Ultrastructure of Lesion Formation in Rhizoctonia-Infected Bean Hypocotyls

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ABSTRACT

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Hypocotyls of *Phaseolus vulgaris* 'Red Kidney' were inoculated with *Rhizoctonia solani,* a fungus known to produce endopolygalacturonase and other enzymes capable of degrading cell wall polymers. Young, intermediate, and mature lesions together with the tissue adjacent to lesions were examined by electron microscopy. Hyphae within infection cushions were surrounded by a moderately dense material, tentatively described as mucilaginous. Penetration did not involve swelling or changes in staining properties of the cuticle. Epidermal and cortex cell walls near the pathogen were swollen and their cytoplasm was collapsed. At a distance of 2–4 cells from the pathogen, the cytoplasm accumulated toward the infection. Walls of these cells were moderately swollen and the middle lamella was densely stained and granulated. The plasmalemma was retracted from the walls and other subtle cytoplasmic changes were evident. Cytoplasmic changes also were found in cells with densely stained walls showing no obvious swelling. Changes in fungal ultrastructure were uncommon except in mature lesions. The results suggest that changes in host structure and physiology associated with this disease are not caused solely by wall degradation and the consequent osmotic bursting of protoplasts.

Stem canker of bean, which is caused by *Rhizoctonia solani* Kühn, is characterized by discrete lesions on the hypocotyls of young bean plants. Water-soaked areas appear within 24–36 hr after inoculation and become dry, sunken, and brown at maturity. Once the primary leaves are fully expanded, bean plants are no longer susceptible.

The pathogen produces numerous polysaccharide-degrading enzymes which cause extensive cell wall degradation within lesions. Endopolygalacturonase is the predominant enzyme found in young lesions (39). Partially purified preparations of this enzyme induce permeability changes in host cells as well as wall degradation (27). Permeability changes and electrolyte leakage are the earliest physiological effects of this disease. Later changes include increased respiration and phytoalexin accumulation (7,37).

Soft rot pathogens also cause host cell death as well as tissue maceration and these pathogens and their enzymes have been used as model systems for studies on the mechanism of killing. The simplest hypothesis states that as the cell wall is degraded, the protoplast expands, and the plasma membrane ruptures as a result of osmotic stress. Experiments using purified pectic enzymes support this hypothesis. Basham and Bateman (3) found that electrolyte leakage was directly related to wall degradation when potato disks were treated with a purified pectic lyase of *Erwinia carotovora.* Death of cells is prevented by plasmolysis (2). Such experiments provide useful information about in vitro enzyme treatment, however it is likely that many factors contribute to cell death in vivo.

Numerous cytological studies have been conducted on diseases in which cell walls are degraded (13,15,17,20,39,40). Most of these have been concerned with penetration mechanisms and evidence of wall dissolution. Fewer ultrastructure investigations have been published. Hänssler et al (24) found extensive disorganization of bean cell walls and necrotic cells adjacent to hyphae of *Sclerotium rolfsii,* but did not report whether cytological changes occurred at a distance from the pathogen. Fox et al (20,21) reported extensive cell wall degradation and cytoplasmic damage up to 500 μm from the site of infection in potato tubers inoculated with *Erwinia carotovora* var. *atroseptica.* Cytoplasmic changes such as swelling of organelles, including nuclei, the appearance of myelin bodies derived from the plasmalemma and numerous cytoplasmic vacuoles were associated with only small-to-moderate swelling of the cell wall. Calonge et al (13) studied the effects of several faculative parasites which produce cell-wall-degrading enzymes during infection and reported extensive wall swelling, but early stages of injury were not evaluated.

Numerous physiological changes in *Rhizoctonia*-infected beans have been described (7,37), thus, this disease is excellently suited for studies relating changes in ultrastructure to changes in function.

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Such studies will further our understanding of maceration and killing of cells, features of cells bordering lesions and changes which occur as lesions age.

