Variation Among Isolates of *Phoma medicaginis* var. *medicaginis* in Spore Production in Vitro and Symptom Expression on Excised Leaves of Alfalfa

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ABSTRACT

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Excised trifoliolate leaves from a susceptible alfalfa (*Medicago sativa*) genotype were inoculated in vitro with 34 isolates of *Phoma medicaginis* var. *medicaginis*. Pathogenicity and virulence of isolates were assessed by their ability to produce leaf spot, petiole blight, and leaf chlorosis. Significant differences ($P = 0.05$) in the ability of isolates to produce symptom types were observed. There was a significant relationship between the ability to produce leaf spot and petiole blight ($r = 0.33$) but not between the ability to produce chlorosis and petiole blight ($r = -0.12$) or leaf spot ($r = 0.22$). Petioles were affected more than leaves. Density of spores produced on PDA varied among isolates ($P = 0.05$) from 3.5×10^5 to 6.0×10^8/ml. However, no association between virulence and in vitro spore production or color of spore suspension was observed among the isolates.

Spring black stem of alfalfa (*Medicago sativa* L.), caused by *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema (syn. *P. herbarum* Westend. var. *medicaginis* Fckl. and *Ascochyta imperfecta* Peck; 2), occurs commonly in the United States, Canada, and Europe (3). It is most destructive in temperate regions, particularly when alfalfa is grown under irrigation, and causes losses in both forage yield and quality. Leaves, petioles, and stems are equally attacked (3). Spring black stem has been shown to be particularly destructive in seed production (8) and is the most important foliar disease of irrigated alfalfa in Wyoming (4). Advanced stages of the disease may consist of a decay of the crown and upper root of mature plants (3). Although host resistance is the major component of any alfalfa disease control program, no cultivars are currently available with an acceptable level of resistance to this disease. A preliminary screening procedure has been described (6), but no resistant materials have been released.

Various disease rating systems have been suggested for measuring the severity of spring black stem on alfalfa (4,5,7, 9,11,12). A 1–5 step scale (1 = no disease, 5 = severe disease) has been suggested for use in evaluating alfalfa lines for resistance (11) and has been used in several field studies (4,5). The rating includes leaf spot, leaf defoliation, stem blackening, and stem dieback. This rating system has been modified to rate specifically for leaf defoliation (4). Other systems used for rating disease on single whole plants have utilized leaf spotting and stem lesions on a 0–10 step scale (12). A more precise system developed for leaves utilized the percentage of leaf area covered (7). Studies with detached or excised leaves have used a 0–5 step scale (0 = none, 5 = dead) which, although not described, photographically included multiple symptoms on leaves (9) as well as a 0–10 step scale that used the size and number of like lesions on leaves (12).

Previous researchers studying the pathogenicity and virulence of isolates of *P. m.* var. *medicaginis* on whole plants (6,8,9) and on excised leaves (9,12) have all standardized inoculum spore concentrations before inoculation. Concentrations of 1×10^6 spores per millileter were used on detached leaves by Ward (12), while a concentration of 4×10^6 was used to screen young seedlings for possible resistance (6). Spore concentration has not been given in other studies (8,9). Although standardizing spore concentrations may provide a means of comparing spore infection efficiency among isolates, it does not measure the effect of spore production efficiency (primary inoculum potential) on virulence. Primary inoculum of *P. m.* var. *medicaginis* is produced in the spring on dead alfalfa stems from the previous year's growth (3). Whether or not differences in spore production occur among isolates in the field and what effect it may have on their virulence in nature has not been studied. Observations in our earlier studies (5) indicated that variations in the ability of isolates to produce spores in vitro did occur. However, if an effective screening and selection technique for spring black stem is to be developed, a thorough understanding of the host-pathogen system is essential.

Our studies were conducted to assess the variability among isolates of *P. m.* var. *medicaginis* from alfalfa seed grown in Wyoming for pathogenicity, virulence, and spore production, as well as to assess the type of symptom(s) produced on excised leaves of alfalfa. The study was designed to allow for differences among isolates in their ability to produce spores in vitro to determine what effect this might have on their virulence. Because our preliminary studies indicated that three types of symptoms (leaf spot, petiole blight, and leaf chlorosis) could be differentiated on excised leaves, ratings were made for all three symptoms to determine if variations in symptom type produced occurred among isolates.

