Ascospore Formation and Discharge by Calonectria crotalariae

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Journal Series No. 4453 of the North Carolina Agricultural Experiment Station, Raleigh.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

The authors wish to thank S. A. Johnston for technical assistance and L. F. Grand and J. E. Menge for advice during the course of this study.

Accepted for publication 16 October 1974.

ABSTRACT

Development and ejection of ascospores from perithecia of *Calonectria crotalariae* occurred between 20-30 C with an optimum at ca. 25 C. Ascospore ejection was stimulated by a reduction in relative humidity, but was not affected by raising the humidity. Perithecia were capable of a second discharge 4-6 hours after being exhausted of mature ascospores. Forcibly-ejected ascospores were highly sensitive to desiccation. Perithecial ontogeny and ascospore discharge mechanisms are described.

Phytopathology 65:393-398

Additional key words: Arachis hypogaea, Cylindrocladium crotalariae, Cylindrocladium black rot.

Cylindrocladium black rot (CBR), caused by *Cylindrocladium crotalariae* (Loos) Bell & Sobers, is a relatively new disease of peanuts *(Arachis hypogaea* L.) and other legumes in the southeastern United States (1, 4, 13). The fungus causes a severe rot of all subterranean tissues, and poses an annually increasing threat to the peanut industry (3, 13, 14).

Perithecia of the perfect state, *Calonectria crotalariae* (Loos) Bell & Sobers, form abundantly on infected stems and hypocotyls at the soil line, if sufficient moisture is present (1, 4, 13) (Fig. 1). In North Carolina, perithecia have been observed in the field as early as mid-June, and are usually present until harvest in October, with peak numbers in August and early September (13, 14).

The role of the perfect state in CBR epidemiology has not been determined. Ascospores can be seen in the field oozing from 2-3-week-old perithecia in a cream to bright yellow, viscous droplet that clings to the tip of each perithecium (13, 16) (Fig. 1). Spores discharged in this manner are presumably dispersed by rain-splash. Linderman (9) recently reported forcible ejection of ascospores by *C. crotalariae* and related species, and postulated that ascospores may also be dispersed by air currents. Infectivity of ascospores of various species of *Calonectria* has been demonstrated (9, 12, 15, 16).

The purpose of this study was (i) to investigate the formation and discharge of *C. crotalariae* ascospores, including temperature and humidity effects, and (ii) to assess the importance of ascospore inoculum in the development of this disease.

MATERIALS AND METHODS.—*Perithecial production.*—Peanut stems were collected from the field at harvest, air dried, cut into 4-cm sections, fumigated with propylene oxide for 48 hours and stored over $CaCl_2$ in sterile desiccator jars at ca. 25 C. Stems were soaked for ca. 15 minutes in sterile distilled water before use, and floated on warm water-agar plates just prior to solidification. A 5-mm-diam plug taken from a 8- to 12-day-old, potato-dextrose agar (PDA) plate of *C. crotalariae* was placed at the center of each stem. Cultures were incubated at ca. 25 C under continuous cool-white, fluorescent light at 110-150 hlx. Mature perithecia formed abundantly on the stems in ca. 2 weeks.

Inoculation techniques.—Mature perithecia with oozing ascospores were collected from the field in early September. Ascospores were washed from the perithecia with sterile distilled water, filtered through six layers of cheesecloth, and the concentration adjusted to ca. 6,500 spores/ml of solution. Seven-week-old peanut plants (cultivar Florigiant) grown in methyl-bromide-fumigated soil in 10-cm-diam pots were inoculated by pipetting 1 ml of the suspension into the soil at the base of each plant. Plants were covered with plastic bags for 24 hours, and then incubated in a greenhouse for 3 months at 24-30 C.

Field-grown peanuts (cultivar Florigiant) were inoculated beginning in mid-June, ca. 6 weeks after planting, and at 2-week intervals thereafter until mid-September. A 1-cm-diam plug taken from an 8- to 12-day-old, PDA plate of *C. crotalariae* was placed next to the tap root of each plant 3-4 cm below the soil surface.

