

Comparative variation of *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema for cultural characteristics and virulence to roots and crowns of alfalfa^{1,2}

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ABSTRACT

The in vitro and in vivo variation of selected isolates of *Phoma medicaginis* var. *medicaginis* was studied in controlled environmental conditions. Isolates were relatively stable in morphology and pigmentation of the colony but varied more in final diameter of the colony, rate of growth and sporulation. The isolate, the organ and geographic location from which it was obtained were significant sources of variation. All isolates caused root and crown rot symptoms in growth chamber and greenhouse experiments. Differences in virulence among isolates were expressed in the length of root lesions. Plant dry weights were reduced by 82% in greenhouse trials.

Key words: spring black-stem, *Phoma medicaginis* var. *medicaginis*, alfalfa, *Medicago sativa* L.

RESUMEN

Variación comparativa de *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema para características culturales y virulencia a las raíces y coronas de alfalfa

La variación in vitro e in vivo de aislados seleccionados de *Phoma medicaginis* var. *medicaginis* se estudió en condiciones ambientales controladas. Los aislados fueron relativamente estables en morfología y pigmentación de la colonia pero variaron más en el diámetro final de la colonia, la tasa de crecimiento y la esporulación. El aislado, el órgano y la localidad geográfica de donde se obtuvo fueron fuentes de variación significativa. Todos los aislados causaron síntomas de pudrición en las raíces y la corona en los experimentos en las cámaras de crecimiento y en el invernadero. Diferencias en la virulencia de los aislados se expresaron en el tamaño de las lesiones en la raíz. En las pruebas de invernadero el peso seco de las plantas se redujo en un 82%.

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Palabras clave: tallo-negro de primavera, *Phoma medicaginis* var. *medicaginis*, alfalfa, *Medicago sativa* L.

INTRODUCTION

For many years, variation in *Phoma medicaginis* var. *medicaginis* (*P.m.* var. *m.*) has received considerable attention, particularly in studies comparing this fungus with the spring black-stem pathogen of red clover (*Trifolium pretense* L.). These studies showed conflicting results regarding the cultural and pathogenic variation of this pathogen in alfalfa. Schenck and Gerdemann (1956) found that 18 isolates of *P.m.* var. *m.* were uniform in appearance, and Johnson and Valleau (1933) reported cultural variability only in the red clover pathogen. Sprague (1929) observed that spores produced on the host varied in morphology, but those produced in culture did not. Conversely, Ellingboe (1959b) grouped isolates of *P.m.* var. *m.* from several locations into six cultural types, which he did not describe, and found differences in the pathogenicity and in the virulence of monoconidial lines from these isolates. Reitz (1948) reported similar observations, but data were not presented. Edmunds and Hanson (1960) reported that geographical origin and years of collection were sources of variation for the pathogenicity of *P.m.* var. *m.*; however, the concentration of spores in the inoculum was not standardized.

Variation due to organ of origin was observed by Cormack (1945), who reported slight to moderate foliar infections induced by isolates from the leaf, stem, root, seed and soil. The differential response of leaves excised from three varieties of alfalfa to seed isolates of *P.m.* var. *m.* led Mead and Cormack (1961) to conclude that parasitic strains occurred in this fungus. Similarly, Gray et al. (1990) in their study with excised alfalfa trifoliates concluded that isolates from different organs and locations varied significantly in their ability to induce symptoms and in the density of spores produced in potato dextrose agar (PDA). However, these same authors acknowledged a significant difference in spore production, but no attempt to standardize the inoculum was reported. Conversely, Kernkamp and Hemerick (1953) found no differences in the pathogenicity of isolates from different geographical areas and plant organs.

Previous studies (Rodríguez et al., 1990, 1992) showed the important role that this foliar pathogen can have in the root and crown rot diseases of alfalfa. Because of disagreement in the literature concerning variation in *P.m.* var. *m.*, this study was conducted to determine the variation in cultural characteristics of this fungus, and to find whether differences in pathogenicity and/or virulence to the roots and crowns of alfalfa were evident.

