Histopathology of Colletotrichum gloeosporioides f. sp. aeschynomene on Northern Jointvetch

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ABSTRACT

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The histopathological relationship of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* with its host, *Aeschynomene virginica*, was investigated by microscopic examination of diseased seedlings and inoculated explants. Inoculation of *A. virginica* seedlings with suspensions of *C. gloeosporioides*, resulted in the formation of pinpoint lesions (0.5 to 1 mm diam) on stems or explants within 48 hr after inoculation. Lesions found within 48 hr after inoculation resulted from direct penetration of trichome bases. Spores of the fungus germinated and produced appressoria within 4 to 5 hr after inoculation, but did not penetrate the stem epidermis via the appressoria until 48 hr after inoculation. Infection resulted in the formation of stem lesions, 2 to 3 cm long, which encircled the stem within 6 to 8 days after inoculation. Intracellular mycelium grew within the cortex, cambium, xylem, and pith ray tissues. Death of *A. virginica* seedlings was caused by collapse of infected stem tissues. Coalescence of lesions enhanced girdling of stems and hastened death. The fungus sporulated abundantly on lesion surfaces.

Additional key words: biological control, myco-herbicide, weed disease.

A leguminous plant, northern jointvetch, *Aeschynomene virginica* (L.) BSP infests many rice fields in Arkansas (20). In 1973, an endemic fungus, *Colletotrichum gloeosporioides* (Penz) Sacc. f. sp. *aeschynomene* (nomennudum), was reported to incite an anthracnose disease of northern jointvetch (5). The fungus typically infects the stems of northern jointvetch but also infects petioles and leaflets. Stem lesions killed northern jointvetch seedlings.

The forma specialis designation is used because the fungus infects only species of plants within the genus *Aeschynomene*: *A. indica* L. and *A. virginica*. *Aeschynomene indica* is more resistant to the disease.

The fungus has potential for use as a biological control agent or myco-herbicide for northern jointvetch in Arkansas rice fields because it is host specific and rapidly kills seedlings of *A. virginica* (5). Control of the weed by this fungus depends upon successful inoculation and infection in field environments. The environmental requirements for high levels of infection and control of the weed have been defined (21). Nonetheless, identification and observation of the infection processes are needed for a full understanding of their dependence on environment. The objectives of this research were to determine the mode of penetration and infection of northern jointvetch by the fungus and to study the histopathology of the disease.

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MATERIALS AND METHODS

Seedlings of northern jointvetch were grown from seeds collected from field plants grown near Stuttgart, Arkansas. Seeds were treated with 0.05% NaOCl, scarified, re-treated with the NaOCl solution, and germinated on moist filter paper on petri dishes for 24 hr at 28 to 32 C before planting. Germinated seeds were planted 0.5 cm deep in pasteurized field soil (fine silt loam) in 7.12-cm diameter plastic pots. All plants were grown in a growth chamber (Environmental Growth Chambers, Model M-31, Chagrin Falls, OH 44022) at 28 C, 7,642 lux, 15-hr daylength, and 40 to 100% RH. Plants were inoculated when they were 15 cm to 18 cm tall, 18 to 21 days after sowing.

Inoculum in all experiments was obtained from 3- to 4-day-old liquid V-8 juice cultures of *C. gloeosporioides* f. sp. *aeschynomene* grown at 28 C (5). Spores were collected by filtration of the culture fluid through Fisher 9-795C (Fisher Scientific Co., Pittsburgh, PA 15219) filter paper. The spores in the filtrate were washed three times by centrifugation (1,050 *g*) and resuspended. Washed spores were diluted to a concentration of 1×10^6 spores/ml in distilled water as determined by comparison

to previously determined concentration curve at 525 nm on a Bausch and Lomb Spectronic 20 spectrophotometer.