MATERIALS AND METHODS

Beans *(Phaseolus vulgaris* L. 'Red Kidney') were planted in steamed soil in 15-cm-diameter pots. Plants were kept in a growth chamber at 25 ± 2 C with a 14-hr photoperiod and a light intensity of 1.3 lumens per cubic centimeter. *Rhizoctonia solani* Kühn, isolate RB (provided by D. F. Bateman) was grown in potato dextrose broth. Five-day-old plants were inoculated by dribbling a mycelial suspension over emerging hypocotyls according to the procedure of Van Etten et al (39). Control plants were treated with distilled water.

Pale brown lesions were evident 24 hr following inoculation. Wedges of tissue containing both lesions and adjacent, noninfected areas were sampled at 24, 36, and 48 hr after inoculation. Control tissue was sampled at 36 hr. Tissue was fixed in 6% glutaraldehyde in 0.03 M potassium phosphate, pH 7.4 for 2 hr, followed by 2% OsO_4 for 12 hr, or in 3% $KMnO_4$ for 2 hr. Samples were dehydrated and embedded in Maraglas (22). Thin sections were usually post-stained in lead citrate and examined with an AEI-6B electron microscope. Adjacent thick (0.5-μm) monitor sections were affixed to glass slides and stained with 0.05% toluidine blue O in phosphate buffer, pH 6.8, for light microscopic examination.

RESULTS

Infection cushions consisted of numerous multinucleate, branched hyphae closely appressed to the cuticle surface (Fig. 1). A moderately dense, homogenously stained material surrounded the hyphal tips in the cushion. This material was more densely stained with $KMnO_4$ than with OsO_4 and is tentatively identified as mucilage.

Penetration of the cuticle was accomplished by numerous hyphae, the tips of which were constricted to form infection pegs of varying diameters (Figs. 1 and 2). The cuticle was invaginated and broken at points of entry but did not appear swollen compared with uninfected controls (Fig. 3). The underlying epidermal wall was completely degraded beneath the center of infection cushions after 24 hr. Hyphae at the margins of infection cushions had little or no mucilage surrounding them but bacteria frequently were observed between the hyphae and cuticle (Fig. 4). Both the outer epidermal wall and the anticlinal wall between contiguous epidermal cells beneath these hyphae were deteriorated.

Cell walls of noninfected tissue were electron translucent (Fig. 3). The outer epidermal wall consisted of tangentially oriented, parallel layers, and the cuticle was a thin, moderately stained layer. Epidermal cells contained a large central vacuole and a thin peripheral layer of cytoplasm. Nuclei were frequently lobate and contained one to several invaginations.

After 24 hr the pathogen had penetrated to the second layer of cortical cells, after 36 hr to the sixth cortical layer, and after 48 hr to the ninth cortical layer. Mycelial growth occurred both inter- and intracellularly. Extensive cell wall swelling was found in all regions adjacent to and up to two cells away from the pathogen. The middle lamella was densely stained with potassium permanganate fixation (Fig. 5). Such walls appeared to offer little or no resistance to penetration since hyphal constrictions were not common. In cases of moderate cell wall swelling at the advancing front of lesions, hyphae formed one or more infection pegs within densely-stained primary host walls (Fig. 6). In these cases the hyphae were swollen during penetration of the middle lamella and were constricted again during passage through the primary wall of the adjacent cell. With $KMnO_4$ fixation, hyphal walls typically consisted of two distinct layers, a densely stained inner layer and a lighter outer layer (Fig. 5).

Severely degraded walls of the outer cortex had a lamellated appearance consisting of loosely parallel strands (Fig. 7). Densely stained granules were concentrated in the region of the middle lamella and were sparsely distributed in the remainder of the primary wall (Fig. 5). At higher magnification, short rods and granules were observed interspersed between the longer strands, resulting in a herringbone pattern (Fig. 8). In epidermal cells adjacent to the infection cushion, the wall beneath the cuticle consisted predominantly of densely stained granules (Fig. 4). Fibrils were restricted to the layer of primary wall adjacent to the cytoplasm.

At the outer margin of developing lesions, three-to-five cell layers from the pathogen, a sharp demarcation in cell wall staining was found (Fig. 9). These walls were intensely stained with toluidine blue in light microscopic observations and were more densely stained after $KMnO_4$ than with glutaraldehyde-OsO_4 fixation. The increased electron density also was observed in sections that were not post-stained with lead citrate. In permanganate-fixed material, these dense walls contained fractures. The dense material appeared to originate within the middle lamella of cells distal to the infection and spread to cover the entire wall in proximal cells. In the same area, occasional wall appositions occurred (Fig. 10). These structures seemed to form most frequently near plasmodesmata.

