Pathological Changes in Ultrastructure: Tobacco Roots Infected with *Phytophthora parasitica* var. *nicotianae*

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ABSTRACT

Within 3 hours after inoculation of roots of a resistant burley tobacco, line L-8, with zoospores of a virulent isolate (race 0) of *Phytophthora parasitica* var. *nicotianae,* a hypersensitive type of response was evident in epidermal and adjacent cortical cells. Necrotic cells with coagulated protoplasm were found in advance of the fungus. In one susceptible combination, Burley 21 inoculated with zoospores of race 1, less drastic effects on ultrastructure were evident in advance of the fungus. In this case, injury in advance may have been caused by either a higher amount of infection than that which occurred with other susceptible combinations or by a slight resistant reaction by the host. In two other susceptible combinations (Burley 21 and race 0; L-8 and race 1), changes in advance of the pathogen were not observed. The earliest effects in these susceptible combinations were a swelling of the endoplasmic reticulum and the formation of structures on cell walls. Some of the structures resembled cell wall lesions in victorin-treated oats, whereas others resembled structures termed sheath, collar, papilla, and lomasome described in other diseased plants. Their occurrence together support the previous suggestion that all such structures may be similar in origin and function. Later effects included increased vesicular activity by the Golgi apparatus and a decreased electron density of the vacuoles. Changes in vacuolar density may have resulted from effects on membrane permeability. Phytopathology 61:33-39.

Cell wall modifications have been reported frequently in ultrastructure studies of diseased plants (1, 5, 8, 15, 18). Blisterlike structures were first found in great abundance in outer root cap cells of victorin-treated oats by Luke et al. (11). In further studies, Hanchey et al. (8) showed that portions of the structures originated just beneath the inner surface of the cell wall, and thus termed the structures "cell wall lesions". Cell wall lesions, as they enlarge, consist predominantly of an electron-lucid matrix in which membranous or irregular dense inclusions are embedded. When fully developed, they appear to separate the cell wall from the plasmalemma, and their origin from the wall is not obvious. A later study (6) demonstrated that the wall lesions remained attached to the cell wall when the protoplast underwent "false plasmolysis". This suggested that these structures contained a material of firm consistency. Structures in rust-infected wheat, termed lomasomes, were suggested by Ehrlich et al. (5) to be closely allied to cell wall lesions. The origin of lomasomes was not determined in the latter study. Wall structures similar to cell wall lesions and to lomasomes, but which occur only in direct association with hyphae or haustoria, have also been reported in other diseases (1, 2, 15).

The significance of wall modifications such as wall lesions and lomasomes in pathogenesis remains unresolved. The hypothesis that the structures may either function in or be related to host resistance has elicited opposite viewpoints. Ehrlich et al. (5) could find no relationship between host reaction to *P. graminis* var. *tritici* and the occurrence of lomasomes. Temmick & Campbell (15), on the other hand, suggested that wall structures, termed by them papillae, may function in the containment of *Olpidium brassicae* in lettuce. In a study of *Albugo candida* in radish, Berlin & Bowen (1) suggested that wall structures, termed by them sheath, may wall-off necrotic haustoria.

To further examine the significance of cell wall modifications in plant disease, we have studied their occurrence in tobacco roots, *Nicotiana tabacum* L., infected with *Phytophthora parasitica* (Dast.) var. *nicotianae* (Breda de Haan) Tucker, the causal agent of black shank. Some of these results were published in an abstract (7), and confirm and extend Nusbaum's light microscopic study of this disease (12).

MATERIALS AND METHODS.—Plants of burley tobacco cultivars Burley 21 and L-8 were grown in the greenhouse in vermiculite in 50-ml plastic tubes. The plants were watered with Hoagland's solution. Two races (9) of the pathogen were used: No. 1452, a race 1 burley isolate, and No. 1156, a race 0 burley isolate. Burley 21 is susceptible to both races, whereas L-8 is highly resistant to race 0 and susceptible to race 1 (14).

Zoospores were obtained from mycelium grown on oatmeal agar (10). Roots from intact seedlings 5 weeks old were rinsed free of vermiculite and inoculated by placing the roots into distilled water that contained zoospores. One, 3, and 6 hr after inoculation, root tips with approx 200 zoospores attached to the apical meristem were excised, and the apical 3 mm were fixed in 1% $KMnO_4$ for 45 min. Dehydration and embedding methods were those previously described (8). Sections were examined and photographed with a JEM-7A electron microscope.

