A COMPARISON OF THE ULTRASTRUCTURE OF COMPATIBLE AND INCOMPATIBLE INTERACTIONS OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* IN COTTON LINES

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INTRODUCTION

This investigation is composed of two manuscripts written and accepted by Phytopathology, the Journal of The American Phytopathological Society. Each manuscript is written as a separate section. The first manuscript (part 1) entitled "Ultrastructural Studies of a Compatible Interaction Between Xanthomonas campestris pv. malvacearum and Cotton'. This is concerned with ultrastructural changes in cells and organelles of susceptible line Ac 44 cotton leaves following inoculation with the pathogenic bacteria. The second manuscript entitled "Cotyledon and Leaf Ultrastructure of a Bacterial Blight-Immune Cotton Inoculated with a low level of Xanthomonas campestris pv. malvacearum (part II). This work demonstrated what ultrastructural changes occur in leaf tissue of a resistant line cotton and to inoculated bacteria when low inoculum concentrations of bacteria are infiltrated into the tissues of the leaves.

Approval for presenting the thesis in this manner is based upon the Graduate College's policy of accepting a thesis written in manuscript form and is subject to the Graduate College's approval of the major professor's request for a waiver of the standard format.

PART I

Ultrastructural Studies of a Compatible Interaction Between *Xanthomonas campestris* pv. *malvacearum* and Cotton

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ABSTRACT

Al-Mousawi, A. H., Richardson, P. E., Essenberg, M., and Johnson, W. M. 1982. Ultrastructural studies of a compatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Phytopathology 72:

The ultrastructural changes in leaves and cotyledons of the susceptible cotton line Acala 44 inoculated with Xanthomonas campestris pv. malvacearum were studied for up to 6 days post-inoculation. Early changes included formation of vesicles between the plasmalemma and cell wall, followed by disappearance of chloroplast granal and stromal membranes. Degeneration of mitochondria occurred after ultrastructural undergo structural degeneration were the nucleus and plasmalemma. Fibrillar material was present at the external cell surfaces near bacteria at 5 days post-inoculation. By day 6 many cell walls were broken and fragmented. Bacteria had entered the mesophyll cells, and specific organelles could no longer be distinguished. Large accumulations of electron-dense fibrillar material were present in the intercellular spaces at 6 days post-inoculation, when water soaking symptoms were apparent.

INTRODUCTION

Bacterial blight caused by Xanthomonas campestris pv. malvacearum (Smith) Dye is an important disease of upland cotton (Gossypium hirsutum L.) (3, 15). A histological study by Thiers and Blank (20) demonstrated bacteria surrounded by slime in intercellular spaces of infected cotton leaves. The bacteria invaded both spongy mesophyll and palisade cells. A light microscopic investigation of the blight susceptible cotton line Acala 44 (Ac 44) (5), demonstrated no structural defense reaction by the host plant against X. campestris pv. malvacearum. A later study (9) showed that final bacterial populations in compatible leaves of Ac 44 were several orders of magnitude higher than the final populations in leaves of the blight-immune line.

Ultrastructural studies of compatible interactions between

bacterial phytopathogens and other crops such as apple, potato, tobacco, and bean have been reported (10, 11, 16, 17). The only published ultrastructural study of bacterial interactions with cotton (6) was primarily concerned with the incompatible interaction and was restricted to the first 24 hr post-inoculation. No ultrastructural changes were observed in the compatible host Ac 44 during that period. In this study we monitored ultrastructural changes in leaf and cotyledon cells and in bacteria for 6 days post-inoculation of X. campestris pv. malvacearum into host tissue. A preliminary report of this work has been published (2).

MATERIALS AND METHODS

Host growth environment. Ac 44, a breeding line which contains no major genes for resistance to bacterial blight (3), is susceptible to infection by all 18 races of X. campestris pv. malvacearum. Acid delinted seeds were grown in 15 cm clay pots filled with a commercially prepared soilless mix of peat moss and vermiculite ("Jiffy Mix Plus," Jiffy Products of America, West Chicago, Illinois). Plants were kept in a greenhouse with a daily maximum temperature of 32 $\pm$ 3° C and night temperature of 20 $\pm$ 3° C. Relative humidity was 100% at night, while the daily mean humidity was 59%. Seedlings had fully expanded cotyledons 2 weeks from germination, and at 3 weeks the second and third foliage leaves were expanded. The fully expanded young leaves, as well as fully expanded cotyledons, were used in this study.

Methods of inoculation and bacterial culture. A highly aggressive strain of X. campestris pv. malvacearum race 3 was used as inoculum at 10^6 bacteria/ml. It was isolated in 1978 by W. M. Johnson from a cotton plant in a field at Altus, Oklahoma, and identified as race 3 using

a standard set of differentials (14). The maintenance and growth of bacteria followed the method of Essenberg et al. (9). Cotyledons were inoculated with bacteria suspended in a sterile, saturated solution of $CaCO_3$ using a sterile hypodermic syringe. The syringe without a needle was pressed gently against the abaxial leaf surface and inoculum of 10^6 bacteria/ml. was injected until complete water soaking of the cotyledon was observed. Foliage leaves were vacuum infiltrated using the procedure described by Essenberg et al. (9). Two controls each were used for cotyledons and leaves. One control was infiltrated with saturated $CaCO_3$ solution, the other control was infiltrated with distilled deionized water. These procedures introduced inoculum doses of (0.6-1.0) X 10^4 bacteria/cm^2 of leaf area. Bacterial population levels were determined by dilution plate counting. Population determinations were made each day from the same inoculated leaves used for electron microscopy (EM). Leaves which had been stored for 7 years were also examined. These leaves had been harvested and dried after developing confluent water soaking.

Tissue preparation for EM. Leaf segments of about 1 mm^2 were fixed every 12 hours for 6 days with 0.1 M potassium phosphate-buffered 4% glutaraldehyde (pH 7.3) at 4 C for 2 hr. Tissues were subsequently washed and postfixed with 2% osmium tetroxide for 4 hr, dehydrated in a graded series of water-ethanol solutions and embedded in the firm formulation epoxy resin of Spurr (19).

Silver reflective thin sections were cut with a diamond knife using a Sorvall MT-2 ultramicrotome. Sections were collected on uncoated grids and stained with 0.5% uranyl acetate and 0.4% lead citrate (21), and were examined with an RCA EMU-3G electron microscope at 100 KV.

RESULTS

Bacterial populations increased logarithmically in leaves for 3 days post-inoculation and stabilized at approximately 10^6 bacteria/cm^2, as previously reported (9).

A similar sequence of ultrastructural changes was apparent in both cotyledons and leaves that were inoculated with X. campestris pv. malvacearum whereas no progressive degenerative changes were observed in the two types of controls. On the first day post-inoculation, bacterial cells were observed in the intercellular spaces (Fig. 1) of leaf sections in which cell organelles and their membrane systems were indistinguishable from control sections.

