Development of *Cladosporium caryigenum* in Pecan Leaves

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ABSTRACT

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Conidia of *Cladosporium caryigenum* germinated on leaves of pecan (*Carya illinoinensis*) in the dark within 3 hr at 23 C and 100% relative humidity. After 12 hr, penetration of host cuticle was achieved by hyphae originating from appressoria. Subcuticular hyphae branched repeatedly, became wider, and grew over host anticlinal epidermal cell walls. Melanized bulbous cells on branches of subcuticular hyphae were observed at 144 hr. Conidiophores originating from these bulbous cells erupted through the cuticle. Conidia were first observed 168 hr after inoculation. Mycelium of *C. caryigenum* did not grow superficially on pecan leaves.

Additional keywords: *Fusicladium effusum*, pecan scab.

Scab of pecan (*Carya illinoinensis* (Wang.) K. Koch) is a major limiting factor in production of most commercial cultivars throughout the southeastern United States (15,16,22). The scab fungus, *Cladosporium caryigenum* (Ell. et Lang.) Gottwald (9), causes severe leaf-spotting, current-season twig infection, and shoot dieback of hypersensitive cultivars. In addition, lesions develop on the nut shuck (involucre), resulting in lowered quality to total loss of the nuts (12,13,22). Leaves and current-season shoots of susceptible cultivars are subject to infection from budbreak until maturity (4).

There have been no detailed histological investigations of infection and establishment of *C. caryigenum*. Nolen (20) reported that mycelium from which conidiophores arose formed on the surface of pecan leaves. According to Demaree (5), infection is superficial and extends only slightly below the epidermis. Conidiophores push through the cuticle and, subsequently, the epidermis and cuticle are destroyed by anastomosing hyphae. Conidiophores developing later are reported to be produced from newly-formed stromata (5).

The present study was undertaken to determine mode of infection, establishment, and conidiation of *C. caryigenum* on pecan leaves.

MATERIALS AND METHODS

General. Greenhouse-grown, 5-yr-old pecans (cv. Schley), maintained at a shoot-tip height of 40–50 cm above soil level in 12.3-L pails, were used. Conidia of *C. caryigenum* were washed in sterile tap water from infected leaves of these trees and filtered twice through four layers of cheesecloth (17). The suspension was adjusted to 1×10^6 conidia per milliliter of water containing polyoxyethylene sorbitan monolaurate (Tween-20, Sigma Chem. Co., St. Louis, MO) at the rate of three drops per liter. The suspension of conidia was applied to the leaves of test trees until runoff. The trees were placed in dew chambers programmed to 23 C, 100% relative humidity, and darkness for 36 hr. This program was followed by a 12-hr incubation at 23 C, >80% relative humidity, and illumination from fluorescent and incandescent lights (30 μE m^{-2} sec^{-1}). Subsequently, the trees were returned to the greenhouse, where temperatures ranged from 22 to 29 C. Daylight illumination mean was 725 μE m^{-2} sec^{-1} with a 14-hr photoperiod.

Tissue samples. Two 10-mm-diameter leaf discs were excised from each of six trees at 3-, 6-, and 9-hr intervals, then at 12-hr intervals through 96 hr, and subsequently at 24-hr intervals through 240 hr. We repeated experiments two times using different trees for each sampling. Leaf discs were immersed in saturated chloral hydrate solution (250 grams per 100 ml of water) to remove chlorophyll. Translucent leaf discs then were stained for 12–16 hr with a 2% acid fuchsin solution in 70% ethanol diluted with a mixture of saturated chloral hydrate and 95% ethanol (7). After satisfactory staining, leaf discs were destained in a fresh saturated chloral hydrate solution until host and pathogen could be easily differentiated. Leaf discs then were counterstained for 5 sec in a 2% bismark brown solution in 70% ethanol. Leaf discs were dehydrated through a series of 85%, 95%, and three changes of absolute ethanol. After dehydration, leaf discs were cleared in methyl salicylate and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ).

Leaf discs stained with acid fuchsin in chloral hydrate gave adequate differentiation of subcuticular hyphae for observation.

