# THE ULTRASTRUCTURAL RESPONSES OF TWO COTTON CULTIVARS INOCULATED WITH *XANTHOMONAS MALVACEARUM* (E.F. SM.) DOWS.

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# THE ULTRASTRUCTURAL RESPONSES OF TWO COTTON CULTIVARS INOCULATED WITH XANTHOMONAS MALVACEARUM (E.F. SM.) DOWS.

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# CHAPTER I

## INTRODUCTION

Bacterial blight of cotton is an important disease in nearly all of the major cotton producing areas of the world. The causitive agent, Xanthomonas malvacearum (E.F. Sm.) Dows., is a gram-negative, rod-shaped bacterium that is host specific for the genus Gossypium, and is represented by at least seventeen major physiological races (4). Succulent above ground parts of cotton plants are attacked by the bacteria following natural inoculation by wind driven rain or sprinkler irrigation. Under optimal conditions, the disease can defoliate plants, predispose bolls to secondary rotting fungi, and eventually lead to destruction of a crop.

Bacterial diseases are difficult to control since most of the available pesticides that are used to control plant diseases are ineffective against bacteria. Bactericidal chemicals, such as antibiotics, will control the pathogen, but are costly and require frequent reapplication. Other control methods that have been employed are general sanitation, use of disease-free and acid delinted seed, all of which can help prevent an outbreak of blight. However, the single most important avenue of disease control has

been the breeding for and selection of resistant lines of cotton. Breeding has been successful in controlling diseases of other crop plants, and bacterial blight of cotton apparently is not an exception. Experimental breeding stock and cultivars are now available which give disease reactions ranging from susceptibility to immunity. As a result, we are in a position to investigate the interaction of the host and pathogen at the ultrastructural level in an attempt to gain an understanding of the factors responsible for compatibility and incompatibility in this relationship.

In the cotton-Xanthomonas system, the incompatible relationship is most commonly studied with a cultivar which is not attacked by any known race of the pathogen. This cultivar has been described as being immune. Under field conditions the cultivar never shows macroscopic disease symptoms, even when nearby susceptible plants are severely attacked. However, under laboratory conditions when immune plant leaves are inoculated with abnormally high levels of bacteria, greater than $10^7$ cells/ml, the inoculated areas will become necrotic within 12 to 24 hours depending on the environmental conditions. This response is known as the hypersensitive reaction and has been demonstrated in other plant-bacteria relationships (28). Conversely, similar inoculations on susceptible plants will produce disease symptoms within 4 to 7 days. Different reactions produced with high levels of bacteria have enabled the plant breeder

to quickly screen populations of plants and classify them as to reaction type. This has also been a useful tool to structural, physiological, and biochemical workers because artificial inoculations produce uniform, repeatable responses in the host tissue. Previous independent studies by Cason et al. (9) and Turner and Novacky (52), have shown that the host response is not basically different from that produced with lower levels of inoculum likely to be encountered under natural field conditions. The intensity of the reaction is apparently directly related to the magnitude of the pathogen population density in host tissue.

Thus the hypersensitive reaction observed following artificial inoculation with high levels of incompatible bacteria produces a confluent necrosis that is reproducible and provides the subject for a detailed structural study of resistance. Earlier work with the light microscope has shown that alterations in tissue structure occur within 2 to 4 hours following infiltration with bacteria (9). Ultrastructural studies have shown that dramatic and rapid changes occur in leaf cell organelles following inoculation with an incompatible pathogen, and most recently the cell wall has become the object of considerable attention. Complementary physiological studies have shown that bacterial enzymes may be responsible for necrosis induction (21).

With a critical ultrastructural study of the affected host tissue, it may be possible to determine the sequence

of events occurring in a single cell that leads to necrosis. This information would be useful to either the biochemist or physiologist. Subtle structural symptoms produced early in the reaction might aid investigators in the identification of factors responsible for necrosis.

In this study, a thorough ultrastructural investigation of the progressive changes that lead to necrosis in cotton leaves has been attempted. When the incompatible reaction was compared with the compatible reaction, pronounced differences were noted. To complement the findings in the structural portion of this study, physiological experiments were performed to monitor the vital activities of respiration and photosynthesis in similarly treated tissues. The results appear consistent with published data in other plant-bacteria systems, however some new ideas are presented.

# CHAPTER II

## REVIEW OF SELECTED LITERATURE

Bacterial blight of cotton was first described by Atkinson (1). He reported symptoms of watersoaking in the angular interveinal areas of leaves. One year later he reported large quantities of bacteria associated with the watersoaked lesions (2). Because of the distinct angular appearance of the lesions, the disease was known for many years as "angular leaf spot of cotton".

In 1901, Erwin F. Smith (47) was able to isolate in pure culture the bacteria associated with the lesions, and subsequently produce the disease in healthy plants by reinoculation from cultures. Smith described the organism as Bacterium malvacearum and reported that the gram-negative cells produced large quantities of bright yellow slime.

In 1928, Massey (35) described the action of the pathogen in the intercellular spaces of host leaves, and reported that the bacteria spread through the host tissue in viscous slime. In the process, walls and middle lamellae of the host cells were dissolved. Massey first hypothesized that the massive tissue destruction was a result of bacterially produced enzymes which he

categorically termed "cytases". Until 1932, all of the histological information on angular leaf spot indicated that vascular tissue was not attacked, even during the severest disease conditions. However, Bryan (7) described an atypical condition in which bacteria actively invaded vessels and phloem of diseased leaves and petioles causing a systemic pathology. The typical "angular" lesions were not formed.

The first comprehensive histological study of angular leaf spot was published by Theirs and Blank (51) in 1951. This study was unique for the time because it was the first to compare histological disease responses of resistant and susceptible plants. The resistance on which the study was based was due to the single gene $B_7$, which confers an intermediate level of resistance. The authors concluded that there were no anatomical differences in the responses of resistant and susceptible plants to angular leaf spot except for lesion size and pathogen populations. Resistance was suggested to be due to physiological factors rather than morphological or functional ones, and the destructive action of the bacteria was again attributed to "bacterial cellulases". No isolation or biochemical confirmation of the presence of cellulytic enzymes was attempted.

Resistance to bacterial blight, the contemporary term for angular leaf spot, has been assigned to a set of "B" (blight) genes that confers resistance, and at least sixteen have been designated (32). These genes occur

naturally throughout the various species of the genus Gossypium. Eight genes are found in the cultivated tetraploid Gossypium hirsutum L. Two additional genes are found in G. hirsutum var. punctatum (5). Single genes in cotton have traditionally conferred blight resistance, and to overcome this resistance, a single point mutation to virulence is all that was required of the pathogen (38). Breeding lines and cultivars are now available that are not attacked by any of the known seventeen races of X. malvacearum (24,3). This immunity has maintained itself for several years at experiment stations in major cotton growing areas of the world (6). Immunity appears to be due to at least two major dominant "B" genes, a recessive "B" gene, and probably additional minor polygenes. To overcome the immunity, a three point mutation as well as dominance over the minor genes would have to occur in the pathogen genome; an unlikely event (38).

Hypersensitivity in plant-bacteria systems is a relatively recent concept. It was proposed by Muller (36) and defined by Klement and Goodman (28) as being a defense mechanism of plants that occurs in a relationship where the host and pathogen are incompatible. Host tissue necrosis rapidly occurs leading to the physical immobilization of the pathogen. The first report of a rapid host response in plant tissue challenged with incompatible bacteria appeared in 1961 when Klement and Lovrekovich (30) observed browning within 24 hours in inoculated areas of bean pods. The

following year they published a quantitative study on the same phenomenon and reported that a hypersensitive response occurred in bean pods when bacterial cell suspensions greater than $10^4$/ml were used as inocula (31). The results were obtained when a Pseudomonas and two Xanthomonas species were used individually; none of which were pathogenic on beans, but were virulent on other plants. No response was observed when a non-plant pathogen (ie. Escherichia coli (Migula) Castellani and Chalmers) were used. They concluded that the phenomenon was not due to a direct action of the bacteria, but to a reaction of the plant tissues, and described the response as a post-infection defense mechanism. Klement et al. (27) provided additional information on the hypersensitive reaction when they produced a rapid necrosis in tobacco leaf panels by inoculating with Pseudomonas pisi Sack., a pathogen on pea plants. A technique for rapid, uniform inoculation of tobacco leaf panels was employed using a needle equipped syringe (26). The needle was inserted into the mesophyll parenchyma and the intercellular spaces were infiltrated with the bacterial suspension. Individual leaf panels were inoculated in this manner which provided a simple, rapid means for assaying host reactions to various levels of inoculum. Inoculated areas became necrotic within 12 to 24 hours following infiltration with bacterial suspensions ranging from $10^5$ to $10^8$ cells/ml. These concentrations are greater than pathogen populations encountered in nature,

and he reported that the macroscopic morphology of the necrotic areas was dependent upon inoculum concentration. The higher inoculum concentrations produced confluent necrosis while low concentrations produced spotted necrosis. A quantitative study of the same phenomenon was published by Turner and Novocky (52) who sectioned tobacco leaf tissue inoculated with $5 \times 10^3$ bacterial cells/ml and observed randomly located dead host cells within two hours. The observations were made in sections of fixed and embedded tissue stained with toluidine blue. They further assayed cell death directly in fresh tissue by floating leaf sections in a solution of Evan's blue (12), a vital stain that differentiates dead cells. Conclusions were that there was a random distribution of bacteria in leaf intercellular spaces and that a minimum ratio of one pathogen cell to one plant cell was sufficient to cause host cell death. Recently similar results were reported to occur in cotton (10). A one to one correspondence was demonstrated between random dark cells in fresh tissue and necrotic cells in sectioned material taken from leaves inoculated with low inoculum levels.

Stall and Cook (50) reported hypersensitivity in pepper following inoculation with Xanthomonas vesicatoria (Doidge) Dows. The authors observed a confluent necrosis within 24 hours following injection with high concentrations of bacteria. Observations of sectioned material revealed collapse of mesophyll tissue with gumming in the

intercellular spaces. They did not observe these symptoms in tissues inoculated with suspensions containing less than $10^5$ bacteria/ml.

Ultrastructural studies covering various aspects of compatibility and incompatibility in plant-bacteria systems have been performed on pepper (43), bean (46), apple (15), and tobacco (18). Much of the available information concerns the incompatible relationship of tobacco and P. pisi. Goodman and Plurad (18) studied ultrastructural changes in tobacco leaves undergoing the hypersensitive reaction and reported dramatic changes in major organelles. There findings were that widespread membrane damage occurred, and they reported that the plasmalemma, tonoplast, bounding membranes of chloroplasts and mitochondria as well as the external membranes of microbodies were profoundly deranged within 7 hours. The damage eventually extended to the cytoplasm and ground substance of organelles and coincided with tissue collapse. In a later paper, Goodman et al. (17) reported a unique phenomenon in which the host cell walls reacted to the presence of incompatible bacteria. They reported that the outer cell wall cuticle became detached and formed a covering over the bacterial cells. The same response was not observed when compatible bacteria were injected. The authors suggested that this enveloping phenomenon was a mechanism by which the host cells immobilized pathogen cells. Similar results were reported in tobacco by Sequeira et al. (44). They further observed

that heat-killed cells were enveloped in the same manner as avirulent cells, but without host cell collapse. A second inoculation 24 hours later with living bacteria failed to produce necrosis. The authors were unable to detect host wall disruption with the secondary bacteria and they concluded that prevention of necrosis appeared to be related to the lack of bacterial attachment to host walls.

Physiological studies on the hypersensitive reaction in tobacco have supported the ultrastructural studies particularly with respect to membrane damage. Several of these studies have shown that electrolyte leakage occurs in tobacco infiltrated with P. pisi (13,14). These studies correlate the onset of electrolyte leakage with ultrastructural membrane disruption. Huang et al. (23), published direct evidence that an alteration of the structural proteins in extracted thylakoid membranes of tobacco leaf cell chloroplasts was caused by incompatible bacterial pathogens.

Studies on apple shoots inoculated with incompatible bacteria indicate findings similar to those in the tobacco system. In a 1968 paper, Burkowicz and Goodman (8) reported that tissue browning coincided with electrolyte leakage, and in a subsequent paper (15) they presented electron micrographs that indicated disorganization of chloroplasts, degeneration of mitochondria and microbodies as well as general disorganization of other subcellular organelle membranes. In a recent paper, Goodman et al. (16)

reported that incompatible bacteria were immobilized in vessels of apple petioles while compatible bacteria were not immobilized. Horino (22) presented similar results in resistant and susceptible rice inoculated with Xanthomonas oryzae (Ureda.) Dows. The bacteria were enveloped with an electron-dense material in the vessels of the resistant cultivar, whereas the bacteria multiplied in the susceptible plant vessels and surrounded themselves with electron-transparent products of their own origination. These phenomena may be related to the immobilization response reported to occur in tobacco (17,44).