RESULTS.—The ultrastructure of healthy roots of

Burley 21 and L-8 was similar. Large dense vacuoles similar to those reported in corn (17) were common in epidermal and cortical cells (Fig. 1). Unlike grain roots, however, the epidermal Golgi vesicles in the two tobacco cultivars were electron-lucid and otherwise similar to those formed in interior cells. Other organelles were similar to those described in maize (17) and oats (8).

Ultrastructure of organelles in infected susceptible roots.—Roots of Burley 21 inoculated with race 0 and of L-8 inoculated with race 1 were similar in appearance during disease development. Zoospores became encysted on roots within 10 min, although penetration was seldom observed at 1 hr, the earliest sampling period. Penetration after 3 hr was both intra- and intercellular, but rarely extended beyond the first cortical layer. No drastic effects on mitochondria, dictyosomes, vacuoles, or nuclei of penetrated cells were evident at 3 hr; however, cisternae of the endoplasmic reticulum were slightly swollen (compare Fig. 1 to Fig. 2, 3, 4, 6). No ultrastructural changes were observed in advance of the fungus.

Six hr after inoculation, Golgi secretory activity was stimulated markedly in invaded cells. There appeared to be no concentration of dictyosomes in any one part of the cell, although Golgi vesicles were often in close proximity to the fungus (Fig. 5). Vesicles smaller than those produced by dictyosomes were also abundant in host cytoplasm near capitate haustoria, but serial sections failed to clarify the origin of these vesicles (Fig. 5). The small vesicles, unlike vesicles of the dictyosomes, were not observed to fuse with the plasmalemma. Vacuoles also stained less intensely than those in healthy roots (Fig. 4). This result may reflect the vacuolar swelling reported by Powers (13) in light microscopic studies of this disease.

In contrast to results with Burley 21 infected with race 0 or L-8 infected with race 1, rapid and drastic effects on ultrastructure were evident as early as 3 hr in another susceptible combination, Burley 21 roots inoculated with the race 1 isolate. Effects were not limited to cells in contact with the fungus, and after 6 hr, the effects were evident 3 or 4 cells from the pathogen (Fig. 8). In cells adjacent to haustoria, mitochondria were frequently distorted, although no early effects on mitochondrial cristae were found (Fig. 7). Golgi secretory activity was either unaffected or greatly suppressed. Suppressed activity was particularly evident in severely damaged cells in which the Golgi cisternae assumed a whorled appearance (Fig. 7). Other common effects were swollen endoplasmic reticulum (Fig. 7, 9) and fragmented nuclear envelopes (Fig. 7). Three or 4 cells in advance of the pathogen, the most common changes were a separation of the plasmalemma from the cell wall, rudimentary dictyosomes, dilated endoplasmic reticulum, and a decreased electron density of the vacuolar contents (Fig. 8, 9). The electron density of the cytoplasm of cells in contact with haustoria was usually increased 6 hr after inoculation. The difference between results obtained with this susceptible combination and the two previously discussed is not known; however, a higher number of infections may have accounted for greater injury. Alternatively, the results may reflect a greater degree of resistance.

Ultrastructural changes in infected, resistant roots.—A hypersensitive response was observed in L-8 roots inoculated with race 0. After 3 hr, a necrotic reaction of the cytoplasm extended approximately two cells in advance of the fungus (Fig. 11). Vacuoles in necrotic cells were enlarged, and densely stained cytoplasm usually formed a thin layer against the cell walls. The cytoplasmic contents of epidermal cells adjacent to and apparently not penetrated by the fungus appeared coagulated and densely stained. In contrast to Burley 21 roots inoculated with the race 1 isolate, no gradation of injury was evident in cells not in direct contact with the fungus. Instead, a sharp demarcation separated injured cells from those cells with little damage (Fig. 11).

Although not a consistent effect, modifications of cell walls adjacent to necrotic lesions were occasionally found (Fig. 12). These modifications, which resembled rudimentary cell wall lesions, were characterized by an electron-lucid matrix which contained densely stained materials, and resulted in the separation of the plasmalemma from the cell wall. Golgi secretory activity was unaffected in cells bordering necrotic lesions.