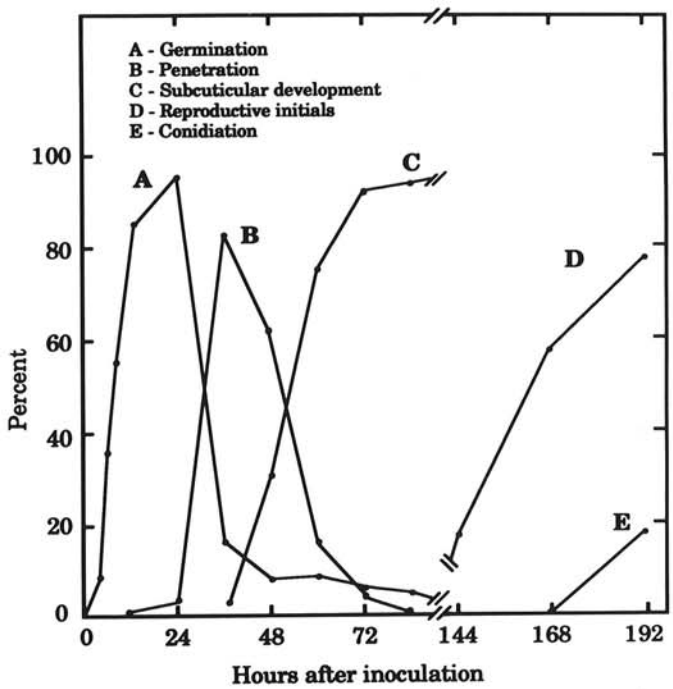
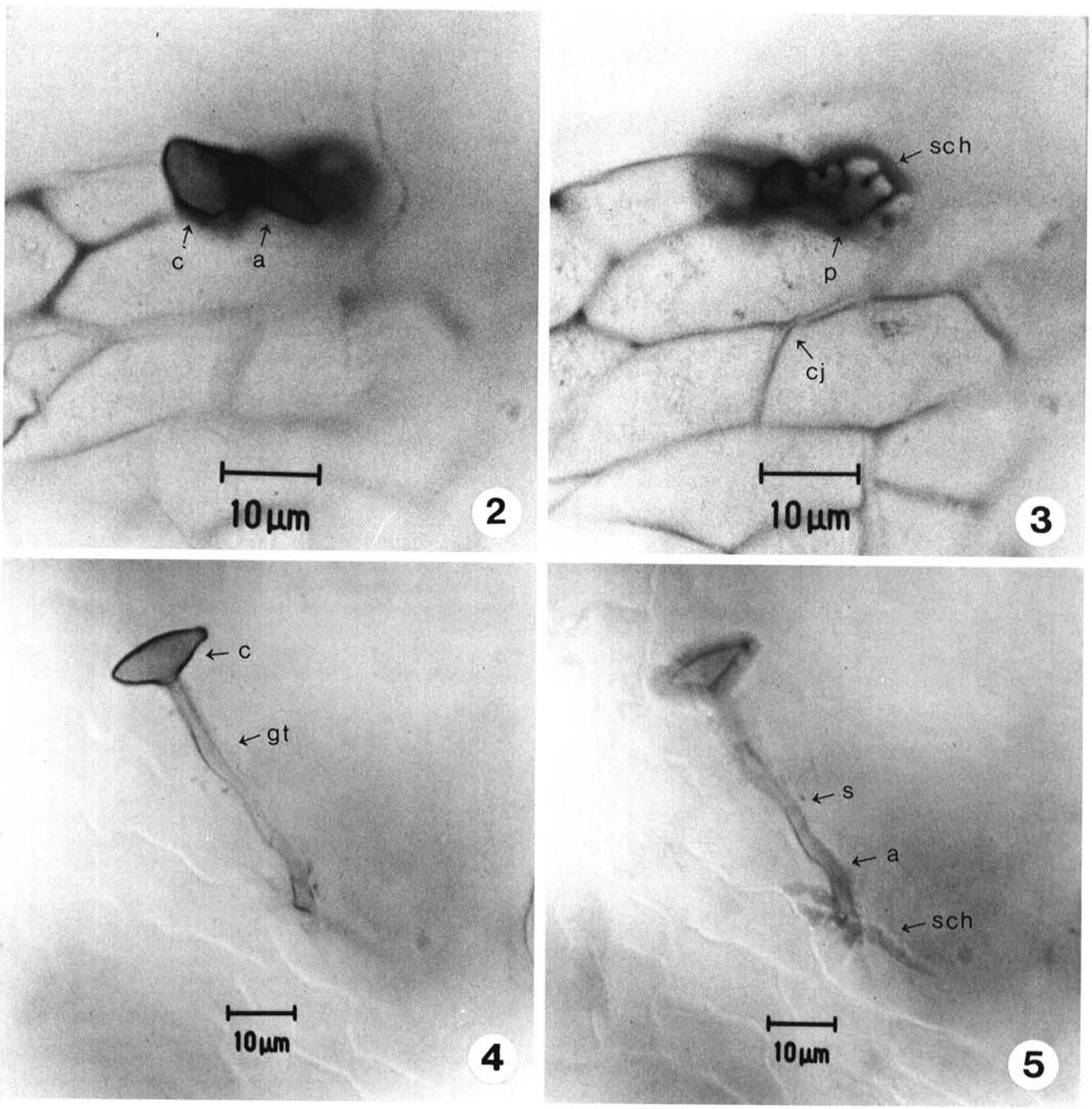


