Histology of the Pathogenesis of *Mycosphaerella graminicola* in Wheat

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ABSTRACT

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Cellular aspects of the pathogenesis of *Mycosphaerella graminicola* in a susceptible and resistant wheat cultivar were studied by light microscopy and scanning and transmission electron microscopy. Experiments were designed as time-sequence studies in two replications with sampling dates at 12-, 24-, and 48-h postinoculation (hpi), and 4-, 8-, 10-, 12-, 14-, and 16-days postinoculation (dpi). A separate experiment was performed to quantify the mycelial biomass in cultivars Shafir and Kavkaz/K4500 l.6.a.4 at the aforementioned intervals using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for *M. graminicola*. The germination frequency of *M. graminicola* conidia was high in both compatible and incompatible interactions, but the infection frequency was low. Infection was strictly stomatal, but appeared to be a random process since many germ tubes crossed stomata without penetrating them. Some germ tubes formed branched structures close to or on top of stomata. These structures were small compared with the size of stomata, were formed irregularly, and were not significantly correlated with successful penetrations of the host. Multiple penetrations of stomata occurred regularly. Hyphae of *M. graminicola* were already observed in the substomatal cavities at 12 hpi and, at 48 hpi, hyphae had reached the nearest mesophyll cells. In the compatible response, colonization was fairly limited until 8 dpi. Hyphae grew intercellularly and in close contact with the mesophyll cells. During the 10- to 12-dpi interval, extensive host cell death occurred, which induced further colonization and, eventually, pycnidium formation in substomatal cavities. Initial and further colonization had marked effects on the number and size of the chloroplasts in the compatible interaction. Nevertheless, leaves remained green until approximately 10 dpi. The resistance response was primarily characterized by very limited colonization, mostly in the vicinity of the substomatal cavity. Quantification of the mycelial mass with ELISA revealed similar mycelial quantities in cultivars Shafir and Kavkaz/K4500 l.6.a.4 until 8 dpi. After 8 days, the mycelial quantity developed exponentially in 'Shafir,' but did not significantly increase in 'Kavkaz/K4500 l.6.a.4.'

Additional keywords: compatibility, incompatibility, intercellular growth, *Septoria tritici*, toxins.

Mycosphaerella graminicola (Fuckel) J. Schröt. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.) is a fungal pathogen that causes septoria tritici leaf blotch on bread wheat (*Triticum aestivum* L., AABBDD, $2n = 42$) and durum wheat (*T. turgidum* (L.) Thell. subsp. *durum* L., AABB, $2n = 28$). The disease occurs particularly in temperate, high-rainfall environments (32), and the symptoms are characterized by necrotic blotches that contain varying densities of pycnidia, the asexual fructifications containing the conidia.

The teleomorph is considered to be largely responsible for the oversummering of the disease in regions where crop rotation is a common agricultural practice, whereas the anamorph mainly contributes to disease development during the growing season (27, 28). However, it was recently shown that *M. graminicola* is able to complete several generations of ascospores after the establishment of the disease in autumn, a finding of considerable epidemiological importance (16). The recently determined heterothallic mating system (16) provides an explanation for the wide genetic variation in *M. graminicola* and particularly for its potential to adapt to host species and cultivars (6,7,14,15,22,23).

Nevertheless, resistance breeding is considered to be an effective strategy to control the disease (32). However, virtually nothing is known about the mechanism of resistance to *M. graminicola* in wheat cultivars (3,12,33). Resistance breeding might, therefore, benefit from a comprehensive study of the pathogenesis in compatible and incompatible interactions of wheat and *M. graminicola*, which was the subject of the present study.

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MATERIALS AND METHODS

Plant materials and *M. graminicola* isolates. Based on previous studies (14), the bread wheat cultivars Shafir and Kavkaz/K4500 l.6.a.4 were selected for the study of compatible and incompatible responses to *M. graminicola* bread wheat isolate IPO87016, respectively. In addition, bread wheat isolate IPO235, durum wheat isolate IPO86022, and the durum wheat cultivar Volcani 447 were occasionally used for comparison.

Experimental procedures and conditions for microscopy. For light microscopy (LM), leaf materials were fixed under low vacuum for 24 h in 5% glutaraldehyde in a 0.02 M phosphate buffer. The samples were dehydrated in a graded ethanol series,

embedded in Technovit 7100 resin (Heraeus-Kulzer, Wehrheim, Germany), and sectioned (2 μm) with a Jung 2040 microtome (Leica, Ryswyk, the Netherlands) equipped with Ralph glass knives. The sections were collected on glass slides, stained with toluidine blue for 10 min, and photographed using a Zeiss Axioplan microscope (Zeiss-Nederland B.V., Weesp, the Netherlands) equipped with an MC100 camera using bright field and differential-interference contrast optics. Nuclei in conidia were stained with 4,6-diamidino-2-phenyl indole (DAPI, 2.5 μg ml^{-1} in McIlvain's buffer, pH 4.4). Leaf material for scanning electron microscopy (SEM) was fixed, critical-point dried with CO_2, and observed using a Jeol 35C scanning electron microscope (JEOL, Ltd., Tokyo). For transmission electron microscopy (TEM), leaves were fixed in 5% glutaraldehyde in a 0.02 M phosphate buffer, postfixed and stained in 2% osmium-tetroxyde in 0.05 M sodium-cacodylate, dehydrated in an alcohol series, embedded in Spurr's standard resin, and viewed using a Jeol 100CX transmission electron microscope (JEOL, Ltd.).

Ten to 15 plants were grown in a line in 7 × 7-cm square pots that were placed in controlled, walk-in climate chambers with a pre- and postinoculation light intensity of 56 μE sec^{-1} m^{-2} for 16 h day^{-1}. Pre- and postinoculation conditions were for temperature (18/16 and 22/21°C [day/night cycle], respectively) and relative humidity (70 and ≥85%, respectively). Inoculum was produced according to procedures described previously (14). Quantitative inoculations were conducted by spraying suspensions of conidia (1×10^7 conidia per ml, 30 ml per isolate, supplemented with two drops of Tween 20 surfactant) on the test cultivars (10 days old) that were placed on a turntable (adjusted to 15 rpm) in an isolation hood equipped with a pressure-driven cleaning device and interchangeable atomizers to avoid contaminations. Plants were left for 1 h on a laboratory bench to dry. Primary leaves that were to be sampled for microscopical observations were then positioned in small, transparent plastic boxes (30), reinoculated along 1 cm of the leaf with 10 to 12 1- to 2-μl drops of inoculum (1×10^7 conidia per ml), and incubated for 12 to 48 h at a light intensity of approximately 3 μE s^{-1} m^{-2}. Control plants were treated with water.

From each isolate-cultivar combination, the twice-inoculated sections of the primary leaves were sampled at different intervals: 12-, 24-, and 48-h postinoculation (hpi), and 4-, 8-, 10-, 12-, 14-, and 16-days postinoculation (dpi). The sampled plants and additional checks were left in the climate chamber for normal disease assessment (necrosis and pycnidium formation scored as percentages on the primary leaves, *N* and *P*, respectively) at 21 dpi. The experiments for LM, SEM, and TEM were performed in two replications for each sampling, with five leaves per replication.

