Histological studies on the development of Phoma root rot of alfalfa[1,2]

Rocío del P. Rodríguez[3]

J. Agric. Univ. P.R. 89(3-4):251-262 (2005)

ABSTRACT

Several stages of the disease cycle of root rot of alfalfa caused by *Phoma medicaginis* var. *medicaginis* were studied by using scanning electron and light microscopy. First activity of the pathogen was the external colonization of the root. The pathogen penetrated directly causing discoloration and tissue disintegration. Inter- and intracellular penetration facilitated by enzymatic degradation was likely the mechanism involved in breaching the barrier of the epidermal cells. Colonization of the cortex was intercellular. Radial access to the xylem elements was achieved through the cortex. Host responses to invasion by the pathogen were suberization of cortical cell walls and occlusion of vessels with pectic substances and wound gum. Cavities in the cortex resulting from tissue degradation were associated with later stages of infection. Intracellular hyphae were observed in dead cells of the cortex and in the xylem.

Key words: histopathology, *Phoma medicaginis* var. *medicaganis*, alfalfa, *Medicago sativa*, host responses

RESUMEN

Estudios histológicos del desarrollo de la pudrición de la raíz por Phoma en alfalfa

Utilizando microscopía electrónica de rastreo y de luz se estudiaron varios estados en el ciclo de la enfermedad de la pudrición de la raíz en alfalfa causada por *Phoma medicaginis* var. *medicaginis*. La primera actividad del patógeno fue la colonización externa de la raíz. El patógeno penetró directamente causando decoloración y desintegración de los tejidos. La penetración inter- e intracelular facilitada por degradación enzimática es el mecanismo encargado de romper la barrera de las células de la epidermis. La colonización de la corteza fue intercelular. El acceso a los elementos del xilema fue radial a través de la corteza. La respuesta del hospedero a la invasión por el patógeno fue suberización de las paredes de las células corticales y oclusión de los vasos con substancias pécticas y goma de herida. Las cavidades en la corteza como resultado de la degradación de los tejidos se asociaron con estados avanzados de la infección. Se encontraron hifas intracelulares en las células muertas de la corteza y en el xilema.

[1]Manuscript submitted to Editorial Board 21 January 2005.

[2]This paper covers work carried out in the USDA Regional Pasture Research Laboratory, University Park, Pennsylvania as a complement of the author's thesis dissertation. Special acknowledgements to Dr. Kenneth T. Leath for his assistance and support with the development of the research.

[3]Phytopathologist (Ad-Honorem), Department of Crop Protection, P.O. Box 9030, University of Puerto Rico, Mayagüez, PR 00681-9030.

Palabras clave: histopatología, *Phoma medicaginis* var. *medicaginis*, alfalfa, *Medicago sativa*, respuestas del hospedero

INTRODUCTION

Root rot of alfalfa occurs worldwide causing considerable damage in this crop. Studies on these host-pathogen relationships have provided information leading to the understanding of some mechanisms in the pathogenesis of root rot fungi and to the identification of possible defense response elicited in the host by the presence of these pathogens. Since a great number of microorganisms are responsible for root rot in alfalfa, characteristic features for each interaction are expected. Penetration can be accomplished by mass hyphal action (Cormack, 1934), penetration pegs (Marks and Mitchel, 1971) and hyphal growth between epidermal cells and appressoria (Parry and Pegg, 1985). Major penetrating sites are the meristematic areas of the roots (Chi et al., 1964), but penetration through uninjured suberized layers of cells in mature roots also occurs (Cormack, 1934).

Typical of root rot diseases is the profuse colonization of the root cortex that eventually leads to the disruption and death of this tissue. The presence of toxins involved in the pathogenesis process has been reported. Cormack (1934) found that *Sclerotinia* spp. and *Plenodomus meliloti* killed the tissues of alfalfa and red clover in advance of hyphal invasion, thus concluding that metabolites toxic to the host are produced in these interactions.

Response of the host to root rot fungi characteristically involved the formation of wound periderm in roots challenged with the pathogens. However, the effectiveness of this mechanism in arresting the infection process varies. Cormack (1934, 1937) found that periderm was ineffective in limiting the progress of infection induced by *Sclerotinia* spp., *Plenodomus meliloti* and *Cylindrocarpon ehrenbergi*. Conversely, Marks and Mitchell (1971) observed that wound periderm restricted colonization of the cortex of taproots of alfalfa infected with *Phytophthora megasperma*, but if the vascular system was already invaded, the wound cork was not formed.

