

## Penetration and Infection of Soybean Leaf Tissues by *Colletotrichum truncatum* and *Glomerella glycines*

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### ABSTRACT

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Conidial germination, infection peg penetration, and establishment of the anthracnose-causing fungi (*Colletotrichum truncatum* and *Glomerella glycines*) on and in soybean (*Glycine max*) leaf tissues, was studied by using bright-field microscopy and scanning electron microscopy. Conidial suspensions of both fungi were atomized onto soybean leaves in a mist chamber at ambient temperature ( $24 \pm 2$  C). Seventy percent of conidia of *C. truncatum* germinated within 4 hr; 25 and 40% of the conidia of *G. glycines* germinated at 6 and 12 hr, respectively. Conidia of both fungi germinated terminally and subterminally with germ tubes that developed appressoria. The germ tubes of both fungi were significantly ( $P = 0.05$ ) longer when conidia germinated on midribs than on leaf laminae. One and

sometimes two appressoria developed from a single germ tube of conidia of *C. truncatum*. Penetration of epidermal cells by infection pegs from appressoria of both fungi was common. Indirect penetration through stomatal openings or direct penetration of guard cells was rare. Hyphae were observed in and between mesophyll cells at 2 days and in the vascular elements 3 days after inoculation. Acervuli were produced by conidia of *C. truncatum* and *G. glycines* at 48 and 72 hr, respectively, and were common on leaf veins and petioles. Discrete veinal necrosis of inoculated leaves was evident only with conidia of *C. truncatum* at 30 hr after inoculation of plants.

*Additional key words:* *Colletotrichum capsici*, *C. fragariae*, *C. gloeosporioides*.

Two anthracnose fungi are associated with soybean [*Glycine max* (L.) Merr.], *Colletotrichum truncatum* (Schw.) Andrus & W. D. Moore and *Glomerella glycines* (Hori) Lehman & Wolf. Both fungi are endophytic. Anthracnose occurs wherever soybeans are grown and can cause significant reduction in stand, seed quality, and yield in the warm, humid tropics and subtropics (13). The disease occurs throughout the U.S. soybean production areas and causes yield reductions of 20% or more in the southern growing areas (2). *C. truncatum* is seedborne (11-13). Staples et al (14) studied appressorium development in *C. truncatum* but not its host penetration.

We studied conidial germination, infection peg penetration, and establishment of *C. truncatum* and *G. glycines* in soybean leaf tissues. No previous studies have been made on the establishment of these two fungi in soybean tissues. Such studies are important in understanding disease initiation by these two fungi.

### MATERIALS AND METHODS

**Inoculation.** Certified Corsoy 79 and Williams 79 soybean seeds (Illinois Foundation Seeds, Inc., Tolono, IL) known to be susceptible to *C. truncatum* and *G. glycines* were placed in 22-cm-diameter clay pots filled with steamed field soil and after emergence were thinned to three seedlings per pot. Seedlings were maintained in a greenhouse until inoculation. Seedlings in the VI growth stage (5) (first trifoliolate leaf) were inoculated with either water alone (control) or aqueous conidial suspensions containing conidia of

either *C. truncatum* or *G. glycines* at  $5.0 \times 10^6$  conidia per milliliter. One isolate of each fungus was used and both were isolated from soybean pods. Seedlings were sprayed until just before runoff and placed in a mist chamber with 12-hr alternating fluorescent-lighted ( $800 \mu\text{Ein}/\text{m}^2/\text{sec}$ ) and dark periods programmed for one 15-min mist application per hr at  $24 \pm 2$  C. There were five replicates (pots) per treatment. Leaflets were harvested at 4, 6, 8, 12, 18, 24, 36, 48 and 72 hr after inoculation. The experiment was done twice.

**Whole leaves.** Leaf samples were cleared and stained by boiling in lactophenol (7) and 1% aqueous solution trypan blue (5:1, v/v) for 5-10 min in a test tube. The cleared leaves were mounted on microscope slides, covered with a coverslip and studied under a bright-field microscope.

