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Cytology of cork layer formation of citrus and limited growth of *Elsinoe fawcettii* in scab lesions

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Abstract

Ultrastructural aspects of host–parasite interactions were investigated in fruits and leaves of citrus (satsuma mandarin) infected with *Elsinoe fawcettii*. Fungal infection induced host tissues to form cork layers bordering the necrotic areas below the infected sites. The cork layers were composed of compact host cells with convoluted cell walls and alternating lamellations, indicating ligno–suberized tissues in the wound periderm. No host tissues below the cork layers were invaded by hyphae. Hyphae grew intercellularly and intracellularly, often causing hypertrophy and compartmentalization of infected host cells. Also, host cells adjacent to invading hyphae showed accumulation of electron-dense materials and the formation of host cell wall protuberances in intercellular spaces. Hyphae had concentric bodies that showed an electron-transparent core surrounded by an electron-dense layer with radiating filamentous structures on their surface. One or more intrahyphal hyphae were found in the cytoplasm of intercellular or intracellular hyphae. These results suggest that the ligno–suberized cork layers in the wound periderm of citrus act as a protective barrier, which leads to restricted growth of *E. fawcettii* in bordered scab lesions. The fungus is thought to form concentric bodies and intrahyphal hyphae as a survival mechanism against the water- and nutrient-deficient environments that occur in the cork layers of necrotic host parts.

Introduction

Citrus scab is one of the most important diseases that reduce the external quality of fruits for the fresh market (Timmer, 2000). The disease produces fruit blemishes and leaf pustules of many cultivars in humid citrus-growing areas. Satsuma mandarin (*Citrus unshiu*), which occupies over 95% of the 26,000 ha used for citrus production in Jeju Island, Korea, is particularly susceptible to citrus scab (Hyun et al., 2001). The disease is caused by an ascomycetous fungus *Elsinoe fawcettii* (anamorph *Sphaceloma fawcettii*) which is found in most tropical, subtropical and temperate regions as a pathogen of many herbaceous or woody plants (Sivanesan and Critchett, 1969).

The ecology, etiology and taxonomy of the disease and/or its pathogen have been intensively studied (Hyun et al., 2001; Jenkins, 1931; Whiteside, 1975), whereas histo- or cytopathological studies have been limited. The infection processes of *Elsinoe* spp. have been regarded as either entrance through natural openings including the space between epidermal cells (Mason and Backus, 1969) or crushing and envelopment of epidermis (Mason and Wilson, 1978). Hyphae of *Elsinoe* spp. invaded host tissues mainly intercellularly, inducing extensive host cell divisions, cell wall thickening and deposition of suberin and lignin-like materials in host tissues (Gabel and Tiffany, 1987; Mason and Backus, 1969; Williamson and McNicol, 1989). However, ultrastructural details of *E. fawcettii* hyphae invading citrus and the cytological processes by

which *Elsinoe* spp. make only limited growth resulting in definite local lesions such as scabs and spots on host plants have not been reported.

Light microscopy of citrus leaves infected with *E. fawcettii* revealed that the formation of phellem (cork layer) and phellogen separated the portion invaded by the fungus, causing the bulging of infected leaves and a shot-hole appearance (Cunningham, 1928). Young citrus fruits are known to develop wound (or necrophylactic) periderm in response to shallow injury by mechanical means, insects and fungal pathogens (Achor et al., 1991; Whiteside, 1988). During the later stages of fruit development, production of wound periderm is less likely, but fruits can survive rind injury by forming a barrier of lignified cells next to the dead tissues. The formation of wound periderm is a non-specific response to detrimental environmental stimuli such as wounding, insect injury and pathogen infection (Kolattukudy, 1984; Simard et al., 2001). In order to better understand the nature of the structural alteration of citrus against pathogen invasion and the fungal survival strategies in local lesions, it is necessary to examine the host–parasite interactions at the ultrastructural level. This study was conducted to (i) observe the ultrastructural characteristics of citrus infected with *E. fawcettii* and (ii) investigate the effect of cork layer formation on fungal growth during scab development.

Materials and methods

Inoculum preparation

Isolates of *E. fawcettii* were obtained from naturally infected satsuma mandarin leaves showing typical scab symptoms in Jeju Island, Korea. The infected leaves were surface-sterilized with 75% ethanol and 1% sodium hypochlorite for 1 min each and rinsed with sterilized distilled water. After drying, each scab pustule was scraped with a scalpel to deposit flakes of diseased tissue onto half-strength potato dextrose agar (12.0 g of potato dextrose broth and 15.0 g of agar in 1 l of distilled water) plates and kept at 27 °C. For inoculum preparation, a typical isolate selected after pathogenicity testing (Hyun et al., 2001) was cultured for 2 weeks, and small pieces of mycelium (5–10 mm^3) were crushed in a petri plate with a spatula. The resulting fragments were stirred into liquid-modified Fries' medium (Whiteside, 1975). The plates were incubated at 27 °C for 2 days, washed three times with sterilized distilled water, flooded with sterilized 0.05% Tween 20 and kept under continuous darkness at 27 °C overnight. Elliptical hyaline conidia in the suspension were harvested by filtering through two layers of cheesecloth and adjusted to 1.0×10^6 conidia/ml.

