THE HISTOLOGICAL RESPONSES OF IMMUNE AND SUSCEPTIBLE COTTON VARIETIES TO THE BACTERIAL BLIGHT PATHOGEN *XANTHOMONAS MALVACEARUM* (E. F. SM.) DOW.

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CHAPTER I

INTRODUCTION

Bacterial blight of cotton is an important disease in nearly all cotton growing areas of the world. The disease was first known as "angular leaf spot" and more recently as bacterial blight of cotton.

The disease is caused by Xanthomonas malvacearum (E. F. Sm.) Dow., a gram negative, motile, rod-shaped bacterium with a single flagellum and is host specific for members of the genus Gossypium. All above ground parts of the plant are attacked by the bacteria and symptoms include defoliation, boll lesions that lead to secondary infection by boll rotting fungi, and lesions on the stem and petioles. Physiological races of X. malvacearum have developed that exhibit varying degrees of pathogenicity on different varieties of cotton (Brinkerhoff, 1963).

Chemical sprays and antibiotics have not been extensively used as a major control measure because the cost of such treatments has been economically prohibitive. Acid delinting, use of resistant varieties, and sanitation have been the most promising control measures. At present, breeding lines of cotton are available which are immune to all known races of X. malvacearum. The immune response is

characterized by the formation of necrotic areas when host tissue is inoculated with high concentrations of inoculum (1.0×10^8 cells/ml in water). When inoculations of lower concentrations (less than 1.0×10^5 cells/ml in water) are made, the hypersensitive reaction does not occur at the macroscopic level. Under field conditions, no apparent reaction is observed on immune plants. Hence, the macroscopic hypersensitive reaction is an experimental phenomenon that is restricted to abnormally high concentrations of inoculum and artificial inoculation. The hypersensitive reaction occurs in immune tissue typically within 12-24 hours after inoculation. The necrosis that results effectively blocks the action of the bacteria by localizing the bacteria in dead, dry tissue.

By contrast, in genetically susceptible cultivars, the bacteria continue to multiply and the lesion enlarges. Leaf veins tend to restrict the spread of bacteria, and as a result the lesions assume the typical angular outline. Action of this nature eventually leads to death of plant tissue and results in leaf abscision and death of very succulent parts.

The goal of this investigation was to determine the nature of the hypersensitive reaction at the tissue level. The included studies complement physiological and biochemical research by other workers on the same phenomenon. Experiments were conducted to show the fate of the cellular components as well as the "tissue" reaction of the leaf or

cotyledon to the pathogen. Both growth chamber and field samples of cotton leaves were examined by means of two histological methods. The experiments were designed to elucidate the interaction of the bacteria with the host tissue and the effects of the hypersensitive reaction upon the cell components and cell structure. The data along with biochemical evidence were then to be used in tracing the sequence of events that occurs during the hypersensitive reaction.

CHAPTER II

REVIEW OF LITERATURE

Bacterial blight of cotton was first described by Atkinson (1891). The symptoms were characterized by water soaked areas of the leaves bounded by veins which gave the lesions an angular appearance. Atkinson (1892) reported an association of large quantities of bacteria with the water-soaked lesions. Smith (1901) isolated the organism and was able to inoculate cotton plants and produce the disease. Smith also described the organism as being able to produce large quantities of slime.

The action of the bacterium within leaf tissue was described by Massey (1928). Bacterial cells spread throughout the host tissue (i.e., intercellular spaces) in a slime mass and later spread over the surface of the lesion. The middle lamella and eventually the cell walls of the adjacent cells were destroyed. Massey postulated that this destructive action was caused by bacterial "cytases". Bryan (1932) described an atypical condition in which bacterial blight was systemic within the leaf tissue. Bacterial cells were found actively spreading throughout the vascular system of infected leaves and petioles. The first comprehensive histological study of bacterial blight of cotton was

conducted by Theirs and Blank (1951). They prepared tissue sections of both a susceptible and a resistant variety (with resistance due to a single gene, B_7) of cotton infected with X. malvacearum, and observed the reactions of these hosts to infection and the action of the pathogen within the host tissues. Their conclusions were that there is anatomically no difference in disease development within susceptible and resistant hosts except for lesion size and pathogen population. Resistance was suggested to be due to physiological rather than morphological factors, and the destructive action of the bacteria was ascribed to be due to highly effective cellulases because of the progressive digestion of the cell walls which they observed. No isolation or biochemical confirmation of the presence of the enzyme was described. Because this work was done prior to description of races of the pathogen, their conclusions may not be valid for all race-cultivar combinations.

Seventeen races of X. malvacearum have been identified by using a set of host differentials developed by Hunter et al. (1968). Resistance in Gossypium has been assigned to a set of B genes (Knight, 1948), and a total of 16 genes are responsible for blight resistance. Resistance genes occur among the species of Gossypium with eight found in the cultivated tetraploid species, G. hirsutum, and two additional genes found in G. hirsutum var. punctatum (Brinkerhoff, 1970). Breeding lines and cultivars with

resistance ranging from susceptible to immune are now available with disease reactions ranging from water soaking to no visible symptoms (Brinkerhoff, 1963).

Hypersensitivity in a plant-bacterial system was proposed by Muller (1959), and defined by Klement and Goodman (1967) as being a defense reaction of plants against pathogens that occurs in an incompatible host-parasite relationship; with necrosis leading to physical localization of the pathogen. The first report of a hypersensitive-like reaction induced by bacterial pathogens was reported by Klement and Lovrekovich (1961) who observed browning in bean pods within one day after inoculation. Further studies were conducted by Klement and Lovrekovich (1962) on the same phenomenon. They observed a hypersensitive reaction in bean pod tissue when inoculum concentrations from 1.0×10^4 cells/ml in water up to 1.0×10^8 cells/ml were injected. One Pseudomonas species and two Xanthomonas species were assayed: none were bean pathogens. The authors concluded that damage was not due to direct action of bacteria but to a reaction evoked by the plant tissues, and described the reaction as a post infection defense mechanism. Klement et al. (1964) further substantiated the observations on the hypersensitive reaction when they produced a similar reaction in tobacco leaves inoculated with Pseudomonas tabaci (Wolf and Foster) Stevens. Necrosis was observed in 12-24 hours when plants were inoculated with bacterial concentrations from 1.0×10^5 cells/ml to 1.0×10^8 cells/ml

in water were used, necrotic areas formed which were substantially reduced in size, and were proportional to the concentration when viewed at 10-12X magnification. The inoculation technique for infiltration of leaves described by Klement (1963) provides a simple, rapid means for assaying host reactions to various levels of inoculum.

Stall and Cook (1966) reported hypersensitivity in pepper when inoculated with Xanthomonas vesicatoria (Doidge) Dow. The authors observed a complete hypersensitive reaction within 24 hours after injection with high levels of inoculum. When injections were made with 1.0×10^8 cells/ml, histological observations revealed cell collapse with characteristic gumming occurring within the tissue. No noticeable effects were seen when inoculum levels of 1.0×10^5 were used. More work on pepper leaves from Stall and Cook's laboratory by Sasser (1968) showed electrolyte leakage and ultrastructural alterations of mesophyll cell organelles undergoing the hypersensitive reaction. A review on the subject of hypersensitivity was published by Klement and Goodman (1967).

More recent studies have linked membrane integrity and electrolyte leakage to the hypersensitive reaction. Stall and Cook (1968) studied the effect of X. vesicatoria on electrolyte loss in pepper leaves and reported that the appearance of the hypersensitive reaction coincides with the loss of electrolytes. Goodman (1968) studied host cell permeability in tobacco and reported an early electrolyte

leakage, loss of membrane integrity and desiccation of inoculated tissue. Chloroplast membranes were shown to be damaged within a period of four hours after inoculation. Goodman (1967, 1968) showed that the -S-S- groups within the structural proteins of the cell membranes could be target sites for chemical reduction. Huang et al. (1974) presented evidence that showed hypersensitivity induced by bacteria alters the structural proteins in thylakoid membranes of tobacco leaf cell chloroplasts.