Ascospore discharge.—Microscope slides covered with a film of petroleum jelly were suspended at distances from 0.5 to 10 cm above perithecia grown on peanut-stem cultures to trap ejected ascospores. Trap slides were left in place for 24 hours. Three drops of lactophenol-cotton-blue stain and a 50 × 22-mm cover slip were then placed on each slide, and clusters of dark-blue-stained ascospores were counted.

To study the effects of temperature on perithecial formation, inoculated peanut-stem cultures were incubated for 2 days at ca. 25 C and then placed in a series of incubators set at two-degree intervals from 12-30 C. The number of perithecia formed per cm of stem was calculated after 3 weeks. Mature perithecia, formed in similar cultures incubated at ca. 25 C for 2 weeks, were used to determine the effects of temperature on forcible ejection of ascospores. Cultures with mature perithecia were placed in a series of incubators set at two-degree intervals ranging from 12-36 C and allowed to equilibrate for 24 hours. Trap slides were then put in place and changed daily for 1 week.

The effect of humidity on forcible ejection of

ascospores was measured using a special spore trap (Fig. 2). Perithecia were grown in 0.473-liter (1-pint), wide-mouth, Mason jars which, when attached to the spore trap, served as Chamber A. Either saturated (ca. 100% RH) or dehumidified (ca. 65% RH) air at 27 C was circulated through the apparatus (see path of dashed arrows). Ejected ascospores were transported in the air stream and impacted on greased microscope slides (C) in chamber B suspended ca. 1 mm above the orifice of the connecting tube (D). Six ascospore samples could be taken without disturbing the apparatus by rotating the circular lid (E) of chamber B and moving the attached greased slides (C) to a new position over the orifice of tube D. Relative humidity in the Mason jar was constantly monitored by an electric hygrometer (Hygrodynamics, Inc., Silver Spring, Maryland) with a narrow-range Hygrosensor (F) mounted in the chamber. When incoming air was switched from saturated to dehumidified air, the humidity in chamber A dropped to ca. 70% RH in ca. 15 min (Fig. 4). When switched from dehumidified to saturated air, the humidity increased to ca. 100% RH in ca. 8 min.

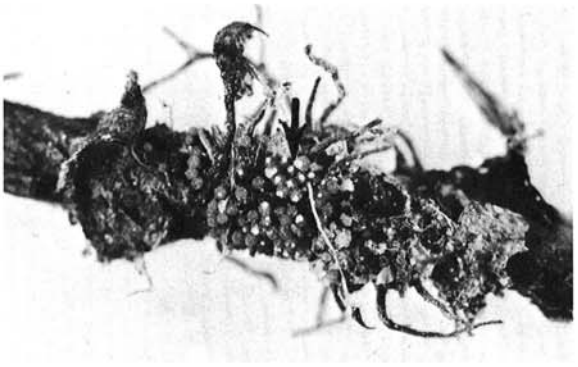


Fig. 1. *Calonectria crotalariae* perithecia with oozing ascospore discharge (arrow) on an infected, field-grown, peanut stem (× 3).