Fig. 1. Chronology of pathogen development in leaves of pecan cultivar Schley inoculated with *Cladosporium caryigenum*. Each point represents the data from 500 host-pathogen encounter sites converted to percent.

However, staining of cell walls usually was inadequate for photography. Therefore, the methods of Pierre and Millar (23) were used to stain leaf discs with 0.1% acid fuchsin in lactophenol. Leaf discs were stained 60 to 90 min, destained when necessary in lactophenol, and mounted in lactophenol. We examined whole mounts by using an Olympus Photomicroscope, Vanox AHBS-F (Olympus Optical Co., Ltd., Tokyo, Japan), equipped with bright-field and Nomarski interference-contrast optics.

For scanning electron microscopy, leaf discs were collected 10, 15, and 30 days after inoculation and fixed in either half-strength Karnovsky's (14) fixative with phosphate buffer, pH 7.0, for 3 hr at 4 C followed by overnight fixation at 4 C in 1% OsO_4 in the same buffer (pH 7.0), or in formalin-acetic acid-70% ethanol (5:5:90) for 24 hr. All samples were dehydrated in ethanol, critical-point dried with liquid CO_2, mounted on aluminum stubs, and sputter-coated with gold-palladium. Samples were examined with an ISI SS-40 scanning electron microscope (International Scientific Instruments, Milpitas, CA) operated at 5 kV and photographed with a Polaroid camera (Polaroid Corp., Cambridge, MA).

Pathogen development. Leaf tissues were examined for development of *C. caryigenum* at the sampling intervals listed above. Fifty host-pathogen encounter sites (actual or potential penetration sites) from a minimum of five leaf discs from each experiment were examined at each sampling time. The following developmental stages (Fig. 1) were quantified in percentages: (A) *germination*, germ tube with a length equal or exceeding the width of the conidium; (B) *penetration*, short, thin, subcuticular hyphae originating at a pore (probable site of infection peg) in the appressorium; (C) *subcuticular development*, enlarged, branched, subcuticular hyphae radiating from the pore, i.e., the pathogen was



Figs. 2–5. Photomicrographs taken with Nomarski interference contrast optics depicting development of *Cladosporium caryigenum* in relation to pecan leaf tissues. **2** and **3**, Conidium and appressorium in two focal planes at 60 hr; **3**, the same site as 2. Subcuticular hyphal growth in relation to small vein (staining technique: acid fuchsin/chloral hydrate). **4** and **5**, Views of the same site in two focal planes at 48 hr; **5**, appressorium delimited by septum with initial subcuticular hyphae in leaf laminae (staining technique: acid fuchsin/lactophenol). Abbreviations: a = appressorium, c = conidium, cj = anticlinal cell wall junctions, gt = germ tube, p = penetration site, s = septum, and sch = subcuticular hyphae.

established in the host; (D) *reproductive initials*, a network of subcuticular hyphae giving rise to conidiophore basal bulbs; and (E) *conidiation*, conidia on conidiophores.

The direction of growth of germ tubes from conidia deposited near trichomes was studied by observing 2,000 conidia at 36, 48, 60, 72, and 84 hr after inoculation. Conidia within one-half of the diameter of a peltate trichome were examined.

