Identification and Effects on *Xanthomonas campestris* pv. *malvacearum* of Two Phytoalexins from Leaves and Cotyledons of Resistant Cotton

Margaret Essenberg, Mary d'Arcy Doherty, Betty K. Hamilton, Veronica T. Henning, Ellen C. Cover, Steve J. McFaul, and William M. Johnson

First six authors: Department of Biochemistry, Oklahoma State University, Stillwater 74078.
Seventh author: Langston University Research Program, Langston University, Langston, OK 73050, and Department of Plant Pathology, Oklahoma State University, Stillwater 74078.
Oklahoma Agricultural Experiment Station Journal Series Article J-3922 and Langston University Journal Series Article 81-4.
We are grateful to Paul B. Grover, Marlece A. Ebbesen, Lisa C. Klaffenbach, Tracy L. Brown, and Jane A. Ramberg for excellent technical assistance; to G. A. Greenblatt for advice on purification; to R. D. Stipanovic for samples of compounds 1–3 from cotton plants; to Peter W. Jeffs for a sample of synthetic lacinilene C 7-methyl ether and for advice on the lanthanide shift experiment; to B. A. Halley, G. R. Waller, R. D. Stipanovic, and A. A. Bell for assistance in interpretation of spectra; to D. R. DiFeo, Jr. and R. M. Caprioli for low-resolution mass spectra; to N. B. Perreira for the high-resolution mass spectrum; to S. O. Sigle for proton magnetic resonance spectra; and to M. G. Rockley for infrared spectra. We acknowledge support by a grant from the Herman Frasch Foundation and by U.S. Department of Agriculture Science and Education Competitive Research Grant 5901-0410-9-0236-0 and Cooperative Research Grant 5460-7. We acknowledge the partial support of the National Science Foundation for funds to purchase the XL(100) nuclear magnetic resonance spectrometer (Grant GP 17641) and the FT (Fourier Transform) accessory (Grant CHE 76-05571).
Accepted for publication 22 December 1981.

ABSTRACT

Essenberg, M., Doherty, M. d'A., Hamilton, B. K., Henning, V. T., Cover, E. C., McFaul, S. J., and Johnson, W. M. 1982. Identification and effects on *Xanthomonas campestris* pv. *malvacearum* of two phytoalexins from leaves and cotyledons of resistant cotton. Phytopathology 72:1349-1356.

Two compounds that inhibit growth of *Xanthomonas campestris* pv. *malvacearum* were isolated from leaves and cotyledons of inoculated bacterial blight-resistant lines of cotton (*Gossypium hirsutum*). Chromatographic and spectral properties indicated that they are 2,7-dihydroxycadalene and its oxidation product, the yellow fluorescent compound lacinilene C. Extracts of inoculated blight-susceptible or uninoculated blight-resistant leaves contained much lower amounts of both compounds. 2,7-Dihydroxycadalene at 0.35 mM caused a 50% reduction in the number of bacterial generations in a liquid culture bioassay. Preparations of lacinilene C from plants of resistant cotton lines WbM(0.0) and Im 216 were optically active, but exhibited ellipticities of opposite signs; they caused 50% inhibition at approximately 0.4 mM and 1.5 mM, respectively. The amounts of 2,7-dihydroxycadalene and lacinilene C extracted from inoculated resistant leaves were approximately one-tenth of the amounts required to account for the observed inhibition of *X. campestris* pv. *malvacearum*, if those compounds were uniformly distributed throughout the tissue water.

Additional key words: bacterial blight of cotton, hypersensitive response, 2,7-dihydroxycadalene, lacinilene C.

In leaves of cotton plants resistant to bacterial blight, single cells of the blight pathogen *Xanthomonas campestris* pv. *malvacearum* (Smith 1901) Dye 1978b (*X. malvacearum*) can establish colonies (7). The bacteria multiply logarithmically within the intercellular spaces of the leaf for 3–4 days and then are inhibited. Results of our earlier work suggested that the inhibition of each bacterial colony is the result of a local resistant response (7) that produces a small bacteriostatic zone around the colony (8).

The objective of the present study was to identify factors responsible for that bacteriostasis.

MATERIALS AND METHODS

Cotton lines. Acala 44 (Ac 44) possesses no major genes for resistance to *X. campestris* pv. *malvacearum* and is susceptible to all 18 known races of this pathogen. The highly resistant line Immune 216 (Im 216) possesses homozygous resistance, including the major resistance genes B_2, B_3, and b_7 on a polygenic background (1,5,6). It is immune to bacterial blight; ie, it is resistant to all known races of *X. campestris* pv. *malvacearum* and shows no macroscopically visible symptoms of natural infection under field conditions.

Near-isogenic blight-resistant (WbM(0.0)) and blight-susceptible (WbM(4.0)) lines were derived from resistant cultivar Westburn M, which was developed by the Oklahoma Agricultural Experiment Station by a complex series of crosses and selections (mimeographed description available from the first author or from the Agronomy Department, Oklahoma State University, Stillwater 74078). The resistance gene content of WbM(0.0) has not yet been determined, but probably it includes most if not all of the genes present in Im 216, as well as possibly B_N. Westburn M does not possess homozygous immunity to bacterial blight. Reselections within Westburn M for resistance and susceptibility to a mixture of races 1, 2, 4, and 10 of *X. campestris* pv. *malvacearum* were made by rigorously screening large numbers of progeny rows derived from individual selfed plants. Progeny not showing full resistance or full susceptibility were discarded. Remaining plants were selfed (fertilized by enforced self-pollination) and the seed from each row was bulked. The resistant line used in this study, WbM(0.0), was the result of four such generations of screening and selfing. The susceptible line, WbM(4.0), was the result of three such generations. Only one of 14 plants and none of 12 plants, respectively, failed the screening tests for full resistance and full susceptibility in the final generations.

Acid-delinted seeds were planted in Jiffy Mix Plus, a commercial mixture of peat moss and vermiculite (Jiffy Products of America, 250 Town Road, West Chicago, IL 60185) in 20 × 32-cm flats. Plants were grown in controlled environment chambers with 12 hr, 30 C days under incandescent plus fluorescent light, 1 to 2×10^4 lux at plant level and 12 hr, 16 C nights. Cotyledons of 2- to 3-wk-old plants or young, fully expanded foliage leaves of 4- to 8-wk-old plants were inoculated.

Bacteria. We used *X. campestris* pv. *malvacearum* race 1 and a highly aggressive strain that was isolated in 1978 from cotton plants in a field at Altus, OK, and identified as race 3 by using a standard

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0031-949X/82/10134908/$03.00/0

set of differentials (13). Stock cultures were maintained on slants of potato-carrot-dextrose agar medium as previously described (7) except that yeast extract was omitted from the medium.

Inoculation. Difco nutrient broth (0.5%) was inoculated with bacteria from the agar slants and incubated on a reciprocal shaker at 30 C for 15 hr. The cultures were centrifuged for 10 min at 1,700 *g* at room temperature and the pellet was resuspended in sterile saturated (0.14 mg/ml) calcium carbonate solution at $1–5 \times 10^6$ colony forming units (cfu) per milliliter. The exact bacterial concentration was determined by dilution and plating on Difco nutrient agar. The lower epidermis of each cotyledon was pricked in four places. A bacterial suspension prepared as described above was loaded into a syringe fitted with a 5-mm length of soft rubber tubing (instead of a needle) that was used to infiltrate the entire intercellular space of each cotyledon through the four small wounds. Leaves were inoculated by vacuum infiltration as previously described (7).

