

## Some Ultrastructural Observations of *Cladosporium caryigenum* Growth in Pecan Leaves

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### ABSTRACT

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After the fungus *Cladosporium caryigenum* infected pecan leaves, hyphae grew subcuticularly over both upper and lower leaf epidermal cells. Hyphae also extended into the leaf interior through the middle lamella of adjacent epidermal cells. All hyphal growth was intercellular. Conidiophores arose from bulbous cells that developed on branches of the subcuticular hyphae. Cell walls of the bulbous cells became melanized

before conidiophore development. Extension of *C. caryigenum* conidiophores occurred by elongation of the bulbous cell and rupture of host cuticle. There was an accumulation of vesicles in the fungal cell near the point of rupture of the host cuticle. A collar, formed by the ruptured cuticle, surrounded the base of the conidiophore. Clusters of concentric bodies were found in conidiogenous cells, but not in vegetative hyphae.

*Additional keywords:* *Fusicladium effusum*, pecan scab, ultrastructure.

*Cladosporium caryigenum* (Ellis & Langl.) Gottwald, the causal organism of pecan scab, is the most serious pathogen of pecan in the southeastern United States. Scab is the principal disease of concern to growers because severely infected nuts may drop prematurely or kernels may not develop even though the nuts remain attached to the branches. Scab may cause twig blight and extensive leaf spotting that may result in tree defoliation (7,13,14,19).

Early light microscope studies indicated that during the infection process *C. caryigenum* hyphae grew on the leaf surface and destroyed the epidermis and cuticle (4,19). A more recent study (15) reported development of *C. caryigenum* in pecan tissues using both light and scanning electron microscopy (SEM). The pathogen infected pecan leaves by direct penetration of the cuticle and developed subcuticular stromata without destruction of the leaf cuticle. Subcuticular hyphae were particularly prevalent over anticlinal walls of the host epidermal cells. Lateral protrusions formed along the main subcuticular hyphae of the mature stroma and produced pairs of brown-colored cells. The darker of the pair was the bulbous basal cell of the conidiophore. Studies made with the scanning electron microscope showed that conidiophores originating from the bulbous cells erupted through the cuticle. Mycelium of *C. caryigenum* was not found growing superficially on pecan leaves.

As part of an ongoing study of *C. caryigenum*, we report some detailed ultrastructural observations of fungal growth and conidiophore development on pecan leaves as seen by transmission electron microscopy (TEM).

### MATERIALS AND METHODS

Methods for inoculation of pecan trees with conidia of *C. caryigenum* were detailed in a previous paper (15).

Tissue samples for electron microscopy were collected 10, 15, and 30 days after inoculation. We repeated experiments two times using different trees for each sampling. For SEM, at all three sampling times, two 10-mm-diameter disks were excised from leaves taken from each of six trees. These samples were fixed and critical point dried according to the methods of Latham and Rushing (15), then examined with an ISI SS-40 (International Scientific Instruments, Milpitas, CA) operated at 5 kV. For TEM studies, approximately 50 pieces of leaf tissue bearing fungal colonies were excised 10 days after inoculation and fixed in 1/2 strength Karnovsky's fixative (11) in sodium phosphate buffer (pH 7.0) for 3 h at 4 C. After several washes in buffer, the tissue was postfixed in 1% osmium tetroxide in the same buffer for 3 h at room temperature. After washing in buffer, the tissue was stained overnight in 0.5% aqueous uranyl acetate at 4 C. After it was dehydrated in a graded ethanol series, the tissue was embedded in Spurr's resin. Thick sections were made from 17 samples of *C. caryigenum* infection sites. Repeated thin-sectioning of these samples yielded leaf sections containing conidiophore clusters and associated subcuticular hyphal growth. These thin sections were stained either with a combination of 2% aqueous uranyl acetate followed by lead citrate or by lead citrate alone. Sections were observed using a Philips 300 TEM (Mahwah, NJ) operated at 60 kV.

### RESULTS

The initial infection of pecan leaves by *C. caryigenum* appeared to occur by direct penetration of the host leaf cuticle. Detailed

observations of these early stages of infection were not included in the present work.

