Pathological anatomy of the upper taproot and stems of alfalfa afflicted with crown rot induced by *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema[1,2]

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ABSTRACT

Internal necrosis in the upper taproot and in the stems is a common symptom associated with Phoma crown rot of alfalfa. Necrotic tissues were examined by using histological and histochemical techniques in permanent and semi-permanent preparations. Necrosis occurred in the woody tissue only, affecting the vascular elements, fibers, and ray parenchyma. In the upper taproot, tissues at the center of the stele were completely degraded with hyperplasia occurring in some parenchyma cells. Occlusion with wound gums and pectin occurred in elements of the secondary xylem. Wound periderm surrounded the infected areas, limiting radial but not longitudinal colonization by the pathogen. In the stems, different types of occlusions were observed, likely of composition similar to that found in the upper taproot. Hyphae were observed within tracheary elements.

Key words: histopathology, occlusions, vascular elements, alfalfa, *Medicago sativa*, host response

RESUMEN

Anatomía patológica de la base de la raíz pivotante y los tallos de alfalfa afectada con pudrición de la corona inducida por *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema

La necrosis interna de la base de la raíz pivotante y de los tallos es un síntoma común asociado a la pudrición de la corona de alfalfa por Phoma. Los tejidos necrosados se examinaron utilizando técnicas histológicas e histoquímicas en preparaciones permanentes y semipermanentes. La necrosis ocurrió solamente en los tejidos leñosos afectando los elementos vasculares, fibras y el parénquima de los rayos. En la base de la raíz pivotante los tejidos del centro de la estela estaban completamente degradados y algunas células de parénquima mostraron hiperplasia. Se observó oclusión con goma y pectina en los elementos del xilema secundario. Las áreas infectadas estuvieron rodeadas de peridermo de herida limitando la colonización radial del pató-

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geno pero no la longitudinal. Se observaron diferentes tipos de oclusión en los tallos posiblemente de la misma composición a la encontrada en la base de la raíz pivotante. Se observaron hifas dentro de los elementos traqueales.

Palabras clave: histopatología, oclusiones, elementos vasculares, alfalfa, *Medicago sativa,* respuesta del hospedero

INTRODUCTION

Crown rot of forage legumes usually involves a discoloration of the crown and upper taproot. Previous reports indicated that although discoloration in most instances is similar, the tissues involved may not be. Gaudet et al. (1980) reported that the discoloration of the stele in the taproot of sainfoin, following crown rot induced by *Fusarium solani*, was associated with accumulation of gels and gums in the vessels and fibers, but cellular degradation was not primarily involved. However, when bacteria were involved, both vessel occlusion and cellular degradation were part of the syndrome. Conversely, Jones and Weimer (1938) observed that taproot necrosis in alfalfa suffering from crown rot induced by *Stagonospora meliloti* was due to infection of the ray parenchyma, and that the fungus did not invade the vessels. In Phoma crown rot of alfalfa, a black necrosis that extended toward the stems and to the upper taproot was commonplace (Rodríguez and Leath, 1992). The present study was conducted to characterize the internal necrosis and identify the tissues involved.

MATERIALS AND METHODS

Seeds of alfalfa (*Medicago sativa* L.) cv. Iroquois were disinfested, plated on water agar and incubated at room temperature for 36 h (Rodríguez and Leath, 1992). Germinated seeds free from microbial growth were selected and planted in plastic pots containing autoclaved vermiculite-sand potting mixture (1:1 vol) moistened with half strength Hoagland's solution. The pots were placed in a growth chamber for two weeks, after which time seedlings were transplanted to slant-boards (Kendall and Leath, 1974). Conditions for growth consisted of 21 ± 1° and 15 ± 1°C day and night temperatures, respectively, and a daily 15-h photoperiod with light intensity of 200 μE/s/m^2. Plants were grown for two months, then inoculated with crown isolate BTC-2 of *Phoma medicaginis* var. *medicaginis* (*P. m.* var. *m*). The fungus was grown on oatmeal agar until sporulating actively. Inoculum was collected by dipping a flamed needle tip into the spore masses. Inoculation was made by stabbing the crown with the spore-infested needle. Controls were treated similarly but without the spores.

