ULTRASTRUCTURE OF INTERACTIONS BETWEEN XANTHOMONAS

CAMPESTRIS PV. MALVACEARUM AND BACTERIAL

BLIGHT-IMMUNE AND -SUSCEPTIBLE COTTON

LINES GROWN IN DARK AS COMPARED

WITH DAY-NIGHT CYCLES

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CHAPTER I

INTRODUCTION

Bacterial blight of cotton, caused by <u>Xanthomonas</u> <u>campestris</u> pv.
<u>malvacearum</u> (Smith 1901) Dye 1978b, was first reported in 1891 by
Atkinson (4). Symptoms of the disease were described as being
comprised of water-soaked lesions bounded by the major veinlets of the
leaf, and known as "angular leaf spot" of cotton. One year later,
Atkinson succeeded in isolating the pathogen, but was unable to repro-
duce the disease in the host. Smith (23) succeeded in inoculating
cotton leaves with the isolated pathogen and producing lesions on
cotton plants. He described the causal agent as short rod-shaped,
actively motile, and non-spore forming bacteria.

Bacterial blight of cotton is a disease of world-wide importance.
The chief means of dissemination are infected seeds, wind, rain, and
surface irrigation. Available pesticides are ineffective against the
pathogen. Acid delinting of seed and sanitation have become the pre-
ferred method of control, but they are not completely successful. The
most important method of control is the development of resistant
cultivars. Cultivars ranging from immune to susceptible are now
available for the study of this disease. Immunity appears to be by
means of three major genes B_2, B_3 and b_7 as well as minor genes (5).
The susceptible line Ac 44 has no major resistance genes to bacterial
blight of cotton.

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To initiate disease, bacteria enter leaves through stomatal openings and then spread through intercellular spaces. Threads of slime develop between mesophyll cells. The pathogen primarily attacks parenchymatous tissues, whereas vascular tissue is rarely invaded (26). Under field conditions immune cultivars do not show any visible symptoms, even when grown near susceptible cultivars, which are severely attacked. However, in the laboratory when immune plants are inoculated artificially with high levels of bacteria (more than 10^8 cells/ml), the inoculated area becomes necrotic within 12-24 hours depending on the inoculum concentration. The intensity of the disease reaction is directly proportional to the population density of bacteria in the host tissues. The observed histological reactions following artificial inoculation with incompatible bacteria accompany the host plant reaction complex known as the hypersensitive response (HR). Klement and Goodman (13) defined HR as a rapidly developing defense reaction which occurs in an incompatible host-parasite relationship, and results in the death of the host plant tissue.

Histological study of cotton lines by Cason et al. (6) has shown that damage to the immune host cotyledonary tissues occurs within 4-6 hours following the inoculation with high doses of pv. malvacearum. Chloroplast damage and tissue necrosis followed by shrinkage are the most obvious histological features accompanying the hypersensitive response. Studies of ultrastructural reactions of immune and susceptible cotton lines by Al-Mousawi et al. (1) and Cason et al. (7) have shown that host cell wall, plasma membrane and chloroplast damage occur in immune (incompatible) plants as early as 4 hours post-

inoculation with pv. malvacearum. Bacteria are found in dead tissues with fibrillar material and envelopments formed around them. The same phenomena are not found in susceptible plant tissues. Here, bacteria multiply to great numbers, are not enveloped and do not cause rapid death of the host tissues.

Gross bacterial blight symptoms did not develop in bean, cucumber, cotton, or soybean when susceptible plants were kept in darkness after inoculation with their respective natural pathogens (24). There is a limited literature concerned with the influence of light/dark cycles on disease reactions induced by bacteria infiltrated into plant tissues. Most earlier studies concerned the interaction of light with other factors such as nitrogen or temperature. Effects of light duration and intensity on bacterial disease development are highly variable. Symptoms resembling the resistant response occurs in soybean leaves inoculated with compatible strains of Pseudomonas glycinea when the leaves were subjected to post-inoculation darkness periods (24). However no bacterial growth yield data were reported. The resistance reaction also occurs in soybean plants inoculated with avirulent races and exposed to darkness. Studies of bacterial blight of cotton and of bean carried out in four light regimes (0, 100-150, and 2,000 ft-c) indicate that water-soaked lesions are largest at the highest light-intensity and do not develop in complete darkness.

In contrast to the above findings, low light intensity is conducive to the best development of disease symptoms in four host-pathogen combinations. Orellana and Thomas (19) and Thomas (27) found that guar plants resistant to X. cyanopsidis and sesame plants resistant to Pseudomonas sesame, show susceptible reactions when grown in low light

intensity of short-day periods. Lozano and Sequeira (16) found when tobacco leaves are infiltrated with race 3 of _Pseudomonas solancearum_ and covered with aluminum foil for periods of 6048 hours the HR does not develop and the bacteria multiply and spread from the infiltrated area to adjacent tissues. On the other hand, Klement and Goodman (14) are of the opinion that light has no effect on HR in tobacco.

The susceptible cotton line Ac 44 exhibits different responses to pv. _malvacearum_ when subjected to continuous post-inoculation darkness. Earlier work showed that when Ac 44 plants were inoculated and immediately placed in continuous darkness, their cotyledons rapidly collapsed. This resembled the response of inoculated resistant Im 216 grown under a normal day/night cycle. Ultrastructural study of Ac 44 was undertaken to determine if the host cell organelles degenerated rapidly as was the case in the incompatible interaction and to see whether the bacteria become enveloped as is always observed during an incompatible reaction, but not during compatible interactions. Immune line Im 216 was included in this study to determine if typical envelopment and rapid necrosis of this cultivar also occurs in the dark. Simultaneous bacterial growth experiments were undertaken to complement the findings of the ultrastructural study, because inhibition of bacterial growth is a characteristic of resistance.

CHAPTER II

REVIEW OF SELECTED LITERATURE

The first comprehensive histological study of cotton bacterial
blight was published by Theirs and Blank (26) and compared the struc-
tural resistance of disease responses with those of susceptible plants.
The resistance on which their study was based was due to a single gene,
which by itself conditions a relatively low level of resistance. They
concluded that there were no anatomical differences in the response
of resistant and susceptible plants to the bacterial blight organism
(pv. malvacearum) except for lesion size and the final bacterial
population size attained.

The hypersensitive interaction (HR) appears important in limiting
pathogenicity of many organisms (17). HR occurs in a relationship
where the host and the pathogen are incompatible or the host is said
to be resistant. Klement and Lovrekovich (15) report browning in
inoculated areas of bean pods after 24 hr post-inoculation with
incompatible bacteria, Pseudomonas or Xanthomonas spp. The browning
does not appear when a non-plant pathogen, Escherichia coli is infil-
trated into the experimental plant's leaves. The authors conclude
that the response is not due to direct action of the bacteria but is
due to plant tissue reactions. Stall and Cook (25) studied hyper-
sensitivity in pepper following inoculation with Xanthomonas vesi-
catoria (Doidge) Dows. Confluent necrosis is observed in this

system within 24 hr following infiltration with high numbers of bacteria (10^8 cells/ml). Turner and Novacky (28) were able to detect dead plant cells in tobacco leaves with the light microscope. Cell death occurred within one to two hours when leaves were inoculated with a low level (5×10^3) of Pseudomonas pisi cells. No further plant cell death occurred after 6 hr. They concluded that a ratio of one bacterium to one plant cell is sufficient to cause host cell death and that contiguous tissue necrosis develops if the ratio of bacterial to total host plant cells is 1:4. Light microscopic studies by Cason et al. (6) of the cotton pv. malvacearum system, demonstrated that alteration in incompatible host tissue occurred within 4-6 hours following infiltration of cotyledons with 10^7 bacteria/ml. Ultrastructural comparisons of developmental patterns in resistant and susceptible cultivar pairs infiltrated with pathogenic bacteria have been carried out on tobacco (11), apple (9), bean (22), and cotton (7) (1). Goodman and Plurad (11) studied the ultrastructural changes in tobacco leaves undergoing the hypersensitive reaction following inoculation with incompatible bacteria (Pseudomonas pisi). They report that plasmalemma, tonoplast, and bounding membranes of chloroplasts, mitochondria, and microbodies are significantly damaged within 7 hr. In a later paper, Goodman et al. (10) found that within 20-minutes after infiltration of incompatible bacteria (Pseudomonas pisi) into tobacco leaves host wall cuticle becomes detached and bacterial cells are enveloped. After 2-6 hr the enveloped material becomes thicker and contains dense-staining fibrils and membrane fragments. Two to four hr after inoculation, the plasmalemma becomes vesiculated, the wall becomes more electron dense and swollen, and the

membranes bounding chloroplasts and mitochondria are severely degraded.
Infiltration of compatible bacteria (Pseudomonas tabaci), does not
induce separation of host wall cuticle at 6 hr, although host cell
cytoplasm is disorganized. Sequeira et al. (20) report that by 4 hr
after inoculation of tobacco leaves with incompatible S_{210} strains of
Pseudomonas solanacearum, granular and fibrillar materials from host
cell walls bounded by a cuticular wall layer envelop the bacteria.
HR develops within 6-12 hr and host cells collapse and their organ-
elles degenerate. In contrast, in a reaction between host tissue and
virulent strains of the pathogen (K_{60}), or compatible interactions,
envelopment does not occur and the bacteria remain free in the
intercellular spaces. No organelle destruction is observed during
the first 12 hr post-inoculation.