In another study, Sigee and Epton (46) dealt with the ultrastructural aspects of the hypersensitive reaction in red Mexican bean plants (Phaseolus vulgaris L.) when inoculated with Pseudomonas phaseolicola (Burk.) Dows. As in Theirs and Blank's work, the authors studied susceptible and resistant plants challenged with virulent bacteria. They reported that rapid and limited cell death occurred in both resistant and susceptible plants, but cells continued to die in the resistant host. Mesophyll cells in the resistant leaves showed degenerative changes in the organelles as well as a decrease in the frequency of free ribosomes. The authors equated watersoaking with a loss of cytoplasmic fluid from these damaged cells. In an earlier paper the same authors reported that the pathogen cells responded differently in the susceptible and resistant hosts (45). In the resistant host the bacterial

nuclear regions degenerated and ribosomal aggregations developed while in susceptible leaves, more clearly defined nuclear regions and aggregations of ribosomal cytoplasm developed. In addition, the bacteria produced vesicles which ruptured liberating their contents in the intercellular spaces of the host.

An unpublished dissertation by Sasser (43) contains the only ultrastructural study reviewed on the hypersensitive reaction in pepper (Capsicum annum L.). As in tobacco, Sasser also demonstrates the close relationship of electrolyte leakage and the disruption of vital cell organelles with physiological and structural data.

The natural question of what agent(s) are responsible for triggering a hypersensitive reaction and whether they are of host or pathogen origin has been studied from several angles. Klement and Goodman (29) observed that membrane damage in tobacco cannot be produced with heat-killed incompatible bacteria, fragmented bacteria or bacterial culture filtrates, and that the hypersensitive reaction has a minimum induction time of only 15-20 minutes. Novocky et al. (37) reported that hypersensitivity could be prevented in tobacco by prior inoculation with low concentrations of living, incompatible bacteria and further that the hypersensitive reaction was light dependent. The protection effect was extended to surrounding untreated tissue. Lonzano and Sequeira (34) reported that the hypersensitive reaction induced by infiltration of tobacco

leaves with cell suspensions (5 X $10^9$ cells/ml) of race 2 isolates of Pseudomonas solanacearum Sm. was prevented by prior infiltration (18 hours) with a dilute suspension of heat-killed cells. A similar protective effect was obtained following preinoculation with heat-killed cells of other plant pathogens, but not with E. coli.

Stall and Cook (49) were able to attenuate the hypersensitive reaction in pepper by pretreatment with a virulent strain of X. vesicatoria. The same effect was obtained with heat-killed cells. However, the protection was only extended against other species of Xanthomonas. A hypersensitive reaction could still be produced by injecting an avirulent Pseudomonas species.

In a recent unpublished report Kiraly (25) claimed that the hypersensitive reaction in tobacco is not necessary to suppress an incompatible pathogen. By prior injection with albumin the hypersensitive reaction was suppressed and the growth rates of incompatible and compatible pathogens were monitored in the host. Data indicated that growth of the incompatible pathogen was inhibited. This suggests inhibition is caused by an unidentified factor; possibly a phytoalexin.

Other biochemical work with immune cotton challenged with X. malvacearum indicates the presence of a phytoalexin which is not produced in the susceptible host (11). More information on phytoalexins should help answer the question

of whether the hypersensitive reaction is an artifact or a real component of resistance mechanisms.

# CHAPTER III

# METHODS AND MATERIALS

Because of the many deviations from and alterations of established procedures, this chapter is extended and detailed. However, others may find the descriptions useful, should similar work be done in the future.

## Plant Growth Conditions

Acid delinted seeds of the immune cultivar Im 216 and susceptible cultivar Ac 44 were obtained for all experiments. No chemical seed protectants were employed during the course of the investigation. Three seeds per pot were planted 1 inch deep in 6 inch diameter clay pots. Potting soil was prepared by mixing local soil, obtained from a field under cotton cultivation, with vermiculite and peat (3:1:1,v/v). All pots and soil were sterilized by autoclaving at 121$^{o}$C at 15 p.s.i. for 30 minutes.

Following planting, each pot was watered with about 250 milliliters of water and incubated in a growth chamber set to regulate a 12 hour light (incandescent and fluorescent at 43,000 lux) and dark period with a temperature of 32$^{o}$C (90$^{o}$F) in the light and 18$^{o}$C (65$^{o}$F) in the dark. The seedlings emerged from the soil within

6 days, and the first foliage leaves appeared within 10-14 days, the state of maturity used in all experiments.

## Bacterial Culture Methods

Race 1 cultures of X. malvacearum were isolated from infected field leaves in 1975 and 1976. The isolates were designated as R1-75 and R1-76 respectively. Virulence and identity of the races were verified by reactions observed on differential host plants (24).

Pieces of infected leaf material were ground in sterile water with a mortar and pestle. The resulting suspension was streaked on nutrient agar plates and incubated at room temperature. Distinct yellow colonies appeared within 2 to 3 days, and single colony transfers were made to potato-carrot-dextrose agar slants for stock culture maintenance.

Inoculum increase was prepared by loop transfer from stock cultures to nutrient broth in 50 ml Erlenmeyer flasks. The liquid cultures were incubated at 30°C in shake culture for 24 hours before harvesting the cells.

Inoculum was prepared by centrifuging the turbid broth at 12,000 X G for 10 minutes at 28°C. The supernatant was discarded and the resulting pellet resuspended in sterile distilled water The suspension was then adjusted spectrophotometrically with distilled water to an absorbance of 0.05 at 600 nm. Plate counts have shown that there are about $5 \times 10^8$ viable bacteria per ml at this absorbance.

## Host Leaf Inoculation

Cotyledon leaves of host plants were used for all experiments and were inoculated by injection (26). Inoculum was drawn into a 1 cc tuberculin syringe fitted with a ½ inch #30 needle. The needle was inserted into the mesophyll parenchyma and inoculum was infiltrated by gently depressing the plunger. The resulting watersoaked area appeared dark green. By watching the progressive water-soaking it was possible to control the extent of inoculation. Typically half-cotyledons were inoculated in this manner.

## Electron Microscopy Methods

### Preparation of Fixatives

A double fixation was employed for all tissues prepared for electron microscopy. The primary fixative, gluteraldehyde, was buffered in two ways: To sodium cacodylate, (0.27 M) adjusted to pH 7.3 with 1 N HCl, was added 8% aqueous highly purified E.M. grade gluteraldehyde (Polysciences, Warrington, Pa.). The final concentration of gluteraldehyde was 3%. The formulation is as follows; 10 ml of 8% gluteraldehyde, 30 ml of 0.27 M cacodylate buffer (pH 7.3), and 0.02 g calcium chloride ($CaCl_2$). This fixative is reported by Sabatini *et al*. (42) to yield excellent results, and avoids the presence of extraneous phosphates encountered with other buffers. The addition

of calcium chloride is reported to help preserve nuclear structure, phospholipids and cytomembranes.

Gluteraldehyde was also buffered with Sorenson's phosphate buffer. Stock solutions of 0.1 M $Na_2HPO_4.2H_2O$ and 0.1 M $KH_2PO_4$ were prepared and portions were titrated to reach a pH of 7.3. To 10 ml of this buffer was added an equal volume of 8% aqueous E.M. grade gluteraldehyde to obtain a 0.05 M buffered 4% gluteraldehyde solution. Calcium chloride was not added to this fixative.

Washing Buffers

Washing buffers are used following gluteraldehyde fixation to remove unbound gluteraldehyde which readily reacts with osmium tetroxide ($OsO_4$) solutions to form electron-dense precipitates. They are adjusted with glucose to match the osmolarity of the gluteraldehyde in the primary fixative. Cacodylate washing buffer was prepared as follows; 60 ml of 0.27 M cacodylate buffer (pH 7.3), 140 ml distilled water and 12.3 g sucrose. Phosphate washing buffer was prepared similarly; 200 ml of 0.1 M phosphate buffer (pH 7.3) and 20.4 g sucrose. These and all buffers were stored at $4^{o}C$.

Osmium Tetroxide Fixing Solution

A buffered osmium tetroxide solution was used as a secondary fixative with gluteraldehyde. Treatment with osmium tetroxide was necessary to stabilize lipids in

membrane structures and microbodies. It also has been reported to react with proteins. Post-fixation with osmium tetroxide was particularly necessary with plant materials since Hayat (19) reported that Hill activity occurred in chloroplasts following fixation with gluteraldehyde.

Ultrapure E.M. grade osmium tetroxide crystals were added to sufficient distilled, deionized water to make a 2% stock solution. Equal volumes of osmium tetroxide solution and either 0.27 M cacodylate or 0.2 M phosphate buffer were mixed to prepare a 1% osmium fixing solution. These solutions were stored in the dark at $4^{o}C$ in air-tight containers until ready for use.

Dehydrating Alcohols

Ethanol solutions of 20, 30, 50, 70, 90 and 95% were prepared for sequential dehydration of fixed tissues. Absolute ethanol and propylene oxide were used as final dehydrants.

Embedding Resins

Two resin formulas were evaluated in this study. An old formulation, Araldite 502, commonly known as "American Araldite" (Ladd Research, Burlington, Vt.), and a more recent formulation by Spurr (48) (Polysciences, Warrington, Pa.) were used to embed dehydrated tissues. The Araldite components were prepared as recommended by Hayat (20). The components were mixed as follows: 27 ml of Araldite

502, 23 ml of DDSA (dodecenyl succinic anhydride) and 0.75 g of DMP-30 (tri-dimethyl amino methyl phenol). These components were measured gravimetricaly because of their thick viscosity. Following thorough mixing, the resin was stored in a tightly capped bottle at -7°C.

Spurr's resin is popular with botanical workers because of the low viscosity of the mixture and consequent ease of infiltration. Several formulations are provided with the resin kit which enables the worker to vary the hardness of the cured plastic. The following formulation, which is midway between the "firm" and "hard" formulations was found to be satisfactory for cotton leaf tissue; 10 g VCD (vinylcyclohexene dioxide), 5 g DER (diglycidyl ether of polypropylene glycol), 26 g of NSA (nonethyl succinic anhydride) and 0.4 g of DMAE (dimethylaminoethanol). These components were mixed and stored in an airtight container at -7°C.

Preparation of Stains

Two heavy metal stains were used in this study. Lead citrate was evaluated for cotton tissue in two preparations. The Reynolds (40) formula was prepared by mixing 1.33 g of lead nitrate ($Pb(NO_3)_2$), 1.76 g of sodium citrate, 30 ml of distilled water and 8.0 ml of 1 N sodium hydroxide (NaOH). This was followed by shaking until the solution cleared. The Venable and Coggeshell (53) formula was also used, and it was prepared by mixing 100 ml of distilled

water, 0.4 g of lead citrate and 1.0 ml of 10 N sodium hydroxide followed by shaking until the solution cleared.

Uranyl acetate was prepared as 2% solutions (w/v) in either distilled water or 50% ethanol. All stains were stored in tightly capped bottles at room temperature in the dark. The shelf life of the lead citrate stains was about one month and about six months for the uranyl acetate stains.

Preparation of Tissue

Tissue segments no larger than 1 $mm^2$ were trimmed in cold ($4^oC$) fixative from excised pieces of inoculated cotyledons. If the tissue pieces floated due to trapped intercellular air, gentle aspiration was applied to cause the samples to sink in the fixing solution. The fixation period was maintained for at least 2 hours at $4^oC$. At the end of this period the fixing solution was removed by aspiration and replaced with cold washing buffer. The washing buffer was changed three times with the tissue left in the last change overnight. The washing buffer was replaced with buffered osmium fixing solution, and post-fixation was maintained for 4 hours at $4^oC$. At the end of the post-fixation the osmium tetroxide was drawn off and replaced with three quick (15 minute) changes of cold washing buffer. The tissue pieces were then dehydrated with successive changes of the graded ethanol solutions. Dehydration was done at $4^oC$ until the tissue samples were

in 100% ethanol. The absolute ethanol was replaced with two changes of propylene oxide. Infiltration was accomplished by passing the samples through successively higher concentrations of resin dissolved in propylene oxide until they were in pure resin. Three resin-to-solvent solutions; one to two, one to one and two to one at 1 hour changes were found to be sufficient. The final change in pure resin was left overnight uncapped in a fume hood to allow residual propylene oxide to evaporate and facilitate complete infiltration.

The tissue samples were cast in molds and polymerized in an oven for 8 hours. For Araldite 502, the polymerization temperature was 60°C (140°F) and for Spurr's resin 70°C (158°F).

Sectioning of Embedded Tissue

Blocks were mounted in a microtome chuck and individually rough trimmed, facewise, to a trapezoidal shape. Rough trimming was done free-hand with ethanol-cleaned industrial razor blades. Initially, the trapezoidal face was trimmed to the dimensions of about 1 to 2 mm on the narrow side, and 2 to 3 mm on the broad side.

Glass knives were prepared on a LKB 7800 knife maker using glass strips manufactured by the same company. The strips were cleaned with ethanol prior to breaking. When possible, the knives were broken shortly before use to

avoid dulling of the edge, that occurs during storage. The tissue blocks were mounted in a Sorvall MT-2 ultramicrotome and "faced" with glass knives. At the same time, thick sections (approximately 240 mμ thick) were cut, collected with a wire loop, and transferred to a drop of distilled water on a clean glass slide. The slide was transferred to a 70°C warming plate until the water drop had dried leaving the sections attached to the slide. A drop of Richardson's (41) toluidine blue in borax solution was spread over the sections and warmed for about 30 seconds. Following a distilled water rinse, the slide was dried and a cover slip was mounted over the sections in immersion oil.

