Ultrastructural Changes in Pepper Cells in Interactions with Phytophthora capsici (Isolate 18) and Monilinia fructicola

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ABSTRACT

Three hours after inoculating fruit of sweet pepper *(Capsicum frutescens)* with *Phytophthora capsici* (isolate 18), hyphae with dense cytoplasm and functional organelles were observed in the outer walls of cells lining the fruit cavity. The second layer of cells was invaded within 5-7 hours and growth was arrested in the third layer 9-12 hours after inoculation. In invaded tissue, the cytoplasm of most hyphae and haustoria became vacuolated and degenerated between 7 and 12 hours. A reinvasion of previously uninvaded first layer cells by juvenile hyphae took place approximately 48 hours after inoculation and fruit tissue was rapidly colonized. The first few cell-layers were invaded in some areas by *Monilinia fructicola* 6-24 hours after inoculation, but little parasitic growth occurred subsequently, even though saprophytic hyphae were common on the inner fruit surface.

In both interactions, pepper cells were rapidly reactivated and disorganized in a characteristic hypersensitive response. This involved the formation of lipid bodies, the vacuolation of the cytoplasm, the invagination and disruption of the tonoplast membrane, the formation of vesicles and their dispersion throughout the cell cavity. The extent of the reaction was limited to cells near to invading hyphae of *P. capsici*, but was widespread throughout the inner surface tissue after inoculation of *M. fructicola*.

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Previous reports have described ultrastructural changes that occur in pepper fruit cells during a compatible interaction with *Phytophthora capsici* (A.T.C.C. 15399) (1), and an incompatible interaction with *Phytophthora infestans* (2). Compatibility was characterized by the rapid physiological stimulation of host cells, subsequent invasion and disorganization of the cytoplasm, unimpeded growth of the fungus, and production of low, noninhibitory concentrations of the phytoalexin, capsidiol. In contrast, incompatibility was expressed by the rapid death of the first invaded cells and slower death of the surrounding cells. The latter were either reactivated, and then invaded with the accompanying enlargement of cell border lesions or were reactivated, but uninvaded. Capsidiol accumulated in this interaction in concentrations that correlated closely with the progressive restriction of hyphal growth (3). Studies of two additional interactions between pepper fruit cells and fungi are described in this paper. One is an incompatible interaction with *Monilinia fructicola*