Light microscopic observations revealed an accumulation of cytoplasm on the sides of host cells toward the infection center for a distance of three-to-five cells from the pathogen. A thin layer of cytoplasm occurred on the remainder of the cell periphery. The cytoplasm was always collapsed in penetrated cells regardless of the stage of lesion development. Cytoplasmic collapse was less frequent in noninvaded cells with moderately swollen walls (Figs. 4 and 5) while other cells had only minor changes (Figs. 4 and 12). In the latter cases, a thin peripheral layer of primary wall adjacent to the cytoplasm remained relatively intact although a lamellated or herringbone pattern was evident. The plasmalemma was separated from the host cell walls and membranous materials were found in the separation zone. No increase in the number of Golgi vesicles was found in these cells. Cells bordering necrotic areas contained cytoplasmic vacuoles (Figs. 11 and 12). These vacuoles appeared to originate from dilations of the endoplasmic reticulum (Fig. 11) and were found both in regions of moderate wall swelling as well as at the outer margin of developing lesions where walls were densely stained, but not swollen. The vacuolar membrane was occasionally discontinuous, but other organelles appeared normal. Cytoplasmic changes were less frequent in cells without increased wall staining.

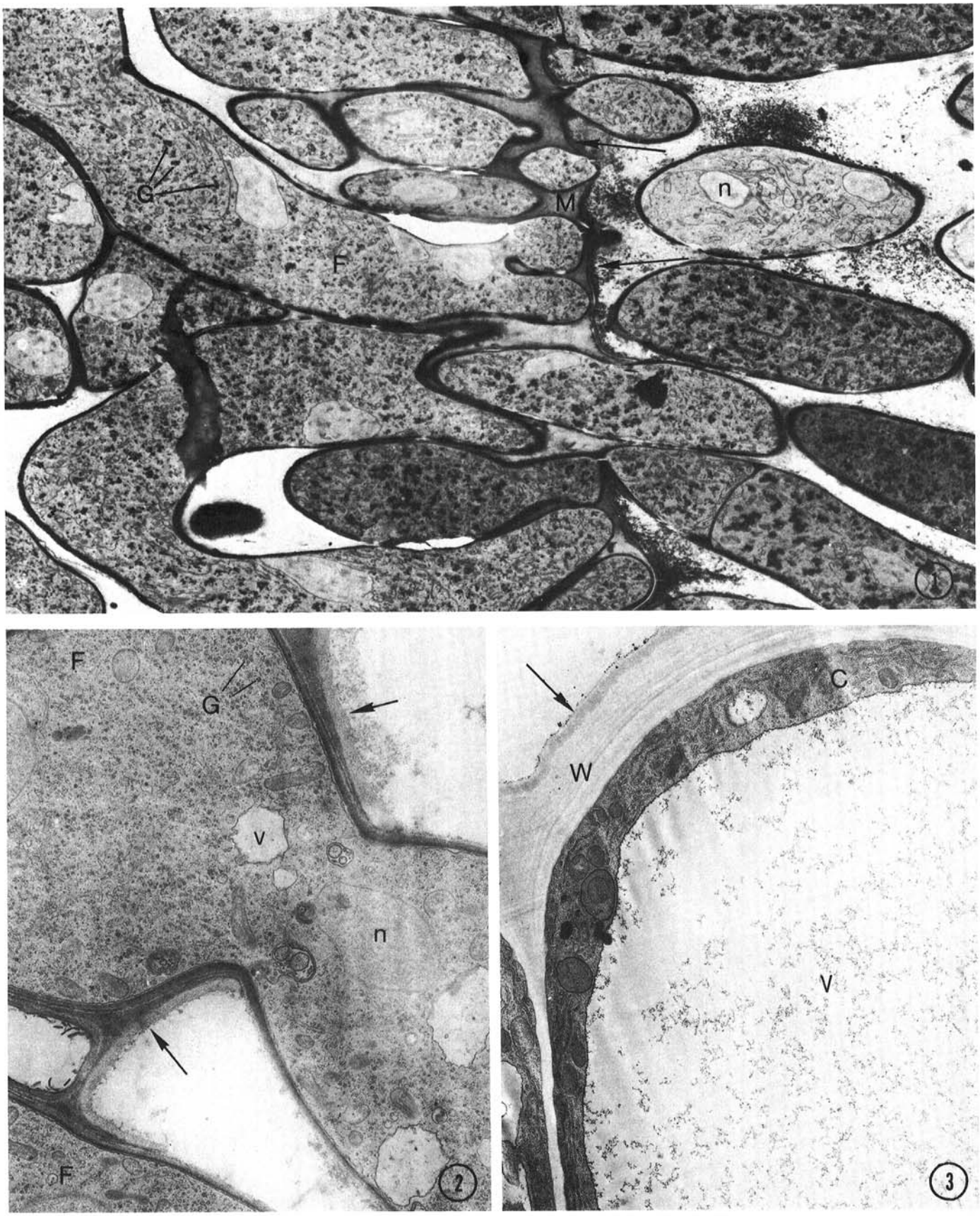
The ultrastructure of hyphae was essentially as reported by Butler and Bracker (12). The distribution of organelles in hyphal penetration tips did not appear to differ from the rest of the hyphae within the infection cushion. Vacuoles were not abundant and were smaller than nuclei (Fig. 2). Mitochondria were numerous and elongate and the endoplasmic reticulum appeared both as individual lamellae and in parallel arrays. Lomasomes and microbodies were found occasionally. Hyphae in the area of penetration contained abundant clusters of glycogen granules (Figs. 1 and 2).