Other studies have compared permeability alterations induced with virulent and avirulent strains of bacteria (Goodman, 1968, and Burkowicz, 1968). The effects of bacterial pH and ammonia production on membrane permeability within tobacco leaf tissue was studied by (Lovrekovich et al. (1970), and Goodman (1972). Novacky (1973) has investigated membrane potentials in cotton cotyledons inoculated with bacterial pathogens, and has shown that incompatible bacteria cause electrolyte leakage in 10-12 hours, whereas with compatible bacteria, leakage is delayed for several days. Klement (1967) observed that membrane damage in tobacco cannot be produced with killed bacteria, fragmented bacteria or bacterial culture filtrates, and also that the hypersensitive reaction is irreversible after only 15-20 minutes.

Structural studies have been few in comparison to physiological studies. Goodman and Burkowicz (1970) studied cellular organelles in apple leaves after injection with

1.0×10^9 cells/ml of Erwinia amylovora (Burril) Winslow, and detected disorganization of chloroplasts, degeneration of mitochondria, and microbodies as well as general disorientation of other subcellular organelle membranes in 50% of leaf cells examined after only 12 hours. By 24 hours after infiltration most of the cellular organelles were not identifiable. Goodman and Plurad (1971) studied ultrastructural changes in tobacco leaves undergoing the hypersensitive reaction, and reported extensive damage to plasmalemma, tonoplast and membranes of chloroplasts and mitochondria within seven hours after injection. Cytoplasm, groundplasm of chloroplast, mitochondria and microbodies, as well as ribosomes were also affected.

Turner and Novacky (1974) studied tobacco leaf tissue inoculated with 5.0×10^3 bacterial cells/ml and observed random cell death within a two hour period. They concluded that there is a random distribution of bacteria within the leaf and that a 1:1 ratio of bacteria to plant cells is sufficient to cause individual cell death. Their results were based on observations of necrotic cells found in stained whole mounts and tissue sections of injected leaves.

CHAPTER III

MATERIALS AND METHODS

Inoculation Methods

The first method tested was the rubber tipped syringe pressure injection technique. This method is accomplished by placing the leaf area to be inoculated between the forefinger and the open end of the syringe and forcing the inoculum into the leaf through the open stomates. This procedure effectively watersoaks an area the size of the tip aperture and infection rates are consistent and high. The second method was designed to eliminate mechanical injury yet facilitate effective inoculation. The procedure consisted of painting a bacterial suspension on the abaxial side of a healthy cotton leaf. A high concentration of bacteria was used (1.0×10^8 cells/ml in water) and application was made with an artist's paint brush. To prevent rapid evaporation of the inoculum, paper discs were moistened and placed inside both halves of a notched, plastic petri dish. The dish was then closed over the inoculated leaf with the petiole entering through the notched opening on the side of the dish. The edges of the dish were sealed with masking tape to prevent drying within the dish, and the apparatus was held close to the plant by a glass rod

anchored in the pot. The treated areas were incubated in a growth chamber on a 12 hour light and dark period with the temperature at 90°F in the light and 65°F in the dark. The third and final method tested is a variation on the procedure described by Klement (1964). The technique involves the use of a standard syringe equipped with a 27 gauge tuberculin needle. Inoculation is accomplished by inserting the needle into the leaf mesophyll (bevel down) and gently injecting the inoculum into the intercellular spaces. The size and distribution of the inoculated areas can be controlled. The amount of inoculum injected can be approximated, and the occurrence and location of the disease reaction is predictable.

Bacterial Culture Methods

Race 1 and 10 cultures of X. malvacearum was obtained from stock cultures for all of the experiments. Inoculum was prepared by transferring a loopful of the bacteria from a potato-carrot-dextrose agar colony to a 160 cc medicine bottle containing 100 ml of sterile nutrient broth. The culture bottle was then placed on a culture shaker and allowed to incubate at room temperature for 24 hours. Dilution plate count verified that under these conditions the concentration of bacteria grew to approximately 1.0 X 10^8 cells/ml of broth in 24 hours. Washed inoculum was prepared by centrifuging 40 ml portions of 24 hour broth cultures at 10,000 rpm for ten minutes and replacing the

supernatant with sterile distilled water. The bacterial pellet was resuspended by agitation with a vortex mixer for 30 seconds. This procedure was repeated twice and the concentration of the final suspension was verified by dilution plate count. Dilute concentrations of inoculum were prepared by serial dilution of a 1.0×10^8 cells/ml broth culture in 10 ml portions of sterile distilled water. A range of concentrations from 1.0×10^8 to 1.0×10^1 was obtained in this manner. Plate counts were made in duplicate of all dilutions including the stock culture by adding 0.25 ml of each solution to a nutrient agar plate and streaking over the surface with a sterile loop. Final calculation of bacteria count was by the formula: (# of colonies/plate) X (4) X (dilution factor). Reisolation of bacteria was done by grinding a diseased area of an Ac 44 leaf in 10 ml of sterile water with a mortar and pestle, plating dilutions of the resulting homogenate and picking an individual colony.

Plant Growth Conditions

Seeds of the susceptible cultivar, Ac 44, and the immune cultivar Im 216 were obtained and planted in six inch clay pots containing a sterilized soil, vermiculite, peat mixture in the ratio of about 8:1:1. The seeds were planted one inch deep, two per pot, with the micropylar end up. The pots were then watered with 250 ml of tap water and covered with saran wrap. All pots were placed in a constant

temperature growth chamber at the settings previously described. The seedlings emerged from the soil within six days, and within twelve days the first foliage leaves appeared, which was the stage of maturity at which all cotyledon injections were conducted.

Paraffin Histology

Plant leaf and cotyledon tissue sections were fixed in cold FPA for 24 hours with gentle aspiration. After fixation was complete, tissues were transferred to a graded series of tertiary butyl alcohol (TBA), water, and ethanol with dehydration beginning in the most aqueous solution (Johansen, 1940). The samples were left in each solution for one hour and overnight in pure TBA after dehydration was completed. All samples were transferred to shell vials for infiltration. Paraffin chips were added to vials containing the samples in TBA and all were transferred to a 37°C warming oven. After 24 hours more paraffin chips were added and the vials were transferred to a 55°C oven for further infiltration. The TBA slowly evaporated, leaving the samples in pure melted paraffin. Three changes of fresh melted paraffin were made at two hour intervals. After infiltration was complete the tissue samples were cast in plastic boats and cooled immediately in an ice water bath. When the blocks solidified they were separated from the boats, trimmed, and mounted on sectioning blocks with melted paraffin.

Sectioning was done on a standard rotary microtome with section thickness of 10 μm. Sections were mounted on clean glass slides with Haupt's gelatin adhesive (Johansen, 1940) and 4% phenol. After drying for 24 hours in a 37°C dust-free oven the slides were carried through a staining series of Gray and Pickel safranin (Gray and Pickel, 1954) and Johansen's Fast Green (Johansen, 1940), cleared in clove oil and mounted in Eukitt (Calibrated Instruments, Inc.).

Plastic Histology

Thick sections were prepared using methods of Feder and O'Brien (1968). Tissue samples of 2 mm X 4 mm were excised from treated cotyledons and fixed immediately in 10% acrolein at 0°C for 24 hours. All samples were dehydrated through a graded series of alcohols at 0°C beginning with three changes of 2-methoxy ethanol at eight hour intervals. The samples were then transferred successively to absolute ethanol, n-propanol, and n-butanol at 24 hour intervals. They were embedded with prepared media JB-4 plastic embedding kit (Polysciences). Dehydrated tissues were infiltrated by immersing them in three changes of solution A for five hours per change. The specimens were then cast in a mixture made up of 42 parts by volume of solution A and one part of solution B. Beem capsules were used for molds and polymerization was completed overnight at room temperature.

Two micrometer sections were made with a standard rotary microtome. The sections were floated on a glass slide with a dilute ammonium hydroxide-water solution and allowed to dry. Sections were stained with 1% acid fuschin in distilled water for two minutes, and then counterstained with .05% toluidine blue in .02 m benzoate buffer at pH 4.4 for 15 seconds (Sidman et al., 1961) and finally rinsed with distilled water. Cover slips were mounted with Eukitt after the sections had air dried.

