The Development of Gloeocercospora sorghi in Sorghum

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ABSTRACT

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The development of *Gloeocercospora sorghi* in sorghum seedlings and mature plants was investigated by light microscopy to provide a background for studying the involvement of hydrogen cyanide (HCN) in zonate leafspot. On primary leaves of 1-wk-old seedlings, penetration was initiated over nonspecialized epidermal cells from appressoria or appressorium aggregations within 8 hr after conidial germination. Lesions on these primary leaves first were observed 12 to 24 hr after inoculation as water-soaked, chlorotic areas surrounding sites of penetration. Generally, pathogen development was restricted to subcuticular hyphae or stroma in the primary leaf epidermis until 36 to 48 hr after inoculation when advanced intercellular colonization became prevalent. In contrast, ingress into 3- or 5-wk-old leaves of 6- or 10-wk-old plants, respectively, occurred most frequently through leaf trichomes. After penetration, pathogen development and lesion appearance in these older leaves of older plants was similar to that in seedlings. However, lesions appeared more slowly and did not coalesce as rapidly on older leaves of older plants.

Zonate leafspot, which is caused by *Gloeocercospora sorghi* (5), often is found in *Sorghum* sp. during warm, wet weather in sorghum-growing regions of the world (26, 30). When cyanogenic plants such as sorghum are subjected to stress such as that of zonate leafspot or other plant diseases, hydrogen cyanide (HCN) is produced by the host (20, 24). Some researchers have speculated that HCN may influence the outcome of pathogenesis by inhibiting pathogen development (20), by injuring host cells and thus allowing more rapid pathogen growth, or by contributing to other aspects of symptom expression (20, 22).

Previously, we reported the kinetics of the decline of hydrogen cyanide potential (HCN-p) from sorghum leaves infected by *G. sorghi* (24). The interaction between sorghum and *G. sorghi* is not known in sufficient cytological detail to associate any aspect of pathogenesis with changes in HCN-p (5, 7, 8). The purpose of this study was to characterize cytologically this host-parasite interaction in sorghum.

MATERIALS AND METHODS

General.—*Gloeocercospora sorghi* D. Bain & Edg. was isolated from diseased sorghum leaves provided by R. A. Fredericksen, Texas A & M University, College Station, TX 77840, USA. The fungus was maintained on V-8 juice agar (21) under continuous fluorescent light at 22 to 25 C, and remained pathogenic during our studies.

Cultivar Grazer, a sorghum [*Sorghum biocolor* (L.) Moench] × sudangrass (*Sorghum sudanense* Stapf. 'Piper') hybrid, was seeded into a sandy loam soil contained in 10-cm diameter clay pots and maintained in a greenhouse with supplemental fluorescent lighting at about 30 C.

Inoculum was prepared by washing conidia from 7- to 13-day-old cultures into distilled water and filtering the suspended spores through three layers of cheesecloth. The spore concentration was adjusted to 2-4 × 10^5 spores/ml in 0.05% aqueous Tween-20 (polyoxyethylene sorbitan monolaurate) (v/v). Plants were sprayed to runoff with this spore suspension and incubated in a moist chamber at 100% relative humidity at 27 C for 4 days.

The HCN-p in sorghum leaves was determined using picric acid in microdiffusion dishes as described previously (24).

Light microscopy.—Inoculated leaves were prepared for observation with the light microscope by three procedures:

Whole mount.—Freshly excised leaves were observed unstained or stained for 5 sec with 0.1% cotton blue (w/v) in lactophenol (31). Unabsorbed stain solution was removed by gentle blotting. Leaf surface fungal structures were observed using a microscope equipped with bright-field optics.

Whole-mount differential staining.—Intact leaves or leaf segments were prepared as fixed whole mounts (19). They were fixed in a solution of absolute ethanol-glacial acetic acid (2:1, v/v) for 24 to 48 hr and then cleared in lactophenol for 24 to 48 hr. Cleared leaves were stained first for 30-60 min in 0.1% acid fuchsin in lactophenol (w/v) (31). Leaves were rinsed briefly in lactophenol to remove excess stain, and then stained for 1 to 5 min in fast

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green [0.5% fast green (w/v) in 2-methoxyethanol (methyl cellosolve)–absolute ethanol–clove oil (1:1:1, v/v)]. Stained leaves were blotted gently and then rinsed briefly in lactophenol to remove excess stain. Prepared leaves were mounted on slides under coverslips in a solution of glycerol, distilled water, and Tween-20 (90:9:1, v/v). With this procedure, fungal structures on the leaf surface were stained green or blue-green, but hyphae beaneath the epidermis were stained pink or red. Thus, the position of fungal structures could be discerned without observing cross sections.