**Scanning electron microscopy.** Uninoculated and inoculated soybean leaflets were washed in 0.1 M phosphate buffer, pH 7.2, and leaf pieces 2-4 mm<sup>2</sup> were cut randomly from leaves and again washed in the buffer solution. Leaf tissues were fixed in 4% gluteraldehyde in 0.1 M phosphate buffer for 4 hr at ambient temperature ( $24 \pm 2$  C). Each specimen for scanning electron microscopy was fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 24 hr at 4 C, dehydrated in an ethanol series for 5 min per step, dried in a critical-point dryer, mounted on aluminum stubs with a TV tube coat, and sputtercoated with gold-palladium (40/60) (3 nm during 40 sec) (6). Observations were made at 10 or 12 kV under a JOEL J.S.M. U3 scanning electron microscope at the Center for Electron Microscopy, UIUC.

**Histopathology.** Leaflets for microtome sectioning were cut into 1-cm pieces, washed in 0.1 M phosphate buffer, fixed in formaldehyde:acetic acid:50% ethanol (5:5:90, v/v) (FAA) 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 50 g of paraffin filtered through sterile absorbent cotton. Sections 6-14  $\mu\text{m}$  thick were cut and stained separately with safranin, then light

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green (7). All stained slides were mounted in Canada balsam and studied under bright-field microscopy.

