Hypersensitive Response of Orange-Colored Robinson Tangerines to Colletotrichum gloeosporioides After Ethylene Treatment

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ABSTRACT

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Mature Robinson tangerines with about 25% external orange color break did not develop anthracnose caused by *Colletotrichum gloeosporioides* when treated with ethylene to obtain a complete orange color. Histochemical tests of the peel removed after ethylene treatment indicated that phenolics and lignin were present in the flavedo in association with infection hyphae produced by appressoria on the peel surface. Ultrastructural changes occurred in cells up to five cell diameters from the point of penetration. Cytoplasm of these cells contained mitochondria with dilated cristae and chromoplasts that often had indistinct envelopes and homogeneous stroma. The cytoplasm also contained numberous vesicles apparently derived from dilated endoplasmic reticulum and dictyosomes. Vesicles formed by the dictyosomes usually were contained within multivesicular bodies. These bodies frequently were associated with the plasmalemma before electron-dense deposits accumulated at the cell wall. Accumulation of deposits occurred concurrently with collapse of the cytoplasm against the cell wall distal from the area of fungal penetration. Many cells eventually became occluded with electron-dense material which surrounded hyphae in the invaded cells.

Additional key words: phenolic materials, postharvest decay, citrus, histochemistry, ultrastructure.

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., is a serious postharvest decay of green-colored but mature Robinson tangerines which require treatment with ethylene to expose the natural orange peel color before marketing (2, 4, 26). Tangerines that already have developed an orange color break of 25% or greater before harvest are more resistant to anthracnose than green-colored fruit during exposure to ethylene (4). Resistance can be induced in the green-colored tangerines if they are first treated with ethylene, to degrade some of the chlorophyll, before inoculating them with *C. gloeosporioides* and treating again with additional ethylene to complete removal of the green color (4).

Penetration of mature, green-colored Robinson tangerines by *C. gloeosporioides* occurs primarily after harvest during the ethylene treatment (2, 4). Latent appressoria on the peel surface form threadlike infection hyphae, which penetrate the cuticle (2, 3). The infection hyphae usually grow into larger primordial hyphae which continue to develop into hyphae. The infection hyphae occasionally enlarge directly into hyphae without a primordial stage (3). Entry of the large intracellular hyphae into host cells does not cause immediate severe cell disruption and the hyphae do not kill cells in advance of invasion (3). Light microscopy studies of the flavedo portion of the peel from orange-colored tangerines that resisted invasion by *C. gloeosporioides* indicated that lignin accumulated in the invaded area of the peel (4), apparently preventing growth of the hyphae into the albedo.

In this study, histochemical tests were undertaken to obtain additional information concerning the nature of the material formed in the flavedo of orange-colored Robinson tangerines in response to penetration by *C. gloeosporioides*. Ultrastructural changes in response to penetration were studied to learn the sequence in which such materials were formed and the manner in which they may prevent disease.

MATERIALS AND METHODS

Robinson tangerines with an orange color break on about 25% of the peel surface were inoculated with *C. gloeosporioides* by procedures described previously (4). After inoculation and subsequent appressorium formation, fruit were treated with 20 μliters of ethylene/liter of air for 0, 1, 2, or 3 days to provide tissue at various stages of resistance. Noninoculated and inoculated peel bearing appressoria were removed from the fruit and prepared for fixation 2 days after completion of the ethylene treatments.

Tissue for histochemical studies was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.0. The tissue was dehydrated with increasing concentrations of *t*-butyl alcohol, embedded in Tissuemat® (Fisher Scientific Co. Pittsburgh, PA 15219) (m. p. 56.5 C) and sectioned at a thickness of 4 μm. Sections of fresh tissue 24 to 36 μm in thickness used in some tests were obtained with the aid of a microtome (11).

