Ultrastructural Changes in Pepper Cells in a Compatible Interaction with Phytophthora capsici

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ABSTRACT

Invasion of pepper fruit tissues was detected 4 h after suspensions of *Phytophthora capsici* zoospores were injected into cavities of ripening fruit of sweet pepper (*Capsicum frutescens*). Cells in the immediate vicinity of invading intercellular hyphae possessed highly lobed nuclei with prominent nucleoli. Where hyphae had penetrated into cell walls, the adjacent host cytoplasm usually was dense with ribosomes. Parallel layers of rough endoplasmic reticulum were common ultrastructural features and often surrounded degenerating chloroplasts.

Six hours after zoospore injection, the cytoplasm of these layers of cells was disorganised by invading hyphae, while cells deeper in the fruit tissue near the apices of advancing intercellular hyphae had ribosome-saturated cytoplasm with rough endoplasmic reticulum layers. These responses were associated with low, noninhibitory levels of the phytoalexin capsidiol, previously found in this compatible interaction.

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It was demonstrated previously that inhibitory concns of the phytoalexin, capsidiol (9) are produced in pepper fruit inoculated with any of a number of fungi, nonpathogenic to peppers (18). Much smaller, noninhibitory, concns accumulated in response to the pathogen *Phytophthora capsici* Leonian and this could not be accounted for by metabolism to other compounds, as demonstrated for other fungi (20). If capsidiol plays a role in the resistance of pepper fruit cells to a number of different fungi, then it is obviously important to determine how its production can be suppressed or bypassed by a successful pathogen such as *P. capsici*. As a preliminary step in the investigation of this problem, a study was made of the sequence of events and the ultrastructural changes taking place during infection by this fungus.

MATERIALS AND METHODS.—Sweet pepper plants, *Capsicum frutescens* L. (*C. annuum* L.) var. *grossum* 'Keystone Resistant Giant', were grown in the greenhouse from seed. Fruits were harvested when half ripe (green, streaked with brown) and used immediately.

Colonies of *P. capsici* (A.T.C.C. 15399) were grown on V8 juice agar in petri dishes at 25 C. Sporangial suspensions were obtained by filtering sterile distilled water washings from these plates through two layers of linen gauze. The suspensions were incubated for 1-2 h at 12 C to allow release of zoospores, which were separated from the empty sporangia by filtration through Whatman's No. 54 filter paper. The resulting zoospore suspensions were diluted and similar numbers of zoospores (65 to 85 $\times$ 10^4) injected into each pepper fruit cavity (18). After incubation for 4 or 6 h at 25 C, the inoculated fruits were cut open and the diffusates removed. Pieces of tissue in the contact area were cut from the fruit and fixed in formaldehyde/glutaraldehyde (2%/2.8%, vol. for vol. in 0.05M phosphate buffer at pH 6.8) for 2 h. A control fruit, injected with sterile distilled water and

incubated for 6 h was treated similarly. After the initial fixation and overnight washing in phosphate buffer, the tissues were postfixed in a 1% solution of osmium tetroxide in phosphate buffer for 2 h. The tissues then were rinsed in several changes of buffer for 0.5 h and dehydrated in an ethanol series. After transfer to propylene oxide, the 4-h sample was infiltrated with Luft's epon resin (13). The 6-h samples were infiltrated with Spurr's resin (17), which had superior sectioning qualities. The infiltrated tissues were incubated at 60 C for 1-3 days. Areas where fungal hyphae were invading the tissue were located by light microscopy before ultrathin sectioning. Sections were cut at right angles to the fruit wall on a Sorvall, Porter-Blum MT-1 microtome using glass knives, stained in saturated aqueous uranyl acetate (0.5-1.0 h), rinsed in distilled water, and further stained in a 0.25% solution of lead citrate for 45 sec (19). The sections were examined and photographed with a R.C.A. (EMU3F) electron microscope.

RESULTS.—*Control.*—Most cells in the control pepper tissues possessed large vacuoles with thin layers of aggregated cytoplasm lining the cell wall. This contained chloroplasts, some showing signs of transformation to chromoplasts, mitochondria, small lipid bodies, and fragmentary rough endoplasmic reticulum (ER) with sparsely and irregularly spaced ribosomes. The nuclei usually were smooth in outline and the general appearance of the cells suggested low metabolic activity (Fig. 1).

