PHYTOALEXIN PRODUCTION IN CONGENIC COTTON LINES CHALLENGED WITH RACES OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Bacterial blight of cotton is an economically important disease. It occurs in nearly all the cotton producing areas of the world and is caused by Xanthomonas campestris pv. malvacearum (Xcm), a gram-negative, rod-shaped bacterium which is a specific pathogen of species of Gossypium. There are at least 19 races of Xcm that are known to attack cotton (Brinkerhoff 1970, Bird et al., 1977, Follin, unpublished).

No effective chemical control method has been developed to date. Xcm can remain virulent in the soil and on leaf debris and can reinfect plants through wind-driven rain.

Infected plants are often defoliated, and boll quality is reduced. Cultivars resistant to most races of Xcm are being developed. Those developed by L. S. Bird at Texas A&M are gaining acceptance by growers. A line of superior resistance in the Westburn M background is being developed at Oklahoma State University by L.'M. Verhalen and is nearly ready for commercial release.

Bacteria multiply 10^5 to 10^7 fold in 11 days in the susceptible line Acala 44 (Ac 44), following low level inoculation with Xcm ($1x10^4$ bacteria ml^{-1}). This inoculum level mimics

natural infections in which bacteria enter through the stomates via wind-driven rain. Some of the early host cellular changes are chloroplast destruction followed by mitochondrial degeneration and later degeneration of the nucleus and plasmalemma. By six days post-inoculation, when watersoaking is macroscopically visible, many cell walls are broken. Bacteria have entered the mesophyll cells, specific organelles can no longer be distinguished, and large accumulations of electron-dense fibrillar material are present in the intercellular spaces (Al-Mousawi et al., 1982).

The highly resistant line Immune 216 (Im 216), when inoculated with low levels of Xcm ($5x10^5$ cells ml^{-1}) shows a different sequence of ultrastructural effects. Bacteria are observed in intercellular spaces near collapsed mesophyll cells around day 5 (Al-Mousawi et al., 1982, Essenberg et al., 1979), and multiply to only 10^7 cfu/cm^2. Two hours post-inoculation with $5x10^8$ cells ml^{-1}, disruptions of the outer surface of host mesophyll cell walls are observed. These disruptions consist of loosening and detachment of surface cuticle and envelopment of adjacent bacteria. The plasmalemma frequently breaks near the enveloped bacteria and the host cytoplasm appears to be clumped and electron-dense. Inoculated tissue becomes necrotic within 24 hours. In susceptible Ac 44 no such bacterial envelopment occurs (Cason et al., 1978, Al-Mousawi et al., 1982). Envelopes form in Im 216 and Ac 44 cotyledon tissue around starch grains, gram-positive Micrococcus lysodeckticus as well as dead Xcm. In Ac 44, where envelopment occurs, the hypersensitive

response is not observed as in Im 216. Envelopes do not form around live Xcm in Ac 44 as is observed in Im 216 (Al-Mousawi et al., 1983).

Envelopes formed around live Xcm in two cotton lines of intermediate resistance, OK 1.2 and OK 2.3. In OK 2.3, the less resistant line, the envelopes were broken by 48 hours post-inoculation and the bacteria were observed to be free of the enveloping structure. In OK 1.2, the more resistant intermediate line, bacteria were observed to be emerging from broken envelopes by 72 hours post-inoculation (Al-Mousawi, personal communication).

Four sesquiterpenoid defense compounds have been isolated and identified from leaves of resistant lines of cotton following inoculation with avirulent races of bacteria. These sesquiterpenoid compounds are 2,7-dihydroxycadalene (DHC), its oxidation product lacinilene C (LC), and their 7-methyl ethers: 2-hydroxy-7-methoxy-cadalene (HMC) and lacinilene C 7-methyl ether (LCME) (Essenberg et al., 1982, and personal communication).

Phytoalexins are low molecular weight, antimicrobial compounds that are produced and accumulate in plants in response to infection (Paxton 1980). ED_{50} values (Table 4) towards Xcm in liquid culture are 0.35 mM (DHC), 0.4 mM (LC from Westburn M (0.0) with $[\theta]_{331}$ $=+1.5\ 10^4$, Table 4), 1.5 mM (LC from Im 216 with $[\theta]_{331}$ $=-1.5\times10^4$),and 0.7 mM (LCME, positive and negative $[\theta]_{330}$). HMC is not inhibitory at its maximum water-solubility of 0.3 mM. Ac b_7, is known to produce predominantly the less inhibitory enantiomer of LC, the one with negative $[\theta]_{331}$ (Essenberg, personal communication). It is presumed that these other Acala background lines will produce

this same enantiomer. The four sesquiterpenoid defense compounds will be referred to collectively as phytoalexins, although HMC has not been demonstrated to be antimicrobial.

LC and LCME emit a yellow-green fluorescence with an emission maximum of 572 nm when exposed to near-ultraviolet light (excitation maximum =375 nm). Clusters of palisade cells in incompatible host-pathogen interactions also fluoresce yellow-green with the same emission maximum as solutions of purified LC. These cells correspond well in spatial distribution with brown necrotic cells shown to be adjacent to bacterial colonies in the incompatible reaction (Essenberg et al., 1979). Pierce (personal communication) used fluorescence microscopy and quantitative extraction of phytoalexins to estimate local concentrations and found them to reach inhibitory levels at the time of bacterial inhibition.

Pierce has also determined that the bulk of tissue phytoalexins are located in the fluorescent cell fraction obtained by rapid sorting of pectic enzyme-liberated cotyledonary cells with a fluorescence-activated cell sorter set to sort brightly green-fluorescent cells from other cells. (Pierce, personal communication).

Phytoalexins may be induced by fungi, nemetodes, bacteria, viruses, toxic chemicals and physical injuries (Misaghi 1982, Deverall 1972, Mansfield 1982). Phytoalexin production is usually associated with host cell death and is related to resistance. Phytoalexin production occurs in compatible interactions as well as incompatible ones but phytoalexin levels often differ. In incompatible interactions, synthesis may be more rapid and phytoalexins

accumulate to higher levels than in compatible interactions. In inoculated bacterial blight-resistant cotton leaves, DHC, LC, LCME and HMC accumulate to 10 to 100-fold higher amounts than in inoculated susceptible leaves (Essenberg, personal communication).

Yoshikawa et al. (1978) found low total tissue levels of glyceollin in resistant and in susceptible soybean hypocotyls infected with race 1 of Phytophthora megasperma var sojae. Local concentrations at or above the ED_{90} value were detected in the hyphal inhibition zone. No such high or localized glyceollin levels are detected in susceptible hypocotyl sections. The authors concluded that glyceollin probably causes cessation of fungal growth in resistant hypocotyls. Moesta et al. (1983) conducted a similar study to that of Yoshikawa et al. (1978), using a radioimmunoassay for Glyceollin I.'' They detected ED_{90} levels of glyceollin I (in 15 um sections) in both incompatible and compatible interactions. Differences in the two reactions were in the distribution of glyceollin I and the extent of fungal penetration. They were not able to correlate these differences with expression of resistance. The radioimmunoassay used was specific for glyceollin I and the location and quantities of isomers II and III were not detected. This reduced measured glyceollin levels about twenty percent.

In interactions between Botrytis spp. and Vicia faba, the phytoalexins wyerone and wyerone acid are produced by living cells at the edge of necrotic lesions (Mansfield 1980) and phytoalexin accumulation seems to be responsible for hyphal growth restriction in

incompatible interactions. Mansfield (1982) also presents evidence that the compatible B. faba metabolizes and detoxifies these phytoalexins and prevents their accumulation to inhibitory levels around its hyphae.

Gnanamanickam and Patil (1977) demonstrated that for red kidney bean plants inoculated with Pseudomonas phaseolicola the phytoalexins phaseollin, phaseollinisoflavan, coumestrol and kievitone were produced. In incompatible interactions these phytoalexins were detected in substantial quantities at about the time of cell death expression. Much lower phytoalexin concentrations were produced in incompatible interactions. In in vitro assays, the four phytoalexins were found to restrict colony formation by P. phaseolicola isolates.

Keen and Kennedy (1974) demonstrate that in incompatible interactions between Pseudomonas glycinea and soybean leaves rapid cell death is associated with rapid isoflavanoid phytoalexin accumulations. In compatible interactions appearance of these phytoalexins is delayed and levels were ten percent or less of levels in resistant leaves. The authors also correlate restriction of bacterial multiplication with accumulations of the isoflavanoids in resistant leaves. Long et al. (1985) have shown a negative correlation ($r = -0.94$) between glyceollin levels and bacterial multiplication for three races of Pseudomonas syringae pv. glycinea (Psg) when inoculated into leaves of three soybean cultivars (under conditions of 100% or ambient humidity). Incompatible interactions have higher glyceollin levels and lower bacterial populations than compatible interactions. Mixtures of incompatible and compatible isolates of Psg elicit visible hypersensitive response and glyceollin

accumulation in soybean similar to incompatible isolates alone but the bacterial restriction was not as great as with incompatible isolates inoculated alone.

More examples of phytoalexin induction by fungi and bacteria as well as by nematodes, viruses and abiotic elicitors can be found in reviews by Mansfield (1982), Deverall (1972), and Misaghi (1982).

Morgham (1983) demonstrated that Xcm inoculated into resistant cotton plants grown in normal light/dark cycles but kept in the dark post-inoculation showed a series of ultrastructural responses similar to the rapid cell death characteristic of typical resistant responses. However levels of Xcm reached those found in compatible interactions and the sesquiterpenoid phytoalexins characteristic of the resistant line grown under normal day/night cycles were not produced. This study suggests that cell death responses alone are probably not sufficient for bacterial growth restriction. The author suggests that part of the resistance mechanisms may be uncoupled.

The present study was undertaken to determine if cotton lines with single genes for resistance to Xcm produce phytoalexins when inoculated with avirulent races of Xcm but do not produce phytoalexins during compatible interactions. The hypothesis that each of the four different B genes triggers production of the same four phytoalexins and therefore determines similar inhibitory mechanisms was also examined. Cotton line Acala Immune (Ac Im) was included to determine if more phytoalexins would be produced by a line with

three single resistance genes than in the single resistance gene lines.

Bacterial mutants from avirulence to virulence as well as cotton lines of similar genetic background were used to reduce the probability that observed differences in phytoalexin production or in bacterial growth were due to genetic differences unrelated to bacterial virulence or host resistance.

CHAPTER 2

MATERIALS AND METHODS

COTTON LINES: Acala 44 is a bacterial blight susceptible cotton line developed by Pressley which has no known genes for resistance to Xcm (Brinkerhoff and Verhalen, 1976). The cotton lines Acala B_2 (Ac B_2), Acala B_3 (Ac B_3), Acala b_7 (Ac b_7) and Acala B_N (Ac B_N) possess the single resistance genes B_2, B_3, b_7, and B_N in an Ac 44 background (Brinkerhoff et al., 1978). Ac Im was developed from a cross between Ac 44 and the highly resistant line Im 216 (Johnson personal communication). Its high level of resistance to race mixtures of Xcm indicate that it has the major resistance genes of Im 216, which are thought to be B_2, B_3, and b_7 (Brinkerhoff and Verhalen, 1976) and may also possess some of the minor resistance polygenes of Im 216.

PLANTS: Four seeds of each cotton line were planted in a commercially prepared soilless mixture of peat-moss and vermiculite (Jiffy-mix Plus) in 6-inch clay pots 1/2 to 3/4 inches deep. The plants were watered daily and fertilized twice monthly with aqueous Stern's Miracle Grow. The plants were kept in a growth chamber set for a 14 hours light, 10 hours dark cycle with an average temperature of $30^{\circ}C$ during the light part and $16^{\circ}C$ in the dark part of the cycle.

The third and fourth foliage produced leaves (after the cotyledons) were used at 5 weeks germination for pretesting and the fifth, sixth and seventh produced leaves were used at 6 to 7 weeks post-germination in the experiments.