After penetration of the host cuticle, the vegetative hyphae of *C. caryigenum* extended subcuticularly over the upper or lower epidermis according to dorso/ventral site of infection, respectively (Fig. 1A). Exposed hyphae were not observed on the leaf surface. Hyphae on both upper and lower leaf surfaces extended intrusively between epidermal cells, through the middle lamella, to form a layer of mycelium below the epidermis, i.e., between cells of the epidermis and the mesophyll (Fig. 1A and B). Hyphae also extended between cells of the mesophyll (Fig. 1A) and several cells deep among cells of leaf veins. All hyphal growth was intercellular; no hyphae were found within the host cells in any stage observed in this study. Woronin bodies (Fig. 1C) were a consistent feature in all stages of hyphal growth. Lobed hyphal cells (Fig. 1D) were common, particularly in the mesophyll. Clusters of hyphae, or stromata, several layers thick, often were observed. Hyphae were observed adjacent to (Fig. 1E) and partially extended over a portion of some stomatal guard cells, in substomatal cavities, and occasionally in stomatal pores (not illustrated here). The stomatal pore region did not, however, appear to be a focal point of hyphal growth. Host walls and cuticle appeared fibrillar in nature adjacent to areas of fungal growth (Fig. 1F).

At the ultrastructural level, the first observable change associated with conidiophore production was the darkening (melanization) of the walls of certain hyphal cells of the subcuticular stromata. Light microscope observations indicated that these darkened cells occurred in pairs with the more darkly staining of the two being the actual cell to produce the conidiophore (15). This arrangement was difficult to observe with TEM due to angles of sectioning, but two cells located end to end, one with more darkly staining walls (Fig. 2A), were observed on at least six occasions. The process of melanization was not determined, but electron-opaque particles (Fig. 2A and B) and vesicles containing a darkly staining substance (Fig. 2C) occasionally were observed during early stages of development. Layers of endoplasmic reticulum located just inside the plasmalemma and extending into the cytoplasm (Fig. 2C and D) also were observed. Microbodies were present in these cells (Fig. 2D) as were lipid bodies (Fig. 2C), lomasomes (Fig. 2D), and microfilaments (Fig. 2D). Early in development, the melanized layer was splotchy and uneven (Fig. 2B) but later became more electron-opaque and of even staining intensity (Fig. 2C and D). After or during melanization, an additional lighter staining wall region became apparent inside the melanized layer (Fig. 2C and D).

These melanized cells formed the base of the conidiophore, with the more heavily melanized cell designated as a bulbous cell. Conidiophores were observed on both the upper and lower leaf surfaces. They were produced only from subcuticular hyphal cells and never were observed to originate from hyphal cells located deeper in the leaf. Conidiophores were not observed to emerge from stomatal pores, although they frequently were found growing from hyphae positioned partially over guard cells. Conidiophores often were produced in clusters as evidenced by apparent clusters of melanized bulbous cells (Fig. 3A) (compare with Fig. 9 in Latham and Rushing [15]).

The actual point of rupture of the cuticle was difficult to observe in thin sections. Although endoplasmic reticulum was common just inside the plasmalemma walls of bulbous cells, the accumulation of other organelles near the outer walls of these cells was not extensive. Vesicles and endoplasmic reticulum were observed, in at least one instance, near a possible point of cuticle rupture (Fig. 3B). The contents of these vesicles were not identified.

Upon rupture of the cuticle, the bulbous cell expanded outward to extend the hyphal cell (Fig. 3A and C). After outward expansion, the melanized wall layer gave the impression of decreasing staining intensity from the base of the conidiophore outward (Fig. 3C-E). Frequently, a new cell cross wall was observed in the bulbous cell at or near the point of rupture (compare Fig. 3A and C). A collar or ring surrounded the base of the conidiophore after rupture of the cuticle and outward expansion of the bulbous cell (Fig. 3E and F). This collar was a remnant of the ruptured

cuticle, and its distinctness depended on the thickness of the leaf cuticle where rupture occurred. A very thin cuticle resulted in a collar that was barely visible, while a thicker cuticle resulted in an obvious ring (Fig. 3E). An additional layer of electron-lucent material with an outer electron-opaque boundary often was observed on the outer surface of the base of the conidiophore, immediately above the point of cuticle rupture and extending along a portion of the conidiophore (Fig. 3C, D, and F).