MATERIALS AND METHODS

Plants of alfalfa showing leaf spots and black-stem symptoms were randomly collected from fields in Bradford, Centre and Lycoming counties in Pennsylvania. Roots and crowns were washed with detergent and rinsed in running tap water. Portions of these organs with rot symptoms were immersed for 5 min in 0.5% sodium hypochlorite, rinsed in sterile distilled water (SDW), and the superficial tissues were excised thus exposing the necrotic interior tissues for isolation. Pieces of tissues from these areas were plated on water agar and incubated at 21°C for two to four weeks. Leaves and stems were treated for 3 min in 0.5% sodium hypochlorite, rinsed and placed in moist chambers for 48 h at room temperature.

Cultures of *P.m.* var. *m.* were also obtained directly on PDA by transferring spores from the picnidia formed in the host tissues. All isolates were stored on silica gel (Perkins, 1962) and the collection was expanded with isolates received from other states and Canada.

Identification of *P.m.* var. *m.* isolates was based on the available descriptions for this pathogen (Barnett and Hunter, 1972; Boerema, 1965, 1976). A typical and some atypical isolates were sent for identification and corroboration to Dr. Gerhard H. Boerema in the Netherlands.

Cultural characters. Nineteen isolates from Pennsylvania representing different locations and plant organs and 10 isolates from other geographical areas were activated from silica gel on PDA. Five-mm-diameter agar disks were cut from the periphery of the colony and transferred to plates containing oatmeal agar (OA). All isolates were treated similarly and incubated at 21°C for 30 days. Actively sporulating cultures were rinsed with SDW and the spore suspensions were further diluted and incubated in water agar (WA) for 48 h. Single-spore cultures were prepared by transferring the germinated spores from the water-agar plates to the centers of plates containing PDA; these cultures were incubated at 21°C for 10 days. Daily growth was measured with a ruler and the rate of growth was estimated by the slope of the lineal equation of regression. For the sporulation measurements, 10 disks of 5-mm diameter were cut at half the radius of the colony and placed in test tubes containing 5 ml of SDW. Test tubes were shaken for 1 min and the number of spores per square millimeter was estimated by using the hemocytometer. Pigmentation of the colony was determined by using the color nomenclature of Rayner (1970) and the appearance of the colony growth was assessed visually.

Experimental design was that of a randomized block, in which each shelf of the incubator constituted a replicate, and the isolates were evaluated in separate trials. The trial with the Pennsylvania isolates

included five replicates with six single-spore cultures per replicate; the trial with isolates from other areas had four replicates with five single-spore cultures per replicate. The variation among isolates was determined by the analysis of variance. Because variation in the experimental conditions among trials may have occurred, an analysis was done to test the significance of the variation among trials. When no significant differences occurred among trials, then a partition analysis was conducted to determine whether organ and location of origin of the isolates were sources of variation. Differences among means for organ and location, as well as the means for isolates, were determined by the Scheffe's test at $P < 0.05$. The relationship between the variables studied was determined by using the correlation analysis.

Root inoculations in the growth chamber. Clonal plants from cultivar Iroquois were used in these experiments. Stem cuttings were disinfested by a 1-min immersion in 70% ethanol, 20-min in hydrogen peroxide and three 10-min rinses in SDW. Rooting occurred in Hoagland solution agar contained in large test tubes (2.1-cm diameter \times 19.8-cm depth). After one month, plants were carefully removed from the test tubes, their roots washed in SDW and placed in the slant-board system (Kendall and Leath, 1974). Plants were grown for an additional two weeks before inoculation. Seventeen isolates from several geographical locations and from different plant organs were included. Monoconidial lines, typical of each isolate and obtained from the previous trial, were used. Inoculum was produced on sterile pieces of stem of alfalfa (Rodríguez et al., 1990). Pieces were used as inoculum after 25 days when fully colonized by the fungus and picnidia were formed.