An additional SEM experiment was performed using 'Shafir' and isolate IPO87016 in two replications to quantify the penetration frequency and investigate the association of penetration with the formation of appressorium-like structures. Inoculated leaves were harvested and fixed at 48 hpi, and stomata were scanned and classified into four categories: i) without hyphal clusters; ii) with hyphal clusters, without penetration; iii) with hyphal clusters, penetration + appressorium-like structures; and iv) with hyphal clusters, penetration – appressorium-like structures.

Experimental procedures and conditions for mycelial quantification. A proprietary double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit based on a polyclonal antibody to a soluble metabolite of *M. graminicola* (Du Pont Co., Stevenage, United Kingdom) was used to quantify the mycelial biomass of *M. graminicola* isolate IPO87016.

Eight pregerminated seeds of 'Shafir' and 'Kavkaz/K4500 l.6.a.4' were sown in a diagonal line across 5 × 5-cm pots containing 125 cm^3 of a 7:3:2 sterile loam/peat/grit mix and grown at 21°C to growth stage 11 (29) with a 16-h light period each day. The pots were arranged in six blocks of 14 pots within a controlled growth cabinet, each block consisting of two rows of seven pots. Cultivars and positions of pots to be harvested at different times were randomized within rows. Harvests of the first leaves of all plants in one pot of each cultivar from each block were made immediately before inoculation (controls). The remaining six pots of each block were simultaneously inoculated with *M. graminicola* isolate IPO87016 on a rotary turntable at 16 rpm (15 ml of inoculum of 10^7 conidia per ml). Pots were enclosed in polythene bags for 48 h after inoculation to allow infection to take place. Primary leaves were harvested at 3, 6, 8, 10, 12, and 14 dpi for quantification of mycelium.

The leaf samples were kept at –20°C until assayed. Leaves taken from two adjacent blocks were thawed, cut into 2- to 3-mm pieces, and homogenized in 2.5 ml of proprietary extraction buffer. Samples were allowed to stand for 1 h and were subsequently centrifuged at 9,000 × *g* for 5 min to sediment plant debris. The supernatant was used according to the protocol of the manufacturer of the ELISA kit. Duplicate assays were made of each sample, except at 3 dpi. The samples of 'Shafir' from 12 and 14 dpi contained more antigen than the most concentrated standard. Hence, these levels were extrapolated from the 10×-diluted samples. Positions of samples and standards on the microtiter plate in which the assay was run were fully randomized. The experiment was analyzed using the generalized linear model facilities in Genstat (9). Variances between replicates were not significantly larger than between duplicate wells containing the same extract. The mean square values between replicates were used to estimate the standard errors.

RESULTS

Macroscopical observations. Checks for disease assessment remained apparently healthy until 8 dpi, after which necrosis developed and pycnidia were formed. Necrosis usually started from the leaf tips. Final disease levels after inoculation with *M. graminicola* isolate IPO87016 were 89% necrosis and 86% pycnidia on 'Shafir' and 5% necrosis and 1% pycnidia on 'Kavkaz/K4500 l.6.a.4' at 21 dpi. The response of 'Shafir' to isolate IPO235 was characterized by relatively high necrosis (40%), but sparse pycnidia formation (5%). These observations were comparable with previous data (14).

Parts of primary leaves left on plants after sampling at 12 and 24 hpi (suboptimal incubation periods) usually did not develop symptoms. All leaves of 'Shafir' in the other treatments, which were appropriately incubated for 48 h, developed necrosis and pycnidia, whereas those of 'Kavkaz/K4500 l.6.a.4' did not. The controls of both cultivars remained free of disease throughout the experiment.

Germination and penetration. The frequency of nongerminated conidia was usually very low, and often all observed conidia on both cultivars had germinated, usually from both ends. Chemotropism or thigmotropism with respect to stomatal structures in the leaves was probably not involved, since germ tubes regularly crossed stomata or grew along the guard cells without entering the stomatal aperture (Fig. 1A). Germ tubes were often branched and commonly developed into clusters of hyphae aggregated in stomatal depressions of the leaves. Penetrations were strictly sto-

TABLE 1. Penetration frequency of germ tubes of *Mycosphaerella graminicola* isolate IPO87016 on 'Shafir' and its association with appressorium-like structures on stomata as observed by scanning electron microscopy at 48-h postinoculation

	No. of scanned stomata[a]
Without hyphal clusters	20
With hyphal clusters, without penetration	88
With hyphal clusters, with penetration + appressorium-like structures	14[b]
With hyphal clusters, with penetration – appressorium-like structures	22[b]

[a] Values are totals of two replications.

[b] Values are not significantly different, $\chi^2 = 1.87$.

matal; direct penetration of the epidermis was not observed. They were achieved either by single germ tubes (Fig. 1B and E) or by the clusters of germ tubes or young mycelium, which resulted in multiple penetrations (Fig. 1F). Penetration was sometimes linked with the formation of appressorium-like structures on the stomatal aperture (Fig. 1C and D). However, in an additional experiment, the frequencies of penetrated stomata with and without the presence of appressorium-like structures were not found to be significantly different (Table 1). The penetration frequency in this experiment was 25%, but it appeared to be much lower in the other experiments.

Colonization. Colonization during the compatible response of 'Shafir' infected with isolate IPO87016 and the effects on the number and shape of cellular organelles of the host were compared with the controls ('Shafir' treated with water) at each sampling date. Since the controls did not deviate from each other at these time intervals, the control of 16 dpi is shown in Figure 2A. In 'Shafir,' mycelium was observed in substomatal cavities from 12 hpi. At 48 hpi, hyphae with clearly visible vacuoles and nuclei reached and continued to colonize the mesophyll intercellularly, usually in close contact with the cell walls (Fig. 2B). During the 48-hpi to 8-dpi interval, mycelial growth remained strictly intercellular, but gradually increased in quantity. At 8 dpi, mesophyll cell walls had an irregular wrinkled appearance and sometimes collapsed (Fig. 2C), a phenomenon that considerably increased at