Two days post-inoculation some vesiculation was seen between the plasmalemma and cell wall (Fig. 2). Plasmolysis was also observed in many cells. Three to 4 days post-inoculation the outer membranes and granal and stromal lamellae of chloroplasts were not intact and chloroplasts had become rounded. Outer mitochondrial membranes and cristae could still be observed (Figs. 3 and 4). The nucleus appeared relatively unaffected, while rough endoplasmic reticulum cisternae appeared swollen (Fig. 3). At this stage cell membranes are disrupted showing vesiculation and irregular outlines. Plastoglobuli within the chloroplasts increased in number as the disease progressed.

Just before final degeneration (5 to 6 days post-inoculation) chloroplasts were irregular in shape with broken outer membranes. Tonoplasts were ruptured and organelles were dispersed throughout the cell (Figs. 5 and 6). Ribosomes were often coagulated (Fig. 5). Nuclei appeared more electron-dense with indistinct outer envelopes

(Fig. 5). Intercellular spaces were expanded due to dissolution of middle lamellae and walls of adjacent cells (Fig. 6). At day 5 post-inoculation fibrillar material appeared to have loosened from host cell walls (Fig. 7) in close apposition to the bacteria. This was followed by breaks in cell walls (Figs. 8 and 9) and entry of bacteria into mesophyll cells (Fig. 10). At this stage no organelles were distinguished within the broken plasmalemma.

The cells of infected leaves did not degenerate at equal rates. At any time during the week following inoculation, cells in several states of degeneration were visible in the same tissue. However, the sequence of changes in each cell was always as described above (Figs. 1-10). Some fibrillar material surrounding bacteria was seen inside and outside of mesophyll cells (Figs. 11-13). At the same time, 5-6 days post-inoculation, intact leaves started to show the water-soaking symptom. Very densely stained aggregates of similar fibrillar material were seen in micrographs of dried diseased leaves many years post infection (Fig. 14).

DISCUSSION

In the compatible interaction between X. campestris pv. malvacearum and G. hirsutum, there was severe damage to the membranes of all organelles, as well as to wall structure of mesophyll cells. Granal and stromal lamellae of chloroplasts were the earliest membranes altered. The most persistant structures were the nucleus and the plasmalemma. Early membrane destruction of chloroplasts and eventual disruption of other host cell membranes have been observed during compatible host-bacterial pathogen interactions (7, 10, 11, 17). Since the ultrastructural changes in host membranes reported here are similar

to changes that occur during leaf senescence (4), they may be due to autolysis of the host in response to the pathogen.

In this compatible system there were no structures enveloping bacteria at host cell wall surfaces such as were observed in the incompatible reaction between cotton and X. campestris pv. malvacearum (1). However, a breakdown of plant cell wall did occur. This was observed to begin with a loosening of fibrillar material in the outer layer of cell wall (Figs. 6 and 7), as observed in other compatible interactions (7, 13, 16, 23, 24 25). Wall loosening was followed by dissolution of the rest of the cell wall (Figs. 8 and 9) leaving fibrillar material throughout the cell. X. campestris pv. malvacearum is known to produce pectinmethylesterase, polygalacturonase, and cellulase (12, 22) which may be responsible for the damage to host walls observed here. The invasion of cotton host cells by X. campestris pv. malvacearum (Fig. 10) was first reported by Thiers and Blank (20). Invasion of xylem parenchyma has been observed in a bacterial disease of vascular tissue (23). Invasion of bean leaf cells by Pseudomonas phaseolicola has been reported (18), but was not observed in other studies of bean leaves following inoculation with P. phaseolicola (17) and P. syringae (7).

Dense fibrillar material was seen inside and outside of the cells at late stages of the disease (Figs 11 -14). Wallis and coworkers have observed somewhat similar fibrillar material in vascular tissue of cabbage (24) and tomato (23) infected with bacterial pathogens. They interpreted the material they saw as shredded host cell wall. Since the fibrillar material in infected cotton cotyledons and leaves was always associated with bacteria, we favor the hypothesis that it is bacterial exopolysaccharide slime (EPS) a substance which is produced

so abundantly in this disease that it sometimes oozes onto surfaces of leaves (20). That EPS can stain as intensely as the fibrillar material we observed was demonstrated when El-Banoby and Rudolph (8) made electron micrographs of bean leaves infiltrated with purified EPS from P. phaseolicola. Identification of the fibrillar material must await further chemical characterization. Since fibrillar material appears late in the disease when host cells are dying and collapsing, it may be part of a medium that enables the bacteria to resist unfavorable envirnomental conditions. Usually when fibrillar material was seen, bacterial cells had developed a capsular coating.

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Figs. 1-3. 1, Twelfth hr post-inoculation with X. campestris pv. malvacearum, cotton foliage leaf mesophyll cell. Cell structure and cytoplasmic components appear as in control, bacterium is close to cell wall (X 20,400). 2, Forty-eighth hr post-inoculation foliage leaf mesophyll cell, vesicles are present between plasmalemma and cell wall, three bacterial cells are close to cell wall (X 19,700), note higher magnification of vesicles (insert)(39,440). 3, Seventy-second hr. post-inoculation, cotyledon mesophyll cell. Chloroplasts appear round while mitochondria and nucleus appear unaltered (X 12,150). B = bacterium, C = chloroplast, Cw = cell wall, Er = endoplasmic reticulum, Is = intercellular space, M = mitochondrion, Mb = microbody, N = nucleus, O = osmiophilic granule, P = plastoglobule, Pl = plasmalemma, T = tonoplast, V = vacuole, Ve = vesicles.

Figs. 4-5. 4, Ninty-sixth hr post-inoculation, cotyledon mesophyll cell. Chloroplast appears round and has lost most of its membranes, mitochondrial membranes appear normal (X 29,000). 5, Fifth day post-inoculation, foliage leaf mesophyll cell with coagulated ribosomes, dense nucleus and chloroplast. Tonoplast membrane is ruptured (arrow) (X 15,600). B = bacterium, C = chloroplast, Cr = coagulated ribosomes, Cw = cell wall, Is = intercellular space, M = mitochondrion, N = nucleus, P = plastoglobule, V = vacuole.