Plastic embedding.—Leaf pieces (2 × 5 mm) were fixed in 3% glutaraldehyde in 0.08 M PIPES (piperazine-N-N'-*bis* 2-ethanol sulfonic acid) buffer (27), pH 6.8. They were postfixed in 2% OsO_4, dehydrated in an acetone series, and embedded in Spurr's medium (29). Blocks were sectioned at room temperature with an American Optical freezing microtome. Sections 10- to 20-μm thick were mounted on glass slides, and were observed in oil without coverslips using a Zeiss microscope with bright-field, dark-field, or Nomarsky interference-contrast optics.

Quantification of histological observations.—For estimating the frequency of a particular aspect of pathogen development at intervals after inoculation, whole mounts of intact primary leaves or leaf segments were examined. For this analysis, 100 host-parasite encounter sites (actual or potential penetration sites) from a minimum of five leaves with 15-25 sites per leaf, were examined at each sampling time. Encounter sites were selected randomly. Data from replicated experiments yielded similar results and were pooled.

Terminology.—For quantifying the microscopic development of the pathogen, six developmental stages (Fig. 1) were recognized and defined. *Germination* (A) was defined as the presence of one or more germ tubes (a minimum of 5 μm in length) per spore. *Ingress* (B) occurred when either the stomatal pore or the cuticle of the leaf epidermis was breached. *Cell penetration* (C) occurred when intracellular hyphae were present. *Initial intercellular development* (D) was distinguished by the presence of several intercellular hyphae at encounter sites where extensive necrosis was not prevalent. *Intracellular colonization* (E) was characterized by extensive ramification of hyphae in one or several adjacent epidermal cells. *Advanced intercellular colonization* (F) was the extensive ramification of intercellular hyphae which occurred only in necrotic tissue.

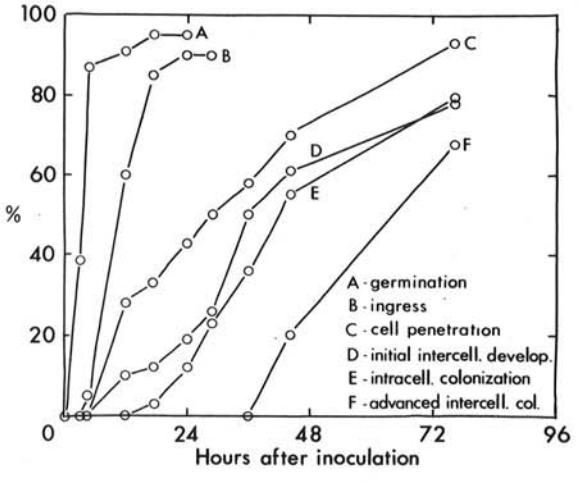


Fig. 1. The chronology of pathogen development in leaves of the sorghum cultivar Grazer inoculated with *Gloeocercospora sorghi*. Each point represents the data from 200 host-parasite encounter sites. Developmental stages (A to F) of the pathogen are: A, germination; B, ingress; C, cell penetration; D, initial intercellular development; E, intracellular colonization; and F, advanced intercellular colonization.

RESULTS

Disease development in leaves differing in HCN potential (HCN-p).—Disease development was observed on leaves of plants differing in HCN-p. The HCN-p ranged from 0 μmoles HCN/g dry wt in the mature leaves of 6- or 10-wk-old plants to 410 μmoles HCN/g dry wt in the primary leaves of seedlings (Table 1).

Symptom development was most rapid on primary leaves of seedlings with high HCN-p. Chlorotic, water-soaked lesions appeared 12 to 18 hr after inoculation; these developed into necrotic lesions between 16 and 24 hr after inoculation which coalesced into characteristically reddish-brown lesions between 48 and 72 hr.

Similar symptoms were observed on the leaves of 6- and 10-wk-old plants with low HCN-p. However, lesions did not appear until 18 to 24 hr after inoculation on mature leaves and between 36 and 48 hr after inoculation on young expanding leaves. There were fewer lesions per unit area on the leaves of older plants, and therefore, lesions did not coalesce as rapidly as on seedlings.

Pathogen development.—The major stages of pathogen development were quantified with time in primary leaves of seedlings (Fig. 1). Selected stages of pathogen development were characterized on the leaves of 6- and 10-wk-old plants. For primary leaves we determined the time after inoculation when each aspect was observed in 50% of the encounter sites. For ease of reference this median time is referred to as the MT_{50}. The MT_{50} values (Table 2) were derived from the data used to construct Fig. 1.

Multicellular, hyaline conidia germinated 4 hr after inoculation by producing one to seven germ tubes (mean 3.0 ± 1.3) of highly variable length (5-250 μm, mean 50 μm) (Fig. 2-A). Germ tubes usually did not branch prior to ingress. If branching did occur ingress usually occurred from each branch.