A previous study showed that *Phoma medicaginis* var. *medicaginis* (*P.m.* var. *m.*) can be a primary root rot pathogen in alfalfa (Rodríguez et al., 1990). Reports herewith cited on the histopathology of other alfalfa-root rot interactions support the concept that each host-pathogen relationship is unique although general features characteristic of this type disease are common for all interactions.

This study was conducted to examine the effect of *P.m.* var.*m.* on the root tissues of alfalfa and to determine how this interaction may differ from those reported for other root rot fungi.

MATERIALS AND METHODS

Clone plants from alfalfa cv. Iroquois were used in all experiments, and were produced in the growth chamber by using the test-tube technique previously described (Rodríguez et al., 1990). Plants were grown in the test tubes for one month before treatments.

Scanning Electron Microscope (SEM) Studies

Plants were carefully removed from the test tubes and their roots washed in sterile distilled water. One set of plants was placed in the slant-board system (Kendall and Leath, 1974), grown for an additional two weeks, and then inoculated. The other set was transferred to sterile petri plates, 150-mm diameter × 15-mm depth, with moist filter paper. These plants were inoculated immediately after transplanting to the petri plates. Inoculum was prepared by scraping the surface of one-month-old cultures growing on oatmeal agar at 21°C, and rinsing with sterile distilled water. The spores thus suspended were collected in flasks after being filtered through sterile cheesecloth. Inoculum concentration was determined with a hemocytometer and adjusted to 2×10^6 spores/ml.

Plants growing on the slant-boards were inoculated by immersing their roots in the spore suspension of the isolate. Controls were treated with sterile distilled water. Inoculation lasted one hour, after which time plants were returned to the slant-boards and placed in a mist chamber at approximately 21°C. Roots of plants were kept exposed throughout the length of the trial.

Plants in petri plates were inoculated by spraying their roots with the spore suspension of *P.m.* var. *m*. After treatment, plants were returned to a growth chamber set at 21°C day and 15°C night temperatures, and at a 15-h photoperiod with light intensity of 125 µE/s/m^2. Half of the lid of the petri plate was covered with black plastic tape to protect the roots from the light.

All trials lasted three days, and samples were taken on a daily basis. Portions of the roots of three plants were collected randomly at each sampling. Half of the root pieces were fixed in 1% gluteraldehyde in phosphate buffer (pH 7; 0.02 M). The other half was plated in potato dextrose agar (PDA) without disinfestations. Root pieces were prepared for SEM studies by using a modified Parry and Pegg (1985) procedure, in which ethyl alcohol was used instead of acetone in the dehydration process and gold instead of platinum when coating the specimens. Samples were critical point dried in a Polaron E3000 dryer and sputter coated (420 Å) in an ISI PS-2 coater. The root surface was observed in an ISI-60 SEM.

Light Microscope Studies

Six-week-old clonal plants developed on the slant-board were inoculated with *P.m.*var.*m.* by using two inoculation methods. The first method consisted of immersing the roots in small tubes of 2-mm diameter × 55-mm depth containing a suspension of 2×10^6 spores/ml. Inoculum was prepared as previously described for the SEM studies. Controls were immersed in sterile water. Roots were kept in treatments for 10 h. The second method consisted of inoculations using the colonized piece of stem technique (Rodríguez et al., 1990). For inoculation, non-wounded roots of single plants were inoculated. The colonized piece of stem was placed approximately 8 cm above the root tip and kept at the inoculated site throughout the length of the trial. Control roots were treated with a clean piece of stem. Samples were taken from the treated site at 24-h intervals for seven days. Six root specimens represented each sampling period. Half of the samples were fixed in formalin-aceto-alcohol (FAA) (Johansen, 1940), and the other half was plated on PDA without disinfestations. The latter were incubated at 21°C and examined after 15 days.

The fixed specimens were prepared by the paraffin method (Johansen, 1940) using tertiary butyl alcohol for dehydration and Paraplast 56°C. The embedded tissues were softened overnight at 4°C in a solution of 1% sodium lauryl sulfate. Sectioning was performed at 10μ with a rotary microtome and sections were mounted on chemically clean slides and affixed with Haupt's adhesive. Two stain procedures were used, Johansen's Quadruple stain and Safranin-Fast green counter stain (Johansen, 1940).

Selected sections were examined for histochemical reactions (Rawlins and Takahashi, 1952). Pectin and gums were tested by using the Iron Absorption method and the phloroglucionol test, respectively. Sections were also assayed for suberin by the Sudan IV test. The zinc-chlor-iodine reaction and polarized light were used to ascertain the presence of cellulose.