Observations on pepper tissue 4 h after inoculation.—Fungal hyphae were frequently observed in the walls of the first few layers of cells bordering the fruit cavity (Fig. 2). Bulges in the host cell walls surrounding some intercellular hyphae protruded into the cell cavity and suggested that softening and spreading of the wall constituents had occurred. In places, hyphae were separated from the host plasmalemma by a modified electron-dense cell
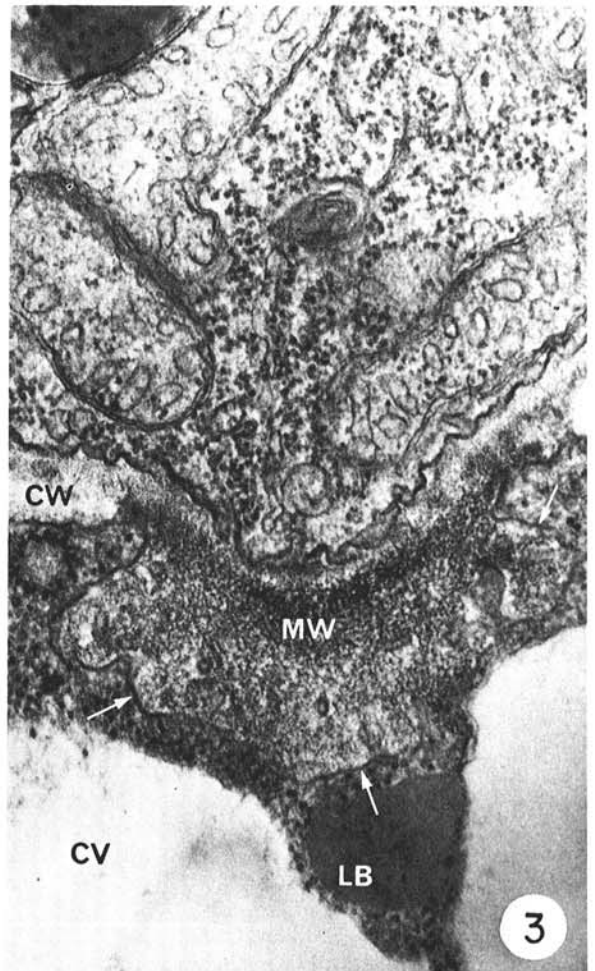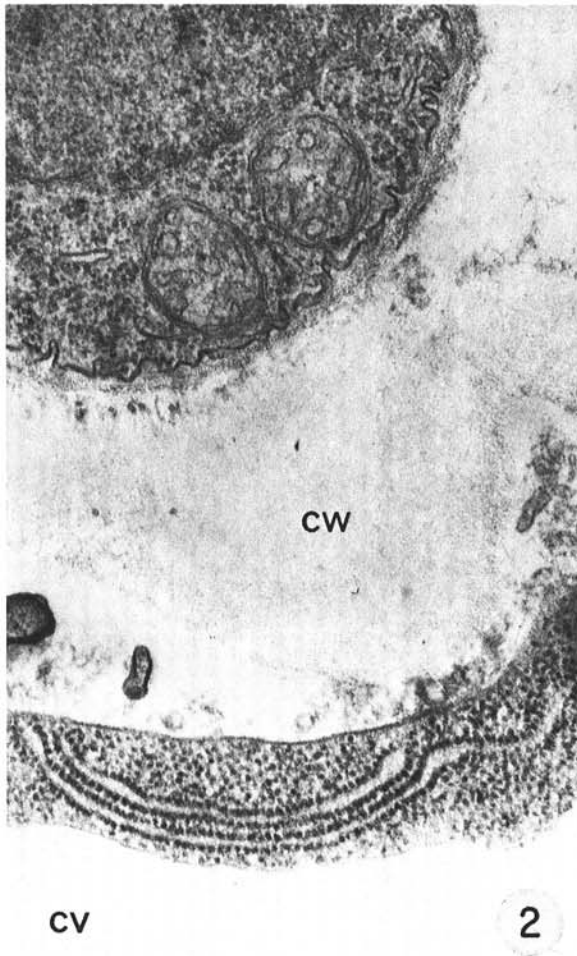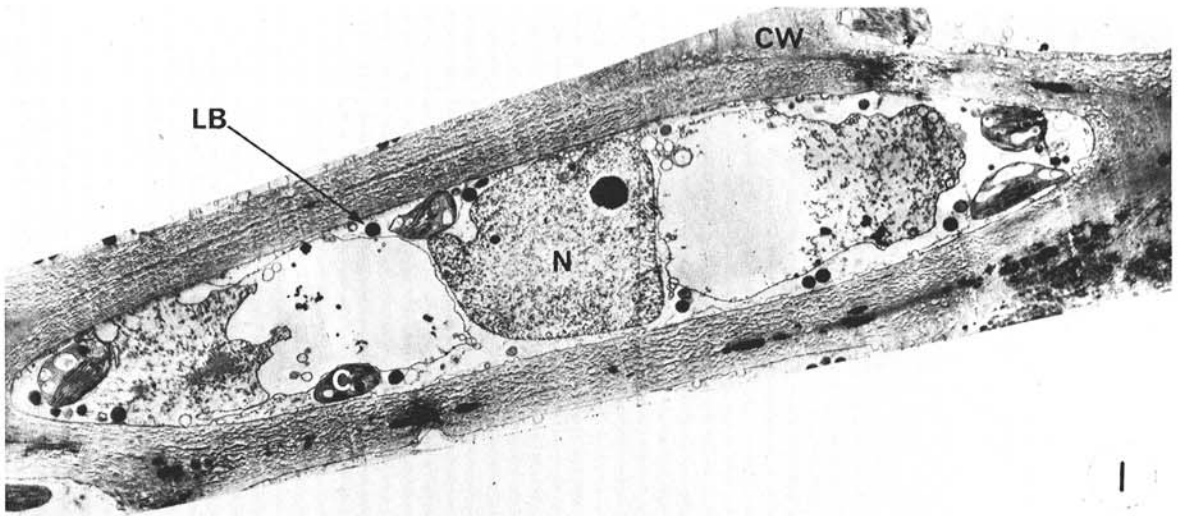
Fig. 1-3. Cells of pepper fruit (*Capsicum frutescens*) **1)** Control pepper cell. Note aggregated cytoplasm, numerous small lipid bodies and