Histochemical tests were conducted on control fruit

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and on inoculated fruit in the region of infection. Tests for phenolic compounds were made with Gibbs' reagent (2,6-dichloro-*p*-benzoquinone-4-chlorimine) (14, 22), Fast Red Salt B reagent (diazotized-5-nitro-2-aminoanisole) (22, 27), and the ferric chloride-potassium ferricyanide reagent (13). Tannins were detected with the nitroso test (20) and the ferric sulfate reaction (21). Tests for lignin were made with phloroglucinol-HCl (12), chlorine water-sodium sulfite (25), toluidine blue O (17), and Schiff's reaction (12). The lacmoid (25) and aniline blue (12) tests were used to detect callose.

Tissue for electron microscopy was removed from control fruit and from infected areas of inoculated fruit. The material was fixed with glutaraldehyde buffered at pH 7.0 in 0.1 M phosphate buffer with and without postfixation in 2% osmium tetroxide in additional buffer. The tissue then received four successive washings in additional fresh buffer. Some tissue was fixed at 4 C with 2% potassium permanganate in distilled water, and rinsed with additional water. Following fixation, all tissue was dehydrated with increasing concentrations of acetone at 4 C. The material was infiltrated with Spurr's resin and thin-sectioned with a diamond knife. Sections were stained with uranyl acetate and lead citrate, or with silver nitrate (18, 19), and viewed with a Philips 201 electron microscope. Micrographs used for the illustrations were prepared from tissue fixed in glutaraldehyde and osmium tetroxide unless described otherwise.

RESULTS

Histochemical tests.—Inoculated fruit without ethylene treatment and noninoculated ethylene-treated fruit did not produce positive staining for phenolics, tannins, lignin, or callose. Cells beneath appressoria of inoculated fruit treated with ethylene stained intensely with ferric chloride-potassium ferricyanide and Fast Red Salt B reagent, indicating the presence of phenolic material. Treatment with the Gibbs' reagent produced only a slight staining response. At some infection sites, accumulation of these compounds occurred only in the epidermal cells. In other areas, epidermal cells and cells one to four layers beneath the epidermis also were involved. A strong, positive response for the presence of tannins was obtained in similar areas of inoculated, ethylene-treated peel with both the nitroso and ferric sulfate stains, but only when the chemicals were applied to fresh tissue. Lignin also was detected in comparable areas of the peel with all of the four stains that were applied. Staining with chlorine water-sodium sulfite produced a more intense red color in fresh than in fixed tissue. Extent of the accumulation of phenolics, tannins, and lignin appeared to be related to the number of cells invaded by *C. gloeosporioides*. A three-day treatment with ethylene consistently did not cause more extensive invasion than did exposure to ethylene for one or two days. The lacmoid and aniline blue tests for callose in inoculated fruit treated with ethylene were negative.

Ultrastructure of the resistant response.—Ultrastructural changes in the peel in response to infection of ethylene treated orange-colored Robinson tangerine by *C. gloeosporioides* were indicative of a hypersensitive reaction. At sites where penetration of infection hyphae was restricted to the epidermal cell wall, cellular changes were restricted primarily to the epidermal cells. Penetration of hyphae into epidermal cells caused changes in cells as far as four cell diameters from the penetrated cell. Cytoplasm of affected cells

