Necrosis Induction in Cotton

D. G. Hopper, R. J. Venere, L. A. Brinkerhoff, and R. K. Gholson

D. G. Hopper: Former Research Associate, Department of Biochemistry, Oklahoma State University, Stillwater 74074. Currently Visiting Scientist, Department of the Air Force, Aerospace Research Laboratories (AFSC), Wright-Patterson Air Force Base, Ohio 45433. R. J. Venere and R. K. Gholson: Research Associate and Professor of Biochemistry, respectively, Department of Biochemistry, Oklahoma State University, Stillwater. L. A. Brinkerhoff: Research Professor, Langston University, Langston, Oklahoma 74052, and Professor, Department of Botany and Plant Pathology, Oklahoma State University, Stillwater 74074.

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ABSTRACT

The necrotic resistant response (hypersensitivity) of upland cotton *Gossypium hirsutum* to *Xanthomonas malvacearum* was examined. Necrosis induced by the antibiotics dactinomycin, chloramphenicol, cycloheximide, mitomycin C, and puromycin, and by the inorganic ions Hg^{++} and Cu^{++}, is similar in appearance to that induced by *X. malvacearum* in resistant cotton cultivars. A necrotic lesion is also produced by sterile culture filtrates of *X. malvacearum* and several other *Xanthomonas* spp. grown on an autoclaved cotton leaf medium. The necrosis induction caused by the culture filtrates is correlated with the activity of enzymes which degrade the pectinaceous constituent of plant cell walls. No necrotic response is observed with culture filtrates from *X. malvacearum* grown on nutrient broth or on a basal salts medium containing any one of several purified carbon sources. The results suggest that the necrotic lesion produced in the cotton host infected with *X. malvacearum* may be initiated by the production of a polymethylgalacturonase by the invading bacteria.

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Additional key words: bacterial blight, host-pathogen interaction.

Compounds and preparations that induce hypersensitive necroses in plants have been the subject of many studies. These compounds are of interest since similar substances may be formed in the early stages of incompatible host-pathogen interactions. Fungi (18) and bacteria (4, 7, 11, 15, 24) are known that produce necrosis-inducing culture filtrates when grown on simple media; however, in many systems a necrosis-inducing capacity separable from live cells has not been observed. This is the case in bacterial blight of cotton.

The present report concerns the production of necrosis in upland cotton *Gossypium hirsutum* L. by two races of the pathogen *Xanthomonas malvacearum* (E. F. Sm.) Dows. The host's responses to antibiotics, inorganic ions, and in vivo and in vitro culture filtrates are investigated in immune, resistant, and susceptible inbred lines of cotton.

MATERIALS AND METHODS.—*Races of the pathogen, inocula, plant lines, and growth conditions.*—Virulent cultures of races 1 and 10 of *X. malvacearum* were used. Virulence was established by inoculation of suspensions of the two races into differential cotton lines according to Brinkerhoff (1).

In some cases, other *Xanthomonas* spp. were used. These included *X. campestris* (Pam.) Dows., *X. oryzae* (Uyjeda and Ishiyama) Dows., *X. phaseolus* (E. F. Sm.) Dows. var. *sojense* (Hedges) Starr and Burkh., and *X. vesicatoria* (Doidge) Dows.

Bacteria were maintained on potato-carrot-dextrose agar slants at 25 C. When needed, the bacteria were transferred to Difco nutrient broth and grown for 24 hours at 25 C in shake culture. The cells were washed by two successive centrifugations at 10,000 *g* for 30 minutes at 4 C. The bacterial pellet was resuspended in sterile tap water equal in volume to the original broth culture. The inoculum cell number was determined by the plate count method. Serial dilutions were made in sterile distilled water, and nutrient agar was used to grow the bacteria. The cell numbers of all other bacterial cell suspensions were determined in this manner.

Cotton plants were grown from our seed collection. *G. hirsutum* cultivars Acala 44 (Ac 44), Acala 161 (Ac 161), and Immune 216 (IM 216) were used. Ac 44 is susceptible to both bacterial races; Ac 161 is susceptible to race 10 and resistant to race 1; Im 216 is resistant to all known races of the pathogen.

Plants were grown in controlled-environment chambers. The light regime was 12 hours of incandescent plus fluorescent light 43,030 lx (4,000 ft-c) preceded and followed by 1 hour of incandescent light only. The light-period temperature was 32 C; and at nighttime, 19 C. These conditions produce sharp, rapid development of blight symptoms in cotton (2).

Cotyledons were inoculated when 3-6 weeks old; leaves were from 1- to 3-month-old plants.