Extraction and purification of inhibitory compounds. *Solvents.* Chloroform, cyclohexane, ethyl acetate, hexane, methanol, and water were either the "glass distilled" grade from Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442, or were "HPLC Reagent" or "Photrex" grade from J. T. Baker Chemical Co., Phillipsburg, NJ 08865.

Method 1. Cotyledons of 50–200 plants (20–80 g fresh weight) were harvested 3 days after inoculation, weighed, and rinsed with deionized water. All subsequent work was carried out under subdued light or under ultraviolet-deficient illumination from General Electric F06T12/GO gold fluorescent lamps. A razor blade was used to cut each cotyledon into distal and proximal halves with a cut perpendicular to the midvein. Extracellular spaces were infiltrated and extracted with 95% ethanol by the procedure of Klement (18). The extract was evaporated to dryness in vacuo at 30 C. The residue was extracted four times with methanol (1 ml/30 g fresh weight of cotyledons) and then seven times with ethyl acetate (2 ml/30 g fresh weight of cotyledons). The methanol and ethyl acetate extracts were combined and centrifuged for 10 min at 1,700 *g* to remove the precipitate. The supernatant fluid was concentrated in vacuo at 30 C to approximately 1 ml and centrifuged as before.

The supernatant fluid was subjected to thin-layer chromatography (TLC) on 250-μm-thick silica gel G plates (Analtech, Inc., Newark, DE 19711) that had been pre-run in methanol and activated for 30 min at 110 C in vacuo. The chromatograms were developed with hexane/ethyl acetate/methanol (60:40:1, v/v) (TLC system 1). Bands of silica gel were scraped from plates with a razor blade and adsorbed substances were eluted with ethyl acetate. The eluates were filtered through glass wool and evaporated with a nitrogen stream at 30 C. The residue was bioassayed for inhibitory activity.

Inhibitory fractions were dissolved in one or two drops of ethyl acetate and 1.0 ml of cyclohexane was added. The sample was subjected to high-pressure liquid chromatography (HPLC) on a Waters model 6000A liquid chromatograph (Waters Associates, Inc., Milford, MA 01757) fitted with a Waters 3.9 mm × 30 cm μPorasil column.

The column was eluted with the following step gradient of 0–100% (v/v) chloroform in cyclohexane: 0% (30 ml), 50% (90 ml), 80% (80 ml), and 100% (20 ml) at a flow rate of 1.0 ml/min.

The inhibitory fractions were further purified separately by HPLC on a Waters 3.9 × 30-cm μBondapak CN column. Compound 1 was injected in, and eluted with, methanol-water (30:70, v/v). Compound 2 was injected in, and eluted with, methanol-water (20:80, v/v) adjusted to pH 5 with HCl.

Method 2. This method is, with a few modifications, the procedure developed by Beier and Greenblatt (2) of Texas A&M University. Leaves or cotyledons were harvested, lyophilized, and ground to powder with a mortar and pestle. The powder was extracted with methanol-water (30:70, v/v, 400 ml/g dry weight of tissue) by stirring for 10 min at room temperature. The extract was filtered through Whatman No. 4 filter paper and the residue was discarded. Sodium chloride (13 g/100 ml) was added to the filtrate and the filtrate was extracted three times with 0.5 volume of hexane-ethyl acetate (9:1, v/v). The combined extracts were rotary evaporated just to dryness and dissolved in 2 ml of methanol-water (85:15, v/v). This solution was passed through a Waters SEP-PAK C_{18} cartridge that had been preconditioned by passing 2 ml of methanol and then 5 ml of water through it. The sample was eluted from the SEP-PAK with 5 ml of methanol-water (85:15, v/v). This eluate and the sample flow-through were combined and extracted four times with 0.25 volume of chloroform. The chloroform extracts were combined, evaporated to dryness with a nitrogen stream at 30 C, and dissolved in 0.4–1.0 ml of methanol-water (25:75, v/v). This solution was subjected to HPLC on a Waters 3.9 mm × 30 cm μBondapak CN column. Substances were eluted with 36 ml of methanol-water (25:75, v/v) followed by 46 ml of methanol-water (40:60, v/v). Ultraviolet absorption spectra of the fractions were taken in these solvents. Samples to be analyzed in other solvents or to be subjected to bioassay were extracted four times with 0.25 volume of chloroform. The four extracts were combined, and the chloroform was evaporated with a nitrogen stream at 30 C. Purified samples were stored either dry or in ethyl acetate solution under nitrogen in the dark at −10 C.

Other thin-layer chromatographic systems. TLC system 2 was hexane-diethyl ether (1:1, v/v) on silica gel G, and TLC system 3 was benzene-methanol (19:1) on silica gel G. The location of compound 1 on TLC plates after chromatography was determined by heating the plates for 10 min at 100 C. This treatment oxidized compound 1 to compound 2, which can be detected by its yellow-green fluorescence under ultraviolet light (365 nm).

Methylation of compound 2. A 1.3-μmole sample of compound 2 was methylated with approximately 10 μmoles of diazomethane in 3.75 ml of methanol-diethyl ether (1:4, v/v) in darkness under nitrogen for 7 hr at room temperature. Progress of the reaction was followed by TLC of 5-μl aliquots in TLC system 2. Remaining diazomethane and solvents were evaporated with a nitrogen stream. The residue was subjected to HPLC on a Dupont 4.6 mm × 25-cm Zorbax-ODS column and was eluted with 32 ml of methanol-water (60:40, v/v) followed by methanol-water (67:33, v/v). Elution volume of the methylated compound was 40 ml. It was extracted with chloroform and dried under a nitrogen stream.

Analytical methods. Molar concentrations of purified compounds were estimated from their ultraviolet absorbances in ethanol, methanol, or aqueous methanol at λ_{max} by using the molar extinction coefficient determined with the same solvent.

Inhibitory activities of the purified compounds relative to *X. malvacearum* were determined as follows: Aliquots ≤0.2 ml were transferred in duplicate to 6 mm × 5-cm test tubes, and the solvent was evaporated with a nitrogen stream at 30 C. Tubes that received solvent only were also prepared. A culture of *X. campestris* pv. *malvacearum* growing logarithmically in 0.8% Difco nutrient broth or, where specified, in Ornston-Stanier salts medium (21) plus 10 mM D-glucose was diluted in the same medium to 5×10^3 cfu/ml, and 20 μl of this suspension was added to each sample tube and also to duplicate clean tubes that served as uninhibited-culture controls. The tubes were agitated for 16 or 48 hr, depending on which medium was used, in a humid atmosphere in darkness at 30 C. Bacterial concentrations at the beginning and end of this incubation were determined by the dilution-plate technique. Each dilution was plated in duplicate. The number of generations (n) that each culture had grown was estimated from the equation:

$$n = \frac{\log [(\text{cfu/ml at end})/(\text{cfu/ml at beginning})].}{\log 2}$$

For an uninhibited culture n was typically seven to eight generations. Values of n from duplicate bioassays were averaged. Bioassay results were expressed as n/n_c, the number of bacterial generations achieved in the presence of a sample compound divided by the number of generations of an uninhibited control culture. Thus, a value of 1.00 indicates no inhibition of bacterial growth, 0.00 indicates a bacteriostatic effect, and values less than zero indicate bactericidal effects.