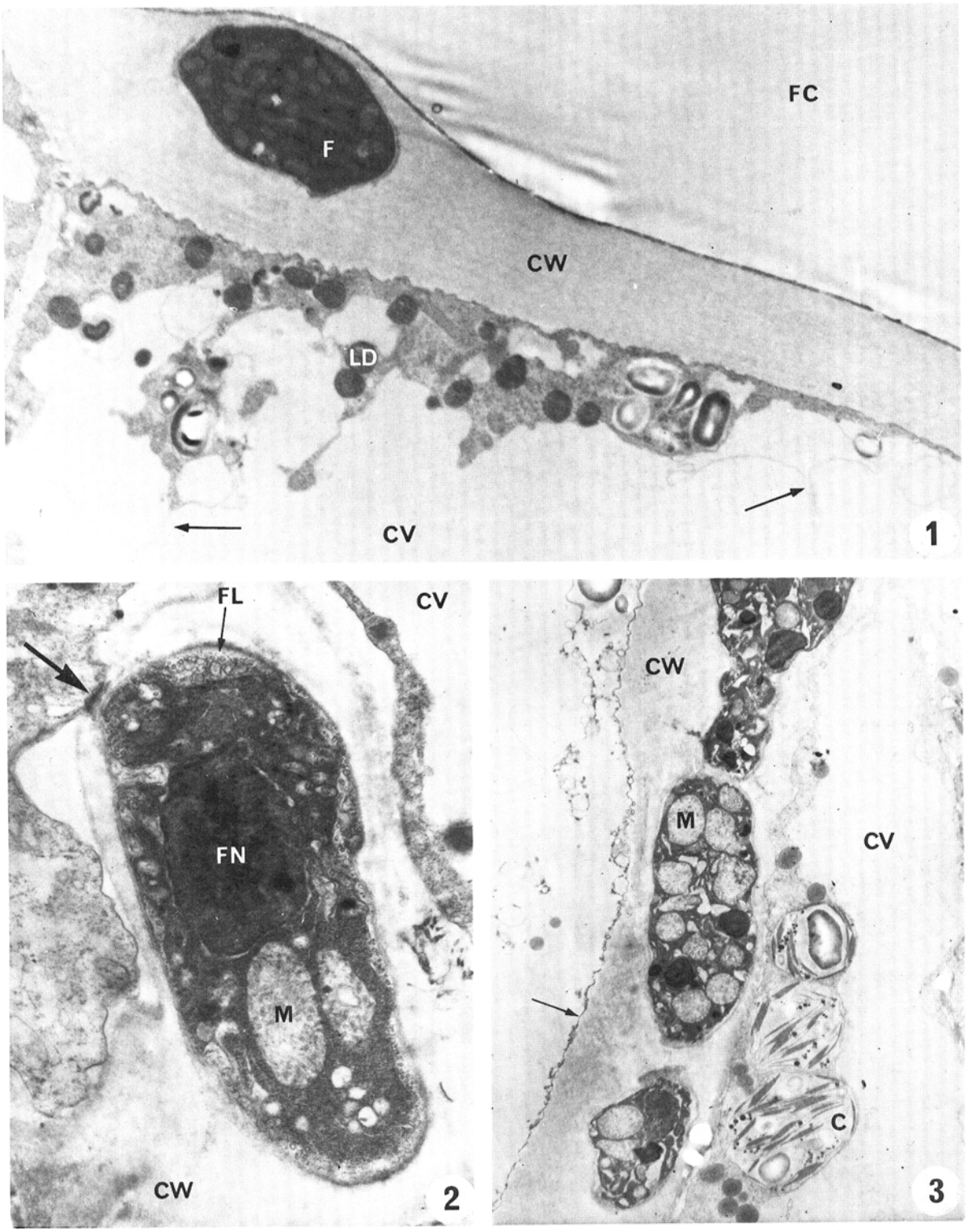


Fig. 1-3. *Phytophthora capsici* (isolate 18) in pepper fruit cell walls. **1)** Hypha in outer wall of cell bordering the fruit cavity 3 hours after zoospore inoculation. Note activated, vacuolating host cytoplasm containing lipid droplets and invaginating tonoplast membrane (arrows) (× 6,900). (2-3) Five hours after zoospore inoculation. **2)** Intercellular hypha between two activated, degenerating cells. Note lomasomes bordering the thin hyphal wall and dark cell wall modification (large arrow) at fungal-host cytoplasm interface. (× 15,450). **3)** Intercellular hypha with numerous mitochondria. Note vesiculated host cytoplasm with dark-staining plasmalemma (arrow) bordering the wall of the cell on the left and activated host cytoplasm containing lipid droplets and chloroplasts with starch granules and osmiophilic droplets in the cell on the right. (× 4,620). Legend: C = chloroplast; CV = cell vacuole; CW = cell wall; F = fungus; FC = fruit cavity; FL = fungal lomasome; FN = fungal nucleus; LD = lipid droplet; and M = mitochondrion.

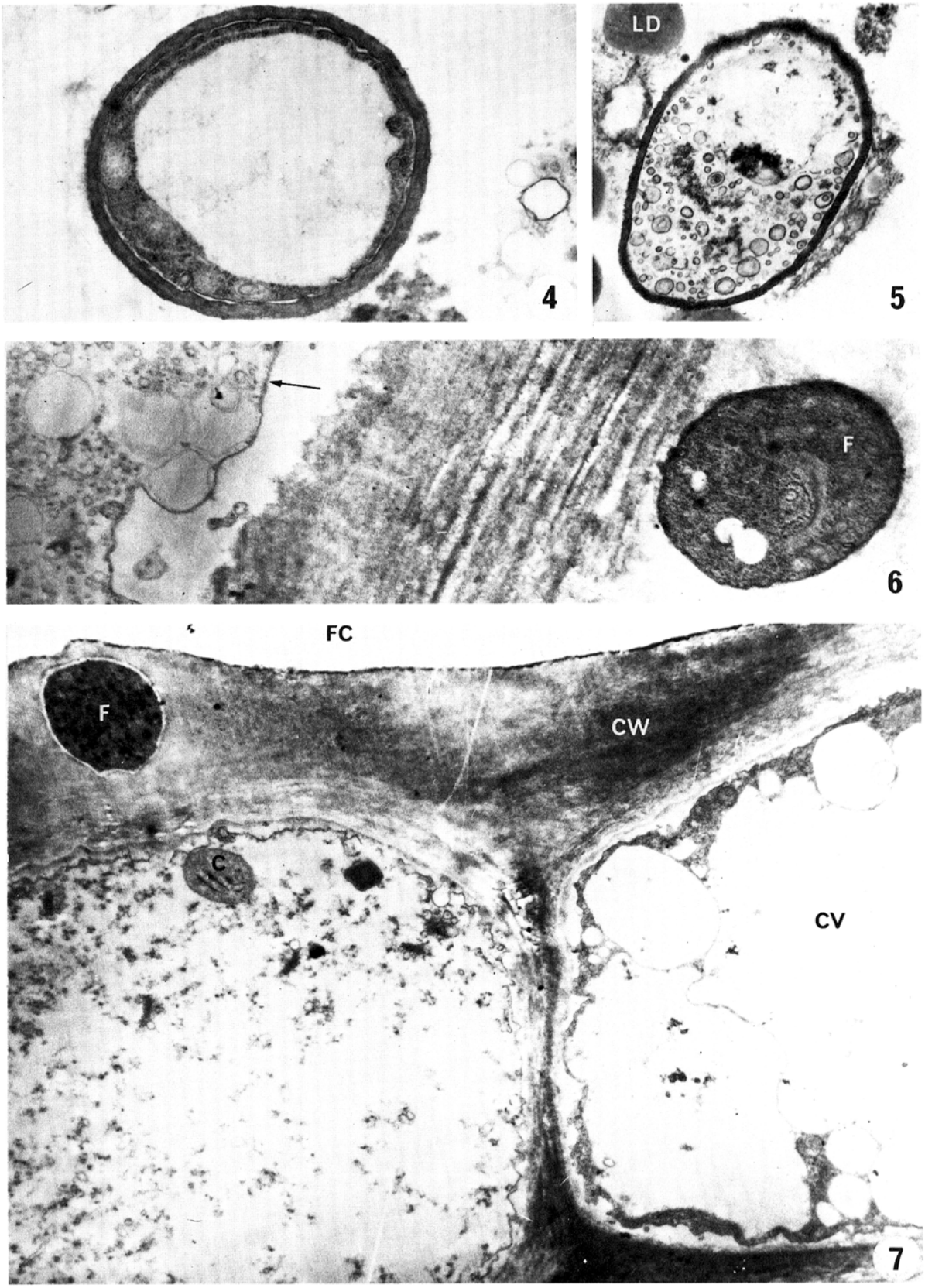
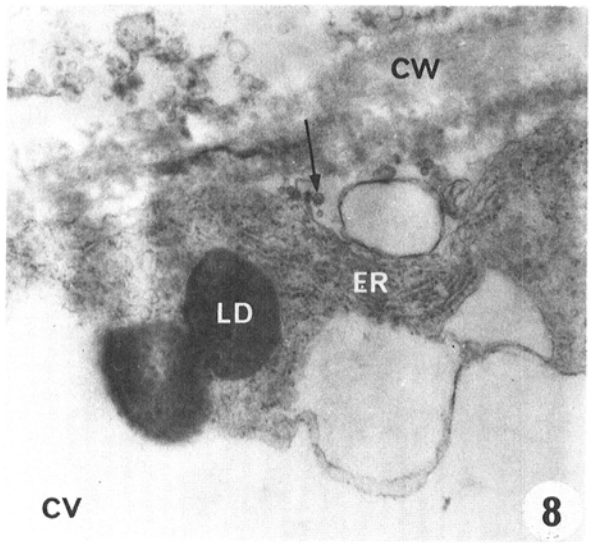


