Spore Germination and Mode of Cotton Infection by Ramularia areola

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ABSTRACT

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Conidia of *Ramularia areola* germinated at temperatures ranging from 16 to 30 C, but the most rapid germination and germ tube development occurred at 25-30 C. Germination and germ tube development were greatly decreased at high spore concentrations. Host penetration by this pathogen was studied under (i) nightly wetting and day drying and (ii) continuous wetting regimes. Excellent penetrations, almost always accompanied by appressorium formation, occurred under nightly wetting and day drying. Severe infection occurred after four consecutive nightly wettings (48 hours) interrupted by daytime drying. Under a regime of continuous wetting there was little penetration and appressoria were not formed. Circumvention of stomatal opening by a germ tube prior to penetration was frequent under continuous wetting. Precocious sporulation of conidial inoculum occurred. Resumption of germ tube growth after the drying period was regulated by the relative humidity of the drying atmosphere.

Additional key words: Cercospora musae, C. beticola, rust fungi.

There is little published information on spore germination by *Ramularia areola* Atk. Although stomatal (15, 19) and cuticular (8) penetrations were reported, details of the penetration process for many members of this genus have not been described. The present study deals with the penetration process and disease severity under continuous wetting versus nightly wetting and day-drying regimes. Tolerance of the growing conidial germ tubes to intermittent drying was determined in vitro, and the influence of temperature and spore concentration on germination and germ tube growth was studied.

MATERIALS AND METHODS

Spore germination.—The effect of temperature on spore germination was studied at 8, 12, 16, 20, 25, 28, 30, and 32 ± 0.5 C. A conidial suspension (5×10^3 conidia/ml) of an Ivory Coast isolate of *Ramularia areola* in sterile deionized water was prepared from 6- to 7-day-old cultures growing on V-8 juice agar. Petri plates, lined with wet blotters, and each containing two depression slides were acclimatized overnight in temperature-regulated incubators. Two drops of a spore suspension were placed in the well (17 mm in diameter) of each slide. Parallel germination tests were conducted on excised host leaves, maintained as above. Plastic cylinders (17 mm in diameter and 7 mm high) were stuck to ventral leaf surfaces and two spore drops were spread over each encircled leaf area. All operations were conducted aseptically to avoid bacterial contamination. Germination counts (per 100 spores) and germ-tube measurements (20 germ tubes) were made after 12-hours of incubation unless otherwise specified. A spore was counted as germinated when the germ tube length exceeded the diameter of the spore. The rate of spore germination and germ tube elongation were followed at 16, 20, 25, 28, and 30 C. Observations were made after 3, 4, 5, 6, 8, and 10 hours of incubation. The effect of six spore concentrations ranging from 5×10^3 to 10^6 conidia/ml on germination and germ tube growth was determined on slides at 25 C. The desired spore concentrations were obtained by serial dilution.

Penetration.—Experiments were conducted in the glasshouse with 16 hours of cool-white fluorescent light (2,000 lux). The temperature fluctuated from 20-30 C. Twenty-day-old potted (12 cm in diameter) cotton plants of cultivar HAR L 321-24-73 (susceptible to grey mildew) were inoculated by atomization with an aqueous conidial suspension (5×10^5 conidia/ml) on both leaf surfaces until run-off. Inoculated plants were enclosed in moist polyethylene bags. The epidermis of the cotyledons could be peeled off in large strips easily from the under surface. Peelings were taken at 24-hour intervals and stained with cotton blue. In the nightly wetting and day-drying treatment, plants were kept covered daily from 0730 hours to 1930 hours. Plants were sprayed with sterile deionized water immediately prior to being enclosed in moist polyethylene bags. Disease severity ratings