Preparation of infected fruits and leaves

Naturally inoculated satsuma mandarin fruits showing typical scab symptoms were collected from a citrus orchard in Jeju Island, Korea 1 month after petal fall to examine the subcellular characteristics of scab lesions. For greenhouse inoculation of leaves, seedlings of satsuma mandarin were grown in a greenhouse maintained at 18–33 °C. They were pruned to stimulate production of uniform flushes of new leaves and inoculated when leaves were a quarter of mature size by spraying with the conidial suspension. Immediately after inoculation, the plants were covered with plastic bags for 2 days to maintain saturated humidity for infection. Symptom severity was checked 3 days after inoculation.

Transmission electron microscopy

Squares (each $2 \times 2\,mm^2$ with ~1 mm thickness of underlying tissues) were excised from scab lesions of the inoculated fruits and leaves using a sterile razor blade. Uninoculated healthy portions of fruits and leaves were also excised as a control. The specimens were fixed with modified Karnovsky's fixative (Karnovsky, 1965) consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C overnight, and washed with the same buffer three times for 10 min each. The specimens were postfixed with 1% (w/v) osmium tetroxide in the same buffer at 4 °C for 2 h, and washed with distilled water twice briefly. The postfixed specimens were stained *en bloc* with 0.5% (w/v) uranyl acetate at 4 °C overnight. They were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 95% and 100%), and three times in 100% ethanol for 10 min each. The specimens were further treated with propylene oxide as a transitional fluid twice for 30 min each, and embedded in Spurr's medium. Ultrathin sections were made with a diamond knife using an ultramicrotome (MT-X; RMC Inc., Tucson, USA). The sections (~50 nm thick) were mounted on copper grids, and stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) for 7 min each. They were examined with a transmission electron microscope (JEM-1010; JEOL Ltd., Tokyo, Japan) operated at an

accelerating voltage of 80 kV. For each specimen, more than 10 ultrathin sections were examined.

Results

Transmission electron microscopy of healthy fruits revealed that epidermal cells were covered with a cuticle and had normal organelles such as nuclei, mitochondria and vacuoles (Figure 1a). The adaxial surface of healthy leaves also had a cuticle and there was no indication of cytoplasmic degradation (Figure 1b). Scab pustules formed initially on the surface of fruits and leaves. Brownish or reddish acervuli of *E. fawcettii* developed at later stages of infection. The adaxial surface of leaves that had been infected was slightly raised and a corresponding depression was found on the opposite surface.

Cytopathology of citrus fruits infected with E. fawcettii

Epidermal cells of infected fruits were sloughed off, exposing the rough or corky surface in lesions (Figure 2a). Several layers of collapsed host cells (cork layer) were evident in the outer side of the lesions. The cork layers consisted of host cells that were almost devoid of cellular contents and the inner regions of the cork layer – several layers below the outermost one – were composed of compact host cells with little intercellular spaces (Figure 2b). These host cells possessed convoluted cell walls having alternating light and dark lamellations. Hyphae of *E. fawcettii* grew mainly intercellularly in the cork layer of infected fruits; however, no host tissues below the cork layers were invaded by hyphae (data not shown). Some hyphae were embedded in host cell walls and wall appositions accumulated locally within the host cells in contact with invading hyphae (Figure 2c). Hyphae had distinct lipid globules, vacuoles and concentric bodies in their cytoplasm. Concentric bodies, ~100–200 nm in diameter, were frequently found in clusters within hyphal cytoplasm and were composed of several distinct layers (Figure 2d). Most of the concentric bodies had an electron-transparent core surrounded by an electron-dense layer that was occasionally encased by a halo. The surface of the concentric bodies was covered with filamentous structures. Although concentric bodies were occasionally associated with nuclei, no consistent locations or spatial topography could be ascertained.

Hyphae penetrated some host cells in the cork layer and the infected host cells were highly vacuolated