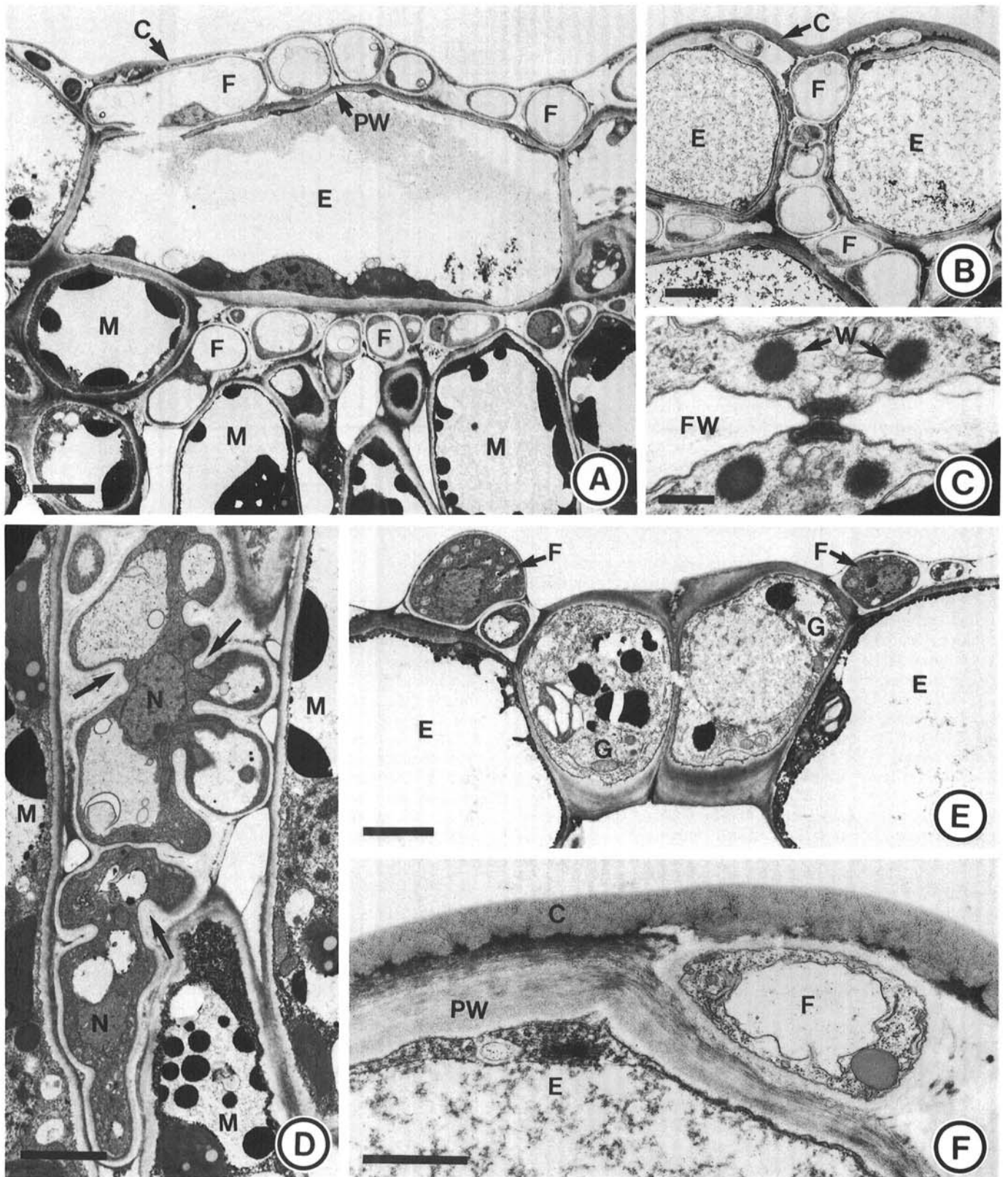
Conidiogenous cells differed very little from vegetative hyphal cells. The walls of conidiogenous cells often were thicker and, as mentioned previously, contained the darkly staining substance assumed to be melanin. These cells also contained concentric bodies, often in clusters of 20 or more (Figs. 3D and E and 4A). There was no apparent consistent location and orientation of the concentric bodies, although they were observed near the base of the lowermost melanized cell and occasionally were associated with the nucleus. They never were observed in vegetative hyphae. The origin and function of these bodies are unknown. Although membranous vesicles and membranous profiles occasionally were observed in the region surrounding the concentric bodies and intermingled with them (Fig. 4A), there was no direct evidence to suggest that these membranes were involved in the development of the concentric bodies. Concentric bodies were comprised of a translucent inner core surrounded by a more densely staining region. This densely staining region contained layers of different staining intensity (Fig. 4B). The surface of the concentric body was covered by lamellae that had a bilayer membrane structure (Fig. 4B) and that in certain sections appeared to originate from within the dense layer. The entire concentric body was surrounded by an electron-lucent region containing no other densely staining features (Fig. 4A and B).