Cell wall structures in infected roots.—The most striking early result of infection in susceptible combinations was the occurrence of modifications to host cell walls. Similar structures were not found in healthy roots in this study. The wall modifications were similar in appearance to structures described previously in other diseases (1, 5, 8, 15). Cell wall structures were found on invaginated portions of the host cell wall surrounding penetrating hyphae (Fig. 2, 9), and in several cases were formed on host epidermal walls before fungal penetration of that cell (Fig. 3). In the latter case, the fibrillar nature of the cell wall was readily apparent and dense granules, similar to those in the wall structure matrix, were distributed in the outer portion of the host cell wall. Intracellular hyphae were sometimes completely surrounded by the wall structures, but unlike cases described by Berlin & Bowen (1) for necrotic *Albugo candida* haustoria in radish, the hyphae of *P. parasitica* var. *nicotianae* were apparently undamaged (Fig. 4). The very irregular nature of the outer border of wall structures, delimited by the host plasmalemma, suggests that they may have a gel-like consistency (Fig. 10).

Wall structures were never observed surrounding small capitate haustoria (Fig. 2, 5, 6), although expanded hyphae were sometimes completely surrounded (Fig. 10). In all cases observed, the structures were shown in either a single section or by serial sectioning to be continuous with the host cell wall. The fungal cytoplasm of capitate haustoria was enclosed by two wall layers distinctly different in electron density (Fig. 5, 6). The layer adjacent to the fungal wall was electron-lucid, pleomorphic, and continuous with the host cell wall. Ehrlich & Ehrlich (4) used the term

