Infection Processes of *Pestalotia subcuticularis* on Leaves of *Hymenaea courbaril*

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ABSTRACT

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Pestalotia subcuticularis causes serious leaf blighting in some species of tropical trees, such as the leguminous species *Hymenaea courbaril*. *P. subcuticularis* forms latent (symptomless) infections as well as leaf spots. Processes of entry, intracellular ramification, latent infection, and lesion formation by *P. subcuticularis* in leaves of *H. courbaril* were followed with light, scanning electron, and transmission electron microscopy. Spores of *P. subcuticularis* germinated within 6 to 12 hr after inoculation onto leaves of *H. courbaril* from greenhouse trees, and epidermal penetration occurred within 12 to 24 hr after germination. Hyphae entered leaves directly without formation of appressoria and did not enter through stomata. During latent infections, hyphae grew beneath leaf cuticles and between mesophyll cells without any apparent damage to plant tissues other than localized cuticular degradation. Inoculated unwounded leaves from greenhouse trees often had symptomless infections for several weeks. Active infections formed within 1 wk if leaves were wounded by cutting or scraping. In active infections, hyphae grew through tracheary elements, causing vein discoloration. Cells were killed before hyphal entry. Affected cells had distorted chloroplast membranes, decreased numbers of starch grains, and cell walls that were degraded and flocculent in appearance. Inhibitory secondary compounds (resins and tannins) in leaves of *H. courbaril* may act to maintain infections in the latent phase.

Pestalotia species (Deuteromycetes, Melanconiales) are common in both temperate and tropical ecosystems and frequently are associated with leaves of woody plants (7). In temperate zones most *Pestalotia* species are saprophytic (10), but in the tropics they have caused serious leaf blighting in trees (4). Tropical species of *Pestalotia* frequently are associated with tea, mango, and mangrove (7,10,17). Several species of *Pestalotia* infect leaves of *Hymenaea courbaril* L., a tropical resin-producing tree. *Pestalotia subcuticularis* Guba is a common species associated with *Hymenaea* over a wide range of ecosystems (4). In the present study we have used scanning and transmission electron microscopy and light microscopy to examine the infection process of *P. subcuticularis* in leaves of *H. courbaril*. Our examination of the leaf-fungus relationship is part of a long-term investigation of resin-producing trees.

The tropics have a greater variety of insect herbivores and fungal pathogens than temperate zones. Because resins are known to deter insect feeding and inhibit fungal growth (33), it has been hypothesized that insects and fungi have acted as selective forces in the maintenance of resin production and variation in resin composition (18–22). Studies of fossil resins (ambers) have shown a striking correlation between resin production in angiosperms and tropical environments (18–22).

Leaf resins in *Hymenaea* are composed of several sesquiterpene hydrocarbons and a few sesquiterpene oxides, as well as diterpenoids (26, Figliuolo and Langenheim, *unpublished*). *Hymenaea* leaf resins have deterrent effects on insects (23–25,34), and one resin component, caryophyllene oxide, inhibits fungal growth, including that of *Pestalotia* spp., in vitro (2,3). Each leaf resin-secreting structure in *Hymenaea* is composed of two parts: the lumen (termed "pocket"), which is a spherical to ovoid intercellular space into which resin is secreted, and a single layer of epithelial cells lining the pocket. Pockets are visible to the naked eye as translucent dots, which, in leguminous genera, often are called punctae. In accordance with Schnepf's (32) terminology, we will refer to *Hymenaea* leaf resin secretory structures as glands. *Hymenaea* leaves also contain phenolic compounds, mainly

condensed tannins (9,27), which are inhibitory to fungal growth (2). Tannins are located in mesophyll cell vacuoles.

A developmental study of *Hymenaea* leaf resins and phenolics (9) has shown that terpene yield is greatest early in leaf development and that tannins are highest in the bud. This pattern may play a significant role in the inhibition of fungal growth in young leaves and the formation of latent infections. Our interest in the relationship between leaves producing secondary compounds and fungi has led us to examine closely the infection process of *Hymenaea* leaves by *P. subcuticularis*. Infection processes by *Pestalotia* spp. in leaves have not been studied in detail. Our objectives were to determine how *P. subcuticularis* penetrates and ramifies in leaves of *H. courbaril*, how latent and active infections affect leaf ultrastructure, and how infections may relate to secondary chemicals.

