Infection of Maize Stigmas by *Ustilago maydis*: Light and Electron Microscopy

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ABSTRACT

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Infection of maize stigmas (silks) by the smut fungus *Ustilago maydis* was documented with micrographs. Stigmas were inoculated with different aqueous suspensions of smut sporidia. When inoculum consisted of individual haploid strains, no mating or infection structures were observed. When stigmas were inoculated with sporidia that had compatible alleles at both mating loci (*a* and *b*), sporidia mated in pairs using a conjugation tube, and each pair formed a dikaryotic infection hypha that grew rapidly across the stigma surface, developed an appressorium, and entered the stigma. When sporidia were compatible at *a* but not at *b*, mating occurred irregularly, and resultant hyphae grew slowly and did not enter stigmas. Sporidia incompatible at *a* did not mate or form infection structures regardless of the *b* alleles they carried. Diploid sporidia with compatible

a and *b* alleles did not mate but formed infective hyphae directly. Diploid and dikaryotic hyphae formed hyaline, slightly swollen appressoria over epidermal cell-wall junctions. Penetrating hyphae arose from appressoria and entered stigmas by growing between epidermal cells. Subsequent growth in stigmas was intracellular, in that hyphae penetrated the walls of host cells, but transmission electron micrographs showed that host-cell plasma membranes remained intact around the hyphae. Hyphae in stigmas were multinucleate, with septa delimiting only vacuolate posterior portions of hyphae from the cytoplasm-filled hyphal tips. Stigma infections were readily effected under the greenhouse growth conditions described and may provide a convenient system for investigating some aspects of this host-pathogen interaction.

Additional keywords: corn smut, pollination, resistance.

The fungus *Ustilago maydis* (DC.) Corda causes common smut disease of *Zea mays* L. (1,4). The disease cycle begins when haploid yeastlike cells called sporidia mate and form dikaryotic hyphae that can infect maize plants. Mating and infection capabilities are regulated by two genetic loci, denoted *a* and *b*. Mating and infection require a pair of haploid sporidia compatible at both loci, i.e., the sporidia must have nonidentical alleles at *a* and *b*. Historically, the *a* locus has been called the mating locus, and the *b* locus has been called the pathogenicity locus. Recently, alleles from both loci have been cloned and sequenced. Based on homology with sequences of known function from other organisms, the *a* alleles are thought to encode pheromones and pheromone receptors (3), whereas the *b* alleles encode DNA-binding proteins, presumably with regulatory functions (12,17). Sporidia with diploid nuclei have been isolated from nature (4) or constructed in the laboratory (9). Sporidia with diploid nuclei containing compatible mating-type alleles reproduce by budding on nutrient media as do haploid sporidia; however, such diploid strains cause disease, whereas haploid sporidial strains are not pathogenic unless they occur with a compatible strain. All meristematic plant tissues are susceptible to infection by *U. maydis*; infection of various plant parts results in characteristic galls composed of abnormal host cells and sporulating fungal hyphae.

Injection of mixed compatible haploid sporidia into 1-wk-old seedlings is convenient for many kinds of studies because leaf galls form 2 wk after inoculation. In the field, however, galls are most commonly found in maize ears, and most yield losses are due to ear damage (4). Because smutted and healthy kernels occur in the same ear, life-history descriptions of *U. maydis* indicate that kernels may be infected through the stigmas (often called silks). Observation of stigma infections is rarely reported, however, and micrographs documenting the process have not been published. Repeated, previous attempts to observe stigma infection were unsuccessful (21), and the authors of a recent study questioned the importance of stigma infection in disease initiation (14).

The first objective of this study was to explore the possibility of stigma infection by *U. maydis* after inoculation with compatible haploid sporidia. We used injection procedures that previously led to infections of immature leaves in the greenhouse (19) and maize ears (14) in the field. Stigma infections were easily effected with these techniques under greenhouse conditions. Therefore, the study was expanded to include inoculations with haploid sporidial strains carrying different mating-type alleles alone and in various combinations. The behavior of a solopathogenic diploid strain also was investigated.

MATERIALS AND METHODS

Seeds of Golden Cross Bantam Hybrid sweet corn, a variety highly susceptible to *U. maydis*, were surface-sterilized in 5% sodium hypochlorite for 5 min, rinsed in several changes of sterile water, and soaked overnight in the final change. Seeds were sown five per 10-cm pot containing seed-starting mix, and pots were watered and placed in a greenhouse. Seedlings usually were allowed to grow until they were about 30 cm tall before they were transplanted individually to 15-cm pots with pasteurized soil mix containing topsoil, sand, vermiculite, perlite, and peat moss (2:1:1:1:1, by volume). Plants were fertilized with liquid fertilizer (20-20-20, N-P-K, diluted 1:8) when transplanted and were fertilized approximately once a week thereafter. Plants treated in this way grew quickly and flowered when they were less than 1 m tall. Pollen-bearing tassels were removed as they developed.

Four different combinations of mating-type alleles were represented by the haploid *U. maydis* strains used: *a1b1*, *a2b2*, *a1b2*, and *a2b1*. A diploid strain (genotype *a2b1 a1b2*) also was used. Kronstad and Leong (11) designated these strains 521, 518, UM031, UM032, and d132 and described their origins.