BACTERIA: Inoculum used in the study was made from a single-colony streptomycin-resistant isolate of race 1 of Xcm and two spontaneous mutants derived from it, race 2 selected for virulence in Ac b_7 and race 4 selected for virulence in Ac B_N (Brinkerhoff 1963). Also used were a streptomycin-resistant isolate of race 3 and two spontaneous mutants derived from it, JH and MP, both selected for virulence in Ac b_7 (Essenberg personal communication). In the standard series of differential cotton lines (Brinkerhoff, 1970) they both are characterized as race 2.' These strains of races 1, 2, and 4 are referred to below as the race 1 family, and race 3 and the JH and MP mutants are referred to as the race 3 family. Collectively, the bacteria will be referred to as races. Bacteria were cultured overnight in Difco nutrient broth (0.8%) at 30°C, centrifuged and resuspended in 14% glycerol, Ornston-Stanier minimal media (Ornston and Stanier, 1966) (v/v), dispensed into 1.2-ml cryotube vials, quick frozen in dry ice:acetone and stored at -70°C.

To start cultures, a cryovial was thawed, 0.01 ml bacterial suspension pipetted into 25 ml Difco nutrient broth and cultured with agitation for 18 hours.

The absorbance of the broth culture was determined at 600 nm and the inoculum prepared by centrifuging the broth for 10 minutes at

1700 xg at room temperature. The bacterial pellet was resuspended in sterile saturated $CaCO_3$ (0.14 mg ml^{-1}) to yield a final volume of 4 liters of each inoculum. Plate counts showed that the inocula contained approximately $5x10^6$ cfu ml^{-1}.

PRETEST INOCULATION METHOD: The third and fourth leaf of each plant was enclosed in a 4x6-inch plastic bag secured around the petiole for at least 1 hour before inoculation to ensure that the stomates were open. Each leaf to be inoculated was visually divided into three sectors. The undersurface of each leaf was sprayed with either race 3, mutants JH and MP (the race 3 family), or race 1, race 2, and race 4 (the race 1 family) (one race per sector) using positive pressure.

Six and ten days after inoculation symptoms were observed and compared with the expected virulence/avirulence pattern for each race (Brinkerhoff 1970). The plants were thinned to two plants per pot; plants that lacked full expected resistance were removed. The third and fourth foliage leaves were removed. The macroscopically visible symptoms characteristic of resistance were localized necrotic areas, chlorosis and desiccation of leaves. That determining susceptibility was water soaking of the leaf. Leaves showing predominantly resistant symptoms with only scattered watersoaked areas were determined to be resistant.

EXPERIMENTAL DESIGN: The experimental design was a randomized Latin Square. One plant in each pot received the race 1 family (races 1, 2 and 4) one race per leaf inoculated into leaves five,

six, and seven. The other plant in the pot received the race 3 family (race 3 and mutants JH and MP), one race per leaf. Foliage leaves five six and seven on three different plants were inoculated with one race each. One plant per cotton line received the $CaCO_3$ control and the other plant in the pot was an untreated control. Two cotton lines were used per experiment. Three experiments were conducted.

BACTERIAL INOCULATION: Four liters of inoculum of each bacterial race were prepared as for the pretest. Leaves to be inoculated were enclosed in plastic bags at least one hour before inoculation.

All leaves that received the same race were inoculated by spraying the undersurface of the leaf with positive pressure until water-soaking was uniform. Leaf discs were excised with a paper punch for determination of bacterial population densities. Inoculated leaves were rebagged to prevent contamination from other inocula and the next set of leaves were then sprayed with inoculum.

BACTERIAL GROWTH POPULATION DENSITIES: Experiment 1 (Ac Im, and Ac B_2): One 0.33 cm^2 disc was taken each day from each leaf up to and including the 11th day post-inoculation. The three discs per race/line combination were placed on moist filter paper and stored over ice until all leaves were sampled. Bacterial population densities were determined as follows. Three leaf discs per race/line combination were rinsed twice for 15 to 20 seconds in sterile saturated $CaCO_3$, homogenized in 1.0 ml of sterile $CaCO_3$ with a mortar and pestle and diluted. Aliquots (37 µl) were spread on Difco nutrient agar plates by a Spiral Plater (Spiral Systems). Plates

were incubated for two days at 30°C before the numbers of bacterial colonies were counted. On the 9th day after inoculation, two discs were sampled from each leaf. Each disc was ground separately in 1.0 ml sterile $CaCO_3$, diluted and plated on nutrient agar plates as before.

Experiments 2 (Ac b_7, Ac B_3) and 3 (Ac B_N, Ac 44): On day 0, two discs were punched from each leaf and ground together. This allowed determination of population densities per leaf. On all other days except day 9, two discs were sampled from each leaf. The six discs per race/line combination were rinsed twice in sterile $CaCO_3$, homogenized together, and plated as before. For day 9, bacterial population densities were determined as for day 9 in experiment one.

PHYTOALEXIN EXTRACTIONS: Eight days after inoculation, one-third of each leaf was excised with a razor blade. The midvein was left intact. A 0.33 cm^2 disc was taken from each excised leaf portion. The three discs for each race/line combination were placed in preweighed foil packets, and fresh weights obtained. The leaf packets were dried at 60°C overnight, placed in a desiccator for 1 hour and dry weights taken. The leaf packets were returned to the oven, redried and reweighed again after one day. This procedure was repeated until the dry leaf weights varied no more than one percent.

Phytoalexins were extracted as follows. Leaf material (0.9 gram fresh weight) was quick-frozen in liquid nitrogen and ground under liquid nitrogen in a prechilled mortar. The resulting powder was transferred to a 7.0 ml tissue homogenizer and homogenized with 5.0 ml methanol-water (80:20, v/v). The homogenized extract was

centrifuged at room temperature, the supernatant decanted and its volume measured. The pellet was reextracted twice with 5.0 ml of the same solvent, centrifuged, and supernatant decanted. The combined supernatants were passed through a Waters C_{18} SEP-PAK pre-rinsed with 10 ml methanol and 10 ml methanol-water (80:20,v/v), and the effluent collected. The SEP-PAK was rinsed with 5.0 ml of the methanol-water solution and the resulting effluent was combined with the previous effluent and diluted with water to a composition methanol-water (40:60, v/v). This mixture was extracted four times with one-quarter its volume of chloroform. The combined chloroform extracts were concentrated with a nitrogen stream, transferred to a 0.4 ml microfuge tube and evaporated to dryness with nitrogen. The residue was dissolved in 0.25 ml methanol-water (60:40, v/v) and microfuged for 5 minutes to sediment any particulates. The supernatant was injected onto a Spectra/Physics high performance liquid chromatography (HPLC) system with a Hibar RP C_{18} column (4 mm x 250 mm, 5 um particle diameter). LC, DHC, and LCME were eluted with 30 ml methanol-water (60:40, v/v) followed by 30 ml methanol water (67:33, v/v) to elute HMC.

ANALYTICAL METHODS: Molar concentrations of purified compounds were determined from their ultraviolet absorbances in aqueous methanol using molar absorptivities determined with the same solvent (LC, $\epsilon_{343} = 5.94 \times 10^3\ M^{-1}\ cm^{-1}$; DHC, $\epsilon_{237} = 7.26 \times 10^4\ M^{-1}\ cm^{-1}$; LCME $\epsilon_{248} = 8.57 \times 10^3\ M^{-1}\ cm^{-1}$; HMC, $\epsilon_{237} = 5.94 \times 10^4\ M^{-1}\ cm^{-1}$). These purified standards were diluted to known concentrations and fluorescence excitation spectra (Perkin Elmer Fluorescence

Spectrophotometer 650-40) were recorded using an emission wavelength of 512 nm for LC and LCME and 239 nm for DHC and HMC. Standard curves of relative fluorescence intensity units (RFIU) versus molar concentrations of diluted standards were prepared. Fluorescence excitation spectra of extracted chromatographed samples were recorded and molar concentrations were determined from the standard curves.

CHAPTER 3

RESULTS

The patterns of resistance and susceptibility of the six cotton lines to the bacterial races studied are given in Table 1.' In the incompatible reactions, bacterial growth was inhibited at 7 days post-inoculation except in the case of Ac Im. In that case bacterial growth was inhibited at 5 days post inoculation (Fig. 1, A-F)

The final population densities of virulent races were significantly different from those of avirulent races with a p value less than 0.01 when an analysis of variance was performed, followed by the protected least squares difference (LSD) test (Steel and Torie, 1980, Table 2). The virulent races' final population densities were greater than 5.93×10^8 cfu/cm^2 whereas the avirulent races' final population densities were less than 3.33×10^8 except for race 3 in Ac B_2 which had an average final population density of 8.56×10^8 (Table 3). Thus final bacterial population densities correlated strongly with the virulence/avirulence assignments (Table 1) based on macroscopically visible symptoms of the inoculated plants.

There were also significant differences within the virulent and avirulent groups. The final population densities of all races inoculated into Ac Im were significantly different from the single B

gene lines inoculated with avirulent races ($p<0.01$), as were the final population densities of all races in Ac 44 when compared to the virulent races inoculated into the single B gene lines ($p=0.01$, Table 2).

An alternative way of expressing the results of the bacterial population density data is in analysis of final growth yield [(average density of days 10 and 11 cfu/cm^2)/(day 0 cfu/cm^2)]. Analysis of growth yields gave similar results with the incompatible interactions in single B gene lines being significantly different from compatible interactions in single B genes, ($p<0.01$).

PHYTOALEXIN STANDARD CURVES: Two independent dilution series of purified LC and LCME and three independent series of purified HMC were analyzed by spectrofluorometry and the RFIU were recorded. Least squares regression lines through the origin [slope $=(\Sigma xiyi)/(\Sigma xi^2)$] were fitted to the data (Fig. 2, A-C; x's are points calculated from regression analysis used to draw the regression line).

For DHC (Fig. 2,C), five independent dilution series were prepared. DHC is very susceptible to air oxidation. Each data set, represented by a separate symbol except x, when analyzed alone using least squares regression, gave a straight line with a correlation coefficient of 0.99. When all 5 data sets were analyzed together, a correlation coefficient of 0.79 was obtained. The average slope was computed as $\tan \bar{\theta} = 330.9$ RFIU/nmol ml^{-1} (θ = angle of regression line from origin). This value was used for estimation of DHC in leaf extracts. It agreed well with the slope of 320 RFIU/nmol ml^{-1} obtained by analysis of all 5 data sets together using a model of

linear regression based on the general linear model but with a multiplicative error.

EXTRACTED AMOUNTS OF PHYTOALEXINS: In all cases, the incompatible reactions give higher yields of phytoalexins than the compatible reactions (Fig. 3, A-D, Table 3). For DHC, the compatible phytoalexin levels were no greater than 0.56 nmol/g dry weight whereas the incompatible levels were no less than 1.44 nmol/g dry weight (Table 3). No individual cotton line exceeded the others in phytoalexin production, nor did any race exceed the others in phytoalexin elicitation.

The races grown on Ac Im had the lowest final bacterial population densities (Table 3). The incompatible race/line interactions with the exception of Ac B_2 and race 3 had lower final bacterial population densities and higher phytoalexin contents than compatible race/line interactions (Fig. 4, A-D). The exception, race 3 in Ac B_2, had final bacterial population densities within the compatible range and accumulated relatively low phytoalexin levels.

CHAPTER 4

DISCUSSION

Visual detection of resistance or susceptibility is correlated with highly significant differences in bacterial growth between compatible and incompatible host pathogen interactions, demonstrating inhibition by the plant in incompatible interactions. In addition, the higher phytoalexin levels in incompatible interactions than in compatible interactions, also support the hypothesis that phytoalexins play a role in the resistant response (see reviews by Mansfield 1982, Deverall 1972, and Misaghi 1982). Every single B gene cotton line when inoculated with avirulent races of Xcm produced quantities of phytoalexins greater than are produced in compatible interactions in single B gene cotton lines. Each such combination also produces more phytoalexin than the fully susceptible Ac 44 (Fig. 4, A-D). These results also indicate that each of the four B genes controls production of the same four phytoalexins. The B genes differ in their specificity for recognition of Xcm races, however they have a common function, once that recognition has occurred. The relative amounts of each of the four phytoalexins when produced in each of the incompatible race/line interactions are quite similar indicating a coordinate control of biosynthesis and perhaps

also of metabolism of these four structurally related sesquiterpenoids.