smooth outline of the nucleus (× 2,540). **Fig. 2, 3)** Pepper cells 4 h after inoculation with *Phytophthora capsici* zoospores. **2)** Hypha (upper left) in bulging cell wall. Note separation of the ribosome-dense cytoplasm with parallel rough endoplasmic reticulum layers (below) from the cell wall (× 40,600). **3)** Intercellular hypha (above) penetrating pepper cell wall. Note modified electron dense wall intruding into host cytoplasm, separating the hypha from the host plasmalemma (arrows) (× 44,500). Legend: C = chloroplast; CV = host cell vacuole; CW = host cell wall; LB = lipid body; MW = modified host cell wall; and N = nucleus.
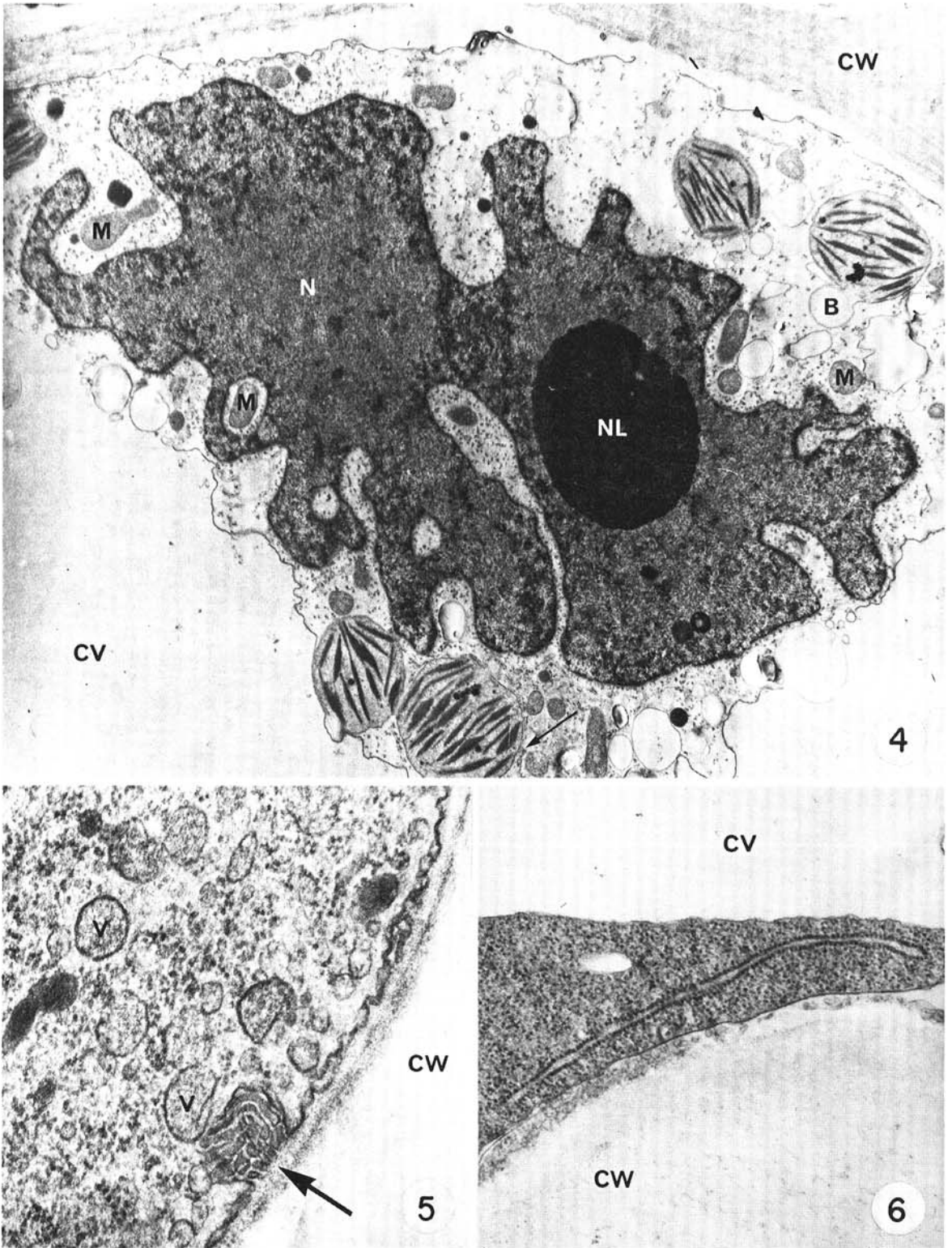