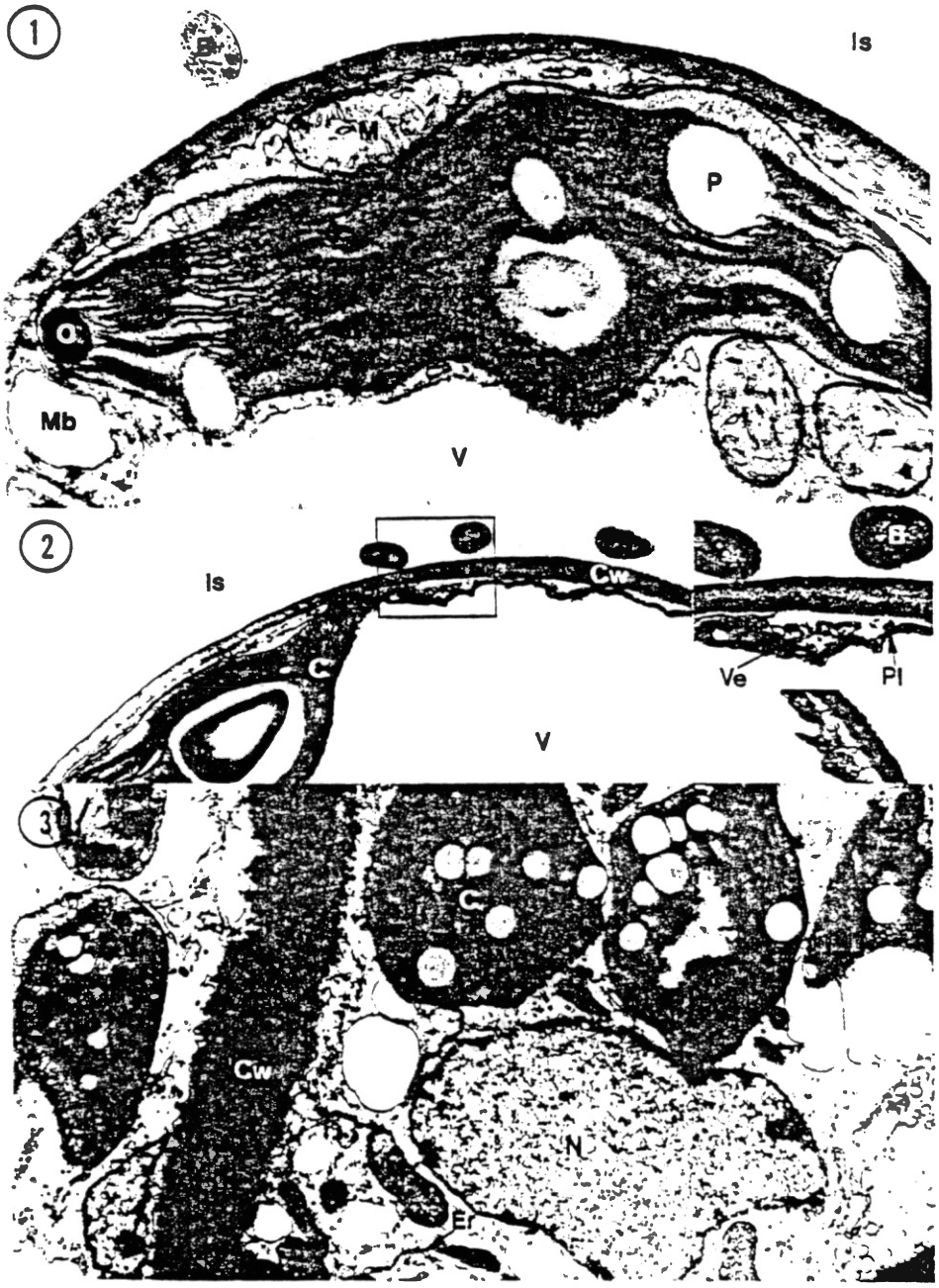
Figs. 6-7. 6, Fifth day post-inoculation, cotyledon mesophyll cells. Note loosening of middle lamella (arrow) (X 11,500). 7, Fifth day post-inoculation, cotyledon mesophyll cell with apparent loosening in outer cell wall near dividing bacterium (X 63,500). B = bacterium,

C = chloroplast, Cw = cell wall, Is = intercellular space.

Figs. 8-10. 8, Sixth day post-inoculation, cotyledon mesophyll cell. Note broken host cell walls (arrows) (X 3,150). 9, Sixth day, cotyledon mesophyll cell. Note broken wall at higher magnification (arrow). Electron-dense fibers appear to separate from cell wall (X 14,300). 10, Sixth day, cotyledon mesophyll cells with bacterial cells inside host cell. Note broken plasmalemma (arrow) X 7,000). B = bacterium, Cw = cell wall, Is = intercellular space.

Figs. 11-12. 11, Sixth day post-inoculation, foliage leaf showing 4 mesophyll cells with fibrillar material filling the intercellular spaces (X 9,200). 12, Sixth day, higher magnification of foliage leaf mesophyll cell with fibrillar material in the intercellular space. Bacterial cells are surrounded by clear capsular material (X 35,500). B = bacterium, C = chloroplast, F = fibrillar material, Is = intercellular space.