The presence of more than one resistance gene in a line seems to impart more efficient control over disease resistance. This is demonstrated by the large significant difference between bacterial growth in single gene resistance lines and in Ac Im. As the amounts of phytoalexins produced were similar, control over the timing of phytoalexin production by each gene might be different, thus allowing for earlier phytoalexin production and possibly a greater phytoalexin to bacteria ratio in Ac Im than in each of the single B gene lines. The probability that the single B genes code for other minor resistance effects is demonstrated in the small but significant difference in bacterial density between fully susceptible Ac 44 and the compatible single gene line interactions. Mutations of avirulent races of Xcm to virulent races evidently led to loss of ability to elicit phytoalexins. These findings support the idea that phytoalexin production is linked to the resistance genes.

Long et al. (1985) showed a negative correlation between glyceollin levels and bacterial growth in the Psg soybean system. As strong a correlation was not demonstrated in the current system. The levels of phytoalexin in incompatible interactions covered a wider range than the levels of glyceollin reported by Long et al. Essenberg (personal communication) inoculated immune [WbM (0.0)] and susceptible [WbM (4.0)] cotton leaves with $5x10^6$ cfu ml^{-1} of Xcm race 3 and studied the production of phytoalexin over time (Fig. 5). The levels of phytoalexins increase slowly at first and peak around 4 days post-inoculation. After 7 days post inoculation, the phyto-

alexin levels rapidly increase for about three more days and then drastically decrease until 15 days post-inoculation at which time the phytoalexin content begins to level out. The phytoalexin data in this study was obtained 8 days post-inoculation. This is on the sharpest part of the phytoalexin curve, where slight variations in time of harvest as well as initiation of phytoalexin synthesis could give rise to very large differences in extractable amounts of phytoalexin.

The time course for glyceollin presented by Long et al. (1985) does not show the rapid increases and decreases seen with the sesquiterpenoid phytoalexins. This variation along with using twice as many strains and twice as many lines could account for the lack of a straight line correlation between amounts of phytoalexin produced and bacterial growth. The variability of phytoalexin production could also result from variable environmental conditions.

Brinkerhoff (1970) demonstrated the effects of environmental conditions on the expression of disease resistance. Under low night temperatures ($20^{o}C$ or below) and high day temperatures ($36^{o}C$ and above) the ability of the single resistance genes to confer resistance in either a susceptible or tolerant upland cotton background was overcome by the pathogen. Immune cotton lines did remain immune under these conditions. In this study, conditions met those for low night temperatures ($16^{o}C$) but not for high day temperatures ($30^{o}C$). This lack of optimal conditions for the expression of resistance by the single B genes may at least in part account for lower phytoalexin yields and higher bacterial growth than under optimal conditions.

These conditions might be why race 3 in Ac B_2, showed visible symptoms of resistance, had higher levels of phytoalexins than compatible interactions but still had bacterial population densities within the compatible range (Figs. 15-18).

An attempt should be made to establish a correlation between cotton lint quality, and quantity and high phytoalexin levels elicited by avirulent races of Xcm in cotton lines having the single B genes. In breeding programs, genes conferring high levels of phytoalexins and genes for possession of good lint qualities should be combined into a line of high resistance to bacterial blight. At the same time, factors for resistance to Verticillium wilt and other cotton diseases must be considered.

Flor (1956) proposed the gene-for gene theory in flax and flax rust in an attempt to explain the genetic control of disease expression (resistance and susceptibility). Flor stated that "for each gene conditioning resistance in the host there is a specific gene conditioning pathogenicity in the parasite" (Flor 1956). The gene for resistance in the host is dominant as is the gene for avirulence in the pathogen. Therefore resistance occurs when the complementary genes in both the host and pathogen are dominant and disease is expressed when either the gene for resistance in the host or the gene for avirulence in the pathogen, or both are recessive.

Loss of an avirulence gene in the blight bacterium seems to give rise to failure to elicit phytoalexin production and leads to disease expression. This relationship makes the cotton/bacterial blight system a good one for studying the gene-for-gene hypothesis.

In at least three other gene-for-gene systems, Psg and soybean (Long et al., 1985), Puccinia coronata f. sp. avenae and oat (Mayama et al., 1982) and Melampsora lini and flax (Keen and Littlefield 1979) high levels of phytoalexins have been correlated with low pathogen growth and therefore high resistance, while intermediate levels of phytoalexins have been associated with intermediate resistance expression. If phytoalexin resistant mutants of Xcm can be selected that cause blight in the presence of phytoalexin levels sufficient for normal resistance, then the association of phytoalexins as a cause of resistance in a gene-for-gene system will be established (Keen 1982, Ellingboe 1982).

Table 1. Bacterial Race and Cotton Line Interactions

	Ac 44[b]	Ac B_2	Ac B_3	Ac b_7	Ac B_N	Ac Im[a]
Race 1	S[c]	R	R	R	R	R
Race 2	S	R	S	S	R	R
Race 4	S	R	R	R	S	R
Race 3	S	R	R	R	R	R
MP	S	R	S	S	R	R
JH	S	R	S	S	R	R

a) Contains genes B_2, B_3, b_7, and perhaps polygenes

b) Contains no known resistance genes

c) S indicates susceptible host, virulent race (compatibility). R indicates resistant host, avirulent race (incompatibility). These relationships were assigned on the basis of macroscopically visable symptoms described under "Pretest Inoculation Method" in the materials and methods section.

Table 2. Analysis of Variance and Protected LSD on the Logs of the Average Final Bacterial Population Densities+

Source	df	SS	MS	F	P
Treatments	3	17.68	5.89	132.91	<.01
Error	33	1.46	0.04		
Total	36	19.15			

Protected LSD

Comparison	t, 33 df	p
Ac Im vs. avir. single B gene lines	33.42	<.01
Ac Im vs. vir. single B gene lines	36.56	<.01
Ac Im vs. Ac 44	32.81	<.01
Avir. single B gene lines vs. Ac 44	12.25	<.01
Avir. single B gene lines vs. vir. single B gene lines	15.20	<.01
Vir. single B gene lines vs. Ac. 44	2.41	0.1

+ See footnotes to Table 3 for explanation of average final population densities.

Table 3. Phytoalexin Levels (nmol/g dry wt.) 8 Days Post-Inoculation and Average Final Population Densities (cfu/cm^2) of Compatible and Incompatible Race-Line Interactions[a].

Incompatible							Compatible						
Line	Race	cfu/cm^2	LC	DHC	LCME	HMC	Line	Race	Cfu/cm^2	LC	DHC	LCME	HMC
Ac Im	R3	3.95×10^6	13.60	17.36	26.02	59.71	Ac 44	R3	1.07×10^9	0.73	0.02	0.55	1.47
	JH	4.25×10^6	38.26	28.84	118.75	132.24		JH	5.94×10^8	0.85	0.15	1.92	3.44
	MP	6.40×10^6	28.54	21.94	83.75	88.81		MP	6.46×10^8	0.33	0.01	0.40	0.09
	R1	7.86×10^6	7.34	4.51	4.15	9.50		R1	1.04×10^9	0.36	0.02	1.09	1.15
	R2	1.35×10^7	20.78	21.87	17.35	28.24		R2	1.08×10^9	0.16	0.09	0.27	0.67
	R4	4.74×10^7	10.98	6.44	35.80	37.71		R4	1.09×10^9	0.83	0.56	2.96	15.22
Ac B_2	R3	8.56×10^8	3.09	1.45	6.84	27.44	Ac B_3	JH	1.04×10^9	0.57	0.02	0.79	0.96
	JH	2.96×10^8	2.32	1.44	4.22	39.41		MP	1.18×10^9	0.32	0.04	2.88	0.11
	MP	3.49×10^8	10.31	9.71	16.02	82.74		R2	1.23×10^9	0.85	0.03	1.11	0.35
	R1	2.14×10^8	12.78	5.55	20.90	97.98	Ac b_7	JH	1.54×10^9	0.26	0.12	0.39	0.37
	R2	2.60×10^8	12.88	5.67	22.28	111.26		MP	1.01×10^9	0.84	0.02	1.27	0.10
	R4	2.56×10^8	8.50	4.36	13.56	48.14		R2	1.24×10^9	0.61	0.02	0.70	0.37
Ac B_3	R3	2.93×10^8	52.06	42.97	53.37	225.75	Ac B_N	R4	1.48×10^9	0.40	0.05	1.80	2.29
	R1	3.26×10^8	24.86	20.15	32.10	113.70							
	R4	1.31×10^8	18.34	17.15	19.91	52.26							
Ac b_7	R3	3.08×10^8	22.27	14.71	27.55	97.47							
	R1	2.63×10^8	28.14	34.15	36.64	156.66							
	R4	1.34×10^8	22.20	15.20	25.62	69.72							
Ac B_N	R3[b]	1.58×10^8	482.65	63.09	358.44	1248.97							
	JH[c]	2.75×10^8	33.80	7.20	52.02	322.33							
	MP[d]	2.84×10^8	4.13	1.78	9.35	93.80							
	R1[e]	2.23×10^8	40.93	3.42	82.15	272.11							
	R2[f]	3.33×10^8	15.01	5.36	14.20	123.08							

a) Some leaves abscised before day 11, therefore the following: b) one leaf out of three on day 10; no day 11 data; c) one leaf out of three on day 11; d) two leaves out of three on days 10 and 11; e) day 9 ctu/cm on one remaining leaf.

Table 4. Abbreviations

[θ]= mean residue ellipticity (degrees cm^2 $decimole^{-1}$). The difference in absorption of right versus left circularly polarized light.

ED= effective dose. For example, ED_{50} is the concentration of phytoalexin needed to cause a fifty percent reduction in the number of bacterial generations.

Figure 1, A-F. Population densities of six strains of Xcm, a) in the susceptible cotton line Ac 44, b) in the single B gene line Ac B_2, c) in the single B gene line Ac B_3, d) in the single B gene line Ac b_7, e) in the single B gene line Ac B_N, f) in the resistant cotton line Ac Im containing genes B_2, B_3, b_7 and perhaps some of the polygenes. Each symbol designates the Xcm strain listed on the graph.

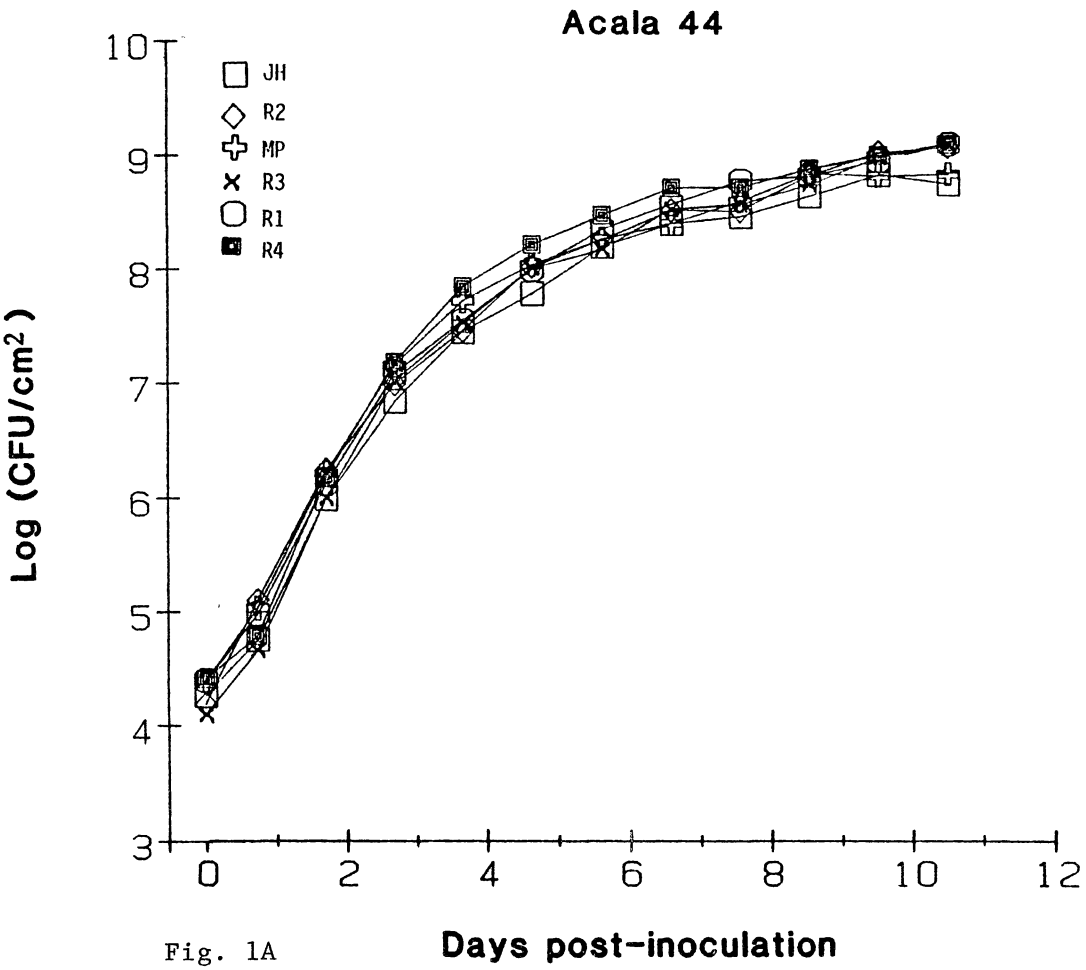

Acala 44
JH
R2
MP
R3
R1
R4
Log (CFU/cm²)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1A