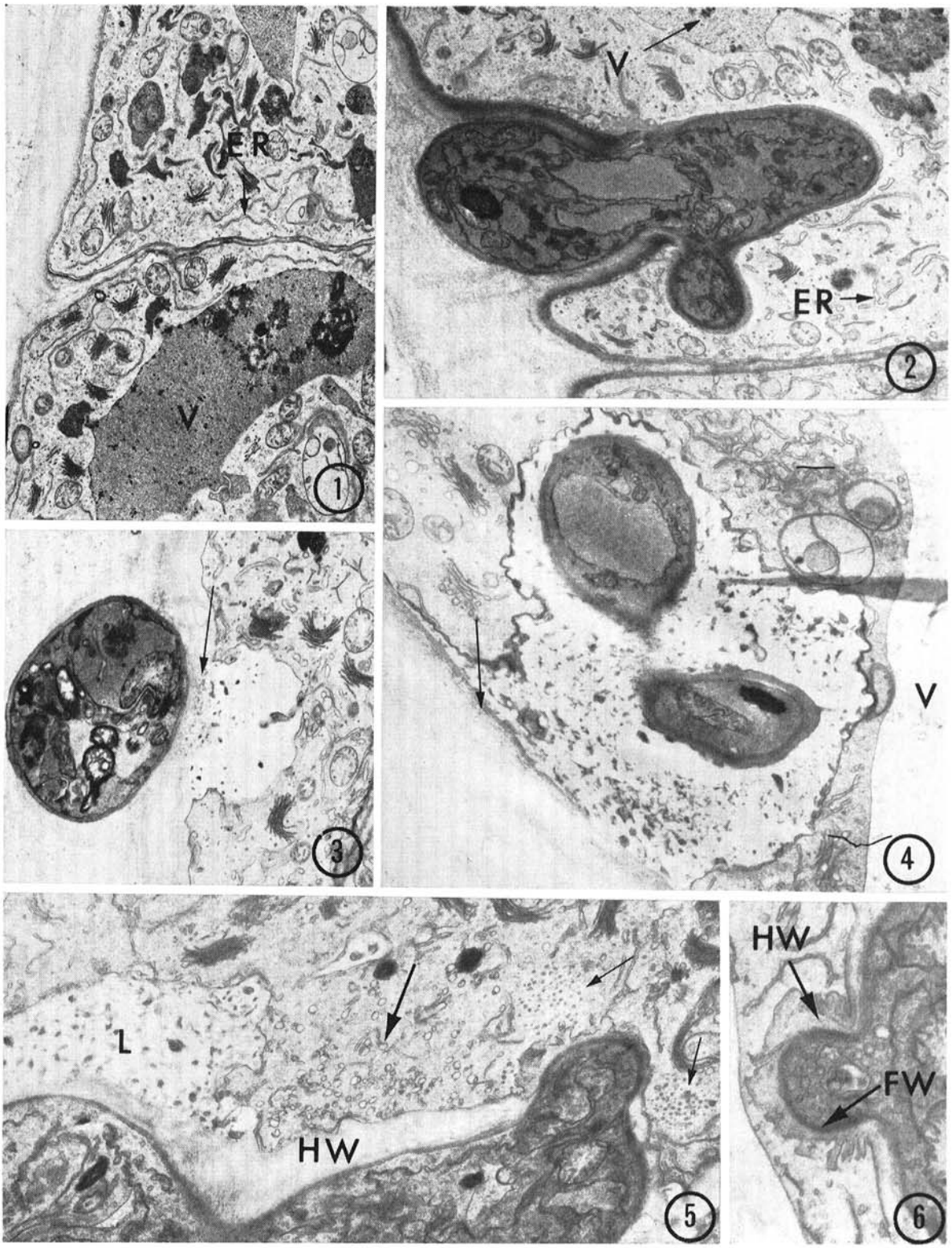

Fig. 1-6. Infection of susceptible roots by *Phytophthora parasitica* var. *nicotianae*. **1)** Epidermal cells of healthy Burley 21 root. Note dense vacuoles (V). (×7,200) **2)** Race 0 hyphae in Burley 21 epidermal cell 3 hr after inoculation. Endoplasmic reticulum (ER) is slightly swollen. (×8,500) **3)** Cell wall lesion formed prior to epidermal penetration (Burley 21 and race 0). Arrow points to granules beneath inner cell wall surface; 3 hr. (×10,000) **4)** Race 0 hyphae enveloped by wall lesion in Burley 21 epidermal cell; 3 hr. (×13,500) **5)** Vesicles 6 hr after inoculation (Burley 21 and race 0). Note Golgi vesicles near cell wall lesion (L) (large arrows) and smaller vesicles near haustorium (small arrows). (×14,700) **6)** Haustorium of race 1 in L-8 tobacco. Fungal wall (FW) is surrounded by modified host cell wall (HW). (×19,200)