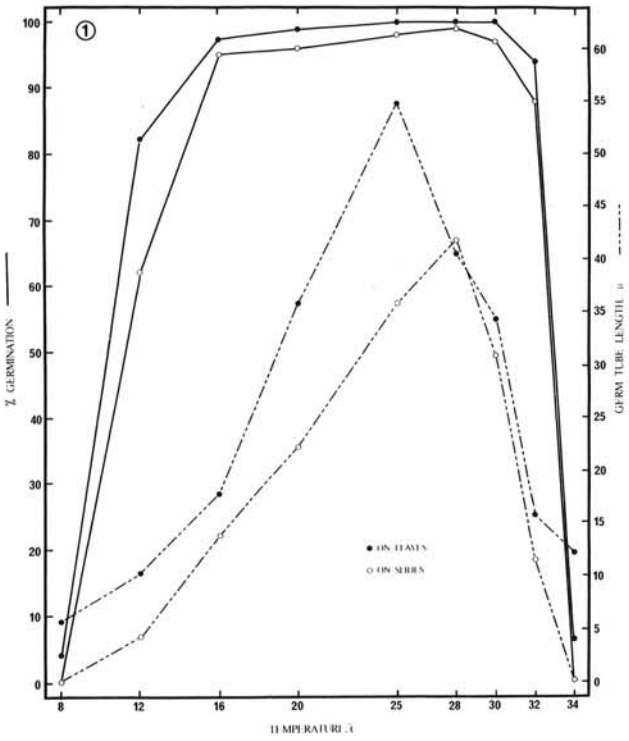

Fig. 1. The effect of temperature on germination and germ tube growth of conidia of *Ramularia areola* on cotton leaves and on glass slides.

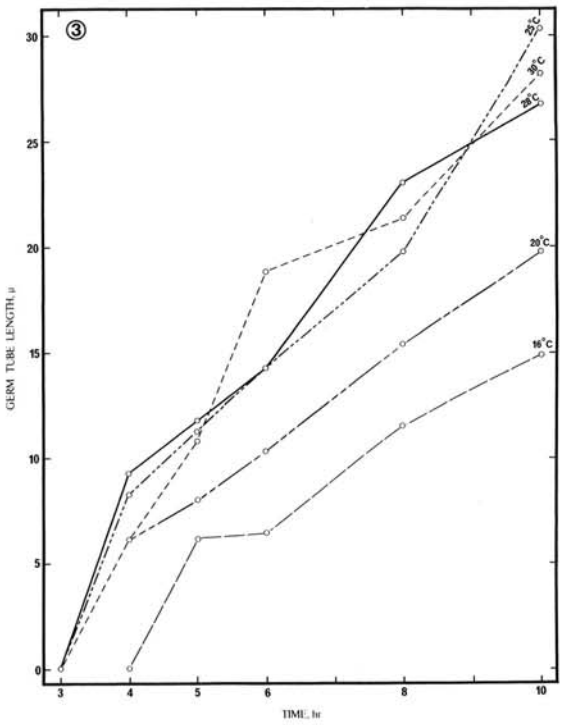

Fig. 3. The effect of temperature on the rate of germ tube development of conidia of *Ramularia areola*.

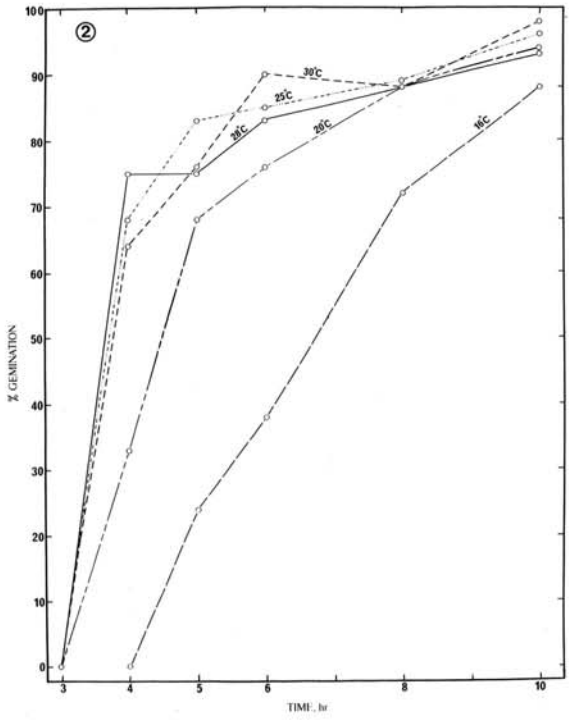

Fig. 2. The effect of temperature on the rate of germination of conidia of *Ramularia areola*.