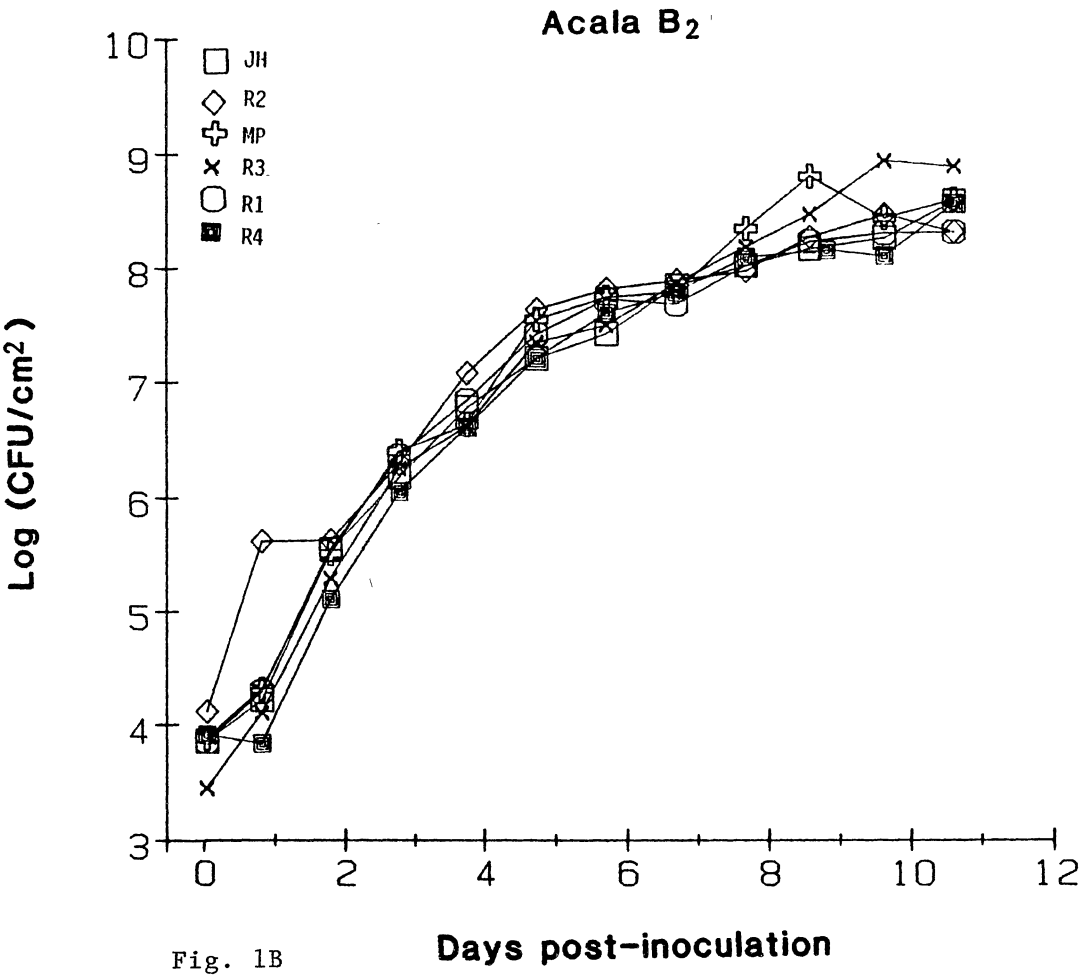

Acala B2
JH
R2
MP
R3
R1
R4
Log (CFU/cm2)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1B

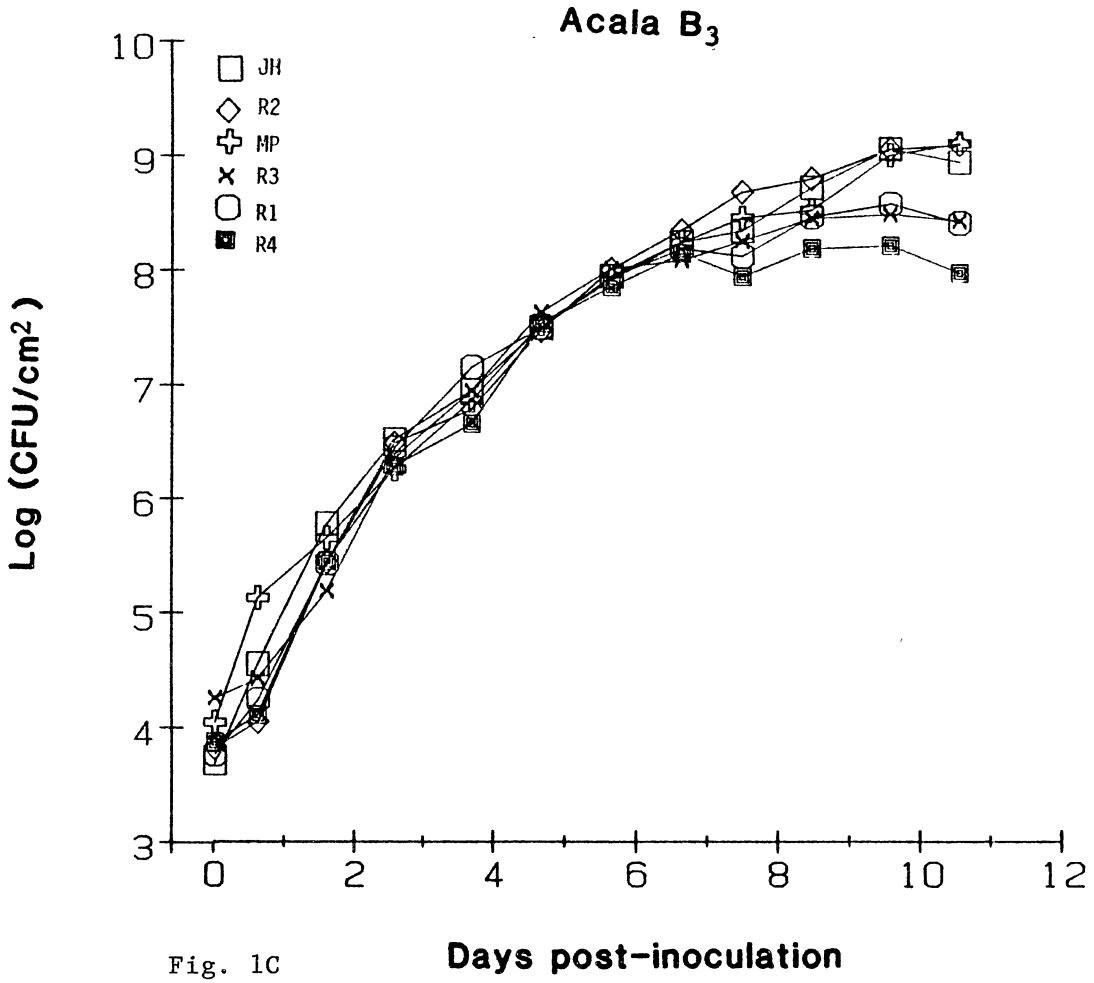

Acala B3
JH
R2
MP
R3
R1
R4
Log (CFU/cm2)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1C

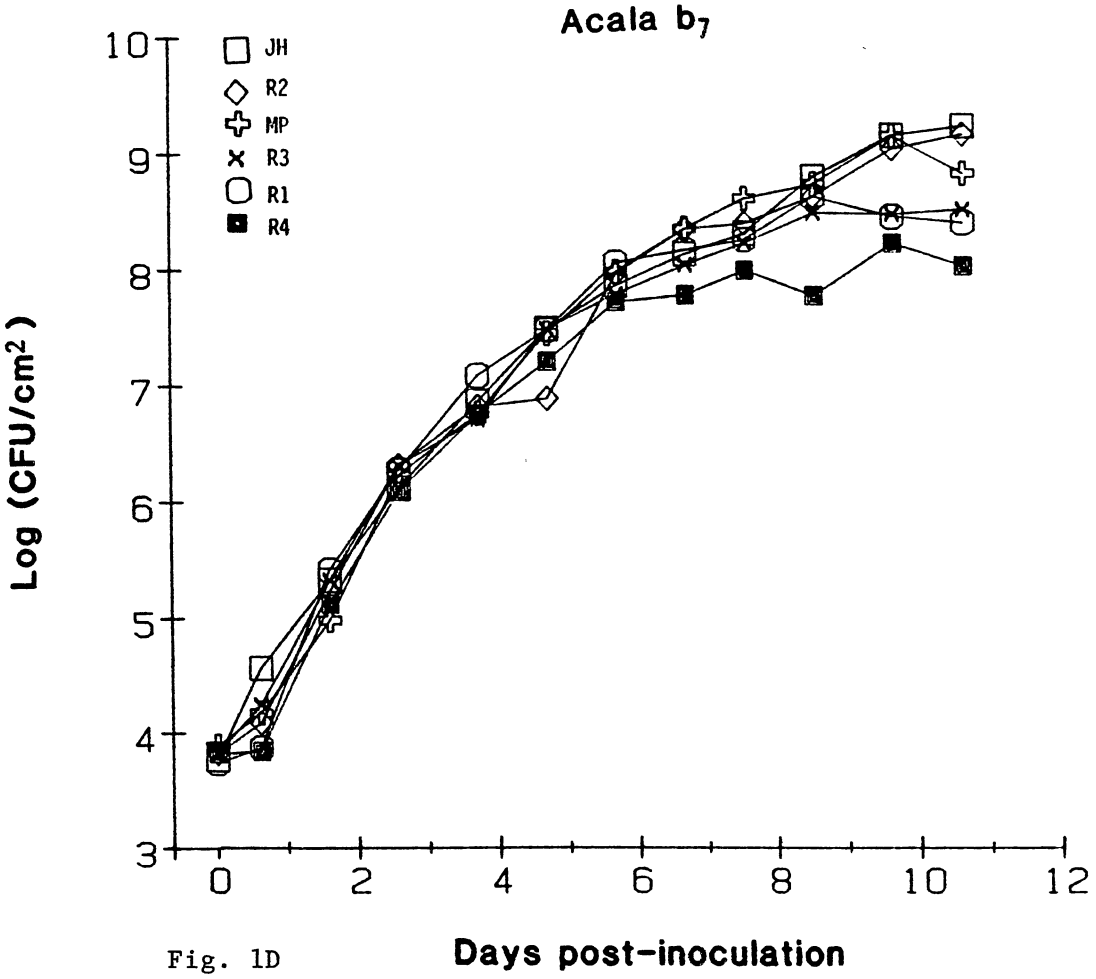

Acala b7
JH
R2
MP
R3
R1
R4
Log (CFU/cm2)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1D

