Ultrastructural Cell Wall Alterations in Immune Cotton Leaves Inoculated with Xanthomonas malvacearum

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ABSTRACT

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Within 2 hr following inoculation of immune cotton cotyledon tissue incompatible bacteria in the intercellular spaces caused disruptions on the outer surfaces of host mesophyll cell walls. These disruptions were characterized by loosening and detachment of the surface cuticle and envelopment of adjacent bacteria. The enveloping structures contained one to several bacteria and were filled with wispy fibrillar material apparently released from the damaged cell wall. The plasmalemma frequently was broken in the vicinity of enveloped bacteria, and host cytoplasm appeared to be clumped and electron-dense. Inoculated tissue became necrotic within 24 hr. Cell walls of similarly inoculated susceptible host tissue were not disrupted during the test period, and intercellular bacteria were not enveloped. These observations suggest that in immune cotton leaves, cell wall damage may lead to membrane breakage and cell necrosis.

Additional key words: Hypersensitive response (HR), *Gossypium hirsutum* L., compatibility, incompatibility.

The host cell wall recently has become a subject of considerable interest, particularly in the study of cellular host resistance to incompatible bacteria. Goodman et al. (5) reported that *Pseudomonas pisi* Sack. cells were immobilized by cuticular elements of tobacco mesophyll cell walls. Sequeira et al. (9) reported a similar phenomenon in tobacco inoculated with avirulent forms of *Pseudomonas solanacearum* Sm., and Sing and Schroth (10) reported saprophytic bacteria were encapsulated by leaf cell walls of red kidney bean. Previously, we reported that cell wall damage was a symptom of the hypersensitive response (HR) in cotton (3). Pectinolytic enzyme activity appears earlier in the cotton incompatible interaction than in the compatible interaction, suggesting that consequent host cell wall damage leads to the necrosis typical of the HR (7, 13).

In this study we present evidence that leaf mesophyll cell walls in immune cotton leaves are damaged in the presence of incompatible bacteria, and that the phenomenon does not occur within the same period in the compatible relationship with susceptible cotton. This difference in host response contributes information on details of the HR in the incompatible relationship, and is consistent with previous observations with tobacco (5, 9).

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MATERIALS AND METHODS

Plant lines and growth conditions.—A blight-susceptible cotton line, Aç 44, and an immune line, Im 216 (2) were used in this study. The immune line is resistant to all known races of *Xanthomonas malvacearum* (E. F. Sm.) Dows. (1), and shows no macroscopically visible symptoms of natural infection under field conditions.

Acid-delinted seeds were planted in 15.5 cm diameter clay pots in a sterile soil-vermiculite-peat mixture (3:1:1, v/v). The seeds were incubated in a plant growth chamber under 12 hr of light (incandescent and fluorescent at 1-2 × 10^4 lux) at 30 C and 12 hr of darkness at 18 C. The seedlings emerged within 6 days, and within 12 days the first foliage leaves were expanded. Cotyledons were selected for inoculation at this stage.

Bacterial culture.—A California field isolate of *X. malvacearum* race 1 was given to us by W. C. Schnathorst. During prolonged culture it spontaneously produced two colony sizes. The larger colony type, which exhibits greater virulence, was used in this study. Under our culture conditions this strain is nonmotile. Inoculum

was cultured by transferring from a single colony on dextrose-agar medium to 125-ml Erlenmeyer flasks containing 25 ml of sterile nutrient broth, and incubating in shake culture at 30 C for 24 hr. Inoculum was prepared by centrifuging 20 ml of broth culture at 12,000 *g* for 10 min and resuspending the pelleted bacteria in distilled water. The suspension was adjusted spectrophotometrically to $A_{600} = 0.05$ which contains ~5 $\times 10^8$ viable cells/ml.

Inoculation method.—Infiltration of the bacterial suspension was by injection as previously described (3). A lateral half of each cotyledon was infiltrated with the inoculum and the other half with sterile distilled water as a control.

Preparation of tissue for electron microscopy.—Tissue pieces were excised from inoculated leaves beginning immediately following injection and at 2-hr intervals for 24 hr. Tissues immediately were fixed by vacuum infiltration with cacodylate-buffered 4% glutaraldehyde (pH 7.3) at 4 C (8). After 2 hr, the samples were washed in cold buffer and postfixed for 4 hr in a similarly buffered 1% osmium tetroxide solution at 4 C. The tissues were dehydrated with a graded series of aqueous ethanol solutions and infiltrated with Spurr's epoxy resin (11).

Thin sections were cut with a diamond knife on either a Sorvall MT-2 or a Reichert OMU-2 ultramicrotome. Sections were collected on noncoated grids and stained successively with 2% aqueous uranyl acetate and Venable's lead citrate (12). The grids were scanned on an RCA EMU-3G electron microscope at 100 kV, and representative sections were photographed.

RESULTS

Controls.—Sections from water-injected tissue revealed cells typical of healthy cotton mesophyll (Fig. 1). Cell walls and cytoplasmic components appeared intact and normal.

