Ultrastructure of Penetration of Ethylene-Degreened Robinson Tangerines by Colletotrichum gloeosporioides

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ABSTRACT

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The appressorium of *Colletotrichum gloeosporioides* produced a narrow, threadlike infection hypha less than 0.5 μm in diameter which emerged through a pore in the wall of the appressorium. The pore was surrounded by a funnel-shaped collar that was formed by extensions of the appressorium wall. The cone, comprised of a distinct layer of wall material, was formed upon the collar and was continuous with the wall of the emerging infection hypha. The infection hyphae penetrated the cuticle, and upon approaching the epidermal cell wall, development proceeded in one of three distinct ways. Most frequently, the hyphae enlarged into primordial hyphae, 1-2 μm in diameter, either between or within the epidermal cell wall or lumen. These hyphae, upon septation, gave rise to larger hyphae 3-5 μm in diameter. In other instances, the infection hyphae formed primordial hyphae in the cuticle which grew subcuticularly along the epidermal cell wall and intercellularly between and below the epidermal cells. Occasionally, the infection hyphae penetrated the cuticle and immediately enlarged into the larger 3-5 μm in diameter hyphae. Only minor organelle changes were evident in advance of the invading large hyphae; the most striking change occurred in the chloroplasts.

Additional key words: postharvest decay, anthracnose, citrus.

Anthracnose, which is caused by *Colletotrichum gloeosporioides* Penz., is a serious postharvest decay of Robinson tangerines *(Citrus reticulata* Blanco) (6). However, the disease only develops when green-colored mature tangerines are exposed to ethylene (2, 3, 6) during degreening. Degreening is a procedure used to improve the color of the fruit by degrading the chlorophyll in the peel, and thereby revealing the orange pigments.

Observations with the light microscope using paraffin-embedded tangerine peel revealed that penetration of *C. gloeosporioides* from latent appressoria was by very narrow (less than 1 μm in diameter) thread-like infection hyphae which were formed when tangerines were treated with ethylene (2). As the infection progressed, these structures gave rise to much larger hyphae ranging in diameter from 2-5 μm.

These ultrastructural studies were undertaken to obtain more detail of the penetration process and ontogeny of the infection hyphae. Observations also were made of the fine-structural, cellular changes that occurred in the host during and after penetration.

MATERIALS AND METHODS

Mature, green-colored Robinson tangerines were prepared for inoculation with *C. gloeosporioides* as previously described (2, 3). Spores were obtained from 7-day-old cultures grown on Difco potato-dextrose agar at 26 C under 6,456 lux of continuous fluorescent light. Spores were removed in sterile water, concentrated by centrifugation at 2,340 *g* for 5 min, and resuspended to a concentration of approximately 250,000 spores/ml of sterile water. Spore droplets then were placed on a 6-mm diameter area of the fruit equator, and the fruit were held overnight near 100% relative humidity at 26 C for formation of the appressoria. Fruit then were treated the following day with ethylene at a concentration of either 50 or 100 μliters/liter of air, using a system previously described (3). Peel was removed at 3 days after inoculation following 0, 8, 15, or 24 hr of exposure to ethylene and at 5 days after inoculation following 96 hr of exposure to ethylene to provide a source of infected tissue with varying stages of fungal penetration.

Infected tissue was fixed with 3% glutaraldehyde in phosphate buffer at pH 7, postfixed with 2% osmium tetroxide in additional buffer, and dehydrated with increasing concentrations of acetone at 4 C. The material was infiltrated with Spurr's resin, thin-sectioned with an ultramicrotome, stained with uranyl acetate and lead citrate, and viewed and photographed with a Philips 201 electron microscope.

RESULTS

Anatomy and germination of appressoria.—The upper surface of the appressorium of *C. gloeosporioides* was enclosed by a double-layered wall covering (Fig. 1). This

covering extended over the surface of the appressorium wall to the slime layer (Fig. 1, 3) at the juncture of the appressorium and the fruit cuticle. The lower portion of the appressorium wall in contact with the fruit cuticle contained the pore (Fig. 2) which provides an opening for the emerging infection hypha. The appressorium wall surrounding the pore extended inward into the appressorium to form a funnel-shaped collar (Fig. 1, 2, 4). The cone (Fig. 1, 4), composed of distinct wall material, was formed upon the collar and extended from beyond the collar edge within the appressorium to the pore where it was continuous with the wall of the emerging infection hypha. The infection hypha did not cause any apparent depression of the cuticle during penetration (Fig. 1, 4).

