A Screening Technique Useful in Selecting for Resistance in Alfalfa to Phytophthora megasperma

F. A. Gray, R. B. Hine, M. H. Schonhorst, and J. D. Naik

First, second, and fourth authors are Research Assistant, Professor, and former graduate student, respectively, Department of Plant Pathology; third author is Professor, Department of Agronomy and Plant Genetics, University of Arizona, Tucson 85721.

Arizona Agricultural Experiment Station Journal Paper No. 2050.

Accepted for publication 27 March 1973.

ABSTRACT

A screening technique, consisting of planting alfalfa seed in infested soil, was developed for selecting plants with resistance to damping-off and root rot caused by *Phytophthora megasperma.*

A high level of resistance to damping-off was obtained in the nonwinter-hardy alfalfa cultivar 'Hayden' after one cycle of selection. The amount of pre- and post-emergence damping-off was influenced by the inoculation technique. High pre-emergence loss occurred when the inoculum was placed with the seed before covering with a heat-pasteurized sand-soil mixture; whereas, high post-emergence loss occurred when the inoculum was blended into the sand-soil mixture before seeding. Seedling disease increased in direct proportion to inoculum concentration. When seed of the cultivar Hayden were planted in flats containing previously infested soil, a 98% seedling stand loss occurred after 2 weeks incubation in growth chambers with a 12-hr light cycle at 24 C and 12-hr dark cycle at 18 C.

Ten isolates of *P. megasperma* recovered from diseased taproots of alfalfa from different geographic areas in Arizona, one isolate from California, and two isolates from Minnesota, were pathogenic to alfalfa grown in the greenhouse in Arizona.

Phytopathology 63:1185-1188.

Additional key word: oospores.

Phytophthora root rot (PRR) of alfalfa, caused by *Phytophthora megasperma*, was described by Erwin (5, 6) as a new disease in 1954. Presently, this disease is thought to be the most common and serious disease of alfalfa in California (*personal communication*, D. C. Erwin, Department of Plant Pathology, University of California, Riverside), as well as in Arizona (10). The disease has also been reported in Minnesota (8), Mississippi (11), Illinois (1), Ohio, Iowa, Wisconsin, South Dakota (9), Washington (4), Australia (14), and Canada (2).

Although several researchers have tested various alfalfa varieties for resistance to *Phytophthora* (2, 7, 10, 11), it was not until 1967 (12, 13) that two *Phytophthora*-tolerant alfalfa germplasms were released by the University of California. During this selection process, plants were inoculated when they were at least 1 month old (*personal communication*, D. C. Erwin). Results of our preliminary studies on seedling age and susceptibility indicated that germlings were more susceptible to damping-off than seedlings of 2, 4, 8, and 16 weeks of age. For this reason, studies were initiated to determine whether an effective screening method could be developed utilizing this highly susceptible stage to concentrate the germplasm for PRR resistance at a rapid rate. The effects of method of inoculation, inoculum level, and temperature, on seedling death are reported in this paper. Portions of this work were previously reported (10).

MATERIALS AND METHODS.—Isolates of *Phytophthora megasperma* were obtained from diseased roots of field-grown alfalfa plants from all of the major alfalfa producing areas in Arizona. Cultures were maintained on a selective antibiotic medium (3). Inoculum was grown at room temperature (22 to 27 C) as standing cultures in liquid V-8 juice medium (100 ml Campbell's V-8 juice, 2 g $CaCO_3$/liter of water) in 32-oz medicine bottles containing 180 ml of the liquid medium. After 2 days of incubation, cultures were shaken vigorously for several seconds to fragment the mycelium, and encourage development of a large quantity of mycelium per bottle. When the cultures were 2 weeks old, the mycelial mats containing oospores were washed, placed in distilled water, and macerated in a Waring Blendor. The inoculum used in all tests consisted of oospores and mycelial fragments of three isolates of *P. megasperma* from the major alfalfa producing areas in Arizona. Oospore counts were made with a Neubauer hemacytometer. For all experiments we used a heat-pasteurized soil-sand mixture (1:1, v/v), with the following characteristics: pH 8.1, 83.0% sand; 13.0% silt; 4.0% clay; 0.06% organic matter; 90 ppm NO_3; 6.9 ppm PO_4; 1,050 ppm soluble salts; and 3.2, 6.2, and 9.0% moisture retention at 15, 0.33, and 0.10 atm tension, respectively. Approximately 2.2 and 14.7 kg of the air-dried soil mixture were placed in 15.2-cm diam plastic pots and in 38 X 54 cm galvanized aluminum flats, respectively. In most studies, growth chambers were maintained on a 12-hr light cycle at 24 C and a 12-hr dark cycle at 18 C. Temperatures in glasshouse studies were maintained at 24-27 C. Daily watering of all experiments insured a high soil moisture condition. Saucers placed under all pots were kept flooded for 2 weeks and then removed. All experiments were replicated four or more times and repeated at least twice. Stand counts were taken 2 to 12 weeks after planting.

