Evidence for a Two-Layered Sheath on Germ Tubes of Three Species of *Bipolaris*

Robert C. Evans, Henry Stempen, and Peter Frasca

First and second authors: associate professors, Biology Department, Rutgers University, Camden, NJ 08102. Third author: research associate professor, Orthopedics Department, Jefferson Medical College, Philadelphia, PA 19107.
Accepted for publication 27 October 1981.

ABSTRACT

Evans, R. C., Stempen, H., and Frasca, P. 1982. Evidence for a two-layered sheath on germ tubes of three species of *Bipolaris*. Phytopathology 72:804-807.

The hyphal sheath of *Bipolaris maydis* race T, as well as those of *B. maydis* race O, *B. zeicola*, and *B. turcicum*, were composed of two layers that were visualized by differential staining and light microscopy. The inner layer reacted with a wide variety of stains, but the outer layer was visualized only by negative staining. In *B. maydis* race T the inner layer first became visible at the tips of the conidia just prior to germination. Later, it formed a fibrous, spherical collar at the base of the germ tube and continued as a thin coating over the hyphal apex. However, once the germ tube became approximately as long as the conidium, this layer was no longer visible in the apical regions of the hypha. Although the sheath was evident in scanning electron micrographs, it was usually torn or closely appressed to the germ tube; the two sheath layers were not distinguishable by this technique regardless of the method of preparation.

Cellular structures that aid in the attachment of germinating conidia to the host plant may play an important role in disease development. Murray and Maxwell (8) described an external, fibrillar surface layer, or "sheath," on appresoria and germ tubes of *Helminthosporium carbonum* and postulated that the sheath facilitates conidial attachment. Sheaths have also been reported on hyphal walls of other pathogens including *Alternaria brassicicola* (1), *Erisyphe graminis* f. sp. *hordei* (2), and *Botrytis cinerea* (5,7).

Wheeler and Gantz (10) used transmission electron microscopy to demonstrate the presence of a sheath on hyphal walls of *Helminthosporium victoriae* and *H. maydis* race T, the southern corn leaf blight pathogen. Potter et al (9), by using light microscopy, showed that a sheath was visible at the base of germ tubes of *H. maydis* race T stained with trypan blue. In addition, scanning electron micrographs revealed a "sheath-fibril complex" that was most obvious at the germ tube tip and at the leading edge of appressoria.

Recently, we (3,4) reported that the sheath in *Bipolaris maydis* (Nisikado) Shoemaker [*Helminthosporium maydis* Nisikado; *Drechslera maydis* (Nisikado) Subram. & Jain] race T was difficult to visualize in its entirety by either light or transmission electron microcopy. However, a sheath much larger than those reported by others was revealed when the hyphae were treated with diaminobenzidine-H_2O_2 or fluorescent antibodies as well as by negative staining with India ink. By using these techniques, we determined that the sheath appeared at the point of emergence of the germ tube just prior to germination and developed as a structural component of the germ tube wall along its entire length, but tapered toward the tip. However, there are some discrepancies among reports in the literature concerning the size and location of the sheath in *B. maydis*. For example, in the study by Potter et al (9), there is no evidence for the type of sheath that we reported; similarly, we (4) found no evidence for their sheath-fibril complex.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0031-949X/82/07080404/$03.00/0