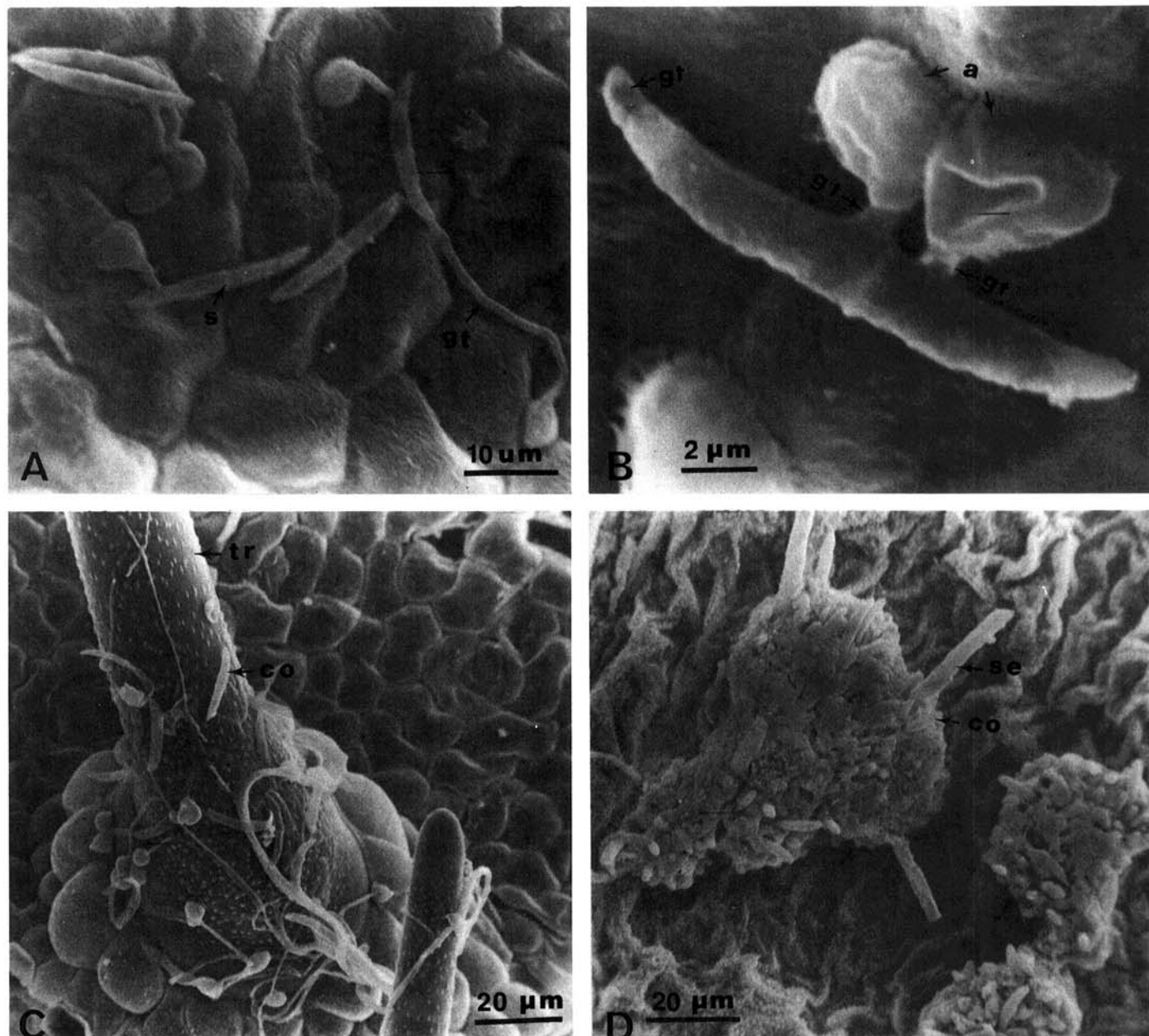
## RESULTS

**Symptoms.** Discrete veinal necrosis was evident in both the cultivars on inoculated leaves 30 hr after inoculation with conidia of *C. truncatum*, which became more pronounced until the leaves dropped at about 48 hr after inoculation. Plants of both the cultivars inoculated with conidia of *G. glycines* developed infrequent grayish, round-to-irregularly shaped lesions, 3–8 mm in diameter with dark margins, on the leaf lamina 48 hr after inoculation.

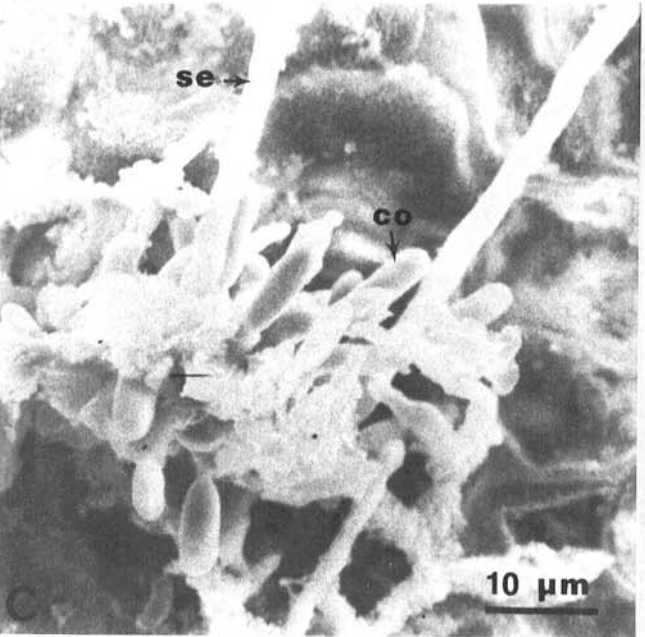
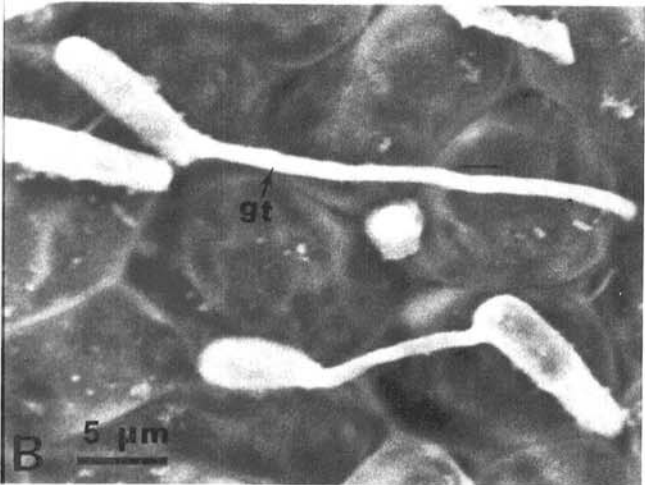
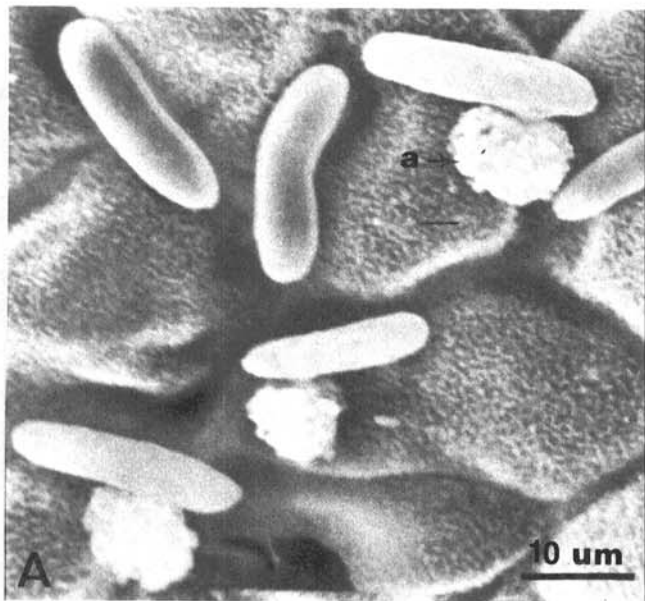
**Scanning electron microscopy.** In both cultivars, ungerminated conidia of *C. truncatum* on plant parts appeared as nonseptate single cells, but during germination, one septum and occasionally two or three septa developed in each conidium. Germ tubes were formed at one or sometimes at both ends and more rarely at the middle (Fig. 1A and B). Seventy percent of the conidia of *C.*