TABLE 1. Hydrogen cyanide potential (HCN-p) in leaves of the sorghum cultivar Grazer at time of inoculation with *Gloeocercospora sorghi*

Plant age (wk)	Leaf position[a]	Leaf development stage	HCN-p (μmoles/g of leaf dry wt)
1	1	expanding	410
6	3	mature	0
6	7	expanding	12
10	6	mature	0
10	9	expanding	25

[a]Leaf 1 was the first true leaf produced, leaf 3 the third leaf produced, etc. Leaf 3 on a 6-wk-old plant and leaf 6 on a 10-wk-old plant were 4 to 5 wk old.

Ingress was initiated from a single appressorium or from appressorium aggregations. Two kinds of appressoria were observed, "typical" appressoria and protoappressoria. Typical appressoria were swollen, darkly staining penetration structures, separated from the germ tube by a septum (Fig. 2-C, D). Protoappressoria [sensu Emmet and Parberry (12)] were smaller, swollen germ tube apices that produced penetration hyphae, but were not delimited from germ tubes by septa. In our estimates of numbers of appressoria, we did not distinguish typical appressoria from protoappressoria.

Appressorium aggregations consisted of associations of appressoria (Fig. 2-B) which commonly originated from appressoria produced by the germ tubes of many different conidia and not from the branching of a single germ tube [Fig. 3-(A to F, H)]. In the later stages of development, appressorium aggregations sometimes were surrounded by proliferating surface hyphae (Fig. 3-