MATERIALS AND METHODS

Cultures of *P. subcuticularis* (American Type Culture Collection 60263) originally collected from leaves of *H. courbaril* near Puerto Marquez, Guerrero, Mexico, were maintained in petri dishes on half-strength potato-dextrose agar at room temperature. Fresh cultures were made weekly. Acervuluslike spore clusters formed at the edges of mycelia within 1 wk after inoculation on agar. Conidia for inoculations were collected with sterile loops from 0.5-mm spore clusters of 7- to 9-day-old cultures and suspended in sterile distilled water. For inoculations, two spore clusters, each measuring 0.5 mm, were suspended in 10 ml of distilled water. Counts of suspensions with a hemacytometer showed that they contained about 10^4 spores/ml. Mature leaves to be inoculated were chosen randomly from 2.5-m-tall greenhouse saplings of *H. courbaril* (JHL collection number 4969) grown from seeds collected near Puerto Marquez.

Mature detached leaves or 2-cm leaf disks were surface disinfested with 3% sodium hypochlorite for 5 min and incubated in a damp chamber at 20 C with spore suspensions on either the adaxial or abaxial surface. The spore suspension was placed carefully in the center of leaf disks or detached leaves, which were floated on water containing 5 μg/ml of kinetin to postpone senescence.

Inoculations were made on greenhouse trees by enclosing one leaflet of a leaf with 2 ml of spore suspension in a plastic bag. Attached leaves to be inoculated first were disinfested with cotton dipped in 3% sodium hypochlorite followed by a sterile water rinse. Before inoculation of attached and detached leaves, some leaves were wounded by gently scraping the cuticles with a sterile spatula. Infection was verified at various intervals after inoculation by surface disinfestation with sodium hypochlorite and incubation of leaves in a damp chamber. For light microscopy, fresh hand sections or whole mounts were stained with lactophenol containing 1% cotton blue and mounted in light corn syrup. Some leaves first were cleared with 10% sodium hydroxide, followed by aqueous chloral hydrate (250 g/100 ml), and then stained with lactophenol containing cotton blue. For localization of phenolics, fresh sections were stained with 5% aqueous ferric chloride. Leaf resins were localized by staining fresh sections with 1% aqueous Nile Blue at 37 C for 1 min and then rinsing in 1% acetic acid.

Small leaf areas (less than 1 mm^2) for examination by transmission electron microscopy (TEM) were sampled from healthy and infected attached leaves 10 days after inoculation. Samples from infected leaves were taken from four areas: lesions containing acervuli; lesion edges, where some green tissue was evident; discolored veins near lesions; and areas outside lesions, where tissue was apparently healthy.

Because *Hymenaea* leaves are thick and have heavy cuticles, good TEM fixation was difficult to obtain, and several protocols were attempted. The best fixation resulted from a 4-hr primary fix of 3% glutaraldehyde and 3% acrolein in 0.1 M phosphate buffer, followed by overnight postfixation in 1% osmium tetroxide in a refrigerator. Vials were kept on ice and under vacuum during the primary fixation and were swirled every 10 min. After osmium postfixation, tissue was dehydrated in an acetone series followed by three changes in propylene oxide, then embedded in Epon/Polybed resin. Thin sections (about 90-nm thick) were cut with a Reichert OM U3 microtome (Reichert Scientific Instruments, Buffalo, NY) and mounted on uncoated copper grids. Sections were stained 15 min in uranyl acetate (saturated solution in 50% ethanol) and 10 min in Millonig's lead stain (29). Micrographs were made with a Siemens 101 transmission electron microscope (Siemens Mfg. Co. Inc., Freeburg, IL) operated at 80 kV.

Leaf samples (about 1 or 2 mm^2) for scanning electron microscopy (SEM) were cut from infected attached leaves, fixed 2 hr at room temperature on a rotator in 3% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, and dehydrated in an acetone series before drying in a critical point drier (Balzers, Hudson, NH). Specimens were mounted on stubs with silver paint and coated with 100 Å gold-palladium with an SPI Module Vac/Sputter coater.