After an examination of the sections with a light microscope, the block face was fine trimmed to about one-half of its size so that only critical areas of tissue remained to be thin-sectioned. The block was remounted in the microtome and the machine adjusted to cut thin (less than 90 mμ) silver-gray sections. A fresh knife was used each time a block was sectioned. When several sections had been cut or the knife became too dull to section satisfactorily, the sections were expanded by passing a chloroform soaked toothpick over them. The sections were collected on #300 mesh uncoated copper grids that had previously been cleaned with glacial acetic acid, water and chloroform. Actual collection of the sections was done by the "spoon" method where the grid is first wetted with filtered water, plunged into the water-filled boat and lifted with forceps

beneath the sections. Residual water on the grid was drawn away with the edge of a filter paper disc, and the sections air-dried on the grid.

Staining of Sections

The sections were first stained by placing the grids, section side down, on drops of uranyl acetate in a clean dental wax-lined syracuse dish. The dish was covered and the sections were stained for 1 hour. The grids were removed with forceps and rinsed with a gentle stream of distilled water which removes all unbound uranyl acetate. The grids were quickly blotted with filter paper and transferred to drops of lead citrate. Pellets of sodium hydroxide were arranged around the lead citrate drops to trap any atmospheric carbon dioxide. Since the lead citrate stain has a high pH, it reacts with carbon dioxide to form insoluble carbonates which can contaminate sections. The grids were stained with lead citrate for 30 minutes followed by a rinse with a gentle stream of 0.02 N sodium hydroxide and finally with distilled water. After blotting, the grids were stored in dust-proof containers before examination on the electron microscope. The grids were scanned on a EMU-3G RCA electron microscope. Photographs were made on Kodak #4489 electron image film with a photometer exposure reading of +25 at 100 KV. Film plates were developed for 4 minutes at 20°C (68°F) in D-19 (Eastman Kodak, Rochester, N.Y.) diluted one:two with

water. Prints were made on Kodak polycontrast F single weight photographic paper exposed with a +4 polycontrast filter and developed in dektol (Eastman Kodak, Rochester, N.Y.) diluted one:two.

## Manometric Measurements of Respiration

Oxygen uptake was measured manometrically with a Gibson model G-20 differential respirometer (Gibson Medical Electronics, Middleton, Wisc.). Cotyledons were inoculated as previously described and 1 $cm^2$ discs were removed with a cork borer for respiration studies. Ten discs were removed for each reaction vessel; one disc from each of ten plants. The discs were placed in the reaction vessel along with 0.2 ml of water to maintain humidity. They were arranged symmetrically around the centerwell. A carbon dioxide trap was made by placing 0.2 ml of 5% potassium hydroxide (KOH) in the centerwell, greasing the rim with lanolin, and inserting a folded 2 $cm^2$ filter paper into the alkali. Stand pipes were fitted to the auxiliary openings of the reaction vessel and the main opening was attached to the manometer joint. All ground glass fittings were sealed with anhydrous lanolin and leak tests were routinely run. Each manometer gauge was set at 250 or a beginning point, and after an initial 30 minutes equilibration, the system was closed to the atmosphere and readings were taken at 15 minute intervals.

The respiration assay was performed in two ways: The vessels were initially loaded and monitored for several hours, and alternately the vessels were loaded at time intervals of 2 hours and short readings were taken. The data points were plotted (µl of $O_2$ vs. time) on standard graph paper.

## Oxygen Evolution Measurements

Oxygen evolution measurements were taken with a YSI (Yellow Springs Inst. Co., Yellow Springs, Ohio), biological oxygen monitor equipped with a teflon membrane oxygen electrode. Susceptible and immune plants were inoculated, and ten 0.5 cm leaf discs were removed for each test. Discs from uninoculated and water inoculated control plants were also tested. The discs were placed in the reaction tube along with 3 ml of distilled water and a magnetic stirring device. Nitrogen was bubbled through the reaction water for 2 minutes to expell the dissolved oxygen. The probe was inserted into the tube, care being taken not to unduly agitate the reaction mixture. The reaction tube was then inserted in a circulating 25°C water bath and allowed to equilibrate for 3 minutes. The fluorescent light banks (750 ft. candles of intensity) were then activated and oxygen evolution was recorded on a Beckman recording device set to record one inch per minute at the 100 millivolt setting. Results were expressed in percent saturation per unit of time.

# CHAPTER IV

# RESULTS

## Macroscopic Responses of Inoculated Plants

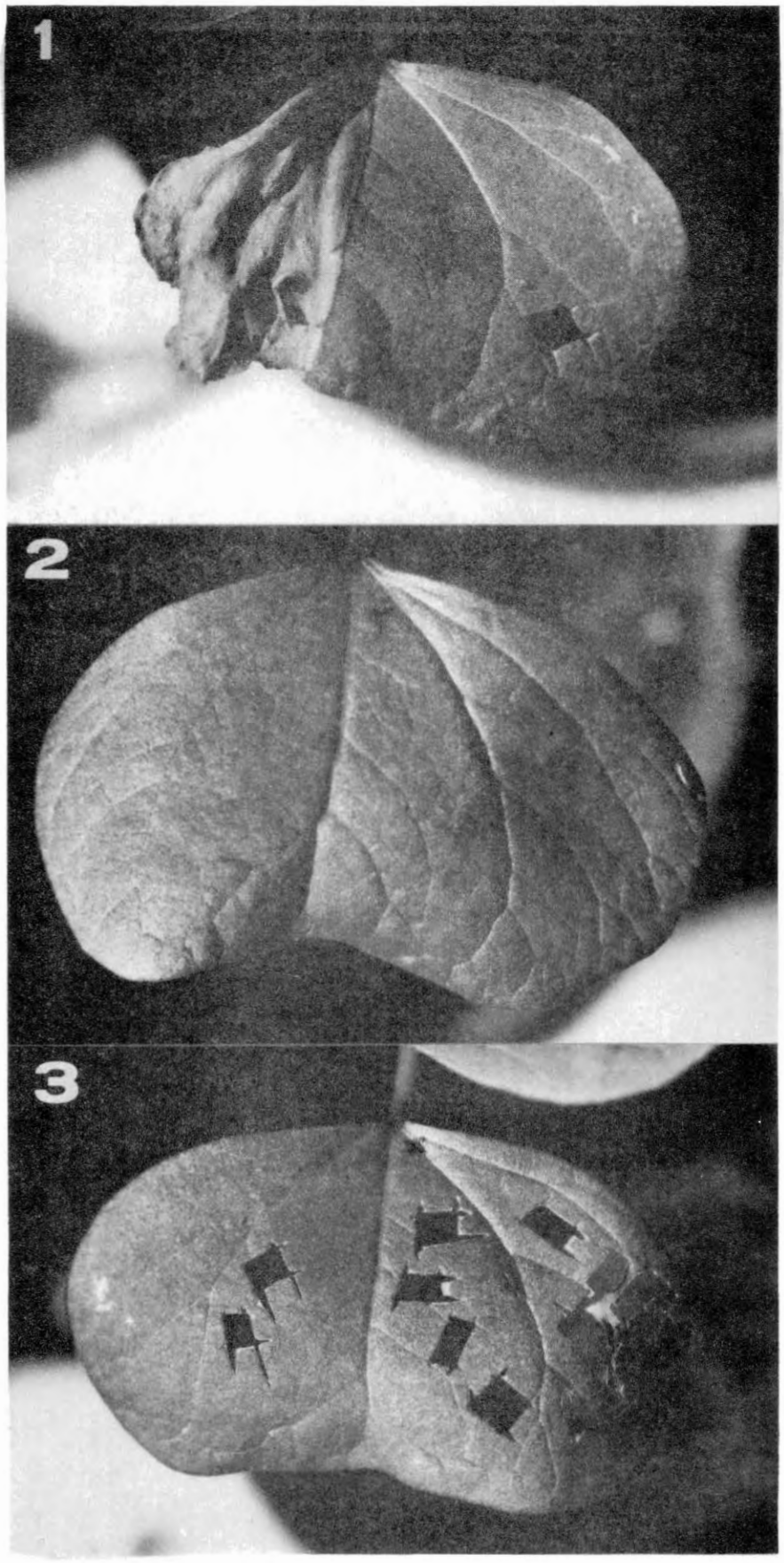
Within 24 hours inoculated portions of cultivar Im 216 became necrotic (Figure 1). This was preceeded by a loss of leaf turgor, a change in color (referred to as "silvering"), and elasticity of the tissue. As is evident in figure 1, only inoculated regions were so affected. Uninoculated regions immediately adjacent to and bordering treated tissues remained healthy for the duration of the experiment. Likewise, controls of uninjected and water-injected leaves remained healthy (Figure 2).

Inoculated leaves of cultivar Ac 44 did not respond with a hypersensitive reaction as did leaves of Im 216. Instead, the Ac 44 leaves remained healthy for the first 24 hours even though they were treated in the same manner as Im 216 (Figure 3). In most cases, Ac 44 leaves remained symptomless for 3 to 4 days when spotted areas of water-soaking appeared. At times, areas of necrosis formed, but these were never confluent, and generally they appeared near inoculation sites, and may have been due to mechanical

Figure 1. An Im 216 Cotyledon Showing Necrosis of the Inoculated Half 24 Hours Following Infiltration. Excised Areas are Where Tissue Samples Were Removed for Histological Purposes.

Figure 2. One of the Control Cotyledons 24 Hours Following Infiltration. The Right Half Was Infiltrated With Water and the Left Half Was Uninfiltrated.

Figure 3. An Ac 44 Cotyledon 24 Hours Following Infiltration With Bacteria. The Right Half Was Inoculated With the Bacterial Suspension and the Left Half With Sterile Water. The Excised Areas are Where Tissue Samples Were Removed for Histological Purposes.


1
2
3

damage. The excised areas in figures 1 and 3 indicate the manner in which tissue samples were taken for histological processing. There were no detectable systemic effects of this type of sampling, and it was preferred to sampling from different leaves because it eliminated possible leaf to leaf physiological variations that might have confounded the results.

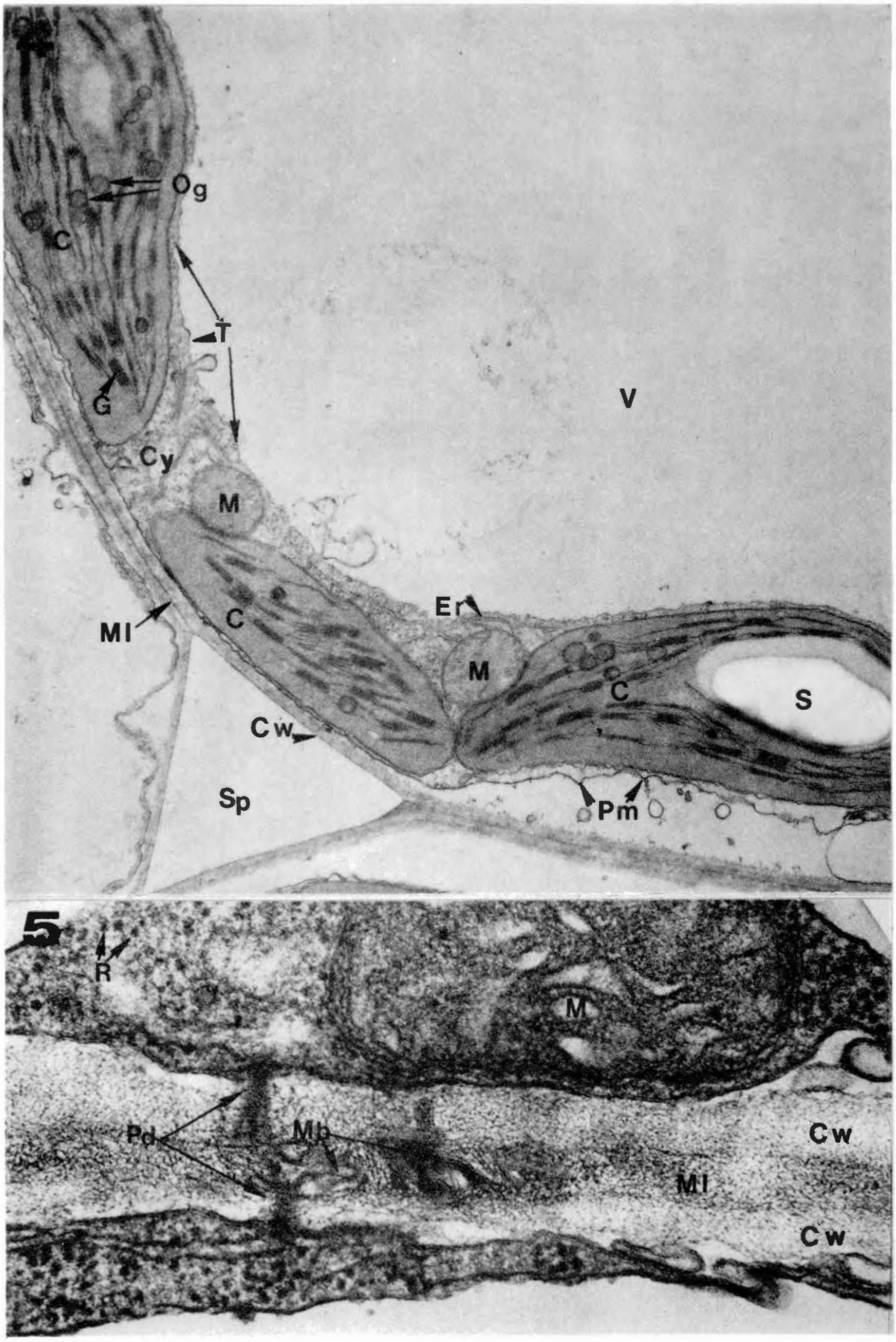
## Electron Microscopy of Inoculated Plants

### Controls

Electron micrographs of mesophyll parenchyma cells resembled descriptions of typical higher plant leaf cells (33). Figure 4 illustrates the components of mesophyll cells in cotton leaves. This electron micrograph was taken from control tissue and is representative of the appearance of cells in uninoculated and water-inoculated leaves fixed with cacodylate-buffered gluteraldehyde. Specimens of phosphate buffer-fixed tissue are not entered here because the results of this type of fixation were generally inferior to the former. Images yielded with a phosphate-buffered gluteraldehyde fixation were generally more granular in appearance than cacodylate-gluteraldehyde fixation, and for this reason cytoplasmic detail suffered in cells fixed with phosphate-gluteraldehyde.