Plants were randomly selected for histological sampling one month after inoculation. The stems and the upper taproot were sampled ap-

proximately 2 and 3 cm from the crown, respectively. Half of the samples from the upper taproot were fixed in formaldehyde-aceto-alcohol (Johansen, 1940), and the other half was plated on potato dextrose agar after being disinfested in 1.25% sodium hypochlorite for one min and then rinsed in sterile distilled water. The fixed specimens were dehydrated with tertiary butyl alcohol (Johansen, 1940) and embedded in Paraplast 56°C. The embedded tissues were softened overnight at 4°C in a solution of 1% sodium lauryl sulfate, and then sectioned at 10µ with a rotary microtome. Sections were mounted on chemically cleaned slides, affixed with Haupt's adhesive, and stained with Johansen's Quadruple stain (Johansen, 1940). Selected sections were histochemically assayed for pectin, wound gums, and suberin by using the iron absorption method, the phloroglucinol test, and Sudan IV, respectively (Rawlins and Takahashi, 1952). Microscopic examination of stem samples was made in transverse and longitudinal free-hand sections mounted in sterile distilled water.

RESULTS AND DISCUSSION

The upper taproot had undergone secondary growth, and the distribution of tissues was similar to that previously described (Simonds, 1935). The vascular bundles were separated by multiseriate rays composed of approximately six columns of parenchyma cells (Figure 1A). These rays were well defined in the wood tissue but not in the phloem, where they were mixed with the parenchyma cells. At the center of the stele, pith-like cells occurred intermingled with the primary xylem; thus the parenchymatous tissue in the upper taproot formed a continuum, broken in some areas only by the cambium. Usually the cambium was more evident in the vascular bundle, and seldom was this tissue discernible in the rays. The periderm was already formed and was comprised of five layers of suberized cells (Figure 1A).

Symptoms of infection were evident in the tissues at the center of the stele and in the woody tissue at one side of the upper taproot cylinder. The other side showed anatomy typical of the controls (Figure 1A). The secondary xylem along with the axial parenchyma and the fibers were the tissues most affected (Figure 1C, D). Initial signs of disorganization of cells in the ray parenchyma were observed particularly when they were located between two infected bundles (Figure 1B). The phloem and the periderm did not show symptoms.

Occlusion of xylem elements and degradation of cells in the axial parenchyma were common in the infected areas (Figure 1B-D). Histochemical tests were positive for wound gum and for pectin in the lumen of the elements. Some elements were negative for both tests,

