Infection and development of *Phoma medicaginis* on moderately resistant and susceptible alfalfa genotypes

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**Abstract:** In North America, spring black stem and leaf spot, caused by *Phoma medicaginis*, is one of the most important diseases of alfalfa (*Medicago sativa*). Symptoms appear initially as small, dark brown to black spots that eventually enlarge and on leaves the spots are associated with chlorosis and defoliation. The infection process of *P. medicaginis* was previously investigated on susceptible alfalfa genotypes, but little is known about this process with alfalfa plants with differing degrees of resistance. We used light and scanning electron microscopy to follow infection and development of *P. medicaginis* from pycnidiospore deposition on leaf surfaces through pycnidia formation on selected susceptible and resistant alfalfa genotypes from the USDA core collection. In both old and young leaves, spore germination, penetration, development of mycelium, and pycnidia formation were delayed by approximately 24 h on moderately resistant alfalfa genotypes compared with susceptible genotypes. A similar delay in visible symptom development occurred until about 192 h after inoculation, after which the amount of chlorosis in all genotypes was similar.

**Key words:** alfalfa, *Medicago sativa*, *Phoma medicaginis*, infection process, spring black stem and leaf spot.

**Résumé :** En Amérique du Nord, la maladie des tiges noires, causée par le *Phoma medicaginis*, est une des maladies les plus importantes de la luzerne (*Medicago sativa*). Les premiers symptômes sont d’abord de petites taches brun foncé à noires qui s’élargiront à la longue; sur les feuilles, les taches sont associées à la chlorose et la défoliation. Le processus d’infection du *P. medicaginis* fut précédemment étudié sur des génotypes sensibles de luzerne, mais ce processus est mal connu chez la luzerne avec d’autres degrés de résistance. Nous avons utilisé la microscopie photonique et la microscopie électronique à balayage pour suivre l'infection et le développement du *P. medicaginis* à partir du dépôt des pycniospores à la surface des feuilles jusqu’à la formation des pycnides sur des génotypes sensibles et résistants choisis dans la collection principale de l’USDA. Sur les vieilles comme sur les jeunes feuilles, la germination des spores, la pénétration et le développement du mycélium, et la formation des pycnides furent retardés de 24 h environ sur les génotypes de luzerne moyennement résistants comparativement aux génotypes sensibles. Un retard semblable dans le développement des symptômes visibles fut observé jusqu’à environ 192 heures après l’inoculation, après quoi la quantité de chlorose fut semblable dans tous les génotypes.

**Mots-clés :** luzerne, *Medicago sativa*, *Phoma medicaginis*, processus d’infection, tiges noires.

**Introduction**

Spring black stem and leaf spot, caused by *Phoma medicaginis* var. *medicaginis* Malbr. & Roum., is one of the most important diseases of alfalfa (*Medicago sativa* L.) in temperate regions of North America and Europe. In addition, the fungus causes phoma black stem on annual *Medicago* species (Barbetti and Nichols 1991), and dark spotting on many other legumes (Edmunds and Hanson 1960; Kernkamp and Hemerick 1953). Leaf symptoms appear initially as small, dark brown to black spots, called tarspots, which typically develop without chlorosis. Lesions enlarge and coalesce, becoming dark and irregularly shaped. Lesion formation is accompanied by leaf chlorosis and abscission (Leath 1990). The disease frequently results in defoliation, starting at the base of plants and progressing upward. Lesions on stems appear as a smooth dark discoloration, which can girdle and kill young stems (Leath 1990).
The fungus also attacks roots (Rodriguez et al. 1990; Rodriguez 2005) and crowns (Rodriguez and Leath 1992).

The frequency of resistance to *P. medicaginis* within populations of alfalfa is low, and effective resistant varieties are not available commercially (Gray et al. 1990; Leath et al. 1988) even though selection methods were developed to screen for resistance (Elgin et al. 1988; Haag and Hill 1974; Wang et al. 2004). Within the annual *Medicago* species, a wide range of responses to *P. medicaginis* has been described, and resistant accessions have been identified in several species (Barbetti 2007; Ellwood et al. 2006; O’Neill et al. 2003).

