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Structural Changes Associated with Resistance of Soybean to *Heterodera glycines*[1]

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Abstract: Subcellular responses to infection by Race 3 of *Heterodera glycines* in susceptible ('Lee') and resistant ('Forrest' and 'Bedford') soybean cultivars were compared. Syncytial formation, initiated in susceptible as well as resistant soybean cultivars, was characterized by wall perforations, dense cytoplasm, and increased endoplasmic reticulum. In susceptible plants, syncytia developed continuously until nematode maturity. This included hypertrophy of nuclei, increase of rough endoplasmic reticulum in early stages of infection, and formation of wall ingrowths at a late stage of infection. In the resistant reaction in Forrest, a necrotic layer surrounded syncytium component cells demarcating them from surrounding normal cells and leading to syncytial necrosis. Wall appositions were prominently formed near the necrotic layer, and the cytoplasm of the syncytium component cells was extremely condensed. The whole syncytium became necrotic at a late stage of infection. Bedford had nuclear degeneration prior to cytoplasmic degradation. Chromatin was often scattered throughout the syncytial cytoplasm. Finally the whole syncytium became degenerated with plasmalemma completely detached from the syncytial cell walls. The differences in resistant responses reflect a difference in genetic composition of the soybean cultivars tested.

Key words: cytopathology, *Glycine max*, *Heterodera glycines*, resistance, soybean, soybean cyst nematode, syncytium, ultrastructure.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is one of the most important parasites of soybeans (9,24). Parasitism of this nematode has been well documented. It involves the transformation of host cells into syncytia that provide nutrition for the developing nematode (5,8,27). Inhibition of syncytium formation and growth has been related to plant resistance. A proposed mechanism for resistance was the sealing off of the syncytium by the formation of cell-wall thickenings (27). Other suggested mechanisms for resistance have been early degeneration of syncytia (6) and necrosis of cells surrounding the undeveloped juvenile nematode in the absence of syncytial formation (28). Proposed mechanisms for resistance have been limited to the soybean cultivar 'Peking' that has served as a genetic source for developing resistant cultivars (13). Other soybean cultivars have not been examined ultrastructurally for their expression of resistance to SCN.

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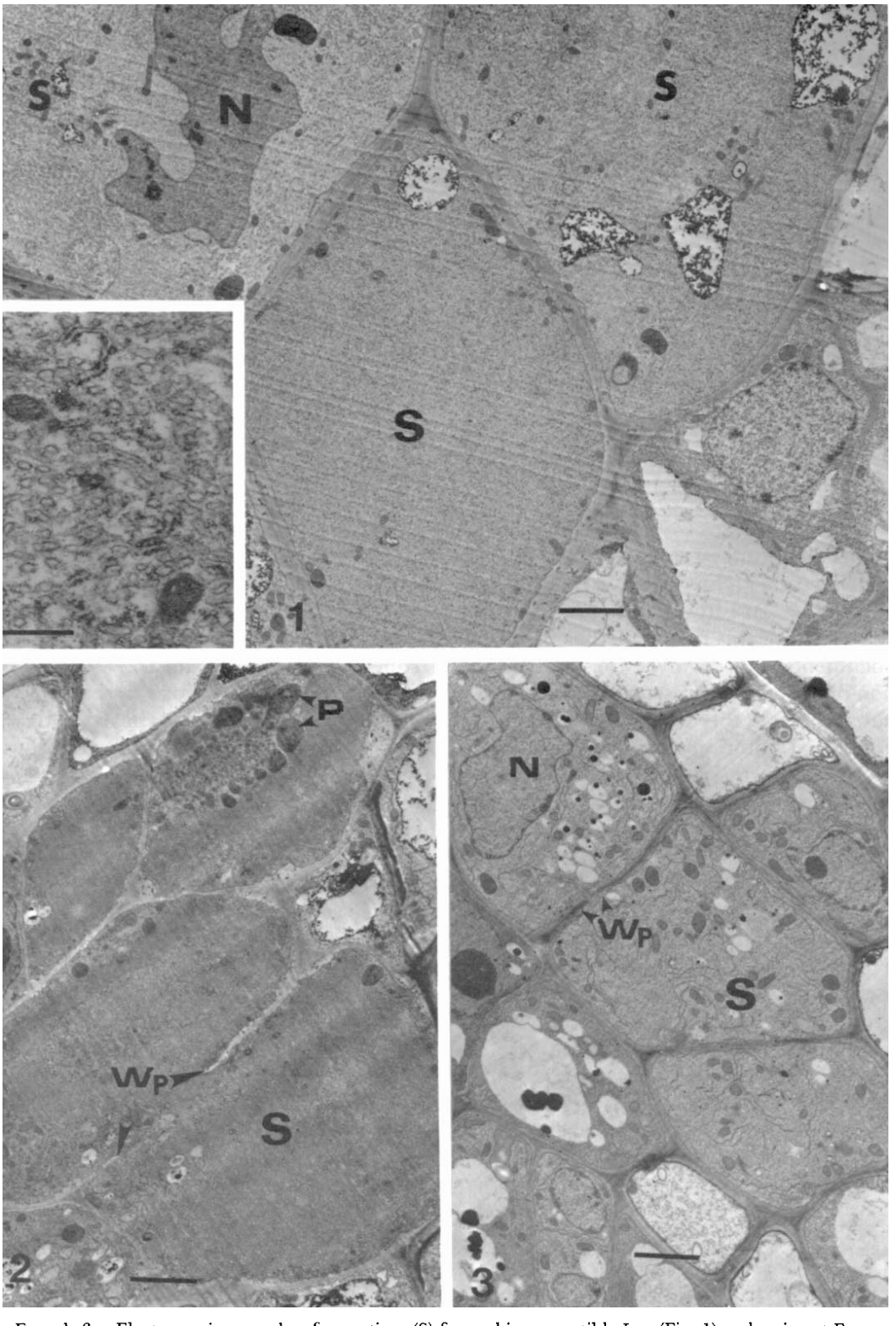
Acedo et al. (1) studied the histopathology of susceptible (compatible) and resistant (incompatible) soybean cultivars infected by nematode populations selected from the susceptible hosts. They found various degrees of necrosis in the compatible and incompatible soybean cultivars, suggesting that the necrotic reaction was not the sole barrier to parasite development. However, the details of pathogenesis were not compared among the plants. In other host plants infected by other species of cyst nematodes, the mechanisms of resistance varied and depended on host–parasite associations (4,33,34).

