Histopathology of Cylindrocladium Black Rot of Peanut

S. A. Johnston and M. K. Beute

Former Graduate Research Assistant and Associate Professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27607. Present address of senior author: Plant Biology Department, Cook College, P. O. Box 231, New Brunswick, New Jersey 08903.

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ABSTRACT

Early stages of pathogenesis of peanut (*Arachis hypogaea*) by *Cylindrocladium crotalariae* involved the formation of infection cushions on the epidermis, followed by complete hyphal colonization of the cortex. Three alternative courses in pathogenesis were observed: (i), the fungus overcame host defense mechanisms, breached the periderm, and completely colonized the hypocotyl resulting in death of the plant; (ii), the host effectively limited the pathogen to the cortex and recovered from disease; or (iii), the fungus initially overcame host defense mechanism, but the host formed an additional periderm in advance of the necrosis, which limited the pathogen to colonized tissues and enabled the plant to recover from the disease. An eight-stage scheme for peanut hypocotyl pathogenesis is proposed on the basis of these observations. Exogenous nutrients enhanced pathogenesis in hypocotyls and resulted in earlier death of infected peanut plants.

The fungus intercellularly penetrated the cortex of fibrous roots 24 hours after inoculation. Complete hyphal colonization and death of these roots occurred within 72 hours.

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Additional key words: *Calonectria crotalariae*, periderm formation, microsclerotia.

Cylindrocladium black rot (CBR) of peanut (*Arachis hypogaea* L.) caused by *Cylindrocladium crotalariae* (Loos) Bell & Sobers was first observed in Georgia in 1965 (2). Since its appearance in North Carolina in 1970 (6), the fungus has become established in all peanut-producing counties, and represents a serious threat to peanut production in the state (10, 11).

Symptoms of CBR include chlorosis and wilting of the main axis, followed by complete wilting of the remaining foliage, and death of the plant. Hypocotyls and tap roots of diseased plants become necrotic and the entire root system eventually is destroyed leaving a blackened, fragmented hypocotyl. Reddish-orange perithecia commonly appear on diseased stems just above the groundline.

The purpose of this study was to determine (i) the mode of infection of peanut by *C. crotalariae*, and (ii) the histological effects of the pathogen on hypocotyl and root tissues.