Sporidial cultures were maintained in dishes of potato-dextrose agar. Flasks (50 ml), each containing 10 ml of potato-dextrose

broth, were autoclaved and allowed to cool, and both cultures were started from the dishes. Flasks were placed in a wrist-action shaker, and cultures were incubated at room temperature with continuous vigorous agitation for 12–24 h. These cultures were not used to inoculate stigmas, because aggregates of thick-walled, hyphalike sporidia remaining from the plate cultures obscured mating interactions. Subcultures were made by transferring drops from the original cultures into flasks of fresh medium. These were incubated, as before, until sporidial concentrations reached 10^5–10^6 sporidia/ml (~18 h). Cultures were transferred to 50-ml polyethylene centrifuge tubes and centrifuged at 7,000 rpm for 6 min in a benchtop centrifuge. The culture supernatant was discarded, and the loosely pelleted sporidia were resuspended in 10 ml of sterile water. These aqueous sporidial suspensions were used immediately, either individually or in combination, as described below, to inoculate maize stigmas. Inocula were injected with a 22-gauge needle and syringe (14) through the husks into the space surrounding immature ovaries and stigmas until fluid was forced out the tops of the ears. Usually 1–3 ml of inoculum was sufficient. We initially inoculated plants before ears emerged from sheathing leaves (20) but later found that inoculation was easier and equally effective when stigmas were just visible in the ear apices. After 12–48 h, ears were removed from plants and brought into the laboratory for further study. Stigma pieces ~5 mm long were cut from the ears with a razor blade and either immediately mounted in water for observation with differential interference microscopy (DIC) or fixed in 1% glutaraldehyde/ 2% formalin in 0.1 M phosphate buffer at pH 7.4. Some fixed stigma pieces were prepared for scanning electron microscopy (SEM) by post-fixing in 1% OsO_4 for 2 h, rinsing in water, dehydration in ethanol, critical-point drying, and coating with gold-palladium. A Philips 505 scanning electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) was used to observe and photograph specimens. Other stigma pieces were prepared for transmission electron microscopy (TEM) by similar post-fixation and dehydration, followed by infiltration and embedment with epoxy resin (19). Sections ~80 nm thick were cut with a diamond knife, stained with ethanolic uranyl acetate and aqueous lead citrate, and observed with a Zeiss EM10 transmission electron microscope (Carl Zeiss, Inc., Thornwood, NY). Thin sections through smut hyphae in early stages of penetrating stigmas were obtained by cutting 12-μm-thick sections with glass knives, reembedding these in drops of plastic on microscope slides, and examining them with differential interference contrast light microscopy (DIC). Penetration sites were located and marked, and the sections containing them were glued to preformed resin blocks, which could be trimmed and thin-sectioned for TEM (19).

Some stigmas were prepared differently for sectioning and observation by light microscopy. Pieces up to 1 cm long were fixed in 1% glutaraldehyde/2% formalin in phosphate buffer, dehydrated in alcohol, and embedded in JB4 embedding medium (Polysciences, Inc., Warrington, PA). Sections were cut 2–5 μm thick with glass knives and placed on drops of water, dried onto slides at ~60 C, and stained with hematoxylin and methylene blue-basic fuchsin (6) to contrast the nuclei.

RESULTS

When immature maize stigmas were observed 12 h after inoculation with individual cultures of haploid sporidia, most fungal cells had a budding morphology. Some sporidia formed narrow hyphae that were not observed to enter host cells (Fig. 1). When stigmas were inoculated with sporidial strains in compatible mating-type allele combinations ($a1b1 \times a2b2$ or $a1b2 \times a2b1$), sporidial fusions were observed on stigmas 12 h later (Figs. 2–4). Fusion tubes of various lengths sometimes were connected laterally to sporidia (Fig. 2), but more often they were attached at the tips (Figs. 3 and 4). A straight hypha emerged from the fusion tube (Fig. 4) or from one of the two sporidia. Similar events were seen when inoculum consisted of sporidia that were compatible at a but not at b ($a1b1 \times a2b1$ or $a2b2 \times a1b2$);

however, irregular types of fusion also occurred. Sometimes more than two sporidia were involved, and the hypha that emerged from mated sporidia was usually short and convoluted (Fig. 5). When sporidia were incompatible at a, no fusions were observed, regardless of the compatibility of the b alleles. Behavior of co-inoculated sporidia incompatible at a was indistinguishable from that of a single-haploid culture of sporidia. Diploid sporidia on stigmas did not mate; instead, a straight, rapidly growing hypha emerged from one end of the sporidium (Fig. 6) 12–14 h after inoculation.

A characteristic crook in the hypha preceded formation of the slightly swollen appressorium that marked the end of growth over the stigma surface (Figs. 7–9). Appressoria were observed on the surfaces of young-maize ovaries (Fig. 7) as well as on stigmas (Figs. 8–11). Appressoria on stigmas formed most commonly over junctures between the long anticlinal walls of epidermal cells (Fig. 9). Maize stigmas were bilobed in cross section, and penetrating hyphae were observed most often along the flattened surface where the lobes were connected. Occasional penetrations of the receptive trichomes that lined the edges of stigmas also were noted. Trichomes were difficult to observe with SEM because of their tendency to charge under the electron beam; furthermore, trichomes were only 1–2 cells thick, and hyphal growth inside versus outside the trichomes was difficult to discern with light microscopy. As a result, trichome penetrations may have occurred more frequently than our observations indicated.

Fusions between sporidia with compatible a and incompatible b alleles produced convoluted hyphae that grew only for short distances. These hyphae sometimes formed appressoriumlike swellings, but the structures tended to be irregularly swollen and vacuolate (Fig. 10) and were not observed to penetrate stigmas.