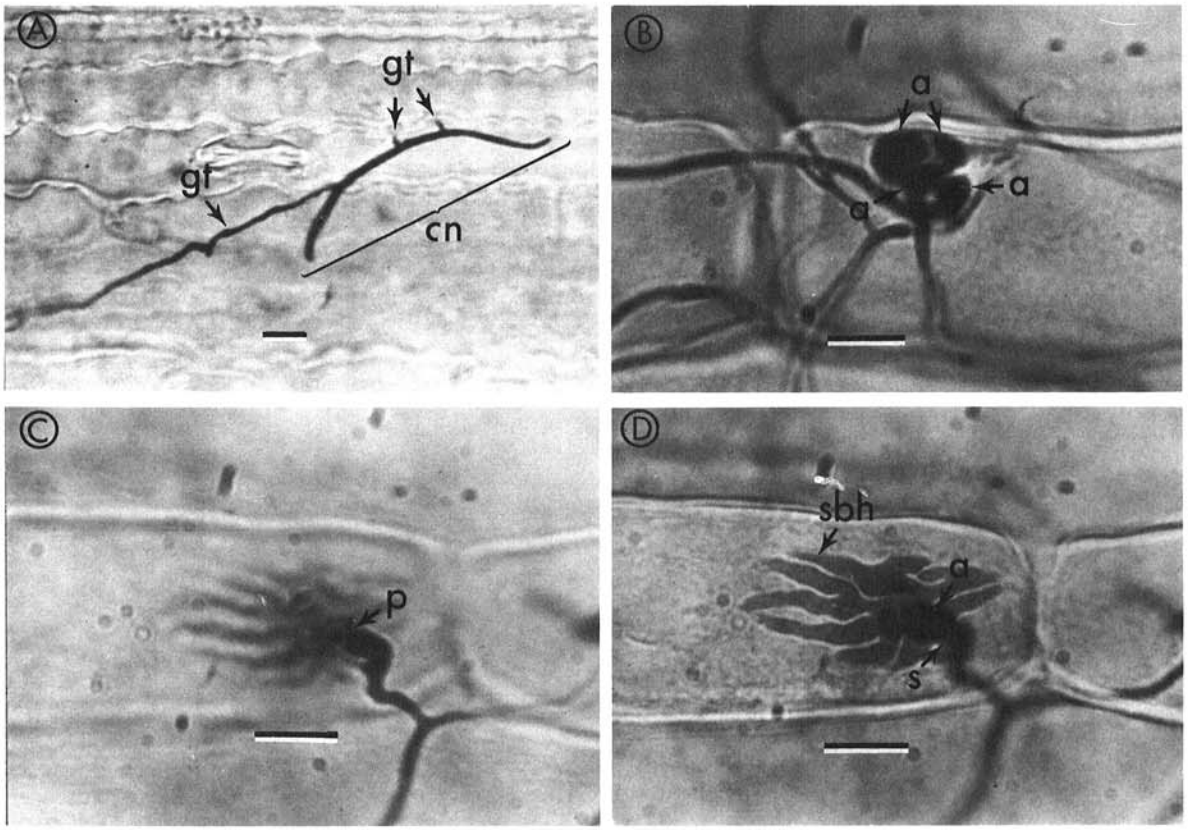
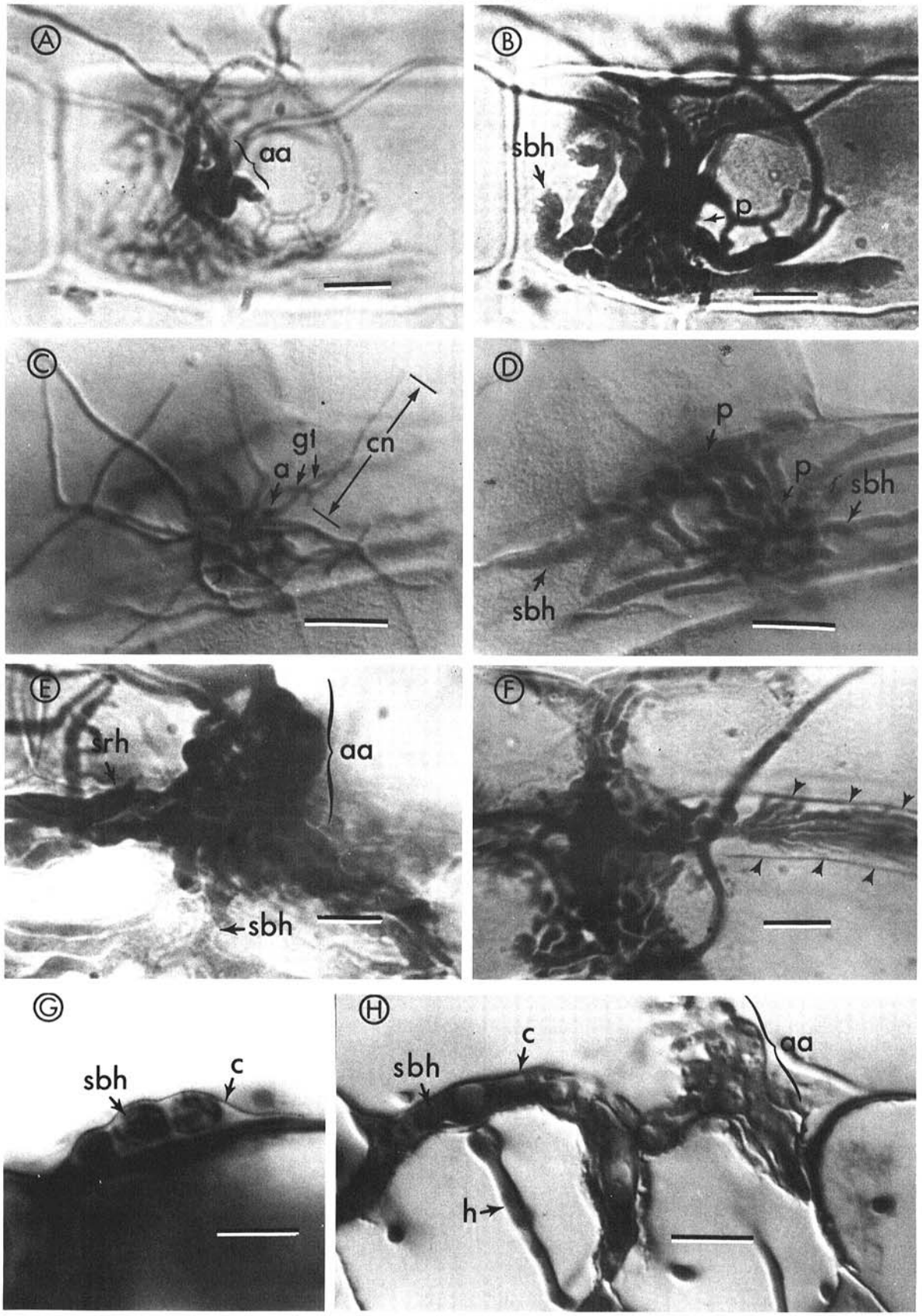


Fig. 2-(A to D). Photomicrographs taken with bright-field optics depicting the development of *Gloeocercospora sorghi* in leaves of sorghum cultivar Grazer prepared by a whole-mount differential staining technique. **A)** A germinating conidium on leaf. **B)** Appressorium aggregation composed of four closely associated appressoria. **C and D)** Views of the same encounter site in two focal planes: C, the encounter site; D, the same site as C. Germ tube delimited by septum, with an appressorium, and finger-shaped subcuticular hyphae. Legend: a = appressorium, cn = conidium, gt = germ tube, p = penetration site, s = septum, and sbh = subcuticular hypha. Scale bars = 10 μm.