*truncatum* germinated on the leaf cuticle within 4 hr after inoculation and produced appressoria, which ranged from clavate to globose to spherical. Germinating conidia produced a mucilaginous substance that became most conspicuous after germ tube emergence. Noncellular, fibrous, mucilaginous strands were associated with regions of contact between appressoria and cuticle. Germ tubes rarely developed more than one appressorium. Germ tubes without appressoria appeared to penetrate the cuticle directly. Appressoria generally were delimited by a septum, but in some instances no such structure formed. More conidia germinated when in clumps than when isolated or in small numbers. Appressoria were formed when conidia were associated with epidermal cells over midveins and multicellular trichomes (Fig. 1C) and only rarely when conidia were on stomatal guard cells or unicellular trichomes.

Similar observations were recorded in leaf samples of both cultivars taken at 6, 8, 12, and 24 hr after inoculation with conidia of *C. truncatum*. No significant differences were noted in the percentage of germinated conidia of *C. truncatum*. After 36 hr,



**Fig. 1.** Scanning electron photomicrographs of soybean leaves showing germination, appressorium formation, and sporulation by conidia of *Colletotrichum truncatum*. **A**, Septum (s) formed in conidia and germ tube (gt) growing from a conidium during germination; **B**, three germ tubes (gt) and two appressoria (a) formed by a conidium; **C**, germinating conidia (co) at the base of the trichome (tr); **D**, acervuli with conidia (co) and setae (se).

