Sporidial mating and infection process of the smut fungus, *Ustilago hordei*, in susceptible barley[1]

G.G. Hu, R. Linning, and G. Bakkeren

Abstract: *Ustilago hordei* (Pers.) Lagerh. causes covered smut of barley and oats. Sporidial mating and the infection process on compatible barley plants, cv. Hannchen, were investigated using light microscopy and scanning and transmission electron microscopy. Within 2 h after mixing of sporidia of opposite mating types on water agar, polar conjugation tubes emerged that subsequently fused, producing infection hyphae at the junctions. Similar events occurred on germinated barley shoots, although sporidia regularly produced several conjugation tubes, of which only one was involved in mating. Tubes emerging from the sides of cells were also observed. Infection hyphae emerged from either the conjugation tube or conjugated cell body. Hyphae elongated along the shoot surface until characteristic crook and appressorium-like structures were formed. An invading hypha emerged beneath this structure and directly penetrated the underlying epidermal cell. Hyphae extended both intra- and inter-cellularly into tissues, without much branching, before becoming established in the shoot meristematic region. Plant plasma membranes remained intact during pathogen ingress and an electron-dense matrix of unknown origin appeared in the interface between plant plasma membrane and invading hypha. A large fungal biomass was generated in the host spike tissue at 42–63 days postinoculation during the development of the floral meristem.

Key words: *Hordeum vulgare*, pathogen, sporidia, teliospores, ultrastructure, Ustilaginales.

Résumé : L'*Ustilago hordei* (Pers.) Lagerh. cause le charbon vêtu chez l'orge et l'avoine. A l'aide de la microscopie photonique et de la microscopie électronique par balayage et par transmission, les auteurs ont examiné l'accouplement sporidial et le processus d'infection chez des plants d'orge compatibles du cv. 'Hannchen'. Moins de 2 h après avoir mélangé des sporidies de types de compatibilité opposés sur eau gélosée, des tubes de conjugaison polaire émergent et se fusionnent subséquemment en produisant des hyphes d'infection à leur jonction. On observe des évènements similaires sur des tiges d'orge germée, bien que les sporidies produisent régulièrement des tubes de conjugaison dont quelques uns seulement sont impliqués dans des croisements. On observe également des tubes qui se forment sur les côtés des cellules. Les hyphes d'infection émergent soit du tube de conjugaison ou du corps cellulaire conjugué. Les hyphes s'allongent le long de la surface de la tige jusqu'à la formation de structures en crosse et appressorioïdes caractéristiques. Une hyphe d'envahissement émerge sous cette structure et pénètre directement la cellule épidermique sous-jacente. Les hyphes s'étendent à la fois dans et entre les cellules à l'intérieur des tissus, sans trop de ramifications, avant de s'établir dans la région méristématique de la tige. Les membranes plasmiques de la plante demeurent intactes au cour de la progression du pathogène, et une matrice dense aux électrons d'origine inconnue apparaît à l'interface entre la membrane plasmique et l'hyphe envahissant. Il y a formation d'une grosse masse fongique dans les tissus des épis 42–63 jours après l'inoculation, au cours du développement du méristème floral.

Mots clés : *Hordeum vulgare*, pathogène, sporidie, téliospores, ultrastructure, Ustilaginales.

[Traduit par la Rédaction]

Introduction

The plant pathogenic fungus *Ustilago hordei* (Pers.) Lagerh. causes covered smut in barley (*Hordeum* spp.) and oats (*Avena* spp.) and is the most studied representative of a group of related, small grain-infecting smuts (Fisher and Holton 1957; Bakkeren et al. 2000). This group is responsible for significant losses worldwide (e.g., Mathre 1997; Thomas and Menzies 1997). *Ustilago hordei* is a dimorphic fungus that grows asexually by budding on a variety of artificial media. Mating of basidiospores (sporidia) is controlled by a bipolar mating system consisting of a single mating-type locus (*MAT*) with two alleles in nature: *MAT-1* (formerly called *a*) and *MAT-2* (formerly called *A*; Bakkeren and Kronstad 1993, 1994, 1996; Lee et al. 1999).

In nature, barley seeds come into contact with wind-dispersed teliospores or teliospores from infested neighboring seeds. Teliospores overwinter under the seed

Received 21 May 2002. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 26 October 2002.

G.G. Hu, R. Linning, and G. Bakkeren.[2] Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Highway 97, Summerland, BC VOH 1Z0, Canada.

[1]Contribution No. 2160 of the Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, B.C.

[2]Corresponding author (e-mail: BakkerenG@agr.gc.ca).

 DOI: 10.1139/B02-098

hull and then germinate with the seeds in the spring. The diploid teliospore undergoes meiosis producing a basidium on which four haploid basidiospores form. This has been described in detail for the related, tetrapolar corn (*Zea mays* L.) smut, *Ustilago maydis* (Ramberg and McLaughlin 1980). In the bipolar smuts, the two mating types (*MAT*) segregate in a 1:1 manner (Thomas 1988, 1991). Under favorable conditions, two basidiospores of opposite mating type fuse through slender mating hyphae (conjugation tubes). At this stage, mating with progeny from other teliospores is possible ("outbreeding"). Fusion produces a thick, dikaryotic infection hypha that is the obligate parasitic form of the fungus representing the other morphological stage. In *U. maydis*, the genetic control of the mating process has been thoroughly studied (for reviews see Bölker 2001; Martinez-Espinoza et al. 2002 and citations therein), and detailed microscopic observations of this mating process in vitro and on corn have been published (Snetselaar and Mims 1992; Snetselaar 1993; Snetselaar et al. 1996).

The morphology and process of sporidial fusion of *U. hordei* in vitro have been described using light microscopy (Martinez-Espinoza et al. 1993). On a suitable medium, such as water agar, mixed basidiospores of compatible mating types produce conjugation tubes, also called mating hyphae. Tube growth is directed toward the tip of tubes emerging from cells of the opposite mating type by low molecular weight, diffusible mating factors (pheromones) that are involved in conjugation tube formation and sporidial fusion (Anderson et al. 1999). To date, it is not known if the same sequence of events occurs during the mating process of *U. hordei* on host barley plants.

The infection process of *U. hordei* in barley has been described in general terms using light microscopy (Kiesling 1952; Chatterjee 1956; Kozar 1969). Similar light microscopy studies have documented different smut pathosystems such as *Ustilago avenae* and *Ustilago kolleri* in oat (Western 1936), *Tilletia tritici* in (*Triticum*) wheat (Woolman 1930), and *Sphacelotheca reiliana* in sorghum (Wilson and Frederiksen 1970). A study of the sporulation stage of *Ustilago nuda* in barley has been conducted using electron microscopic analysis (Luttrell 1987). Modern ultrastructural techniques have not been previously employed to study the *U. hordei* – barley interaction and there is no detailed description of the infection process on host plants.

In general terms, a successful infection occurs when *U. hordei* hyphae penetrate the germinating barley seedling. Many of the bipolar, small grain-infecting smuts have a small window of opportunity just after seed germination to infect seedlings. Without inducing any symptoms, the fungus advances in plant tissue establishing itself in plant nodes and in the meristematic region of the coleoptilar growing point. Much later, during the development of the inflorescence, the fungus proliferates and differentiates into sexual teliospores that replace the seeds. The infection process of *U. maydis* in corn has been described in detail microscopically (Snetselaar and Mims 1993, 1994), but this process contrasts sharply with that of the small grain-infecting smuts. *Ustilago maydis* can infect any part of the corn plant where cell division and elongation occur, inducing galls in which it proliferates and sporulates. Successful infection by *U. hordei* at later stages in plant development cannot be established. The molecular basis of this resistance remains to be elucidated.