Fig. 3-(A to H). Photomicrographs taken with bright-field optics (A, B, E, and F) or Nomarsky interference contrast optics (C, D, G and H) depicting the development of *Gloeocercospora sorghi* in leaves of the sorghum cultivar, Grazer, prepared by a whole-mount differential staining technique (A to F), G and H by plastic embedding. **A and B)** An appressorium aggregation, shown in two focal planes which produced elaborate, branching subcuticular hyphae before penetrating the periclinal wall of a host epidermal cell. **C and D)** Approximately 14 appressoria loosely associated into an appressorium aggregation and shown at two focal planes. Penetration and the production of subcuticular hyphae occurred both over the periclinal epidermal cell wall and at the anticlinal junction of two contiguous walls (D). Granular, epicuticular waxes can be seen over some of the subcuticular hyphae. **E)** A massive dome-shaped appressorium aggregation which, typical of later stages of aggregate development, is covered by proliferating hyphae on the leaf surface. **F)** A penetration site at which growth of subcuticular hyphae has caused a separation of two formerly contiguous anticlinal epidermal walls (arrows). **G)** Cross sections of three subcuticular hyphae. **H)** Cross section of an appressorium aggregation and some subcuticular hyphae, some of which are partly intercellular. Note the intracellular hypha (h) which traversed an epidermal cell. Legend: a = appressorium, aa = appressorium aggregation, c = cuticle, cn = conidium, gt = germ tube, h = hypha, p = penetration site, sbh = subcuticular hypha, srh = surface hypha. Scale bars = 10 μm.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E, H). Each appressorium component produced a penetration hypha in some aggregations. Whether these penetration hypha were produced synchronously or at different times during aggregate formation was not determined.

During the first 18 hr after inoculation, ingress was more frequent from single appressoria than from appressorium aggregations (Fig. 4). Beginning about 20 hr after inoculation, the proportion of single appressoria to appressorium aggregations decreased (Fig. 4). Since ingress and initial cell penetration occurred before 24 hr after inoculation, the many appressorium aggregations formed after 24 hr may not have been important penetration structures.

Initial pathogen ingress from appressoria or appressorium aggregations occurred both by direct penetration of the nonspecialized epidermis, guard cells, or leaf trichomes [Fig. 2-(C, D), 3-(A to F, H), 5-(A, B)] and indirectly through stomates. On all leaves examined, direct penetration was more common (77-90%) than was indirect penetration (Table 3). However, the frequency of penetration of various types of epidermal cells depended on the age of the plant and the age of the leaf (Table 3). In the primary leaves of 1-wk-old seedlings, penetration of the elongate, rectangular, nonspecialized epidermal cells was more common (avg 84) than penetration of leaf trichomes (avg 2), whereas the average frequencies of penetration of the nonspecialized epidermis and leaf trichomes were similar in the leaves of 6- or 10-wk-old plants (Table 3).

Penetration of the cuticle (about 11 hr after inoculation of primary leaves) and growth of the pathogen as subcuticular hyphae preceded entry of the pathogen into the cell lumen. Cuticular penetration occurred most frequently over the anticlinal wall junctions of epidermal cells (75% of invasion sites) (Fig. 3-D, F), and less often over the periclinal cell walls (25% of invasion sites) [Fig. 2-(C, D), 3-(A to D)]. Initial growth of subcuticular hyphae usually was over the anticlinal wall junction. Subcuticular hyphae sometimes were considerably larger than surface hyphae, were cytoplasmically richer, and often developed into extensive, palmate patterns by profuse branching (Fig. 2-D, 3-B, F). These hyphae either remained subcuticular, grew within the epidermal cell wall, or became intercellular (Fig. 3-G, H). Extensive growth of subcuticular hyphae between anticlinal cell walls caused wall separation (Fig. 3-F).

TABLE 2. Time after inoculation of primary leaves of sorghum cultivar Grazer with *Gloeocercospora sorghi*, when each stage of pathogen development occurred at 50% of the encounter sites examined (MT_{50})[a]

Stage of development	(MT_{50}) (hr)[b]
A Germination	4
B Ingress	11
C Cell penetration	30
D Initial intercellular development	36
E Intracellular colonization	41
F Advanced intercellular colonization	65

[a]Two hundred encounter sites were examined at each of nine intervals after inoculation: 3, 5, 12, 18, 24, 30, 36, 45, and 76 hr.

[b]Times were obtained by interpolation of the data plotted in Fig. 1 (Myers, D.F., and W. E. Fry, 1978. Phytopathology 68:1147-1155).