For inoculation, wounded and non-wounded roots of 6-week-old plants were inoculated with the isolates. For wounding, roots were stabbed with a 0.15-mm-diameter needle, and the colonized piece of stem was placed on the wounded area. Plants were kept in the growth chamber for seven days, after which time length of the lesions were measured with a ruler. Each isolate was tested on one plant with six inoculated roots, and half of the roots were wounded. This experiment was repeated three times. The data were subjected to an analysis of variance, and the means compared with the Tukey Studentized test at $P < 0.05$.

The sporulation of each isolate in the piece of stem was estimated by immersing three stem pieces in a test tube containing 5 ml of SDW and shaking for 1 min. The number of spores per milliliter in this suspension was estimated with the hemocytometer and divided by three. This procedure was repeated four times, and the data analyzed with the analysis of variance.

Root inoculation in the greenhouse. Clonal plants of cultivar Iroquois were produced in the greenhouse. Seven isolates were selected on the

basis of their performance in the growth-chamber studies. Inoculum was prepared by scraping the surface of the 30-day-old single-spore cultures growing on OA and then rinsing with SDW. For all isolates, concentration of inoculum was adjusted to 2×10^6 spores/ml by using the hemocytometer. For inoculation, two-month-old plants were carefully removed from the rooting medium and their roots washed in tap water. Roots of test plants were wounded by pressing them with a file-card (wire brush used to clean files) immediately prior to immersion for 1 h in the designated spore suspension. Control plants were immersed in SDW. After treatment, plants were planted in clay pots containing 450 g of a vermiculite peat-potting mixture, placed in the mist chamber for two days at approximately 21°C, then returned to the greenhouse for one month.

Treatments were arranged in a randomized block design with seven replications. Dry weights of foliage and roots were analyzed with the analysis of variance, and the means of isolates compared with the Tukey Studentized test at $P < 0.05$.

Crown inoculations. Seed plants from cultivar Iroquois were grown in slant boards. Monoconidial lines from isolates BTL-2 (leaf), BTS-2 (stem), BTC-2 (crown), and BTR-2 (root) were grown on OA until sporulating actively. Inoculum consisted of a mass of spores on the tip of a flamed dissecting needle, and plants were inoculated by being stabbed in the crown with the needle-borne spores approximately 3 mm deep. Control plants were similarly stabbed but with a needle free of spores. The plants were returned to the growth chamber for one month. For evaluation, stems and roots were serially cross-sectioned, starting from the tip and progressing toward the crown until necrosis was observed. The length of internal discoloration from the inoculation site was measured with a ruler.

The experimental design consisted of four isolate treatments replicated six times with six plants per replication arranged in a randomized block design. Data on length of internal necrosis were subjected to the analysis of variance, and the means of isolates compared by using the Tukey Studentized test at $P < 0.05$.

For all in vivo studies, random samples were obtained from the inoculated plants and disinfested in 0.25% sodium hypochlorite for 1 min, and rinsed in SDW. Pieces of these samples were plated on PDA and examined after 15 days at 21°C for microbial contaminants.

RESULTS AND DISCUSSION

Phoma medicaginis var. *medicaginis* was readily isolated from the leaves, stems and roots of alfalfa. Bacteria were more frequently associated with diseased crown tissues. The cultural characters of most fungal colonies obtained from the isolations were typical of *P.m.* var. *m.* Colonies were dark grey with a tint of olive (olivaceous grey—No. 121),

with smooth regular margins, and sparse aerial mycelium that became evanescent near sporulation. Abundant gregarious fertile picnidia were formed scattered over the surface of the colony. Crystals beneath the colony (Figure 1A) were produced by all isolates that showed the typical cultural features of the black-stem pathogen of alfalfa. Type isolates with corroborated identification were used as a reference for subsequent identifications.