In this paper we report the results of a detailed microscopic investigation using light microscopy and scanning and transmission electron microscopy techniques; this study was undertaken to (*i*) describe the mating process of *U. hordei* on the host plant and compare the difference in mating behavior of *U. hordei* in vivo and in vitro, and (*ii*) describe the complete infection process during a compatible interaction of the pathogen in a barley host plant.

Materials and methods

Fungal strains and inoculations

Ustilago hordei strains Uh359 (*MAT-1*) and Uh362 (*MAT-2*), progeny from Canadian Prairies teliospore collection No. 4854 (J. Menzies, Agriculture & Agri-Food Canada, Cereal Research Centre, Winnipeg, Man.), were cultured on potato dextrose agar plates at 22°C. Single colonies were transferred into liquid potato dextrose broth or YEPS (1% yeast extract, 2% Bacto peptone, 2% sucrose) and incubated at 22°C on an orbital shaker until the culture reached mid-log phase. The basidiospores were collected by centrifugation for 5 min at 3000 × *g* and then resuspended in sterile double-distilled water at a density of 1×10^6 cells/mL. For in vitro mating experiments, 100 µL of cells from each of the two mating types were mixed and plated on 2% water agar on glass slides. The slides were placed on moist filter paper in Petri dishes and incubated at 22°C until viewed directly under the microscope.

For in vivo mating experiments, seeds of barley cv. Hannchen were dehulled and surface sterilized for 1 min in 70% ethanol followed by 10 min in 1% NaOCl and several

Fig. 1. Mating process of *Ustilago hordei* in vitro and in vivo. (A) One or two polar conjugation tubes are produced per sporidial cell within 2 h after the mixing of compatible sporidia (Uh359 *MAT-1* and Uh362 *MAT-2*). Scale bar = 10 µm. (B) Conjugation bridges (arrows) form after two conjugation tubes, representing opposite mating types, make contact (12 h after mixing). Scale bar = 15 µm. (C) Lateral conjugation tubes (arrow) develop from the side of a sporidial cell. Note that the conjugation bridge is formed (12 h after mixing). Scale bar = 6 µm. (D) Infection hyphae (arrow) emerge from the conjugation bridge (12 h after mixing). Scale bar = 10 µm. (E) A polar conjugation tube forms from the sporidial cell (S) on the surface of a seedling (2 h postinoculation). Scale bar = 5 µm. (F) A polar conjugation tube fuses with a lateral tube. Seemingly, an infection hypha (arrow) emerges from one of the cell bodies, although this could also be a budding daughter cell (6 h postinoculation). Scale bar = 3 µm. (G) An infection hypha grows out from the conjugation bridge and elongates along the surface of the young shoot (first leaf). A septum (Sp; arrow) can be observed (6 h postinoculation). Scale bar = 3 µm. (H) A lateral conjugation tube fuses with a polar tube and forms a conjugation bridge from which an infection hypha emerges (6 h postinoculation). Note septa (arrows). Scale bar = 5 µm. (I) Three sporidial cells are possibly involved in the mating, and one infection hypha emerges. Note septa (arrows). Scale bar = 6 µm. Figures 1A–1D are differential interference contrast light micrographs of mating on water agar, and Figs. 1E–1I are scanning electron micrographs on barley shoots.