Fig 4-7. *Phytophthora capsici* (isolate 18) in pepper fruit tissue. (4-5) Haustoria in disorganized host cells 9 hours after zoospore inoculation. **4)** Vacuolated fungal cytoplasm separating from haustorial wall. (× 18,140). **5)** Vesiculated fungal cytoplasm (× 16,700). **6)** Intercellular hypha 12 hours after zoospore inoculation. Note vesiculated host cytoplasm (left) and separation of the host plasmalemma (arrow) from the dark-staining cell wall (× 7,670). **7)** Intercellular hypha in dark-staining outer wall of cell bordering the fruit cavity 48 hours after zoospore inoculation. Note disorganized cell with fragmenting, vesiculated cytoplasm directly beneath the fungus and vacuolating, activated host cytoplasm in adjacent cell to the right (× 7,360). Legend: C = chloroplast; CV = cell vacuole; CW = cell wall; F = fungus; FC = fruit cavity; and LD = lipid droplet.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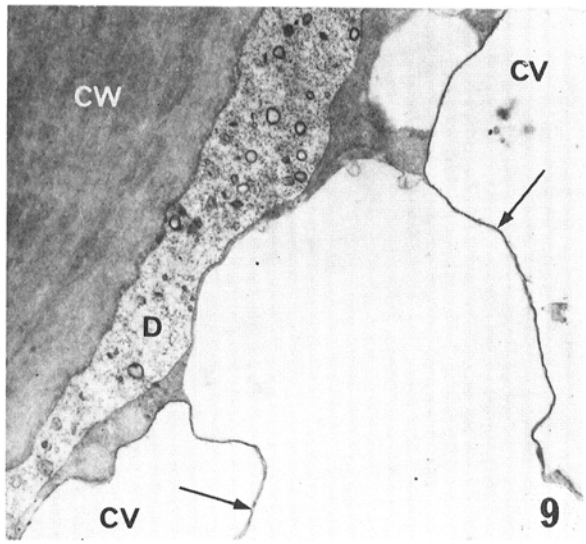
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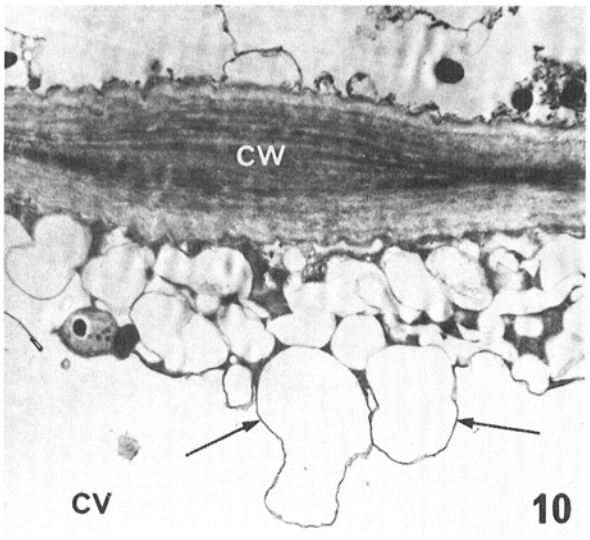
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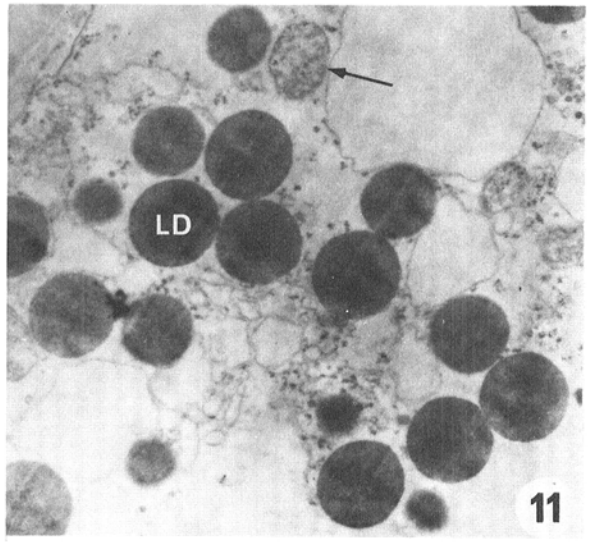
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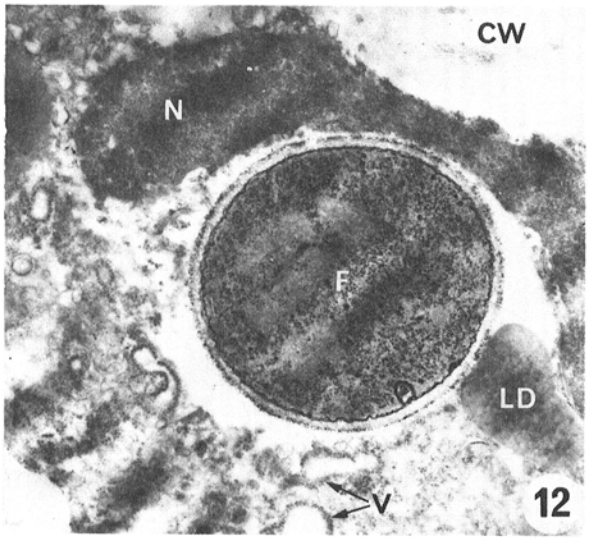
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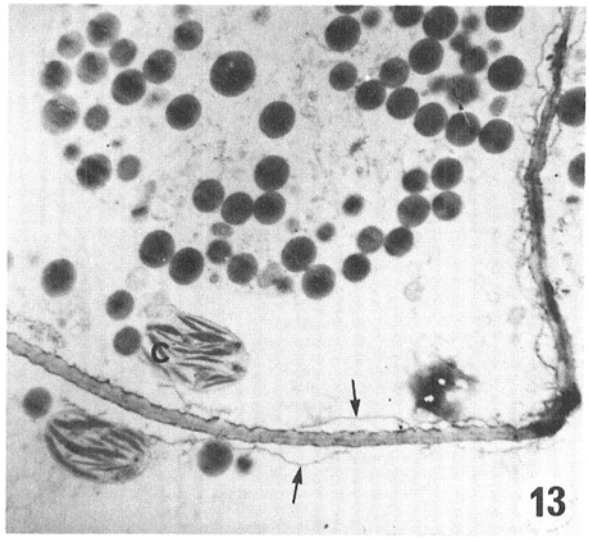
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(Wint.) Honey, a fungus widely used for the nonspecific induction of phytoalexins. The other is an interaction with an isolate of *P. capsici* (isolate 18), which is of especial interest for it was originally described as avirulent on peppers (5). Our observations indicate however, that it is pathogenic on pepper fruit tissue, although less so than *P. capsici* (A.T.C.C. 15399). This paper reports ultrastructural events; details of capsidiol accumulation during these interactions will be presented in the following paper (4).