Plants were inoculated with spore suspensions until run-off with an atomizer (Crown Industrial Products Co., Hebron, IL 60034). After inoculation, the plants were transferred to a dew chamber at 28 C for 24 hr before being returned to the growth chamber. Portions of the plant stems were collected for examination at 0, 4, 8, 12, and 24 hr and 2, 3, 4, 5, and 6 days after inoculation. Plants inoculated with distilled water served as controls.

In other experiments, 1-cm-long stem segments, hereafter called explants, were excised from stems and placed on moist filter paper in petri dishes (14). After inoculation with one droplet (50-μliters) of the spore suspension, explants were incubated at 28 C, and collected and fixed hourly from hours 1 through 8 and at 12, 24, 48, and 72 hr after inoculation. Explants inoculated with distilled water served as controls.

All plant material was fixed in formalin/acetic acid/alcohol immediately after collection, dehydrated in a *n*-butyl alcohol series, and embedded in paraffin (18). Sections (8 to 12 μm) of the diseased tissues were made with a rotary microtome and were stained with safranin-cotton blue, safranin-fast green, or Johansen's quadruple stain (8, 18).

RESULTS

Symptomology.—The first symptoms of infection in intact plants appeared 48 hr after inoculation and were associated with trichomes on the stems. These symptoms were pinpoint, brown lesions approximately 0.5 mm to 1.0 mm in diameter at the trichome bases. Trichome lesions rapidly elongated, reaching 2 to 3 cm by 6 to 8 days after inoculation, and became black, necrotic, and sunken. As the lesions elongated, they also expanded around the stem, frequently girdling it within 6 days after inoculation. Most plants were dead within 10 days after inoculation.

Sporulation of the fungus occurred on the lesions within 4 days after inoculation.

Water-soaked lesions, not associated with trichomes, sometimes appeared on stem tissue 3 to 4 days after inoculation. Their subsequent development was similar to that of the trichome-associated lesions.