Immune host line Im 216.—Water soaking that resulted from infiltration disappeared within 20-30 min, and observations from tissue fixed shortly following infiltration revealed intercellular bacteria near the outer surfaces of host cell walls (Fig. 2). Observation of sections fixed after 2 to 4 hr revealed that the host cell walls had become disrupted in the vicinity of bacteria (Fig. 3). Loosening and detachment of the cell wall surface cuticle (Fig. 4) and envelopment of bacterial cells (Fig. 5, 6, 7) followed. The detachment process of the surface cuticle of the host cell wall apparently caused the release of numerous wispy strands of the wall substratum (Fig. 4). The liberated wall material appeared to be gathered about the bacterial cells and filled the spaces between bacteria inside the enveloping structures (Fig. 5, 6, 7). In tissues fixed 6 to 8 hr following inoculation there were numerous areas in which the host plasmalemma had become broken and vesiculated. In many instances damaged membranes were found near enveloped bacteria (Fig. 6). By 10 hr after inoculation the cytoplasm of many host cells appeared coagulated and was electron-dense. Organelles were deranged and swollen (Fig. 7). By 12 to 14 hr numerous necrotic host cells were observed with collapsed and coagulated cytoplasm.

Susceptible host line Ac 44.—Neither cell wall disruption nor envelopment of bacteria was observed in Ac 44. Host cell walls appeared unchanged in the presence of bacteria for the 24-hr test period. Bacterial cells did not become enveloped even after close contact with the host cell wall (Fig. 8, 9). Some evidence of bacterial cell division was apparent (Fig. 9), but this was observed in only a small percentage of the bacteria. No disruptive effects were observed on host cell membranes for the test period.

DISCUSSION

The envelopment phenomenon appears to be closely associated with necrosis of host cells in the incompatible *Gossypium-Xanthomonas* interaction. Neither envelopment of bacteria nor host cell necrosis was observed in the compatible relationship. In a previous

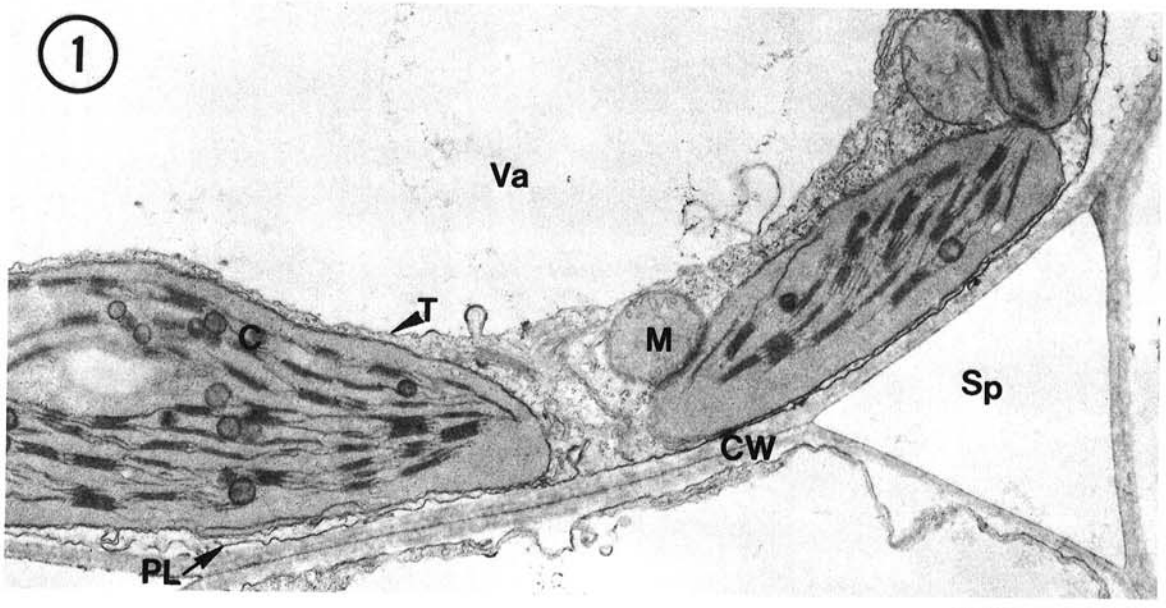
Abbreviations for electronmicrographs: C = chloroplast; CW = cell wall; Cu = cell wall cuticle; Cy = cytoplasm; D = disruption; DM = dense material; E = envelopment; R = rupture; Sp = intercellular space; T = tonoplast; Va = vacuole; V = vesicle; and Xm = *Xanthomonas malvacearum* bacteria.