Cultural characters. After 10 days of incubation, single-spore cultures of isolates from Pennsylvania showed moderate cultural stability for color and morphology of the colony. Predominantly, they were similar to the parent culture of the isolate, but variants occurred at low frequency. Shades different from the predominant grey olivaceous pigmentation and dark sectors were observed (Figure 1B). Variation in morphology was shown by some cultures in which the mycelium grew tightly packed together thus giving the colony a leathery texture. Cultures showing this growth habit had smaller diameters but appeared thicker than the typical type, and the surface of the medium at the margins of the colony was slightly depressed (Figure 1C). Another distinct variant occurred in the stem isolate from Rock Springs. This culture was olive with a tint of grey (grey olivaceous No. 107), produced abundant fluffy aerial mycelium, and did not sporulate (Figure 1B).

Single-spore cultures from isolates from other geographical locations varied less in morphology than those from Pennsylvania, and variants were rarely observed. All isolates conformed to the morphological characteristics typical of *P.m.* var. *m.*; however, differences in shades of pigmentation among some isolates also occurred. Isolates from Wyoming were consistently light olivaceous grey, thus suggesting a standard pigmentation in these isolates.

The analysis of Pennsylvania isolates for diameter of the colony, rate of growth and sporulation of single-spore cultures showed that the isolates were the major sources of variation and that variation due to the experimental conditions among trials was not significant (Table 1). Organ of origin significantly affected diameter of the colony, rate of growth and sporulation. Similarly, location was a significant source of variation for diameter of the colony and for rate of growth, but it had no effect on the *in vitro* sporulation. Location had more effect on the diameter of the colony and the rate of growth than did organ of origin, as evidenced by the higher mean square values obtained for the location of the isolates.

Overall means for organ of origin showed that isolates from the crown and root grew faster, attaining larger colony diameters than those from the leaf, and that root and stem isolates sporulated more than leaf and crown isolates (Table 2a, b, and c). Means for location indicated that isolates from Rock Springs had smaller diameters than those from other