Fluorescence Methods

Eighteen fluorochromes were screened for staining of X. malvacearum (Table I). All stains were prepared by making a 0.1% solution in dionized twice distilled water. Staining was carried out by mixing 5 ml of a 1.0×10^8 cells/ml broth bacteria culture and 0.5 ml of stain solution in centrifuge tubes resulting in a final stain concentration of .01%. Initial pH readings of stain-bacteria mixtures were recorded. Tubes were labeled and shaken with a vortex mixer then centrifuged at 7,000 rpm for ten minutes. The supernatant was decanted and replaced with an equal amount of distilled deionized water. The pellet was resuspended with the vortex mixer and the mixture was recentrifuged. The washing was done twice with the final supernatant being replaced with water.

After resuspension each sample was tested for cell viability by preparing a nutrient agar streak. Fluorescence

TABLE I

FLUOROCHROMES

Methylene Blue	National Aniline Div. Allied Chem.
Aniline Blue	National Aniline Div. Allied Chem.
Methyl Violet	National Aniline Div. Allied Chem.
Thionin	Harleco
Neutral Red 1% aq.	Carolina Biol.
Congo Red 1% aq.	Carolina Biol.
Titan Yellow	Eastman Kodak
Berberine Sulphate	Sigma
Acriflavin Neut.	Sigma
Acriflavin HCl	Sigma
6-9-diamino-2-ethoxy Acridine Lactate	Sigma
Rhodamine	Allied Chemical
Acridine Orange	Allied Chemical
Nile Blue	Allied Chemical
Basic Fuchsin	Allied Chemical
Acid Fuchsin	Allied Chemical
Janus Green	Coleman and Bell
Tinopal 4-B-M Conc'	Ciba-Geigy

and staining was tested by preparing wet mounts of bacteria and examining on an American optical fluorescence Series 10 Microstar equipped with a mercury arc HBO 200 watt light source, an ultraviolet exciter filter Schott BG-12, and a Schott OG1 barrier filter.

Histochemical Methods

Free-hand sections were cut from test materials and placed in moist petri dishes to prevent desiccation. Sections were then transferred to an alkaline hydroxylamine reagent (Reeve, 1958) prepared by mixing 14 g of NaOH and 14 g of hydroxylamine hydrochloride in 100 ml of distilled water. Sections were allowed to react for ten minutes. After the hydroxylamine treatment the sections were transferred to an equal volume of 33% aqueous solution of ferric chloride containing 0.1 N HCL. After the color reaction, the sections were transferred to microscope slides, mounted in glycerine, and cover slips applied. Pectic substances were identified and localized by a red color in the middle lamella.

CHAPTER IV

RESULTS

Histology of Inoculation Methods

A rubber-tipped syringe used with pressure injection was effective in inoculating leaf and cotyledon tissues. Subsequent infection and hypersensitive reactions were high. The method was determined to be unacceptable for histological study because of tissue damage that resulted from the mechanical pressure (Figure 1). Painting inoculum on the abaxial side of the cotyledon caused the least mechanical injury of all methods tested, and no histological alterations could be detected. However, infections were not uniform and areas that were attacked could not be predicted. At no time during the test did an entire treated area become watersoaked as a result of pathogenesis or did an entire treated area become necrotic as a result of hypersensitivity. The hypodermic injection method (Klement, 1964) was as effective as the rubber-tipped syringe in producing a plant reaction and produced no observable changes in cell arrangement or internal leaf anatomy (Figure 2). Histologically the tissue sections were indistinguishable from uninoculated controls.

Histology of Host Reactions

Cotyledons of Im 216 and Ac 44 were inoculated with a nutrient broth culture of 1.0×10^8 cells/ml race 1 X. malvacearum. The plants were incubated under growth chamber conditions of a 12-hour photoperiod with temperature settings of 90°C during the light period and 65°C during the dark period. After 24 hours incubation the immune variety exhibited the typical hypersensitive reaction. The injected areas on the cotyledons had wilted and dried and were dark green to gray in color. No symptoms of disease development or hypersensitivity were observed in the susceptible variety. Small tissue sections were excised from the necrotic area on the immune cotyledon and the inoculated area of the susceptible cotyledon and processed for histology with the paraffin method.

Tissues from immune plants exhibited striking structural changes in anatomy. Perhaps the most obvious and dramatic change was the compression in cotyledon tissue and the disorganization of cell structure and arrangement (Figure 3). Compression was due to desiccation, which is characteristic of the hypersensitive reaction. As a result of collapse, the cell walls were severely disrupted. Palisade parenchyma which is oriented perpendicular to the plane of compression was crushed with the cell walls assuming a folded accordion-like configuration (Figure 3). Spongy parenchyma was similarly affected. Bundles appeared to be least affected with the xylem elements apparently

normal and functional (Figure 4). The phloem and bundle sheath cells appeared damaged with the phloem being functionally useless. Guard cells and epidermal cells appeared to be less affected but were decidedly damaged (Figure 5). Chloroplasts were found throughout the necrotic tissue both within and outside of palisade and spongy parenchyma. Some appeared to be spherical and swollen with transparent bubble-like areas in the matrix (Figure 17).

Six days after inoculation disease symptoms became evident on the Ac 44 cotyledons. A dark, shiny watersoaked appearance with a sticky slime covering the outside of the lesion was evident on the areas injected with the bacteria. Samples of diseased tissue were taken as before and processed for paraffin histology. Histology of the water-soaked lesion was much as described by Theirs and Blank (1949). Bacteria moved through the intercellular spaces of the palisade and spongy parenchyma cells with no apparent reactions of host cells to pathogenesis (Figures 6 and 7). Larger veins physically checked the spread of the bacterial masses (Figure 8). Of particular interest was the formation of large masses of red staining gum-like substances in the phloem (Figure 9). No bacteria were found in the xylem, and apparently the xylem was not attacked even in areas of advanced pathogenesis. The epidermis did not stop the spread of the bacteria and slime. Large areas of slime were observed on the lower and upper surfaces of the leaf (Figure 7).

Figure 1. Damage Resulting from the Rubber-Tip Syringe Inoculation Method Showing Leaf Cross Section with Palisade Parenchyma (p) Collapsed from Mechanical Pressure. Magnification: 270X.

Figure 2. Damage Free Section Inoculated with a Syringe and Needle Indistinguishable from Uninoculated and Water Inoculated Controls Showing Epidermal Cells (e), Guard Cells of a Stomate (g), Palisade Parenchyma (p), Spongy Parenchyma (s), and a Vascular Bundle (b). Magnification: 270X.

Figure 3. Cross Section of a Im 216 Leaf Inoculated with 1.0×10^8 Cells/ml in Broth Showing Structural Changes as a Result of Hypersensitivity. The Indicated Features are: Vertical Collapse (v), Folded Palisade Cell Wall (f), and Laminar Spongy Parenchyma (l). Magnification: 650X.

Figure 4. Cross Section of a Im 216 Cotyledonary Vascular Bundle Showing Little Damage from the Hypersensitive Reaction. Features are: Xylem (x), Phloem (p), and Bundle Sheath Cells (b). Magnification: 650X.