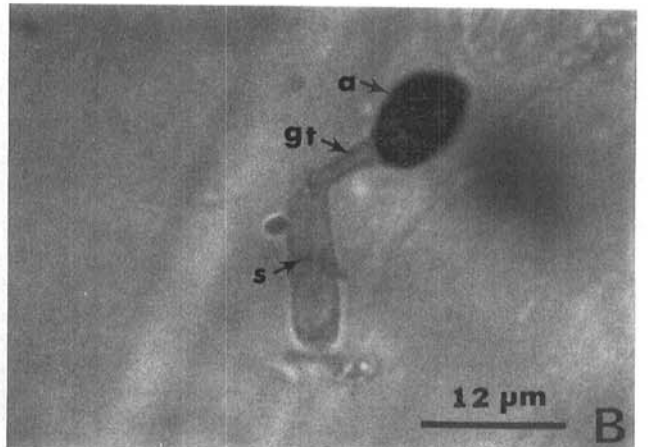
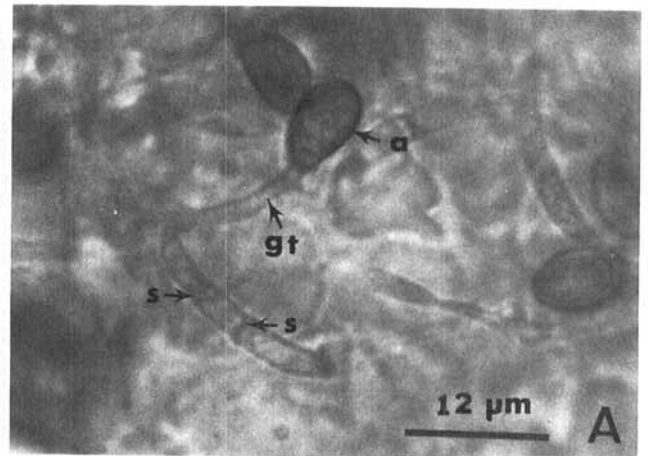


**Fig. 2.** Scanning electron photomicrographs of soybean leaves showing germination, appressorium formation and sporulation by *Glomerella glycines*. **A**, Germinating conidia with appressoria (a); **B**, conidia with long germ tubes (gt); **C**, acervulus with conidia (co) and setae (se).

acervulus initials were observed. Mature setose acervuli formed between 48 and 72 hr (Fig. 1D). Infected epidermal cells began to collapse after 36 hr and were entirely collapsed within 3 days of inoculation. Acervuli generally were more common on petioles and leaf veins than on the interveinal lamina.