Figure 4. An Electron Micrograph Showing the Components of Typical Cotton Leaf Mesophyll Cells. An Intercellular Space (Sp), is Enclosed by Cell Walls (Cw). The Middle Lamella (Ml), is Indicated Between Adjacent Cells. Within the Cell Wall the Plasma Membrane (Pm), Contains the Cytoplasm (Cy), and Organelles. The Chloroplasts (C), Contain Grana (G), Osmophilic Granules (Og), and Starch Grains (S). Mitochondria (M), and Endoplasmic Reticulum (Er), are Also Evident. To the Inside of the Cytoplasm the Tonoplast (T), Contains the Vacuole (V). X 12,000.

Figure 5. A High Magnification Electron Micrograph Showing Two Plasmodesmata (Pd), Traversing the Cell Walls (Cw), and Middle Lamella (Ml), of Adjacent Cells. Membrane Bodies (Mb), Appear Within the Plasmodesmata Which May Form a Continuum Between the Two Cells. Ribosomes (R), and a Mitochondrion (M), are Indicated in the Upper Cell. X 77,000.


Og
C
T
G
V
Cy
M
Er
Ml
C
M
C
S
Cw
Sp
Pm
5
R
M
Pd
Mb
Cw
Ml
Cw

Cell walls, stained with uranyl acetate, were rendered a medium gray tone. Generally, when walls were sectioned in near cross section, a fibrillar network of cellulose could be resolved running parallel to the long axis of the wall. A thin electron-dense covering made up the outermost layer of the cell wall. The author refers to this structure as the primary cell wall, however it has been described by others as cuticular (17). This primary cell wall appeared to be continuous with the middle lamella of adjoining cells. Plasmodesmata were frequently found in areas where adjoining cells were closely appressed. In some cases they appeared to contain a membrane continuum of the two adjacent cells (Figure 5).

To the inside of the cell wall was found the plasma membrane or plasmalemma. This and all cell membranes stained distinctly dark, due to the action of osmium tetroxide fixation of lipids and their affinity for lead citrate stains. In high magnification micrographs, membrane structure appeared as a trilayer; dark, light and dark bands. This appearance was only observed when a membrane was cut exceedingly thin in or near cross section. The integrity of the plasma membrane was an indicator of general cell viability.

The cytosol with its organelles and biosynthetic apparatuses were contained by the plasmalemma. Many ribosomes were seen as spherical black dots dispersed throughout the cytoplasm. At times they could be seen in

linear arrangement on the endoplasmic reticulum network that was also found throughout the cytosol. Golgi bodies were encountered infrequently, and were as described by Ledbetter and Porter (33). Mitochondria were also numerous and their preservation and normal structure were taken as an indication that fixation was successful. Mitochondria are double membrane organelles with an outer bounding membrane and an inner membrane that forms vesicles throughout its matrix. Deviations from this structure indicate that the cell is in stress.

Chloroplasts were large and numerous in cotton leaf mesophyll cells. Like mitochondria, they are double membrane structures. In cotton mesophyll cells the chloroplasts were elongated and contained a granular matrix with numerous stacks of thylakoid lamellae, osmophilic granules, and often large starch grains. Chloroplasts, although not generally recognized to be as sensitive to stress as mitochondria, were found to be more sensitive to disruption in the Gossypium-Xanthomonas interaction.

Nuclei, which are very large organelles were encountered very infrequently. They typically appeared granular and often were in section with the nucleolus which appeared as a dark, electron-dense mass in the center of the nucleus. The nuclear envelope was typical of other bounding membranes in the cell.

The tonoplast-bound vacuole occupied by far the greatest volume in the cell. In fact, the wall-cytoplasm

continuum appeared as little more than a highly structured network against open areas of the intercellular spaces and the vacuoles.

Inoculated Im 216 Leaves

Immediately following inoculation, bacterial cells could be found dispersed throughout the intercellular spaces suspended in the inoculum water. This water was quickly lost to the atmosphere and partially absorbed by the plant cells. As the intercellular water cleared, (usually within 20 to 30 minutes) the suspended bacteria were deposited on the outer surface of the host cell walls (Figure 6). As the bacteria neared the host cell, some unidentified factors caused a disruption on the host cell wall surface (Figure 7). Cellulose fibrils loosened and gathered about a central clearing that was formed directly below the bacterial cells (Figure 8). The cause for this phenomenon is not known but may be due to metabolic products of the pathogen. As the bacteria came in contact with the host cell, the disrupted wall appeared to peel away releasing numerous fine strands of wall material that surrounded the pathogen cells (Figure 9). Eventually the components released from the host cell wall entirely covered the bacteria and formed what appeared to be a pustule (Figures 10 and 11). These pustules always appeared quite turgid and contained from one to five bacterial cells. No more than one pustule was ever

Figure 6. Bacterial Cells (Xm) Shortly Following Infiltration, in the Intercellular Space (Sp), of an Inoculated IM 216 Leaf. The Host Cell Wall (Cw), Appears Intact and the Cytoplasmic Contents Chloroplasts (C), Mitochondria (M), Appear Normal. X 40,500.

Figure 7. A Bacterial Cell (Xm), Very Near an IM 216 Cell Wall (Cw), Showing a Disrupted Area (D), on Both Sides of the Bacterium. X 58,500.

Figure 8. Bacterial Cells (Xm), and a Damaged IM 216 Cell Wall (Cw). Disrupted Areas (D), are Clearly Shown About a Central Clearing Directly Below the Bacterium. The Disruptions (D), Appear to be Cell Wall Fibrillar Material (F). X 77,000.

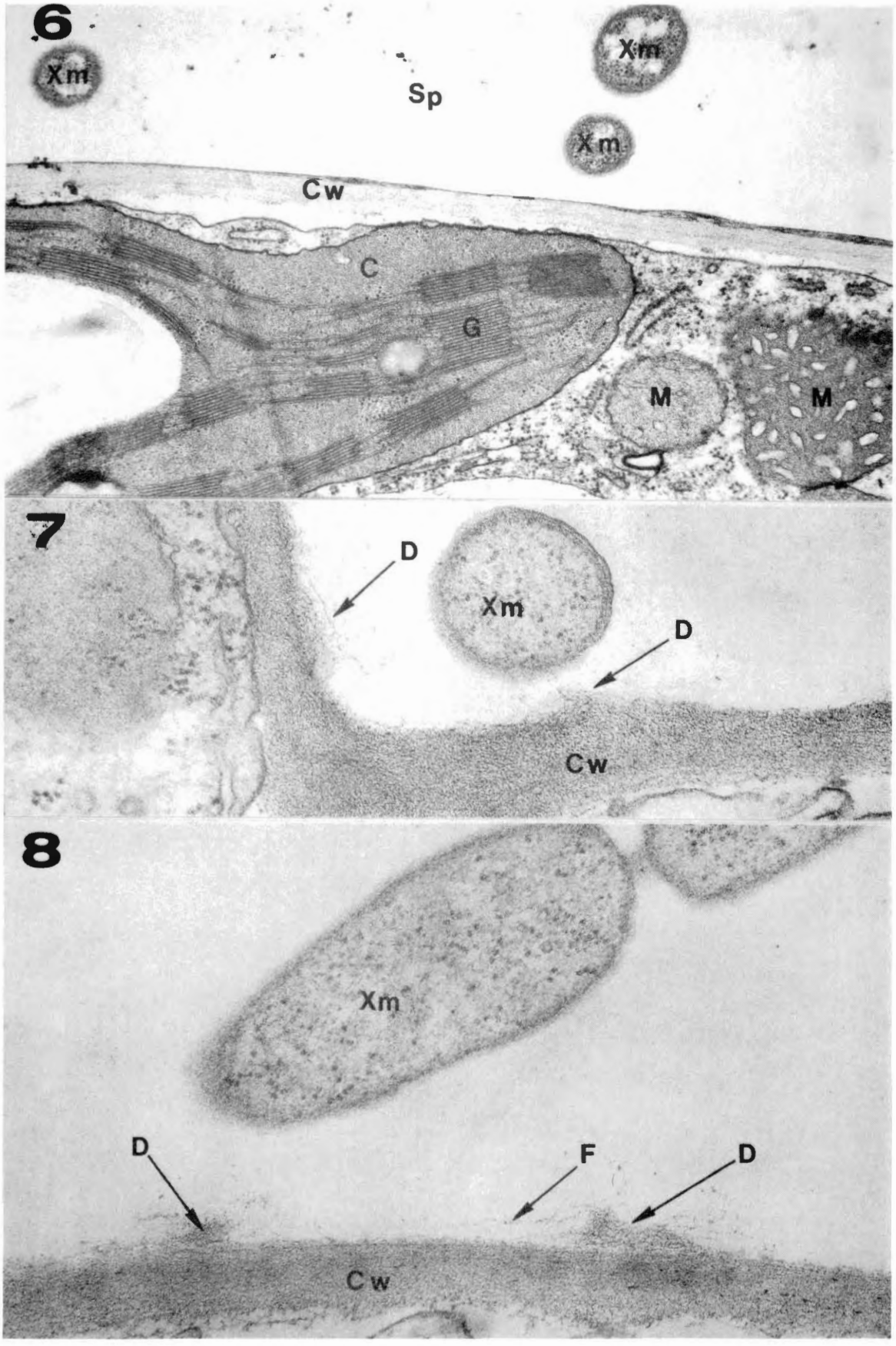
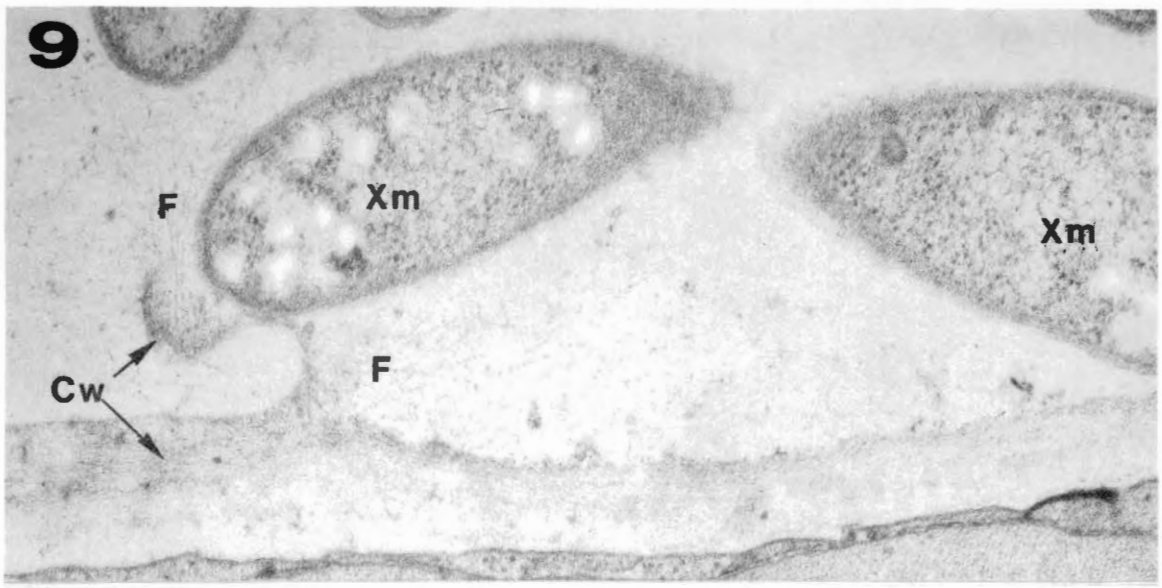

6
Xm
Sp
Xm
Xm
Cw
C
G
M
M
7
D
Xm
D
Cw
8
Xm
D
F
D
Cw

Figure 9. Bacterial Cells (Xm), in Contact With a Strip of Im 216 Cell Wall Material (Cw), That Appears to be Peeling Away From the Substrate. Whispy Strands of Fibrillar Material (F), are Apparently Released in the Process. Fixed at 8 Hours Following Infiltration. X 58,500.