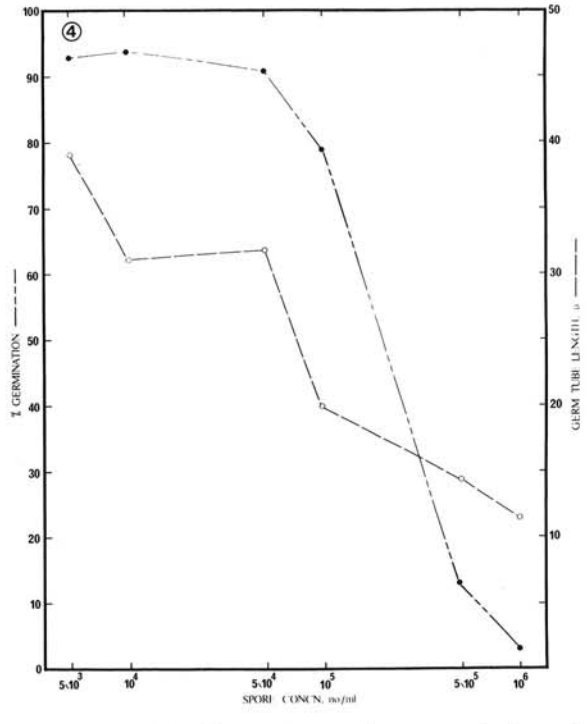

Fig. 4. The effect of spore concentration on germination and germ tube growth of *Ramularia areola*.

(percentage of leaf area diseased) were determined for the most severely infected leaf of each plant (9). Although the infection experiments were repeated three times the results of only one trial in which the inoculations were most successful are reported.

RESULTS

Spore germination.—Germ tube development was more sensitive to temperature than was spore germination (Fig. 1). Both processes were generally better on excised leaves than on slides. Germination on excised leaves, although low, occurred at the lowest as well as the highest temperatures tested, in contrast with that on slides. The temperature between 25 to 30 C provided the most rapid germination (Fig. 2) and germ tube elongation (Fig. 3). Both processes were relatively slow at 16-20 C. Maximum germ tube growth occurred at the lowest tested concentration (5×10^3), and above that concentration germ tube length decreased as the spore concentration increased (Fig. 4).

Penetration under continuous wetting.—About 98% of the conidia germinated on the leaf surface 12 hours after inoculation, initiating two germ tubes per conidium from apical and basal cells. The earliest recorded penetration was at 16 hours after inoculation. Germ tubes grew at random attaining up to 600 μm in 36 hours. They continued to elongate without branching, sometimes traversing stomata without penetrating. Six days later, germ tube growth was extensive. It was impossible to follow growth that developed from a conidium. Lysis of conidia and germ tubes by some bacterial contaminants was frequently observed.

Penetrations were effected without appressoria through open stomata that the germ tubes encountered by chance (Fig. 5-A). A substomatal vesicle was not formed after entry by the germ tube. In another type, stomatal penetrations were initiated by side branches arising from the main germ tube at the proximity of a stoma (Fig. 5-B).

Circumvention of stomatal pores by germ tubes.—When plants were exposed to continuous wetting, the growing tip of germ tubes was attracted to the rim of the outer stomatal chamber and then penetrated at one end of the stomatal slit (Fig. 5-C) or grew away without penetrating (Fig. 5-B). This is somewhat comparable to the coiling at the stomatal entrance described for some other fungi (4). These phenomena represent a tactile response of the germ tubes to nightly closure of the stomata.

Penetration under nightly wetting and day drying.—Nightly wetting and day drying intensified penetrations by the pathogen. Here the germ tube growth was discontinuous, and consequently it was limited as compared with that in continuous wetting. However, this was accompanied by an increased number of germ tubes per spore, which often were branched.

Penetrations under this wetting regime were usually accompanied by an appressorium, but in several instances an appressorium was not formed. Numerous penetrations were observed 4.5-7 days after inoculation. Germ tubes usually reached stomata (proximal to germinating spore) in two or three consecutive nightly wettings. Later side branches developed from the main germ tube near the stoma which upon reaching the stomatal pore terminated in an appressorium, fitting along the crack between the closed guard cells (Fig. 6-A).

