External Growth, Penetration, and Development of *Cercospora zeae-maydis* in Corn Leaves

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ABSTRACT

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The influences of plant age, genotype, leaf age, and moisture on symptom expression of gray leaf spot caused on corn (*Zea mays*) by *Cercospora zeae-maydis* were studied. External growth, penetration, internal colonization, and sporulation were characterized histologically. Spores germinated after 24 hr at 22–30 C when plants were exposed to 12 hr of mist. Where no free water accumulated, germ tubes showed positive stomatal tropism. The presence of free water on the upper leaf surfaces reduced tropistic responses toward stomata, appressorium formation, and subsequent penetration. On the lower leaf surfaces, abundant appressoria formed over stomata 4–5 days after inoculation and penetration occurred after 6–7 days. In mature plants, chlorotic dots formed at 9 days, elongated discolored streaks at 12 days, and necrotic and sporulating lesions at 16–21 days. Young corn plants were also susceptible and developed sporulating lesions 3–4 days earlier than mature plants. Leaf age and plant genotype had little effect on symptom expression under greenhouse conditions. Internal colonization was intercellular and confined to the mesophyll. Delimitation of hyphal growth lateral to the vascular system by sclerenchyma tissue surrounding the major veins resulted in typical long, narrow, parallel bordered lesions. Fungal stroma developed in the substomatal cavities coincident with necrosis of the lesion and gave rise to numerous conidiophores and conidia. These results suggest that sustained periods of high relative humidity are more important for the development of *C. zeae-maydis* than plant or leaf age. The typical late-season appearance of this disease in the field is likely due to extended periods of high humidity provided by the canopy of mature corn plants.

Additional key word: histopathology.

Cercospora zeae-maydis Tehon & Daniels causes a foliar disease of corn known as gray leaf spot. The disease was first reported in Illinois in 1924 (8), but has never been considered a problem in that state. It is most prevalent in the mountainous regions of Kentucky, Tennessee, Virginia, North Carolina, and South Carolina. The disease has been more serious recently, and losses as great as 20% have been reported in Tennessee (2). Increase in the prevalence of gray leaf spot is thought to be associated with the increase of minimum or no-till farming practices (7). Concern exists that gray leaf spot may move into other corn-producing states as the acreage of minimum tillage corn increases.

Individual lesions of gray leaf spot are long and narrow with parallel borders and are typically tan to brown, sometimes assuming a silvery or gray cast. Under favorable conditions, the number of lesions increases rapidly and their coalescence causes extensive necrosis of leaf tissue. Gray leaf spot epidemics are typified by rapid disease progression late in the growing season (P. M. Beckman, *unpublished*). Apical progression of disease from lower leaves late in the season suggests that host plant maturity and leaf age affect disease severity.

Study of gray leaf spot has been limited by difficulties in producing substantial disease under greenhouse conditions (4). The present study was conducted to describe symptom expression under varying conditions and to histologically characterize external growth and penetration, internal colonization, and subsequent sporulation of the fungus in corn leaves. Greenhouse studies were used to examine the influence of plant age, plant genotype, leaf age, and moisture on symptom development. These results are discussed in relation to the occurrence of gray leaf spot in the field.

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MATERIALS AND METHODS

Inoculation. Special care was necessary to maintain sporulating cultures of *Cercospora zeae-maydis*. Fresh cultures were started by homogenizing cultures in a Sorvall Omni-Mixer (Sorvall, Inc., Norwalk, CT 06856) for 45 sec and pouring the homogenate onto V-8 juice agar (300 ml V-8 juice, 700 ml H_2O, 3 g $CaCO_3$, and 17 g agar). Excess liquid was decanted, and the plates were incubated in stacks of 10 for 9 days under constant fluorescent light with the top plates receiving 27 lux. The plates were then placed in darkness for 2–3 days. Conidia were harvested in 0.01% Tween-20 with a camel's hair brush (Fisher Scientific, Pittsburgh, PA 15219). A conidial suspension was either atomized or brushed with a camel's hair brush onto both leaf surfaces of corn plants. After inoculation, the plants were held in a greenhouse maintained by wet-pad cooling at 22–30 C. The plants were exposed to a fine mist for 6 sec at 4-min intervals from 0900 to 2100 hours for the duration of the experiment. Inoculations were made at dusk (0800 hours). Ten corn cultivars were examined, but most of the data reported are for Pioneer Brand (PB) 3334, which is moderately susceptible.

