Ovarian Infection of Sporobolus poiretii by Bipolaris ravenelii

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Electron micrographs by Ann Bird.
Supported by CSRS Special Grant 177-15-03.
Accepted for publication 21 August 1975.

ABSTRACT

False smut of *Sporobolus poiretii*, caused by the hyphomycetous fungus *Bipolaris (Helminthosporium) ravenelii*, is a local lesion disease in which infection is restricted to ovaries. Germ tubes from airborne conidia form appressoria and penetrate cells of the stigma, style, or ovary. Hyphae of the intracellular mycelium traverse host cell walls without constriction. They invaginate the host plasmalemma and become ensheathed during a brief period in which a compatible relationship exists between fungus and host. Hyphae mass within the host cells, which are soon completely absorbed. The pedicel of the floret remains green, but the ovary is replaced by a sclerotioid, pseudoparenchymatous fungus stroma. Hyphae arising from the stroma emerge between remnants of the palea and lemma and form olivaceous to black mats of long, branched conidiophores. The disease cycle may be completed in 3-4 days. Dew periods of 2-12 hours are sufficient for infection. Discoloration of the ovary is evident in 48 hours. Sporulation may occur within a minimum latent period of 60 hours, if moisture is available during the final 12 hours.

Phytopathology 66:260-268.

Additional key words: disease cycle, fungus-host relationships, replacement diseases.

Local infections of plant ovaries that result in replacement of host tissues by the causal fungus are best known in the ergot diseases of grasses (1, 13) in which the pathogens are Ascomycetes in the genus *Claviceps*. A similar type of infection has been reported among Basidiomycetes in the smut fungi *Melanopsichium eleusinis, Neovossia indica, Tilletia horrida,* and *Tolyposporium penicillariae* (3) and among Deuteromycetes in *Cercospora seminalis,* cause of false smut of *Buchloë dachtyloides* (14). Preliminary observations indicated that infection is similar also in the false smut, or sooty heads, disease of *Sporobolus poiretii* which is caused by the hyphomycete *Bipolaris (Helminthosporium) ravenelii* (Curt.) Shoemaker. *Sporobolus poiretii* is an introduced weed grass common throughout the southeastern United States along roadsides and in lawns, pastures, field borders, and waste land. It is a bunch grass that resembles tall fescue *(Festuca elatior* var. *arundinaceae)* in habit and produces a succession of compact, spike-like panicles from early summer until late fall. The panicle gradually emerges from the sheath of the flag leaf over a period of a week or more, and the florets open and mature progressively from apex to base of the panicle. Infection of the panicles with *B. ravenelii* is so consistent that *S. poiretii* has been given the common name of smut grass. Further studies of false smut of *S. poiretii* were made to secure data for a comparative analysis of local-lesion replacement diseases of ovaries.

MATERIALS AND METHODS.—Diseased panicles of *S. poiretii* were collected in central and southern Georgia and in northern Florida. The fungus was isolated by transferring dry conidia from infected florets to 18% V-8 juice agar plates on the dry tip of a dissecting needle. Host plants transplanted from the field to 20-cm diameter pots in the greenhouse produced vigorous plants with normally developing panicles. In inoculations, suspensions of conidia from culture were applied to run-off with a chromatography sprayer attached to a portable electric compressor. Inoculated plants were placed in a mist chamber for 2-48 hours before they were returned to greenhouse benches. In tests involving smaller plants in 15-cm diameter pots, inoculated plants were put into a sheet metal dew chamber placed inside an incubator in which constant temperatures were maintained.

Microscopic observations were made on squashes and free-hand sections of dissected ovaries and florets mounted in water, which was sometimes replaced with lactophenol-cotton blue. For paraffin sections, florets were fixed in formalin-propionic acid-alcohol, and stained with Heidenhain's hematoxylin. For electron micrographs, ovaries were excised into a drop of fixative on a sheet of dental wax, sliced longitudinally or transversely, and run through the following schedule: fixed in 2% gluteraldehyde-2% acrolein in 0.025 M phosphate buffer for 1.5 hours, postfixed in 2% OsO_4 for 2 hours, left overnight in 1.5% uranyl acetate in 30% ethanol, dehydrated in an ethanol series, and embedded in Spurr's medium. Sections were stained on the grids with lead citrate for 2 minutes.

OBSERVATIONS AND RESULTS.—*The disease.*—The disease was characterized by dense, velvety mats of long, branching conidiophores and conidia covering individual florets. The mats spread and coalesced until large areas of the panicle, or entire panicles, were completely covered (Fig. 1). The color at first was greenish-brown but became dark-olive and finally black. On dead and dying panicles, the mat surface was dull-black and almost carbonaceous. Often, especially late in the season, the mats were partially or completely overgrown by the white mycelium and orange conidia of the hyperparasitic fungus *Trichothecium*