Fig. 1-3. **1)** Control cotton palisade parenchyma cell fixed shortly following infiltration with sterile distilled water. Cell structure and cytoplasmic components appear normal and healthy (×12,000). **2)** Bacterial cells near immune host cell wall shortly following infiltration. Host cell wall and cytoplasm appear normal (×40,500). **3)** Bacterial cell and a damaged immune host cell wall. Disrupted areas are clearly shown about a central clearing directly below the bacterium. The disruptions appear to be cell wall fibrillar material. Fixed at 2 hr following infiltration (×77,000).

Fig. 4-5. **4)** Bacterial cell in contact with a detached strip of immune host cell wall surface cuticle. Wispy strands of fibrillar material are apparently released from the wall and appear to gather about the bacterial cells. Fixed 8 hr following infiltration (×58,500). **5)** An envelopment site containing four bacteria. The structure appears turgid and filled with fibrillar material which also extends beyond the structure. The covering of the structure appears to be continuous with the surface of the host cell wall and originates at disrupted areas. Fixed at 10 hr following infiltration (×77,000).

Fig. 6-7. **6)** A bacterium enveloped between adjacent walls of two host cells. The plasmalemma appears broken in several areas with the formation of numerous membrane-bound vesicles as a result. Host cytoplasm appears dense and clumped. Fixed at 10 hr following infiltration (×77,000). **7)** A single bacterium enveloped on the surface of an immune host cell wall. The host cell wall cuticle is distinct and continuous with the surface of the envelopment. A rupture is apparent with fibrillar material escaping into the intercellular space. Host cell cytoplasm is electron-dense and clumped. Fixed at 10 hr following infiltration (×58,500).

