PECTIC ENZYME: AN ELICITOR OF NECROSIS IN COTTON INOCULATED WITH BACTERIA

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When bacterial blight-resistant and blight-susceptible cotton cotyledons were infiltrated with bacterial blight pathogen, hydrolytic pectic enzyme(s) appeared in extracts of the resistant cotyledons sooner than in extracts of the susceptible cotyledons. In each interaction the pectic enzymatic activity preceded necrosis.

A bacterial phytopathogen, nonpathogenic against cotton, elicited a response in bacterial blight-susceptible cotton which was enzymatically and macroscopically similar to the response of blight-resistant cotton to the bacterial blight pathogen.

Enzymatic hydrolysis of cell wall pectin appears to be one of the factors contributing to foliar necrosis in cotton.

INTRODUCTION

Foliar symptoms of cotton bacterial blight, induced by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye, are first manifested by water-soaking bounded by leaf veinlets (1). The water-soaked areas eventually collapse and become dark, dry necrotic lesions surrounded by irregular chlorotic or lighter-colored necrotic areas. Cotton resistant to bacterial blight shows no water-soaking. Instead, leaf areas infiltrated with concentrated bacterial inocula ($\geq 10^7$ cells/m*l*) become dry and necrotic. Bacterial suspensions containing $<10^7$ cells/m*l* induce microscopic necrotic lesions when they are infiltrated (1,2). Histological studies describing the development of bacterial blight in susceptible cotton indicate that macroscopic symptoms become evident only after bacteria produce a copious quantity of slime and cause the disorganization of a large number of host cells (3,4). A similar pattern of tissue necrosis was also seen in resistant cotton hosts (5). In both hosts, necrosis was preceded by an alteration of the middle lamella and the cell wall. Both Fushtey (6) and Tribe (7) showed that a PG^+ could macerate and kill nonplasmolyzed cells of cucumber, turnip, carrot, and potato. Also, Basham and Bateman (8) reported that an endopectate lyase produced by *Erwinia chrysanthemi* pv. *chrysanthemi* Burkholder, McFadden & Dimock caused maceration and cell death of intact nonplasmolyzed potato medullary tissue.

The objective of this study was to determine the time course of pectic enzyme appearance in bacterial blight-resistant and -susceptible cotton following infiltration with *X.* pv. *malvacearum* and in the susceptible line following infiltration with the pea pathogen *Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye and Wilkie.

MATERIALS AND METHODS

Preparation of inocula

Virulent cultures of *X.* pv. *malvacearum,* race 1, or of *P.* pv. *pisi* were used as bacterial inocula. These phytopathogenic bacteria were maintained on potato-carrot-dextrose agar (2). When needed, bacteria were transferred from the agar to 100 m*l* nutrient broth and grown in shake culture for 24 hr at 25 ± 1 C. Bacteria were recovered from the broth by centrifugation (12×10^3 g. for 10 min) followed by resuspension in sterile distilled water equal in volume to the broth culture. This procedure provided an inoculum suspension of about 10^8 bacteria/m*l* as determined by plate counts.

Growth of cotton plants and inoculation procedure

Bacterial blight-susceptible Acala 44 (Ac 44) and bacterial blight-resistant Immune 216 (Im 216) were grown from acid-delinted seed in 15.5-cm-diameter clay pots containing a sterile soil:vermiculite:peat mixture

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(3:1:1 v/v). These plants were grown in controlled-environment chambers set to provide a light regime consisting of 12 hr. incandescent plus fluorescent light (4.3×10^4 lux) preceded and followed by 1 hr of incandescent light only. The temperature during the light period was 32 ± 1 C and 19 ± 1 C during the dark period. When plants were about two weeks old, the fully expanded cotyledons were infiltrated with bacterial inocula. A 5-m*l* glass syringe, fitted with a 2.5-cm piece of thick-walled rubber tubing over the barrel tip, was used to treat the cotyledons through their open stomata. The intercostal area of the entire cotyledon was infiltrated by introducing inoculum into each cotyledon at four locations, 0.2-0.3 m*l* inoculum being used per location. Control cotyledons were inoculated in the same way but with a heat-killed bacterial suspension (autoclaved at 120 C and 1.05 kg/cm^2 for 10 min).