Spectral studies on compounds 1 and 2 were carried out by analyzing the amorphous solids resulting from extraction and purification method 1 or 2. Samples were weighed in 6 mm × 5-cm

test tubes on a Mettler Micro Balance (Mettler Instrument Co., Hightstown, NJ 08520). Molar absorptivities were determined with 458 μg of compound 1 and 171 μg of compound 2. These samples were dissolved in ethanol, and their ultraviolet and visible-light absorption spectra were determined with a Hitachi 100-80 computerized spectrophotometer (Hitachi Scientific Instruments, Mountain View, CA 94043). Sample solutions were made alkaline by addition of 0.01 to 0.02 volumes of aqueous 1.0 N NaOH. For 70 eV electron impact mass spectra, a Finnigan model 3200 mass spectrometer fitted with a model 6000 data system (Finnigan/MAT, Sunnyvale, CA 94086) was used. For chemical ionization mass spectra a Finnigan model 3300 mass spectrometer fitted with a model 6100 data system was used with 1 torr of methane. The high-resolution mass spectrum was determined on 359 μg of compound 1 with a Consolidated Electrodynamics model 21-110B spectrometer (Consolidated Electrodynamics Corp., Division of DuPont, Monrovia, CA 91016) linked to a Data General Nova System 3/12 (Data General, Southboro, MA 01772). Proton magnetic resonance (pmr) spectra (100 MHz) of 151 μg of compound 1 and 393 μg of compound 2 were determined with a Varian HR XL-100-15 spectrometer (Varian Associates, Aerograph Div., Walnut Creek, CA 94597) interfaced to a Nicolet TT-100 Fourier transform accessory (Nicolet Technology Corp., Mountain View, CA 94041). Compound 1 and compound 2 solutions were 0.05–0.2% in approximately 25 μl of deuteriochloroform containing a trace of tetramethylsilane as internal reference and were sealed in vacuo in 1.5–1.8 × 90-mm melting-point capillaries. Seven thousand scans were performed. For the lanthanide shift experiment, the sample weights given in Table 2 were dissolved in 0.1 ml of deuteriochloroform with a trace of tetramethylsilane and were transferred to a nuclear magnetic resonance tube with a sample chamber 5 mm in diameter. Samples were held at 10 C under nitrogen. For the methyl derivative of compound 2, 500–600 scans were performed. For the synthetic compound, single scans were performed. The infrared spectrum was of 90 μg of compound 2 deposited onto a sodium chloride window by evaporation of a solution and was determined with a Digilab FTS-2DC spectrometer (Digilab, Cambridge, MA 02139) interfaced to a Data General Nova System 3/12. Circular dichroism spectra were determined with a Cary 61 spectropolarimeter (Cary, Monrovia, CA 91016).

RESULTS

Extraction and thin-layer chromatography. Since *X. campestris* pv. *malvacearum* grows and becomes inhibited within the intercellular spaces of immune leaves and cotyledons, any effective phytoalexins must be extracellular. Our purification method 1 was a modification of the procedure of Klement (18) for extraction of compounds with water from intercellular spaces of cotyledons. Although we performed the extraction with 95% ethanol, which probably disrupts host membranes and extracts some cellular solutes as well, extracted amounts of photosynthetic pigments were much lower than in whole-tissue extracts obtained by homogenization. Cotyledons were used instead of leaves because the many anastomosing veins of leaves prevented effective use of Klement's extraction method.

Cotyledons of Im 216 and Ac 44 were harvested 4 days after inoculation with race 1 or with sterile inoculation medium (saturated calcium carbonate solution). At this time, bacterial multiplication had stopped in Im 216 cotyledons, but it still continued in the cotyledons of susceptible Ac 44 (Fig. 1). Thus, phytoalexins that were accessible to the bacteria would be expected to be present in higher concentrations in inoculated Im 216 cotyledons than in inoculated Ac 44 cotyledons. Intercellular extracts prepared by method 1 were subjected to TLC, system 1. Illumination of the chromatogram with ultraviolet light revealed many fluorescent bands from the extract of inoculated Im 216 cotyledons (Fig. 2). The most brilliantly fluorescent band was yellow-green and chromatographed with an R_f of approximately 0.4. Extracts of inoculated Ac 44 cotyledons (Fig. 2), or of the Im 216 control cotyledons, exhibited fewer and fainter fluorescent bands except in the region of R_f 0–0.05, where the three extracts were similar. Yellow-green fluorescence at R_f 0.4 was faint in the

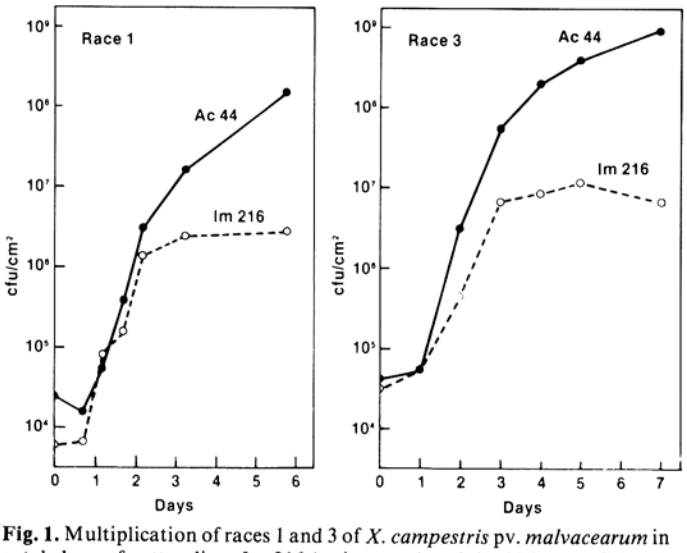

Fig. 1. Multiplication of races 1 and 3 of *X. campestris* pv. *malvacearum* in cotyledons of cotton lines Im 216 (resistant, o) and Ac 44 (susceptible, •). Inoculum concentrations were 1.6×10^6 cfu/ml (race 1) and 3.3×10^6 cfu/ml (race 3).