Information on the morphology of the resistant system has been limited and generalized. The host–parasite relationship of the variation in soybean–SCN interaction which has been reported through the years (2,21,25,26,30) has not been adequately elucidated. Therefore, the purpose of these subcellular studies was to investigate differences in pathogenesis of susceptible and resistant soybeans and cytological changes related to the mechanisms of resistance.

Materials and Methods

'Lee', 'Forrest', and 'Bedford' soybean seeds were germinated in sterilized vermiculite. At the primary leaf stage, the



FIGS. 1–3. Electron micrographs of syncytium (S) formed in susceptible Lee (Fig. 1) and resistant Forrest (Fig. 2) and Bedford (Fig. 3) soybean cultivars 5 days after inoculation with *Heterodera glycines* Race 3. Each syncytium is characterized by dense cytoplasm, hypertrophy of syncytium component cells containing tightly

seedlings were transplanted to sterilized, fine sand in 10-cm-d pots and inoculated with second-stage juveniles (J2) of SCN Race 3. Approximately 500 J2 were placed in each pot. One day after inoculation, soybean roots were rinsed with tap water to remove the nematodes that had failed to invade the root tissue and plants were transplanted into sterilized sand in pots.

Infected root segments were collected 5, 10, 15, and 20 days after inoculation. Root segments were fixed with a modified Karnovsky's fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.0) for 2 hours and rinsed in 0.05 M cacodylate buffer three times for 20 minutes each. The segments were postfixed with 1% osmium tetroxide (OsO_4) in 0.05 M cacodylate buffer for 2 hours. These segments were prestained with 0.5% uranyl acetate overnight at 0–4 C and dehydrated in an ethanol series. The segments were further dehydrated with 100% propylene oxide and embedded in Spurr's epoxy resin (29).

Thick sections (0.5–2.0 μm) cut with a glass knife were mounted on glass slides, dried on a warming table at 30 C, and stained with 1% toluidine blue. The sections were examined with a phase-contrast light microscope (LM) to monitor the infection sites for electron microscopy.

Silver-gold sections (ca. 800 Å) were cut with a glass or diamond knife on a Sorvall Porter-Blum ultramicrotome. Sections were collected on 400-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a SIEMENS 1A or JEOL 100 CX electron microscope.

Results

Structural features of syncytia: Syncytia were found 5 days after inoculation in both susceptible and resistant soybean cultivars. Syncytia were characterized by cell-wall perforations and dense cytoplasm in the hypertrophied syncytium component cells (Figs. 1–3). Syncytial cells in Lee were considerably larger than those in the resistant cultivars. The dense cytoplasm of syncytia was composed of much more numerous endoplasmic reticulum (ER) and ribosomes and other cell organelles such as mitochondria and plastids. Small cytoplasmic vacuoles were observed in the syncytia of all the soybean cultivars in which electron-dense spherical inclusions occurred.

Development of syncytia in the susceptible soybean: In Lee soybean (susceptible to Race 3), continuous syncytial development was observed over a period of 20 days after inoculation. The cytoplasm increased in electron density, and the central vacuole was reduced to several smaller secondary vacuoles separated by cytoplasmic strands (Fig. 4). Electron-dense spherical inclusions occurred first in the cytoplasm of syncytium-component cells (Fig. 4) and appeared to be transported into the vacuoles through the tonoplast (Fig. 5). Frequently the inclusions appeared somewhat crescent shaped and often aligned at the boundary of the vacuole (Fig. 6).

Five days after inoculation, nuclei in the syncytium of the susceptible host were always extremely hypertrophied with prominent nucleoli and highly convoluted nuclear envelopes (Figs. 1, 7). The most prominent cytoplasmic feature at this stage of infection was an increased quantity of membranes resembling endoplasmic reticulum, which will be referred to as ER hereafter, and ribosomes that apparently contribute to the appearance of dense cytoplasm (Figs. 1, 5, 6). Other cell organelles, such as mitochondria and plastids, were not increased in number or size.