Infection hypha penetration and ontogeny.—During penetration of the cuticle, the infection hypha maintained its original diameter of less than 0.5 μm until it approached the wall of the epidermal cell. Further development of the infection hypha then proceeded in one of three distinct patterns.

The most frequent manner of penetration occurred by extension and enlargement of the infection hypha to form a primordial hypha, 1-2 μm in diameter, which extended further to form larger hyphae, 3-5 μm in diameter. Development of the infection, primordial, and large hyphae is seen in sections (Fig. 5, 6) of the cuticle and epidermis. The primordial hypha was formed either at or in the anticlinal wall (Fig. 6), within the periclinal cell wall of the epidermis (Fig. 7), or within the lumen of the epidermal cell (Fig. 8). Wall appositions occasionally were observed on the inner surface of the epidermal cell wall (Fig. 8). The primordial hypha was separated from the large hypha by a septum (Fig. 6, 7) which was seen to be perforated when viewed in additional sections. The large hyphae continued to grow throughout the peel and were responsible for most of its decay.

Less frequently, the infection hypha formed the primordial hypha in the cuticle next to the epidermal cell wall (Fig. 9), and continued to grow subcuticularly (Fig. 10). Upon reaching the juncture of two epidermal cells (Fig. 11), the hypha grew between the epidermal cells and subsequently between the epidermal and second layer of cells causing the cell walls to separate along the middle lamella (Fig. 12)

Occasionally, the infection hypha continued to penetrate the cuticle, and did not form the primordial

