Histopathology of Calonectria crotalariae on Highbush Blueberry

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ABSTRACT

The histological relationship of *Calonectria crotalariae* in infected blueberry leaves, stems, and roots was investigated. Penetration of leaf and stem tissue was primarily through stomates; however, direct penetration was observed. Appressoria were formed 8 h after inoculation and measured 5-6 μm in diam. Infection of succulent stems by *C. crotalariae* caused a complete breakdown of cortex and phloem tissue. Hyphae measured 1-5 μm in diam, and grew intracellularly in the vascular tissue of stems and roots. Death of blueberry plants is due primarily to the occlusion of the xylem elements by mycelia, gel deposits, and tyloses that impede or restrict the flow of water. Perithecial development is initiated in the stem cortex with the formation of a large stroma. The somatic hyphal cells push through the epidermis and give rise to the mature perithecium.

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Additional key words: *Cylindrocladium*, histopathology, *Vaccinium corymbosum*.

Calonectria crotalariae (*Cylindrocladium crotalariae*) Loos, Bell & Sobers, was first described in Georgia in 1966 as the cause of a peg, pod, and root necrosis of peanuts (1). Since that time, the disease has become widespread in North Carolina, and now poses a serious threat to the peanut industry (9). In addition to peanuts, the fungus is pathogenic to several plants (7), including blueberry (*Vaccinium corymbosum* L.) (6). The stems, leaves, and roots of blueberry plants are all susceptible to infection by *C. crotalariae*. Symptoms first appear on blueberry seedlings as browning of the stem near the soil line, followed by a wilting of the plant. Within 1.0 wk the entire stem turns brown and dies. The pathogen also causes root necrosis and a leaf spot consisting of brown circular lesions surrounded by a red border measuring 1-3 mm in diam. The research reported herein was undertaken to determine the mode of infection, and the histological effects of the pathogen on various plant tissues.

MATERIALS AND METHODS.—Cultures of *C. crotalariae* were obtained from infected blueberry seedlings and grown on potato-dextrose agar (PDA). Conidia were harvested from 7-day-old PDA cultures by washing the surface of a culture with sterile distilled water. The conidial suspension was sprayed onto young, succulent stems and leaves of the blueberry cultivar Jersey, and placed in a moist chamber at 25 C. Inoculations with ascospores were made by crushing *C. crotalariae* perithecia in sterile distilled water on a glass slide, and applying the spores to the leaf and stem surface with a camel's-hair brush. Leaves and stems to be examined for germination and penetration by the pathogen were excised, placed in petri dishes with moist filter paper, and incubated at 25 C. Root systems were inoculated by dipping them into a mycelium-and-spore suspension, and then transplanting the inoculated plants into sterilized sand in 10-cm diam clay pots. Roots were removed after four days and examined histologically.

To study spore germination and appressorium formation, inoculated leaves and stems were cut into 5 mm^2 sections, cleared, stained with cotton blue in lactophenol (8), and observed after 2, 4, and 8 hr. Fungal penetration and disease development were determined 8, 12, 24 h, and 4, 7, and 14 days after inoculation. Leaves, stems, and roots were cut into 10 mm sections, cleared, and fixed in Carnoy's solution, dehydrated in tertiary butyl alcohol, and embedded in paraffin. Sections 15-μm thick were mounted on slides with Haupt's adhesive and stained with safranin and fast green (4).

Root discoloration was observed 4 days after inoculation. Histological examination of infected tissue showed large amounts of hyphae in the cortical cells and