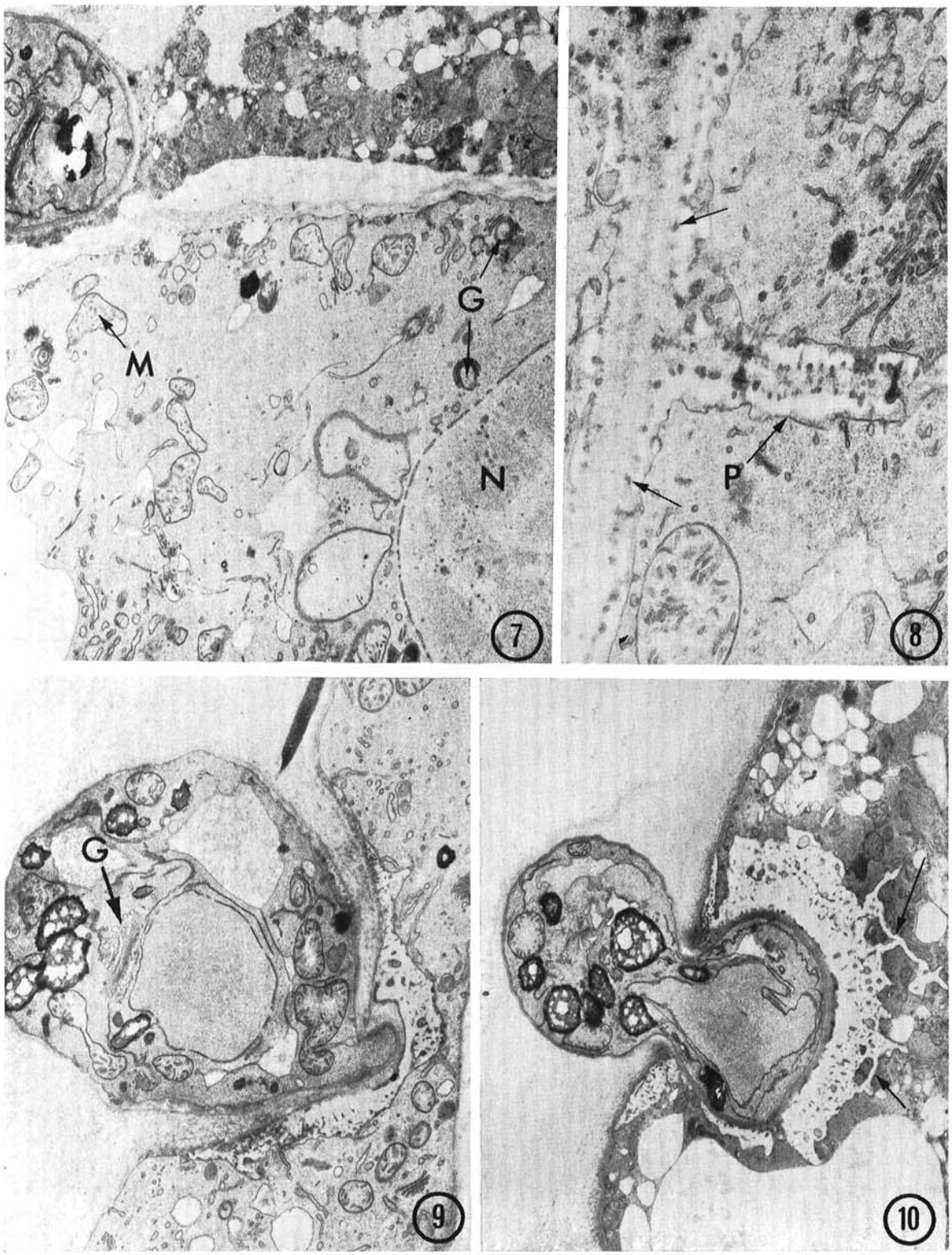

Fig. 7-10. *Phytophthora parasitica* var. *nicotianae* (race 1) in Burley 21 roots 6 hr after inoculation. **7)** Cellular damage is evident to mitochondria (M), dictyosomes (G), and envelope of nucleus (N). (×11,000) **8)** Separation of plasmalemma (P) from wall apparently three cells in advance of fungus. Outer portion of cell wall contains dense granules (arrows). (×23,000) **9)** Cell wall lesion in penetrated epidermal cell. Note fungal perinuclear Golgi apparatus (G, arrow). (×12,000) **10)** Dense cytoplasm and electron-lucid vacuoles in damaged epidermal cell. Outer border of lesion is reticulate (arrows), suggesting a fluid consistency. (×7400)

