Pathogenicity of *Phoma medicaginis* var. *medicaginis* to Crowns of Alfalfa

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ABSTRACT

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The pathogenicity of *Phoma medicaginis* var. *medicaginis* to crowns of alfalfa was determined by inoculating clonal and seedling plants of the cultivar Iroquois with two isolates of the pathogen. Crowns and stubble were inoculated. Crown inoculations with either isolate of *P. m. medicaginis* caused a black necrosis that extended up into stem bases through one internode and down into upper taproots and occasionally into lateral roots. Wounding of crowns was necessary for infection. Stem stubble provided a suitable infection court for the pathogen to invade crown tissues. Crown and stubble inoculations caused fewer axillary buds, smaller crown diameters and foliar dry weights, and an increased number of dead stems. Pycnidia formed abundantly on dead and dying stubble. *P. m. medicaginis* was demonstrated to be pathogenic to alfalfa crowns.

Additional keywords: lucerne, *Medicago sativa*, spring blackstem

Crown rot is a major cause of yield loss and stand decline in alfalfa (*Medicago sativa* L.) (9,24) and occurs under a wide variety of environmental conditions, as evidenced by its worldwide distribution and persistence throughout the growing season (20). Such versatility may result from the diversity of organisms involved in the disease etiology. Numerous fungi, including *Phoma medicaginis* Malbr. & Roum. in Roum. var. *medicaginis* Boerema (1,3,14,21, 23,25,26), have been implicated in the crown rot syndrome under varied geographic conditions. Bacteria (17,27,28) have also been associated with crown rot of alfalfa. The varied and often combined action of these organisms, concomitant with other stresses in the crop (5,8), have resulted in the crown and taproot deterioration being referred to as the crown and root rot complex (16).

Effects of crown rot first become apparent during the third year after planting (4,7) and increase in incidence and severity with stand age (4). A thorough knowledge of the primary causal agents of crown rot in alfalfa is prerequisite to the development of control measures. Disease control in alfalfa depends chiefly on breeding for resistance. Therefore, in the selection process it is imperative to expose plants to the specific pathogens associated with crown rot in specific geographic areas.

Wilcoxson et al (29) associated *P. m. medicaginis* with crown rot of alfalfa, and the pathogen caused the highest crown disease index in tests conducted by Perez (18). These reports suggest that *P. m. medicaginis* is a primary pathogen in the crown rot disease of alfalfa, but definitive studies are lacking.

The objectives of this research were to determine the pathogenicity of *P. m. medicaginis* to alfalfa crowns, the effect of wounding on crown infection, and whether infection altered plant growth. The results of a series of related experiments utilizing seedling and clonal plants, two pathogen isolates, and different inoculation methods are reported.

MATERIALS AND METHODS

General. Pathogenicity of *P. m. medicaginis* was investigated in a series of growth chamber and greenhouse experiments. All experiments tested the *P. m. medicaginis* pathogenicity to alfalfa crowns or the effect of crown or stubble infection on plant performance. Experiments were similar but varied for pathogen isolate, type of plant, and inoculation method used. A randomized complete block design was used throughout, all data were subjected to an analysis of variance, and treatment means were compared by the Bonferroni multiple comparison technique (12). Presence or absence of *P. m. medicaginis* in plant tissue was determined by serial plating of crown tissue on potato-dextrose agar at 21 ± 1 C. Five randomly selected plants from each treatment of each experiment were assayed.

Plant culture. All plants were from the cultivar Iroquois. Clonal plants were produced by taking regrowth stem cuttings from a large field-grown plant that had exhibited susceptible leaf and stem symptoms in the field. Cuttings were rooted over a 30-day period in sand in the greenhouse, then potted in 500-ml pots containing a peat–vermiculite potting mix (Terra-Lite Redi-Earth, Peat-Lite mix, W. R. Grace & Co, Cambridge, MA). Plants grew for another 30 days before inoculation. Seedlings were produced from surface-disinfested seed. Seeds were washed in flowing tap water for 1 hr, immersed in 70% ethanol for 5 min and in mercury bichloride (1 g per liter of water) for 20 min, and given three 20-min rinses in sterile distilled water. Seeds were germinated on 1% water agar and monitored for microbial contamination. After 48 hr, seeds that were germinated and free of microbial growth were transferred to other growth containers.