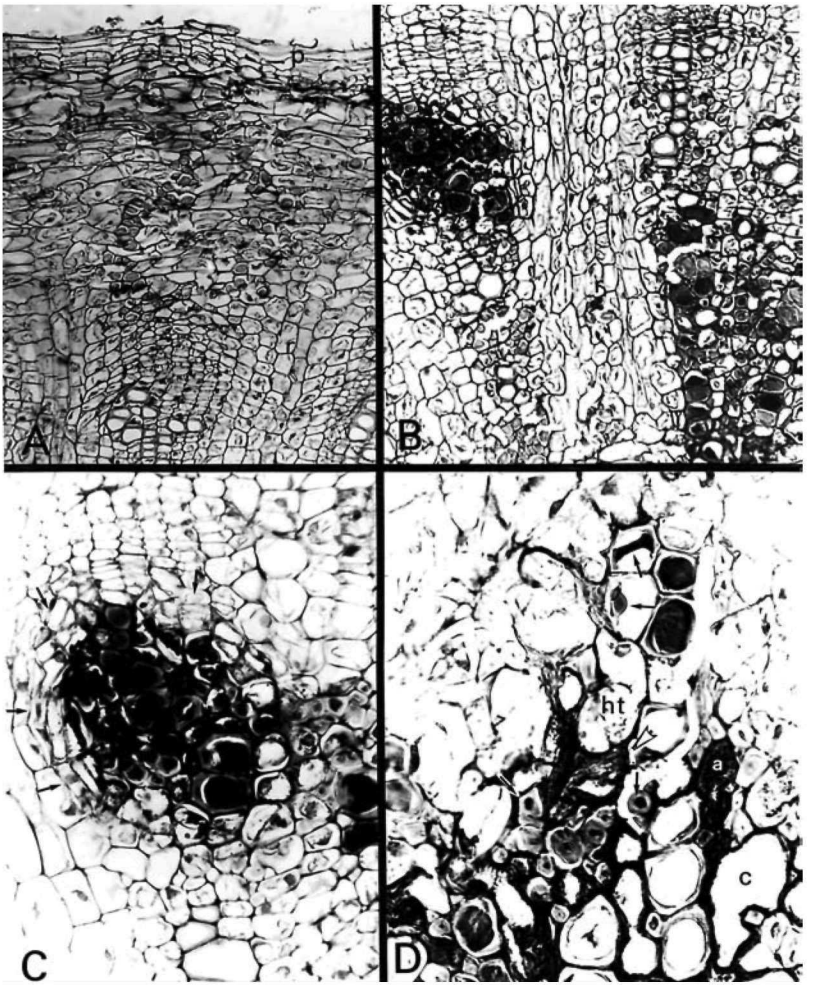

FIGURE 1. Photomicrographs of permanent preparations from the upper taproot phase of crown rot of alfalfa one month after inoculation with *Phoma medicaginis* var. *medicaginis*. A. Cross-section from non-infected areas illustrating the periderm (p), and the continuity of the ray parenchyma (r), X170. B. Cross section of affected side of the organ showing occlusion of tracheary elements and distortion of cells in ray parenchyma (r) located between two affected bundles, X124. C. Xylem tissue at higher magnification showing the severity of infection in this tissue and the formation of wound periderm (arrows) surrounding the infected area. Notice the containment of the infection on the side of the periderm and the extension of necrosis in areas not included within this tissue, X330. D. Cross-section of taproot showing the extreme disorganization of the tissue in areas closer to the center of the stele and the radial extension of the infection in areas not associated with the wound periderm. Note the cavities (c), death of axial parenchyma (a) hypertrophy (ht) of cells crushing adjacent cells (arrowhead), occlusion at the perforation plate of the vessel and fibers (arrows), and another non-identified structure in the lumen of the element (arrow), X309.

thus suggesting that occlusions might be phenolic (Pegg, 1985). Wound gum was the predominant type of occlusion, occurring in fibers and secondary xylem closer to the center of the stele and also in that near the cambium. Xylem elements positive for pectin were located between these two portions of the cylinder.

The center of the infected stele was extremely disorganized with an abundance of cavities (Figure 1D). Parenchyma cells appeared larger than normal, and hyperplasia also occurred. Those cells undergoing the abnormal growth crushed the adjacent cells, and a dark staining substance formed in and around the cavities and within the xylem elements. Histochemical tests were positive for pectin. Parenchyma cells surrounding some bundles in the lateral xylem (Figure 1C) and in the xylem tissue close to the center of the stele differentiated to form a well-defined periderm layer with walls testing positive for suberin. The primary xylem was not occluded; however, some of the elements were distorted.