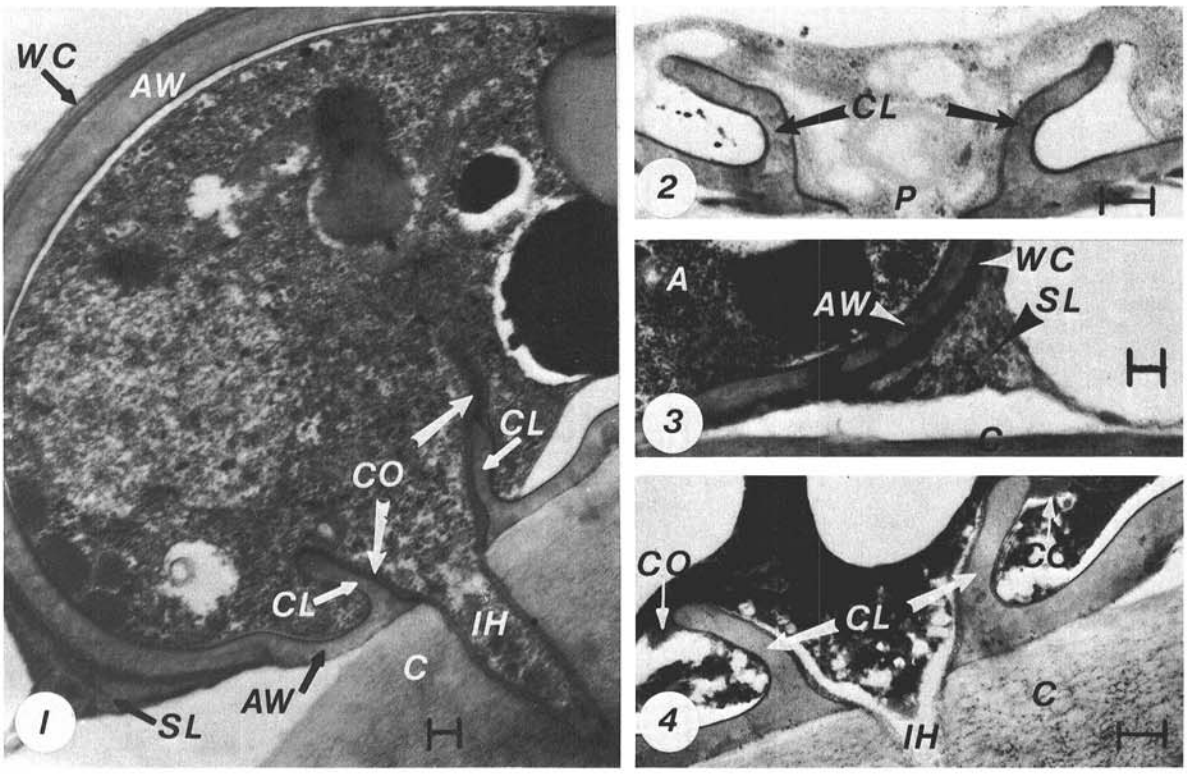

Fig. 1-4. Anatomy and germination of the appressoria of *Colletotrichum gloeosporioides;* calibration bar = 0.2 μm. **1)** Appressorium exhibiting the thick cell wall with its surrounding wall covering. The appressorium contains the collar which is formed by extensions of the appressorial wall. The cone which is formed on the collar consists of wall material that is continuous with the wall of the newly formed infection hypha. **2)** Collar surrounding the pore of an appressorium detached from the fruit cuticle. **3)** Slime layer which may help adhere the appressorium to the fruit cuticle. The layer has been separated from the cuticle during tissue preparation. **4)** A non-median section through an appressorium during early penetration of the cuticle by the infection hypha. Note that the cuticle is not depressed. Legend: AM = appressorial membrane; AW = appressorial wall; CL = collar; CO = cone; C = cuticle; IH = infection hypha; and P = pore.