Hyphae within lesions (Figs. 5 and 6) typically contained fewer glycogen granules than those found in the infection cushion (Fig. 1). Necrotic hyphae were more common in 48-hr infections than in 24-hr infections. As the lesion aged, the most common hyphal alteration was a clearing and vesiculation of the cytoplasm and the occurrence of numerous lomasomes (Fig. 7). In some cases, fungal cytoplasm was densely stained and organelles were difficult to distinguish. Occasionally, intrahyphal hyphae similar to those described by Butler and Bracker (12) also were found.

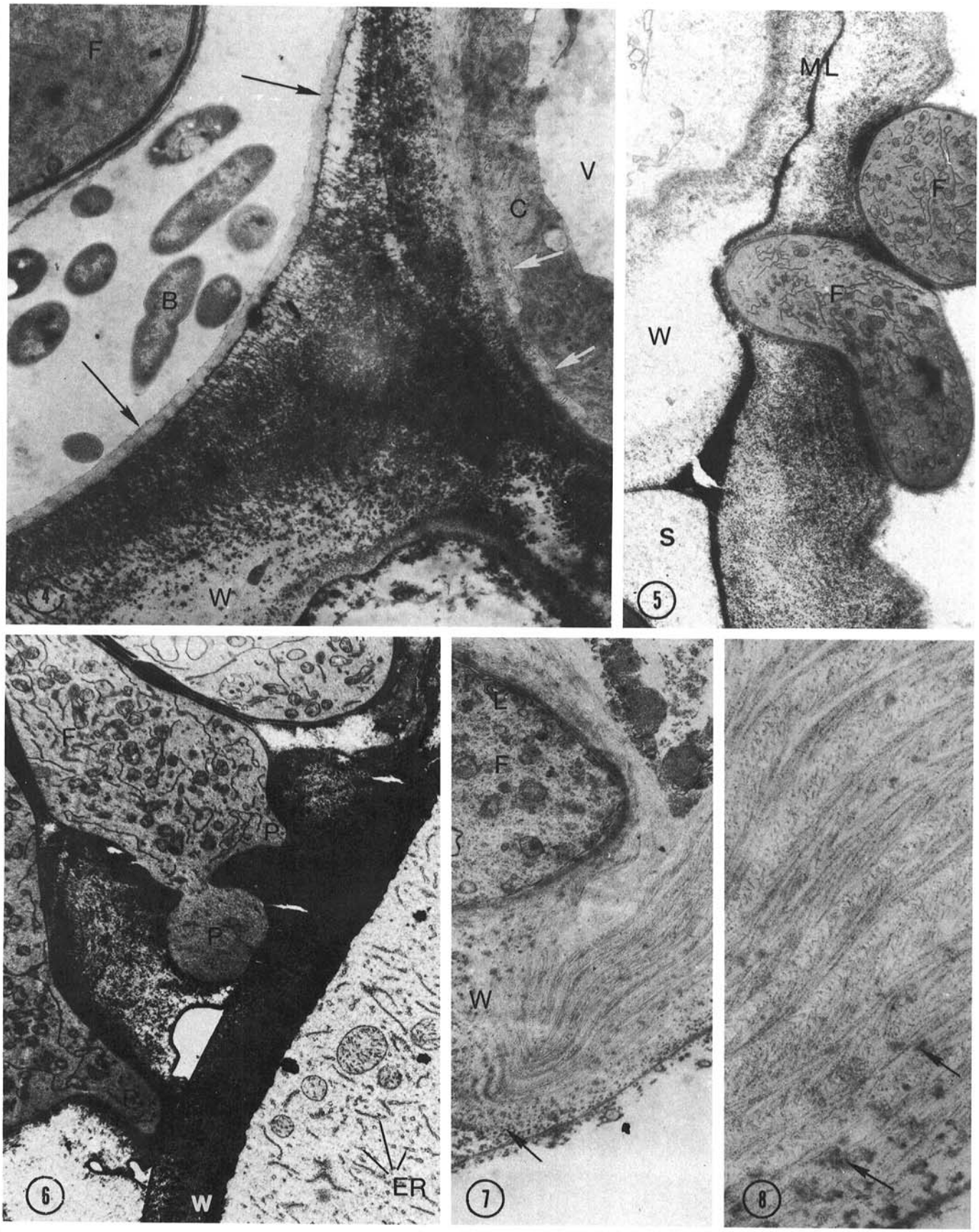
DISCUSSION

The isolate used in this study formed infection cushions and penetration pegs similar to those described for other isolates (15,17,18,19). One difference was the formation of a mucilaginous-appearing material. Mucilage was described by Flentje (19) on *Rhizoctonia*-infected cabbage, lettuce, and tomato, and may facilitate attachment of the infection cushion to the host. This material has not previously been described on bean.

