Histopathology of *Cercospora sojina* in Soybean Seeds

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ABSTRACT

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Soybean seeds were collected from plants either uninoculated or inoculated separately with one of eight isolates of *Cercospora sojina*. Seeds infected by *C. sojina* were discolored gray to dark brown. Histopathological and scanning electron microscope studies showed the presence of hyphae of *C. sojina* within the seed coat tissues of seeds from plants inoculated with all but one isolate. The fungus penetrated seeds both indirectly through pores and cracks in the seed coat and directly through hilar tracheids. In seeds inoculated with four of the isolates and in infected seeds from naturally inoculated plants, hyphal mats in parenchymatous seed coat tissues as well as hyphal aggregates, which varied in size and number, were associated with fungal hyphae. Hyphal aggregates were abundant in the hilar region, moderately common in the seed coat layers, found occasionally on the seed surface and in the space between the seed coat and embryo, and rarely observed in the hypocotyl-radicle axis. Fungal infection was not found in the cotyledons. Hyphae without hyphal aggregates were found in seeds from plants inoculated with three of the isolates.

Additional key words: *Cercospora kikuchii*, *Glycine max*, *Phomopsis* spp.

Many soybean (*Glycine max* (L.) Merr.) pathogens are seedborne (8,11). *Cercospora sojina* Hara (syn. *C. daizu* Miura), causal agent of frogeye leaf spot of soybean, is seedborne and reduces seed quality because of seed discoloration (7,10,14). The disease is found worldwide and causes yield reductions in the U.S. of 12–15% (6). Soybean seeds infected with *C. sojina* develop conspicuous light to dark gray to brown areas that vary from minute specks to large blotches covering the entire seed coat. Some lesions show alternating bands of light and dark brown. Occasionally, brown and gray lesions diffuse into each other. Usually the seed coat cracks or flakes. The symptoms are distinct from those produced on soybean seeds by *C. kikuchii*, *Colletotrichum truncatum*, the *Diaporthe-Phomopsis* complex, and *Fusarium* (11). No studies have been reported on the host-parasite relationship between *C. sojina* and soybean seed tissues. We present results from scanning electron microscopy and histopathological studies on the penetration and distribution of seven isolates of *C. sojina* in soybean seeds harvested from plants inoculated separately in the field with different isolates of the fungus.

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MATERIALS AND METHODS

The soybean seeds came from samples from studies by Yorinori (13–15) and were preserved for 3 yr in test tubes under ambient laboratory conditions. Seeds from a variety of cultivars were examined under a dissecting microscope and sorted into those with and without symptoms caused by *C. sojina*. The seed lots came from field-grown plants that each had been spray-inoculated 34, 40, 47, and 52 days after emergence with a conidial suspension (13) of one of eight isolates of *C. sojina* labeled F2, MS14, TN1, TN2, TN4, LA1, LA2, and LA5 (which are ATCC 44531, 44083, 44084, 44085, 44087, 44088, 44089, and 44090, respectively) at Urbana.

To verify the presence of *C. sojina*, randomly selected seeds with and without symptoms of infection by *C. sojina* were surface

sterilized with 0.5% NaOCl (10% Clorox), washed with three changes of sterile deionized water, and plated on acidified (pH 4.5) potato-dextrose agar (PDA; Difco) at five seeds per 9-cm-diameter plate before and after the histopathology studies.

Studies of whole tissues. Twenty-five seeds each of asymptomatic and symptomatic seeds from plants either uninoculated, naturally infected, or inoculated separately with one of the isolates of *C. sojina* were used. The seeds were boiled in deionized distilled water for 2–3 hr and then dissected into tissue groups of seed coats, endosperms (aleurone layer), cotyledons, and hypocotyl-radicle axes. Each tissue group was cleared and stained by boiling in lactophenol (5) and trypan blue (5:1, V:V) for 5–10 min in a test tube. Seed coats, aleurone layers, and hypocotyl-radicle axes were mounted separately on microscope slides, cotyledons were squashed under a coverslip, and all were observed under bright-field microscopy.