Electron microscopic study of apple leaves by Goodman and Bur-
kowicz (a) in which they inoculated with virulent and with avirulent
strains of Erwinia amylovora demonstrated a similar disorganization
of chloroplasts, mitochondria degeneration, and microbodies also
degenerated in tissues when they were infiltrated with virulent and
avirulent strains respectively.

Sigee and Epton (22) studied the ultrastructureal changes of
resistant and susceptible varieties of Phaseolus vulgaris infiltrated
with Pseudomonas phaseolicola (Burk) Dows. In resistant leaves, most
spongy mesophyll cells are found to be dead with very dense contents
24 hr after infiltration, while susceptible mesophyll cells have
enlarged chloroplast stroma with electron-dense granules. The chloro-
plasts also become more spherical. The same authors reported earlier
(21) that bacterial cells respond differently in susceptible than in

resistant hosts. In the former, bacterial nuclear regions are defined with ribosomes aggregated in the cytoplasm. In the latter, the nuclear regions degenerate and fewer ribosomal aggregations develop.

Ultrastructural studies of the Gossypium hirsutum, pv. malvacearum interaction by Cason et al (7) indicated that in immune or incompatible plants, loosening of host cell wall surface cuticle and envelopment of adjacent bacteria occur by 2 hr post-inoculation. Six to eight hours after inoculation the plasmalemma of the host becomes broken with the resulting formation of numerous membrane-bound vesicles. Cytoplasm appears concentrated and electron-dense. In susceptible, compatible, plants bacteria are not enveloped and cell organelle degeneration is not observed within 24 hr.

Another study, Al-Mousawi et al (2) deals with ultrastructural aspects of bacterial blight-immune cotton line leaves inoculated with a low level of pv. malvacearum. Collapsed mesophyll cells were observed within 2 days post inoculation. All bacterial cells were found associated with fibrillar material, but this did not completely envelop the bacteria in all cases. On the other hand, a study of susceptible or compatible plants by Al-Mousawi et al (1) reported no envelopment and no fibrillar material associated with the bacteria. Severe damage to the granal and stromal lamellae of chloroplasts is observed 4 days post-inoculation. The number of the plastoglobules within the chloroplasts increases as the disease progresses. At day 5 and 6 looseness of cell wall fibrillar material, rupture of the tonoplast, condensation of ribosomes and breakage of plasmalemma were observed.

A physiological study of the relation between membrane potential

and ATP level in pv. <u>malvacearum</u> infected cotton cotyledons (18)

demonstrates that 24 hr after inoculation the membrane potential

(E_m) of infected susceptible cotyledons is maintained at a lower

value in the dark than in the light. Since the ATP level is higher

in diseased than in healthy tissue and higher in the dark than in the

light, the low value of E_m in diseased tissue placed in the dark is

not due to inhibition of mitochondrial energy production caused by

the bacteria.

CHAPTER III

MATERIALS AND METHODS

Plant Growth Environment

Seeds of the susceptible cultivar Ac 44 and the immune Im 216 were used in all experiments. Three seeds were planted one inch deep in 6 inch diameter clay pots containing a commercially prepared soilless mix of peat moss and vermiculite (Jiffy mix-plus). The pots were watered with tap water and kept in a growth chamber set for a 14 hr light 10 hr dark cycle with an average temperature of $30^{\circ}C$ during the light and $19^{\circ}C$ in the dark part of the cycle.

Seedlings emerged from the soil within seven days and cotyledons had fully expanded within 10 days. Cotyledons of 3-week old plants were used. In other experiments the fourth and fifth foliage leaves were used at 5 weeks from germination.

Bacterial Culture

Xanthomonas campestris pv. malvacearum race 3 was obtained from a stock culture. Inoculum was prepared by transferring 2-3 loopfuls of bacteria from stock cultures to medicine bottles containing 25 ml sterile nutrient broth. The culture bottle was incubated at $30^{\circ}C$ with shake culture for 18 hours.

The absorbance of the broth culture was determined spectrophotometrically at 600 nm, and the inoculum was prepared by

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centrifuging the turbid broth for 10 min. at room temperature. The supernatant was replaced by an equal volume of sterile saturated $CaCO_3$ solution and the bacterial pellet was resuspended by using a vortex mixer for 30 sec. The suspension was transferred to 100 ml of sterile $CaCO_3$. Plate counts showed that the resulting suspension contains approximately 6×10^8 bacteria/ml under these conditions.

Inoculation Methods

Cotyledons of Im 216 and Ac 44 were inoculated with 6×10^8 bacteria/ml using a 1cc sterile syringe with a needle. The needle was inserted into the mesophyll parenchyma and the inoculum was infiltrated by gently depressing the plunger until complete water soaking appeared. The fourth and the fifth leaves were inoculated using a syringe without a needle. The syringe was pressed gently against the abaxial leaf surface. Control leaves were infiltrated with saturated $CaCO_3$ solution. A control set of plants of each line was kept in a light/dark chamber and another set was placed in a continuous dark chamber following inoculation.

Electron Microscopic Methods

Preparation of Fixatives

Glutaraldehyde which is used as a primary fixative, was buffered with phosphate buffer. Ten ml of 0.1 M KH_2PO_4 were added to 40 ml of 0.1 M Na_2HPO_4 $2H_2O$ and the pH was adjusted to 7.3. To 10 ml of this buffer was added 10 ml of 8% aqueous EM grade glutaraldehyde to obtain a 0.05 M buffered 4% glutaraldehyde solution.

Osmium tetroxide solution was used as a post fixative. To 10 ml of 4% osmium tetroxide (O_sO_4) were added 10 ml of sterile water and 20 ml of phosphate buffer to obtain 1% O_sO_4 fixing solution. This was done under a fume hood and the solution was stored in the dark at 4^oC.

Washing Buffer

Phosphate washing buffer was prepared by adding 20.4 g of sucrose to 200 ml of the phosphate buffer. It was used after fixation with glutaraldehyde to remove unbound glutaraldehyde which otherwise reacts with O_sO_4 to form electron dense precipitates.

Dehydrating Alcohols

A series of aqueous ethanol solutions 20, 30, 50, 70, 90, and 95% were used for the dehydration of fixed tissue. 100% ethanol and propylene oxide were used as final dehydrants.

Embedding Resin

Spurr's resin was prepared by mixing 10 g vinyl cyclohexene dioxide (VCD), 5 g diglycidyl ether of polypropylene glycol (DER), 26 g of nonenyl succinic anhydride (NSA), and 0.4 g of dimethylaminoethanol (DMAE). The mixture was stored in a 10 cc syringe at -7^oC.

Stain Preparation

Uranyl acetate and lead citrate were used to stain the thin sections. Uranyl acetate was used as a post stain and prepared by adding 0.2 g uranyl acetate to 40 ml of distilled water and vigorously shaking

for 10 min, until the solution appeared clear, at which time it had a pH of ∿3.7.

Lead citrate was prepared by mixing 1.33 g lead nitrate. 1.76 g sodium citrate, and 30 ml of distilled water. The mixture was shaken for 30 min in a 50 ml flask. 8 ml of 1N sodium hydroxide were added and the solution diluted to 50 ml and mixed by repetitive inversion until the lead citrate dissolved.

Preparation of Tissue

Tissue segments ($1mm^2$) were trimmed in cold glutaraldehyde and placed in fixing solution for 2 hours at 4^oC. After 2 hours the fixing solution was removed and replaced with cold washing buffer. The washing buffer was changed two times over a period of one hour and then left overnight in the third change of buffer. Washing buffer was replaced by O_SO_4 for four hours at 4^oC. At the end of this period, the tissues were washed with 3 changes of washing buffer and left overnight. The tissue pieces were then dehydrated by using a series of graded solutions of ethanol/H_2O for 30-60 min. each. The last solution of the ethanol series, absolute ethanol, was followed by 2 changes of propylene oxide. Tissue segments were then infiltrated with propylene oxide mixed with Spurr's resin in the proportion of 1:3, 1:1, 3:1 for one hour each and finally infiltrated with pure resin overnight. This was done with the resin in an uncapped vial under a fume hood. This allowed the residual propylene oxide to evaporate.

Tissue samples were transferred to fresh Spurr's resin, cast in molds and polymerized in a 70^oC oven for 8 hours.

Sectioning of Embedded Samples

Block faces were rough trimmed, to a trapezoid shape with clean razor blades. Glass knives were prepared on an LKB-7800 knife maker. The knives were broken shortly before use to avoid dulling of edges. Thick sections (270 mμ) were cut using a Sorvall MT-2 ultramicrotome and stained with toluidine blue. Thick sections were examined with a light microscope and areas of interest were chosen. The trapezoidal face was re-trimmed to the dimension of 1 mm on the broad side and only the area of interest was retained for thin-sectioning.

Thin sections were cut using a diamond knife. Silver sections, 60-90 mμ, were expanded by passing xylene vapor over them. Sections were collected on uncoated 300 mesh copper grids that had been cleaned with 95% ethanol and distilled water. The grids were stored in dust-proof containers.

Staining of Sections

Sections were first stained with uranyl acetate. The grids were placed section side down on a drop of the stain in a clean dish. Sections were stained for 30 min. Then grids were washed by dipping them in three vials containing sterile water for 10-15 sec. each. This removed stain not bound by the tissues. Grids were blotted with filter paper and transferred to a drop of lead citrate for 20-30 min. Pellets of sodium hydroxide (NaOH) were arranged around the drops of lead citrate. This absorbed CO_2, which otherwise would react with lead citrate to form insoluble carbonates and contaminate the sections. Grids were then washed with water, blotted on filter paper, and

stored. Grids were scanned on a Philips EM 200. Photomicrographs were taken using Kodak EM film. Film plates were developed for 1 min at $30^{\circ}C$ in D-19. Prints were made on Kodak polycontrast ER photographic paper and developed in Dektol developer diluted 1:2.