A septum separated the appressorium from the germ tube. The shape of appressoria varied considerably but generally were ellipsoidal (in lateral view) averaging 13 μm in length (Fig. 6-B). Occasionally two or three appressoria, from a single germ tube or from different germ tubes, were formed over a single stoma. The appressorium, gripping the stomatal slit, gave rise to an infection peg from its ventral surface. The latter was typically thick-walled, brown, and pierced through the crack between the guard cells and then swelled into a spherical to oval (average 10 μm in diameter) substomatal vesicle which in turn gave rise to an infection hypha (Fig. 6-C). A few appressoria were double-celled, and in some cases both the cells independently functioned as appressoria (Fig. 6-D).

Frequently appressoria were formed at epidermal sites other than stoma. These appressoria were ineffective, but they germinated producing sequentially an infection peg, vesicle, and infection hypha (Fig. 6-E, F) as in *Puccinia antirrhini* (10).

Precocious sporulation.—Precocious sporulation frequently was observed on the surface of inoculated leaves 12 hours after inoculation. The conidial inoculum, instead of initiating germ tubes budded laterally or at times terminally (Fig. 6-G) producing solitary dauthter conidia that might be detached leaving a scar. The daughter conidia may be borne on intermediate short projections over parent conidia (Fig. 6-H). In a less-pronounced type, observed generally under continuous wetting, conidial germ tubes grew for 36 hours and then bore conidia apically, in chains (Fig. 6-I).

Precocious sporulation occurred principally when inoculations were made during dry weather when there was no moisture film on the leaves. When inoculations were made during cloudy and damp weather a perfect moisture film persisted and all conidia germinated normally. A similar type of sporulation frequently was observed when conidia were germinated on cellophane membranes (maintained on moist filter paper) upon which no moisture film occurred (Fig. 6-J). Precocious spores germinated mostly in situ by germ tubes when conditions were favorable.

Effect of continuous wetting versus intermittent wetting and drying on germ tube elongation.—Boiled cellophane pieces (2×1.5 cm) were loaded with a drop of conidial suspension (10^4 conidia/ml) and placed over moist filter paper in petri dishes at 25 C. Germ tube development, under continuous wetting was determined at five 8-hour intervals. In alternate wetting and drying periods, each 8-hour wet period (at 25 C) was interrupted by a 16-hour dry period at 35 C. The wetting duration was fixed at 8 hours based on the presumption that dew persists for about 8 hours per day under field conditions. The effect of drying on germ tube growth was determined at three relative humidities (RH); viz., 23, 60, and 80% at 35 C in a hygrometric oven, except for the RH 23% that was tested in an ordinary oven in the laboratory. After the dry period, membranes bearing the germinated spores were placed directly over moist filter paper. The percentage of pregerminated conidia that could resume growth after the intervening dry periods was determined as previously described (14), based on 25 conidia per

treatment. Germ tube measurements (50 germ tubes) were made at the termination of each wet period based on the longest one on each conidium. All conidia resumed growth even after four successive dry periods at RH 23%, but the germ tube growth rate was slightly reduced compared to that in continuous wetting. The ability of germinated conidia to resume growth declined with increase in the RH of the drying atmosphere (Fig. 7). The vulnerability of the germinated spores increased through the consecutive dryings at 60% RH. Ninety-nine and 100% of the spores failed to resume growth after the first and second dryings, respectively, at 80% RH. Preliminary trials were conducted with 50% RH at 40 C and growth was little affected (5%) even after the second drying.

Inoculation studies.—Disease severity was compared on plants exposed to (i) nightly wetting and day drying and (ii) continuous wetting for 24, 36, 48, 72, 96, 120, and 144 hours. Three-week-old potted Réba B 50 cotton