Fig. 8-9. **8)** A single bacterium near the host cell wall surface in Ac 44 shortly following infiltration. No disruptive effects on the host cell wall are evident (×77,000). **9)** A bacterial cell touching the host cell wall surface in Ac 44 24 hr following inoculation. The bacterium was not enveloped and the host cell structure appears normal. The bacterium appears to be in early stages of division. (×58,500).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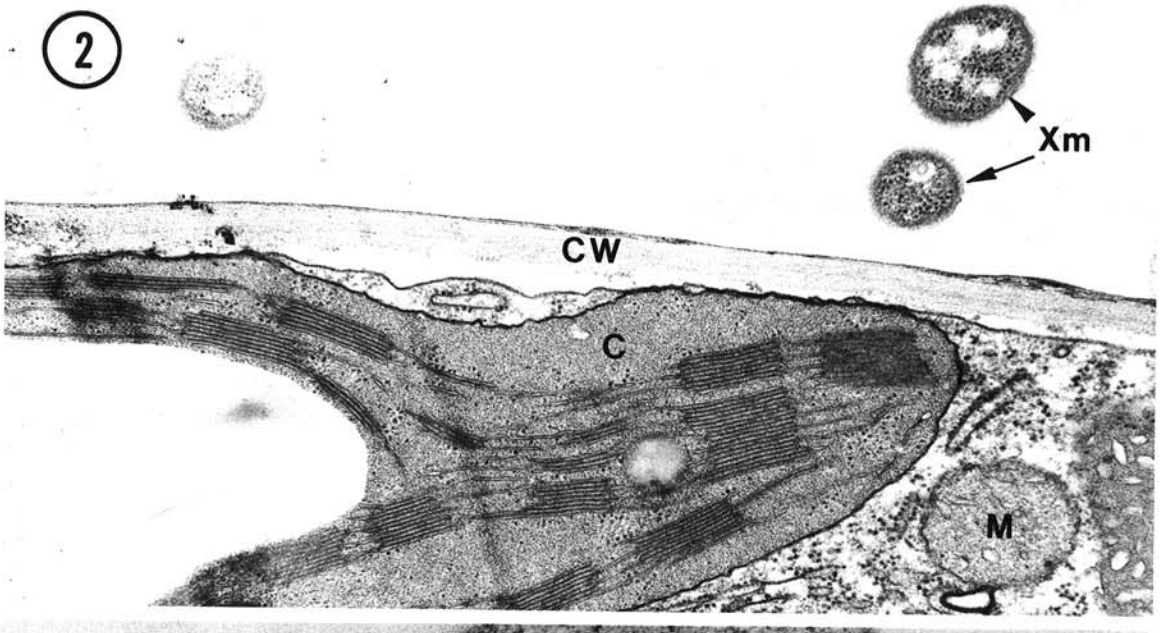