RESULTS

Germination of conidia and penetration of host cuticle. Examination of whole-leaf discs indicated that conidia remained unicellular during germination. Germ tubes extending from conidia were observed 3 hr after inoculation. At 12 hr after inoculation, 83.2% of conidia had germinated, and, at 0.4% of the encounter sites, association of an appressorium or germ tube terminus with subcuticular hyphae was apparent (Figs. 1, 2–5).(μg F m^{-3}).At 24 hr after inoculation, 95.2% of the conidia were germinated and 2.6% of the encounter sites showed subcuticular hyphae.

Some conidia produced a germ tube that enlarged immediate to the conidium and became an appressorium (Figs. 2 and 3), and others produced a germ tube that elongated to produce an appressorium distally (Figs. 4 and 5). In some instances, germ tube growth occurred without any swelling or formation of a septum to differentiate the appressorium. True appressoria were darker in color than germ tubes and were delimited by a septum in 31.8% of the encounter sites. Germ tubes became slightly to substantially inflated distally 74.8% of the time. One or more light-reflecting spots or pores in an appressorium wall were observed near the juncture of epidermal, anticlinal cell walls (Fig. 3). These pores apparently were loci where subcuticular hyphae originated. The tip of a germ tube either assumed an oblong to ellipsoid shape or became lobed with a pore sometimes occurring in more than one lobe. Germ tubes grew along leaf surfaces randomly and were straight or occasionally curved. Germ tubes were frequently short and swollen when they were located on veins. An analysis of germ tube length on veins versus on laminae, however, showed no significant differences (data not shown). Germ tubes ranged in length from 10 μm to 111 μm, including an appressorium. Of conidia evaluated during the first 36 hr of incubation, 93% germinated from one side, 3.6% from both sides, and 3.4% from the end. Maximum penetration of pecan tissues by *C. caryigenum* was observed at 36 hr (Fig. 1). Subcuticular hyphae adjacent to the site of penetration averaged 20 μm in diameter and ranged from 11 to 27 μm in five leaf discs.

Establishment of pathogen. At 48 hr after inoculation, 62.2% of the germ tubes had successfully penetrated the cuticle and given rise to hyphae, with 29.4% exhibiting enlarged, branched growth (Fig. 1). After 60 hr, lateral protrusions were observed along enlarged or mature hyphae. From 72 to 84 hr, protrusions had enlarged to form finger-like entities. These were present in 85.3% of the subcuticular colonies.

After 72–84 hr, subcuticular hyphae in leaf discs stained more readily and deeper with acid fuchsin than when younger. Subcuticular hyphal growth followed the anticlinal cell walls of the epidermal cells as if superimposed upon them.

The majority of germ tubes of *C. caryigenum* grew toward the base of trichomes. Of 320 conidia located within a one-half diameter of the trichome, 82.2% produced germ tubes that grew directly to the trichome base and produced an appressorium that wrapped around part of the base. In contrast, after contacting the trichome base, 9% continued halfway around, departed from the base, and grew on to a site where attachment and infection occurred. The remainder, 8.8%, did not grow to the trichome base but was located close to veins.

Reproductive initials and conidiation. After 144 hr of incubation, short, lateral protrusions were observed along the main subcuticular hyphae. Subsequently, a pale brown cell was delimited distally and followed by an additional terminal, larger, dark brown bulbous cell (Fig. 6). A conidiophore emerged by rupturing the leaf cuticle immediately above a bulbous cell (Figs. 7, 8). Dark brown bulbous cells occurred at 17.2% of encounter sites after 144 hr (Fig. 1). An average of six bulbous cells was recorded in each colony. At many encounter sites, only the appressorium and part of the germ tube could be used for assessing pathogen activity, because many spent conidia and germ tubes had become detached and apparently lost during the staining and rinsing procedures. Of the appressoria examined at the 168-hr incubation interval, 98.4% gave rise to subcuticular hyphae and 56.2% of the colonies produced conidiophore basal bulb cells at an average of 30.5 each. The percentage of colonies showing basal bulb formation had increased to 76.8% after 192 hr. At this time, 17.8% had conidia still attached to conidiophores (Figs. 8, 9). Conidia developed holoblastically in acropetal succession from the apices of simple or branched conidiophores (Fig. 10). There was no growth of hyphae on the leaf surface (Figs. 8, 9).