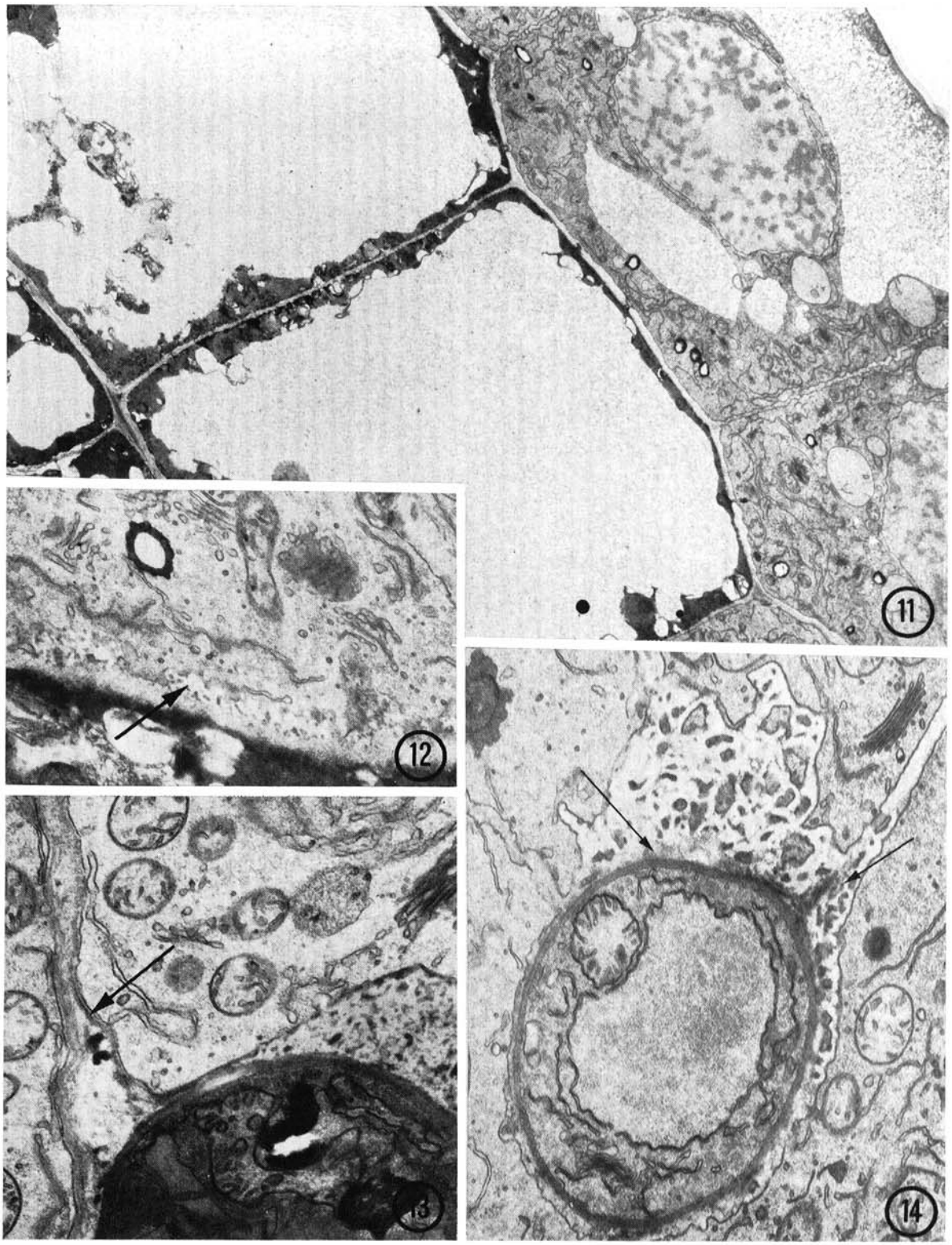

Fig. 11-14. Infection of L-8 roots by *Phytophthora parasitica* var. *nicotianae*, race 0; 3 hr. **11)** Necrotic cortical cells in advance of fungus surrounded by apparently normal cells. (×4200) **12)** Cell wall lesionlike structure adjacent to necrotic cell. (×21,000) **13, 14)** Wall dissolution in wall lesions (arrows). Burley 21 and race 0; **13)** 6 hr after inoculation; (×21,000) **14)** 3 hr after inoculation. (×21,000)

"encapsulation" to designate the outermost layer. This term describes a structure which is neither typical host or fungal cell wall. While certainly not normal in appearance, its origin from the host cell wall as indicated both in this and in the Ehrlich study makes questionable the use of the term encapsulation. Both haustorial-induced invaginations of the cell wall and cell wall structures appear to result from cell wall modifications which resemble softening, although the nature of such modifications may differ in the two cases. Haustoria were infrequently observed in the Burley 21-race 1 or L-8-race 0 combinations.

Cell walls adjacent to intercellular hyphae were also conspicuous because of the presence of wall structures (Fig. 5, 13, 14). In many cases, a splitting of the cell walls was evident (Fig. 13). Whether such splitting was caused by fungal pressure or enzymatic activity was not determined. In other cases, a softening of the cell wall was suggested, with a gradual spreading of host wall material into the wall structure area (Fig. 14).

Fewer wall structures were present in the susceptible combination Burley 21 and race 1 and the resistant combination L-8 and race 0. Since both of these combinations resulted in early cellular disruption, the latter result suggests that lesion development was dependent on some activity of living, relatively undamaged, cells.