## DISCUSSION

Infection of pecan leaves by *C. caryigenum* occurred by direct penetration of the leaf cuticle (15). In this study, we did not investigate the process of initial infection in detail. However, our previous light microscope and SEM observations (15) and these TEM observations of fungal growth did not reveal any mechanical injury of the leaf through which the pathogen may have entered, nor was penetration of the leaf through stomatal openings observed.

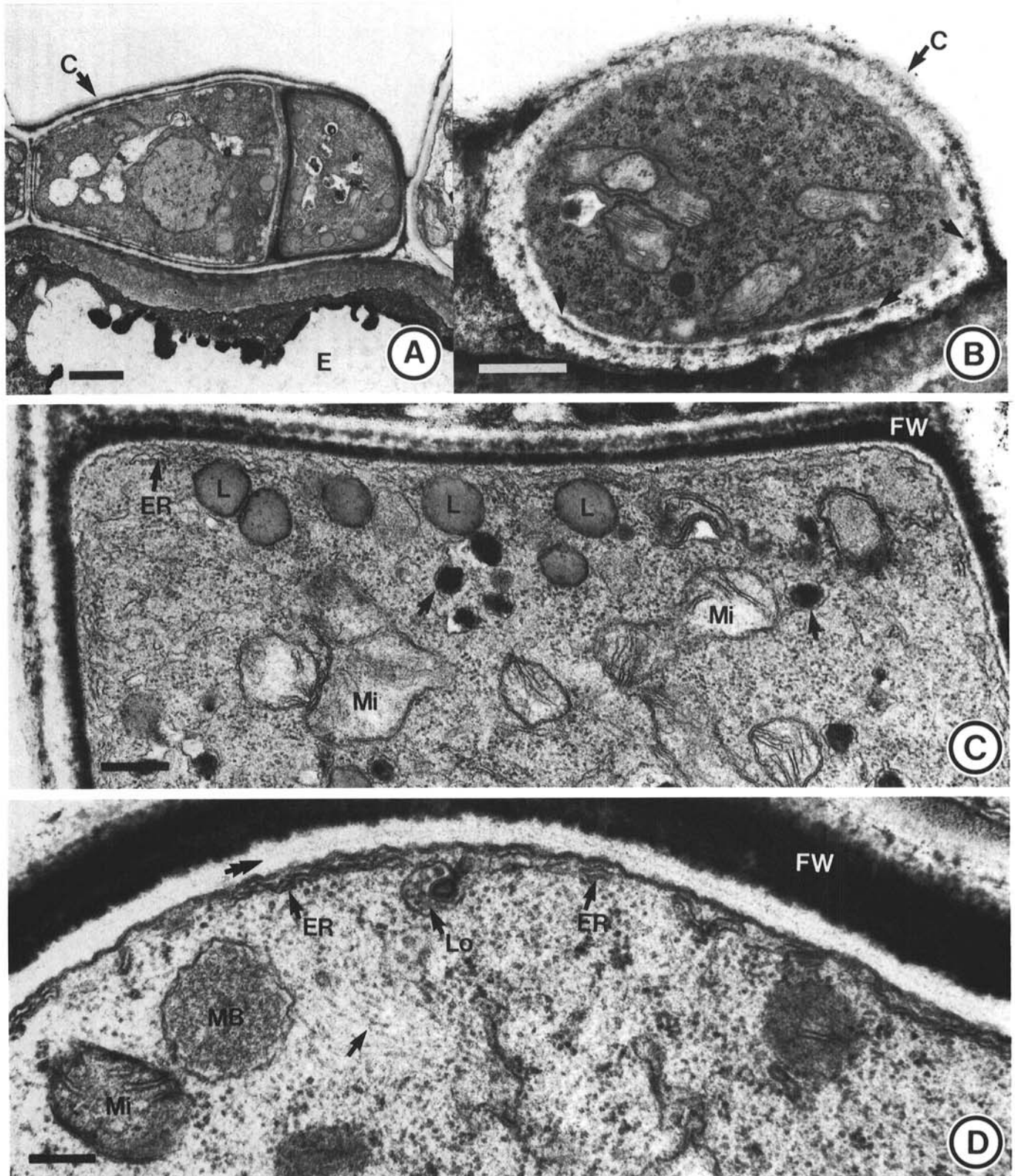
The formation of conidiophores in *C. caryigenum* was preceded by the melanization of the cell walls of certain cells of the subcuticular hyphae. Ellis and Griffiths (5,6) found lateral walls of conidiogenous cells to be extensively melanized in several species of *Torula*, but apical regions of these same cells were pigmented only lightly. They concluded that this facilitated a system whereby rigid lateral walls directed internal pressure to the apical region of the cell, enabling rupture of the cuticle and outward extension of the fungal cell to occur. A study by Howard and Ferrari (10) showed that melanin enabled appressoria of *Magnaporthe grisea* to maintain high internal pressures, making host penetration possible. In *C. caryigenum*, all walls of the basal bulbous cells appeared to be equally melanized as evidenced by uniform staining intensity. It is possible that the physical rupture of the host cuticle involved melanization and the resulting mechanical stresses caused by the buildup of internal pressures. Due to the accumulation of vesicles near possible points of rupture (Fig. 3B), however, the role of enzymatic activity resulting in a weakened cuticle at these points of rupture cannot be overlooked. The decreasing staining intensity from the base to the tip of the extended conidiophore (Fig. 3A) suggests that expansion of the conidiophore may be due to stretching of the existing wall rather than to addition of new wall material.