Experimental design. Observations on symptom progression and tissue samples for histological studies were taken from two major inoculation trials in the greenhouse. The first experiment was designed to examine the influence of leaf age and plant genotype on symptom expression. Susceptibility of greenhouse-grown plants was also compared to plants of the same age and cultivar grown in pots outdoors. Two plants from each of 10 corn cultivars (Coker 19, PB3369A, PB3368A, PB3147, PB3184, PB3334, P-A-G PX79-34126, DeKalb XL72B, Northup-King SX17A, and Funk G4848) were inoculated at anthesis with 4×10^4 conidia per milliliter. At least six leaves representing all leaf positions were inoculated on both surfaces in August 1980, and observations were recorded on all leaves at 1- to 3-day intervals for 25 days. A second experiment compared and characterized differences in development of disease and symptom expression between young and mature plants. Ten young plants (PB3334) with

the eighth leaf in the whorl and 10 mature plants at anthesis were inoculated with 2×10^5 conidia per milliliter on 5 September 1980, and two plants of each age group were sprayed with water as controls. Observations were made and tissue samples were taken daily until the lesions were mature (supporting sporulation). Tissue samples approximately 20 cm^2 for each plant age group were taken from an inoculated leaf at a median position up the plant on three plants in separate areas of the greenhouse bench. A particular plant was not resampled for two consecutive days. In both experiments, additional plants of PB3334 were included to examine the effect of mist on lesion development. Two plants each were exposed to no mist, mist for 1 day, or mist for 3 days after inoculation.

Histology. Histological observations were made on whole mounts, epidermal peelings, and paraffin-embedded sections. Leaf pieces removed at 24-hr intervals were fixed in a standard solution of formalin-propionic acid-propanol (FPP) (3). Epidermal peelings were made either before or after fixation. Whole-mount tissue either fresh, fixed, or cleared in Farmer's solution, and epidermal peelings were stained in trypan blue on glass slides. Staining was enhanced by passing the slide over an alcohol flame for 5–15 sec. Whole-mount tissue selected for more detailed photographic examination was cleared in Farmer's solution and either embedded in paraffin or prepared for scanning electron microscopy (SEM). Paraffin-embedded sections were stained with

