Formation of Microsclerotia of Cylindrocladium spp. in Infected Azalea Leaves, Flowers, and Roots

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ABSTRACT

Lesions on attached azalea leaves infected with *Cylindrocladium scoparium, C. theae,* or *C. floridanum* contained pigmented hyphae or cells, but no microsclerotia. Following abscission, each species of *Cylindrocladium* rapidly invaded the entire leaf when held at a high relative humidity. *Cylindrocladium scoparium* and *C. floridanum* formed abundant microsclerotia in leaf mesophyll parenchyma within 2 weeks after abscission, whereas *C. theae* formed relatively few. Microsclerotia were not specifically associated with stomata. Both *C. theae* and *C. floridanum* produced sclerotiumlike stromata in leaves on which perithecia of their *Calonectria* stages developed. Flower tissues infected with each of the three *Cylindrocladium* spp. contained microsclerotia and smaller, thick-walled, pigmented cell aggregates. Perithecia of *C. theae* and *C. floridanum* were observed on the stamens of infected flowers. Azalea roots infected with *C. scoparium* or *C. floridanum*, examined after the onset of wilt symptoms, contained relatively few microsclerotia. Roots infected with *C. theae* contained some small, pigmented cell aggregates, but no microsclerotia.

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Additional key words: survival structures, flower blight, leaf spot, root rot, azalea wilt.

Within the last decade, *Cylindrocladium* has become an increasingly important pathogen of many ornamental and forest crops. Timonin & Self (7) first described blight and wilt of azalea cuttings caused by *C. scoparium*. The extensive work of Cox (4) demonstrated the exceptional pathogenic capabilities of *C. scoparium* in causing damping-off, root rot, crown canker, and needle blight on conifers. Bugbee & Anderson (2) demonstrated histologically that microsclerotia of the pathogen were formed in infected needles, and that such microsclerotia were a link between the above- and belowground phases of the disease on spruce. Reis (6) found microsclerotia of *C. scoparium* in substomatal chambers in leaf spots on azaleas 10 days after inoculation. Because I could not find microsclerotia of *C. scoparium* in lesions on azalea leaves cleared immediately after detachment from the plant, I undertook a more detailed study of infected leaves before and after abscission. In addition to *C. scoparium* Morgan, *C. floridanum* Sobers & Seymour, and *C. theae* (Petch) Alfieri & Sobers, which also infect azaleas (1, 5), were included. The flower blight and root rot phases of the disease were also examined.

MATERIALS AND METHODS.—*Inoculation procedures.*—Azalea plants [*Rhododendron obtusum* (Lindl.) Planch.] were inoculated separately with conidial suspensions of the three *Cylindrocladium* spp. applied as a spray to both upper and lower leaf surfaces and to flowers when present. Inoculated plants were covered with a plastic bag and placed in a lighted incubator at 29.5 ± 1 C (85 F). The plastic bag was removed 24 hr after inoculation, and the plants were covered with a ventilated 15-cm clear plastic pot to reduce air flow around the plant, and kept in the 29.5 ± 1 C incubator (12-hr photoperiod at 300 ft-c). Large, flat pans of water were kept in the incubator to increase the relative humidity. Leaf and flower samples were collected at various intervals after inoculation. Abscised leaves were collected 1 week after inoculation and placed in a moist chamber, and three leaves were removed periodically to be cleared. Azalea cultivars Whitewater, Roadrunner, and Kingfisher were used primarily in these studies.