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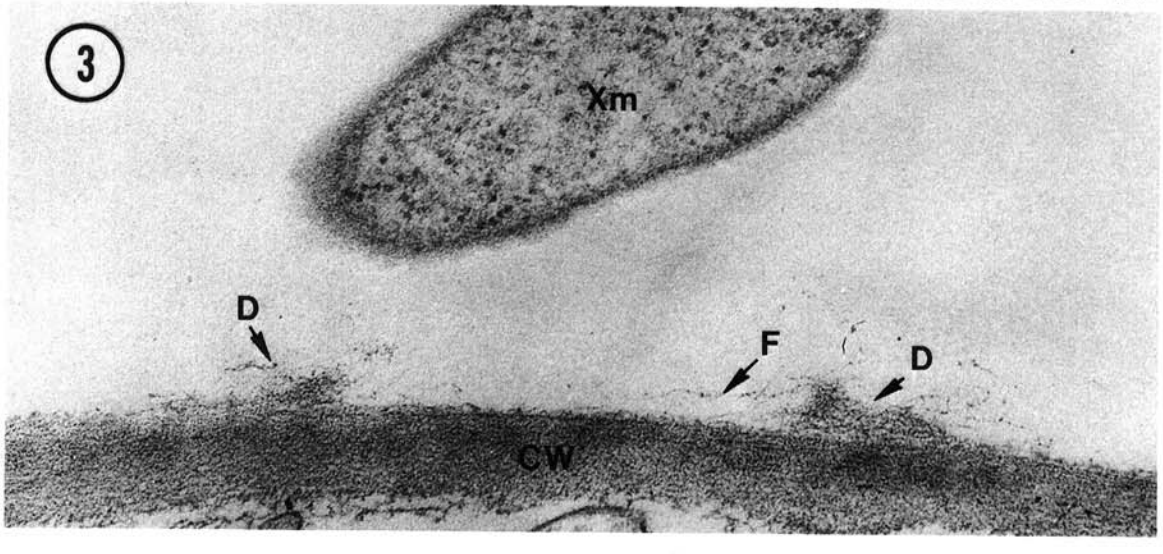
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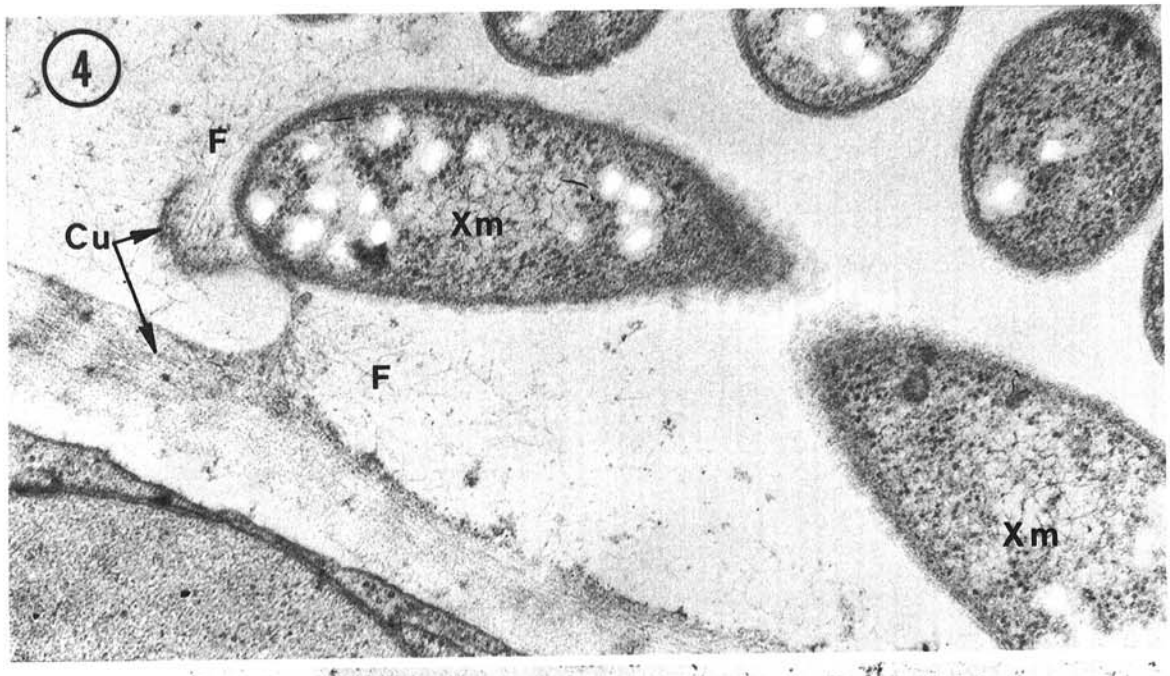
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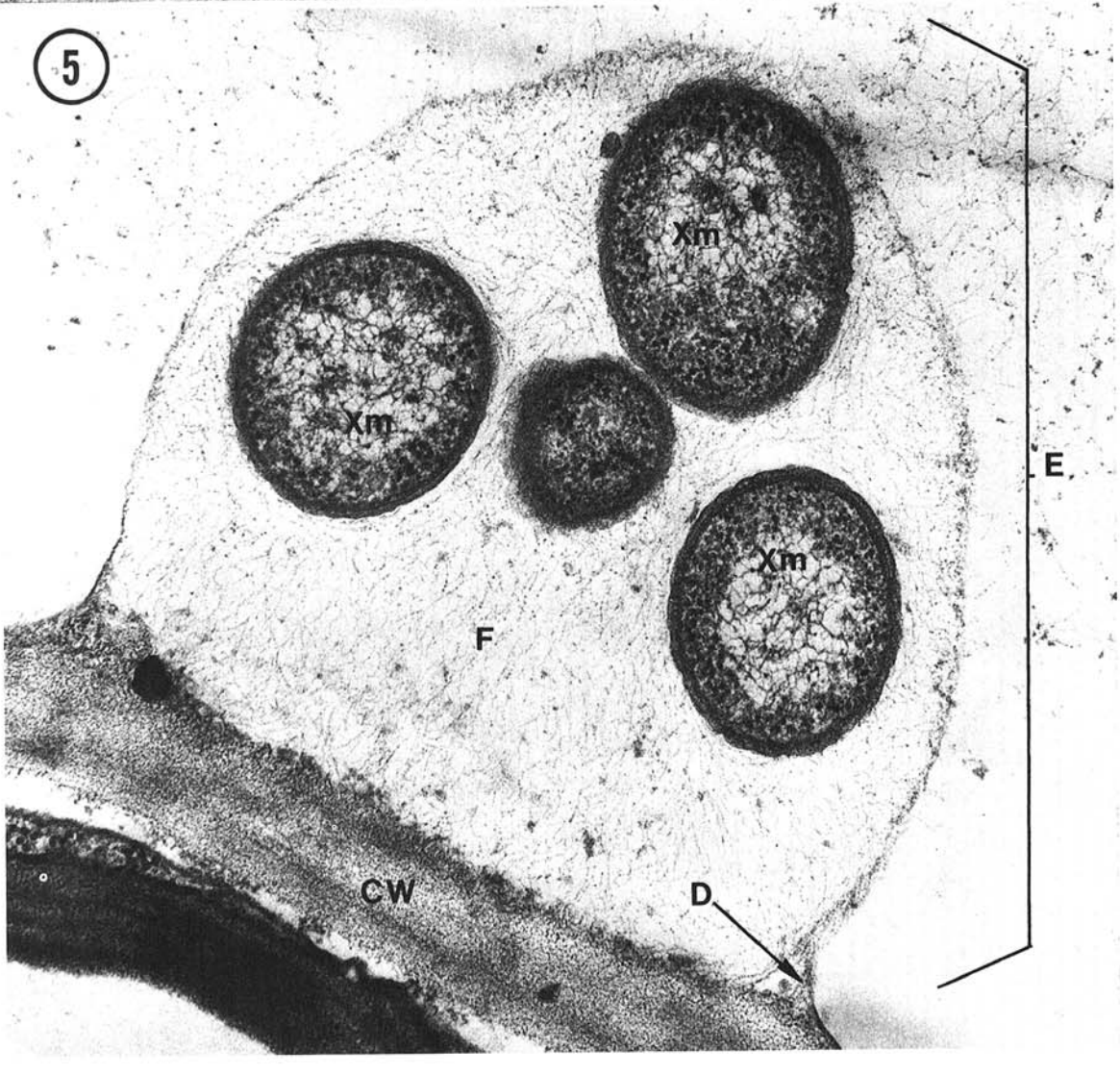