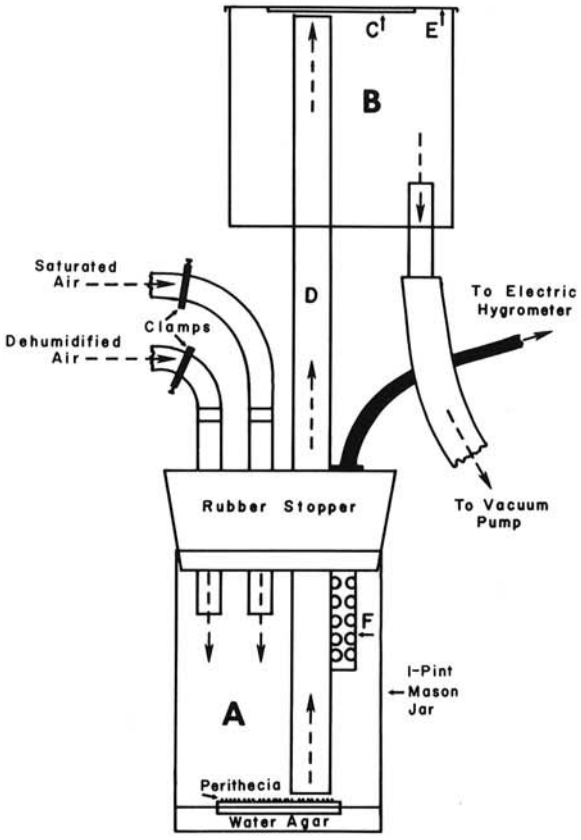


Fig. 2. Spore trap designed to measure the effects of fluctuating humidity on forcible ejection of ascospores from perithecia of *Calonectria crotalariae*. Humidity changes in chamber A were constantly monitored by a Hygrosensor (F). Ascospores discharged from perithecia were carried in the airstream (dashed arrows), up the connecting tube (D) and impacted on greased microscope slides (C) in chamber B. Rotation of circular lid (E) enabled six spore samples to be taken without disturbing the apparatus.

The sensitivity of forcibly-ejected ascospores to desiccation was measured by collecting spores on ungreased slides in the previously described spore trap. Ascospores were then incubated for intervals from 1 to 60 minutes at ca. 73% RH and 33 C, simulating day-time field conditions in North Carolina during July and August. Three drops of sterile distilled water and a cover slip were added to each slide after the desiccation period. Control spores were not desiccated. Percent germination was calculated after 4 hours of incubation at 30-33 C.

Histology.—One-cm-long peanut-stem sections with attached perithecia were collected from peanut-stem cultures at 2-day intervals beginning 5 days after inoculation. Tissues were fixed in a solution of formalin:alcohol:acetic acid:water (2:10:1:7, v/v) (FAA), aspirated for 20 minutes in a disiccator jar and stored overnight under a partial vacuum. Specimens were dehydrated with a tertiary-butyl-alcohol series and embedded in TissuePrep (m.p. 56.5 C, Fisher Scientific Co., Pittsburgh, Pa.). Sections 12-μm thick were cut with a rotary microtome. Preparations were mounted with Haupt's adhesive and 4% formalin, and stained with Triarch Quadruple stain (Triarch, Inc., Ripon, Wisc.).

RESULTS.—Peanut plants inoculated basally with suspensions of *C. crotalariae* ascospores showed typical CBR symptoms after 8 weeks, and ca. 25% were dead after 12 weeks. At that time, perithecia were visible at the bases of the more severely diseased plants.

Ascospores of *C. crotalariae* were forcibly ejected from perithecia prior to being exuded in a viscous ooze. Ascospores were routinely captured in large numbers in still air on greased microscope slides suspended 5 mm above the perithecia, although a few were caught as high as 2 cm. These distances are similar to the range for most pyrenomycetous fungi that forcibly discharge ascospores (6, 7).

Development and ejection of ascospores occurred readily between 20-30 C and optimally at ca. 25 C (Fig. 3). This thermal response correlates closely with that for vegetative growth (Fig. 3) and disease development (2). Light was not required for perithecial formation (4), but continuous exposure to cool-white, fluorescent light resulted in quicker and more abundant perithecial development.

Linderman (9) reported forcible ascospore ejection by *C. crotalariae* at humidities ranging from 16.9-100% RH, but indicated that fewer spores were discharged at 100% RH. Most of the studies reported here were done in covered petri dishes containing agar where the air was saturated as evidenced by condensation. In all cases, large numbers of ascospores were forcibly ejected in 24 hours.