Figs. 13-14. 13, Sixth day post-inoculation, foliage leaf mesophyll cells with fibrillar material and bacteria inside mesophyll cell (X 35,000). 14, Seven-year-old, dried, diseased leaf showing dense fibrillar material (X 39,800). B = bacterium, C = chloroplast, Cw = cell wall, F = fibrillar material, Is = intercellular space.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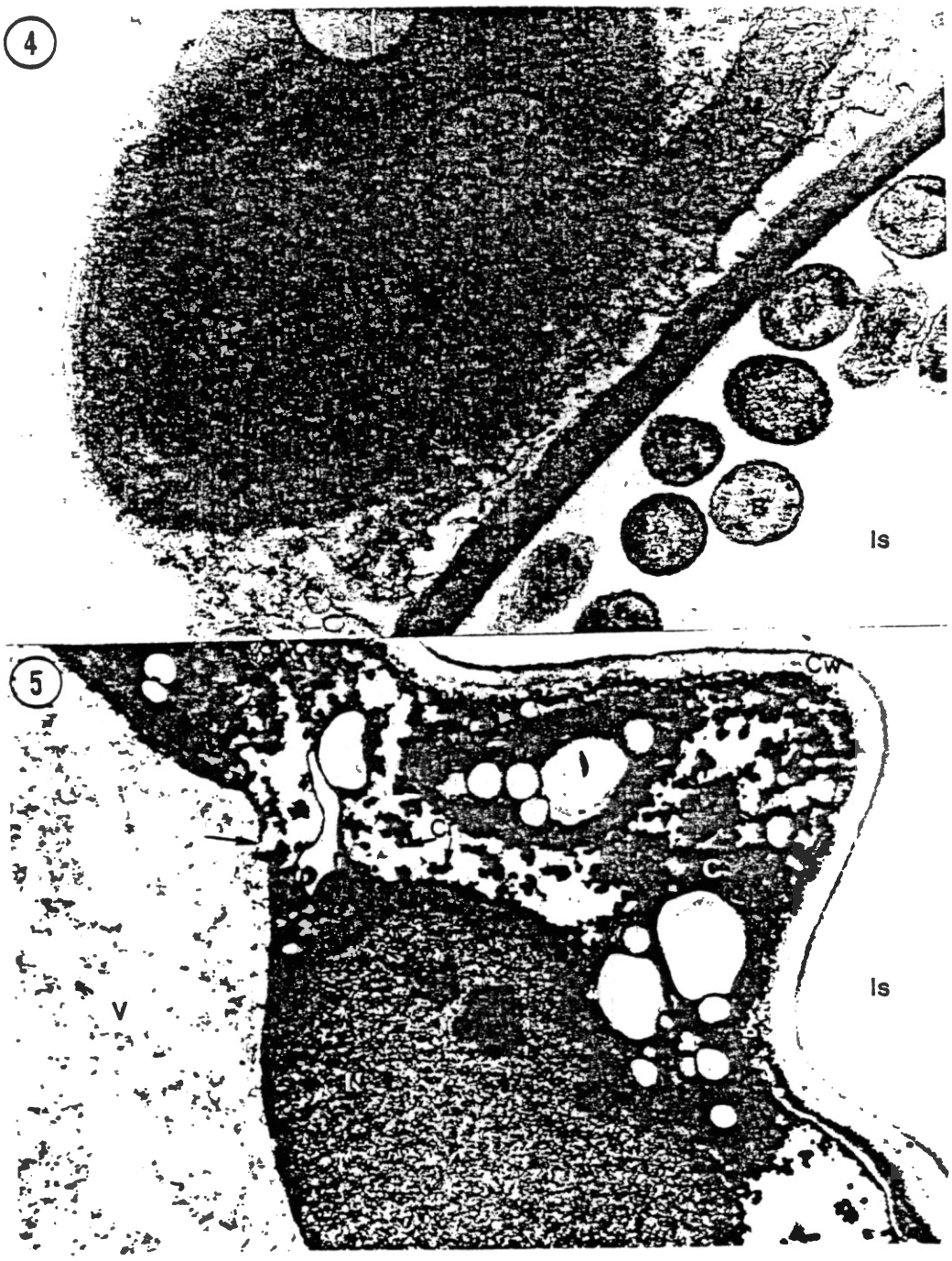