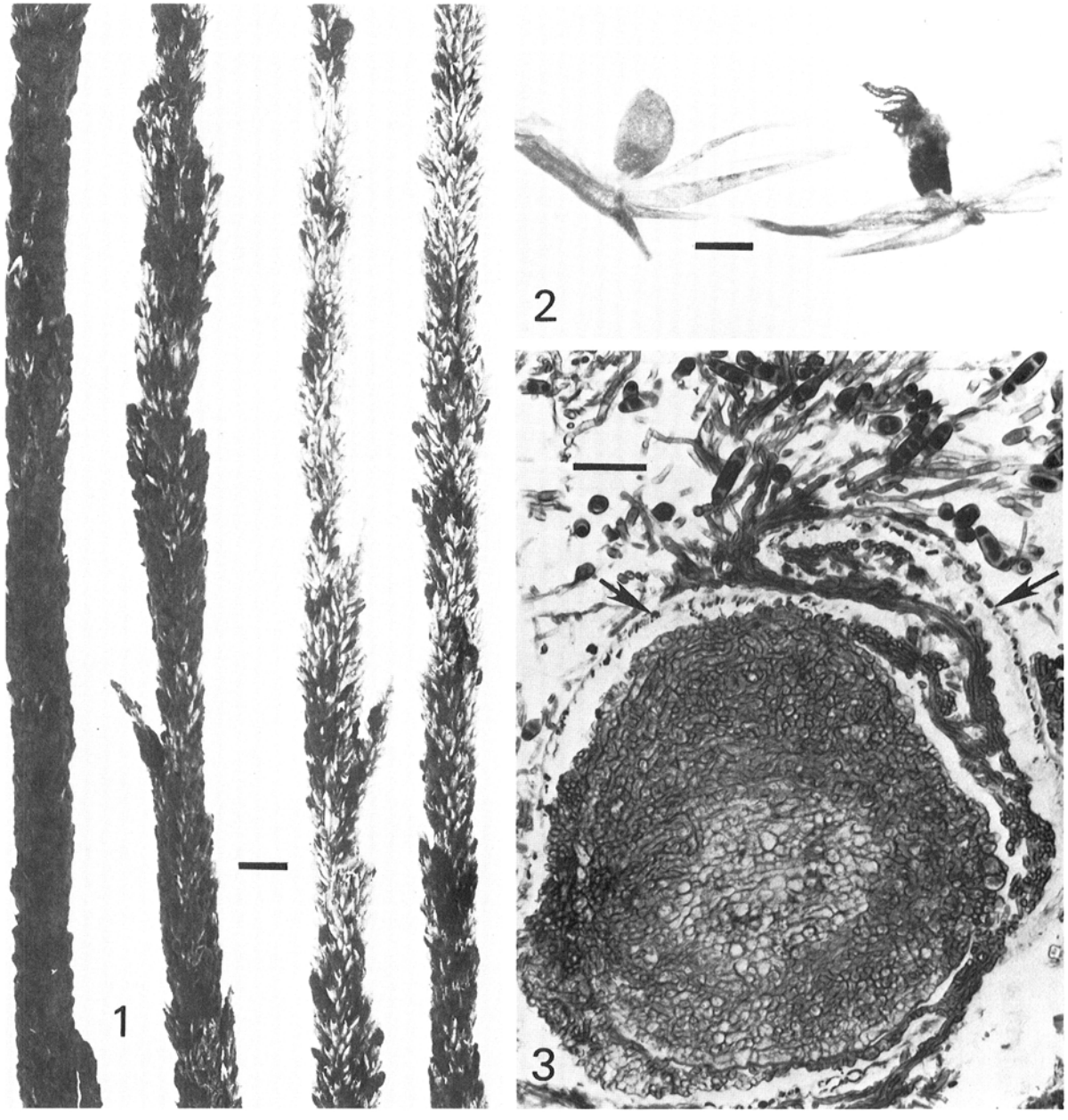


Fig. 1-3. 1) Panicles of *Sporobolus poiretii* infected to varying degrees with *Bipolaris ravenelii*; bar = 0.5 mm. **2)** Healthy ovary (left) and infected ovary (right) 72 hours after inoculation; bar = 0.5 mm. **3)** Cross section through infected floret near apex of ovary showing complete replacement of ovarian tissue by a sclerotioid fungus stroma and conidiophores emerging between remnants of the lemma and palea (arrows), bar = 50 μm.

helminthosporii (Thuem.) Sacc. The ovary was entirely replaced by a brown sclerotioid mass of fungus hyphae enclosed only by the remnants of the outer epidermal cell walls of the lemma and palea, between which columns of conidiophores emerged (Fig. 3, 4). The pedicel, however, remained green. Prior to emergence of conidiophores symptoms of earlier stages of infection could be detected with a hand lens as darkening of ovaries within infected florets. Infected ovaries were shrunken and dark-olivaceous, whereas healthy ovaries were green (Fig. 2). The discoloration started at the apex of the ovary and proceeded to the base, where it halted at the receptacle of the floret.

In dense stands of the grass along roadsides, the disease was so consistently present and involved all panicles so completely throughout the summer as to suggest systemic infection of the type commonly occurring in head smuts of cereals. When, however, completely