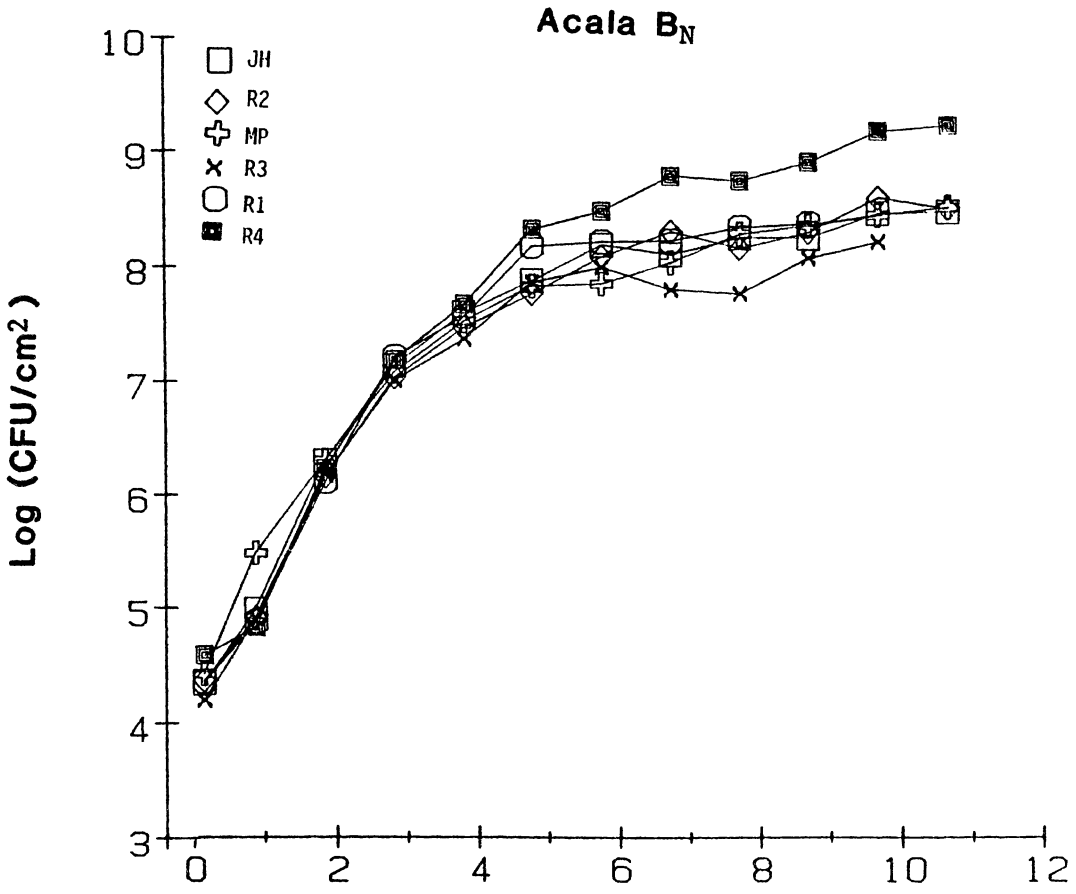

Acala B_N
JH
R2
MP
R3
R1
R4
Log (CFU/cm^2)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1E

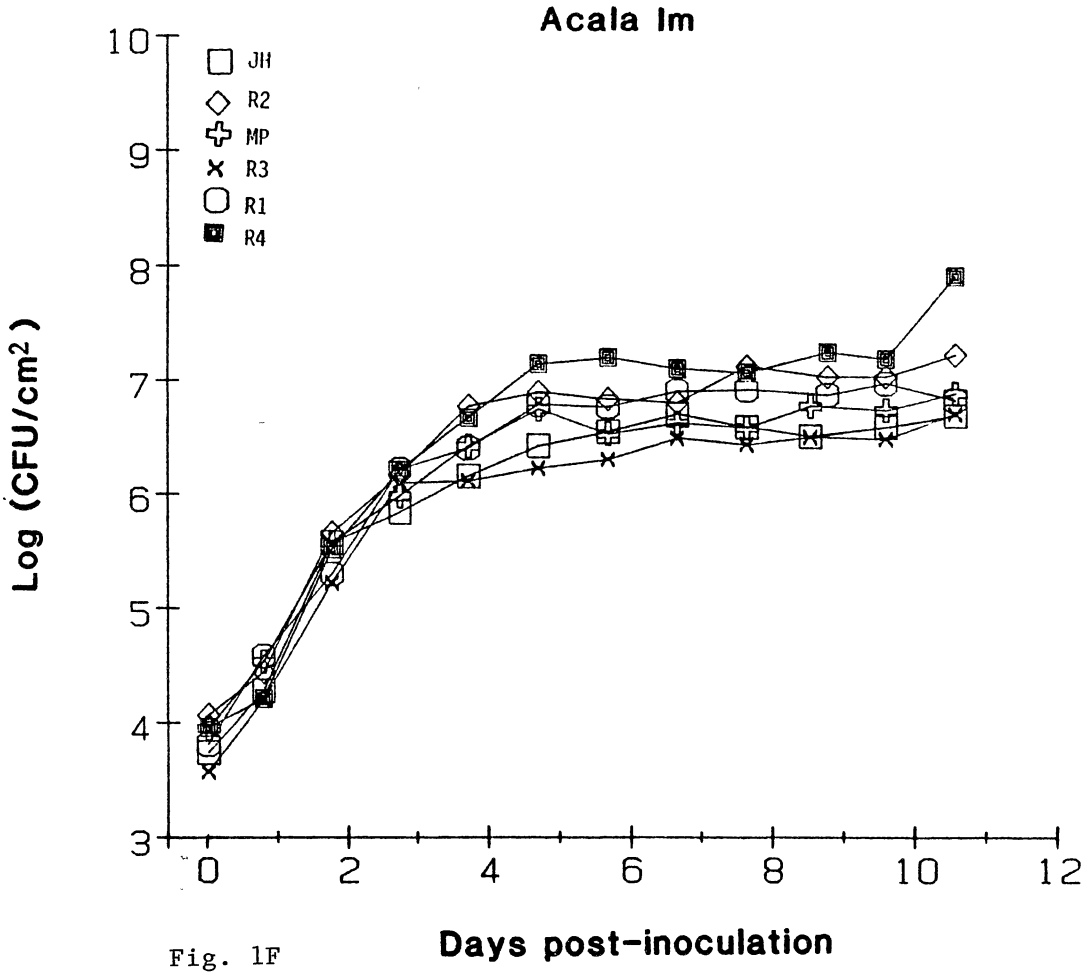
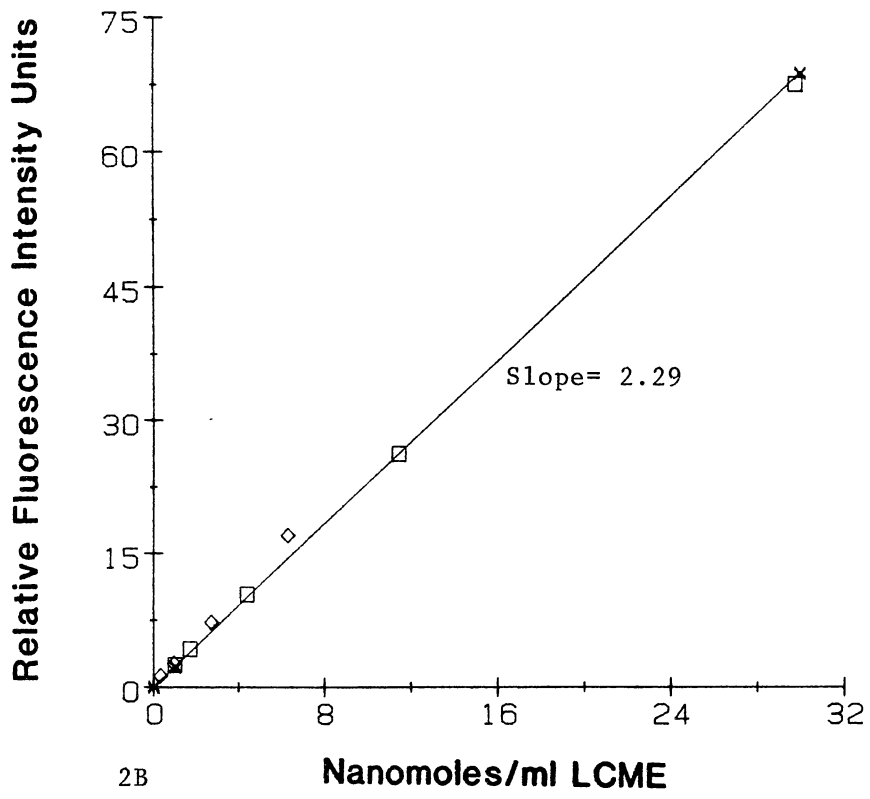

Acala Im
JH
R2
MP
R3
R1
R4
Log (CFU/cm²)
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
Days post-inoculation

Fig. 1F

Figure 2, A-D.'Standard Fluorometric curve for phytoalexin, a) Lacinilene C, b) Lacinilene C 7-methyl ether, c) 2,7-dihydroxy-cadalene, d) 2-hydroxy-7-methoxycadalene. Each symbol except x represents a different data set. X= points used to draw the regression line.


Relative Fluorescence Intensity Units
75
60
45
30
15
0
0
8
16
24
32
Slope= 2.29
Nanomoles/ml LCME

2B

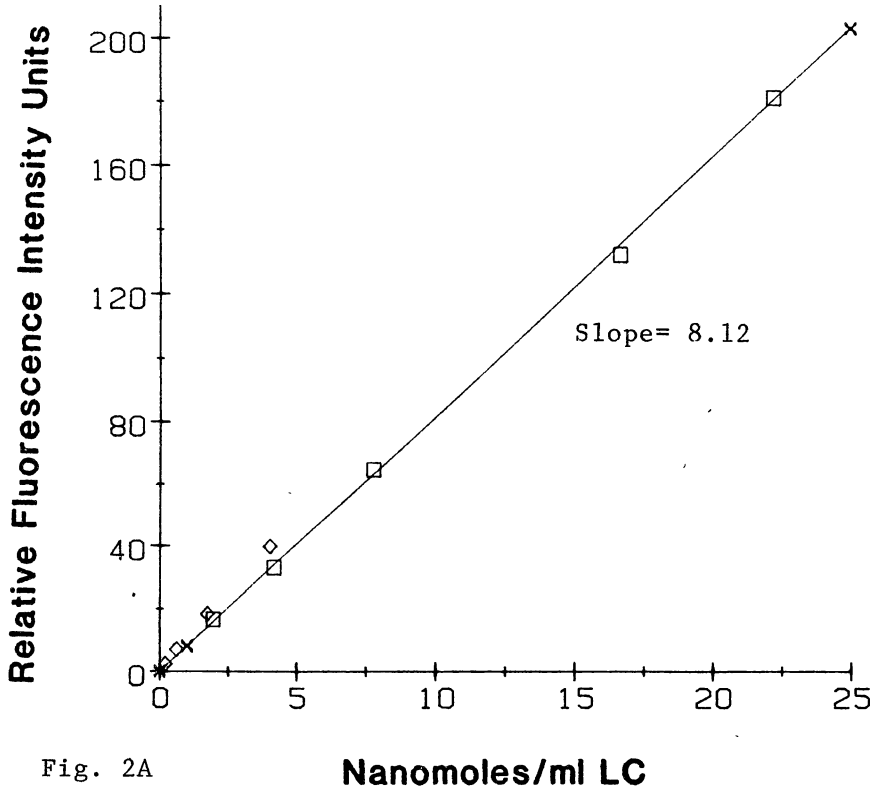
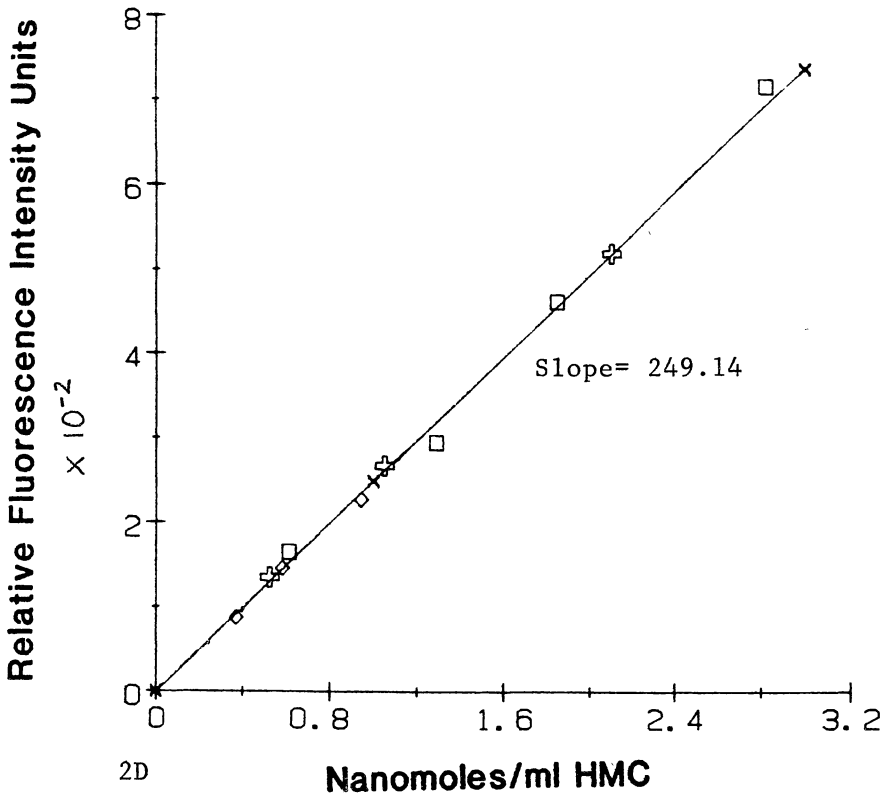