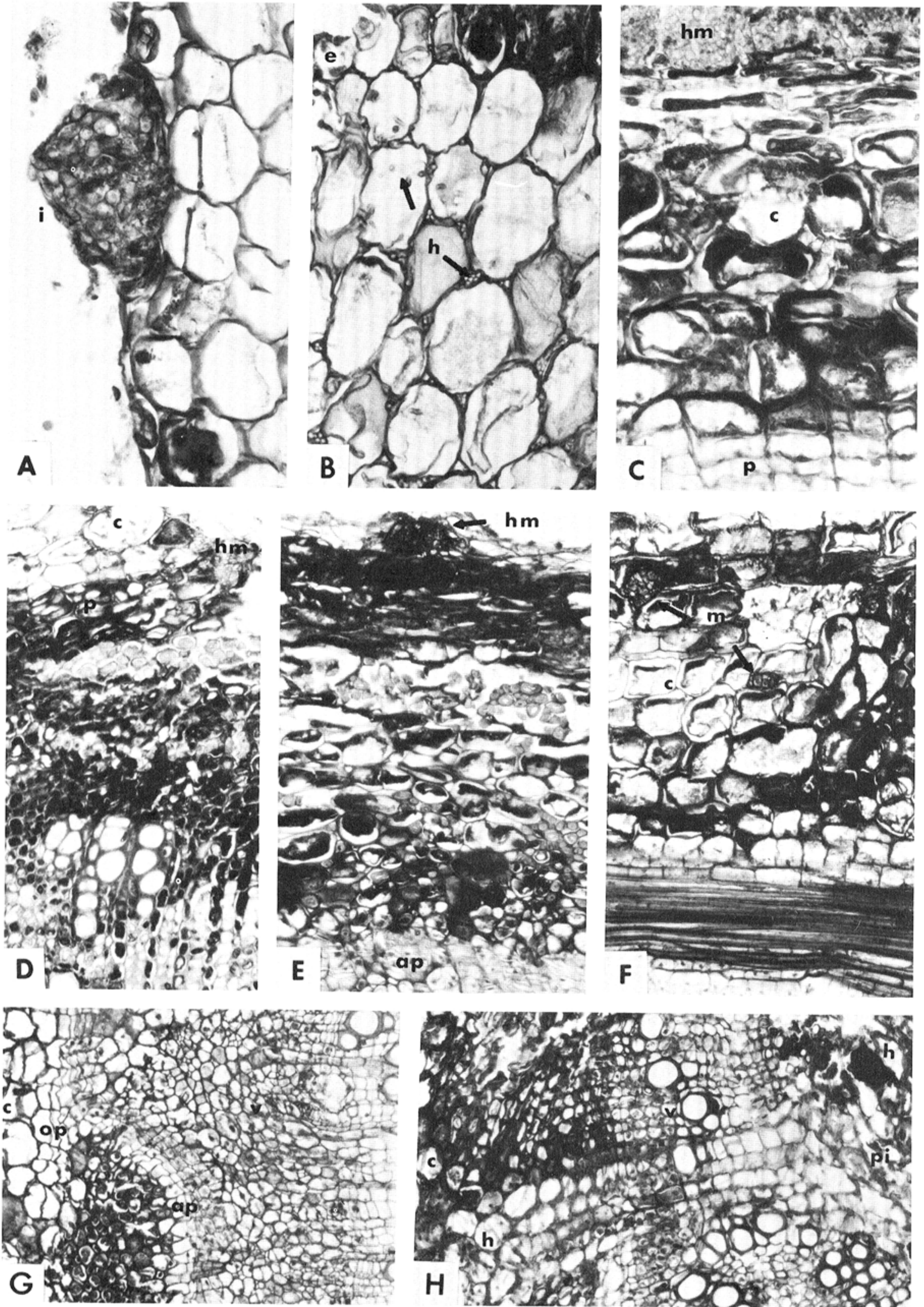
MATERIALS AND METHODS.—Isolates Boone and K-3 of *C. crotalariae* were obtained from peanut hypocotyls collected from two CBR-infected peanut fields in North Carolina. Microsclerotia used for inoculum were produced by growing the fungus in 225-ml flasks containing approximately 40 ml of 2% malt extract broth. After 6 weeks, the cultures were comminuted in a Waring Blendor for 2 minutes, and then passed through a series of nested 15-cm diameter sieves with 246, 149, and 104-μm openings (60-, 100-, 150-mesh, respectively). Microsclerotia collected from the sieves were rinsed with water to remove any remaining mycelial fragments. Concentration was determined by agitating the microsclerotial suspension, adding 1 ml to a dish, and counting the number of microsclerotia.

Inoculation techniques.—Peanut seedlings were germinated in a moist chamber and transplanted into 10-cm diameter clay pots (two per pot) containing a sand and soil mixture (2:1, v/v). One week after transplanting, 10 ml of Rhizobium inoculum mixture containing 2 g of commercial powder ('Nitragin' the Nitragin Company, Inc., Milwaukee, Wisconsin 53209) per 100 ml of water were added to each pot. Greenhouse grown plants were inoculated 10 days after planting by injection of microsclerotia 3 cm below the soil surface adjacent to the hypocotyl.