Seeds for use in greenhouse experiments were transferred directly from agar to peat–vermiculite potting mix in 500-ml pots. Seeds for use in slant-board culture (13) were first planted into autoclaved vermiculite–sand mix (1:1, v/v) moistened with half-strength Hoagland's solution (11), and maintained in growth chambers for 2 wk at 21 ± 1 C day and 15 ± 1 C night temperatures, with a 15-hr photoperiod and a light intensity of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. Seedlings were then transplanted onto slant boards and grown in the same environment for 45 days before inoculation.

Inoculum production. Two isolates of *P. m. medicaginis* were used. Both had been isolated from diseased crowns of alfalfa growing on separate farms in Bradford County in north central Pennsylvania, in an area in which premature stand decline and severe crown rot of alfalfa was prevalent. Inoculum for use on slant boards consisted of 0.25-cm^2 pieces of polyester cloth (15,22) that had been colonized by *P. m. medicaginis* for 30 days on 1.5% water agar. Inoculum for other experiments was produced on 1-cm-long alfalfa stem segments that had been sterilized by immersion for 5 min in 70% ethanol and 20 min in mercury bichloride, followed by rinsing in sterile distilled water and microwaving for 4

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min. Sterility of stem pieces was verified by lack of microbial growth in nutrient and potato-dextrose broths. Inoculated stem pieces were maintained in the dark at 21 ± 1 C for 30 days. Inoculum was also produced by growing *P. m. medicaginis* on oatmeal agar under the same conditions. Conidial suspensions were prepared by placing colonized stem pieces into sterile distilled water and shaking. Concentrations were adjusted on the basis of hemacytometer counts. Response to treatments was based on symptom presence and assessment of plant characteristics (e.g., bud number, stub dieback, crown diameter, and foliar weight) 30 days after inoculation.

Slant-board experiments. Both experiments were done with seedling plants. Isolate 1 of *P. m. medicaginis* was used in both experiments. Wounding was done by stabbing a dissecting needle into the crown to a depth of 2 mm. Treatments were 1) wounded control, 2) inoculated without wounding, and 3) inoculated with wounding. Each treatment was replicated six times, and each replication consisted of one slant board containing six plants. Inoculation in the first experiment was done by placing an infested cloth square against an intact or wounded crown. Inoculation in the second experiment was made by applying spores of *P. m. medicaginis* to the surface of an intact crown or by stabbing into a crown with a spore-infested needle. Spores for this inoculum were produced on oatmeal agar and collected by dipping the needle tip into the spore masses. In both experiments, crowns were split longitudinally after 30 days, and the extent of internal rot evaluated.

Greenhouse experiments. The first experiment was done with clonal plants and isolate 2 of *P. m. medicaginis*. Crown inoculation treatments were the same as for slant-board experiments, and inoculations were made using the infested-needle technique. For stubble inoculations, stems were cut back to 4 cm, and all newly cut ends were touched with a toothpick infested with a mass of conidia. After inoculation, plants were kept in the dark at 21 ± 1 C for 36 hr, then returned to the greenhouse with ambient temperatures between 20 and 29 C. Treatments for the stubble inoculations were 1) sterile-toothpick control and 2) spore-infested toothpick. Each treatment in the stubble experiment was replicated five times, and each replication consisted of one pot containing two plants. Plants were evaluated 30 days after inoculation for symptoms and for effects on plant characteristics (e.g., the number of axillary buds, the amount of stub dieback, crown diameter, and foliar weight).

The second experiment was done with seedling plants and isolate 1 of *P. m. medicaginis*. Crown inoculation treatments were the same as those already described, but a dissecting needle was used instead of a toothpick to inoculate stubble. Each treatment was replicated four times, and each replication consisted of 25 pots each with a single plant. Plants were evaluated for symptom development and effects on plant characteristics, as previously described.

A very concentrated spore mass was used for all stubble inoculations, so to ensure that this was not solely responsible for the marked symptom development, a comparison was made with less concentrated spore suspensions. This greenhouse experiment was done with clonal plants and isolate 1 of *P. m. medicaginis*. Stubble was inoculated either by spray applications with three concentrations of a spore suspension or with a topical needle application of spore mass that was obtained directly from an oatmeal agar culture. Treatments were 1) sterile needle control, 2) 1×10^5 conidia per milliliter, 3) 5×10^5 conidia per milliliter, 4) 1×10^6 conidia per milliliter, and 5) an unknown concentration of conidia in spore mass. Each treatment was replicated three times, and each replication consisted of five pots each containing one plant. The number of dead stems per plant 30 days after inoculation was used to evaluate treatments.