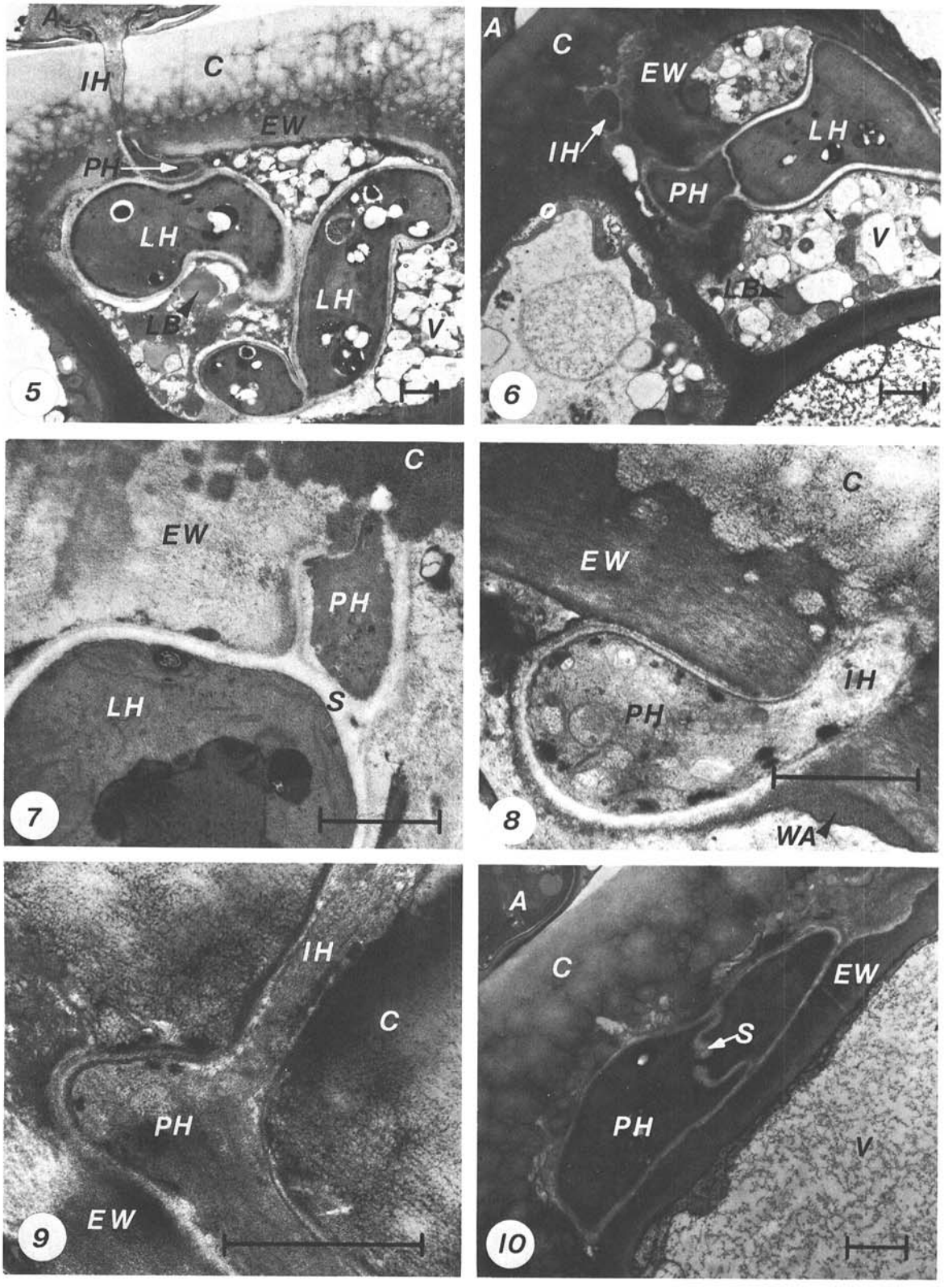

Fig. 5-10. Infection hypha ontogeny of *Colletotrichum gloeosporioides;* calibration bar = 1.0 μm. **5)** Appressorium with infection hypha penetrating the cuticle and epidermal cell wall. The hypha has enlarged into the primordial hypha which, in turn, has formed the large hyphae. Note the numerous vacuoles in the host cell. **6)** Curved and swollen infection hypha causing deterioration of the cuticle before forming the primordial hypha that has forced the epidermal cell wall inward during penetration. Host cell contains many vacuoles with numerous lipid bodies and swollen mitochondria. **7)** Primordial hypha formed in the epidermal cell wall separated from the large hypha by a septum. **8)** Enlarged view of the infection hypha penetrating the epidermal cell wall and forming the primordial hypha. Note wall apposition formed on the cell wall. **9)** Infection hypha showing development into the primordial hypha which is growing subcuticularly. **10)** Subcuticular primordial hypha with perforated septum. Note disorganization of the surrounding cuticle. Legend: A = appressorium; C = cuticle; CV = cell vacuole; EW = epidermal wall; IH = infection hypha; LB = lipid body; LH = large hypha; PH = primordial hypha; S = septum, and WA = wall apposition.