MATERIALS AND METHODS

Thirty-four isolates of *P. m.* var. *medicaginis* obtained from alfalfa seed (unsprayed controls of cultivar Ranger) during a previous study (4) and American Type Culture Collection (ATCC) isolate 12087 of *A. imperfecta* were cultured on potato-dextrose agar (PDA), maintained in the dark at 21 C, and subcultured monthly. Five cultures of each isolate were maintained for 9 mo to determine if crystals were produced (2,10). For inoculum production, a 0.6-cm-diameter culture plug of each isolate was transferred to each of five petri dishes containing PDA and placed on the laboratory bench for 4 wk at 21 C. One day before inoculation of alfalfa leaves, cultures were flooded with 5 ml of sterile, distilled water and pycnidiospores were dislodged by gently rubbing the surface of cultures with a rubber policeman. This procedure was repeated, and the resulting aqueous spore suspensions were increased with sterile, distilled water to 50 ml and poured through 16 single layers of cheesecloth and stored at 7 C until used. Pycnidiospore concentration for each isolate was determined. A hemacytometer was used to count two 1-ml aliquots of the spore suspension for each isolate, and the two values were aver-

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aged. Additionally, because spore suspensions varied considerably in color, a Munsell Soil Color Chart was used to assign color ratings (1).

An excised leaf technique was used to assess virulence of isolates. Alfalfa is genetically heterozygous, so a single Ladak alfalfa plant, susceptible to spring black stem, was vegetatively propagated for use in the study. Selection of the most severely diseased plant was made following inoculation of 25 Ladak plants with a spore mixture of 10 randomly selected *P. m.* var. *medicaginis* isolates. The alfalfa clone was maintained in a controlled environmental chamber at 25 C with a 14-hr photoperiod of incandescent and fluorescent light with a photon flux of 520 $\mu E \cdot m^{-2} \cdot s^{-1}$. Three weeks before establishing the tests, plants were clipped back to provide a new crop of leaves. Trifoliolate leaves, located between nodes two and five and numbered basal to the stem apex, were removed and petioles were trimmed to 1 cm. The entire trifoliolate leaf was disinfested by submersion for 2 min in 0.05% of sodium hypochlorite, rinsed in sterile, distilled water, and allowed to dry. Leaves were then placed in a moisture chamber consisting of a 2.3-cm-deep × 10-cm-wide glass petri dish containing an inverted, smaller glass petri dish (top only, 1.4-cm-deep × 6.2-cm-wide). Ten ml of sterile, distilled water was placed in each chamber to provide high relative humidity. One trifoliolate leaf was placed on top of the inverted smaller dish and the chamber was closed. The height of the large petri dish was such that the trifoliolate leaf did not contact the upper glass surface.

For inoculation, each trifoliolate leaf was removed with forceps, sprayed with the spore suspension (concentrations varied among isolates) until runoff, and returned to its chamber. One drop of Tween 80 was added, and each spore suspension was placed on a magnetic stirrer for 1 min before inoculation. A portable aerosol sprayer (Crown Spra-Tool 8011 Power Pak, nonflammable propellent, Fisher Scientific, Pittsburgh, PA) was used to apply the spore suspensions. Five trifoliolate leaves were sprayed for each isolate. Leaves were sprayed again after 24 and 48 hr, for a total of three inoculations. Inoculum was stored at 7 C between sprays. Following inoculation, the chambers were placed in a Precision (Model 818) dual program illuminated incubator, kept in the dark for the first 72 hr to enhance infection (6), and in a 14-hr photoperiod of fluorescent light with a photon flux of 54 $\mu E \cdot m^{-2} \cdot s^{-1}$/10-hr dark cycle for the remaining 96 hr.

After 7 days of incubation, leaves were removed from chambers and rated for disease symptoms. Each trifoliolate leaf was rated separately for leaf spot, petiole blight (darkening and necrosis), and leaf chlorosis. A seven-step scale similar to the leaf rating system of James (7) was used to rate leaf and petiole systems, representing 0, 1, 5, 20, 50, 70, and 80% of leaf or petiole tissues diseased. Leaf chlorosis was rated on a six-step scale representing 0, 10, 35, 65, 90, and 100% chlorotic leaf area. Additionally, the sum of the three symptom ratings were averaged, following transformations described below, to provide an average severity index (ASI) rating.

The experiment was arranged in a completely randomized design with five replicates and a cyclic rotation of moisture chambers every 2 days during incubation to avoid possible border effects caused by a door-mounted light bank. Prior to analysis of variance, the arcsine transformation was used to transform percentage data. In addition, transformed data for leaf spot and petiole blight were divided by 18 degrees in order to convert data to a six-step scale comparable to the chlorosis rating scheme and rounded to the nearest whole number. The LSD test was used to compare means among isolates. Correlation coefficients among mean symptom ratings (leaf spot, petiole blight, and leaf chlorosis) and between spore densities and disease ratings were calculated.

The experiment was performed twice, and the two tests were compared by examining mean disease ratings rounded to the nearest whole number of the six-step, 0–5 scale.