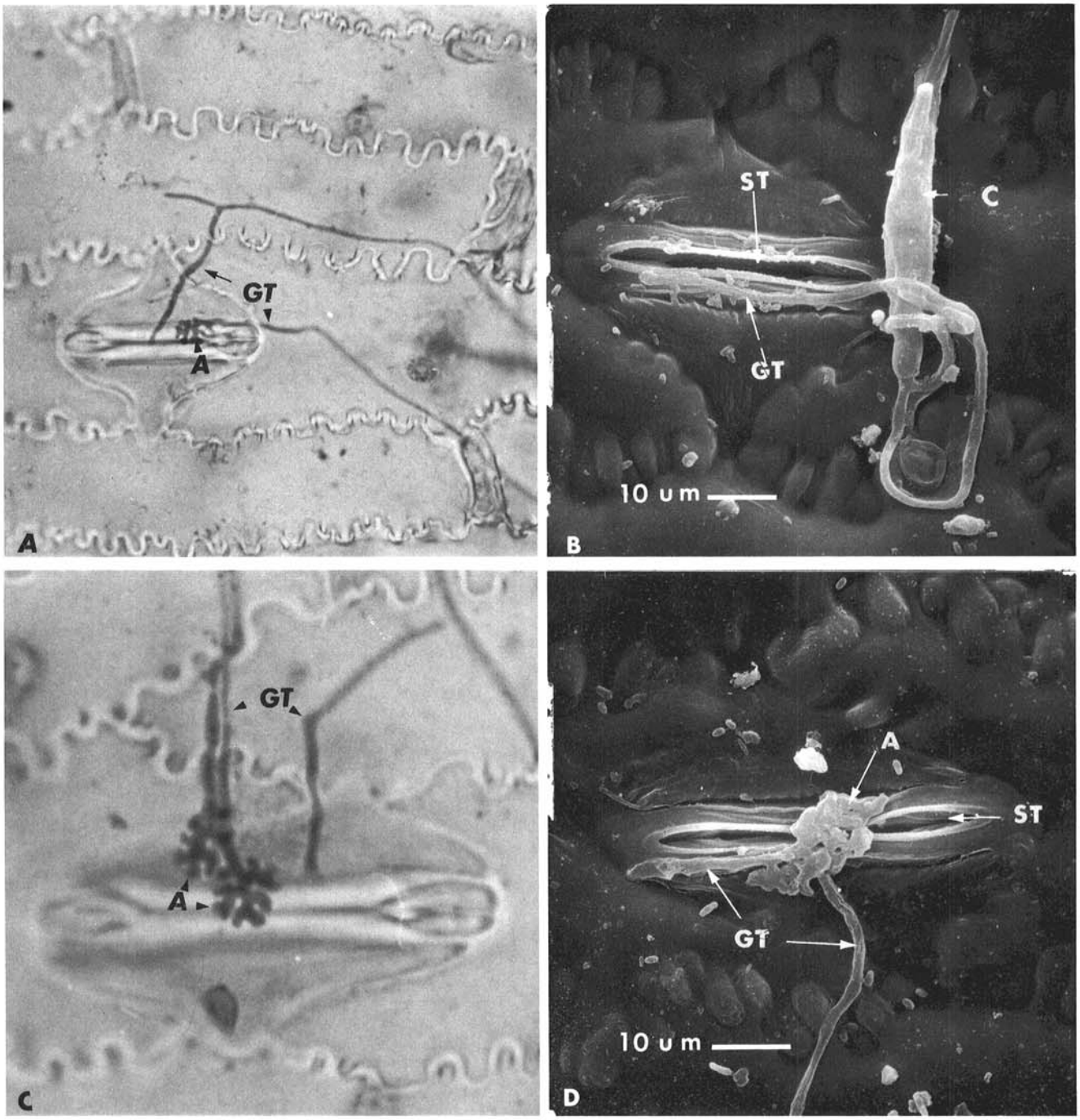

Fig. 1. Stomatal tropism and appressorium formation by *Cercospora zeae-maydis* on corn leaves. **A and B,** Characteristic branching and growth of germ tubes toward stomata. **C,** Amorphous lobes of two appressoria and the typical areas of development on guard cells near the stomatal aperture and at the junction with an epidermal cell. **D,** Aggregated and convoluted nature of an appressorium and the extension of a germ tube beyond the appressorium. **A** and **C** are of stained epidermal peelings photographed through bright-field optics (×250). **B** and **D** are scanning electron micrographs. A = appressorium, C = conidium, GT = germ tube, ST = stoma.

a modified Conant's stain (3) or with cotton-blue in lactophenol. Sections for SEM were dehydrated in a graded ethanol-Freon 13 series (1), gold sputtered, and observed under an ETEC scanning electron microscope.

RESULTS

Symptoms. The first macroscopic symptom of the disease appeared as chlorotic dots 9 days after inoculation. Characteristic stages of symptom expression from initial chlorotic dots to sporulating lesions could be loosely grouped into 3-day periods. In 9–12 days after inoculation, chlorotic dots enlarged and in some cultivars developed a watery or greasy appearance. Rapid elongation of the dots after 13–16 days resulted in long, narrow lesion initials that took on the characteristic parallel-bordered, blunt-ended shape of a mature lesion. During the elongation or streak stage, the color of the streak changed to a rusty-brown color. Streaks ranged from 1–3 mm wide and 5–35 mm long. Necrosis progressed gradually from the center of the fully elongated streaks, and in 3 days all but the ends of the lesion had turned to a tan or brown color. Conidiophores and conidia were produced 1–3 days after the lesion became necrotic and about 16–20 days after