Root clearing.—Fibrous roots from CBR-infected peanut plants collected in the field and from inoculated plants in the greenhouse were cleared (3) to determine the presence of microsclerotia. Several necrotic, fibrous roots were removed from each plant and boiled in two changes of 1N KOH for 15 minutes. Roots were first rinsed in two changes of distilled water, then treated with 25 ml of an acidified 5% sodium hypochlorite solution (two drops of 5N HCl in 25 ml) until roots became a pale straw color (30-60 seconds). They were immediately rinsed three times with distilled water and mounted in lactophenol for microscopic examination.

Histological techniques.—Hypocotyl and root pieces were fixed in formalin-alcohol-acetic acid, dehydrated in tertiary butyl alcohol (7), embedded in Fisher's Tissue-Prep, sectioned at 12 μm, and stained with Triarch's quadruple stain.

General procedures.—Experiment 1 was performed to determine the mode of hypocotyl penetration. Peanut seedlings (cultivar Florigiant) were transplanted to 1-liter plastic pots containing vermiculite and subirrigated. Ten days later the seedlings were inoculated by placing a silica gel (5) plug containing microsclerotia of the K-3 isolate against the hypocotyl 2.5 cm below the vermiculite surface. Hypocotyls were harvested 1, 2, 4, 8, and 16 days after inoculation. Each treatment was replicated three times.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Experiments 2, 3, and 4 were conducted to determine what tissues were infected, and the location and time of propagule production. Disease progression with the weakly virulent isolate, Boone, was studied in Experiment 2. Infection of peanut hypocotyls by the Boone and the severe K-3 isolate was compared under greenhouse conditions in Experiment 3. Experiment 4, similar in design to Experiment 3, was conducted in a temperature-controlled water bath held at 28 C (1).

In Experiment 2, each plant was inoculated with approximately 100 microsclerotia. Hypocotyls of inoculated and noninoculated plants were harvested each week for 6 weeks. Each plant was inoculated with approximately 300 microsclerotia in Experiment 3. In each of three replicates five plants were inoculated with the Boone isolate; five plants with the K-3 isolate; and five plants were noninoculated checks. Each replication was initiated 2 weeks after the previous one. Hypocotyls of inoculated and noninoculated plants were harvested 2, 4, 6, 8, and 12 weeks after inoculation.

Approximately 900 microsclerotia were added to each plant in Experiment 4. Hypocotyls and fibrous roots of two inoculated and noninoculated plants were harvested 2, 4, 6, and 8 weeks after inoculation. Several necrotic fibrous roots from infected plants in Experiments 3 and 4 were cleared and observed for the presence of microsclerotia at each harvest.

OBSERVATIONS AND CONCLUSIONS. — *Symptom development*. — A general chlorosis was associated with most infected plants throughout the test. Generally, no additional above-ground symptoms were noted until the more advanced stages of CBR were reached, at which time the foliage wilted completely.

In the chlorotic plants, numerous lateral roots were usually necrotic in the vicinity of the inoculation point within 2 weeks after inoculation. Inoculated plants with no above-ground symptoms had few necrotic lateral roots. Inoculated plants with perithecia on the cotyledons, however, had completely necrotic root systems. Wilted plants had extensive root necrosis, and the hypocotyls were severed in some cases. Plants not killed within 6 weeks after inoculation survived the entire test; even though hypocotyls were not necrotic, root systems were discolored with numerous necrotic lateral roots.

Stages in hypocotyl pathogenesis.—Observations were divided into eight distinct stages. Stage 1 of pathogenesis involved the random growth of hyphae over the hypocotyl surface. Stage 2 involved the formation of infection cushions (approximately 93 mm in diameter × 55 μm in thickness) on the epidermis 4 days after inoculation (Fig. 1-A). Several cell layers beneath the cushions became pre-necrotic as was noted by enlarged nuclei and increased staining intensity of these cells. Hyphae emerged from the infection cushion and invaded the surrounding necrotic cells both inter- and intracellularly in Stage 3 (Fig. 1-B). Complete cortical colonization occurred at Stage 4a. Hyphal progression stopped at the periderm just exterior to the phloem region (Fig. 1-C). If conditions were favorable for pathogenesis at Stage 4a, hyphae aggregated into large clumps on the periderm surface with necrosis spreading through the periderm into the vascular system (Stage 5a) (Fig. 1-D).