MATERIALS AND METHODS.—*Phytophthora capsici* (isolate 18), originally isolated from a pepper plant in North Carolina (5), was kindly supplied by R. K. Webster of the Department of Plant Pathology, University of California, Davis. It was grown routinely on V8 juice agar plates at 25 C. For zoospore production, mycelial plugs were transferred to flasks containing 25 ml of sterile liquid V8 juice and incubated in the dark at 25 C. After 1-2 weeks, the mycelial mats were separated from the medium and macerated with a little sterile distilled water in a sterile Waring Blendor jar for 30 seconds. Drops of the resulting mycelial suspension were spotted onto the surface of water-agar plates using a sterile syringe with a No. 15 needle. The plates were incubated for 3 days at room temperature in the light from a single fluorescent tube (daylight, 40W) at a distance of 12 cm. The plates were then flooded with 6-ml of chilled, sterile distilled water and incubated for 1-2 hours at 18 C. The resulting zoospore suspension was filtered through Whatman's No. 54 filter paper and spore concentrations were determined using a haemocytometer.

Monilinia fructicola, originally isolated from a peach fruit, was grown on V8 juice agar plates for 7-8 days at 25 C. Plates were flooded with sterile distilled water and spores were removed from the colonies by agitation. The resulting suspension was filtered through two layers of sterile gauze to remove mycelial fragments and diluted to the desired spore concentration.

Ripening fruits of *Capsicum frutescens* L. (*Capsicum annuum* L.) var. *grossum* 'Keystone Resistant Giant' were harvested from greenhouse-grown plants and spore suspensions immediately inoculated into the cavities of each fruit using a syringe. Between $1\text{-}3 \times 10^6$ *P. capsici* zoospores and $5\text{-}10 \times 10^6$ *M. fructicola* spores were injected into each fruit, which were incubated at 25 C for the various periods indicated in the results. After fruits were opened, pieces of tissue cut from the contact area were fixed, stained, embedded, sectioned, and examined by light- and electron microscopy, as previously described (1, 2).