RESULTS AND DISCUSSION

For the study of early stages in the pathogenesis, root samples were collected up to 72 h. Colonization and the host response were observed in specimens collected from roots inoculated with stem pieces. Results from inoculations in all systems closely agreed, so overall features of the alfalfa-*P.m.*var.*m.* interaction are reported.

Pre-penetration and Penetration Stages

The epidermal cells of healthy roots of alfalfa are long and smooth textured. Adjacent walls of cells do not meet at the surface level but at a lower plane forming grooves along the longitudinal walls of the cells (Figure 1A). One day after inoculation, spores began to germinate, pro-

ducing one or two germ tubes. Changes on the surface of epidermal cells began with the germinating spores. Areas of the cells closely associated with the germ tubes showed signs of erosion (Figure 1B). After germination, the fungus externally colonized the roots of alfalfa, forming a hyphal network with no apparent preference for particular areas of the root surface. Hyphae of the fungus grew transversely as well as longitudinally, and mycelium of the fungus appeared equally abundant at the root tip as on more mature portions of the roots (Figure 1C, D). Seemingly, the pre-penetration phase of root rotters involves an extensive colonization of the root surface, probably sustained by the root exudates (Hancock and Huisman, 1981). Parry and Pegg (1985) reported that *Fusarium avenaceum* and *Fusarium culmorum* grew extensively on the seedling's root surface, but *F. oxysporum* did not, and that the scarce hyphae observed were oriented between the walls of the epidermal cells. Contrary to previous reports (Gerik and Huisman, 1985), root tips were not differentially colonized by *P.m.* var. *m.* Apparently the conditions prevailing in these experiments, the roots growing in nutrient solution, were conducive to equal colonization of the root surface.

During the external colonization phase, the growing thallus may form aerial mycelium (Figure 1E) or it may grow close to the epidermal cells. It is in the latter situation that the penetration mechanisms appear to be disclosed. Commonly, hyphae of *P.m.* var. *m.* grew toward the grooves between epidermal cells (Figure 1F). This observation is in agreement with that of Baayen and Rukenberg (1999), who reported germ tubes of *F. oxysporum* f. sp. *lilii* moving toward the anticlinal grooves of the epidermal cells. In addition, Bishop and Cooper (1983) found that for all wilt pathogens studied intercellular penetration was achieved by hyphal tips growing inward between epidermal cells. It has been reported that the grooves formed between the longitudinal walls of adjacent epidermal cells are possible reservoirs of nutrients that support growth of microorganisms on the root surface (Bowen, 1979). Therefore, the observed growth of the hyphae of *P.m.* var. *m.* toward these areas on the surface represents a chemical tropism that eventually may lead to intercellular penetration of hyphae between epidermal cells. Along with this tropism, the surface of epidermal cells in contact with the hyphae appeared eroded and rough (Figure 1G). Reports from basic studies of the root surface have shown that the root is covered with mucilage, particularly the root cap and elongation zone (Greaves and Darbyshire, 1972). However, the appearance of the root surface in the controls did not show the same texture (Figure 1A). Therefore, these alterations in the texture of the cell surfaces indicate that other changes are occurring at the hyphae-root surface interface.

Light microscopy studies of these early stages in the infection process revealed that walls of cells closely associated with the fungus reacted