The infection process of *P. medicaginis* on alfalfa was investigated on susceptible alfalfa genotypes (Bantari and Wilcoxon 1963; Hijano 1979; Schenck and Gerdemann 1956; Rodriguez 2005), but little is known about the infection process with resistant alfalfa plants. The objective of this study was to describe the infection and colonization process of *P. medicaginis* from the time of pycnidiospore deposition on the leaf surface to pycnidia formation on alfalfa plants with differing degrees of resistant to the pathogen. To achieve this, we selected alfalfa genotypes within the USDA alfalfa core collection with differing levels of resistance in alfalfa varieties (Rodriguez et al. 1990; Rodriguez and Leath 1992). The fungus was grown for 3–4 weeks on potato dextrose agar (Difco Laboratories, Sparks, Md.) at room temperature (approximately 21 °C) with 12–14 h/day of ambient light. Twenty millilitres of sterile double distilled water was added to the culture for 1 h before gently scraping the colony surface with a sterile wire loop to release the spores. The concentration of spores was measured using a hemacytometer and adjusted to 1.2 × 10⁶ – 1.6 × 10⁶ spores/mL using sterile water. Tween 20 (polyoxyethylene sorbitan monolaurate) was added as a surfactant at a concentration of 20 µL in 1 L. Plants were inoculated using a 1 quartz (1 quart = 0.946 L) clear plastic manual trigger sprayer with a total amount of spore solution per petri dish of approximately 1 mL. After inoculation, leaves were kept at room temperature (22 ± 1 °C) with 12–14 h/day of ambient light and moistened by periodic sprays with sterile double distilled water.

For screening of the core collection, leaves were visually scored 6 days after inoculation. The visual scale used was based on the percentage of chlorosis and presence of tar spots, as follows: 1, no symptoms; 2, no chlorosis and <10 tar spots; 3, <25% chlorosis and <25 spots; 4, 25%–49% chlorosis and ≥25 spots; 5, 50%–74% chlorosis; 6, ≥75% chlorosis. Plants from accessions that exhibited low (accession Nos. PI467926, PI399551, PI467901, PI440517, and PI315461) and high scores (accession Nos. PI467917, PI468013, PI478510, and PI478565) were evaluated two additional times for selecting the final plants for microscopy and phenotypic evaluations. Plants with similar growth habits were then selected for comparisons of symptoms and pathogen development. Thus, two plants from accession Nos. PI399551 and PI464733, now identified as 113 and 145, respectively, were moderately resistant and two plants from accession Nos. PI467917 and PI478565, now termed 157 and 155, respectively, were selected as susceptible types. These moderately resistant and highly susceptible plant types were used in light and scanning electron microscopy (SEM) studies.

**Materials and methods**

**Screening the USDA alfalfa core collection**

The USDA alfalfa core collection (Basigalup et al. 1995) was obtained from the USDA Agricultural Research Service, Regional Plant Introduction Station, Pullman, Washington. Seeds of each of the 189 accessions were planted into 5 cm × 20 cm plastic cones (Stueve & Sons, Corvallis, Ore.) in a mixture of steam-pasteurized soil, sand, and vermiculite (2:1:1, v/v/v). Seedlings were thinned to one per cone 2–3 weeks later.

Three plants from each accession were randomly selected for inoculation. Leaves of 6- to 10-month-old plants at early to late flower stages (Fick and Mueller 1989) were used. Three healthy leaves from each selected plant were removed for a detached leaf inoculation procedure from the lowest five nodes. The three leaves of each plant were placed on sterile filter paper (Whatman No. 1) in a sterile 100 mm × 15 mm polystyrene petri dish moistened with sterile double distilled water. Although three plants per accession did not fully represent the wide genetic diversity within each population, space and time limitations made screening of only a small number of genotypes practical. Freshly harvested spores from *P. medicaginis* isolate 866 (obtained from K.T. Leath, USDA Agricultural Research Service, U.S. Regional Pasture Research Laboratory, University Park, Pa.), were used to inoculate detached leaves of alfalfa accessions. This isolate was used previously in standardized tests to evaluate resistance in alfalfa varieties (Rodriguez et al. 1990; Rodriguez and Leath 1992). The fungus was grown for 3–4 weeks on potato dextrose agar (Difco Laboratories, Sparks, Md.) at room temperature (approximately 21 °C) with 12–14 h/day of ambient light. Twenty millilitres of sterile double distilled water was added to the culture for 1 h before gently scraping the colony surface with a sterile wire loop to release the spores. The concentration of spores was measured using a hemacytometer and adjusted to 1.2 × 10⁶ – 1.6 × 10⁶ spores/mL using sterile water. Tween 20 (polyoxyethylene sorbitan monolaurate) was added as a surfactant at a concentration of 20 µL in 1 L. Plants were inoculated using a 1 quart (1 quart = 0.946 L) clear plastic manual trigger sprayer with a total amount of spore solution per petri dish of approximately 1 mL. After inoculation, leaves were kept at room temperature (22 ± 1 °C) with 12–14 h/day of ambient light and moistened by periodic sprays with sterile double distilled water.

