Observations by Scanning Electron and Bright-Field Microscopy on the Mode of Penetration of Soybean Seedlings by *Phomopsis phaseoli*

MARTIN M. KULIK, Research Plant Pathologist, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705

ABSTRACT

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Cotyledons, hypocotyls, and unifoliolate leaves of soybean seedlings at growth stage V1 grown in pots in the greenhouse were inoculated by spraying with an aqueous suspension of 100,000 alpha conidia of *Phomopsis phaseoli* per milliliter. Plants were incubated in a moist chamber at 21–27 C for 24–144 hr. Scanning electron and bright-field microscopy were used to examine plant material collected at 24-hr intervals beginning 24 hr after inoculation. Conidia produced germ tubes 24–48 hr after inoculation. Germ tubes from conidia situated directly over or immediately adjacent to stomata sometimes produced appressoria. Germ tubes penetrated leaves and cotyledons via stomata but did not penetrate directly through the cuticle. Conidia were not observed on hypocotyls. A reticulum associated with conidia that may serve to anchor the conidium to the host surface also was observed.

Additional key words: soybean pod and stem blight

Phomopsis phaseoli (Desm.) Sacc. (syn. *P. sojae* Lehman; teleomorph = *Diaporthe phaseolorum* (Cke. & Ell.) Sacc.) is the principal member of the *Diaporthe/Phomopsis* disease complex of soybeans (*Glycine max* (L.) Merr.) responsible for pod and stem blight and seed decay or mold (8). This fungus is known to invade soybean plants at all growth stages, and heavy infection of seedlings often occurs (6). *P. phaseoli* is also capable of invading a number of other plants (7); however, the mode of penetration of *P. phaseoli* of host vegetative tissues is not known. An understanding of this vital step in the infection process is important not only from an empirical viewpoint but because of its usefulness in any study of host-parasite interactions. It also bears on future work in the areas of disease control, epiphytology, and plant breeding.

Means of fungal entry into plants include direct penetration by enzymatic action or mechanical pressure, through wounds, or via natural openings such as lenticels or stomata (10). The present study provides evidence that *P. phaseoli* penetrates the unifoliolate leaves and cotyledons of soybean seedlings via the stomata.

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MATERIALS AND METHODS

Inoculation. Seeds of the soybean cultivar Blackhawk were planted in pots of a synthetic soil mix in the greenhouse. Cotyledons, hypocotyls, and unifoliolate leaves of seedlings at growth stage V1 (2) were inoculated with an aqueous suspension of 100,000 alpha conidia of *P. phaseoli* per milliliter. The isolate of *P. phaseoli* used in this study came from a stem of a soybean plant grown in the field at Beltsville, MD, and conformed in its morphology to *P. sojae* sensu Lehman (8). Alpha conidia to be used as inoculum were produced on autoclaved soybean petioles in culture tubes incubated on a laboratory bench. All inoculum used in this study was produced at one time and

stored at 3 C until needed. The germination of a portion of each conidial suspension used to inoculate plants in the greenhouse was routinely checked on water agar with a bright-field microscope. Immediately after inoculation, plants were placed in a shaded moist chamber on a greenhouse bench (21–27 C) and left for 24–144 hr. Inoculated plant material was collected beginning 24 hr after inoculation and continuing at 24-hr intervals for up to 144 hr.

Electron microscopy. Plant material to be examined by scanning electron microscopy (SEM) was cut into pieces 2–3 mm and fixed in 2.5% glutaraldehyde in sodium cacodylate buffer. Vacuum was applied if sections failed to sink in the fixative solution. After 2–3 hr at laboratory temperature (20–25 C), the sections were placed at 5 C overnight. Sections to be freeze-fractured were postfixed in 1% osmium tetroxide/sodium cacodylate buffer after rinsing in cold 50 mM buffer for 1–2 hr at laboratory temperature. The dehydration schedule consisted of 10–15 min in each of 30, 50, 70, 95, and 100% EtOH. The sections were taken through three changes of 100% EtOH and stored at 5 C. Critical-point drying was carried out with liquid CO_2 in a Tousimis Samdri model 780A instrument. Sections were placed on aluminum sample stubs and sputter-coated with gold-palladium in an argon atmosphere with a Technics Hummer V instrument. Sections were examined with an Hitachi S-430 scanning electron microscope.