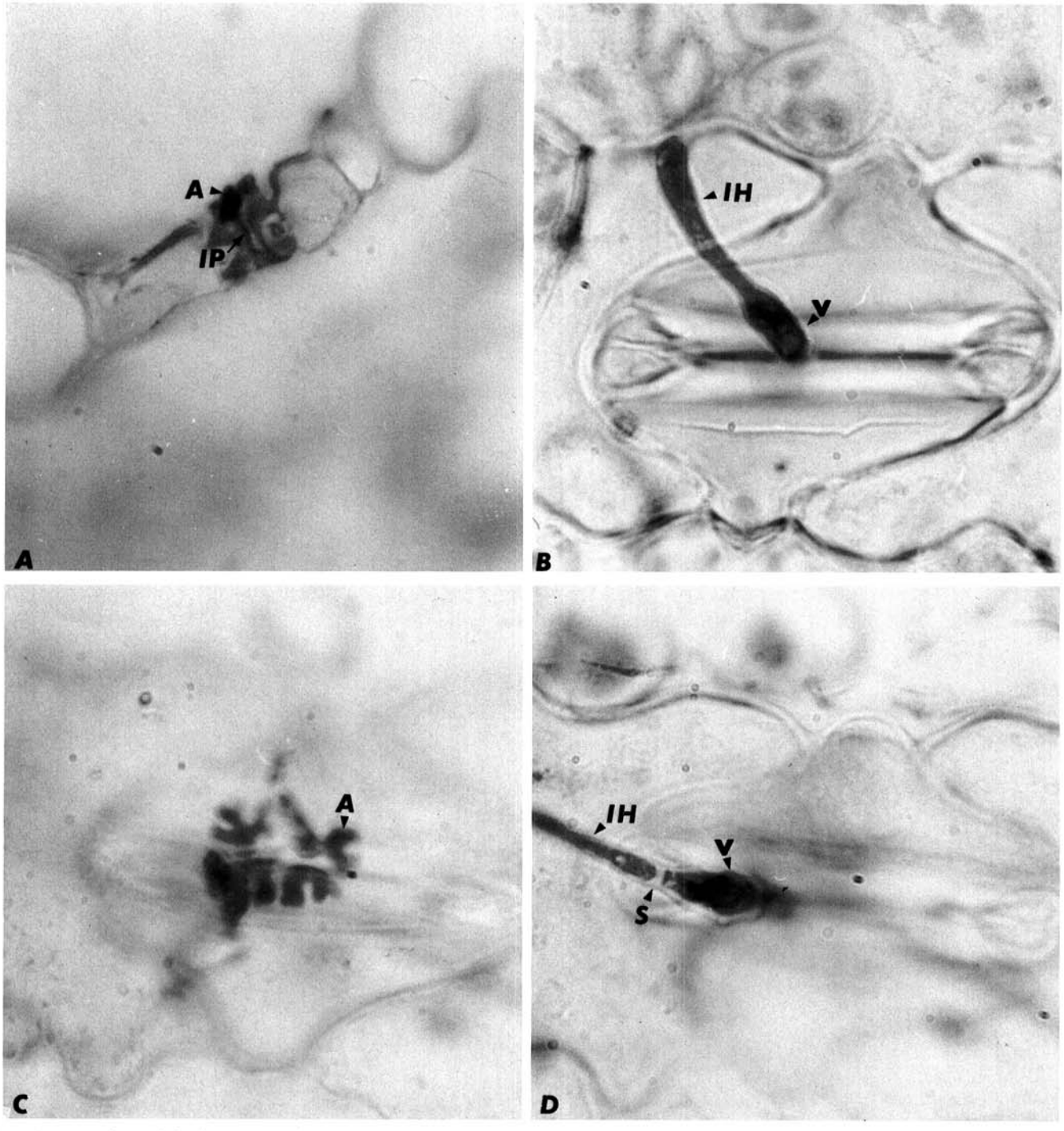

Fig. 2. Appressoria and infection hyphae of *Cercospora zeae-maydis* on corn leaves observed under bright-field optics (×1,000). **A,** Cross section of an appressorium and infection peg showing penetration through a stoma. **B,** View of a stained, inverted epidermal peeling of a corn leaf showing characteristics of vesicle and primary infection hyphae. **C and D,** View of inverted epidermal peeling of a corn leaf showing an appressorium photographed through the guard cells and the corresponding vesicle and its infection hyphae separated by a septum. A = appressorium, IH = infection hyphae, IP = infection peg, S = septum, V = vesicle.

inoculation. Conidiophores bearing abundant conidia were limited to necrotic tissue.