Although secondary wall thickenings

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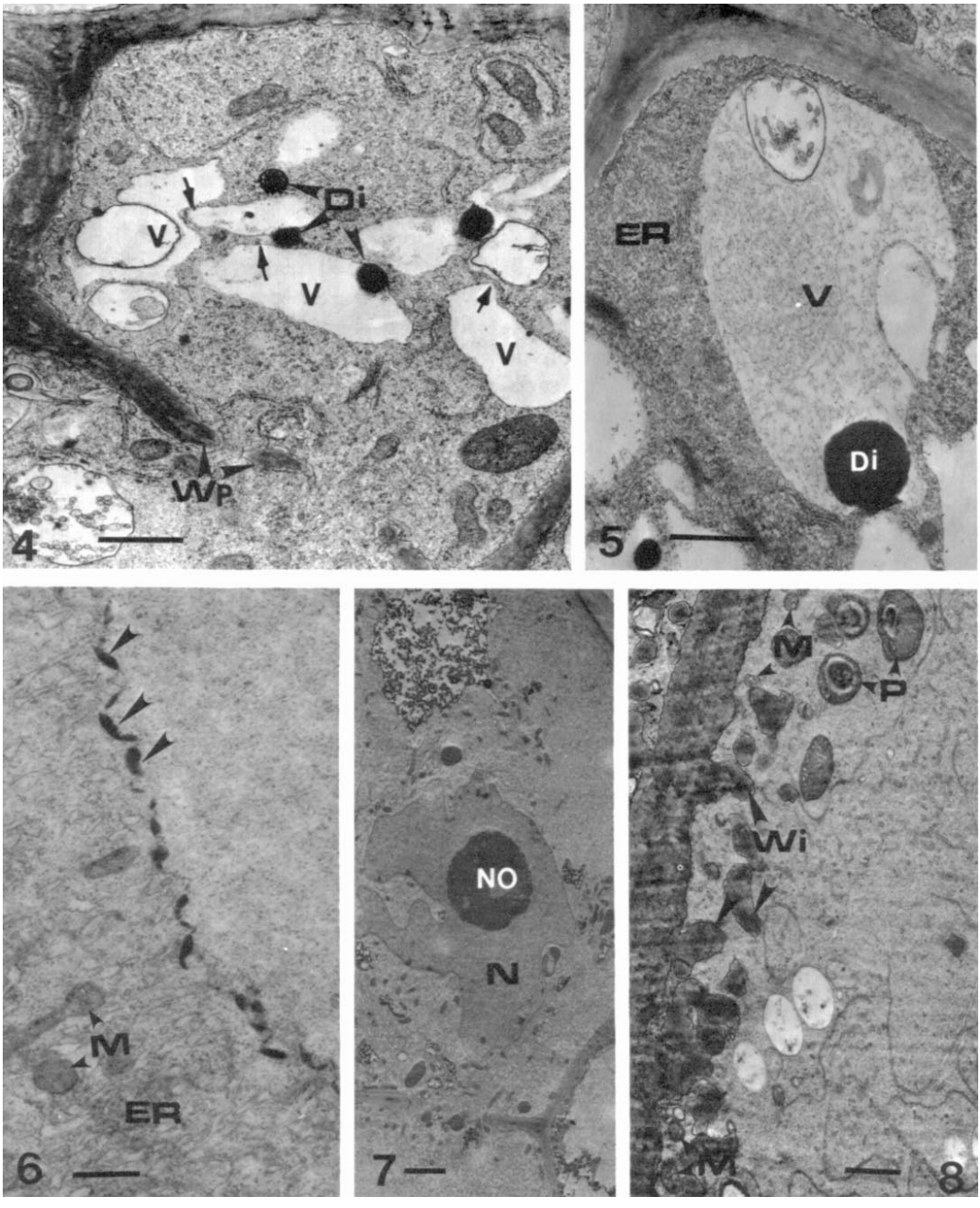
packed endoplasmic reticulum and ribosomes, and cell-wall perforations (Wp) between cells. Resistant soybeans (Figs. 2, 3) have relatively smaller syncytium-component cells than the susceptible Lee soybean (Fig. 1). N = nucleus. P = plastids. Scale bars = 2.5 μm. Inset in Figure 1: Higher magnification of a portion of dense cytoplasm of syncytium formed in Lee soybean showing highly proliferated membranes including rough endoplasmic reticulum and ribosomes. Scale bar = 0.5 μm.