Enzyme extraction

Immediately (zero-time control), and at various times after infiltration, control and experimental cotyledons were excised from the cotton plants. One-g samples (fresh weight) were macerated with 2.0 g of insoluble polyvinylpyrrolidone in a Sorvall Omnimixer with 2.0 to 10.0 m*l* of 0.01 *M* sodium phosphate buffer, pH 7.0, 0.10 *M* in NaCl and 0.20 *M* in 2-mercaptoethanol. Each macerate was strained through two layers of cheesecloth and the filtrate so obtained was centrifuged at 12×10^3 g for 10 min. Supernatants were collected by decanting and then dialyzed 16 hr against 100 volumes of distilled water. These extracts were prepared at 4 ± 1 C.

Enzyme assay methods

The hydrolytic pectic enzymes, polymethylgalacturonidase (PMG) and polygalacturonidase (PG), both of which are classified as polygalacturonide glycanohydrolase E.C. 3.2.1.15, were routinely assayed with either 0.1% (w/v) citrus pectin or 0.1% (w/v) polygalacturonic acid, respectively, in 0.10 *M* Tris-acetate buffer, pH 5.0 or pH 7.0. The hydrolase activity was assayed by measuring the increase of reducing sugar (9). The activity of pectate lyase (polygalacturonide glycanolyase E.C. 4.2.99.3) was followed by measuring the change in absorbance at 230 nm (10) or by the periodate:thiobarbituric acid method (11). Reaction mixtures were incubated at 30 ± 1 C for 1 hr. Control tubes were treated exactly like the tubes containing experimental reaction mixture but contained boiled extract (100 C for 10 min) in place of unboiled extract.

A unit of activity is defined as that amount of enzyme which catalyzes the release of 0.01 μmole D-galacturonic acid per hour. Specific activity is the number of units/(mg protein).

Assays designed to measure viscosity changes were done at 30 ± 1 C in Cannon-Fenske viscometer tubes. The reaction mixtures consisted of 4.0 m*l* of either a 1.0% (w/v) solution of citrus pectin or a 1% (w/v) solution of polygalacturonic acid and 1.0 m*l* of deionized water; when the extract was assayed for lyase activity at pH 8.0 or pH 9.0, a 10^{-3} *M* solution of $CaCl_2$ replaced the deionized water. To complete the reaction mixture, 2.0 m*l* of the cotyledonary extract was used.

Controls consisted of boiled extract (100 C for 10 min) in place of the unboiled extract. The mixtures were incubated at 30 ± 1 C for up to 1 hr. The protein concentration of the extracts was estimated from the equation reported by Layne (12).

Aliquots of all reaction mixtures from each host-pathogen interaction were subjected to descending paper chromatography on Whatman No. 1 paper with a developing solvent consisting of pyridine:ethyl acetate:acetic acid: water (36:36:7:21 v/v). Reaction products were located by an alkaline permanganate spray (13).

Polyuronide content

The polyuronide concentration of control cotyledons was measured by the procedure described by Hancock (14), whereas the polyuronide content of the experimental cotyledons was determined by the method reported by McCready and McComb (15).

Bacterial growth

Two leaf discs, 1.0 cm in diameter, were collected from two cotyledons that had been infiltrated with viable phytopathogenic bacteria. Each disc was surface sterilized with Clorox:water (10:90 v/v) for five min, rinsed with sterilized tap water, and macerated in 1.0 m*l* of sterilized tap water with a presterilized mortar and pestle. The bacterial population of each disc was determined by a dilution plate count method in which serial dilutions were made with sterilized distilled water and the bacteria were grown on plates containing nutrient agar. Plate colonies were counted after 4 days growth at 25 ± 1 C.