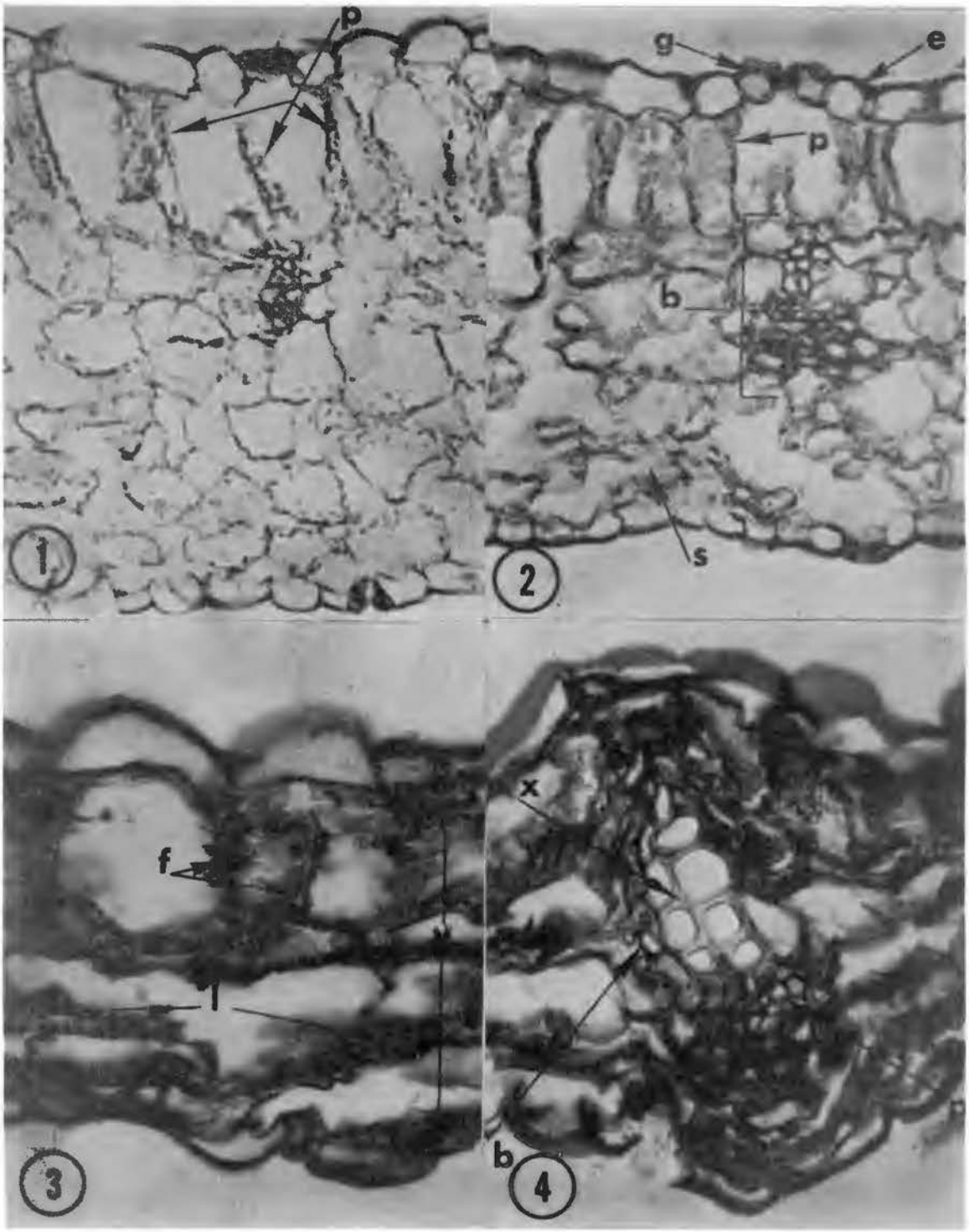
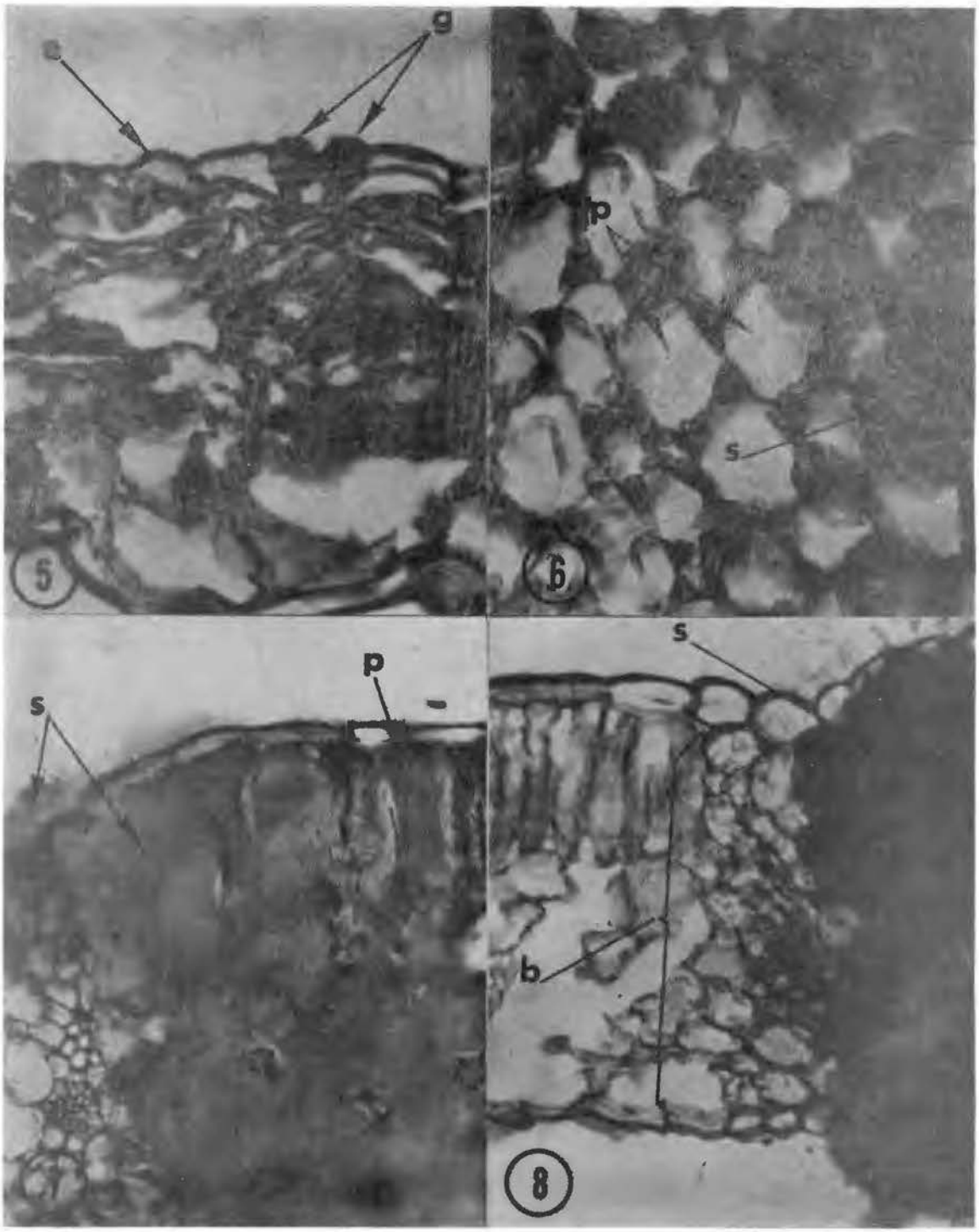

p
1
g
e
p
b
s
2
f
3
x
b
4

Figure 5. Necrotic Im 216 Cotyledon Showing Guard Cells (g) and Epidermal Cells (e). Magnification: 650X.

Figure 6. Paradermal Section of Ac 44 Leaf One Week After Inoculation Showing Pathogenesis of Susceptible Tissue. Features Indicated are: Bacterial Slime (s), Palisade Parenchyma Cells (p). Magnification: 270X.

Figure 7. Cross Section of Ac 44 Leaf Showing Pathogenesis of Susceptible Tissue. Features Indicated are: Bacterial Slime (s) and Remnants of Palisade Parenchyma (p). Magnification: 270X.

Figure 8. Vascular Bundle Showing Blockage of Pathogenesis in Susceptible Ac 44 Leaf Tissue. Features Indicated are: Bacterial Slime (s) and Vascular Bundle (b). Magnification: 270X.


g
5
p
s
6
p
s
s
b
8

Fluorescence Staining of Bacteria

The paraffin method failed to differentiate bacteria cells from host tissue in the immune tissue. An attempt to pre-stain bacteria before injection with a fluorochrome was tried (Wilson, 1965). Eighteen fluorochromes were screened for bacterial staining and toxicity. The results are summarized in Table II. Of all fluorochromes tested, aridine orange gave the strongest fluorescence and was not toxic to the bacteria. Toxicity was assayed for by inoculating a nutrient agar streak plate with the stained bacteria and checking for growth.