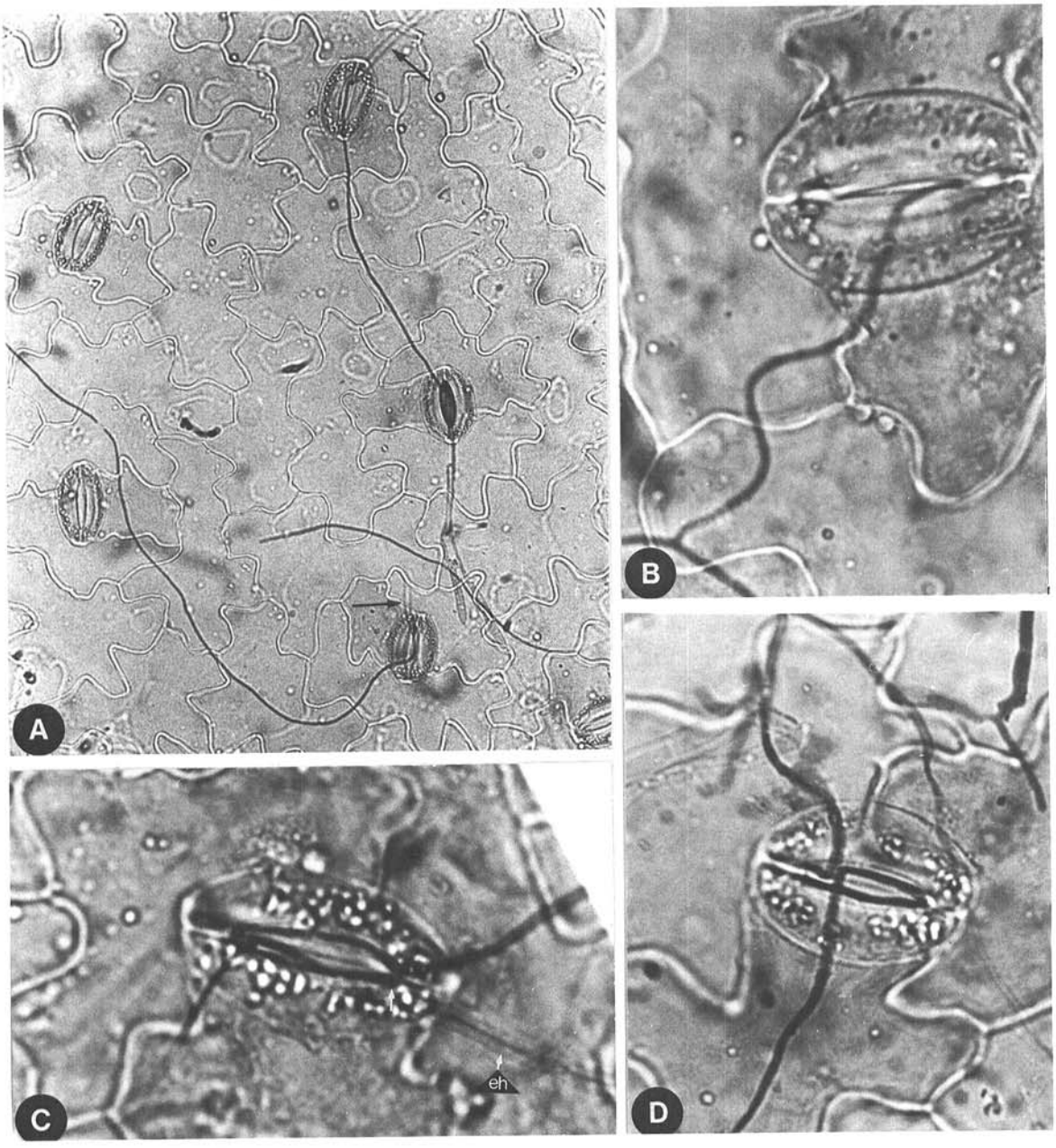

Fig. 5-(A to D). Penetration of cotton leaves by *Ramularia areola* under continuous wetting. **A)** Typical penetrations; note the hyphae under the epidermis (arrows). **B)** Side branch of a germ tube entering at one end of a partially opened stoma. **C and D)** Circumvention of stomatal opening by germ tubes; note the penetration at one end of stoma (C) showing endophytic hypha (eh).