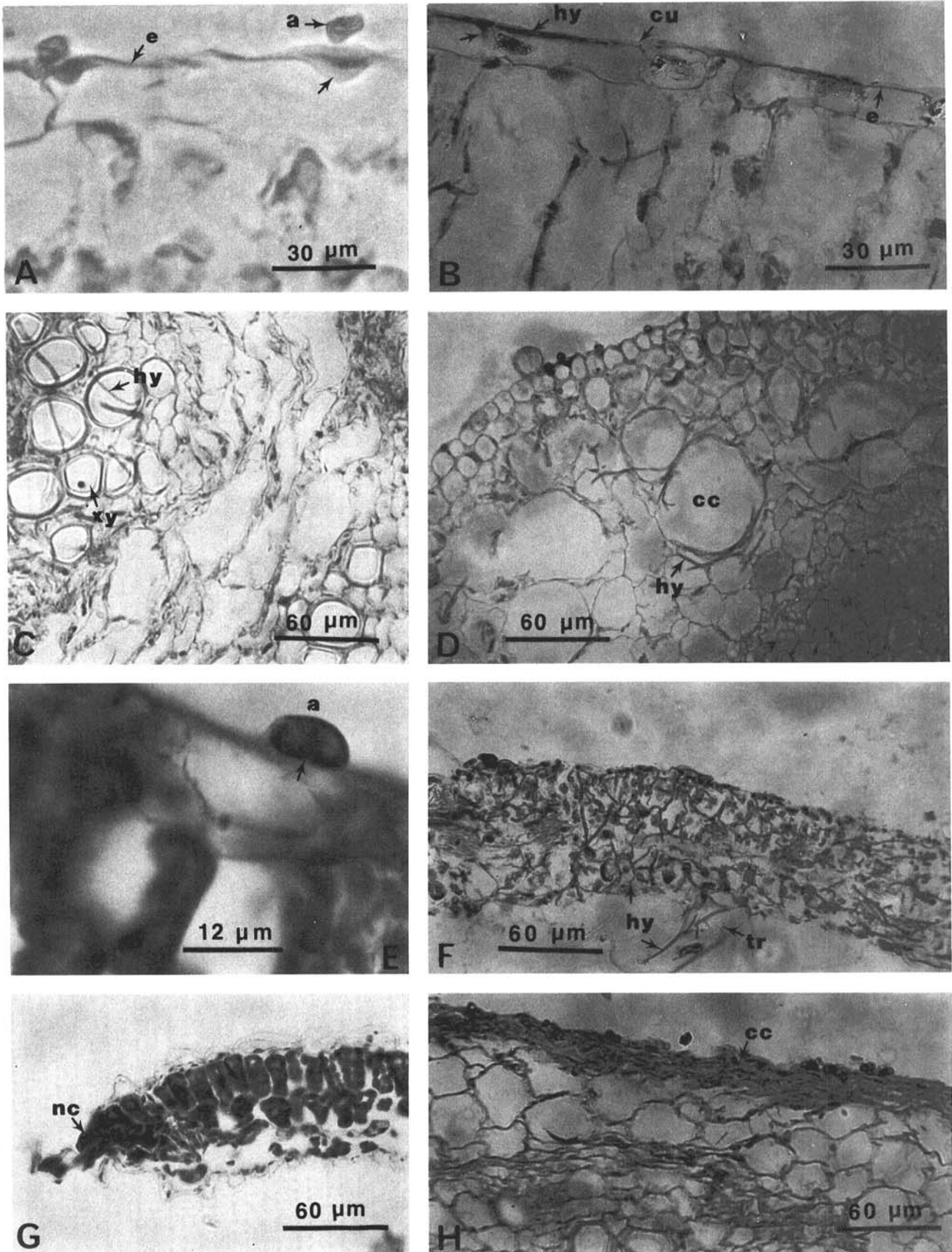
Conidial germination of *G. glycines* (Fig. 2A and B) was observed within 4 hr, with 25% of the conidia germinated at 6 and 40% at 12 hr after inoculation in both cultivars. Conidial germination and germ tube formation was similar to that for conidia of *C. truncatum*. Appressoria were delimited by a septum, and septa were observed in the germ tube. Appressoria developed more frequently from conidia on the epidermis and leaf veins and less frequently on guard cells and trichomes. Similar observations were recorded at 12, 18, and 24 hr. Acervuli developed after 72 hr (Fig. 2C). More acervuli formed along the leaf vein and on the petioles than on the leaf blade.

**Bright-field microscopy.** Observations on conidial germination from the SEM studies were confirmed for both fungi in both cultivars by using bright-field microscopy (Fig. 3A and B). Based on 20–30 measurements each, germ tubes formed by germinating conidia of *C. truncatum* and *G. glycines* were significantly longer ( $P=0.05$ ) (15.2 and 10.9  $\mu\text{m}$ , respectively) on leaf midribs than on leaf laminae (2.5 and 2.4  $\mu\text{m}$ , respectively). Direct penetration of the cuticle and epidermal cell walls by infection pegs was observed. Hyphae from appressoria occasionally grew through stomatal openings, but more frequently penetrated directly. At the site of appressorial development, the epidermal cell wall thickened and turned dark, and a lesion developed (Fig. 4A). Hyphae from appressoria generally penetrated the upper tangential and radial walls of epidermal cells. Infection pegs formed infection hyphae between the cuticle and outer epidermal cell walls (Fig. 4B).



**Fig. 3.** Germinating conidia on the surface of cleared leaf lamina showing one or two septa (s), germ tube (gt) and appressoria (a). **A**, *Colletotrichum truncatum*; **B**, *Glomerella glycines*.





