

Histopathology of Immune and Susceptible Cotton Cultivars Inoculated with *Xanthomonas malvacearum*

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ABSTRACT

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Histological observations showed that, with large doses of inoculum, damage to immune host tissue occurred within 4-6 hr after inoculation. Chloroplast damage, cell compression, and tissue shrinkage followed by necrosis and desiccation were the most obvious features of the hypersensitive response. The susceptible cultivar exhibited none of these responses; the pathogen directly disintegrated host cells and produced foliar lesions. A direct relationship between inoculum concentration and a cellular staining

phenomenon was observed in the immune cultivar. Thick sections of plastic embedded tissue showed bacteria localized among host cells. High numbers of bacteria were found in sub-stomatal cavities and between palisade and spongy parenchyma cells. Growth of the pathogen in the immune host tissue occurred during the hypersensitive reaction with the resultant appearance of localized colonies of bacteria in polysaccharide slime among necrotic host cells.

Additional key words: plasmolysis, flaccid, intensely-stained, water-soaked, tissue-shrinkage.

Xanthomonas malvacearum (E. F. Sm.) Dows. causes bacterial blight of cotton, a disease of world-wide importance. The most promising method of control has been the development of resistant cultivars. Breeding lines and cultivars with blight resistance ranging from susceptible to immune are now available with disease reactions ranging from severe water-soaking to no visible symptoms (1). The immune cultivars show a hypersensitive necrotic reaction following injection with inoculum levels $>10^6$ bacteria/ml (5).

Much work has been done on the physiology of the hypersensitive reaction in plant-bacteria systems, but there have been few histological studies. In this study, the histological nature of the hypersensitive reaction in cotton was examined. Experiments were performed to show effects on individual host cells as well as the reaction of leaf and cotyledon tissues when inoculated with varying concentrations of virulent bacterial cells. The distribution of bacteria in the host tissue following artificial inoculation, and pathogen multiplication during the hypersensitive and susceptible reactions also were studied.

MATERIALS AND METHODS

Plant growth conditions.—Seeds of the susceptible cotton cultivar Ac 44 and the immune cultivar Im 216 were planted in 15.5-cm diameter clay pots in soil-

vermiculite-peat mixture (8:1:1, v/v). The pots were covered with plastic wrap and incubated in a constant-temperature growth chamber set to regulate 12 hr of light (incandescent and fluorescent at 21,200 lux) at 32 C and 12 hr of darkness at 24 C. The seedlings emerged from the soil within 6 days, and within 12 days the first foliage leaves appeared. Cotyledons were selected for injection at this stage of maturity.

Bacterial culture method.—Race 1 and race 10 (6) of *X. malvacearum* were obtained from stock cultures. Inoculum was prepared by needle transfer from a single colony on dextrose-agar medium to medicine bottles containing 100 ml of sterile nutrient broth and grown for 24 hr at 25 C in shake culture. Washed inoculum was prepared by centrifuging 40-ml portions of broth culture at 12,000 g for 10 min and resuspending the cells in sterile distilled water. This procedure was repeated twice and the concentration of the final suspension was verified by dilution plate count. Dilute concentrations of the initial inoculum were prepared by serial dilution. A range of concentrations from 10^1 to 10^8 cells/ml was obtained in this manner. Duplicate plate counts were made for all concentrations.

Inoculation method.—The inoculation method was a variation of the procedure described by Klement (8). A standard 1-cc syringe equipped with a 0.4-mm diameter (27-gauge) needle was inserted into the mesophyll and inoculum was injected into the intercellular spaces. This method allowed visual approximation of the amount of inoculum introduced and localization of the disease reaction. Cotyledons of susceptible and immune plants