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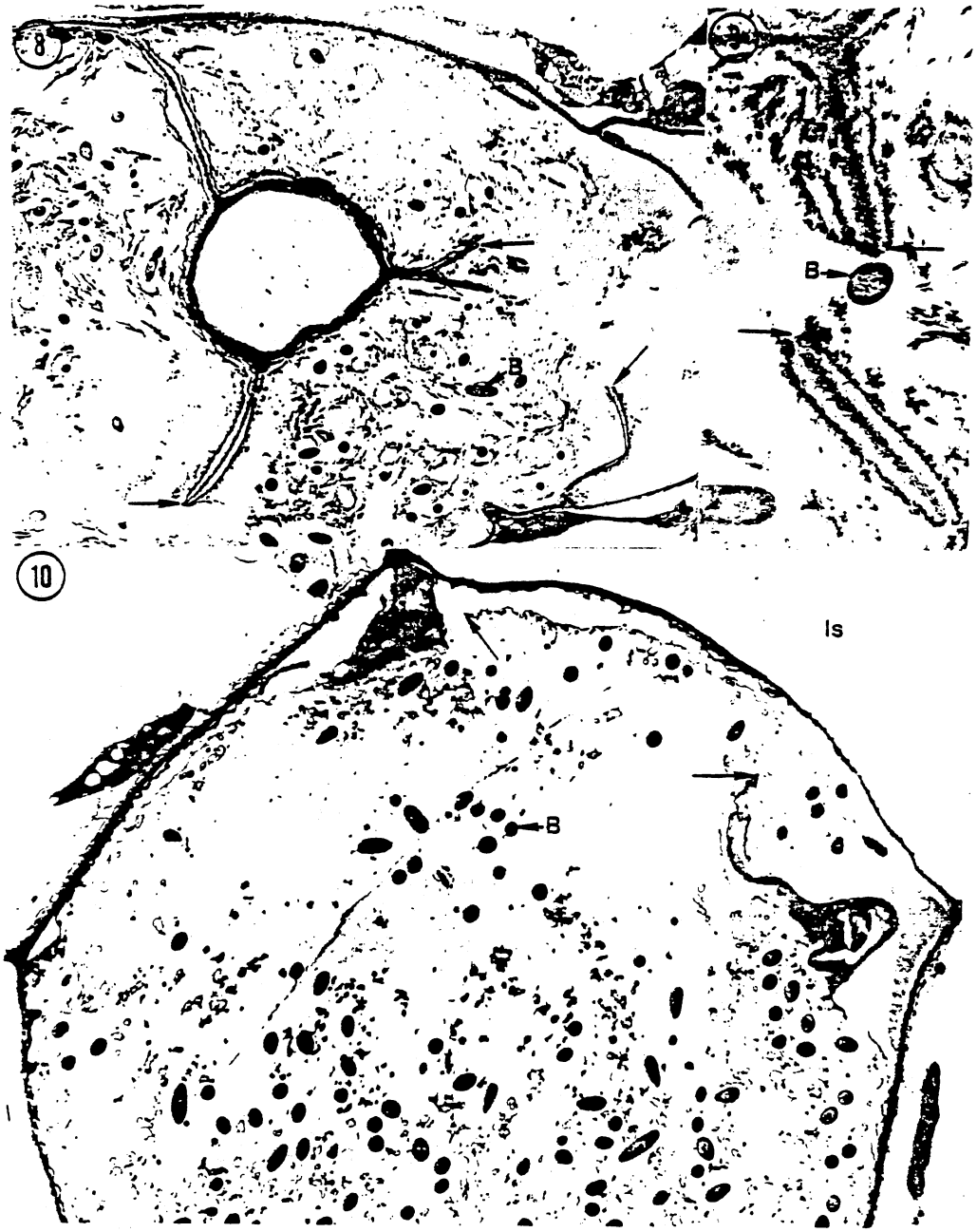
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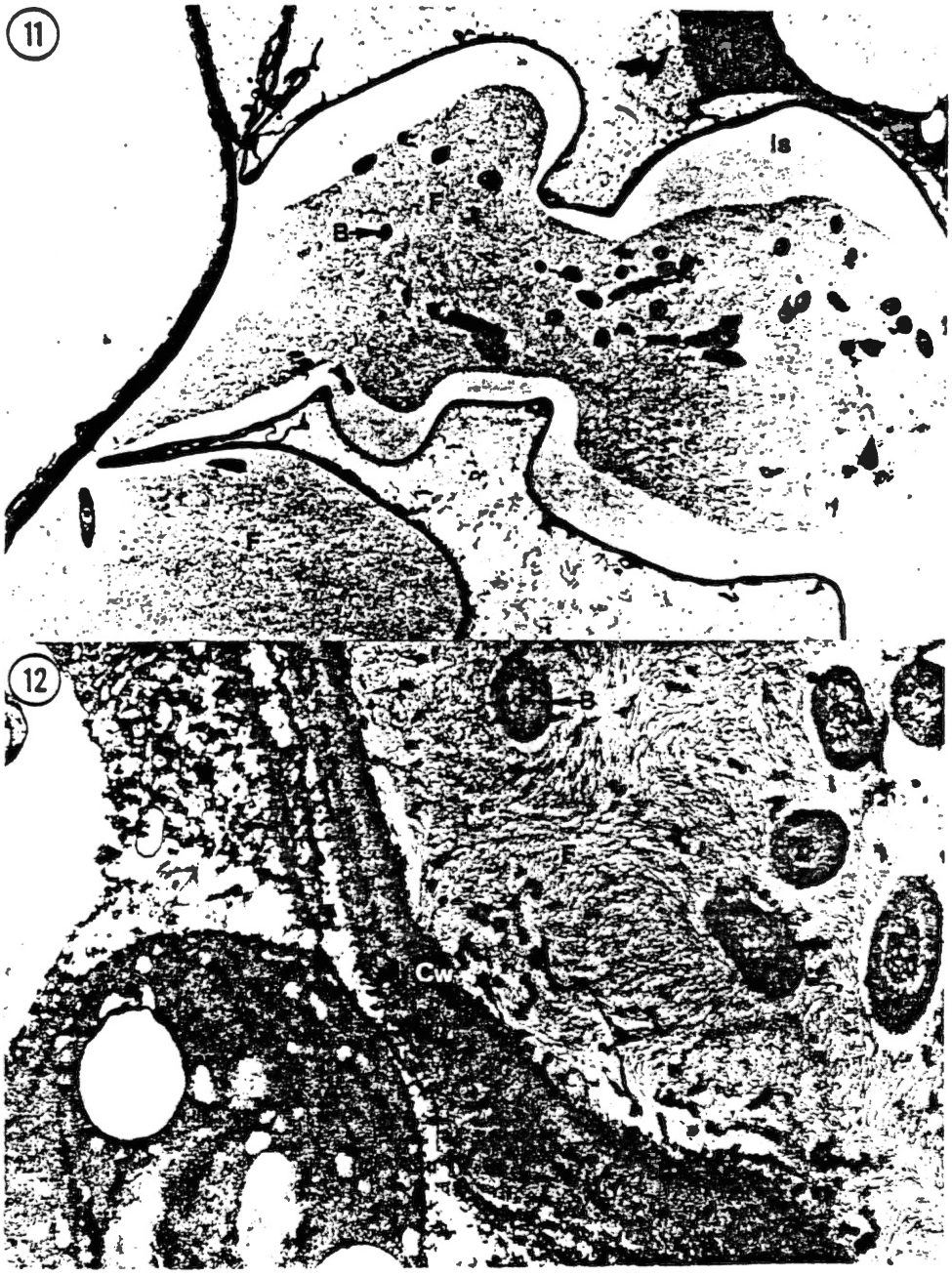
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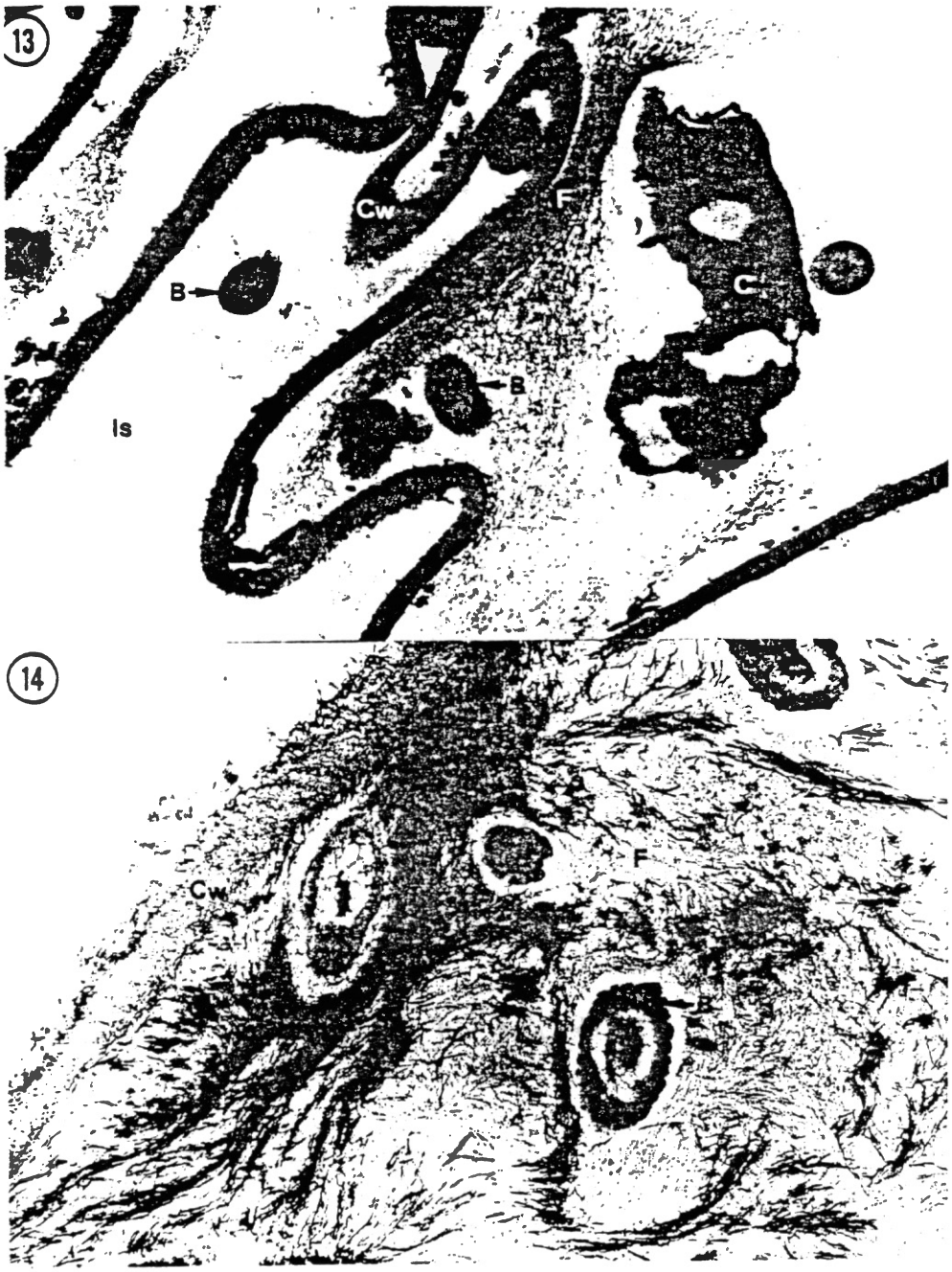
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PART II

