Substrate Effects on *Helminthosporium maydis* Race T Conidium and Germ Tube Morphology

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ABSTRACT

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Conidia of *Helminthosporium maydis* race T were produced on glucose asparagine agar and ground maize-leaf agar media, and on maize leaf lesions. Comparisons by light microscopy revealed that conidia produced on the glucose asparagine agar were shorter, had fewer septations, and germinated more slowly than conidia from maize-leaf agar or maize leaf lesions. Observations with scanning electron microscopy showed that conidia from the glucose asparagine agar have an almost-smooth wall surface and become flattened in response to vacuum while conidia from the other two substrates have rougher walls and become wrinkled in response to vacuum. Substrate effects on conidial morphology and germ tube response indicate that fungus nutrition should be considered in morphological, taxonomic, and epidemiological studies of *H. maydis*. Germ tubes of conidia from all three substrates formed a sheath-fibril complex when the conidia were inoculated onto maize leaves. The complex also was observed on germ tubes from conidia germinating on cellulose triacetate membrane filters resting on either moist filter paper or maize leaves. The sheath-fibril complex may function to attach germ tubes and appressoria to a substrate.

In 1970, *Helminthosporium maydis* Nisikado (*Cochliobolus heterostrophus* Dreschler) race T caused extensive damage to the dent maize (*Zea mays* L.) crop in the United States. This new race of *H. maydis*, which causes Southern corn leaf blight, became the object of many intensive investigations. Many of these studies focused on environmental factors affecting the germination of the conidia and dissemination of the pathogen (7,15,24) and on the cytology of the fungus and the suscept-pathogen relationship (1–4,22,26,27). The inoculum used in many of these studies was grown on artificial media, including media specially developed to enhance sporulation (8,9). Trainor and Martinson (23) reported that the conidia formed on most artificial media were more darkly pigmented, broader, and shorter and had less inoculum potential

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than did conidia formed on maize leaves or on an agar medium containing ground maize leaves. The decreased inoculum potential was related to the prepenetration phenomena of lower conidial germination rates, slower conidial germination, fewer germ tubes, and poor appressorial formation. Trainor and Martinson (23) did not study conidial or germ tube morphology in any detail.

The objectives of this study were to determine by light and scanning electron microscopy whether the substrates for sporulation affected the morphology of conidia both before and after germination.

TABLE 1. Length and number of septations of *Helminthosporium maydis* conidia produced on different media

Medium[x]	Mean spore length (range)[y]	Mean no. of septa (range)
GAA	56.6 (30–95) a[z]	4.9 (3–7) d
GMLA	69.7 (40–90) b	7.0 (5–9) e
Maize leaves	78.3 (49–108) c	8.0 (5–11) f