Histopathology. For microtome sectioning, 35 seeds of each lot were boiled in water, fixed in 70% ethanol for 48 hr, dehydrated through a tertiary butyl alcohol series, and embedded in paraffin (Paraplast; Sherwood Medical Industries, Inc., St. Louis, MO) containing 2–5 g of beeswax per 500 g and filtered through sterile, absorbant cotton. One or two transverse incisions were made with a razor blade into each seed to assure dehydration, infiltration, and embedding. After solidification, the paraffin blocks were trimmed to expose the tissues. The blocks were softened by immersion in aqueous 1% sodium lauryl sulphate for 24 hr, then washed in water and transferred to a mixture of glycerol and glacial acetic acid (1:1) for 7 days (12). Serial microtome sections were cut 10–20 μm thick and stained with safranin and light green (5). All stained sections were mounted in Canada balsam.

Scanning electron microscopy (SEM). Only symptomatic seeds from plants either naturally infected or inoculated with isolate LA1 were used. The seed coats were removed after being soaked in water for 1 hr at 60–70 C. Pieces of tissue 2–3 mm square were cut at random from the seed coats and cotyledons, washed in 0.1 M phosphate buffer (pH 7.2), and fixed for 4 hr in 4% glutaraldehyde. Tissues from the hilar region and embryo axes were fixed separately. Each specimen was fixed for SEM in 1% osmium tetroxide for 24 hr at 4 C, dehydrated in an ethanol series, dried in a critical-point drier, mounted on an aluminum stub with Tube Koat (T. Pella, Inc., Tustin, CA), and sputter-coated with gold-palladium 30 nm/40 sec (4). Observations were made at 15–17 kV under a JEOL J.S.M.-U3 SEM, Center for Electron Microscopy, UIUC.

RESULTS

Studies on whole seeds. Seeds from plants inoculated with isolates LA1, LA2, TN2, and TN4 showed light to dark gray discoloration usually with a brown cast about the hilum and cracking perpendicular to the hilum. Concentric alternating light and dark rings were noted (Fig. 1). Some seeds were papillate as described by Sherwin and Kreitlow (10). Seeds from plants inoculated with isolates F2, LA5, and TN1 showed a light brown discoloration and those with MS14 showed no discoloration for all cultivars. Asymptomatic seeds were noninfected and were used as controls in the histopathological studies. Occasionally, seeds from uninoculated plants had small gray specks with brown margins on the seed coat, other than in the hilar region, and cracks associated with the discolored areas. No fungus other than *C. sojina* was recovered from seeds plated on PDA before or after the histological studies.

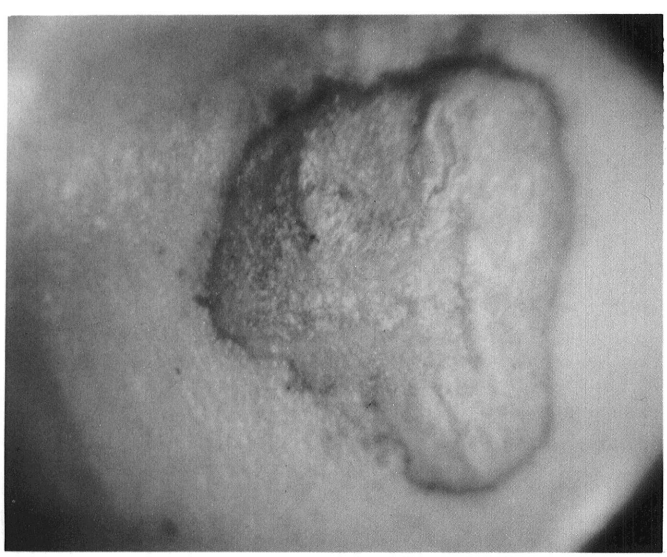

Fig. 1. Soybean seed showing symptoms of infection by *Cercospora sojina*.

Studies on whole tissues. Soybean seeds consist of a seed coat, endosperm (aleurone layer and parenchymatous cells) and an embryo made up of two large, fleshy cotyledons, a plumule, and a hypocotyl-radicle axis (1). The seed coat has three layers: epidermis (palisade cell layer), hypodermis (hourglass cell layer), and a parenchyma cell layer (1).