Conductivity determinations

Diffusion of electrolytes from the cells of the cotton host was also used to provide a

measure of cellular damage (16,17). For the control, one disc, 1.0 cm in diameter, was cut from the intercostal area of each of five cotyledons that had been infiltrated with heat-killed bacteria (120 C at 1.05 kg/cm^2 for 10 min). Five experimental samples were also cut from five cotyledons that had been infiltrated with viable bacteria.

Each set of five discs was bathed in 25.0 m*l* of sterilized distilled water. The average initial conductivity of this water was about 1.0 μmho. Flasks containing the leaf tissue discs were incubated on a platform shaker subjected to a controlled ambient temperature of 25 ± 1 C. The conductivity of the bathing fluid was measured again after 1 hr. Changes in conductivity are reported as the difference between the average increase in the conductivity for three samples of fluid used to bathe the five control discs and the average increase in the conductivity of three samples that had been infiltrated with viable phytopathogenic bacteria (experimental minus control).

All conductivity measurements were made with a Yellow Springs Instrument Co. Model 53 conductivity meter.

RESULTS

Enzyme activity

Tissue extracts of both resistant and susceptible cotton cotyledons that had been infiltrated with *X*. pv. *malvacearum* released reducing sugars when incubated with pectin (polymethylgalacturonic acid) or polygalacturonic acid (Table 1). Extracts of control cotyledons which had been infiltrated with heat-killed cells contained no detectable pectinase activity. Since D-galacturonic acid was the only reaction product detected on paper chromatograms of aliquots from reaction mixtures of all three host-pathogen interactions at any of the sampling times of Table 1, one can conclude that the increase in reducing sugars observed during the assay incubation was due to action of PG or PMG in the extracts upon the respective substrates, rather than to the action of enzymes upon other substrates that might have been present. This release was most likely due to hydrolase activity, since no lyase activity was detected at either pH 8.0 or pH 9.0.

Viscosimetric assays of the hydrolytic pectic enzymes detected in each host pathogen combination was carried out at 0.5-pH-unit intervals from pH 0.5 to pH 9.0. Viscosimetric assays for lyase activity was also carried out at pH 8.0 and pH 9.0 No changes in viscosity were observed in any assay. This indicates that the pectic enzymes detected in the extracts are exo-enzymes rather than endo-enzymes. This result was unexpected since previous observations had shown that pectic enzymes that cause necrosis are usually of the endo type.

Incubation for up to 24 hr of those reaction mixtures in which no enzymatic activity was detected during the usual incubation time of 1 hr still revealed no activity by the reducing sugar, viscosimetric, or lyase assays.

The PMG(s) detected prior to necrosis in bacterial blight-resistant interactions was (were) more active at pH 7.0 than at pH 5.0 However, the PG(s) detected prior to necrosis in a bacterial blight-susceptible interaction was (were) more active at pH 5.0 than at pH 7.0.

TABLE 1. *Polygalacturonase and polymethylgalacturonase activities in extracts from cotyledons of Ac 44 and Im 216 following inoculation with* Xanthomonas campestris *pv.* malvacearum

Cultivar	Time after inoculation, hr[a]	Enzyme activity[b] (specific activity)[c] Polygalacturonase pH 5.0[d]	Polygalacturonase pH 7.0	Polymethylgalacturonase pH 5.0	Polymethylgalacturonase pH 7.0
Im 216	1	0.03±0.02	0.50±0.02	0.37±0.01	3.54±0.05
	3	2.39±0.22	3.06±0.37	4.50±0.25	15.44±1.02
	24	0.46±0.14	0.10±0.00	0.30±0.01	3.70±0.26
Ac 44	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	24	26.00±1.02	2.00±0.36	0.92±0.01	1.20±0.36
	48	0.10±0.02	0.00±0.00	0.00±0.00	0.00±0.00

[a]Time at which cotyledons were collected and macerated.
[b]Enzyme activity was determined by measuring the increase in the reducing sugars of reaction mixtures (9). Values given are the average ± standard error of the mean.
[c]Each number is the mean of values obtained from at least two experiments. In each experiment, duplicate determinations were made.
[d]pH of assay buffer.