**Fig. 4.** Photomicrographs of transverse sections of soybean leaf tissues stained with safranin and light green showing germination and penetration by *Colletotrichum truncatum* and *Glomerella glycines*. **A**, At the point of contact between the appressorium (a) of *C. truncatum* and epidermis (e), upper tangential epidermal cell wall thickened, dark, and developing a lesion (arrow); **B**, hypha (hy) of *C. truncatum* between the cuticle (cu) and upper tangential and anticlinal (arrow) epidermal cell wall (e) in the cotyledon; **C**, hyphae (hy) of *C. truncatum* in the xylem vessels (xy) of the petiole; **D**, hypertrophied cortical cells (cc) of the petiole surrounded by hyphae (hy) of *C. truncatum*; **E**, of appressorium (a) of *G. glycines* showing the thin wall (arrow) at point of contact with the epidermis; **F**, abundant hyphae (hy) of *G. glycines* in the lamina and trichome (tr); **G**, necrotic cells (nc) in the margin of lamina inoculated with *C. truncatum*; **H**, collapsed cortical cells (cc) in the petiole inoculated with *C. truncatum*.

Hyphae grew through the anticlinal walls. At an advanced stage of colonization, infected cells showed wall thickening and cytoplasmic plasmolysis. After 2 days, inter- and intracellular hyphae were observed in the mesophyll cells. In some sections, hyphae could be seen in the vascular elements of the leaf and petiole (Fig. 4C). Cells in the margin of laminae were necrotic (Fig. 4G). No tyloses were observed. No papillae were observed (1).

There was a tendency for conidia of *C. truncatum* in both cultivars to accumulate around the bases of trichomes, produce appressoria and penetrate. Hyphae penetrated the cortex and vessels of seedling stems and petioles. When hyphae were abundant in cortical cells, the cells appeared hypertrophied (Fig. 4D), and often collapsed (Fig. 4H). Hypertrophied and collapsed cells were never observed in control tissues. Heavy colonization of petioles and stems resulted in seedling death.

The germination and infection processes of *G. glycines* differed little from those of *C. truncatum* except that usually fewer hyphae developed in tissues infected with *G. glycines*. In microtome sections, the walls of appressoria of *G. glycines* appeared thin at the point of contact with the host cell wall (Fig. 4E). Cells beneath appressoria were darker and sunken similarly to those infected with conidia of *C. truncatum*. Occasionally both inter- and intracellular hyphae were observed in abundance in mesophyll cells of the leaf tissues (Fig. 4F). Hyphae were seen in xylem vessels only when the tissue was disintegrated.

## DISCUSSION

There was no difference in conidial germination, appressorium and acervulus formation, penetration and colonization by both fungi in either cultivar. In both fungi, there was the tendency for a high frequency of germination of conidia associated with trichomes, formation of long germ tubes on the midrib, formation of short germ tubes on laminae, and development of acervuli along leaf veins and on petioles. These latter associations in part may be due to the large amount of free moisture associated with these structures. Conidia of *C. truncatum* and *G. glycines* germinated at 4 hr on host tissue, and the number of germinated conidia of *G. glycines* was lower than that of *C. truncatum* at any one time. The difference may be due, in part, to differences in optimum temperature for conidial germination. Staples et al (14) reported conidial germination in *C. truncatum* within 5 hr at 24 C on sterile bean extract agar, development of a central septum in conidia after germination began, and the development of germ tubes from terminals of conidia. We observed the formation of conidial septae in *C. truncatum* and *G. glycines* and the development of two or three germ tubes by *C. truncatum*. Three germ tubes also were recorded for *Colletotrichum capsici* (Syd.) Butl. & Bisby (10).

Cell damage and distortion associated with appressorium and infection peg formation suggests that enzymes or other toxic materials may be produced during infection. In the conidia of both *C. truncatum* and *G. glycines*, appressoria occasionally were directed toward and formed in a stomatal cavity, suggesting that stomata are also a site of entry. Penetration of leaf cells by the formation of appressoria and infection pegs is the more common mode of entry. Similar observations were reported for *C. gloeosporioides* Penz. on papaya (4) and citrus fruit (3). We also observed direct penetration by germ tubes. After infection, hyphae develop between the cuticle and epidermis, as also observed by Chau and Alvarez (4). Roberts and Snow (10) reported indirect penetration through stomata and direct penetration of cotton bolls by *C. capsici*.