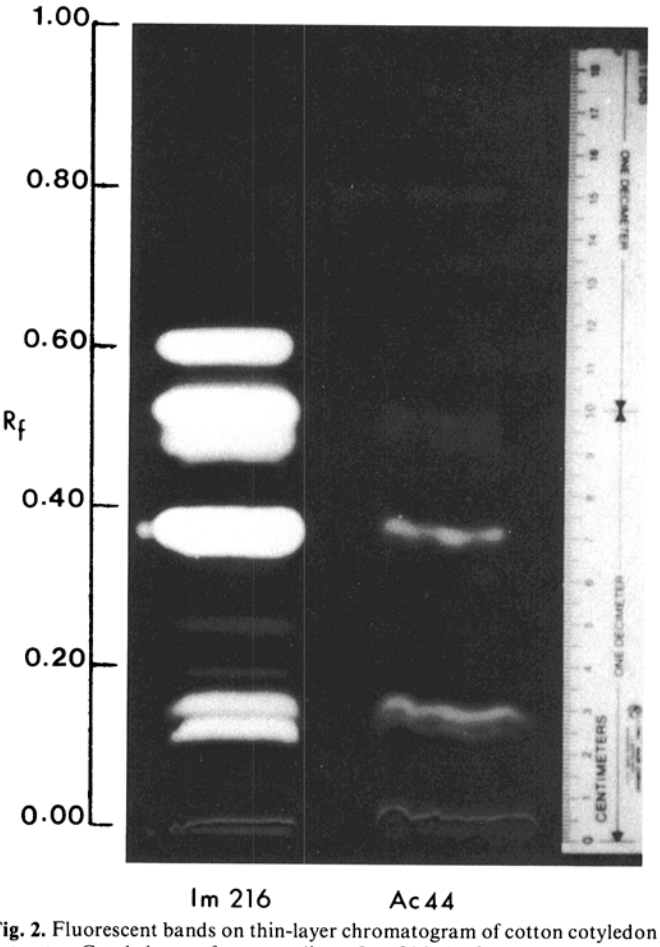

Fig. 2. Fluorescent bands on thin-layer chromatogram of cotton cotyledon extracts. Cotyledons of cotton lines Im 216 (resistant) and Ac 44 (susceptible) were infiltrated with a suspension of 10^6 cfu/ml of *X. campestris* pv. *malvacearum* race 1. Four days later cotyledons were harvested. Extracts were prepared and chromatographed by method 1. Shown above are bands that fluoresced when the chromatogram was illuminated with ultraviolet light (365 nm). Fluorescence from the band of $R_f = 0.20$ and all bands of $R_f > 0.35$ was yellow-green. Fluorescence from other bands was blue.

extract of inoculated Ac 44 and undetectable in the Im 216 control extract.

Entire thin-layer chromatograms were divided into fractions, which were eluted and bioassayed for inhibitory activity to *X. campestris* pv. *malvacearum*. Typical results are shown in Fig. 3. The chromatogram of the extract from inoculated Im 216 had strong inhibitory activity in the fractions near the origin and in the fraction that contained the bright yellow-green fluorescence. The chromatogram of extract from inoculated Ac 44 was also strongly inhibitory in the fractions near the origin, but was only weakly inhibitory in all other fractions. Two bright-blue fluorescent bands at R_f 0.15 and 0.17 from both extracts (Fig. 2) were only weakly inhibitory.

There was a positive correlation between yellow-green fluorescence and inhibitory activity in various preparations. The most active preparations were obtained from Im 216 plants that had been inoculated with cultures of *X. campestris* pv. *malvacearum* highly virulent to Ac 44. In a screening test, Im 216 was inoculated with two to four isolates each of races 1, 2, 3, and 4, to all of which Im 216 is resistant. An isolate of race 3 elicited the most yellow-green fluorescent material and the most inhibitory activity. Therefore, this strain was used in the remainder of the work reported here.

The central portion of a thin-layer chromatogram was divided into fractions for bioassay (Fig. 4). The greatest inhibitory activity was in fraction 4, a band of slightly higher R_f than the yellow-green fluorescent band (fraction 3). Very little inhibitory activity was detected in corresponding fractions from an extract of inoculated Ac 44.

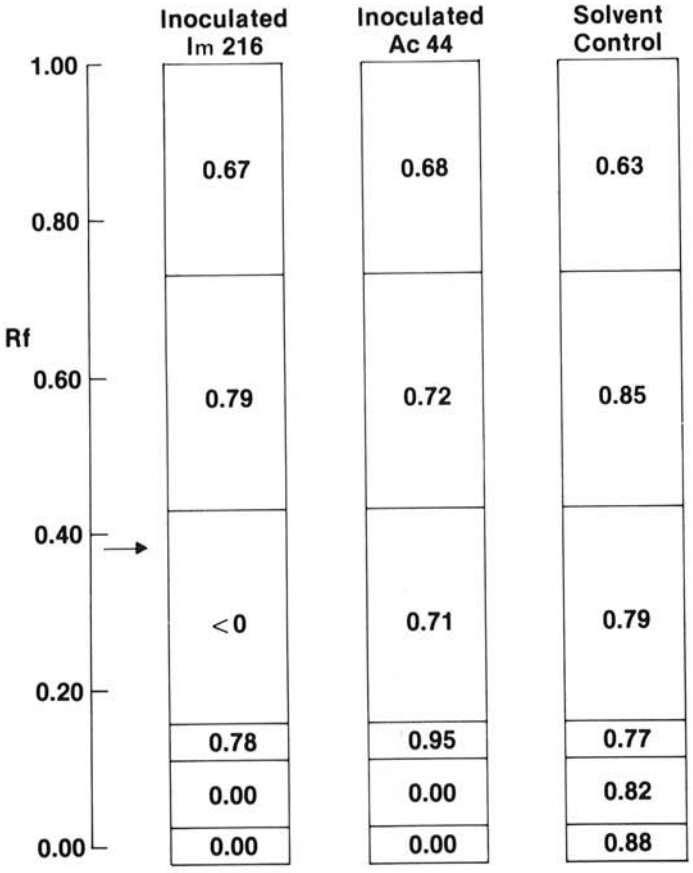

Fig. 3. Effects of fractions eluted from a thin-layer chromatogram of cotton cotyledon extracts upon growth of *X. campestris* pv. *malvacearum*. A chromatogram similar to the one in Fig. 2 was divided into the fractions indicated, and these were eluted with ethyl acetate. Material in each fraction derived from 0.1 g (fresh weight) of cotyledons was bioassayed with race 1. Equal areas of a portion of the chromatogram on which no extract had been applied were eluted and bioassayed for the solvent control. The numbers in the figure are n/n_c ratios (see Materials and Methods). The arrow at R_f 0.38 indicates the position of the brightest yellow-green fluorescent band.

High-pressure liquid chromatographic purification of compound 1. Fraction 4 was subjected to HPLC on μPorosil, and aliquots of the eluted fractions containing materials from 0.6 g (fresh weight) of cotyledons were bioassayed. The only HPLC fraction from TLC fraction 4 that showed inhibitory activity (n/n_c less than 0.6) in three individual chromatographic runs using extracts from two different inoculations was the largest 340-nm-absorbing peak that eluted during the 50% chloroform step. The inhibitory substance in this peak is referred to as compound 1. Upon rechromatography in the same system, the fraction containing the principal 340-nm-absorbing peak retained inhibitory activity. An equivalent fraction from a control extract of uninoculated Im 216 showed no 340-nm absorbance and no inhibitory activity.

At this stage of purification compound 1 exhibited ultraviolet and fluorescence spectra which were later found to be characteristic of the pure compound. However, pmr spectra indicated the presence of impurities. Adequate purification was realized by further chromatography on μBondapak CN as described above (elution volume, 27–29 ml).

High-pressure liquid chromatographic purification of compound 2. The yellow-green fluorescent compound 2 of TLC fraction 3 was eluted from μPorosil by 80% chloroform. All fractions were bioassayed using aliquots containing material from 0.6 g (fresh weight) of cotyledons, but none were inhibitory at this concentration. The lowest n/n_c value was 0.7. Later work indicated that in this bioassay the concentration of compound 2 was less than 1 mM, which is too dilute for detecting inhibitory activity. The compound was further purified by chromatography on μBondapak CN (elution volume, 19–20 ml).