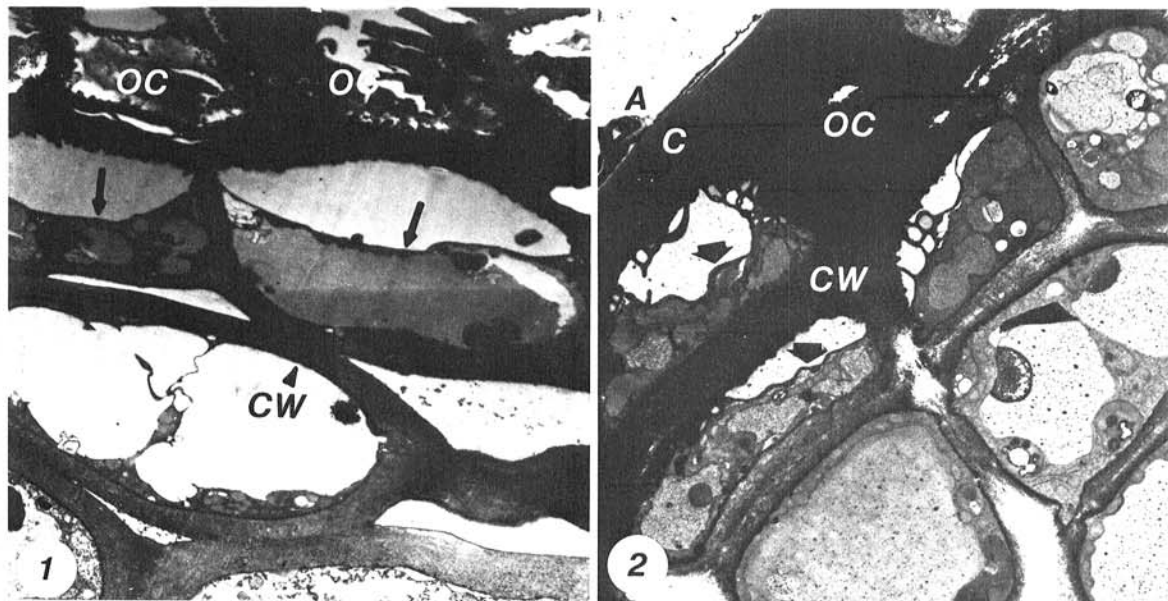

Fig. 1-2. 1) Tissue of Robinson tangerine peel showing occluded epidermal cells (OC) near the area of fungal penetration and collapse of the cytoplasm (arrows) in adjacent cells. Walls (CW) of these affected cells were more electron-dense than walls of cells farther from the penetration site (× 3320). **2)** Flavedo cells at the infection site stained with silver nitrate for polysaccharides. Note the greater affinity for silver of contents within the occluded cell (OC) and the surrounding cell walls (CW). Cytoplasm (arrows) is collapsed away from the point of fungal penetration at the appressorium (× 4370). Legend: A = appressorium; C = cuticle.

collapsed and the cells often became occluded by electron dense material, particularly those cells in and near the epidermis (Fig. 1, 2). Ultrastructure of cells outside the hypersensitive area and cells of control fruit was similar to that reported for orange (28). Ethylene, in the absence of the fungus, did cause changes in chromoplast ultrastructure that are associated with ripening (28).

Cytoplasm of cells involved in the early stages of the hypersensitive reaction contained more cell organelles than cytoplasm of cells outside the affected area. Rough endoplasmic reticula (RER) were more abundant and they were often associated with ribosomes in the surrounding cytoplasm (Fig. 4). Cytoplasm of cells involved in the hypersensitive reaction contained short pieces of both smooth endoplasmic reticulum (SER) and RER. The shortened pieces were dilated, often constricted near the ends, and frequently were associated with lightly-stained vesicles in the surrounding cytoplasm (Fig. 3). Mitochondria were numerous and the cristae usually were dilated (Fig. 4, 6). Chromoplasts within the cytoplasm of the cells involved in the hypersensitive response exhibited several ultrastructural changes. Thylakoids often dissolved leaving a dense, homogeneous stroma with lipid bodies stained with different intensities (Fig. 4), and the envelope of the chromoplasts often became indistinct (Fig. 5).

Dictyosomes (Fig. 5) occurred more frequently in the cytoplasm of hypersensitive cells and produced vesicles of varying sizes. These vesicles were more dense in appearance than those formed by the endoplasmic reticula. Granular contents of dictyosome-formed vesicles varied extensively in electron density (Fig. 5, 6, 9, 10), whereas the vesicular membrane consistently was electron dense but often indistinct. The vesicles usually were contained within a multivesicular body (Fig. 6, 9) where they were dispersed within a granular matrix. Material resembling lipid frequently was associated with the multivesicular bodies (Fig. 6,9).

Electron dense granular deposits, resembling phenolic material, accumulated between the invaginated plasmalemma and the cell wall (Fig. 7, 8). Additional accumulation produced deposits of material with electron lucent, lipid-like centers and granular, nondelineated margins (Fig. 9, 10), often in combination with granular material and vesicles (Fig. 10). The lipid-like areas were not preserved in comparable electron-dense material in tissue fixed only with glutaraldehyde (Fig. 11). Multivesicular bodies often were found near wall deposits of electron-dense material (Fig. 6,9).