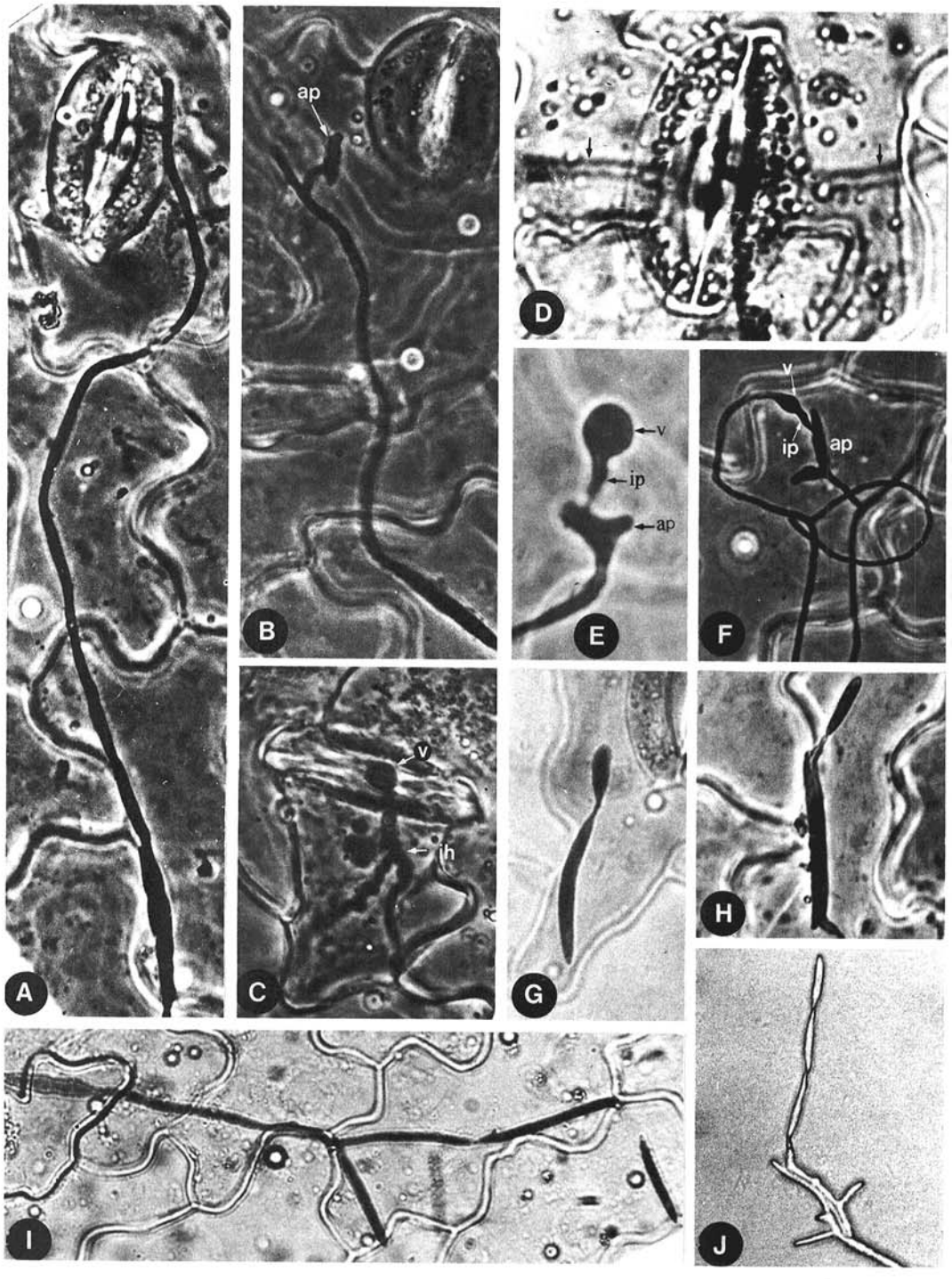

Fig. 6-(A to J). Penetration of cotton leaves by *Ramularia areola*. (A-D) under nightly wetting and day drying: **A)** Typical penetration; note the appressorium fitting along the slit between the closed guard cells. **B)** An appressorium (ap) detached from stomata. **C)** Substomatal vesicle (v) and infection hypha(ih). **D)** A single appressorium effecting two penetrations; note the infection hyphae below the epidermis (arrows). **E and F)** Germination of appressoria (ap), formed on the epidermis not over a stomata, infection peg (ip), vesicle (v). (G, H, and I) Precocious sporulation of the inoculum conidia on the leaf surface. **G)** Terminal budding of a conidium. **H)** Precocious conidia on short projections over mother conidia. **I)** Precocious conidia formed in a chain; note the detached spore. **J)** Precocious sporulation on cellophane membrane.

plants were inoculated with an *R. areola* isolate from Madagascar. The inoculum concentration was adjusted to 10^5 conidia/ml.