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**Light and scanning electron microscopy**

Individual plants of alfalfa genotypes 113 (PI399551), 145 (PI464733), 157 (PI467917), and 175 (PI478565) were propagated by rooting stem cuttings and grown under greenhouse conditions of 16 h of light per day at 22 ± 2 °C during the day and 19 ± 2 °C at night. Leaf samples from the lowest five nodes for each genotype were removed for inoculation when plants were at the early to late blossom stage (Fick and Mueller 1989).

Isolate 13.3.LL.1 of *P. medicaginis*, collected from alfalfa leaves in southern Minnesota, was used for inoculation. In preliminary experiments, this isolate was more aggressive than isolate 866. The fungus was grown on potato dextrose agar for 3–4 weeks at room temperature (21 ± 2 °C). Spores were collected from cultures as previously described. Spore viability was tested before inoculation by plating spores on water agar medium for 24 h. Spore concentrations varied between 1.5 × 10⁶ and 4.0 × 10⁶ spores/mL, depending on the experiment.

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3 Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products and vendors that might also be suitable.
For each time interval and genotype, 15 leaves were inoculated, and 15 were inoculated with water as controls.

Inoculated and control leaves were used for assessing fungal development at 24 h intervals, beginning 1 h after inoculation (hai) and continuing through 240 hai. An additional time point, 12 hai, was used in one experiment. Four experiments were run during the course of eight months. The first three experiments were repetitions using alfalfa genotypes 157 (susceptible) and 113 (moderately resistant), whereas the last was done using the alfalfa genotypes 175 (susceptible) and 145 (moderately resistant). This last set of genotypes was used to evaluate spring black stem and leaf spot symptoms in old and young leaves. Genotype 175 (susceptible) replaced 157 and had a similar developmental pattern and matured like genotype 145.

For light microscopy, whole leaves were fixed in a mixture of glacial acetic acid and absolute ethanol (1:1, v/v) for 24 h, rinsed twice with sterile double distilled water for 5 min each, and preserved in a mixture of lactic acid, glycerol, and sterile double distilled water (1:1:1, v/v/v). To stain fungal structures, inoculated leaf samples were covered with a boiled mixture of 30% lactophenol cotton blue (Fluka, Sigma-Aldrich, St. Louis, Mo.) in 95% ethanol for 8–10 min, and then rinsed in sterile double distilled water to remove excess stain. Samples were mounted on glass slides with sterile water for observation.

For SEM, rectangular leaf samples of approximately 3–4 mm × 4 mm and 3–4 mm × 8–10 mm were affixed to aluminum stubs with carbon double-sided tape, and the sample borders coated with carbon conductive paint. The samples were frozen for stabilization by cooling at –120 °C with liquid nitrogen and immediately transferred to a cryostage on a Hitachi S-3500N SEM. Frozen samples were then coated with gold using a sputter caster that was contiguous with the cryostage. A low accelerating voltage as specified by Ahlstrand (1996) was used to produce secondary electron images.

For observation of putative appressoria, an artificial substrate was employed. Sheets of Parafilm® “M” (American National Can™, Chicago, Ill.) were sprayed with a spore suspension as described above. At 24 and 48 hai, pieces of inoculated parafilm were affixed to aluminum stubs and observed by SEM as detailed above.