During the petri dish studies, it was noted that a sudden reduction in humidity stimulated ascospore ejection. Using the spore trap illustrated in Fig. 2, it was found that reducing the relative humidity from 100% to 65% triggered massive discharge of ascospores (Fig. 4). Discharge began immediately and proceeded rapidly by the time the humidity was lowered to 90%. Most ascospores were discharged within 15-20 minutes and perithecia were exhausted within 1 hour (Fig. 4). The experiment was routinely repeated with similar results.

Raising the humidity did not trigger ascospore ejection. Mason-jar cultures incubated overnight in desiccator jars at 90% RH failed to discharge when the humidity was raised and held at 100% for 1 hour. Subsequent dehumidification, however, did trigger ejection. Perithecia exposed to ca. 65% RH for 4 hours ejected ascospores when the humidity was initially reduced from 100%, but failed to discharge when the humidity was again raised to 100% for 1 hour. Subsequent dehumidification again resulted in ascospore ejection. Atomizing perithecia with distilled water in the above procedure, to simulate rain or dew, also failed to trigger ascospore ejection.

Two-week-old perithecia, exhausted of mature ascospores by reducing the humidity to ca. 65% for 1 hour, were capable of another large discharge of ascospores after 4-6 hours of incubation at a constant humidity. Incubation at either 65 or 100% RH resulted in equal recovery.