Fig. 4-6. Four hours after inoculation with *Phytophthora capsici* zoospores. **4)** Invaginated pepper cell nucleus with a large prominent nucleolus. Note mitochondria surrounding nuclear margin and bulging chloroplast outer membrane. Layers of endoplasmic reticulum (ER) can be seen parallel to chloroplast surface (small arrow, center bottom) (× 6,400). **5)** Intercellular hypha with lomasome (arrow) and associated small vesicles. (× 54,300). **6)** Ribosome-dense pepper cell cytoplasm with single layer of rough ER (× 28,500). Legend: B = bulging chloroplast outer membrane; CV = host cell vacuole; CW = host cell wall; M = mitochondria; N = nucleus; NL = nucleolus; and V = vesicles.
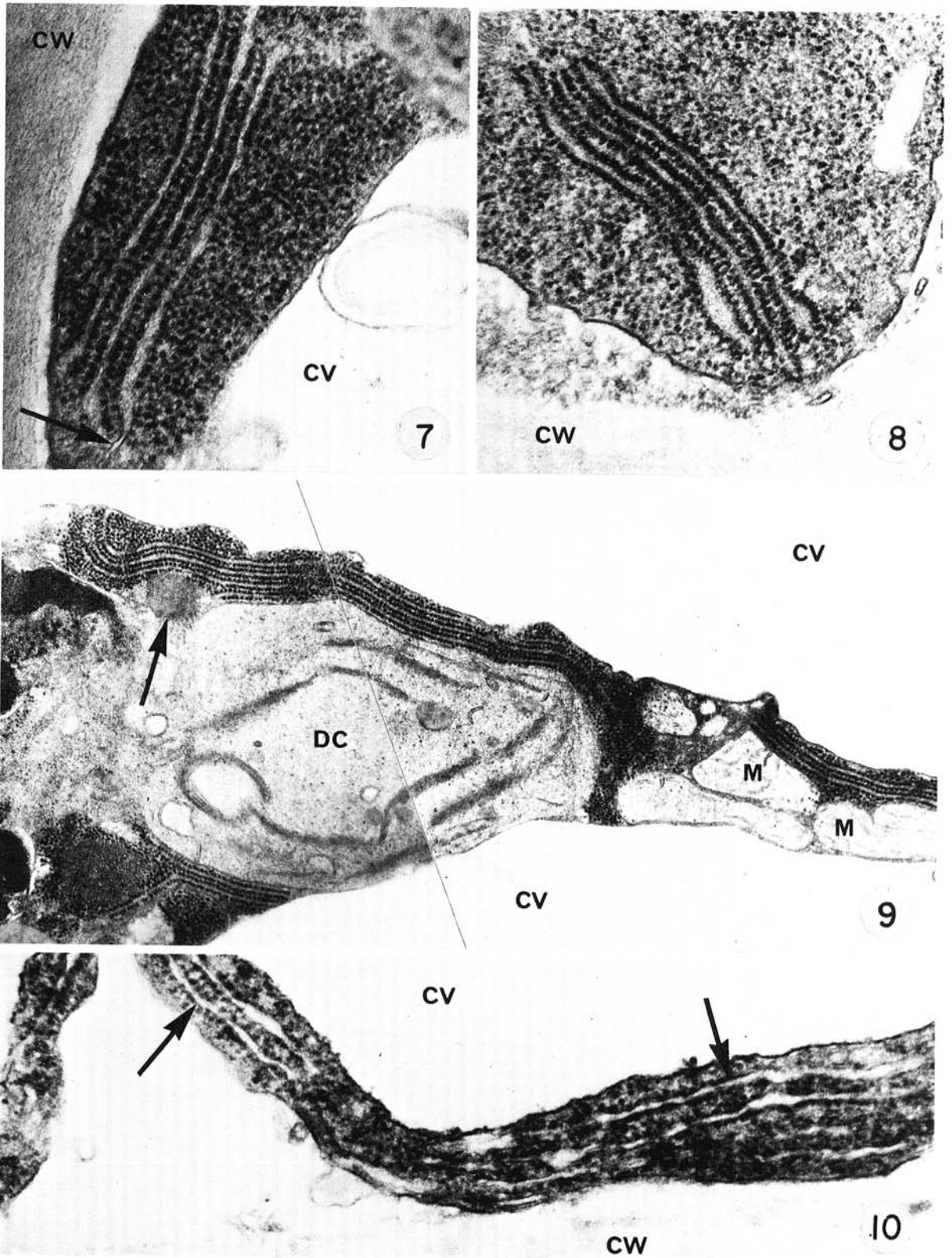