Antibiotics and salts.—The antibiotics and the concentrations in deionized water of each used were: dactinomycin (0.0008-0.08 mM), chloramphenicol (6.2 mM), cycloheximide (7.1 mM), mitomycin C (0.3 mM), puromycin (4.3 mM), and streptomycin (3.4 mM). All of the antibiotics were purchased from Sigma Chemical Co., and were used without further purification.

The reagent-grade chloride salts of Cu^{++}, Hg^{++}, and Na^{+} were from Eastman Kodak Co. The concentrations of the salt solutions employed were 1-3 mM, 1-5 mM and 1-420 mM for Cu^{++}, Hg^{++} and Na^{+}, respectively. The concentrations of the salt solutions, as well as the

solutions of the antibiotics, are essentially the same as those used in a study of the induction of necrosis and of pisatin synthesis in excised pods of pea *Pisum sativum* L. (22, 23).

Media.—Four types of culture media were employed in experiments designed to detect the production of an active necrosis-inducing principle by *X. malvacearum*. These included: (i) Difco nutrient broth, (ii) basal salts plus a defined carbon source, (iii) an aqueous extract of the intercellular fluid of healthy (IEH) or of artificially inoculated (IEI) cotyledons, and (iv) basal salts plus autoclaved Ac 44 and Im 216 cotton leaves.

The basal salts solution was composed of the following salts in g/liter: $MgSO_4 \cdot 7H_2O$, 0.1; $NH_4H_2PO_4$, 1.0; K_2HPO_4, 1.0; and NaCl, 5.0. The pH was adjusted to 6.5 with 10% (w/v) NaOH. This solution was dispensed in 90-ml portions into 200-ml screw-capped prescription bottles, and then autoclaved at 1.0 atmosphere (15 lb) for 15 minutes. Before cooling, 10 ml of a 25% (w/v) solution of a filter-sterilized carbon source was aseptically added to each bottle. Carbon sources used were glucose, cellobiose, cellulose, gluconic acid, galacturonic acid, and citrus pectin, all from Sigma Chemical Co.

The IEH medium was obtained as described for the extraction of intercellular fluid from tobacco leaves (12). Cotyledons were water-soaked by injection of sterile tap water, and the intercellular fluid was withdrawn by centrifugation of half-cotyledons at 3,000 *g* for 20 minutes at 4 C. The fluid was collected and recentrifuged at 15,000 *g* for 30 minutes at 4 C, and then further sterilized with a Swinney filter of 0.45-μ pore size attached to a 5-ml syringe.

The IEI medium was prepared as above except that the cotyledons were thoroughly water-soaked with a washed suspension of a race 1 nutrient broth culture of *X. malvacearum* (10^8-10^9 cells/ml) 24 hours before extraction of the intercellular fluid. The IEI fluid was yellow from Ac 44 and Ac 161 cotyledons and yellow-brown from Im 216 cotyledons. The yield was 6 ± 1 ml per 80 cotyledons of each plant line.

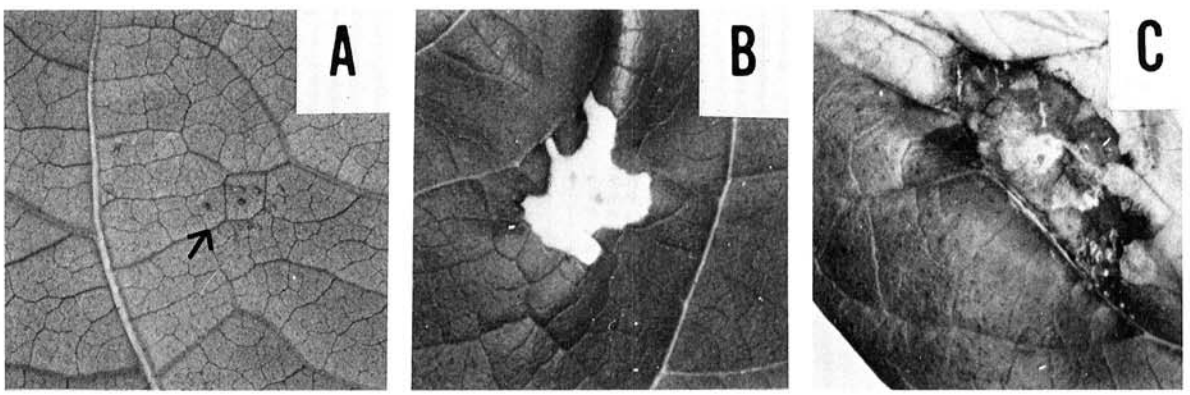

Fig. 1-(A to C). Symptoms after three weeks from infiltration of leaf tissue of blight resistant, immune and susceptible cotton cultivars. **A)** Tap water in resistant Ac 161. **B)** Race 1, 10^8 cells/ml, in immune (Im 216). **C)** Race 1, 10^8 cells/ml, in susceptible Ac 44. (B) showed only necrosis, whereas (C) developed watersoaking at first and then necrosis. The very small dark spots are from pin pricks made at the time of inoculation to facilitate watersoaking.