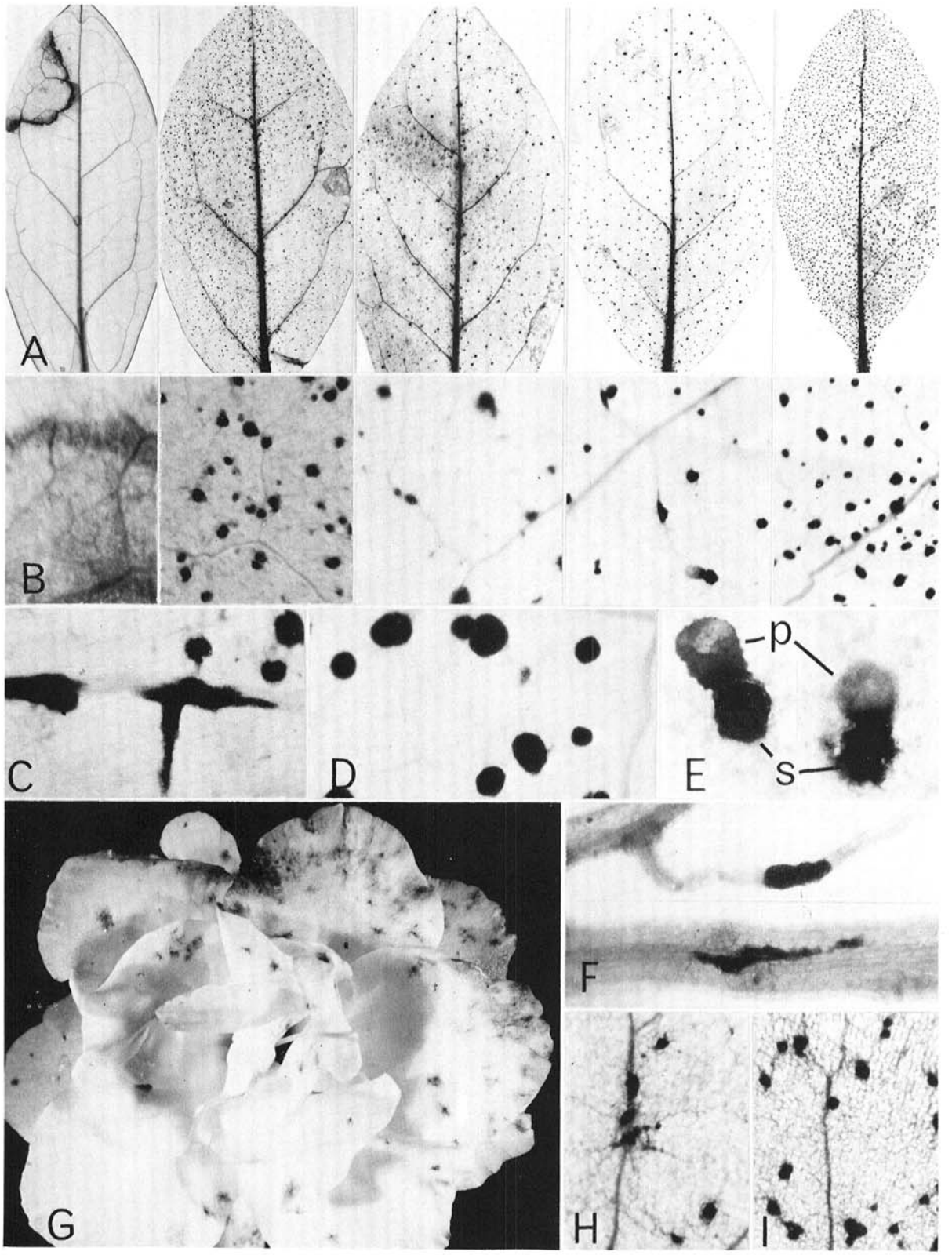
I made root inoculations by transplanting 2-month-old rooted cuttings in a 1:1:1 mixture of peat, soil, and perlite, or field soil infested separately with vermiculite cultures of each of the three *Cylindrocladium* spp. tested. Control plants were potted in greenhouse soil mix or field soil to which noninoculated vermiculite was added. Root samples were collected from plants which had died at different times. Thus, the time from death to collection day varied from 2 to 16 weeks. Dead plants were maintained on the greenhouse bench during that period.