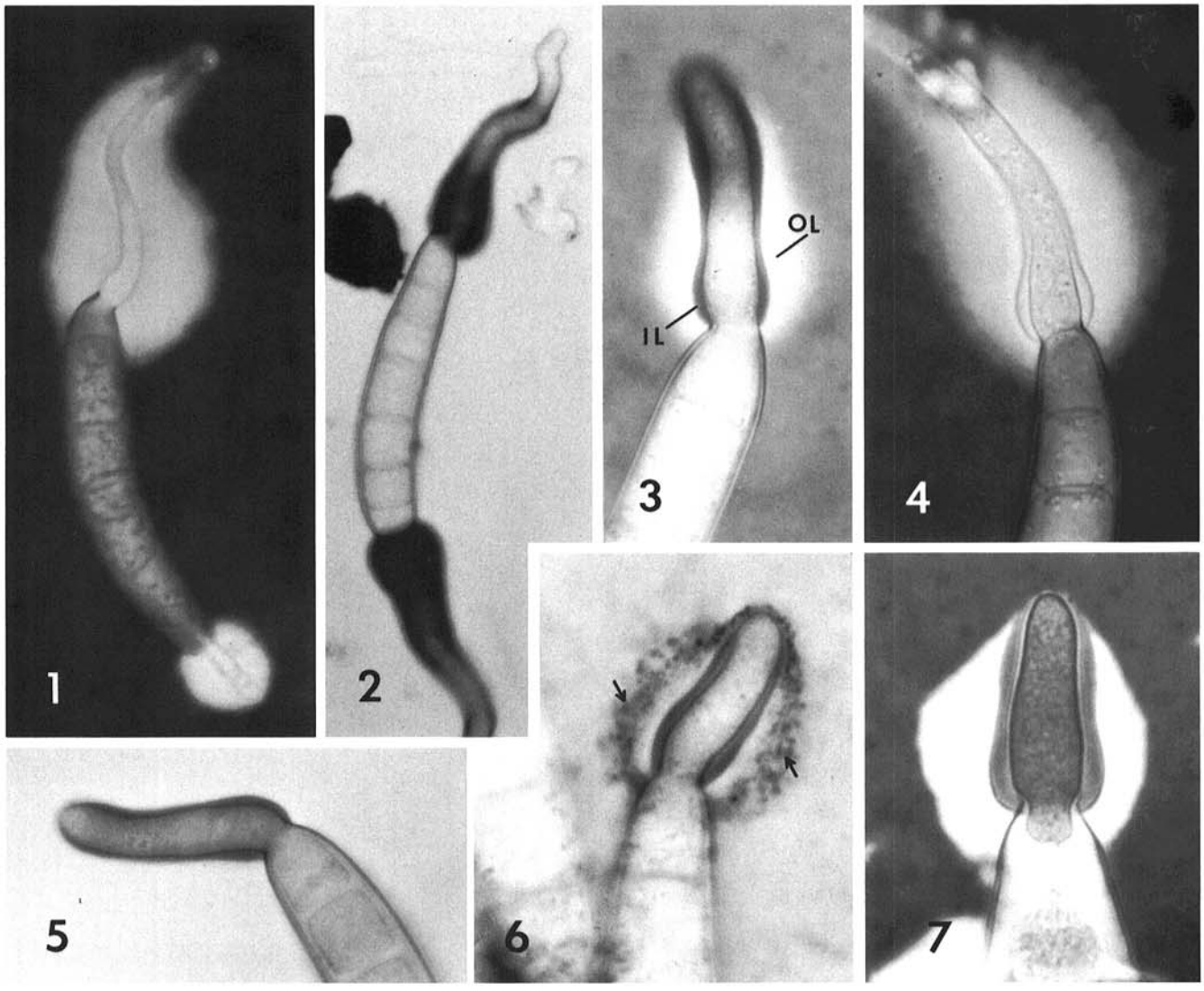
In this report we reconcile these observations by presenting light microscopic evidence for the existence of a two-layered sheath in three *Bipolaris* species. We also trace the development of the inner sheath layer in *B. maydis* race T and supplement the work done previously on the outer layer.

MATERIALS AND METHODS

Cultures of *B. maydis* race T, *B. maydis* race O, *B. zeicola* (Stout) Shoem. (*Helminthosporium carbonum* Ullstrup), and *B. turcicum* (Pass.) Shoem. [*Helminthosporium turcicum* Pass.; *Drechslera turcica* (Pass.) Subram. & Jain], as well as the basal glucose-xylose-asparagine (GX) maintenance medium, and the incubation conditions were described previously (4). Conidia were harvested by flooding the cultures with sterile distilled water, scraping lightly with a glass rod, and transferring 0.1 ml portions of the conidial suspension to GX medium and 2% water agar for various time periods. In some cases, a section of the plate was touched lightly with a drop of water contained in an inoculating loop, and the detached conidia were transferred to experimental media.

Conidia were placed directly on the agar surface, on the surface of a cellophanelike film (courtesy of the DuPont Chemical Co., Wilmington, DE), or on pieces of cellulosic, 4.8 μm (porosity) dialysis tubing (Fisher Scientific, King of Prussia, PA) lying on the agar surface. For liquid culture, conidia were placed in 250-ml flasks containing 50 ml of either distilled water or GX medium lacking agar. The flasks were incubated on a rotary shaker at 100 rpm in the light at 25C.