Relative Fluorescence Intensity Units
200
160
120
80
40
0
0
5
10
15
20
25
Slope= 8.12
Nanomoles/ml LC

Fig. 2A


Relative Fluorescence Intensity Units
X 10-2
8
6
4
2
0
0
0.8
1.6
2.4
3.2
Slope= 249.14
Nanomoles/ml HMC

2D

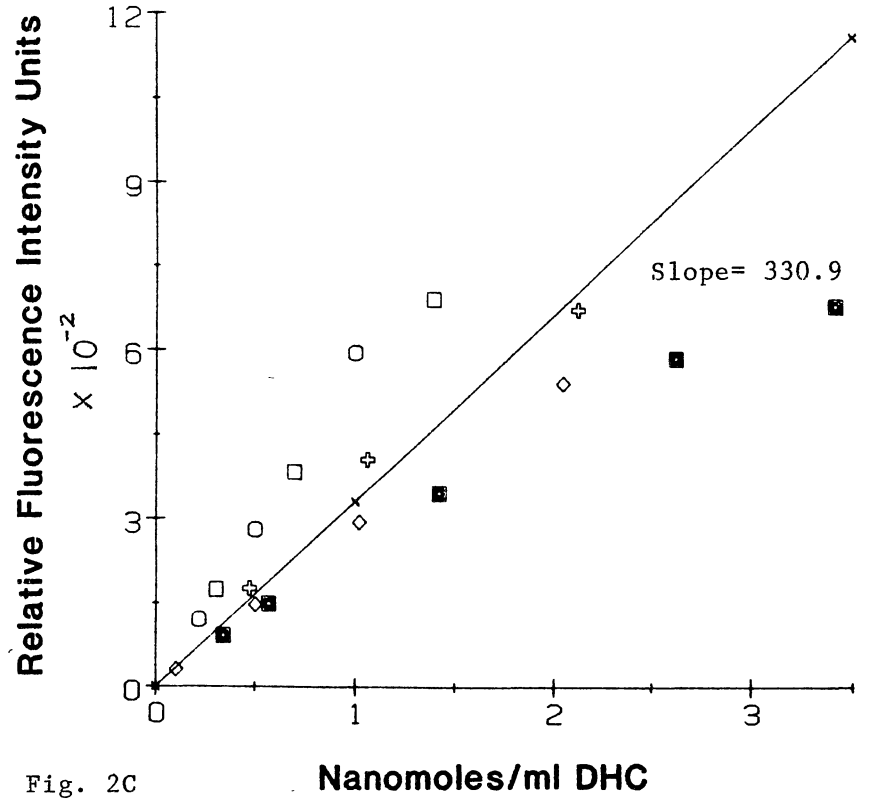

Relative Fluorescence Intensity Units
X 10-2
12
9
6
3
0
0
1
2
3
Slope= 330.9
Nanomoles/ml DHC

Fig. 2C

Figure 3, A-D. Phytoalexin production in cotton lines inoculated with races of Xcm, a) Lacinilene C, b) 2,7-dihydroxycadalene, c) Lacinilene C 7-methyl ether, d) 2-hydroxy-7-methoxycadalene. Each symbol designates the Xcm strain listed on the graph.

Fig. 3A

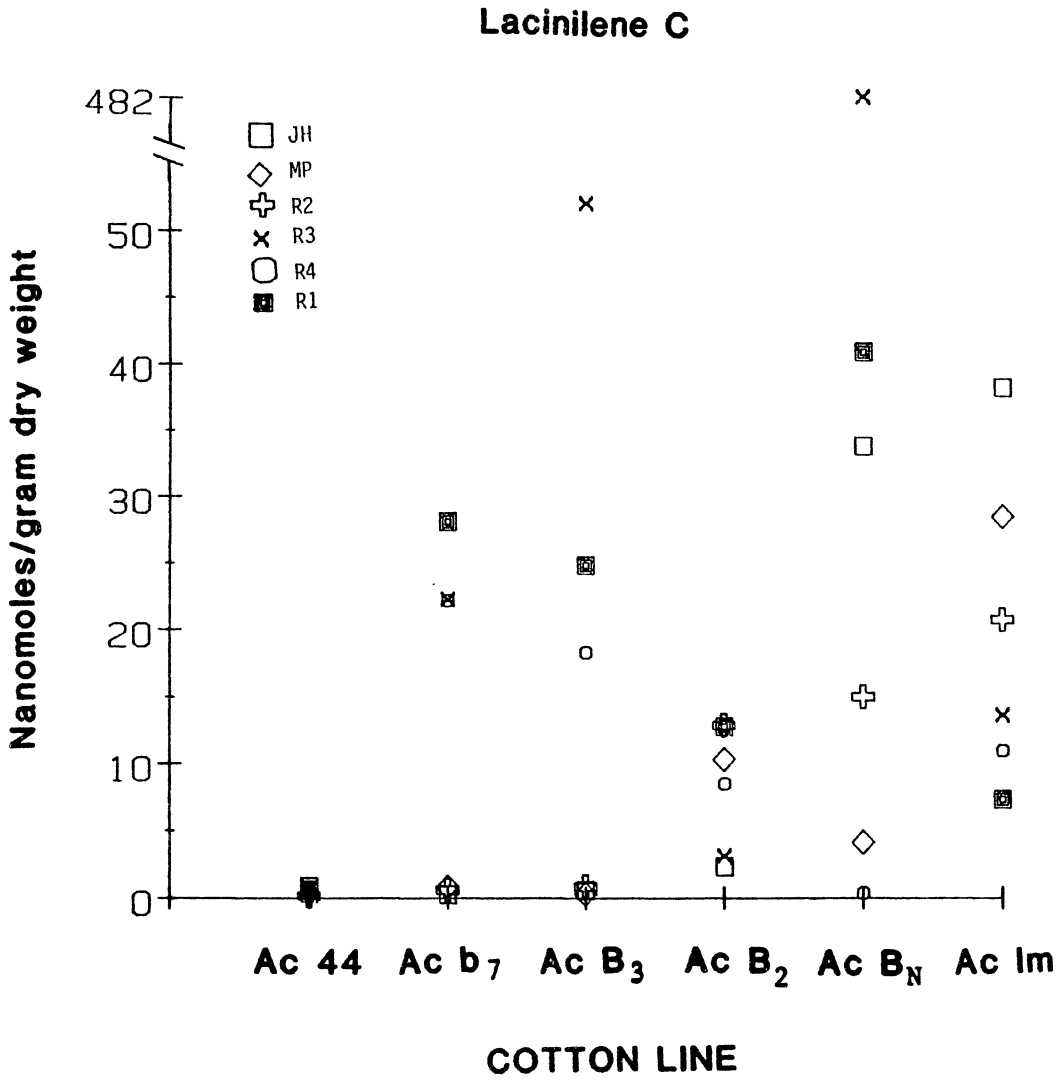

Lacinilene C
482
50
40
30
20
10
0
JH
MP
R2
R3
R4
R1
Nanomoles/gram dry weight
Ac 44
Ac b7
Ac B3
Ac B2
Ac BN
Ac Im
COTTON LINE

Fig. 3B

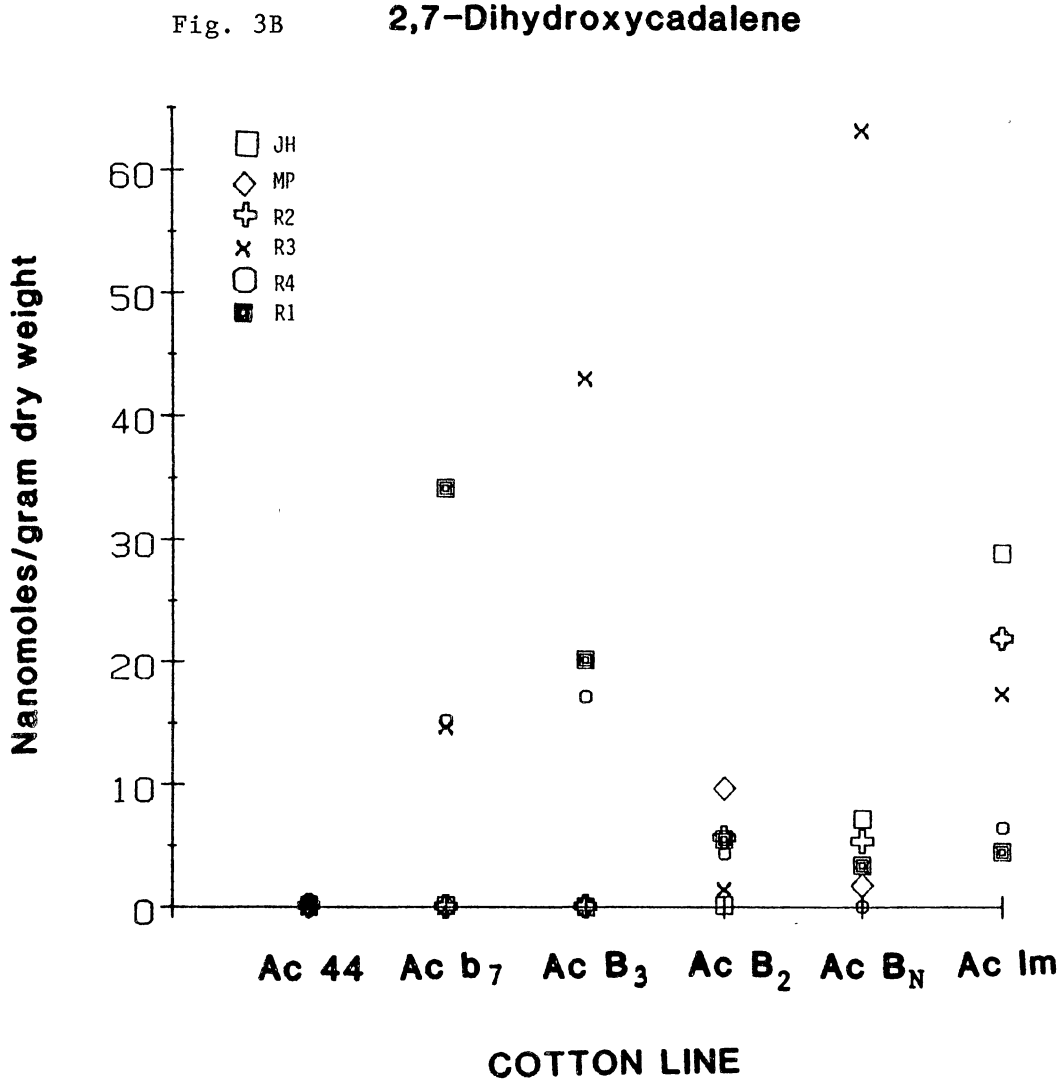

2,7-Dihydroxycadalene
JH
MP
R2
R3
R4
R1
Nanomoles/gram dry weight
60
50
40
30
20
10
0
Ac 44
Ac b7
Ac B3
Ac B2
Ac BN
Ac Im
COTTON LINE