Fig. 7-10. Cells of pepper fruit (*Capsicum frutescens*) 4 h after inoculation with *Phytophthora capsici* zoospores. **7)** Ribosome-dense pepper cell cytoplasm with parallel layers of rough endoplasmic reticulum (ER). Note ER (arrow) visible in area devoid of ribosomes (×59,500). **8)** Ribosome-dense pepper cell cytoplasm with parallel layers of rough ER (×52,000). **9)** Rough ER layers orientated parallel to the surface of a degenerating chloroplast, and also near mitochondria. Note microbody (arrow) which contains a crystal similar to Fig. 13 (×31,300). **10)** Rough ER (arrows) with irregularly spaced ribosomes in pepper cell cytoplasm (×50,000). Legend: CV = host cell vacuole; CW = host cell wall; DC = degenerating chloroplast; and M = mitochondria.
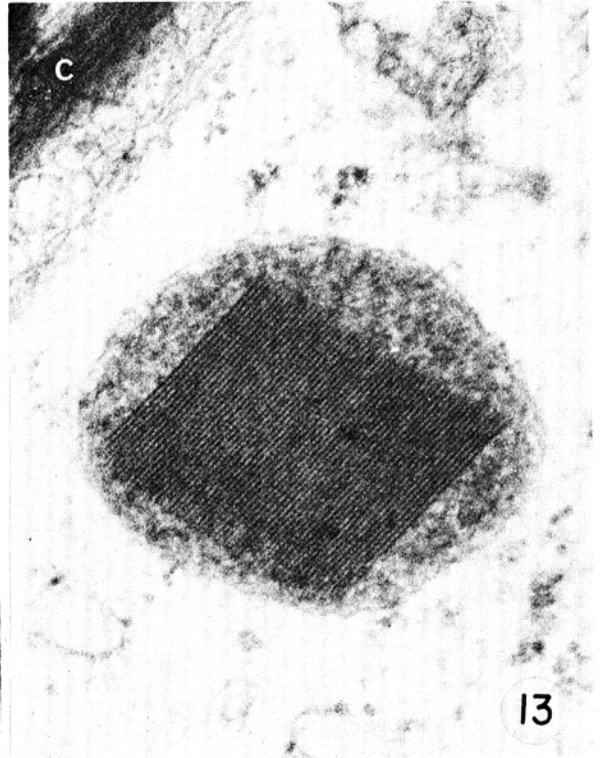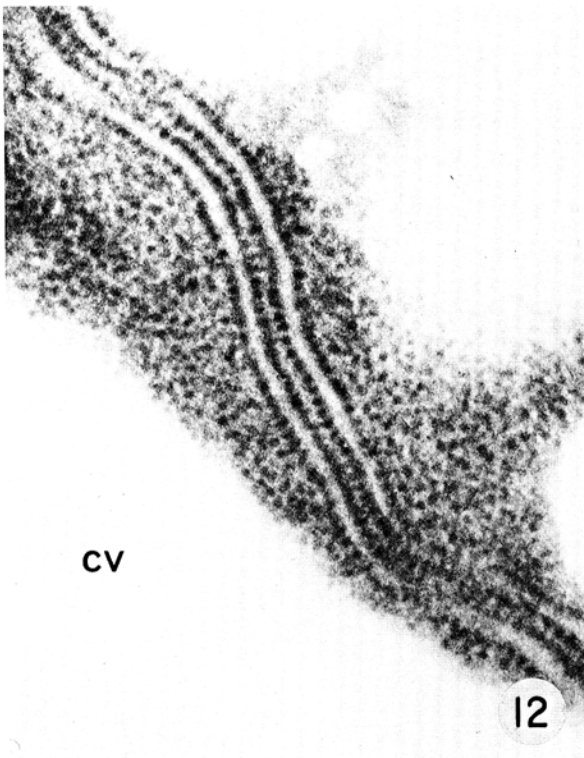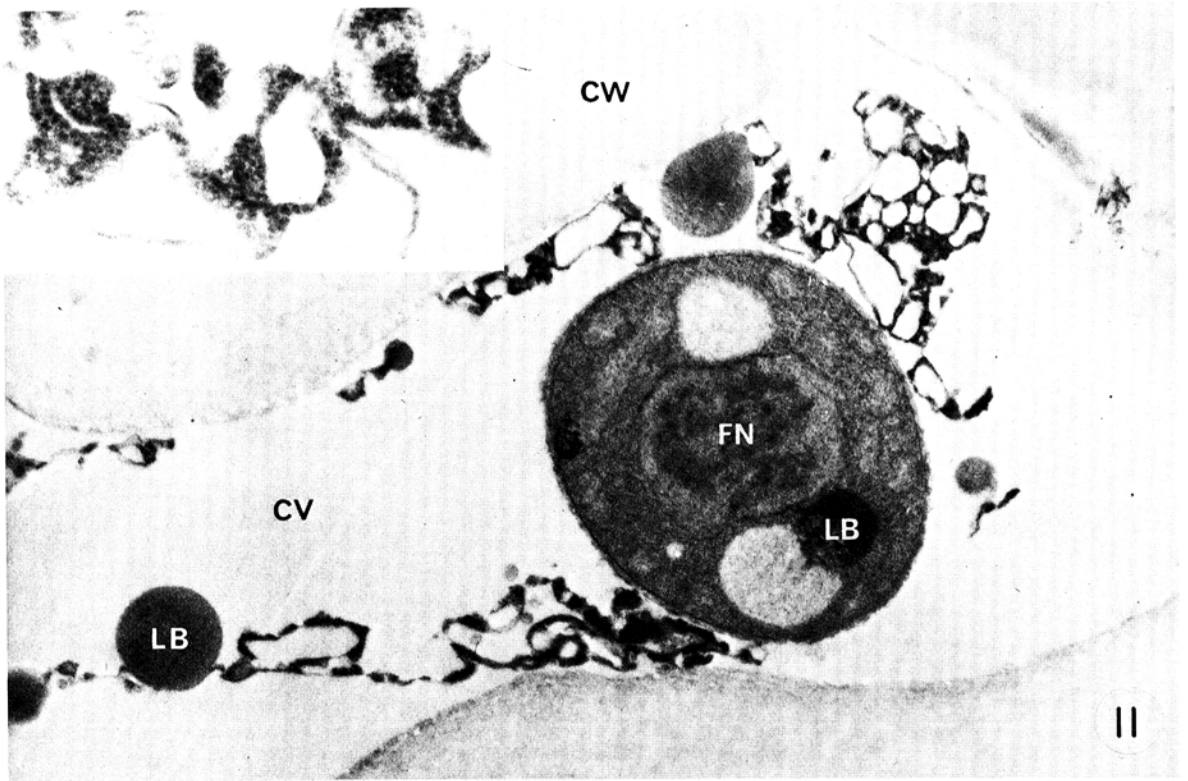
Fig. 11-13. Six hours after inoculation with *Phytophthora capsici* zoospores. **11)** Intracellular hypha surrounded by remains of pepper cell cytoplasm (× 12,200.) (Inset) Magnification showing ribosomes in disorganized host cytoplasm (× 51,400). **12)** Ribosome-dense pepper cell cytoplasm with parallel rough endoplasmic reticulum layers (× 76,000). **13)** Crystal containing microbody in pepper cell cytoplasm (× 74,400). Legend: C = chloroplast; CV = cell vacuole; CW = cell wall; FN = fungal nucleus; and LB = lipid bodies.