Vascular occlusion is a normal response of plants to biotic and abiotic stresses, and for some interactions it is considered a mechanism of resistance impeding the invasion of the vascular system (Beckman and Talboys, 1981). Beckman and Halmos (1962) found that non-pathogenic organisms in the vascular system could induce responses comparable to those of plants resistant to *Fusarium oxysporum* f. *cubense*; similarly, the invasion of the vascular tissues by these saprophytes was successfully prevented by the host responses. However, Jones (1929) observed occlusion of vessels in the upper taproot of alfalfa affected by winter injury, and he found a higher incidence of bacterial wilt after winter injury. It is then likely that injuries and other microorganisms entering through these wounds might also be involved in eliciting the above mentioned host responses. In angiosperm trees the formation of gum is related to esterified pectic polysaccharides secreted across the pit membranes into the lumen of the vessel; this response could be a reaction to age, injuries or infections (Rioux et al., 1998). The absence of vascular plugging in the control specimens, even though bacteria occasionally were associated with the plated tissues, indicates that the response herewith documented in the inoculated plants was due to the alfalfa—*P. m.* var. *m.* interaction and not to the inoculation method or other microorganisms.

Frequently, cells in a host respond to infection or injury by forming wound cork around the affected tissues (Essau, 1977). In some alfalfa-root pathogen interactions, the wound periderm has been considered a defense mechanism of varying effectiveness (Cormack, 1934, 1937; Marks and Mitchell, 1971). In the alfalfa–*P. m.* var. *m.* interaction the periderm may have prevented radial proliferation (Figure 1C, D), but on the basis of the continuity of the necrosis beyond the sampled area (Rodríguez and Leath, 1992) the periderm had limited effectiveness

against longitudinal colonization. Similar to the vessel occlusion defense mechanism (Beckman and Talboys, 1981), the effectiveness of wound periderm in arresting the invasion of healthy tissues is a function of time. Although *P. m.* var. *m.* was consistently isolated from all upper taproot pieces showing necrosis, fungal hyphae were not observed microscopically. Likely the darkly stained necrotic tissues obscured the colonizing hyphae.

Similar to that in the upper taproot, necrosis in the stems was associated only with the woody tissues. The vascular elements, fibers, and ray parenchyma were affected. Light yellow, tyloses-like occlusions were associated with some of the vessels (Figure 2A), and other vessels were occluded with a black substance that fully or partially blocked the lumen of the vessel. This substance was usually deposited along the lateral walls, involving the pits (Figure 2B), but occasionally it accumulated within the vessel element and the fibers of the xylem tissue. Histochemical tests were not conducted in these specimens; however, it is likely that the composition of these substances accumulating in the lumen of the tracheary elements is similar to that found in samples from the upper taproot. Zhao et al. (1999) found tyloses only in diseased tissues and believed it could be a restraining mechanism to prevent pathogen colonization. The primary wall of the tyloses could secrete pectic substances to completely occlude the vessel element (Rioux et al., 1998). Hyphae were detected inside the tracheary elements (Figure 2B). Cells in rays were distorted and necrotic (Figure 2C). Comparable sections from the controls showed the integrity of the ray tissue and the clear appearance of the fibers and the conductive tissue (Figure 2D).

Phoma medicaginis var. *medicaginis* was consistently isolated from all stem pieces showing internal discolorations. Occasional colonies of Gram-positive bacteria (potato soft rot tests were negative) and non-pathogenic fungi were found associated with the plated tissues. Because of their low frequency and presence in the control tissues, it was concluded that these organisms did not influence the results herewith presented.

These results indicate that the internal necrosis of the upper taproots and the stems associated with crown rot induced by *P. m.* var. *m.* was due to occlusion with wound gum and pectin of tracheary elements and fibers, and to cellular degradation. Beckman and Halmos (1962) stated that the difference between compatible and non-compatible interactions in vascular diseases rests on the ability of the pathogen to move systemically by the disruption of the host's defense responses. Evidently *P. m.* var. *m.* was able to breach these protective barriers as evidenced by the length of necrosis in the woody tissue of infected plants (Rodríguez and Leath, 1992). Although the response of the host was similar to that reported for vascular diseases, and hyphae of the