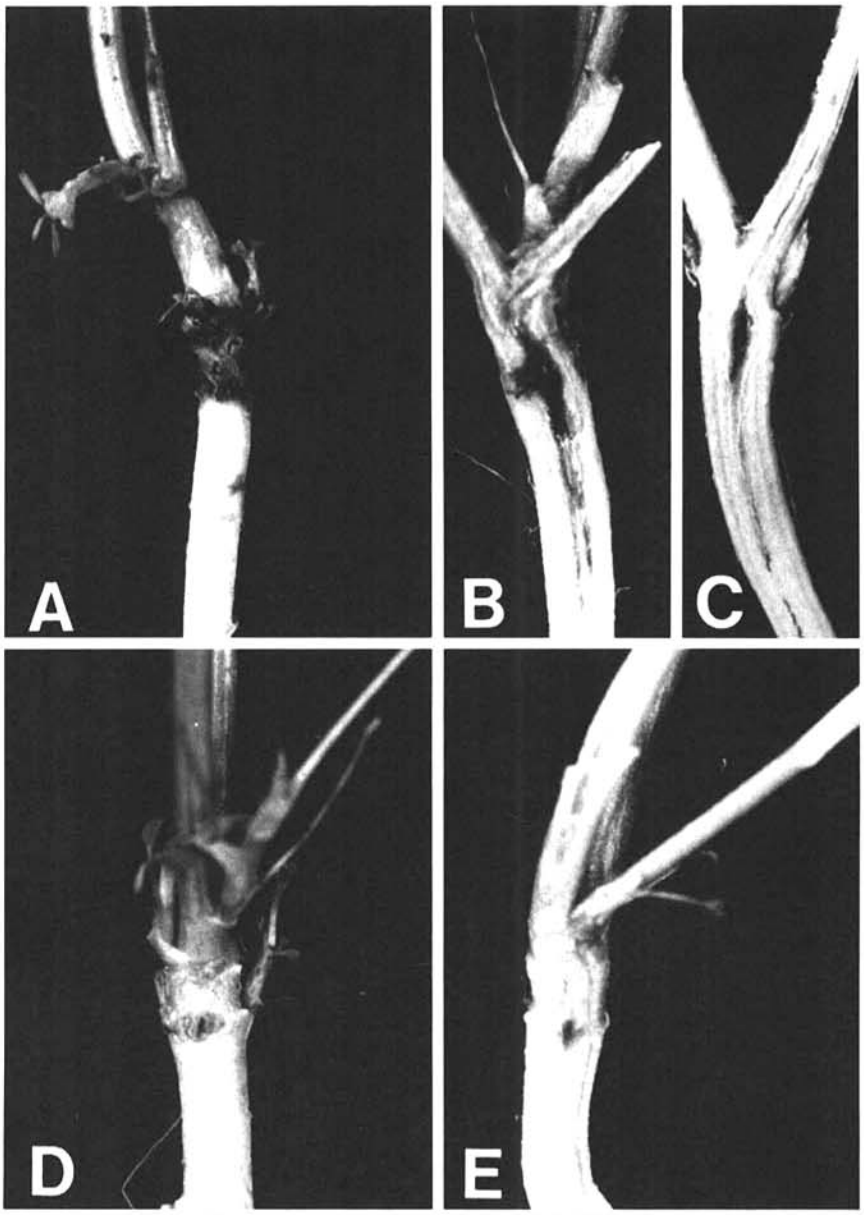

Fig. 1. Symptoms in stem, crown and upper taproot of slant-board grown seedling alfalfa 1 mo after inoculation with isolate 1 of *Phoma medicaginis* var. *medicaginis*. **(A)** Collapsed and cracked surface of diseased crown (×2). **(B,C)** Internal rot in crown and extension of necrosis into the upper taproot stele and stem of infected plant (×2). **(D,E)** Control plants illustrating the absence of rot symptoms in treated areas (×2).

RESULTS

General. In all experiments, rot symptoms developed in wounded, inoculated crowns. No rot developed in control plants. The two isolates of *P. m. medicaginis* were equally pathogenic in both crown and stubble inoculations.

Symptoms did not develop in inoculated crowns unless they were wounded. No differences were observed in disease development between clonal and seedling plants, and *P. m. medicaginis* was consistently isolated from symptomatic tissues. *Trichoderma, Penicillium*, and *Chaetomium* fungi were occasionally isolated from both control and inoculated plants. Infrequently, gram-positive bacteria also were isolated. Potato soft-rot assays to indicate the pathogenic potential of these bacteria (2) were always negative.