Appressoria and penetration sites formed by diploid hyphae (Fig. 11) were similar to those formed by dikaryotic hyphae. Diploid and dikaryotic infection hyphae sometimes grew long distances over many epidermal cells on the stigma before forming appressoria, but only the hyphal tips contained cytoplasm. Older hyphal regions were vacuolate, often collapsed, and were separated from cytoplasm by crosswalls (Figs. 9 and 11). Usually, we could not follow a dikaryotic hypha back to the fused sporidia from which it had presumably emerged, because of hyphal collapse and entanglement with hyphae from other fusion events.

Numerous penetrations of stigmas were observed by light and electron microscopy, and although variations occurred, most penetration sites exhibited an appressorium, beneath which a relatively broad hypha entered the stigma between epidermal cells and then turned sharply to enter one of the cells (Fig. 12). Subsequent growth in stigmas was intracellular. Electron-dense fibrous material was typically observed in the plant cell wall around a penetrating hypha as well as around the appressorium (Fig. 12). Electron-dense strands sometimes connected surface hyphae to epidermal cells (Fig. 13).

Whole, live infected stigmas were observed with DIC. Hyphae grew through living host cells, and host cytoplasm streamed around the hyphae. Plastids and other organelles seemed to aggregate around the hyphae, and the host-cell nucleus was often near the hyphal tip. Rapidly moving organelles in the host cytoplasm surrounding the hypha appeared as longitudinal striations in DIC photomicrographs (Fig. 14). Hyphae typically grew diagonally across the large vacuoles of stigma cells but usually turned and penetrated cell walls at a right angle (Fig. 14). Hyphae sometimes grew through end walls of stigma cells. Hyphae grew rapidly through stigma cells and often seemed to be growing in the general direction of the ovary. In one instance, a hypha extended through more than 12 cells along the stigma axis 24 h after inoculation. Nuclei in the hyphae could not be identified with certainty in unstained living material; however, stained sections revealed multinucleate hyphae (Fig. 15) with no obvious pairing of nuclei or septations other than those delimiting vacuolate hyphal compartments.

Transmission electron micrographs showed that host-cell plasma membranes and cytoplasm were continuous around invading hyphae (Figs. 16–18). Even hyphae growing through

large vacuoles were separated from the vacuole by the host plasma membrane and a thin layer of cytoplasm (Fig. 16). The tonoplast presumably delimited the vacuole but was not obviously intact in these sections. Host-cell walls were ruptured by the hyphae, and deformation of the cell wall around hyphae growing from one cell into another suggested mechanical penetration (Fig. 17). An electron-dense substance was observed between the host plasma membrane and the fungal cell wall on both sides of the breached plant cell wall (Fig. 18). The presence of many collapsed hyphae in stigmas 36 h after inoculation provided further evidence
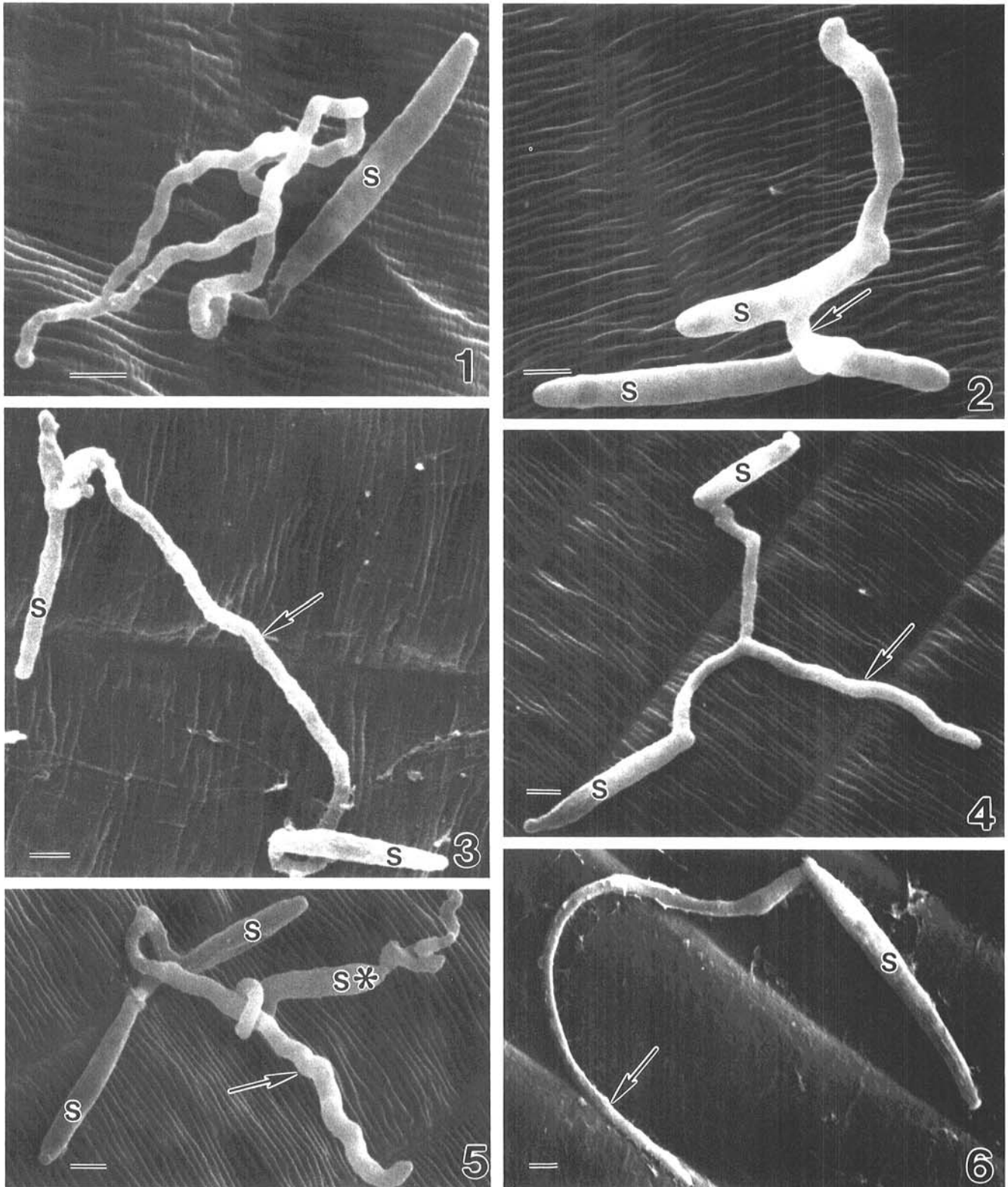
Figs. 1–6. Scanning electron micrographs of maize stigmas 12 h after inoculation with *Ustilago maydis* sporidia. **1,** This sporidium (S), on a stigma inoculated with a single-haploid culture, formed a narrow, coiled hypha. **2,** Mated, fully compatible sporidia (S) connected by a short fusion tube (arrow). **3,** Fully compatible sporidia (S) connected by a long fusion tube (arrow). **4,** An infection hypha (arrow) emerged from a fusion tube that connected two sporidia (S). **5,** Mated sporidia (S), compatible at *a* but not at *b*. A third sporidium (*) is apparently attached to the convoluted hypha (arrow). **6,** An infection hypha (arrow) emerged from a diploid sporidium (S). All bars = 2 μm.