Plots of the time course for the appearance of pectic enzyme activity in extracts from bacterial blight-susceptible and blight-resistant cotton interactions show that this activity appeared earlier in extracts from the resistant-plant interactions (Fig. 1A-1C).

Both PMG(s) and PG(s) could be detected in extracts from all three interactions shown in Fig. 1. In each interaction the maximal specific activities of both hydrolases occurred at the same time. However, only the behavior of the pectic enzyme hydrolase having the predominant activity is plotted in Fig. 1. Further analysis of the plots drawn in Fig. 1 showed that pectic enzyme activity of 10 units/mg protein, which appears to be the minimal specific activity required for necrosis induction, appeared 18 hr earlier in the resistant cotton (Im 216) - *X.* pv. *malvacearum* interaction than in the susceptible cotton (Ac 44) - *X.* pv. *malvacearum* interaction.

Pectic enzyme activity appeared earlier in a bacterial blight-susceptible cotton variety undergoing interaction with *X.* pv. *pisi* than in the same variety infected with *X.* pv. *malvacearum* (Fig. 1B, 1C). Hydrolytic activity of at least 10 units/mg protein was detected in extracts from this resistant Ac 44 - *P.* pv. *pisi* interation about 10 hr after infiltration with bacteria. No lyase activity was detected and viscosimetric assays indicated that the hydrolytic activity was again due to exo rather than endo cleavage.

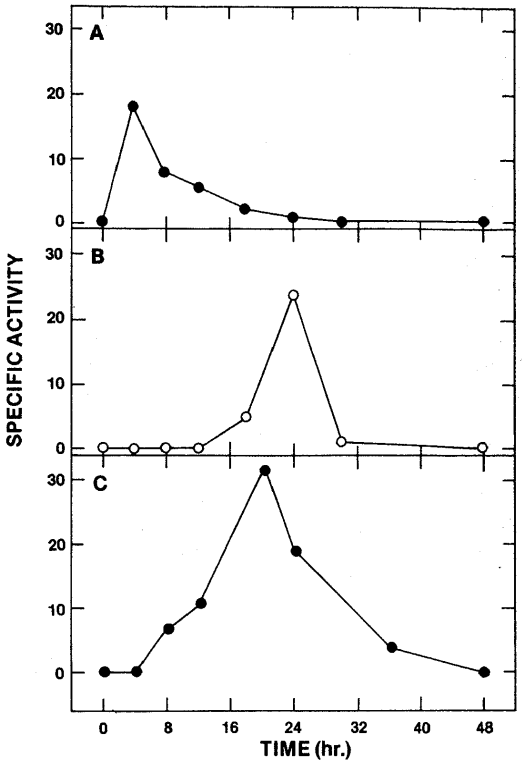

FIGURE 1. Polygalacturonase (PG) and polymethylgalacturonase (PMG) specific activities (units x mg. protein^{-1}) in extracts of cotyledons sampled at different times after inoculation. A) PMG in extracts of the Im 216-*X.* pv. *malvacearum* interaction, pH 7.0 B) PG in extracts of the Ac 44-*X.* pv. *malvacearum* interaction, pH 5.0 C) PMG (○) in extracts of the Ac 44-*P.* pv. *pisi* interaction, pH 7.0.