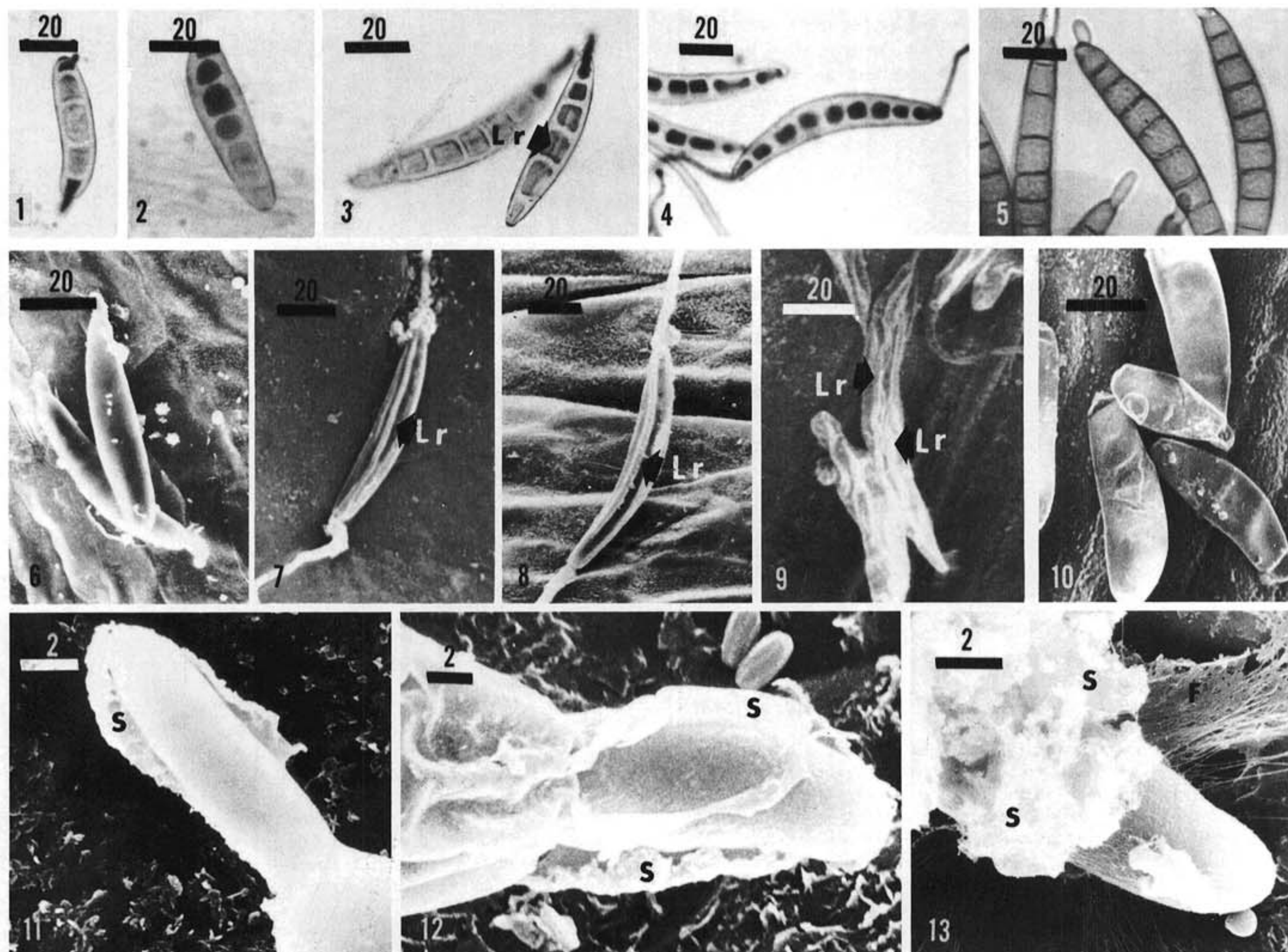
[x]GAA = glucose asparagine agar; GMLA = ground maize leaf agar.
[y]AT least 100 conidia were examined from each medium. Spore lengths are in micrometers.
[z]Means followed by a common letter do not differ ($P = 0.05$) according to Duncan's multiple range test.

MATERIALS AND METHODS

The *H. Maydis* race T isolate used in these experiments was derived from a single spore from a lesion on an ear of maize collected near Mitchellville, IA, in 1969 (23). The stock culture was maintained by transferring conidia in sterile distilled water to sterile petri dishes containing ground maize-leaf agar (GMLA) (23) or glucose asparagine agar (GAA) (8).

Inoculum for the experiments was prepared by harvesting conidia from the stock culture in sterile distilled water and transferring them to sterile dishes of GMLA or GAA or misting them onto young maize plants (W64A cmsT) in a moist environment. After 2–3 days, leaves with immature lesions were detached from the plants and placed in a moist chamber to induce sporulation. The inoculated petri plates or maize leaves were placed under diffuse fluorescent light at room temperature until conidia formed. The conidia produced on either of the artificial media or the maize leaves were harvested in sterile distilled water and used in the experiments.

For studies of conidial germination, droplets containing the freshly harvested conidia were placed on the surface of GMLA, GAA, maize leaves, or cellulose triacetate membrane filters (Metricel GA, Gelman Instrument Company, Ann Arbor, MI)