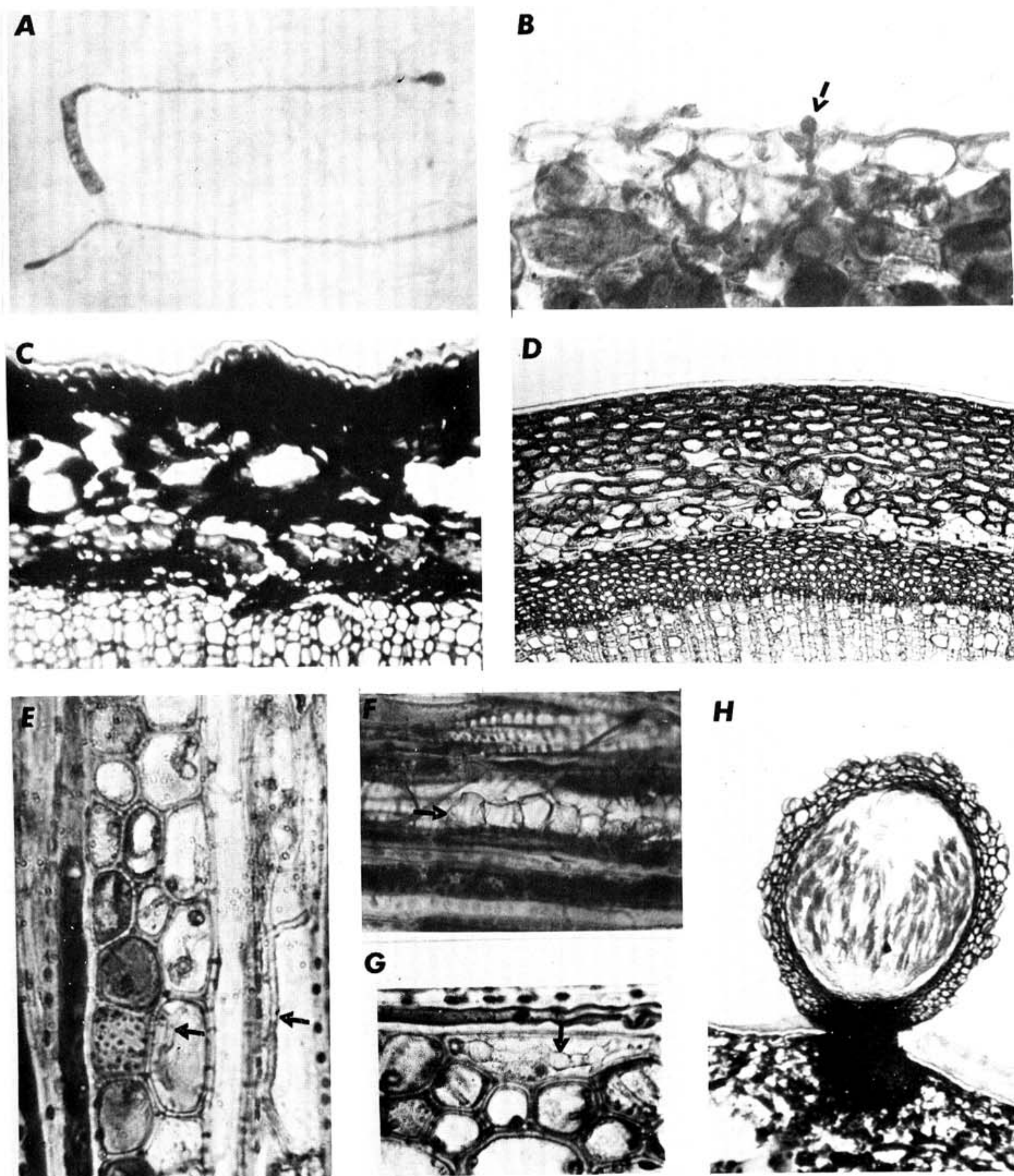

Fig. 1-(A to H). Histological relationships of blueberry (*Vaccinium corymbosum* L.) and *Calonectria crotalariae*. **A)** Conidium, germ tube, and appressorium on lower leaf surface 8 h after inoculation (× 82.4). **B)** Appressorium and penetration hyphae through leaf stomate from germinated ascospores 12 h after inoculation (× 82.4). **C)** Transverse section of infected blueberry stem showing disorganized cortex and phloem 7 days after inoculation (× 20.6). **D)** Transverse section of a healthy blueberry stem (× 20.6). **E)** Tangential section of infected blueberry stem showing hyphae inside the vascular tissue (arrow) (× 82.4). **F)** Tangential section of an infected blueberry stem showing complete occlusion of vessel by tyloses (arrow) (× 82.4). **G)** Microsclerotia development inside parenchyma cell of a xylem ray (arrow) (× 82.4). **H)** Transverse section of infected blueberry stem showing mature perithecium. Note large stroma inside cortex (× 20.6).

in the xylem vessel elements of the roots. Hyphae grew intracellularly in the vessels and after 4 days had penetrated ca. four to five cells. Gel-like deposits, apparently formed by the host in response to fungal infection, were also observed in the xylem vessels. Penetration from one vessel into another was primarily through pit openings. Tyloses formed near the center of the root in advance of the hyphae. No microsclerotia were observed in the infected roots. Inoculated plants wilted and died after seven days.

Spore formation.—Conidia were produced when 7-day-old infected stems were placed on moist filter paper in a petri dish for 24 h. Transverse sections of the infected stem showed that the fungus formed a stroma in the substomatal cavity. As the stroma developed and spores were produced, the epidermal cells were pushed upwards and finally ruptured, releasing a mass of hyphae and conidia.

Ascospores were produced within an oval-to-globose perithecium borne on a short, dark stroma. Perithecial development was initiated ca. 60-80 μm below the epidermis in the cortex where large (10-15 μm diam), thick-walled fungal cells formed a stroma ca. 150 μm wide. As the stroma enlarged, the fungal cells pushed through the epidermis and continued to divide, forming the mature perithecium. The perithecium wall is composed of large, thick-walled, irregular-shaped cells (15-30 μm in diam) about 8 to 10 cell layers thick (Fig. 1-H). The inner cell wall is very compact with the outer layers more loosely arranged. Inside the perithecial cavity, a mass of fungal tissue developed at the base, from which long-stalked asci were produced. Asci are hyaline, clavate, 75-120 μm × 12-20 μm, and contain eight ascospores. At maturity, and under favorable environmental conditions, ascospores are released through an ostiole.

DISCUSSION.—On highbush blueberry, *C. crotalariae* is a highly pathogenic, nonspecialized fungus that invades the leaf, stem, and roots, and causes serious damage to all three organs.