Polyuronide content

Following the appearance of necrosis, about 72 hr after infiltration of Ac 44 cotyledons with viable cells of *X.* pv. *malvacearum*, the cotyledons contained as much as 20% more polyuronide than controls. The polyuronide content of necrotic Im 216 cotyledons sampled 24 hr after infiltration with viable cells of *X.* pv. *malvacearum* was reduced as much as 33% compared to controls (Table 2) and very little bacterial polysaccharide was present.

Bacterial growth

Growth curves of phytopathogenic bacteria multiplying in bacterial blight-susceptible or -resistant cotton are shown in Figure 2A-C. Maximal bacterial numbers were recovered from the susceptible Ac 44 - *X.* pv. *malvacearum* interaction. In all interactions,

TABLE 2. *Content of polyuronide in control and* Xanthomonas campestris *pv.* malvacearum *-- inoculated Ac 44 and Im 216 cotyledons*

Cultivar	Exp. No.	Polyuronide Content[a,b] Control	Inoculated	Change (%)[c]
Ac 44	1	53.3±2.5[d]	51.5±4.5	- 3.4
	2	52.5±0.7	63.0±0.1	+ 20.0
Im 216	1	57.2±1.5	50.7±2.0	- 11.4
	2	59.0±0.2	39.6±0.4	- 32.9

[a]Values are expressed as mg galacturonic acid per g dry weight of cotyledons.
[b]Ac 44 and Im 216 cotyledons were extracted 48 and 24 hr after inoculation, respectively.
[c]Values represent the change in mean percent between healthy and infected cotyledons.
[d]Average and standard error of the mean of three replications per experiment.

the bacterial population did not decline until a specific activity of pectic enzyme of at least 10 units/mg protein was reached. In susceptible and resistant cotyledons, *X*. pv. *malvacearum* continued to grow several hr after pectic enzymes had attained this level of activity before a decline in bacterial numbers was observed (see arrows in Figures). However, *P*. pv. *pisi* showed an almost immediate reduction of the bacterial population after the specific activity of pectic enzyme reached a value of 10.

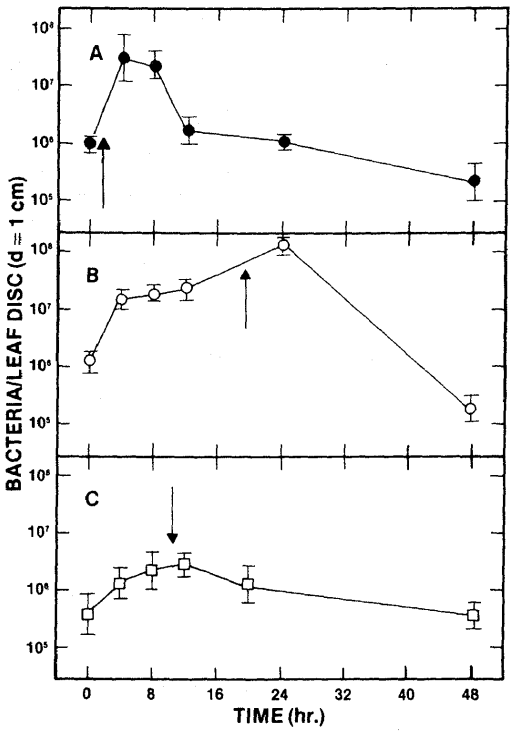

FIGURE 2. Multiplication of bacteria in intact cotyledons. A) Growth of *X*. pv. *malvacearum* in Im 216. B) Growth of *X*. pv. *malvacearum* in Ac 44. C) Growth of *P*. pv. *pisi* in Ac 44. Each point represents the average counts of two sets of duplicate plates in two experiments. The vertical lines indicate the standard deviations and the arrows indicate the time when pectic enzyme specific activity reached a value of 10 units/mg protein.

Host tissue damage

Macroscopic lesion development caused by *X* pv. *malvacearum* on cotyledons of the resistant host (Im 216) was first expressed by chlorosis. This occurred after the specific activity of PMG(s) reached a value of 10 units/mg protein. Turgor loss, wilting, and browning were subsequently observed. By 24 hr, the cotyledons had become necrotic.