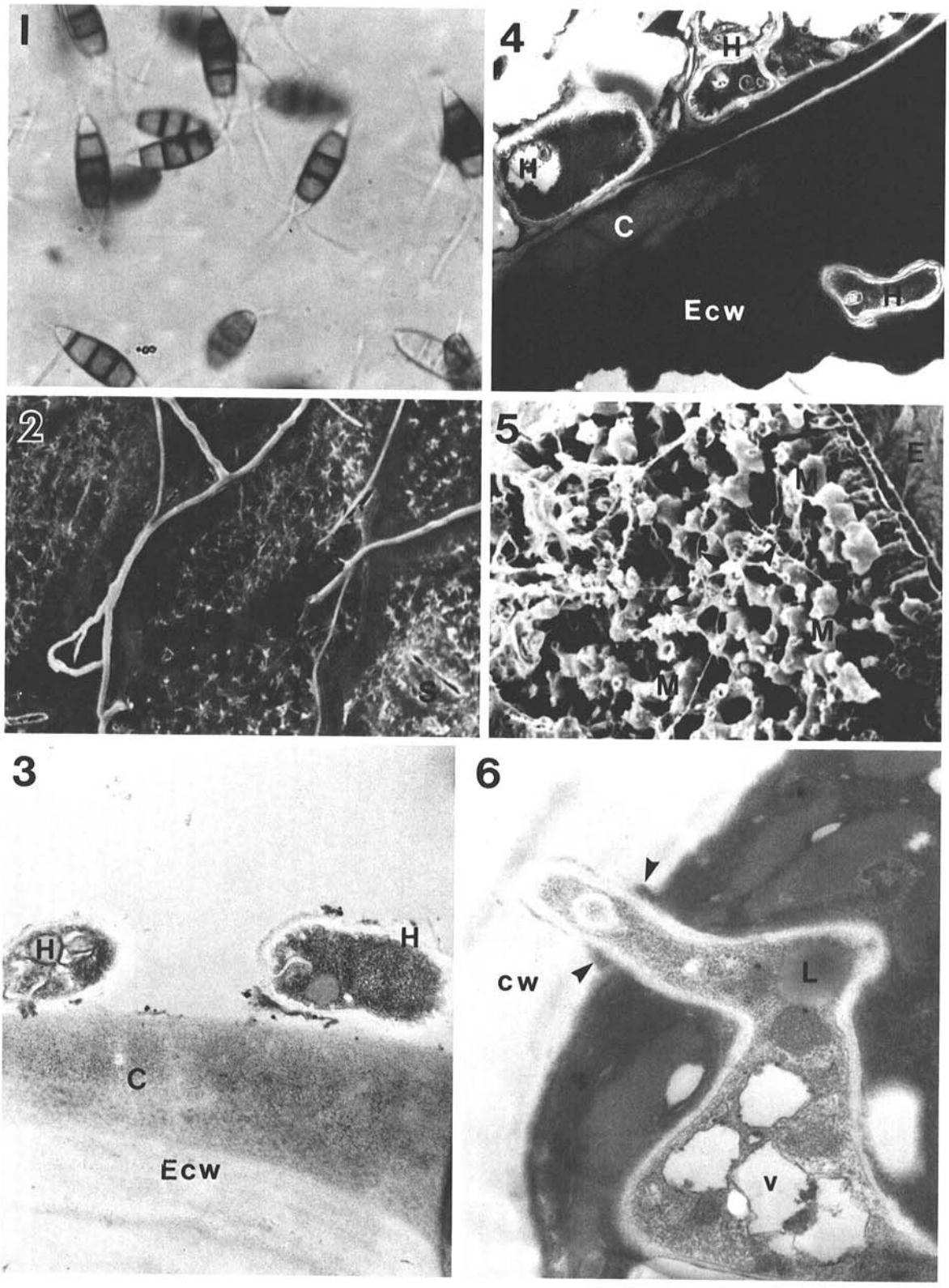
RESULTS

The sequence of events in penetration and ramification was the same for all leaf treatments but occurred more rapidly in leaf disks, detached whole leaves, or attached wounded leaves. In unwounded leaves of *H. courbaril* still attached to a greenhouse sapling, *P. subcuticularis* caused no obvious symptoms, other than some degradation of the cuticle, over the course of several weeks. Active infections, characterized by hyphae that ramified rapidly and produced lesions or damaged organelles, occurred when inoculated, unwounded attached leaves were wounded or detached. Detached leaves and leaf disks always exhibited lesions within 4 days of inoculation. Wounding of attached leaves before inoculation usually resulted in lesion formation within 1 wk. Our descriptions of the ultrastructure of active infections have been made from both wounded and unwounded attached leaves.