Inoculum production.—Cultures of *Cylindrocladium* were grown on potato-dextrose agar plates from which conidia were washed from 2-week-old cultures, grown from three single condidia/plate, into water suspension. Concentrations of conidial suspensions were not measured, but usually resulted in one-five lesions/leaf when sprayed on test plants. Vermiculite cultures for soil infestation were grown in 1,000-cc jars containing 500 cc vermiculite saturated to runoff (ca. 200 cc) with Czapek-Dox broth. The species of *Cylindrocladium* used in these studies were: *C. scoparium* and *C. theae* isolated from azalea; and *C. floridanum* isolated from redbud (*Cercis* sp., isolate 31) and from soil at Beltsville, Md. (isolate 56).

Leaf, flower, and root clearing.—Inoculated flowers were cleared in 100% methanol for at least 24 hr before examination. Leaves and roots were cleared in 5% NaOH for 7 to 10 days (changed daily) until relatively clear, washed with water, and transferred to 100% methanol.

RESULTS.—*Leaf infections.*—Examination of cleared leaves of cultivars Kingfisher, Whitewater, and Roadrunner inoculated with either *C. scoparium, C. floridanum,* or *C. theae* showed no pigmented microsclerotia visible in lesions on attached leaves collected weekly up to 4 weeks after inoculation. Pigmented hyphae or cells were present in the lesion, but most of the discoloration was associated with the host response to infection. After abscission, each of the three *Cylindrocladium* spp. spread throughout the leaf tissue, resulting in browning of the whole leaf within 1 week. In the cultivars tested, *C. scoparium* and *C. floridanum* produced a few (less than 50/leaf) small microsclerotia within 1 week after leaf abscission, but by 2 to 3 weeks, the size and number of microsclerotia had increased to over 1,000/leaf (Fig. 1-A,B). In contrast, *C. theae* produced relatively few (less than 200/leaf) large microsclerotia in infected abscised leaves, but many thick-walled pigmented hyphae or cell clusters were visible. The latter may have been immature microsclerotia. When *C. theae* produced what appeared to be sclerotia, such structures were usually stromata on which perithecia

Fig. 1. A, B, C, D, E) Azalea leaves of cultivar Kingfisher inoculated with *Cylindrocladium* spp. and cleared with 5% NaOH 3 weeks after inoculation. **A, B)** (left to right) leaf inoculated with *C. scoparium* but still attached to the plant; leaf inoculated with *C. scoparium* but 2 weeks after abscission from the plant; abscised leaf inoculated with *C. theae*; abscised leaf inoculated with *C. floridanum* (isolate 31); abscised leaf inoculated with *C. floridanum* (isolate 56). Note that microsclerotia or perithecial stromata form only in abscised leaves, not on leaves still attached to the plant (A = X 1.5, B = X 10). **C, D)** Microsclerotia (X 40) in abscised leaves inoculated with *C. scoparium* (C), some of which are closely associated with vascular bundles, whereas those of *C. floridanum* (isolate 56) are not (D). **E)** Perithecia (p) of the *Calonectria* stages of *C. floridanum* (isolate 31) (left) and *C. theae* (right) borne on small sclerotiumlike stromata (s) (X 40). **F)** Roots of Kingfisher azalea, inoculated with *C. floridanum* (isolate 31) and cleared with 5% NaOH, showing microsclerotia in the cortex. **G)** Flower of Whitewater azalea showing small lesions 48 hr after inoculation with *C. scoparium* conidia. **H, I)** Microsclerotia of *C. scoparium* (H) and *C. floridanum*-isolate 56 (I) (both X 20) produced in infected petals of Whitewater azalea flowers.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of its *Calonectria* stage developed (Fig. 1-E). Isolate 31 of *C. floridanum* produced its *Calonectria* stage in a similar manner, but also produced microsclerotia unrelated to perithecial formation.