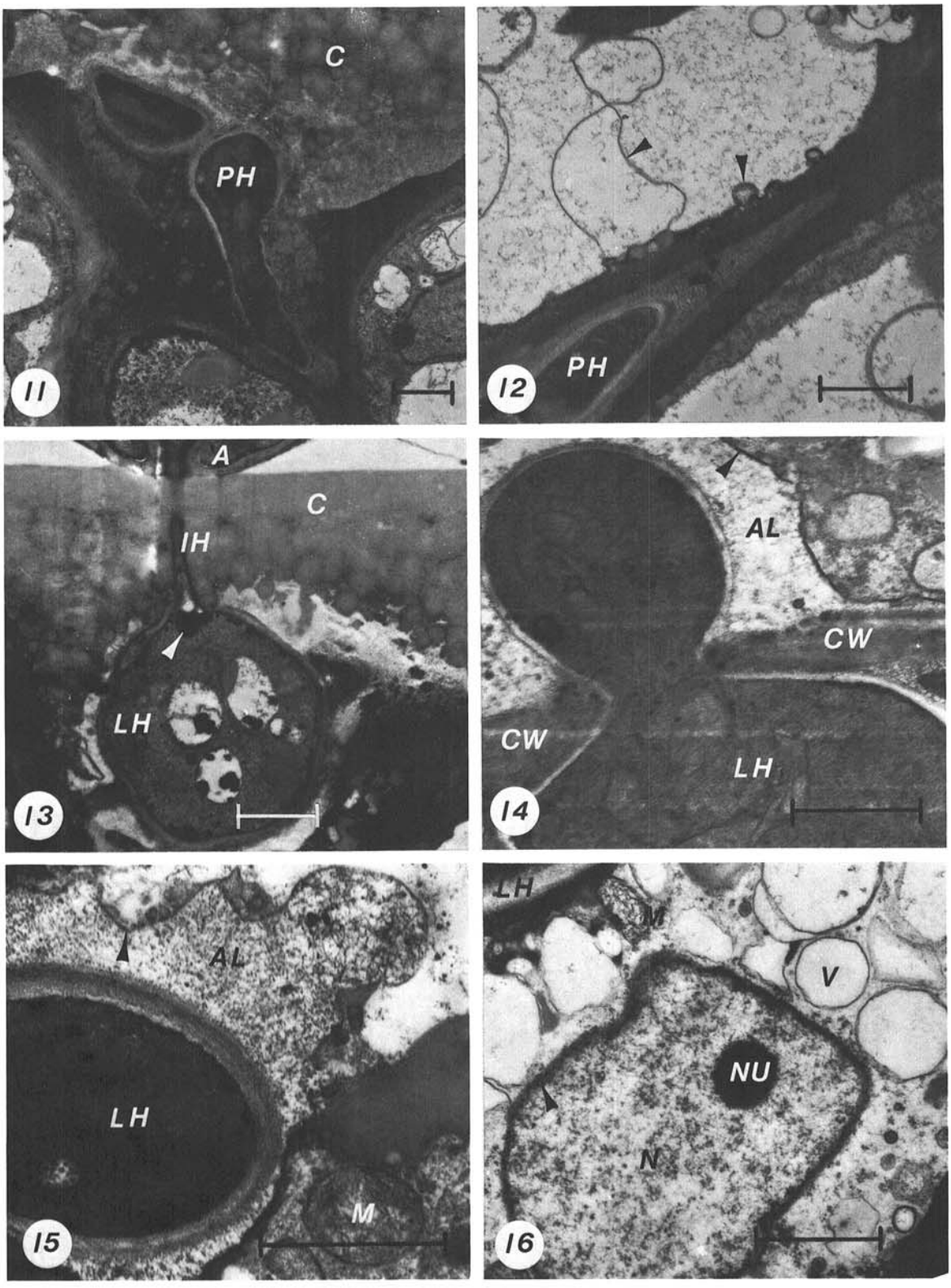

Fig. 11-16. Infection hypha ontogeny and cellular invasion by *Colletotrichum gloeosporioides;* calibration bar = 1.0 μm. **11)** Primordial hypha growing in the cuticle and penetrating between the epidermal cells. **12)** Primordial hypha growing between the epidermal and second cell layer. Note separation of the cell walls along the middle lamella in advance of the hypha and the invaginated, dark-staining tonoplast (arrows) of vacuoles in the adjacent cell. **13)** Infection hypha which has enlarged directly into a large hypha upon penetration of the cuticle. **14)** Constriction of the large hypha as it penetrates the host cell wall. Plasmalemma (arrow) of the invaded cell is invaginated and an appositional layer is present. **15)** Invaginated host plasmalemma (arrow) surrounding a large hypha and separated from it by the appositional layer. **16)** Nucleus with deteriorating nuclear envelope (arrow) near a large hypha. Note the numerous vacuoles and the deteriorated mitochondrion. Legend: A = appressorium; AL = appositional layer; CW = cell wall; C = cuticle; IH = infection hypha; LH = large hypha; M = mitochondrion; NU = nucleolus; N = nucleus; PH = primordial hypha; and V = vacuole.