Spore germination, appressorium formation, and penetration are completed within 12 h. Following establishment of *C. crotalariae* in blueberry tissues, death occurs within 7 days. The fungus has other advantages as a pathogen. Conidia and ascospores of *C. crotalariae* are capable of producing three, sometimes four, germ tubes from a single spore, and can penetrate host tissue directly through the cuticle and into the epidermal cell, or through natural openings, such as stomates or lenticels. If an abundance of mycelium is available at the infection site, hyphae penetrate directly.

The formation of microsclerotia by different *Cylindrocladium* spp. has been observed in roots, leaves, and flowers of several hosts (2,3,5). Large aggregates of pigmented microsclerotia were not observed in infected blueberry roots, stems, or leaves. However, enlarged hyphal cells, often borne in chains, were observed in the vascular tissue of the stem, suggesting the possible development of microsclerotia. Only once were these enlarged cells observed in leaf tissue, and this occurred in the upper epidermis where a mass of hyphae had penetrated. Microsclerotium formation has primarily been reported to occur in the root cortex (2,5); however, Cordell et al. (3) reported the formation of sclerotia in the root stele of yellow-poplar roots infected with *C. floridanum*. No microsclerotia were observed in infected blueberry roots 4 days after inoculation. Whether or not they are formed after a longer period of time, is not known.

To my knowledge, formation of tyloses has not been previously reported for Cylindrocladium-infected plants. *Calonectria crotalariae* apparently is a vascular parasite on highbush blueberry capable of inducing tyloses and gel formation in the stem and root tissues. Death of blueberry plants infected with *C. crotalariae* appears to be due primarily to the occlusion of the xylem vessels by mycelium, tyloses, and the formation of gel deposits which impede or restrict the flow of water.

Perithecial development is initiated in the cortex of the stem ca. 3 wk after infection. The somatic hyphae form a pseudoparenchymatous tissue, which continues to enlarge until it emerges through the epidermis, and later forms the mature perithecium. More than one perithecium may develop from a single stroma.

RESULTS—*Germination.*—Ninety percent of the ascospores and conidia of *C. crotalariae* germinated after 2 h on blueberry leaves. Germ tubes emerged from both ends of the ascospores and conidia, and sometimes as many as three germ tubes were observed. Germ tubes measured 5-40 μm, and 100-400μm after 2 and 8 h, respectively. Germ tubes averaged 2μm in diam. In several instances, large irregular-shaped structures (5-10 μm in diam) developed at the end of germ tube branches. Hyphae would occasionally grow from these structures and develop appressoria. Appressoria observed 8 h after spore germination (Fig. 1-A) were unicellular, generally globose, and measured 5 to 6 μm in diam.

Penetration.—After 8-12 h conidial inoculations resulted in the formation of appressoria and infection hyphae which penetrated directly through leaf or stem epidermis, or indirectly through a stomate. Because the blueberry stem cuticle was ca. 10-μm thick, and impervious to the stain, the penetrating hyphae were not always visible. However, infection hyphae were located inside the epidermal cells. Ascospore inoculations resulted in penetration of leaf tissue from an appressorium through open or closed stomates (Fig. 1-B). The hypha became constricted to 0.5 μm or less in diam when it penetrated through a closed stomate, and enlarged when it entered the substomatal cavity. Direct penetration through the stem cuticle by germinated ascospores was observed, although the hyphae were not seen penetrating the epidermal cell.

Disease development.—After penetration through a stomate, the hyphal tip enlarged and later became branched. Hyphae in the mesophyll tissue were intra- and intercellular and measured 2-4 μm in diam. In one instance, large, round to irregular-shaped fungal cells 10 μm in diam formed in the upper epidermis where a mass of hyphae had penetrated, suggesting formation of microsclerotia. Epidermal cells and adjacent parenchyma cells in the mesophyll became necrotic and collapsed 24 h after inoculation.

Growth of the mycelium in the stem tissue caused the cortex and phloem to become disorganized after 7 days

(Fig. 1-C, D). Hyphae measured 1-5 μm in diam and grew intracellularly in the cortex, pericycle, phloem, xylem, and pith parenchyma (Fig. 1-E). Hyphae also grew intercellularly in the air spaces of the cortex. Hyphae in the stem vessels spread longitudinally, with several strands occupying a single vessel. Extensive branching of the hyphae occurred within the vessels, resulting in invasion of adjacent vessels and tracheids. Hyphae grew from tracheids into the parenchyma cells of xylem rays, or from one vessel into another by penetration directly through cell walls, or indirectly through pit openings.

Tyloses were abundant in the xylem vessels and ray cells of naturally infected blueberry stems. These protrusions caused partial or complete occlusion of the vascular tissue (Fig. 1-F). Tyloses and hyphae were observed to occupy the same vessel. No tyloses or hyphae were observed in healthy stems.

In addition to tyloses, enlarged hyphal cells (microsclerotia) were formed in the tracheids and ray cells. Early formation of these microsclerotia were observed when hyphal strands in the ray cells and tracheids enlarged into irregular-shaped cells often borne in chains and measuring 5-10μm in diam (Fig. 1-G). Plugging of the xylem vessels by the formation of gel deposits was also observed.

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