To assess spore swelling prior to germination, spore length and width was measured at 1, 24, and 48 hai by light microscopy and SEM. Percent germination of spores was selected for additional evaluations. The results of these evaluations are shown in Table 1. The final selection of moderately resistant genotypes was based on mean disease scores of 3.5 or less (less than 25% chlorosis).

Single plants from four highly susceptible accessions were also evaluated a second time. The disease scores of these plants were 5.8 ± 0.2 (PI467917), 5.7 ± 0.2 (PI468013), 5.2 ± 0.1 (PI478510), and 6.0 ± 0.0 (PI478565), confirming that these genotypes were indeed highly susceptible.

The distribution of disease scores on alfalfa accessions and of spores length and width were analyzed using JMP start statistics software version 3.2.1 (SAS Institute Inc. 1996). Differences in symptom development were tested using an exact Wilcoxon sum rank test (Hollander and Wolfe 1998) under the null hypothesis: H0: μ1 = μ2, and the alternative H1: μ1 < μ2, where μ1 is mean chlorosis of the moderately resistant genotype and μ2 is the mean value for the more susceptible genotype. Data were analyzed using R software version 1.7.1 (R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org).

### Results

#### Identification of moderately resistant and susceptible genotypes from the alfalfa core collection

Symptoms of spring black stem and leaf spot were evaluated on three plants from each of the 189 alfalfa accessions. Mean disease scores, based on the amount of chlorosis, varied from 1.7 to 6.0, with an overall value of 4.5 ± 0.9 (mean ± SE). There was a high frequency of susceptible genotypes in the collection (Fig. 1). Single plants from accessions with mean scores less than 4.0 (approximately 25% of the accessions) were selected for additional evaluations. The results of these evaluations are shown in Table 1. The final selection of moderately resistant genotypes was based on mean disease scores of 3.5 or less (less than 25% chlorosis).

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#### Symptom development

Percent leaf chlorosis was determined every 24 h from 1 to 240 hai using detached leaves from moderately resistant (145; PI464733) and susceptible (175; PI478565) alfalfa genotypes. Older leaves, from the five lower nodes, of the moderately resistant genotype developed significantly lower amounts of chlorosis (P ≤ 0.05) than did the older leaves from the susceptible genotype at 72, 96, 120, 144, and 168 hai (Table 2). Thus, there was a delay in symptom development on the older leaves of the moderately resistant alfalfa genotype compared with the susceptible plants.

Symptoms on young leaves of both genotypes appeared at 96 hai (Table 2). The amount of chlorosis was less (P ≤ 0.05) on younger leaves of the moderately resistant genotype compared with the susceptible genotype at 96 to 240 hai, with the exception of 192 hai when differences were not significant.

In summary, symptoms were less severe on leaves of the moderately resistant genotype than the susceptible genotype. Symptoms developed more slowly on younger leaves than older leaves of both genotypes.

#### Fungal development on susceptible and moderately resistant alfalfa genotypes

Ninety-five percent of spores germinated on water agar by 24 h after plating. They had germ tubes up to 4 mm in length. At 1 hai, spores were present on inoculated epidermal leaf cells and absent on the controls. Spores were often
collapsed and partially dehydrated, but their general shape was well maintained. Spores occurred in clusters. Most were located at the junctions of epidermal cells but were occasionally observed on nonjunction areas. Spores were elliptical in shape and mostly unicellular. Spore surfaces were mostly smooth, with very small occasional surface irregularities (Fig. 2A). Using SEM, most spores had uniform sizes with a length of 5.28 ± 0.17 μm and a width of 2.07 ± 0.06 μm. A few spores were larger (8.25 μm x 2.85 μm), and some of these were bicellular. Clustered spores had diverse sizes and shapes and were connected at spore ends or sides (Fig. 2A).