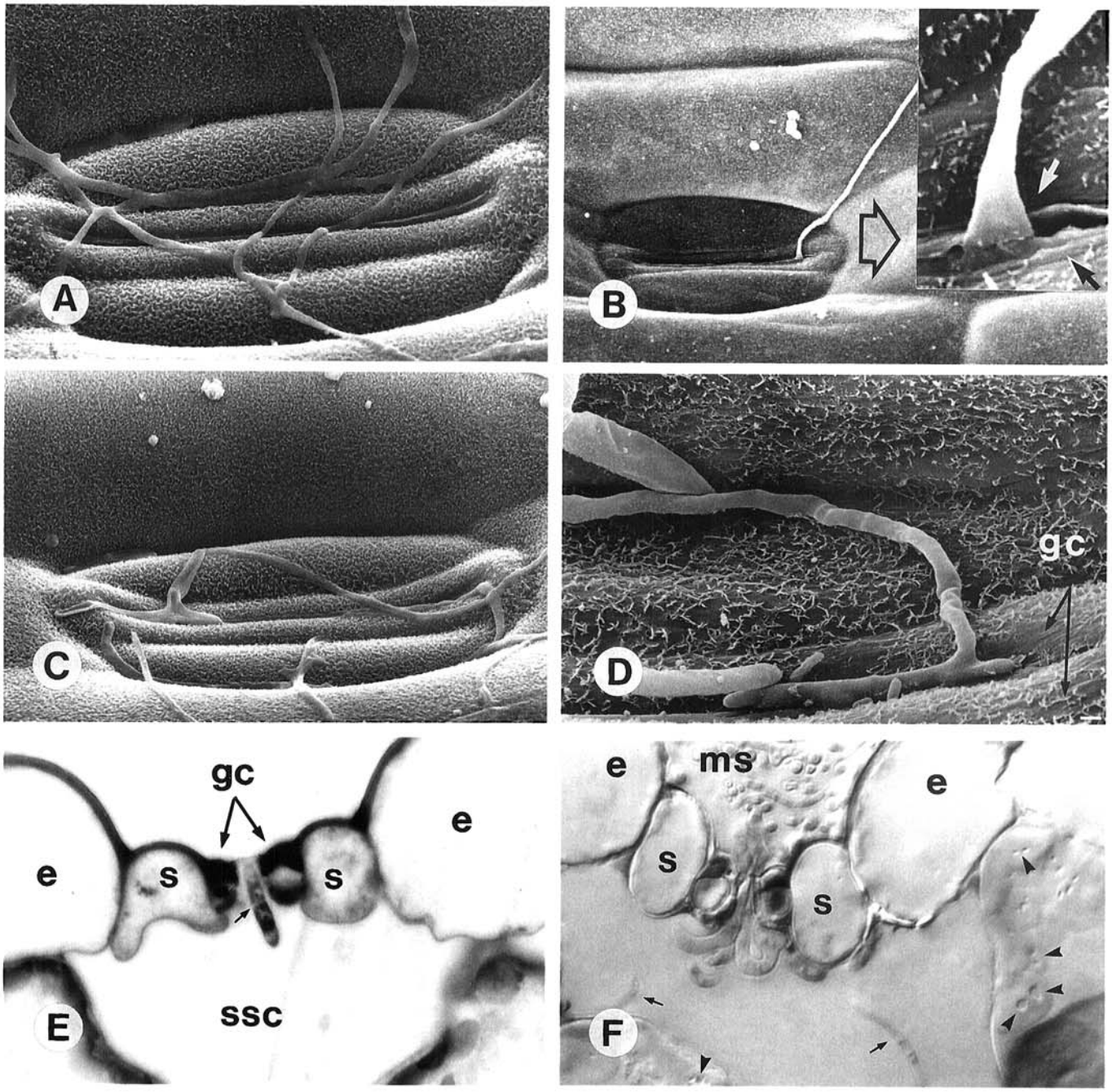
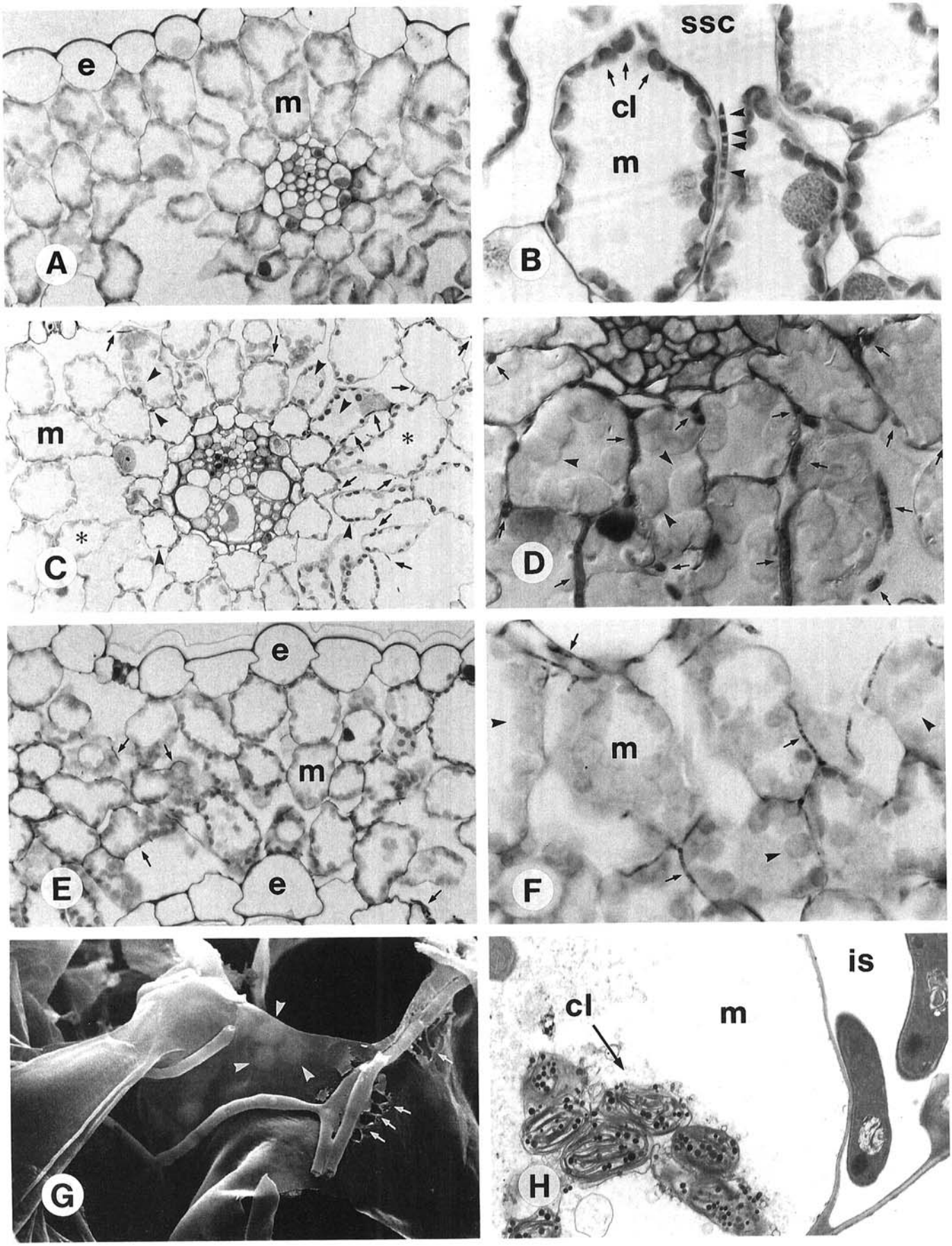