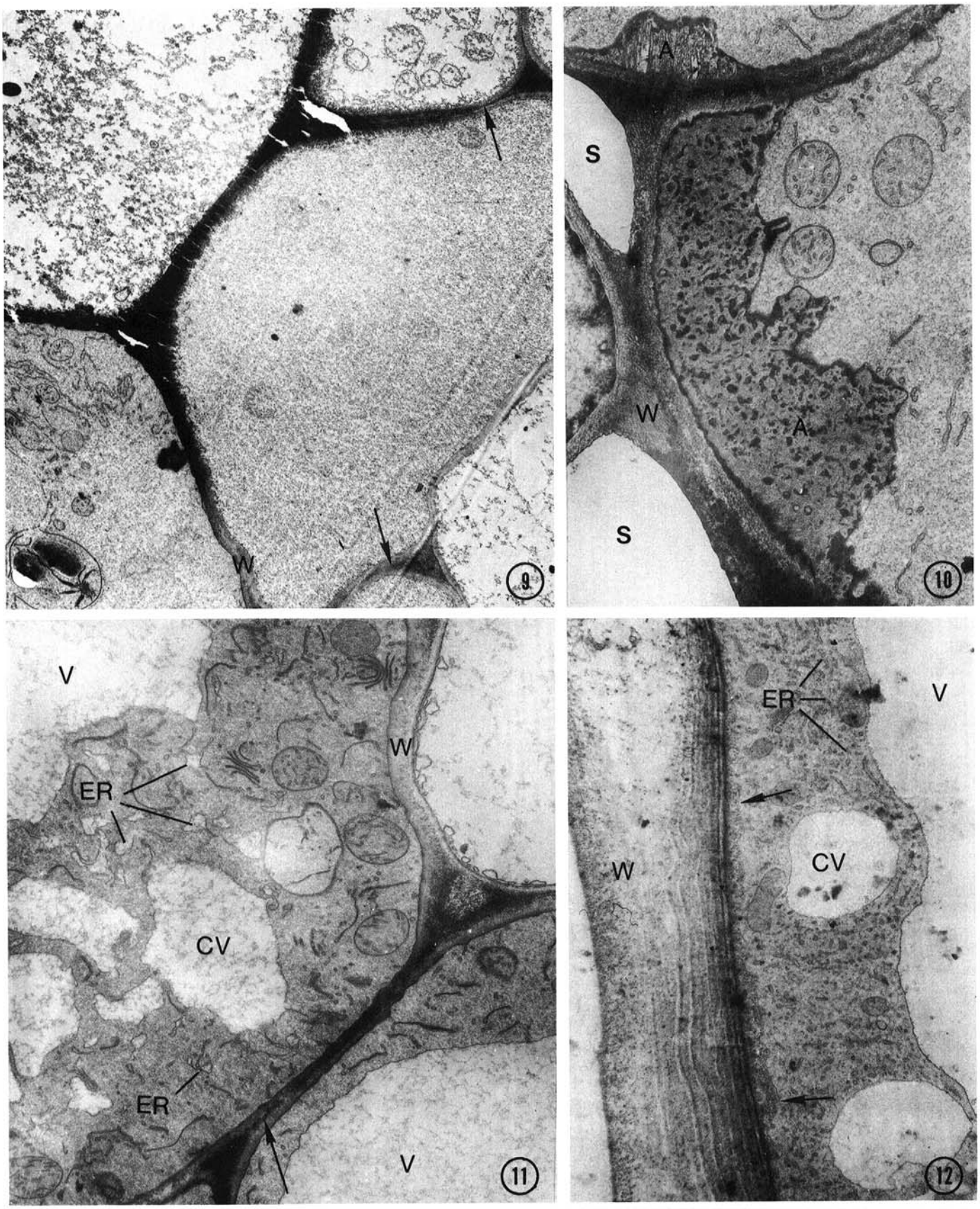
Rhizoctonia produces cutinases in vitro, but the role of these in pathogenesis is not clear (1). In this study, cuticular penetration



Figs. 1-3. Cross sections of hypocotyl epidermis from infected and healthy plants. **1,** Infection cushion from a 36-hr lesion. Hyphae, perpendicular to the long axis of the plant, are penetrating host cuticle (arrows). Epidermal cell walls beneath the cuticle (right) are completely degraded. Mucilaginous-appearing material (M) can be seen between hyphae (F) outside epidermis; glycogen (G) is abundant within hyphae. n = fungal nucleus. $KMnO_4$ fixation, ×4,000. **2,** One hypha (F) of an infection cushion penetrating host cuticle (arrows) which does not appear swollen or degraded. Epidermal cell wall beneath cuticle (right) is completely degraded. G = glycogen, v = fungal vacuole, n = fungal nucleus. Glutaraldehyde-OsO_4 fixation, 36 hr, ×14,000. **3,** Healthy host epidermal cell fixed with glutaraldehyde-OsO_4. Outer epidermal wall (W) is electron translucent and exhibits a lamellate appearance. The cuticle (arrow) stains more densely than the cell wall. C = host cytoplasm, V = host central vacuole (×9,500).