At 240 hr, the diameter of 50 subcuticular hyphae in colonies was evaluated from five leaf discs. Diameters ranged from 18 to 55 μm, with a mean of 34 μm.

DISCUSSION

Our histological investigations of *C. caryigenum* show contrasts and similarities with results from studies of some other *Cladosporium* spp. (1,2). *Cladosporium fulvum* and *C. cucumerinum* penetrate tomato and cucumber leaves, respectively, through stomata without an appressorium, and conidiophores originate from intercellular mycelium invading subepidermal tissues (2). In contrast, *C. carpophilum*, which causes scab of *Prunus* spp., infects leaves by penetration of the cuticle (1), as does *C. caryigenum*. A well-defined appressorium was not, however, found in *C. carpophilum* (1). Furthermore, according to Bond (2), *C. carpophilum* is similar to *C. caryigenum* in forming

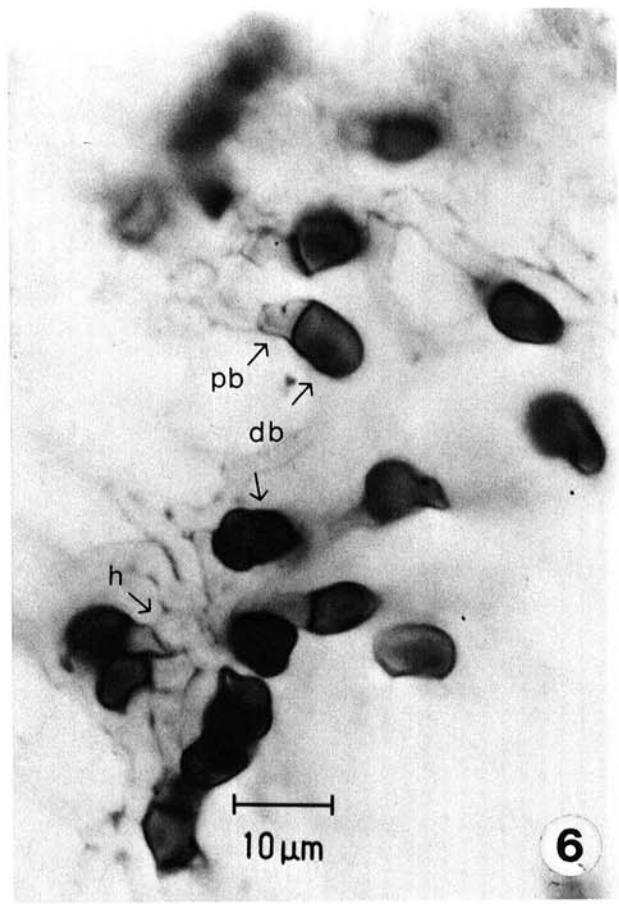
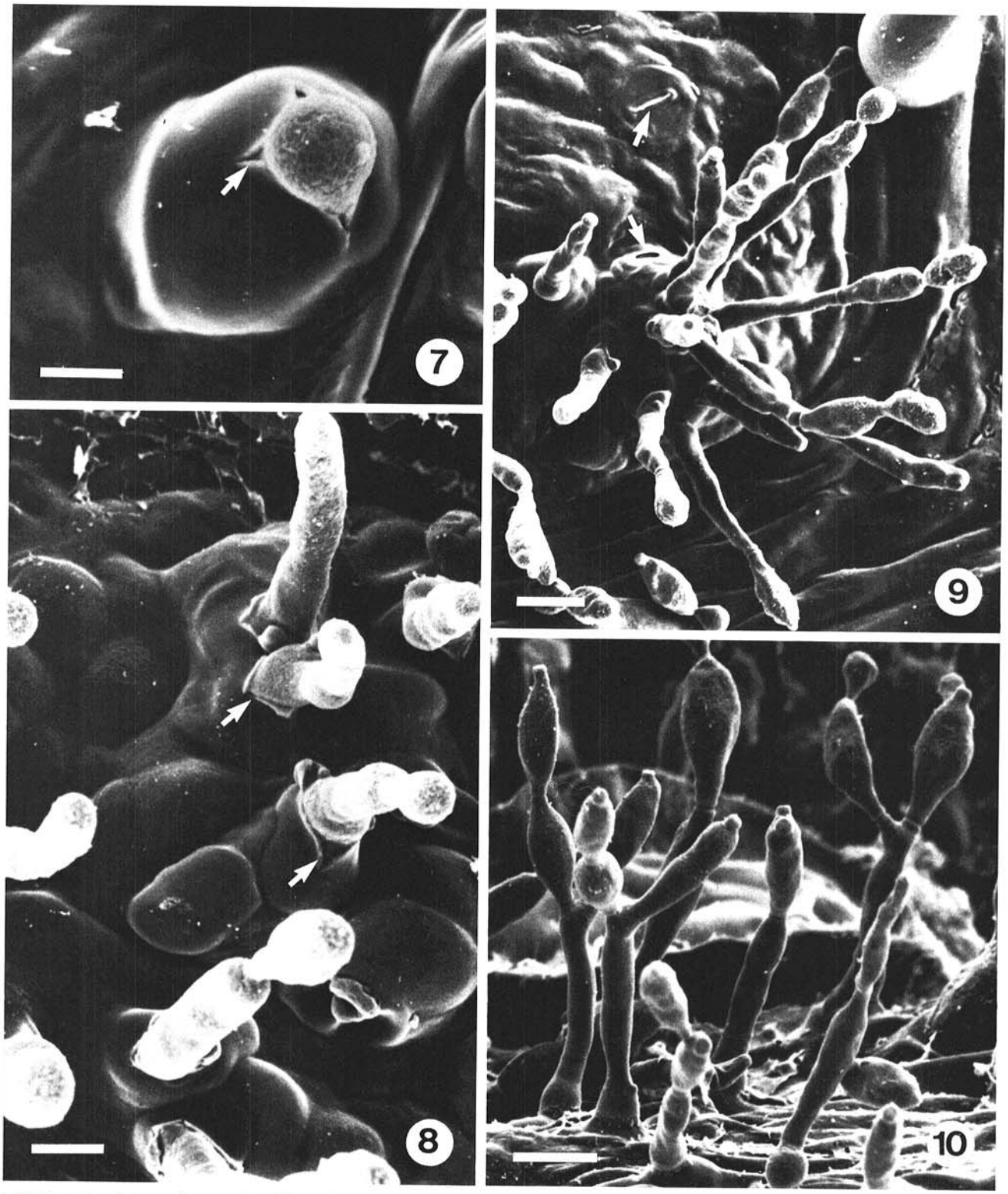