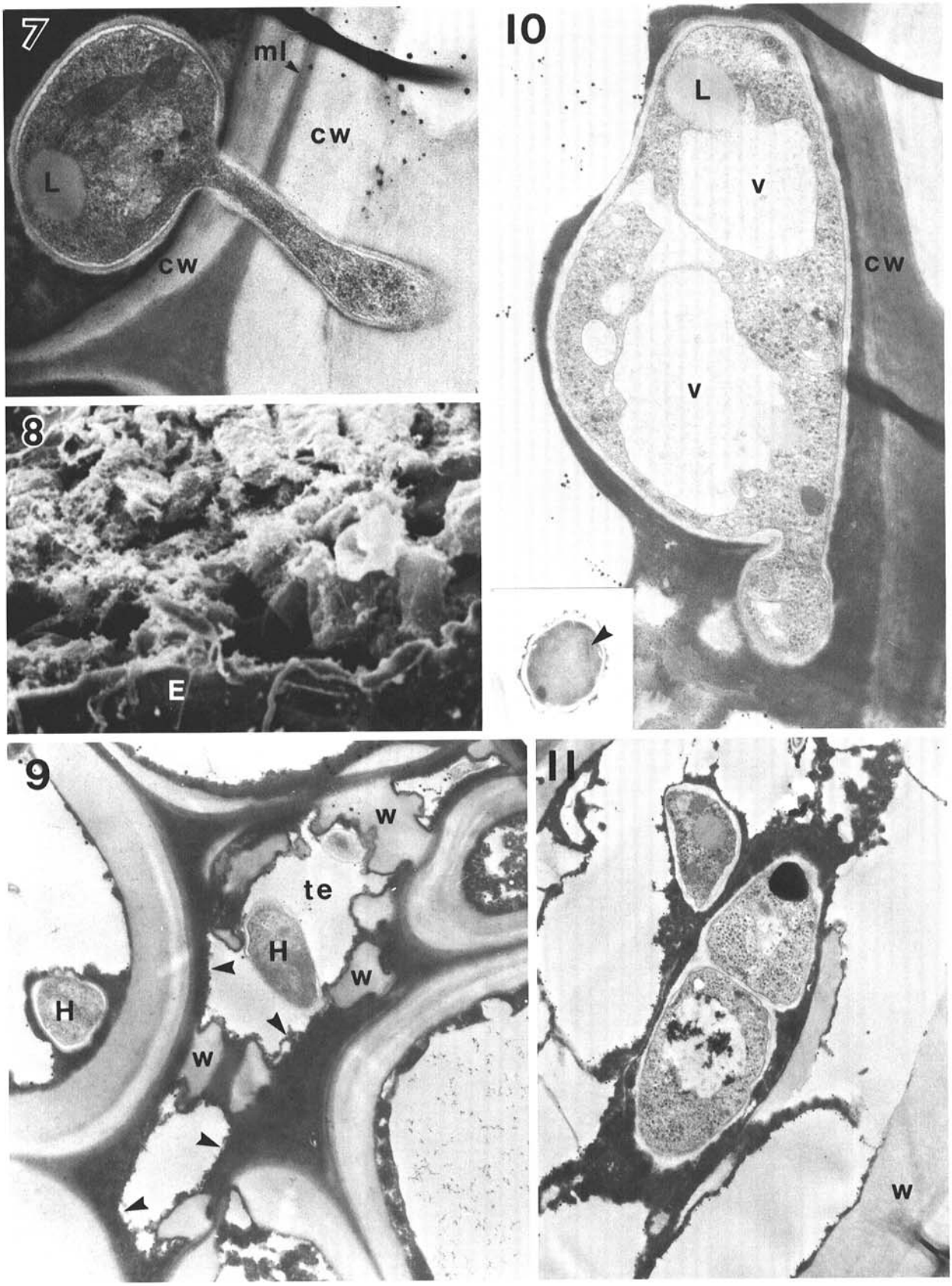
The five-celled spores of *P. subcuticularis* ranged in size from 22 to 27 μm long and 6 to 7 μm wide. Each spore was crowned by three or four setulae, about 22 to 31 μm long (Fig. 1). Spores germinated on leaf surfaces within 6 to 12 hr after inoculation, and epidermal penetration by conidial germ tubes occurred within 12 to 24 hr after germination. Hyphae entered wounds readily but were not seen entering via stomata. In the absence of wounds, entry occurred by direct penetration of the epidermis, without the formation of appressoria. Hyphae on leaf surfaces grew in and beneath the cuticle. The waxy cuticle in the area of growing hyphae was visibly degraded, as shown by SEM (Fig. 2). The cuticle became electron dense in the areas of growing hyphae (Fig. 3). A previous study of healthy tissue showed that cuticle is normally electron translucent (Fail and Langenheim, *unpublished*). As the fungal biomass on the leaf surface increased and hyphae ramified through the cuticle and epidermal outer cell walls, electron density of the cuticle increased, and the walls of epidermal cells also became electron dense (Fig. 4).