Hyphae of *C. sojina* could be distinguished from those of other fungi found in soybean seed coat tissues by comparing hyphal widths and reaction to stains (3,9,12; and I. K. Kunwar, T. Singh, and J. B. Sinclair, *unpublished*). The hyphal width of *C. sojina* ranges from 0.8 to 1.6 μm, that of *C. truncatum* from 3 to 11 μm, and that of *Phomopsis* from 3.8 to 8.7 μm. Immature hyphae of *C. sojina* are light green when stained with safranin and light green and blue with trypan blue. Mature hyphal cells of *C. sojina* appear dark brown without staining and the cytoplasm in hyphal cells occasionally stains light green when stained with safranin and light green. Mature hyphae of *C. sojina* do not take trypan blue stain.

Hyphae of *Alternaria*, *C. truncatum*, *Fusarium*, and *Phomopsis* were not found in any of the tissues studied. In other studies, mature and immature hyphae of *Alternaria* were dark brown without staining. Mature hyphae of *C. truncatum* were brown without staining and contained oil globules, immature hyphae appeared green when stained with safranin and light green or blue with trypan blue. Immature and mature hyphae of *Fusarium* (12) and of *Phomopsis* were hyaline; they stained green when stained with safranin and light green and blue with trypan blue.

No hyphae were observed in asymptomatic seeds or in seeds from plants inoculated with isolate MS14. Hyphae typical of *C. sojina* and hyphal aggregates were observed in the seed coats of seeds from naturally infected plants and in seeds from plants inoculated with isolates LA1, LA2, TN2, and TN4, but only hyphae were found in the aleurone layers. Only hyphae were observed in the seed coats from plants inoculated with isolates F2, LA5, and TN1. Hyphae were not found in any embryo tissues of any other seed sample.

Histopathology and scanning electron microscopy. In bright-field microscopy of microtome sections and in SEM, hyphae and hyphal aggregates of isolate LA1 of *C. sojina* were found on the seed coat surface of infected seeds at many locations, but particularly near and in the hilar region (Figs. 2B and 3D). Hyphae penetrated through the seed coat pores (Fig. 2A), the palisade layer (Figs. 2C and 3A and B) and hilar tracheids (Figs. 2B and 3D). Abundant hyphae of *C. sojina* and their aggregates were observed in the hilar region of some seeds (Fig. 3D), in the hilum and seed coat palisade cell layers, and aggregated in the stellate parenchyma where the host cells appeared to have disintegrated (Fig. 3D). Hyphae were observed in the hilum tracheids and were more abundant in the top then in the base (Fig. 3E), and the stellate parenchyma surrounding the tracheids had disintegrated (Fig. 3C and D).

At certain locations, the seed coat was eroded and the hypodermis was exposed and hyphae could be observed in that cell layer. Hyphae were abundant in the palisade cell layer (Fig. 3A and B), and in the lumen and intercellular spaces of the hypodermis (Figs. 2D and 3A). Hyphae of *C. sojina* in seed coat tissues stained dark brown and formed thick, brown hyphal mats in the parenchymatous region of the seed coat, and nearby host cells were lysed (Fig. 3A). The hyphae penetrated the seed coat tissues, grew inter- and intracellularly along the long axes of the cells of the palisade cell layer, irregularly in the hypodermis, and along the

parenchymatous cell layer. Hyphae penetrated the aleurone layer, which appeared to remain intact, but the cytoplasm of some cells had coagulated, producing a vacuole-like space which was not seen in the noninfected seeds.

Hyphal aggregates were observed in the tissues of seeds from naturally infected plants and from plants inoculated with isolates LA1, LA2, TN2, and TN4, but not in tissues of asymptomatic seeds or in seeds from plants inoculated with other isolates. Hyphal aggregates were formed generally in the hypodermis directly below the lower surface of the palisade layer and occasionally in the palisade cell layer (Fig. 3A). Cells in the palisade cell layer near hyphal aggregates always appeared lysed (Fig. 3A). Cells of the hypodermis were not affected. Some seeds had hyphal aggregates in the seed coat tissues but not on the seed surface (Fig. 3B). Hyphal aggregates were larger and more numerous within seed coat tissues than on the seed surface, and were spherical and ranged from 56 to 89 μm in diameter when within the seed tissues. Hyphal aggregates in seeds from plants inoculated with LA1 were more numerous and larger (100.8–156.8 μm in diameter) than those in seeds from plants inoculated with isolates LA2, TN2, and TN4. Hyphal aggregates occurred less frequently and were smaller in seeds from naturally infected plants than in seeds from inoculated plants.