of rapid tip growth and subsequent posterior hyphal collapse (Fig. 19). These collapsed hyphae would be extremely difficult to visualize with light microscopy.

DISCUSSION

The inoculation methods described here reliably produced stigma infections in dozens of greenhouse-grown plants over a period of 2 yr. Inoculated ears allowed to mature contained kernel galls similar to those found in field-infected plants, although the smaller greenhouse-grown ears contained fewer kernels than did ears from the field. No attempts were made to inoculate or investigate field-grown plants; however, some of our observations may prove useful to those designing experiments involving ear inoculations for a variety of purposes.

We can sympathize with the difficulties experienced by previous
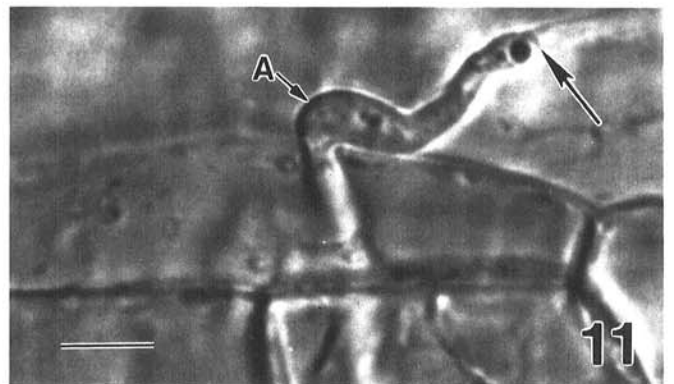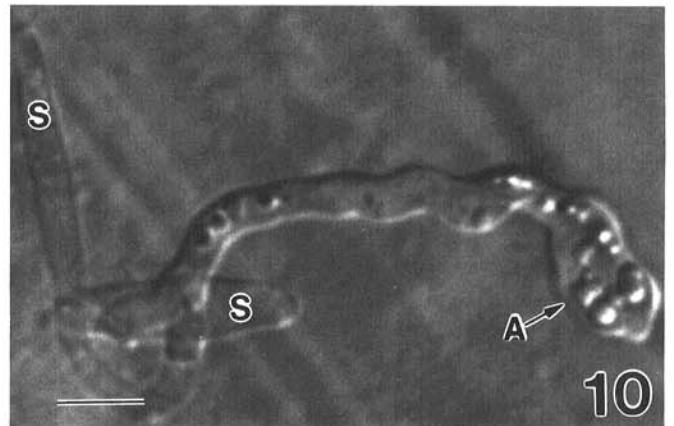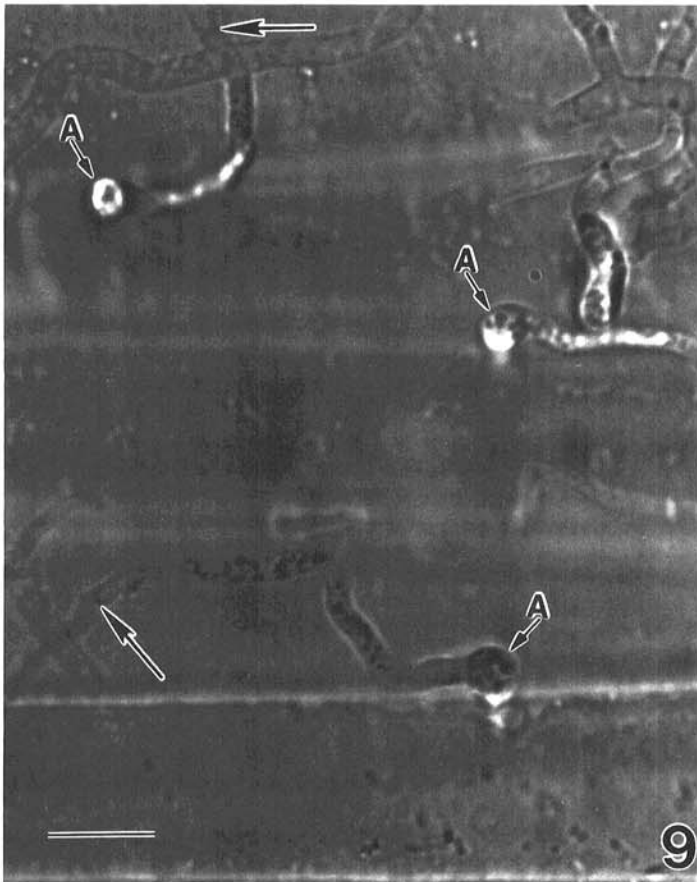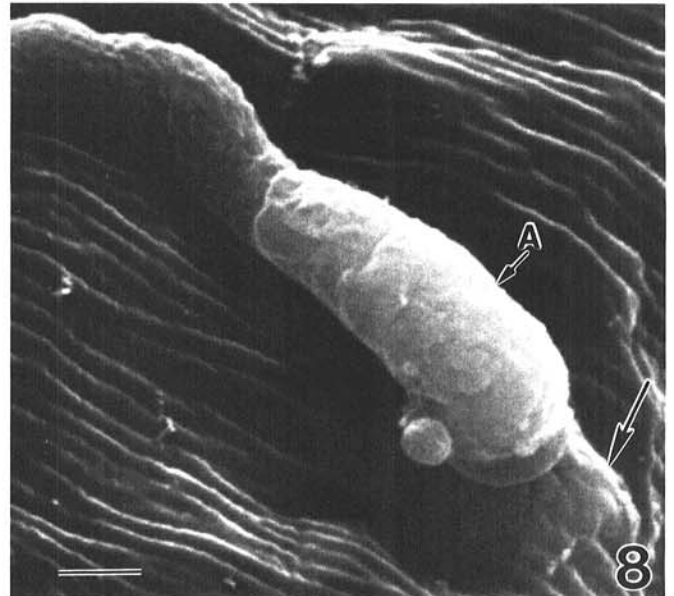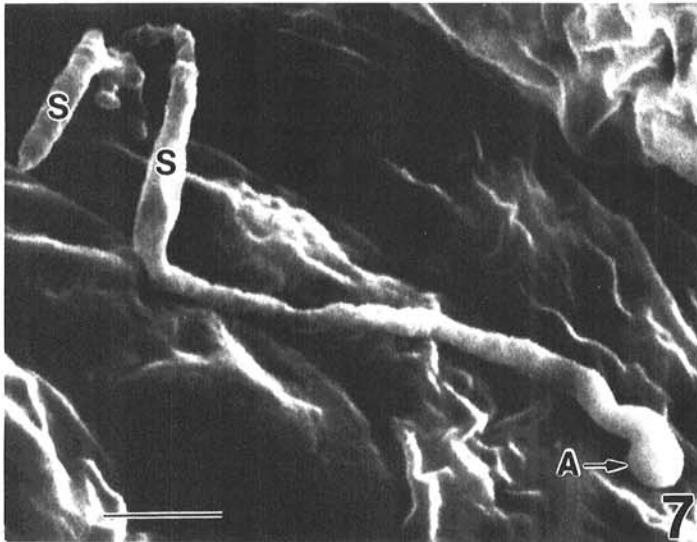
Figs. 7-11. Scanning electron micrographs (7,8) and differential interference microscopy (9-11) of fixed *Ustilago maydis* appressoria and infection hyphae 12–14 h after inoculation. 7, The surface of a maize ovary; 8-11, stigma surfaces; 7-9, fully compatible matings; 10, mated sporidia compatible at *a* but not at *b*; and 11, a diploid infection. 7, Fused sporidia (S) with infection hypha emerged from one of the mated sporidia. The curving of the hypha behind the slightly swollen appressorium (A) is characteristic. Bar = 5 μm. 8, Appressorium (A) and apparent penetration of maize stigma cell. The rim (arrow) between the appressorium and the stigma surface may correspond with the fibrous material shown in Figure 12. Bar = 1 μm. 9, Three appressoria (A) and penetration sites were all at junctions between adjacent epidermal cells. Septa (arrows) separated vacuolate and cytoplasmic hyphal compartments. Bar = 20 μm. 10, A short, twisted hypha separated the fused sporidia (S) from the large, irregularly shaped appressorium (A). Bar = 5 μm. 11, Appressorium (A) and septum (arrow) formed by diploid hyphae were indistinguishable from dikaryotic infection structures. Bar = 10 μm.
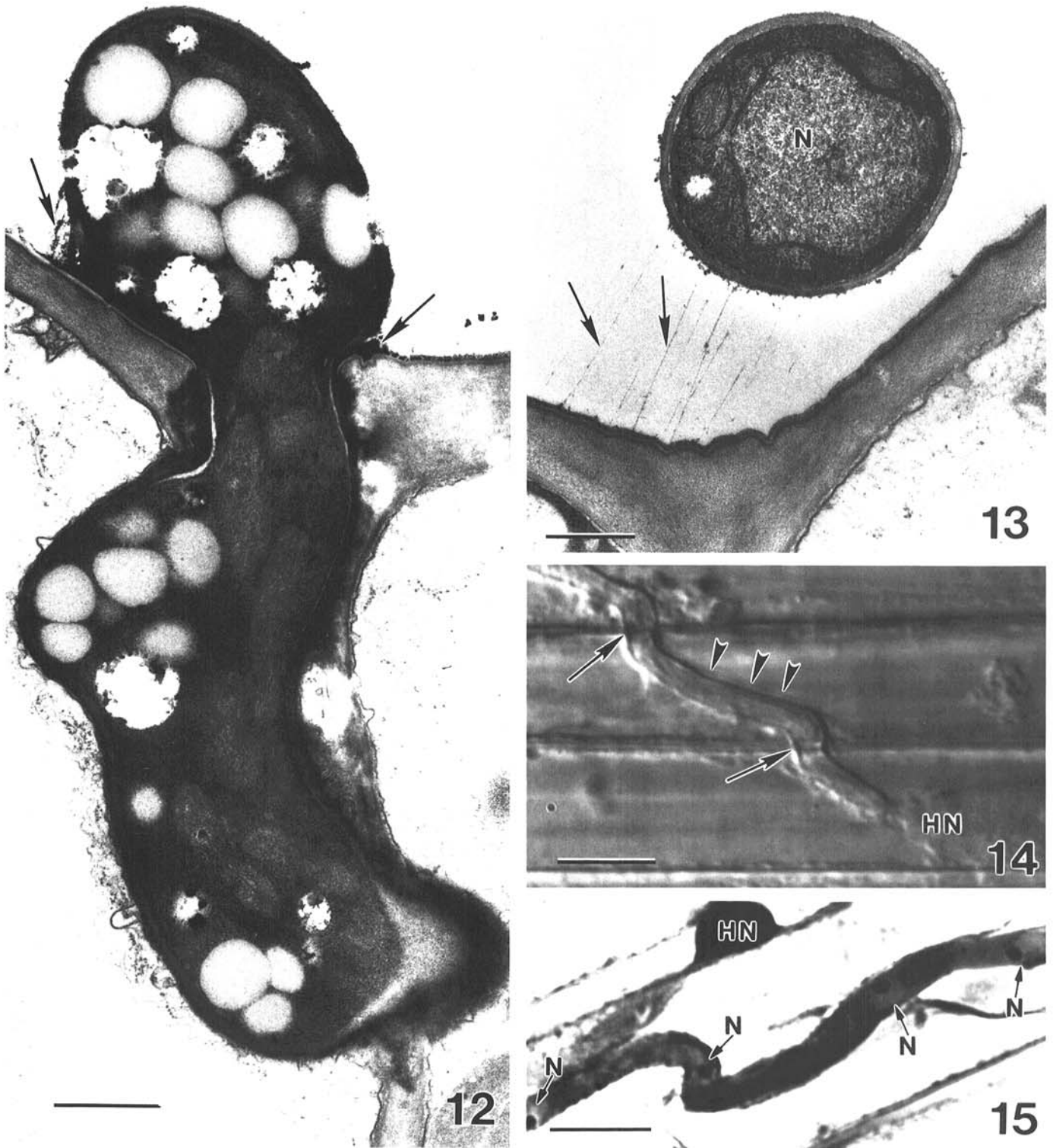
Figs. 12–15. Micrographs of maize stigmas 14–16 h after inoculation with compatible *Ustilago maydis* sporidia. **12 and 13,** Transmission electron micrographs of hyphae on stigmas; **14,** a differential interference microscopy image of a live stigma; and **15,** a light micrograph of a stained, sectioned stigma. **12,** A hypha emerged from the appressorium, entered the stigma between two epidermal cells, grew a short distance into the cell on the left, and then grew into the cell on the right and out of the plane of this section. Fibrous material occurred between the appressorium and host-cell wall (arrows). Bar = 1 μm. **13,** Cross section of hypha that grew along the stigma surface, connected to the surface of the cell wall underneath by strands (arrows). N = fungal nucleus. Bar = 0.5 μm. **14,** Smut hypha grew through interior stigma cells. The hyphal tip was near the host nucleus (HN). Apparent striations along the hypha (arrowheads) are the record