Two sets of host plants were used for injection with stained bacteria. One group was made up of normal green plants and the other group was etiolated plants grown in the dark. The etiolated plants were used in an attempt to avoid possible autofluorescence of host chlorophyll when the tissue sections were viewed with fluorescence microscopy. Cotyledons from both groups of plants were injected with a 1.0×10^8 cells/ml concentration of stained bacteria, and tissue sections were excised and processed for histology with the paraffin method. Although the acridine orange stained bacteria fluoresced brightly when viewed with a wet mount preparation, efforts to locate individual bacteria or clumps of bacterial cells in host tissues were unsuccessful. Autofluorescence from chlorophyll and other substances in green tissue masked the presence of bacteria. There was no autofluorescence by chlorophyll in etiolated tissues, but

TABLE II

REACTIONS TO FLUOROCHROMES

Fluorochrome	Solution pH	Bacterial Viability	Fluorescence	Color
Methyl Violet	4.3	-	-	-
Methylene Blue	4.9	+	-	-
Aniline Blue	3.5	+	-	-
Thionin	5.6	-	-	-
Neut. Red 1% aq.	4.5	+	++	Y/G*
Congo Red 1% aq.	8.5	-	-	-
Acridine Orange	4.9	+	++++	R&G**
Titan Yellow	6.5	+	-	-
Berberine Sulphate	5.7	+	+	Y/G*
Acriflavin Neut.	5.9	-	++++	Y/G*
Acriflavin HCl	2.4	-	++++	Y/G*
Acridine Lactate	5.7	+	+++	Green
Rhodamine	2.9	-	++	Y/G*
Nile Blue	3.6	+	-	-
Basic Fuchsin	5.4	-	-	-
Acid Fuchsin	5.4	+	-	-
Janus Green	3.8	+	-	-
Tinopal 4-B-m conc'	6.5	+	-	-

*Yellow/Green.

**Red and Green.

other unidentified wall and cytoplasmic components fluoresced brightly and prevented visual localization of any bacterial cells.

Monitoring the Hypersensitive Reaction

Ten-day-old Im 216 plants were injected with both washed and broth cultures of race 10, X. malvacearum. The inoculum concentration of each (1.0×10^8 cells/ml) was verified by dilution plate count. Tissue samples of 3 mm X 3 mm were taken for paraffin histology at 0, through 22 hours at two hour intervals after injection. Control samples were also taken that had been injected with sterile distilled water and sterile nutrient broth.

The hypersensitive reaction occurred approximately 12 hours earlier in tissue injected with broth cultures than in tissues injected with washed cells. In tissue fixed immediately after injection there were no observable effects of the presence of the bacteria upon the internal structure of the cotyledon. The tissue resembled the control tissue both in spatial arrangement of cells and overall integrity of the sections (Figure 10). Palisade parenchyma was turgid with numerous chloroplasts lining the tertiary cell wall. Both epidermal strips appeared normal with functional guard cells and glandular hairs being unaffected. Spongy parenchyma was loosely packed and interspersed with numerous air spaces. Distinct nuclei and chloroplasts could be detected when viewed at 450X magnification. Two hours after

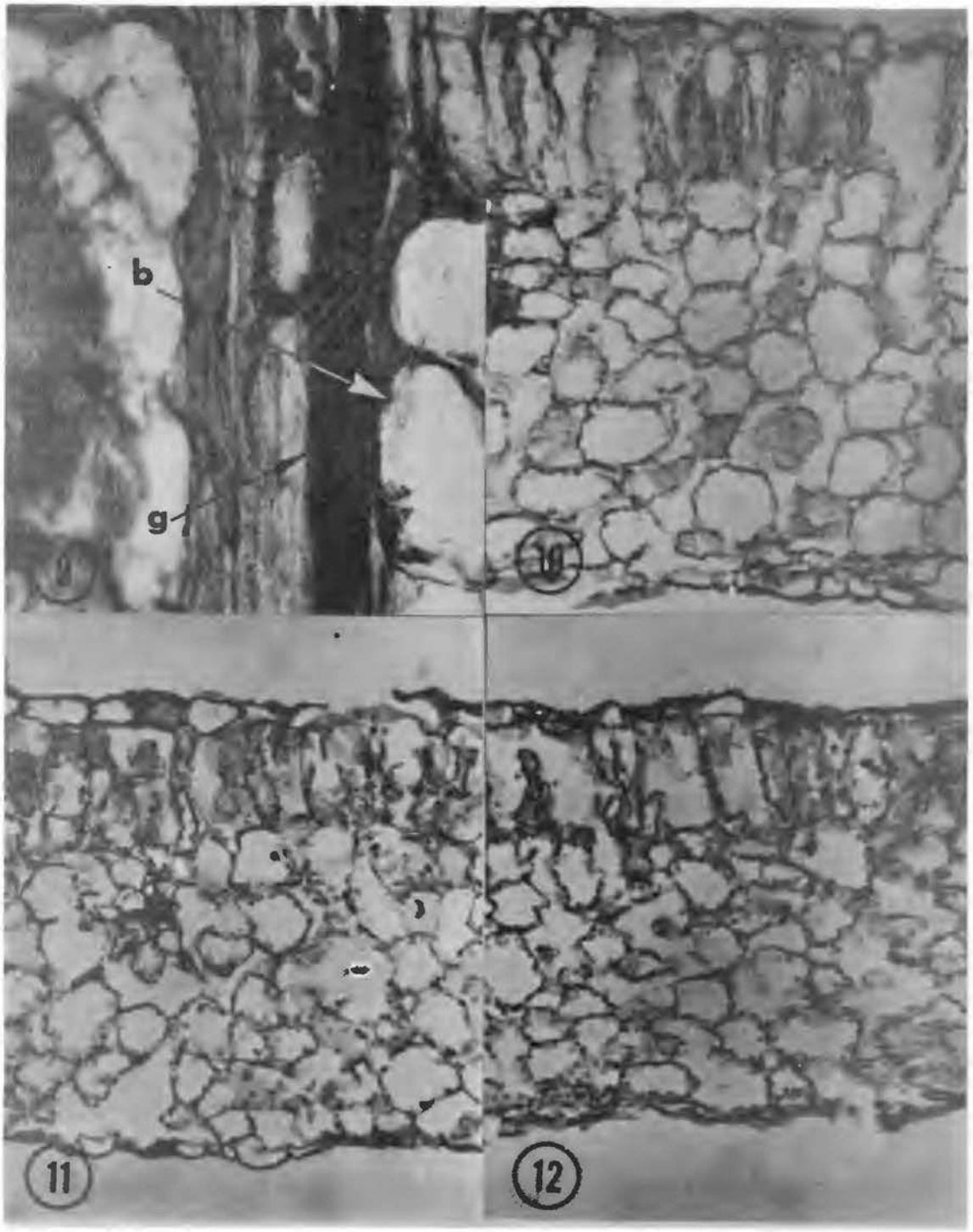
injection few if any changes in the cotyledon tissue were apparent. One noticeable difference was that some of the chloroplasts seem to have moved from the interior cell wall towards the center of both the palisade and spongy parenchyma cells (Figure 11). Cell integrity and internal structure otherwise appeared normal. This stage corresponded to 14 hour samples in the cotyledons injected with washed cells. Four hours after injection with broth cultures the first gross symptoms appeared. Injected cotyledons appeared to have a slight loss of turgor when compared to control specimens. Histologically there was a slight vertical tissue collapse of about 10%. This is presumably the result of desiccation. Internally, cells appeared to have structural damage with the cell walls being the most obviously affected structures (Figure 12). A slight infolding as well as compression in the vertical plane were characteristic of most cells examined. Epidermal cells appeared unaffected. Bundles examined were nearly normal with xylem elements least affected and phloem slightly compressed. This stage resembled specimens examined 16 hours after injection with washed cells. Six hours after injection the cotyledons were flacid, and spongy parenchyma cells had assumed oval shapes from vertical compression (Figure 13). Chloroplasts were found loose in the cytoplasm. Apparent rapid desiccation was giving rise to the vertical collapse and resultant compression of the cells. Eighteen hour samples in the washed

Figure 9. Paradermal Section of Ac 44 Leaf One Week After Inoculation Showing Gumming (g) Found in Phloem Cells (p) of a Vascular Bundle. Magnification: 650X.