Cotyledon and Leaf Ultrastructure of a Bacterial Blight-Immune Cotton Line Inoculated with a Low Level of *Xanthomonas campestris* pv. *malvacearum*

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ABSTRACT

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Cotyledons and foliage leaves of the cotton line Im 216, which is immune to bacterial blight, were inoculated with a low level inoculum (5×10^5 cells/ml) of Xanthomonas campestris pv. malvacearum, and examined by light microscopy and transmission electron microscopy. Degenerative changes in some of the mesophyll cells were apparent 2 days after inoculation. Bacteria were found in samples taken 5 and 6 days after inoculation, and were located in intercellular spaces near collapsed mesophyll cells. Fibrillar material was associated with all bacteria observed but did not completely envelop all the bacteria. Clusters of bacteria were larger than observed in an earlier ultrastructural study of Im 216 cotton cotyledons inoculated with a higher cell number but were similar to compact masses of bacteria observed previously in light microscopic studies of Im 216 cotyledons inoculated with 7×10^5 cells/ml of X. campestris pv. malvacearum.

INTRODUCTION

Envelopment of pathogenic and saprophytic bacteria by fibrillar material at the surfaces of host cells has been observed by transmission electron microscopy (TEM) in leaves of tobacco (12, 13), cotton (2, 4), rice (10), and bean (5, 9). In these studies, leaves were inoculated with bacterial suspensions sufficiently concentrated (10^8 - 10^9 cells/ml) to elicit hypersensitive responses (HR), resulting in confluent necrosis, (5, 7, 11) in incompatible hosts. Such high concentrations are probably rare under field conditions. Our bacterial blight-immune cotton (Gossypium hirsutum L.) line Im 216 has been grown in field rows adjacent to rows of diseased susceptible lines for more than 10 years without showing macroscopically visible HR to naturally transmitted infection.

In tobacco, cotton, and rice, envelopment of bacteria has been observed with incompatible pathogens and saprophytes, but not with compatible pathogens (2, 4, 10, 12, 13). It has been proposed that attachment and envelopment are essential steps in the expression of host resistance (8, 13). In bean, however, envelopment is less specific (5, 9).

Infiltration of leaves of a cotton line Im 216, which is immune to bacterial blight, with suspensions of greater than 10^7 cells/ml of Xanthomonas campestris pv. malvacearum (Smith) Dye caused confluent necrosis (7). Following infiltration with low concentrations of X. campestris pv. malvacearum, leaves remained alive and turgid and only small clusters of mesophyll cells became brown and necrotic (7). Bacteria were observed by light microscopy (LM) in the intercellular spaces adjacent to the necrotic cells. Some of these bacteria were in compact masses on the surfaces of mesophyll cells (7, plates 11-13) or between collapsed mesophyll cells (7, plate 15).

We report here TEM observation of Im 216 cotton line leaves and cotyledons inoculated with 5×10^5 cells/ml of X. campestris pv. malvacearum, which introduced about 2×10^3 cells/cm^2 of leaf area (7). At this inoculum concentration, resultant bacterial colonies were widely separated, and growth was inhibited 3-4 days after inoculation by the host's localized resistance response (7). Other workers have considered that studies with such dilute inocula would be infeasible for TEM studies (5). The objectives of the present study were to determine if envelopments previously described (4) with an artificially high inoculum level also occurred with lower levels and to demonstrate with TEM whether compact masses of bacterial cells produced after

introduction of dilute inocula were similar to those described in a previous LM study (7).

MATERIAL AND METHODS

Cotton line Im 216 was inoculated with a highly aggressive isolate of X. campestris pv. malvacearum race 3 (6). The Im 216 cotton line possesses a high resistance to all known races of X. campestris pv. malvacearum. Growth conditions for host plants and bacteria were as described in Al-Mousawi et al (1) and Essenberg et al (6), respectively. The inoculum was a suspension of 5 x 10^5 bacteria/ml in sterile, saturated calcium carbonate solution. Cotyledons of four two week-old plants were infiltrated with the inoculum using a hypodermic syringe without a needle. Nine fully expanded leaves of six three week-old plants were inoculated by vacuum infiltration. Samples were taken from cotyledons and leaves every 24 hr for 6 days after inoculation. The tissues were fixed and embedded as previously described (1). Sixty blocks prepared from eight cotyledons and nine leaves were sectioned and examined. Thick sections (0.4-0.5 µm) were taken from the blocks, stained with Azure B bromide (7) and examined with a light microscope. Block containing darkly-stained mesophyll cells that were characteristic of cellular responses to bacterial infection (7) were selected, trimmed to include them, and thin sectioned for TEM (1).

RESULTS

Two days after inoculation, some mesophyll cells showed collapsed, concave cell walls and distrupted organelles and membranes. During the following days after inoculation, affected mesophyll cells contracted further and became more densely stained.

Bacteria were detected by light microscopy in 46 of the 60 blocks. Approximately 250 grids with thin (silver-reflective) sections were examined by TEM. Bacteria were found in a few grids containing sections of five cotyledons and three leaves, taken 5 and 6 days after inoculation. All bacteria observed were associated with collapsed mesophyll cells in intercellular spaces.

Groups of bacteria were surrounded by fibrillar material and appeared to be trapped by collapsing mesophyll cells (Figs. 1 and 2). Other clusters of bacteria occurred in intercellular spaces at the junction of two mesophyll cells (Fig. 3) or on the surfaces of cells (Figs. 4 and 5). Fibrillar material was always observed to be associated with bacteria.

DISCUSSION

In earlier studies of Im 216 cotton cotyledons after inoculation with 10^8 cells/ml of X. campestris pv. malvacearum, bacterial cells were observed to be completely enveloped by fibrillar material that was connected to mesophyll cell walls (4). In the present study of Im 216 cotton leaves after inoculation with 5×10^5 cells/ml, fibrillar material was associated with all bacteria observed, but it did not appear to envelop all bacteria completely. This is true except in the case of Fig. 1, which is totally enclosed space, completely filled with the fibrillar material. In most cases, with the exception of the bacterial mass in Fig. 5, the fibrillar material extended to mesophyll cell walls. It is possible that the bacterial mass of Fig. 5 was attached to the host wall in a plane that was not sectioned.

The larger groups of bacteria seen in this study were consistent with the larger bacterial growth yield from inocula of 5×10^5 cells/ml

(approximately 600-fold), which produce widely spaced bacterial colonies to which cotton leaves appear to respond locally, requiring 3-4 days to inhibit bacterial growth (7). In contrast, an inoculum of 10^8 cells/ml elicited a more rapid and general HR which inhibits bacterial growth within one day, permitting a growth yield of only approximately 6-fold (7). The shape, location, and number of bacteria in the groups appeared similar to compact masses of bacteria demonstrated previously by photomicrography of Im 216 leaves that had been inoculated with 7×10^5 bacteria/ml (7).

In our earlier ultrastructural study of the general HR to a concentrate inoculum (4), fibrillar materials were associated with X. campestris pv. malvacearum and appeared to attach it to host cell walls. We conclude from the current study that fibrillar material is also present during the localized resistance response of Im 216 cotton leaves to individual bacterial colonies. The greater looseness of the fibrillar material in the observations presented here causes us to doubt that it directly restricts bacterial multiplication, as has been suggested by others (8).