Bacterial Growth Experiments

Two discs of 0.33 cm^2 area each were removed from 2 leaves of each cotton line at various intervals (0, 10, 24, 48, 72 hours) after inoculation with pv. malvacearum. Each point on growth curves was calculated from the bacterial populations in two leaf discs from duplicate leaves.

Bacterial population number was determined as follows: discs were rinsed twice in sterile saturated $CaCO_3$ for 15-20 sec, homogenized in a mortar in 1 ml $CaCO_3$, diluted and spread on nutrient agar plates with the aid of a Spiral Plater (Spiral System). Plates were incubated for 2 days at $30^{\circ}C$ before counting the numbers of bacterial colonies. The same procedures were used to calculate the bacterial population in plants kept under continuous darkness or under light/dark conditions.

CHAPTER IV

RESULTS

Control samples for transmission electron microscopy were taken of both Ac 44 and Im 216 cotton line leaf tissue. There was a series of plants kept in the usual light/dark regime as well as a set of control plants placed in continuous darkness after infiltration with saturated $CaCO_3$ solution followed by killing and fixing in the usual manner. Organelles were normal, and cell wall and cytoplasmic components were intact 24 h post infiltration. This was the case with Ac 44 leaf tissue when plants were grown under a normal light/dark regime (plate 1) as well as leaf tissue of Im 216 plants grown under continuous darkness (plate 2).

Susceptible Cotton Line Ac 44

At 4 h post inoculation in the plants placed in the dark, all bacterial cells were enveloped in a thick fibrillar material (plate 3) or with loosened fibrillar material (plate 4). Host cell organelles and their membrane systems appeared intact and normal. Cell wall surfaces appeared loosened and detached and fibrillar material was released from the cell wall surface (plate 5). By 24 h post-inoculation, the wall cuticle was distinct and continuous with the envelope. Bounding membranes of the chloroplasts and the plasma

membrane appeared to be unaffected (plates 6-8). Some cells responded
to the presence of bacteria more rapidly than others (plate 8). One
cell bounded by bacteria often appeared normal, while another neigh-
boring bounded cell contained condensed cytoplasm (plate 8). Changes
in granal and stromal lamellae of chloroplasts were among the earliest
alterations noted. Lamellae tended to become indistinct or disappear
and the stroma condensed and became electron dense. After 2 days
thick fibrillar material was associated with groups of bacterial cells.
Severely damaged cell walls and some vesicles were found near enveloped
bacteria (plate 9). Enlarged envelopes were filled with electron
dense material and bacterial cells were surrounded by a clear capsular
coating (plate 10). At 2 days the nucleus appeared relatively
unaffected, but cytoplasm and ribosomes were condensed (plate 11).

By the third day, the numbers of bacteria per envelopment had
increased and host cells were in a necrotic condition (plate 12). By
this time most of the envelopes had broken and bacterial cells were
released into intercellular spaces (plate 13). Bacterial cells
appeared free in intercellular spaces near collapsed host cells
(plate 14). While there was a slight increase in numbers of bacteria
per envelopment in earlier (4-24 h) post-inoculation samples (table1),
a large increase appeared after two to three days. Enveloping films
ruptured by the third day possibly as a result of increasing size of
the envelope due to increases in numbers of bacteria.

Ac 44 plants inoculated under normal day/night conditions exhib-
ited a different ultrastructural response to pv. malvacearum. Tissue
fixed 30 min. post-inoculation showed no sign of envelopment of
bacterial cells. Although some material was deposited on the cell wall

surface, the cell wall appeared intact and normal (plate 15). At 1 h post inoculation, bacterial cells appeared free in the intercellular space near the junction of two cells and host cell organelles were intact (plate 16). At 24 to 48 h post inoculation bacterial cells were free in the intercellular spaces. The plasmalemmae and granal and stromal lamellae of chloroplasts were not significantly different from those of control sections. (plate 17-20). After 3 days, the chloroplasts had lost most of their membranes and the number of plastoglobuli within the chloroplasts increased. The cytoplasm of host cells contained larger amounts of electron dense material (plate 21). Some cells became necrotic and the thylakoid lamellae of their chloroplasts were separated (plate 22).

Immune Cotton Line Im 216

Ultrastructural preparations of Im 216 in the dark at 4 h post inoculation revealed that the host cell wall was eroded at various locations close to bacterial cells, and wispy fibrillar material probably released from the damaged host cell walls gathered around the bacterial cells (plate 23). Bacterial cells embedded in thick fibrillar material were surrounded by a clear zone (plate 24). Bacterial cells found at the junction of two cotton leaf cells were ensheathed by a complex which apparently contained host cell wall cuticle (plate 25). Numerous vesicles often formed near broken host plasmalemmae adjacent to bacteria (plate 25). Chloroplast membranes appeared unaffected (plate 23). At 12 h chloroplasts had lost most of their membrane structure, plasma membranes were no longer apparent in many cells, mitochondria appeared relatively unaffected and vesicles had

formed between plasmamembranes and host cell walls (plate 26-27).
At 24 h post-inoculation host target cells were severely damaged with
separated chloroplast thylakoid lamellae (plate 28). In some severely
degraded host cells the chloroplast fretwork was in total disarray,
and bounding membranes and stroma were destroyed (plate 29). By the
second day, bacterial cells appeared structurally abnormal and were
still enveloped near collapsed cells (plate 30-31).

Leaf tissues of Im 216 grown under the normal light/dark cycle
and fixed shortly after inoculation (30-60 min) showed envelopment of
bacterial cells (plate 32-33). Four hours later thick fibrillar
material was found associated with bacteria and chloroplasts appeared
relatively unaffected (plate 34-36). Within 12 hr, wispy fibrillar
materials probably at least partly of damaged host cell wall origin,
appeared and cell organelles were severely degraded (plate 37-38).
Chloroplasts within severely damaged host cells were rounded and
membrane systems disarranged (plate 39). One day after inoculation,
groups of bacteria were found in collapsing mesophyll cells (plate 40).

Bacterial Population Densities

The growth rate and growth yield of pv. malvacearum in Ac 44 in
the dark were very similar to growth rate and growth yield in the
light/dark cycle (Fig. 1). In Im 216 bacteria grew much slower in
the light than in the dark, and growth showed strong inhibition
within 2 days (Fig. 2). The bacterial population density in Im 216
in the dark was as high as that in plants that show a susceptible
(compatible) reaction in the light. The low average number of bacteria

per envelope in Ac 44 grown in the dark and sampled at early times post-inoculation (4-24 h) (table 1) correlated well with the low growth yield of bacteria (Fig. 1). Low numbers of bacteria per envelope of Im 216 grown under day/night conditions were correlated with low bacterial population densities.

CHAPTER V

DISCUSSION

When ultrastructural study of the compatible interaction between
X. campestris pv. malvacearum and Gossypium hirsutum L., susceptible
line Ac 44, was carried out in leaf tissues, of plants grown under
normal day/night conditions, there was no development of fibrillar
materials or enveloping films around the bacteria in samples taken
shortly after inoculation or at later times post-inoculation (plates
15-22). The susceptible cotton line, Ac 44, leaf tissue exhibited
a different ultrastructural pattern as a response to pv. malvacearum
when grown under continuous darkness following inoculation. Bac-
terial cells appeared to be enveloped within fibrillar material at
the host cell wall surfaces four hours post-inoculation (plates 3-4).
Fibrillar material from host cell walls appeared to be released and
to gather around the bacterial masses (plate 5). This is similar to
the phenomenon that has been observed in an incompatible interaction
between Im 216 and pv. malvacearum (plate 23). Host cell organelles
of Ac 44 kept in normal day/night conditions following bacterial inoc-
ulation did not degenerate very rapidly. They appeared to be somewhat
altered at 3 days post-inoculation (plates 21-22). Ac 44 kept in the
dark following bacterial inoculation showed, in addition to envelopment
of bacteria, host cell organelle degeneration at one day post-inocula-
tion (plates 8-11). My interpretation of the ultrastructural study

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reported here was that cell wall, plasma membrane, cytoplasm, and
chloroplast degeneration occurred earlier than is seen in Ac 44
infiltrated with pv. malvacearum and kept under normal day/night
conditions (1).

Envelopment of bacteria was observed as early as 4 h post-inocu-
lation with Ac 44 in the dark (plates 3-4) and wall cuticle loosened
and became detached with liberation of fibrillar material from the
cell wall (plate 5). This corresponded to the host cell wall damage
observed in an incompatible reaction at 4 h (plate 23). After 3
days envelopes appeared very fragile, enveloping material broke down
and bacteria were released into intercellular spaces (plate 13).
Reaction of pv. malvacearum-inoculated Ac 44 kept in the dark mimicked
those of OK 2.3, a cotton line of intermediate resistance derived from a
cross between resistant Im 216 and susceptible Ac 44 cotton lines (3).
In a study carried out with OK 2.3, envelopes were formed around pv.
malvacearum but broke down at later stages post-inoculation. The
behavior of OK 2.3 is due to the presence of only one resistant gene
(3). Since similar envelopments of bacteria and breakage of envelopes
at later stages post-inoculation occurred in Ac 44 grown in the dark
and in a cotton line of intermediate resistance grown under normal
day/night conditions, Ac 44 susceptible cotton line grown under contin-
uous darkness following inoculation with pv. malvacearum gave some of
the ultrastructural responses of partially resistant plants. However,
bacterial multiplication in Ac 44 did not differ significantly between
dark and light/dark conditions (Fig. 1).