After entry into a leaf, hyphae remained for a variable period of time between mesophyll cells and caused no damage obvious with the light microscope. This intercellular growth period was short (a day or two) in detached leaves or disks but continued for up to 6 wk in leaves still attached to the tree. Figure 5 shows intercellular colonization in an unwounded attached leaf 2 wk after inoculation. Mesophyll cell walls at this stage appeared unaffected by the pathogen. The leaf area from which this sample was taken appeared green and healthy. Mycelial growth on the leaf surface of an unwounded leaf continued even after penetration of the leaf.

When a leaf was wounded and an infection became active, leaf cells were killed three to 10 cells in advance of the fungal mycelial front. Hyphae grew quickly into the disrupted cells and often caused a localized increase in electron density of the cell wall (Fig. 6). Hyphae frequently constricted at the point of entry, then expanded after cell walls had been breached (Fig. 7). *Pestalotia* apparently did not enter living cells. SEM examination showed that cell walls became degraded and flocculent in appearance during an active infection (Fig. 8). Severe cell wall degradation such as this did not occur in a latent infection.



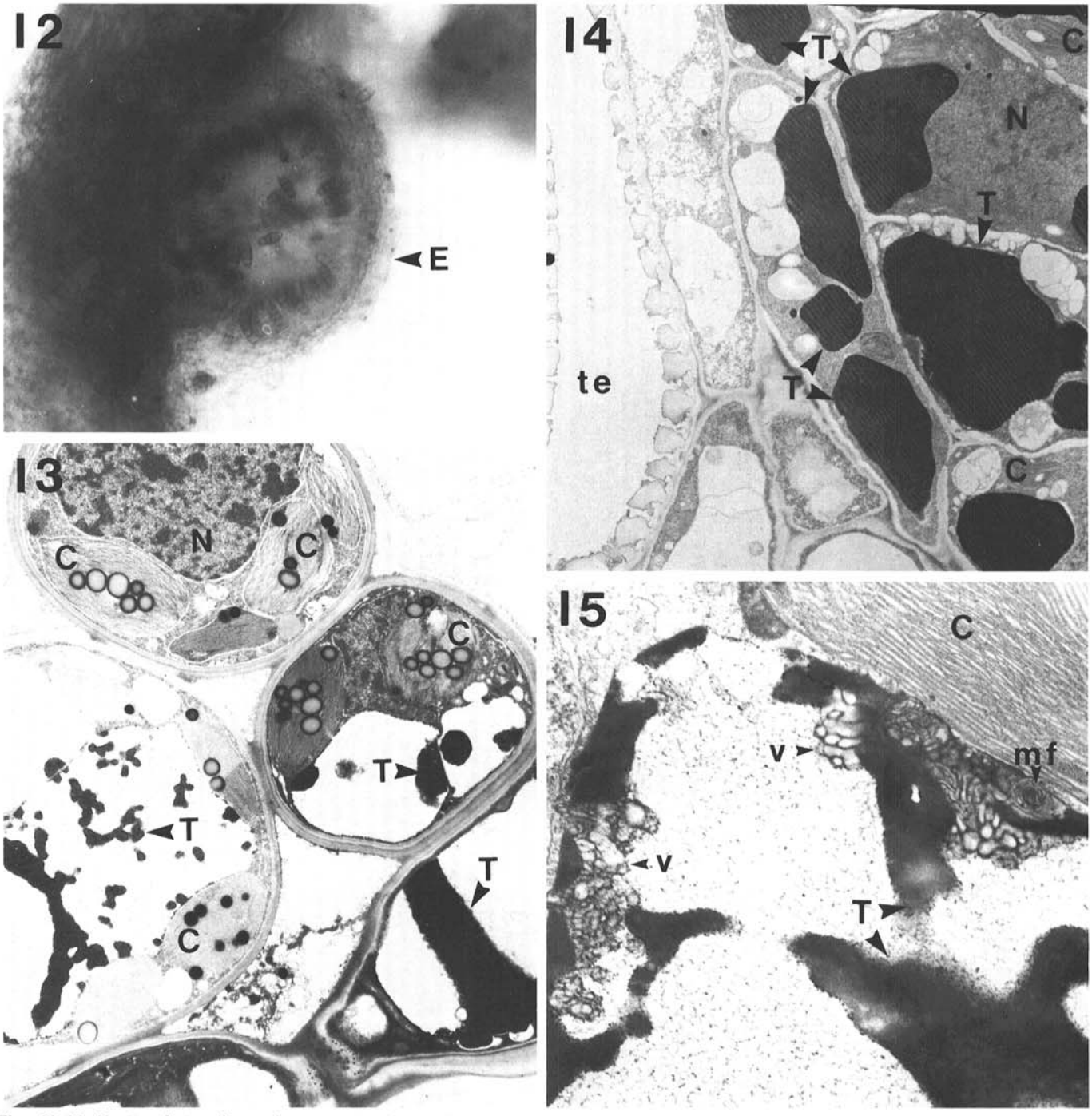
Figs. 1–6. 1, Five-celled conidia of *Pestalotia subcuticularis* are 22–27 μm long and 6–7 μm wide. Each spore is crowned by three or four setulae, each 22–31 μm long. The three central cells have dark brown pigmented walls. From an unstained wet mount. ×850. **2,** Leaf surface of *Hymenaea courbaril* with hyphae of *P. subcuticularis* on and in the cuticle. Cuticular waxes have been degraded in the vicinity of the hyphae. S = stomatal cells. ×319. **3,** Hyphae (H) of *Pestalotia* on surface of leaf of *H. courbaril* during early stage of active infection. Cuticle (C) has increased in electron density due to fungal enzyme activity. Ecw = outer epidermal cell wall. ×11,900. **4,** Advanced active infection, with hyphae (H) on leaf surface and in epidermal wall (Ecw). Division between epidermal wall and cuticle (C) is unclear, and both layers are extremely electron dense. ×11,900. **5,** Leaf with a latent infection. The lower epidermis was peeled away to expose spongy mesophyll cells (M). Fine strands between mesophyll cells are fungal hyphae. A portion of upper epidermis (E) is visible to the right, with the palisade layer (P) immediately beneath it. Sample was taken from an apparently healthy leaf. ×319. **6,** Fungus in a mesophyll cell is entering the adjoining cell. Hyphae do not enter living cells. Electron-dense deposits have formed in the plant cell wall (cw) where the hypha is penetrating. The mesophyll cell is dead, and its cytoplasm is degraded and electron dense. L = lipid, v = vacuole. ×25,500.