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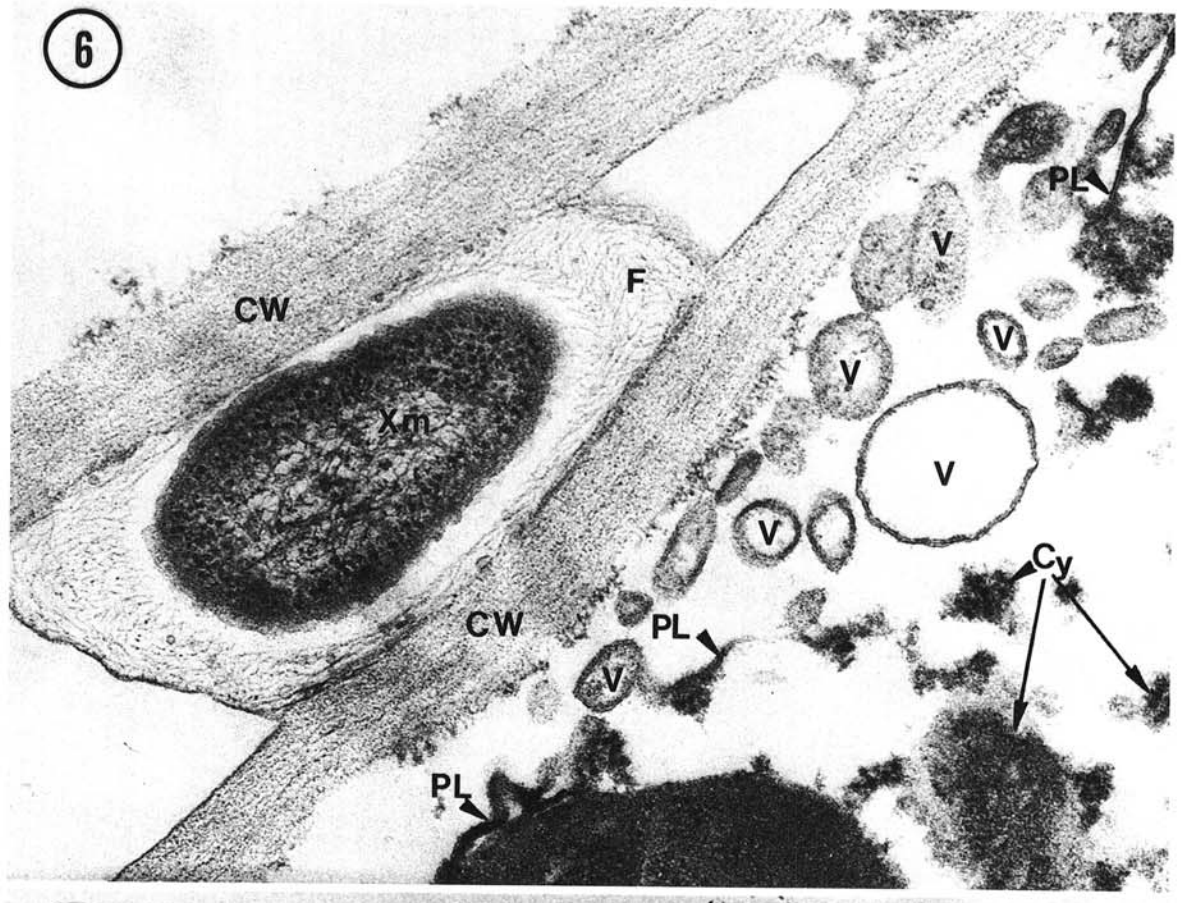
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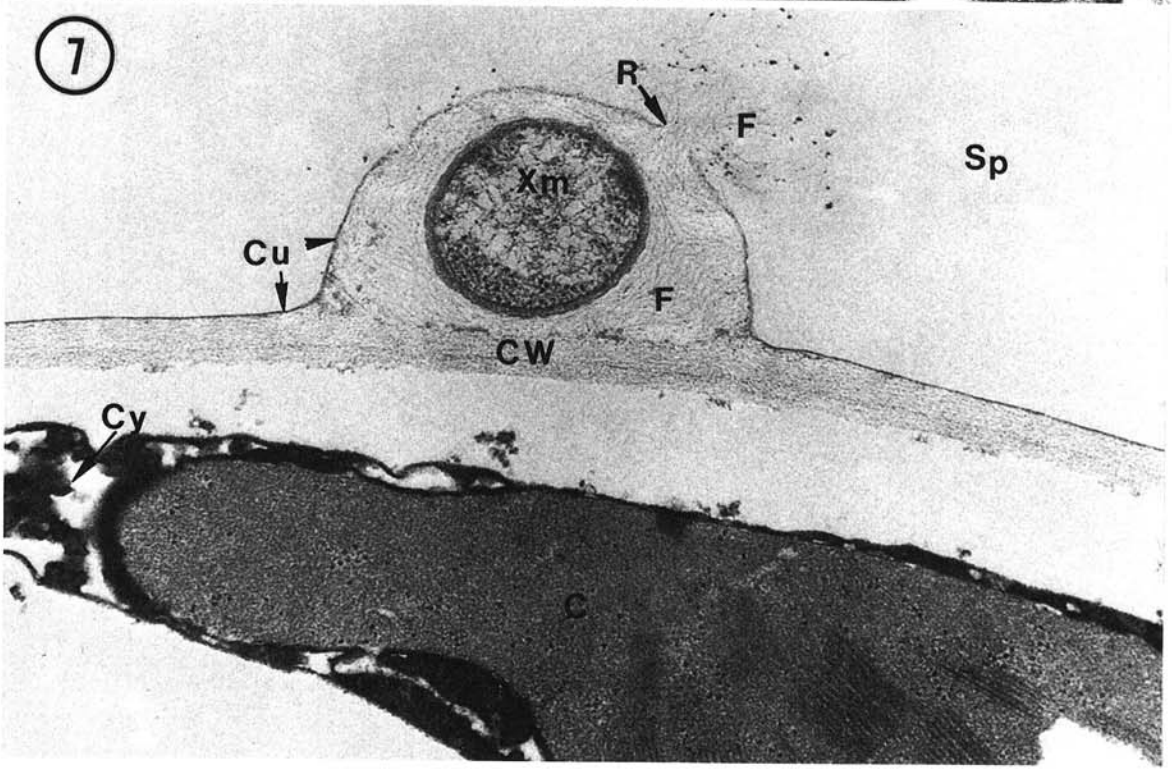
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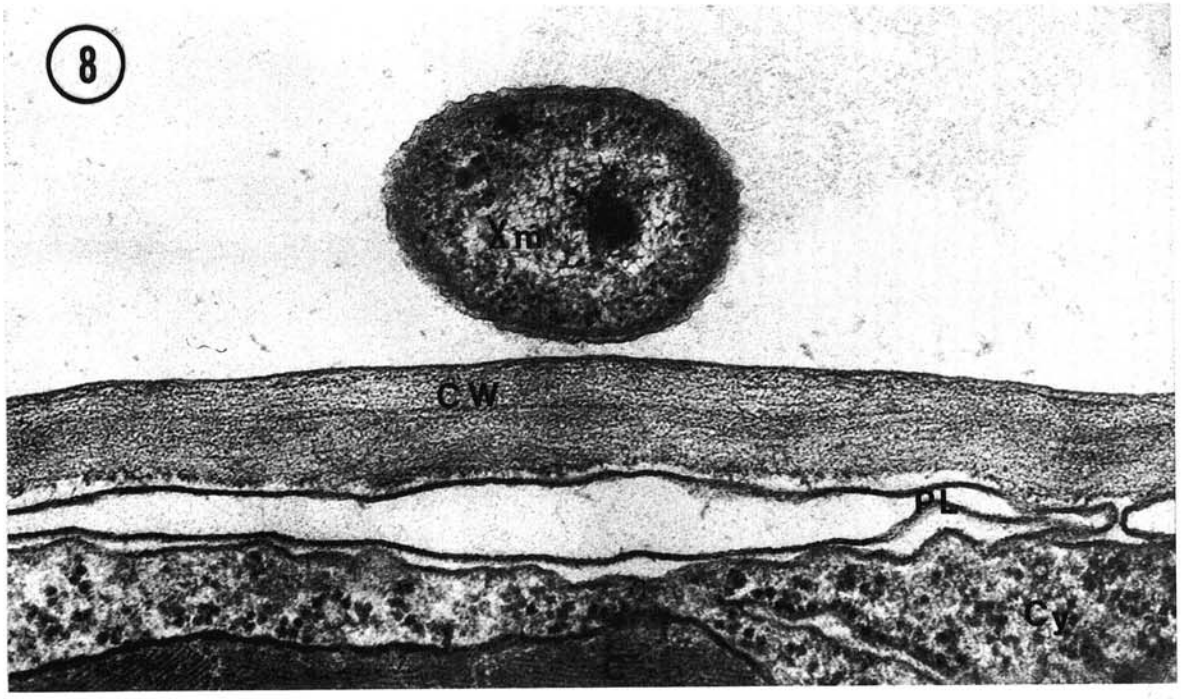