Colonization of embryo tissues by *C. sojina* was found in two seeds from plants inoculated with isolate LA1; both seeds had

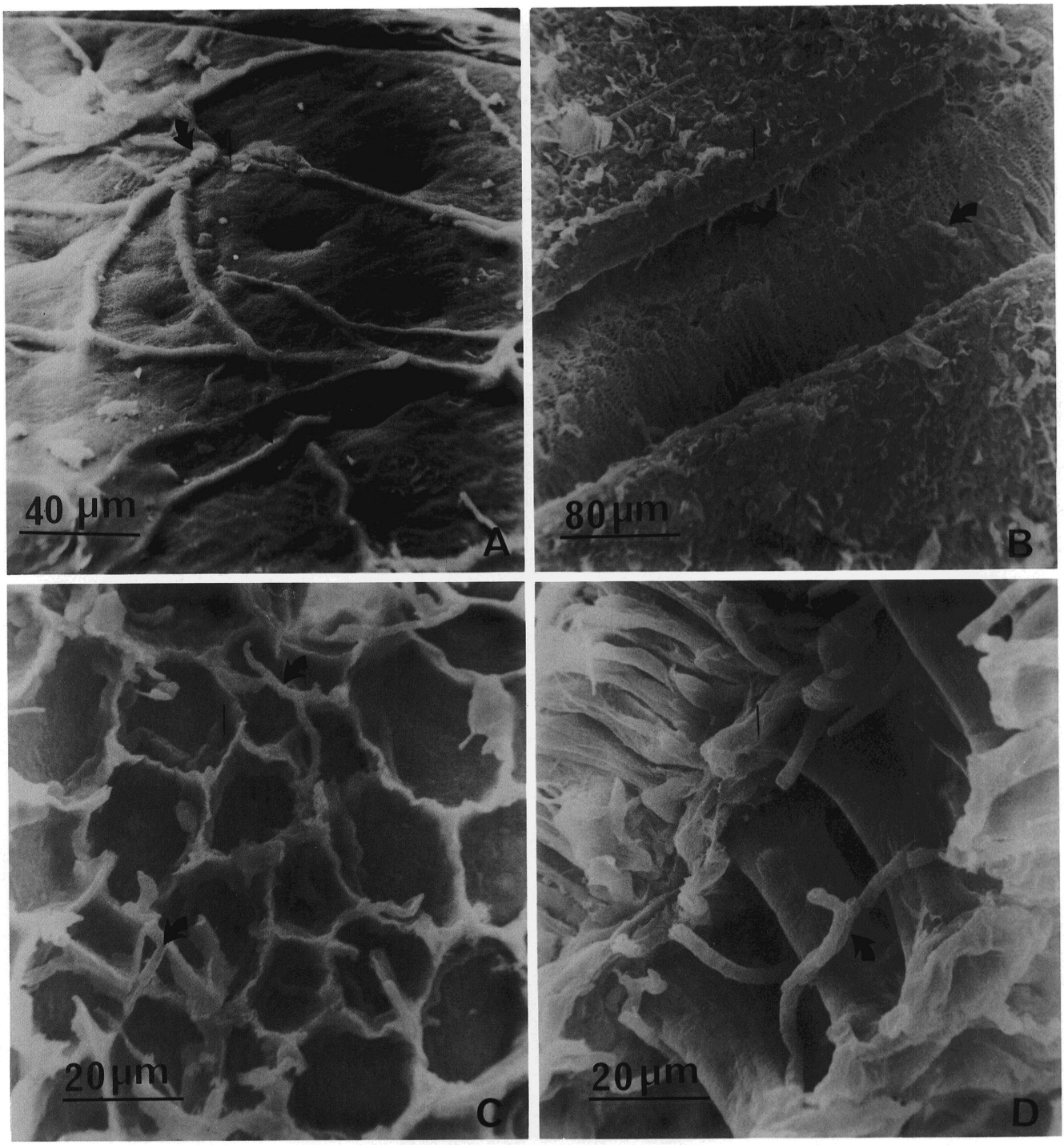

Fig. 2. Scanning electron photomicrographs of soybean seeds from plants inoculated with isolate LA1 of *Cercospora sojina*: **A,** hyphae (arrows) on the seed coat surface and hyphae penetrating through seed pores; **B,** hyphae (arrows) in hilar region and in hilar tracheids; **C,** tangential section showing hyphae (arrows) colonizing the honeycomblike palisade cell layer; and **D,** cross section of a seed coat showing hyphae (arrow) in the hypodermal (hourglass) cell layer.

hyphae in the embryonic axis. Cells near the colonized region either lysed or had coagulated cytoplasm leaving vacuole-like spaces (Fig. 3F and G). The walls of such cells were thickened and stained red. In some seeds, hyphal aggregates occurred in the space between the aleurone layer and hypocotyl-radicle axis (Fig. 3B). No hyphae or aggregates were observed in cotyledonary tissues in histology studies, but in SEM studies, hyphae were observed on the surface of cotyledonary tissue.