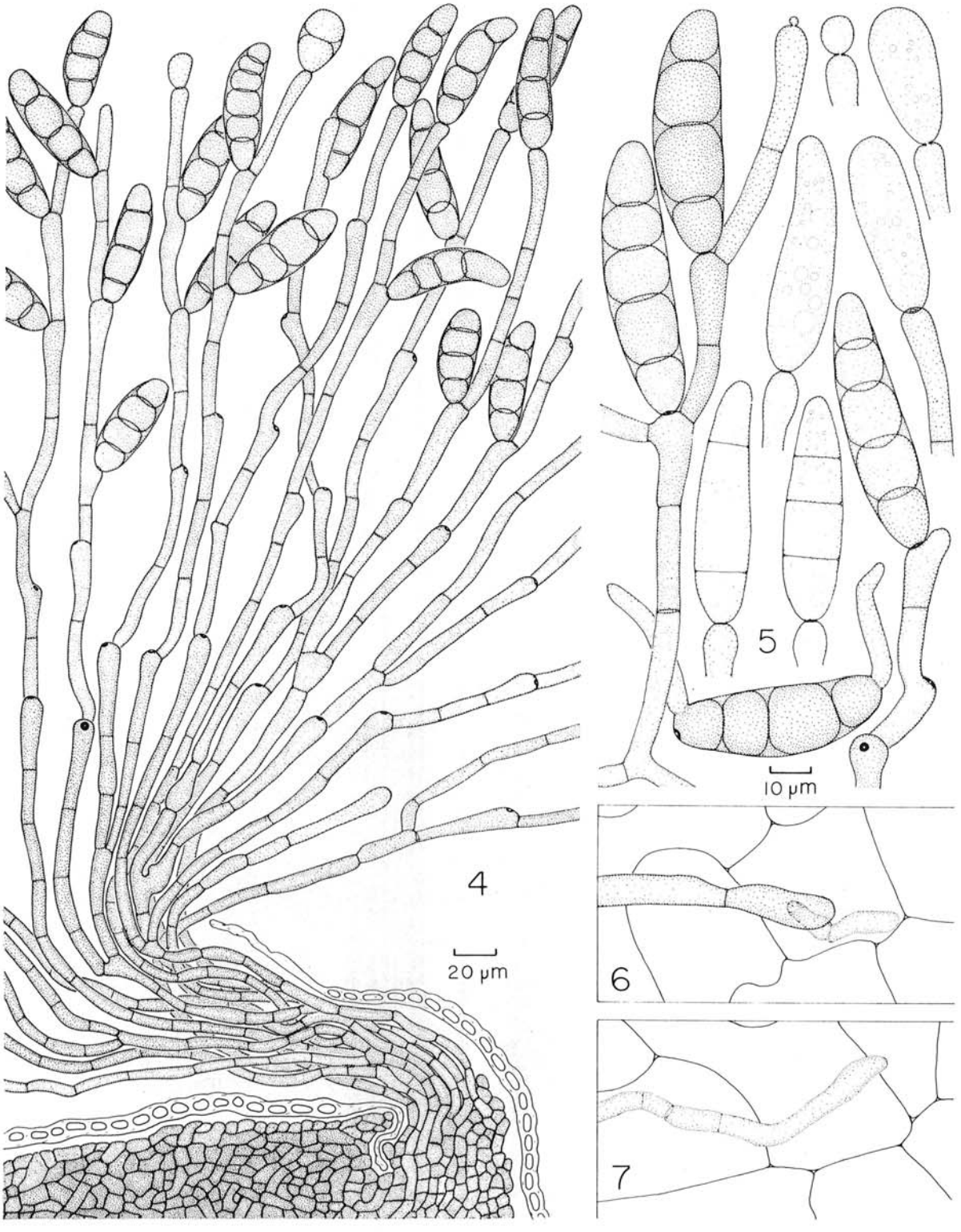


Fig. 4-7. *Bipolaris ravenelii*. **4)** Freehand section of column of conidiophores arising from stroma and emerging between lemma and palea which have been reduced to the outer epidermal cells and cuticle. **5)** Development and germination of conidia..**6)** Appressorium on surface of ovary epidermis sending penetration tube into epidermal cell. **7)** Intracellular hypha penetrating lateral walls of ovarian epidermal cells. All figures from fresh material mounted in water. Figure 4 to the scale in Fig. 4; Fig. 5-7 to the scale in Fig. 5.

infected plants were transplanted to pots in the greenhouse and cut back, all new heads remained free of infection. Only individual florets close to anthesis became infected when panicles and leaves were inoculated. Developing kernels were resistant. Kernels removed from beneath mats overgrowing them from surrounding infected florets were free of the fungus. When panicles that appeared to be completely infected were laid on moist paper in petri dishes, some grains germinated in place and produced healthy seedlings. In more sparse, isolated host populations in waste land, especially in open woods and woods borders, infection was less consistent, and larger quantities of seed were produced.

The fungus.—The inconspicuous, ellipsoidal, black, sclerotioid stromata occupying the space between the lemma and palea measured 0.8 - 1.3 × 0.4 - 0.5 mm. They were composed of angular cells with dark-brown walls that measured 7 - 29 × 7 - 23 μm (Fig. 3). Conidiophores, which were at first hyaline but soon became colored a moderately dark olivaceous brown, arose as outgrowths from the sides and apex of the stroma and emerged in sheets or columns through the juncture of the enclosing lemma and palea (Fig. 4). They were cylindrical, multiseptate, often branched, 5-11 μm in diameter, and 300-500 (-900) μm long. At the points where detached conidia left inconspicuous scars in the form of dark-ringed pores, the conidiophores were slightly nodulose and geniculate. Conidia (Fig. 4-5) were ellipsoid, inequilateral, at first hyaline but soon colored a moderate olivaceous brown, 45-77 × 13-19 μm, and 2- to 5-septate (means of 25-spore samples 56-64 × 15.7 - 17.0 μm and 3.3- to 3.8-septate). In culture, they were shorter and broader, up to 66 μm long and 25 μm wide, and 2- to 6-septate. The inconspicuous flush hilum at the base appeared as a faintly ringed pore. Conidia were pseudopleurogenous, being produced at the tips of successive lateral proliferations. As a result of approximately synchronous growth, at least in the early stages, conidia or conidial scars appeared in successive tiers in the mats of conidiophores (Fig. 4). Conidia originated as ovoid extrusions through apparent pores in the outer wall at the conidiophore apex and approached their mature size and shape before septa appeared (Fig. 5). In a typical 3-septate conidium, the basal and apical septa were laid down at approximately the same time, and the middle septum appeared slightly later. Germ tubes originated from the polar cells but grew as frequently from the sides as from the tips of the cells.