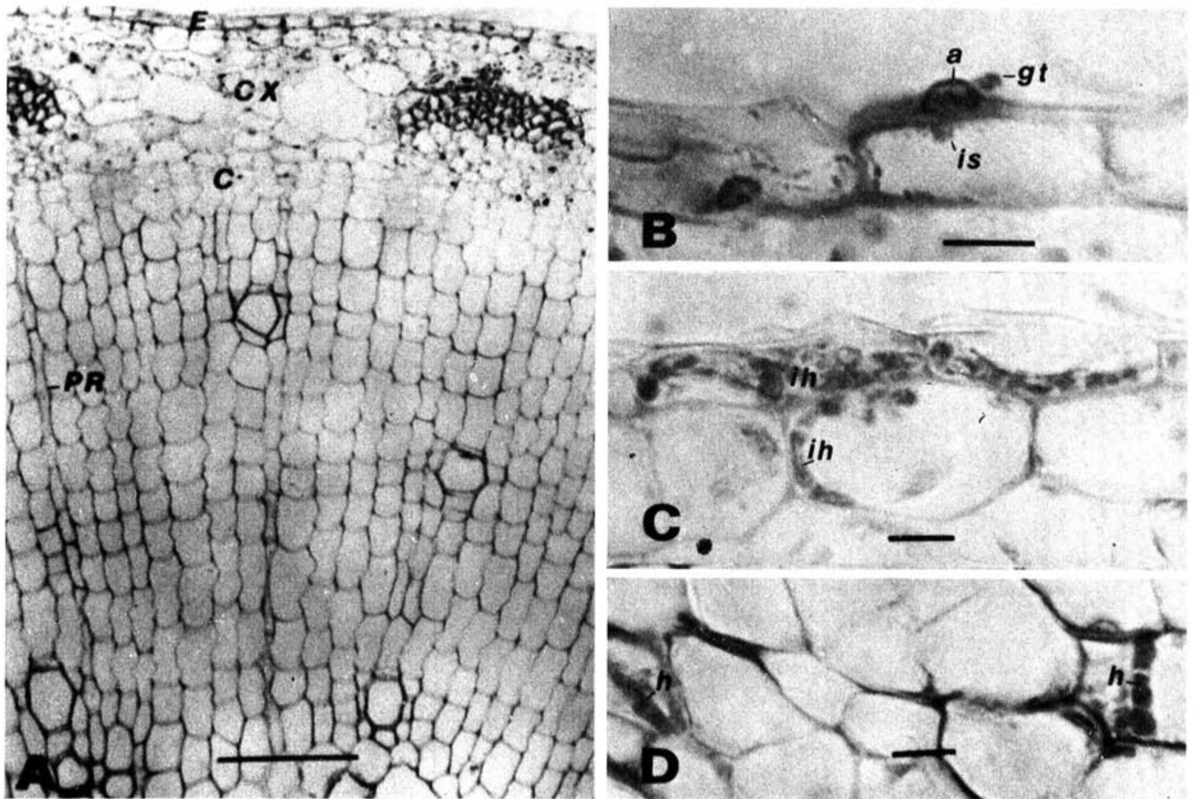

Fig. 1-(A to D). Early stages in the penetration and colonization of stems of northern jointvetch by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. **A)** Cross-section of a healthy northern jointvetch stem illustrating normal tissue arrangements. **B)** Appressorium of *C. gloeosporioides* f. sp. *aeschynomene* on the surface of northern jointvetch. An infection peg has penetrated the cell wall and has produced an intermediate swelling within the host epidermal cell. **C)** Ramification of the fungus as infection hyphae into cells of the epidermis and cortex adjacent to the cell originally penetrated. **D)** Colonization of cortical cells near a trichome base penetrated by the fungus. Legends: a = appressorium, C = cambium, CX = cortex, E = epidermis, gt = germ tube, h = hyphae, ih = infection hyphae, is = intermediate swelling, and PR = pith ray. Bars represent 0.1 mm in Fig. 1-A and 10 μm in Fig. 1-(B to D).

Symptoms of infection on explants also appeared 48 hr after inoculation as lesions 0.5 mm to 1.0 mm in diameter. These lesions were similar to those described earlier for whole plants, and also were associated with the trichomes. Inoculated explants incubated for 72 hr also were watersoaked beneath the entire inoculum droplet, but control explants treated with distilled water were not.

A cross section of a healthy stem of northern jointvetch is illustrated in Fig. 1-A. Comparison of noninoculated stem and explant cross sections showed that excision had little visible effect on cellular integrity except at the cut ends up to 72 hr after excision.

Pre-penetration activities.—Spores of *C. gloeosporioides* f. sp. *aeschynomene* that were sprayed onto intact plants or in droplets on explants germinated aithin 2 to 4 hr after inoculation. Spores became two-celled after germination, but typically produced only one germ tube, which originated near one end of the spore. Appressoria were produced within 4 to 5 hr after inoculation and were usually brown and rounded on the dorsal surface. Germ tubes which did not produce appressoria continued to grow and branch profusely, but were never observed to penetrate the epidermis of the plant.

Penetration.—Infection of northern jointvetch resulted from penetration of the epidermis via appressoria and via penetration of stem trichomes. Lesions produced within 48 hr after inoculation were always associated with trichomes. Appressoria were not found on the surfaces of infected trichomes when observed as thin sections or as whole mounts. Nevertheless, hyphae were observed in cells of the cortex near the edges of the trichomes within 12 hr after inoculation (Fig. 1-D). Within 72 hr after inoculation, the fungus had ramified to include adjacent cortex cells.

Penetration of the epidermis via the appressoria occurred directly beneath appressoria and was observed on stained sections taken 48 hr after inoculation. Penetration of host epidermal cells had not occurred beneath any of more than 200 appressoria observed on sections taken 24 hr after inoculation. About 10% of more than 200 appressoria observed in sections taken at 48 hr after inoculation had infected the host cells. Even after 72 hr only about 10% of the appressoria had penetrated the epidermis.