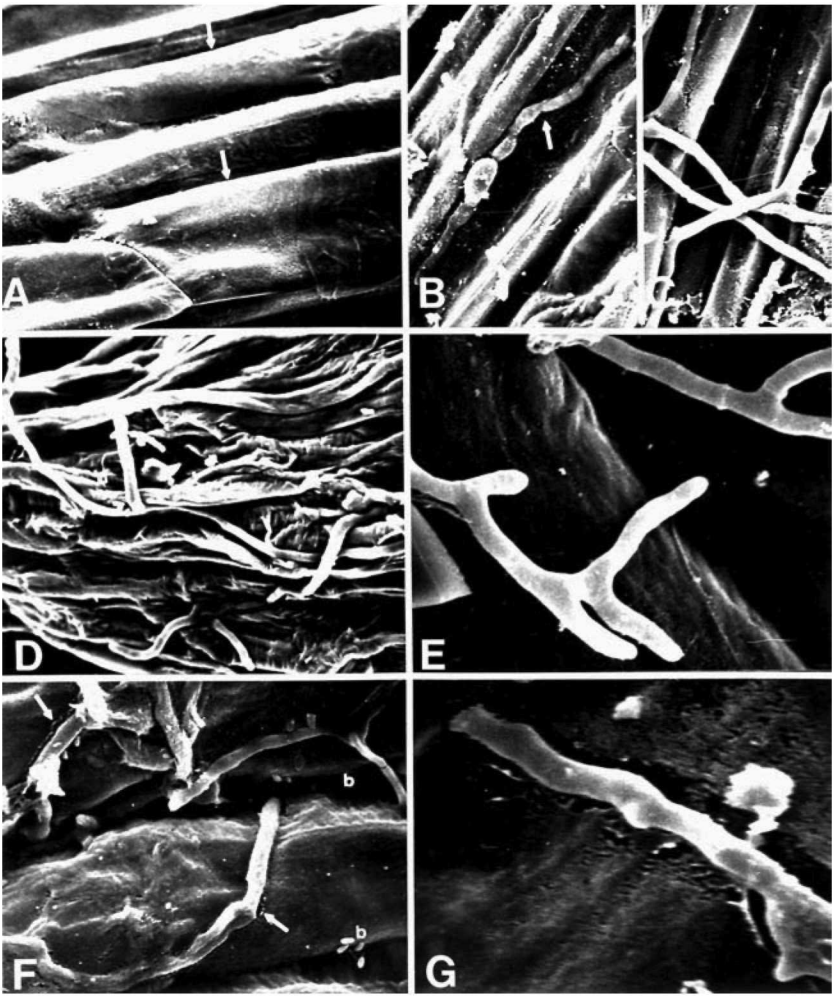

FIGURE 1. Scanning electron photomicrographs of early stages of the alfalfa root-*Phoma medicaginis* var. *medicaginis* interaction. A. Control showing grooves in the longitudinal walls of the epidermal cells (arrows), X1680. B. Germlings of the pathogen with germ tubes close to the surface (arrows), X1500. C. External colonization of mature portions of the roots, X1500. D. Surface colonization of the root tip, X 1090. E. Aerial hyphae of the pathogen at the root surface, X6400. F. Hyphae of the pathogen growing inward through the grooves between epidermal cells, X2750. G. Erosion on the surface of the root when in contact with the pathogen, X7900.

differently to the Johansen's Quadruple stain. These cells showed a dark red-purple coloration, different from the light yellow-green observed in cells not associated with hyphae or in those from the controls (Figure 2A,

B). Tests for the presence of cellulose were negative in these areas associated with propagules of the pathogen (Figure 2C, D). These results strongly support the conclusion that alterations in the cellulose component of the cell wall occurred as a result of this host-pathogen interaction. Since neither appressoria nor evidence for mechanical penetration was observed, it becomes evident that a chemical mechanism is involved in the penetration of *P.m.* var. *m.* to the roots of alfalfa.

Colonization

After penetration, the fungus moves intracellularly between the epidermal cells. Dark-staining portions were consistently observed in the walls associated with what appeared to be the penetration site between adjacent cells (Figure 3A). Bishop and Cooper (1983) reported that host cell walls in contact with the penetrating hyphae were more electron opaque than adjacent wall areas. In addition they observed a matrix between the host walls and the fungal hyphae, and beneath these regions of contact, alterations in the host wall ultrastructure occurred. This finding led them to conclude that this matrix may be related to fungal degradative enzymes. The observed reaction in the alfalfa cell walls associated with the penetration site might be indicative of cell wall degradation by the *P.m.* var. *m.* hyphae. As the infection progressed, the epidermal cells began to deteriorate (Figure 3B). Subsequently, these cells collapsed and the cortical cells immediately below showed signs of deterioration.

Colonization of the root cortex by *P.m.* var. *m.* is intercellular (Figure 3D). The progress in the cortical tissues is primarily parallel to the main axis of the root. The ability with which this fungus colonized the intercellular spaces indicates that these areas provide a suitable environment for fungal growth. Hancock and Huisman (1981) contend that nutrients in the apoplast are at levels that can support the growth of intercellular parasites. Bishop and Cooper (1983) provided evidence that support this statement, they found degradation of the intercellular material around the hyphae colonizing the root tips.