Microsclerotia of *C. scoparium* and *C. floridanum* which were the result of growth within abscised, infected leaves were not specifically associated with stomata. Microsclerotia were formed in interveinal areas of parenchyma as well as in bundle parenchyma (Fig. 1-C, D). Microsclerotial formation was also observed in detached leaves of at least eight other cultivars and 17 P.I. numbered accessions inoculated with conidia of *C. scoparium*

Flower infections.—Flowers inoculated with each of the three *Cylindrocladium* spp. developed visible brown lesions within 24 hr. Flowers were somewhat more susceptible than foliage because when inoculum levels were too low to give significant amounts of leaf spot, there was always considerable flower blight. Flower infections were at first a localized petal blight (Fig. 1-G), but within 1 week, the lesions coalesced so that infected petals became uniformly brown to whitish buff (depending on the cultivar), flaccid, and the flowers often abscised. Within 2 weeks after inoculation, most infected flowers collapsed. Microsclerotia developed in infected flowers 2 weeks after inoculation, regardless of whether or not the flower was attached to the plant. These microsclerotia were usually smaller than those produced in leaves. Flowers infected with *C. theae* contained fewer microsclerotia than those infected with *C. scoparium* or *C. floridanum*. Perithecia of *C. theae* and *C. floridanum* (isolate 31) formed in infected flowers, but usually only on the stamens. Flower infections by *Cylindrocladium* spp. appeared much like *Botrytis* infections, but methanol-clearing easily distinguished them, as *Botrytis* produces no microsclerotia in infected petals.

Root infections.—Most of the infected azalea roots contained no pigmented microsclerotia, even in roots collected many weeks after the onset of wilt symptoms. I established the presence of the pathogen by culturing roots similar to those cleared, or by clearing roots from which the pathogen had grown onto culture plates. Microsclerotia when found, however, occurred in the cortex of all sizes of roots and generally on plants which had been dead at least 2 months. Larger roots whose cortex has sloughed had no microsclerotia in the stele, even though it was usually darkly discolored. Microsclerotia occasionally adhered to the stele after the cortex had sloughed. Microsclerotia were found in roots inoculated with either *C. scoparium* or *C. floridanum*, but not *C. theae*. Small, pigmented cell aggregates were observed, however, in *C. theae*-inoculated roots. It is not known whether these clusters were immature microsclerotia, or mature but of a different structure than the microsclerotium.

DISCUSSION.—In his histological study of azalea leaf spot caused by *Cylindrocladium*, Reis (6) examined sections of infected leaf tissue removed from inoculated plants 5 or 10 days after inoculation. He reported microsclerotia in substomatal chambers, 10 days after inoculation. The microsclerotia enlarged until they broke through the cuticle. The subcuticular fungal masses which he called microsclerotia appear to be only small aggregates of cells or hyphae rather than microsclerotia. In my studies, no pigmented microsclerotia formed in infected leaves until after leaf abscission. Bugbee & Anderson (2) showed after infected spruce needles clearly contained microsclerotia only 3 days after inoculation. These microsclerotia were large enough to crush parenchyma cells surrounding substomatal chambers. The formation of microsclerotia in azalea leaves occurs as a result of saprophytic growth in parenchyma of detached leaves, and an association with stomata would not be expected.

Bugbee & Anderson (2) reported microsclerotia present in the cortex of *Cylindrocladium*-infected spruce roots 26 days after inoculation. In my study, few microsclerotia occurred in azalea roots infected with *C. scoparium* or *C. floridanum*, and only small cell clusters occurred with *C. theae*. Each *Cylindrocladium* sp. could be readily isolated from infected roots, however, so presumably such roots contained mycelium or chlamydospores that could not be detected by use of the clearing procedures. Cordell et al. (3) reported no microsclerotia in the cortex of *Cylindrocladium*-infected yellow-poplar roots. Whether the pathogens can survive in infected roots in soil without forming microsclerotia is not known.

With respect to the disease epidemiology, the importance of the sequential development of the saprophytic growth phase of *Cylindrocladium* in azalea leaves, after the parasitic leaf spot phase, cannot be over-emphasized. The microsclerotia, as well as perithecia, which result from the saprophytic growth in leaves or flowers, on or in the soil, may play major roles in the epidemiology of the wilt-phase of the disease, as well as leaf or flower blights. Fallen leaves, flowers, or roots containing microsclerotia may become incorporated into the soil or may be carried to noninfested areas. Of potentially more significance, however, is the role such sources of inoculum may play in disease development during the propagation of cuttings.

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