Fig. 1. Scanning electron and light micrographs of *Mycosphaerella graminicola* germ tube growth and penetration in 'Shafir.' **A,** Germ tubes of isolate IPO87016 crossing the stomatal aperture without penetration at 48-h postinoculation (hpi), ×1,550. **B,** Penetration of isolate IPO235 of a stoma observed at 12-days postinoculation (dpi). Note the flattened appearance of the germ tube, which is caused by closing of the guard cells (arrows), ×2,030 (inset ×35,000). **C and D,** Appressorium-like structures of IPO87016 on guard cells at 48 hpi, ×3,125 and ×3,515, respectively. **E,** Penetration of a stoma by IPO87016 at 48 hpi. Note the septum in the germ tube (arrow), ×1,050. **F,** Mycelial stroma or hyphal cluster on outside of stoma giving rise to multiple penetrations of IPO86022 observed at 4 dpi. Note the hyphae (arrows) in the substomatal cavity and the starch granules in the chloroplasts (arrowheads), ×1,050. Abbreviations: e = epidermal cell, gc = guard cell, ms = mycelial stroma, s = subsidiary cell, and ssc = substomatal cavity.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10 dpi (Fig. 2D). Hyphal growth in the mesophyll remained intercellular until cell death (Fig. 2C through H). Walls of cells in the colonized area were no longer stained by toluidine blue, particularly at 8 to 12 dpi (Fig. 2E and F). Rapid, massive host cell death, with the mycelium largely attached to the degenerated cell walls, was observed during the 10- to 12-dpi interval (Figs. 3A and B). This was followed by mycelial proliferation, apparently resulting from the release of nutrients from the mesophyll cells after their collapse (Fig. 3B and C).

The shape of chloroplasts changed significantly during the 48-hpi to 12-dpi interval. Compared with the controls (Fig. 2A), they condensed and were, together with the nucleus, relocated toward the cell wall, probably as a result of the enlargement of the central vacuole (Fig. 2B and C). However, just prior to cell collapse, chloroplasts seemed to expand again (Fig. 2D, E, and F).

Compatible responses of the durum wheat cultivar Volcani 447 to isolate IPO86022 (data not shown) were analogous to the pathogenesis of isolate IPO87016 in 'Shafir.'

The incompatible response of 'Kavkaz/K4500 l.6.a.4' to isolate IPO87016 was primarily characterized by the virtual lack of colonization. Hyphae were only occasionally observed between mesophyll cells, and only in the vicinity of substomatal cavities (LM observations; 3.5 and 22% of fungus-containing sections at 8 and 10 dpi, respectively). In contrast to observations at these stages in 'Shafir' after inoculations with isolates IPO87016 and IPO235, the appearance of chloroplasts in mesophyll cells of 'Kavkaz/K4500 l.6.a.4' was similar to the controls (Fig. 4A). There was no evidence for compartmentalization, hypersensitive responses, or processes related to cell wall strengthening by polyphenolic compounds or lignin (stained by toluidine blue).

Resistant responses of 'Shafir' to isolate IPO86022 (data not shown) appeared to be different from those of 'Kavkaz/K4500 l.6.a.4' to isolate IPO87016, as it involved slightly more colonization and incidental alterations of mesophyll cell walls.

Inoculation of 'Shafir' with isolate IPO235 revealed a similar cytological picture with respect to isolate IPO87016, though 'Shafir' is more resistant to isolate IPO235 and was less colonized (Fig. 4B). The chloroplasts contained clearly identifiable starch granules (Fig. 4B and C) that seemed to be released from the chloroplast just before cell collapse (Fig. 4C). At 10 dpi, the appearance of chloroplasts was notably different from the controls. They were either condensed and intensely stained or swollen and destained. However, these responses seemed to be provoked by soluble compounds produced by *M. graminicola*, since there was usually no mycelium in the vicinity of the cells with these defects (Fig. 4D, E, and F).

Pycnidium formation. Young pycnidia initials of isolate IPO87016 were already observed from 8 dpi onward in the substomatal cavities of 'Shafir' (Fig. 5A). Thereafter, diverse stages of pycnidium formation were observed. Young initials evolved to mycelial stromata, typically located underneath the stoma (Figs. 2E and 5A), and developed into prepycnidia that eventually matured (Fig. 5B, C, and D). The development of a young pycnidium

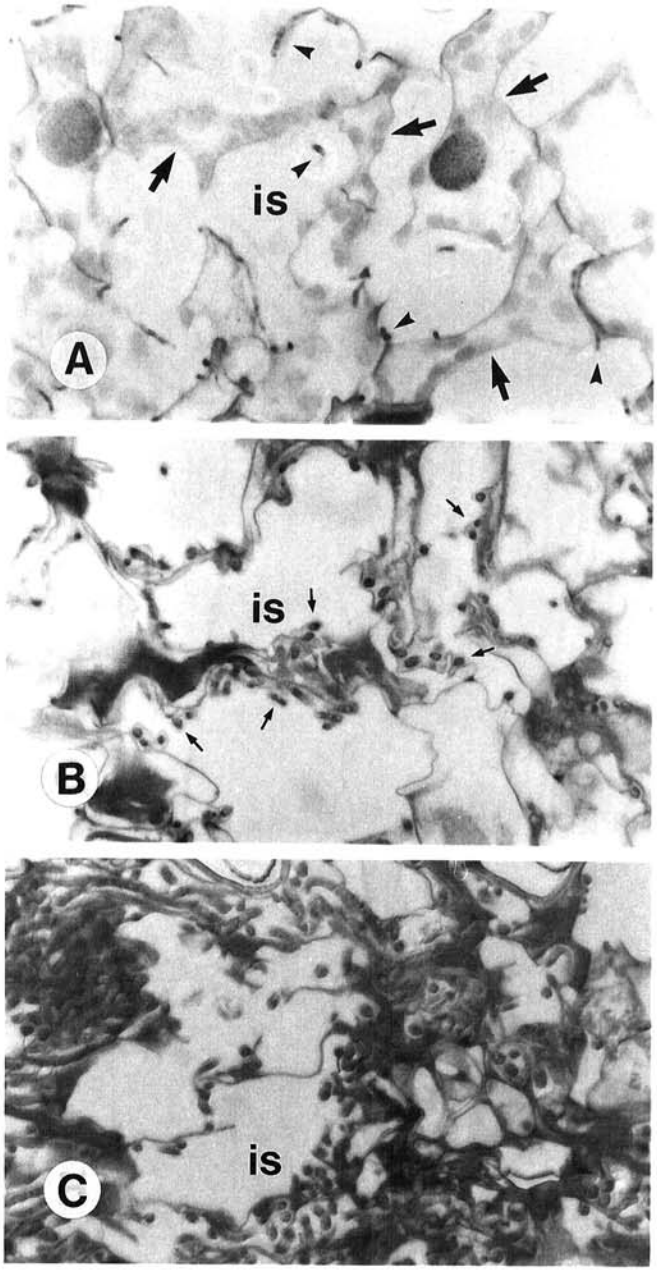

Fig. 3. Mesophyll cell collapse and its effect on mycelial proliferation of *Mycosphaerella graminicola* isolate IPO87016 in 'Shafir.' **A,** Beginning of mesophyll cell collapse at 12-days postinoculation (dpi). Note the irregular shape of the cells (arrows), the destained cell walls, which are in close contact with hyphae (arrowheads), and the hardly stained chloroplasts, ×625. **B,** Massive mesophyll cell collapse at 14 dpi. Many hyphae (arrows) are found in the vicinity of the degenerated cells (also Fig. 5C), ×625. **C,** Colonization at 16 dpi. Advanced stage of mesophyll cell collapse, ×625. Abbreviation: is = intercellular space.