Continuous darkness following inoculation with pv. malvacearum
appeared to have little effect on the cause of ultrastructural changes

in the resistant plants, Im 216. The observed ultrastructural responses of Im 216 in the dark are very similar to those in the light. Rapid host cell organelles degeneration occurred affecting chloroplasts, cell walls and membranes (plates 26, 37, and 38) and bacterial envelopment persisted throughout the observation period (plates 30-31 and 39-40). However, the bacterial growth rate and yield in Im 216 in the dark were as high as in susceptible Ac 44 plants that showed a compatible reaction (Fig. 2). Under conditions similar to those described here, Cover, Pierce and Essenberg detected terpenoid PTO phytoalexins in Im 216 kept in light/dark conditions, but not in Im 216 kept in dark (unpublished work). In the light/dark conditions the growth of incompatible bacteria was inhibited as is usually the case (8). In the absence of light the resistant plants responded to inoculation of incompatible bacteria with ultrastructural features characteristic of its typical necrotic HR. However, the HR appeared to be ineffective and bacterial growth was not inhibited. It is suggested that this may be the result of uncoupling of parts of resistance mechanisms HR as is said to occur in a number of systems (12).

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TABLE 1

AVERAGE NUMBERS OF BACTERIAL CELLS OBSERVED

PER ENVELOPE[a] IN THIN SECTIONS

| | Hr post-inoculation | | | | |
Treatment	4	12	24	48	72
Ac 44-dark	2.55 ±1.8[b]	2.6 ±1.9	3.3 ±2.4	9.25 ±5.8	34.15 ±14.6
Im 216-dark	1.7 ±1.1	2.3 ±2	3.3 ±1.2	6.3 ±3.2	
Im 216-light	1.6 ±.74	2.1 ±1.6	2.7 ±1.3		

[a]within apparently intact envelopes or near broken ones

[b]standard deviation of the mean

Fig 1. Population densities of pv. <u>malvacearum</u> in leaves of Ac 44 infiltrated with 6 x 10^8 bacteria/ml and grown under continuous darkness (●) or under light/dark cycle (0). Each plotted point represents the mean bacterial population density of two replicate discs.
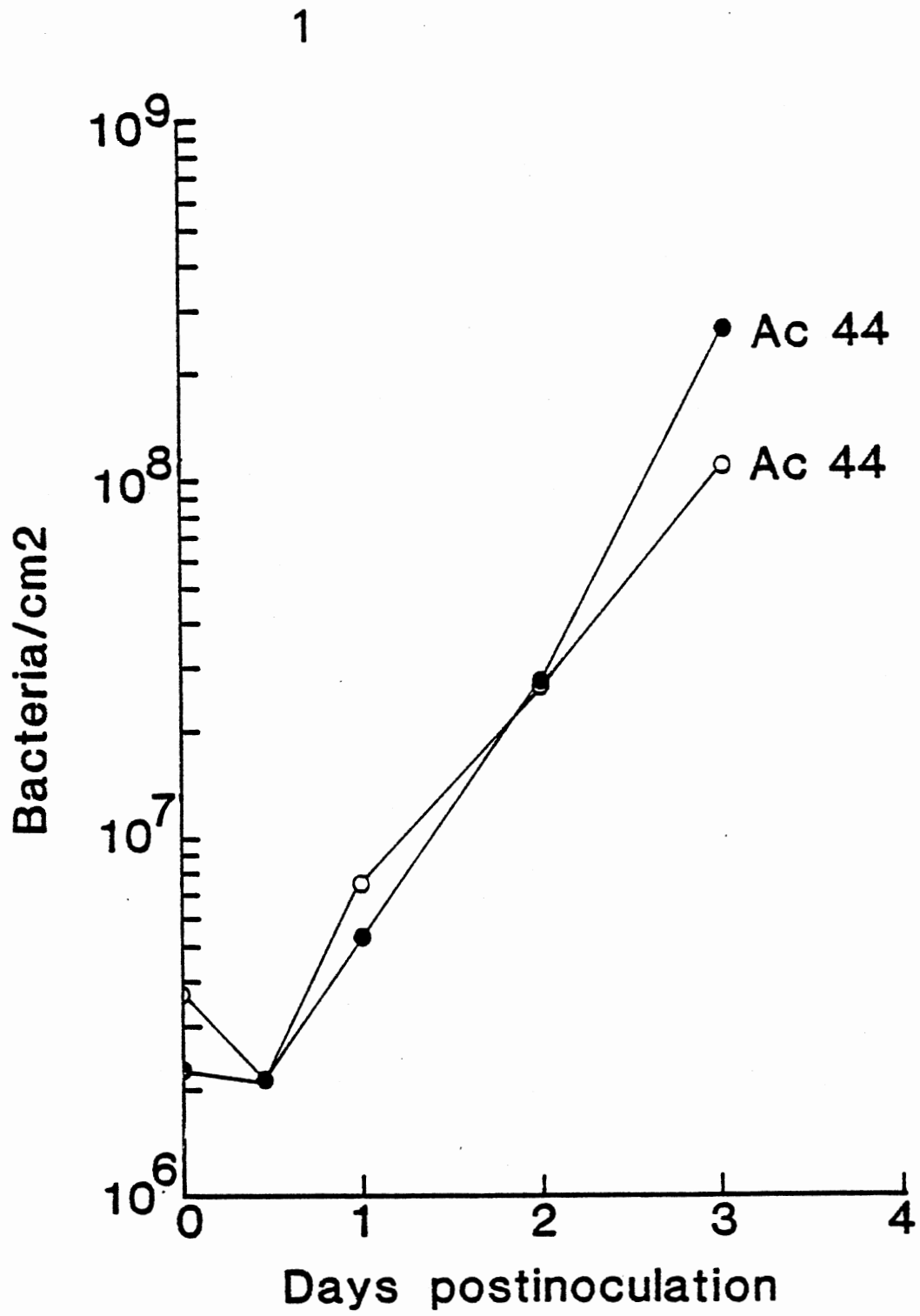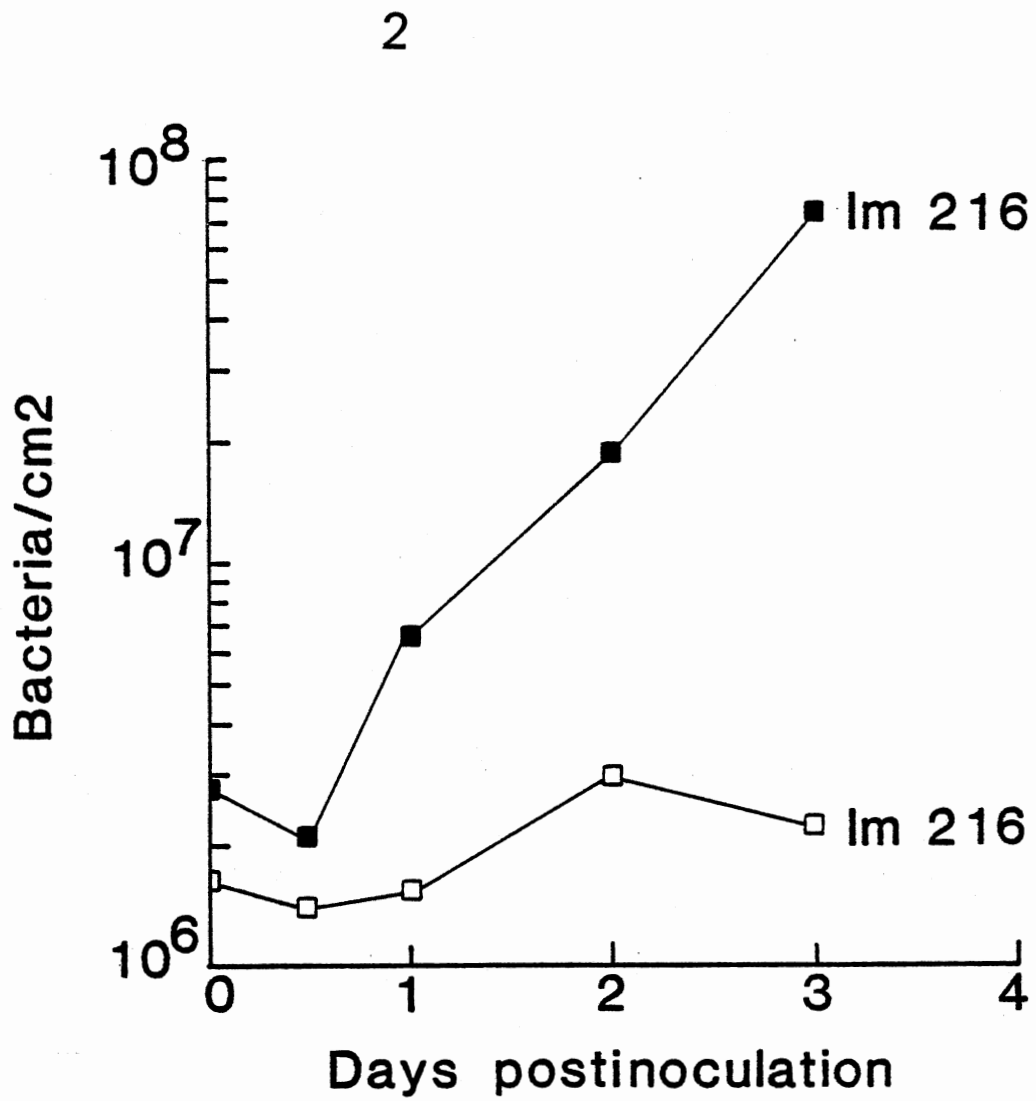
Fig. 2. Population densities of pv. <u>malvacearum</u> in leaves of Im 216 infiltrated with 6 x 10^8 bacteria/ml and grown under continuous darkness (■) or under normal light/dark cycle (□). Each plotted point represents the mean bacterial population density of two replicate discs.