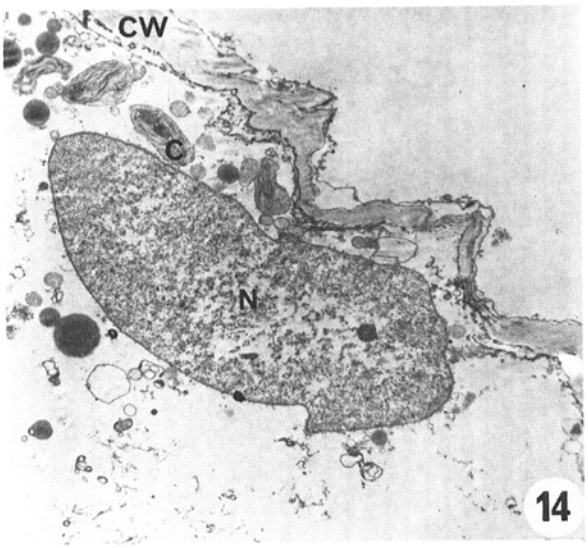
RESULTS.—*Light microscopy.*—Encysted *P. capsici* zoospores, germ tubes and appressoria were seen in depressions on the inner fruit cavity surface 3 hours after inoculation, and by 12-24 hours, an extensive network of germ tubes and hyphae had developed in some fruit. Between 24 and 48 hours, the cell walls and contents of a number of surface cells closely associated with hyphae turned dark brown. Most pepper fruit tissue in the contact area was completely permeated by fungal hyphae six days after zoospore inoculation.

Monilinia fructicola spores were visible in depressions on the inner fruit cavity surface soon after inoculation, but less than 50% had germinated by 36 hours. Germ tubes were usually short, but in some fruits profuse mycelial growth was observed. Surface host cell layers under these growths appeared to lose their rigidity. Cells with brown walls were first noticed 24 hours after inoculation and their numbers subsequently increased. The interaction was incompatible and fruits remained whole and relatively unaffected during an extended observation period of up to one month. Areas of the ripening fruit adjacent to the inoculum remained green to the naked eye for longer than other areas, which rapidly turned red.

Uninfected, control host cells.—The ultrastructure of cells in control tissues was as previously described and illustrated (1, 2).

Interaction with P. capsici.—Infection hyphae were observed in the outer walls of some cells lining the fruit cavity 3 hours after inoculation (Fig. 1). The fungal cytoplasm was dense with mitochondria, ribosomes, smooth and rough endoplasmic reticulum (ER), and contained a number of lipid droplets. By 5 hours, intercellular hyphae were present in the first two cell layers bordering the fruit cavity (Fig. 2, 3). In a few disorganized host cells, hyphalike haustoria were observed. These arose as side branches from intercellular hyphae and were constricted slightly at the point of cellular penetration. The fungal cytoplasm usually appeared active and extensive lomasome systems were evident, especially in haustoria and where intercellular hyphae were in close proximity to host cell cytoplasm (Fig. 2). However, the separation of cytoplasm from the fungal wall was apparent in some profiles and fragmenting, highly vacuolated cytoplasm was observed in a few hyphae. After 7 hours, the rate of spread had slowed and more hyphae and haustoria were seen with