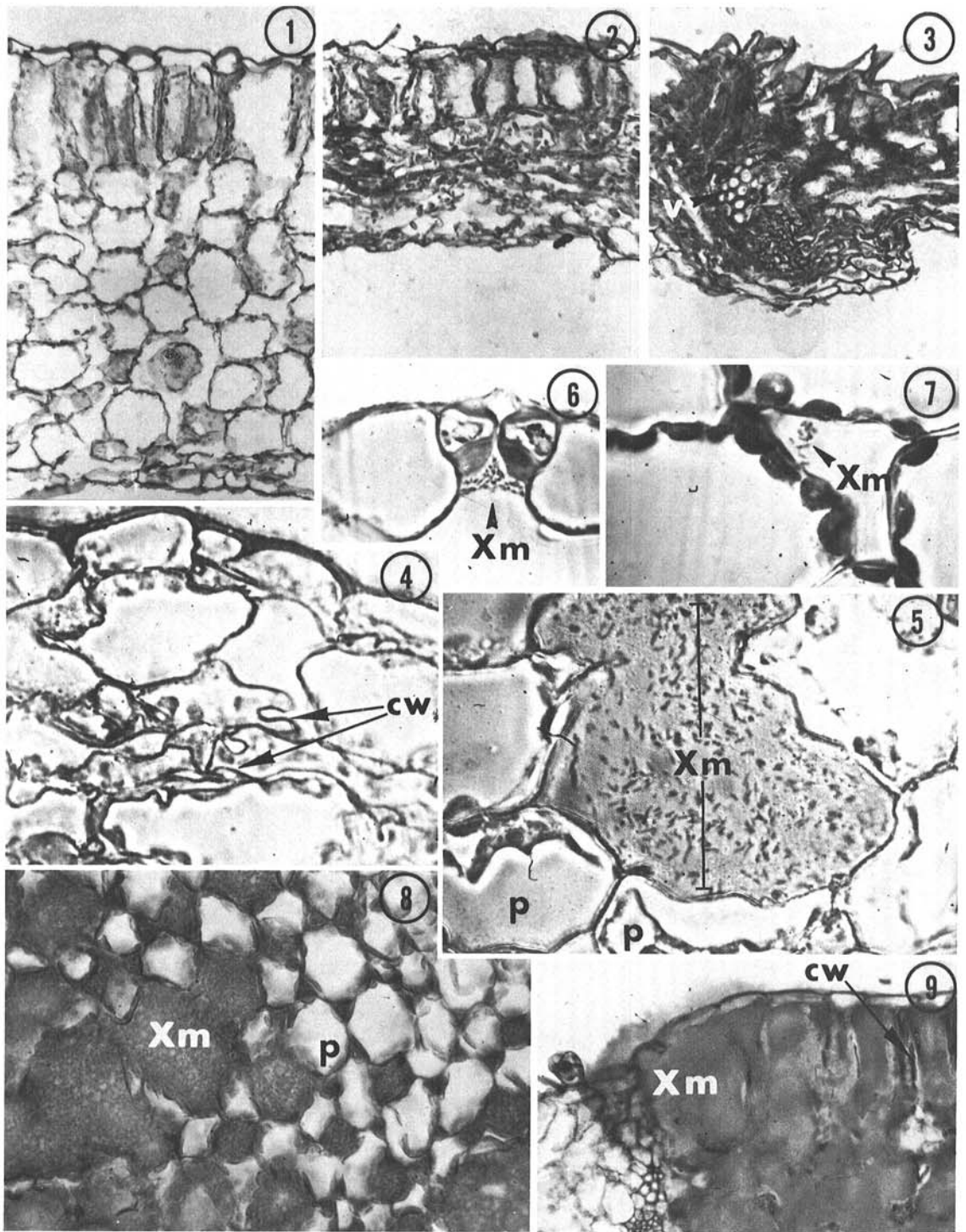


Fig 1-9. Histopathology of immune and susceptible cotton cotyledon tissue inoculated with *Xanthomonas malvacearum*. 1) Immune cotyledon tissue immediately following infiltration with a 10^8 cells/ml suspension of bacteria. Tissue appears normal with no evidence of damage. 2) Similar tissue 8 hr following infiltration (symptoms include: cell disruption, tissue shrinkage, and intense staining) and 3) 10 hr following infiltration with severe symptoms evident. Mesophyll appears amorphous and dense with vascular tissue (v) relatively unaffected (Fig. 1-3, $\times 200$). 4) Mesophyll cells of immune cotyledon 12 hr following inoculation showing folding and layering of the cell walls (cw) during the hypersensitive reaction, $\times 550$. 5) Intercellular space 12 hr following inoculation; filled with bacteria (Xm) in polysaccharide slime with plasmolyzed host cells (p) immediately adjacent. 6) Sub-stomatal cavity of immune cotyledon immediately following inoculation showing numerous bacterial cells (Xm). 7) Intercellular space of similar tissue showing bacterial cells (Xm), (Fig. 5-7, $\times 700$). 8) Paradermal section of susceptible cotyledon 6 days following inoculation showing invading bacteria (Xm) filling the intercellular spaces and dissolving host palisade cells (p). 9) Cross section of same tissue showing bacterial masses (Xm) and remains of host palisade cell walls (cw), (Fig. 8-9, $\times 320$).

were inoculated separately with bacteria in broth culture and washed bacteria resuspended in distilled water. Check injections were made with sterile distilled water and sterile culture broth.