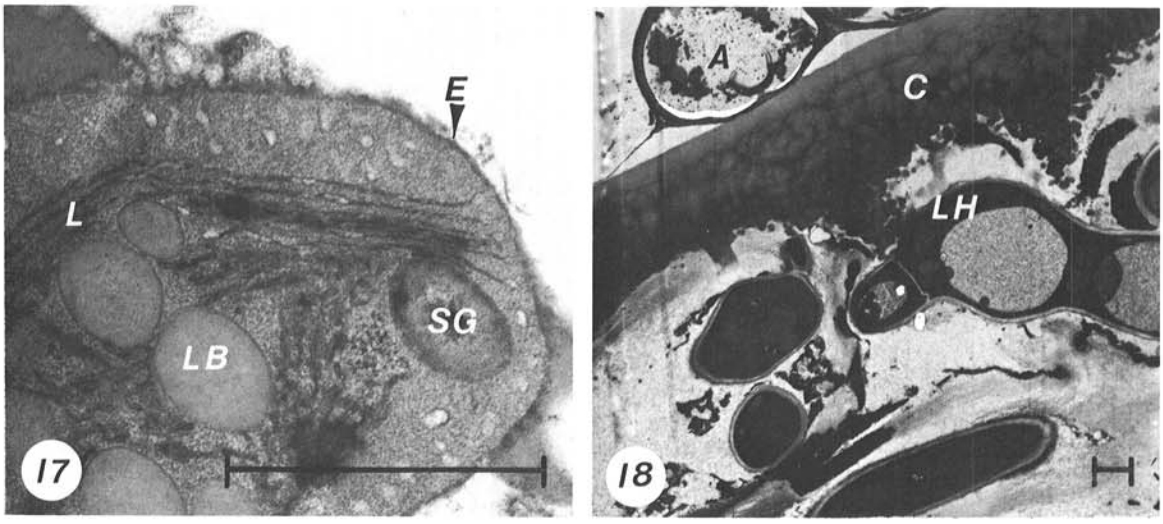

Fig. 17-18. Cellular invasion by *Colletotrichum gloeosporioides;* calibration bar = 1.0 μm. **17)** Chloroplast in cell in advance of invading large hyphae. Note dissolution of the envelope, internal membrane (lamellae) system, and starch grain, and the lack of intense staining of the lipid bodies. **18)** Destruction of the two layers of cells beneath the intact cuticle by the large hyphae. Legend: A = appressorium; C = cuticle; E = envelope; L = lamellae; LH = large hypha; LB = lipid body; and SG = starch grain.

hypha, but immediately enlarged into the large hypha either between (Fig. 13) or within the lumen of the epidermal cells.

Cellular changes associated with infection.—Intracellular large hyphae of *C. gloeosporioides* did not kill host cells in advance of penetration or cause severe disruption of invaded cells. The hyphae were constricted at the site of host wall penetration as the fungus grew from one cell into the next (Fig. 14). In early stages of penetration, the hyphae were surrounded by the invaginated host plasma membrane and an intervening appositional layer of varying thicknesses (Fig. 14, 15). Host nuclei maintained the normal double-membraned envelope even after hyphal cell penetration, but as pathogenesis advanced, disintegration of the nuclear envelope became apparent (Fig. 16). Associated with this response was the development of a cell with numerous vacuoles (Fig. 5, 6, 12, 16), lipid bodies (Fig. 5, 6), and degenerating mitochondria (Fig. 16). Chloroplasts appeared most sensitive to infection and responses were noted therein one to two cells in advance of invading hyphae (Fig. 17). Lamellae of affected chloroplasts were swollen and later disintegrated, and lipid bodies stained less intensely (Fig. 17). With further development of the large hyphae, the cell walls of invaded cells were destroyed, but the overlying cuticle remained intact (Fig. 18).

DISCUSSION

Three discrete paths of invasion may be followed by *C. gloeosporioides* during infection of Robinson tangerines treated with ethylene for color enhancement. Development of the infection hyphae during penetration of the cuticle and peel produced various ultrastructural manifestations. The infection hyphae most frequently formed primordial hyphae which, in turn, gave rise to the larger hyphae. Less frequently, the primordial hyphae grew below the cuticle and intercellularly between and below the epidermal cells without formation of the larger hyphae. Occasionally, the infection hyphae penetrated the cuticle and immediately enlarged into the large hyphae.

The ultrastructure of the appressorium and infection hypha of *C. gloeosporioides* resembled in several ways that described for *C. lindemuthianum* (4). The terms collar and cone, which are used to identify structures produced by *C. lindemuthianum,* also were used in this study to describe similar structures produced by *C. gloeosporioides. Colletotrichum lindemuthianum* also produced very narrow infection hyphae, 0.5 μm in diameter or less, during penetration of the host cuticle (4). Additional enlargement occurred during penetration of the epidermis in a fashion similar to the primordial hyphae of *C. gloeosporioides,* but without subcuticular or intercellular growth before expansion into large subepidermal hyphae.