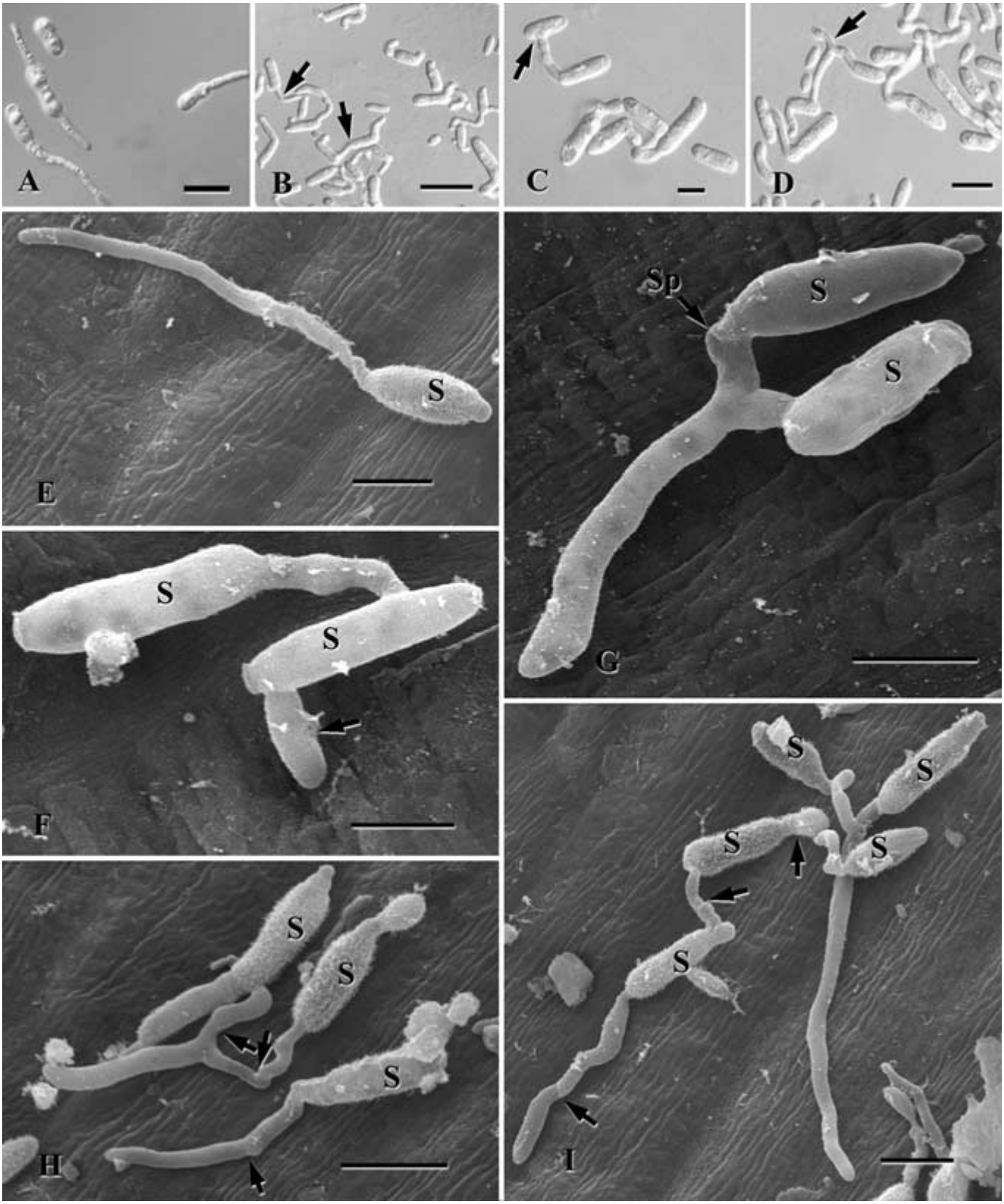


Fig. 2. Mating and infection process of *Ustilago hordei* on barley seedlings. (A) More than two polar conjugation tubes have emerged from one sporidial cell (S), but only one of the tubes mates to form a conjugation bridge (arrow; 6 h postinoculation). Scale bar = 10 µm. (B) Conjugation tubes elongate for a considerable distance from sporidial cells (S) to form the conjugation bridge (arrow; 12 h postinoculation). Scale bar = 15 µm. (C) A hyphal tip encounters a juncture at the anticlinal wall, forms a crook, and develops an appressorium-like structure (A) (12 h postinoculation). Scale bar = 2 µm. (D) An invading hypha (F) penetrates into the epidermal cell (Ec) directly from beneath the appressoriumlike structure (A). It passes through several host cells intracellularly after which both intra- and inter-cellular growth can be observed. Note an intercellular hypha (arrow) and an intracellular hypha (2 days postinoculation). Scale bar = 12 µm. (E) Detail of direct penetration into the epidermal cell by the invading hypha from beneath an appressorium-like structure. No discernable necrotic or abnormal reaction was seen in invaded epidermal cells. The host cytoplasm and vacuole (V) surround the invading hypha (2 days post inoculation). Scale bar = 2 µm. A, appressorium-like structure; EW, epidermal wall; L, lipid body; F, mycelium. (F) Intercellular hypha between mesophyll cells of the young outer leaf of the shoot. Two distinct nuclei (N) can be seen in the mycelium (F). Note that the plant cell wall appears to be degraded (double arrows), presumably because of enzymatic action from the hypha (6 days postinoculation). Scale bar = 2 µm. IS, intercellular space. (G) Intracellular hyphae. Two nuclei (N) are present in the mycelium (F). The host cytoplasm surrounds the mycelium (arrow: see enlargement in Fig. 3A; 6 days postinoculation). Scale bar = 3 µm. Figures 2A–2C are scanning electron micrographs; Fig. 2D is a light micrograph; and Figs. 2E–2G are transmission electron micrographs.

washes with sterile, double-distilled water. They were subsequently allowed to germinate under sterile conditions in Petri dishes lined with water-soaked filter paper at 22°C in a dark incubator. At 48 h, roots had emerged, and shoots varied in length from 5 to 15 mm. Previous experiments showed that barley cv. Hannchen is susceptible to infection by the pathogenic hyphae from mated Uh359 and Uh362 cells, routinely producing up to 70% infected plants in the greenhouse (data not shown). The previously described mixed cell suspension was rubbed gently onto the crown area surface of the barley seedlings with sterile cotton-wrapped stubs. Inoculated seedlings were overlaid with moist, sterile filter paper and were kept in the same Petri dishes at 22°C in a dark incubator. Shoots from treated seedlings continued to elongate and were collected at 2, 6, and 12 h postinoculation (hpi) at which point they were processed for scanning electron microscopy (see below). To investigate the complete infection process, at 48 hpi, the inoculated barley seedlings were planted in trays (one per 5-cm pot) in a 1:1 vermicullite – peat moss mixture and fed weekly with 3 g/L 20:20:20 (N:P:K) general purpose fertilizer (Plant Prod, Plant Products Co. Ltd., Brampton, Ont.). They were grown in a climate-controlled growth chamber having a daylength of 18 h at approximately 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation and 22°C and a nighttime temperature of 18°C. The main plant culm (not emerging tillers) was sampled at 2, 4, 6, 9, 42, 54, and 63 days postinoculation (dpi). At 63 dpi, plants started to flower.

To further follow the infection process using light microscopy, strain Uh359 was stably transformed with an integrated construct containing a *Ustilago*-specific β-glucuronidase (GUS) expressing cassette. In this construct, we fused the GUS coding region (Jefferson 1989) to the promoter of the *U. maydis* glyceraldehyde-3-phosphate dehydrogenase gene (Smith and Leong 1990; details of the construct available on request). Tissue samples were incubated directly in X-gluc staining buffer (0.1 M $NaPO_4$ buffer (pH 7.5), 0.5 mM $K_3(Fe(CN)_6)$, 0.5 mM $K_4(Fe(CN)_6)$, 10 mM EDTA, 0.5% (v/v) Triton X-100, 20% methanol, 1 mg/mL X-glucuronide; ClonTech Laboratories) at 37°C.

Sample processing for light and electron microscopy

Samples for microscopic analysis were prepared as described in Hu and Rijkenberg (1998*a*, 1998*b*). Briefly, the harvested shoots were sliced into 3 × 3 mm pieces and fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) overnight. Subsequently, the samples were washed twice in buffer, postfixed for 2 h in 2% osmium tetroxide in the same buffer at room temperature, rewashed in buffer, and then dehydrated in a graded ethanol series. For scanning electron microscopy (SEM) observations, the specimens were critical point dried in a CPD 020 dryer (Balzers Union) with carbon dioxide as a transition fluid and mounted on copper stubs. All specimens on stubs were gold–palladium coated in a Polaron sputter coater. The exterior of the coated samples was examined with a JEOL S-350 scanning electron microscope operating at 10.0 kV. The remaining samples were embedded in Spurr's resin (Spurr 1969). For differential interference contrast (DIC) microscopy, 1-µm thin sections were cut with a glass knife and heat-mounted on glass slides. The sections were stained with a drop of 1% toluidine blue in sodium acetate and examined with DIC. For transmission electron microscopy (TEM), ultrathin sections were cut with a diamond knife, stained with 2% uranyl acetate for 15 min, washed in double-distilled water and post-stained in lead citrate for 15 min. They were subsequently washed again in double-distilled water and viewed with a JEOL 100CX transmission electron microscope at 80 kV.

Data processing

SEM images were captured using Quartz PCI software, version 4.0. Light microscope and TEM images were made by digitizing black and white photographic negatives made from Kodak TMAX 100 film or by digitizing 35-mm color slides and converting them to black and white. Adobe Photoshop version 6.0 was used to adjust image contrast and to compose all plates.

Results and discussion

In vitro mating process of *U. hordei*

Within 2 h after the mixing of *U. hordei* cells of opposite mating types (strains Uh359 and Uh362) on water agar, conjugation tubes were seen (Figs. 1A and 1B). These narrow, meandering conjugation tubes usually emerged from one end of the sporidial cell but were occasionally initiated from both ends of the cell (Fig. 1A). Conjugation tubes then elongated and some extended over considerable distances. It is