Spores observed by light microscopy were stained with cotton blue. Cotton blue stained the spore cytoplasm, which was surrounded by a nonstained area, probably a part of the cell wall (Fig. 2B). Spore germination was observed at 12 hai by formation of a small protrusion between the middle and the tip of spores (Fig. 2C). By 24 hai, some spores produced a short germ tube and host penetration had already been initiated. Small globular structures occurred occasionally on spore surfaces on both alfalfa genotypes (Fig. 2D). However, perfectly smooth spore surfaces were also observed later when the fungus appeared to be penetrating epidermal cells (Fig. 2E). A distinct, easily recognizable appressorium was lacking, but germ tube tips expanded when in contact with the plant surface in many cases (Fig. 2E). Germination on a parafilm sheet revealed similar swellings at the terminus of the germ tubes, with the surface beneath the swelling depressed in the centre (Fig. 2F). The fungus penetrated directly through the surface of epidermal cells and at epidermal cell junctions.

Germination and penetration of spores was asynchronous on both alfalfa genotypes. Penetration through stomata was never observed. An extracellular mucilaginous matrix was frequently seen surrounding spores and germ tubes in contact with epidermal leaf surfaces (Fig. 2E). Often, the ma-
Fig. 2. Light (LM) and scanning electron micrography (SEM) of development of *Phoma medicaginis* on alfalfa leaves. (A) SEM of spores 1 h after inoculation (hai). Spore surfaces are smooth with occasional, small, irregular, globular structures (open arrow). (B) LM of a spore 1 hai stained by cotton blue. The spore cytoplasm is surrounded by a nonstained area (100×). (C) A protusion from a spore at 12 hai. (D) SEM of spore surfaces at 24 hai with occasional, small globular structures (open arrow). (E) SEM of apparent spore penetration (solid arrows) and fusion with a neighboring spore (open arrow). (F) SEM of spore germination on parafilm. (G) SEM showing fusion of spores and hyphae at 72 hai. (H.1) LM at 72 hai of fungal subcuticular growth (unstained, solid arrow) and stained cytoplasm of surface-bound spores clustered on the susceptible alfalfa genotype (100×). (H.2) LM of germinating spores at 72 hai and apparent penetration on the moderately resistant alfalfa genotype (100×). (I) SEM at 96 hai. Subcuticular hyphal growth has raised cuticular areas (solid arrow). The width of subcuticular mycelia was wider than the initial spore germ tube. Collapsed epidermal cells were invaded as well as few adjacent cells (open arrow). (J) SEM of a pycnidium with spore ooze from the ostiole at 144 hai. The pycnidium is on the surface of the susceptible genotype. (K) SEM of immature pycnidia at 144 hai on a leaf of the moderately resistant genotype. Scale bar = 1 µm in Figs. 2A–2F and 2H; scale bar = 10 µm in Figs. 2G and 2I–2K).
the moderately resistant genotype than when on leaves of the susceptible genotype. At 6 days after inoculation, leaves of the susceptible genotype were approximately 95% chlorotic, whereas on the moderately resistant genotype was clearly delayed compared with the initial size of spores. In addition, spores on the susceptible genotype were larger at 24 hai than were spores on the moderately resistant genotype. On susceptible leaves, 196 spores had a length of 5.82 ± 0.07 µm and a width of 2.65 ± 0.04 µm, whereas 110 spores measured on the moderately resistant alfalfa genotype were 5.56 ± 0.09 µm long and 2.39 ± 0.06 µm wide. Spore lengths between moderately resistant and susceptible genotypes were significantly different (P = 0.01) as was the difference of spore width between the genotypes (P = 0.00).

The percent germination of spores at 24 hai was determined for both alfalfa genotypes using light microscopy. Germination on the susceptible genotype was 47.2% (142 germinated of 301 spores counted), whereas germination on the moderately resistant genotype was 27.1% (114 germinated of 420 spores counted). Thus, spores swelled and germinated more rapidly when on leaves of the susceptible genotype than when on leaves of the moderately resistant genotype.

At 48 hai, germination and penetration continued, and mycelial growth beneath epidermal cells was observed by light microscopy. Most germinating spores were associated with mucilaginous material, which was not observed near ungerminated spores. Spores showed variable morphology at 48 hai, which was most likely associated with activities during germination. Spores in clusters apparently fused or were connected, possibly by conidial anastomosis tubes. A few spores with long germ tubes were seen on both alfalfa genotypes. Penetration of epidermal cells was greater at 48 hai than at 24 hai, and the site of penetration was surrounded by abundant extracellular material. There was an increase in percent spore germination observed by light microscopy compared with 24 hai in both genotypes. Again, germination was greater on the susceptible genotype (64.9% germination; 48 germinated spores of 74 counted) than on the moderately resistant genotype (33.7% germination; 31 germinated spores of 92 observed). At 48 hai, tarspots were observed on a few leaflets of both genotypes.