RESULTS.—*Pathogenicity studies.*—Three isolates of *P. megasperma* from major alfalfa producing areas in Arizona (Buckeye, Laveen, and Gilbert), were equally pathogenic to established 2-week-old 'Hayden' alfalfa plants in glasshouse studies. In an experiment with ten replications, 25 seeds were planted in each 15.2-cm plastic pot. Seedling stands were thinned to three plants/pot. Two weeks after planting, each pot received 25 ml of inoculum consisting of mycelial mats from two culture bottles (32 oz), blended in 300 ml of distilled water. Several holes, extending downward to the root system, were made in the soil surface to facilitate root infection. After 12 weeks, the original stands were reduced by approximately 23, 33, and 23% with the isolates from Buckeye, Laveen, and Gilbert, respectively. Root damage caused by the three isolates was similar to that caused on field-grown plants.

In another study, ten isolates from Arizona, including the three in the above test, were pathogenic as evidenced by their ability to incite damping-off in seedlings of Hayden alfalfa grown in the glasshouse. The inoculum of each isolate was standardized by adjusting the wet weight of the mycelial mats. Each mat was blended and the volume brought up to 250 ml with distilled water. Fifty ml of this inoculum were poured on the soil surface of each pot. Fifty seed of the variety Hayden were then scattered on the soil surface and covered with approximately 6 to 12 mm of soil. Damping-off reached a maximum approximately 3 weeks after seedling emergence. The Gilbert isolate was less pathogenic, causing an 8% stand loss compared to over 98% caused by the other nine isolates. Further seedling studies were run in controlled growth chambers using the above experimental procedures to compare the pathogenicities of an isolate of *P. megasperma* from California (P-349), two isolates from Minnesota (DC1-6 and DC1-2), and an isolate from Laveen, Arizona. Stand losses after 2 weeks with the P-349, DC1-6, DC1-2, and the Laveen isolate were 100, 100, 96, and 96%, respectively.

Inoculation methods compared.—Pots were seeded with 40 Hayden seeds/pot. Each pot received 50 ml of a prepared inoculum suspension consisting of mycelial mats from three culture bottles (32-oz) blended in 660 ml of distilled water. When the inoculum was either (i) blended into the soil prior to seeding, (ii) placed on the soil surface prior to seeding and covering or (iii) poured on the soil surface after seeding and covering, the pre- and post-emergence damping-off differed greatly according to treatment. After 3 weeks pre-emergence and post-emergence stand losses for the three inoculation methods (as listed above), were 0, 52, 85%, and 84, 36, 14%, respectively. These results show that the degree of pre- and post-emergence damping-off can be altered

TABLE 1. Incidence of seedling damping-off of 'Hayden' alfalfa caused by *Phytophthora megasperma* at three inoculum levels

Inoculum levels[a]	Surviving plants 14 days after inoculation[b]	% Stand loss
0 dilution	29	98
10-fold dilution	191	68
100-fold dilution	495	17
Check	593	0

[a] Inoculum levels consisted of approximately 70, 7, and 0.7 oospores/g of air-dried soil at the 0, 10-fold, and 100-fold dilutions, respectively, plus an undetermined number of mycelial fragments.

[b] Values represent the average of three replications.

greatly by the inoculation method.

Inoculum level studies.—Stock inoculum consisting of macerated mycelial mats from nine culture bottles (32 oz), placed in 5 liters of distilled water, was either undiluted or diluted 10-fold and 100-fold. One liter of each of the three concentrations was blended into the soil of each flat. There were approximately 70, 7, and 0.7 oospores/g of air-dried soil, respectively, as well as an undetermined number of mycelial fragments. Each flat was then seeded with seven rows (100 seed/row) of the variety Hayden. After 2 weeks the percent damping-off for the three inoculum levels were 98, 68, and 16% (Table 1).

Since knowledge of the role of either oospores or mycelial fragments of the alfalfa isolate of *P. megasperma* as inoculum was lacking in the literature, several experiments were conducted to answer these questions. To obtain oospores free of viable mycelium, 2-week-old cultures grown in a liquid V-8 juice medium, were macerated and dried for 10 days at 25 C. In growth chamber studies, oospores in numbers as low as 10/g of air-dried soil caused seedling stand losses greater than 93% after 2 weeks. In another test, inoculum consisting of mycelial fragments, free from oospores and sporangia, was obtained by growing cultures in a potato-dextrose broth medium for 2 weeks. When this inoculum was placed with alfalfa seed at the time of planting, a 100% seedling stand loss occurred after 2 weeks.