The origin of melanin in *C. caryigenum* is unclear. No dictyosomelike organelles, which could be implicated in the formation of melanin, have been encountered in this fungus. Recent studies of fungi using freeze substitution have given better preservation of membrane systems (18,23), however. In fact, Golgi bodies have been identified in the conidiophore of *Aspergillus nidulans* fixed by freeze substitutions (18). This method may reveal interesting membranous organelles in *C. caryigenum* as well. Other organelles



**Fig. 1.** Vegetative hyphae of *Cladosporium caryigenum* on pecan leaves. **A**, An overall view of fungal hyphae growing between the leaf cuticle and the outer epidermal cell wall and below the epidermis extending into the mesophyll. **B**, Fungal hyphae between the plant cuticle and the outer epidermal wall, between epidermal cells, and below the epidermis. Fungal hyphae extend through the middle lamella region of adjacent epidermal cells to reach the inner regions of the leaf. **C**, Woronin bodies are common in these fungal cells and typically are located near or within the septal pore. **D**, An extensively lobed (arrows) hyphal cell extending into the mesophyll. Lobed hyphal cells were observed frequently. **E**, Fungal hyphae extending over part of the guard cells but not within the stomatal pore. **F**, A hyphal cell extending between the cuticle and the plant cell wall. The plant cell wall in this region is fibrillar. C, cuticle; E, epidermis; F, fungus; FW, fungal cell wall; G, guard cells; M, mesophyll; N, nucleus; PW, plant cell wall; W, Woronin body. Bars = 3.0, 3.0, 0.2, 2.0, 3.0, and 1.0  $\mu$ m, respectively.