Penetration was noted on trichomes and in the tissues surrounding the leaf veins. Hyphae were not found in vascular elements of leaf veins of young leaves, but were found in disintegrated older leaves and in the petiole. This could possibly be a characteristic of an advanced stage of infection. Milholland (9) reported that ingress of *Colletotrichum fragariae* Brooks into the crown of strawberries from petioles could be traced to spread along infected vascular tissue, and that after 14 days the intracellular spaces in the cortex were thickened, and tannin was deposited in parenchyma cells in resistant cultivars.

Mercer et al (8) reported that veinal necrosis in bean plants inoculated with *C. lindemuthianum* (Sacc. & Magn.) Scrib. was the result of preferential conidial deposition, appressorial formation, and susceptibility at veins relative to other leaf tissues. The veinal area of soybean appears to be the preferential site for invasion by conidia of *C. truncatum*, this area remains wet longer than interveinal tissues in the mist chamber.

This is the first description of penetration and establishment by conidia of *C. truncatum* and *G. glycines* in soybeans. The isolate of *C. truncatum* that was used was more vigorous than that of *G. glycines* in conidial germination, infection peg penetration, disintegration of host cells, and colonization of vascular elements. It seems doubtful that either fungus moves from one part of the plant to another through the vascular system, since hyphae appear to be localized in disintegrated tissues and were not seen in living tissues of inoculated plants.

## LITERATURE CITED

1. Aist, J. R. 1976. Papillae and related wound plugs of plant cells. *Annu. Rev. Phytopathol.* 14:145-163.
2. Backman, P. A., Williams, J. C., and Crawford, M. A. 1982. Yield losses in soybeans from anthracnose caused by *Colletotrichum truncatum*. *Plant Dis.* 66:1032-1034.
3. Brown, G. E. 1975. Factors affecting postharvest development of *Colletotrichum gloeosporioides* in citrus fruits. *Phytopathology* 65:404-409.
4. Chau, K. F., and Alvarez, A. M. 1983. A histological study of anthracnose on *Carica papaya*. *Phytopathology* 73:1113-1115.
5. Fehr, W. R., and Caviness, C. E. 1977. Stages of soybean development. CODEN, IWSRBC 80:1-12.
6. Jakstys, B. P., and Crang, R. E. F. 1982. Laboratory Techniques for Biological and Clinical Electron Microscopy. Center for Electron Microscopy, University of Illinois at Urbana-Champaign. 92 pp.
7. Johansen, D. A. 1940. *Plant Microtechnique*. McGraw-Hill, New York. 523 pp.
8. Mercer, P. C., Wood, R. K. S., and Greenwood, A. D. 1971. Initial infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Pages 381-389 in: *Ecology of Leaf Surface Micro-organisms*. T. F. Preece and C. H. Dickinson, eds. Academic Press, New York.
9. Milholland, R. D. 1982. Histopathology of strawberry infected with *Colletotrichum fragariae*. *Phytopathology* 72:1434-1439.
10. Roberts, R. G., and Snow, J. P. 1984. Histopathology of cotton boll rot by *Colletotrichum capsici*. *Phytopathology* 74:390-397.
11. Rodriguez-Marcano, A., and Sinclair, J. B. 1978. Fruiting structures of *Colletotrichum dematium* var. *truncata* and *Phomopsis sojae* formed in soybean seeds. *Plant Dis. Rep.* 62:873-876.
12. Schneider, R. W., Dhingra, O. D., Nicholson, J. F., and Sinclair, J. B. 1974. *Colletotrichum truncatum* borne within the seed coat of soybean. *Phytopathology* 64:154-155.
13. Sinclair, J. B. 1982. *Compendium of Soybean Diseases*. The American Phytopathological Society, St. Paul, MN. 104 pp.
14. Staples, R. C., Laccetti, L., and Yaniv, Z. 1976. Appressorium formation and nuclear division in *Colletotrichum truncatum*. *Arch. Microbiol.* 109:75-84.