Fig. 6. Photomicrograph taken with Nomarski interference contrast optics depicting subcuticular hyphae and conidiophore basal bulbs. Abbreviations: h = hyaline branch, pb = pale brown cell, db = dark brown bulbous cell.

subcuticular stromata from which conidiophores develop. Our work confirms his observations on *C. caryigenum*.

Investigators studying *C. caryigenum* on pecan have reported lesion development after 10 days of incubation at orchard temperatures (6), 7–10 days incubation at 24 C (27), and 7–9 days incubation at 10–35 C (11). Gottwald (10) reported maximal infection occurred after 36 hr of continuous leaf wetness and 20–30 C, a temperature range considered optimal. Maximum lesion development occurred following a 48-hr infection period and continuous leaf wetness at 15–25 C. In our investigations, some pecan trees developed a necrotic leaf scorch when incubation was continued in the dew chamber after the 48-hr infection period. During this post-infection period, a 12-hr photoperiod (30 μE m^{-2} sec^{-1}) was maintained (data not published). Furthermore, lesions of *C. caryigenum* supported prolific growth of bacteria that became a problem in scanning electron miscroscopy evaluations of



Figs. 7–10. Scanning electron micrographs of *C. caryigenum* on pecan leaves. **7**, Initial emergence of conidiophore by rupture of the leaf cuticle (arrow) above the bulbous cell; scale = 2.5 μm. **8**, Later stage showing rupture of the leaf cuticle (arrows) and conidiophore development. There is no surface mycelium present. Scale = 5 μm. **9**, Cluster of conidiophores emerging from the leaf surface but not through the stomata (arrows). No surface mycelium is evident. Scale = 10 μm. **10**, Simple and branched conidiophores with attached conidia. Scale = 10 μm.

fungal developments. In contrast, trees that were moved to the greenhouse after the 48-hr infection period had vigorous, bright green leaves, and conidiation occurred 7–8 days after inoculation. The time for lesion development in our study was similar to that reported by others (6,10,11,27), even though we moved trees from dew chambers at 23 C to a greenhouse where temperatures ranged from 22–29 C.