A nightly wetting and day drying was the most favorable condition for infection (Fig. 8, 9). The minimum wet periods for infection under nightly wetting and day drying, and continuous wetting regimes were 24 and 36 hours, respectively. The percentage of infection under nightly wetting and day drying increased markedly as wetness was lengthened to 36 and 48 hours. There was no significant increase in infection when the wet period was increased from 48 to 144 hours. Severely infected leaves became dry, curly, and dropped prematurely. Unlike the typical disease spots no fungal growth was observed on the underside of the leaves.

DISCUSSION

The rapid germination and germ-tube growth probably contribute to rapid disease development under conditions of warm humid weather (5). The precocious sporulation of this pathogen was similar to that described for some other fungi (2, 11, 12). Based on the present observations, it was hypothesized that rapid drying of the inoculum film on the leaf surface is the cause of precocious sporulation. Paralleling the present observations, Whiteside (20) reported greater numbers of appressoria and a consequent increase in disease severity under daily wetting and drying than under continuous wetting. The infection process is comparable to that of *Cercospora beticola* (17) in which when continuously wet, the pathogen is principally dependent upon open stomata for host entrance. Under a nightly wetting and day-drying regime, which is representative of field conditions, the pathogen could penetrate closed stomata. The latter process closely resembled the classic mode of penetration by some rust fungi (18) in which the uredial germ tube forms sequentially: an appressorium, infection peg, substomatal vesicle, and infection hypha. Based on the present results, it is concluded that nocturnal wetting is the most effective for infection by this pathogen, as for *Cercospora musae* (7).

The results reported here suggest that germinated conidia of *R. areola* resist several successive dry periods of intermediate relative humidity (up to 60%) during the warm dry time, as does *C. musae* (3). The germinated conidia of *Ramularia* and its allied genus, *Cercospora,* which are relatively slow penetrating (about 3-4 days), are probably extremely tolerent of desiccation (6, 14). Whereas fungi in the Peronosporales (e.g., *Phytophthora infestans*) that penetrate their hosts rapidly [2 hours (13)]

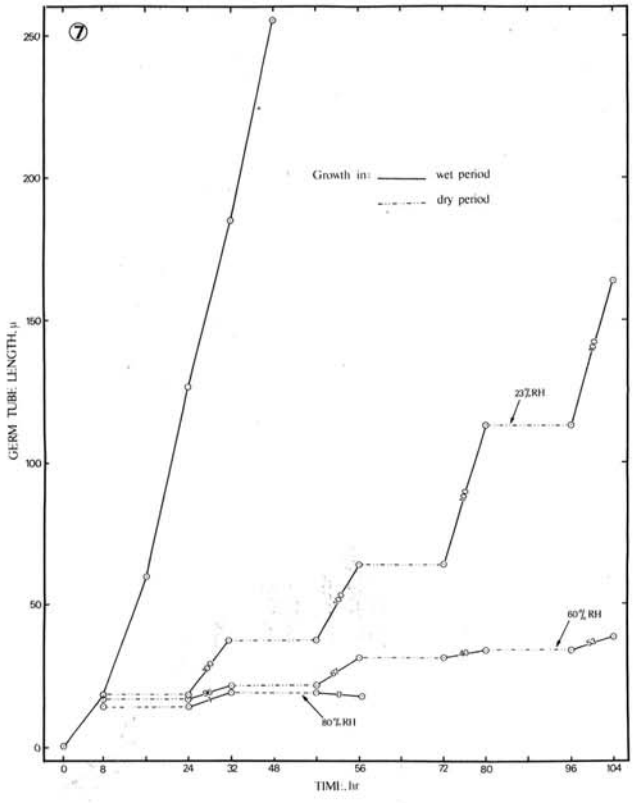

Fig. 7. Germ tube development by conidia of *Ramularia areola,* exposed to continuous wetting (25 C) and alternate wetting (25 C) for 8 hours and drying (35 C) for 16 hours at various relative humidities (RH). Figures interrupting the solid lines include the per cent spores resuming growth after the drying exposure.