Figs. 1–13. Photomicrographs and scanning electron micrographs of *Helminthosporium maydis* conidia and germ tubes. Figs. 1–5. Photomicrographs of spores stained with trypan blue (Figs. 1–4) and unstained (Fig. 5). **1,** Conidium produced on glucose asparagine agar (GAA), 1 hr after seeding a maize leaf. **2,** Conidium produced on GAA, 2 hr after seeding. **3,** Conidia produced on ground maize-leaf agar (GMLA), 1 hr after seeding on a maize leaf; Lr=longitudinal ridge on spore wall. **4,** Conidia produced on GMLA, 2 hr after seeding. **5,** Conidia harvested from fresh lesions on maize leaves, 1 hr after seeding on slide. Figs. 6–10. Scanning electron micrographs of *H. maydis* conidia germinating on maize leaves. Figs. 6–8. **6,** Fixed conidia produced on GAA; **7,** on GMLA and **8,** on maize leaf lesions; note longitudinal ridge (Lr) on spore wall. Figs. 9–10. Response of fresh unfixed conidia **9,** from GMLA and **10,** GAA to vacuum. Figs. 11–13. Scanning electron micrographs of germ tubes from conidia produced on **11,** GAA, **12,** GMLA, and **13,** on a maize leaf lesion; all are germinating on maize leaves and have been fixed. Sheath (S) and fibrils (F) are indicated. The number above the scale bar in each figure represents the proportional scale bar length in micrometers.

with 0.80-μm porosity. The spores were allowed to germinate at room temperature in a humid crisper under diffuse light. Samples were taken 0.5, 1, 2, and 4 hr after transfer by use of a 7-mm-diameter cork borer.

Both bright-field and phase-contrast optics were used for light microscopic examination of unfixed and fixed conidia. The unfixed, living conidia were examined germinating in distilled water or on agar-coated slides. Conidia germinating on maize leaves were fixed, and the leaves were cleared in ethanol and glacial acetic acid using the techniques described by Knox-Davies (13), stained with trypan blue in lactophenol, and mounted in either piccolyte (General Biological Supply House Inc., Chicago, IL 60620) or 10% glycerin.

Samples for observation with a scanning electron microscope (SEM) were prepared by one of two methods. Fresh, unfixed samples were coated with carbon and gold and observed immediately. Alternatively, samples were fixed overnight in 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer of pH 7.2 (12). After being rinsed in the phosphate buffer, the samples were postfixed in 1% osmium tetroxide in the same buffer and again were rinsed in buffer. After dehydration through an ethanol and freon TF series, the samples were critical-point dried in CO_2 and coated with carbon and gold. At least 20–30 conidia were examined from each sample. All SEM observations were made with a JEOL JSM-35 SEM.

RESULTS

The size and shape of the conidia were greatly influenced by the substrate on which the conidia were produced. Conidia grown on GAA were shorter and less curved with more blunt ends (Figs. 1, 2, 6) than were conidia produced on either GMLA (Figs. 3, 4, 7) or maize leaves (Figs. 5, 8; Table 1). Observation of at least 100 conidia from each of the sources indicated that conidia from GAA had fewer septations (Figs. 1, 2) than did conidia from either GMLA (Figs. 3, 4) or maize leaves (Fig. 5; Table 1).

Trypan blue stain was absorbed slowly by conidia from GMLA and absorption generally occurred only after initiation of germination (Figs. 3, 4). Conidia from GAA, however, absorbed the stain before any evidence of germ tube formation (Fig. 2). Germination of the conidia from GAA was slower than germination of conidia from the other two sources. Germination was assumed to have occurred when the germ tube was at least as long as it was wide. One hour after inoculation on maize leaves, 95% of the conidia produced on GMLA had developed germ tubes, but only 81% of the conidia from GAA had germinated even after 2 hr. After 4 hr, 97% of the conidia from GAA had germinated, but only 29% showed bipolar germination; conidia from GMLA or maize leaves showed approximately 76% bipolar germination.

When examined with the SEM, conidia formed on lesions (Fig. 8) and on GMLA (Fig. 7) had at least one or two visible longitudinal ridges along the spore surface, but spores from GAA had no ridges (Fig. 6). This structural feature was only faintly visible with the light microscope (Fig. 3) with spores produced on GMLA (Leitz Labolux at ×900–1,000; 1.30 NA condenser; and 1.40 NA objective or 1.30 NA phase objective). During SEM examination, fresh, unfixed conidia from GMLA became wrinkled and distorted by the vacuum, but the folds in the conidial wall remained roughly in a longitudinal direction (Fig. 9). Conidia from lesions also appeared to have been wrinkled by the vacuum, but conidia from GAA appeared to be flattened and empty (Fig. 10).