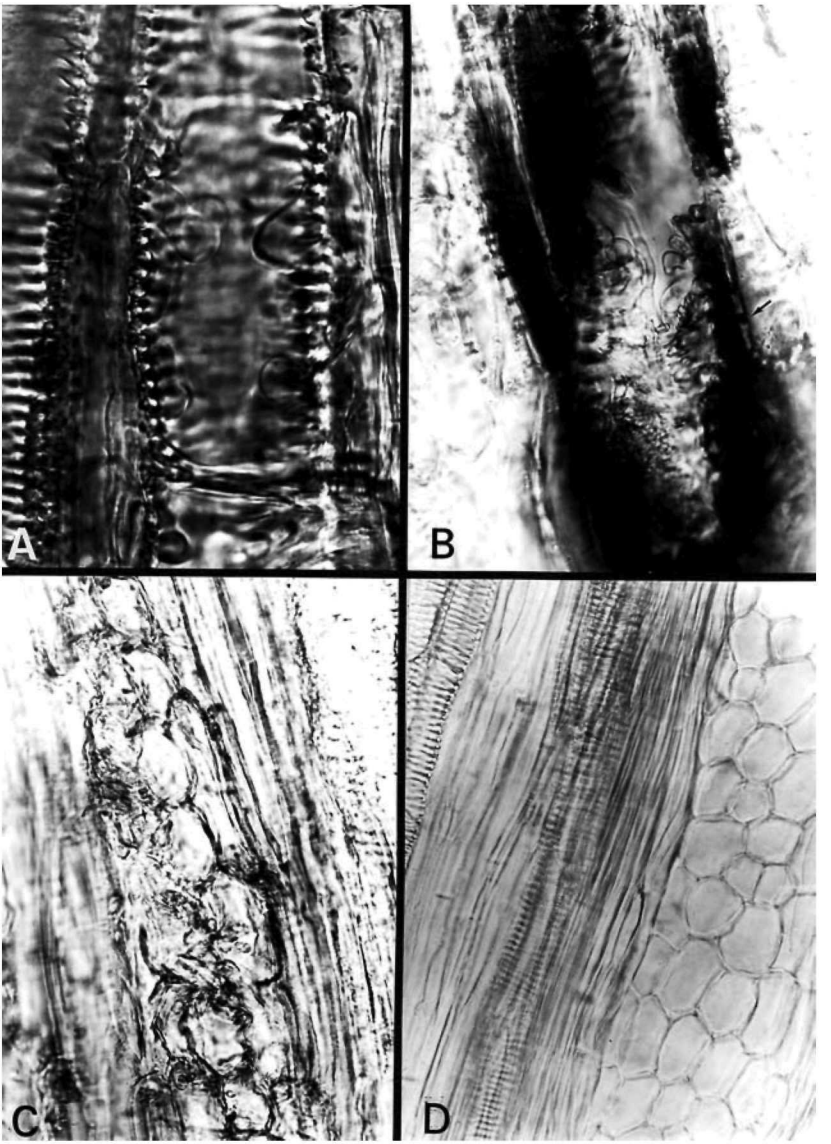

FIGURE 2. Photomicrographs of free-hand sections from fresh alfalfa stems one month after inoculation of the crown with *Phoma medicaginis* var. *medicaginis*. A. Tyloses-like occlusions emerging from the lateral walls of the vessel, X660. B. Dark substance concentrated on the lateral walls of the vessel. Note the plugs in the pit chamber (arrow) and the fungal hypha inside the vessel, X743. C. Distortion of cells in ray parenchyma associated with more advanced stages of necrosis, X389. D. Longitudinal section of stem from control plants illustrating the clear appearance of the xylem tissue, X300.

fungus were detected inside the vessels of the stems, the involvement of parenchymatous tissue in the response suggests that colonization may also occur through this tissue, and possibly that the fungus is not confined to the treachery elements.

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