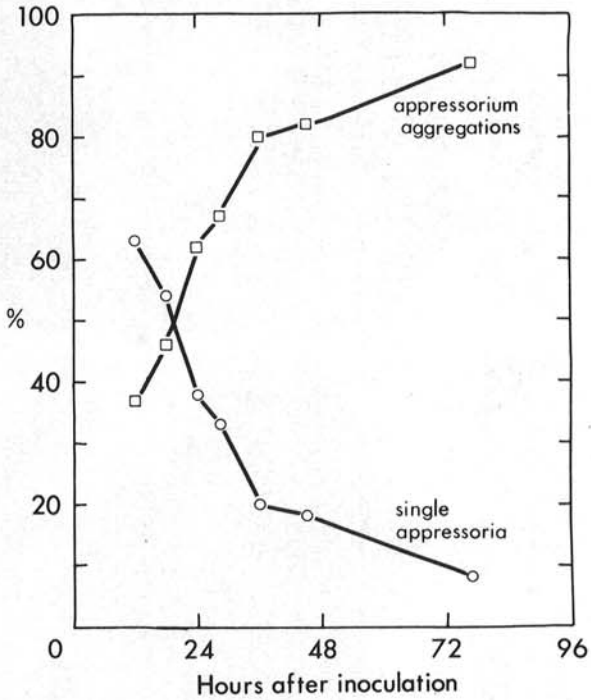
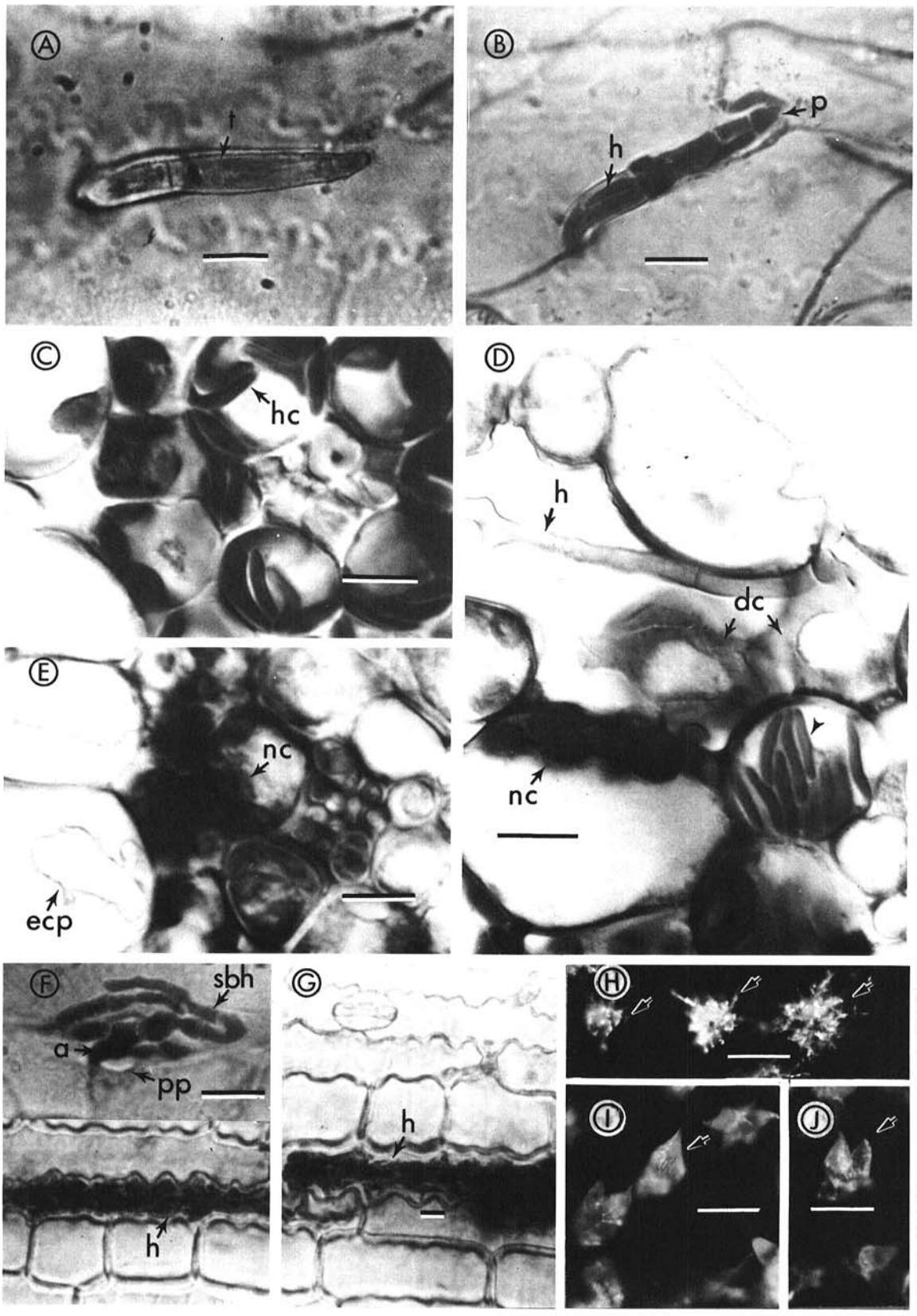


Fig. 4. Changes in the incidence of single appressoria and appressorium aggregations with time on leaves of the sorghum cultivar, Grazer, inoculated with *Gloeocercospora sorghi*. Each determination is based upon observations of 200 encounter sites.