Fig. 2. Light, scanning, and transmission electron micrographs of colonization during the compatible interaction between 'Shafir' and *Mycosphaerella graminicola* isolate IPO87016. **A,** Water-treated control at 16-days postinoculation (dpi). Note the regular shape of the mesophyll cells and their organelles, ×313. **B,** Intercellular growth between mesophyll cells just beyond the substomatal cavity at 48-h postinoculation (hpi). Note the changed shape of the chloroplasts (arrows) and the vacuoles in the hypha (arrowheads), ×625. **C,** Cross section through a primary leaf at 8 dpi. Note the intercellular growth of the hyphae (arrows), the wrinkled shape of the mesophyll cell walls, and the strong condensation of chloroplasts (intensely stained). A comparison of chloroplast stainability can be seen in Figure 2A, D, E, and F. Some mesophyll cells are on the verge of collapse (asterisks), ×250. **D,** Intercellular growth (arrows) at the point of cell collapse at 10 dpi. The stainability and shape of the chloroplasts (arrowheads), which fill nearly the whole cell lumina, has changed compared with 8 dpi (Fig. 2C) and the control (Fig. 2A). The vascular bundle and bundle sheath cells appear to be affected, ×625 (also Fig. 2H). **E and F,** Overview (×250) and detail (×625), respectively, of a cross section through a primary leaf at 12 dpi. Note that hyphae (arrows) grow in close contact with the mesophyll cell walls, which are destained when being in contact with mycelium (also Fig. 3A) and only clearly visible when they are adjacent. **F,** Hyphae (arrows) are in close contact with mesophyll cells, and chloroplasts (arrowheads) are swollen, hardly stained, and comparable with those in Figure 2D. **G,** Scanning electron micrograph of intercellular growth of hyphae at 12 dpi. The hyphae grow in close contact with the cell walls, attached to the cell wall by an extracellular matrix (arrows). Chloroplasts are vaguely visible through the cell wall (arrowheads), ×6,250. **H,** Transmission electron micrograph of intercellular growth of hyphae at 10 dpi. Note the degenerate chloroplasts and the intact cell wall, ×10,000. Abbreviations: cl = chloroplast, e = epidermal cell, is = intercellular space, m = mesophyll cell, and ssc = substomatal cavity.

into a conidium-producing mature pycnidium was completed in 2 to 3 days. Ripe pycnidia were invariably located under the stomata, usually one per substomatal cavity, in accordance with the linear arrangement of pycnidia in diseased leaves. Pycnidia were subglobose, with the ostioles confined by and in the shape of the stomatal openings without any additional internal or external structures such as the paraphyses (Fig. 5B, C, and D) observed in the hymenia of sexual fructifications. Microspores were not observed.

Conidiogenesis and sporulation. Initially, aseptate conidia were formed from conidiophores on the wall of the pycnidium (Figs. 5D and 6A). The wall of mature conidia consisted of two layers, as also evident from the ultrastructure of the septae that divide the conidia in segments (Fig. 6B and C). Conidia were exuded through the pycnidial ostiole in a cirrhus (Fig. 7A) because of the hygroscopic characteristics of the matrix in the pycnidium. Each cell of a mature septate conidium contained one nucleus (Figs. 6B and 7B and C).