The macroscopic lesion caused by infiltration of *X* pv. *malvacearum* into cotyledons of the susceptible cotton host was first expressed as a clearly discernable water-soaking without obvious wilting. Water-soaking was seen on the cotyledons before the specific activity of a pectic enzyme (PMG and/or PG) reached a value of 10 units/mg protein. Specific activities of this magnitude were not measured until about 20 hr after infiltration with viable cells of *X*. pv. *malvacearum*. In this instance, PG(s) rather than PMG(s) attained this activity level. The water-soaked lesion was usually observed about 18 hr after inoculum infiltration. Loss of turgor, wilting, browning, and finally necrosis followed. Necrosis occurred only after PG(s) reached a specific activity of 10 units/mg protein (Fig. 1 B). However, this (these) PG(s) probably could not degrade the cell wall, which includes pectin in its composition, as efficiently as the PMG(s) that were detected in resistant interactions. Pectin is a methylated derivative of polygalacturonic acid. Preliminary studies indicated that pectin methyl esterase activity was absent. Hence, the slowness of necrotic lesion production in susceptible tissue may be due to a lower ratio of PMG(s) to PG(s) activity. By 72 hr, cotyledons of the susceptible cotton host exhibited a degree of tissue collapse and desiccation identical in macroscopic properties to that of the bacterial blight-resistant host cotyledons observed 24 hr earlier.

The pectic enzymatic activity detected in infected plants of both susceptible and resistant varieties was extremely labile. Activity was rapidly lost during purification. Because of this lability, pectic enzyme (PMG and PG) from these sources was difficult to further characterize.

Macroscopic lesion development on cotyledons of the resistant Ac 44 - *P*. pv. *pisi* interaction resembled that in the resistant Im 216 - *X*. pv. *malvacearum* interaction both in appearance and in that PMG was more active than PG. No water-soaking of the lesion was seen. At about 10 hr after infiltration with viable cells of *P*. pv. *pisi* the cotyledons became chlorotic and PMG(s) having a specific activity of at least 10 was(were) detected. Loss of turgor and wilting began about 12 hr after infiltration. By 36 hr, the cotyledons were collapsed, desiccated, and necrotic to about the same visible extent as cotyledons of the Im 216 - *X*. pv. *malvacearum* interaction at 24 hr.

Control Ac 44 and Im 216 cotyledons that had been infiltrated with heat-killed *P*. pv.

pisi or *X*. pv. *malvacearum* remained fully turgid and normal in appearance.

The lesion development observed on foliar tissue of all three interactions was associated with leakage of electrolytes (increase in conductivity of surrounding fluids). Electrolyte leakage is plotted in Fig. 3. The most rapid and largest increase was observed in the resistant Im 216 - *X*. pv. *malvacearum* interaction.

In both interactions of the resistant variety, a significant increase in electrolyte leakage occurred only after the specific activity of extracted PMG(s) reached a value of at least 10 units/mg protein. An increase in such leakage was also observed in the susceptible Ac 44 - *X*. pv. *malvacearum* interaction after the specific activity of pectic enzyme reached this value. As mentioned above, PG(s) rather than PMG(s) was(were) more active in the extracts of cotyledons obtained from this interaction. The lower rate of electrolyte leakage in the susceptible interaction than in either resistant interaction maybe due to less efficient degradation of pectin-containing cell walls by PG than by PMG.