wall, which intruded into the host cell cytoplasm (Fig. 3). This modification was similar to wall structures, believed to be deposits, formed in other host-parasite interactions and has been termed a papilla or cell wall lesion (2). As the pepper cell was subsequently invaded by hyphae (see later), the modification was evidently short-lived, appearing just prior to actual penetration of the cell cytoplasm.

The fungal cytoplasm contained mitochondria, ribosomes, ER (Fig. 2,3), occasional small lipid bodies and a few small vacuoles. Numerous lomasomes and associated cytoplasmic vesicles were observed on and near the fungal cell membrane (Fig. 5).

Nuclei in pepper cells near to, but not penetrated by, fungal hyphae had irregular, deeply, invaginated outlines and large prominent nucleoli (Fig. 4). They were usually associated with groups of chloroplasts, some with bulging outer membranes, and mitochondria were common around their margins (Fig. 4). Most pepper cells adjacent to intercellular hyphae contained cytoplasm dense with large numbers of free and ER-attached ribosomes (Fig. 2,6). The rough ER had regularly spaced ribosomes and was frequently arranged in long parallel layers (Fig. 2,7,8,9). These layers often were parallel to the outer membranes of degenerating sac-like chloroplasts which contained disorganised and disintegrating lamellae (Fig. 9). In cytoplasm devoid of other organelles, the rough ER layers usually were arranged parallel to the cell wall (Fig. 2,7), though not always (Fig. 8). In some sections, the cytoplasm contained fewer ribosomes, most of which were spaced irregularly on the ER (Fig. 10). Chloroplasts in these areas retained their internal organization though they had lost their typical bi-convex form. Generally the extent of chloroplast degeneration appeared to be related to increases in the numbers of ribosomes and to the degree of organization of the rough ER. Although chloroplasts were the organelles first and most noticeably affected, mitochondria also were seen in various stages of degeneration. The separation of the cytoplasm and plasmalemma from the cell wall was often observed (Fig. 2,4,6,7,8,10).