Fig. 5-(A to J). Photomicrographs taken with bright field optics (A, B, F, and G), Nomarsky interference contrast optics (C to E) and dark field optics (H to J) depicting the development of *Gloeocercospora sorghi* in the sorghum cultivar, Grazer. Figures A, B, F and G were prepared by a whole-mount differential staining technique, C to E by plastic embedding, and H to J were from fresh material. **A)** Trichome of a healthy leaf and **B)** Trichome penetrated and colonized by hyphae. **C)** Cross section of a healthy primary leaf depicting typical bundle sheath chloroplasts oriented peripherally. **D)** Cross section of an infected primary leaf 48 hr after inoculation. Some cells are disintegrated near the intercellular hypha in the substomatal cavity. Chloroplasts in a nearby mesophyll cell are necrotic and have diffuse margins, while chloroplasts in an adjacent bundle sheath cell have lost their typical peripheral location (arrow). **E)** Cross section of a primary leaf between 48 and 72 hr after inoculation. Chloroplasts are partly disintegrated and have a granular internal structure. Epidermal cells are plasmolyzed. **F)** Penetration site with a host papilla. **G)** A portion of sorghum epidermis showing epidermal cells completely filled by ramifying intracellular hyphae. **H)** Three sporodochial initials (arrow) on necrotic leaf. **I and J)** Single (I) and multiple (J) cone-shaped sporodochia (arrows) on necrotic leaf. Legend: a = appressorium, dc = disintegrating cell, ecp = epidermal cell plasmalemma, h = hypha, hc = healthy chloroplast, nc = necrotic chloroplast, p = penetration site, pp = papilla, t = trichome. Scale bars in A to G = 10 μm, those in H to J = 100 μm.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Epidermal cell penetration was initiated from subcuticular hyphae at about 30 hr after inoculation of primary leaves of seedlings (Fig. 3-H) and occasionally was accompanied by the formation of host papillae (Fig. 5-F). Intracellular hyphae did not immediately ramify into the subepidermal mesophyll, but generally were restricted to individual epidermal cells until 36 hr after inoculation. Colonies within epidermal cells were located beneath closely associated subcuticular hyphae (Fig. 5-G).

At all host-parasite encounter sites, host cells were affected prior to pathogen colonization. Macroscopically, the effect was manifested as water-soaking, chlorosis, or necrosis of affected tissue at a distance from the pathogen. This effect was characterized by disruption of chloroplasts and cell collapse [Fig. 5 (C to E)].

Necrotic mesophyll was colonized intercellularly between 36 and 48 hr after inoculation. The fungus entered mesophyll tissue from stomata, from subcuticular hyphae between the anticlinal walls of two adjacent epidermal cells, or from intracellular hyphae in colonized, unspecialized epidermal cells or leaf trichomes.

Sporulation in necrotic tissue was similar to an earlier description (5). Hyphae grew from stomata and formed initials which produced sporodochia (Fig. 5-H). Conidia, borne on short conidiophores, were closely packed in salmon-colored, conical-shaped sporodochia often produced in clusters (Fig. 5-I, J).

DISCUSSION

In our study, the development of *G. sorghi* in sorghum differed significantly from that described by other investigators; the fungus produced appressoria, appressorium aggregations, and subcuticular hyphae and gained ingress via stomata or by direct penetration into nonspecialized epidermal cells and leaf trichomes. In contrast, Bain and Edgerton (5) and Dean (7) reported that *G. sorghi* entered leaves only through stomata and produced no appressoria; they also observed no subcuticular hyphae. There are at least two reasons for these differences. First, factors governing pathogen development are not reproduced precisely by different investigators studying the same host-parasite interaction. For example, the presence of nutrients and the degree and periodicity of leaf wetness can affect the formation of appressoria and infection cushions (10, 33). The leaf surface was kept wet in our study, but possibly not in other studies (5, 7). Thus, the formation of appressoria or appressorium aggregations may have been influenced by leaf wetness or the presence of nutrients in guttation fluid. Second, different interpretations of similar results may have been made. Bain and Edgerton (5) indicated that germ tubes were slightly swollen in contact with the stomatal aperture. Similar development was seen at stomatal apertures and over the cuticle of epidermal cells in our study. We interpret these swellings to be proto-appressoria (12), because they directly preceded ingress and were not delimited by septa.