Figure 10. A Pustule (P), Containing Four Bacterial Cells (Xm). The Pustule Appears Turgid and Filled With Whispy Fibrillar Material (F), Which is Also Seen Extending Beyond the Pustule. The Im 216 Cell Wall (Cw), Appears Damaged (D), as a Result of Pustulation. Fixed at 10 Hours Following Infiltration. X 77,000.


9
F
Xm
Xm
Cw
F

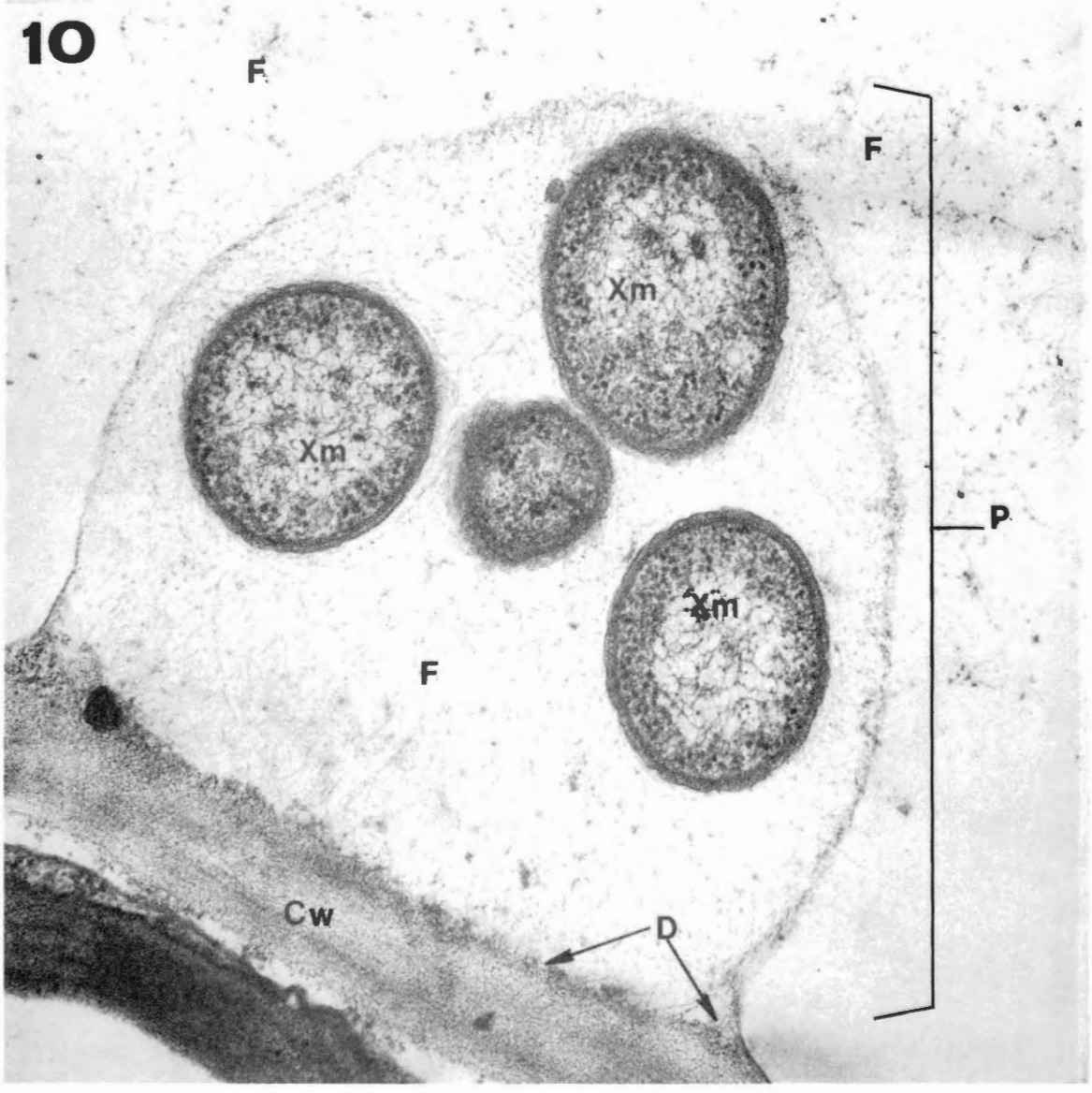
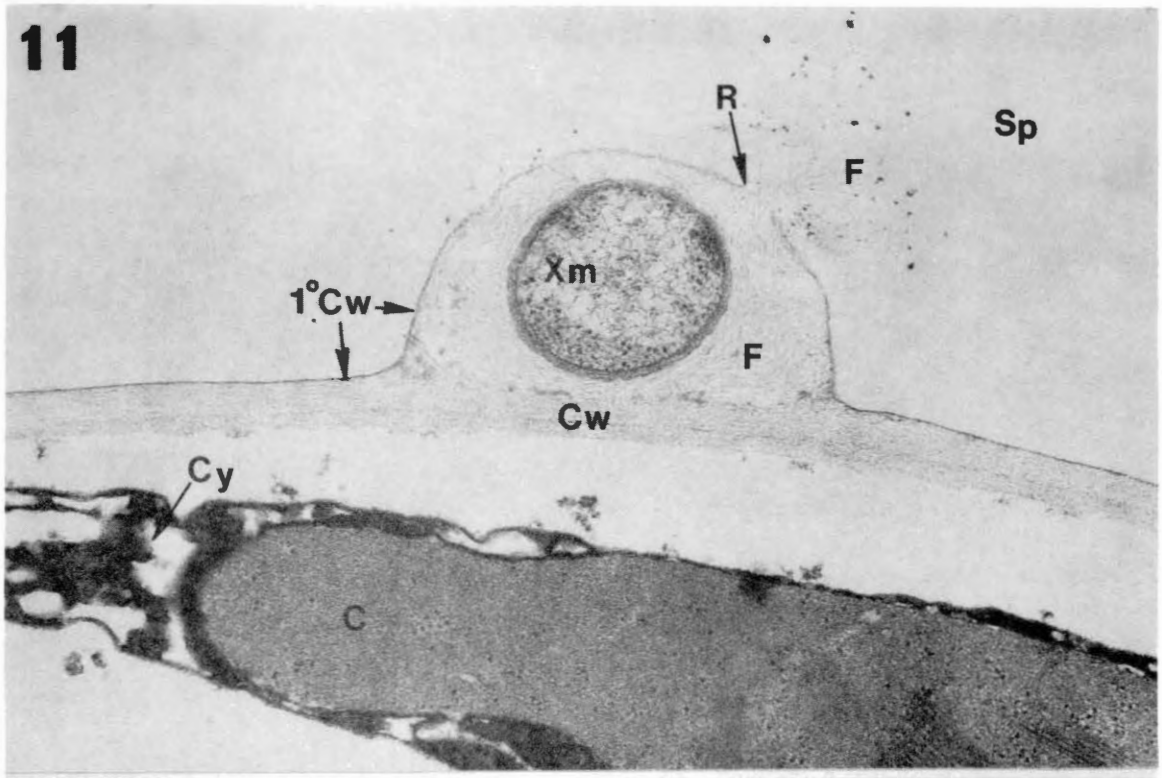
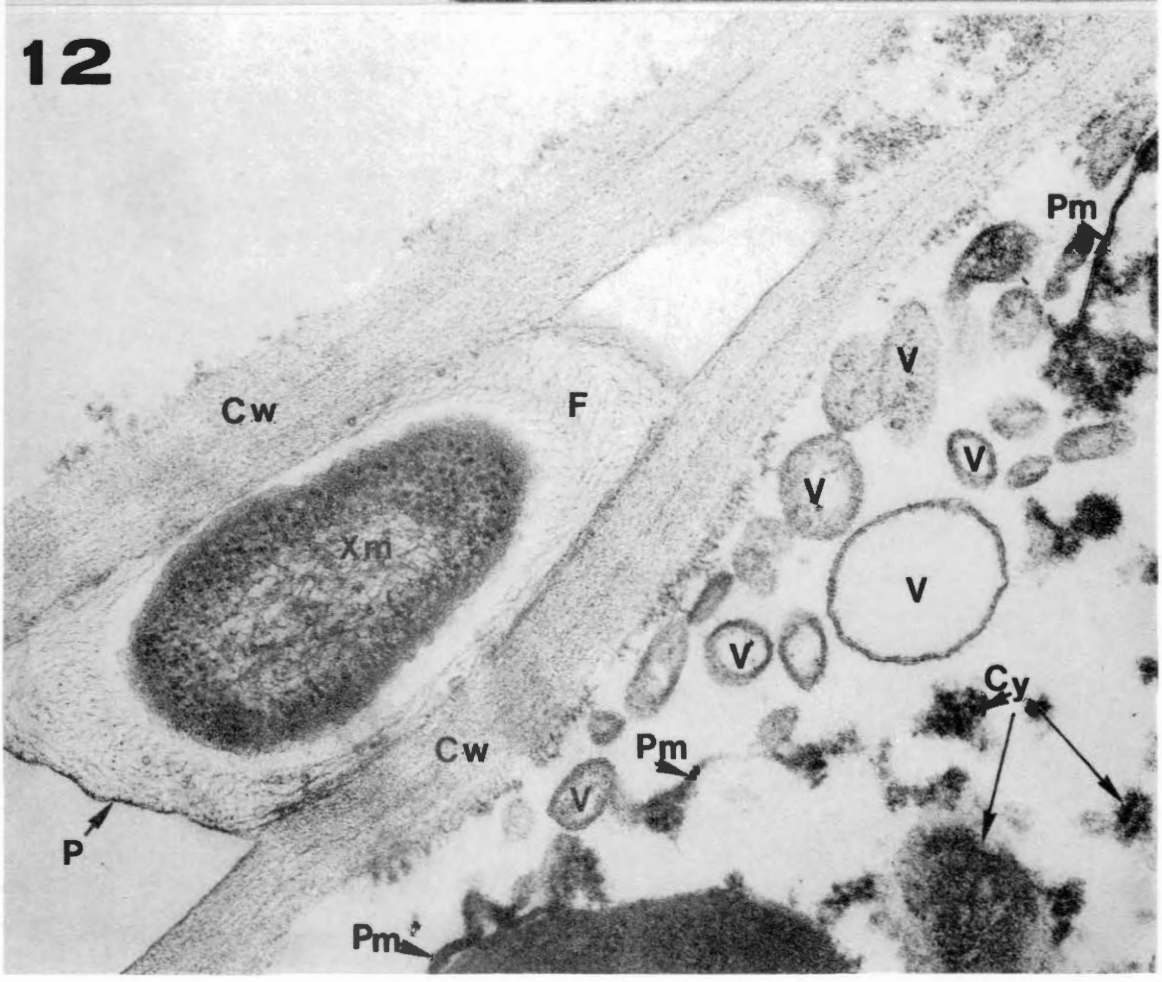

10
F
F
Xm
Xm
Xm
P
F
Cw
D

Figure 11. A Pustule Containing a Single Bacterial Cell (Xm). The Primary Cell Wall ($1^{o}$Cw), is Distinct and Continuous With the Pustule. A Rupture (R), is Seen With Fibrillar Material (F), Escaping Into the Intercellular Space (Sp). Necrotic Im 216 Cytoplasm (Cy), Appears in the Cell Along With a Granular Chloroplast (C). Fixed at 10 Hours Following Infiltration. X 77,000.

Figure 12. A Pustule (P), is Seen on Im 216 Cell Walls (Cw), of Adjacent Cells. The Plasma Membrane (Pm), Appears Broken in Several Areas. Numerous Vesicles (V), Have Apparently Formed as a Result of the Breakages. Cytoplasm (Cy), Appears Coagulated and Clumped. Fixed at 10 Hours Following Infiltration. X 77,000.


11
R
Sp
F
Xm
1°Cw
F
Cw
Cy
C


12
Pm
V
Cw
F
V
V
Xm
V
V
Cy
Cw
Pm
V
P
Pm

observed on a host cell. Apparently pustules can form in a short period of time since they were found in tissue fixed at 2 hours following inoculation. However, various stages of pustule formation were seen in tissue fixed up to 12 hours following inoculation. Other bacteria were found on host cell walls with no apparent response to their presence. The reason for this is unclear. However, it may be that a pustule can form only at specific sites on the cell surface (44).