In leaves and cotyledons of susceptible cotton, no attachment of X. campestris pv. malvacearum to host cell walls by enveloping fibrillar material has been observed (1); however, in two cotton lines that possess lower levels of genetically determined resistance than Im 216, we have observed X. campestris pv. malvacearum enveloped and attached to host cell walls within a few hr after inoculation. Later, however, the envelopes ruptured as the bacteria multiplied (our currently unpublished work).

We cannot at present reject either of the hypotheses that the attachment observed in resistant cotton plays a role in the incompatible interaction or, alternatively, that it plays no essential role in resistance but for some reason is not observed during a compatible interaction.

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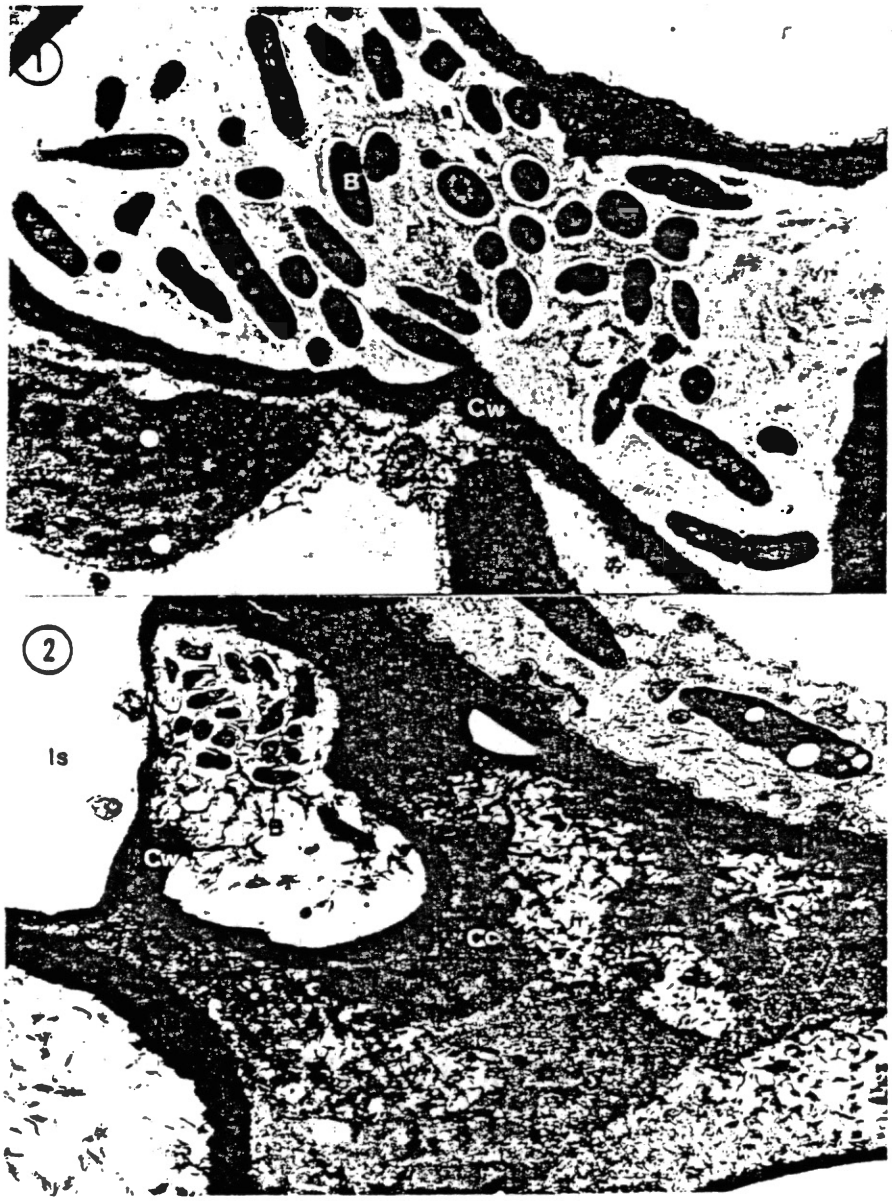
Figs. 1-2. 1, Mesophyll cells of immune cotton cotyledon 5 days after inoculation with X. campestris pv. malvacearum. Bacterial cells within fibrillar material in the intercellular space, bacteria surrounded by capsular material (arrows) (X15,120). 2, Mesophyll cells of foliage leaf 5 days after inoculation, bacteria apparently trapped in mass of fibrillar material near collapsed mesophyll cell (X11,350). B = bacterium, Cc = collapsed cell, Cw = cell wall, F = fibrillar material, Is = intercellular space.

Figs. 3-4. Mesophyll cells of foliage leaf 6 days after inoculation. 3, Bacterial cells between two mesophyll cells. The upper mesophyll cell has abnormal cytoplasmic contents (X43,800). 4, Bacterial cells surrounded by fibrillar material attached to two collapsed mesophyll cells (X10,100). B = bacterium, Cc = collapsed cell, Cw = cell wall, F = fibrillar material, Is = intercellular space.

Fig. 5. Bacterial cells enveloped by fibrillar material. Bacteria close to collapsed foliage leaf mesophyll cell (X24,500). B = bacterium, Cc = collapsed cell, Is = intercellular space.