Penetration pegs of *C. gloeosporioides* f. sp. *aeschynomene* were not readily visible by light microscopy. After penetrations of the epidermal cell wall, intermediate swellings (4) were observed immediately within the host cell walls (Fig. 1-B). Although some host cell walls became refractile directly beneath some

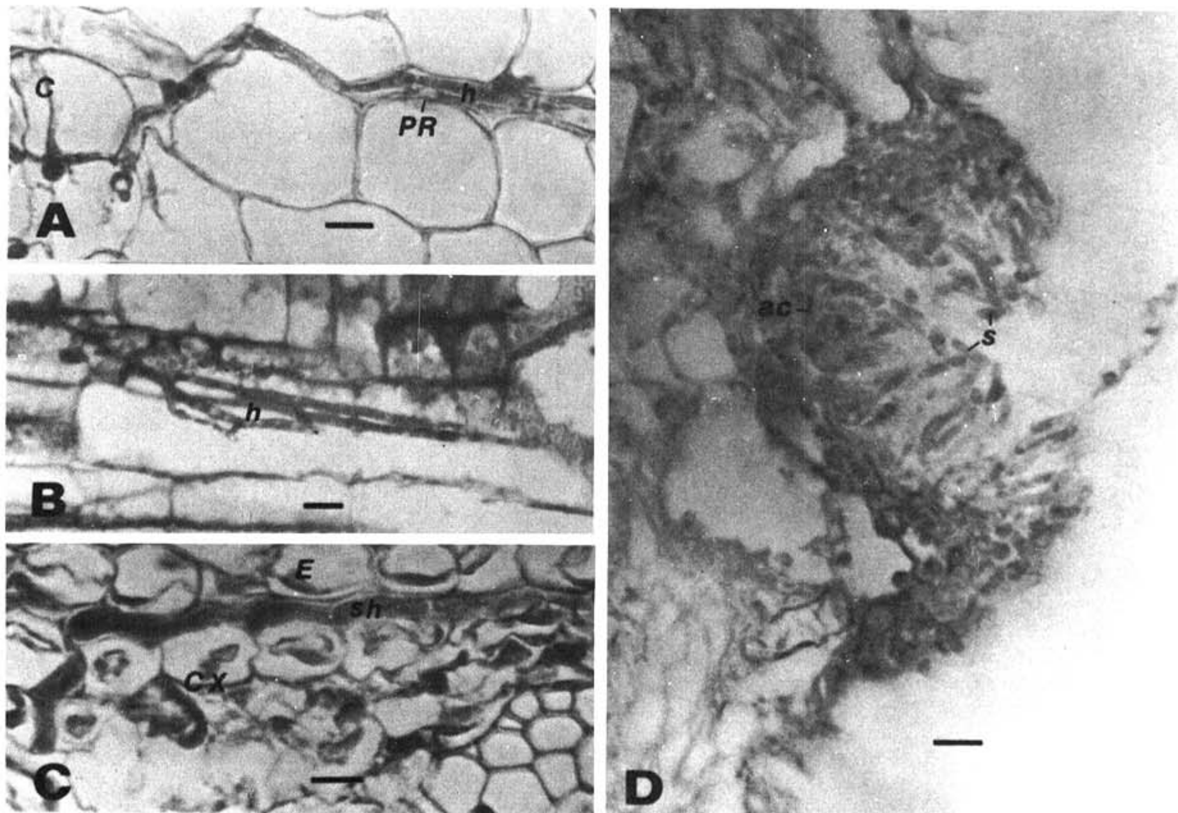

Fig. 2-(A to D). Colonization of northern jointvetch stems by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. **A)** Mycelium of the fungus within a pith ray in a stem cross-section (×600). **B)** Colonization of host tissue near the cambium region (×500). **C)** Sporogenous hyphae beneath the epidermis of the stem at the edge of a lesion (×670). **D)** Cross section through an acervulus collected 4 days after inoculation. Epidermal and cortical regions are necrotic (×620). Legend: ac = acervulus, C = cambium, E = epidermis, h = hyphae, PR = pith ray, s = spores, and sh = sporogenous hyphae. Bars represent 10 μm.

appressoria when viewed under phase contrast conditions, papillae were not observed beneath appressoria (1, 2).