Figs. 4-8. Hyphal penetration and host cell wall damage. **4,** Outer epidermal wall (W) with intact cuticle (black arrows). Dense granular material occurs in the middle lamella and the outer portion of epidermal wall. The lower cell is necrotic; the plasma membrane is displaced in the adjacent cell (white arrows). B = bacteria, C = host cytoplasm, V = host central vacuole, F = hypha. Glutaraldehyde-OsO_4 fixation, 36 hr, ×9,000. **5,** Hypha (F) penetrating extensively swollen host cell wall (W). The middle lamella (ML) stains densely. S = intercellular space. KMnO_4 fixation, 36 hr, ×5,500. **6,** Hyphal penetration in fourth to fifth cortical cell layer. Pegs (P) are formed against host wall (W). Host cytoplasm is relatively undamaged. F = hypha, ER = host endoplasmic reticulum. KMnO_4 fixation, 36 hr, ×6,500. **7,** Vacuolated hypha (F) with lomasomes (L) adjacent to swollen, lamellated host cell wall (W). Granular material can be seen (arrow). Glutaraldehyde-OsO_4 fixation, 24 hr, ×11,000. **8,** Higher magnification of degraded host cell wall in Fig. 7. Layers of microfibrils form a herringbone pattern interspersed with granules (arrows). Glutaraldehyde-OsO_4 fixation, 24 hr, ×45,000.