Dark deposits occurred in the intercellular spaces of cortical cells (Figure 3C). In addition, sectors in the walls of cortical cells, as well as those in cells intimately associated with intercellular hyphae, were highly chromophilic with safranin (Figure 3E). Similarly, red "droplets" emerging from the cortical cells and directed toward the hyphae were observed (Figure 4A). Reactions resulting from histochemical tests for suberin were positive for all depositions (Figure 4B). The presence of this substance close to the intercellular hyphae suggests that some fungal activity might be responsible for this reaction. Possibly this response may represent attempts by the host to contain the progress of the infection. Beckman and Talboys (1981) reported that barriers intended to delimit infections some-

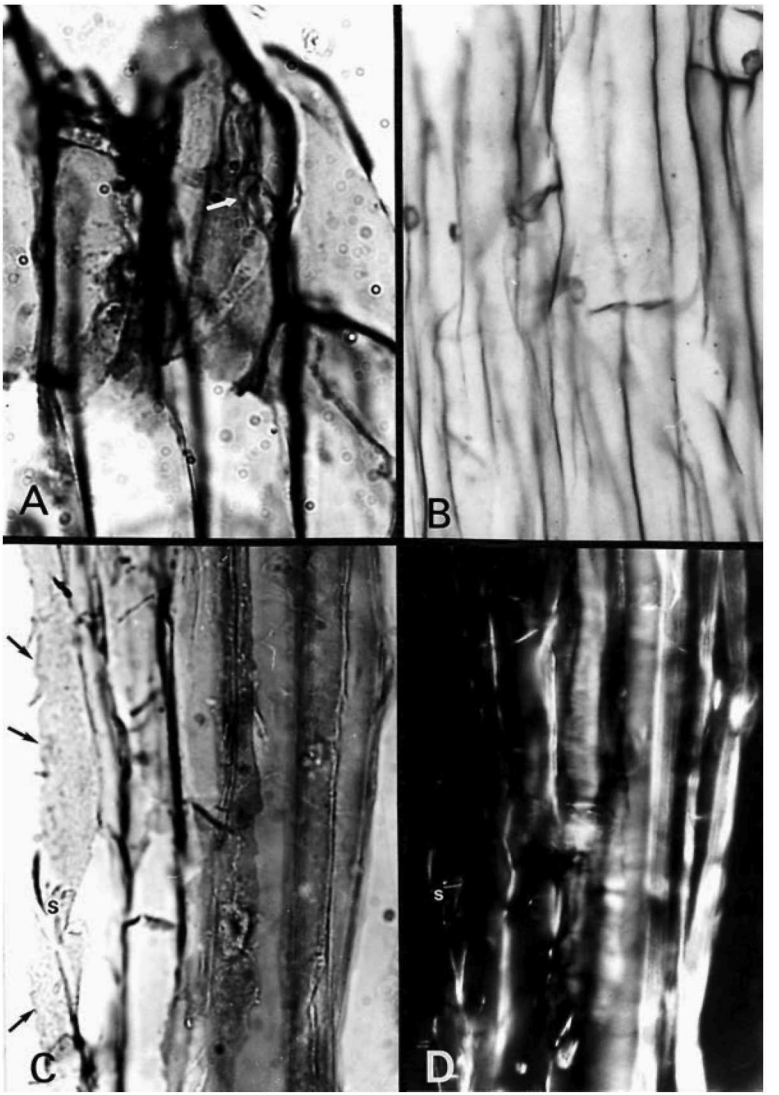

FIGURE 2. Light photomicrographs of the response of alfalfa root to inoculation with *Phoma medicaginis* var. *medicaginis,* three days after inoculation. A. Dark appearance of the epidermal cells associated with the germlings (arrow) of the pathogen, X1700. B. Comparable section from the control showing the clear appearance of the cells after stained with the Johansen Quadruple stain, X495. C. Discriminating response of epidermal cells to test for cellulose. Dark stained portions (right), indicate a positive zinc-chloriodine reaction. Notice the negative reaction of the inoculated area (arrows), (s) germlings, X495. D. Polarized light test of the previous section. Notice the absence or low birefringence in the corresponding inoculated areas, (s) germlings, X495.

