Localization of Protein in the Hyphal Sheath of *Bipolaris maydis* Race T

Robert C. Evans and Henry Stempen

Biology Department, Rutgers University, Camden, NJ 08102.
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ABSTRACT

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Experiments using rabbit anti-*Bipolaris* antiserum followed by ferritin conjugated anti-rabbit IgG antiserum showed that the antigenic component of the hyphal sheath of *Bipolaris maydis* race T is localized in discrete patches within the fibrillar matrix of the sheath and more evenly distributed on the outer surface of the hyphal wall proper. Pretreatment with papain significantly reduced the localization of the ferritin label, indicating that the principal antigenic component is proteinaceous. A reaction product that accumulated when germinated conidia were incubated with diaminobenzidine tetrahydrochloride and H_2O_2 was localized in the fibrillar portion of the sheath. The amount of product present was not diminished by prior treatment with papain, heat, or antibody. Thus, the portion of the sheath where this reaction occurs is apparently not associated with the proteinaceous patches.

Additional key words: cell wall, southern corn leaf blight.

Germ tubes and hyphal tips of *Bipolaris maydis* (Nisik.) Shoemaker (*Helminthosporium maydis* Nisik.; *Drechslera maydis* (Nisik.) Subram. & Jain) race T, the southern corn leaf blight pathogen, possess a granular/fibrillar, sheathlike extension of the cell wall (4,15). Similar hyphal sheaths have been found on other fungi, including several wood-decay hymenomycetes (13,14), *Dactylaria brochopaga* Drechs. (2), and *Botrytis cinerea* Persoon & Fries (10). The function of the sheath is not known, but for pathogenic species it has been speculated that the sheath facilitates infection by enabling the pathogen to adhere to the host (8,12).

In *B. maydis* race T the sheath is composed of a thin inner layer that reacts with a variety of stains and a thick outer layer that is visualized only by treatment with fluorescent antibodies or with a combination of diaminobenzidine tetrahydrochloride and H_2O_2 (DAB/H_2O_2) as well as by negative staining with India ink (5,6).

Aside from the observation that the sheath of *B. maydis* contains antigenic material, the chemical nature of the sheath is unknown. Day and co-workers (1,7) have reported proteinaceous fibrillar structures (fimbriae) on the wall surfaces of many species of smut fungi and yeasts. They postulate that these structures may be involved in facilitating pathogen invasion of the host. In *B. maydis*, the reaction of the sheath with the cosubstrates DAB/H_2O_2 suggests that one or more of the antigenic components may catalyze peroxidative reactions. These cosubstrates have been used histochemically to visualize several enzymes such as peroxidase and catalase as well as nonenzyme hemoproteins such as hemoglobin, myoglobin, and cytochrome c (3).

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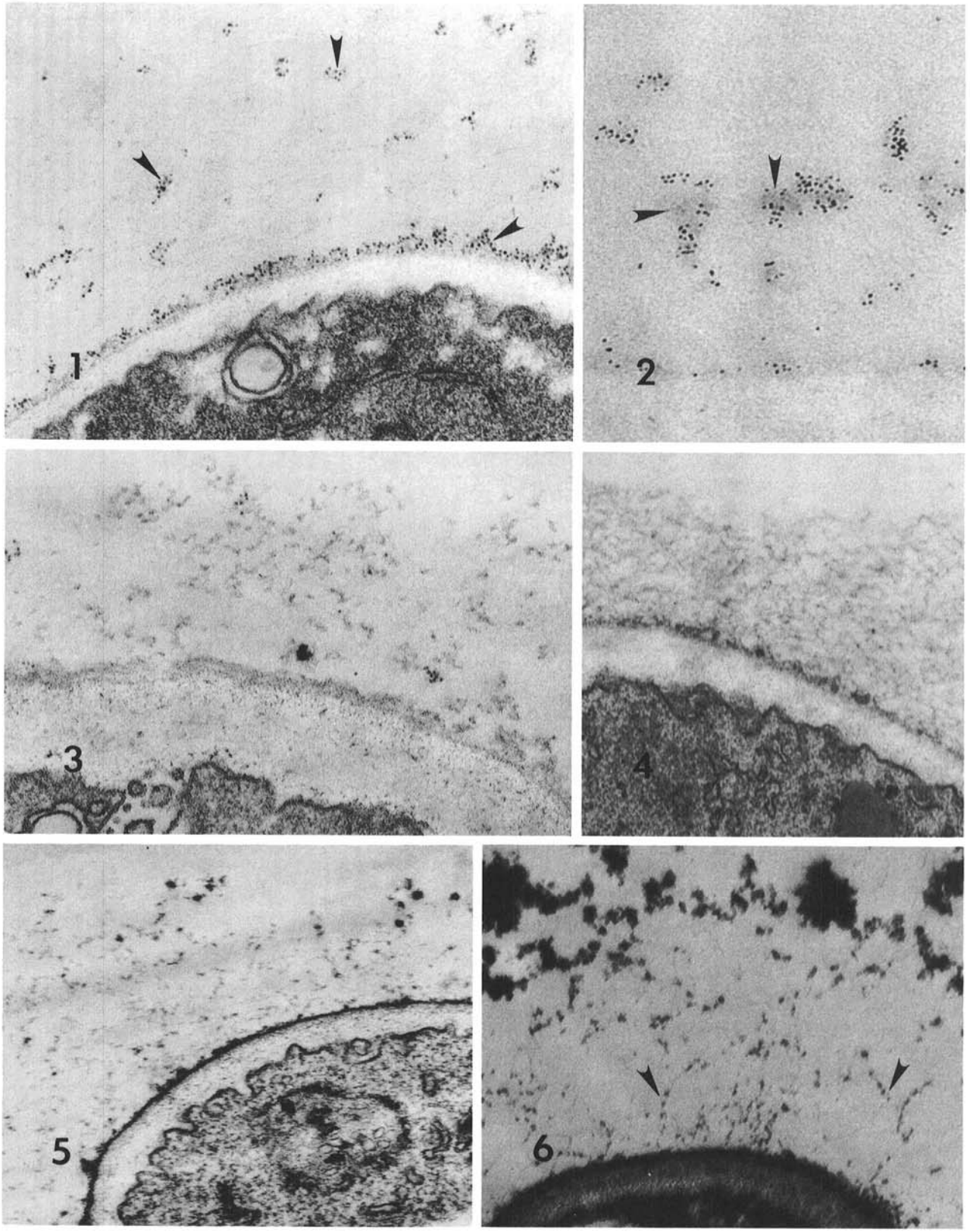
In this paper we describe the distribution and nature of the antigenic component of the hyphal sheath.