Symptom expression in mature plants in this study was typical of symptom expression in plants grown in the field. In young plants, however, host discoloration in the chlorotic dot and streak stages were less discernible, and necrosis developed rapidly without progression through well-defined stages. Generally, initial macroscopic symptoms occurred 1–3 days earlier, and conidiophore production occurred 3–4 days earlier in younger plants. In contrast, no consistent differences in susceptibility were

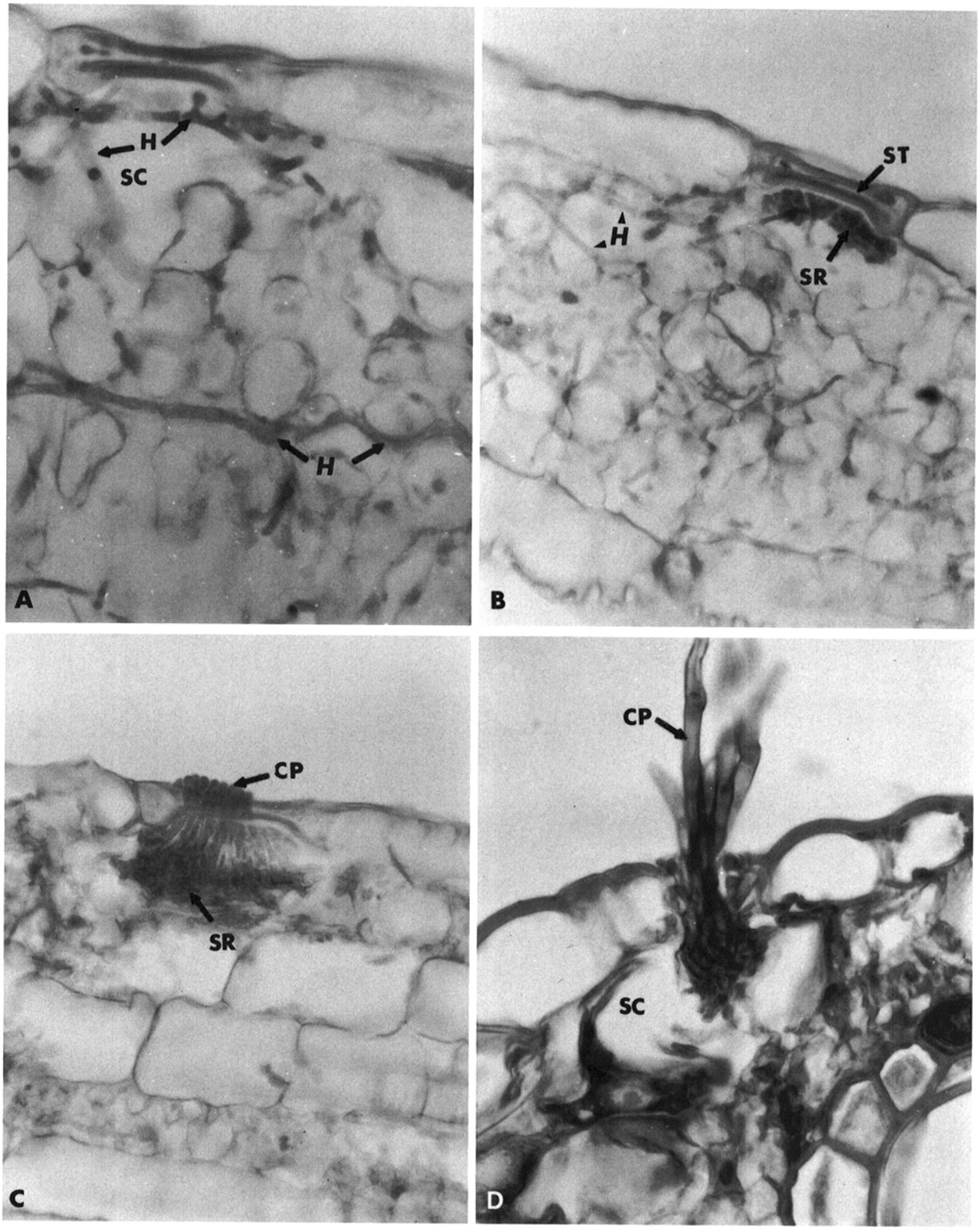

Fig. 3. Fungal stroma and conidiophores of *Cercospora zeae-maydis* in necrotic corn leaf tissue. **A,** Ramification of hypha in leaf tissue and initial stroma development below guard cells. **B,** Fungal stroma partially filling the substomatal cavity. **C,** Fully developed stroma in substomatal cavity and conidiophores protruding from a stoma. **D,** Fully developed conidiophores. Tissue was paraffin-embedded, cut into 10 μm sections, and observed with bright-field optics (×400). CP = conidiophore, H = hyphal stroma, SC = substomatal cavity, SR = stroma, ST = stoma.