Fig. 8-13. Pepper fruit cells. (8-11) Host cytoplasm in first few cell layers bordering the fruit cavity 5-7 hours after inoculation with zoospores of *Phytophthora capsici* (isolate 18). **8)** Activated cytoplasm with smooth and rough endoplasmic reticulum (sparsely and irregular arranged ribosomes) and lipid droplets. Note separation of the plasmalemma from the disintegrating cell wall and the vesicles (arrow) in the separation zone (× 19,600). **9)** Diffuse deposit containing small membrane-bound vesicles between the host plasmalemma and cell wall. Note highly invaginated, dark-staining tonoplast membrane (arrows) and vacuolating cytoplasm (× 10,960). **10)** Highly vacuolated host cytoplasm separated from the cell vacuole by an invaginated, dark-staining tonoplast membrane (arrows). Note dark, swollen cell wall (× 5,070). **11)** Disorganized host cytoplasm after the breakdown of tonoplast membrane continuity. Note lipid droplets, membrane-bound vesicles of various sizes and a degenerating mitochondrion (arrow) (× 15,700). (12-13) Host cytoplasm after inoculation of *Monilinia fructicola* spores into the fruit cavity. **12)** Intracellular hypha in host cell bordering the fruit cavity 6 hours after spore inoculation. Note close association of the host nucleus with the fungus and lipid droplets, ribosomes and small ribosome-lined vesicles in the host cytoplasm (× 19,600). **13)** Disorganized host cytoplasm containing lipid droplets and small vesicles 24 hours after spore inoculation. Note plasmalemma (arrows) bordering the cell wall and chloroplasts with osmiophilic droplets (× 4,950). Legend: C = chloroplast; CV = cell vacuole; CW = cell wall, D = diffuse deposit; ER = endoplasmic reticulum; F = fungus; LD = lipid droplet; N = host nucleus; and V = ribosome-lined vesicles.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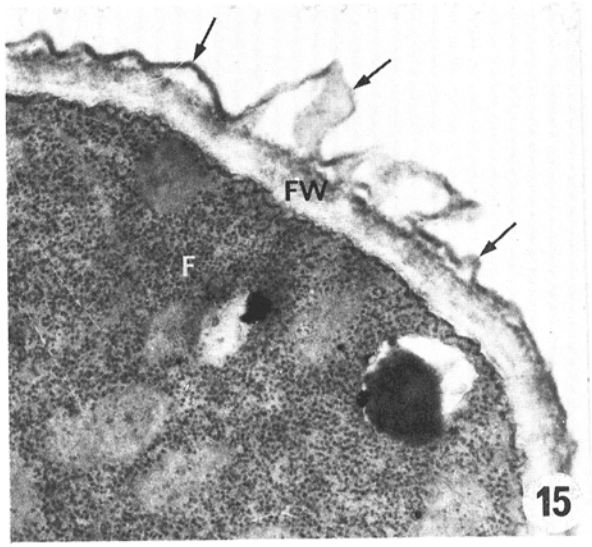
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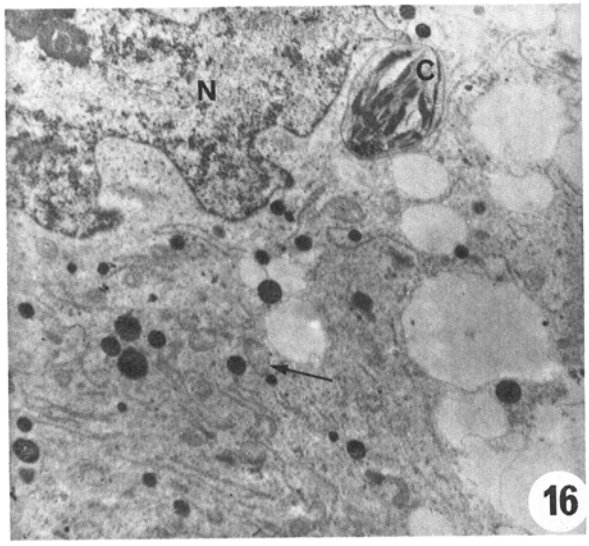
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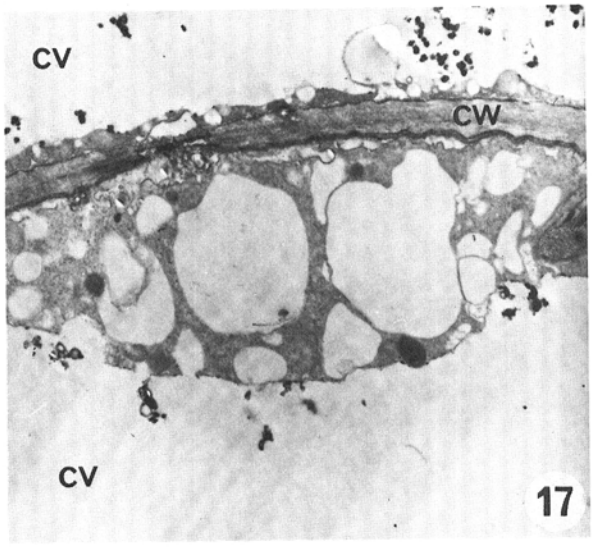
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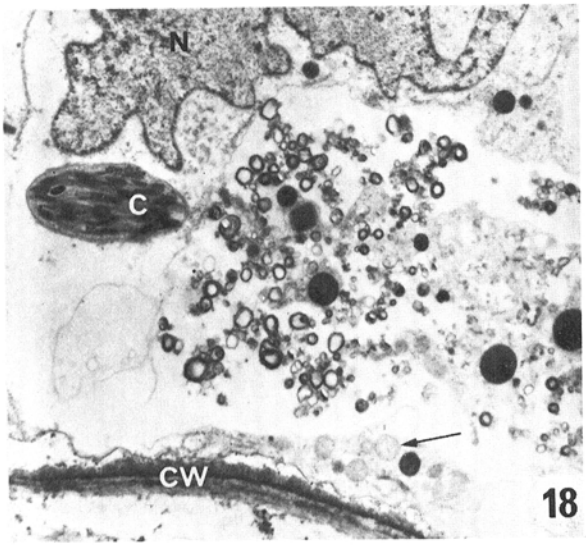
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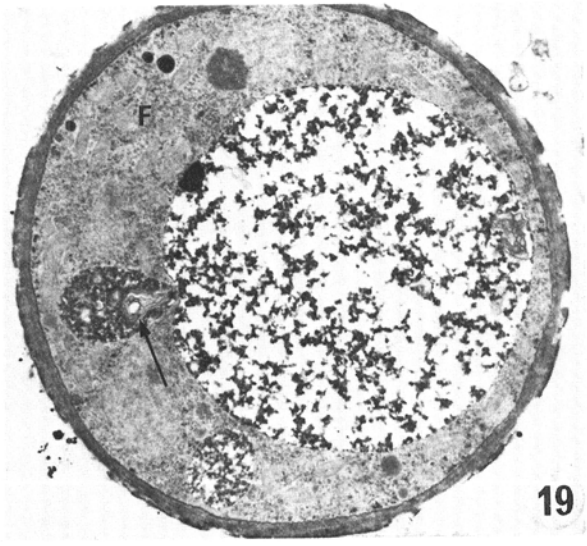
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vacuolated cytoplasm. Invasion had spread to the third cell-layer 9 hours after zoospore inoculation and many cells in the first two layers were invaded by haustoria, the majority of which, however, were highly vacuolated (Fig. 4) or vesiculated (Fig. 5). The separation of the fungal cytoplasm from the haustorial wall was again noticeable in some profiles (Fig. 4) and mitochondria appeared to be disintegrating internally. However, in most intercellular hyphae the cytoplasm was dense and lomasomes were common at the periphery. By 12 hours, invasion of the tissues had either stopped or slowed considerably, hyphae again being confined to the first three cell layers. Many hyphae and haustoria were vacuolated, but some radial sections, especially near the apices of intercellular hyphae, revealed dense cytoplasm with normal mitochondria and active Golgi bodies (Fig. 6). After 24 hours, a larger proportion of intercellular hyphae and haustoria were vacuolated and the fungus had not penetrated to deeper layers. However, after 48 hours, the number of hyphae with dense cytoplasm had increased and renewed growth was observed. Juvenile hyphae in the outer cell walls of previously uninvaded cells bordering the fruit cavity indicated that new infections had occurred, presumably from hyphae growing saprophytically in the fruit cavity (Fig. 7). Subsequent invasion of the tissue was relatively rapid and as observed earlier, hyphae emerged from the outer surface of the fruit 6 days after inoculation.