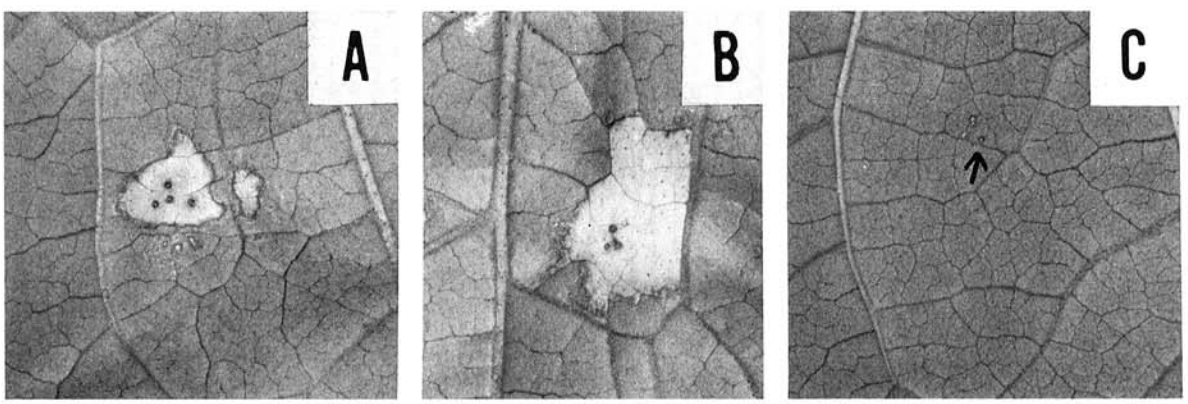

Fig. 2-(A to C). Symptoms after 3 weeks induced by injection of chloride salts in blight susceptible Ac 44 leaf tissue: **A)** 1 mM $CuCl_2$; **B)** 1 mM $HgCl_2$; **C)** 1 mM NaCl. Note lack of injury from NaCl.