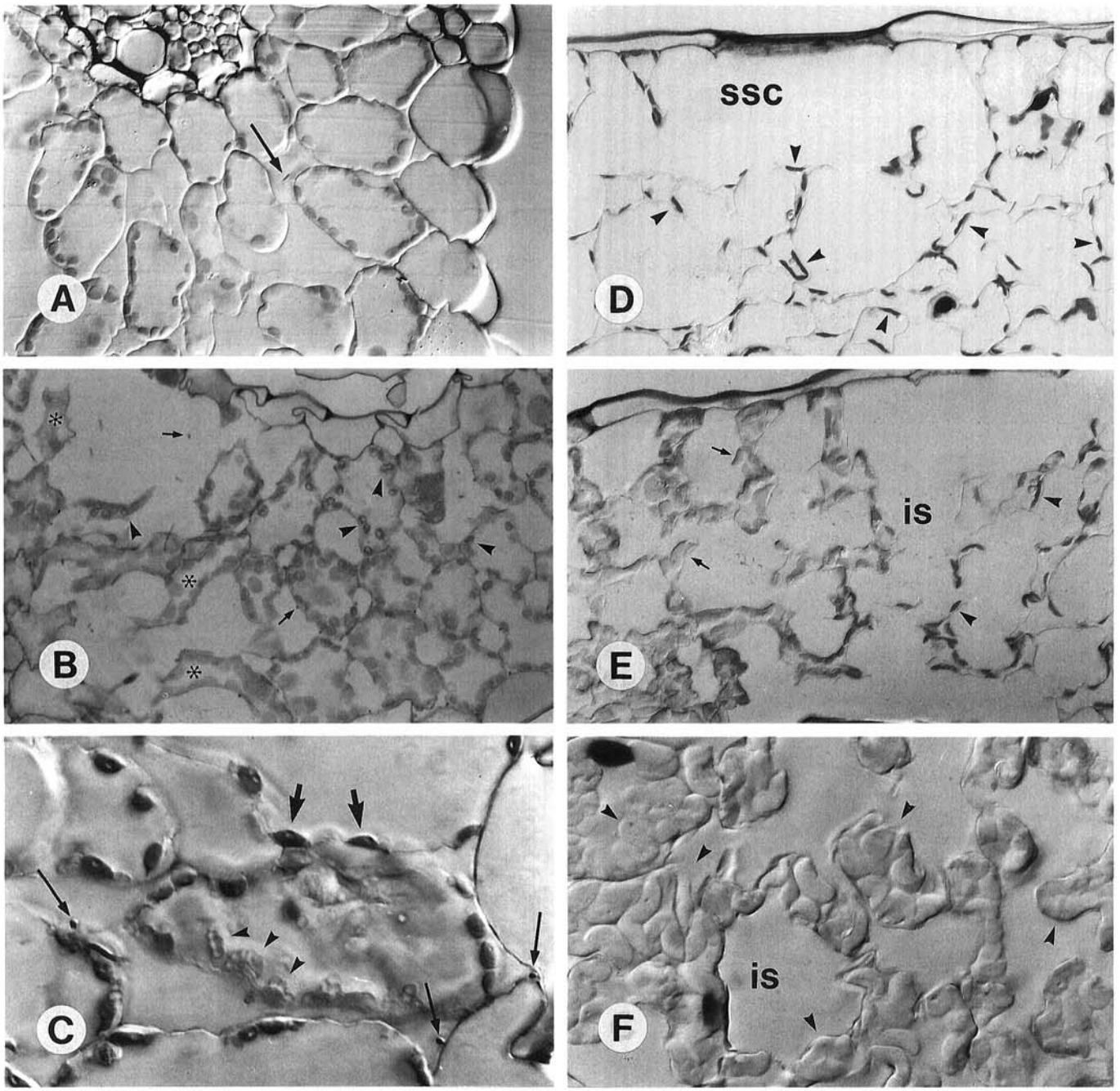

Fig. 4. Light micrographs of the incompatible and intermediate responses of 'Kavkaz/K4500 l.6.a.4' and 'Shafir' toward *Mycosphaerella graminicola* isolates IPO87016 and IPO235, respectively. **A,** Intercellular hypha (arrow) observed in 'Kavkaz/K4500 l.6.a.4' at 10-days postinoculation (dpi). The shape of the mesophyll cells and the number and size of the chloroplasts are similar to those observed in the controls and significantly dissimilar from those observed in 'Shafir' at the 8- to 10-dpi interval (Fig. 2C and D), ×313. **B,** Overview of a cross section of a primary leaf of 'Shafir' inoculated with isolate IPO235 at 12 dpi. Colonization (arrows) is still fairly limited, but several cells are at the verge of collapse (asterisks). Note the irregular shape of the mesophyll cells and the large starch granules (arrowheads) in the chloroplasts, ×313. **C,** Details of mesophyll cells of 'Shafir' at 14 dpi with isolate IPO235. Note the intercellular mycelium (arrows), the intensely stained chloroplasts (arrows), and the starch granules that appear to be released from the chloroplasts (arrowheads), ×940. **D through F,** Details of chloroplast alteration in 'Shafir' at 10 dpi with isolate IPO235. Note that none of the mesophyll cells are in contact with or close to hyphae of the fungus. **D,** Small, intensely stained and condensed chloroplasts (arrowheads), ×400. **E,** Transitional zone showing mesophyll cells with intensely stained chloroplasts (arrowheads) and cells with swollen, hardly stained chloroplasts (arrows), ×313. **F,** Swollen, hardly stained chloroplasts (arrowheads) of cells at the verge of collapse, ×625. Abbreviations: is = intercellular space and ssc = substomatal cavity.

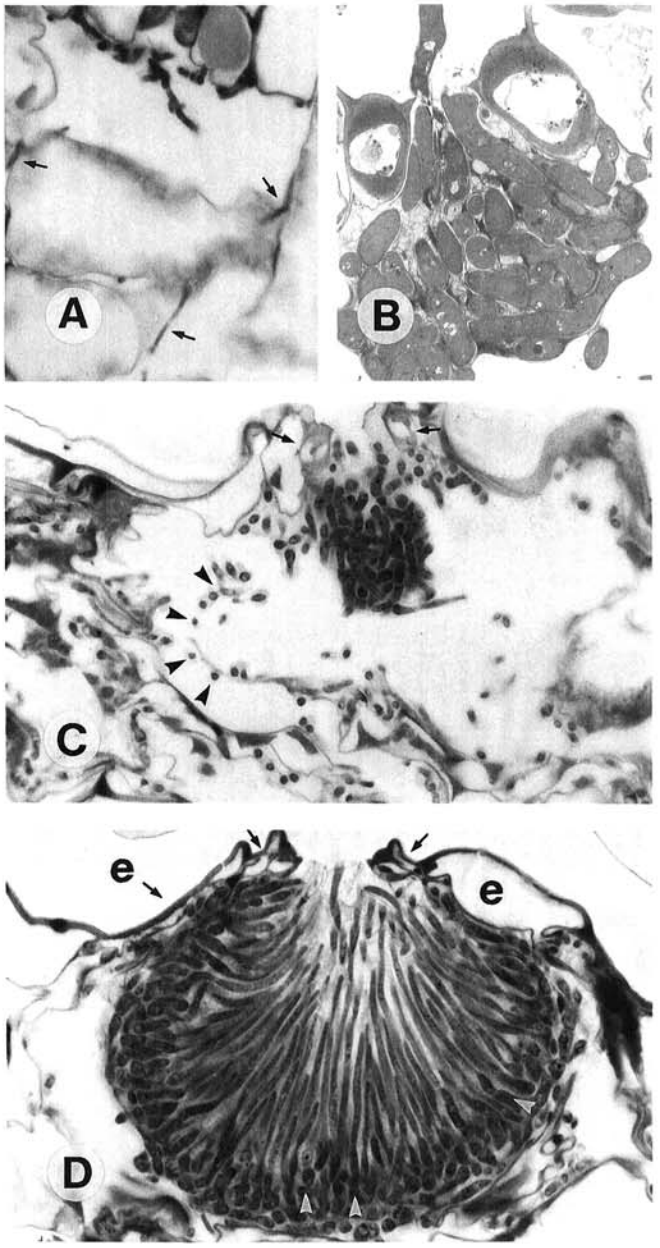

Fig. 5. Light and transmission electron micrographs of asexual fructification of *Mycosphaerella graminicola* isolate IPO87016 in 'Shafir.' **A,** Initiation of a pycnidial development under the stomatal aperture at 12-days postinoculation (dpi). Note the intercellular hyphae (arrows), ×625. **B and C,** Formation of a prepycnidium under the stomatal aperture (arrows) after massive mesophyll cell collapse at 10 and 14 dpi, respectively. Note **B,** the plasmolysis in the guard cells, subsidiary cells, and epidermal cells and **C,** the restricted distribution of mycelium, which is still largely confined to the cell walls of collapsed cells (arrowheads), ×6,000 and ×625, respectively. **D,** Mature pycnidium in the substomatal cavity at 14 dpi. The ostiole of the pycnidium is located under the stomal slit. The pycnidial wall consists of a layer of conidiophores (arrowheads), each of which can sequentially produce conidia (also Fig. 5A). Note that both epidermal cells remained uncolonized, ×625. Abbreviation: e = epidermal cell.