Figs. 9-12. Changes in host cell walls and cytoplasm at the lesion edge. **9,** Dense staining in host cell walls (W) appears to originate in the middle lamella (arrows). The infection center is towards the upper left. $KMnO_4$ fixation, 36 hr, ×4,000. **10,** Large appositions (A) against the host cell wall (W). S = intercellular space. $KMnO_4$ fixation, 48 hr, ×14,000. **11,** Cortical cell showing numerous cytoplasmic vacuoles (CV) and dilated endoplasmic reticulum (ER). Intense wall (W) staining appears to begin in the middle lamella region (arrow). V = central vacuole. $KMnO_4$ fixation, 48 hr, ×12,000. **12,** Third cortex cell showing swollen wall (W), cytoplasmic vacuoles (CV) and short profiles of endoplasmic reticulum (ER). Pockets of granular or vesicular material are found between the cell wall and plasma membrane (arrows). V = central vacuole. Glutaraldehyde-OsO_4 fixation, 24 hr, ×10,000.

was not accompanied by swelling or changes in staining properties of this layer as was found with *Fusarium solani* f. *pisi* on pea (34). Weinhold and Motta (40) also found no evidence of cuticle degradation on *Rhizoctonia*-infected cotton, although the cuticle separated from the cell wall beneath infection cushions. This separation also was described by Christou (15) in infected beans and was observed in the present study. A scanning electron microscope study by Lisker et al (29) also showed no swelling, although cuticular cracking beneath infection cushions on beans was observed. These results suggest mechanical cuticular penetration by isolate RB, however, specific localization methods for cutinases would be required to detect more subtle changes (33,34).

Extensive wall swelling was limited to the region surrounding the pathogen and up to two cells in advance, 24 or 48 hr after inoculation. Radiating from this area for a distance of an additional three cells was an increased wall staining by permanganate, OsO_4, and toluidine blue. These results indicate that the highest concentration of cell-wall-degrading enzymes occurs near the hyphae and that diffusion in advance of the hyphae is relatively limited.

Constrictions formed by the pathogen, even when host walls are swollen, offer additional support for chemical and structural differences between the outer layer of primary wall and the rest of the wall. Granules were seen less commonly in these areas than in wall layers closest to the middle lamella. Pectins have been found to decrease and cellulose to increase with distance from the middle lamella (6). The pattern of digestion of the epidermal wall (Fig. 4) also supports the hypothesis that these granules represent degraded pectic materials. The granules follow the same pattern as do pectic materials in outer epidermal walls, with a layer of pectins nearest the cuticle and down the middle lamella, and cellulose microfibrils nearest the protoplast (16). Similar granules have been found in digesting walls during protoplast isolation (30). Further studies localizing pectic materials in developing lesions are in progress and should clarify the nature of these changes.

Extensively degraded host cell walls near the pathogen consisted of a herringbone pattern of fibrils and smaller densely-stained granules. Chafe and Doohan (14) found that the thickened sieve cell wall of white pine had a similar herringbone pattern in oblique sections. They suggested that this appearance resulted from a crossed helical arrangement of microfibrils. In a transverse section, longitudinal sections of layers alternate with cross sections of microfibrils in adjacent layers. The sections are thick enough (60–90 nm) to allow for an overlap of microfibrils in adjacent lamellae, resulting in the "V" pattern seen in this study. A similar pattern has been described in other diseased plants (13,21) and in cell walls of ripening fruits (10,31). Pesis et al (31) interpreted the cross-fibrils to be hemicellulose. The lamellated and herringbone pattern of walls in diseased plants is presumably caused by degradation of the wall matrix by fungal enzymes. Removal of this matrix, which consists primarily of xyloglucans, arabinogalactans, and rhamnogalacturonans, would result in a loosening and separation of the layers of cellulose microfibrils. Microfibrils do not appear degraded and thus, cellulase is probably not important in initial phases of pathogenesis. The loss of birefringence described by Bateman (5) in infected beans and by Weinhold and Motta in infected cotton (40) may be the result of disorientation rather than dissolution of microfibrils.