1
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Cw
2
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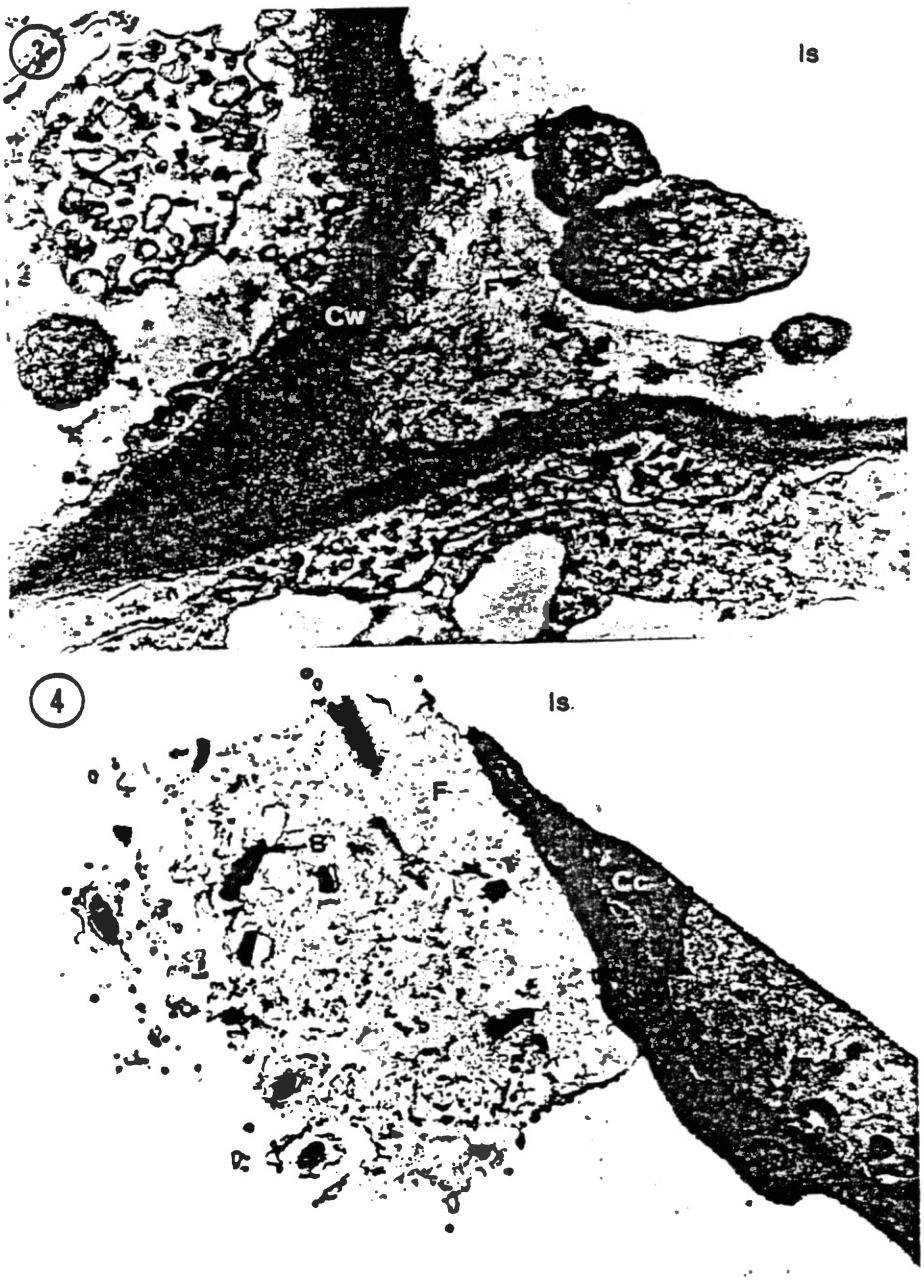
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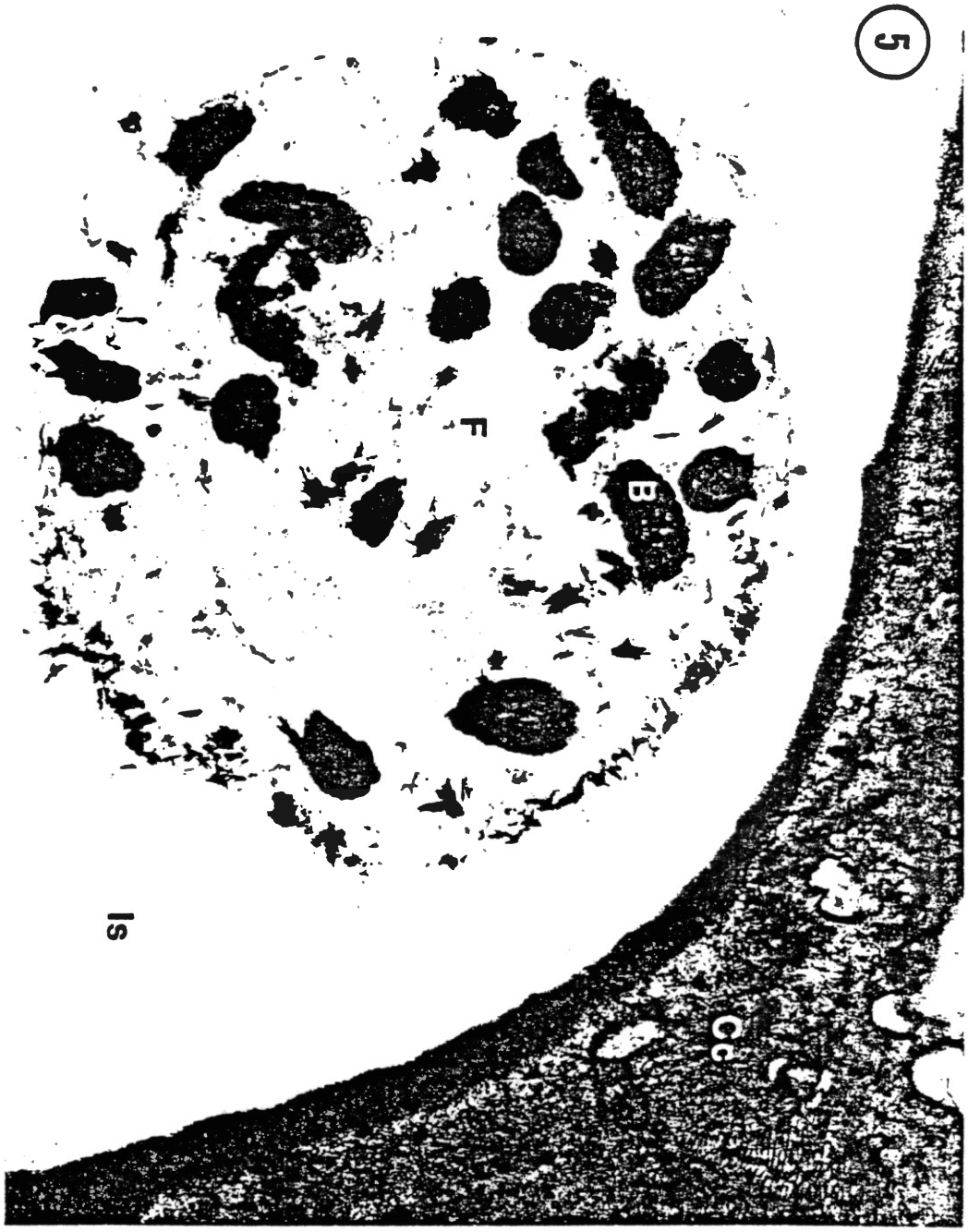
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PART III

APPENDIX

During the period of this study, other work was also done, and will be published later. In one of these studies live and dead bacteria of Xanthomonas campestris pv. malvacearum were injected separately into cotyledons of susceptible and resistant cotton lines. Polystyrene latex beads, starch grains, and Gram (+) Micrococcus lysodeikticus were injected separately into resistant line cotton cotyledons. There were envelopes formed around starch grains, Gram (+) bacteria, and dead bacteria in resistant plant tissues (Im 216). However, there were no envelopes formed around hydrophobic latex beads in either line.

In another study, two other lines of cotton were used. These were lines whose resistance to the bacterial blight is intermediate between cotton lines Im 216 and Ac 44, the susceptible cotton line. Envelopes were formed around bacteria in both lines within the first day after inoculation. By 48 hours most envelopes were broken in OK 2.3, the less resistant intermediate line, and multiplying bacteria were emerging from them. Envelopes were broken after 72 hours following inoculation in OK 1.2, the more resistant intermediate line.

In a third study, fine structure of X. campestris pv. malvacearum was studied after injection into Ac 44, Im 216, and in nutrient broth.

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