Deposits of the electron-dense material extended from the cell wall into the lumen in association with the collapse of the cytoplasm (Fig. 12,13). In some cells, an electron-lucent lamellate layer of material was present between the dense deposit and the cytoplasm, and remained associated with the cytoplasm during its collapse (Fig. 14). Remnants of darkly stained dense material, often in the form of projections, usually remained attached to the cell wall and extended into the lumen in the void created by the collapse of the cytoplasm (Fig. 14). Collapse of the cytoplasm occurred away from the dense wall deposits toward the opposite side of the cell lumen (Fig. 1, 2). Some accumulation of electron-dense material also occurred in the cytoplasm where individual vesicles of extreme density were associated with larger deposits of this material (Fig. 15). Electron-dense material also accumulated on the tonoplast and within the vacuole (Fig. 15). Quite extensive vacuolar accumulations of electron-dense material were evident in some cells after accumulation of dense deposits at the cell wall (Fig. 16). These cells, particularly epidermal cells, eventually were occluded with electron-dense material and dense, degenerated protoplasm (Fig. 1, 2). Hyphae within such cells were surrounded by the electron-dense contents (Fig. 17).

Walls of cells in the process of accumulating dense material at the plasmalemma usually stained more darkly after fixation with either glutaraldehyde and osmium tetroxide (Fig. 1) or potassium permanganate than walls of cells further removed from the penetration site or cell walls of healthy fruit. Silver used to stain glutaraldehyde and osmium tetroxide fixed tissue also had greater affinity for the walls and contents of cells involved in the hypersensitive response (Fig. 2), than for cells not undergoing ultrastructural changes.

DISCUSSION

Flavedo cells of orange-colored Robinson tangerines apparently resist invasion by *C. gloeosporioides* through localization of the hyphae by means of a hypersensitive response. Changes were apparent within protoplasts of cells in the hypersensitive tissue well in advance of cell penetration by the pathogen. Ultrastructural changes during accumulation of electron-dense substances within the hypersensitive cells of Robinson tangerines were similar to those observed in phenolic-storing cells of cotton during phenolic synthesis (16), in callus cultures of slash pine during tannin accumulation (1), in pine tissue cultures containing tannin in response to infection by *Cronartium ribicola* (23, 24), and in mycorrhizal associations during phenolic (7) or tannin accumulation (5). However, initial deposits of dense, material, presumably phenolic in nature, accumulated at the wall of tangerine cells in contrast to the cited instances where phenolics accumulated within the vacuole.

Lightly-stained vesicles and individual dense vesicles or multivesicular bodies appeared consistently in the cytoplasm of the hypersensitive tangerine cells. The vesicles appeared to be incorporated into the cell wall and into phenolic deposits at the wall and within the cytoplasm. The usual association of the vesicles with cells containing deposits of phenolic material within the cytoplasm or at the cell wall suggests that the vesicles played a major role in the synthesis of the phenolic material and in the subsequent hypersensitive response. In fact, vesiculation previously has been associated with lignin (30) and tannin (23) synthesis.

The cell wall usually became more electron-dense as wall deposits of phenolic substances accumulated. Increased density of the cell wall probably is an indication that lignin was being synthesized and incorporated into the cell wall during phenolic accumulation at the cell wall surface. Evidence for the presence of lignin within the cell wall was obtained with the histochemical tests and with enhanced staining by fixation with potassium permanganate (10). The red color that developed in resistant tissue stained with chlorine water-sodium sulfite