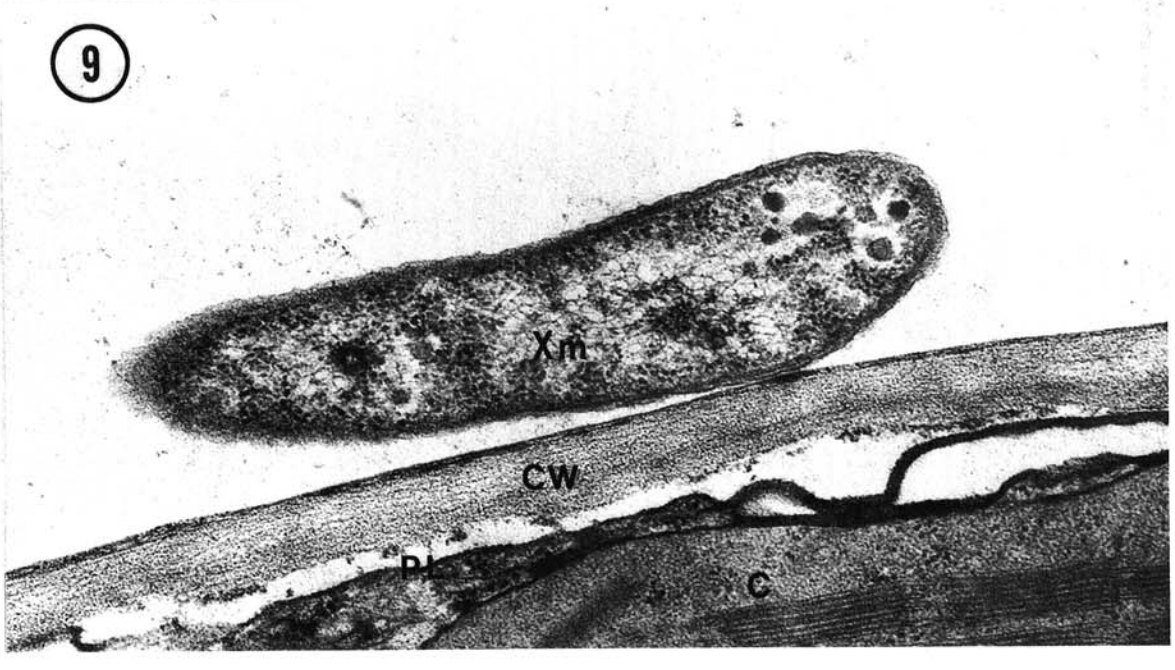

paper (7) the suggestion was made that induction of necrosis in cotton and activity of polymethylgalacturonase appear to be related, and it is possible that this enzyme, and very likely others (13), may be associated with the disruptive effects on host cell walls that lead to envelopment of the bacteria. Considering that this process may be a wounding phenomenon, it is conceivable that these sites of enveloped pathogen cells may be areas in which bacterial products diffuse into the host cell wall. It was noted that the immune host cell plasmalemma was broken and vesiculated adjacent to enveloped bacteria. This indirectly suggests that plasmalemma damage may have been caused by extracellular products of nearby enveloped bacteria. This disruption of the membrane may be responsible for the observed degeneration of cytoplasmic contents and death of the cell. Two previous reports (4, 6) have indicated that the plasmalemma in tobacco is broken or becomes discontinuous during the early stages of the HR. This report shows that a similar phenomenon occurs in cotton.

Sequeira et al. (9) demonstrated that necrosis occurs only when living bacteria are enveloped by the host cell wall in tobacco leaves and interpreted that to mean that metabolic products of bacterial origin probably are necessary to cause death of the host cell. The same requirement may be valid for the *Gossypium-Xanthomonas* interaction. Envelopment of bacteria on the host cell wall might facilitate the movement into the host cell of bacterial products that cause membrane disruption.

Goodman et al. (5) suggested that incompatible bacteria may be immobilized by wall elements of tobacco cells and that this phenomenon may constitute a new type of defense reaction against incompatible bacteria. We do not believe that the enveloping phenomenon described in this study is necessarily a means for immobilization of incompatible bacteria because the structures appear fragile and are susceptible to rupture (Fig. 7). They appear to be morphologically dissimilar to the enveloping wall elements described in tobacco (5, 9) although the two structures may have homologous origins. The structures we have described typically are turgid and electron-lucent compared to those enveloping elements described in tobacco which appear thick and electron-dense. Possibly however, the observed dissimilarities may be due to differences in methods of tissue preparation.

Wall disruption and envelopment of intercellular bacteria by liberated wall material notably were absent in the compatible relationship (Fig. 8, 9). This observation is consistent with the previously observed delay in appearance of pectinolytic enzyme activity in the compatible host until 24 hr after inoculation (13). If wall disruption and envelopment are necessary steps in the HR, early induction of pectinolytic enzymes may determine incompatibility and a delay in enzyme induction may permit a compatible interaction. Studies are now in progress to characterize further the ultrastructural differences between the compatible and incompatible relationships.

LITERATURE CITED

1. BRINKERHOFF, L. A. 1970. Variation in Xanthomonas malvacearum and its relation to control. Annu. Rev. Phytopathol. 8:85-110.
2. BRINKERHOFF, L. A., and L. M. VERHALEN. 1976. Inheritance of immunity to bacterial blight in an upland cotton cross. Page 31 *in* 1976 Proc. Beltwide Cotton Prod. Res. Conf., 5-7 January, Las Vegas, NV.
3. CASON, E. T., JR., P. E. RICHARDSON, L. A. BRINKERHOFF, and R. K. GHOLSON. 1977. Histopathology of immune and susceptible cotton cultivars inoculated with Xanthomonas malvacearum. Phytopathology 67:195-198.
4. GOODMAN, R. N. 1972. Electrolyte leakage and membrane damage in relation to bacterial population, pH, and ammonia production in tobacco leaf tissue inoculated with Pseudomonas pisi. Phytopathology 62:1327-1331.
5. GOODMAN, R. N., P.-Y. HUANG, and J. A. WHITE. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, Pseudomonas pisi, in tobacco leaf tissue. Phytopathology 66:754-764.
6. GOODMAN, R. N., and S. B. PLURAD. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1:11-15.
7. HOPPER, D. G., R. J. VENERE, L. A. BRINKERHOFF, and R. K. GHOLSON. 1975. Necrosis induction in cotton. Phytopathology 65:206-213.
8. SABATINI, D. D., K. G. BENSCH, and R. J. BARNETT. 1962. New fixatives for cytological and cytochemical studies. *In* Proc. Fifth Int. Congr. Electron Microsc. 3:1-3. (Academic Press, New York).
9. SEQUEIRA, L., G. GAARD, and G. A. DE ZOETEN. 1977. Interaction of bacteria and host cell walls: its relation to mechanism of induced resistance. Physiol. Plant Pathol. 10:43-50.
10. SING, V. O., and M. N. SCHROTH. 1977. Bacteria-plant cell surface interactions: active immobilization of saprophytic bacteria in plant leaves. Science 197:759-761.
11. SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
12. VENABLE, J. H., and R. COGGESHELL. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407.
13. VENERE, R. J., L. BRINKERHOFF, and R. K. GHOLSON. 1974. The role of pectinolytic enzymes in the production of a necrotic lesion in cotton bacterial blight. Proc. Am. Phytopathol. Soc. 1:78-79 (Abstr.).