DISCUSSION.—The similarity in position and especially of appearance of wall modifications reported in many diseased plants strongly suggests that all such modifications represent a common phenomenon. But the diversity of terminology used to describe cell wall structures such as those found in this study complicates any discussion of their nature. Cole & Lin (3) discussed membranous structures: plasmalemmasomes, mesosomes, and lomasomes occurring adjacent to cell walls. Bracker (2) discussed the various terms applied to structures surrounding haustoria and chose the term "collar" for structures apparently identical to those found in *Phytophthora*-infected tobacco in this study [see Fig. 8, 21, and 32 of citation (2)]. This term appears too restrictive, since it was defined as an extension of the host cell wall that encloses haustoria. Such a definition would exclude identical structures induced at a distance from the pathogen or formed as a result of a toxin. For simplicity, we prefer the term wall lesion for all those structures for which it can be demonstrated that wall fragments occur in a matrix between the plasmalemma and cell wall. Although the latter term was first used to describe structures which originated from a splitting of an outer layer of the cell wall, such an origin may be difficult to demonstrate and thus need not be a qualifying characteristic of wall lesions. The demonstration of wall fragments, on the other hand, should require no more than a few variations in fixation procedures.

Although wall lesion formation may not be related to the disease reaction of the plant as a whole, several considerations suggest that they may be related to the sensitivity of individual cells to injury. In victorin-treated oat roots, cells which contained wall lesions but few other ultrastructural abnormalities were surrounded by severely damaged cells which lacked wall lesions (8). The reason for the absence of necrosis in such cells is not known; however, the possibility exists that lesion material may have functioned protectively against cell injury. Ehrlich et al. (5), by varying wheat rust infection type with temp, observed massive lesions (termed lomasomes) in infection centers at low temp (65 and 70 F). The boundary formations were smaller and less frequent at temperatures of 72 F and above. The authors suggested that the lesions were not fully associated with either resistance or susceptibility, but did not consider the question of whether their presence was related to the degree of cell injury.

The present study of *P. parasitica* var. *nicotianae* in tobacco roots indicates that a higher frequency of wall lesion formation occurred in those infections which resulted in little cell damage. Two possible reasons for this relationship might be (i) wall lesion formation is a nonspecific response to cell injury, but is dependent on some activity of intact, relatively undamaged cells; or (ii) the formation of wall lesions is an integral part of cell defense or repair mechanisms, and may delay or prevent extensive cell damage. The fact that changes in Golgi activity and vacuolar staining properties occurred later than wall lesion formation suggests that these boundary formations do not prevent development of other abnormalities in infected or injured cells. But the question of whether they play a minor role in delaying extensive damage or merely occur as a coincidence of infection awaits further study.

The sequence of events leading to wall lesion formation was not as apparent in these studies as it was in an earlier study of victorin-treated oats (8). No evidence was found that vesicles formed in the cytoplasm played any role in the initiation of these structures, although vesicles may have played a role in subsequent enlargement (Fig. 5). Likewise, no evidence of early cell wall degradation such as that shown in victorin-treated oats was found; however, the occurrence of electron dense granules in outer portions of cell walls, which were similar in appearance to those in the lesion matrix (Fig. 3, 4), in addition to the demonstration of cell wall fragments in the matrix (Fig. 5, 13, 14), suggests that portions of the matrix are wall materials. Minor cell wall dissolution, accompanied by the imbibition of water, could account for the apparent gel-like nature of the structures.

The loss of vacuolar staining density in all combinations studied and the increased cytoplasmic staining which was frequently accompanied by a coagulation of the cytoplasm in the resistant and moderately susceptible combination may be due to changes in the localization or concentration of phenolic oxidation products. Veech (16) found little change in the activity or pattern of peroxidase zymograms in an inoculated tobacco cultivar resistant to black shank. Moderately diseased susceptible plants, on the other hand, showed increased activity of several isozymes. No differences in peroxidase localization were detected between inoculated susceptible and resistant plants; however, light

microscope resolution was probably insufficient to determine changes in cytoplasmic localization. The loss of staining in vacuoles suggests a dilution of vacuolar contents by either the entry of water or the loss or alteration of the densely stained materials. Powers' report (13) that cells of infected stems showed increased vacuolation supports the suggestion that water entry to the cell was increased. Although changes in the vacuole further suggest concomitant effects on the permeability of the plasmalemma and/or tonoplast, no disruptions or other gross changes in these membranes were observed. These findings are in contrast to studies on victorin-treated oats in which an early effect on the resolution of the plasmalemma unit structure was found (8).

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