Identification of compound 2 as lacinilene C. Chromatographic and spectral characteristics of compound 2 were similar to those reported by others (26–28) for lacinilene C (1,7-dihydroxy-4-

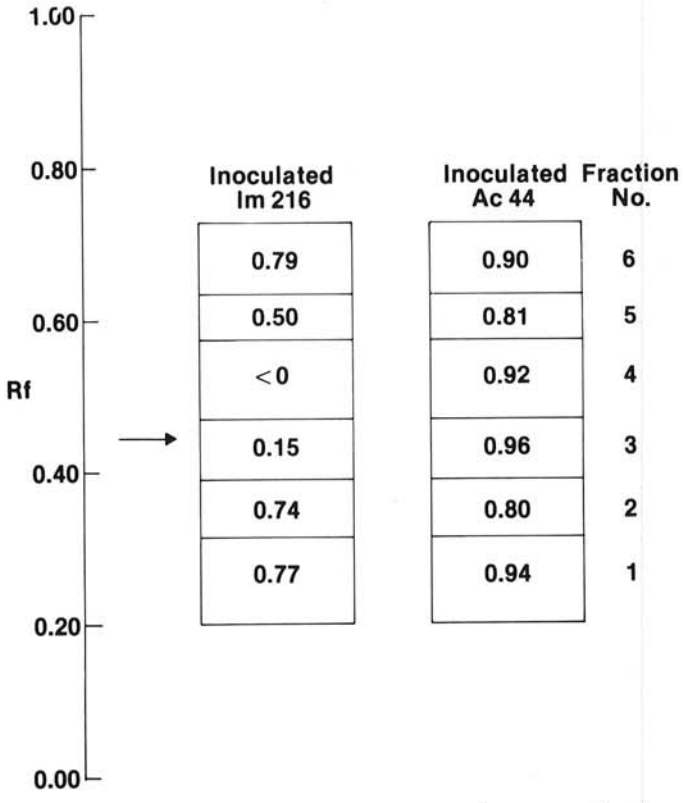

Fig. 4. Effects upon growth of *X. campestris* pv. *malvacearum* of fractions eluted from a thin-layer chromatogram of cotton cotyledon extracts. The procedure was as described in Fig. 3 except that the inoculum was 5×10^6 cfu/ml of race 3, cotyledons were harvested 3 days after inoculation, and the quantities used for bioassay were derived from 0.3 g fresh weight. The arrow at R_f 0.44 indicates the position of the brightest yellow-green fluorescent band.

isopropyl-1,6-dimethyl-2(1*H*)-naphthalenone, $C_{15}H_{18}O_3$, shown in Fig. 5). Compound 2 co-chromatographed in TLC systems 2 and 3 with authentic lacinilene C. The proton magnetic resonance spectrum of compound 2 provided evidence for presence of an isopropyl group in which the methyl groups are diastereotopic (pair of overlapping doublets centered at δ 1.27 and 1.29, each with J = 6.7 Hz, total 6H, and an apparent quintuplet (expected: septet) at δ 3.23, J = 6.7 Hz, 1H), two methyl groups (δ 1.52, singlet, 3H, and δ 2.28, singlet, 3H), a vinyl hydrogen atom (δ 6.04, singlet, 1H), and two aromatic hydrogen atoms (δ 7.17, singlet, 1H, and δ 7.37, singlet, 1H). The infrared spectrum indicated hydroxyl protons (broad signal centered at 3,300 cm^{-1}) and a conjugated ketone (1,658 and 1,610 cm^{-1}). The ultraviolet absorption maxima and molar absorptivities in ethanol were 223 nm (ϵ 11,200), 252 (12,100), 290 (sh), 343 (6,500), and 375 (sh, 5,000). A large bathochromic shift occurred upon addition of sodium hydroxide: 245 (11,400), 274 (9,400), 307 (sh, 2,700), and 443 (11,800). The 70 eV electron impact mass spectral ion intensities at m/e above 150 were: 246 (19%), 218 (20%), 203 (40%), 188 (14%), 176 (17%), 175 (100%), and 161 (33%). The chemical ionization spectrum obtained with methane provided further evidence that the molecular weight is 246, as it contained peaks at m/e 287 $(M + C_3H_5)^+$ (3%), 275 $(M + C_2H_5)^+$ (2%), and 247 $(M + 1)^+$ (79%) (9). The base peak at 229 $[(M + 1) - 18]^+$ is probably due to the facile loss of water by alcohols in chemical ionization spectra (9). On the basis of its spectral and chromatographic properties, we conclude that our compound 2 is lacinilene C.

Optical activity of lacinilene C. Our preparations of lacinilene C from inoculated resistant cotton cotyledons were optically active. A sample extracted from Im 216 cotyledons two days after inoculation and analyzed 12 days after extraction exhibited the following circular dichroic parameters: $\lambda^+ = 377$ nm, $[\theta]_{max} = +1.93 \times 10^4$; $\lambda^0 = 354$ nm; $\lambda^- = 331$ nm, $[\theta]_{max} = -2.11 \times 10^4$; $\lambda^0 = 271$ nm; $\lambda^+ = 255$ nm, $[\theta]_{max} = +1.70 \times 10^4$. Surprisingly, lacinilene C from WbM(0.0) leaves had ellipticities of opposite signs. A sample extracted 10 days after inoculation and analyzed 5 days after extraction exhibited parameters: λ^- 377 nm, $[\theta]_{max} = -1.18 \times 10^4$; $\lambda^0 = 354$ nm; $\lambda^+ = 331$ nm, $[\theta]_{max} = +1.48 \times 10^4$; $\lambda^0 = 269$ nm; $\lambda^- = 255$ nm, $[\theta]_{max} = -9.4 \times 10^3$. Preparations of lacinilene C racemized slowly upon storage. It is not known which of our preparations was of the same configuration as the lacinilene C of positive $[\alpha]_D$ isolated from field-dried cotton bracts by Stipanovic et al (25).

Confirmation of the structure of lacinilene C. Methylation of compound 2 gave a product which co-chromatographed in TLC systems 2 and 3 with authentic lacinilene C 7-methyl ether (structure 3, Fig. 5). A complete absence of bathochromic shift in its ultraviolet absorption spectrum upon addition of sodium hydroxide indicated that the sample was not contaminated with lacinilene C 2-methyl ether. Proton magnetic resonance spectra of the methylated compound 2 and of a sample of chemically synthesized lacinilene C 7-methyl ether (1-hydroxy-7-methoxy-4-isopropyl-1,6-dimethyl-2(1H)-naphthalenone (15) were very similar (Table 1). The lanthanide shift reagent, tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octane-4,6-dionato)europium ($Eu(fod)_3$), was added to the methylated compound 2 and to the synthetic lacinilene C 7-methyl ether, and pmr spectra were recorded. The relative shifts in proton signals (Table 2) were consistent with complexation of the europium at the 1-hydroxyl group and provided further evidence for structure 3 (Fig. 5), as discussed by Jeffs and Lynn (14). In experiments of this kind, the shift in each proton signal increases with increasing ratio of shift reagent to sample. However, in any single spectrum, shifts of the proton

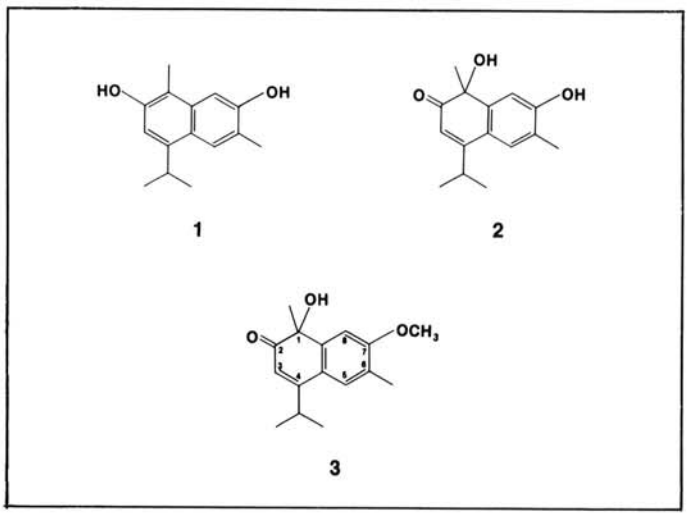

Fig. 5. Structures of 2,7-dihydroxycadalene (1), lacinilene C (2), and lacinilene C 7-methyl ether (3). It is proposed that compound 1, compound 2, and the methyl ether derivative of compound 2 have structures 1, 2, and 3, respectively.