Figs. 7–11. 7, Fungus in a dead mesophyll cell, entering a tracheary element. The hypha has contracted at the point of entry and has begun to expand inside the tracheary element. L = lipid, cw = cell wall, ml = middle lamella. ×22,050. **8,** Leaf with an active infection. Part of the upper epidermis has been removed to show that mesophyll cell walls are damaged and appear flocculent and that individual cells cannot be distinguished. A part of the epidermis (E) is visible at the lower edge. Fungal hyphae can be seen on the epidermal surface. Compare with Figure 5, a similar view of a latent infection. **9,** Cross section of a vein from a leaf with an active infection. Tracheary element (te) contains both fungal hyphae (H) and electron-dense phenolic deposits (arrows). w = spiral wall thickenings of tracheary element. ×9,450. **10,** Fungus growing in a dead mesophyll cell. Hypha contains a relatively dense cytoplasm with many ribosomes, small vesicles, and vacuoles (v). Hyphae commonly contain lipid droplets (L) and crystals (arrow, at inset). cw = cell wall. ×17,010. **11,** Septate hypha growing in a tracheary element. Electron-dense deposits surround the fungus. w = cell wall. ×10,260.

As active infections progressed, veins became a light brownish orange in color, visible to the unaided eye. When infected tissue was treated with ferric chloride, tracheary elements stained intensely, indicating the presence of phenolic compounds. Healthy veins did not stain with ferric chloride. Tracheary elements of infected veins contained hyphae and electron-dense deposits (Fig. 9). During latent infections, no vein discoloration occurred, and no hyphae were seen in tracheary elements.

Hyphal cytoplasm was less electron dense than the necrotic plant tissue surrounding it and contained numerous ribosomes, vesicles, and vacuoles (Fig. 10). Hyphae were septate (Fig. 11) and often contained lipid bodies (Figs. 6, 7, and 10) and crystals (Fig. 10, inset). Active infections produced lesions, which increased slowly in size until most of the leaf was affected. Acervuli of *P. subcuticularis* formed at the edges of lesions, where they appeared first as slightly raised areas of the leaf surface (Fig. 12). At maturity, acervuli erupted through the epidermis and cuticle to release masses of spores. Each five-celled spore has two pigmented cells, which makes acervuli visible to the unaided eye as dark brown spots at the edges of necrotic areas. Samples taken from areas immediately adjacent to lesions were often so damaged that no useful information could be gained with TEM.