Depending upon the severity of infection, necrosis was either localized beneath the hyphal aggregate or general in the stele. If necrosis was general in the vascular system, the plant invariably died (Stage 6) (Fig. 1-H). Although infrequent, gel formation was observed in some necrotic vessels. Hyphae were detected in the vascular system only after plant death. Perithecia generally formed on the colonized cortical surface at the soil line. Occasionally, plants which had perithecia produced on the hypocotyl had no foliar symptoms. When perithecia were produced under these conditions, however, necrosis had spread through the periderm into a localized area in the vascular system.

If conditions were not favorable for pathogenesis at Stage 4a, the periderm appeared effectively to protect the hypocotyl from further invasion (Stage 4b). Hyphae accumulated in the cortex and eventually microsclerotia were formed (Fig. 1-F). Cells within the periderm remained healthy with no signs of progressive necrosis.

If Stage 5a occurred and necrosis spread within the periderm, occasionally an additional periderm formed in advance of the necrosis which appeared to hinder further spread (Stage 5b) (Fig. 1-F, G). At both Stage 4b and 5b the plant recovered from the disease. Although both the Boone and K-3 isolates behaved similarly, the K-3 isolates induced more rapid and severe development of disease symptoms.

Effects of exogenous nutrients on pathogenesis. — The K-3 isolate of the fungus was grown on potato-dextrose agar (PDA) for 7 days. Three-week-old plants grown in 10-cm diameter clay pots were inoculated by placing a 1-cm diameter plug of mycelium approximately 5 mm thick adjacent to the hypocotyl 3 cm below the soil surface. Check plants were inoculated in a similar manner with an autoclaved K-3, PDA culture. Hypocotyls were harvested 7, 11, 14, and 37 days after inoculation.

No foliar symptoms were observed 7 days after inoculation, but hypocotyl tissues in cross section were discolored up to the pith on the side of inoculation. Most *C. crotalariae*-inoculated plants harvested 11 or more

Fig. 1-(A to H). Stages in hypocotyl pathogenesis by *Cylindrocladium crotalariae*. **A)** formation of infection cushion on hypocotyl surface in stage 2 (× 232); **B)** inter- and intracellular invasion of cortical tissue in stage 3 (× 232); **C)** hyphal mat on surface, and necrosis of cortex contained at the periderm in stage 4a (× 232); **D)** necrosis of periderm cell layers spreading into vascular region from the hyphal aggregation at the periderm surface in stage 5a (× 93); **E)** containment of infection by additional periderm as viewed from hyphal mat on cortex in stage 5b (× 93); **F)** microsclerotium formation in colonized cortical tissue subsequent to containment of infection at stage 4b (× 93); **G)** formation of additional periderm which contains infection after breechment of original periderm in stage 5b (× 93); **H)** collapse and disruption of ground tissue system and colonization of ground tissue system and intact vascular system in stage 6 (× 93). Legend: cortex (c), epidermis (e), hyphae (h), hyphal mat (hm), infection cusion (i), additional periderm (ap), microsclerotia (m), original periderm (op), periderm (p), pith (pi), vascular system (v).

days after inoculation were completely wilted.

Histological observations indicated that fungal invasion was more rapid in the presence of exogenous nutrients (PDA plug) than when microsclerotia were used in inoculations. Hyphae had not penetrated the periderm at 7 days, but the entire region within the periderm including the pith was deeply stained, indicating necrosis or pre-necrosis of these tissues. At 11 days, all tissues of completely wilted plants were disorganized, except the xylem. Hyphae were present in all tissues including the xylem, and perithecia formed on the colonized cortical surface at ground level.