The first cytoplasmic response observable by light microscopy was a redistribution of cytoplasm toward the infection center three to five cells from the pathogen. This phenomenon, termed traumatotaxis by Küster (in 11), is a common result of injury and disease. Previous light microscopic studies have not shown cytoplasmic changes in advance of the pathogen (15,17,18). However, different fixation methods were used in the present study.

Lai et al (27) found permeability changes in cells within and at a distance from lesions. The first detectable ultrastructural effect in comparable areas was a separation of the plasma membrane from the cell wall, a result contrary to the expected response if wall dissolution results in protoplasmic swelling. This separation was observed adjacent to walls which were extensively swollen (Fig. 12) and along walls which showed only increased staining (Fig. 11). Other early responses included dilation of the endoplasmic reticulum, cytoplasmic vacuolation and occasional tonoplast discontinuity. Similar effects were described by Jones et al (26) in a resistant reaction of *Phytophthora infestans* on pepper and in pectin lyase-treated apple cells (25). These changes also were observed by Lazarovits and Higgins in resistant tomatoes infected with *Cladosporium fulvum* (28). They suggested that swollen ER and increased dictyosome activity were associated with increased glucan synthesis and callose deposits along the walls. In the present study, swollen ER was not necessarily associated with such appositions. Lobate nuclei have been reported for several other diseases (26,32,35) and have been interpreted as evidence of increased nuclear activity. However, they occurred with equal frequency in healthy cells in this study. Further changes indicating a gradual progressive disorganization of the cytoplasm before total collapse were not observed, suggesting that water entry is rapid after a certain degree of wall swelling is reached.

Previous studies have not detected any marked changes between host cells within lesions and cells bordering lesions (15,18,19,39) as were seen here. Bateman suggested that limitation of lesion size probably results from several factors. He found a dialyzable, inorganic inhibitor of enzyme action within developing lesions. Further studies showed that ^{45}Ca accumulated at the lesion site, and in two of four tests exogenously supplied Ca inhibited infection in excised hypocotyls (4,8). Walls surrounding lesions and in older plants were resistant to enzymatic degradation and both had a higher calcium content than young, susceptible walls (4,8,9). These results support the hypothesis that accumulation of calcium ions during disease development results in the formation of calcium pectates, which are resistant to hydrolysis by endopolygalacturonase.

In the present study, a change in the pectic substances was observed in advance of the pathogen. Histochemical tests for calcium localization are in progress to determine if this resulted from pectin degradation or from the formation of calcium pectates.

Another possible cause of lesion delimitation is the accumulation of fungitoxic or fungistatic compounds (37,38). Van Etten and Bateman (38) reported that the phytoalexins phaseollin and kievitone in young lesions increased to levels inhibitory to the pathogen. Light microscopic studies have shown that phytoalexins affect both fungal and host cytoplasm (23,36,38). Hyphae exposed to phaseollin for 1 min became granular in appearance, protoplasmic streaming ceased, and metabolite leakage occurred (38). It would be reasonable to expect similar alterations in hyphal ultrastructure in vivo, possibly the cytoplasmic vacuolation observed in hyphae in this study. Although changes in fungal ultrastructure were not common until 48 hr after infection, they may be related, in part, to phytoalexin production by the host. Host cytoplasmic changes occur independently of wall digestion. These changes also may be caused by similar toxic compounds (23). Ultrastructural studies of the effects of phytoalexins on host and pathogen would be helpful.

The present work confirms cytoplasmic effects independent of extensive wall swelling and suggests that leakage of electrolytes is probably not simply an osmotic effect. The host cytoplasmic changes probably result from more than one cause.

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