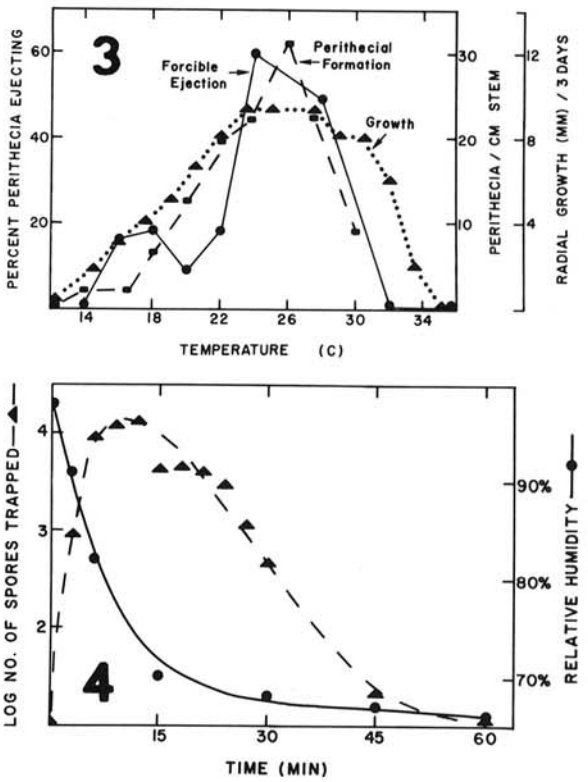


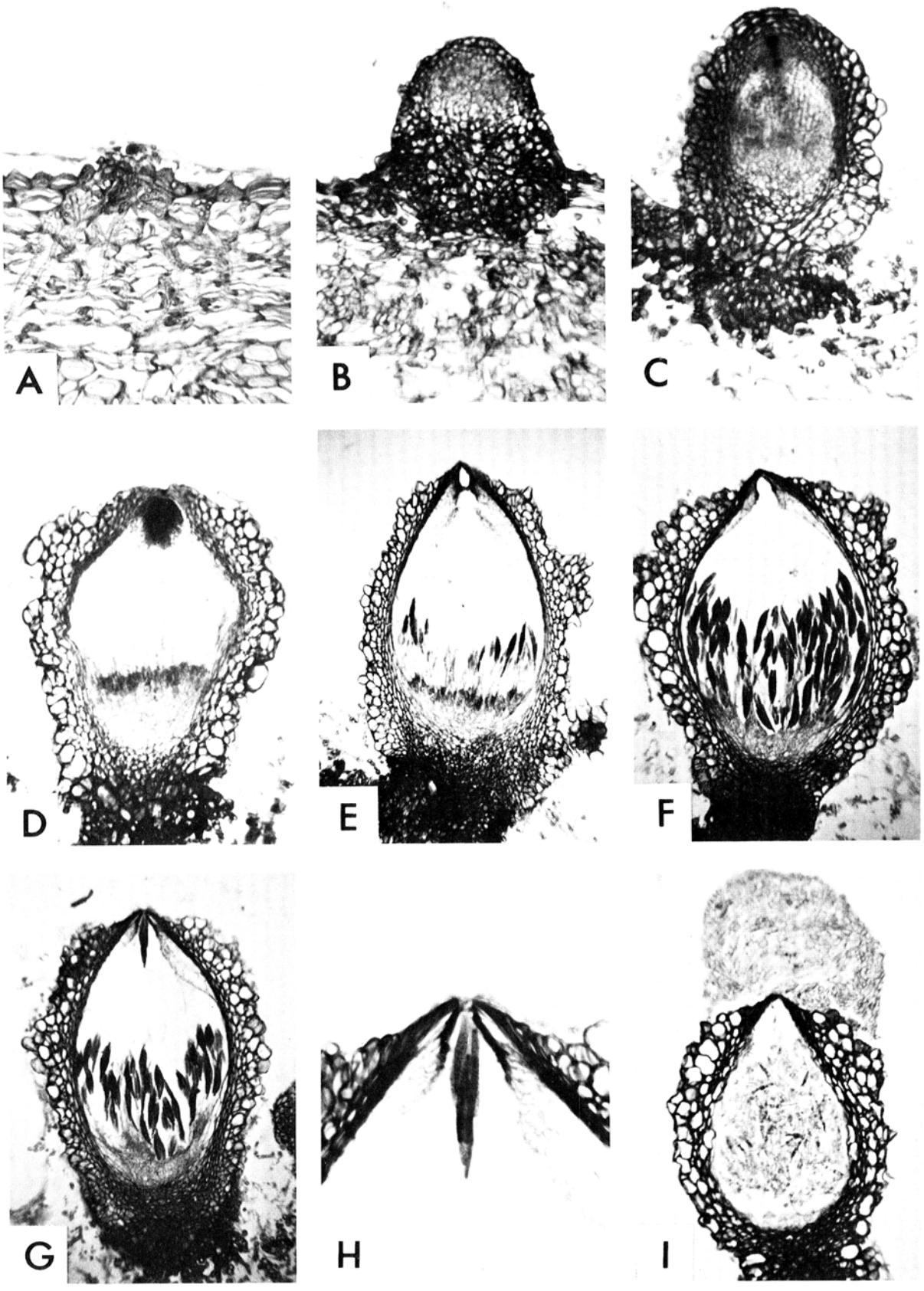
Fig. 3-4. 3) The effect of temperature on growth, perithecium formation and forcible ejection of ascospores by *Calonectria crotalariae*. 4) Forcible ejection of ascospores by perithecia of *Calonectria crotalariae* in response to lowering humidity.

Perithecial ontogeny and ascospore discharge were followed histologically (Fig. 5). Mycelia aggregated into perithecial initials on the surface of colonized peanut stems 5-7 days after inoculation (Fig. 5-A). Perithecial initials then began to enlarge and the interior filled with rows of parenchymatous cells (Fig. 5-B). Nine days after inoculation, the internal parenchymatous cells had enlarged and a plug, which stained dense red with safranin, began to appear in the position of the developing ostiole (Fig. 5-C). By the 11th day, the perithecial cavity began to form by disintegration of the enlarged, parenchymatous cells and asci began to elongate into the cavity from the darkly-staining hymenium. The densely-staining ostiole plug had considerably enlarged at this time (Fig. 5-D). Thirteen days after inoculation, the perithecial cavity was completely open, the ostiole had formed by dissolution of the densely-staining plug, and a few asci were nearly mature (Fig. 5-E). Fully mature asci containing mature ascospores (Fig. 5-F) were commonly observed from 2-3 weeks after inoculation. A single ascus, broken loose from the hymenium, was seen lodged in the ostiole in ca. 70% of the 86 mature perithecia observed (Fig. 5-G, H). This detached-ascus type of discharge has been previously reported (5, 7), but has usually been associated with long-necked perithecia. Asci within a perithecium did not mature simultaneously, but, as in the case with many pyrenomycetes (7), continued to mature and be discharged in this manner for ca. 2 weeks (Fig. 6). After 3-4 weeks at 100% RH, remaining undischarged asci deliquesced within the perithecium and the ascospores were exuded in a viscous ooze (Fig. 1, 5-I, 6). Frequent subjection of the perithecia to 60-70% RH caused this to occur much sooner.