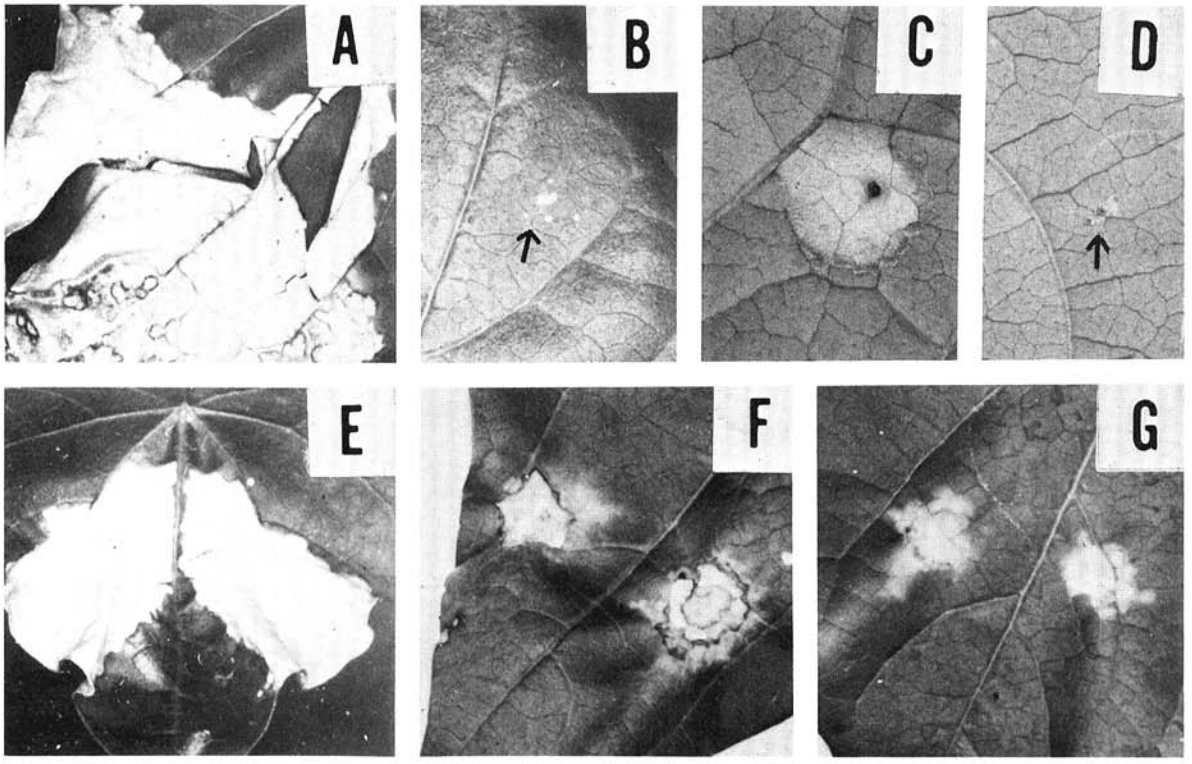

Fig. 3-(A to G). Symptoms induced after three weeks by injection of antibiotics into immune or susceptible cotton leaf tissue: **A)** Large spreading lesion elicited by chloramphenicol in Immune, Im 216. **B)** Very small lesion (ar arrow) induced by chloramphenicol in resistant, Ac 161. **C)** Necrotic lesion induced in Ac 44 by 0.08 mM dactinomycin. **D)** Dactinomycin in Ac 44 at 0.008 mM. **E)** Necrosis in Ac 44 induced by cycloheximide. **F)** Necrosis in Ac 44 induced by mitomycin C. **G)** Necrosis in Ac 44 induced by puromycin. The response of immune, resistant and susceptible plant lines was the same to the antibiotics with the exception of chloramphenicol (A and B). This difference was consistent in several tests.

The cotton leaf medium was prepared by grinding together 50 g each of Ac 44 and Im 216 leaves in 1.0 liter of the basal salts solution with a Waring Blendor (set at high speed) for 2 minutes at 4 C. The slurry was dispensed in 90-ml portions into 200-ml screw-capped prescription bottles, and then autoclaved at 1.0 atmosphere (15 lb) for 20 min. After cooling, the bottles were aseptically inoculated with 10 ml of a bacterial suspension prepared from a 24-hour nutrient broth culture. Cultures were grown with shaking (80 oscillations/ min) at 25 C for up to 21 days.

Culture filtrates.—Sterile filtrates were prepared from the various culture media by centrifugation at 15,000 *g* for 30 minutes at 4 C followed by filtration of the supernatant through Morton filters at 25 C. Filtrates were either tested immediately, or held at 5 C until needed. In some cases, filtrates were dialyzed under toluene for 16-18 hours against 150 volumes of sterile deionized water at 4 C. After dialysis, the filtrates were again centrifuged and filtered before use to insure against any possible microbial contamination.

Inoculation technique.—Solutions, bacterial suspensions, and culture filtrates were aseptically inoculated into leaves through a pinhole made in the lower surface with a sterile needle. A 5-ml glass syringe fitted with a short piece of thick-walled rubber tubing over the tip of the barrel was used to transfer the liquid into the test plants. Several application points were necessary when complete watersoaking was desired.

Necrosis induction test.—Solutions of antibiotics and salts were tested for necrosis induction by injection into eight or more 1-cm^2 leaf areas of healthy plants having at least 1-2 true leaves; at least two well-separated areas were inoculated on each cotyledon. A washed and resuspended 24-hour nutrient broth culture of *X. malvacearum* and sterile deionized water served as controls. A portion of the filtrate heated for 15 minutes in a boiling water bath and then cooled to 25 C before injection was used as an additional control when culture filtrates were tested. Inoculated plants were incubated under the normal growth conditions for up to 14 days.

Enzyme assays.—The pectic enzyme activity of each culture filtrate was determined by measuring reducing groups released (20). This assay is much more sensitive for exo-pectic enzymes than for the endo-forms. Pectin lyase activity was measured by determination of products reacting with the thiobarbiturate reagent (21). The substrate in each case was 1% (w/v) citrus pectin in 50 mM sodium citrate, pH 7.0; 1% merthiolate was added to prevent growth of any microbial contaminants during

incubation. Reaction mixtures consisted of 1.0 ml substrate, 1.0 ml culture filtrate and 0.5 ml of either deionized water or 0.1 mM $CaCl_2$ solution. Controls consisted of 1.0 ml of the filtrate heated 15 minutes in a boiling water bath, and then cooled to 25 C in place of the unboiled filtrate. The samples were incubated in screw-capped culture tubes (20 × 150 mm) for 24 hours in a water bath at 30 C. The reactions were stopped by addition of 1.0 ml alkaline copper reagent, or 5.0 ml of 0.5 N HCl, to separate 1.0-ml aliquots of each sample. Reducing group determinations were made with a Klett-Summerson colorimeter employing a No. 42 blue filter. The pink color produced with the thiobarbiturate reagent was read at 560 nm on a Beckman Model DB spectrophotometer. Readings were made against a reagent blank. Results are corrected for values obtained with the control samples. Activity is expressed as the total Klett-reading per milliliter of filtrate per 24 hours of incubation for the release of reducing groups and as the total OD at 560 nm per ml filtrate per 24-hour incubation for the release of products by pectin lyase. Both assays were linear with time during the 24-hour incubation period.