Host cells bordering the fruit cavity with infection hyphae in their outer walls showed signs of cytoplasmic reactivation 3 hours after inoculation (Fig. 1). As *P. capsici* invaded underlying tissue, some host cells appeared to disintegrate completely without any apparent reactivation, while other cells remained comparatively normal and resembled those in control tissue. However, these and most other cells near and ahead of intercellular hyphae eventually disorganized and died in a manner described below.

The initial cellular response to intercellular hyphae was the invagination of the nucleus and the appearance of dilated smooth ER, dilated rough ER with irregularly spaced ribosomes, and numerous lipid droplets in the host cytoplasm (Fig. 1, 8). The separation of the host plasmalemma from the cell wall also became noticeable in some places, especially at plasmodesmata, where small membrane-bound vesicles sometimes accumulated in the separation zones. This separation was extensive in some cells and occasionally large diffuse deposits and small vesicles were observed between the plasmalemma and cell wall (Fig. 9). Associated with these responses was the development of a highly invaginated, dark-staining tonoplast membrane and the vacuolation of the cytoplasm (Fig. 1, 9, 10). The vacuolation led to the formation of numerous vesicles of various sizes, which were released, together with the lipid droplets, degenerating mitochondria, microbodies with crystalline inclusions (1), swollen chloroplasts, and cytoplasmic fragments, into the cell cavity as continuity of the tonoplast membrane was lost (Fig. 11). It is probable that many of the vesicle membranes were formed from the fragmenting, invaginated tonoplast membrane. Occasionally, chloroplasts with lipidlike granal accumulations (2) were seen in disorganized cell cavities, but usually the grana appeared normal and osmiophilic droplets were present in the stroma, indicating a differentiation to chromoplast. The outer layers of some irregular cell walls appeared to be disintegrating (Fig. 8), while outer walls were swollen and darkly stained (Fig. 6). A dark-staining plasmalemma was usually discernible closely associated with the walls of vesiculated, dead cells, (Fig. 2, 3, 6, 7, 11). Haustoria were always observed to be surrounded by fragmented, vesiculated cytoplasm (Fig. 4, 5), indicating that these responses occurred before or during cellular penetration. Dark deposits seen at some penetration sites (Fig. 2) indicated that some cells may have initiated a limited papilla-like response before vesiculation and disorganization.

Interaction with M. fructicola.—A few hyphae, dense with cytoplasm containing functional organelles, were observed in the cell-layer bordering the fruit cavity 6 hours after spore inoculation (Fig. 12). Invaded cells were usually disorganized, but in one section the host protoplasm had accumulated around an intracellular hypha, suggesting its movement to this possible penetration site (Fig. 12) Ribosomes, lipid bodies and small ribosome-studded vesicles were evident in the host cytoplasm. Surrounding cells and other cells bordering the fruit cavity appeared to be in various stages of collapse and cell walls were stained darker in some areas. After 24 hours, the host cytoplasm in the first three cell-layers bordering the fruit cavity was in various stages of vacuolation (as was described earlier for cells reacting to *P. capsici*, isolate 18) and where tonoplast membrane continuity was broken, vesicles, lipid droplets, and swollen chloroplasts, were dispersed throughout the cell cavity (Fig. 13). Dead host nuclei were smooth in outline (Fig. 14) and the dark-staining host plasmalemma was discernible bordering, yet usually detached from, the cell wall, which was variously comparatively normal, darkly stained, swollen, or irregularly shaped (Fig. 13, 14). The few hyphae that were seen in these cells were dense with cytoplasm and were usually surrounded by the invaginated host plasmalemma (Fig. 15). Observations between 44 and 48 hours after spore inoculation revealed that most of the cells in the first 1-3 layers lining the fruit cavity were completely disrupted; cell walls having

Fig. 14-19. Interaction of pepper fruit cells with *Monilinia fructicola*. (14-15). Twenty-four hours after inoculation with spores. **14)** Disorganized host cell with an irregular cell wall. Note the relatively smooth-surfaced nucleus and the fragmenting, vesiculated cytoplasm (× 3,280). **15)** Intracellular hypha bordered by invaginated, dark-staining plasmalemma (arrows) (× 29,900). (16-19) Forty-four to forty-eight hours after spore inoculation. **16)** Activated, vacuolating host cytoplasm containing small, dark, lipid droplets, mitochondria (arrow), and smooth endoplasmic reticulum. Note invaginated nucleus (× 5,000). **17)** Vacuolating host cytoplasm (× 5,000). **18)** Small membrane-bound vesicles and dark lipid droplets in disorganizing, activated host cytoplasm. Note invaginated nucleus and mitochondria (arrow) (× 5,000). **19)** Membranous lysosomelike structure (arrow) adjacent to vacuole containing disintegrating fungal cytoplasm (× 7,260). Legend: C = chloroplast; CV = cell vacuole; CW = cell wall; F = fungus; FW = fungal wall; and N = host nucleus.

collapsed and in some areas disappeared. Cells deeper in the tissue, approximately 3-7 cell-layers from the cavity, were reacting in a similar manner to those in the first three layers 24 hours after inoculation. Many possessed highly invaginated nuclei and lipid droplets, ER, microbodies with crystalline inclusions, and chloroplasts, some with stromal osmiophilic droplets and others with lipid-like granal deposits, were evident in the vacuolated, vesicle-forming cytoplasm (Fig. 16, 17, 18). Cells below these reactivated, disorganizing layers appeared normal and resembled control tissue. As before, few fungal hyphae were seen in the tissue, but those that were, appeared to be confined to the disintegrating first few layers and showed signs of internal vacuolation and fragmentation, possibly initiated by lysosomal activity (Fig. 19). Hyphae growing saprophytically in the cell cavity also began degenerating at this time.