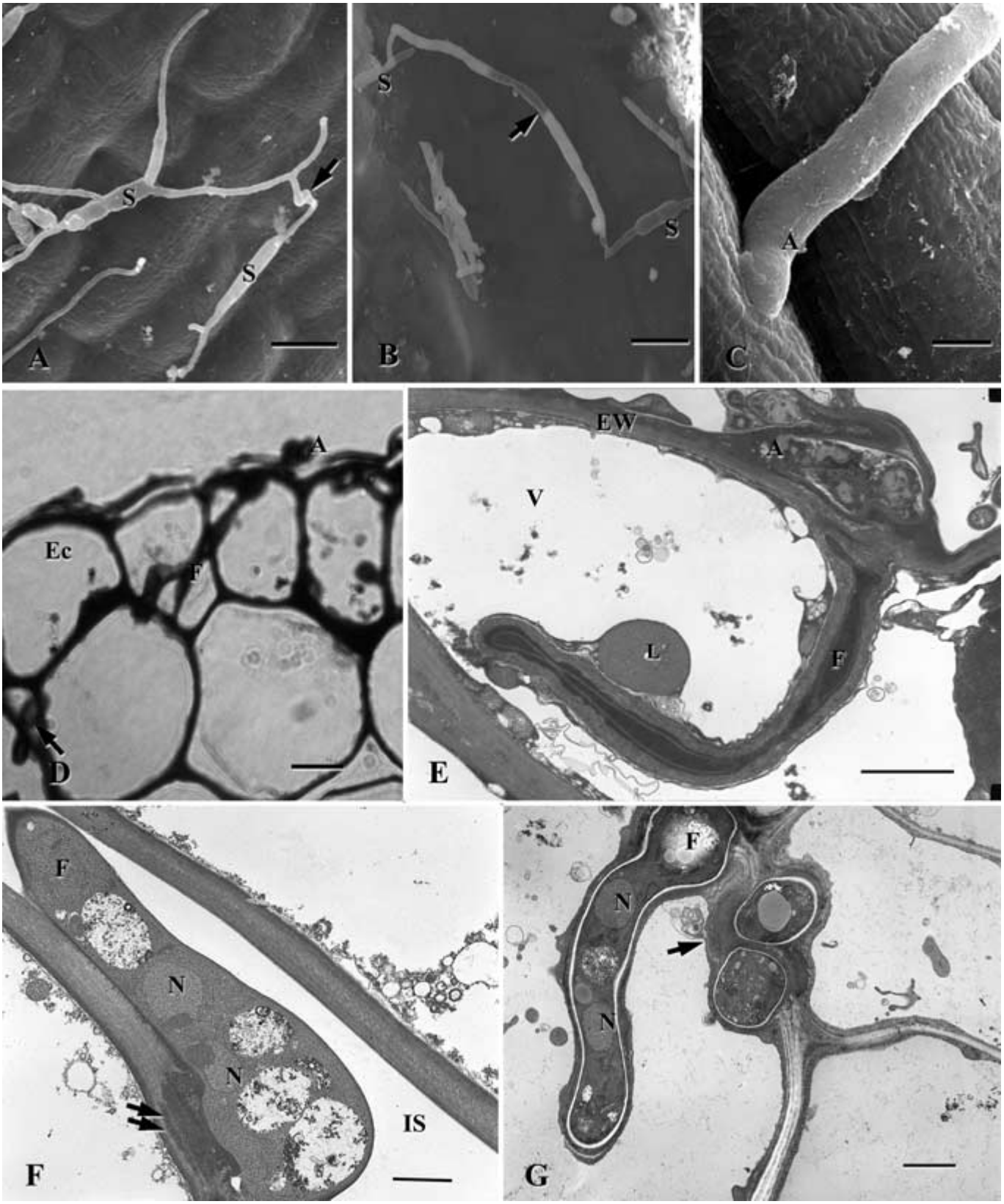


Fig. 3. Early infection process of *Ustilago hordei* in barley seedlings. (A) Enlargement of part of Fig 2G. The invading hypha has breached the plant cell wall (PW) but does not break the host plasma membrane (arrows). Mitochondria (M) and lipid bodies (L) are present in the mycelium (6 days post inoculation). Scale bar = 1 μm. (B) A layer of cell wall apposition (arrows) surrounds the mycelium (F) (6 days postinoculation). IS, intercellular space. Scale bar = 2 μm. (C) Mycelia progress to the epidermal cells of the opposite side of first leaf. Both intra- (single arrow, F) and inter-cellular mycelium (double arrows, F) can be observed (6 days postinoculation). 1st Le, first leaf; 2nd Le, second leaf. Scale bar = 5 μm. (D, a and b). Fungal mycelium (F) from the first leaf crosses over to enter the epidermal cell of second leaf (9 days postinoculation). Scale bar = 5 μm. (E) Mycelium (F) advances further, but without much proliferation, to the bundle sheath area (9 days postinoculation). Scale bar = 5 μm. (F) Mycelium (F) is present in some bundle sheath cells, but xylem cells (Xy) seem to be free of fungal hypha in this section. Some hypha appear collapsed in the bundle sheath cells (arrows; 9 days postinoculation). Scale bar = 5 μm. Figures 3A, 3B, 3E, and 3F are transmission electron micrographs, and Figs. 3C and 3D are light micrographs.

thought that these tubes emerge in response to and grow towards a gradient of pheromone produced by the opposite mating type (Martinez-Espinoza et al. 1993; Snetselaar et al. 1996). Conjugation tubes of opposite mating type fused, often at their tips, once contact was made (Fig. 1B). Frequently, conjugation tubes emerged from the side of sporidial cells (Figs. 1C and 1D). By 12 h after mixing, many conjugation bridges had formed from the fusion of conjugation tubes (Fig. 1B) and thicker, more vigorously growing hyphae emerged from the conjugation bridge and elongated further (Fig. 1D).

Our results indicate that sporidial mating in *U. hordei* occurs in the same way as in *U. maydis* (Bowman 1946; Snetselaar and Mims 1992; Snetselaar 1993), in *Ustilago violacea* (Pers.) Rouss. (Day and Jones 1968; Poon et al. 1974), and in other smut fungi (Fisher and Holton 1957). As previously observed in vitro on water agar in *U. hordei* (Martinez-Espinoza et al. 1993) and in *U. maydis* (Snetselaar and Mims 1992; Snetselaar 1993), conjugation tubes may grow 70–80 μm in distance before fusing. Our present investigation of *U. hordei* mating on water agar confirms the findings of Martinez-Espinoza et al. (1993). That is, after mating, infection hyphae always emerged from the conjugation bridge, the point at which the conjugation tubes fused at their tips. Usually, a single polar conjugation tube was produced per cell, although occasionally two polar tubes were seen. However, we also observed conjugation tube initiation at the sides of sporidial cells on both water agar and host plant surfaces.

Mating process on the plant surface

On the surface of barley shoots, we observed a similar sequence of events as those that occurred on water agar. A conjugation tube was normally produced at the end of the sporidial cell within 2 h after shoot inoculation, and it subsequently elongated over the shoot surface (Fig. 1E). On many occasions, conjugation tubes emerged from the sides of cells (Fig. 1F). A conjugation bridge formed when two conjugation tubes of opposite mating type made contact and fused (Figs. 1F and 1G). Typically, a thicker, more vigorously growing hypha arose from the conjugation bridge and elongated along the shoot surface (Figs. 1G and 1H). However, hyphae produced from one of the mating cell bodies were also observed (Fig. 1F). This has also been observed in *U. maydis* (Snetselaar and Mims 1992). Many conjugation tubes failed to encounter and fuse with a tube from a potential mating partner and eventually collapsed on the shoot surface. On a few occasions, three cells were involved in the mating process and the hypha emerged either from the conjugation bridge or from one of the mated cells (Fig. 1I), similar to the mating process described for *U. violacea* (Poon et al. 1974). More than two conjugation tubes were also observed to form from one single mating cell, but only one of the tubes was involved in the fusion (Fig. 2A). Conjugation tubes can extend for a considerable distance (approximately 70 μm in Fig. 2B) before forming a conjugation bridge. In summary, the mating process on the host surface seems more profuse than in vitro, possibly because of plant factors having been co-opted in the process to maximize the chance of finding a mating partner.

Our study using mixed basidiospore cultures as inoculum does not rigorously mimic the natural conditions of early stage infection. Although inoculation with teliospores was not investigated, the production of basidiospores and subsequent mating would very likely proceed as described in this study. Other studies have employed forced injection of compatible mating type cell mixtures of *U. maydis* into corn (Snetselaar and Mims 1992, 1994) or *S. reilianum* into sorghum (Wilson and Frederiksen 1970).

Infection process of *U. hordei*

The hyphae arising from the conjugation bridge or from one of the mated cells elongated on the shoot surface and were capable of growing considerable distances (on average more than 30 μm) over many epidermal cells without apparent differentiation or cueing by plant cells. Close contact was seemingly maintained, but it was unclear whether the hyphae were actually attached to the host surface. When the hyphal tip grew over a juncture between the long anticlinal walls of epidermal cells, it usually formed a characteristic crook. Immediately adjacent to this bend, the fungal tip became slightly swollen (Fig. 2C). At high magnification, we observed that, in some instances, this structure possibly released some mucilage-like material, presumably for attachment. These appressorium-like structures most likely are the sites of direct penetration. Compared with other plant pathogenic fungi, such as *Puccinia triticina* (Hu and Rijkenberg 1998*a*), the appressorium-like structure of *U. hordei* is not morphologically differentiated, but it can be recognized by its slightly swollen appearance and crook just preceding it; its appearance resembles that of *U. maydis* (Snetselaar and Mims 1992).