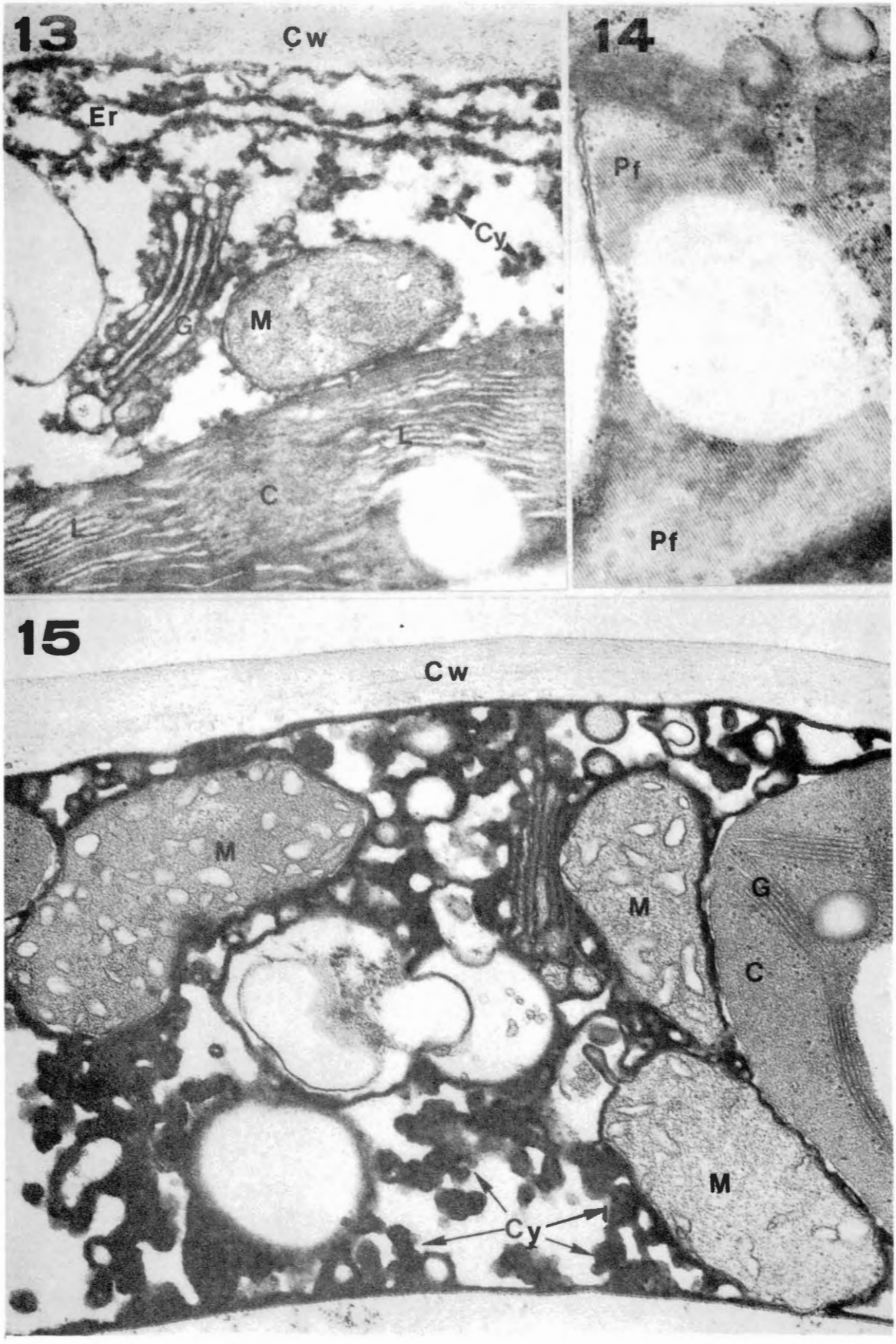
The plasma membrane was frequently found broken in the vicinity of bacteria in pustules (Figure 12). In the areas where breaks in the plasmalemma occurred, numerous single membrane bound vesicles were found. These vesicles are presumably of plasma membrane origin and formed as a result of the break.

When the plasma membrane loses integrity, the cytoplasm and its contents must inevitably also lose integrity and functionality. The beginning of this process is clearly evident in figure 12 where cytoplasmic ribosomes are seen in clumps of electron-dense material that appears to be in stages of coagulation. This condition was observed as early as 6 hours following inoculations and was apparently preceeded by breakage of the plasma membrane. Subsequently, the cytoplasm became heavily condensed and electron-opaque (Figure 13). Remnants of endoplasmic reticulum and major organelles were found in various stages of disorganization.

Figure 13. Necrotic Cytoplasm (Cy), of a Damaged Im 216 Cell Containing a Golgi Apparatus (G), a Mitochondrion (M), Endoplasmic Reticulum (Er), and a Damaged Chloroplast (C), With Separated Thylakoid Lamellae (L). Fixed at 14 Hours Following Infiltration. X 40,500.

Figure 14. The Crystalline Structure of Phytoferritin (Pf), in a Damaged Im 216 Chloroplast. X 117,000.

Figure 15. Highly Coagulated Cytoplasm (Cy), of a Damaged Im 216 Cell Containing Numerous Organelles. Mitochondria (M), do not Appear Greatly Disrupted. A Granular Chloroplast (C), Appears on the Right With Faint Remains of Grana (G). Fixed at 14 Hours Following Infiltration. X 77,000.

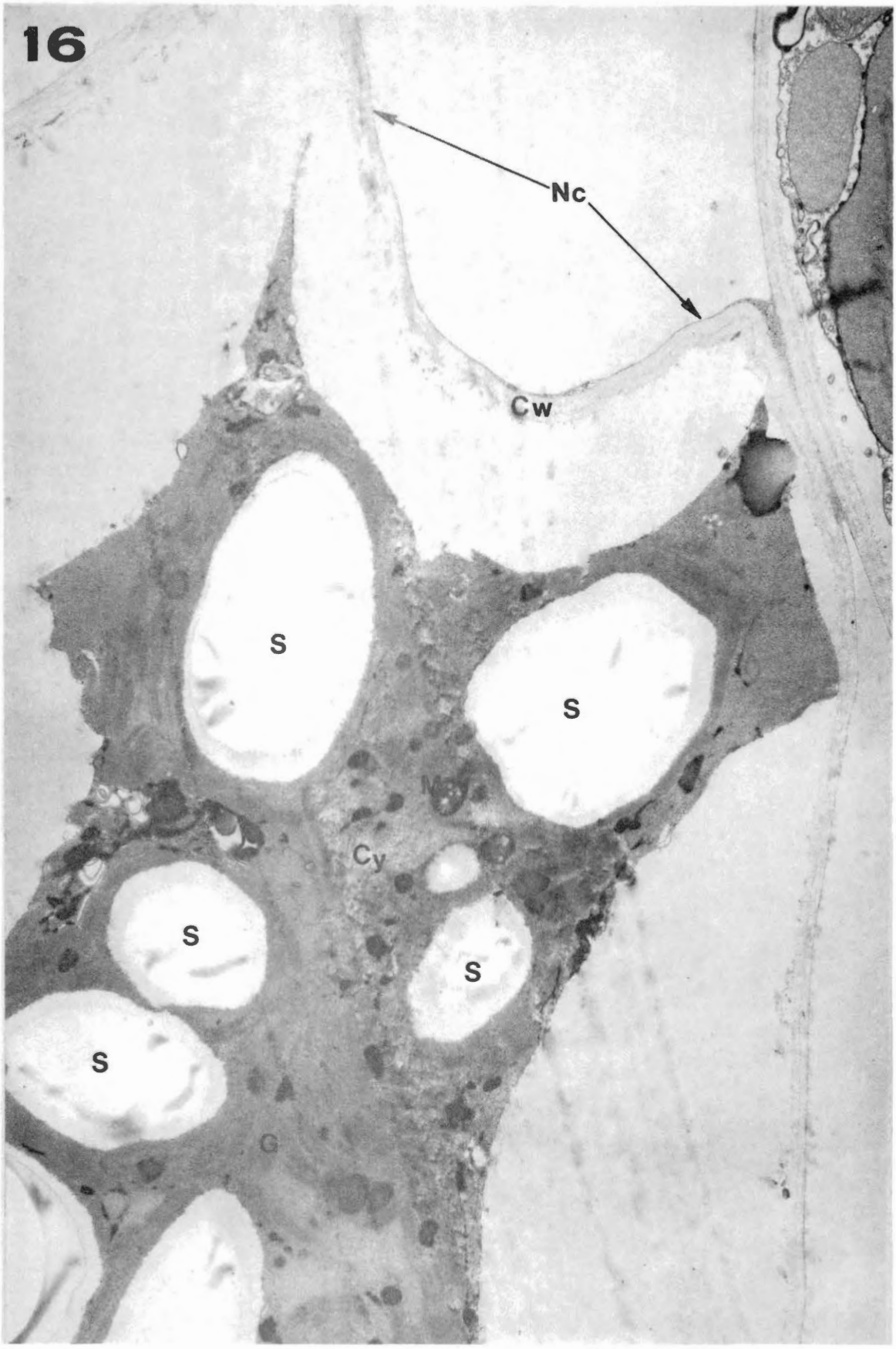

13
Cw
Er
Cy
G
M
L
C
L
14
Pf
Pf
15
Cw
M
M
G
C
M
Cy

Among the major organelles to exhibit signs of disruption, the chloroplasts appeared to be the most sensitive. Chloroplasts first exhibited abnormal structure shortly following breakage of the plasma membrane. A disruption in the highly ordered structure of the grana was frequently observed (Figure 13) as a consequence of stress. Frequently the chloroplast appeared quite granular and the internal membrane structure became indistinct. In severely deranged chloroplasts, crystalline structures of phytoferritin became apparent in the chloroplast matricies (Figure 14).

Mitochondria are apparently more resistant than chloroplasts in the cotton-Xanthomonas system. Figure 15 illustrates mitochondria in tissue 12 hours following inoculation. Even in conditions of severe coagulation of cytoplasm and destruction of nearby chloroplasts, mitochondria appeared to be relatively undisrupted. Not until the later stages of cell necrosis did the mitochondria finally lose their structure and blend into the amorphous contents of a necrotic cell (Figure 16).

Nuclei were so infrequently encountered that it was extremely difficult to describe their fate in stepwise fashion. However, it is assumed that nuclei generally followed the same pattern of degeneration as other organelles; loss of bounding membrane integrity, disruption of internal features, and necrosis.

Figure 16. A Necrotic Im 216 Cell (Nc), Containing a Large Area of Necrotic Cytoplasm (Cy). Few Cytoplasmic Components are Recognizable. Identified are: Starch Grains (S), and Some Chloroplast Grana (G). A Mitochondrion is Tentatively Identified at (M). Fixed at 20 Hours Following Infiltration. X 12,000.

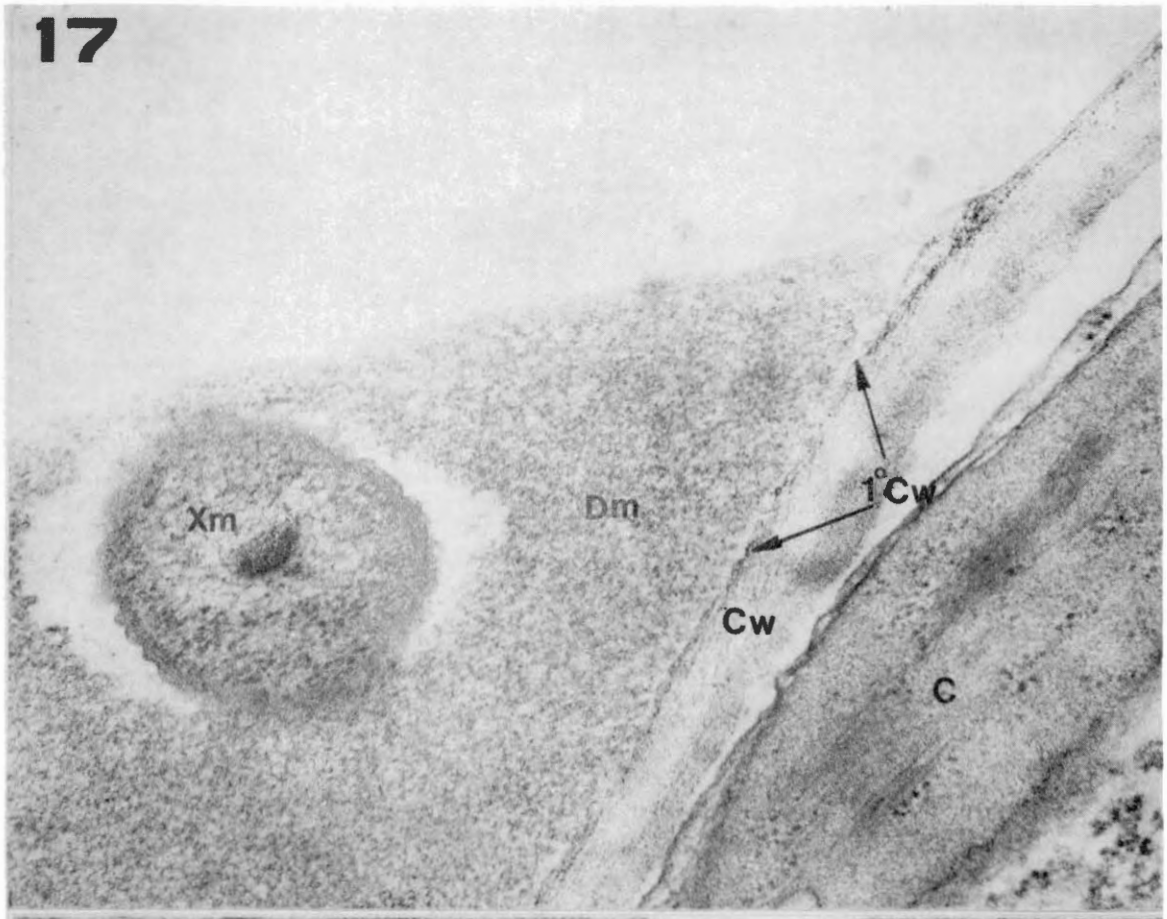

16
Nc
Cw
S
S
S
S
S
Cy
G

Inoculated Ac 44 Leaves

Cultivar Ac 44, as expected, did not respond with the same ultrastructural changes that occurred in Im 216 undergoing the hypersensitive reaction. Instead, the cells were generally passive to the presence of the bacteria. Pustle formation did not occur as in cultivar Im 216, but bacteria were found in cell wall junctions and in small areas between cells. Close examination of these bacteria revealed that they were usually surrounded by electron-dense material that did not resemble the material found inside the immune pustules (Figure 17). Closer examination further revealed that the host outer cell wall layer was intact and had not been disrupted. The appearance of the electron-dense matrix surrounding the bacteria did not appear until 8 hours following inoculation. This is in conjunction with the fact that the host cell walls appeared to be intact, suggests that the electron-dense material is of bacterial origination and was synthesized de novo. During the 24 hour test period no stress symptoms were detected in cells of inoculated Ac 44 leaves (Figure 18). There was some evidence that pathogen cells multiplied, but on a limited basis. Sections of inoculated Ac 44 leaves taken 1 week following treatment revealed that inoculated host cells had undergone degeneration, but not necrosis. Many of the cells had very electron-dense material deposited in the vacuoles (Figure 19). The

Figure 17. A Single Bacterial Cell (Xm), Embedded in Electron-Dense Material (Dm), on the Outside of an Ac 44 Cell Wall (Cw). The Primary Cell Wall ($1^{o}$Cw) is Apparently Intact. Fixed at 8 Hours Following Infiltration. X 117,000.