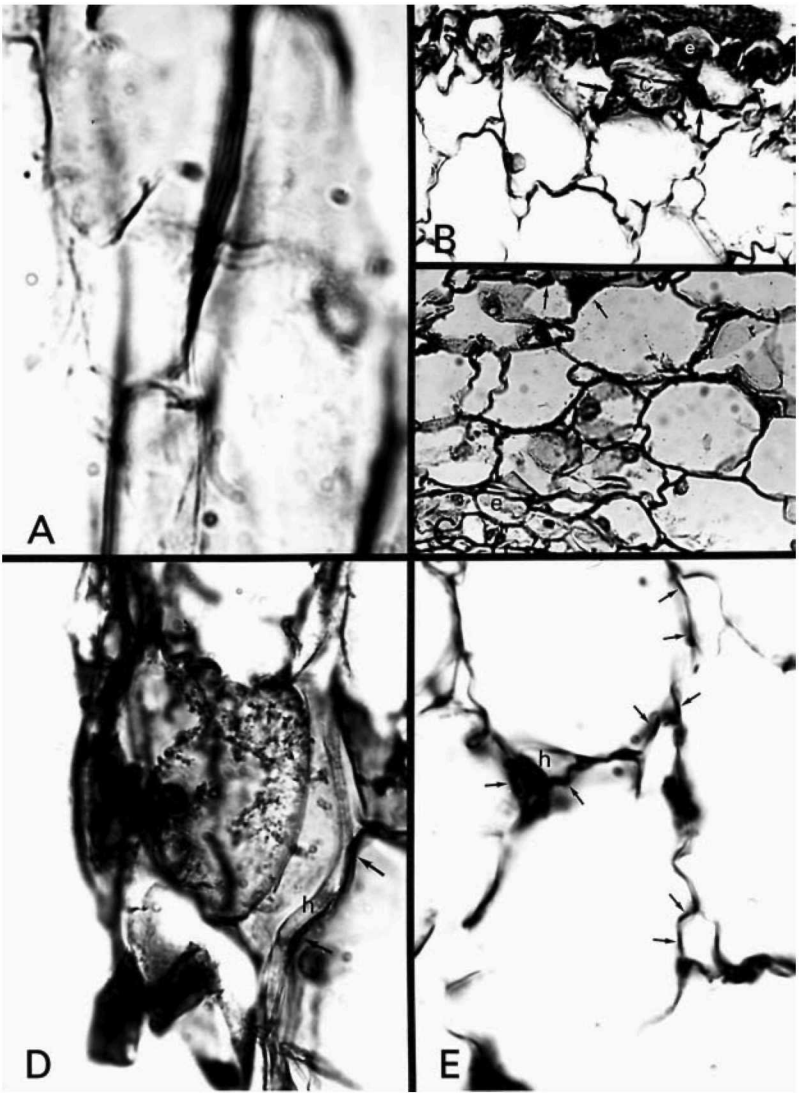

FIGURE 3. Colonization by *Phoma medicaginis* var. *medicaginis* in roots of alfalfa. A. Dark staining areas in the wall of adjacent cells associated with penetrating hyphae, X2228. B. Advance of the infection front including deterioration of cells in the epidermis (e), initial signs of infection in cells of the cortex, and occlusion of intercellular spaces (arrows), X500. C. Transverse section of the root showing endodermis (e) and occlusion of intercellular spaces (arrows) in the cortex, X515. D. Longitudinal section showing intercellular hyphae (h). Notice the dark staining areas of the cell wall in association with the hyphae (arrows), X1114. E. Cross section of the root showing intercellular hyphae (h) and dark staining sectors in the walls of cells in the cortex, X1485.

times involve only depositions of new cell wall materials that may be suberized. Murillo et al. (1999) reported similar defense-related ultrastructural changes induced by *F. moniliforme* in maize seedlings. This was the only detected response of the cortical cells to the presence of *P.m.* var. *m.* As the disease progressed, cells of the cortex started to collapse, reducing the radial width of the root cylinder at the inoculation site. Intracellular hyphae were detected only in dead or dying cells (Figure 4C).

Seven days after inoculation, hyphae of the fungus were detected inside the xylem elements (Figure 4E). This observation agrees with the general view that root rotters are primarily cortical pathogens, and only at later stages of infection do they gain access to the stele (Pennypacker, 1981). In the cortical rot Fusaria, this invasion into the vascular tissues occurred primarily through the meristematic region (Chi et al., 1964) or as a consequence of the weakening and death of the root tissue (Parry and Pegg, 1985). Because of the inoculation procedures, *P.m.* var. *m.* gained access to the xylem elements through the radial colonization of the cortex and not through the meristem. However, a response of the conductive tissues similar to that reported for wilt pathogens was observed (Beckman, 1987). Plugging of the pits chamber and occlusion of the xylem elements occurred (Figure 4F, G). This substance in the lumen of the xylem elements was positive for pectin and wound gums, but regions in the elements did not label for both substances, thus suggesting the presence of phenolic occlusions (Pegg, 1985).