Fig. 3C

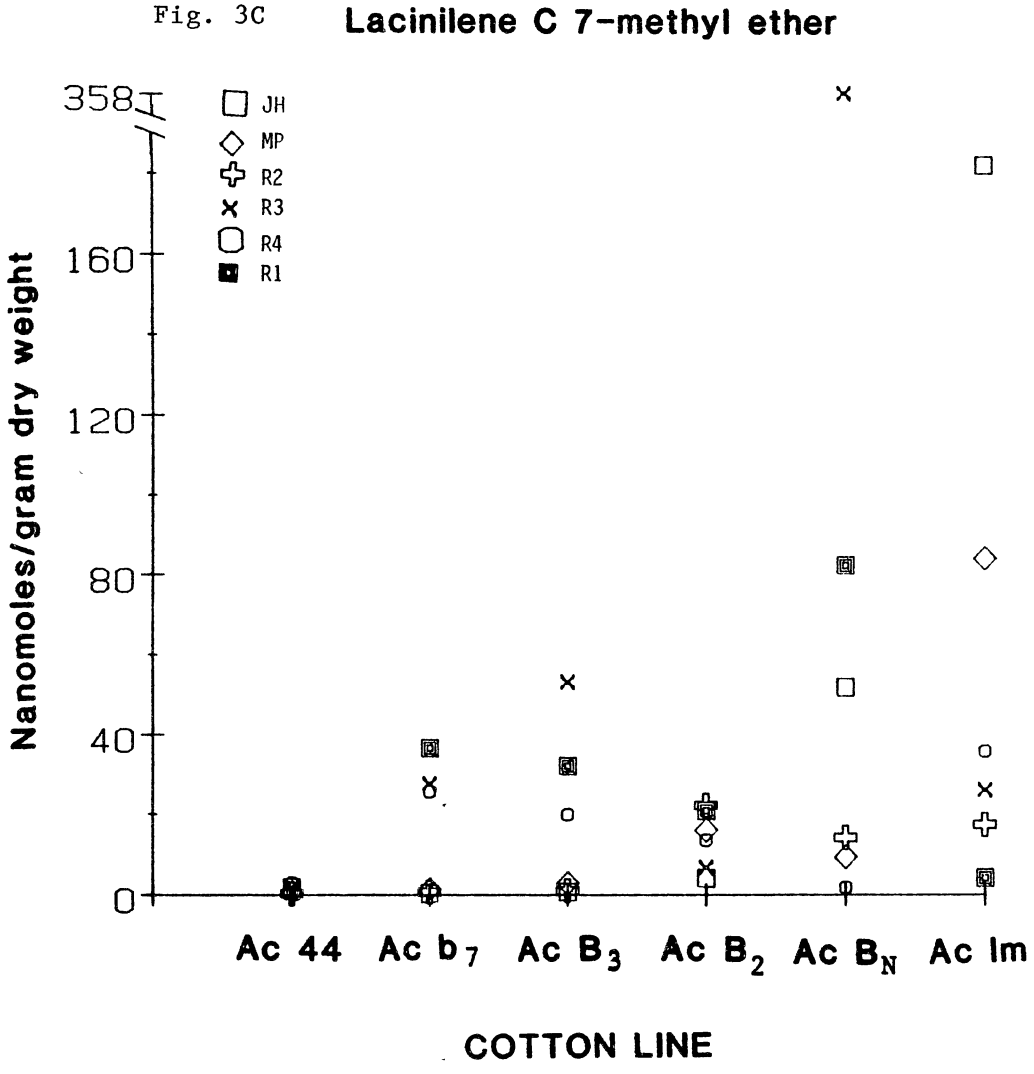

Lacinilene C 7-methyl ether
358
160
120
80
40
0
Nanomoles/gram dry weight
JH
MP
R2
R3
R4
R1
Ac 44
Ac b7
Ac B3
Ac B2
Ac BN
Ac Im
COTTON LINE

Fig. 3D

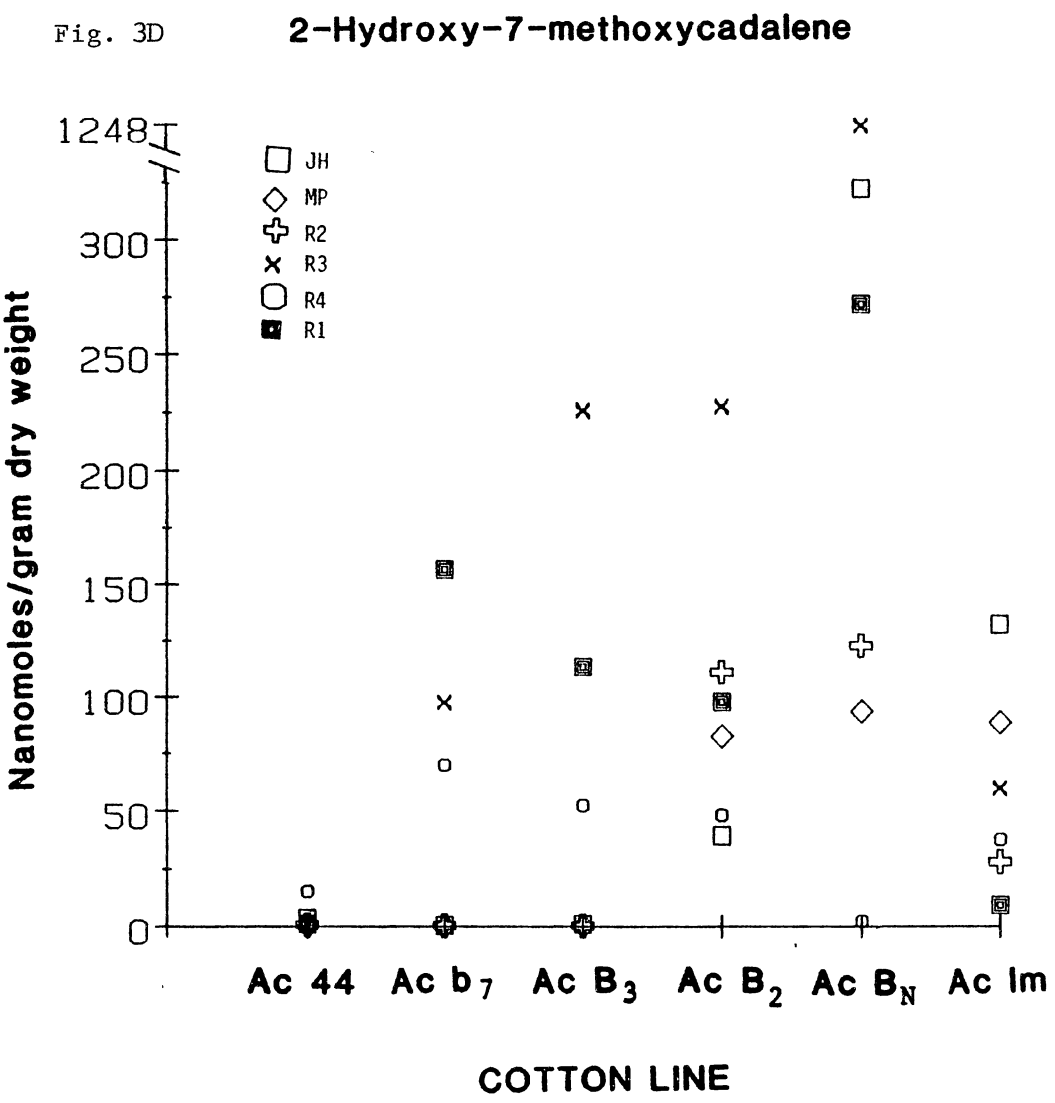

2-Hydroxy-7-methoxycadalene
1248
300
250
200
150
100
50
0
Nanomoles/gram dry weight
JH
MP
R2
R3
R4
R1
Ac 44
Ac b7
Ac B3
Ac B2
Ac BN
Ac Im
COTTON LINE

Figure 4, A-D. Average final Xcm population densities versus phytoalexin production in six cotton lines inoculated with each of the six strains of Xcm, a) Lacinilene C, b) 2,7-dihydroxycadalene, c) Lacinilene C 7-methyl ether, d) 2-hydroxy-7-methoxycadalene. Each symbol designates the cotton line listed on the graph. J= mutant JH, M= mutant MP, 1, 2, 3, 4= race 1, 2, 3, 4 respectively.

All points to the left of dashed lines are from compatible interactions; points to the right are from incompatible interactions.

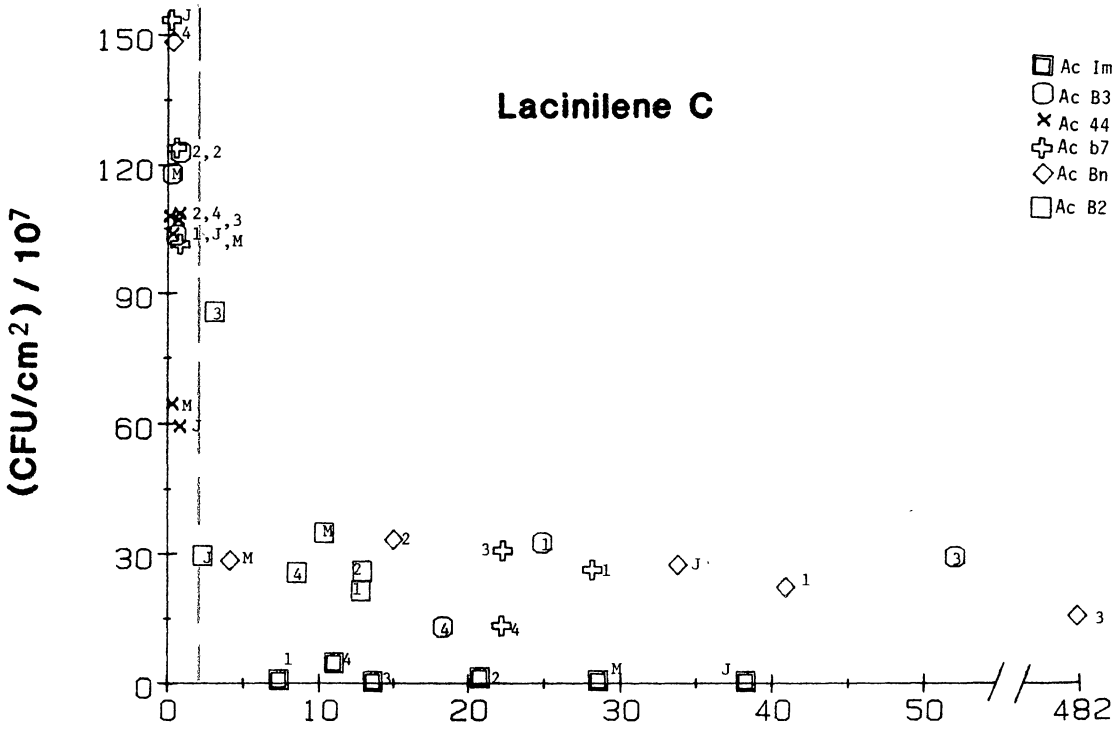

Lacinilene C
Ac Im
Ac B3
Ac 44
Ac b7
Ac Bn
Ac B2
(CFU/cm2) / 10^7
150
120
90
60
30
0
0
10
20
30
40
50
482
Nanomoles/gram dry weight