At intervals, samples of conidia were removed from each of the test media, stained in various ways, and examined microscopically. Conidia germinated on sections of cellophane or dialysis tubing could be stained easily by manipulating the entire section with forceps. A number of stains with standard formulations as used in bacteriology were used (all in aqueous solution). These were Hucker's crystal violet, alcian blue, Loeffler's alkaline methylene blue, acid methylene blue, periodic acid-Schiff reagent (6), Tyler's capsule stain, erythrosin, nigrosin, and Sudan black B. Other dyes used were basic fuchsin (0.2%), malachite green (5%), toluidine blue (0.1–0.5%), trypan blue (1%), Janus green B (0.01%), ruthenium red (0.01%), and Ponceau S (0.2% in 3% trichloroacetic acid). For negative staining, Higgins India ink (#4415) (Faber-Castell Corp., Newark, NJ) and Higgins Black Magic India ink (#4465) were used in a 1:4 dilution in water. All specimens were examined immediately after staining. Observations were made of



Figs. 1–7. Photomicrographs of *Bipolaris maydis* race T conidia and germ tubes incubated in dialysis tubing overlaying GX medium. **1–2.** A broad hyphal sheath was present on specimens mounted in **1,** India ink; a narrower sheath was stained by **2,** nigrosin (×800). **3,** Mounting the specimen in India ink plus nigrosin revealed a negatively stained outer layer (OL) and a nigrosin-stained inner layer (IL) of the sheath (×1,350). **4,** Both sheath layers are visible but unstained on specimens mounted in Black Magic India ink (×1,350). **5,** Specimen mounted in trypan blue showing the inner layer (×1,350). **6,** Germinated conidium mounted in Black Magic India ink plus trypan blue. Note the aggregation of carbon particles (arrow) at edge of outer sheath layer (×1,350). **7,** Specimen mounted in nigrosin, erythosin and Black Magic India ink. The inner sheath layer does not appear to be a continuation of the conidial wall (×1,350).

the specimens as wet mounts in the staining solution. Additional trials using alcian blue, toluidine blue, periodic acid-Schiff reagent, and ruthenium red were made by exposing the organism to the stain and then washing. There was no apparent difference between washed and unwashed specimens.

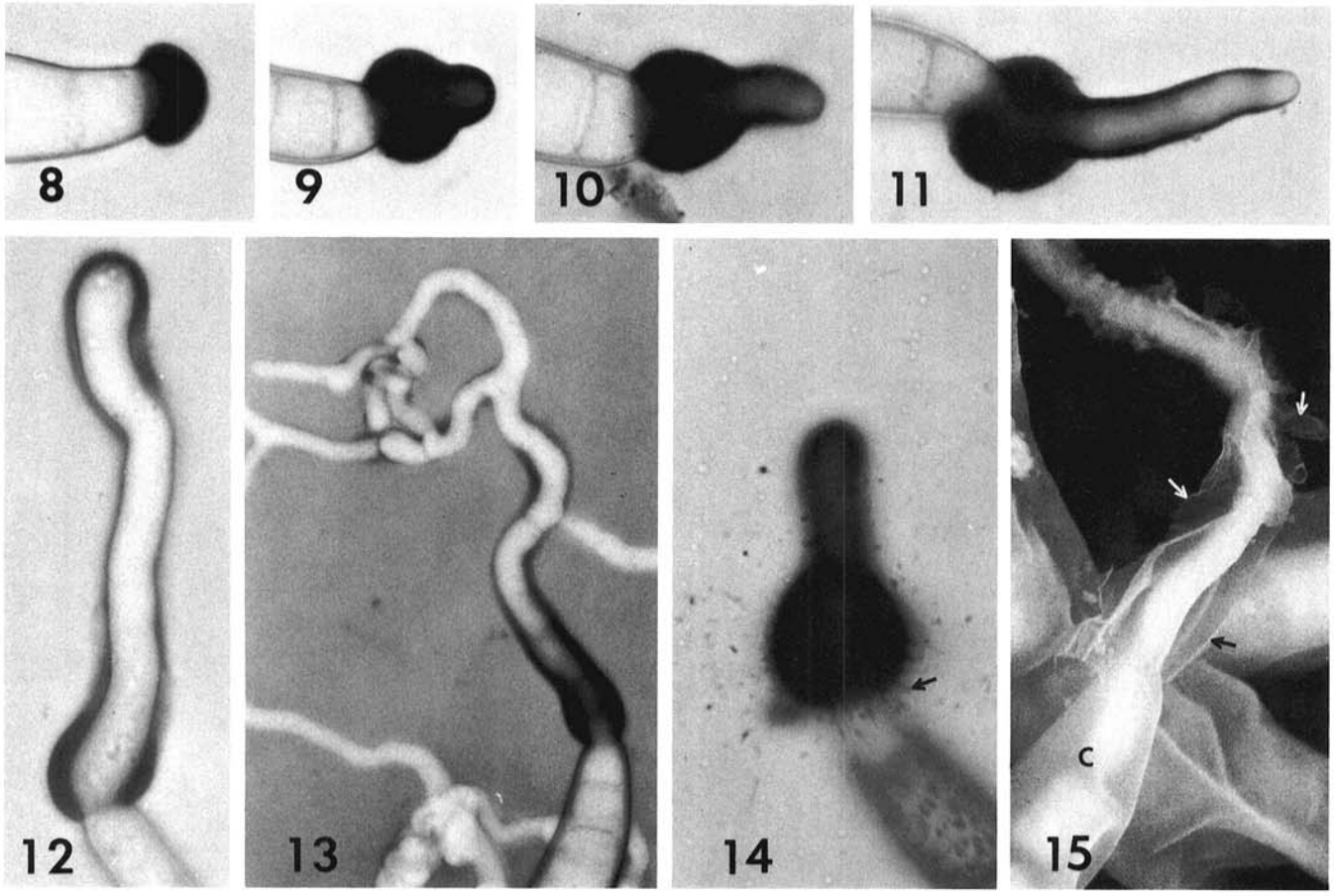
Germinated conidia of *B. maydis* race T were prepared for scanning electron microscopy (SEM) in several ways. Material was washed in water and either: air-dried; placed directly in liquid nitrogen and frozen; fixed 30 min in 4% glutaraldehyde in 0.1 M Na cacodylate (pH 7.2), dehydrated in a graded ethanol series, and air-dried; or dehydrated in a graded acetone series and critical-point dried with CO_2. In all cases, samples were run with and without fixation in 1% osmium tetroxide at 4 C in 0.1 M Na cacodylate (pH 7.2) prior to dehydration. All samples were sputter coated with either copper or gold or coated by carbon evaporation and examined by using a JEOL JSM-35C scanning electron microscope.