Vascular plugging occurs as a plant response to biotic and abiotic stresses (Beckman, 1987). The clear appearance of the lumen in xylem elements of the control specimens (Figure 4H) indicates that vascular plugging was elicited by the activities of *P.m.* var. *m* and not by stresses due to experimental conditions.

Eventually, cavities were formed in areas where cortical cells existed (Figure 4D), and occasionally hyphae of the fungus were observed in these pockets. These may represent a possible mechanism for the dissemination of this pathogen to the rhizosphere. Formation of reproductive structures was not observed. The last sample was taken only seven days after inoculation; it is possible that in time the fungus might have sporulated in dead tissues of the host thus completing the cycle.

Isolations from inoculated areas yielded cultures of *P.m.* var. *m.*, and other fungi were not detected. Colonies of bacteria were found associated only with the slant-board specimens, and potato soft rot trials were negative. These results indicate that the pathological anatomy and the pathogenesis processes herein reported were the result of the alfalfa-*P.m.* var. *m.* interaction.

Overall, the pathogenesis of *P.m.* var. *m* in the roots of alfalfa was typical of a root rot pathogen. However, the unique response of the host

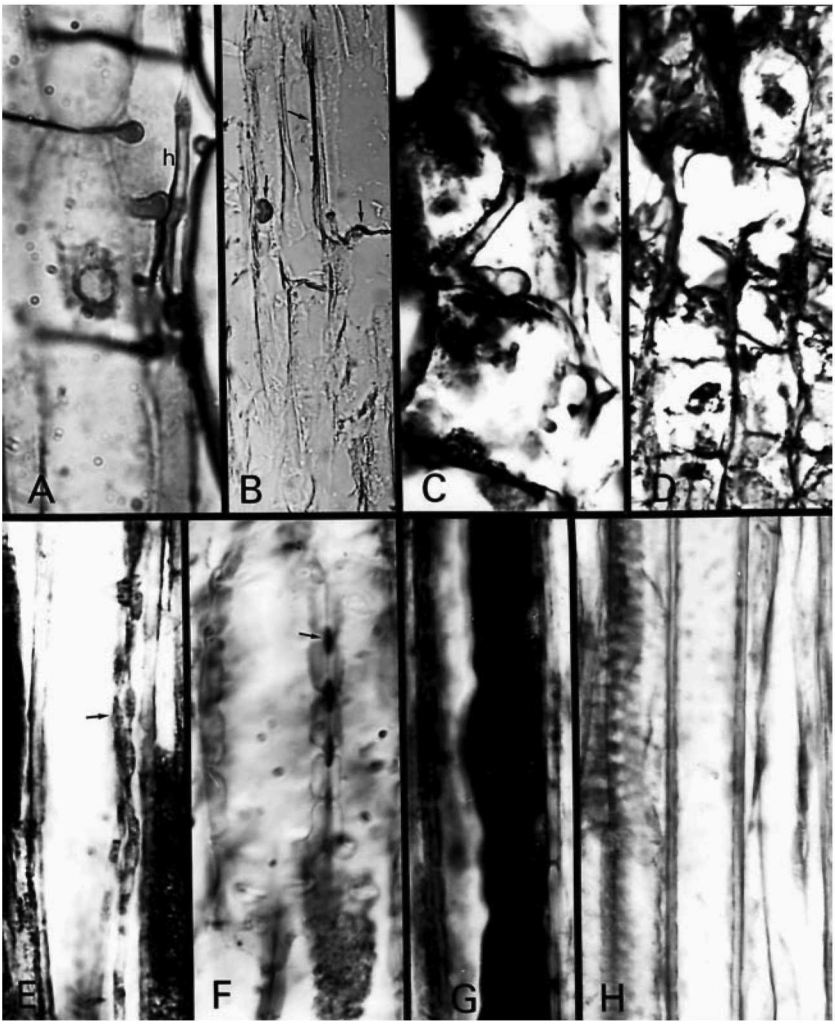

FIGURE 4. Responses of the alfalfa cortical and vascular tissues to invasion by *Phoma medicaginis* var. *medicaginis*. A. "Droplets" in cortical cells associated with intercellular hyphae (h), X1113. B. Longitudinal section of the root treated with Sudan IV showing the positive reaction to the stain of the "droplet" and the cell wall (arrows), X223. C. Intracellular hyphae (arrow) in dead cell of the cortex tissue, X1155. D. Cavities and deterioration of cortical cells seven days after inoculation, X300. E. Hyphae (arrow) inside a tracheary element, X866. F. Plugs of pit chambers (arrow) in xylem element of young portions of the roots three days after inoculation, X1090. G. Occlusion of tracheary element that labeled positive for pectin, X866. H. Longitudinal section of a root in the control showing the clear appearance and the integrity of the xylem tissues, X750.

to invasion by this pathogen indicates that the Phoma root rot disease in alfalfa involves features different from those reported in other alfalfa-root rot pathogen interactions.