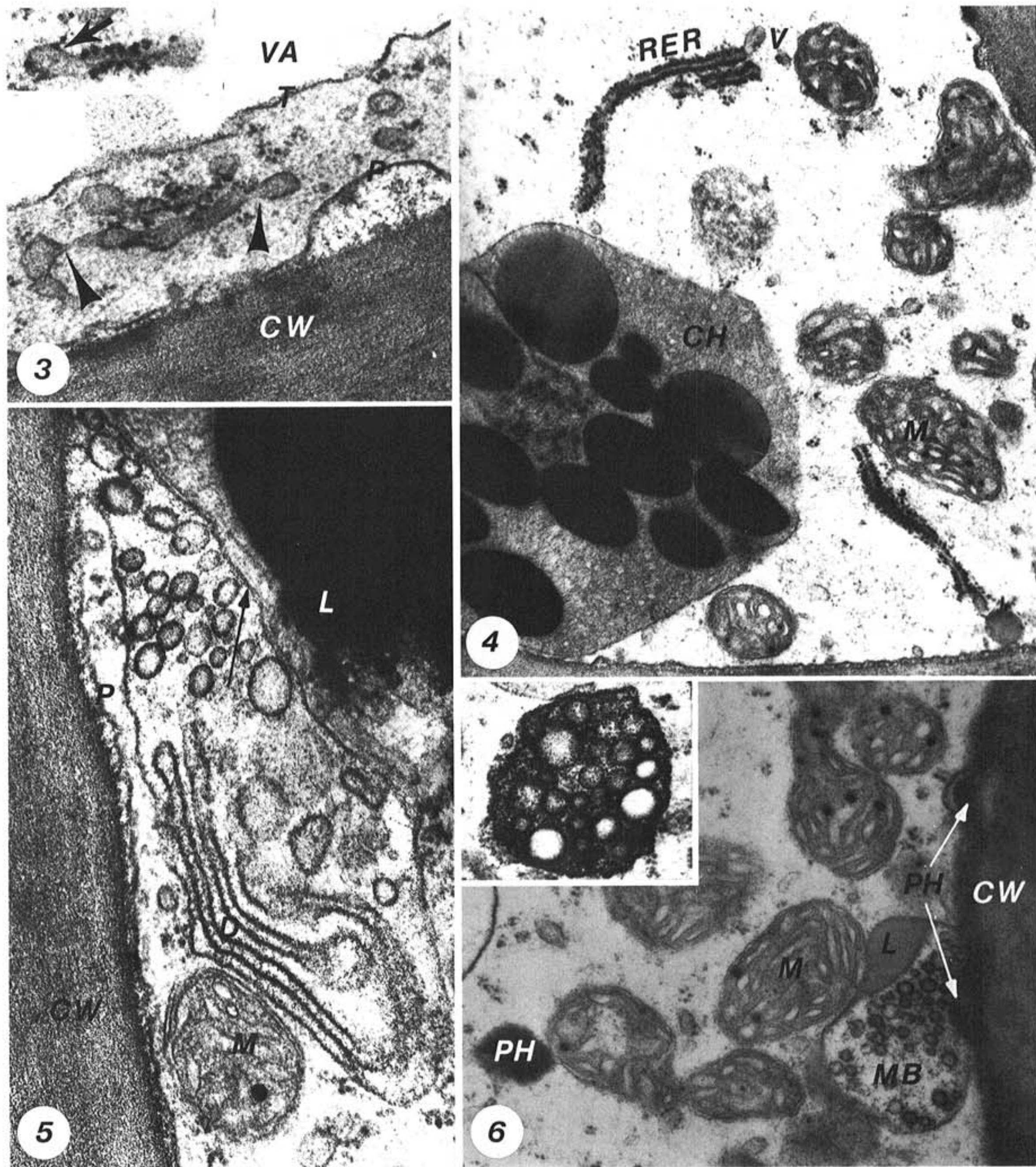

Fig. 3-6. 3) Dilated and essentially smooth endoplasmic reticulum with constrictions (arrows) at either end. Lightly stained vesicles are present in the cytoplasm (× 49,850). (Inset) Dilated rough endoplasmic reticulum with few ribosomes at the enlarged end (arrow). (× 52,800). Legend: CW = cell wall; p = plasmalemma; T = tonoplast; VA = vacuole. **4)** Rough endoplasmic reticulum (RER) in a cell involved in the resistant response. Vesicle (V) associated with the rough endoplasmic reticulum. The stroma of the chromoplast (CH) is quite homogeneous and cristae of the mitochondria (M) are swollen (× 27,225). **5)** Dictyosome (D) associated with vesicles surrounded by a membrane (arrow). Osmiophilic material (L) in an adjoining chromoplast (× 66,825). Legend: CW = cell wall; M = mitochondrion; P = plasmalemma. **6)** Multivesicular body (MB) containing lipid-like material (L). The body is associated with initial deposits of electron-dense (PH) material at the cell wall (CW). Similar material (PH) is also present within the cytoplasm (× 37,950). (Inset) Multivesicular body within the cytoplasm containing vesicles of varying degrees of electron opacity (× 52,800). Legend: M = mitochondrion.