RESULTS AND DISCUSSION

All 34 isolates of *P. m.* var. *medicaginis* recovered from seed grown in Wyoming produced symptoms of spring black stem on excised leaves of alfalfa. Isolates differed significantly ($P = 0.05$) in the ability to produce leaf spot, petiole blight, and leaf chlorosis (Table 1). When disease ratings were converted to ASI, isolates

Table 1. Symptom ratings on excised trifoliolate leaves of alfalfa and cultural characteristics of 34 isolates of *Phoma medicaginis* var. *medicaginis* from alfalfa

Isolate	Symptom rating[a]				Crystals produced	Inoculum density spores/ml[b]	Color of spore suspension[c]
	Leaf spot	Petiole blight	Leaf chlorosis	ASI			
1	2.3	2.1	3.0	2.4	−	2.5×10^8	olive
2	1.0	2.9	2.4	2.2	+	1.0×10^7	yellow
3	2.7	3.4	2.4	2.9	+	1.8×10^7	light gray
4	2.2	3.1	2.6	2.6	−	1.0×10^8	olive-brown
5	1.1	3.4	1.6	2.2	+	1.1×10^7	light gray
6	1.4	3.1	2.0	2.3	−	3.3×10^8	light gray
7	1.5	2.6	2.6	2.2	−	1.7×10^8	light yellowish brown
8	1.9	3.2	1.8	2.4	−	1.4×10^7	olive-gray
9	1.8	3.2	1.4	2.3	+	1.2×10^7	light gray-gray
10	1.4	3.0	0.8	1.9	+	1.2×10^7	light gray
11	1.5	2.2	1.8	1.9	+	1.0×10^7	light gray
12	1.9	3.4	1.6	2.4	+	1.0×10^7	light olive-gray
13	0.9	3.3	3.2	2.5	−	2.0×10^8	olive-yellow
14	0.5	1.9	2.6	1.8	+	1.0×10^7	white
15	1.7	2.6	0.8	1.9	−	1.1×10^7	light gray
16	2.4	2.4	2.0	2.3	−	3.2×10^8	pink
17	2.0	2.3	2.4	2.3	−	3.5×10^8	pale brown
18	0.8	1.9	2.2	1.8	−	3.5×10^5	white
19	1.9	3.2	1.0	2.2	−	1.5×10^7	light gray
20	1.4	2.4	3.0	2.3	−	4.1×10^8	light olive-brown
21	1.7	2.5	1.8	2.1	−	1.8×10^7	olive-brown
22	1.7	2.4	2.4	2.2	−	2.0×10^8	reddish yellow
23	1.5	3.3	1.4	2.2	−	2.3×10^7	olive-brown
24	1.6	3.0	0.8	2.0	−	6.0×10^8	light yellowish brown
25	0.5	2.9	1.2	1.7	−	3.4×10^7	pale olive
26	0.4	2.0	0.6	1.2	+	1.1×10^7	white
27	1.5	2.8	1.8	2.1	−	5.1×10^8	very pale brown
28	1.3	2.3	1.8	1.9	−	3.1×10^8	reddish yellow
29	1.0	2.2	2.0	1.8	−	1.4×10^8	olive
30	0.7	2.2	2.4	1.9	−	1.5×10^8	olive
31	0.4	2.0	0.2	1.2	−	3.2×10^6	white
32	1.2	3.4	1.6	2.2	+	1.1×10^7	white
33	1.3	2.3	1.8	1.8	−	5.7×10^7	olive
34	0.4	2.9	1.8	1.9	−	1.1×10^7	white
35[d]	1.6	3.4	1.4	2.2	+	1.1×10^7	white
LSD_{05}	0.9	1.0	1.4	0.7			

[a] All disease ratings are based on a scale of 0–5, representing 0, 10, 35, 65, 90, and 100% of tissues exhibiting symptoms. ASI is the average of leaf spot, petiole blight, and leaf chlorosis ratings prior to rounding to the nearest tenth. To convert to percentages, use the following formula: $\{[\sin(18 \times R)]^2\}100$, where R is the mean disease rating. LSD values should be used for mean comparisons only with data as presented.

[b] Values are based on spores recovered from five cultures suspended in 50 ml of water. Values are the mean of two counts.

[c] Color of spore suspensions determined by comparison with the Munsell Soil Chart.

[d] *Ascochyta imperfecta* Peck ATCC 12087.