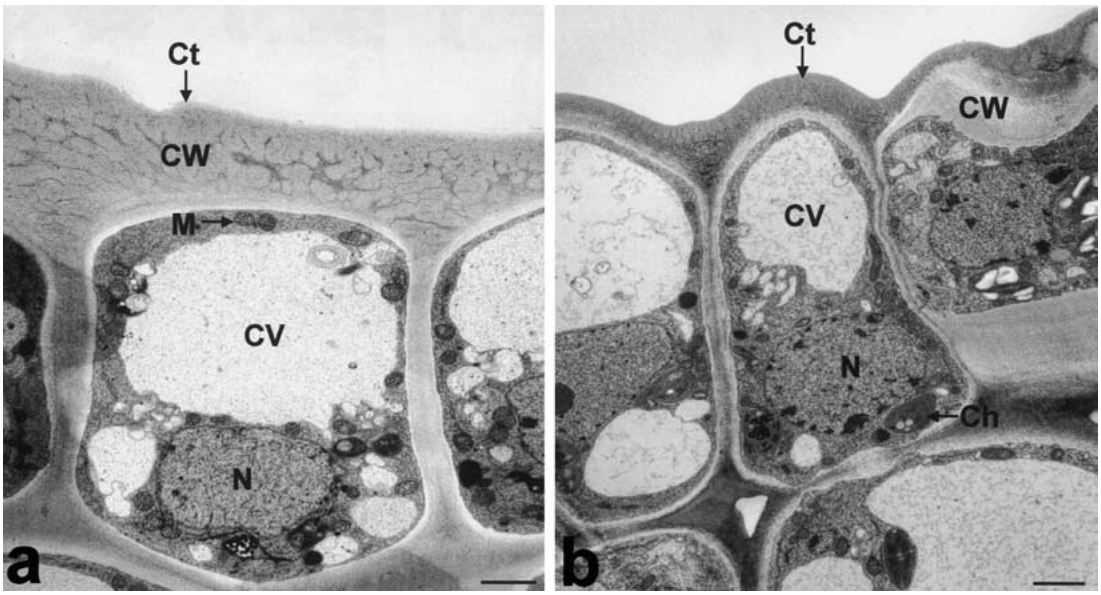

Figure 1. Transmission electron micrographs of uninoculated fruits and leaves of satsuma mandarin. (a) Epidermal cells of the fruit rind. The cells contained intact cytoplasm with a nucleus (N), mitochondria (M) and a central vacuole (CV). Ct = cuticle, CW = host cell wall. Bar = 1.0 μm. (b) Epidermal cells of the adaxial leaf surface. Ch = chloroplast. Bar = 1.5 μm.