of rapidly moving organelles in the host cytoplasm ensheathing the hypha. The hypha made a 90° angle and a wide pore (arrows) as it grew from one cell into another. Bar = 10 μm. **15,** Hypha in a stigma 36 h after inoculation was multinucleate with no septa; four nuclei (N) with large, dark nucleoli are visible in this section of hypha. HN = host nucleus. Bar = 10 μm.
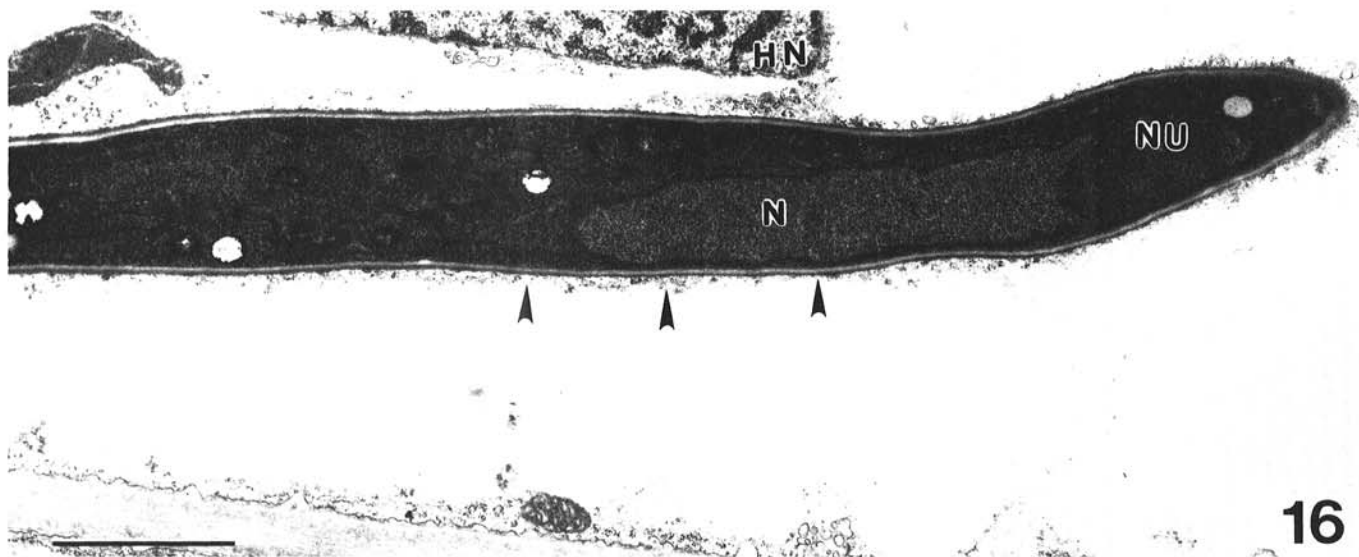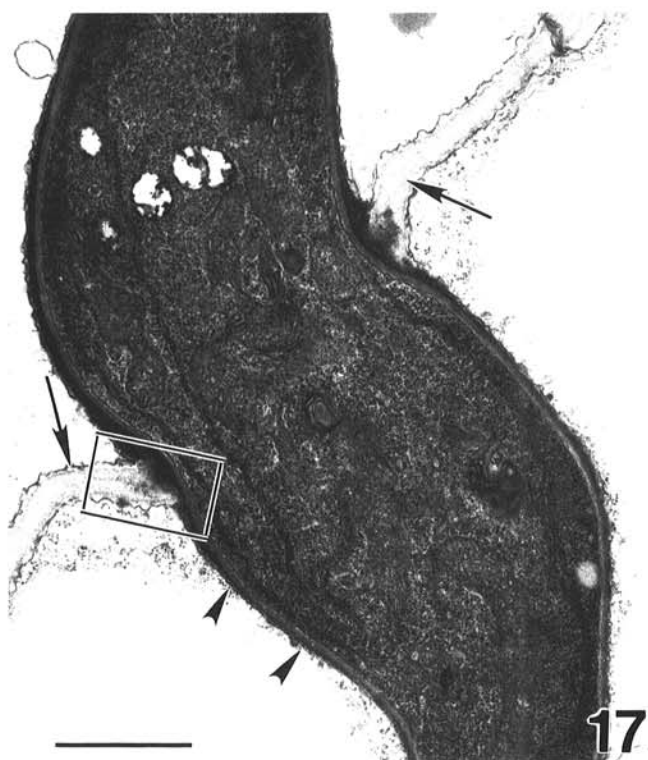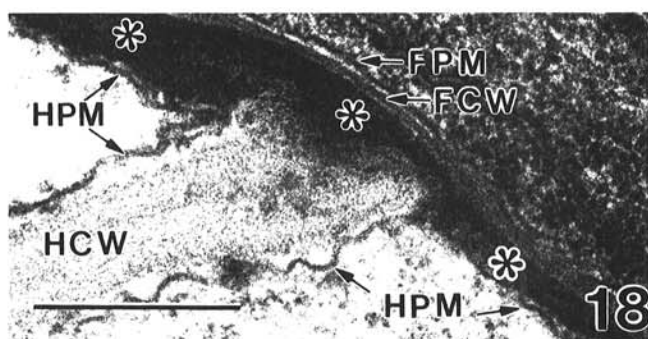
Figs. 16–19. Transmission electron micrographs of *Ustilago maydis* hyphae in stigma cells 36 h after inoculation. **16,** A hypha with a long nucleus (N) and prominent nucleolus (NU) traversing the large vacuole of a stigma cell was surrounded by host plasma membrane and a thin layer of cytoplasm (arrowheads). HN = host nucleus. Bar = 2 μm. **17,** Serial sectioning confirmed that this hypha grew from the upper left to the lower right cell; the nature of the cell-wall deformation on either side of the penetrating hypha (arrows) suggests that mechanical forces were involved in cell-wall penetration. The host cytoplasm and plasma membrane (arrowheads) surrounded the hypha. Bar = 1 μm. **18,** Higher magnification of the marked square in Figure 17. An electron-dense substance (*) was between the host plasma membrane (HPM) and the fungal cell wall (FCW). The host plasma membrane was continuous around the hypha, and the fungal plasma membrane (FPM) also was visible. HCW = host-cell wall. Bar = 0.5 μm. **19,** Seven collapsed fungal hyphae (arrows) in a section of stigma cells. Bar = 2 μm.