Although single perithecia are active for only 2-3 weeks (Fig. 6), they are present in varying numbers in North Carolina peanut fields from mid-June through October, except during severe drought periods. Inoculation of field-grown peanut plants at 2-week intervals throughout the growing season resulted in abundant perithecial production 4-6 weeks later during the summer months. Perithecial development was reduced considerably in October, possibly by unfavorably cool night temperatures (Fig. 7).

Although ascospores are viable when either forcibly ejected or discharged in a viscous ooze, they are extremely sensitive to desiccation. Percent germination of forcibly-ejected ascospores, when exposed to ca. 73% RH and 33 C, simulating day-time field conditions in North Carolina during July and August, dropped to $< 10\%$ in 2 minutes and $< 0.1\%$ in ca. 30 minutes (Fig. 8). Studies done at ca. 25 C gave similar results.

DISCUSSION.—Perithecial development in *C. crotalariae* follows closely the *Nectria*-type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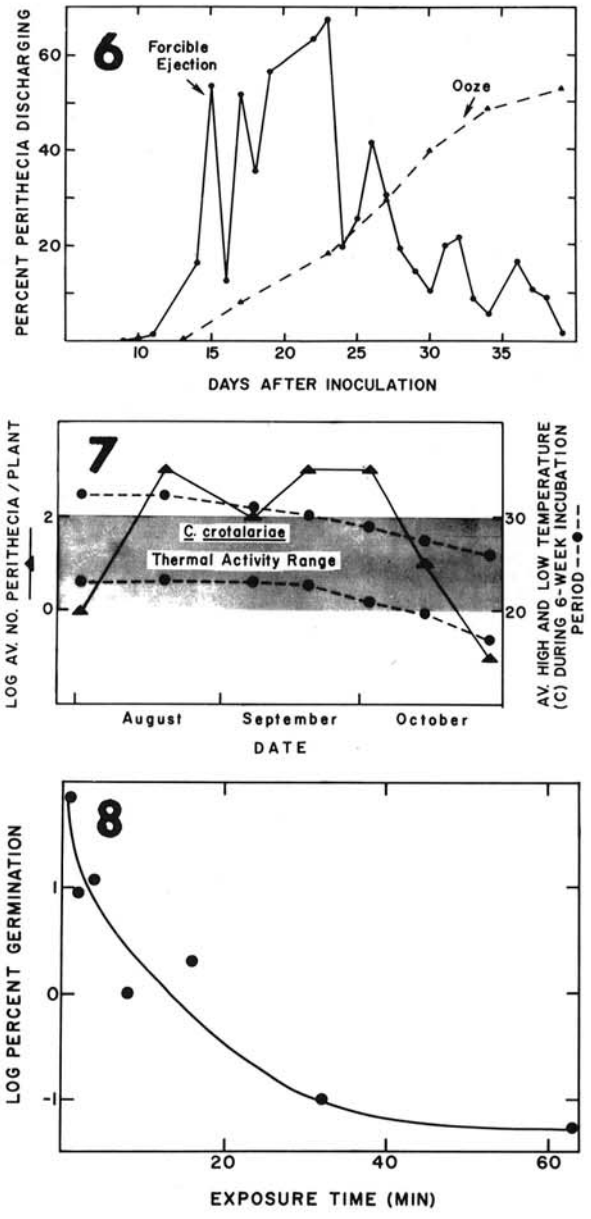