Paraffin embedment.—Tissue samples from immune cotyledons were fixed in cold formalin/propionic acid/ethanol (1:1:18, v/v) fixative at 2-hr intervals beginning immediately after inoculation. Tissue samples from susceptible cotyledons were taken daily for 1 wk and fixed in a similar manner. Following fixation the samples were embedded as previously described (7). Sections 10 μ m thick were cut and mounted on clean glass slides with gelatin adhesive and 4% phenol. After drying for 24 hr in a 37 C dust-free oven, the slides were carried through a staining series of Gray and Pickel's safranin (4) and Johansen's fast green (7), and cleared with clove oil.

Plastic embedment.—Tissue samples of inoculated and check cotyledons were prepared for histology by the method of Feder and O'Brien (2) with prepared media from the JB-4 plastic embedding kit (Polysciences, Warrington, Pa.). Sections 2 μ m thick were cut on a standard rotary microtome with a steel knife. Sections were stained with 1% acid fuchsin in distilled water for 2 min, then counterstained with 0.05% toluidine blue in 0.02 M benzoate buffer at pH 4.4 (9) for 15 sec.

RESULTS

Monitoring the hypersensitive reactions in cultivar Im 216.—The hypersensitive reaction occurred in tissues injected with the highest concentrations of bacteria (10^8 , 10^7 cells/ml). Both races of *X. malvacearum*, when injected as a broth or water suspension, elicited a strong host reaction. All of the hypersensitive host reactions were histologically identical. The hypersensitive reaction in cotyledons injected with washed bacteria was delayed slightly compared to similar inoculations made with broth suspensions.

In tissues injected with $\sim 10^8$ cells/ml broth suspension, no signs of tissue alteration were observed when tissue was fixed immediately after inoculation (Fig. 1). Progressive deterioration of cells and general tissue structure occurred from 4 hr after injection. Symptoms included organelle disruption, plasmolysis, and tissue-shrinkage. By 8 hr following injection the cotyledons were flaccid, and appeared gray-green. Tissue-shrinkage was about 50% of the original thickness, and the spongy parenchyma appeared as a laminar mass with chloroplasts dispersed throughout (Fig. 2). By 10 hr, the internal structure of the tissue appeared as an amorphous mass of cell walls and chloroplasts (Fig. 3). Desiccation was complete by 12 hr and the tissue was paper thin and brittle to the touch.

Observations on sections from plastic-embedded tissues clarified observations of symptoms in paraffin-embedded tissues. Soon after inoculation, the mesophyll cells began to show plasmolysis. Cell walls collapsed and plastids became deformed and swollen following a loss of orientation along the inner cell wall. These symptoms were intensified with progressive tissue-shrinkage and desiccation causing the walls of the palisade and spongy parenchyma to fold in a layering fashion (Fig. 4). Areas containing numerous bacteria were found in the

intercellular spaces between plasmolyzed and necrotic cells (Fig. 5).

Distribution and multiplication of bacteria in cultivar Im 216.—Tissue sections from plastic-embedded tissue injected with washed bacteria or broth culture at $\sim 10^8$ cells/ml concentration provided data on the initial distribution of bacterial cells in the host tissue following infiltration. Crevices and small confined areas within the cotyledon tended to trap and hold the bacteria as the inoculum was forced into the mesophyll. Large numbers of bacteria were found in sub-stomatal cavities (Fig. 6) and between mesophyll cells (Fig. 7). The bacteria trapped in these areas later gave rise to small colonies of bacteria as previously described (Fig. 5).