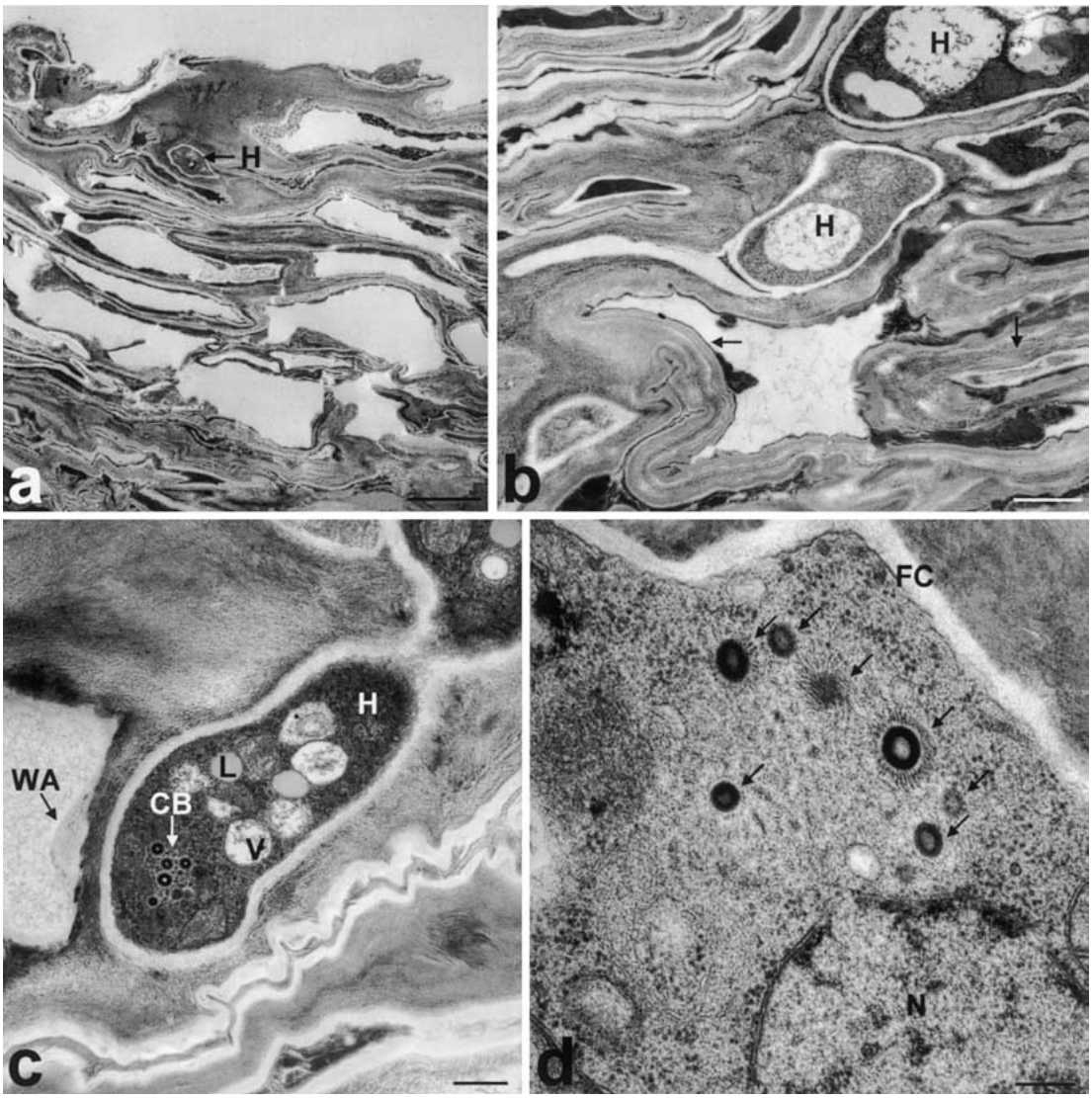

Figure 2. Transmission electron micrographs of satsuma mandarin fruits infected with *E. fawcettii*. (a) Host cells in the cork layer. The epidermis had become detached to reveal a corky surface below an infected site. A hypha (H) was embedded in host cell walls. Bar = 2.0 μm. (b) A higher magnification of host cells in the cork layer. Note the convoluted host cell walls with alternating lamellations (arrows) and hyphae (H). Bar = 1.0 μm. (c) A hypha (H) having organelles and inclusions. Cell wall appositions (WA) accumulated locally within the host cell. CB = concentric bodies, L = lipid globule, V = vacuole. Bar = 0.5 μm. (d) A higher magnification of concentric bodies (arrows). They had an electron-transparent core surrounded by an electron-dense layer with radiating filamentous structures on their surface. FC = fungal cell wall, N = nucleus. Bar = 150 nm.

and abnormally enlarged, exhibiting hypertrophy when compared with neighboring host cells (Figure 3a). Also, compartmentalization often took place, showing that host cells were further divided by cell walls which were usually lamellate and thinner than others (Figure 3b). Most of the cell divisions were made at right angles to the fruit surface (anticlinal), but occasionally thin cell walls were centripetally laid down at various angles. Electron-dense materials accumulated in the peripheral regions of cytoplasm in infected host cells. One or more intrahyphal hyphae were often found in intracellular hyphae (Figure 3c). Electron-transparent hyphal cell walls clearly delimited intrahyphal hyphae from