Table 2. Distribution of mean symptom ratings of excised alfalfa leaves inoculated with 35 isolates of *Phoma medicaginis* var. *medicaginis*

Disease symptom	Disease category[a]					
	0	1	2	3	4	5
Leaf spot	3	13	16	1	0	0
Petiole blight	0	0	14	21	0	0
Leaf chlorosis	1	9	19	6	0	0
ASI	0	2	30	3	0	0

[a] Disease categories are 0–5, representing 0, 10, 35, 65, 90, and 100% of tissues affected. Mean disease ratings from the first of two tests were rounded to the nearest whole number for categorization in this table. ASI represents the mean of leaf blight, petiole blight, and leaf chlorosis ratings.

also differed significantly ($P = 0.05$). On the 0–5 scale, the range of mean ratings for leaf spot, petiole blight, leaf chlorosis, and ASI was 0.4–2.7, 1.9–3.4, 0.2–3.2, and 1.2–2.9, respectively. Although most isolates (31 of 35) had an ASI rating between 1.5 and 2.5, isolates varied markedly in the type and severity of symptoms produced. This is quite apparent when frequency distributions of isolate numbers within the six disease categories for the three symptom types and ASI are examined (Table 2). ASI ratings of most isolates fell into disease category 2. However, isolates varied more for leaf spot, leaf chlorosis, and petiole blight symptoms (Table 2). Petioles were affected more than leaves.

Correlation coefficients (r) calculated to measure relationships between or among isolates and the three disease symptoms were 0.33 for leaf spot-petiole blight, 0.22 for leaf spot-leaf chlorosis, and −0.12 for leaf chlorosis-petiole blight; only the first was significant ($P = 0.05$). Thus, the ability of isolates to produce the given types of symptoms does not appear to be related.

Spore production among isolates ranged from 3.5×10^5 spores/ml in isolate 18 to 6.0×10^8 in isolate 24. Approximately one-half of the isolates (47%) produced between 1.0×10^7 and 1.9×10^7 spores/ml. Two isolates produced less than 1.0×10^7 while 17 produced more than 2.0×10^7. Correlations between leaf spot, petiole blight, and leaf chlorosis or overall disease (ASI) and in vitro spore production among isolates was nonsignificant ($P = 0.05$). Therefore, isolates with high spore-producing efficiency showed no advantage over those with low spore-producing efficiency relative to their virulence on excised leaves. The advantage in nature however, may be their ability to increase disease incidence (colonization of more host tissue), assuming environmental conditions necessary for spore dispersal are present.

All isolates grown on PDA were olive-green or blackish olive-green in color. Isolates that produced large numbers of spores had more pycnidia and less aerial mycelium than isolates that produced lower numbers of spores. There was a definite association between the color of spore suspensions and spore density (Table 1). Isolates with a spore suspension that was white or gray (white, light gray, light olive-gray, olive-gray) had the lowest spore densities (3.5×10^5 to 1.4×10^7 spores/ml), while isolates with a spore suspension that was brown, yellow, or pink had the highest spore densities (1.6×10^7 to 6.0×10^8 spores/ml). There was no apparent relationship between spore color and isolate virulence.

Crystals, previously reported to occur in cultures of *P. m.* var. *medicaginis*, were present in 9-mo-old cultures in 11 of the 35 isolates (Table 1).

The experiment was repeated, and ASI disease measurements were reasonably consistent between tests. In the second test, 71% of the isolates received the same ASI ratings as in the first test (means rounded to the nearest whole number on the 0–5 scale), and 97% of the isolates received ratings within one disease category of that recorded in the first test. However, there were considerable differences between tests for severity of individual symptoms produced, particularly for leaf spot. When rounded mean disease ratings for leaf spot were compared, only 37% of the isolates received identical ratings and 80% received ratings within one disease category. The same comparisons for petiole blight and leaf chlorosis were 57% and 100%, and 51% and 94%, respectively. Thus, ASI was the most consistent measure of disease.

Our results are similar to those reported by Mead and Cormack (9). Although they standardized the spore densities of all isolates used and we did not, all isolates of *P. m.* var. *medicaginis* from alfalfa in both studies were pathogenic and the majority produced a moderate level of disease. Apparently, spore concentration in all isolates was sufficiently high for disease development. Isolate 18 had the lowest spore concentration of 3.5×10^5 and produced an ASI of 1.8. Our studies were not designed to evaluate the effect of spore concentration within a given isolate on virulence. Whether virulence of isolate 18 would have been increased by increasing the concentration of spores is not known.

Ward (12) stated that using detached leaves was a reliable method for evaluating resistance to spring black stem in alfalfa. From the results of our study, if trifoliolate leaves are to be used to assess or select for resistance, ratings of leaf spot, petiole blight, and leaf chlorosis should be included because each appears to play an equally important but separate role in pathogenesis and isolates vary in their ability to produce these symptoms. Although separate ratings of all three symptoms would be more difficult with whole plants, we feel that all three should be included in any disease foliage rating for spring black stem. Selection of one or more isolates for use in a screening program should be based on the ability of isolates to produce moderate to severe levels of leaf spot, petiole necrosis, and leaf chlorosis, as well as on the ability to produce a large number of spores in vitro. Although isolates having a higher spore-producing capacity may not produce a higher level of disease, they would provide a greater amount of spore inoculum for screening purposes.

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