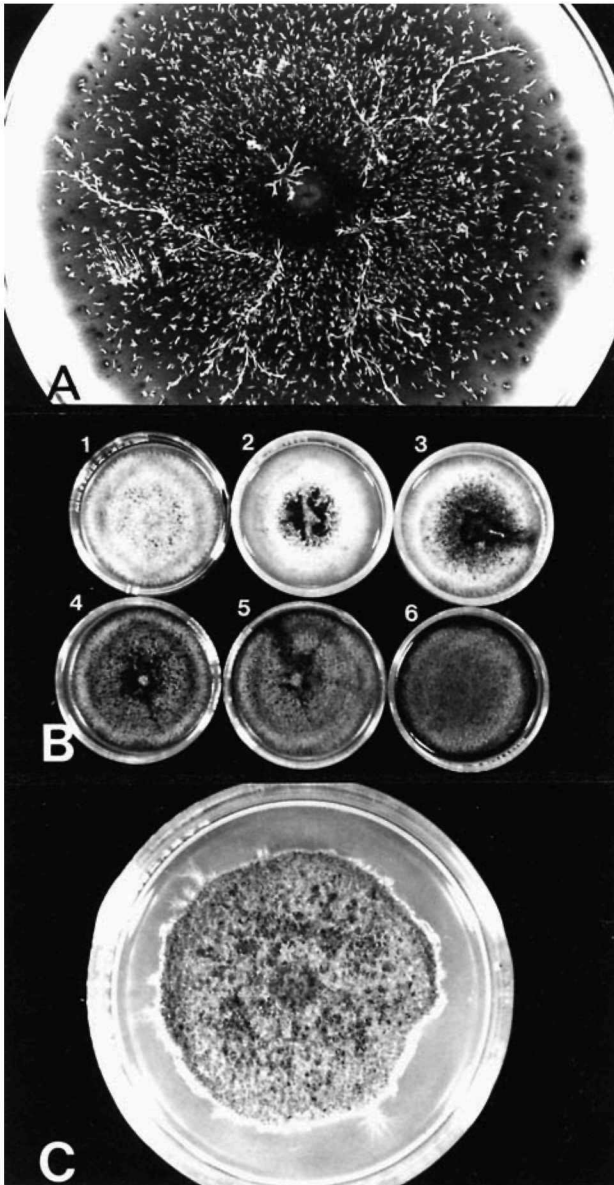


FIGURE 1. Cultural characteristics of *Phoma medicaginis* var. *medicaginis* and variants. A. Crystals formed at the bottom of the colony typical of the fungus. B. Variants in pigmentation of single-spore cultures. 1 to 3. Lighter shades showing stromatic and moist center associated with high sporulating zones; 4 and 5. Cultures with characteristic features of this fungus; 6. Mycelial type from the Rock Springs isolate. C. Morphological variant.

TABLE 1.—Statistics from the analysis of variance for diameter of the colony, rate of growth and sporulation of isolates from Pennsylvania of *Phoma medicaginis* var. *medicaginis* grown on potato dextrose agar at 21°C.

Source ¹	DF	Mean Square		
		Diameter	Rate	Sporulation
Trials	3	0.1920	0.0024	81.77
Rep*Trials	16	0.0927	0.0212	130.95
Isolates	15	3.6048**	0.1207**	1466.09**
Location	5	8.0731**	0.2261**	111.39
Organ	3	1.3483**	0.0612**	4881.53**
Location*Organ	7	1.3803**	0.0709**	969.98**
Culture (Is)	95	0.1838	0.0042	50.42
Error	440	0.1363	0.0033	46.77

¹Rep = Replications.

**Significant at $P < 0.01$.

locations, and isolates from the Taylor farm grew more slowly than the others (Table 2a, b). Interactions between organ and location were detected for diameter of the colony, rate of growth and sporulation (Table 1). This interaction indicates that deviations occurred from the pattern suggested by the overall means; hence the organ of origin of the isolate was not always the major source of variation.

The correlation analysis showed a linear relationship between the colony diameter and growth rate ($r = 0.72$, $P < 0.01$). Fast growing cultures tended to have larger diameters and vice versa. The first three days after spore transfer had a great influence on the rate of growth and the final diameter of the colony. Cultures with a prolonged lag phase tended to have colonies of smaller diameters. Similar observations were reported by Ellingboe (1959a), who found that differences in growth among single-spore cultures of *P.m.* var. *m.* reside in the first 24 to 72 h after transfer. Some of the cultures showing an extended lag phase had smaller colony diameters at the end of the trial. Others overcame the lag period with a rapid growth phase that yielded a final colony diameter similar to that of cultures without a prolonged lag phase. These cultures thus had higher growth rates than did cultures that produced the same size colony but had shorter lag periods.

Sporulation of the isolates was not correlated with diameter of the colony ($r = 0.04$, $P > 0.01$), but a negative correlation with the rate of growth was found ($r = -0.16$, $P < 0.01$). However, only 3% of the variation in the sporulation was explained by the rate of growth, thus indicating that faster growing isolates did not always sporulate less and vice versa. This finding indicated that other factors were more determinative for conidial production.