At 72 hai, abundant hyphal growth and plant cell collapse on the susceptible genotype were evident. Fusion of germings and hyphae were observed (Fig. 2G). Infection of the moderately resistant genotype was clearly delayed compared with infection on the susceptible genotype. On the susceptible genotype, the fungus had penetrated the leaf surface, and mycelial growth was seen under the cuticle of the epidermal cells by 72 hai; however, on the moderately resistant genotype, spores were still in the process of germination and initial penetration (Figs. 2H.1 and 2H.2). The length and width of fungal mycelia under the cuticle exceeded the size of the spores. At 72 hai, the first visible symptoms of chlorosis were observed on the leaves of the susceptible genotype, whereas the moderately resistant genotype had almost no chlorosis. Collapse of penetrated epidermal cells occurred on inoculated susceptible leaves. Neither chlorosis nor cell collapse was observed on control samples.

At 96 hai, there was greater subcuticular hyphal development and more collapsed epidermal cells than at 72 hai. The width of subcuticular mycelia was now more than three times larger than the spore germ tube. Epidermal cells initially invaded had collapsed, as had adjacent epidermal cells, indicating that the fungus invaded or affected these cells as well (Fig. 2I). Based on visual observations, invasion of epidermal cells on the moderately resistant genotype was slower compared with the susceptible genotype and hyphae growing over the leaf surface were more common than on the susceptible genotype. Light microscopy revealed fungal infection and development occurring subcuticularly and probably inter- or intra-cellularly. The amount of chlorotic leaf area visible was always greater on the susceptible genotype compared with the moderately resistant genotype.

At 120 hai, SEM revealed abundant hyphae growing over and under the cuticle of the leaf epidermal cells on the susceptible genotype. Pycnidia bursting through the leaf cuticle were evident on susceptible leaves but not on moderately resistant leaves. Epidermal cells collapsed, and the stomata on the abaxial leaf surface were wide open. With light microscopy, more hyphae were seen invading leaf epidermal cells of the susceptible genotype compared with the moderately resistant genotype. Chlorosis was threefold greater on leaves of the susceptible genotype (78%) than on the moderately resistant genotype (25%).

Observations by SEM at 144 hai revealed mature pycnidia releasing spores on the leaf surface of the susceptible genotype (Fig. 2J). Pycnidia were surrounded by hyphae and were often associated with abundant electron-dense material. Under conducive conditions on the susceptible genotype, the fungus was able to complete its life cycle in 6 days. In contrast, pycnidia were just emerging on the surface of the moderately resistant genotype at 6 days after inoculation (Fig. 2K). This electron-dense material was also present on leaves of the moderately resistant genotype, where it surrounded developing pycnidia. Under light microscopy, mature pycnidia (releasing spores) were observed on leaves of the susceptible genotype, whereas immature pycnidia (not releasing spores) on the moderately resistant genotype. At 6 days after inoculation, leaves of the susceptible genotype were approximately 95% chlorotic, whereas leaves of the moderately resistant genotype were approximately 50% chlorotic.

Between 144 and 240 hai, P. medicaginis continued to cause cell collapse. It ramified within leaves and produced abundant pycnidia. Electron-dense material was frequently associated with hyphae that surround pycnidia on both genotypes, but it was also seen on other areas of the leaf surface. Under light microscopy, this material stained with cotton blue as did portions of fungal hyphae and, thus, appears different from the material surrounding spores in the first stages of the infection process. The electron-dense material may originate from plant cell contents released from collapsed cells. From 144 to 240 hai, the differences in percent chlorosis between leaves of the two genotypes were less distinct. Similar symptoms were observed at 192 and 240 hai, and the leaves became flaccid.
Discussion

Spore germination, fungal penetration and infection, and development and maturation of pycnidia were delayed by about 24 h on both of the moderately resistant alfalfa genotypes compared with the susceptible genotypes. Invasion of epidermal cells on the moderately resistant genotypes was slower and less frequent compared with the susceptible genotype and hyphae growing over the leaf surface were more common than on the susceptible genotype. Symptom development on the moderately resistant genotypes was delayed 24 h until about 192 hai, after which the amount of chlorosis in moderately resistant and susceptible genotypes was similar.