2

Figure showing Bacteria/cm2 versus Days postinoculation, with curves labeled Im 216 and Im 216.

Abbreviations for transmission electron micrographs: B, bac-
terium; C, chloroplast; Cw, cell wall; V, vacuole; P, plastoglobule;
Is, intercellular space; M, mitochondrion; Mb, microbody; Ve, vesicles;
F, fibrillar material; Cc, collapsed cell; FB, free bacterium, BE,
broken envelope; N, nucleus; Cy, cytoplasm.

Plates 1-2. Control plant palisade parenchyma cell fixed 24 h
after infiltration with $CaCO_3$. Cell structure and cytoplasmic com-
ponents appeared intact and normal. 1) Leaf tissue from Ac 44 plants
grown under normal day/night conditions. X20,812. 2) Leaf tissue
from an Im 216 plant grown under continuous darkness and inoculated
with no bacteria. X21,830.

plates 3-4. 3) Leaf cells from tissues of Ac 44 plants grown
in darkness following inoculation, 4 h after infilbration with pv.
malvacearum. A single bacterium enveloped by thick fibrillar material.
X50,320. 4) Mesophyll cells of Ac 44 leaves placed in the dark and
fixed at 4 h post-inoculation. Group of bacterial cells adjacent to
two cells were enveloped by loose fibrillar material. Host cell
organelles appeared intact and normal. X30,192.

plate 5. Mesophyll cells of Ac 44 plants grown in darkness and
fixed 12 h after inoculation. Wispy fibrillar material partly of
host wall origin appeared to be gathering around the bacterium.
X25,641.

plates 6-8. 6) Single bacterium shown enveloped in the inter-
cellular space at a junction of two leaf cells of an Ac 44 plant grown
under continuous darkness for one day post-inoculation with pv. mal-
vacearum. X10,391. 7) Bacterial cell enveloped at the surface of Ac
44 leaf cell wall one day after inoculation, cell wall cuticle was

distinct and appeared continuous with the surface of the envelopment. X20,812. 8) Cells of a leaf of Ac 44 one day post-inoculation. One host cell appeared normal near the enveloped bacterium, but the other abutting cell showed a damaged chloroplast and electron-dense cyto-plasm. X12,574.

plates 9-10. 9) Group of bacterial cells enveloped at the sur-face of Ac 44 leaf cells two days after infiltration. Vesicles were formed between the damaged cell wall and broken plasma membrane. X25,641. 10) Two days post-inoculation a large envelope contained a group of bacterial cells which are surrounded by clear capsular material. X13,120.

plate 11. Two days post-inoculation, a leaf mesophyll cell of Ac 44 maintained in continuous darkness after inoculation, showed coagulated ribosomes (arrow), chloroplasts had lost most membranes, but the nucleus appeared normal. X8,325.

plates 12-14. 12) Mesophyll cells of Ac 44 maintained in dark-ness 3 days after inoculation. There was a large group of bacterial cells within an envelope, and the host cells were devoid of cytoplasm. X8,325. 13) A broken envelope with bacteria released into inter-cellular space at 3 days post-inoculation. X15,188. 14) Free bac-terial cells in intercellular spaces near collapsed cells of Ac 44 which had been kept in the dark, 3 days after inoculation. X13,875.

plates 15-16. 15) Thirty min post-inoculation with pv. mal-vacearum into leaf of Ac 44 plant kept under normal light/dark cycle. Bacterial cells were free in the intercellular spaces. Adjacent host cell wall and chloroplast were normal. X31,135. 16) one h after inoculation, there was no sign of envelopment of bacteria. X32,088.

plates 17-18. 17) Mesophyll cell of Ac 44 maintained on a normal light/dark cycle, 24 h after inoculation. Cell structure and cytoplasmic components appeared similar to those in control. X18,315. 18) Bacterial cells near cell wall of Ac 44 mesophyll cell one day post-inoculation. Host cell walls, membrane structure, and granal and stromal lamellae of the chloroplasts appeared normal. X14,189.

plates 19-20. 19) Leaf cells of Ac 44 two days after inoculation with pv. malvacearum leaves were kept on a normal light/dark cycle. Cell organelles appeared intact and normal. X18.315. 20) Bacterial cell in similar leaf tissue in the intercellular spaces. X21,978.

plates 21-22. 21) Similarly inoculated Ac 44 leaves three days post-inoculation. Cytoplasm contained much electron-dense material and a chloroplast had lost its granal lamellae. X21,978. 22) Thylakoid lamellae of chloroplasts were separated. Three days post-inoculation. X28.416.

plates 23-25. 23) Four h after inoculation of Im 216 leaf tissue maintained under continuous darkness. Release of fibrillar material from the damaged cell wall. X57,720. 24) Thick electron-dense material surrounded a group of bacteria at 4 h post-inoculation. X15,984. 25) Broken plasma membrane and numerous vesicles were formed in a host cell near an enveloped bacterial cell. X25,437.

plates 26-27. 26) Twelve h after inoculation into Im 216 leaves kept in continuous darkness. Mitochondria appeared normal but there was increased number of plastoglobuli within the chloroplasts. X20,812. 27) Some vesicles were formed between the cell wall and the plasma membrane 12 h post-inoculation. X27,750.

plates 28-29. One day post-inoculation with Im 216 in Im 216 leaves maintained in the dark. 28) Enveloped bacterial cells near damaged host cell. X16,650. 29) Chloroplast fretwork was spread and cytoplasm appeared dense and clumped. X35,224.

plates 30-31. Similarly inoculated and maintained Im 216 at 2 days post-inoculation. 30) Two days after inoculation with Im 216. Bacterial cells looked abnormal near collasped cells. X12,485. 31) Enveloped bacterial cells near damaged host cells. X16,650.

plates 32-33. 32) Thirty min post-inoculation into Im 216 plants grown under normal light/dark cycle. Little fibrillar material was associated with enveloped bacteria. X29,304. 33) One h after inoculation single bacterium enveloped at the juncture of two mesophyll cells. Granal and stromal lamellae appear unaltered. X16,483.

plates 34-36. 34) Leaf of Im 216, 4 h after inoculation. Bacteria were ensheathed in thick fibrillar material. X29,304. 35) Four h post-inoculation. Chloroplasts appeared normal and cytoplasm was dense. X14,696. 36) mesophyll cells were altered with electron-dense cytoplasm and chloroplasts that had begun to lose their membranes. X17,372.