FIGS. 4–8. Lee soybean syncytium observed 5 (Figs. 4, 5, 7), 10 (Fig. 6), and 15 (Fig. 8) days after inoculation. 4) The central vacuole has become several secondary vacuoles (V) divided by cytoplasmic strands (arrows). Several electron-dense spherical inclusions (Di) occur in the cytoplasm. Wp = cell-wall perforation. Scale bar = 1 μm. 5) A large dense inclusion (Di) in a secondary vacuole apparently transported from the cytoplasm. Dense cytoplasm consists mainly of endoplasmic reticulum (ER) and ribosomes. Scale bar = 1 μm. 6) At the tonoplast, the dense inclusions (unlabeled arrowheads) appear somewhat crescent shaped and are aligned throughout the tonoplast. The cytoplasm is packed with endoplasmic reticulum (ER). M = mitochondria. Scale bar = 0.5 μm. 7) A typical enlarged and convoluted nucleus (N) of an early syncytium in susceptible host, Lee soybean, containing prominent nucleolus (NO). Scale bar = 2.5 μm. 8) Formation of cell-wall ingrowths (Wi) sectioned at different angles observed 10 days after inoculation. Mitochondria (M) and plastids (P) are scattered near these wall ingrowths. Scale bar = 1 μm.

were present on the syncytial cell walls 10 days after inoculation, finger-like projections were rarely observed at the intermediate stages of infection (5–10 days after inoculation). Wall ingrowths, some of which were elongated finger-like forms, were commonly observed 15 days after inoculation (Fig. 8). Mitochondria and plastids were arranged alongside cell-wall ingrowths (Fig. 8). At 20 days after inoculation, fully developed wall ingrowths in syncytia adjacent to xylem vessels produced branches to form numerous labyrinths.

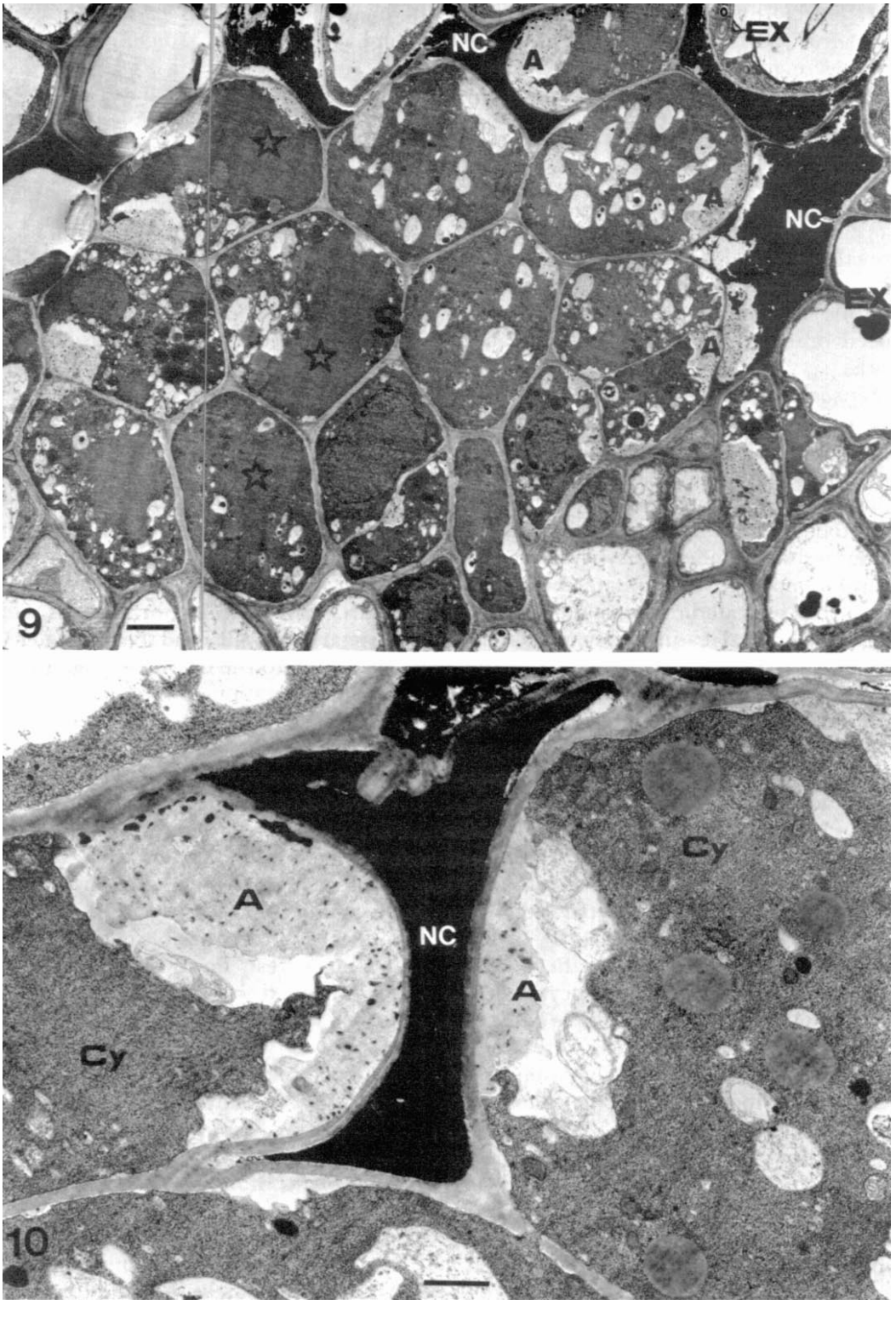
Formation of necrosis and hypersensitive reaction in Forrest: Necrosis was frequently found around syncytia or modified cells in the Forrest soybean 5 days after inoculation. Necrosis was initiated adjacent to the nematode lip region, extended through the tissue, and surrounded the syncytium. The necrosis appeared to separate the syncytium from surrounding normal cells (Fig. 9). The structural features of syncytial cells varied with their location relative to the necrotic layer. Syncytium-component cells near the necrotic layer had either very small wall perforations or none, extremely dense cytoplasm, and prominent cell-wall appositions adjacent to the necrotic layer (Figs. 9, 10). Wall appositions formed inside the wall of syncytial cells appeared to further separate component cells from surrounding normal cells; additional separation was due to the necrotic layer (Fig. 10). In the syncytium-component cells farthest away from the necrotic layer, or in syncytia without prominent necrosis, cell walls had numerous perforations and cells were much enlarged with abundant endoplasmic reticulum and ribosomes as in the early Lee syncytium (Fig. 11). As infection progressed, however, the whole syncytium became necrotic (Fig. 12).