Figure 10. Immune Im 216 Cotyledon Tissue Immediately After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.

Figure 11. Immune Im 216 Cotyledon Tissue Two Hours After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.

Figure 12. Immune Im 216 Cotyledon Tissue Four Hours After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.


b
g
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11
12

cell test corresponded to this stage. Eight hours after broth culture injection, the symptoms observed in previous samples were intensified. The vertical collapse was about 50% of the original thickness. The macroscopic symptoms of the cotyledons were flacidity to the point of limpness and a grayish green color. Virtually all intercellular spaces were now absent and spongy parenchyma appeared as a laminar mass with chloroplasts dispersed throughout (Figure 14). Palisade parenchyma cells were squat and cuboidal, and in some sections the cells appeared to have ruptured. Bundles were least affected, with the xylem normal and the phloem compressed. Ten hours after inoculation the internal structure of the cotyledon appeared as an amorphous mass of cell walls and chloroplasts (Figure 15). At this point the physiological portion of the hypersensitive reaction was assumed to be ended with only further desiccation of the cotyledon remaining to complete the reaction. The eight and ten hour samples corresponded to the 20 and 22 hour samples in the water washed inoculum test. From 12 hours after the broth culture injection, desiccation was complete and the tissue was paper thin and brittle to the touch. Areas surrounding the inoculated area were dark brown with red pigments at the fringes.

Response to High and Low Inoculum Levels

The typical hypersensitive reaction so commonly associated with injection of inoculum of 1.0×10^8 cells/ml

is not observed following injection with lower levels of inoculum. The response with reduced bacterial concentration is characterized by little if any macroscopic reaction. An experiment was designed to provide information on possible subtle cellular changes which might occur with injection of X. malvacearum bacteria in concentrations of less than 1.0×10^8 cells/ml.

A series of inocula was prepared with concentrations from 1.0×10^8 to 1.0×10^1 cells/ml in water. Preparation was by the serial dilution method and verification of concentration was by dilution plate count. Inoculations were made in the morning after stomata had opened, usually around 9:30 a.m., on Im 216 plants growing under irrigation in the field. Fully expanded leaves were selected (the fourth leaf down from the apex) for inoculation and labeled. A 1 cm^2 area on the abaxial side of the leaf was water-soaked with each dilution of inoculum and encircled with a felt tipped ink pen and numbered according to the dilution factor. Thus each leaf was inoculated with a full set of dilutions including the 1.0×10^8 cells/ml concentration for the hypersensitive response. Five replications of inoculations were prepared on five different plants. After necrosis was observed in the areas injected with 1.0×10^8 cells/ml concentration, tissue samples were taken from each area and fixed immediately for paraffin histology.

Macroscopically the appearance of the injected areas varied from complete necrosis to no visible symptoms.

A summary of the macroscopic observations is summarized in Table III. Under 10X magnification no symptoms of hypersensitivity could be detected in leaves inoculated with water or bacteria of 1.0×10^1 and 1.0×10^2 cells/ml. With concentrations of 1.0×10^3 and 1.0×10^4 cells/ml, a slight browning reaction could be observed in an area next to the point of injection. This seems to indicate that there is a higher concentration of bacteria in these areas. With inocula of 1.0×10^5 and 1.0×10^6 cells/ml, a distinct browning of the tissue throughout the treated area was observed. There did not appear to be significant vertical collapse or tissue destruction as is seen in necrosis with high concentrations of inoculum. Areas treated with inoculum concentrations of 1.0×10^7 and 1.0×10^8 cells/ml were distinctly necrotic and the reaction resembled that obtained previously when similar inoculations were performed.

The gradation of histological reactions was similar to the macroscopic reactions just described. No cellular damage was detected in those areas injected with inoculum concentrations of 1.0×10^1, 1.0×10^2, and 1.0×10^3 cells/ml. Areas injected with inoculum concentrations of 1.0×10^4, 1.0×10^5, 1.0×10^6, and 1.0×10^7 cells/ml contained some cells which stained much more intensely than surrounding cells. There was a quantitative relationship between the number of intensely staining cells and the concentration of the inoculum used in each area. The relationship between leaf cells affected and inoculum concentration

TABLE III

MACROSCOPIC RESPONSES TO HIGH AND LOW LEVELS OF INOCULUM IN IM 216 LEAVES VIEWED AT 10X MAGNIFICATION

Inoculum Level	Macroscopic Reaction
10^1 bacterial cells/ml in water	no visible reaction
10^2 bacterial cells/ml in water	no visible reaction
10^3 bacterial cells/ml in water	slight browning at injection site
10^4 bacterial cells/ml in water	slight browning at injection site
10^5 bacterial cells/ml in water	browning of injected tissues
10^6 bacterial cells/ml in water	browning of injected tissues
10^7 bacterial cells/ml in water	necrosis and desiccation of injected tissues
10^8 bacterial cells/ml in water	necrosis and desiccation of injected tissues

is graphically illustrated in Figure 21 in the appendix. There appeared to be a linear relationship between the concentration of inoculum injected and the number of plant cells affected with the data point at eight on the X axis representing 100% of the plant cells heavily stained. The slope of the line (0.9) suggests that there is a one to one relationship between numbers of bacteria and numbers of plant cells affected. The data also shows that an individual cell, with lower levels of inoculum, can stain similarly to the way that all cells stain in a necrotic area treated with 1.0×10^8 cells/ml. This may mean that an individual cell that stains intensely may have undergone the hypersensitive reaction.

Plastic Embedding Histology

Thick sections of Im 216 cotyledon tissue were prepared using methods of Feder and O'Brien (1968), to more closely study the reaction of the host cells and the multiplication of the pathogen within the host. Cotyledons were injected with a washed suspension of 1.0×10^8 cells/ml race 1 X. malvacearum and tissue samples were harvested at two hour intervals from 0 time through 22 hours. Small 2 mm X 4 mm samples were excised to facilitate complete fixation and infiltration in the plastic method.

Plastic sections of 2 µm provided data on the distribution and multiplication of bacteria in the cotyledons undergoing the hypersensitive reaction. Of particular