Temperature studies.—Hayden seeds, 50/pot, were planted in 15.2-cm pots containing infested soil (the high inoculum level in the above study). These pots were then placed in growth chambers with the following light-dark temperature regimes; 24-13 C, 30-18 C, and 35-24 C. Observations of seedling survival were made periodically. In a similar test, Hayden alfalfa seedlings were grown in 15-cm pots containing perlite and inoculated when 2 weeks old. Two weeks after inoculation, the total plant weights were recorded. Data from the above two studies are shown in Table 2 and illustrate the marked effect of temperature on incidence and severity of this disease.

TABLE 2. Effect of temperature on the pathogenicity of *Phytophthora megasperma* on alfalfa

Temp (C)[a]	% Stand loss[b]	% Reduction in total plant weight[c]
35 - 24	6	0
30 - 18	85	70
24 - 13	81	57

[a] Values represent the temperatures of the light cycle (12 hr) and the dark cycle (12 hr), respectively, in the growth chambers.

[b] Readings made 11 days after seeding and expressed as a percentage of the control. Results are the averages of five replications.

[c] Readings taken 2 weeks after inoculating 2-week-old seedlings, calculated as percent of control. Results are the averages of five replications.

TABLE 3. Increase in seedling resistance to *Phytophthora megasperma* in the alfalfa variety 'Hayden' after one cycle of selection

	Test 1[y]			Test 2[z]	
	Surviving plants				
Entries	Check	Inoculated	Stand loss (%)	Stand count	Average stem height (mm)
UC-47	123 a	119 a	3 a		
Hayden PX	147 a	118 a	20 a	275	70
Hayden Certified	174 b	37 b	79 b	137	31

[y] UC-47 is a *Phytophthora*-resistant germplasm released by the University of California. Means with the same letter are not significantly different at .05 level of probability. Hayden PX seed was from a polycross of 100 Hayden alfalfa plants which had undergone one cycle of selection for *Phytophthora* resistance. Values represent the total of four replications, each consisting of placing germinated seed in six rows (50 seed/row), into infested soil in growth chambers maintained at a 12-hr light cycle (24 C) and a 12-hr dark cycle (18 C). Values are expressed as a percentage of the control. Data were taken 2 weeks after seeding.

[z] Values represent the total of 12 replications, each consisting of three rows (100 seed/row) of scarified seed in flats containing infested soil. Data were taken 12 weeks after seeding.

Resistance in Hayden alfalfa after one cycle of screening.—Over 10,000 seedlings of the variety Hayden were screened for resistance by seeding into flats (100 seed/row and seven rows/flat) containing infested soil. The inoculum rate was similar to that which gave 98% damping-off in the inoculum level study. The inoculum was hand-blended into the soil of each flat followed by seeding. Flats were placed in growth chambers and removed to glasshouse benches after 2 weeks. Plants surviving (approximately 2%) after 2 months were removed from the flats and examined for Phytophthora root rot. Plants showing signs of root infection were discarded and the remaining plants (100) were potted and maintained in the greenhouse. Plants were randomly intercrossed by hand. Seed produced from these plants represented

the first cycle Hayden polycross (PX) PRR resistant germplasm. Subsequent experiments consisted of comparing seedling stand losses in the Hayden PX and Hayden Certified seed to determine comparative levels of resistance. The experimental procedures were the same as those mentioned above in the variety screening test with the following exceptions: seed of all entries were scarified before planting or were germinated in petri dishes until the radicles were 5 to 10 mm in length and then planted. The *Phytophthora*-tolerant alfalfa germplasm, UC-47 (12, 13), was used as a resistant check in one test. When germinated seed of Hayden Certified, Hayden PX, and UC-47 were placed in flats containing infested soil, stand losses at 2 weeks were 79, 20, and 3%, respectively (Table 3). Hayden PX and UC-47 had significantly less seedling damping-off than did Hayden Certified. In a similar replicated test in which 1,200 seeds of Hayden Certified and Hayden PX were planted in previously infested soil, Hayden PX had significantly less seedling damping-off and greater average stem heights per plant than Hayden Certified (Table 3).

DISCUSSION.—The destructiveness of Phytophthora root-rot to field grown alfalfa is well documented (1, 2, 5, 8, 9, 10, 11, 14). The need for screening techniques useful in breeding programs for Phytophthora root rot resistance is obvious. This study therefore, was conducted to develop useful techniques for this purpose.