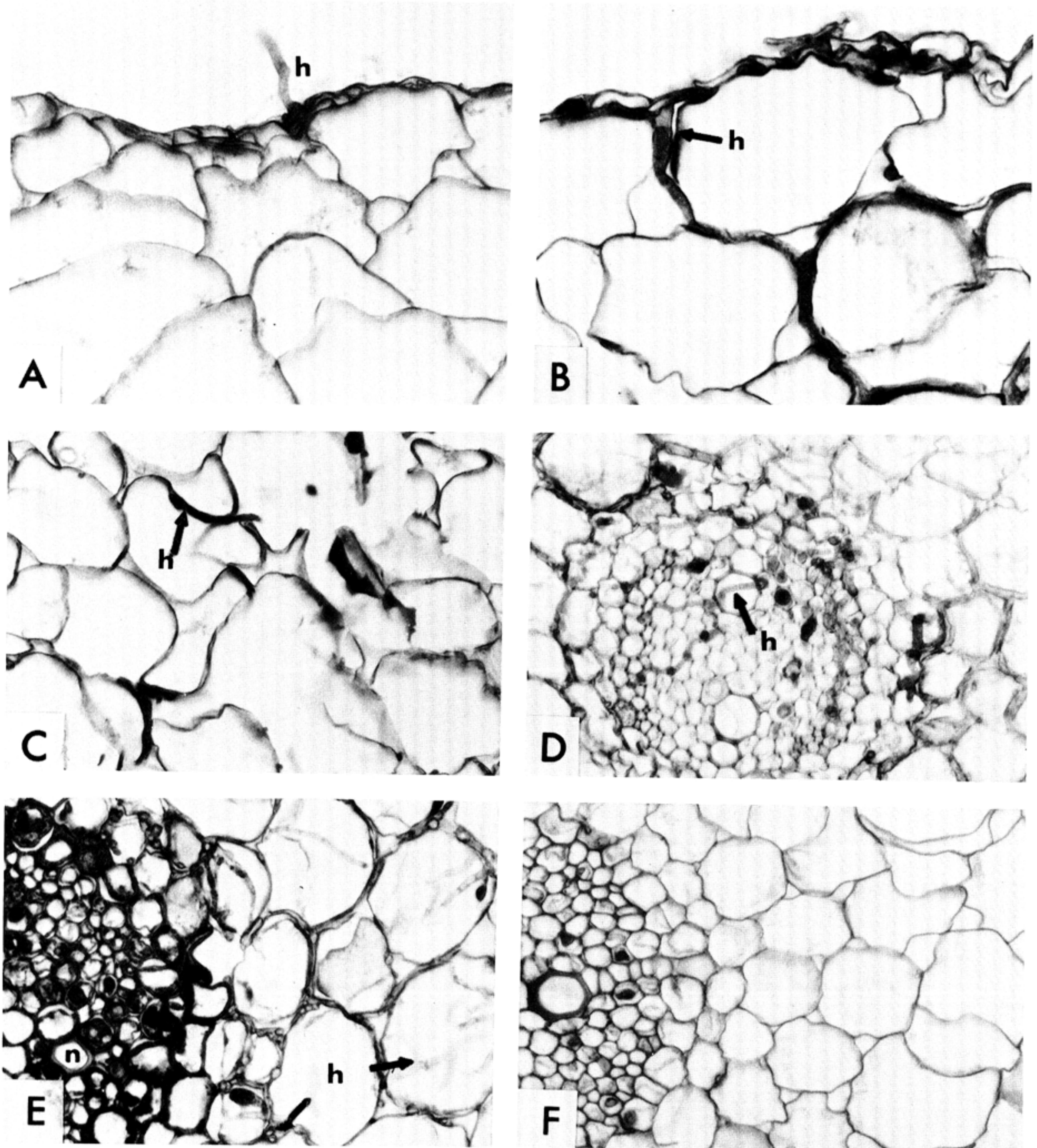

Fig. 2.-(Ato F). Stages in pathogenesis of fibrous roots by *Cylindrocladium crotalariae*. **A)** hyphal growth on surface of root 12 hours after inoculation (× 459); **B)** intercellular hyphal invasion 24 hours after inoculation (× 459); **C)** cortical hyphal colonization 36 hours after inoculation (× 286); **D)** hyphal colonization of vascular tissue system 48 hours after inoculation (× 286); **E)** cortical hyphal colonization and vascular necrosis 72 hours after inoculation (× 286); **F)** healthy fibrous root after 72 hours of incubation (× 286). Legend: hyphae (h), necrotic stele (n).

Fibrous root pathogenesis.—Roots of 10-day-old plants were washed and entire plants were placed in moist chambers. Several 3-mm diameter water-agar plugs containing mycelium of the K-3 isolate were positioned with mycelium in contact with fibrous roots. Two inoculated root segments were removed 6, 12, 24, 36, 48, and 72 hours after inoculation. Noninoculated check roots were removed at the last harvest.

Pathogenesis of fibrous roots differed in several aspects from that of hypocotyls. Intercellular penetration occurred between 12 and 24 hours after inoculation, and no hyphal cushions were observed (Fig. 2-A, B). Hyphae penetrated to the stele by 36 hours (Fig. 2-C) and had grown through the stele by 48 hours after inoculation (Fig. 2-D). At 72 hours, the cortex was completely colonized and the stele was necrotic (Fig. 2-E).

DISCUSSION.—Collapse of the underlying epidermal cells and necrosis of surrounding cortical cells beneath infection cushions appears to be a prerequisite for fungal invasion, which suggests the possible involvement of phytotoxins. Natural periderm formation appears to effectively limit *C. crotalariae* growth to the cortex under certain circumstances. Moreover, plants with only cortical decay are capable of recovery. If *C. crotalariae* induces cell necrosis within the periderm, however, invasion can continue until the host either dies or initiates an additional periderm to check fungal advance. Fibrous root pathogenesis may be more rapid than hypocotyl pathogenesis, because no periderm is present to stop fungal invasion. Based on observations of CBR-pathogenesis of peanut in this study, rapid formation of additional periderm could be a characteristic to be considered in a breeding program.