Colonies in culture were similar to the mats produced on infected florets; they were at first pale olivaceous but darkened to brown or nearly black. Growth was relatively slow (2.7 mm/day of linear radial growth on 18% V-8 juice agar at 25 C in darkness), and multiple-point inoculations were made on plates to produce inoculum. Conidium production was severely inhibited by light. Very few conidia developed under constant illumination from daylight fluorescent tubes even at intensities below 1,080 lux (100 ft-c), and fewer developed under alternating light and darkness than in constant darkness.

Matings of isolates in culture on sterilized squares of dead corn leaf on Sach's agar failed to produce ascocarps. No ascocarps were found in field material, and the only apparent source of primary inoculum was conidia on stromata overwintered on dead panicles. Mats of conidiophores on overwintered panicles collected on 10 May, after the first new panicles had appeared, still bore conidia. Many conidia were dead or contained dead cells, and some were occupied by the mycelium of another fungus. Approximately 10% germinated in drops of water on glass slides left overnight in a moist chamber. When end cells were dead, germ tubes arose from the intercalary cells at the septal pores and emerged through the dead end cells. Regeneration of conidiophores and production of new conidia failed to occur when overwintered panicles were placed in moist chambers. Under these conditions, the conidiophore mats of *B. revenelii* were quickly overgrown by colonies of *Cladosporium herbarum* Pers. ex Link.

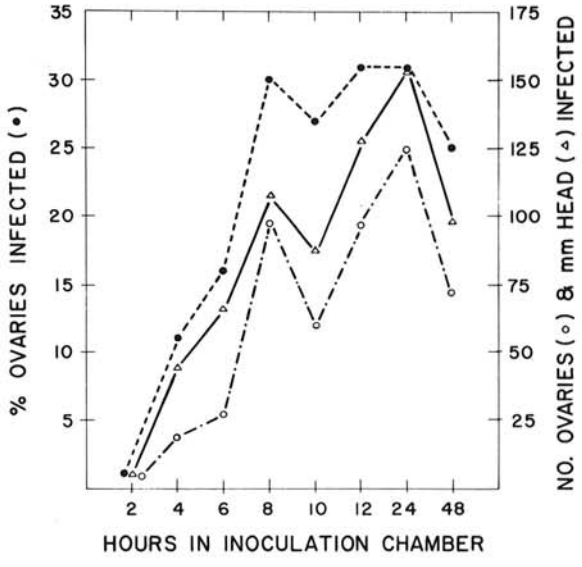


Fig. 8. Ovarian infection of *Sporobolus poiretii* as determined by those producing conidia of *Bipolaris ravenelii* at 76-96 hours on panicles (heads) sprayed with conidia and held in a mist chamber for periods of 2-48 hours following inoculation.

Disease cycle.—Length of the infection period was determined by holding inoculated plants in an inoculation chamber under conditions suitable for germination of conidia and penetration of host tissue for 2-48 hours (Fig. 8). Plants with three to four blooming panicles each in eight pots were sprayed with conidia, placed in a mist chamber at 27-28 C, and removed singly at appropriate intervals. Subsequent sporulation on ovaries of intact plants later returned to the mist chamber, and on cut panicles placed in moist chambers for 48 hours, was the criterion of infection occurring during the varying periods in the inoculation chamber. Results recorded were: length of panicle (head) from uppermost to lowermost florets with infected ovaries, number of ovaries infected in this segment, and percentage of ovaries infected (Fig. 8). A mean of four ovaries per panicle in a mean length of 5 mm of panicle became infected on plants held in the inoculation chamber for 2 hours. Numbers of ovaries infected increased with longer periods in the inoculation chamber to a peak of 125 ovaries per panicle at 24 hours. Increase in length of panicle segments in

which ovaries were infected followed the same course. This factor resulted in flattening of the curve for percent ovaries infected (Fig. 8) after a peak of 30% at 12 hours in the inoculation chamber. With increasing time in the inoculation chamber, infection extended higher into the panicle, where a larger proportion of the ovaries were older and more resistant to infection. Ovarian infection rates as high as 70% were obtained when calculations were based on counts made only in the lower half of the infected segment of the panicle. The sharp drop in all measurements of infection on plants held in the inoculation chamber for 48 hours probably resulted from the use of subsequent sporulation as the index of infection. Long periods in the inoculation chamber, followed closely by an additional 24-hour moist period to induce sporulation, increased the growth of contaminants (chiefly *Cladosporium herbarum*) that suppressed sporulation of the pathogen.

In tests to determine the incubation and latent periods and the moisture requirements of the disease cycle, inoculated plants were placed in a dew chamber at 25 C for 12 hours and then removed to dry conditions. Immediately after removal from the dew chamber and at 12-hour intervals thereafter up to 84 hours, sample panicles were cut and placed in petri-dish moist chambers (Fig. 9). Regardless of moisture conditions following the initial 12-hour dew period, the first symptoms of infection on ovaries appeared 48 hours after inoculation. Areas of shrunken, olivaceous tissue appeared at the apex and extended downward over as much as 25% of the ovary. At 60 hours, this discoloration extended to the base of many ovaries (Fig. 2), although others showed only initial symptoms. With an immediately preceding moist period of as much as 12 hours, the first mature conidia were present 60 hours after inoculation; even ovaries discolored only in the apical region produced conidia. Dry periods of 12-36 hours intervening between the initial and final 12-hour moist periods failed to delay sporulation. In fact, intervening dry periods of 24-36 hours increased sporulation (Fig. 9, treatments 3, 4). With continuous moisture (Fig. 9, treatment 1), the panicles were so overgrown by contaminants that conidia of the pathogen could not be detected under the dissecting scope; this was true also of some panicles exposed to only a 12-hour intervening dry period (Fig. 9, treatment 2). At intervals of 60 hours or longer (Fig. 9, treatments 5-8), following dry periods of 48 hours or more, sporulation occurred within a subsequent moist period of 12 hours. After 84 and 96 hours (72 and 84 hours dry), sporulation was induced by 7 hours of moisture (Fig. 9, treatments 7, 8). On panicles held dry in the greenhouse for 4 weeks following removal from the dew chamber, the black stromata produced conidiophores in 4 hours and the first conidia in 7 hours after being placed in moist chambers. Conidia were rated "mature" when they could be distinguished by color under the dissecting scope. At this time they were three-septate and full-sized, but only pale yellow as opposed to the darker brown of fully matured conidia. Germinability was not a criterion since obviously immature conidia were easily detachable and capable of germination.