RESULTS.—Injection of tap water, distilled or demineralized water, nutrient broth, or the basal salts solution produced no visible alteration within the inoculated region in all three plant lines (Fig. 1-A).

Induction of necrosis by the pathogen.—Injection of a *X. malvacearum* suspension of either race 1 or 10 at a concentration of 10^7 - 10^9 viable cells/ml of inoculum produced a necrotic reaction in all three plant lines. The lesions in both the immune and resistant plant lines remained dry and confined in most part to the original 1.0-cm^2 inoculated area (Fig. 1-B). However, in the susceptible Ac 44 leaf, the lesion progressed from the original inoculation site in a radial manner as a water soaked margin surrounding the previously invaded necrotic tissue (Fig. 1-C). Visible necrosis also appeared much more rapidly in resistant and immune plants. Thus, necrosis was evident within 24-48 hours in Im 216, within 3-6 days in Ac 161, while at least 7 days were necessary before necrosis was observed within the originally water-soaked lesion in Ac 44.

Necrosis induction by ions.—The chloride salts of Cu^{++} and Hg^{++}, but not Na^+, induced rapid necroses in the leaves and cotyledons of all three plant lines. Following inoculation chlorosis appeared within 1 hour, black spots and watersoaking within 3 hours, and both of these symptoms were replaced by necrosis at 24 hours. The complete formation of a desiccated necrotic lesion required about 96 hours. The necroses produced by Cu^{++} and Hg^{++} on Ac 44 leaf tissue are shown in Fig. 2-A and 2-B, respectively. These lesions were visibly indistinguishable from lesions induced by viable *X. malvacearum* cells in Im 216 plants (Fig. 1-B). There was no visible response to 0.02 mM $CuCl_2$, 0.05 mM $HgCl_2$, or to NaCl at concentrations of 1 mM (Fig. 2-C) or 420 mM.

Induction of necrosis by antibiotics.—Dactinomycin, chloramphenicol, cycloheximide, mitomycin C, and puromycin produced necroses in cotyledons and leaves of immune, resistant, and susceptible plant lines. No visible response was observed with streptomycin at a concentration of 3.4 mM.

The response of Im 216, Ac 161, and Ac 44 to injection of these antibiotics was essentially equivalent except with chloramphenicol. At 24 hours, the chloramphenicol-induced necroses were equivalent in size and appearance in all three plant lines. This similarity lasted about 2 weeks. Afterwards, the necrosis in Im 216 spread over almost the entire cotyledon or leaf (Fig. 3-A), whereas the lesions in Ac 44 and Ac 161 remained the same size (Fig. 3-B).

Dactinomycin treatment produced a visible lesion within 48 hours in all three plant lines. Necrosis was complete in 4 days. The size of the necrotic area was correlated with dactinomycin concentration. At 0.08 mM the lesion in Ac 161 (Fig. 3-C) coincided with the area inoculated. At 0.008 mM, the lesion was much smaller than the area inoculated (Fig. 3-D). No lesion was visible at 0.8 μM.

The necrosis caused by cycloheximide was visible in 3 days, and spread extensively in a radial manner for an additional 2 days (Fig. 3-E).

Mitomycin C induced very rapid necroses, a visible response appeared within 1.0 hour. The necrotic lesions, which were fully developed within 4 days, were limited to the inoculated areas, but were completely encircled by distinct chlorotic zones (Fig. 3-F).

Puromycin produced lesions similar to those caused by mitomycin C, but at least 2 days were required before a visible response was detected. The lesions were fully developed by the end of 4 days (Fig. 3-G).