Appressorium aggregation is a term new to the plant pathological literature. Generally, appressoria are classified as simple or compound (12). Emmett and Parberry (12) recognized two types of compound appressoria, lobate appressoria and infection cushions. Some appressorium aggregations superficially resemble infection cushions, but differ from them in mode of development. Infection cushions develop by branching of either several adjacent hyphae or a single hypha (1, 9, 10, 16, 32). In the appressorium aggregation, each appressorium originates from a single hypha, with many conidia thus involved in the formation of a single appressorium aggregation. This origin and mode of development is most clearly observed when the individual appressoria of an aggregation are loosely associated (Fig. 3-C). In the later stages of development, some appressorium aggregations are dome-shaped like some infection cushions, because of the growth of hyphae around the aggregate.

Mangin (17) described the association of numerous appressoria produced by *Leptosphaeria herpotrichoides* de Not. and *Gaeumannomyces graminis* (Sacc.) Arx & Olivier as infection plaques. These structures are similar in appearance to the appressorium aggregations of *G. sorghi*, and the appressoria of *L. herpotrichoides* apparently were produced from many conidia. Since the term plaque is today associated with lysis of bacterial colonies by bacteriophage, we suggest that the term appressorium aggregation be used for the closely associated appressoria produced by *G. sorghi* and *L. herpotrichoides* as well as equivalent compound

TABLE 3. Relative epidermal and stomatal penetrations of sorghum cultivar Grazer leaves by *Gloeocercospora sorghi*[a]

Site	1-wk-old plant	6-wk-old plant		10-wk-old plant	
	Leaf 1	Leaf 3	Leaf 7	Leaf 6	Leaf 9
Epidermis:					
Nonspecialized cell	84 w	47 x	29 yz	40 y	31 z
Leaf trichome	2	27	46	42	52
Guard cell	4	4	2	4	1
(Total)	(90)	(78)	(77)	(86)	(84)
Stomate	10	22	23	14	16

[a]Numbers of observations based on the mean of two experiments consisting of five leaves per experiment and 20 ± 5 determinations per leaf. Penetration patterns of leaf positions followed by the same letter are not significantly different ($P = 0.05$) according to the chi-square test. Superscript letters adjacent to the first number of each vertical column refer to all values in a column.

appressoria which develop from many individual conidia in other species.

Our use of a new whole-mount staining procedure facilitated the distinction of surface hyphae from subcuticular hyphae. Because subcuticular hyphae can be difficult to identify by most whole-mount procedures, it is not surprising that Dean (7) did not observe them in his study of zonate leafspot. Use of our procedure as well as other modified whole-mount procedures (11) should aid the identification of subcuticular hyphae in other host-parasite interactions.

Subcuticular hyphae have been observed in other plant disease interactions (2, 3, 6, 15, 23, 28). The palmate patterns of the subcuticular hyphae of *G. sorghi* were similar to those produced by *Helminthosporium turcicum* Pass. in corn (15). The growth of the subcuticular hyphae of *G. sorghi* over epidermal cell wall junctions and their stromatic appearance also were similar to the subcuticular development of *Rhynchosporium secalis* (Oud.) Davis in barley (3, 14).

Since *G. sorghi* spent a relatively long time period in the subcuticular phase during disease development, subcuticular hyphae may have an important role in pathogenesis. While in the subcuticular phase, the pathogen apparently caused a change in the permeability of both underlying and more distant host cells as evidenced by water soaking and necrosis in these tissues. Ayres and Owen (4) associated resistance of barley to *R. secalis* with inability of the pathogen to establish subcuticular hyphae. In the susceptible interaction, the development of subcuticular hyphae resulted in an increase in the permeability of underlying mesophyll cells followed by the collapse and death of these cells (3, 13).

Gloeocercospora sorghi often penetrated leaf trichomes on older plants. Penetration of trichomes as a major site of fungal ingress is uncommon (18, 25). Mason (18) showed that *Gibberidea heliopsidis* (Schw.) Shear commonly penetrated *Helianthus strumosus* L. leaves through leaf trichomes and remained primarily intracellular. When *G. sorghi* penetrated leaf trichomes, the pathogen remained intracellular initially and did not immediately colonize the underlying mesophyll. Lesions appeared later on leaves of older plants relative to their formation on seedlings. Since pathogen development was slower when trichomes were penetrated, the higher frequency of trichome penetration on older plants may account for the delay in initial lesion appearance on older plants.

One hypothesis regarding the possible role of HCN in plant disease is that HCN production may affect symptom development (20, 22). Our studies do not provide evidence to support this hypothesis. Lesions on primary leaves of seedlings with high HCN-p were similar to those on older leaves with low or nondetectable HCN-p. On primary leaves of seedlings, *G. sorghi* generally is restricted to one or several epidermal cells for the first 24 hr after inoculation. We detected little or no HCN production during this time (24). The majority of HCN production began 36 hr after inoculation (24) and this was associated with initial intercellular development and with intracellular colonization. A plausible explanation is that HCN is produced as result of colonization of host cells and has little effect on *G. sorghi* at this stage of development.

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