Bright-field microscopy. For examination by bright-field microscopy, plant material was collected fresh and immediately sectioned and immersed in CRAF III (chromium acetic acid/Formalin). The sections measured 3–8 mm on a side. If the plant sections failed to sink in the fixative, they were placed under vacuum and left in the CRAF III fixative for at least 24 hr at laboratory temperature. The samples were rinsed in distilled water and dehydrated through

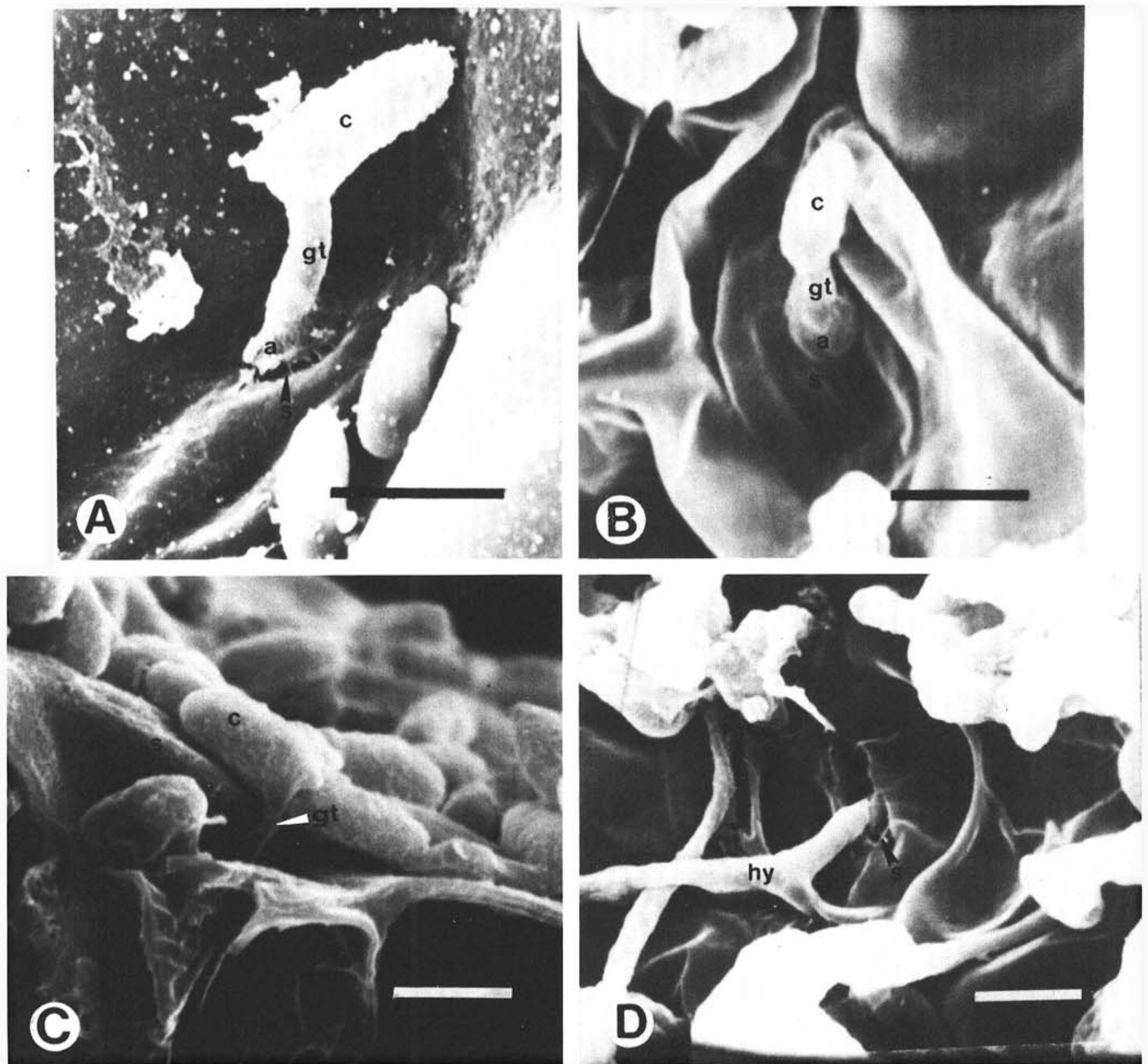

Fig. 1. Scanning electron photomicrographs of soybean vegetative organs showing appressorium formation and penetration via the stomata by *Phomopsis phaseoli*. Scale bars = 5 μm. **(A)** Germinated conidium (c) with germ tube (gt) and appressorium (a) over a stoma (s) of a cotyledon. **(B)** Germinated conidium with germ tube and appressorium over a stoma of a leaf. **(C)** Freeze-fracture cross section of a stoma on a cotyledon showing a germinated conidium and germ tube that appears to penetrate the stoma. **(D)** Hypha (hy) from a germinated conidium not near the stoma that it has penetrated.