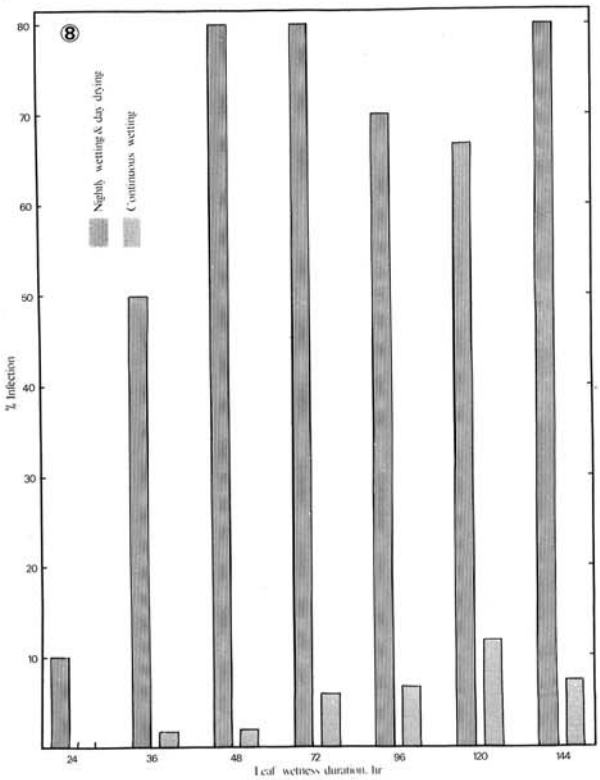

Fig. 8. The effect of various leaf wetness periods under continuous wetting and under nightly wetting and day drying on the infection severity of *Ramularia areola* on cotton.

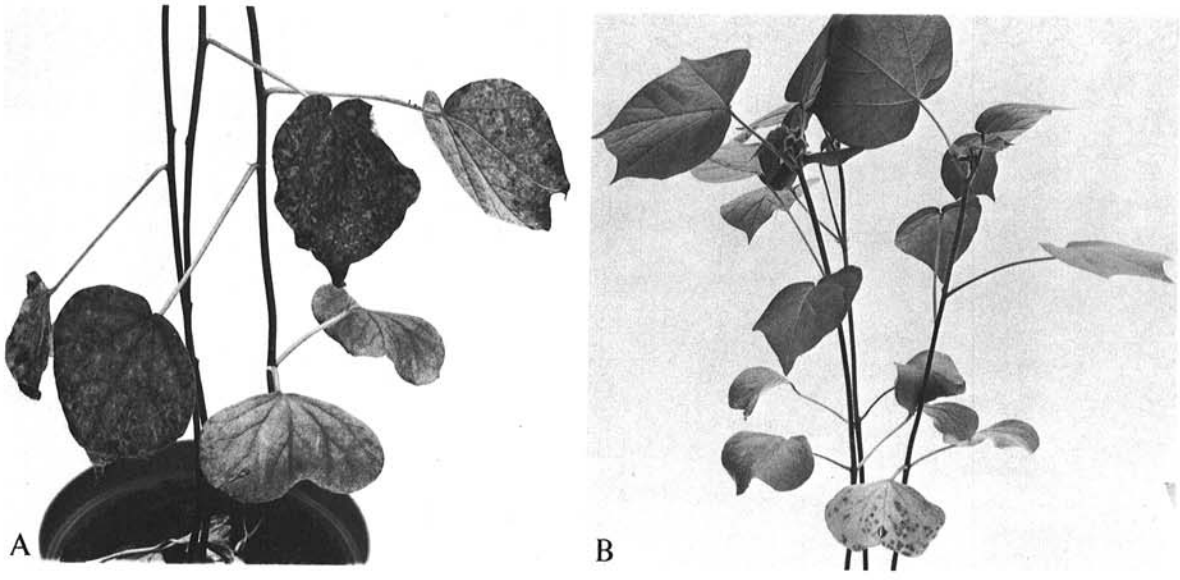

Fig. 9. Infection severity of cotton by *Ramularia areola* under 48 hours night-time wetting (A) and under continuous wetting of the same duration (B). Photographs were taken 16 days after inoculation.

are highly vulnerable to drying (1). In this study the conclusion was that two factors; viz. (i) ability of germ tubes to survive day drying and (ii) hydrotropism, directing germ tubes into stomatal opening as suggested also for *C. musae* (7), enabled the nightly wetting and day drying regime to enhance penetrations by this pathogen.

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