Figure 18. A Portion of an Ac 44 Cell Showing Three Bacterial Cells (Xm), Embedded in Electron-Dense Material (Dm). The Primary Cell Wall ($1^{o}$Cw), Appears Intact. To the Inside of the Cell Wall (Cw), the Plasma Membrane (Pm), Chloroplasts (C), Cytoplasm (Cy), and Mitochondrion (M), Appear Normal. Fixed at 24 Hours Following Infiltration. X 77,000.


17
Xm
Dm
1°Cw
Cw
C

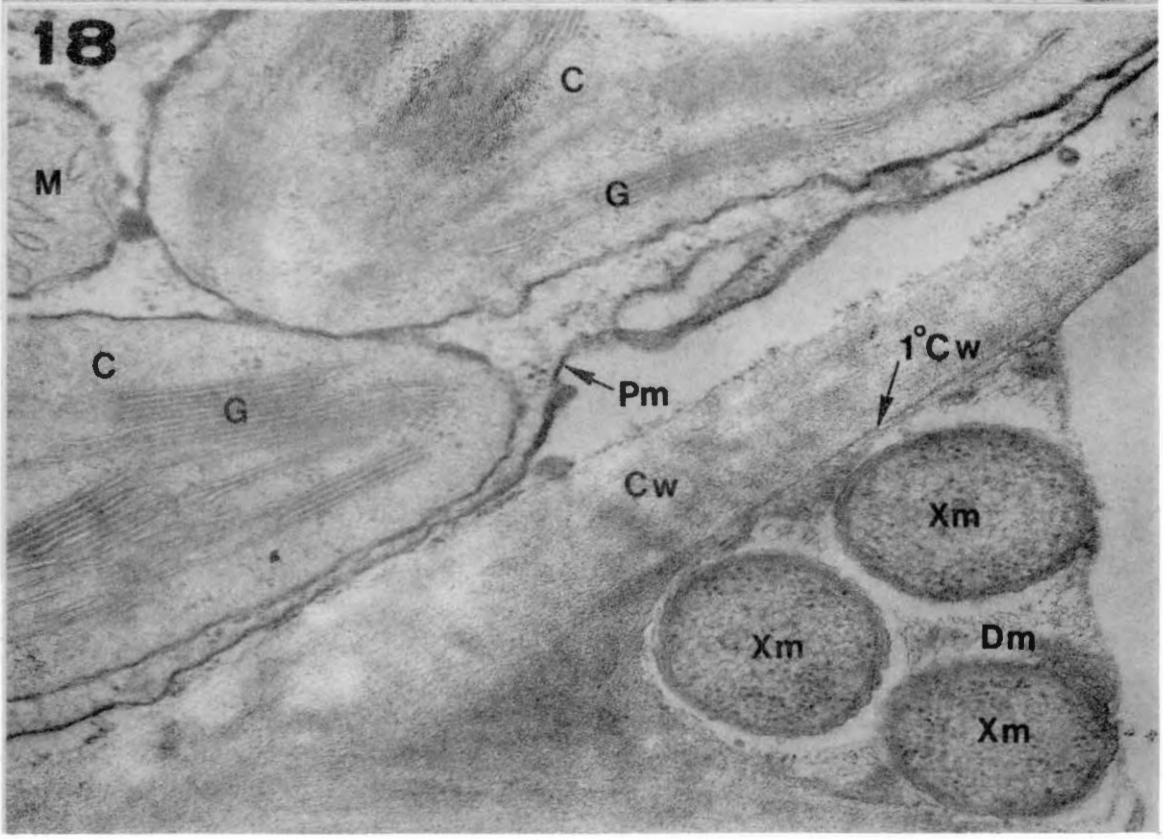
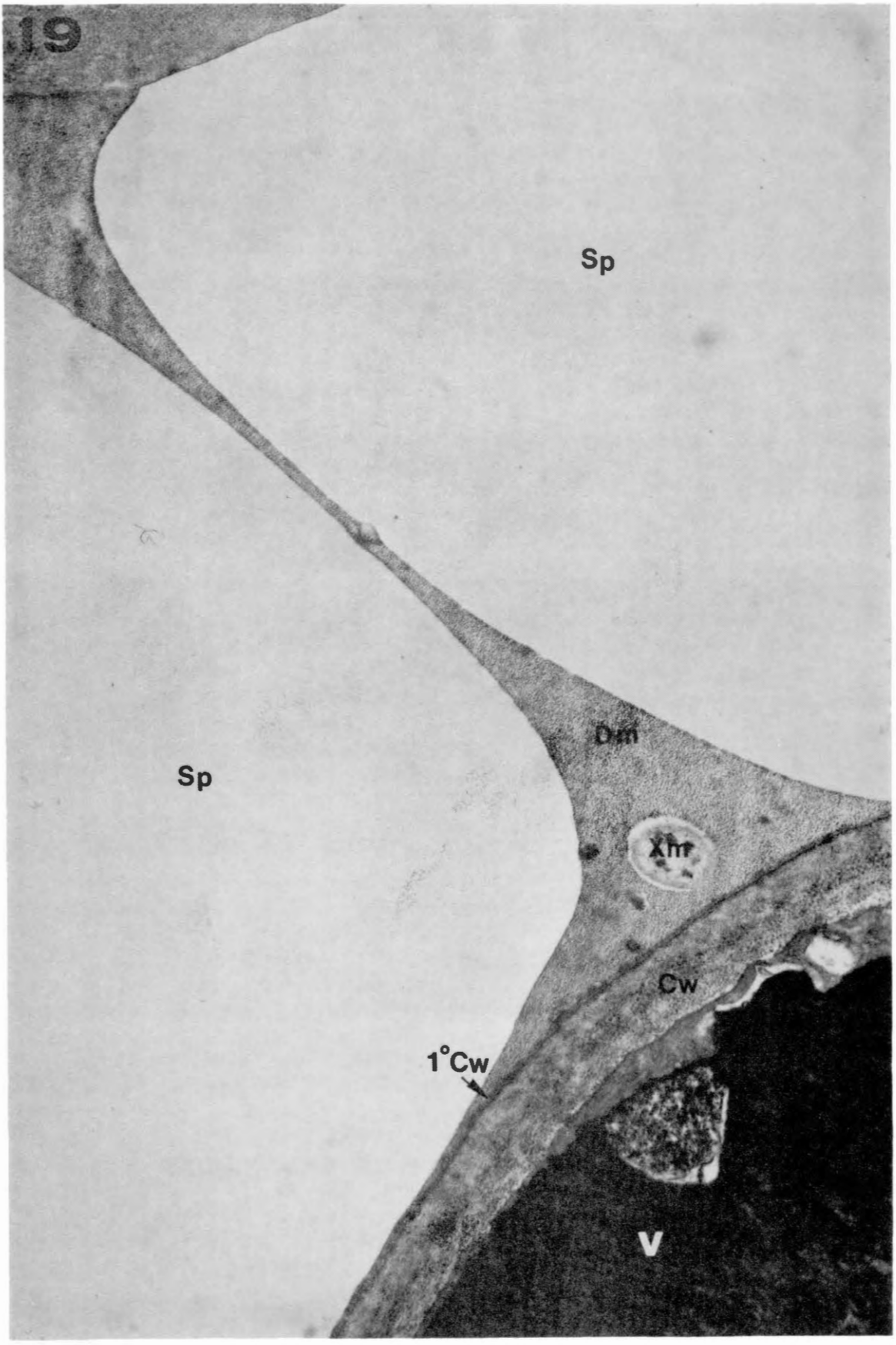

18
C
M
G
C
G
Pm
1°Cw
Cw
Xm
Xm
Dm
Xm

Figure 19. A Single Bacterium (Xm), is Shown Embedded in Electron-Dense Material (Dm), That Traverses the Intercellular Space (Sp), of Two Ac 44 Cells. The Tissue Was Fixed One Week After Inoculation. The Primary Cell Wall ($1^{o}$Cw), Appears Intact. Inside the Cell Vacuole (V), is Electron-Opaque Material That May Be Tannin Deposition. X 19,500.


19
Sp
Sp
Dm
Xm
Cw
1°Cw
V

chemical nature of this material is unknown, but the author wishes to suggest that this material might be tannin deposition. This suggestion is based on previous published electron micrographs that demonstrate a similar blackening of the central vacuole due to tannin accumulation (33).

## Electron Microscopy of *Xanthomonas malvacearum*

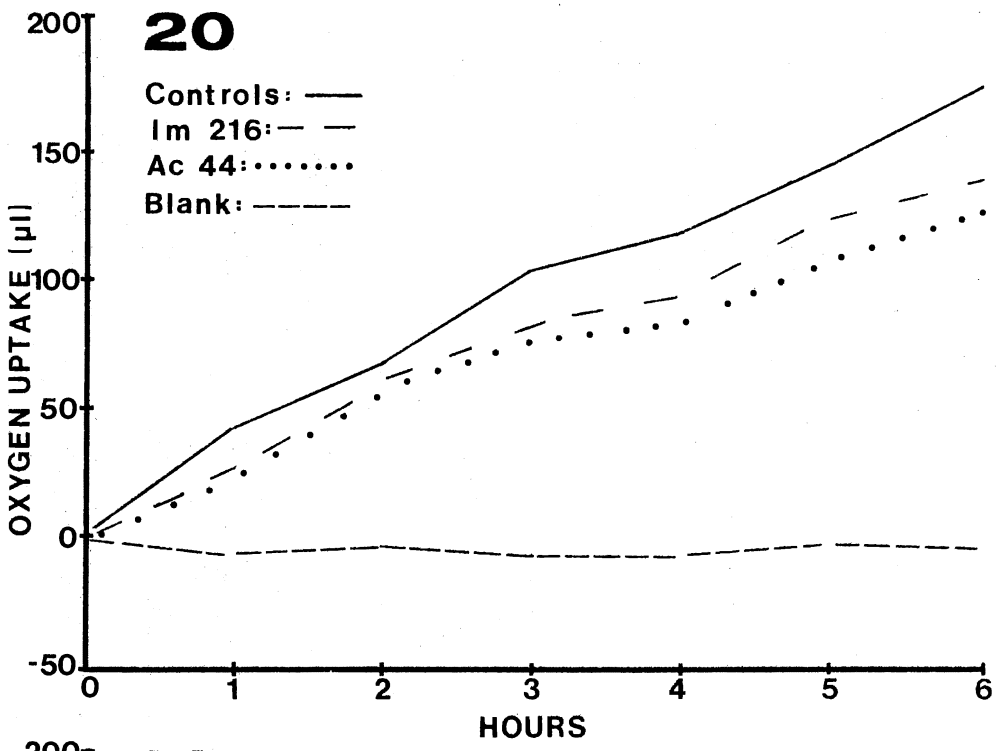
Sigee and Epton (45) have presented evidence that the ultrastructure of *P. phaseolicola* is different when incubated in susceptible and resistant varieties of bean plants. Their work indicates that differences in numbers and aggregations of bacterial ribosomes occur, as well as morphology of the nuclear region. Although the present study was not primarily directed to the structural changes in the pathogen, observations of thinly sectioned bacteria did not reveal major differences in the prokaryotic ultrastructure of *X. malvacearum* following incubation in immune and susceptible plants. In most sectioned material, the typical structure of gram-negative bacteria was intact (39). However, a more critical investigation may reveal differences.