TABLE 2.—*Effect of location and organ of origin on colony diameter, rate of growth, and sporulation of Phoma medicaginis var. medicaginis grown on potato dextrose agar at 21°C.*

Location County-Farm	Organ				
	Crown	Root	Stem	Leaf	Mean
a. Diameter of the colony ¹ (mm)					
Bradford-Lewis	45 a ²	45 a	49 a	46 a	46 ab
Mifflin-_____	—	—	48 a	—	48 a
Bradford-Thomson	49 a	47 a	47 ab	46 a	47 a
Bradford-Taylor	50 a	49 a	45 ab	37 b	45 b
Lycoming-Young	46 a	47 a	43 bc	44 a	45 b
Centre-Rock Springs	—	—	42 c	43 a	43 c
Mean	48 A ³	47 A	46 A	43 B	
b. Rate of growth ¹ (mm).					
Bradford-Lewis	5.4 a ²	5.3 a	6.2 a	5.4 a	5.6 a
Mifflin-_____	—	—	6.1 a	—	6.1 b
Bradford-Thomson	6.0 a	5.7 a	5.6 ab	5.4 a	5.7 ab
Bradford-Taylor	6.1 a	5.9 a	4.5 c	3.6 b	5.0 c
Lycoming-Young	5.4 a	5.5 a	4.6 c	6.1 a	5.4 a
Centre-Rock Springs	—	—	5.1 bc	5.7 a	5.4 a
Mean	5.7 A ³	5.6 A	5.4 B	5.2 B	
c. Sporulation ¹ (× 1000)					
Bradford-Lewis	5.1a ²	17.4 a	8.9 a	10.7a	10.5 a
Mifflin-_____	—	—	7.8 a	—	7.8 a
Bradford-Thomson	4.0 a	21.0 a	11.9 a	2.2 a	9.8 a
Bradford-Taylor	4.6 a	10.8 a	14.1 a	5.9 a	8.9 a
Lycoming-Young	3.2 a	8.1 b	26.8 b	5.7 a	11.0 a
Centre-Rock Springs	—	—	13.2 a	6.5 a	9.9 a
Mean	4.2 A ³	14.3 B	13.8 B	6.2 A	

¹Average of 30 single-spore cultures per isolate.

² Means in a column with the same letter do not differ significantly at P < 0.05.

³Means in row with the same letter do not differ significantly at P < 0.05.

The comparison among isolates from other geographical locations showed influence of organ of origin on the parameters measured (Table 3). Leaf isolates tended to have higher growth rates whereas seed isolates grew more slowly and produced fewer spores. The stem isolates sporulated more and had smaller colony diameter. Variations from this trend also occurred, evidenced by the differences in the diameter of the colony of the Pennsylvania leaf isolate from those from New York and Kansas, as well as the differences in diameter of the colony of the root isolates from Prince Edward Island.

Root inoculations in the growth chamber. All isolates were pathogenic to the roots of alfalfa (Table 4). Wounding significantly enhanced

TABLE 3.—*Effect of geographic location and organ of origin on colony diameter, rate of growth, and sporulation of Phoma medicaginis var. medicaginis grown on potato dextrose agar at 21°C.*

Isolate	Location ¹	Organ	Diam. ² (mm)	Rate ² (mm)	Sporulation ² (× 1000)
KPL-2	KS	Leaf	50 a ³	6.4 a	10.0 a
NY-873	NY	Leaf	50 a	7.1 a	3.4 b
PA-866	PA	Leaf	44 c	6.7 a	1.7 bc
ILL-365	IL	Stem	45 c	6.0 ab	12.4 a
MiS	MS	Stem	45 c	4.7 b	13.7 d
AS-6	AB	Stem	43 c	4.1 b	11.6 a
PEIR-366	PE	Root	43 c	5.2 b	2.9 b
PEIR-381	PE	Root	48 b	5.0 b	1.4 c
WSe-5	WY	Root	48 b	3.7 bc	1.1 c
WSe-11	WY	Root	47 b	3.2 c	1.3 c

¹AB = Alberta-Canada, IL = Illinois, KS = Kansas, MS = Mississippi, NY = New York, PA = Pennsylvania, PE = Prince Edward Island, WY = Wyoming.

²Average of 20 single-spore cultures per isolate.