workers attempting to observe stigma infection with light microscopy, because small size and lack of pigmentation in sporidia and appressoria made observation of infection sites difficult to achieve with bright-field light microscopy. The availability of DIC optics was critical to observation of penetration sites and intracellular hyphae. Another factor aiding our study of stigma infections was concurrent investigation of sporidial mating on leaves and in vitro (19). Log-phase sporidia grown in shaken liquid medium, suspended in water, and combined immediately mate readily, and mating structures have a relatively consistent morphology. Sporidia grown on solid medium or for long periods of time in liquid culture often have irregular shapes, become somewhat hyphal, and may not be capable of mating without first undergoing a mitotic division (K. Snetselaar, *unpublished data*). These factors could contribute to erratic infection and difficulty in observing infection structures. Many studies do not indicate the condition of sporidia used as inoculum. The timing of observation is also important; mating sporidia and appressoria were numerous 12–18 h after inoculation of stigmas, but after 24–48 h, most dikaryons were inside stigmas. The empty sporidia and hyphae left on the surface were difficult to see, as were collapsed portions of hyphae inside stigma cells.

Our observations indicated that fusion of fully compatible haploid sporidia preceded infection of stigmas. Sporidia compatible at *a* but not at *b* mated but did not infect stigmas, thus confirming in vitro observations (16; K. Snetselaar, *unpublished data*). Although haploid sporidia sometimes formed narrow hyphae on stigma surfaces, we saw no evidence of penetration by haploid hyphae and subsequent fusion in the plant as described by some authors (5,20). Smut fungi are notoriously variable, however, and we used only four related haploid strains. Other *U. maydis* strains may behave differently. Sporidial fusion clearly is not always required for infection; diploid sporidia formed infective hyphae without mating first.