TABLE 1. Comparison of the proton magnetic resonance spectra of the methyl ether derivative of compound 2 and of synthetic lacinilene C 7-methyl ether (1-hydroxy-7-methoxy-4-isopropyl-1,6-dimethyl-2(1H)-naphthalenone, compound 3)

	Chemical shift (ppm)							
	3-H	5-H	8-H	1-CH_3	6-CH_3	7-OCH_3	*i*-propyl H	*i*-propyl CH_3's
Methyl derivative of compound 2	6.03	7.37	7.21	1.54	2.25	3.93	3.23 (J = 6.8 Hz)	1.27, 1.28 (J = 6.7 Hz)
Synthetic compound 3	6.04	7.37	7.21	1.54	2.25	3.93	3.25 (J = 6.7 Hz)	1.27, 1.28 (J = 6.8 Hz)

TABLE 2. Shifts induced by $Eu(fod)_3$ in the proton magnetic resonance spectra of the methyl derivative of compound 2 and of synthetic 1-hydroxy-7-methoxy-4-isopropyl-1,6-dimethyl-2 (1H)-naphthalenone (compound 3)

	Induced shift in 3-H signal (Hz)	Induced shifts relative to induced shift in 3-H signal							
		3-H	5-H	8-H	1-CH_3	6-CH_3	7-OCH_3	*i*-propyl H	*i*-propyl CH_3's
Methyl derivative of compound 2									
0.06 mg + 0.066 mg $Eu(fod)_3$	+ 84	1.00	0.24	0.87	···[a]	0.11	0.16	0.22	0.25, 0.15
0.06 mg + 0.066 mg $Eu(fod)_3$ + 0.03 ml D_2O[b]	+ 44	1.00	0.25	0.89	1.28	0.12	0.18	0.16	0.25, 0.16
0.1 mg + 0.2 mg $Eu(fod)_3$	+532	1.00	0.24	0.92	1.42	0.11	0.17	0.30	0.26, 0.16
Synthetic compound 3									
2 mg + 0.2 mg $Eu(fod)_3$	+ 11	1.00	0.23	0.87	1.26	0.07	0.15	0.2	0.3, 0.3
2 mg + 0.4 mg $Eu(fod)_3$	+ 32	1.00	0.23	0.85	1.29	0.11	0.16	0.25	0.27, 0.19
2 mg + 0.6 mg $Eu(fod)_3$	+ 54	1.00	0.24	0.85	1.26	0.10	0.17	0.26	0.24, 0.16

[a] This signal was obscured by a signal from an impurity.
[b] Addition of D_2O decreased the induced shifts.

signals relative to each other are independent of this ratio and depend on their positions in the molecule relative to the europium atom. The relative shifts induced by $Eu(fod)_3$ in all proton signals of the methylated compound 2 agree with the relative shifts induced in proton signals of the synthetic lacinilene C 7-methyl ether (Table 2). We conclude that the two compounds are identical and, therefore, that compound 2 is lacinilene C (structure 2, Fig. 5).

Autoxidation of compound 1 to lacinilene C. Freshly purified compound 1 was evaporated to dryness and heated at 110 C for 45 min. An ultraviolet absorption spectrum of the resulting material was more complex than would be expected of a mixture of compounds 1 and 2. The mixture was chromatographed on μBondapak CN with methanol-water (40:60, v/v). Seven peaks were eluted, including one at the elution time characteristic of lacinilene C. The latter had the ultraviolet absorption spectrum of lacinilene C and co-chromatographed with authentic lacinilene C in TLC systems 2 and 3. Using the ultraviolet extinction coefficients for lacinilene C and compound 1 (see below), we estimate that 25 nmoles of lacinilene C and 64 nmoles of unreacted compound 1 were recovered after heating 149 nmoles of compound 1. Thus, lacinilene C is one of several products obtained by heating compound 1 in the presence of air.

Identification of compound 1 as 2,7-dihydroxycadalene. Chromatographic and spectral characteristics of compound 1 were similar to those reported by Stipanovic et al (25) for a precursor to lacinilene C which they isolated from cotton bracts. Their spectra indicated that the precursor is 2,7-dihydroxycadalene (4-isopropyl-1,6-dimethyl-2,7-naphthalenediol, $C_{15}H_{18}O_2$, shown in Fig. 5). Compound 1 cochromatographed in TLC systems 2 and 3 with a sample of the precursor.

The pmr spectrum of compound 1 indicated the presence of an isopropyl group (doublet centered at δ 1.34, $J = 6.8$ Hz, 6H, and an apparent quintuplet (expected: septet) at δ 3.65, $J = 6.8$ Hz, 1H), two methyl groups in nearly identical environments (singlets at δ 2.39, 3H, and δ 2.40, 3H), and three aromatic hydrogen atoms (δ 6.84, singlet, 1H, δ 7.18, singlet, 1H, and δ 7.80, singlet, 1H). Two broad, one-proton signals at δ 4.82 and 5.07 that decreased when D_2O was added to the sample were probably due to the phenolic protons. The electron impact mass spectral ion intensities at m/e above 150 were: 230 (80%), 216 (15%), 215 (100%), 201 (13%), and 200 (25%).

Our compound 1 has been more fully characterized than the lacinilene C precursor of Stipanovic et al (25). All results are consistent with the structure they proposed. The chemical ionization spectrum obtained with methane confirmed the molecular weight of 230, as it contained peaks at m/e 271 $(M + C_3H_5)^+$ (3%), 259 $(M + C_2H_5)^+$ (4%), and 231 $(M + 1)^+$ (100%). The high-resolution 70 eV electron impact mass spectrum confirmed the molecular formula of $C_{15}H_{18}O_2$ (calculated: m/e 230.1308, found: 230.1315) and indicated that the m/e 215 and 200 peaks resulted from losses of one and two methyl groups, respectively, which yielded $C_{14}H_{15}O_2$ (calculated: m/e 215.1072, found: 215.1093) and $C_{13}H_{12}O_2$ (calculated: m/e 200.08372, found: 200.0803). The ultraviolet absorption maxima and molar absorptivities in ethanol were: 218 nm (sh), 237 (ε 67,000), 292 (6,000), 304 (6,000), 318 (sh), and 333 (3,700). Addition of sodium hydroxide produced a bathochromic shift, indicative of phenolic groups: 226 nm (sh), 253 (64,000), 302, 315, and 346.

On the basis of its spectral characteristics and its autoxidation to lacinilene C, we conclude that compound 1 is 2,7-dihydroxycadalene (structure 1, Fig. 5).

Quantities extracted from cotyledons and leaves. Table 3 shows the quantities of dihydroxycadalene and lacinilene C that were obtained by method 1. Neither compound was detectable in the extracts of inoculated susceptible or uninoculated resistant cotyledons.