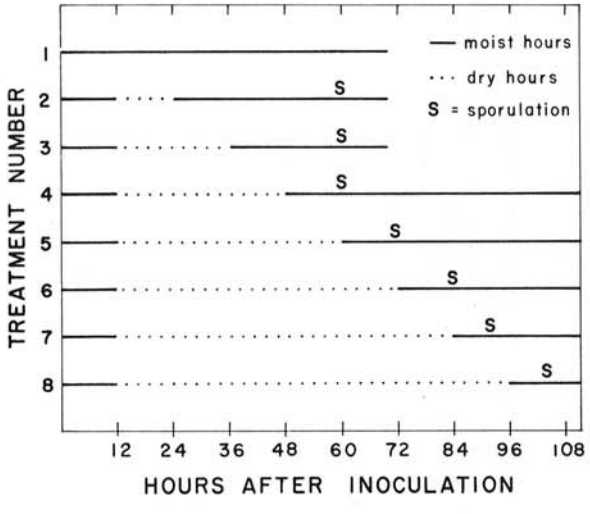
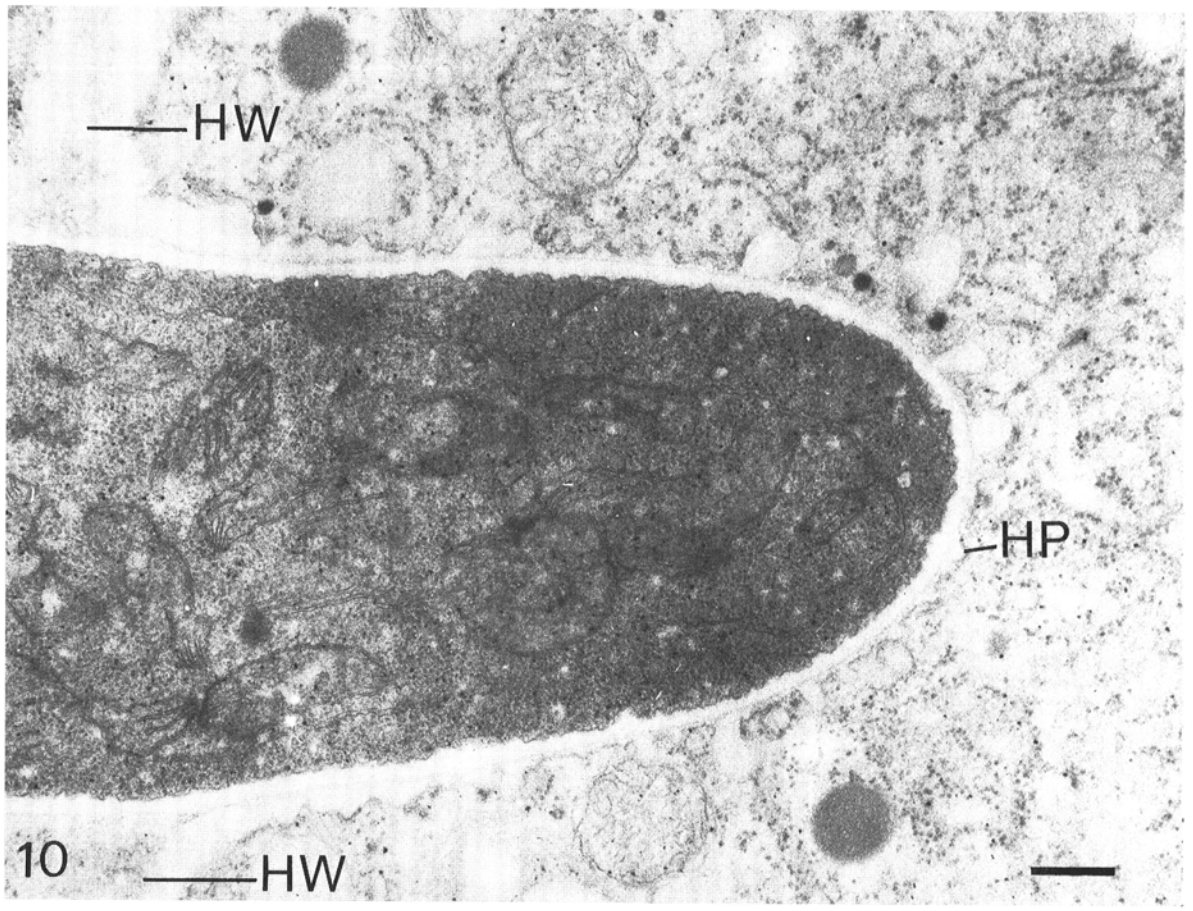


Fig. 9. Time from inoculation to sporulation of *Bipolaris ravenelii* on ovaries of *Sporobolus poiretii* with a constant 12-hour dew period after inoculation followed by 0- to 84-hour dry periods before panicles were cut and placed in moist chambers.