plates 37-38. Similarly inoculated Im 216 plants maintained on a normal light/dark cycle. Twelve h post-inoculation. Fibrillar material had been released from damaged host cell wall. X12,709. 38) Electron-dense cytoplasm with damaged chloroplasts. X11,655.
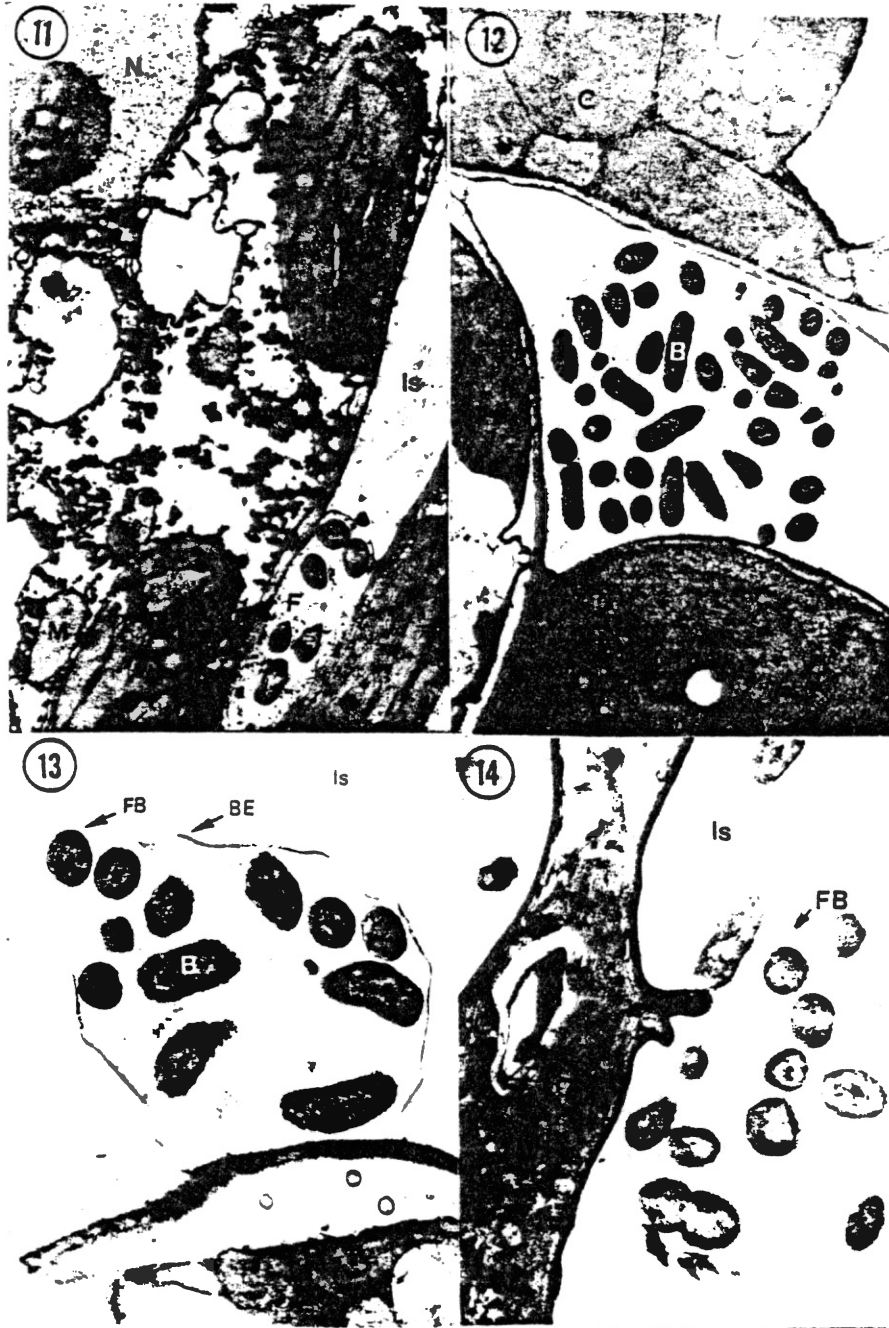
plates 39-40. Im 216 leaf mesophyll cell one day post-inoculation kept in normal light/dark