At 2 dpi, invading hyphae were found penetrating the epidermal cell of the first young leaf of the shoot directly beneath the appressorium-like structure (Fig. 2D). Our data confirm previous observations that infection hyphae of *U. hordei* penetrate the epidermal cell directly (Kiesling 1952; Kozar 1969). Such direct penetration by fungi within

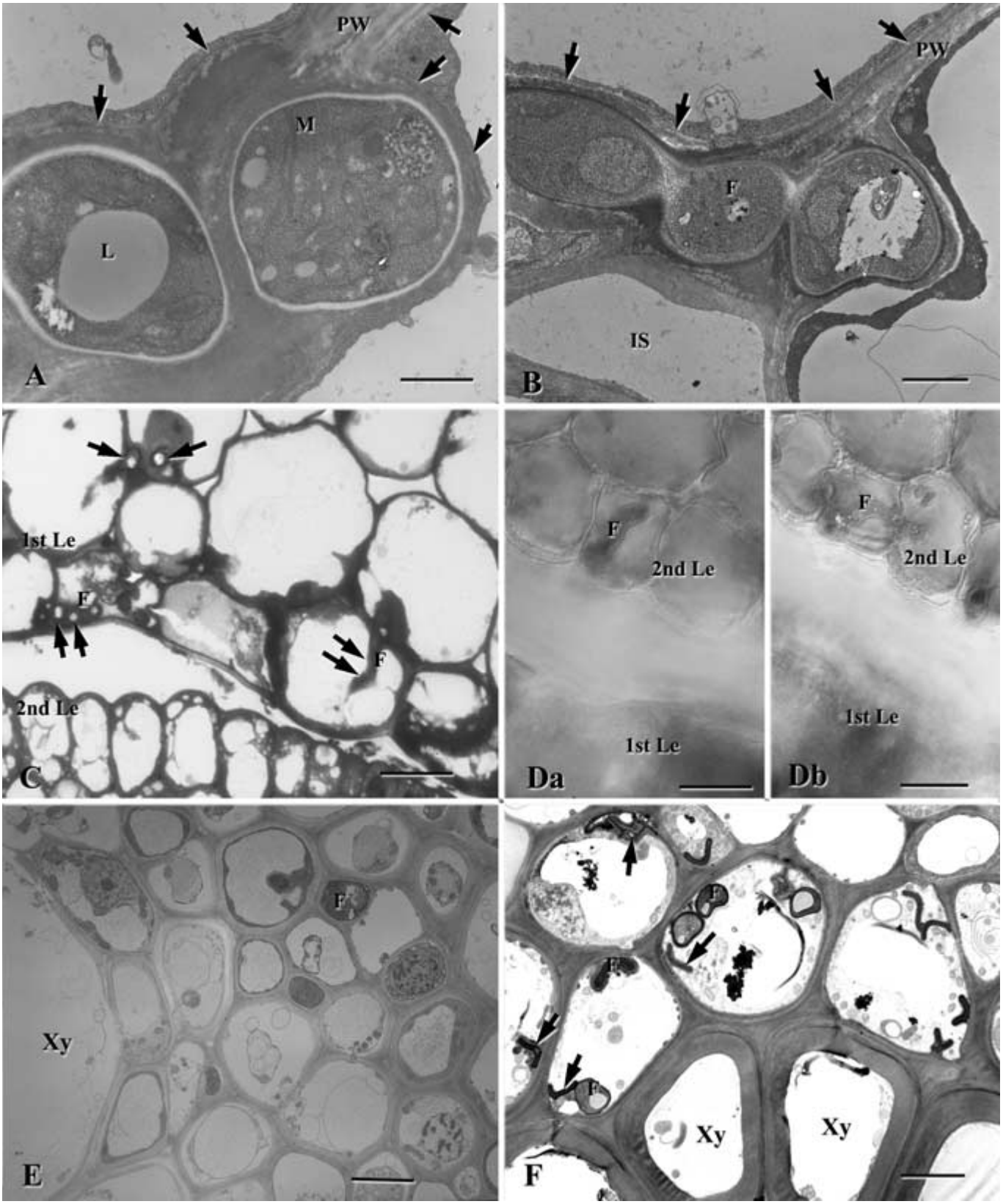


Fig. 4. Infection process of *Ustilago hordei* in barley inflorescences. A. Cross section through apical meristem. Hyphae advance to the apical meristem region (St) without much proliferation (12 days postinoculation). Scale bar = 50 μm. (B) Enlargement of boxed area in Fig. 4A showing sections of fungal hyphae (F) in plant cells. Scale bar = 5 μm. (C) Transmission electron micrograph showing that fungal mycelia (F) are in the region close to the growing point (12 days postinoculation). Scale bar = 5 μm. (D) At 42 days postinoculation, established mycelia (arrows) in the growing point region proliferate. Branched hyphae are present in the stem and young flag leaf. At the base of the upper second leaf, a trace of fungal mass is also visible. Le, leaf; Fle, flag leaf; St, floral meristem region. Scale bar = 10 μm. (E) Fungal mass in the ear tissue (arrows; 42 days postinoculation). Scale bar = 50 μm. (F) Enlargement of part of Fig. 4E showing future spikelets infected with fungal hyphae (arrows). Scale bar = 10 μm. Figures 4A, 4B, and 4D–4F are light micrographs, and Fig. 4C is a transmission electron micrograph.

the Ustilaginales has also been described for *U. maydis* (Snetselaar and Mims 1992). The plant plasma membrane was not compromised upon invasion and remained intact, surrounding the invading hypha (Fig. 2E). No necrotic reactions or other abnormal cell morphology were observed. This indicates that the fungus has the ability to evade the host resistance and defense mechanisms and to establish a biotrophic relationship with the host.

Subsequent fungal growth at this stage appeared to be mainly intracellular. Invaginated fungal hyphae were frequently surrounded by a layer of an electron-dense substance, the interfacial matrix (Luttrell 1987), and the host cytoplasm, but no necrotic reaction was observed (Figs. 2E, 3A, and 3B). Similar dense matrices have been observed previously in light microscope studies and have been described as "sheaths" by others investigating the *U. hordei* – barley (Kiesling 1952; Kozar 1969) and *Tilletia tritici* – wheat interactions (Woolman 1930). Similar structures have also been reported in cells of festucoid grasses infected with the leaf stripe smut fungus, *Ustilago striiformis* (Mims et al. 1992). The biochemical composition, origin, and role of these matrices in host-parasite interactions have yet to be elucidated.