Slant-board experiments. Plants from both experiments were evaluated for crown rot symptoms, which were similar in both experiments. Symptoms and data reported are from the first experiment. Inoculation of intact crowns produced no rot symptoms, but inoculation of wounded crowns produced severe symptoms. Development of necrosis around wound sites in the absence of *P. m. medicaginis* was only slight (Fig. 1D and E). Inoculation of wounded crowns caused external and internal rot symptoms (Fig. 1A–C). Often the crown epidermis split vertically from the inoculation site (Fig. 1A), but this did not occur in controls. A dry, black necrosis developed internally from the wound site with either isolate and extended deeper into crown tissues. Internal necrosis extended into the taproot as far as 70 mm from the inoculation site; the mean length of necrosis was 25 mm. Root necrosis was confined to the stele and often extended to the zone of lateral root proliferation, occasionally causing necrosis in lateral roots. Internal necrosis also extended into basal portions of stems through the first internode, with a maximum extension of 63 mm and a mean of 23 mm.

Greenhouse experiments. Rot symptoms from inoculation of crowns of clonal and seedling plants with either isolate in both experiments corroborated those shown in Figure 1 for slant-board grown plants; therefore, no description of symptoms from these experiments is presented. Topical inoculations of freshly cut stubble with either isolate caused infections in nearly every stem. Symptoms resulting from stubble inoculation with isolate 2 are shown in Figure 2. Symptom development began as a black necrosis at the cut end of the stem. Necrosis advanced down the stub from the infection point, with the dead stub later becoming bleached or tan behind the blackened leading edge (Fig. 2B). Necrosis stopped at the node, and the stub portion below the node became chlorotic. Pycnidia developed in the bleached area of the stub (Fig. 2A). An internal necrosis extended down the stub into the crown (Fig. 2D), and infection also caused death of axillary buds (Fig. 2C).

Effects on plant development of inoculating crowns or stubble of alfalfa plants were similar in both greenhouse experiments. Both inoculations reduced the number of axillary buds, crown diameters, and foliar dry weights and increased the amount of dead stems. Results from inoculations with *P. m. medicaginis* isolate 1 are presented in Table 1.

No differences were found among stubble inoculations with several spore concentrations in another greenhouse experiment. The numbers of diseased stems with concentrations of 1×10^5, 5×10^5, and 1×10^6 conidia were not different from each other or that obtained with the spore mass treatment, and thus the high concentration of conidia in the spore mass inoculations was not prerequisite to infection and symptom development.

DISCUSSION

Recently *P. m. medicaginis* was shown to be a primary pathogen of alfalfa roots (22), and evidence presented here indicates that it should be considered a primary crown pathogen as well. It was shown to cause crown necrosis and to impair plant performance following controlled inoculations in the absence of other pathogens.

P. m. medicaginis was consistently associated with internal and external necrosis in crowns, and necrosis observed in our experiments was similar to that