MATERIALS AND METHODS

Cultures of *B. maydis* race T (ATCC 36180) were incubated on a glucose-xylose-aparagine agar medium (6) for 7 days in the dark at 28 C. Conidia were harvested by flooding the cultures with sterile distilled water and scraping lightly with a glass rod (5).

Antiserum preparation. Aliquots (10 ml) of an aqueous conidial suspension (10^5 conidia per milliliter) were placed in 250-ml flasks and incubated at room temperature on a rotary shaker at 100 cycles per minute for 2–3 hr at room temperature. Formalin was then added to give a final concentration of 0.5% (w/v). This preparation contained germinated and ungerminated conidia as well as hyphal fragments. The inactivated suspension from one flask was centrifuged and resuspended in 1 ml of Formol-saline [0.5% (w/v) formalin in 0.9% (w/v) NaCl], mixed with an equal volume of complete Freund's adjuvant, and 0.5 ml injected subcutaneously into each of two rabbits. *B. maydis* mycelia in two other flasks were similarly inactivated and then homogenized in a Polytron homogenizer. Starting 2 wk after the subcutaneous injection, each rabbit received nine intraperitoneal injections of increasing doses (0.1–0.5 ml) of homogenate in a 5-wk period. Blood was drawn by cardiac puncture 1 wk after the last injection.

Ferritin-labeled antibody studies. Conidia were germinated as described above, washed three times with 0.15 M K-phosphate buffer, pH 7.2, in a 15-min period, and fixed in 3% glutaraldehyde in phosphate buffer for times ranging from 30 min to overnight. The fixed conidia were then washed, divided in three batches, and incubated at 37 C for 45 min as follows. To one batch was added anti-*Bipolaris* antiserum diluted 1:60 with phosphate-buffered saline (0.15 M NaCl in 0.15 M phosphate buffer, pH 7.0)



Figs. 1–6. Electron micrographs of *Bipolaris maydis* race T germ tubes and hyphae showing the sheathlike extension of the cell wall. **1,** Section of hypha showing localization of ferritin label on the hyphal surface and in patchlike areas (arrows) within the sheath after treatment with rabbit anti-*Bipolaris* and labeled goat anti-rabbit antisera (×91,000). **2,** Detail of sheath to show localization of ferritin label in granular regions of sheath (arrows) (×136,000). **3,** Control preparation treated with normal rabbit serum followed by ferritin-labeled anti-rabbit IgG antiserum. Very little nonspecific staining is observed (×91,000). **4 and 5,** Sections of hyphae and sheath treated with papain followed by rabbit anti-*Bipolaris* and goat anti-rabbit ferritin-labeled antisera showing reduced localization of the ferritin label (×91,000). **6,** Section of sheath from preparation previously treated with DAB and H_2O_2. Note the tendency toward linear patterns created by reaction product deposits (×45,000). During the course of the reaction, a precipitate forms in solution that tends to aggregate and form dense clumps that adhere to the outer region of the sheath.

containing 1:20 normal goat serum. To a second batch was added normal rabbit serum diluted 1:60. To a third batch was added phosphate-buffered saline plus diluted goat serum alone. After incubation all samples were washed three times in a 15-min period with phosphate-buffered saline and diluted goat serum and then reacted with goat anti-rabbit IgG (whole molecule) ferritin-labeled antibody (U.S. Biochemical Corp., Cleveland, OH) 45 min at 37 C.

The samples were then washed three times with 0.1 M Na-cacodylate buffer, pH 7.2, in a 15-min period, postfixed in cold 1% (w/v) OsO_4 in cacodylate buffer, and then washed, dehydrated, embedded in Spurr's resin, sectioned, stained with uranyl acetate and lead citrate, and examined with the electron microscope (6).