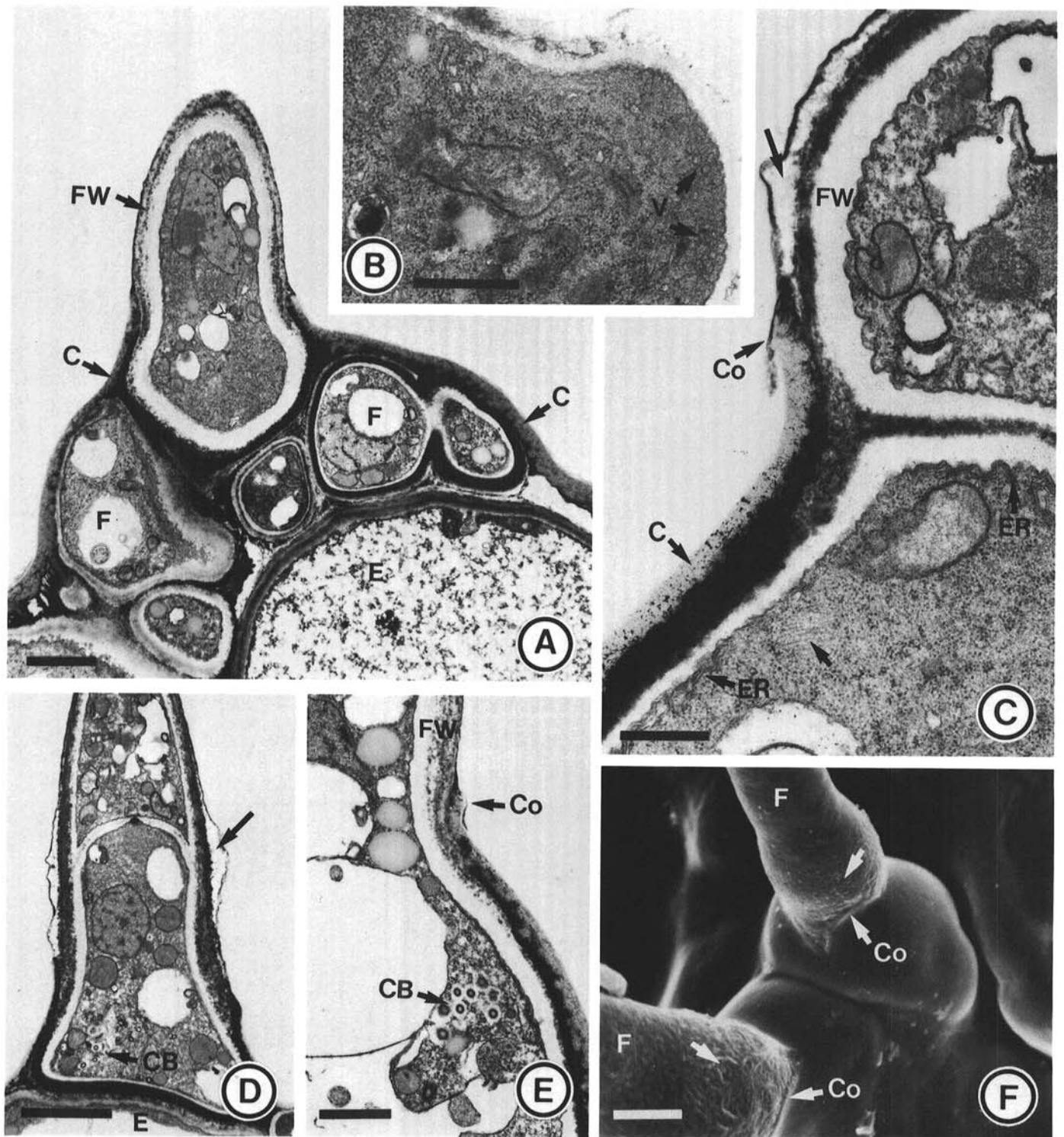




**Fig. 2.** Stages in melanization of the cell wall of *Cladosporium caryigenum*. **A**, A pair of melanized fungal cells between the plant cuticle and the outer epidermal cell wall. **B**, An early stage of fungal cell wall melanization showing the splotchy deposition of melanin (arrows). Vesicles with osmiophilic contents and other smaller osmiophilic deposits are present in the fungal cytoplasm at this stage. **C**, A slightly later stage of melanization showing endoplasmic reticulum just inside the plasmalemma. Mitochondria, lipid bodies, microbodies, and vesicles with osmiophilic inclusions (arrows) also are present. Note the narrow electron-lucent inner region of the fungal cell wall. **D**, A later stage of melanization of the fungal wall showing a darkly staining wall region with an electron-lucent inner region (double arrow). Fine filaments (arrow) frequently are found in the cytoplasm. C, cuticle; E, epidermis; ER, endoplasmic reticulum; FW, fungal cell wall; L, lipid body; Lo, lomasome; MB, microbody; Mi, mitochondrion. Bars = 1.0, 0.5, 0.5, and 0.2  $\mu\text{m}$ , respectively.

that may be associated with melanization include endoplasmic reticulum, present in these cells as extensive systems just within the plasmalemma, and microbodies, reported by Maxwell et al (17) to be involved in the process of melanization. Numerous

large electron-opaque bodies and smaller round bodies were observed in the cytoplasm of the bulbous cells of *C. caryigenum*, but we have no direct evidence of their relationship to the cell wall.