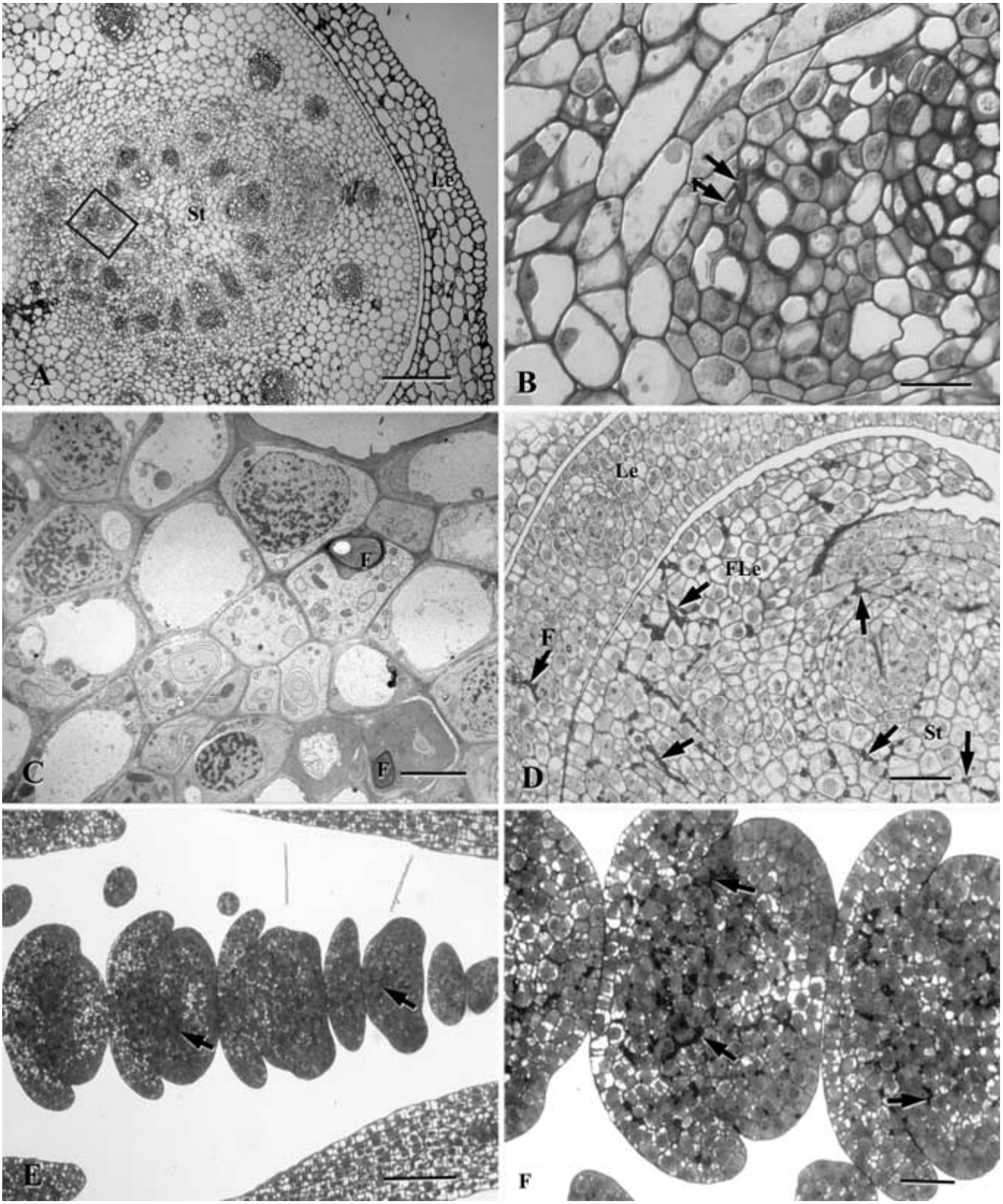
After the fungal hypha passed through several cells, fungal ingression turned to both intercellular (Fig. 2F) and intracellular growth (Figs. 2G, 3A, and 3B). Fungal hyphae appeared to be dikaryotic (Figs. 2F and 2G) and contained mitochondria, lipid bodies, endoplasmic reticulum, and vacuoles (Fig. 3A). The invading hyphae entered plant cells without breaking the plant plasma membrane, and the electron-dense interfacial matrix continued to be laid down in the interface between the invading hyphae and the host plant plasma membrane (Fig. 3B).

The invading hyphae grew further without much branching, penetrating several mesophyll cells in the first leaf of the shoot before reaching the epidermal cells on the inner side of the first leaf (Fig. 3C). There seemed to be very little proliferation and increase in fungal biomass. At 6 dpi, hyphae grew over the inner epidermal cell of the first leaf and penetrated into the epidermal cell of the second leaf (Fig. 3D, *a* and *b*). After the hypha had entered the second leaf, it penetrated further into the meristematic tissue of the growing point of the coleoptile. Even at this stage, fungal proliferation was limited (Fig. 3E). Often hyphae were difficult to find, and no obvious path of ingress was observed. We did not attempt to track such a path through serial sectioning. It has been suggested by Woolman (1930) that older (empty) hyphal remnants might be digested by plant enzymes.

Fungal hyphae entered bundle sheath cells and possibly phloem cells but were rare in xylem cells (Figs. 3F, 4A, and 4B). Although spaces between leaves and phloem cells might be used for short longitudinal growth (Kiesling 1952), ingress was mainly perpendicular to the long axis, growing towards the nodal and apical meristems. Possibly the fungus responds to a chemical gradient of factors produced by these undifferentiated tissues. At 9 dpi, only a few hyphae were observed in the proximity of the growing point and it appeared that the fungus still had not proliferated much (Figs. 4A, 4B, and 4C). No necrotic or apparent abnormal reaction or structures were observed in the host cytoplasm at this stage (Figs. 4B and 4C).

Kozar (1969) used the term "haustoria" to describe the fungal fragments in plant meristem tissues. Wilson and Frederiksen (1970) also use this term in describing the sorghum – *S. reilianum* interaction. We found that the intercellular hyphae of *U. hordei* could send several branches into plant cells, some of which seemed to terminate within the cells. However, they did not have a consistent appearance and were different from the haustoria described in other fungi, such as rusts (Hu and Rijkenberg 1998*b*). On many occasions the intracellular hyphae of *U. hordei* passed through several cells. As concluded by Luttrell (1987) and Snetselaar and Mims (1994) we choose not to refer to them as haustoria. Whether some of the observed terminal hyphal cells have haustoria-like functions remains to be determined. The very minimal growth of this smut inside host tissue would probably not require extensive nutrition.

Fungal hyphae eventually reached the region of the apical meristem and nodes of the coleoptile again with little proliferation by 42 dpi. Beyond 42 dpi, there was an obvious increase in fungal biomass in the meristematic region (Fig. 4D) as the fungus established itself. This coincides with host floral initiation, which is intriguing as it suggests that the fungus is cued by plant factors. At 54 dpi, all barley leaves had developed, and the meristematic region had differentiated into a floral meristem, the future spike. Many hyphae were present in the flag leaf, while the upper second leaf had small amounts of fungal mass at its base (Fig. 4D). During its development, the barley ear became progressively filled with ramifying mycelium, and many hyphal fragments were present in the analyzed sections (Figs. 4E and 4F). TEM showed that there were many fungal branches from both intra- and inter-cellular hyphae (Fig. 5A). Electron-dense material was often deposited around the intracellular hyphae (Fig. 5B). It appears that the fungus remains mainly quiescent in other tissues even during inflorescence development when the fungus proliferates in this organ. This was demonstrated using the GUS-expressing strain (Fig. 6A). Similar events have been described during the infection of sorghum by *S. reilianum*, another bipolar fungus causing head smut (Wilson and Frederiksen 1970).