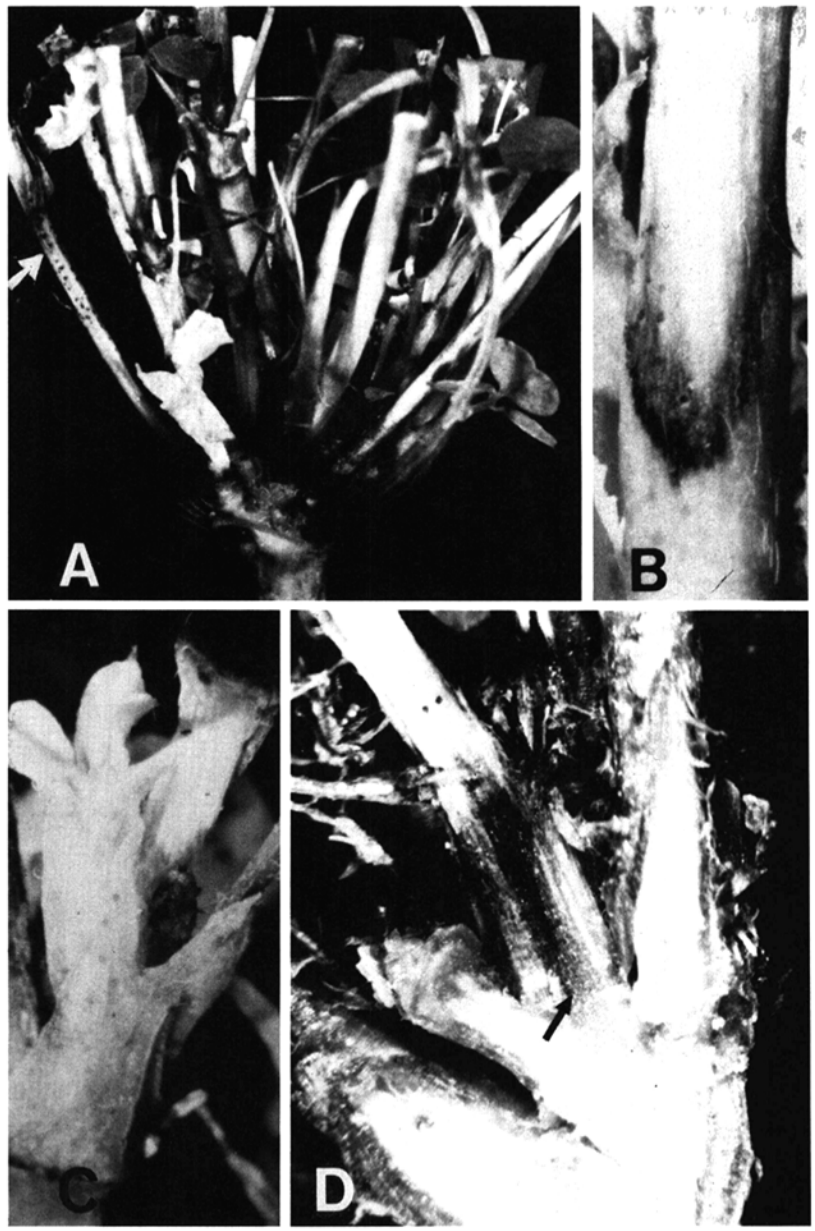

Fig. 2. Symptoms in stubble and crowns of greenhouse-grown seedling alfalfa plants 1 mo after inoculation with isolate 1 of *Phoma medicaginis* var. *medicaginis*. **(A)** Stubble-inoculated plant showing dieback and pycnidia (arrow) formed in the dead portion of the stub. **(B)** Typical stub dieback symptom with black leading edge and bleached, dead portion (×5). **(C)** Dead axillary bud associated with an infected stub (×5). **(D)** Internal necrosis (arrow) extending into crown from dead, inoculated stub (×3).

Table 1. Characteristics of greenhouse-grown alfalfa seedlings 30 days after inoculation with *Phoma medicaginis* var. *medicaginis* isolate 1

Inoculation site Treatment	Plant characteristic[a] Bud (no.)	Stub dieback (no.)	Crown diameter (mm)	Foliage dry weight (mg)
Crown				
Wounded control	4	0	3.9	263
Wound + *P. m. medicaginis*	2*[b]	1*	3.2*	190*
Stubble				
Sterile needle control	4	0	4.0	256
P. m. medicaginis spore mass	2*	2*	3.2*	190*

[a] Means of 100 plants.
[b] Asterisk indicates values that are significantly different from control within crown or stubble treatment at $P < 0.05$.

previously described (4,6,10,27). *P. m. medicaginis* spread from crown infections down into the upper taproot, which also has been previously observed (19,25), and also upward into the stems, which has not been previously described.

Wounding was recently shown to enhance root infection but was not prerequisite (22); however, wounding was necessary for crown infection in these experiments. A requirement of wounding for crown infection should not compromise the infective capabilities of *P. m. medicaginis* under field conditions, because injuries from harvest machinery, insects, and freezing are commonplace. Stems wounded by cutting were readily colonized by *P. m. medicaginis*, and it is quite likely that freshly cut stubble following harvesting is a major infection court for the pathogen's entry into crowns under field conditions. Dead or dying stubs provided effective sites for pycnidial formation and sporulation.

At 30 days after inoculation, alfalfa was impaired in several ways. With a perennial crop like alfalfa, it is likely that crown infections by *P. m. medicaginis* would cause severe stunting over a longer time and possibly dispose plants to greater injury from climatic stress.

All disease symptoms in our experiments were attributed to the pathogenicity of *P. m. medicaginis*. This fungus was the only organism consistently associated with the symptoms. *P. m. medicaginis* has the capability to cause serious disease problems in alfalfa crowns and should be considered a major individual contributor to the crown rot complex of this crop.

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