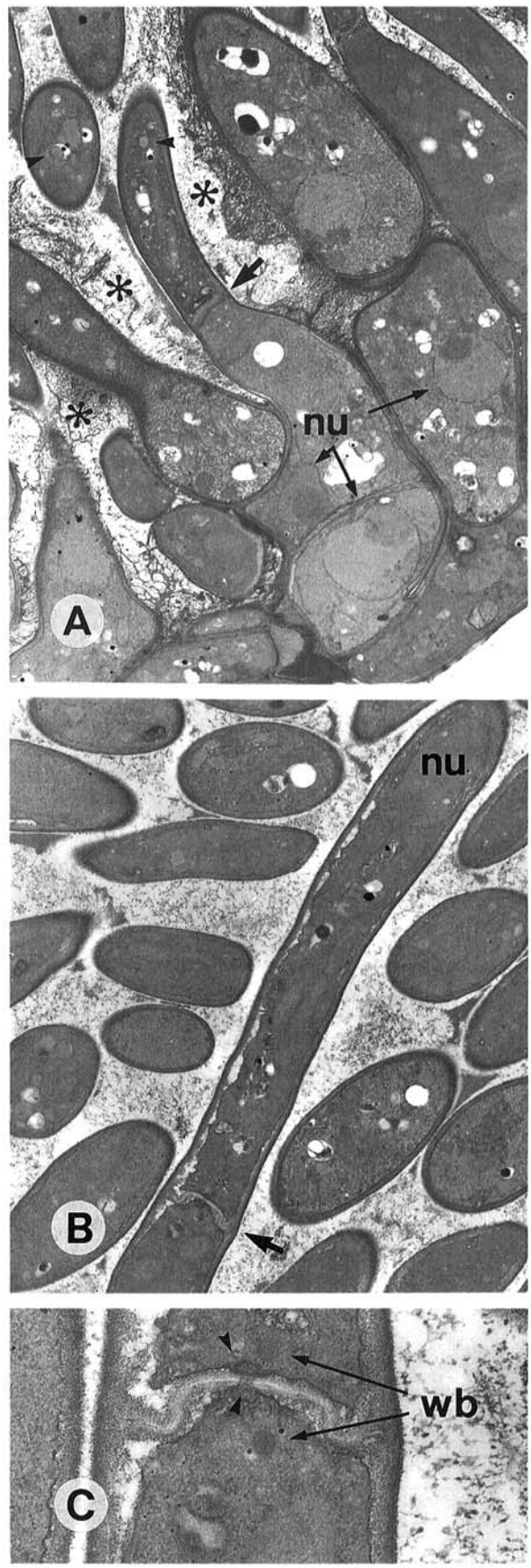

Fig. 6. Transmission electron micrographs of spore formation of *Mycosphaerella graminicola* isolate IPO87016 in 'Shafir.' **A,** Section through a young pycnidium with conidiophores and conidia. Note septum (arrow) between immature conidium and conidiophore, lipids (arrowheads) and cell organelles, as well as the hygroscopic matrix supporting the conidia (asterisks), ×6,700. **B,** Longitudinal and transverse section through young conidia. Note first conidial septum (arrow) and the nucleus and other cell organelles in the upper segment of the longitudinal section, ×8,800. **C,** Detail of the septum in **B** shows the wall-layering in the septum, the septal pore (arrowheads), and woronin bodies, ×35,000. Abbreviations: nu = nucleus and wb = woronin body.

Mycelium quantification. The development of antigen within the leaves was immediately detectable in extracts from inoculated plants, but the level of antigen did not change significantly on either cultivar until 6 dpi. Thereafter, the antigen level in 'Shafir' increased exponentially at a constant rate until the end of the experiment (14 dpi). The antigen level in extracts from 'Kavkaz/K4500 l.6.a.4' remained at a constant low level throughout the experiment (Fig. 8). The assay was not detectably nonlinear against log (antigen concentration) within the range of the standards provided by the kit.

DISCUSSION

Topographic structures or chemical signals are necessary for germ tube orientation and penetration in many pathogens (13). Many rust species rely on a combination of chemotropism and thigmotropism for the production of appressoria on stomata, which are necessary for successful penetration of the host (1). Firm attachment of germ tubes to leaf surfaces is a prerequisite for oriented growth and appressorial induction, hence for pathogenicity, and is often visualized by erosion tracks after dissolution of host cuticular components such as waxes (24). Although we observed such tracks, germ tubes appeared to grow largely at random over the leaf surface, regularly crossing stomata without penetration. Hence, under our experimental conditions, thigmotropic or chemotropic signal perception in *M. graminicola* did not seem to be of importance. The difference of mycelial content in leaves sampled at 12 or 24 hpi and those sampled at 48 hpi is additional evidence for this conclusion. In spite of the observed presence of hyphae in substomatal cavities at 12 hpi, only plants that were incubated for 48 h developed *M. graminicola* symptoms. This observation accorded with data published by Shaw (26), though he observed differences between a susceptible and a moderately susceptible cultivar, as well as the effects of light on the infection frequency. An incubation period of 48 h might enhance the probability of penetration. The fungus apparently requires an incubation period of 48 h to reach the mesophyll cells, a prerequisite for further colonization. The appressorium-like structures observed on stomata were not necessarily involved with successful penetration and were, therefore, not considered to be appressoria in the true sense, i.e., organs necessary for penetration as in many rust species. They may be organs that help the fungus to attach to the plant surface rather than to penetrate the host, particularly because they were also observed on epidermal cells (3,33). Our experiments provided evidence only for stomatal penetration of *M. graminicola* and did not confirm reports of direct penetration (3,33).

Differences between responses on susceptible and resistant cultivars were already evident from 48 hpi and increased thereafter, particularly with respect to the form and number of chloroplasts and nuclei in the susceptible and intermediate responses of 'Shafir' to isolates IPO87016 and IPO235, respectively. These effects also occurred without the presence of hyphae in the proximity of the mesophyll cells, particularly with the latter isolate. The condensation of chloroplasts could be an early response to soluble compounds excreted by the fungus, subsequently followed by intense swelling in cells at the verge of collapse. In addition to the chloroplast alterations, starch granules seemed to be released from the chloroplasts, which was not observed in the interaction with isolate IPO87016. The role of this phenomenon is unknown, but starch may be processed in an attempt to delimit further colonization. In our study, the process of chloroplast condensation and degeneration was initially not linked with necrotization and did not significantly influence the efficiency of photosynthesis as measured by chlorophyll fluorescence until stages later than 10 dpi (M. J. Prins, C. Kliffen, and G. H. J. Kema, *unpublished data*). Such changes, therefore, might be symptomatic of disturbed sink-source relationship, and reflect the sensitivity of wheat mesophyll cells to stress factors, since they were also observed in wheat leaf tissues infected with wheat streak mosaic rymovirus and *Pyrenophora tritici-repentis* (8,18). The aforementioned alterations were not observed in incompatible responses in which hyphae were only occasionally observed, without any visible detrimental effect on mesophyll cells.

The strictly intercellular growth of the fungus provides a clue for the elucidation of the pathogenesis of *M. graminicola* in wheat. It implies that communication between the plant and the fungus takes place in the apoplast, which creates perspectives for analysis of intercellular washing fluids and for elucidation of resistance and virulence in this pathosystem (5). Similar approaches for the interaction of wheat with *P. tritici-repentis* revealed necrosis-inducing proteins, which appear to be pathogenicity factors (17). Our observations suggest the involvement of soluble toxic compounds in the wheat-*M. graminicola* pathosystem, since i) in compatible interactions, mesophyll cells were severely affected without the presence of mycelium in the vicinity, and ii) cell collapse occurred within a short span of time. However, cell collapse was not observed before 8 dpi, which might indicate that a critical amount of fungal biomass or a specific physiological state is required to induce necrosis. Further TEM analysis of the interaction between *M. graminicola* isolate IPO87016 and cultivars Shafir and Kavkaz/K4500 l.6.a.4 is in progress.