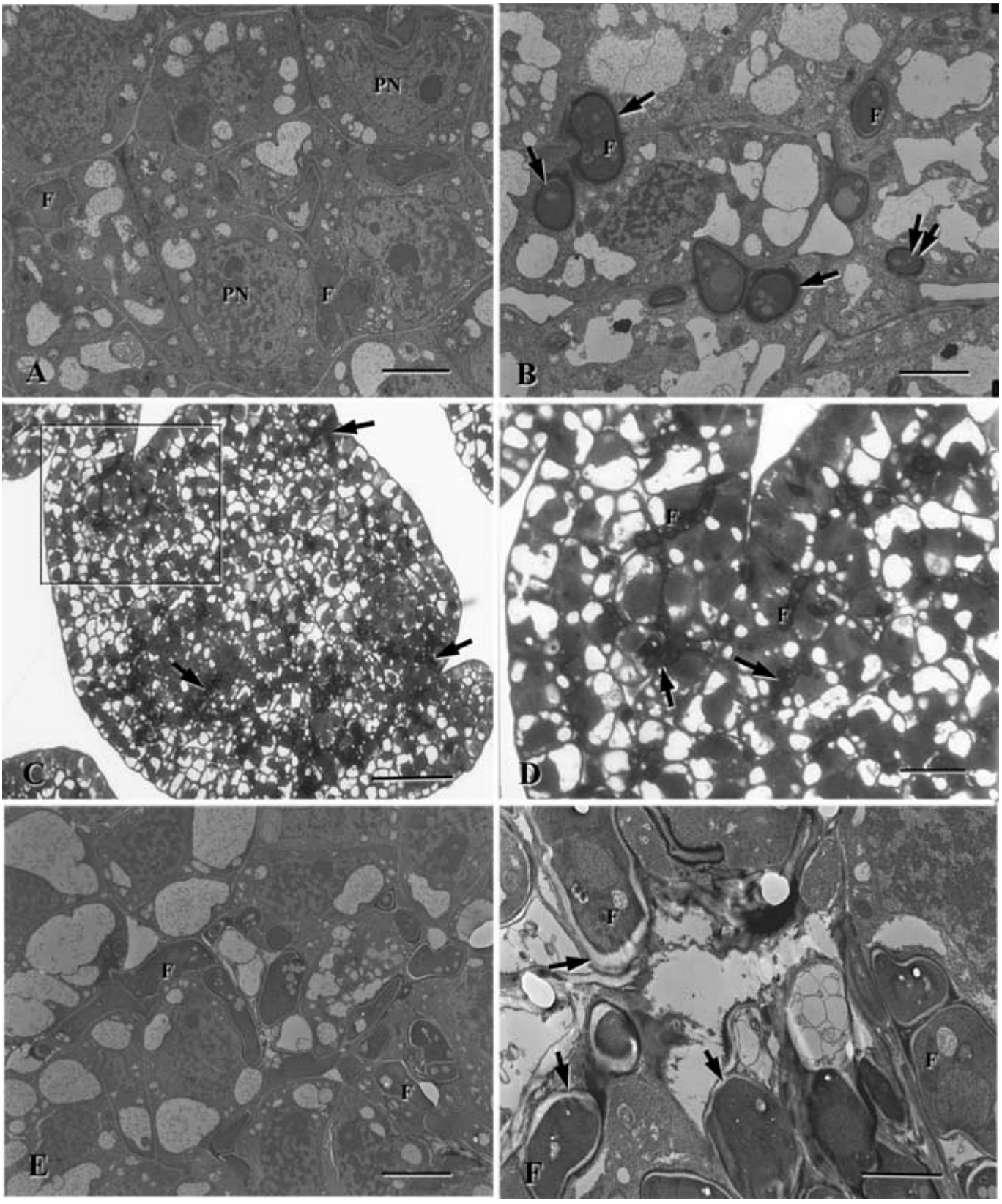
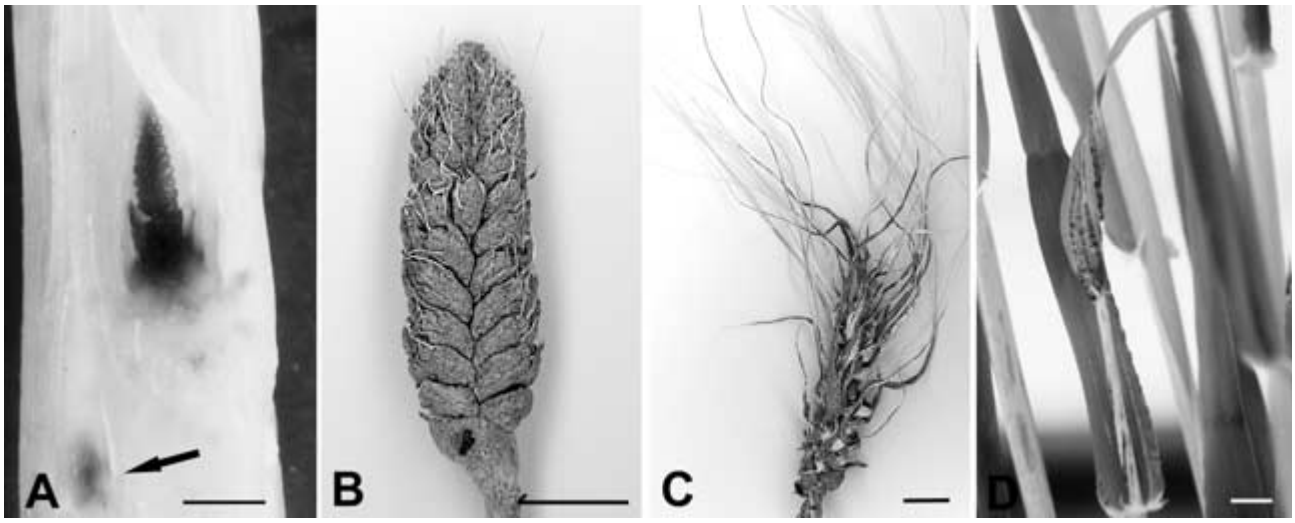


Fig. 5. Barley spike tissue infected with *Ustilago hordei*. (A) Transmission electron micrograph showing both intracellular and intercellular hyphae (F) are present in the ear tissue (54 days postinoculation). PN, plant nucleus. Scale bar = 5 μm. (B) Frequently, an electron-dense "interfacial matrix" was found in the interface between plant cytoplasm and fungal hyphae (arrows; 54 days postinoculation). Scale bar = 5 μm. (C) Fungal mass (arrows) in ear tissue (63 days postinoculation). Scale bar = 10 μm. (D) Enlargement from Fig. 5C. Septate fungal hyphae (arrows) are present in some sections (63 days postinoculation). F, mycelium. Scale bar = 5 μm. (E) Transmission electron micrograph showing the elongated fungal hyphae (F) in the ear tissue. A large fungal mass has been built up (63 days postinoculation). Scale bar = 5 μm. (F) The cell wall of fungal hyphae is thickening. Some plant cells harboring thickened fungal branches disintegrate (63 days postinoculation). Scale bar = 2 μm. Figures 5A, 5B, 5E, and 5F are transmission electron micrographs, and Figs. 5C and 5D are light micrographs.

Fig. 6. Late stages of the infection cycle during a compatible *Ustilago hordei* – barley, cv. Hannchen interaction. (A) Location of fungal biomass at 47 days postinoculation. Longitudinal section through inflorescence colonized by Uh359 stably transformed with a β-glucuronidase (GUS) expressing construct, mated with Uh362. Tissue was stained directly with β-glucuronide. Note that most biomass resides in the immature spike and its base; some fungal material can be found in lower tissues (arrow). Scale bar = 2 mm. (B) Fully emerged, heavily infected barley spike with all seeds replaced with teliospore-containing sori. Note the virtual absence of awns. Scale bar = 5 mm. (C) Partially infected barley spike; note deformed awns with sori. Scale bar = 5 mm. (D) Flag leaf with streaks of smut sori; spike has not yet emerged from the boot. Scale bar = 10 mm.



At 63 dpi, spike tissue was filled with abundant septate fungal hyphae (Figs. 5C, 5D, and 5E). In this early stage of sporogenesis, some fungal cells started to exhibit thickened walls, indicating the prelude to teliospore formation (Fig. 5F). Smut sori replacing seeds could often be seen after heading (Figs. 6B, 6C, and 6D). In the young flag leaf of barley, at 42–63 dpi, masses of both inter- and intra-cellular mycelia were observed, whereas only a trace of fungal hyphae were discernable in the upper second leaf (Fig. 6A). Frequently, parallel streaks of sori could be seen on veins of the flag leaf, often weeks ahead of emergence of smutted spikes (Fig. 6D). Kozar (1969) also described flag leaves containing fungal mass. Using a polymerase chain reaction based detection method, Willits and Sherwood (1999) demonstrated the presence of *U. hordei* in leaves of both susceptible and resistant barley varieties. In fact, they found *U. hordei* DNA present in the first three or four leaves of resistant varieties.