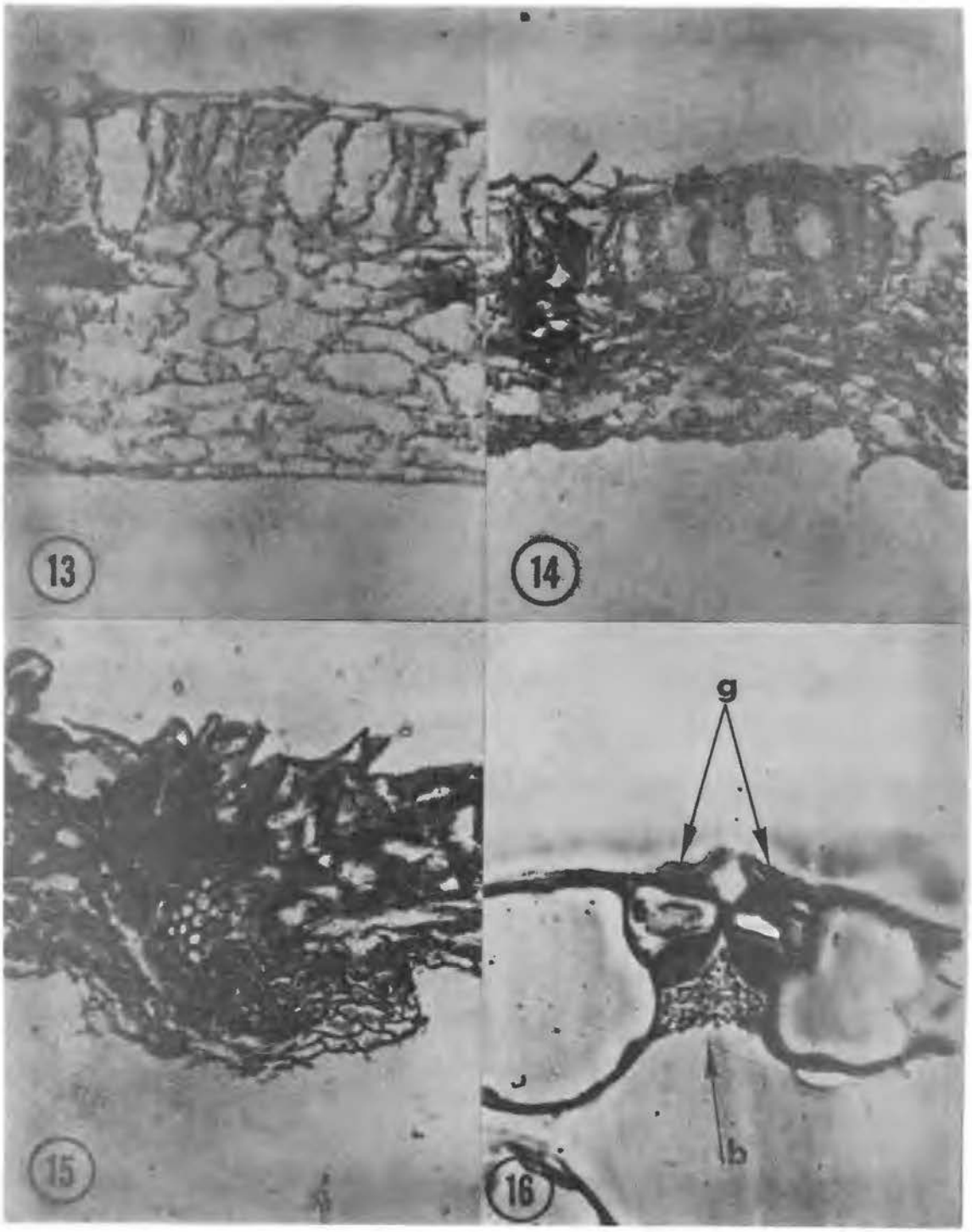
interest was the distribution of bacteria in the cotyledon tissue immediately after injection. Apparently crevices and small confined areas tend to trap and hold the bacteria as the inoculum is forced into the mesophyll. Areas of the internal structure where large numbers of bacteria lodged were the substomatal cavities under the guard cells (Figure 16) between palisade parenchyma (Figure 17) and in small areas between spongy parenchyma cells (Figure 18). These locations later gave rise to small colonies of multiplying bacteria. Sections taken from samples from time 0 through 22 hours after injection were essentially the same with respect to internal structure and distribution and numbers of bacteria. Fourteen hours after injection the beginning of the hypersensitive reaction was first observed. The reactions in the cotyledon were closely parallel to those observed with the paraffin method. The cells began to collapse and also many of the plastids became deformed and swollen against the cell wall. There was also an increase in the numbers of bacteria in the intercellular spaces. Sections taken 16 hours after injection were characterized by an intensification of the effects seen in the 14 hour samples with the addition of vertical compression. Samples from 18 and 20 hour tissue were very necrotic with compression causing the walls of palisade and spongy parenchyma to fold over in a layering fashion (Figure 19). Clumps of slime teeming with bacteria were found in areas similar to those where numbers of bacterial cells had been located in

Figure 13. Immune Im 216 Cotyledon Tissue Six Hours After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.

Figure 14. Immune Im 216 Cotyledon Tissue Eight Hours After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.

Figure 15. Immune Im 216 Cotyledon Tissue Ten Hours After Inoculation with 1.0 X 10^8 Cells/ml in Broth. Magnification: 220X.

Figure 16. Bacteria Found in a Substomatal Cavity of an Im 216 Cotyledon Immediately After Injection with 1.0 X 10^8 Cells/ml in Water. Features are: Bacterial Cells (b), and Guard Cells (g). Magnification: 1400X.


13
14
15
16
g
b

earlier samples (Figure 20). Further examination of tissue 22 hours after injection revealed additional cell destruction, greater vertical collapse, and eventual desiccation of tissues.

Pectin Localization

In an attempt to determine if a loss of pectin occurred in leaf tissues undergoing the hypersensitive reaction, a histochemical test for pectin (Reeve, 1958) was performed on thinly sectioned fresh Im 216 leaf tissue undergoing the hypersensitive reaction. Both control uninoculated tissue and inoculated tissue (1.0×10^8 cells/ml in water race 1 X. malvacearum) were tested. Samples were taken from the inoculated tissue eight hours after the inoculation.

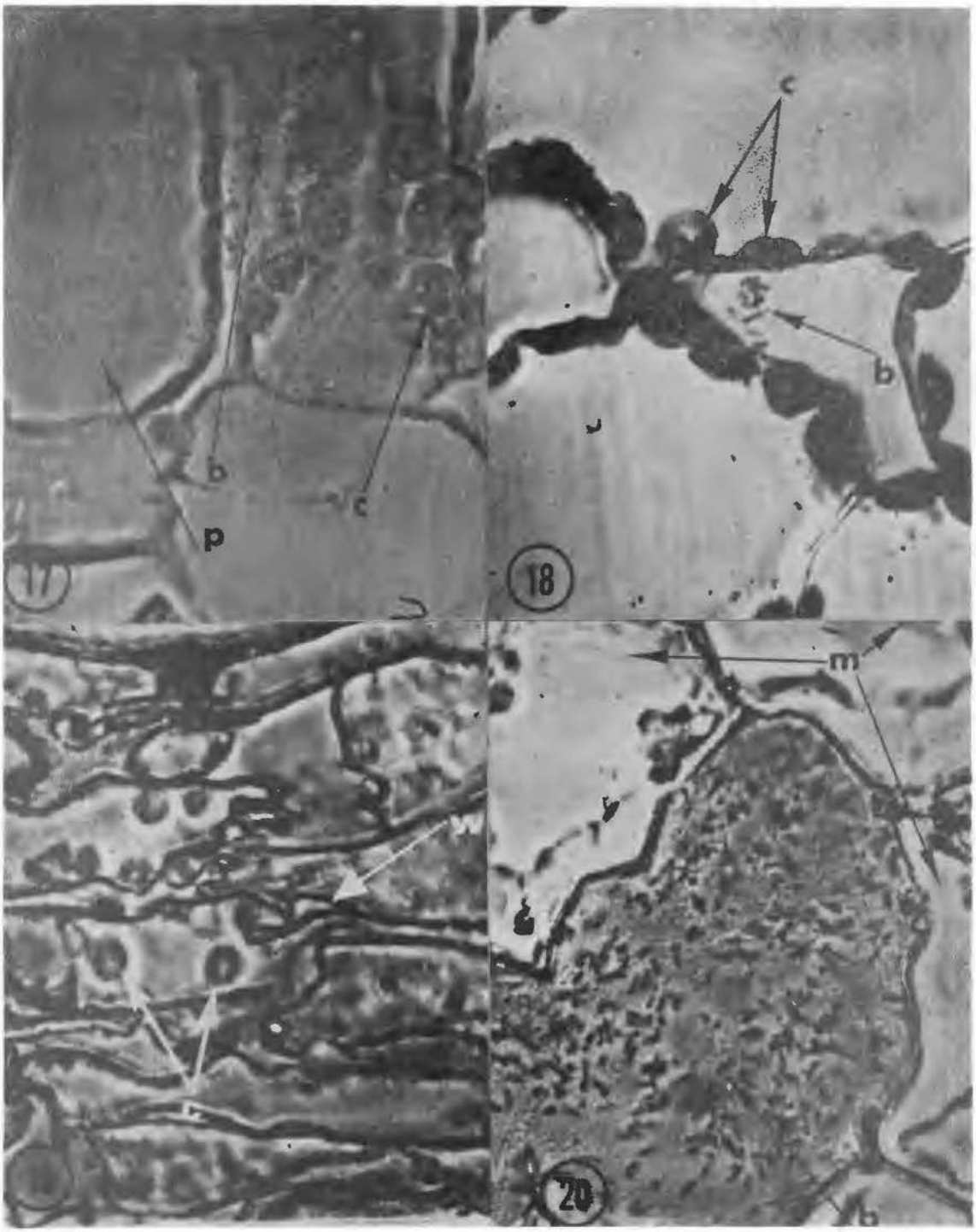
After evaluation no quantitative differences in pectin content could be detected between control and experimental tissues. All samples gave a positive test for pectin, but the intensity of staining was inconsistent.