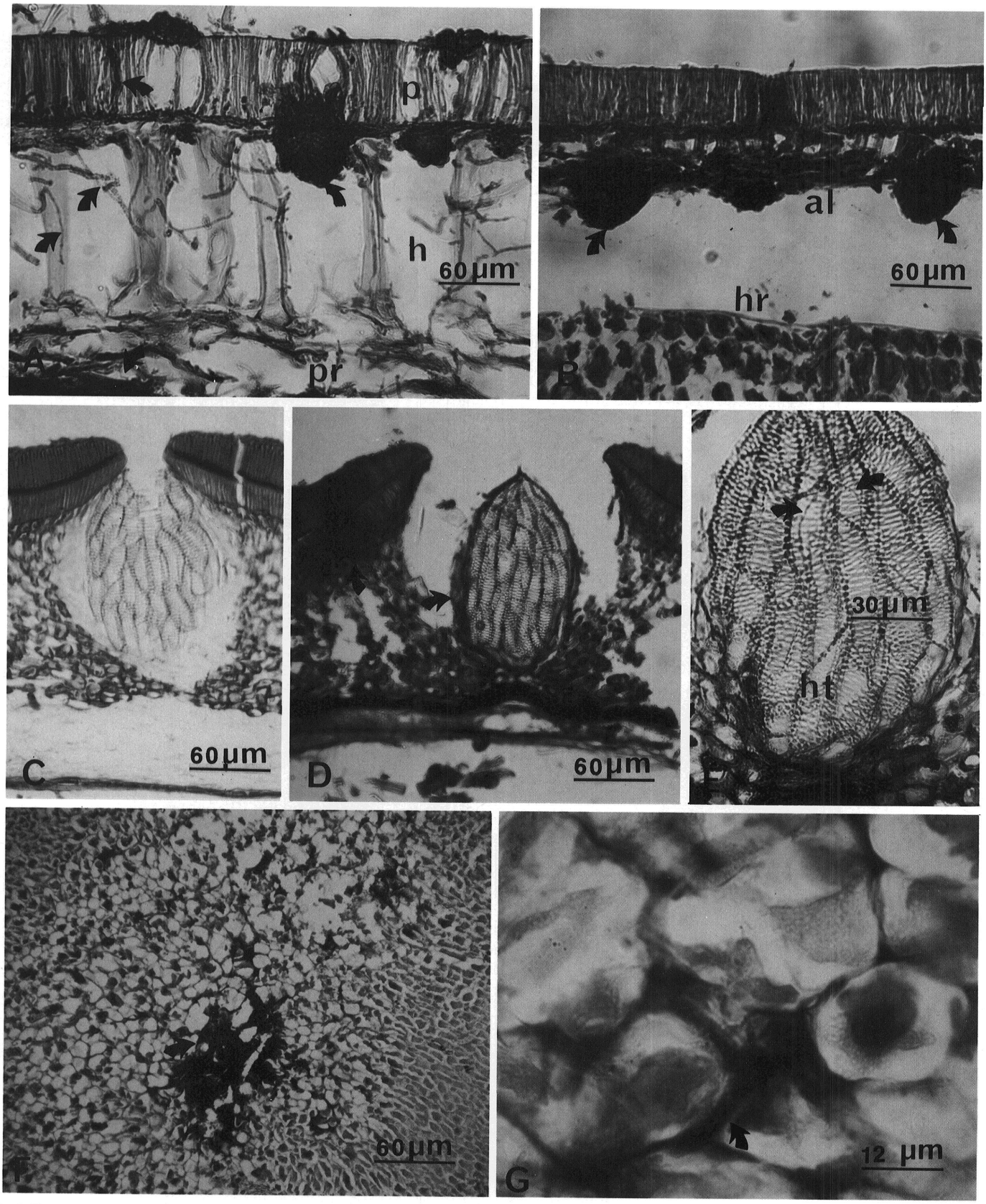

Fig. 3. Transverse sections of soybean seeds from plants inoculated with isolate LA1 of *Cercospora sojina*: **A,** hyphae (arrow) and hyphal aggregate (arrow) in palisade layer (p), hypodermal layer (h) and hyphal mat (arrow) in parenchyma (pr); **B,** hyphae and hyphal aggregates (arrows) in the space between the aleurone layer (al) and hypocotyl radicle axis (hr); **C,** hilar region of a noninfected seed; **D,** hilar region of an infected seed showing hyphae and hyphal aggregates (arrows); **E,** hyphae (arrows) in the hilar tracheids; **F and G,** hyphae (arrows) in hypocotyl-radicle axis.

DISCUSSION

This is the first description of the penetration and colonization of soybean seeds by *C. sojina*. The SEM studies show that the fungus can penetrate through seed coat pores and cracks or through hilar tracheids. Penetration through soybean seed pores was similar to that described by Hill and West (2), who did not identify the fungus involved. Bright-field microscopy showed that fungus hyphae appeared to colonize the hilar tracheids and stellate parenchyma, then spread into the other tissues of the seed coat. The concentration of hyphal aggregates at the hilar region may be due to the high moisture content of these tissues and the high level of gaseous exchange that takes place during seed development (1). The variation in occurrence among seeds and amount of hyphal aggregates in a seed may be due to environment-related causes, differences among cultivars, or variability among isolates of the test fungus.