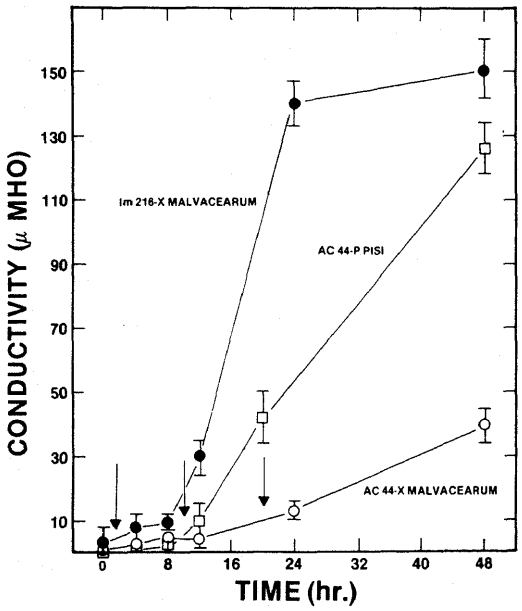

FIGURE 3. Electrolyte leakage from discs of cotyledons sampled at different times after inoculation with viable bacteria. Each point represents the average conductivity from two experiments, in each of which three determinations were made at each indicated time. The vertical lines indicate the standard deviations and the arrows indicate the time when pectic enzyme specific activity reached a value of 10 units/mg protein.

DISCUSSION

Since *in vitro* pectic enzyme was correlated with necrosis induction in an earlier study (18), it was decided that an examination of the time course for the appearance of this enzymic activity *in vivo* was necessary. The results of this study confirmed the previous statement that ". . . earlier necrosis in Im 216 is probably the result of the rapid appearance of pectinolytic activity in the invaded host tissue." (19).

The more rapid development of macroscopic symptoms, reduced bacterial population, and the changes in conductivity measured in the resistant Im 216 - *X*. pv. *malvacearum* interaction, compared to the magnitude of these events in the susceptible Ac 44 - *X*. pv. *malvacearum* interaction, provides evidence for the hypersensitive nature of resistance in cotton.

Perry (20) stated that resistance to bacterial blight of cotton operated only when tissue dissolution was observed. The appearance of high levels of PMG in the cotyledons of Im 216 infiltrated with *X*. pv. *malvacearum* and Ac 44 infiltrated with *P*. pv. *pisi* before turgor loss, increased leakage of electrolytes, and reduced bacterial populations supports the hypothesis that the presence of the appropriate pectic enzyme in the host may be one of the initiating factors for tissue necrosis. Pectic enzymes have been implicated in the production of necrotic lesions in at least two other diseases affecting cotton. Mussell (21) concluded that an endopolygalacturonase was responsible for the foliar necrotic symptoms of *Verticillium* wilt. Wang and Pinckard (22) indicated that pectic enzyme caused necrosis of cotton fruit infected with *Diplodia gossypina* (Cke.). Also, Hopper et al. (18) demonstrated a correlation between pectic enzyme produced *in vitro* by *X* pv. *malvacearum* and necrosis induction in cotton infiltrated with culture filtrates of this organism. Pectic enzyme has also been associated with the cell death of other plant tissues in addition to cotton (8).

Extracts prepared from the resistant cotton cotyledons turned brown very rapidly. These extracts contain a peroxidase that can catalyze catechin oxidation (23). Hunter has shown that catechin reaction products are inhibitory to pectic enzyme of *Rhizopus solani* (24). It is possible that catechin reaction products formed by peroxidase catalysis may have inhibited the pectic enzyme in these cotton--phytopathogenic bacteria inter-

actions and thus caused the determination of less than the true amounts of enzyme activity.

In the work described here, we observed significant differences between the susceptible and resistant interactions in the time course of appearance of pectic enzymes and in the pH range and substrate specificity of those enzymes. Taken together, these results suggest that the predominant type of pectic enzyme present in an interaction may determine whether the response is resistant or susceptible.

It has been reported that pectic enzymes can elicit phytoalexins in soybean and castor bean (25,26). Also, pectic fragments of soybean and of castor bean cell walls are able to elicit phytoalexins (27,28). Essenberg et al. (29) have identified two terpenoid phytoalexins from extracts of Im 216 leaves and cotyledons following infiltration with *X*. pv. *malvacearum*.

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