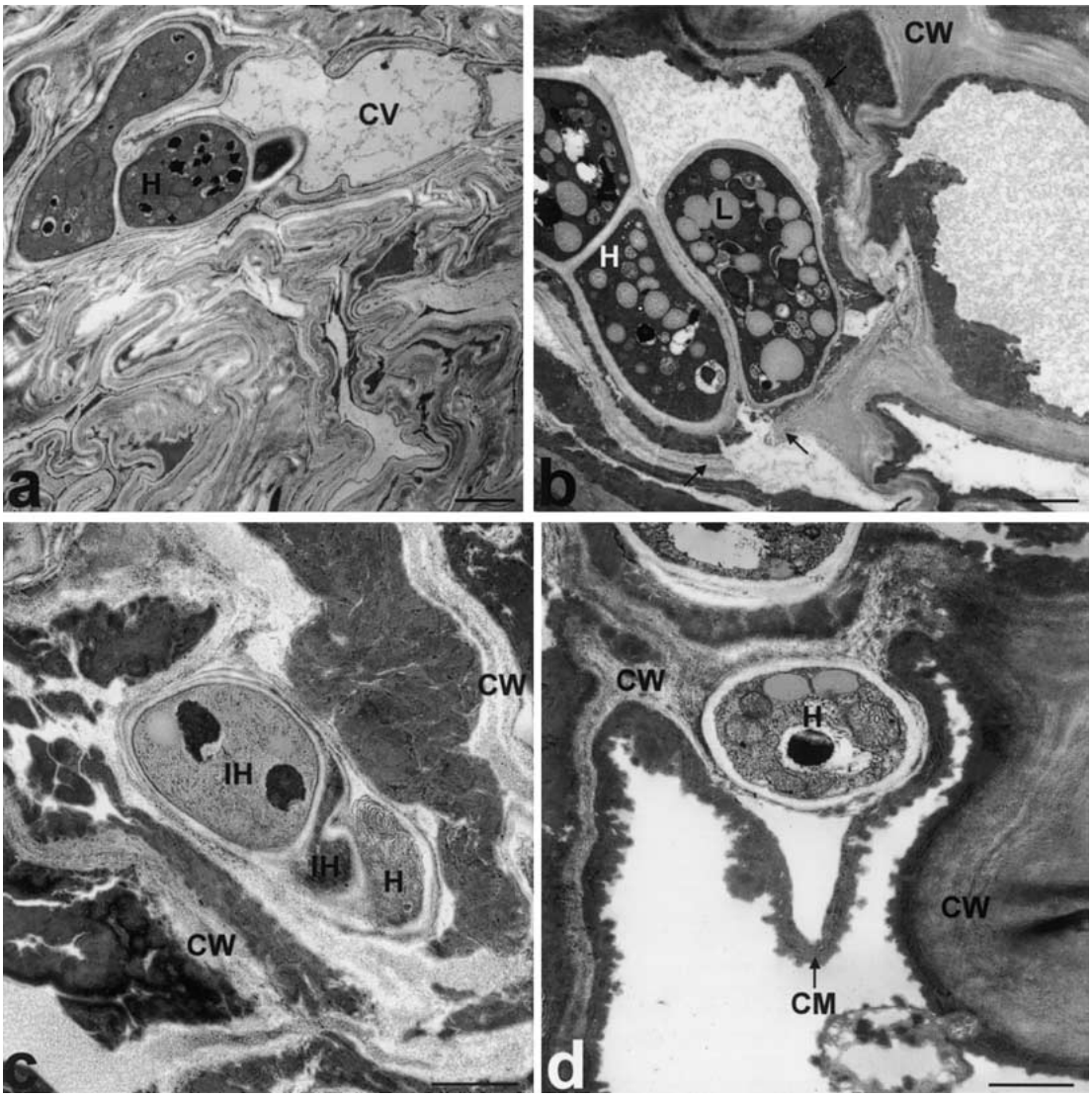

Figure 3. Transmission electron micrographs of satsuma mandarin fruits infected with *E. fawcettii*. (a) Host cell penetration by a hypha (H). The infected host cell showed hypertrophy. CV = central vacuole. Bar = 1.5 μm. (b) Host cell compartmentalization. Thin cell walls (arrows) were centripetally laid down in the infected host cell. CW = host cell wall, L = lipid globule. Bar = 1.0 μm. (c) Intrahyphal hyphae (IH) within an intracellular hypha (H) in subepidermis. Note the lamellar structure within an enclosing hypha. Bar = 1.0 μm. (d) Separation of host cell membrane (CM) from cell wall around an invading hypha (H). Bar = 1.0 μm.

the cytoplasm of enclosing hyphae, and the outermost enclosing hypha occasionally had a lamellar structure in the cytoplasm. Some infected host cells showed separation of cell membrane from cell wall in regions where the host cells were in contact with hyphae (Figure 3d). The damaged host cells were almost devoid of cellular contents, and the membranes were deposited with electron-dense materials.

Cytopathology of citrus leaves infected with E. fawcettii

Hyphae formed dense subepidermal stromata on the adaxial surface of leaves and some hyphae grew subcuticularly (Figure 4a). Epidermal and subepidermal cells in the palisade mesophyll tissue were extensively colonized by hyphae. The infected cells