Degeneration of nucleus and cytoplasm in Bedford: Five days after inoculation, some syncytium-component cell nuclei showed signs of degeneration; i.e., the entire nucleoplasm of some nuclei became homogeneously heterochromatic (Fig. 13). Other nuclei contained a large mass of clumped chromatin (Fig. 14). The nucleoplasm around the clumped chromatin mass was usually electron lucent and often contained cytoplasmic organelles, such as mitochondria (Fig. 14), and membraneous debris, indicating the breakdown of the nuclear envelope. In more developed larger syncytia, chromatin material was scattered randomly in the cytoplasm (Fig. 15). No structurally recognizable nuclei were observed in such syncytia, indicating that nuclei probably had degenerated (Fig. 15). Although nuclei of early syncytial cells were undergoing degeneration, the cytoplasm appeared to be intact, exhibiting characteristic cytoplasmic features of early syncytia, such as a large amount of endoplasmic reticulum, ribosomes, cell-wall perforations, and small secondary vacuoles (Fig. 15). In addition, the cell walls of such syncytia were much thickened and the plasmalemma was widely separated from the cell walls. The adjacent normal cells had thin primary cell walls, and the plasmalemma was bound to the cell walls (Fig. 15).

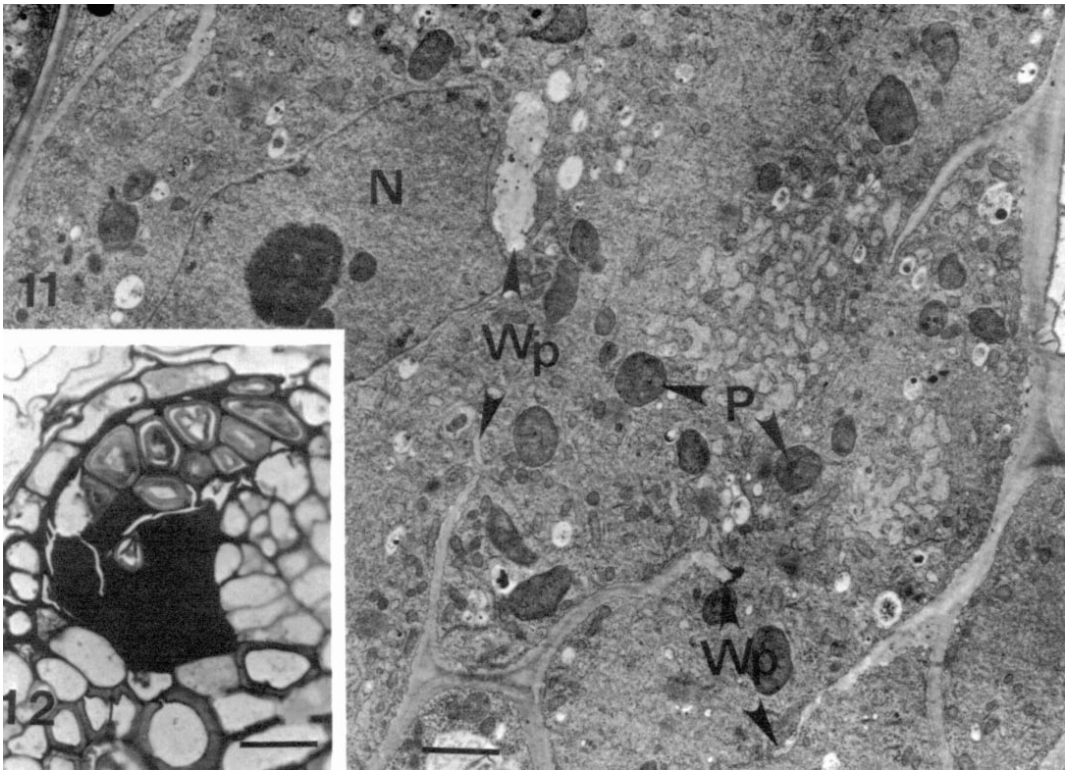
After degeneration of nuclei, the cytoplasm also appeared to undergo degradation. Syncytium-component cells of many syncytia 10 and 15 days after inoculation showed such cytoplasmic degradation. The plasmalemma was completely detached from the cell wall, and the cytoplasm appeared as irregular membraneous debris in the middle of cells (Fig. 16). No cell organelles were recognizable in these cells. Unlike in Forrest, however, no necrotic layer was formed around the syncytia (Fig. 16).