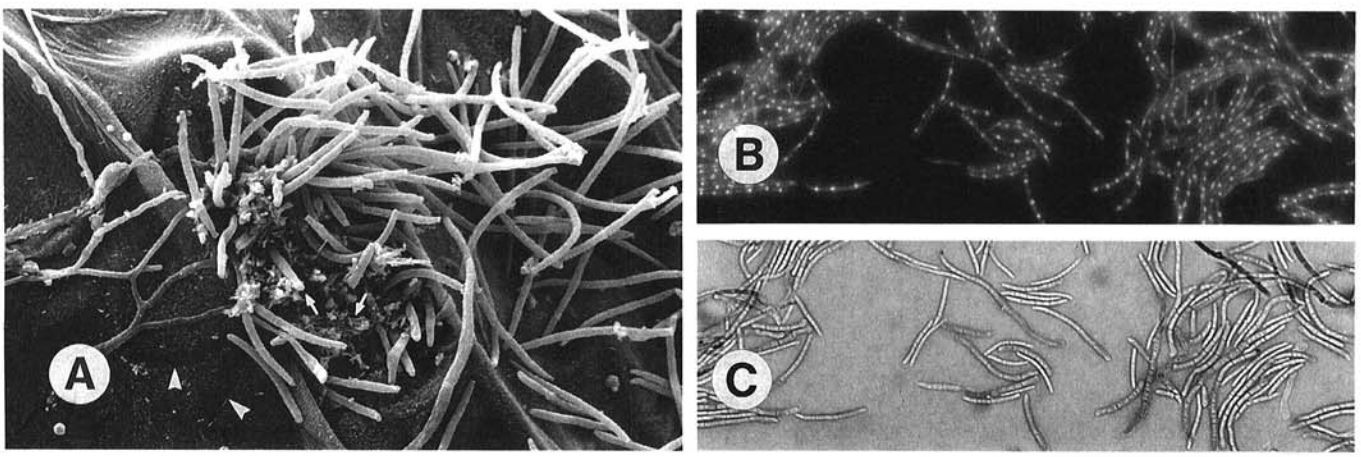

Fig. 7. Scanning electron and light micrographs of asexual conidium liberation in *Mycosphaerella graminicola*. **A,** Micrograph of a mature pycnidium at 12-days postinoculation (dpi). Remnants of a conidial cirrhus after critical-point drying preparation. Erosion tracks after the dissolution of epicuticular wax (arrowheads) caused by germ tubes and old mycelium on the leaf, ×2,413. Exuded conidia stained with 4,6-diamidino-2-phenyl indole and viewed with **B,** UV epifluorescent microscopy and **C,** regular light microscopy, illustrating that each conidial cell is uninucleate, ×1,000.

The pathogenesis of *M. graminicola* in wheat differs from that of other perthotrophic fungi on cereals such as *P. tritici-repentis* and *Rhynchosporium secalis* (17–21). These pathogens do not penetrate the stomatal aperture, but colonize the epidermal cells producing appressoria to penetrate the anticlinal cell walls, periclinal cell walls, or both. Cell collapse, due to necrosis-inducing phytotoxic compounds (10,17), in these model systems occurs within a few days after inoculation. Hyphae of *R. secalis* were observed between mesophyll cells only at very late stages of pathogenesis (19) and resistance was associated with the production of papillae, which were not observed in the current experiments with *M. graminicola.*

Pectin-degrading enzymes may play a role in the pathogenesis of *M. graminicola* in a susceptible host, which can be appropriately studied by using toluidine blue that stains acid pectin. In contrast to the mesophyll cell walls of the water-treated controls of 'Shafir,' mesophyll cell walls in the proximity of hyphae of isolate IPO87016 were hardly stained. However, papillae or any other cell wall strengthenings were not observed in conjunction with the occasional presence of *M. graminicola* isolate IPO87016 between mesophyll cells of the resistant cultivar Kavkaz/K4500 l.6.a.4. The resistance mechanism, therefore, does not seem to rely on defense responses to fungal cell wall-degrading enzymes, but rather on the production of compounds that preclude fungal colonization and, hence, pycnidium formation. In our experiments, pycnidium formation only occurred after massive cell collapse, which presumably provided the necessary release of nutrients for further colonization and eventual production of these asexual fruiting bodies in the substomatal cavities, which was already described in classical *M. graminicola* literature (4,12,33). The resistance mechanism of 'Kavkaz/K4500 l.6.a.4,' therefore, does not seem to be linked with arrested pycnidium formation as suggested by Cohen and Eyal (3).

Minimal increase of fungal tissue, if any, in 'Kavkaz/K4500 l.6.a.4' was also evident from the ELISA experiment. Significant differences between 'Shafir' and 'Kavkaz/K4500 l.6.a.4' occurred at 8 dpi and later, thus after the first cell collapse, which initiated rapid colonization. The slight increase of fungal tissue observed microscopically in 'Shafir' during the 48-hpi to 8-dpi interval, before the first cell collapse, was not detected by ELISA. Nevertheless, ELISA of the antigen of *M. graminicola* provides a quantitative measure of fungal proliferation within a leaf. In addition to the cytological information of the pathogenesis of *M. graminicola* in wheat, the antigen level seems likely to be proportional to the fungal biomass in the leaf and not preferentially produced during certain physiological states of fungal growth. The assay, therefore, may contribute considerably to the understanding of the interaction between *M. graminicola* and wheat, as was recently reported for the *Leptosphaeria maculans-Brassica napus* pathosystem (2) and offers prospects as a rapid alternative to evaluate inoculation experiments to characterize varying levels of resistance in the host and virulence in the pathogen.

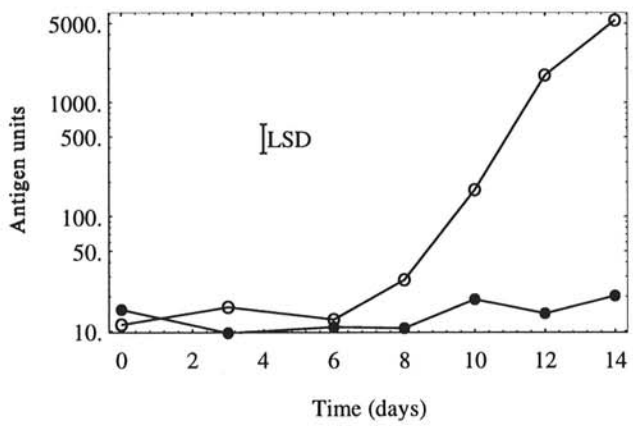

Fig. 8. Antigen quantification of *Mycosphaerella graminicola* isolate IPO87016 with a proprietary kit based on enzyme-linked immunosorbent assay at different time intervals in compatible and incompatible interactions with the wheat cultivars Shafir (○) and Kavkaz/K4500 l.6.a.4 (●), respectively.

The role of phytoalexins in disease resistance is evident in diverse pathosystems (11,31), and phytoalexin detoxification might be responsible for phytoalexin tolerance and, as such, indispensable for the expression of virulence (25,31). Hence, virulence in isolate IPO87016 to 'Shafir' might be controlled by detoxification of phytoalexins, which enables colonization of the mesophyll tissue and, eventually, the production of sufficient toxic compounds to kill host cells. In that case, pycnidium production might be considered as a strictly autonomous process following cell collapse. Kema et al. (14,15) suggested that necrosis induction and pycnidia production were under different genetic control, since they observed a poor correlation between these parameters, which is exemplified in the interaction of 'Shafir' with isolate IPO235. Such a response might be explained by incomplete inactivation of triggered antifungal compounds, which allows restricted colonization and eventually toxin production, but cell collapse evidently does not initiate mycelial proliferation, resulting in limited, local pycnidium formation. Analysis of intercellular washing fluids appears to be essential to resolve those aspects of host-pathogen interactions in the wheat-*M. graminicola* pathosystem.

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