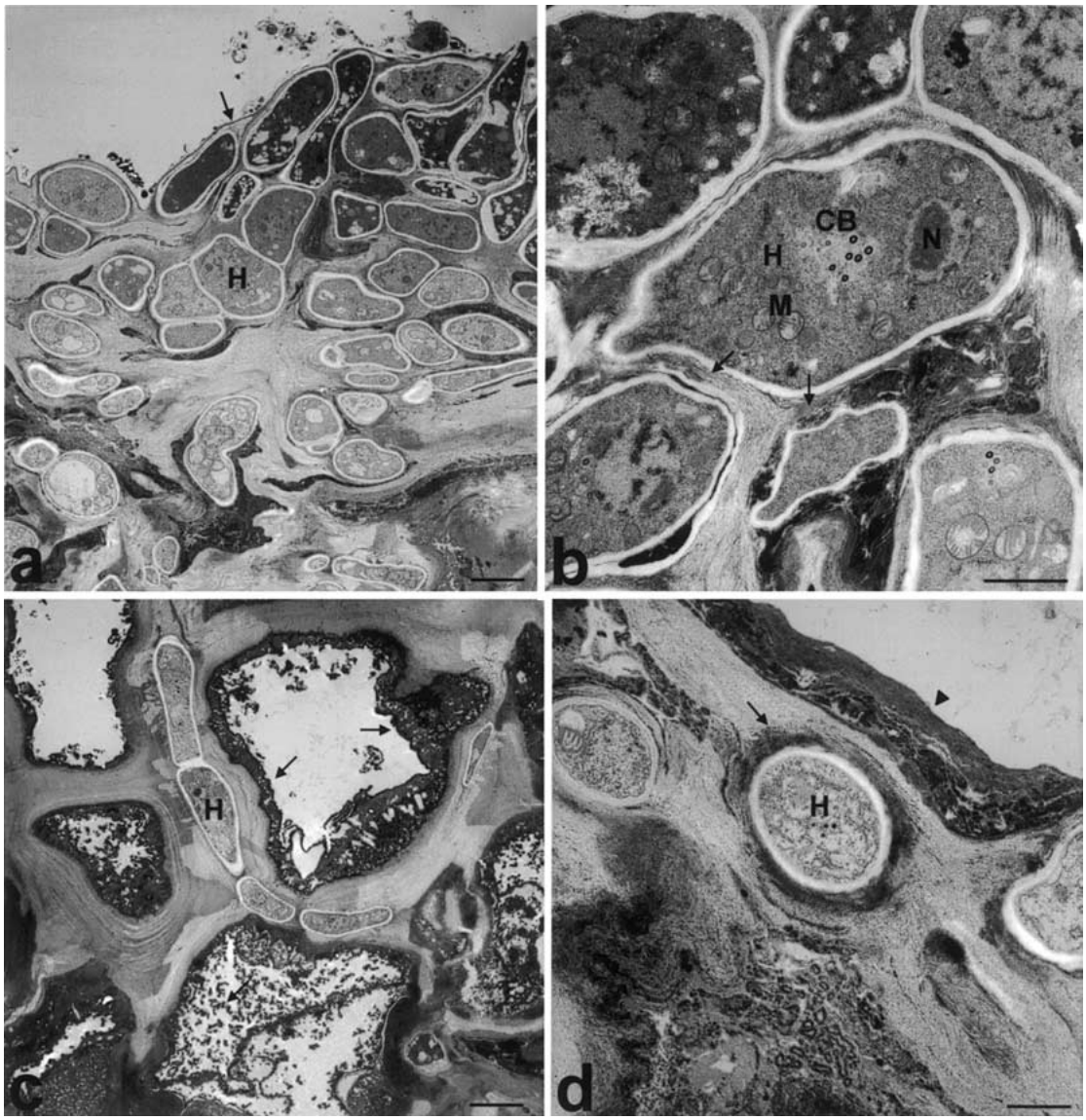

Figure 4. Transmission electron micrographs of satsuma mandarin leaves infected with *E. fawcettii*. (a) Hyphal colonization of the palisade mesophyll tissue. Hyphae (H) grew intercellularly and intracellularly. Note the cuticle (arrow) above the epidermal cell wall. Bar = 3.0 μm. (b) Host cell penetration by a hypha (H). Note the host cell wall modifications (arrows) adjacent to the hypha. CB = concentric bodies, M = mitochondria, N = nucleus. Bar = 1.0 μm. (c) Invasion of the palisade mesophyll tissue by hyphae (H). Electron-dense materials (arrows) accumulated in host cells adjacent to the hyphae. Bar = 2.5 μm. (d) Intercellular hyphae (H) in the palisade mesophyll tissue. Note the varying electron density of fibrillar matrix (arrow) of the host cells around hyphae. Electron-dense materials (arrowhead) accumulated in the neighboring host cell. Bar = 1.0 μm.

were markedly distorted when compared with those of uninfected cells in fruits (Figure 1b). Fungal penetration of host cells involved the localized dissolution of host cell walls, showing changes of fibrillar matrix of host cell walls adjacent to hyphae (Figure 4b). Invading hyphae had electron-transparent cell walls, nuclei, mitochondria and concentric bodies. Fungal invasion was often observed in intercellular spaces of the palisade mesophyll tissue of leaves (Figure 4c). Fungal invasion was usually accompanied by the accumulation of electron-dense materials in host cells adjacent to invading hyphae. In particular,