Papain treatment. Germinated conidia were washed three times in 0.01 M MES buffer, pH 6.2, and then incubated for 1 hr at 28 C in 1 mg/ml papain (Sigma Chemical Co., St. Louis, MO) dissolved in MES buffer.

DAB studies. Conidia were streaked onto glass coverslips and incubated in a moist chamber for 3 hr at 28 C. Coverslips with adhering conidia were then washed once in 0.01 M Na acetate buffer, pH 4.25, and floated conidial side down on a solution of 5 mM DAB-tetrahydrochloride and 0.02% (v/v) H_2O_2 in the same buffer contained in 35- × 10-mm polystyrene petri dishes. The DAB and H_2O_2 were prepared immediately before use. The dishes with germinated conidia were incubated in the dark for 1 hr at 28 C. The coverslips were then removed, the conidia washed with water, and examined using light microscopy.

In those cases where conidia were examined by electron microscopy, the conidia were germinated in flasks on a shaker as described for the antibody studies, then reacted with DAB/H_2O_2, fixed, dehydrated, and embedded as described previously (6). In some cases, germinated conidia were treated with *Bipolaris* antiserum as described above and then treated with DAB/H_2O_2.

Conidia to be given a heat treatment were germinated as usual on coverslips and the coverslips placed in a 100 C oven for up to 1 hr. Conidia were then exposed to DAB/H_2O_2 at room temperature and examined by light microscopy as described above.

All experiments were performed at least three times.

RESULTS AND DISCUSSION

Experiments using rabbit anti-*Bipolaris* antiserum followed by ferritin-conjugated anti-rabbit IgG antiserum showed the ferritin label concentrated in patches within the sheath proper and more evenly distributed on the outer surface of the hyphal wall (Figs. 1 and 2). The patches appeared similar in both longitudinal and transverse sections. The patches were associated with slightly electron-dense granules previously shown to be scattered through the fibrillar portion of the sheath (6). Antigen activity at the surface of the hyphal wall is probably due to some of the granular material concentrated on the wall surface. Structures resembling the fimbriae reported for members of the ustilaginales, ascomycetous yeasts, and basidiomycetous yeasts (7) were not observed.

Others have observed nonspecific binding of rabbit serum proteins to pituitary gland cells when the sera were used in high concentration (11). Preliminary trials showed that dilution (1:60) of the *Bipolaris* antiserum and the addition of normal goat serum to the buffered saline (1:20) reduced the nonspecific binding of rabbit serum proteins to a minimum (Fig. 3).

The hyphae on whose surface the ferritin label was found were presumed to be actively elongating because of the presence of a sheath, the abundance of mitochondria, and/or the thin cell wall. Other hyphae with a pelliclelike covering (4) were free of ferritin label as was the surface of the conidia.

When germinated conidia were treated with papain there was a visible reduction in the localization of ferritin label in the sheath (Figs. 4 and 5). The principal antigenic component is thus proteinaceous in nature. The extent of binding by ferritin-labeled antibody was estimated by counting the number of electron opaque spots, which represent the ferritin label, per unit area of representative sections. Sections treated with anti-*Bipolaris* antisera contained (per 0.25 μm^2) 53.9 ± 5.9 spots, sections treated with normal goat serum alone contained 3.4 ± 0.6 spots, and those treated with papain followed by anti-*Bipolaris* antisera contained 2.8 ± 0.7 spots. However, when trypsin was substituted for papain, the results from one experiment to the next were inconclusive. Preliminary experiments using pepsin, chymotrypsin, and protease showed no apparent effect of these proteolytic enzymes on the binding of antibody to the sheath. It is possible that inadequate washing after papain treatment could have left a residuum of enzyme that hydrolyzed the anti-*Bipolaris* antibody. However, the resulting product would still contain components capable of binding sheath antigen and be available for reaction with the ferritin-labeled anti-rabbit antiserum that was specific for whole molecule rabbit IgG.

Experiments with DAB/H_2O_2 showed that reaction product accumulated primarily in the fibrillar portion of the sheath (Fig. 6). When germinated conidia were incubated with antibody before DAB/H_2O_2 treatment, the intensity of the DAB reaction, as measured by the density of the reaction product, was unaffected. These data suggest that the regions associated with the DAB/H_2O_2 reaction are not the same as the regions where antibody binds.

Experiments designed to determine the nature of the DAB/H_2O_2 reaction revealed that H_2O_2 was necessary for the reaction to occur and that the component of the sheath that reacts with H_2O_2 was heat resistant. Even when the germinated conidia were incubated at 100 C for 1 hr the density of reaction product was not diminished. In addition, the accumulation of reaction product was not diminished by prior papain treatment. Thus, the DAB/H_2O_2 reaction does not appear to be enzymatic. A nonenzymatic oxidative system involving H_2O_2 and iron for the decomposition of wood by brown-rot basidiomycetes has been proposed by Koenigs (9). Further studies are necessary to determine if the mechanism for oxidizing DAB in *B. maydis* involves iron.

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