Figure 17. Bacteria Found in Palisade Parenchyma of Im 216 Cotyledon Immediately After Injection with 1.0 X 10^8 Cells/ml in Water. Indicated Features are: Bacterial Cells (b), Palisade Cell (p), and Chloroplasts (c). Magnification: 1400X.

Figure 18. Bacteria Found in Spongy Parenchyma of Im 216 Cotyledon Immediately After Injection with 1.0 X 10^8 Cells/ml in Water. Indicated Features are: Bacterial Cells (b) and Chloroplasts (c). Magnification: 1400X.

Figure 19. Layering of Cell Walls in Im 216 Cotyledon Twenty Hours After Injection with 1.0 X 10^8 Cells/ml in Water. Indicated Features are: Cell Walls (w) and Chloroplasts (c). Magnification: 1400X.

Figure 20. Bacterial Colony Found in Im 216 Mesophyll Tissue Twenty Hours After Injection with 1.0 X 10^8 Cells/ml in Water. Indicated Features are Bacterial Colony Showing X. malvacearum Cells in a Matrix of Slime (b) Surrounded by Host Mesophyll Cells (m). Magnification: 1400X.


b
c
p
17
c
b
18
m
w
20
b

CHAPTER V

DISCUSSION

Histological studies of the hypersensitive reaction in the Im 216 variety of upland cotton revealed several interesting observations. Structural changes, as a result of the hypersensitive reaction in leaf and cotyledon tissue, can be detected in as little as four to six hours after injection with a broth culture of X. malvacearum. The first and probably most dramatic of all of the reactions observed is the vertical collapse and rapid desiccation of leaf or cotyledon tissues. This rapid drying is probably due to loss of membrane integrity and selectivity allowing release of cytoplasmic solutes into the intercellular spaces, followed by water loss as osmotic pressure of the cell cytoplasm falls. Membrane alteration is further evidenced by the fact that chloroplasts seem to become swollen and drift away from the inner wall of the cell. Membrane studies are beyond the range of the light microscopist. Ultrastructural studies by electron microscopy would be useful in confirming these suggestions of membrane damage. Of particular interest and probably the most useful part of this study was the observation that individual cells may be undergoing the hypersensitive reaction with lower levels

of inoculum than concentrations that are commonly used to elicit macroscopic necrosis. This conclusion is based on the observation that in sections taken from necrotic areas all of the internal parenchyma cells stain heavily with safranin and that the occurrence of these cells decreases linearly with decreasing concentrations of inoculum. This could explain why the hypersensitive reaction is never seen under natural conditions, because natural inocula are dilute. Data also indicated that the hypersensitive reaction occurred 12 hours later in tissues inoculated with washed cells than in tissues inoculated with broth culture cells. This suggests that something in the broth or polysaccharide slime (which were lost during washing) might have a role in early initiation of the host response.

The distribution of the bacteria within the host tissue was found to be unequal. Observations were made of 50 or more bacterial cells in pockets such as substomatal cavities and a few to single bacteria in the parenchymatous tissue of the mesophyll. The distribution is probably the result of the inoculation method and the filtering action of the host cells, but this phenomenon may also occur under natural conditions. The quantitative relationship between host and pathogen cells probably can be determined with further study of statistical relationships between bacterial numbers and necrotic cells (Turner, 1974).

Pathogen growth within the host tissue was also examined and bacterial growth within the immune leaves and

cotyledons occurred during the hypersensitive reaction. Microscopic pockets of bacterial cells and slime were found in immune tissue near the end of the hypersensitive reaction. These pockets probably develop from the large clumps of bacteria observed in certain parts of the leaf tissue immediately after inoculation. The destruction of host tissue effectively arrested the spread of these slime pockets. By contrast, in susceptible tissues bacteria multiplied and spread through the intercellular spaces dissolving host cells with only vascular bundles checking pathogenesis of new tissue.

CHAPTER VI

SUMMARY

Histological studies of the host responses in immune and susceptible cotton varieties to X. malvacearum indicated the following:

1. Vertical collapse of the tissue, cell compression, cell wall folding, chloroplast damage, and desiccation were all characteristic of the hypersensitive reaction in immune leaf and cotyledon tissue. None of these reactions was observed in the susceptible variety during the six-day period it was observed, but pathogenesis and cell destruction by dissolution did occur.

2. The hypersensitive reaction could be detected histologically in four to six hours after inoculation with unwashed cells and within 16 hours after inoculation with washed cells.

3. A linear relationship was observed between inoculum concentration used and numbers of host cells that abnormally stained intensely red.

4. Bacteria were found dispersed throughout the leaf after inoculation with certain areas containing greater numbers of bacteria than other areas.

5. Bacterial growth was observed during the hypersensitive reaction with the formation of bacterial slime pockets in the intercellular spaces.

6. The hypersensitive reaction effectively stops pathogenesis in host tissue by bacteria.

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APPENDIX

GRAPH OF HOST CELL AND PATHOGEN INTERACTION

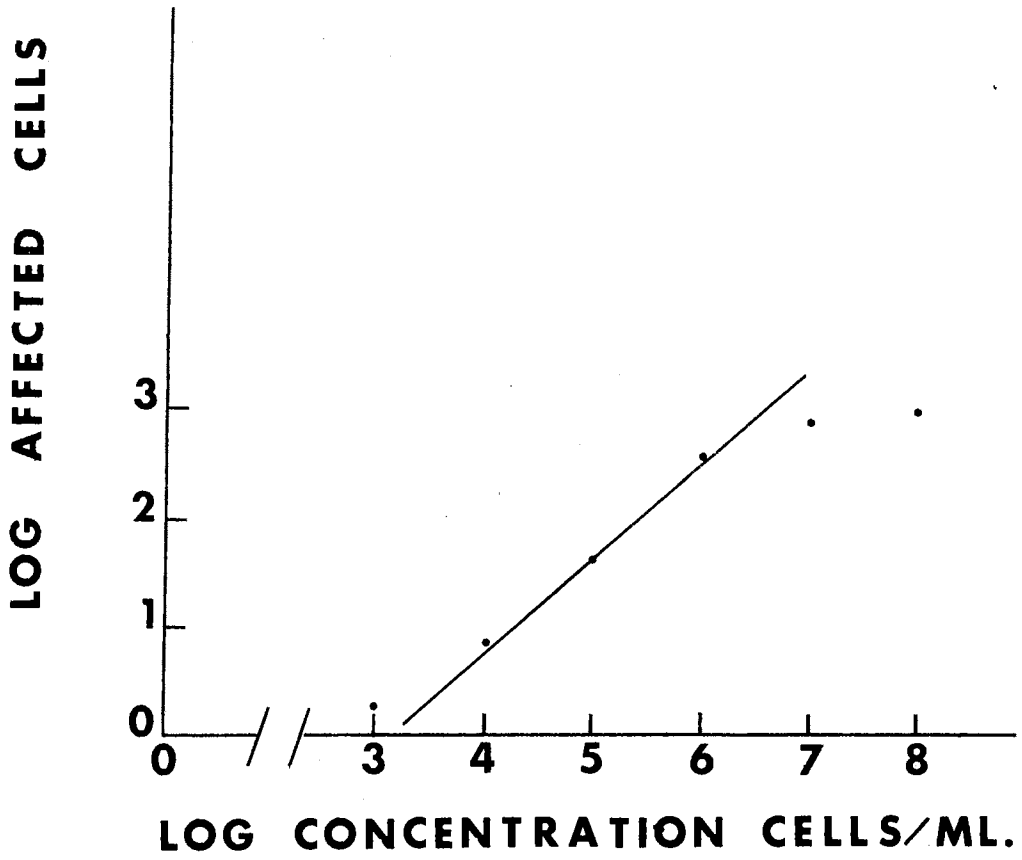

Figure 21. Graphic Illustration of the Relationship Between the Log of the Inoculum Concentration and the Log of the Number of Host Cells That Stained Intensely

VITA

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