Discussion

Results of this study suggest that syncytium formation is initiated in both susceptible and resistant soybeans. In the susceptible plant, however, the structural modifications associated with syncytial development appeared to be a continuous process. In resistant plants, inhibition of syncytium development suggested that inhibition is the mechanism of resistance. After initiation of similar syncytia among soybean cultivars, including wall perforations,



FIGS. 9, 10. Forrest syncytium (S) observed 5 days after inoculation. 9) Low magnification view of a syncytium which is surrounded by a layer of necrotic and collapsed cells (NC) that separated syncytium from the external normal cells (EX). The small syncytium-component cells near the necrotic layer consist of



FIGS. 11, 12. Electron and light micrographs of Forrest syncytium. 11) Syncytium-component cells farthest away from necrotic layer containing dense cytoplasm with increased endoplasmic reticulum and wall perforations (Wp). N = nucleus. P = plastids. Scale bar = 2 μm. 12) Light micrograph showing an entire syncytium that became necrotic at 15 days after inoculation. No individual component cell can be recognized. Scale bar = 5 μm.

dense cytoplasm, and increased ER, different malfunctions of syncytia were noted in the resistant soybean cultivars.

At 5 days after inoculation, cytoplasm in susceptible plants appeared to be in the process of de-differentiation into primary meristem cell types; i.e., enclosing of new cytoplasm by fusing of vacuoles, presence of dense cytoplasm, and increase of membrane structures (3,18). The structural features of syncytia in susceptible cultivars also resembled secretory and absorptive (transfer) cells. The increase in amount of endoplasmic reticulum and deposition of electron-dense material in vacuoles resembled the characteristic features of a farina gland cell (12) which is a secretory cell. Formation of cell-wall ingrowths supports the hypothesis that syncytia are absorptive. Jones (14) reported movement of solutes from giant cells to a nematode. Membrane potential fluctuation was suggested because giant cells are secretory as well as absorptive (17). Accumulation of solutes through the ingrowths was found in transfer cells (11). Similar accumulation may also occur in giant cells or syncytia with the amplified membrane surface resulting from the formation of the wall ingrowths (15,16). Thus, a syncytium in the susceptible soy-

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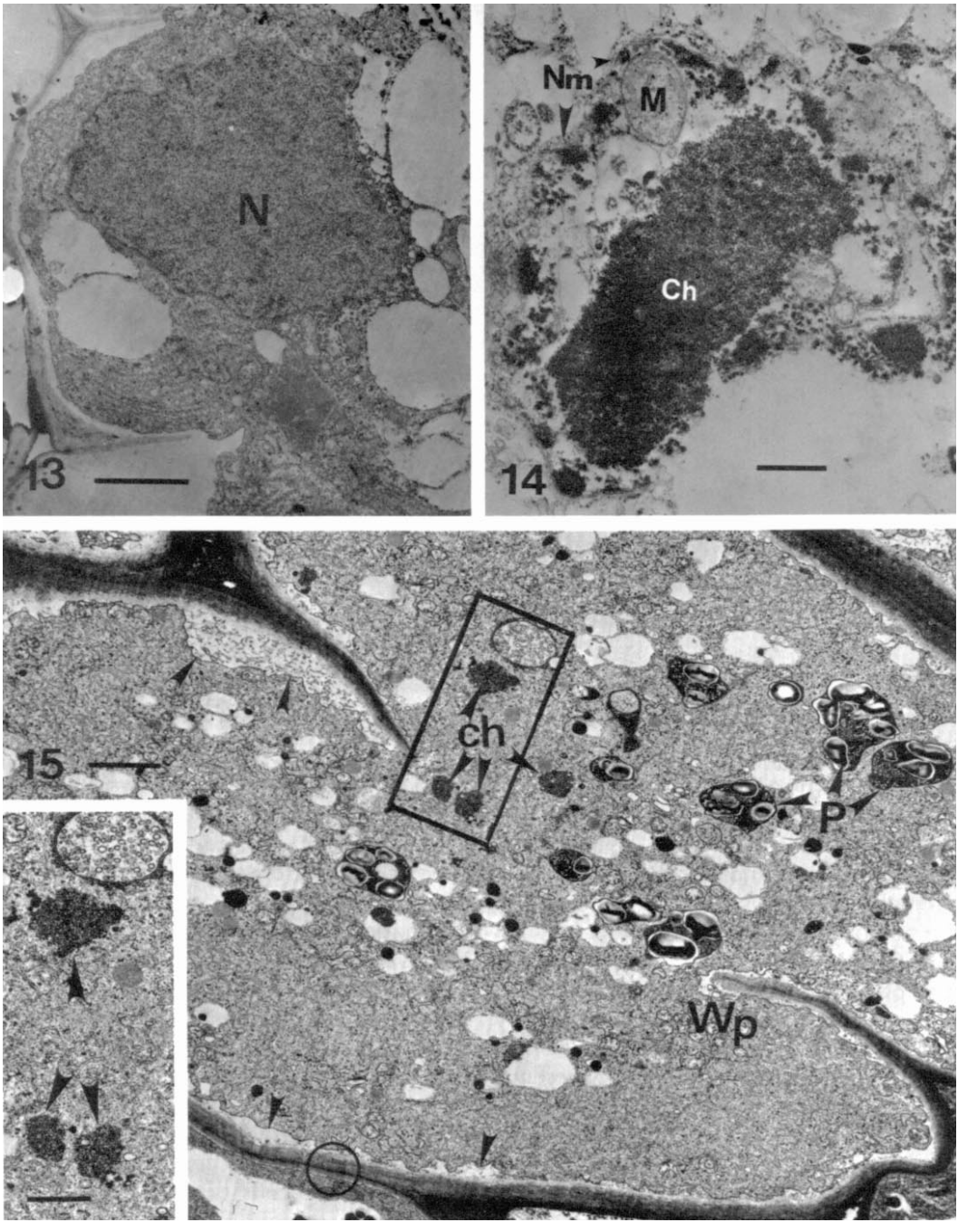
extremely dense cytoplasm (stars) and small vacuoles. The cells adjacent to the necrotic layer contain prominent cell-wall appositions (A). Scale bar = 2.5 μm. 10) A higher magnification of an area of two syncytium-component cells adjoining necrotic layer (NC) showing the details of cell-wall appositions (A) and the dense cytoplasm (Cy). Scale bar = 1 μm.