Cells not in the immediate vicinity of intercellular hyphae resembled cells in the control tissue. They did not have ribosomes in large numbers, their nuclei had relatively smooth outlines and other cell organelles appeared morphologically normal.

Observations on pepper tissue 6 h after inoculation.—Hyphae had penetrated through the cell walls and into the vacuoles of the first few cell layers. The cytoplasm was detached from the cell walls and appeared dispersed and vacuolated, but the remnants still contained large numbers of ribosomes (Fig. 11).

The fungal cytoplasm, while containing more lipid bodies and vacuoles than formerly, had numerous mitochondria, ribosomes, and lomasomes, and evidently was still active (Fig. 11).

Intercellular hyphae had penetrated cell layers deeper in the fruit tissue and cells adjacent to these had characteristically ribosome-dense cytoplasm and rough ER layers (Fig. 12).

Microbodies containing crystalline inclusions occasionally were seen in pepper cells in infected and uninfected tissues, usually near chloroplasts (Fig. 9, 13), and resembled microbodies found in other plant cells (8). The bulges observed in some chloroplast membranes (Fig. 4) may be assiocated with the formation of these or similar microbodies.

DISCUSSION.—The invasion of pepper fruit tissue by *P. capsici* seemed to physiologically reactivate most of the morphologically mature pepper cells in the vicinity of intercellular hyphae, stimulating production of large numbers of free and ER-attached ribosomes. Whether this stimulation was caused by actual physical contact between the fungus and pepper cell cytoplasm or by the effects of fungal metabolites diffusing from invading hyphae, has not been absolutely determined. The evidence would appear to support the latter possibility, as organized ribosome-dense cytoplasm was observed only in cells adjacent to intercellular hyphae. The separation of the cytoplasm from cell walls and degeneration of cell organelles prior to penetration was probably also the result of the effects of fungal metabolites.

The pepper cell nucleus appeared to be the first organelle to react to the stimulus. The nucleolus became prominent and the nuclear margins became highly lobed and invaginated which, by increasing nuclear cytoplasmic contact, presumably indicated heightened nuclear metabolic activity (Fig. 4). Such activity accords with the known function of the nucleus as the site of synthesis of ribosomal protein and assembly of ribosomal RNA (3) and with the spectacular increases in ribosome numbers reported here. It was estimated from ultrastructural differences between tissues at 4 and 6 h that at least part of the cell cytoplasm became saturated with ribosomes within 2 h after stimulation. Areas of cytoplasm containing fewer ribosomes, mostly attached irregularly to the ER (Fig. 10), were probably at an intermediate stage in the ribosome saturation process. The number and organization of the rough ER layers also suggests that increases occurred de novo, rather than by redistribution of existing ER. Close association between some rough ER arrangements and chloroplasts was evident in cytoplasm containing few ribosomes (Fig. 4), and was pronounced in ribosome-dense cytoplasm (Fig. 9). This distinctive rough ER layering was not observed around other cell organelles to such an extent, though there was occasional contact between rough ER and mitochondrial surfaces (Fig. 9). It is possible that the outer membranes of chloroplasts (large organelles) acted as convenient orientation surfaces for the ER layers. The ER in some plant cells is, however, continuous with the outer membranes of chloroplasts, and it has recently been suggested that some ER may possibly be derived from chloroplast membranes (4). This suggestion could account for the close rough ER-chloroplast association.

The arrangement of the rough ER in parallel layers with regularly spaced ribosomes (Fig. 2,7,8,9,12) is an unusual feature in mature plant cells, but is common in animal secretory cells, where it is

associated with the formation and channelling of proteinaceous compounds (3). Dense concns of host ribosomes and rough ER have not been reported in susceptible interactions with other *Phytophthora* species (6,10). However, in susceptible interactions with various obligate parasites, increases in ribosomes (e.g. 1,14), rough ER (e.g. 7, 11), and occasionally both (5) have been found in host cytoplasm adjacent to penetration sites or haustoria. Ribosome-dense cytoplasm is indicative of a high capacity for protein production and the rough ER indicates that the products of this activity, if formed, could be channelled. The close association of rough ER with degenerating chloroplasts could also indicate the channelling of metabolites, possibly carbohydrates. Transport of sucrose has been suggested for similar rough ER sheathing found around plastids in *Acer pseudoplatinus* phloem companion cells (21).

In the compatible interaction described here, low concns of the phytoalexin capsidiol are induced (18). This interaction should be comparable therefore, to compatible interactions in other host-parasite combinations, in which phytoalexins are induced in low levels. These are characterized by an apparent suppression of a hypersensitive response, normally correlated with phytoalexin production, and by the establishment of a phase of compatibility between host and parasite. Thus in bean (*Phaseolus vulgaris*) infected with *Colletotrichum lindemuthianum*, no deleterious changes were detected in host cells for at least 20 h following penetration (15,16) and potato cells similarly failed to show any deleterious changes to *Phytophthora infestans* for 22 h (12). In pepper fruit however, the situation is somewhat different, for within 6 h after inoculation with *P. capsici*, host cells first exhibit a series of changes, indicative of a very active response, and then rapidly become disorganized by penetrating hyphae and presumably die. This leads to the conclusion that for this system, rapid death of host cells does not constitute a typical hypersensitive reaction, nor does it result in phytoalexin production. Furthermore, even though the fungus induces profound changes in the host cell, either these are not associated with enhanced capsidiol production, or the ensuing cytoplasmic disorganization rapidly renders them ineffective. Finally, it is evident that any dynamic response of the host cell leading to resistance must be mobilized and become active within a very short time span.

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