Concluding remarks

Although some aspects of mating, the infection process, and the completion of the obligate parasitic stage of the life cycle of the smut fungus *U. hordei* in a susceptible barley host have been investigated before, our study presents novel insights into mating interactions on the plant surface and provides a detailed ultrastructural and cell-morphological analysis of the complete infection process. In summary, it appears that, initially, *U. hordei* infects its host by stealth cueing in very subtly on various plant factors and stages of development to establish an intimate relationship with its host. As a hemibiotroph, it has filled a very successful niche in nature by virtue of its synchronization with its host's life cycle. Although differences in resistance to *U. hordei* are present among various barley cultivars (Tapke 1945), the nature of this resistance is still unknown. The current investigation provides an understanding of the sequential events during the *U. hordei* – barley interaction. Moreover, by studying the early infection process, a basis for comparison of future ultrastructure studies of cellular resistance in incompatible interactions involving gene-for-gene resistant reactions during invasion of *U. hordei* races harboring specific avirulence genes has been established. It is anticipated that such studies will reveal that each of these genes can trigger specific resistance reactions potentially blocking pathogen development at different stages.

Acknowledgements

We gratefully appreciate the technical assistance by M. Weis and thank Dr. H. Sanfaçon and Dr. K. Snetselaar for comments on the manuscript.

References

Anderson, C.M., Willits, D.A., Kosted, P.J., Ford, E.J., Martinez-Espinoza, A.D., and Sherwood, J.E. 1999. Molecular analysis of the pheromone and pheromone receptor genes of *Ustilago hordei*. Gene, **240**: 89–97.

Bakkeren, G., and Kronstad, J.W. 1993. Conservation of the *b* mating-type gene complex among bipolar and tetrapolar smut fungi. Plant Cell, **5**: 123–136.

Bakkeren, G., and Kronstad, J.W. 1994. Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi. Proc. Natl. Acad. Sci. U.S.A. **91**: 7085–7089.

Bakkeren, G., and Kronstad, J.W. 1996. The pheromone cell signaling components of the *Ustilago a* mating-type loci determine intercompatibility between species. Genetics, **14**: 1601–1613.

Bakkeren, G., Kronstad, J.W., and Lévesque, C.A. 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. Mycologia, **92**: 510–521.

Bölker, M. 2001. *Ustilago maydis*, a valuable model system for the study of fungal dimorphism and virulence. Microbiology, **147**: 1395–1401.

Bowman, D.H. 1946. Sporidial fusion in *Ustilago maydis*. J. Agric. Res. **72**: 233–243.

Chatterjee, P. 1956. Nature of resistance to penetration and infection of *Ustilago hordei* (Pers.) Lagerh. in seedlings of four hull-less spring barley varieties. Ph.D. thesis, Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich.

Day, A.W., and Jones, J.K. 1968. The production and characteristics of diploids in *Ustilago violacea*. Genet. Res. **11**: 63–81.

Fisher, G.W., and Holton, C.S. 1957. Biology and control of the smut fungi. Ronald Press, New York.

Hu, G.G., and Rijkenberg, F.H.J. 1998*a*. Scanning electron microscopy of early infection structure formation by *Puccinia recondite* f.sp. *tritici* on and in susceptible and resistant wheat lines. Mycol. Res. **102**: 391–399.

Hu, G.G., and Rijkenberg, F.H.J. 1998*b*. Ultrastructural studies of the intercellular hypha and haustorium of *Puccinia recondite* f.sp. *tritici*. J. Phytopathol. **146**: 39–50.

Jefferson, R.A. 1989. The GUS reporter gene system. Nature (London), **342**: 837–838.

Kiesling, R.L. 1952. Histological studies on covered smut of barley. Ph.D. thesis, Department of Plant Pathology, University of Wisconsin, Madison, Wis.

Kozar, F. 1969. The pathway of infection of *Ustilago hordei*. Can. J. Genet. Cytol. **11**: 977–986.

Lee, N., Bakkeren, G., Wong, K., Sherwood, J.E., and Kronstad, J.W. 1999. The mating type and pathogenicity locus of the fungus *Ustilago hordei* spans a 500-kb region. Proc. Natl. Acad. Sci. U.S.A. **96**: 15 026 – 15 031.

Luttrell, E.S. 1987. Relations of hyphae to host cells in smut galls caused by species of *Tilletia*, *Tolyposporium*, and *Ustilago*. Can. J. Bot. **65**: 2581–2591.

Martinez-Espinoza, A.D., Gerhardt, S.A., and Sherwood, J.E. 1993. Morphological and mutational analysis of mating in *Ustilago hordei*. Exp. Mycol. **17**: 200–214.

Martinez-Espinoza, A.D., Garcia-Pedrajas, M.D., and Gold, S.E. 2002. The Ustilaginales as plant pests and model systems. Fungal Genet. Biol. **35**: 1–20.

Mathre, D.E. 1997. Compendium of barley diseases. American Phytopathological Society Press, St. Paul, Minn.

Mims, C.W., Snetselaar, K.M., and Richardson, E.A. 1992. Ultrastructure of the leaf stripe smut fungus *Ustilago striiformis*: host–pathogen relationship and teliospore development. Int. J. Plant Sci. **153**: 289–300.

Poon, N.H., Martin, J., and Day, A.W. 1974. Conjugation in *Ustilago violacea*. I. Morphology. Can. J. Microbiol. **20**: 187–191.

Ramberg, J.E., and McLaughlin, D.J. 1980. Ultrastructure study of promycelial development and basidiospore initiation in *Ustilago maydis*. Can. J. Bot. **58**: 1548–1561.

Smith, T.L., and Leong, S.A. 1990. Isolation and characterization of a *Ustilago maydis* glyceraldehyde-3-phosphate dehydrogenase-encoding gene. Gene, **93**: 111–117.

Snetselaar, K.M. 1993. Microscopic observation of *Ustilago maydis* mating interactions. Exp. Mycol. **17**: 345–355.

Snetselaar, K.M., and Mims, C.W. 1992. Sporidial fusion and infection of maize seedlings by the smut fungus *Ustilago maydis*. Mycologia, **84**: 193–203.

Snetselaar, K.M., and Mims, C.W. 1993. Infection of maize stigmas by *Ustilago maydis*: light and electron microscopy. Phytopathology, **83**: 843–850.

Snetselaar, K.M., and Mims, C.W. 1994. Light and electron microscopy of *Ustilago maydis* hyphae in maize. Mycol. Res. **98**: 347–355.

Snetselaar, K.M., Bölker, M., and Kahmann, R. 1996. *Ustilago maydis* mating hyphae orient their growth toward pheromone sources. Fungal Genet. Biol. **20**: 299–312.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–43.

Tapke, V.F. 1945. New physiologic races of *Ustilago hordei*. Phytopathology, **35**: 970–976.

Thomas, P.L. 1988. *Ustilago hordei*, covered smut of barley and *Ustilago nuda*, false loose smut of barley. *In* Advances in plant pathology. Vol. 6. *Edited by* G.S. Sidhu. Academic Press, London. pp. 415–425.

Thomas, P.L. 1991. Genetics of small grain smuts. Annu. Rev. Phytopathol. **29**: 137–148.

Thomas, P.L., and Menzies, J.G. 1997. Cereal smuts in Manitoba and Saskatchewan, 1989–95. Can. J. Plant Pathol. **19**: 161–165.

Western, J.H. 1936. The biology of oat smuts. Ann. Appl. Biol. **23**: 245–263.

Willits, D.A., and Sherwood, J.E. 1999. Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. Phytopathology, **89**: 212–217.

Wilson, J.M., and Frederiksen, R.A. 1970. Histopathology of the interaction of *Sorghum bicolor* and *Sphacelotheca reilianum*. Phytopathology, **60**: 828–832.

Woolman, H.M. 1930. Infection phenomena and host reactions caused by *Tilletia tritici* in susceptible and nonsusceptible varieties of wheat. Phytopathology, **20**: 637–652.