea to *P. ultimum* and subterranean clover to *P. irregulare* (1,3,19). Moreover, recurrent selection of disease tolerance in alfalfa was applied successfully in Alberta using soils with replant problems where *Pythium* spp. are suspected of causing losses (8). In this investigation, shoot growth of different cultivars of alfalfa was affected differently by rootlet infection by *P. irregulare*. The basis for these differences should be explored further: genetic resistance or tolerance could be applied to management of *Pythium* root diseases of alfalfa.

There does not appear to be cross-resistance of alfalfa to *Phytophthora* and *Pythium* root diseases. For example, the synthetic germ plasm line A77-10B that was rated as highly resistant to *Phytophthora megasperma* f. sp. *medicaginis* (7) was very susceptible to *P. irregulare*.

Table 3. Relationship between inoculum densities of *Pythium irregulare*, rootlet infection, and root-length densities of alfalfa (cv. Moapa 69)^a

Inoculum densities (propagules/g soil)	Infections		Root-length density (cm/g soil)	
	No./100 cm root	Percent colonization	Live	Necrotic
0	0.0	0.0	11.3	0.22
42	20.8	7.7	9.3	0.25
133	20.8	7.7	9.6	0.25
158	26.8	13.8	5.5	0.23
350	36.4	18.0	6.3	0.20
617	51.2	20.8	6.9	0.18
r^b	0.93**	0.90*	-0.61	-0.84*

^a Isolate 83-6 of *P. irregulare* was used in this experiment. The soil (Yolo silt loam) was treated with metham-sodium prior to reinfestation. Five 2-cm-diameter cores were taken from the top 5 cm in pots from all treatments between the first and second harvests for determination of degrees of rootlet infection and root-length densities. Data represent means of five replicates.

^b Regression analyses were calculated as propagules per gram of soil (independent variable) versus the dependent variables. * = $P < 0.05$, ** = $P < 0.01$.

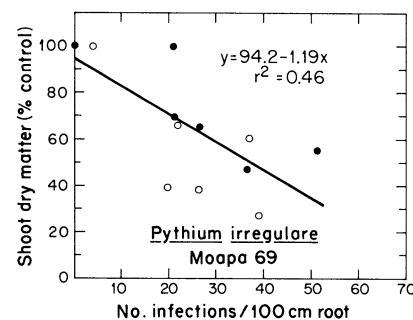


Fig. 1. Relationship between root infection by *Pythium irregulare* and yield of shoot dry matter of alfalfa (cv. Moapa 69). Regression of combined results from the third harvests from two experiments in a greenhouse. Each data point is the mean of six replicates (the open dots represent one experiment and the closed dots represent the other experiment).

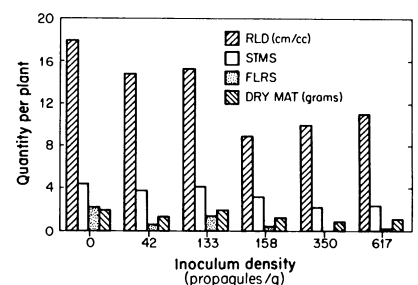


Fig. 2. Relationship between inoculum densities of *Pythium irregulare* and various yield components of alfalfa (cv. Moapa 69, third harvest) in a greenhouse experiment. Data are mean values of six replicates. RLD = root-length densities (cm/cm³ soil), STMS = mean number of stems per plant, FLRS = mean number of flowers per plant, and DRY MAT = mean dry matter (grams) per plant.

Table 4. Influence of root infection of various cultivars of alfalfa by *Pythium irregulare* on yield of shoot dry matter^a

Cultivar ^b	PRR Rating ^c	Dry matter (g)			
		Experiment 1		Experiment 2	
		Noninfested	Infested	Noninfested	Infested
Moapa 69	S	15.5	3.2	6.0	5.2
Lahonton	MR	13.3	4.8	6.5	6.2
Pierce	R	15.1	6.0	7.4	6.3
Condor	HR	17.4	4.2	6.2	5.5
WL 516	HR	16.3	3.5	7.2	6.1
WL 605	HR	14.5	7.5	6.3	5.7
A77-10B	HR	8.1	4.4
LSD (<i>P</i> = 0.05)		1.58		0.91	

^aData from two separate experiments conducted outdoors at Berkeley in the summer and fall of 1988 and 1989 with directly seeded alfalfa. Soil (Hanford sandy loam) was autoclaved prior to infestation with *P. irregulare* (83-6). Data sets are of mean dry matter yields of five replicate pots (five plants per pot) at the second harvests taken 6 October 1988 and 16 October 1989 for experiments 1 and 2, respectively. *Pythium* infections averaged 13 and 7 in noninfested soils and 58 and 25 in infested soils (per 100 cm of root) in soil samples taken between the first and second harvests in experiments 1 and 2, respectively.

^bMoapa 69 and Lahonton are public cultivars; Condor and Pierce were supplied by Northrup-King, Inc., Fresno, CA, and WL 516 and WL 605 were supplied by W-L Research, Inc., Bakersfield, CA. Seed of the germ plasm line A77-10B was supplied by D. C. Erwin, University of California, Riverside.

^cPhytophthora root rot resistance (PRR) ratings are based on the system used by the National Certified Alfalfa Variety Review Board and signify S = susceptible, MR = moderately resistant, R = resistant, and HR = highly resistant. The ratings listed are according to the seed producer or published information.

Indeed, there was little apparent relationship between rankings of Phytophthora root rot resistance and rootlet infection by *P. irregulare* among the commercial cultivars of alfalfa (Table 4). Apparently, the *Pm* gene that conditions for resistance to Phytophthora root rot does not impart general resistance to other pythiaceae fungi (9,18,21).

Root infection by *P. irregulare* has been implicated repeatedly as affecting early growth of both annual and perennial crops (15,27,30). The carryover effects of seedling-stage diseases are seldom assessed, but apparently a retardation in the rate of expansion of young root systems can affect shoot development. However, in the later developmental stages of plants, root stresses may have proportionately greater effects on fruit development than on shoot growth. For example, Roncadori et al (27) observed that infection of cotton roots by *P. irregulare* initially retarded shoot growth in the field, but that by the end of the season plants were equal in size. Nonetheless, these investigators found that seed cotton yield was reduced 11–14% in infested plots. These findings, and the observation in this study that flowering was retarded, suggest that root infection by *Pythium* spp. could affect seed yield in alfalfa forage-seed production crops.

Replant problems and other non-specific types of root maladies of perennial crops are thought to be caused by pathogens that infect host roots and significantly increase their inoculum densities in soil over time (23,29). The replant disease situation with alfalfa is

complex in that several soilborne fungi infect alfalfa roots and may contribute to poor stand establishment in subsequent plantings of this crop (13,17). *P. irregulare* forms a major component of the root-infecting mycoflora associated with alfalfa in California, and findings here and by others strongly suggest that because it causes both seedling and root diseases, it could be a major contributor to replant diseases of this crop.

ACKNOWLEDGMENTS

I thank Dolores Doyle for providing excellent technical assistance and T. A. Gordon, A. H. McCain, and A. R. Weinhold for critical review of the manuscript.

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