Figs. 12–15. 12, *Pestalotia subcuticularis* acervulus, just before rupture of leaf epidermis (E) and spore release. Hand section of an infected leaf, stained with lactophenol cotton blue. ×500. **13,** Necrotic cells in an active infection. Chloroplasts (C) have disorganized membranes, no starch grains, and numerous lipid droplets. Tannins (T) are degraded. Compare with healthy tissue in Figure 14. N = nucleus. ×1,800. **14,** Healthy mesophyll with tracheary element (te) and many vacuoles containing tannins (T). "Washboard" pattern is due to sectioning chatter, caused by the extreme density and hardness of tannins after fixation. N = nucleus, C = chloroplast. ×1,800. **15,** Closer view of tannins in an infected leaf. Tannin breakdown is accompanied by the formation of many small vesicles and myelin figures. C = chloroplast, v = vesicle, T = tannin vacuole, mf = myelin figure. ×32,000.

Cells located in green tissue about 1 cm away from lesions showed many signs of cytoplasmic damage, including distorted thylakoids in chloroplasts, a decrease in size and number of starch grains, and increased numbers of lipid bodies (Fig. 13).

Vacuoles of *Hymenaea* leaves often contained condensed tannins, which changed during the infection process. After infection, tannin vacuoles appeared degraded (Fig. 13). In healthy leaves tannin vacuoles were electron dense and had a striated appearance, caused by block vibration of the extremely dense vacuoles during sectioning (Fig. 14). Formation of many small vesicles and myelin figures in the tannin vacuoles was associated with the process of degradation (Fig. 15).

DISCUSSION

P. subcuticulans naturally colonizes newly emerging leaves of *H. courbaril* and may remain latent until leaves are mature (4). Leaf-infecting fungi may enter tissues by direct penetration or through stomata or wounds. Whether direct penetration occurs by the mechanical pressure of infection structures or by production of enzymes has been long debated. Host cuticles and cell walls can be degraded enzymatically during primary penetration (1,16). *P. subcuticularis* produces cutinases (Kolattukudy, *unpublished*), and cuticular degradation of infected leaves of *H. courbaril* is seen with SEM. Hyphae have not been seen entering stomata, and specialized structures such as appressoria have not been found. Entry into the leaf occurred between epidermal cells or through wounds. Wounding the leaf shortened the latent phase of an infection, sometimes decreasing the time required for lesion formation from several weeks to a few days. During the latent phase of an unwounded leaf, subcuticular hyphae ramified extensively and entered intercellular spaces as well.

The stimulus that causes a latent infection to become active is unknown. *P. subcuticularis* did not enter healthy, living cells but quickly ramified in areas where tissues were damaged. In a natural situation, the fungus may live in the cuticles and intercellular spaces of leaves until leaves senesce or are damaged, as by insect herbivory. This situation apparently occurs in Honduran oil palms where *Pestalotiopsis* (*Pestalotia*) lesions are associated with insect feeding wounds (36). *Pestalotia* lesions and acervuli have been seen, however, on otherwise healthy and intact leaves of *Hymenaea* collected from many parts of Central and South America (2), which suggests that insect damage or senescence is not a prerequisite for lesion formation.

The long-lived leaves of tropical trees are thought to be subjected to intense biotic selection pressures from fungi. Phylloplane populations are more dense and more diverse in tropical ecosystems than in temperate zones (5,31). However, there are few detailed examinations of fungus-leaf interactions in tropical trees, and, of these, most have been confined to crop plants such as rubber and coffee (11,15). In tropical conditions the early colonization of leaves by fungi may be very common (14,31). Leaf penetration may initiate a parasitic relationship, but in some cases there is a dormant phase after fungal entry, leading to a latent infection. Latent infections have been noted for many tropical pathogens (37), but little is known about them. Most of the work on tropical latent infections has involved fruits, in which the transition from latent to active infection accompanies ripening (6,8,30). Verhoeff (35) suggests that the decrease in toxic secondary compounds such as tannins which occurs during ripening may allow previously quiescent fungi to become pathogenic.