Fig. 4A

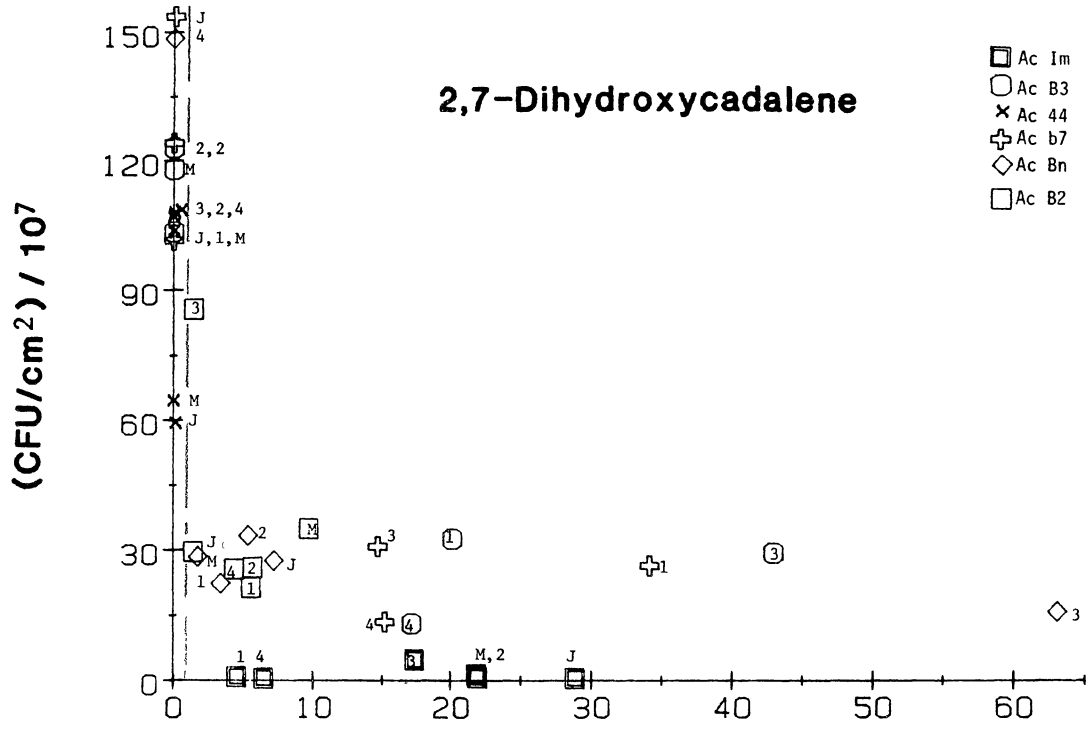

2,7-Dihydroxycadalene
Ac Im
Ac B3
Ac 44
Ac b7
Ac Bn
Ac B2
(CFU/cm2) / 10^7
150
120
90
60
30
0
0
10
20
30
40
50
60
Nanomoles/gram dry weight

Fig. 4B

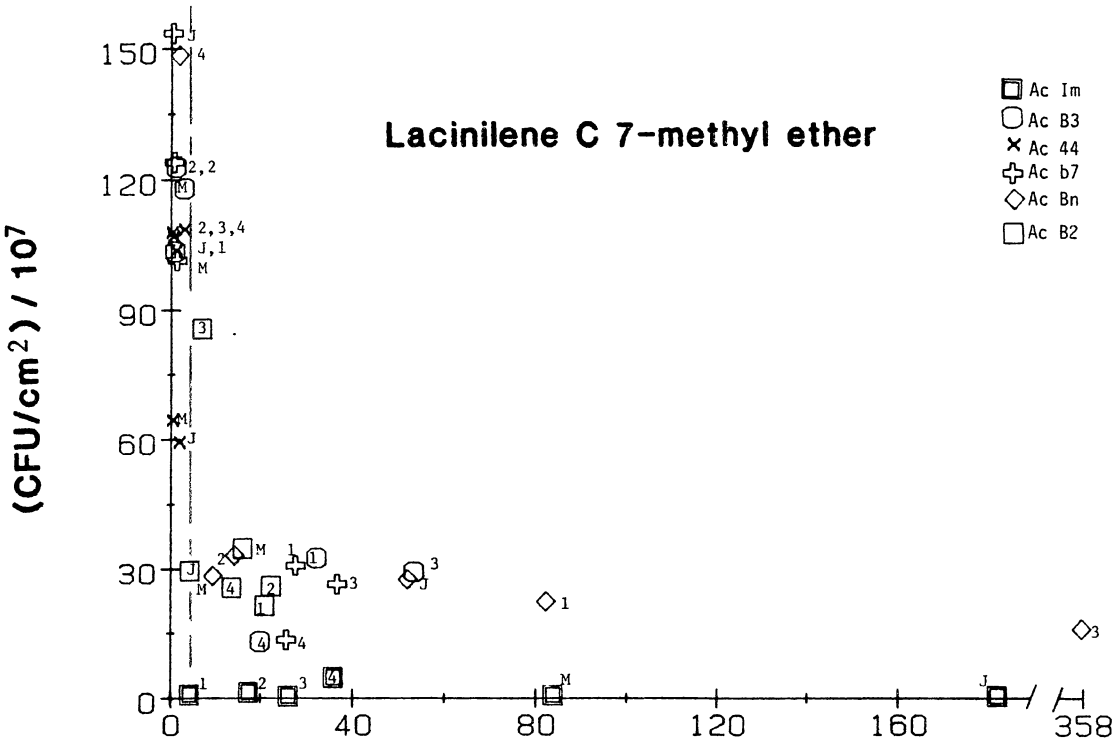

Lacinilene C 7-methyl ether
Ac Im
Ac B3
Ac 44
Ac b7
Ac Bn
Ac B2
(CFU/cm^2) / 10^7
150
120
90
60
30
0
0
40
80
120
160
358
Nanomoles/gram dry weight

Fig. 4C

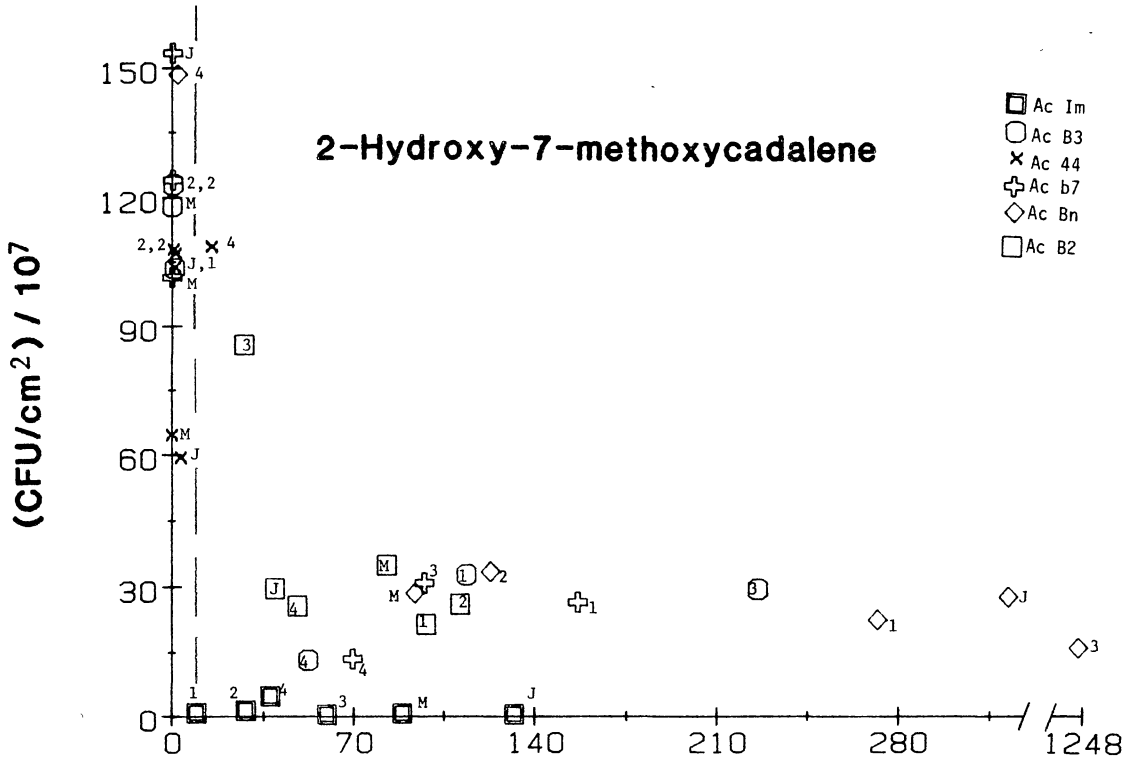
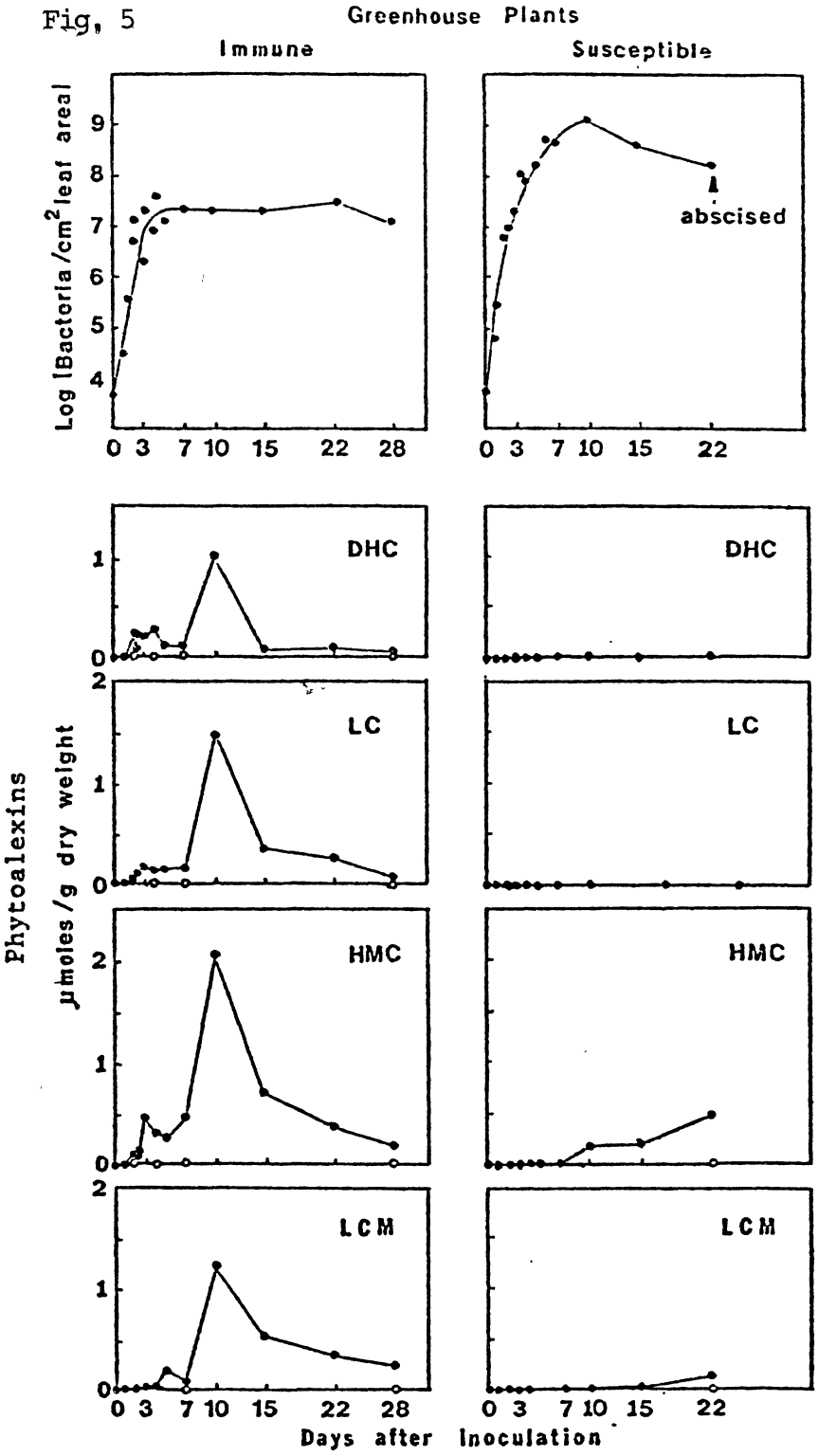

2-Hydroxy-7-methoxycadalene
(CFU/cm2) / 10^7
Nanomoles/gram dry weight
Ac Im
Ac B3
Ac 44
Ac b7
Ac Bn
Ac B2
150
120
90
60
30
0
0
70
140
210
280
1248

Fig. 4D

Figure 5.' Bacterial population densities and quantities of phytoalexins extracted by the acetonitrile method from leaves of congenic immune WbM (0.0) and susceptible WbM (4.0) cotton lines after infiltration with Xcm race 3 (adapted from Essenberg, personal communication).

Fig, 5


Greenhouse Plants
Immune
Susceptible
Log [Bactoria /cm² leaf areal
abscised
DHC
LC
HMC
LCM
Phytoalexins
µmoles /g dry weight
Days after Inoculation

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VITA 2

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Candidate for the Degree of

Master of Science

Thesis: PHYTOALEXIN PRODUCTION IN CONGENIC COTTON LINES CHALLENGED WITH DIFFERENT RACES OF XANTHOMONAS CAMPESTRIS PV. MALVACEARUM

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