cycle. Chloroplasts appeared round and had lost most of their membranes. X12,487. 40) Enveloped bacteria near collapsed cells. X9,990.
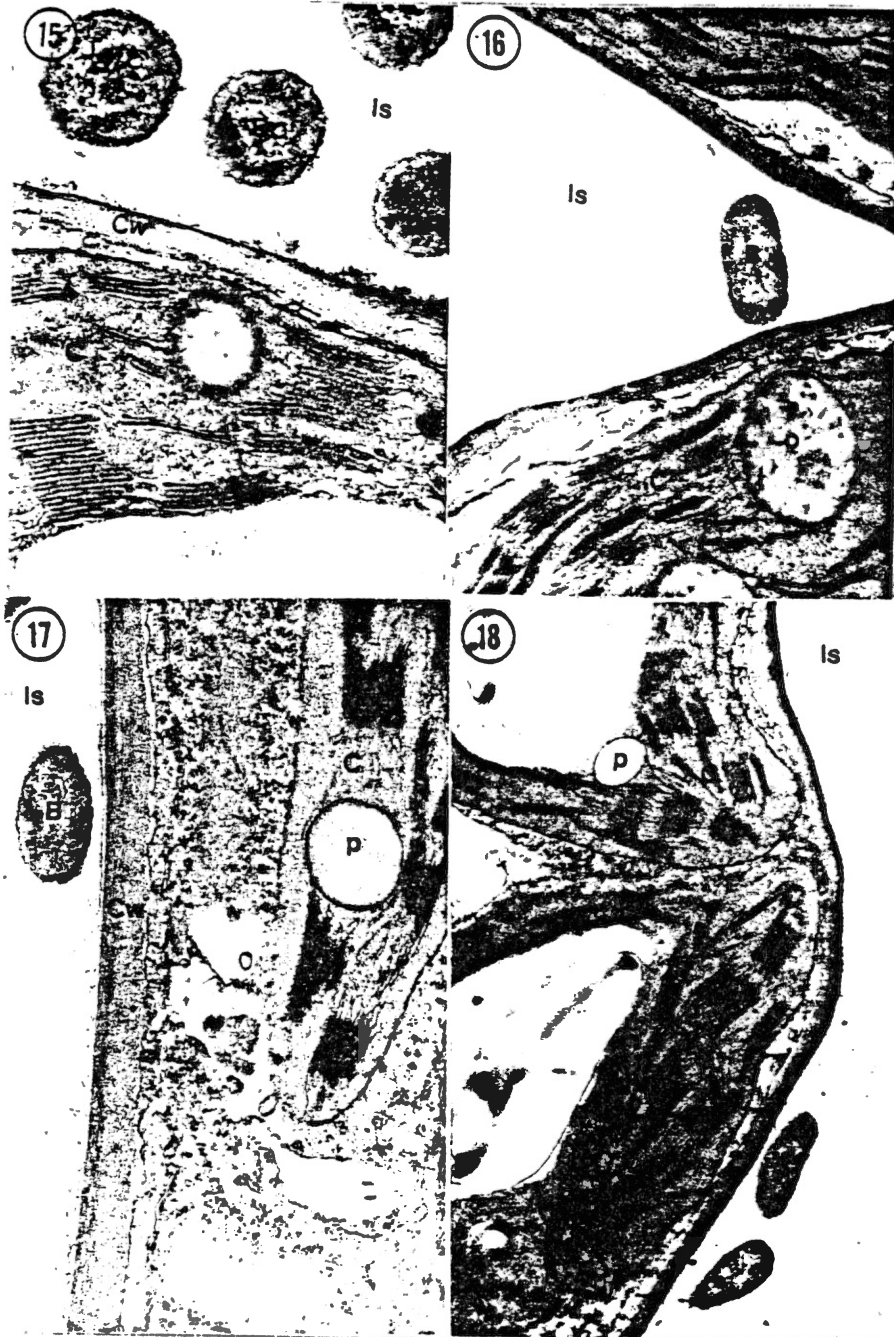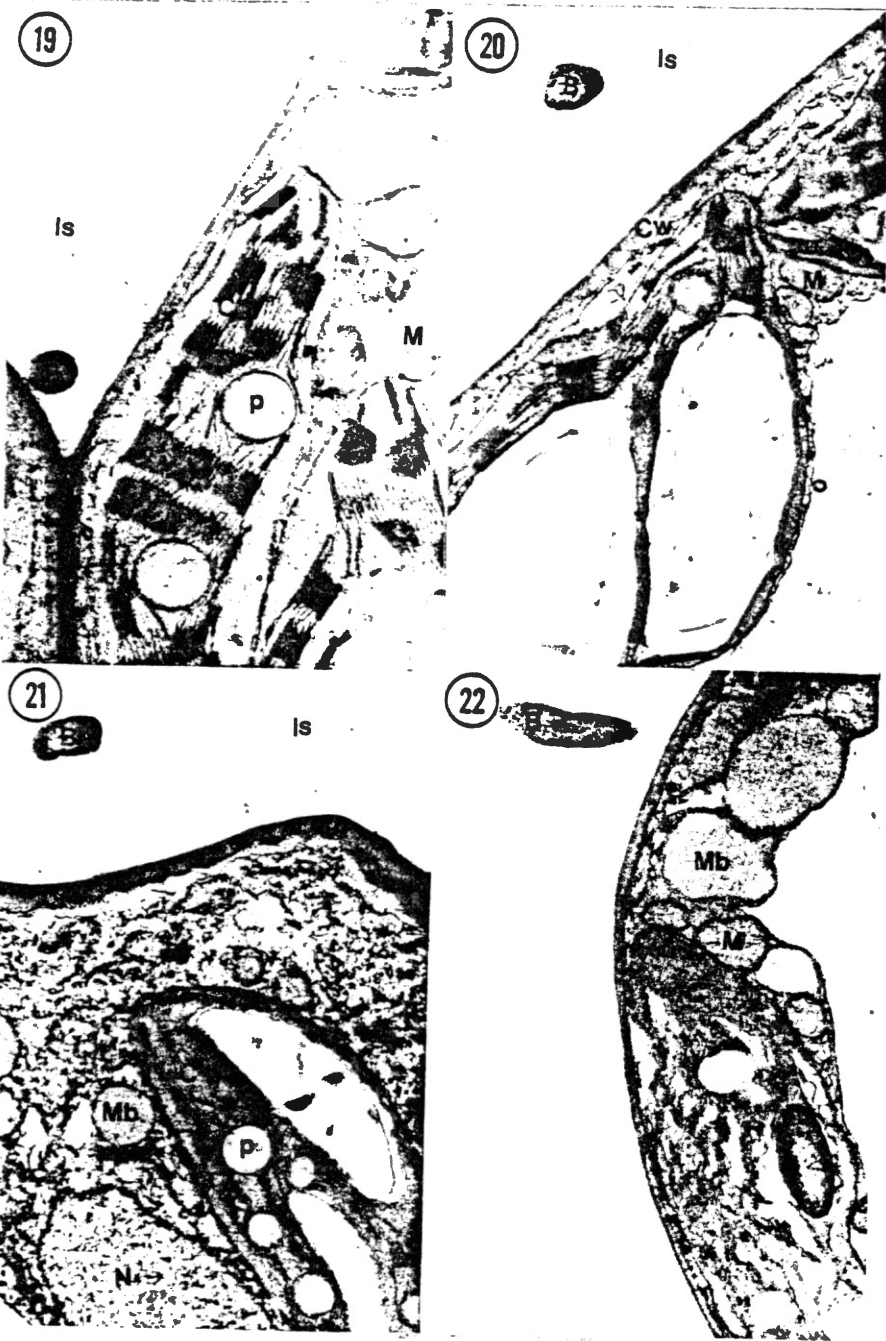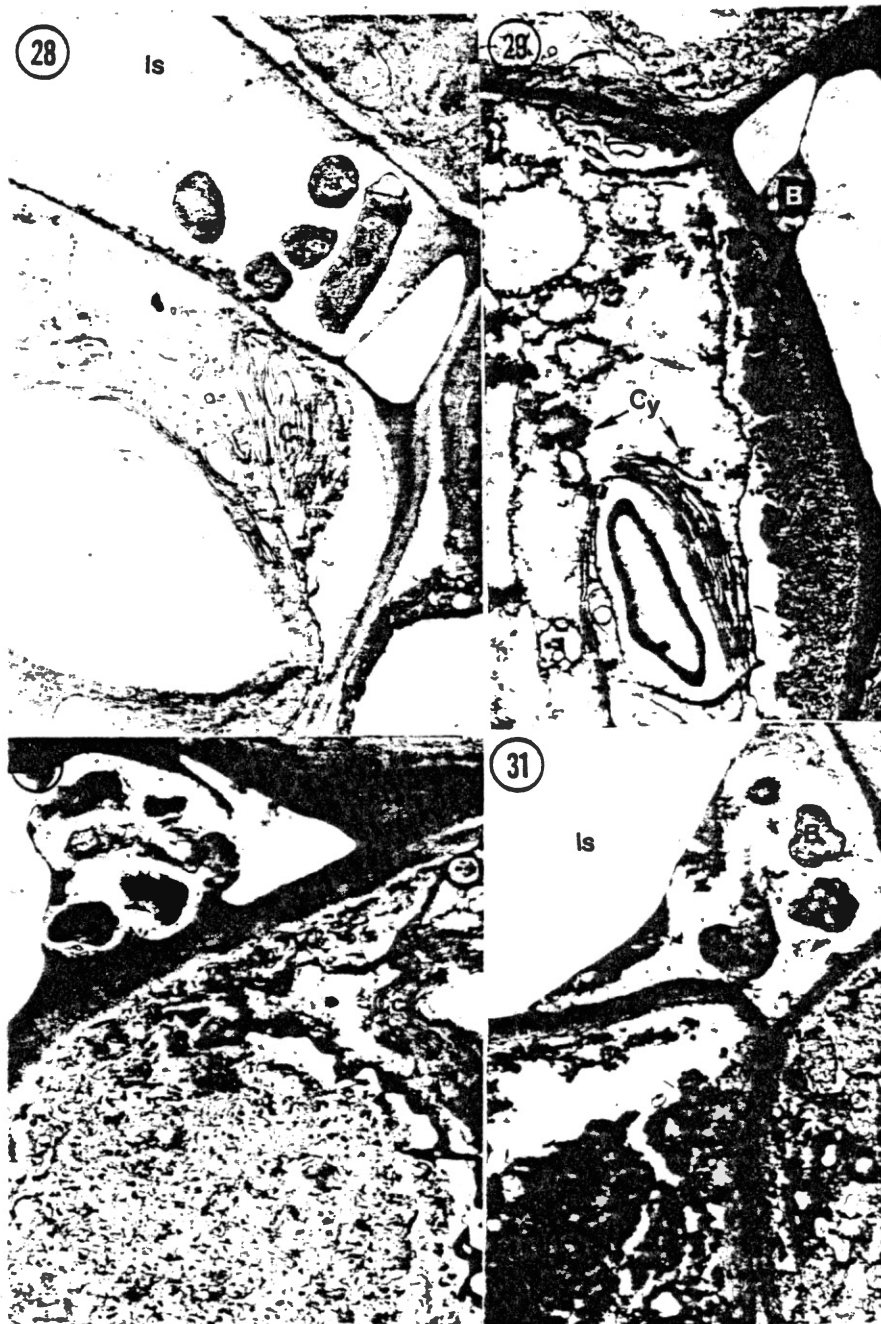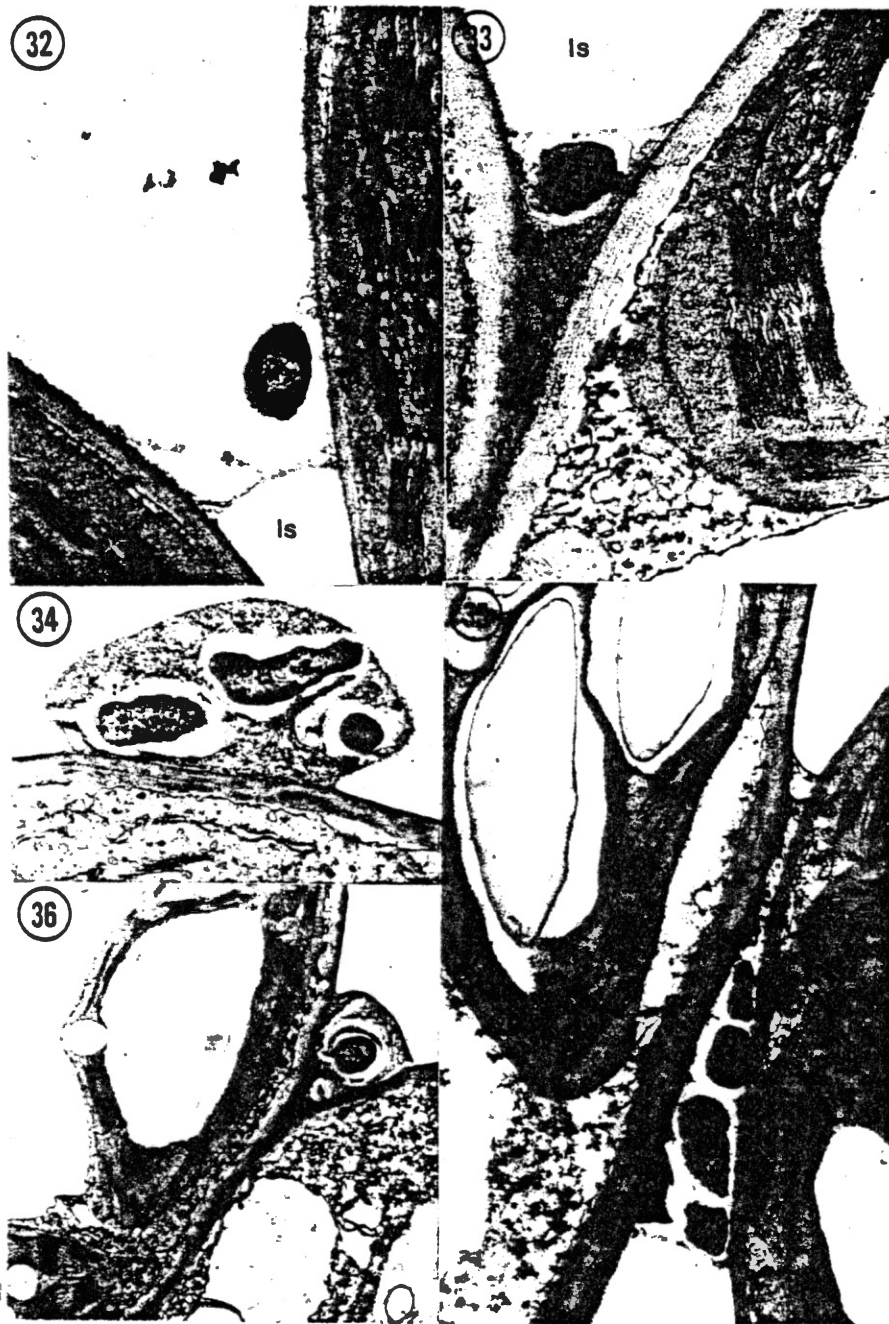
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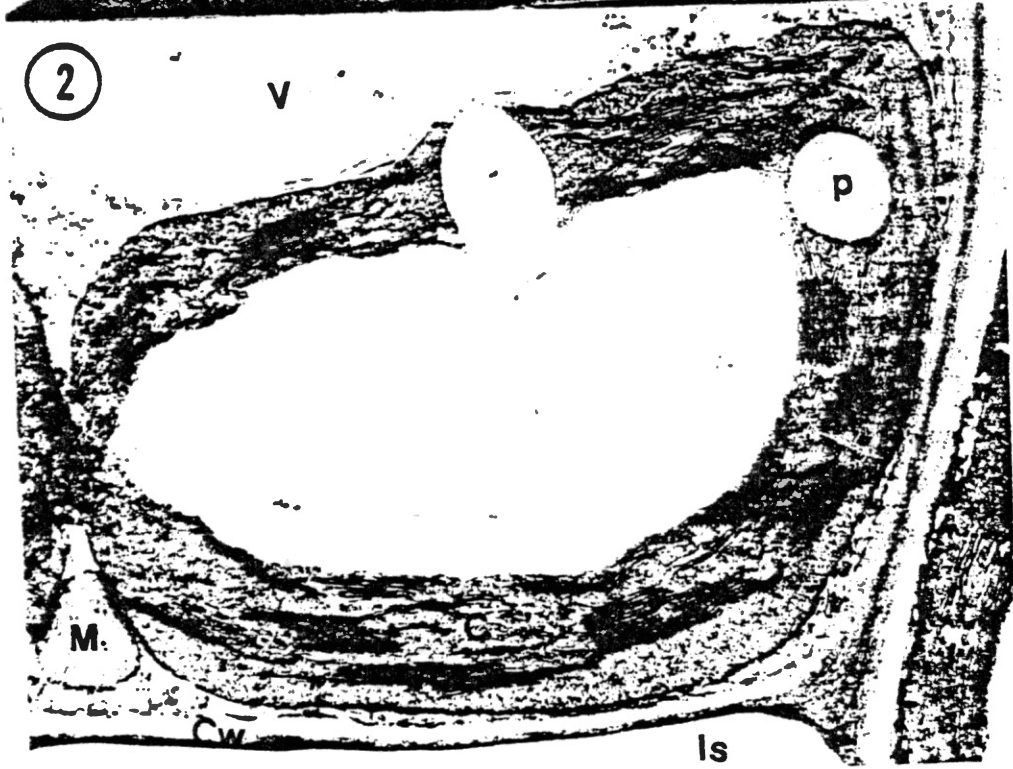