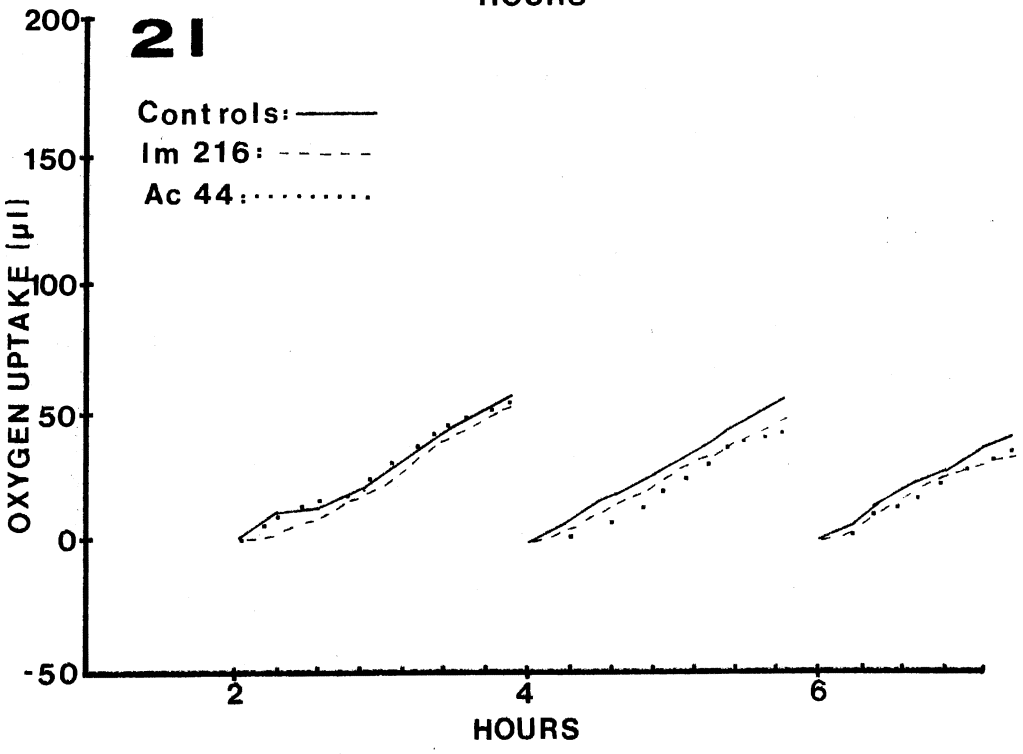
## Respiration of Inoculated Tissues

Data from respiration experiments generally supports the observation that mitochondria are resistant organelles in inoculated tissues. Figure 20 represents averaged data

Figure 20. A Graph Showing the Respiration Rates of Inoculated Im 216 and Ac 44 Leaf Discs. In This Test the Discs Were Loaded Into Manometer Flasks of a Gilson Differential Respirometer and Monitored Continuously for 6 Hours. Data are Averaged Curves for 5 Tests.

Figure 21. A Graph Showing the Comparative Respiration Rates for Inoculated Im 216 and Ac 44 Leaf Discs. The Gilson Flasks Were Loaded at 2 Hour Intervals and Monitored for Sufficient Time to Establish a Curve.


20
Controls: ——
Im 216: — —
Ac 44: .....
Blank: ———
OXYGEN UPTAKE (µl)
200
150
100
50
0
-50
0
1
2
3
4
5
6
HOURS

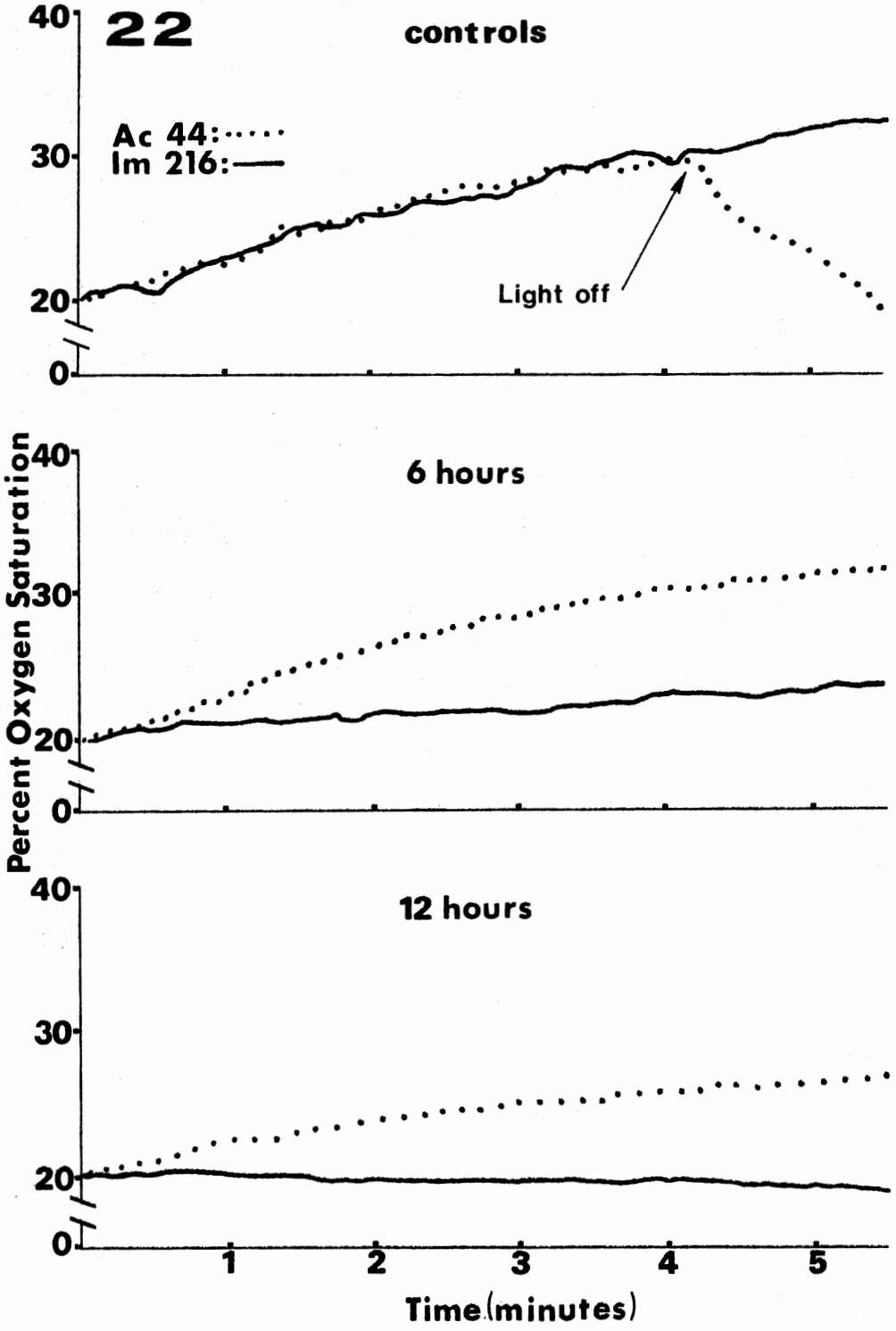

21
Controls: ——
Im 216: - - - - -
Ac 44: .....
OXYGEN UPTAKE (µl)
200
150
100
50
0
-50
2
4
6
HOURS

from several experiments in which inoculated tissues were continuously monitored for several hours beginning immediately following inoculation. It was observed that the tissue discs removed from watersoaked leaves remained so during the entire course of the experiment and that respiration rates were not significantly different. In another test, samples were taken at 2, 4 and 6 hour intervals following inoculation. These data also failed to demonstrate major deviations in respiration rates of inoculated tissue (Figure 21). This test was considered more valid than the former since continued watersoaking of inoculated tissues is atypical and may prevent the interaction of the bacteria and the host cell wall. A preliminary test indicated that small populations of inoculum bacteria contributed minor and insignificant amounts to the total oxygen consumption, and this total was almost immeasurable.

## Oxygen Evolution in Inoculated Tissues

Figure 22 represents oxygen evolution data from inoculated and uninoculated tissues at 6 and 12 hours following inoculation. In the 6 hour test, oxygen evolution is lower in inoculated Im 216 discs than Ac 44 discs, and in the 12 hour test the figures indicate that these trends are intensified. The net oxygen evolution in Im 216 at 12 hours is essentially zero and may indicate a slight net consumption. This data supports observations

Figure 22. Multiple Graphs Showing Oxygen Evolution from Inoculated Leaf Discs of Ac 44 and Im 216. Controls are Representative of Values Obtained From Water Infiltrated and Non-infiltrated Controls at the 6 and 12 Hour Time Intervals. The Evolution Rates of the Two Test Cultivars are Nearly Identical. A Sudden Drop at 4 Minutes With Ac 44 in the Control Curve Indicates the Effect of Removing Light. At 6 Hours Ac 44 Gives a Steady Evolution Rate While Im 216 Shows a Significant Decrease. At 12 Hours the Trend is Intensified. Ac 44 Gives a Steady but Somewhat Decreased Evolution Rate While Im 216 Indicates Slight Oxygen Uptake.


22
controls
Ac 44:·····
Im 216:——
Light off
6 hours
12 hours
Percent Oxygen Saturation
40
30
20
0
1
2
3
4
5
Time (minutes)

that chloroplasts degenerate rapidly when the plasmalemma is broken and functionality is probably lost as a result.

# CHAPTER V

## CONCLUSIONS

It is evident from these data that there are structural and physiological differences in immune and susceptible cultivars with respect to infection responses. The ultimate goal of this and other research on this problem is to identify the factors that cause resistance in one cultivar and susceptibility in the other.

Perhaps one of the most significant findings in this study was the section dealing with the host cell wall response in the immune cultivar Im 216. The fact that pustule formation is absent in the Ac 44 cultivar could account for the lack of rapid necrosis and consequent susceptibility.

The author feels that this important step may be a factor that begins the sequence of events that lead to necrosis. The evidence is in support of this idea: Data has shown (11) that the hypersensitive reaction is irreversible in immune cotton leaves after 20 to 30 minutes following inoculation. Electron microscopy in this study revealed that pustules were formed within 2 hours. Since no samples were taken prior to 2 hours following inoculation it is uncertain exactly how quickly pustule formation

occurs. Figure 9 clearly demonstrates that the primary host cell wall is damaged in the process of pustule formation. The author feels that the damaged area of the wall, which is the basal part of the pustule, may be an avenue for exchange of materials between the host and pathogen cells contained within the pustules. It is conceivable that this damaged area might provide the pathogen easy access to nutrient materials contained in the host cell wall and cytoplasm. It also might facilitate the movement of host defense products toward the pathogen cells. Another idea is that pustule formation might serve as a means by which the host cells immobilize pathogen cells and prevent their spread (17). This idea might be partially correct in that a necrotic cell collapses, and in the process, bacteria become trapped in dead tissue, but it is doubtful that pustules alone could immobilize because they appear fragile (Figure 11).

Once the pustule has been established on the host cell, destructive bacterial enzymes might pass the wounded area of the cell wall and cause damage to the plasma membrane. Since the plasma membrane is vital to the cell, and damage to it might cause death of the cell, this may be a factor that leads to degeneration of organelles, cytoplasm and ultimate necrosis. The respiration and photosynthesis studies only serve to emphasize this idea. Loss in photosynthesis capacity was determined to occur several hours following inoculation and this coincided with

structural data on the integrity of chloroplasts. These effects were seen long after damage to the plasma membrane had occurred. Similarly the degenerative sequence occurred with the mitochondria, but apparently these organelles were more resistant to unfavorable conditions in the cytoplasm and their structure remained intact long after chloroplasts had degenerated. Respiration experiments indicated little differences in oxygen uptake rates between hosts, and this indirectly supports the above structural data. This observation was unexpected since mitochondria are typical indicators of stress conditions in plant cells. Interesting as these observations are, the author feels that these events are a consequence of damage to the plasma membrane, and that the pathogen cells directly damage the cell wall rather than the host cell components.

The question of why pustules are found on immune cells and not on susceptible cells naturally comes to mind. The answer to this may be in the fact that work on pectinases in this system has shown enzyme activity rises rapidly in inoculated immune leaves and slowly in susceptible leaves. This may be due to available nutrients found in intercellular spaces. One line of thought is that available sugars in Ac 44 could delay the induction of bacterial pectinases. If pectinase activity remained low then the sudden and dramatic damage to host cell walls might not occur. However, high levels of enzyme activity in Im 216 might account for the disruptive effects that occur during

pustule formation. It is interesting to note that pustules were not observed in tissue that did not become necrotic, but they were always observed in association with tissue that did necrose.

Pathogen activity in susceptible tissue apparently is not inhibited as in immune tissue. Since host cells do not become necrotic, pathogen cells multiply to great numbers. This relationship is an example of refined parasitism: Pathogen cells benefit at the expense of host cells without causing rapid death of the host. This is not to say that there is no response of the host to the pathogen. If the vacuolar deposition in figure 21 is tanniniferous, its presence may be a general mechanism to discourage the invasion of pathogens (33).

The author feels that these concepts provide a reasonable hypothesis in light of the evidence. It will be interesting to see how accurate these ideas are as more information on this system becomes available in the future.

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VITA 2

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