In greenhouse studies conducted in Arizona, *P. megasperma* produced both seedling damping-off and root rot of older alfalfa plants (10). This is in contrast with field observations in Arizona in which only root rot of older plants has been observed. Our screening technique incorporated the selection for resistance to both damping-off and root rot. More emphasis, however, was placed on selection for damping-off resistance since this represented a faster and more economical means of screening thousands of plants. Approximately 200 of 10,000 Hayden alfalfa seedlings screened showed resistance to damping-off. Of these, approximately 100 were free from root infections and were retained. Seed produced from an interpollination of these selections had 59% less damping-off when compared with seedlings of Certified Hayden. A correlation between resistance to damping-off and root rot was shown in another study (*unpublished data*, authors) using the *Phytophthora*-tolerant germplasm UC-47. UC-47, selected for resistance to root rot, exhibited a comparatively high level of damping-off resistance when compared with several alfalfa cultivars. Since validation of any screening program involving the selection for resistance to an organism can be verified only under field conditions, field tests are now under way to determine what level of field resistance to Phytophthora root rot has been obtained.

Earlier reports have not indicated the presence of alfalfa seedling resistance to damping-off caused by *P. megasperma*. Erwin (7), indicated all seedlings were killed when seed of several alfalfa varieties were planted in infested soil. In our preliminary studies, however, damping-off resistance was shown not only to exist in UC-47 but also in the Plant Introduction (PI) entry P.I. 141462 from Iran. This entry was the only one of 25 tested having resistance indicating the rarity of seedling resistance in existing alfalfa entries. Differences in inoculum densities and methodology may account for the discrepancy between this and other reports related to alfalfa seedling resistance to *P. megasperma*. The relationship between inoculum density and distribution in the soil to disease severity was shown in our studies. Hand-blending the inoculum into the soil prior to seeding was an effective means of inoculum dispersal as indicated by the random occurrence of surviving plants remaining in the flats after a 2-month incubation period.

Erwin (7), reported a loss in pathogenicity of a California isolate of *P. megasperma* from alfalfa after several years of culturing. In our studies, ten isolates from Arizona were pathogenic to seedling alfalfa. However, one isolate from Gilbert, Arizona, was less pathogenic than the other nine. A loss of virulence in the Gilbert isolate occurred after 18 months of continuous culturing in the laboratory. The need for periodic verification of pathogenicity of isolates utilized in screening programs should therefore be taken into consideration.

LITERATURE CITED

1. BUSHONG, J. W., & J. W. GERDEMANN. 1959. Root rot of alfalfa caused by Phytophthora cryptogea in Illinois. Plant Dis. Reptr. 43:1178-1183.
2. CHI, C. C. 1966. Phytophthora root rot in alfalfa in Canada. Plant Dis. Reptr. 50:451-453.
3. ECKERT, J. W., & P. H. TSAO. 1962. A selective antibiotic medium for isolation of Phytophthora and Pythium from plant parts. Phytopathology 52:771-777.
4. ELGIN, JR., J. H., D. W. EVANS, & J. M. KRAFT. 1972. Occurrence of Phytophthora root rot of alfalfa in Washington. Plant Dis. Reptr. 56:830-831.
5. ERWIN, D. C. 1954. Root rot of alfalfa caused by Phytophthora cryptogea. Phytopathology 44:700-704.
6. ERWIN, D. C. 1965. Reclassification of the causal agent of root rot of alfalfa from Phytophthora cryptogea to P. megasperma. Phytopathology 55:1139-1143.
7. ERWIN, D. C. 1966. Varietal reaction of alfalfa to Phytophthora megasperma and variation in virulence of the causal fungus. Phytopathology 56:653-657.
8. FROSHEISER, F. I. 1967. Phytophthora root rot of alfalfa in Minnesota. Plant Dis. Reptr. 51:679-681.
9. FROSHEISER, F. I. 1969. Phytophthora root rot of alfalfa in the upper Midwest. Plant Dis. Reptr. 53:595-597.
10. HINE, R. B., F. A. GRAY, & M. H. SCHONHORST. 1972. Phytophthora root rot of alfalfa in Arizona. Plant Dis. Reptr. 56:472-473.
11. JOHNSON, H. W., & F. L. MORGAN. 1965. Phytophthora root and crown rot of alfalfa in the Yazoo-Mississippi Delta. Plant Dis. Reptr. 49:753-755.
12. LEHMAN, W. F., D. C. ERWIN, & E. H. STANFORD. 1967. Root rot tolerance in new alfalfa strains now available to plant breeders. Calif. Agric. 21(4):6.
13. LEHMAN, W. F., D. C. ERWIN, & E. H. STANFORD. 1969. Registration of Phytophthora-tolerant alfalfa germplasm, UC-38 and UC-47. Crop Sci. 9:527.
14. PURSS, G. S. 1959. Root rot on lucerne. Queensl. Agric. J. 85:767-770.