To determine the length of the infectious period, emerging panicles with apical florets already infected and producing conidia were tagged on five potted plants placed out of doors. After 2 weeks, the initially infected florets were still producing conidia abundantly, and infection had progressed to the base of the panicle, where a few florets were still blooming. Sporulation slowly declined; but after 5 weeks, traces were still evident on the panicle tips that remained alive, and sporulation was abundant on the lower portions. Sporulation continued as long as the branches of the panicle remained green and, presumably, functional. Death of the panicle was not obviously hastened by infection.

Host relationships.—Conidia sprayed onto florets lodged in the branches of the stigma and germinated. The germ tubes, after attaining various lengths, and often after repeated and apparently random contacts with stigmatic cells, formed terminal appressoria and penetrated directly into the host cells. Some germ tubes grew as far as the apex of the ovary before forming appressoria and penetrating the epidermal cells (Fig. 6). Growth within the host was intracellular, and the hyphae penetrated the host cell walls without constriction (Fig. 7). Invading hyphae entered the apex of the ovary within 10 hours after inoculation. Penetration of the epidermal cells of the ovary, and of the apparently more delicate cells of the ovule, was by lateral branches arising behind the irregular front of hyphae growing downward through the ovary wall. Cells of the ovule were more resistant to invasion and remained intact after the ovary wall was largely destroyed (Fig. 14). Even after these cells disintegrated, the space formerly occupied by the ovule was only loosely filled by fungus hyphae. Progress of colonizing hyphae in the ovarian wall stopped at the base of the ovary. Cells in the stalk of the ovary were invaded, but not destroyed; the receptacle of the floret was not invaded (Fig. 14).

Hyphal tips passed directly through host cell walls and pushed back the plasmalemma of the invaded cell (Fig. 10). The host-cell nuclei, mitochondria, and endoplasmic



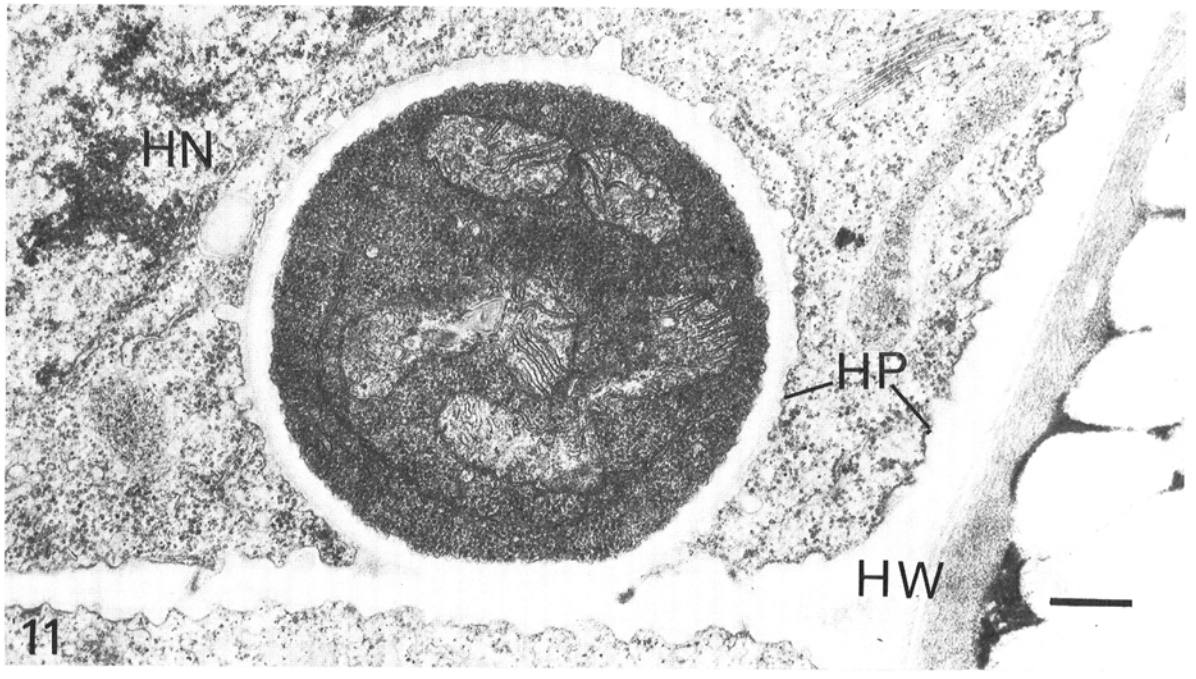


Fig. 10-11. Intracellular hyphae of *Bipolaris ravenelii* in *Sporobolus* ovarian wall tissue. **10)** Hyphal tip penetrating host wall (HW) and pushing back host plasmalemma (HP); bar = 0.5 μm. **11)** Cross section of hypha lying between host cell wall and invaginated host plasmalemma (HP) which is depositing sheath against the fungus cell wall; host nucleus (HN) with intact membrane and cytoplasm nearly normal; bar = 0.5 μm.

reticulum remained intact (Fig. 10, 11). A sheath was deposited between the invading hypha and the invaginated and irregularly lobed host plasmalemma (Fig. 10-12). In some inner cells of the ovary wall, an encasement of material, similar to that forming the secondary walls of host cells, surrounded the fungus hyphae outside of the sheath (Fig. 13). The encasement was apparent around hyphal segments that occupied the center of host cells, as well as around those lying against host cell walls. In adjacent cells of the ovule, and in the outer cells of the ovary wall, the encasement was lacking. This was true even in host cells in which disintegration of the protoplast indicated an advanced stage of infection. Host protoplasts were quickly absorbed as the hyphae proliferated within the cells and became compacted into a pseudo-parenchyma (Fig. 14). Host cell walls then were digested, and no trace of ovarian tissue remained (Fig. 3). Even with death of the protoplast, few histological symptoms of necrosis were apparent. The discoloration at the apex of the ovary, which was the first external symptom of infection, resulted from browning of the hyphae massed within the cells rather than from discoloration of the host tissue.