observed among leaves on the same plant. Lesion development was similar on plants grown both in the greenhouse and outdoors. In the greenhouse, mist was essential for lesion development in the winter, but was not always necessary in the summer. Symptom development was similar on all 10 cultivars tested.

Histological studies. On both leaf surfaces 90–100% of the conidia germinated in a 12-hr moisture period. Germ tubes grew for a period not less than 7 days on the lower leaf surface where little or no free water accumulated. Germ tube growth on the lower surface and on nonmisted leaves showed a definite positive tropism toward stomata (Fig. 1A and B). Of 100 appressoria observed from 31 randomly chosen microscopic fields on five tissue pieces of different leaves, lateral side-branching toward stomata was obvious in approximately 25% of the germ tubes that had formed appressoria over stomata. Stomatal attraction was evident from either the direct growth of germ tubes toward stomata or a definite change in direction of germ tubes toward stomata with the subsequent formation of appressoria. Germ tube growth in the presence of free water on upper leaf surfaces was more extensive up to 5 days after inoculation than in the absence of free water. After this time, the germ tubes stained less readily. Stomatal tropism was not observed on the upper surface where free water accumulated. Five days after inoculation only two appressoria were formed by 390 germinated conidia examined on tissue pieces from seven leaves. Neither plant age nor cultivar had any effect on germ tube growth.

On the lower leaf surface, swelling of germ tube terminals or intercalary branches was observed 2–3 days after inoculation. Appressoria were observed occasionally at 2–3 days and were abundant over stomata after 4–5 days. Appressoria varied greatly in size, complexity, and shape. Appressorium formation was a process of growth from a single germ tube cell that appeared to continue over several days. Swellings from this cell developed into amorphous projections that spread laterally or became convoluted to form aggregates of varying shapes and dimensions (Figs. 1A, C, D, and 2C). Appressoria typically ranged from 17–45 μm in diameter, but occasionally, appressoria 60 μm in diameter were observed. Single conidia produced as many as eight appressoria over different stomata, but the formation of two to five appressoria per conidium was the most common. The fungal aggregates were usually found over stomata and occasionally over guard cells (Fig. 1C) and the junction of epidermal cells.

Penetration by *C. zeae-maydis* was determined by examining inverted and stained epidermal peelings taken from three plants each of young and mature corn. Penetration occurred only from appressoria formed over stomata (Fig. 2A–D). Initial penetration occurred at 5 days with a mean penetration of 5% (15 of 300) at 6–7 days after inoculation of young plants and 7–8 days after inoculation of mature plants. These values may be conservative due to possible breakage of infection hyphae during epidermal peeling. After ingress, appressoria failed to stain as readily. However, dark-staining appressoria were observed continuously from the time of initial ingress until about 2 wk after inoculation when production of a secondary cycle of conidia confounded observations on extramatrical growth. New penetrations continued to be observed during this period. Of 300 appressoria observed on tissue pieces from three plants, approximately 5% supported penetration into the substomatal cavity in a given 24-hr period.

No septation was observed in the aggregate structure. Although only one infection peg developed from a single appressorium, more than one appressorium was commonly found over a stoma. Thus, one or more infection hyphae could be observed entering the substomatal cavity. Ingression was always associated with some degree of hyphal swelling or aggregation into an appressorium.