Ramification.—After the epidermal cell wall was penetrated, the intermediate swellings beneath the appressoria rapidly grew into infection hyphae which penetrate adjacent cells (Fig. 1-C). The cytoplasm of the infected cells was visibly disrupted and disorganized by 48 to 72 hr after inoculation. Cells adjacent to infected cells, however, were not disrupted. Hyphae were visible within cortical, cambial, and pith ray tissues by 96 hr after inoculation (Fig. 2-A,B). Brown material, which was observed in a few xylem vessels 3 days after inoculation, usually was associated with the presence of hyphae in the cortex adjacent to the discolored areas in the xylem. Within 4 days after inoculation, plant tissues beneath the surface of the lesion had collapsed and mycelial colonization of epidermal, cortical, vascular, cambial, and pith ray tissues was extensive. Hyphae were observed beneath the epidermis at this time on the edges of the lesions (Fig. 2-C). These hyphae appeared to be sporogenous, producing the acervuli that did not contain setae. Sporulation occurred on the lesion surfaces by 4 days after inoculation. Sporulation was abundant, but occurred only on the lesions surface (Fig. 2-D).

DISCUSSION

Colletotrichum gloeosporioides f. sp. *aeschynomene* is highly pathogenic to northern jointvetch as evidenced by the rapid rate of infection and colonization of host tissues. Lesions girdled the plant stem and killed the seedlings. Coalescence of adjacent lesions hastened girdling and death of the seedling.

The fungus has several attributes that contribute to its efficacy as a biological control agent in field environments. Spores of the fungus germinated and produced appressoria within 4 to 5 hr after inoculation. In this regard, it is similar to several other species of *Colletotrichum* that produce appressoria soon after inoculation (3, 9, 11, 12, 16, 17). All *Colletotrichum* species, however, do not produce appressoria as quickly as the northern jointvetch anthracnose fungus; *C. pisi* Pat. requires 26 hr to produce appressoria after inoculation (1). A second attribute of *C. gloeosporioides* f. sp. *aeschynomene* is that it penetrated the stem of northern jointvetch directly via the appressoria or through the bases of the numerous trichomes on the stems. Infection developed more rapidly at the trichomes than at the other sites, and initial symptoms of infection always were associated with trichomes. Appressoria were not observed at the trichome infection sites in whole-mounts nor in stained sections. However, other species of *Colletotrichum* such as *C. phomoides* (Sacc) Chester infect plants by appressorial infection of trichomes (7).

Few appressoria found on the plant surfaces actually produced infection hyphae even after 72 hr, although appressoria were produced within 4 to 5 hr after inoculation. Likewise, previous research reported relatively few infections from appressoria produced by *C. gloeosporioides* on citrus fruits (3) or from *C. pisi* on garden pea (15). Inhibition of appressoria penetration may have been caused by unfavorable incubation temperatures. Skoropad (19) reported that infection by appressoria of *C. graminicola* (Ces) Wils. occurred only within a very narrow range of temperatures, much narrower than temperatures required to stimulate appressoria formation on plant surfaces. The research reported herein was conducted at a constant temperature of 28 C; other temperatures may increase or decrease infection of plant cells by appressoria.

Penetration of the epidermis of northern jointvetch by appressoria did not occur until 48 hr after inoculation. Usually, at this time only one epidermal cell was infected. Many species of *Colletotrichum*, other than *C. gloeosporioides*, penetrate their hosts much earlier (10, 13, 17). Stomates, although abundant on the stems of northern jointvetch were not penetrated by appressoria as was reported for *Colletotrichum lindemuthianum* (Sacc. & Moan) Scribner (6).

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