The resistant cotton line Im 216 and the susceptible line Ac 44 used in the work described above are not closely related lines. An experiment was also conducted with near-isogenic susceptible and resistant lines WbM(4.0) and WbM(0.0). Results are given in Table 4. Compounds with identical mobilities in TLC systems 2 and 3 and on Zorbax-ODS and with identical UV spectra to 2,7-dihydroxycadalene and lacinilene C were isolated from inoculated WbM(0.0). Much greater amounts of the two compounds were obtained from inoculated resistant WbM(0.0) than from inoculated susceptible WbM(4.0), or from uninoculated resistant WbM(0.0). This observation supports the hypothesis that production of these compounds is an expression of the genes for resistance to bacterial blight.

Inhibitory activities of the isolated compounds towards *X. campestris* pv. *malvacearum*. Fig. 6 shows the effects of 2,7-dihydroxycadalene and lacinilene C at various concentrations on the growth rate of *X. campestris* pv. *malvacearum* race 3. At the lowest concentrations tested, both compounds appeared to stimulate growth slightly, but at higher concentrations they were inhibitory. 2,7-Dihydroxycadalene at approximately 0.35 mM (80 μg/ml) caused 50% reduction in the number of bacterial generations during the bioassay (Fig. 6A). It was bactericidal at 0.6 mM (140 μg/ml).

The circular dichroism parameters demonstrated that the predominant form of lacinilene C from inoculated WbM(0.0) was enantiomerically related to that from Im 216; ie, the two substances were of opposite configuration at C-1. Therefore, it is interesting that the substance from WbM(0.0) appeared to be markedly more inhibitory (Fig. 6B). It is consistent with that observation that lacinilene C from Im 216 gained inhibitory activity as it racemized in storage to an equimolar mixture of the two enantiomers (Fig. 6B).

TABLE 3. Quantities of 2,7-dihydroxycadalene and lacinilene C in extracts from fresh cotton cotyledons[a]

	nmoles/g dry weight[b]	
	2,7-Dihydroxycadalene (Compound 1)	Lacinilene C (Compound 2)
Inoculated Im 216	111	92
Inoculated Ac 44	2[c]	2[c]
Im 216 control	1	1

[a] Cotyledons were infiltrated with 2×10^6 cfu/ml of *X. campestris* pv. *malvacearum* race 3 in saturated calcium carbonate solution or with sterile, saturated calcium carbonate solution (Im 216 control). The cotyledons were harvested 3 days later. 2,7-Dihydroxycadalene and lacinilene C were extracted and purified according to method 1.

[b] One gram dry weight is equivalent to approximately 5 g fresh weight.

[c] These low values were calculated from the ultraviolet absorbance of the samples at 237 and 343 nm, respectively. However, ultraviolet-absorbing impurities were present, and, therefore, the calculated values are upper limits.

TABLE 4. Quantities of 2,7-dihydroxycadalene and lacinilene C in whole tissue extracts from freeze-dried, ground leaves of near-isogenic resistant and susceptible cotton lines[a]

	nmoles/g dry weight[b]	
	2,7-Dihydroxy-cadalene (Compound 1)	Lacinilene C (Compound 2)
Inoculated WbM(0.0) (resistant)	171	112
Inoculated WbM (4.0) (susceptible)	2	2
WbM(0.0) control	1	6

[a] Foliage leaves were vacuum infiltrated with 3×10^6 cfu/ml of *X. campestris* pv. *malvacearum* race 3 in saturated calcium carbonate solution or with sterile, saturated calcium carbonate solution (control). The leaves were harvested 2 days later. Compounds 1 and 2 were extracted and purified according to method 2.

[b] One gram dry weight is equivalent to approximately 5 g fresh weight.

DISCUSSION

By using a bioassay to detect substances that inhibit multiplication of *X. campestris* pv. *malvacearum* in liquid culture, and by using standard analytic procedures we have isolated two inhibitory compounds and identified them as 2,7-dihydroxycadalene and lacinilene C. There is some evidence that lacinilene C 7-methyl

ether is a causative agent in byssinosis, a chronic respiratory distress of some cotton mill workers, which results from inhaling the dust generated from cotton plant fragments during mechanical processing of cotton fibers (17). The observations reported here suggest that in living cotton plants these compounds function in resistance to bacterial blight.

The compounds have the same cadinane carbon skeleton as other sesquiterpenoids from cotton, hemigossypol, 6-deoxyhemigossypol, desoxyhemigossypol, and their methyl ethers (4,24) and differ from them in positions of hydroxyl groups and in oxidation states. Levels of the gossypol-related sesquiterpenoids correlate with resistance of cotton plants to the fungal pathogens *Verticillium dahliae* Kleb., *Fusarium oxysporum* Schlecht f. sp. *vasinfectum* (Atk.) Snyd. & Hans., and *Rhizoctonia solani* Kühn (3). It has been reported that hemigossypol and desoxy-6-methoxyhemigossypol (called vergosin in that report) are inhibitory to *Verticillium* (30).

Our observations do not exclude a role for sesquiterpenoids other than 2,7-dihydroxycadalene and lacinilene C in resistance to bacterial blight. However, if other sesquiterpenoids are present, their inhibitory activities are less than that of 2,7-dihydroxycadalene. Zaki et al (31) reported R_f values for gossypol, hemigossypol, and desoxy-6-methoxyhemigossypol of 0.28, 0.28, and 0.58, respectively, in the same TLC system used for Figs. 2 and 3. Using a commercial sample, we observed the same R_f for gossypol in this TLC system. 6-Deoxyhemigossypol and the methyl ethers of all of these compounds would be expected to have higher R_f values than the corresponding phenols. The only inhibitory activity that we observed at R_f greater than 0.2 was in fractions 3 and 4 (Figs. 2 and 3), and when these fractions were further resolved by HPLC, the only fraction that was inhibitory at the concentration bioassayed contained 2,7-dihydroxycadalene. The evidence, although not complete, suggests that the gossypol-related compounds function in resistance of cotton plants to fungal pathogens and that the differently substituted version of the same cadinane carbon skeleton, 2,7-dihydroxycadalene, functions in resistance of cotton to bacterial blight.

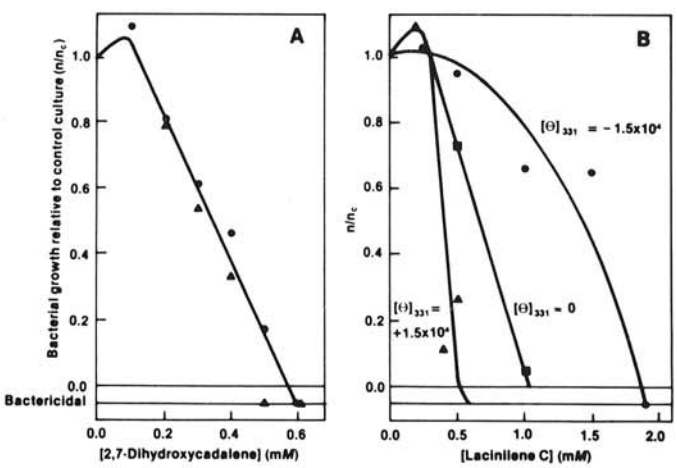

Fig. 6. Effects of 2,7-dihydroxycadalene (compound 1) and lacinilene C (compound 2) at various concentrations upon growth of *X. campestris* pv. *malvacearum* race 3 in liquid salts plus D-glucose medium (21). On the *y*-axis are plotted the numbers of generations the bacteria grew in the presence of the purified compounds (n) divided by the numbers of generations achieved by control cultures (n_c). Each point is the average of n/n_c values obtained from four or six bioassay tubes. Since weakly inhibitory substances sometimes eluted from our HPLC column, control bioassays were simultaneously performed with equal volumes of column effluent collected shortly before or after the final chromatography of the compounds. Most of these control bioassays resulted in $n/n_c > 0.8$; the lowest value was $n/n_c = 0.53$. **A,** 2,7-Dihydroxycadalene. The circles and triangles represent different preparations from inoculated Im 216 cotyledons. **B,** Lacinilene C. Circles represent lacinilene C from inoculated Im 216, molar ellipticity $[\theta]_{331} = -1.5 \times 10^4$. Squares represent the same preparation after racemization during 11 wk of storage, $|[\theta]_{331}| < 3 \times 10^2$. (It was repurified by HPLC the day before bioassay.) Triangles represent lacinilene C from inoculated WbM(0.0), $[\theta]_{331} = +1.5 \times 10^4$.