We have hypothesized that higher resin levels in young leaves may initially inhibit mycelial growth because one resin component, caryophyllene oxide, inhibits fungal growth in vitro (3,12,13). This compound occurs normally in varying amounts in *Hymenaea* leaf resin glands and also will form when caryophyllene (a major resin component) is exposed to air. Damage to resin glands by fungal growth may release small amounts of caryophyllene into intercellular spaces where it can become the inhibitory oxide. We saw no apparent leakage of resin from pockets of infected tissues with Nile Blue staining, but the phenomenon may be extremely localized and transitory. The cell walls of all mesophyll cells, including those of resin gland epithelial cells, eventually were destroyed after an infection passed the latent stage and became active. At this point, fungal biomass in a leaf was presumably large enough to overcome any inhibitory effects of resin released from glands.

Other secondary compounds, such as tannins, also may initially inhibit the growth of mycelia in young leaves of *H. courbaril* because these tannins inhibit the growth of mycelia of *Pestalotia* spp. in vitro (2). When the tannin vacuoles of infected leaves of *H. courbaril* were visibly degraded during the active infection process, tissues became extremely electron dense. This could have resulted partly from tannin leakage into the cytoplasm, which causes increased contrast in plant tissues (28). In active infections, hyphae ramified through the tracheary elements, which developed deposits shown to be phenolics by ferric chloride staining. This phenolic material could have been plant tannins that leaked into the veins, or *P. subcuticularis* may have produced phytotoxic phenolics as hyphae grew in the veins. If the phenolics are fungal in origin, they may have killed plant cells near the veins, from which the hyphae ramified into dead cells.

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CHEVRON CHEMICAL CO., San Ramon, CA

CIBA-GEIGY CORPORATION, Agric. Div., Greensboro, NC

DEKALB-PFIZER GENETICS, DeKalb, IL

DEL MONTE FOODS USA, Walnut Creek, CA

DNA PLANT TECHNOLOGIES, INC., Oakland, CA

E. I. DUPONT DE NEMOURS & CO., INC., Agric. Chem. Dept., Newark, DE

ELI LILLY & CO., Lilly Res. Labs, Greenfield, IN

FERMENTA ASC CORPORATION, Mentor, OH

FERRY MORSE SEED CO., San Juan Bautista, CA

FUNK SEEDS INTERNATIONAL, INC., Bloomington, IL

GREAT LAKES CHEMICAL CORPORATION, West Lafayette, IN

GRIFFIN CORPORATION, Valdosta, GA

GUSTAFSON, INC., Des Moines, IA

HARRIS MORAN SEED CO., Hayward, CA

H. J. HEINZ CO., Bowling Green, OH

HOECHST ROUSSEL AGRI. VET. CO., Somerville, NJ

ICI AMERICAS, INC., Mountain View, CA

ICI AMERICAS, INC., Richmond, CA

ILLINOIS CROP IMPROVEMENT ASSOCIATION, Urbana, IL

ILLINOIS FOUNDATION SEEDS, INC., Champaign, IL

ISTITUTO DI FITOVIROLOGIA, Torino, Italy

JANSSEN PHARMACEUTICA, Piscataway, NJ

LANDIS INTERNATIONAL, Valdosta, GA

LOXTON RESEARCH CENTRE, Loxton, Australia

MERCK & CO., INC., Rahway, NJ

MOBAY CORPORATION, Kansas City, MO

MONSANTO CO., St. Louis, MO

NOR-AM CHEMICAL CO., Wilmington, DE

NORTHFIELD LAB—DEPT. OF AGRICULTURE, Adelaide, Australia

NORTHRUP KING CO., Woodland, CA

PEST PROS, INC., Plainfield, WI

PETOSEED CO., INC., Woodland, CA

PFIZER, INC.–TEKCHEM, Chem. Div., New York, NY

RHONE-POULENC AG COMPANY, Research Triangle Park, NC

RICERCA, INC., Painesville, OH

RJR NABISCO INC., Winston-Salem, NC

ROHM & HAAS CO., Philadelphia, PA

SAKATA SEED AMERICA, INC., Salinas, CA

SANDOZ CROP PROTECTION CORP., Des Plaines, IL

O. M. SCOTT & SONS, Marysville, OH

TWYFORD INTERNATIONAL, INC., Sebring, FL

UNIROYAL CHEMICAL CROP PROT. R&D, Bethany, CT

UNOCAL CHEMICALS, West Sacramento, CA

VALENT USA CORPORATION, Tallahassee, FL

W-L RESEARCH, INC., Evansville, WI