RESULTS AND DISCUSSION

Conidia from *B. maydis* race T incubated for 2 hr on GX agar medium overlain with cellophane and negatively stained with India ink exhibited a thick sheath associated with the germ tube (Fig. 1). The sheath was not visible on germ tubes from conidia mounted in water alone. Conidia incubated under the same conditions but mounted instead in nigrosin also showed no evidence of this large, enveloping sheath. Instead, there was visible a stained region that formed a collarlike sphere at the base of the germ tube and continued as a thin layer to the tip of a hypha (Fig. 2). Mounting the conidia in India ink plus nigrosin revealed that these nigrosin-positive regions represented an inner layer of the sheath (Fig. 3). Both the outer, India ink layer and the inner, nigrosin-positive layer were visible on germ tubes of conidia of *B. maydis* race T, *B. maydis* race O, *B. zeicola*, and *B. turcicum* incubated on water-agar and GX agar medium with or without the presence of cellophane or dialysis tubing. They were also present on germ tubes of *B. maydis* race T incubated in liquid GX medium and in distilled water; the other species were not tested under these conditions.

The use of a wide assortment of stains in this study failed to aid in determining the chemical composition of the sheath layers. The nigrosin-positive layer was visualized with all the stains tested except Sudan black B. The principal difference observed was that the width of the basal region was slightly larger in the presence of some stains than others. The outer sheath layer that was made visible by negative staining with India ink did not react with any of the dyes tested. The use of Black Magic India ink not only made the outer sheath visible by negative staining, but also outlined the inner layer (Fig. 4). The reaction with trypan blue (Fig. 5) was similar to that of nigrosin, but with the combination of trypan blue and Black Magic India ink a heavy aggregation of carbon particles formed at the edge of the outer sheath layer (Fig. 6). When the specimens were stained with a combination of erythrosin, nigrosin, and Black Magic India ink, the sheath layers and the conidial wall were clearly distinct (Fig. 7).