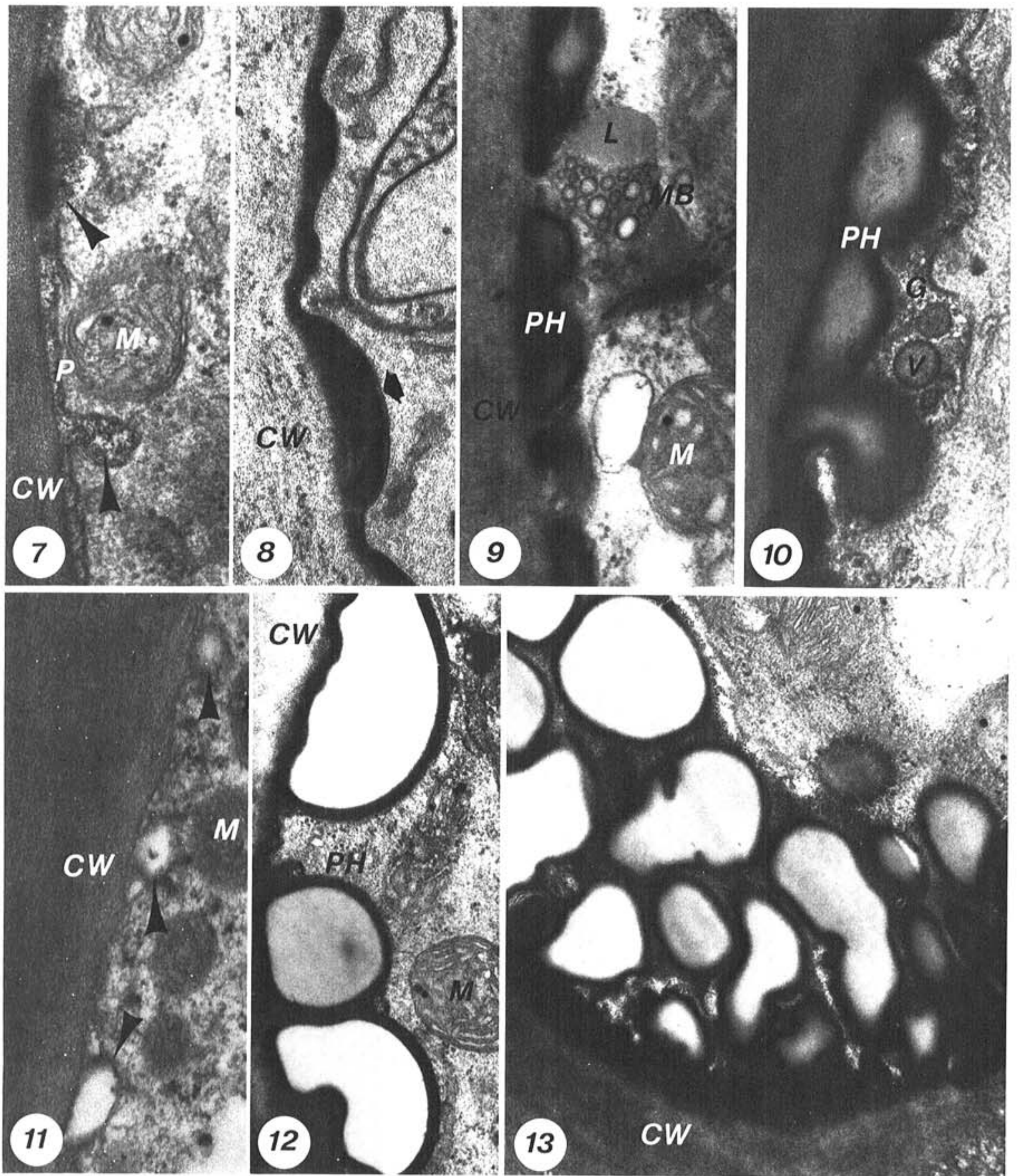

Fig. 7-13. 7) Accumulation of granular electron-dense material (arrows) between the cell wall (CW) and plasmalemma (P) during early stages in the deposit of such material at the cell wall (× 58,050). Legend: M = mitochondrion. **8)** Accumulation of dense material (arrow) at the cell wall (CW) in tissue fixed with potassium permanganate (× 36,630). **9)** Deposits of dense material (PH) with slightly lucid cores at the cell wall (CW) near mitochondria (M) and a multivesicular body (MB) containing lipid-like material (L) (× 43,560). **10)** Increased deposit at the cell wall consisting of granular material (G), vesicles (V), and electron-dense material (PH) with lucid cores (× 63,000). **11)** Deposits of electron-dense material (arrows) at the cell wall in tissue fixed with glutaraldehyde. The lipid-like core was not preserved (× 49,500). Legend: CW = cell wall; M = mitochondria. **12.** Wall deposits (PH) have enlarged into sphere or lobe-like structures with essentially electron-lucid cores but quite dense margins (× 33,240). Legend: CW = cell wall; M = mitochondrion. **13.** Frothy network at the cell wall interspersed with sphere and lobe-like lucid areas surrounded by dark margins and separated by dense, granular material (× 36,300). Legend: CW = cell wall.

indicated the presence of syringyl units within the lignin (31). Extrusion of the phenolic substances into the cell lumen was associated with withdrawl and collapse of the cytoplasm. Such ultrastructural changes in the wall deposits of phenolic material may indicate possible polymerization of the phenolics to form lignin. Ultrastructure of electron-lucent material found in some collapsed cells (Fig. 14) indicates that such material may be suberin (6).