FIGS. 13–15. Bedford syncytium observed 5 days after inoculation. 13) A syncytial nucleus (N) that has become homogeneously heterochromatic. Scale bar = 2 μm. 14) A nucleus containing greatly increased chromatin (Ch) that is clumped into a large mass occupying a large area of nucleoplasm. This nucleus also contains a mitochondrion (M) indicating that it entered the nucleus through the disrupted nuclear envelope (Nm). Scale bar = 0.5 μm. 15) Cytoplasm of syncytial cells containing scattered chromatin (Ch) as electron-dense masses of various sizes. The cell wall of syncytium appears much thicker than adjacent normal cell wall (circle), and the plasmalemma is widely separated from the cell wall (unlabeled arrowheads). Wp = wall perforations. P = plastids. Scale bar = 2.5 μm. Inset: Higher magnification of boxed area in Figure 15 showing the details of chromatin structure (arrowheads). Scale bar = 1 μm.

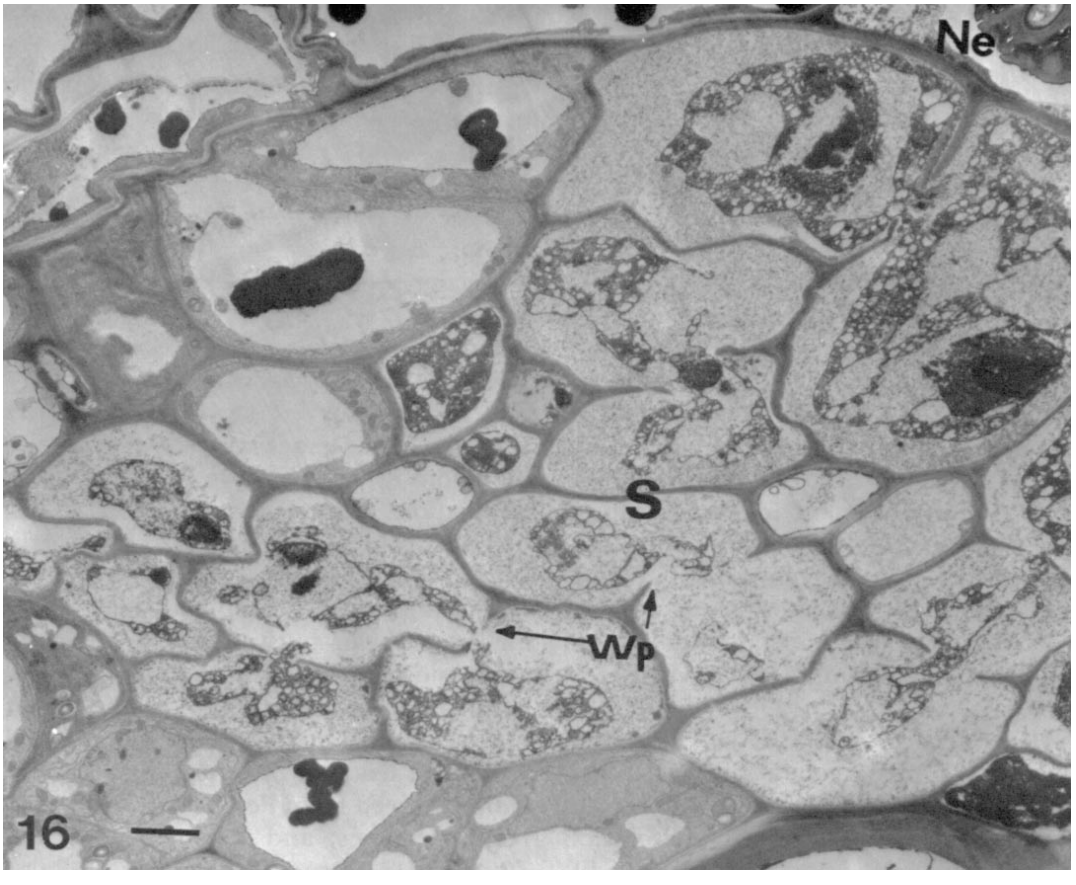


FIG. 16. Bedford syncytium (S) at late stage of infection. Entire syncytium-component cells are undergoing degeneration. No cell organelles are recognizable. The plasmalemma is completely detached from the cell wall and the cytoplasm occurs as an irregular membranous mass in the middle of each cell. However, no necrotic layer surrounds this degenerating syncytium. Wp = wall perforation. Ne = nematode. Scale bar = 2.5 μm.

bean may be a well-designed system in transporting nutritional materials from the host tissue to the infecting nematode.

In Forrest, a resistant soybean variety, a necrotic layer surrounded the syncytium possibly blocking movement of nutrient materials into syncytial cells. Poor development of syncytial cells, including small size and lack of wall perforations, support this hypothesis. Also, the necrotic layer may inhibit syncytium-inducing materials from reaching normal cells and prevent incorporation of new syncytium-component cells. The dense cytoplasm and prominent cell-wall appositions adjacent to the necrotic layer are similar to the first stage of hypersensitive reactions of cells affected by the root-knot nematode (23). Riggs et al. (27) stated that the wall thickenings (wall appositions) associated with boundary formation or paramural bodies (7,19) walled off the plasmodesmata and prevented cell-to-cell movement of materials which would lead to a deficit in food supply or build-up of toxic byproducts within syncytia. The sealing-off of plasmodesmata by wall thickenings also prevented cell-to-cell movement of virus particles (31). Plasmalemma commonly separated from the cell wall in Bedford, another resistant variety of soybean. This membrane separation might break the cytoplasmic strand connections through plasmodesmata and prevent further development of syncytial cells, leaving the external normal cells intact.

Increased nuclear activity seems to be