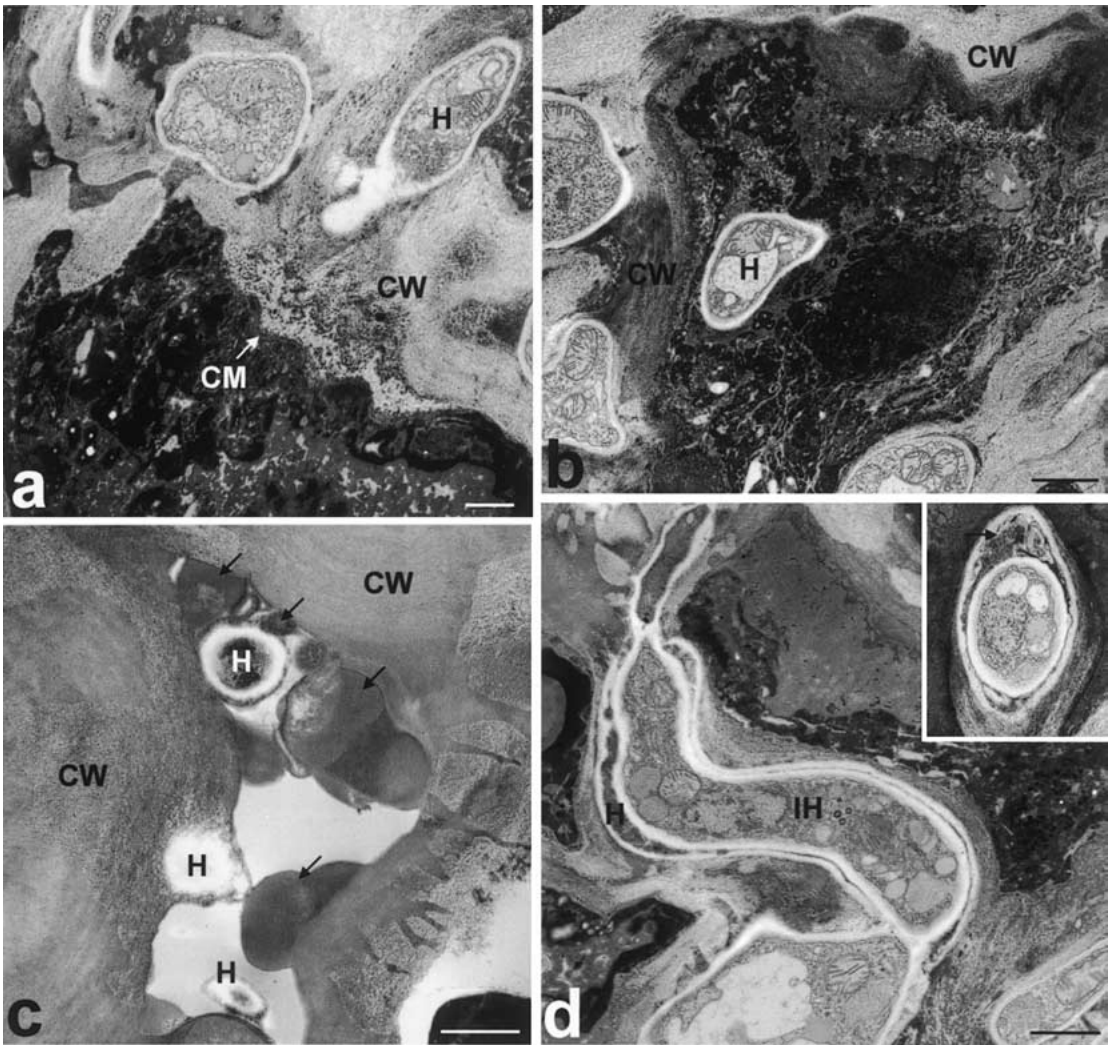

Figure 5. Transmission electron micrographs of satsuma mandarin leaves infected with *E. fawcettii*. (a) Host cell penetration by hyphae (H). The host cell membrane (CM) locally retracted from the cell wall (CW). Bar = 1.0 μm. (b) Intracellular growth of a hypha (H). Note the necrotic cytoplasm of the infected host cell. CW = host cell wall. Bar = 1.5 μm. (c) Formation of host cell wall protuberances (arrows) around hyphae (H) in the intercellular space. Bar = 1.0 μm. (d) An intrahyphal hypha (IH) in an enclosing hypha (H). The enclosing hypha had degenerated cytoplasm (arrow) as shown in the rectangle. Bar = 1.0 μm.

electron-dense fleck-like aggregates were found in the central vacuoles of host cells. Host cell walls in contact with invading hyphae often showed varying electron density of fibrillar matrix around hyphae (Figure 4d). Electron-dense multi-textured materials accumulated locally around host cell walls in close proximity to hyphae.

Infected host cells with necrotic cytoplasm showed separation of cell membrane from cell wall adjacent to invading hyphae (Figure 5a). Some hyphae were found in host cells and showed intact hyphal cytoplasm (Figure 5b). Host cell degeneration was severe, making organelles indiscernible around intracellular hyphae. Cell wall protuberances occasionally formed outside host cell walls adjacent to invading hyphae (Figure 5c). Some hyphae were surrounded by cell wall protuberances of varying shape and size in intercellular spaces of the palisade mesophyll tissue. Longitudinal sections of intercellular hyphae showed that they had one or more intrahyphal hyphae in cytoplasm (Figure 5d). While the enclosing hypha showed degenerated cytoplasm, its

intrahyphal hypha(e) possessed intact cytoplasm with distinct organelles and inclusions.

Discussion

This study demonstrated ultrastructural aspects of cork layer formation of citrus against invasion by *E. fawcettii* and the postpenetration behavior of the fungus during scab development. Fungal infection induced citrus fruits to form cork layers bordering necrotic areas below the infected sites. Such a structural alteration of infected host tissues is an indication of typical wound periderm formation (Achor et al., 1991; Simard et al., 2001), even though other components of wound periderm, such as phellogen and phelloderm, were not present. It is likely that the cell walls having alternating lamellations in infected citrus fruits contain suberin, as suberized cell walls are composed of fine alternating lamellae of phenolics and waxes which appear electron-dense and electron-translucent, respectively (Kolattukudy, 1984). Suberization usually involves the deposition of polymeric materials including lignin-like materials in cell walls, providing a protective water-impermeable layer in a variety of plant parts (Kolattukudy, 1984). Consequently, cork layers in wound periderm act as a physical barrier to pathogen growth, block the spread of pathogen-secreted toxic substances and prevent the apoplastic movement of water and nutrients from healthy tissues to infected ones, eventually depriving pathogens of water and nourishment (Achor et al., 1991; Agrios, 1997; Simard et al., 2001). The observation that fungal invasion was confined to cork layers also supports the effectiveness of cork layers as a protective barrier to invasion by *E. fawcettii*, as reported in other studies concerning wound periderm formation (Achor et al., 1991; Simard et al., 2001). Considering the formation of a cork layer and phellogen of scab-infected citrus leaves in a previous study (Cunningham, 1928), wound periderm formation is thought to be involved in the development of scab lesions in citrus by pushing the necrotic tissues outwards and removing the pathogen from the infected host parts completely (Agrios, 1997). The infected host cells in the cork layer of citrus fruits, however, are considered to have been unsuberized and served as a supply route for water and nutrients (Williamson and McNicol, 1989).