Peroxidase, associated with plant resistance (31), has

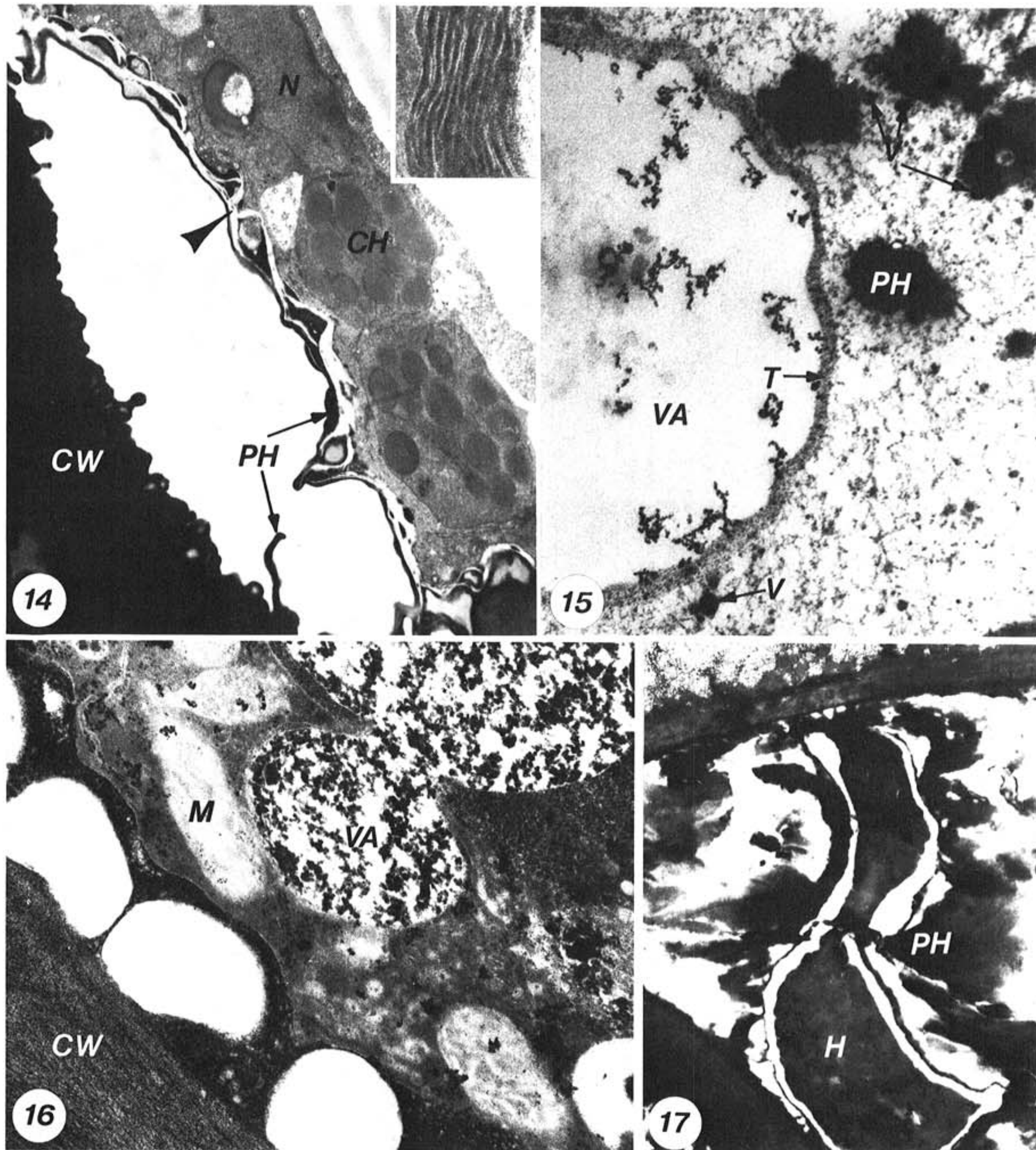

Fig. 14-17. 14) An electron-lucent fibrous layer of material (arrow) (Inset) (× 108,920) next to the electron-dense margin of material (PH) associated with the withdrawn cytoplasm. Dense material (PH) attached to the cell wall (CW) extends into the void created by the collapsed cytoplasm (× 11,550). Legend: CH = chromoplast; N = nucleus. **15)** Electron-dense vesicles (V) near additional dense material (PH) within the cytoplasm. Fine, granular material has accumulated at the tonoplast (T) and larger particles are present in the vacuole (VA) (× 33,000). **16)** Glutaraldehyde fixed tissue containing electron-dense material at the cell wall (CW) and within the vacuole (VA) (× 35,340). Legend: M = mitochondria. **17)** Epidermal cell containing a primordial hypha (H) of *Colletotrichum gloeosporioides* lacking internal detail and surrounded by electron-dense material (PH) (× 11,180).

been strongly implicated in lignin biosynthesis (9). Transport of peroxidase and lignin precursors (phenolic substances) via vesiculation to the plasmalemma-cell wall interface may eliminate the compartmentalization that possibly exists within the cytoplasm or vacuole between the oxidative enzymes and phenolic substrates. In fact, cell integrity is not altered when phenolics accumulate within the vacuole (16), suggesting that vacuolar accumulations may be protected from enzyme action. In resistant tangerine peel, vacuolar accumulations were associated with cell necrosis, but only after accumulation of phenolics had occurred at the cell wall.

In tangerine, lignin may not be directly involved in preventing the invasion by hyphae of *C. gloeosporioides*. Toxic substances formed during the hypersensitive response which cause cell necrosis also may inhibit growth of the hyphae. Further occlusion of affected cells may result from the formation of melanin, a dark-colored pigment that is associated with hypersensitivity (8) and also stains with silver nitrate (15).

It is not known why mature Robinson tangerines with a good orange color break have the capacity to resist infection by *C. gloeosporioides* during treatment with ethylene, while pale, green-colored fruit do not. Synthesis of phenolic substances in phenol-producing cells may be associated with the plastids (16). If such is the case, degradation of chlorophyll in chloroplasts of Robinson tangerines, either naturally or with ethylene (4), may be required before phenolic synthesis can be initiated.

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