The hypodermal and parenchymatous cell layers contain stored protein and other nutrients (9), which may account for the heavy colonization of these tissues by the fungus. The pattern of colonization is similar to that described for *Phomopsis* in soybean seeds (3). Sherwin and Kreitlow (10) suggested that the discoloration caused by *C. sojina* in soybean seeds frequently extended beneath the seed coat and into the cotyledons and embryo and assumed that these tissues were infected. We did not find hyphae in the cotyledonary tissues and found incipient infection of the hypocotyl-radicle in only two seeds and only from plants inoculated with isolate LA1.

The highest seed transmission (33%) and reduction in 100-seed weight (28%) of the various isolates was obtained from the 29 cultivars inoculated with isolate LA1 (14). We found that seeds from plants inoculated with isolate LA1 had larger and more hyphal aggregates than those from plants inoculated with any of the other seven isolates of *C. sojina*.

Of the eight isolates, the least reduction in 100-seed weight (5.7%), the lowest seed transmission (1%) and reduction in seed number was from 29 cultivars inoculated with isolate MS14 (14). We did not observe hyphae of *C. sojina* in any seeds from plants inoculated with MS14 probably because of its low seed transmission.

The establishment of *C. sojina* in soybean seeds differs from that described for *C. kikuchii* (Mats. & Tomoy.) M. W. Gardner and *Phomopsis* (3), in that *C. sojina* is restricted to the seed coat tissues and the space between the seed coat and embryo with the exception of two seeds inoculated with isolate LA1. The fungus may grow into seedling tissues during germination and emergence.

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