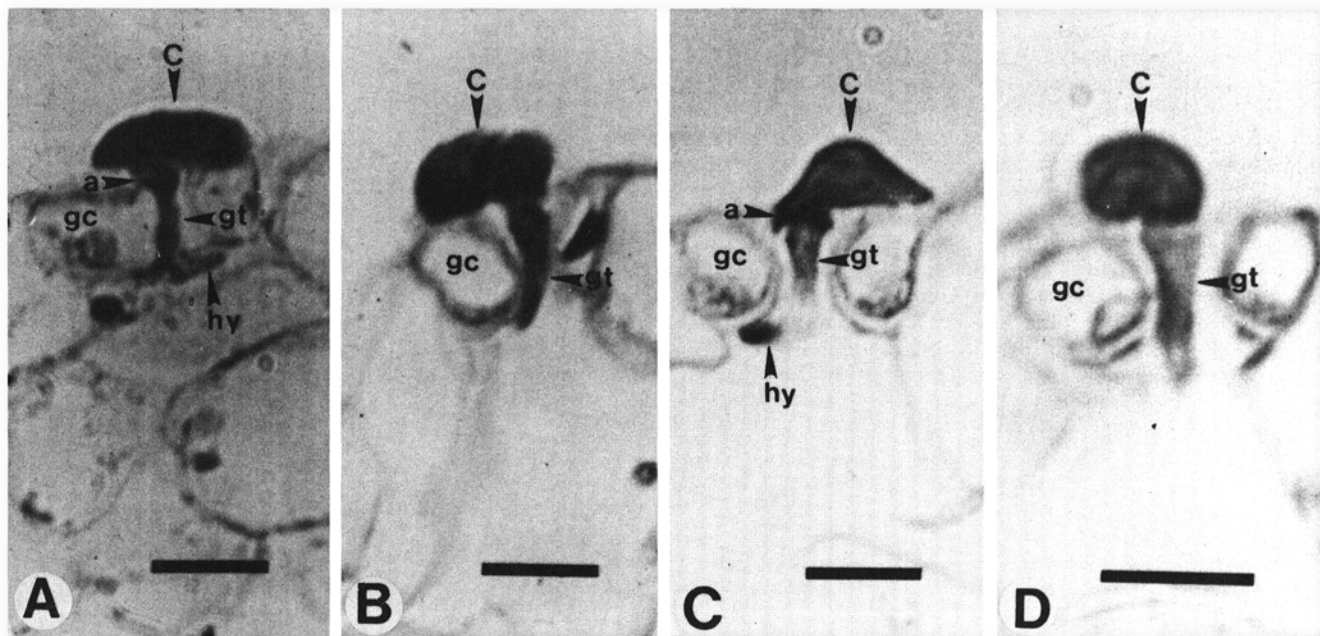

Fig. 2A–D. Photomicrographs showing cross sections of stomata on soybean cotyledons being penetrated by germ tubes (gt) from germinating conidia (c) of *Phomopsis phaseoli* (a = appressorium, hy = hypha, and gc = guard cell). Scale bars = 5 μm.

an EtOH series: 1 hr in each of 5, 10, 15, and 30% EtOH. Dehydration was continued in a tertiary butyl alcohol (TBA) series for 1–2 hr in each solution of TBA:EtOH:distilled water (v/v/v): 1:4:5, 1:2.5:1.5, 21:30:9, 11:5:0, and 3:1:0. The samples were then put through three changes of 100% TBA, remaining in the last change overnight. Infiltration was begun by adding TBA:paraffin oil (1:1) to the samples. After 1 hr at laboratory temperature, they were placed in a hot-air oven for 1 hr at 40 C. Paraffin chips then were added slowly until the mixture was saturated. The samples then were placed at 56–58 C until the paraffin had melted. The mixture containing the samples was discarded and replaced with pure, melted paraffin. The samples were placed back at 56–58 C, and the paraffin was changed hourly until no more TBA was evident in the samples. The samples were left overnight, and the paraffin again was replaced. Samples were embedded in Paraplast Plus and sectioned on a Leitz rotary microtome at 6–10 μm. Sections were mounted on slides with Haupt's adhesive, and the paraffin was removed with xylene. The sections were stained with safranin-fast green (12). Slides were examined under a Leitz Ortholux microscope.