Other host cell changes associated with fungal invasion included the accumulation of electron-dense materials in host cells. Based on their texture and electron density, these materials probably correspond to phenolic compounds (Scalet et al., 1989). Wall appositions formed inside host cells in contact with hyphae and appeared to interfere with host cell wall penetration by hyphae. Cell wall protuberances, which are formed outside host cells, probably deter host cell wall penetration and intercellular invasion by hyphae through cell wall thickening and intercellular plugging (Kim et al., 2001; Murillo et al., 1999).

The invaded host cell walls were characterized by the fibrillar dissolution of host cell walls around invading hyphae, indicating fungal secretion of cell wall-degrading enzymes and their diffusion at a distance from the point of fungal contact. Moreover, fungal attack induced host cells to lose membrane turgor and permeability, as shown in the separation of cell membrane from cell wall of infected host cells. The hypertrophy and compartmentalization of infected host cells suggest that *E. fawcettii* causes an imbalance in the growth regulatory system of citrus, bringing about abnormal cellular responses. Similar anatomical changes have been reported from host plants infected with *Elsinoe* spp., and are thought to be responsible for the bulging of infected host parts (Gabel and Tiffany et al., 1987; Mason and Backus, 1969). Zeigler et al. (1980) contended that an anamorph of *Elsinoe* sp. causing super-elongation disease of cassava produced gibberellin A_4 under *in vitro* conditions. However, the exact mechanisms remain to be elucidated in further studies of *E. fawcettii*–citrus interaction. Even the compartmentalized host cells possessed lamellate cell walls, which suggested the formation of suberized cell walls possibly limiting hyphal spread in divided cells.

The most intriguing finding in hyphal cytoplasm of *E. fawcettii* was the frequent occurrence of concentric bodies. Originally described as ellipsoidal bodies (Brown and Wilson, 1968), concentric bodies have been considered of general occurrence in fungi (Granett, 1974; Honegger, 2001; Rushing and Latham, 1991; Sanders and Ascaso, 1997). Their proposed functions include membrane synthesis, material transport (Brown and Wilson, 1968) and tolerance to desiccation stress (Classen et al., 2000; Honegger, 2001). In particular, features shared by most ascomycetous fungal cells having concentric bodies is their desiccation tolerance and relative longevity. Although *E. fawcettii* is not found in extremely dry habitats, fungal growth in cork layers bears the risk of desiccation stress because of the water impermeability of cork layers. As concentric bodies are not membrane bound and have a gas-filled

core with a proteinaceous electron-dense outer layer and radiating filamentous structures, it has been suggested that they may be the remains of denatured proteins involved in cytoplasmic cavitation processes for desiccation tolerance by keeping desiccation-inflicted fungal protoplasts in contact with cell walls (Honegger, 2001). Considering that fungal survival depends on the ability of hyphae in scab pustules to undergo drying periods, it is possible that the ability of *Elsinoe* spp. to colonize host tissues and survive long periods without sufficient host substrates in pustules on detached fruits and leaves (Timmer, 2000) might be partially associated with the occurrence of concentric bodies in hyphal cytoplasm.

Some hyphae of the fungus were characterized by the formation of intrahyphal hyphae. This is the first report that *E. fawcettii* forms intrahyphal hyphae. They have been observed in a wide variety of fungi and may enable fungi to survive when adverse conditions lead to the degeneration of hyphae (Kim et al., 2001; Lim et al., 1983; Shankar et al., 1998). Through the formation of intrahyphal hyphae, hyphae are assumed to protect their vegetative structures and make permeability barriers to antifungal compounds or fungistatic environments which may be lethal to unprotected hyphae. The degenerated cytoplasm of the enclosing hyphae may reflect their protective roles against defense responses during invasion and colonization of host tissues.

In conclusion, this study increased our knowledge of citrus infection by *E. fawcettii* and the fungal survival-related characteristics at the ultrastructural level. Factors that govern cellular responses of fungal growth to host environments, and their epidemiological implication for citrus scab development remain to be investigated. It was evident in this study that wound periderm developed in satsuma mandarin, a cultivar susceptible to citrus scab, as a major histological response to invasion by *E. fawcettii*. Therefore, the development of citrus cultivars that induce wound periderm formation more rapidly and have a more complete bordering of infected areas may contribute to reducing the severity of scab.

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