Only the dactinomycin lesion (Fig. 3-C) was visually indistinguishable from the necrotic lesion produced by the bacteria in the resistant plants (Fig. 1-B). The other antibiotic-induced necroses differed from pathogen-

TABLE 1. Growth of *Xanthomonas malvacearum,* race 1, in the inoculated intercellular fluid (IEI) extracted from upland cotton cotyledons[a] and in nutrient broth

Media	Viable cells per ml[c] at sample time (hours)		
	0	7	24
IEI - Im 216[b]	$1.3 \pm 0.2 \times 10^4$	$0.8 \pm 0.2 \times 10^5$	$4.5 \pm 0.5 \times 10^7$
IEI - Ac 44[b]	$1.5 \pm 0.5 \times 10^4$	$0.9 \pm 0.1 \times 10^5$	$1.3 \pm 0.3 \times 10^8$
Nutrient broth	$2.2 \pm 0.8 \times 10^4$	$0.9 \pm 0.2 \pm 10^5$	$4.0 \pm 2.0 \times 10^7$

[a]The cotyledons were completely watersoaked by hand injection with a 10^8 cells/ml suspension of *X. malvacearum* 24 hours before harvest of the cotyledons and extraction of the fluids.

[b]*X. malvacearum* produces a hypersensitive reaction in Im 216 and a susceptible reaction in Ac 44.

[c]Cell numbers were determined by plate count of serial dilutions of IEI in sterile distilled water plated on nutrient agar. Plates were incubated at 25 ± 1 C for 4-5 days.

TABLE 2. Reducing sugar activity (RS), pectin lyase activity (PL) and necrosis induction by sterile filtrates from the growth of *Xanthomonas malvacearum* (races 1 and 10) on a ground cotton leaf medium

Culture age (days)	Viable *X. malvacearum* (count[c] × 10^{-8})		RS[a]		PL[b]		Time for necrosis induction (days)			
							Plant line			
							Ac 44		Im 216	
	Race		Race		Race		Race		Race	
	1	10	1	10	1	10	1	10	1	10
0	0.5	0.1	0	0	0	0	NVR[d]	NVR	NVR	NVR
2	2.6	1.3	390	488	0	0	7-9	7-9	7-9	7-9
4	20.0	100.0	390	292	0	0	7-9	8-10	7-9	8-10
8	34.0	38.0	488	0	0	0	7-9	NVR	7-9	NVR
14	2.8	2.3	98	0	0	0	NVR	NVR	NVR	NVR

[a]Activity is expressed as the total Klett reading above controls per ml of filtrate per 24 hours of incubation at 30 C.
[b]No reaction products were detected that exhibited maximum absorption at 560 nm after reaction with the thioburbiturate-HCl reagent.
[c]Cell numbers were determined by plate count of serial dilutions in sterile distilled water plated on nutrient agar. Plates were incubated at 25 ± 1 C for 4-5 days.
[d]NVR = no visible response during the 14-day observation period after inoculation of the sterilized filtrates into the leaves of the test plants.

TABLE 3. Reducing sugar activity (RS), pectin lyase activity (PL), and necrosis induction in undialyzed (UD) and dialyzed (D) culture filtrates from four *Xanthomonas* spp. grown for 21 days on a ground cotton leaf medium

Organism	Assay				Time for necrosis induction (days)			
					Plant line			
	RS[a,c]		PL[b,c]		Ac 44[c]		Im 216[c]	
	UD	D	UD	D	UD	D	UD	D
X. campestris	0	0	0	0	NVR[d]	NVR	NVR	NVR
X. oryzae	200	390	0	0	8-10	8-10	7-9	7-9
X. phaseolus	170	3,670	0.16	3.30	8-11	2-3	8-10	2-3
X. vesicatoria	1,920	1,885	4.88	6.00	2-4	2-3	2-3	2-3

[a]Results are expressed as the total Klett reading above controls per ml filtrate per 24 hours of incubation at 30 C.
[b]Results are expressed as the total optical density at 560 nm above controls per ml filtrate per 24 hours of incubation at 30 C.
[c]UD = filtrate tested prior to dialysis; D = filtrate tested after dialysis under toluene for 16 hours against 150 volumes of sterile deionized water at 4 C.
[d]NVR = No visible response during the 14-day observation period after inoculation of the sterilized filtrates into the leaves of the test plants.

induced necroses either in time of appearance, spreading of lesions beyond the area initially inoculated and/or gross appearance of lesions [compare Fig. 1-(B,C) with Fig. 3-(C to F)].

Induction of necrosis by culture filtrates.—Culture filtrates prepared from 0- to 7-day-old nutrient broth cultures of race 1 or race 10 of *X. malvacearum* produced no visible lesions when inoculated into leaves and cotyledons of all three cotton cultivars. There were also no visible responses to culture filtrates prepared from the basal salts medium supplemented with either glucose, glucuronic acid, galacturonic acid, cellobiose, cellulose, or pectin. No detectable pectic enzyme activity was present in any of these filtrates. Both bacterial races grew well on all these media during the 7-day culture period. Inoculation of leaves with the cells (about 10^6 - 10^8 cells per ml) obtained from each medium produced typical necroses in Im 216 and a water-soaking susceptible response in Ac 44.