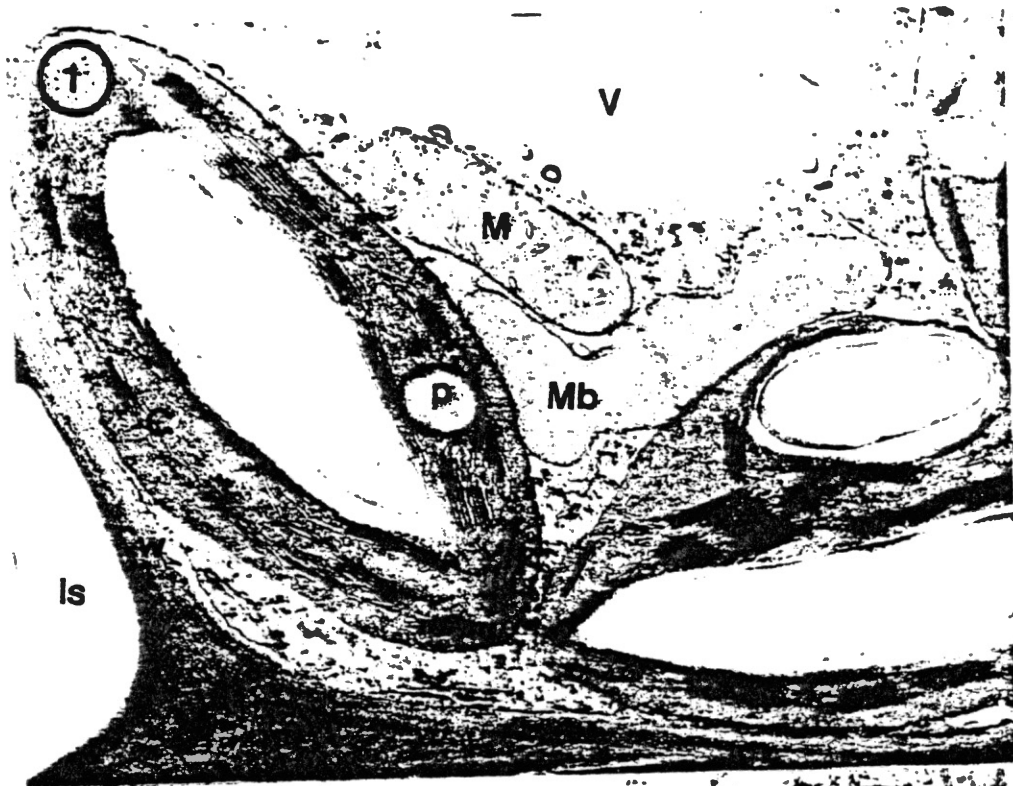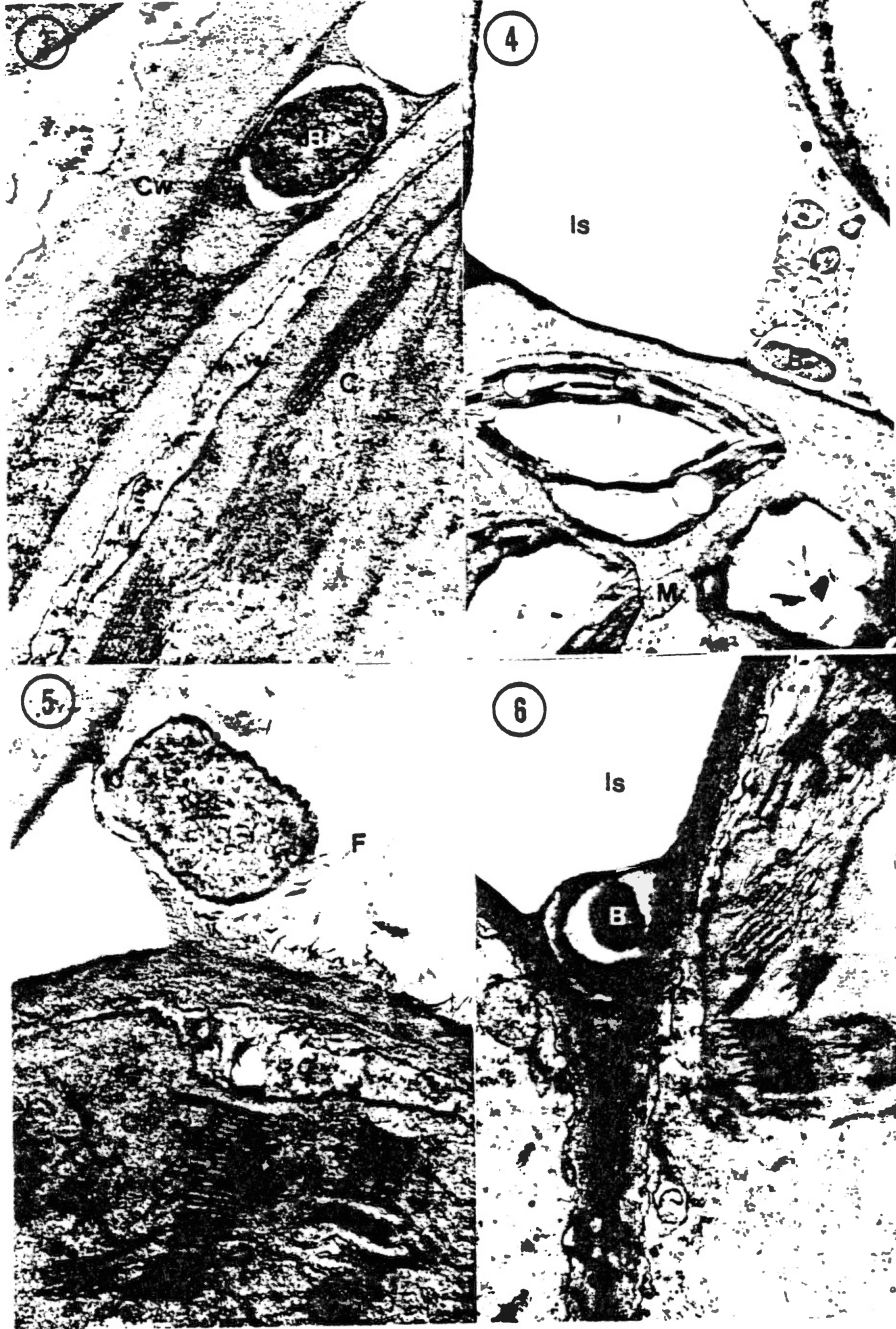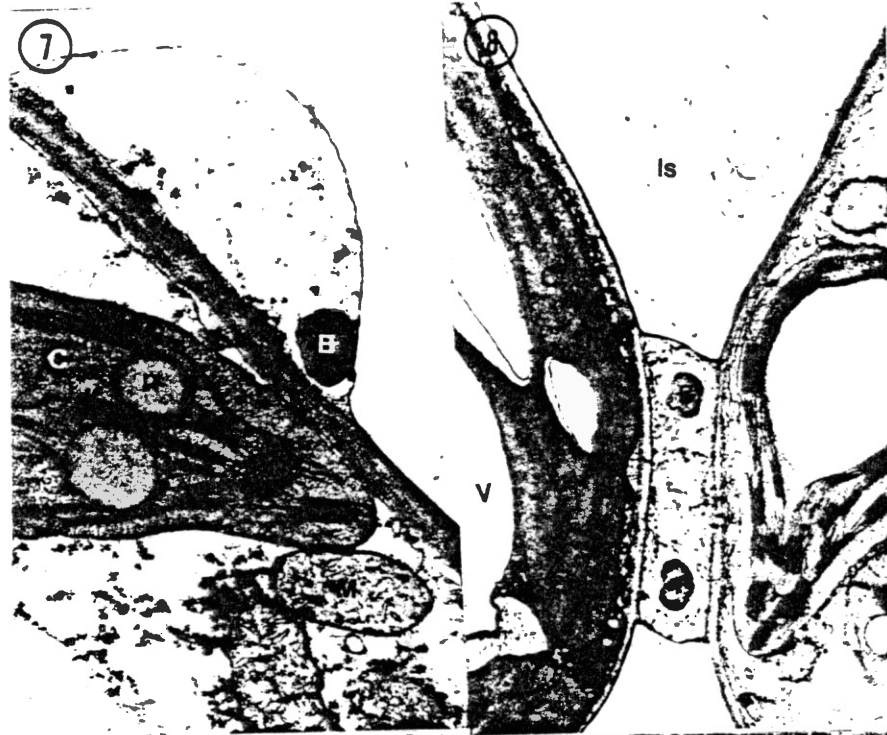
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Thesis: ULTRASTRUCTURE OF INTERACTIONS BETWEEN XANTHOMONAS CAMPESTRIS PV. MALVACEARUM AND BACTERIAL BLIGHT-IMMUNE AND -SUSCEPTIBLE COTTON LINES GROWN IN DARK AS COMPARED WITH DAY-NIGHT CYCLES

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