Closer examination of the conidial walls and germ tubes of chemically fixed conidia also indicated that the conidia from GAA were relatively smooth, and the emerging germ tubes were sometimes covered with a sheath, which occasionally split as the tube elongated (Fig. 11). Walls of conidia from GMLA were rough and convoluted, and the germ tubes from these conidia were partially covered with a granular sheath (Fig. 12). Conidia from lesions closely resembled those from GMLA but were slightly longer and slimmer with a flocculent sheath partially surrounding the germ tube (Fig. 13).

The age of individual conidia varied due to sequential development of conidia on each conidiophore, but samples were taken from cultures that had been seeded 7–8 days earlier. The conidia observed in the different situations were, therefore, in a range of comparable ages. No differences were observed between these conidial populations and those harvested from cultures that were 2–3 wk older. There was no indication that the interval between culture transfer and the removal of conidia had any effect on the appearance of the conidia. More conidia usually were harvested from older cultures.

Observations of conidia germinating on maize leaves, potato dextrose agar (PDA), or membrane filters indicated that the appearance and characteristics of the elongating germ tubes were not greatly influenced by the media on which the conidia were produced, but were strongly affected by the substrate on which they were growing. Conidial concentration on PDA, maize leaves, or membrane filters did not appear to significantly influence the rate of germination or germ tube elongation. Germ tube elongation was slightly more rapid in distilled water than on either agar or on maize leaves, possibly indicating some dependence on available free water.

On maize leaves, a sheath around the emerging germ tube (Fig. 14) appeared to spread from the germ tube and to form a thin film over the immediate leaf surface as elongation occurred. In light micrographs, this film was made visible because of the debris and bacteria that became embedded in it (Figs. 15, 16). The sheath was present on 75–80% of the germ tubes examined and appeared to be partly formed of fibrils that were visible in the SEM (Figs. 13, 18, 19). The fibrils extended from the germ tube to the leaf surface and appeared to anchor the germ tube to the leaf (Figs. 17, 18, 22). The sheath-fibril complex was most obvious at the tip of the actively growing germ tube or at the forming appressoria (Figs. 19, 20).

Germ tubes of conidia germinating on agar did not form either sheath or fibrils. The germ tubes were free of most contamination (Fig. 21) and did not form appressoria even 4 hr after transfer. Conidia on maize leaves began to form appressoria within 1.5–2.0 hr, and penetration of the epidermis had occurred at the anticlinal wall junctions within 4 hr after inoculation (Fig. 15).

When conidia germinated on membrane filters resting on either maize leaves or moist filter paper, the sheath-fibril complex was present, and appressoria formed (Figs. 23, 24). The appressoria formed by these conidia closely resembled the appressoria formed by conidia germinating on maize leaves. When the conidia germinated on membrane filters resting on a moist glass surface, a diffuse, mucilaginous layer surrounded the germ tubes, but no fibrils were visible (Fig. 26). When the membrane filters were placed on brass disks in a moist environment, elongated swellings, which resembled appressoria, formed at the end of the germ tubes, but no sheaths or fibrils were visible (Fig. 25).