Infection hyphae usually developed a slightly enlarged, generally one-celled, vesicle immediately upon penetration (Fig. 2B and D), but sometimes it appeared to be two-celled. A robust primary hypha with septations (Fig. 2D) grew from the vesicle until it encountered the mesophyll or parenchyma tissue surrounding the substomatal cavity. Internal colonization of the corn leaves was confined to the air spaces and intercellular spaces within the parenchyma tissue of the mesophyll (Fig. 3A). Discoloration of host cells was usually, but not exclusively, associated with the presence of abundant internal mycelium. Chlorotic or greasy dot areas were highly colonized by the fungus. Tissue in elongated streaks often had relatively less mycelial colonization. Host discoloration associated with sparse internal hyphae, the exclusively intercellular growth, and the slight halo effect

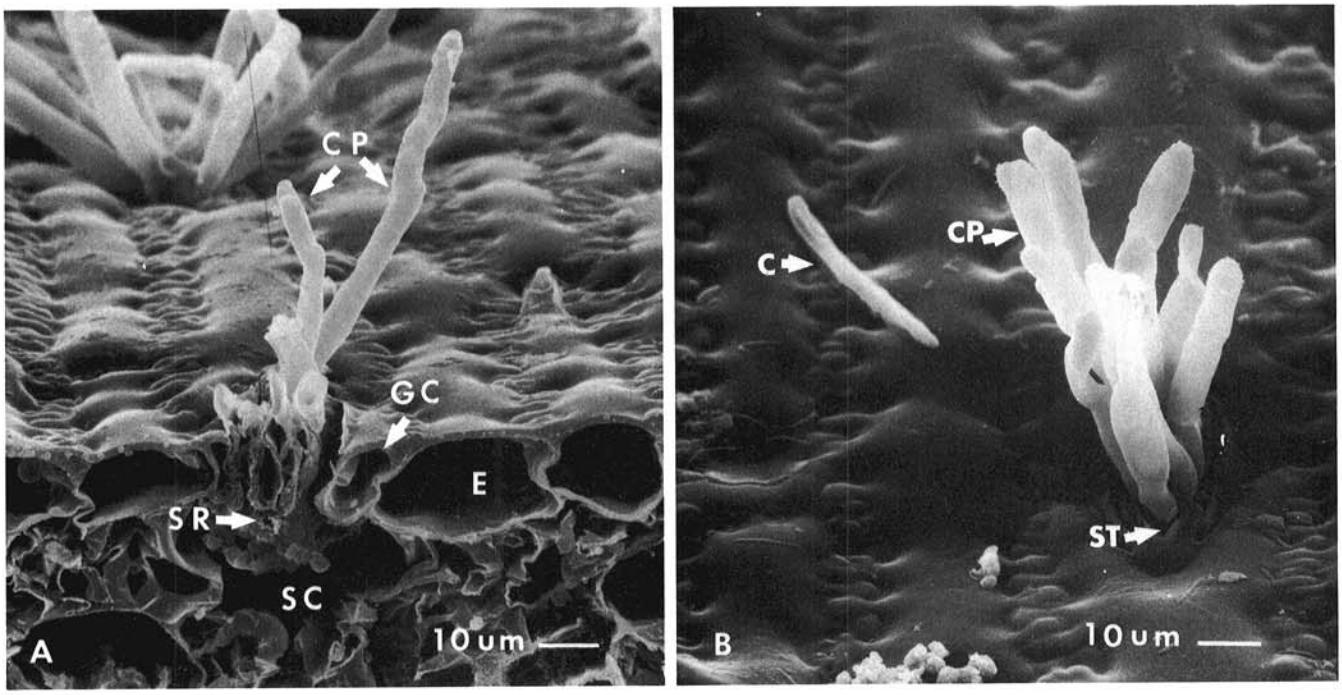

Fig. 4. Scanning electron micrographs of stroma and conidiophores of *Cercospora zeae-maydis* in necrotic corn tissue. **A,** Cross section of necrotic corn tissue showing fungal stroma partially filling the substomatal cavity and giving rise to conidiophores. **B,** Surface of corn leaf with conidium and conidiophores of *C. zeae-maydis*. C = conidium, CP = conidiophore, E = epidermal cell, GC = guard cell, SC = substomatal cavity, SR = stroma, ST = stoma.

surrounding lesion initials may indicate the presence of a fungal toxin. Hyphal growth lateral to the vascular system was generally delimited by the sclerenchyma tissue surrounding the major veins. The confinement of fungal colonization by the major veins resulted in the characteristic long, parallel borders of the gray leaf spot lesions.