Milholland recently reported both direct and indirect penetration of highbush blueberry by *C. crotalariae* by 12 hours after inoculation (9). Stem invasion resulted in disorganization and breakdown of the cortex and phloem. Death of the blueberry plant was attributed to the occlusion of the xylem elements by mycelial deposits and tyloses. In contrast to the findings of Milholland with blueberry, *C. crotalariae* does not enter the vascular system of the infected peanut hypocotyl until the plant is completely wilted. However, necrosis appears to spread into the vascular system from the approaching hyphae, resulting in death of living xylem and phloem cells. This dysfunction of the vascular system undoubtedly results in the wilting and rapid death of the peanut plant.

Inoculation of peanut hypocotyls with the fungus growing on PDA plugs resulted in more rapid necrosis of peanut hypocotyls than in tests in which only microsclerotia were used as inoculum. Necrosis was observed farther in advance of fungal growth, suggesting an increase in some diffusible toxic fungal metabolites. The presence of *C. crotalariae* microsclerotia in plant debris from previously infected crops (12), or the colonization of noninfested organic debris during infection, may enhance spread of the disease in the field.

Production of microsclerotia was similar to that reported for other *Cylindrocladium* spp. (4, 8). Although microsclerotial production was prolific in all tests, they were only found in the cortex of plants. They were produced in necrotic fibrous roots of both *C. crotalariae*-inoculated greenhouse plants and *C. crotalariae*-infected field plants. Microsclerotia were detected 56-84 days after inoculation in greenhouse plants. The abundant production of microsclerotia in the peanut root system may contribute to a rapid increase and spread of the pathogen in infested fields, even when infected plants do not succumb to the disease.

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