Fig. 6-8. 6) Duration of forcible ejection and oozing of ascospores by perithecia of *Calonectria crotalariae*. 7) Perithecial production on field-grown peanuts 6 weeks after inoculation with *Calonectria crotalariae*. 8) Survival of *Calonectria crotalariae* ascospores exposed to desiccation at ca. 73% relative humidity and 33 C.

developmental scheme described by Luttrell (10). Development and discharge of ascospores seems to be more closely related to the water relations of the fungus than to temperature. Relatively moist conditions are a prerequisite to perithecial development. Discharge of ascospores then occurs as the fairly humid conditions prevailing in North Carolina peanut fields fluctuate during the diurnal cycle.

Ingold and Marshall (8) noted that ascospore ejection in the pyrenomycete *Sordaria fimicola* (Rob.) Ces. and de Not. was also stimulated immediately upon a reduction in atmospheric humidity. Although no mechanisms explaining this phenomenon were postulated, they felt it would be biologically advantageous, in that a reduction in humidity is likely to be correlated with air turbulence favoring dispersal. It seems that a possible discharge mechanism in *C. crotalariae* could involve the development of hydrostatic pressure within the perithecial cavity. A sudden drop in humidity may cause a slight desiccation and contraction of the large parenchymatous cells composing the outer wall of the perithecium (Fig. 5). Since the perithecial cavity in Pyrenomycetes is not empty but filled with a mucilaginous liquid (6, 7), this contraction would exert a positive hydrostatic pressure on the perithecial contents. When pressure from within is applied to a detached ascus lodged in the extremely narrow ostiole of this perithecium (Fig. 5-H), ascospores would be forcibly ejected through the opening.

Because of the extreme sensitivity of *C. crotalariae* ascospores to desiccation, it is highly unlikely that they would remain viable after long-distance transport by air currents. The most likely role of this propagule in CBR epidemiology is short-distance, within-field spread of the disease. Forcible ejection of ascospores probably occurs most frequently in the early morning. During the night, when the humidity is ca. 100%, ascospore maturation would occur, followed by massive discharge when the humidity began to drop at dawn. Similar patterns have been reported for other fungi (11). Early morning dew, commonly present during summer in North Carolina peanut fields, would preclude ascospore desiccation during the subsequent infection period. Discharge of ascospores in viscous ooze probably also retards desiccation and may facilitate further ascospore dispersal by rain-splash or insects.

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Fig. 5-(A to I). Perithecial development in *Calonectria crotalariae* at various intervals after inoculation. **A)** Perithecial initial forming by hyphal aggregation on the surface of inoculated peanut stem (5 days) (× 183); **B)** Perithecial initial enlarging by the internal development of parenchymatous cells (7 days) (× 211); **C)** Perithecial cavity forming by the development and enlargement of rows of parenchymatous cells. Note ostiole beginning to form by the development of a densely-staining plug near the perithecial apex (9 days) (× 168); **D)** Perithecial cavity beginning to pen by disintegration of the rows of enlarged parenchymatous cells. A few asci are beginning to elongate from the newly-developed, darkly-stained hymenial layer. Note enlarged ostiole plug (11 days) (× 181); **E)** Perithecial cavity completely open, a few asci nearly mature, ostiole formed by dissolution of the darkly-staining plug (13 days) (× 106); **F)** Fully-developed perithecium with mature and immature asci (1-3 weeks) (× 109); **G)** Mature perithecium with detached ascus lodged in ostiole prior to forcible ejection (2-3 weeks) (× 108); **H)** Close-up of detached ascus lodged in ostiole. Note unattached basal strand of ascus (× 358); **I)** Overmature perithecium with ascospores discharged in a viscous ooze. Note asci have deliquesced internally (3-4 weeks) (× 135).

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