Differences in germination rates are most likely related to intrinsic properties of the alfalfa genotypes. Nutrients such as glucose and asparagine, when added to fungal inoculum, were shown to increase P. medicaginis spore germination rates (Renfro and Wilcoxson 1963). Addition of glucose and potato-dextrose broth increased germination rates, at least in the first 24 hai; promoted more abundant, wider, and more vigorous germ tubes; and resulted in a greater number of “appressoria” (Banttari and Wilcoxson 1964). Thus, availability of carbohydrates and amino acids on leaves of the differing alfalfa genotypes may have influenced spore germination.

Antimicrobial compounds present on leaves can also influence spore germination. Detectable levels of the phytoalexin medicarpin and sativan, a related isoflavonoid, were observed in detached alfalfa leaves as early as 8 hai with P. medicaginis (Blount et al. 1992). Medicarpin was detected in inoculum drops of P. medicaginis spores (“diffusate solutions”) on alfalfa leaves at 24 hai in concentrations of 1 µg/mL and in subtending tissue at 3 µg/g (Higgins 1972); correspondingly, germination of P. medicaginis spores was generally poor. In in vitro assays, germinating spores of P. medicaginis degraded medicarpin to nearly undetectable levels after 8 to 24 h of contact (Higgins 1972). If the moderately resistant genotypes produced and (or) released more medicarpin to the diffusate than did the susceptible genotypes in the current experiments, spore germination could have been delayed until the fungus degraded the phytoalexin.

In this study, only direct penetration through the cuticle was observed. In previous work, penetration through stomata was reported (Hijano 1979; Schenck and Gerdemann 1956). Banttari and Wilcoxson (1963, 1964) reported that direct penetration was most frequently observed, with occasional penetration through stomata. At the point of penetration, we observed a swelling at the tip of the short germ tube (Fig. 2F). Previous reports of appressorium formation during direct penetration of epidermal cells on alfalfa have been contradictory. On leaves, Banttari and Wilcoxson (1963, 1964) reported appressoria during penetration and described distinct swellings on the tips of the germ tubes. However, Schenck and Gerdemann (1956) reported that the tip of the hypha of P. medicaginis usually turned clockwise but that penetration occurred without formation of a distinctive appressorium. On stems, Hijano (1979) reported appressoria formation by 72 hai. Other Phoma species have been reported to directly penetrate with or without formation of appressoria (Roustae et al. 2000).

Differences in epidermal cells on different parts of the plant may cause the fungus to develop differently in response to hardness, hydrophobicity, topography, and plant signals (Tucker and Talbot 2001). The lack of differentiation of a fully developed appressorium (without septa) occurs in other fungal pathogens that penetrate the plant surface directly. These fungi need cell wall degrading enzymes for penetration (Mendgen et al. 1996). The presence of an abundant mucilaginous matrix surrounding germinating P. medicaginis spores may facilitate penetration through release of hydrolytic enzymes. Muciligenous material was not detected surrounding spores germinating on an artificial surface. This may indicate that stimuli from plant surfaces are required for matrix production.

At 72 hai, anastomosis of spores and germ tubes and (or) hyphae was seen on the surface of epidermal cells. This was previously observed in P. medicaginis by Ellingboe (1959) working with the fungus on agar plates. Fusion between hyphae of Phoma clematidina (Thüm.) Boerema on leaves of Clematis species has also been reported (van de Graf et al. 2002). Conidial anastomosis is widespread in filamentous fungi, but little is known about the biology or importance of this phenomenon (Roca et al. 2005). In Colletotrichum lindemuthianum (Sacc. & Magnus) Briosi & Cavara, conidial anastomosis increases spore germination and is proposed to increase the pool of resources available for infection (Roca et al. 2003). Hyphal fusion is assumed to be important in translocation of water and nutrients within a fungal colony, for interhyphal communication, and for homeostasis within the colony (Glass et al. 2004). Hyphal fusion is also critical in sexual and parasexual cycles. Sanderson and Srb (1965) reported heterokaryosis and parasexuality after combination of auxotrophic mutants generated by ultraviolet light from a single strain of a synonym for P. medicaginis, Ascochyta imperfecta Peck. If anastomosis of differing P. medicaginis isolates occurs as reported by Ellingboe (1959), the fungus could generate variation through parasexual recombination. However, whether anastomosis between or among fungal structures of the same isolate is advantageous in disease progression by P. medicaginis remains to be tested.