Intercellular extracts from healthy and race 1-infected cotyledons of Ac 44 and Im 216 also did not evoke a visible response when injected into leaves and cotyledons of all three cultivars. Both extracts were excellent growth media for both races of *X. malvacearum*. For example, the intercellular fluids extracted from Ac 44 and Im 216 cotyledons infected for 24 hours with race 1 were comparable to nutrient broth in their ability to support the growth of race 1 (Table 1).

However, necrosis-inducing culture filtrates were obtained from both races grown on the cotton leaf medium. Both filtrates elicited visibly identical lesions in both Im 216 and Ac 44 plant lines (Table 2). The final appearance of all lesions was indistinguishable from the necrotic lesion produced by inoculation of the immune plant (Im 216) with *X. malvacearum*. Both lesions differed from the necrosis produced in the Im 216-*X. malvacearum* interaction in that 7-10 days rather than 2-3 days were required for the complete desiccation of the

inoculated areas. Maintaining the cultures for up to 14 days, did not reduce the time required to produce a desiccated necrotic lesion. On the contrary, the ability of the race 1 filtrate to elicit a necrotic response was lost between 8 and 14 days in culture; this ability was lost between 4 to 8 days for the race 10 culture. In each case, the loss in necrosis-inducing ability was correlated with a decline in the bacterial population, and with the loss of polymethylgalacturonase activity (Table 2).

If the necrosis-inducing culture filtrates were heated for 15 minutes in a boiling water bath, cooled to 25 C, and then inoculated into cotyledons and leaves of the test plants, no visible lesion was detected. Dialysis of the filtrates for 14-16 hours against 200 volumes of distilled and deionized water at 4 C had no effect on the necrosis-inducing ability of either filtrate. These results indicated that the necrosis-inducing sustance(s) present in the filtrates was a nondialyzable, heat-labile molecule, possibly protein in nature.

Since enzymes which degrade the pectin portion of plant cell walls have been implicated in plant cellular death in several studies (3, 5, 6, 9, 17, 19, 25, 28), we examined the necrosis-inducing *X. malvacearum* filtrates for this activity. Assays for polymethylgalacturonase indicated an apparent correlation between this enzymatic activity and necrosis induction (Table 2). The enzyme(s) in the *X. malvacearum* filtrates was probably a hydrolase rather than a lyase since no activity was detected by the thiobarbiturate assay. In addition, the polymethylgalacturonase was not altered by Ca^{++}, which usually enhances the *trans*-eliminative degradation of pectin (27). Absence of in vitro lyase activity in *X. malvacearum* has been previously reported (29).

Four different *Xanthomonas* spp. were employed to further test the correlation between polymethylgalacturonase activity and necrosis induction. A 24-hour washed and resuspended nutrient broth culture containing 10^7 - 10^9 cells per ml of either *X. campestris, X. oryzae, X. phaseolus,* or *X. vesicatoria* induced a necrotic lesion within 24-72 hours after inoculation into the leaves of Ac 44 or Im 216 plants. Filtrates were prepared from 21-day cultures of these organisms grown on the ground cotton leaf medium. At this time, all four cultures were in the later stationary phase of growth. Each filtrate was tested for pectic enzyme activity and necrosis induction. The results are summarized in Table 3. No correlation between the ability of *viable bacteria* to induce necrosis in cotton in vivo, and to produce pectic enzymes in culture was observed. However, *only* those filtrates which exhibited pectic enzyme activity were able to also elicit a necrotic lesion in Ac 44 and Im 216 plants. The filtrate obtained from the *X. vesicatoria* culture exhibited the highest level of enzyme activity. Inoculation of this filtrate resulted in the most rapid formation of a necrotic lesion, comparable in time to the lesion produced during the host-pathogen interaction in immune plants.

Dialysis of only the *X. phaseolus* filtrate caused a significant increase in RS and pectin lyase activity. Note that this increased activity was correlated with a dramatic reduction in the time required for the completion of the necrotic response. Delayed production of necrosis by the undialyzed *X. phaseolus* filtrate is not surprising since in the leaf a low-molecular-weight pectic enzyme inhibitor might diffuse away from the enzyme at a much slower rate than that occurring during dialysis.

Pectin lyase activity was detected only in the *X. phaseolus* and *X. vesicatoria* culture filtrates. As noted, dialysis significantly enhanced the pectin lyase activity of the *X. phaseolus* filtrate, but only slightly enhanced that of the filtrate of *X. vesicatoria.* Filtrates heated for 15 minutes in a boiling water bath were unable to elicit a necrotic response, again showing the heat-lability of the necrosis-inducing factor(s).