Response of cultivar Im 216 to high-and low inoculum concentrations.—Im 216 leaves were inoculated with suspensions of washed race 1 *X. malvacearum* cells varying in concentration from 10^1 to 10^8 cells/ml. The macroscopic appearances of the injected areas 24 hr following inoculation varied from no visible symptoms to necrosis. Under $\times 10$ magnification, no symptoms of hypersensitivity could be detected in areas injected with distilled water or bacteria suspensions of 10^1 or 10^2 cells/ml. With suspensions of 10^3 and 10^4 cells/ml, a slight browning reaction was observed in tissue adjacent to the injection site. This suggested that there was a higher concentration of bacteria in these areas. A distinct browning of the tissue throughout the inoculated areas was observed following infiltration of 10^5 and 10^6 cells/ml suspensions. There was no apparent tissue-shrinkage or massive tissue destruction as seen in tissues injected with higher concentrations of inoculum. Areas injected with inoculum suspensions of 10^7 and 10^8 cells/ml were distinctly necrotic.

Paraffin-prepared sections stained with safranin and fast green failed to reveal cellular damage in areas injected with distilled water or low concentrations of bacteria. Sections of areas injected with suspensions of 10^4 , 10^5 , 10^6 , and 10^7 cells/ml contained isolated mesophyll cells that stained much more intensely with safranin than other adjacent cells. The numbers of these bright red cells increased in direct proportion to the inoculum concentration. None of the intensely stained cells could be detected in tissue injected with suspension of 10^3 bacteria/ml and lower.

Histopathology in the susceptible cultivar Ac 44.—Six days after inoculation, macroscopic disease symptoms became evident on inoculated cotyledons. A dark, shiny water-soaked appearance along with bacterial extracellular polysaccharide was characteristic of the diseased areas. Tissue samples were taken as previously described and processed for paraffin sectioning. Microscopic inspection of sections showed bacteria densely distributed through the intercellular spaces of the palisade and spongy parenchyma cells, with no apparent reactions of the host to pathogenesis (Fig. 8, 9). No bacteria were found in the xylem, and apparently the xylem was not attacked, even in areas with advanced symptoms.

DISCUSSION

Histological studies of the hypersensitive reaction in the Im 216 cultivar of upland cotton revealed that

structural changes in leaf and cotyledonary tissue can be detected in as little as 4-6 hr following inoculation with high concentrations (10^8 - 10^7 cells/ml) of *X. malvacearum* (race I or race 10) in culture broth. The same host response was delayed when similar concentrations of washed bacteria resuspended in distilled water were injected into the immune host. The observed alterations in immune host tissue during the hypersensitive reaction are in many ways similar to reported symptoms in tobacco (3) and pepper (10) undergoing a hypersensitive response to incompatible bacteria.

At present, the mechanism for the hypersensitive reaction in cotton is unexplained, but some workers have suggested the role of pectinases and cell wall damage (5). Our data indicate that cell wall damage is a factor in the overall tissue reaction. Cell wall bending and folding are symptoms of the hypersensitive reaction, whereas direct disintegration of the wall occurs during the susceptible reaction. Early membrane damage also appears to be a contributing factor in necrosis. Photomicrographs indicate that plasmolysis of immune host cells occurs during the hypersensitive reaction. Other workers have observed similar responses in tobacco (3) and there have been many reports of electrolyte loss from tissues inoculated with incompatible bacterial populations.

The possibility that individual host cells may be undergoing the hypersensitive reaction with lower levels of inoculum than those required to elicit a macroscopic response was suggested by a staining phenomenon that differentiated single necrotic cells in tissues inoculated with low concentrations of bacteria. This may explain why the macroscopic hypersensitive reaction is never seen in the Im 216 cultivar under natural conditions. Natural inocula are assumed to be dilute and contain small populations of bacteria; therefore, only microscopic hypersensitive reactions may occur as described for tobacco (12). The uneven distribution of bacteria in host tissue following inoculation is probably the result of the infiltration process, but this phenomenon may occur under natural inoculation conditions. A quantitative host-pathogen cell ratio study was not made in this investigation, but it is possible that localized numbers of bacteria could cause host cells in the immediate vicinity to undergo the hypersensitive response while more distant host cells remained unaffected. Microscopic colonies of bacteria that developed from small localized groups of bacteria were characteristically surrounded by

plasmolyzed host cells in the Im 216 cultivar. In contrast, bacteria multiplied in great numbers in the susceptible Ac 44 cultivar and spread through the intercellular spaces dissolving host cells, a condition characteristic of a high degree of compatibility. The bacteria were localized in the vascular network, giving the macroscopic lesions a characteristic "angular" appearance. Symptoms in the susceptible cultivar were similar to those previously described for susceptible cotton (11) and susceptible pepper (10).

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