**Fig. 3.** Conidiophore formation in *Cladosporium caryigenum*. **A**, A cluster of melanized fungal cells with one conidiophore extending through the cuticle in this plane of section. **B**, A detailed view of a fungal cell at a possible future site of cuticle rupture. In this region, small vesicles are located in the fungal cytoplasm and near the plasmalemma of the fungal cell. **C**, A higher magnification of the point of rupture of a fungal cell through the plant cuticle. A collar forms from the plant cuticle around the base of the extending conidiophore (compare with **F**). An electron-lucent substance (long arrow) covers the base of the conidiophore. Fine filaments (short arrow) are present in the cytoplasm of the fungal cell. **D**, The base of a conidiophore showing the electron-lucent material (arrow) surrounding the lower region above the point of cuticle rupture. **E**, The base of a conidiophore showing the collar formed by the rupture of the plant cuticle. Note there is no electron-lucent material around the base of the conidiophore in this section. **F**, Scanning electron micrograph of the bases of two conidiophores. The collar formed by the plant cuticle is distinct in both (compare with **C** and **E**). The fibrillar material near the base of each conidiophore (arrows) corresponds to the electron-lucent material described in **C** and **D**. **C**, cuticle; **CB**, concentric body; **Co**, collar; **E**, epidermis; **ER**, endoplasmic reticulum; **F**, fungus; **FW**, fungal cell wall; **V**, vesicles. Bars = 0.2, 0.5, 0.5, 2.0, 3.0, and 3.0  $\mu\text{m}$ , respectively.