Increasing host necrosis was evidenced by general lack of staining of mesophyll tissue and a concurrent browning of these cells. A dense, red-staining material often accumulated in or near necrotic tissue especially as lesions aged (Fig. 3D). In the necrotic tissue, cell walls remained intact until lesions aged.

As host tissue became necrotic, hyphal strands appeared to grow toward the guard cell region of the substomatal cavity (Fig. 3A). Hyphae began to aggregate and differentiate producing a stroma that partially or completely filled the substomatal chamber (Figs. 3B, C, D and 4A). Conidiophores erupted through the stomatal opening from the stroma (Figs. 3D, 4A and B). Conidia were initiated apically on conidiophores, which then grew sympodially past the point of conidial attachment for a short distance before initiation of conidia at the new apices. Numerous conidiophores and conidia were produced from a single stroma under conducive conditions.

Eventually, necrotic host tissue deteriorated and hyphae were able to colonize within the host cells. As the host tissue continued to deteriorate, the fungal stroma remained intact and, presumably, would survive over the winter to produce the primary inoculum in the subsequent growing season.

DISCUSSION

Colonization of corn tissue by *C. zeae-maydis* was similar to that reported for many *Cercospora* species on other plants. Germ tubes ramified extensively over the leaf surface before penetrating through stomata. Under conditions of high relative humidity, germ tubes and intercalary branches from germ tubes grew toward stomata, apparently in response to tropistic attraction, and formed numerous appressoria. In the presence of free water, germ tubes grew extensively, but showed no stomatal tropism, appressorium formation was rare, and no penetration of the host was observed. Survival on the leaf surface was also reduced. Rathaiah (6) reported extensive random growth of *C. beticola* Sacc. germ tubes on sugar beet foliage under continuous wetting. Germ tubes passed over or beside stomata, but lateral branching toward stomata was not observed and penetration of the host was rare. When conidia germinating on leaves were exposed to varying periods of drying and levels of humidity ranging from 96 to 98%, Rathaiah (6) found increased stomatal tropism, appressorium formation, and penetration. He concluded that these enhanced responses were probably due to hydrotropism or stomatal attraction due to differences in a water vapor gradient. Similar responses are cited for *C. musae* Zimm. on banana (5).

The extended period between inoculation and lesion development with *C. zeae-maydis* is not unusual for *Cercospora* species. A latent period from 16 to 120 days has been reported for *C. musae* (5). This extended latent period may be due to several factors, but is partially due to the ability of *Cercospora* to sustain mycelial growth on the leaf surface for long periods before penetration occurs. Rathaiah (6) reported that germinated spores of *C. beticola* are capable of withstanding several diurnal cycles on leaves and still penetrate the host. *C. zeae-maydis* was observed to sustain mycelial growth on the leaf surface for at least 1 wk before penetration and new lesions were observed well after initial lesions had developed fully.

The late-season appearance of gray leaf spot (GLS) and the development of lesions beginning on the lower leaves suggests that susceptibility may be increased by plant or leaf age. Results of our studies, however, clearly showed that neither plant age nor leaf age greatly influenced susceptibility. Hilty (2) has reported a similar observation. Disease spread to the upper leaves is more likely to be related to increased inoculum density and favorable environmental conditions than to plant age. Field studies in Raleigh, NC, in 1979 and 1980 (Beckman, P. M., *unpublished*) support the role of environment in development of this disease. Five fields were examined for development of gray leaf spot. Substantial disease developed at only one site, which was located in a sheltered, low-lying area adjacent to a stream, where dew was apparent and humidity remained high until late in the morning. The 1979 and 1980 GLS epidemics in Raleigh indicate that, provided moisture requirements are met, a scarcity of rainfall and high daily temperatures averaging 28 C do not limit disease (Beckman, P. M., *unpublished*). Our present study indicates that disease development probably requires periods of sustained high humidity, provided by the plant canopy, to accommodate extended periods of growth of *C. zeae-maydis* on the leaf surface before penetration. Our overall conclusion on the importance of moisture in gray leaf spot development is consistent with the geographic distribution of severe disease in low areas and valleys of mountainous regions where fields are subject to dews that dry slowly and to lingering late-season fogs.

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