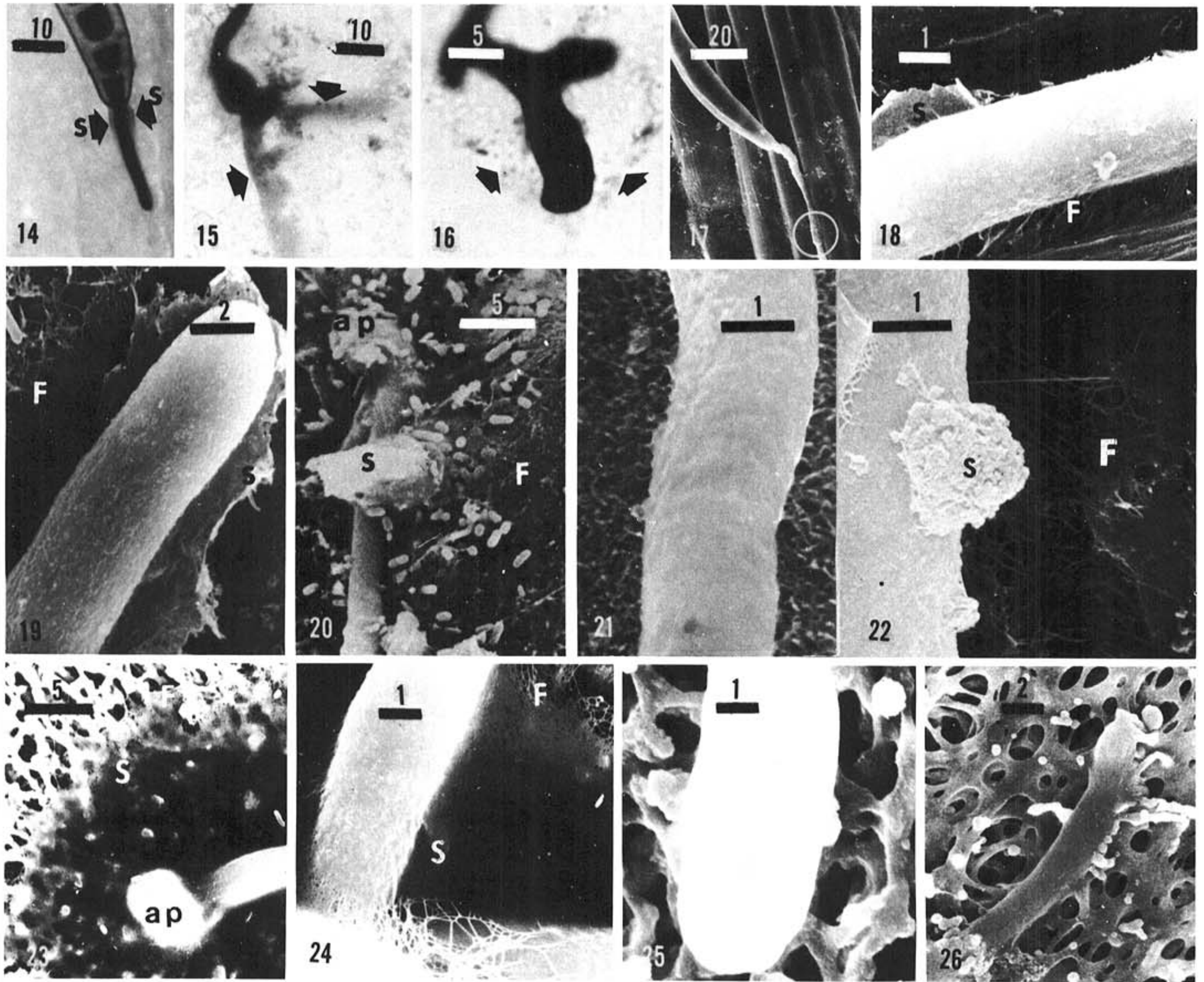
DISCUSSION

Helminthosporium maydis conidia were described originally as strongly curved spores, 30–115 μm in length, with up to 12 septations (5). This study supports the observation of Trainor and Martinson (23) that the cultural substrate greatly influences conidial length, shape, and germination processes. Trainor and Martinson (23) found that the high nitrogen content in synthetic culture media induced morphologically abnormal spores. We found that conidia produced on GAA usually were only slightly curved and that the lengths of conidial population were concentrated toward the lower end of the described length range for *H. maydis* conidia. Trainor and Martinson (23) reported that the conidia produced on GMLA were essentially identical to conidia from lesions, but, in the present study, the conidia from GMLA were smaller with fewer septations than were conidia from maize leaf lesions. However, the texture of the cell walls, the shape of the conidia, and germination rates of the conidia from GMLA and from maize leaves were similar.

Both light- and scanning electron microscopic observations indicated that the substrate on which the conidia were produced affected the conidial cell wall. Enhanced permeability to trypan blue stain in lactophenol of conidia produced on GAA, as well as

their differential resistance to folding and wrinkling in vacuo, suggest that nutrition affects the composition and structure of the conidial wall. Locci and Quaroni (14) did not report morphological differences between *H. maydis* conidia produced on artificial media and on diseased corn leaves, but in some of their SEM photomicrographs, longitudinal ridges such as we observed (Figs. 7, 8) were evident on the conidia. White et al (27) did not mention the presence of the ridges in their study of *H. maydis* conidium ultrastructure using transmission electron microscopy; however, they observed spores produced on synthetic media. We made no attempt to chemically characterize the conidial wall or intracellular components that may vary because of nutrition during sporulation. Trainor and Martinson (23) warned against using spores produced on synthetic media in epidemiological studies because of nutritional effects on inoculum potential. Similarly we would discourage the use of spores produced on synthetic media for studies of morphological or taxonomic characters.