³Means in a column with the same letter do not differ significantly at $P < 0.05$.

the development of disease ($F = 188.90$, $P < 0.01$), and lesions in the wounded inoculated roots were longer than those in the non-wounded inoculated roots. The interaction between isolate and root treatment was not significant. With the two methods of inoculation, differences in virulence among isolates were detected. In non-wounded roots, the root isolate BTR-2 was the most virulent, inducing longer lesions than those induced by the other isolates. In the presence of wounds, isolate BTR-2 and isolate MS showed extremes in virulence. Differences between the remaining isolates were not detected. For each isolate the mean for each inoculation method was calculated, demonstrating that isolate BTR-2 was the most virulent, inducing significantly longer lesions.

Differences were not detected among isolates for sporulation on stem pieces. Therefore, spore concentration was not likely the factor accounting for the differences detected in the length of lesion induced by some isolates. Mycelium functions as inoculum for most fungal pathogens; however, when using the stem pieces as inoculum, only very sparse mycelium was present along with abundant fertile picnidia. Therefore, conidia were considered to be the primary inoculum.

Root inoculations in the greenhouse. All isolates were pathogenic to wounded roots of alfalfa and caused significant loss in dry weights of roots and foliage (Table 5). However, significant differences among isolates were not detected. Different environmental conditions, methods of inoculation, and criteria of evaluation might explain the differences in the behavior of isolates when evaluated in the growth chamber and

TABLE 4.—*Effect of location and organ of origin of Phoma medicaginis var. medicaginis on the length of rot lesions in roots of clonal alfalfa plants, wounded and non-wounded, grown in slant boards in the growth chamber.*

Isolate	Source		Length of lesion ¹ (mm)		
	Location	Organ	NW ²	W ²	Mean
BTR-2	Bradford, Pa	Root	6 a ³	9 a	7.4 a
BTS-2	Bradford, Pa	Stem	4 b	7 ab	5.3 b
LYS	Lycoming, Pa	Stem	3 b	7 ab	5.0 bc
LYR	Lycoming, Pa	Root	4 b	6 abc	5.0 bc
NY-873	New York	Leaf	3 b	6 abc	4.6 bcd
WSe-11	Wyoming	Seed	3 b	5 bc	4.3 bcd
LYL	Lycoming, Pa	Leaf	3 b	5 bc	3.9 bcd
CRL	Centre, Pa	Leaf	3 b	5 bc	3.9 bcd
KPL-2	Kansas	Leaf	3 b	5 bc	3.9 bcd
LYC	Lycoming, Pa	Crown	3 b	5 bc	3.9 bcd
PA-866	Centre, Pa	Leaf	3 b	4 bc	3.8 bcd
BTL-2	Bradford, Pa	Leaf	2 b	5 bc	3.7 bcd
BTC-2	Bradford, Pa	Crown	2 b	5 bc	3.7 bcd
PEIR-366	Prince Ed Island	Root	2 b	5 bc	3.5 bcd
Ill-365	Illinois	Stem	2 b	4 bc	3.3 cd
MiS	Mississippi	Stem	2 b	4 bc	3.3 cd
MS	Mifflin, Pa	Stem	2 b	3 c	2.7 d

¹Average of three replications with three roots in each root treatment.

²NW = non-wounded roots, W = wounded roots.

³Means in column followed by the same letter do not differ significantly at P < 0.05.

in the greenhouse. The more moderate temperatures, the reduced competition with other species, and the constant exposure of roots to inoculum in the growth chamber studies favored the fungus. Lengths

TABLE 5.—*Effect of root inoculations with isolates of Phoma medicaginis var. medicaginis, on the root and foliar dry weights (mg) of clonal alfalfa plants grown in the greenhouse.*

Treatment	Location	Organ	DWR ¹	DWF
Control	—	—	229 a ²	360 a
BTL-2	Bradford, Pa-Thomson	Leaf	75 b	132 b
BTC-2	Bradford, Pa-Thomson	Crown	57 b	109 b
BTS-2	Bradford, Pa-Thomson	Stem	50 b	118 b
WSe-11	Wyoming	Seed	53 b	90 b
PEIR-366	Prince Edward Island	Root	50 b	73 b
BTR-2	Bradford, Pa-Thomson	Root	43 b	83 b
KPL-2	Kansas	Leaf	41 b	67 b

¹DWR = dry weight of roots, DWF = dry weight of foliage.