From 72 to 240 hai, infection and development of pycnidia in moderately resistant alfalfa genotypes was delayed compared with susceptible genotypes. In other studies in alfalfa, medicarpin, a pterocarpan isoflavonoid, was elicited rapidly upon fungal challenge (Paiva et al. 1994). Bioassays revealed that vestitone and medicarpin at 0.5 mmol/L inhibited mycelial growth of P. medicaginis up to 44% and 42%, respectively (Blount et al. 1992). Also, increased medicarpin production through isoflavone O-methyltransferase overexpression in alfalfa conferred an effective resistance against P. medicaginis (He and Dixon 2000). Thus, in the current study, it is possible that rapid, large accumulations of medicarpin and related flavonoids toxic to P. medicaginis accounted for the delay in fungal development in leaves of moderately resistant genotypes.

In addition to phytoalexins, other plant defenses may be active during the earlier periods of infection. For example, P. medicaginis elicits β-1,3-glucanase transcription in leaf tissues and in cell suspensions of alfalfa, reaching a maximum level at 24 hai compared with uninoculated alfalfa...
(Maher et al. 1993). Rapid elicitation of pathogenesis-related proteins in the moderately resistant genotypes may have contributed to the delay in fungal development. Symptoms developed more slowly on younger leaves than older leaves of both genotypes. This is in agreement with previous finding using a detached leaf assay. Wang et al. (2004) found disease severity was significantly greater in older leaves compared with younger leaves when evaluating eight alfalfa cultivars at the seedling stage.

Symptoms on leaves infected by *P. medicaginis* include dark lesions and chlorosis. Whether a phytotoxic fungal metabolite is involved remains unknown, although this is a distinct possibility. Toxins have been identified in several *Phoma* and related *Ascochyta* species (Fogliano et al. 1998; Pedras and Biesenthal 2000; Strange 1997; Vurro et al. 1997). More importantly, a toxin isolated from cultures of *P. medicaginis*, brefeldin A (di Menna et al. 1979; Suzuki et al. 1970; White et al. 1979), caused lesions in alfalfa tissues (Tietjen et al. 1983). It is unlikely that tarspots observed at 48 hai in both genotypes are caused by brefeldin A or its metabolites. Observation by SEM did not reveal alterations of the epidermal cell surfaces; however, there were obvious spore masses germinating and (or) penetrating epidermal cells, and these were surrounded by matrix material. Thus, it is possible that mostly fungal structures themselves contributed to the early 72 hai tarspot phenotype. In addition, Weber et al. (2004) found abundant amounts of brefeldin A only in dead precolonized plant tissue, indicating that it may be used more for cellular habitat conquest. Chlorosis was observed in both genotypes. Its development was delayed in the moderately resistant genotypes. In general, chlorosis preceded mycelial growth and extended farther over leaves than did dark lesions. We cannot rule out that chlorosis is due to a host response to infection and not to a toxic compound. Most damage to leaf function comes from chlorosis and accompanying defoliation, so future studies should focus on identifying the cause(s) of chlorosis.

Our results suggest that resistance to *P. medicaginis* in alfalfa may involve inhibiting or delaying spore germination. Because alfalfa varieties are a composite of genetically heterogeneous plants, resistance mechanisms are likely diverse and expressed to different extents in individual plants. In addition, *P. medicaginis* is highly variable (molecularly, phenotypically, and in virulence) (Castell-Miller 2005; Ellwood et al. 2006). Thus, the effectiveness of inhibition of spore germination as a component of resistance needs to be investigated with multiple isolates and evaluated under field conditions.

**Acknowledgement**

We thank Gilbert Ahlstrand for technical support.

**References**