necessary for susceptible pathogenesis, as indicated by various studies of ectoparasites as well as endoparasites (10,22,32). In *Longidorus elongatus*-infected ryegrass, DNA contents initially increased but nuclear size and DNA contents were rapidly reduced. This reduction was accompanied by nuclear degeneration at a late stage of infection (10), possibly the result of discontinued feeding stimulus from the nematode. In Bedford, the nematode infection probably stimulated nuclear activity, inducing a susceptible type of syncytium. The stimulated nuclear activity might be blocked, however, by the changes in cytoplasmic environment (probably caused by lack of suitable metabolites due to inhibition of cell-to-cell movements of materials through plasmodesmata) and inability of the nucleus to sustain the activated state that subsequently led to degeneration.

Although host resistance or susceptibility is known to be genetically controlled, the direct relationship between certain genes and responding cellular alteration has not been determined. Regardless of differences in the morphology of syncytia among the plants, the fact that the cells infected by the nematode were altered indicated that the nematodes penetrated and were established in resistant root tissue. Basically, there was no significant relationship of nematode penetration to the total expression of resistance (1). Thus, the gene-for-gene relations of host–parasite interactions are not dependent upon the penetration and establishment of nematode juveniles. The structural features of syncytial cells in Peking reported by Riggs et al. (27) are similar to those in Forrest in this study in many respects. These similarities include formation of wall depositions and increase of lipid globules and cytoplasmic features before degeneration or necrotization of syncytia. This suggests that plants having the same type of resistance (both resistant to races 1 and 3 of SCN) may have similar modifications of infected cells; i.e., sealing-off of syncytia with cell-wall thickenings and possible hydrolyzing of cells indicated by profound lipid globules (20). Bedford probably has another gene for resistance, because the resistance mechanism exhibited by cytopathology of syncytia was completely different from those of Forrest and Peking. Therefore, nuclear degeneration followed by cytoplasmic degradation might be related to the major resistant gene expression unique to Bedford.

Rapid selection of variants virulent to the previously resistant soybean cultivars has been observed through the years (2,21,26,30). Demonstrated intraspecies variation in reactions to hosts in SCN populations suggests that use of more differential varieties might result in more diverse SCN races. Genes that control physiological processes in plants may differ among soybean cultivars by affecting the expression of resistance genes and thereby contribute to variations in resistance. This may be implied by the slight differences in structural features of syncytia in Forrest, which derived from Peking as a source for resistant genes, and Peking. Jones and Dropkin (15) suggested that some cellular modification can occur by simple uptake of solutes by nematodes, which may not be related to the specific physiological interactions between host and parasite governed by gene-for-gene relations. Slight variation in host preference of a nematode population (e.g., host races or physiological races) may be associated with variation in host response. Selection pressure acting on such variations in populations may result in rapid breakdown of resistance.

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