Our study also showed that germ tubes from *H. maydis* conidia germinating on maize leaves developed a sheath-fibril complex that appeared to attach the germ tube to the leaf surface. Locci and Quaroni (14), in their SEM study of *H. maydis* infection processes, indicated that *H. maydis* conidia secreted a slime-like material as they germinated on maize leaves, but did not mention any fibril formation. Wheeler (26) observed some diffuse extracellular material surrounding germ tubes in his transmission electron microscopic (TEM) study of *H. maydis* penetration of maize leaves. Transmission electron micrographs of the closely related *H. carbonum* Ullstrup (*Cochliobolus carbonum* Nelson) revealed a fine fibrillar network that surrounded the germ tube (17) or appressoria (18) of conidia germinating on maize leaves. Murray and Maxwell (18) postulated that the fibrils were involved in cementing the appressoria and germ tubes to the leaf surface. A loose granular or fibrillar material also was observed on the appressoria of *Erysiphe graminis* f. sp. *hordei* Marchal spores germinating on *Hordeum vulgare* L. 'Traill' (6). This material, formed between the appressorium and leaf surface, was described as an extension of the outer portion of the appressorium cell wall. Bacteria growing on the surface of wheat (*Triticum aestivum* L.)



Figs. 14–26. Germinating conidia and germ tubes of *Helminthosporium maydis*. Figs. 14–16. Light micrographs of trypan blue-stained *H. maydis* conidia germinating on maize leaves. **14,** Conidium and germ tube with sheath (S). **15,** Germ tube and appressorium; arrows indicate the internal hyphae after penetration via the anticlinal cell walls. **16,** Germ tube with extracellular sheath (arrows) present as a thin film over the leaf surface. Figs. 17–22. Scanning electron micrographs of fixed germ tubes on maize leaf or agar surfaces. **17,** Conidium germinating on maize leaf; circled area is enlarged in Fig. 18. Figs. **18–20,** Germ tubes with sheath (S), fibrils (F) and appressorium (ap). **21,** Germ tube on agar surface. **22,** Germ tube on maize leaf showing sheath (S) and fibrils (F). Figs. 23–26. Scanning electron micrographs of fixed germ tubes and appressorium (ap) on cellulose triacetate membrane filters resting on **23,24,** a maize leaf. **25,** a brass disk (showing enlarged germ tube tip), and **26,** a glass surface. Number above the scale bar represents the proportional scale bar length in micrometers.

roots were embeded in a similar fibrillar network observed in SEM studies by Rovira and Campbell (19,20), but the network was throught to be a product of both the plant and the bacteria. The presence of both sheath and fibrils on the germ tubes and appressoria produced by *H. maydis* conidia germinating on membrane filters indicates that the complex is a product of the fungus and not a product of, or induced by, the plant. The sheath-fibril secretions appeared to attach germinating conidia, germ tubes, and appressoria to the host surface as indicated by the loss, during processing, of most nongerminated conidia and the retention of germinated conidia. Wheeler (26), however, found no evidence that the extracellular material functioned to attach germ tubes or appressoria to the leaf surface.

Although the substrate on which the conidia were produced did affect the conidium appearance and germination rate, there was no significant variation in the germ tube morphology and sheath-fibril formation after germination had occurred. The similarity of germ tube morphology and sheath-fibril formation indicates that these characters are not attributable to prior nutrition of the fungus, but are a response to their immediate environment. Further studies to determine the role and the composition of the sheath-fibril complex may help to clarify the preinfection suscept-pathogen relationships.

It has been demonstrated in several fungal species that nutrition of the sporulating culture can affect spore appearance (10,11,21) and organelle number and activity (16,25). The results of our research reinforces the conclusion that nutrition of the fungus during sporulation must be considered when evaluating morphological, taxonomic, or physiological data.

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