Aliquots of each filtrate and extracts of leaf disks containing the necrotic lesion were plated onto nutrient agar, and no colonies were observed. Thus, an active necrosis-inducing principle(s) must have been present in those filtrates that caused necrotic lesions.

DISCUSSION.—The same antibiotics and heavy metals which elicit phytoalexin production in peas (8, 23) also elicit a hypersensitivelike necrotic reaction when injected into cotton leaves or cotyledons. However, since (with one exception) susceptible, resistant, and immune cultivars all show the same type of reaction, these results more likely are the general phytotoxic effects of these agents, rather than a mimic of the resistance mechanism. In the case of chloramphenicol, a protein synthesis inhibitor, a strikingly greater necrotic effect was produced in Im 216 than in Ac 161 or Ac 44. This finding is interesting, but may not be related to the resistance mechanism, since Im 216 differs from the other two cultivars in many genetic characters, in addition to possessing genes for resistance to *X. malvacearum.*

Neither race 1 nor race 10 of *X. malvacearum* secretes a necrosis-inducing compound(s) when cultured for up to 7 days on (i) nutrient broth, (ii) a basal salts medium supplemented with defined carbon sources, or (iii) the intercellular fluids of healthy or infected cotyledons. These results might be challenged on the basis that any necrosis-inducing compounds secreted might not reach active threshold levels due to dilution by the culture media. However, it should be noted that all of the media used support the growth of *X. malvacearum* to 10^8 - 10^9 cells per ml. Also, washed cell suspensions from the basal salts plus carbon source media retain the ability to produce necrosis in resistant or immune plants, and watersoaking in susceptible plants, as do cells from the nutrient broth culture.

The failure of nutrient broth, or the intercellular fluid of intact tissue, to serve as media on which a pathogenic bacterium produces detectable levels of necrosis-inducing compounds during growth, is not unprecedented (15). Many pseudomonads which induce a hypersensitive reaction in tobacco do not form detectable levels of necrosis-inducing compounds in vivo or in vitro (11, 13-16).

The uninhibited growth of *X. malvacearum* on extracts of the intercellular fluids of previously infected resistant tissue contrasts with the inhibition of *X. vesicatoria* growth on a similar extract from resistant pepper, *Capsicum annuum* L. (26).

In contrast to results with other media, a necrosis-inducing compound(s) was secreted by *X. malvacearum* grown on the ground cotton leaf medium. The necrosis produced by *X. malvacearum* secretions was delayed in

time of appearance, but otherwise visually was identical with the pathogen-induced hypersensitive necrosis when inoculated into cotyledons and leaves of healthy susceptible and immune cotton plants. The principle(s) produced in vitro was heat-labile and non-dialyzable. All active filtrates with necrosis-producing activity also exhibited polymethylgalacturonase activity. This enzyme activity was not detected in any other *X. malvacearum* culture filtrate including one in which pectin was used as the carbon source.

The same correlation was seen with other *Xanthomonas* spp. cultured on the cotton leaf medium. Only culture filtrates possessing polymethylgalacturonase activity were able to cause necrosis. The greater the enzymatic activity, the more rapid was the production of a desiccated necrotic lesion. Therefore, the necrosis induction and enzymatic activity appear to be related.

In earlier work, pectic enzymes were not implicated as necrotizing agents in spite of the numerous reports concerning the ability of these enzymes to kill plant cells (3, 5, 6, 9, 17, 19, 25, 28). However, an endopolygalacturonase was recently correlated with the necrotic symptoms observed in Verticillium wilt of cotton (19), and pectinolytic enzymes were found in lesions caused by *Mycosphaerella pinodes* and *Ascochyta pisi* in detached pea leaflets (10). Pectin and pectinic acid degradative enzymes are used for the isolation of intact plant protoplasts. However, these isolations are done under conditions causing plasmolysis, and plasmolyzing conditions are not expected to exist in the leaf, initially.

If bacterial pectic enzymes are responsible for triggering the hypersensitive response, it follows that these enzymes are not formed (or their formation is greatly delayed) in the susceptible interaction.

Studies are now in progress to determine whether the extracellular pectic enzyme activity secreted into the cotton leaf medium during the culture of *X. malvacearum* is the only compound in these filtrates which can effect necrosis in test plants. Concurrent studies will attempt to establish if the same enzyme is produced in the compatible host-pathogen interactions in vivo in the proper time sequence to account for the great disparity in time of appearance of necrosis in susceptible and resistant infected plants.

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