Although growth within the ovary was intracellular, free hyphae on the exterior were to some extent involved in formation of the stroma. Under moist conditions, hyphae from the discolored tips of ovaries in early stages of infection, in addition to producing conidiophores, also grew rapidly downward over the surface of the ovary. Thus, a hyphal mantle sometimes covered the surface of basal epidermal cells before they were invaded by the internal mycelium.

DISCUSSION.—The consistent infection of panicles of *Sporobolus poiretii* in dense stands by false smut, which has been demonstrated to be a local lesion disease, may be explained by the low moisture requirements of the disease cycle, the short latent period, the long infectious period, and the nature of the host. Under field conditions that provide rain at appropriate intervals, the experimentally determined minimum latent period could result in a 3-day disease cycle. With moisture limited to nighttime dews, the disease cycle would be extended to 4 days. A dew period of 2-12 hours on the first night would be sufficient for infection to occur. Conidia could be produced within a single 12-hour dew period on the third night, and new infections could be established on the fourth night. Although the dispersal period has not been considered, it seems improbable that this would extend the minimum length of the disease cycle for within-plant spread beyond 4 days. Individual infected florets continue to produce conidia for at least a month, and the infectious period for single panicles is lengthened by the progressive basipetal opening of the florets and the consequent progression of infections down the panicle. This, together with the continual production of inflorescences throughout the season, allows the development of overwhelming volumes of inoculum.

Although no data have been obtained on the effect of the disease on individual plants, the damage may well be limited to a diversion of normal reproductive energy from production of seed to production of conidia of the pathogen. Infected plants remain vegetatively vigorous. In established stands, the grass apparently is able to sustain the severe losses in seed production because it is perennial. In sparse stands, and on isolated plants, where seed production is more important, the disease develops later and less consistently, and more seed are produced.

The disease cycle in Sporobolus false smut is essentially like that in the ergot diseases (1, 13), in the localized ovarian infections caused by certain smut fungi (3), and in Buchloë false smut (14) in that infection is restricted to the ovaries, which are destroyed and replaced by fungus stromata or masses of teliospores. A further similarity between Sporobolus false smut, Buchloë false smut, and ergot is the destruction and replacement of the ovary at so early a stage of development as to make it improbable that production of quantities of conidia or massive sclerotia could derive from the relatively small amount of tissue in the young ovary at the time it is consumed. Development of the fungus must depend on a continued flow of nutrients from the host into the fungus stroma. Certainly, in both Sporobolus false smut and in ergot, premature death of these host tissues prevents further development of the fungus. Moreover, anomalous development of seeds at the apex of ergot sclerotia (9) could be interpreted as indicating a flow of nutrients, not only from the pedicel into the developing sclerotium, but also across the fungus tissue into the developing seed.

Despite the similarities in the disease cycles, Sporobolus false smut differs from the ergot and smut diseases and from Buchloë false smut in that the mycelium of the pathogen is intracellular. In its relationships with host cells, *Bipolaris ravenelii* is comparable to *Phyllachora lespedezae* on *Lespedeza striata* (7) and to *Phacidium curtisii* on *Ilex opaca* (8). *Phyllachora lespedezae* and *Phac. curtisii* penetrate the living mesophyll cells of the host leaf, causing no apparent damage other than a reduction in number of chloroplasts (7) and a consequent mild chlorosis. The intracellular mycelium grows in a compatible relationship with the host for weeks or months. Host tissue is destroyed only in restricted areas where it is replaced by stromata or ascocarps of the fungus. All host tissue in these areas is completely digested and absorbed as it is replaced. The tar spot symptoms that characterize infection by both of these fungi result from darkening of the fungus replacement tissues rather than from discoloration of host tissue. These fungi are remarkable in their apparent ability to produce enzymes capable of digesting all host cell components, including cell walls, and to apply these enzymes selectively to their target cells, leaving immediately adjacent cells in the same tissue unaffected. Continued development of stromata or ascocarps, in fact, depends upon survival of the surrounding host tissue. Although events in the process of infection of *Sporobolus* ovarian tissues by *B. ravenelii* must be measured in hours rather than weeks or months as in *Phy. lespedezae* and *Phac. curtissi,* the sequence is the same: penetration of host cells; establishment, however briefly, of a compatible relationship with the host cells; and selective absorption and replacement of host tissue. Furthermore, *B. ravenelii* likewise appears to be dependent, after formation of the stroma, on a continuing compatible relationship with adjacent living host cells, the cells of the receptacle.