Appressoria that formed on stigmas were identical in appearance to those observed on leaf surfaces (19), but infection sites on thin and transparent stigmas were considerably easier to observe than those on leaves. Although *U. maydis* appressoria were unmelanized and morphologically undifferentiated relative to appressoria formed by other pathogenic fungi, they were easily recognized by their swollen appearance and the curved hyphae attached to them. Infective hyphae probably can enter ovaries directly as well as through stigmas, at least when sporidia are delivered by injection as in this study. Appressoria were observed on maize ovaries, but we did not attempt to obtain sections through them.

Dikaryotic hyphae probably were attached to host surfaces by an adhesive material of some kind; not only was fibrous material observed around surface hyphae and appressoria, but we noticed in preparing stigmas that more sporidia and hyphae remained attached to stigmas when inoculum contained compatible sporidia than when single sporidial strains were used. Filaments termed "fimbriae" have been reported in *U. maydis* sporidia (7,22).

After the initial penetration between epidermal cells, hyphal growth in stigmas was intracellular, in the sense Luttrell (13) used the term to refer to hyphae that have breached the host-cell wall but not the plasma membrane. Hyphae grew rapidly without branching and were multinucleate during the early stages of infection described here. Septa separated only posterior vacuolate hyphae from rapidly elongating hyphal tips. In contrast, hyphae in maize ovaries and leaves were characterized by intercellular growth, prolific branching, and paired nuclei in septate hyphal compartments (4,18). Galls formed in ovaries and leaves but not in stigmas. Rapid directional growth of smut hyphae through the stigma may be an important determinant of pathogenicity for *U. maydis*, because hyphae that fail to reach the ovary will not sporulate.

Walter's (21) practice of ensuring the presence of pollen on stigmas may have contributed to his inability to observe stigma infections. Consideration of maize pollination biology (8) suggests that unpollinated stigmas remain susceptible to infection by *U. maydis* longer than do pollinated stigmas. When several pollen tubes have traversed the stigma and entered the ovary, an abscission zone forms at the base of the stigma, and the stigma is eventually shed (8). *U. maydis* infection hyphae, which are obligately biotrophic, probably cannot grow into the ovary across dead cells in the abscission zone. We excluded pollen by removing maturing tassels and by inoculating ears prior to stigma emergence.

Pollination effects on smut infection have not been examined in detail. Increases in smut incidence correlated with detasseling have been attributed to the associated mutilation of the plants (4). Detasseling and other injuries may stimulate increased cell divisions, and tissues with dividing cells are more susceptible to smut infection. Perhaps attempts should be made to separate the effects of injury from the effects of pollen exclusion on smut incidence.

The smut hyphae observed during this study did not grow preferentially in the pollen tracts that surround vascular bundles in maize stigmas (10). The possibility of interaction between pollen tubes and smut hyphae is a fascinating topic for speculation and future study. Characteristics of pollen tube growth may influence the progress of smut infection in the female parent.

Differences in susceptibility to *U. maydis* occur among maize hybrids and breeding lines (2,4), but the nature of resistance is still unknown (20). Some resistance may be morphological; ears tightly wrapped by husks may resist entry by smut propagules (4). The possibility of cellular resistance to *U. maydis* in stigmas has not been investigated. Evidence for differential resistance of stigmas to another fungus, the ear rot pathogen *Fusarium graminearum*, has been reported (15). Among the inbred maize lines tested, the ability of the fungus to degrade stigma tissue was correlated with the severity of ear rot symptoms (15). The procedure for examining stigmas outlined here could be adapted to investigate whether cellular properties of stigmas contribute to smut susceptibility and resistance. We observed that *U. maydis* will infect stigmas attached to ovaries dissected from immature ears and placed in drops of inoculum, so the infective capabilities of different *U. maydis* strains might be compared on genetically identical tissue. Such a protocol would be useful in screening mutant sporidial clones for altered pathogenicity.

Very little is known about the biology of infection and host-parasite interaction in smut fungi. These pathogens are important because of direct economic effects and are useful in experimental systems. To date, *U. maydis* and other smut fungi have been used primarily in studies involving genetics and fungal mating-type genes. The information presented here should be useful to workers interested in using smut fungi to investigate basic aspects of pathogenicity and plant-fungus interactions.

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