The development of the inner sheath layer on conidia of *B. maydis* race T germinated on dialysis tubing and mounted in nigrosin was examined. This layer was first apparent on conidia just prior to germination at the locations where the germ tubes emerged (Fig. 8). It enlarged and formed the characteristic collarlike sphere around the germ tube base as well as the layer that



Figs. 8–15. Photomicrographs and scanning electron micrograph of *Bipolaris maydis* race T conidia and germ tubes incubated on dialysis tubing overlaying GX medium. **8–12**, Representative stages in the development of the inner sheath layer on germ tubes from conidia incubated 0.5, 1.0, 1.5, 2.0, and 3.0 hr, respectively, as demonstrated by nigrosin staining (×1,350). **13,** Conidium incubated 5 hr and mounted in nigrosin. Note that the nigrosin-stained layer does not visibly extend to the apical regions of the germ tube (×800). **14,** Nigrosin-stained specimen showing fibrillar character (arrow) of the inner sheath layer (×1,350). **15,** Scanning electron micrograph of a germinated conidium (C) prepared by critical-point drying showing ragged sheath (arrows) (×2,000).

extended over the hyphal tip (Figs. 9 and 10). The collar soon became less spherical, more tapered, and formed a progressively thinner layer along the hyphal surface (Fig. 11). Although the latter was usually thinnest at the hyphal tip, in some cases it was relatively thick in this area (Fig. 12). When the germ tube became roughly as long as the conidium, the layer was no longer visible at the apex (Fig. 13).

The collar appeared to be composed of fibrous material (Fig. 14) and was found only on germ tubes. When segments of older hyphae were observed in conidial suspensions, the new hyphae that grew from these fragments always possessed an outer sheath (4), but in no instance was an inner layer observed.

Using SEM, we observed fragments with the general appearance of the sheath on some germ tubes regardless of the method of preparation. However, such sheaths were not numerous and were either ragged or closely appressed to the hyphal surface (Fig. 15).

The use of trypan blue (Fig. 5) indicated that the inner layer corresponded to the trypan blue-staining "sheath" described by Potter et al (9). The outer layer may be the structure made visible in their light micrographs because of bacteria and debris embedded in it. Using SEM, they described a sheath formed partly of fibrils. Others (3,4,10), who used transmission electron microscopy, also describe a fibrillar component of the sheath in this species. However, Potter et al (9) observed that this "sheath-fibril complex" in their scanning electron micrographs was most obvious at the tip instead of the base of the germ tube. Although this complex may correspond to the two-layered sheath, based on our observations this complex should be more readily demonstrable in the basal, rather than the apical, portions of the germ tube. In our scanning electron micrographs there was no evidence of a two-layered sheath regardless of the method of preparation.

In addition, using SEM, Potter et al (9) found the sheath-fibril complex only on appressoria and on germ tubes of conidia germinated on maize leaves or on moist filter paper. None was found on germ tubes from conidia germinated on agar. However, in our light microscopic study, sheaths were abundant under these conditions as well as in liquid culture, but we experienced difficulty preparing specimens so that even a single sheath was visible in the SEM. Evidently, the sheath is a delicate structure and thus is easily lost or distorted beyond recognition during sample preparation for SEM.

LITERATURE CITED

1. Campbell, R. 1970. An electron microscopy study of exogenously dormant spores, spore germination, hyphae and conidiophores of *Alternaria brassicicola*. New Phytol. 69:287-293.
2. Edwards, H. H., and Allen, P. J. 1970. A fine structure study of the primary infection process during infection of barley by *Erisyphe graminis* f. sp. *hordei*. Phytopathology 60:1504-1509.
3. Evans, R. C., and Stempen, H. 1980. Evidence for pellicle-bound excrescences on hyphal walls of *Bipolaris maydis*. Mycologia 72:523-533.
4. Evans, R. C., Stempen, H., and Stewart, S. J. 1981. Development of hyphal sheaths in *Bipolaris maydis* race T. Can. J. Bot. 59:453-459.
5. Hawker, L. E., and Hendy, R. J. 1964. An electron-microscope study of germination of conidia of *Botryis cinerea*. J. Gen. Microbiol. 33:43-46.
6. Kligman, A. M., and Mescon, H. 1950. The periodic-acid-Schiff stain for the demonstration of fungi in animal tissue. J. Bacteriol. 60:415-421.
7. McKeen, W. E. 1974. Mode of penetration of epidermal cell walls of *Vicia faba* and *Botrytis cinerea*. Phytopathology 64:461-467.
8. Murray, G. M., and Maxwell, D. P. 1975. Penetration of *Zea mays* by *Helminthosporium carbonum*. Can. J. Bot. 53:2872-2883.
9. Potter, J. M., Tiffany, L. H., and Martinson, C. A. 1980. Substrate effects on *Helminthosporium maydis* race T conidium and germ tube morphology. Phytopathology 70:715-719.
10. Wheeler, H. H., and Gantz, D. 1979. Extracellular sheaths on hyphae of two species of *Helminthosporium*. Mycologia 71:1127-1135.