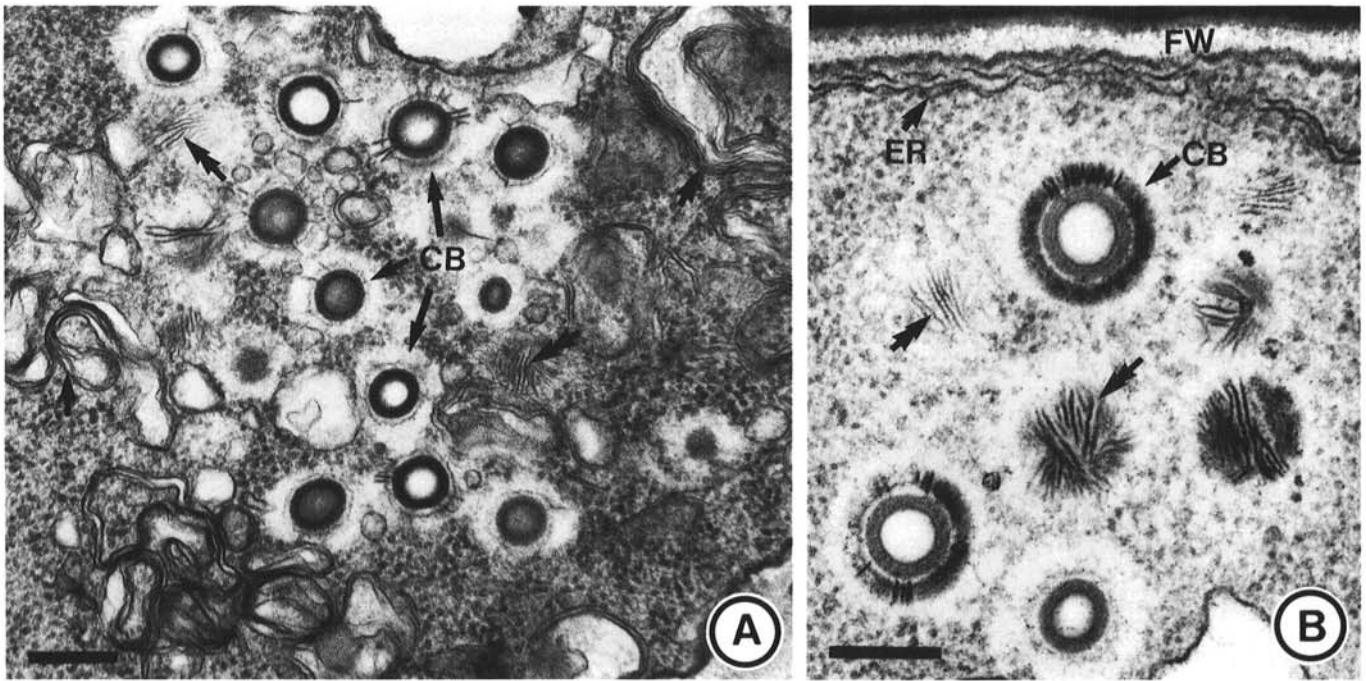


Fig. 4. Concentric bodies in conidiogenous cells of *Cladosporium caryigenum*. A, A cluster of concentric bodies each surrounded by an electron-lucent region. Sections taken near the surface of the concentric body reveal projecting lamellae (double arrow). Convoluted layers of membrane (arrows) may contribute to the formation of concentric bodies. B, A higher magnification revealing the bilayer structure of the lamellae (double arrows) projecting from the concentric body. CB, concentric body; ER, endoplasmic reticulum; FW, fungal cell wall. Bars = 0.2  $\mu$ m.

Concentric bodies were abundant in the conidiophore-producing cells of *C. caryigenum* but not in vegetative hyphae. Our observations of concentric body structure are in agreement with previous descriptions (2,8,9). Concentric bodies were first described from lichenized fungi (2) and have since been observed in the mycobiont of a number of lichens (9) and other fungi (1,8,22). While there are some variations in the ultrastructural details in these accounts, there are also several consistent features. An electron-lucent core is surrounded by a densely staining layer(s) covered by membranous lamellae. It has been suggested that concentric bodies, with their membranous surface lamellae, may provide increased surface area for biochemical reactions (8). Many concentric bodies are usually located together in a zone lacking any other densely staining material. Griffiths and Greenwood (9) found clusters of concentric bodies near or in contact with the nucleus in *Peltigera aphthorsa*. The type of cell in which concentric bodies have been found varies widely among the species examined thus far. In *Venturia inaequalis*, for example, concentric bodies were found in cells of the perithecial wall and occasionally in the vegetative hyphae and conidiophores (8). Griffiths and Greenwood (9) found concentric bodies in all areas of the lichen thallus and in the apothecium, but not in the asci or ascospores. In *C. caryigenum*, concentric bodies were observed only in those cells of the subcuticular stromata in the process of producing conidiophores, i.e., melanized cells and in the resulting conidiophores. We did not make detailed observations of conidia, thus we do not know if concentric bodies also exist in these cells.

We observed no major ultrastructural changes in the host leaf tissue as a result of early infection by *C. caryigenum*. In *C. caryigenum*, as in *C. cucumerinum* (21), pathogen growth was confined to the middle lamella region of adjacent host cells. Lazarovits and Higgins (16), in a study of *Fulvia fulvum* (as *Cladosporium fulvum*) infection of tomato, found very few degenerative changes in tomato host cells in early stages of infection. It was only after or near the time of sporulation that substantive changes were observed. They concluded that a good nutritional relationship was maintained between the host and the pathogen until fungal reproduction commenced. This type of growth, without visible detrimental effects to the host, also characterizes *V. inaequalis* infection of apple tissues (3). *V. inaequalis* derives its nutrition from underlying and surrounding host cells without

the formation of intracellular hyphae and the subsequent destruction of host tissue in early stages of infection. It forms subcuticular stromata several layers thick from which conidiophores arise (20). As in *C. caryigenum*, the host cuticle displays a remarkable capacity for stretching over these subcuticular growths of fungal hyphae. In *C. caryigenum*, destruction of leaf cells associated with intercellular fungal growth was only observed in late stages of pathogenesis (unpublished observations). The host cell wall generally had a more fibrous nature when adjacent to fungal growth (Fig. 1F), however. Paus and Raa (21) reported that in the vicinity of *C. cucumerinum* hyphae, the host cell wall was fibrous in appearance, possibly due to degradation brought about by the presence of the pathogen. This type of growth may indicate that substances from the cell wall, particularly sugars, could be important energy sources for the pathogen (12,21).

The factors that influence and limit growth of *C. caryigenum* on pecan are as yet unknown. One way that plants may resist pathogen attack and subsequent infection is by the production of chemicals that inhibit fungal growth, for example, phytoalexins (24 and references therein). Additional studies of *C. caryigenum*, including enzyme and cytochemical localizations of both the fungus and the host pecan, may not only provide evidence concerning mode of growth but also may give clues to the factors that limit and determine the extent and severity of pathogenesis by this fungus.

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