Invagination of the host plasmalemma by penetrating hyphae and reaction of the host resulting in formation of a sheath (2), as in infection of *Sporobolus* by *B. ravenelii*, might be anticipated in intracellular infections by fungi

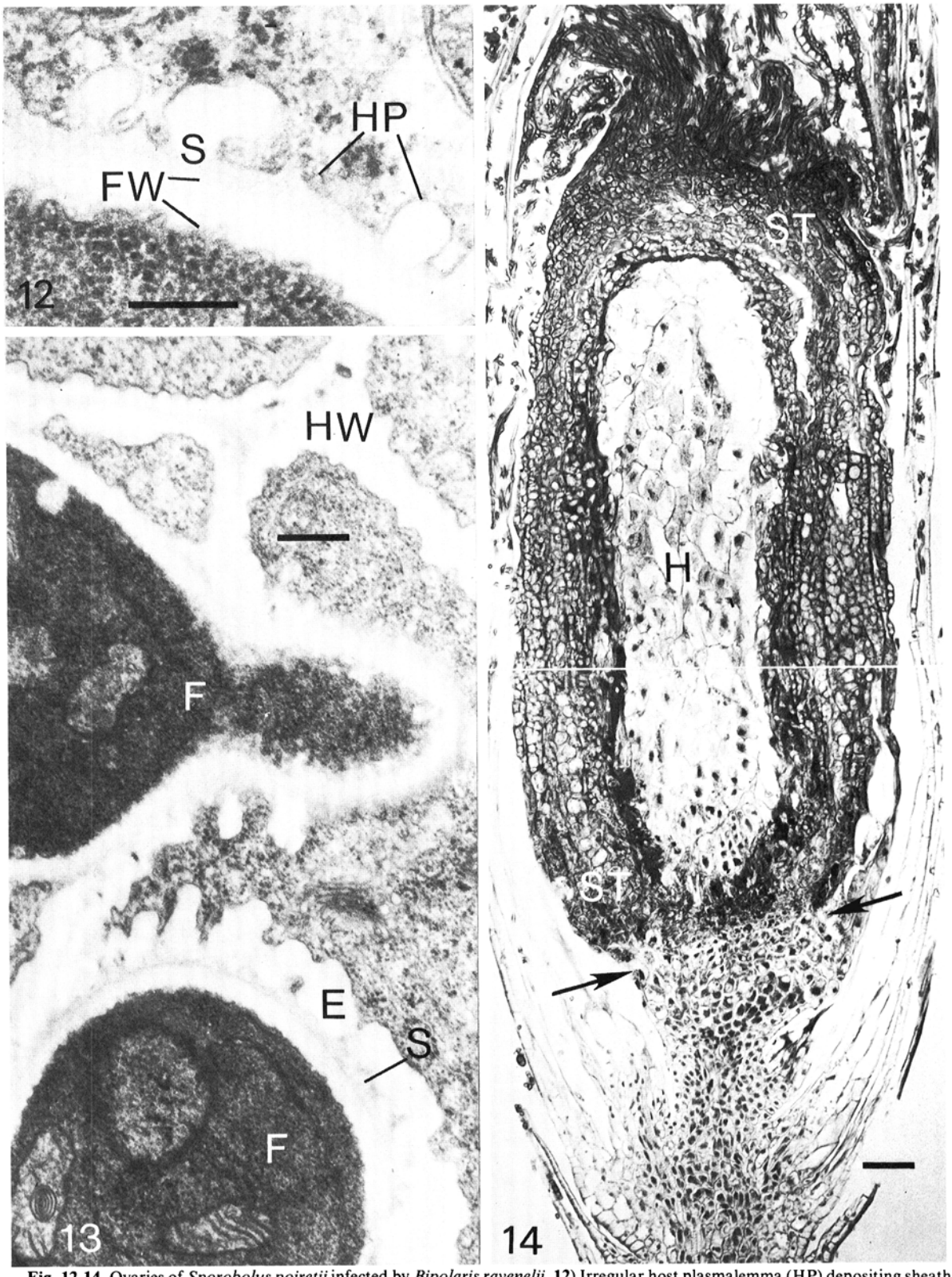


Fig. 12-14. Ovaries of *Sporobolus poiretii* infected by *Bipolaris ravenelii*. **12)** Irregular host plasmalemma (HP) depositing sheath (S) against fungus cell wall (FW); bar = 0.5 μm. **13)** Thick encasement (E) and sheath (S) surrounding intracellular hyphae (F); bar = 0.5 μm. **14)** Longitudinal section of infected floret showing lemma, palea, and bracts enclosing an ovary wall largely replaced by fungus stroma (ST) producing conidiophores at apex, ovule (H) not yet completely disintegrated, and base of ovary (below arrows) and receptacle uninvaded; bar = 50 μm.

that do not cause immediate death of the host cells. This process is similar to haustorium development in mildews and rusts (4); it has been demonstrated previously for the intracellular mycelium of *Gibberidea (Leptosphaeria) heliopsidis* in *Helianthus strumosus* (10), the limited intracellular hyphae of the monokaryotic mycelium of *Puccinia sorghi* in *Oxalis corniculata* (12), and hyphae of various smuts in cells of grasses (4). The irregular lobing of the host membrane surrounding the sheath was attributed by Fullerton (4) to fusion of vesicles with the invaginated plasmalemma. Formation of an encasement around the invading hyphae has been reported also in smut infections; but, according to Fullerton (4), the encasement material is deposited in the sheath, rather than as a distinct layer surrounding the sheath as in *Sporobolus* cells infected by *B. ravenelii*.

The penetration of host cell walls by broad, unconstricted hyphal tips demonstrated in invasion of ovarian cells of *S. poiretii* by *B. ravenelii* has been described also in invasion of thallus cells of the alga *Porphyra perforata* by *Pythium marinum* (6), of *Oxalis* mesophyll cells by *Puccinia sorghi* (12), and of tissues of grass ovaries and nodes by smuts (4). Type of penetration may reflect only the thickness or composition of the host cell walls. Formation of penetration pegs by fungi that kill the host protoplast in advance of, or at the time of, penetration (5, 11) occurs also in fungi that enter a compatible relationship with the invaded cells, as in *Gibberidea heliopsidis* (10) and *Phyllachora lespedezae* (7).

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