Formation of the collar of *C. gloeosporioides* appeared to be simultaneous with the formation of the appressorium. Dissolution of the wall of the appressorium to form the pore, as described during germination of appressoria of *C. graminicola* (5), was not observed. The dissolution process may have occurred at an earlier stage in the development of the appressorium than was studied in this investigation. Wall material which comprised the cone was continuous with the wall of the newly-formed infection hypha. Therefore, the cone could be considered as part of the infection hypha and synthesis of the cone probably represents one of the initial stages in the formation of the hypha.

Penetration of the cuticle by the infection hypha does not appear to be mechanical, as indicated by the lack of an inward depression of the cuticle during penetration. If

penetration is enzymatic, then activity may be extremely limited and restricted only to the tip of the invading hypha as suggested previously (1). The fact that the cuticle surrounding the infection hypha after penetration was not degraded also would suggest that the enzyme activity was restricted only to that area adjacent to the tip of the infection hypha. The production of such a cutinolytic enzyme may be restricted to the initial penetration of the outer part of the cuticle which contains cutin and cuticular wax (7). The observed degradation of the inner portion of the cuticle suggested the production of cellulolytic and/or pectinolytic enzymes which can degrade cellulose and pectin present in this part of the cuticle (7).

During penetration of the outermost part of the cuticle, the infection hypha retains its original shape, perhaps because of restrictions placed upon it by the surrounding cuticular wax and cutin. Enlargement of the infection hypha took place only after its tip had reached the inner portion of the cuticle where cuticular degradation occurred. At this point, the infection hypha formed the primordial hypha. Continued growth of this hypha only occurred subcuticularly or intercellularly. Development into the larger hypha may have been prevented because of pressures exerted upon the primordial hypha as it grew between the cuticle and the epidermal cell wall and between walls of two contiguous epidermal cells.

Colletotrichum gloeosporioides is a typical intracellular fungal pathogen that does not kill cells in advance of invasion or cause severe cell disruption soon after penetration (1). The hyphae normally are surrounded by the invaginated host plasma membrane upon penetration and are separated from the membrane by an appositional layer.

The chloroplasts appeared to be the most sensitive of all organelles in the cell to the invading hyphae of *C. gloeosporioides*. Deterioration of the chloroplasts and subsequent pigment loss produces the silver halo that surrounds appressoria during initial stages of infection as reported earlier (2).

These studies revealed considerably more detail of the infection process than was previously obtained with light microscopy (2). However, the role of ethylene in inducing susceptibility to infection of mature, green-colored Robinson tangerines by *C. gloeosporioides* remains to be defined.

LITERATURE CITED

1. BRACKER, C. E., and L. J. LITTLEFIELD. 1973. Structure of host-pathogen interfaces. Pages 159-313 *in* R. J. W. Byrde and C. V. Cutting, eds. Fungal pathogenicity and the plant's response. Third Long Ashton Symposium, 1971. Academic Press, New York. 499 p.
2. BROWN, G. E. 1975. Factors affecting postharvest development of Colletotrichum gloeosporioides in citrus fruits. Phytopathology 65:404-409.
3. BROWN, G. E., and C. R. BARMORE. 1977. The effect of ethylene on susceptibility of Robinson tangerines to anthracnose. Phytopathology 67:120-123.
4. MERCER, P. C., R. K. S. WOOD, and A. D. GREENWOOD. 1971. Initial infection of Phaseolus vulgaris by Colletotrichum lindemuthianum. Pages 381-389 *in* T. F. Preece and C. H. Dickinson, eds. Ecology of leaf surface microorganisms. Academic Press, London. 640 p.
5. POLITIS, D. J., and H. WHEELER. 1973. Ultrastructural study of penetration of maize leaves by Colletotrichum graminicola. Physiol. Plant Pathol. 3:465-471.
6. SMOOT, J. J., and C. F. MELVIN. 1967. Postharvest decay of specialty hybrid citrus fruits in relation to degreening time. Proc. Fla. State Hortic. Soc. 80:246-250.
7. VAN DEN ENDE, G., and H. F. LINSKENS. 1974. Cutinolytic enzymes in relation to pathogenesis. Annu. Rev. Phytopathol. 12:247-258.