LITERATURE CITED

Baayen, R. P. and F. H. J. Rukenberg, 1999. Fine structure of the early interaction of lily roots with *Fusarium oxysporum* f. sp. *lilii*. *Europ. J. Plant Pathol.* 105:431-443.

Beckman, C. H., 1987. The Nature of Wilt Diseases of Plants. American Phytopathological Society Press, St. Paul, MN. 175 pp.

Beckman, C. H. and P. W. Talboys, 1981. Anatomy of resistance. pp. 487-521. *In:* Fungal Wilt Diseases of Plants. M. E. Mace, A. A. Bell and C. H. Beckman, eds. Academic Press, N.Y.

Bishop, C. D. and R. M. Cooper, 1983. An ultrastructural study of root invasion in three vascular wilt diseases. *Physiol. Plant Pathol.* 22:15-27.

Bowen, G. D., 1979. Integrated and experimental approaches to the study of growth of organisms around roots. pp. 209-227. *In:* Soil-Borne Plant Pathogens, B. Schippers and W. Gams, eds. Academic Press, New York.

Chi, C. C., W. R. Childers and E. W. Hanson, 1964. Penetration and subsequent development of three *Fusarium* species in alfalfa and red clover. *Phytopathology* 54:434-437.

Cormack, M. W., 1934. On the invasion of roots of *Medicago* and *Melilotus* by *Sclerotinia* sp. and *Plenodomus meliloti* D. and S. *Can. J. Res.* 11:474-480.

Cormack, M. W., 1937. *Cylindrocarpon ehrenbergi* Wr., and other species as root parasites of alfalfa and sweet clover in Alberta. *Can. J. Res.* Sec.C. 15:403-424.

Gerik, J. J. and O. C. Huisman, 1985. Mode of colonization of roots by *Verticillium* and *Fusarium*. pp. 80-83. *In:* Ecology and Management of Soilborne Plant Pathogens. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong and J. F. Kollmorgen, eds. The Amer. Phytopath. Soc. Press, St. Paul, MN.

Greaves, M. P. and J. F. Darbyshire, 1972. The ultrastructure of the mucilaginous layer of plant roots. *Soil. Biochem.* 4:443-449.

Hancock, J. G. and O. C. Huisman, 1981. Nutrient movement in host-pathogen systems. *Ann. Rev. Phytopathol.* 19:309-331.

Johansen, D. A., 1940. Plant Microtechnique. MacGraw Hill Book Co. New York. 523 pp.

Kendall, W.A. and K.T. Leath, 1974. Slant-board culture methods for root observations of red clover. *Agron. J.* 14:317-320.

Murillo, I., L. Cavallarin and B. San Segundo, 1999. Cytology of infection of maize seedlings by *Fusarium moniliforme* and immunolocalization of pathogenesis-related PRms protein. *Phytopathology* 89:737-747.

Marks, G. C. and J. E. Mitchel, 1971. Penetration and infection of alfalfa roots by *Phytophthora megasperma* and the pathological anatomy of infected roots. *Can. J. Bot.* 49:63-67.

Parry, D. W. and G. F. Pegg, 1985. Surface colonization, penetration and growth of three *Fusarium* species in lucerne. *Trans. Br. mycol. Soc.* 85:495-500.

Pegg, G. F., 1985. Life in a black hole-The microenvironment of the vascular pathogen. *Trans. Br. Mycol. Soc.* 85:1-20.

Pennypacker, B. W., 1981. Anatomical changes involved in the pathogenesis of plants by *Fusarium*. pp. 400-408. *In: Fusarium*: Diseases, Biology and Taxonomy. P. E. Nelson, T. A. Toussoun and R. J. Cook, eds. The Pennsylvania State University Press, University Park, PA.

Rawlins, T. E. and W. N. Takahashi, 1952. Technics of Plant Histochemistry and Virology. The National Press, Milbrae, CA. 125 pp.

Rodríguez, R., K. T. Leath and R. R. Hill, Jr., 1990. Pathogenicity of *Phoma medicaginis* var. *medicaginis* to roots of alfalfa. *Plant Dis.* 74:680-683.