RESULTS AND DISCUSSION

Under SEM, germination of conidia began sometime between 24 and 48 hr after inoculation of cotyledons and unifoliolate leaves. Conidia were not observed on hypocotyls, possibly because their vertical surface caused the inoculum to run off. Germination on water agar was always 85% or greater, and this percentage was reflected in the large number of conidia that germinated on the host. Of these, the vast majority produced germ tubes that ramified over the host surface but never penetrated during this study (i.e., 144 hr). Penetration, when it occurred, was via a stoma (Fig. 1A,B). It was sometimes accompanied by the formation of an appressorium at the end of a germ tube that arose from a conidium situated directly over or immediately adjacent to a stoma. Three other observations support the conclusion of stomatal penetration. The first is a freeze-fracture cross section that shows the penetration of a stoma by a germ tube (Fig. 1C). The second is the observation that an occasional hypha was seen to penetrate via an open stoma not near the conidium from which the hypha arose (Fig. 1D). The third observation is that cross sections of soybean cotyledons viewed under a bright-field microscope clearly show that germ tubes arising from conidia situated directly over stomata have penetrated the stomata (Fig. 2A–D). These bright-field photomicrographs also show that, once inside the host, the hypha begins to grow laterally, just under the epidermal cells. In other cross sections (not shown), hyphae continued to grow laterally for a short distance from the stomatal point of entry. This agrees with the observation of Hill et al (5) that hyphae of *P. phaseoli* remained "close to the point of inoculation."

Germinating conidia and their germ tubes appeared to be attached to the host surface by means of a reticulum of unknown composition and origin (Fig. 3). Manandhar et al (9) recently reported

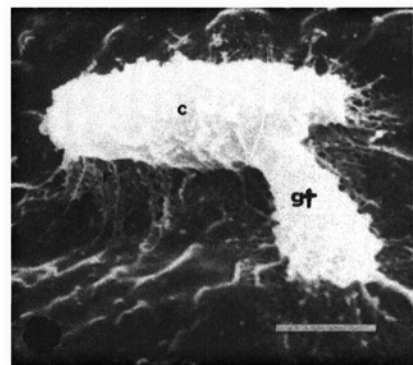

Fig. 3. Scanning electron photomicrograph of a germinating conidium (c) of *Phomopsis phaseoli* on a soybean cotyledon, showing a reticulum (r) that is apparently binding the conidium and germ tube (gt) to the host surface. Scale bar = 2 μm.

that germinating conidia of *Colletotrichum truncatum* (Schw.) Andrus & W. D. Moore (syn. *C. dematium* (Pers. ex Fr.) Grove var. *truncatum* (Schw.) von Arx), incitant of soybean anthracnose, "produced a mucilaginous substance that became more conspicuous after germ tube emergence. Noncellular, fibrous, mucilaginous strands were associated with regions of contact between appressoria and cuticle." The adhesive nature of appressoria was recognized by Frank (4) more than a century ago. Wolf and Wolf (14) mention that a "mucilaginous matrix aids in sticking the spore, appressorium, or germ tube to the cuticle."

The results of the present study suggest that germ tubes of *P. phaseoli* form appressoria when they contact closed stomata. It has been known for a long

time that germ tubes of some fungi will form appressoria when they contact a hard surface (1). Hyphae of *P. phaseoli* arising from conidia located at some distance from stomata sometimes penetrated via stomata but did not form appressoria. This is the typical form of stomatal penetration by an anamorphic fungus observed many years ago by Pool and McKay in their study of *Cercospora beticola* Sacc. (11).

Most fungi enter their hosts by direct penetration of the cuticle, and this penetration often is accompanied by the formation of an appressorium (13). This mode of entry is illustrated by the soybean pathogens *C. truncatum* and *Glomerella glycines* (Hori) Lehman & Wolf, which in rare cases also can penetrate the host via stomata (9). Pathogens in the anamorphic genera *Colletotrichum, Gloeosporium*, and *Marssonia* are especially noted for the production of appressoria (14).

A lesser number of fungi penetrate their hosts via stomata, and most of them form appressoria (3,13); however, according to Wood (15), leaf-infecting anamorphic fungi usually penetrate via stomata. Perhaps, some anamorphic taxa are prevented from penetrating their hosts via stomata because their germ tubes exceed the diameter of the stomatal apertures.

The results of this study show that *P. phaseoli* penetrates soybean unifoliolate leaves and cotyledons via stomata and not the cuticle.

One question that still remains to be answered is whether the ramifying hyphae of *P. phaseoli* on the host surface are attracted by some means to the stomata or whether contact is merely a chance occurrence.

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