Emmett and Parbery (8) indicated morphological variation of appressoria may be induced by factors such as temperature, moisture availability, light intensity, hardness of surfaces, chemicals, and genetics. Also, they pointed out that time was important and suggested that germ tube growth in an environment unsuited for complete appressorial development could account for additional variability. In our studies, nearly 75% of germ tubes examined produced a recognizable appressorium. Whereas some appressoria developed as an inflated structure adjacent to the conidium when germination occurred, others formed germ tubes of varied lengths that extended from the conidium. Also, more than one infection site per appressorium occasionally was observed. Because the trees in this study were subjected to uniform inoculation and incubation conditions, other factors such as nutritional condition of conidia might have caused some variability. Another factor of possible influence on the pathogen's germ tube-appressorial morphology may be the occurrence of physiological races of *C. caryigenum* (3). The high adaptability of this pathogen to newly-bred selections and cultivars has been reported (3,6).

Infection-site specificity was studied by Preece et al (24), who found that 90% of conidia of *Erysiphe polygoni* on red clover leaves and 92% of conidia of *Peronospora parasitica* on cauliflower leaves formed appressoria in the junction areas between the anticlinal walls of adjoining epidermal cells. A greater rate of exosmosis of cellular nutrients over cell junctions is thought to stimulate germination and growth of organisms in these sites (25). Ruinen (26) showed that growth of microorganisms began in the depressions over anticlinal walls of the epidermal cells and spread to cover the entire leaf surface. The twisted and somewhat zig-zag pattern of germ tube growth of *C. caryigenum* suggest attraction to a specific penetration site. All infection sites were observed to occur over adjacent anticlinal cell-wall junctions. After penetration, subcuticular, repent hyphae continued to grow over these cell-wall junctures.

Mercer et al (19), working with *Phaseolus vulgaris* inoculated with *Colletotrichum lindemuthianum*, and McKeen (18), working with *Vicia faba* inoculated with *Botrytis cinerea*, have described and illustrated the development of the germination pore and have shown how a penetration peg arose from the pore region in the appressorium to enter host tissues. A similar condition occurs in *C. caryigenum*, as shown in our results.

Anatomical studies on leaves of scab-resistant and scab-susceptible pecan cultivars by Wetzstein and Sparks (28) showed that susceptible cultivars had a high density of variable-size peltate trichomes. The trichomes frequently abutted on the abaxial surface of scab-susceptible Schley leaves, and secretions were found adjacent to some trichomes. They (28) suggested that high trichome densities might contribute to a microclimate favorable for scab development by supporting a humid environment, or aid in the retention of free water on the leaf surface. Also, secreted substances might promote development and germination of conidia. In our study of the possible effect of trichomes on infection incidence, only 16% of 2,000 conidia-encounter sites were associated with peltate trichomes on the adaxial leaf surface. The count might have been higher had our study included the abaxial leaf surface where more trichomes are found. Nevertheless, 82.2% of those conidia grew to the base of the trichomes where infection occurred.

C. caryigenum developed a subcuticular hyphal network in pecan leaf tissues in a manner broadly similar in terms of position to the development of *Venturia inaequalis* in apple leaf tissues (21). As the subcuticular hyphal network of *C. caryigenum* developed, however, basal bulbs formed as branches along the hyphae, conidiophores ruptured the leaf cuticle, and conidiation commenced. The host cuticle exhibited a high degree of plasticity by expanding with the bulging of the melanized conidiophore basal bulbs while not rupturing. In fact, the only breaks in the cuticle other than stomates were those made by the forced emergence of conidiophores from the basal bulbs. These observations contrast with the report of Demaree (5) that the cuticle of pecan leaves was destroyed by anastomosing hyphae.

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