²Means in a column with the same letter do not differ significantly at P < 0.05.

of lesions probably did not reflect the overall impact of the infection on the host as much as dry weights did.

Crown inoculations. All isolates were pathogenic to the crowns of alfalfa grown in the growth chamber, but differences in the extent of external crown deterioration were not observed. However, differences occurred among the isolates in the length of the internal necrosis of the stem and into the upper taproot (Table 6). Extensive discoloration of the vascular tissues was detected in the stem following inoculations of the crowns with stem and leaf isolates, and only the leaf isolate extensively colonized the vascular tissue of the upper taproot. Fewer invasion of the vascular tissues of both organs was obtained when crowns were inoculated with root and crown isolates.

Problems in isolating the fungus from the inoculated plants were not encountered. For all in vivo studies the fungus was recovered from diseased tissues almost exclusively, thus indicating that the isolates of *P.m. var. m.* were responsible for the results obtained.

The variation in virulence among isolates reflects differences in the rate at which they infect the host tissues and possibly demonstrates differences also in their ability during early stages of the disease cycle to exploit the external nutritional environment provided by the roots. Isolates of *P.m. var. m.* in response to known concentrations of sugars and amino acids expressed changed morphology, growth and sporulation (Chung and Wilcoxson, 1971; Mead, 1962).

Significant differences in cultural characters and virulence to the roots were detected primarily in controlled environments. The relevance of this finding lies in the selection of isolates and inoculation environments for screening in the breeding programs. The cultures used in the in vivo studies were selected on the basis of the overall characteristics of the pathogen; the cultural variants were not tested. If these variants prove to be stable, they can be an important tool for studies on the genetics of this fungus.

TABLE 6.—*Effect of crown inoculations with selected isolates of Phoma medicaginis var. medicaginis on the length of internal necrosis in the stems and taproots of alfalfa plants grown in slant boards in the growth chamber.*

Isolate	Source		Length (mm) ¹	
	Location	Organ	Stem	Taproot
BTL-2	Bradford, Pa-Thomson	Leaf	41 a ²	46 a
BTS-2	Bradford, Pa-Thomson	Stem	40 a	27 b
BTR-2	Bradford, Pa-Thomson	Root	29 b	31 b
BTC-2	Bradford, Pa-Thomson	Crown	29 b	29 b

¹Average of 36 plants.

²Means in a column followed by the same letter do not differ significantly at $P < 0.05$.

Morphology has often been used as an indicator of variability in all living species. Variation among species of *Sclerotium* collected from different geographical areas was so evident and stable that the species could be differentiated by cultural characteristics, histochemical reactions and composition of sclerotia (Punja and Damiani, 1996). Daniel et al. (2003) reported that the phenotypic variations found in their studies with isolates from different geographical locations were derived asexually. Mutations, heterokariosis and parasexuality are proposed mechanisms of variations operating in the asexually reproducing fungi (Hansen and Smith, 1932). Sanderson and Srb (1965) demonstrated heterokariosis and parasexuality in *P.m.* var. *m.* using in vitro induced auxotrophic mutants. Little is known about the frequency or relevancy of heterokariosis in nature and few genera of imperfect fungi exhibit heterokariosis in natural populations (Caten and Jinks, 1966). Taylor et al. (1999) proposed that in nature most fungi are "clonal and recombining". Any of these mechanisms could have caused the variants observed. There is need for more research on the inheritance of *P.m.* var. *m.* to determine the actual mechanism taking place.

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