DISCUSSION.—Ultrastructural events occurring during an incompatible interaction between *P. infestans* and peppers, a nonhost, have been reported previously (2). The observations reported here using two more fungi demonstrate the variability of the interaction. They indicate that although incompatibility is associated with a rapid hypersensitive response, differences in detail exist which presumably relate to differences between the fungal species challenging the fruit tissue.

Phytophthora capsici (isolate 18) was chosen for study because of its reported avirulence on peppers (5). In unpublished experiments using pepper leaves, we also have found this isolate avirulent, but as the results reported here indicate, the situation is different in fruit. The initial interaction was incompatible, growth of invading hyphae being arrested 9-12 hours after inoculation. However, 48 hours after inoculation previously uninvaded cells on the surface of the fruit cavity were invaded (Fig. 7) and the fruit tissue rapidly colonized. During the incompatible phase, penetration of walls of cells bordering the fruit cavity was as rapid as in previously studied interactions with the virulent *P. capsici* (A.T.C.C. 15399) and with *P. infestans* (1, 2). Following wall penetration, the first two cell-layers were invaded within 5-7 hours, after which the rate of most hyphal growth slowed and apparently ceased in the third cell-layer. Between 7 and 12 hours, this was accompanied by the vacuolation of first the haustoria and then most of the intercellular hyphae. With *P. infestans* (2), events took place more slowly. Although hyphal growth was halted in the third cell-layer also, this stage was not reached until 24-36 hours following inoculation. It is of interest that in a recent report of an incompatible interaction between *P. infestans* and its natural host, the potato, the sequence of events leading to the arrest of hyphal growth was more rapid (6). A significant feature of the incompatible interaction of pepper cells with *P. infestans* was the development of smooth ER and polyribosomes in reactivated cytoplasm prior to penetration and the subsequent formation of large cell border lesions between the invaginating host plasmalemma and intruding haustoria. This was not observed in the two incompatible interactions described in this paper, although small aggregations of vesicles or diffuse deposits were observed occasionally in the separation zone between the host plasmalemma and the cell wall (Fig. 8, 9), and the plasmalemma was invaginated to some extent around penetrating intercellular hyphae (Fig. 15).

The interaction with *M. fructicola* differed from the other interactions in the rate and extent of fungal invasion. Hyphae penetrated a few cells in the first layer bordering the fruit cavity 6 hours after inoculation, but subsequent invasion and spread was infrequent and slow. However, appreciable saprophytic growth took place on the surface of cells lining the fruit cavity. Lysosomelike structures bordering vacuoles containing disintegrating cytoplasm 44-48 hours after inoculation (Fig. 19) suggest that internal hyphal disorganization may be self-initiated.

A comparison of the progress of tissue response to *P. capsici* (isolate 18) at 3, 5, 7, and 9 hours after inoculation, indicated that the reactivation of the cell cytoplasm, which usually occurred prior to cellular penetration, and its subsequent disorganization, took place within 1 hour of stimulation. The hypersensitive response consisted of the development of ER and lipid droplets in the cytoplasm (Fig. 8), the invagination of the tonoplast membrane (Fig. 9, 10), the vacuolation of the cytoplasm (Fig. 10), the breakdown of tonoplast membrane continuity, and the dispersion of cell organelles, including microbodies with crystalline inclusions, vesicles, and cytoplasmic fragments throughout the cell cavity (Fig. 11). Evidently these changes were stimulated by metabolites in the immediate environment of intercellular hyphae. However with *M. fructicola*, similar host cell responses (Fig. 13, 16, 17, 18) occurred to a depth of 6-7 cell layers, indicating that in this case the stimulus, if of fungal origin, spreads an appreciable distance from the hyphae in and on the surface cells. Swelling, distention, and darkening of pepper cell walls was also more noticeable in this interaction and their disappearance in some areas suggests that cell-wall degrading enzymes may be involved.

The present, and the two previous investigations (1, 2), cover a range of fungus-pepper combinations from compatible to highly incompatible. Some aspects of these interactions appeared to be general. For example, the formation of dark papilla-like deposits (Fig. 2), as suggested previously (2), may be a nonspecific response to contact between activated pepper cell cytoplasm and fungal hyphae. The usual association of fungal lomasomes with these deposits indicates that their formation may be influenced by local fungal secretions. Other features appear to be specifically related to the fungus-pepper combination. Thus, although some hypersensitive cells were observed (D. R. Jones, *unpublished*), the basic response to *P. capsici* (A.T.C.C. 15399) in the compatible interaction appeared to be one of physiological rejuvenation, the reactivated cytoplasm disrupted only after invasion (2). In the incompatible interactions, a hypersensitive response occurs before or during penetration. The slower degeneration of pepper cells invaded secondarily by *P. infestans* (2) may be an additional distinct response. In all cases, cells were reactivated prior to penetration, indicating the involvement of diffusible stimuli.

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