Several studies have indicated that antifungal phytoalexins are also inhibitory to bacterial pathogens in vitro (11,16,19,20) and that these pathogens elicit production of the phytoalexins in antibacterial concentrations (11,16,19). However, Wyman and VanEtten (29) were unable to confirm the antibacterial activities of some of these phytoalexins and suggested that apparent activities may depend on bioassay conditions. Gnanamanickam and Mansfield (10) recently reported that eight phytoalexins are much more inhibitory to Gram-positive bacteria than to Gram-negative bacteria. Very little work has been reported on specifically antibacterial defense compounds from plants. Hildebrand and Schroth (12) showed that infection of pear blossoms with *Erwinia amylovora* (Burr.) Winslow et al causes hydrolysis of arbutin to a hydroquinone that has antibacterial activity. A number of antibacterial substances have been extracted from plants after inoculation with bacteria (19,22,23), but to our knowledge they have not been identified.

We have only preliminary evidence concerning the importance of the two compounds described in this report in the bacteriostasis observed in resistant cotton plants (Fig. 1). Our results indicate that their concentrations would not be inhibitory if they were distributed equally throughout the leaf tissue. The quantities of 2,7-dihydroxycadalene and lacinilene C that were extracted from inoculated WbM(0.0) were 171 and 112 nmoles per gram dry weight, respectively (Table 4). Those amounts correspond to approximately 40 and 26 nmoles per milliliter of tissue water; ie, 0.04 and 0.026 mM. Those average concentrations are only about one-tenth of the concentrations that would be necessary to inhibit bacterial growth (Fig. 6B).

However, 2,7-dihydroxycadalene and lacinilene C may be locally concentrated near the bacteria. With a fluorescence microscope we have observed yellow-green fluorescence in Im 216 cotyledons 1–6 days after inoculation (B. Hamilton, M. Essenberg, P. E. Richardson, and V. E. Scholes, *unpublished*). The fluorescence was restricted to brown, irregularly shaped host cells, which are known from earlier work (7) to be the sites of the bacterial colonies. Lacinilene C was the most brilliant of the yellow-green fluorescent fractions extracted from these tissues. Since it was optically active, it was not entirely produced by autoxidation of 2,7-dihydroxycadalene during purification, but must have been produced enzymically, and was probably responsible for the yellow fluorescence in the brown host cells. Thus, lacinilene C and its precursor, 2,7-dihydroxycadalene, probably are locally concentrated in the host cells closest to the bacteria.

LITERATURE CITED

1. Barnes, M. 1980. Evaluation of the concept of horizontal and vertical resistance by the cotton—*Xanthomonas malvacearum* host-pathogen system. Masters thesis. Oklahoma State University, Stillwater. 60 pp.
2. Beier, R. C., and Greenblatt, G. A. 1981. A novel solvent strategy for SEP-PAK C_{18} elution of hydrophobic compounds: Applications to lacinilenes and cadalenes from cotton bract. J. Liq. Chromatogr. 4:515-524.
3. Bell, A. A., and Stipanovic, R. D. 1978. Biochemistry of disease and pest resistance in cotton. Mycopathologia 65:91-106.
4. Bell, A. A., Stipanovic, R. D., Howell, C. R., and Fryxell, P. A. 1975. Antimicrobial terpenoids of *Gossypium*: Hemigossypol, 6-methoxyhemigossypol and 6-deoxyhemigossypol. Phytochemistry 14:225-231.
5. Brinkerhoff, L. A. 1970. Variation in *Xanthomonas malvacearum* and its relation to control. Annu. Rev. Phytopathol. 8:85-110.
6. Brinkerhoff, L. A., and Verhalen, L. M. 1976. Inheritance of immunity to bacterial blight in an upland cotton cross. Page 31 in: Proc. Beltwide Cotton Prod. Res. Conf., 5–7 January 1976, Las Vegas, NV. Published by the National Cotton Council, Memphis, TN. 176 pp.
7. Essenberg, M., Cason, E. T., Jr., Hamilton, B., Brinkerhoff, L. A., Gholson, R. K., and Richardson, P. E. 1979. Single cell colonies of *Xanthomonas malvacearum* in susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves. Physiol. Plant Pathol. 15:53-68.
8. Essenberg, M., Hamilton, B., Cason, E. T., Jr., Brinkerhoff, L. A., Gholson, R. K., and Richardson, P. E. 1979. Localized bacteriostasis

indicated by water dispersal of colonies of *Xanthomonas malvacearum* within immune cotton leaves. Physiol. Plant Pathol. 15:69-78.
9. Field, F. H. 1972. Chemical ionisation mass spectrometry. Pages 133–181 and particularly pages 145 and 149 in: A. Maccoll, ed., MTP Intl. Rev. of Science Vol. 5. Mass Spectrometry. Univ. Park Press, Baltimore. 300 pp.
10. Gnanamanickam, S. S., and Mansfield, J. W. 1981. Selective toxicity of wyerone and other phytoalexins to Gram-positive bacteria. Phytochemistry 20:997-1000.
11. Gnanamanickam, S. S., and Patil, S. S. 1977. Accumulation of antibacterial isoflavonoids in hypersensitively responding bean leaf tissues inoculated with *Pseudomonas phaseolicola*. Physiol. Plant Pathol. 10:159-168.
12. Hildebrand, D. C., and Schroth, M. N. 1963. Relation of arbutin-hydroquinone in pear blossoms to invasion by *E. amylovora*. Nature 197:513.
13. Hunter, R. E., Brinkerhoff, L. A., and Bird, L. S. 1968. The development of a set of upland cotton lines for differentiating races of *Xanthomonas malvacearum*. Phytopathology 58:830-832.
14. Jeffs, P. W., and Lynn, D. G. 1975. Isolation and structure of 1-hydroxy-7-methoxy-4-isopropyl-1,6-dimethyl-2(1H)-naphthalenone from cotton. J. Org. Chem. 40:2958-2960.
15. Jeffs, P. W., and Lynn, D. G. 1978. The synthesis of 1-hydroxy-7-methoxy-4-isopropyl-1,6-dimethyl-2(1H)-naphthalenone—A cytokinetic compound from cotton. Tetrahedron Lett. 1617-1618.
16. Keen, N. T., and Kennedy, B. W. 1974. Hydroxyphaseollin and related isoflavanoids in the hypersensitive resistance reaction of soybeans to *Pseudomonas glycinea*. Physiol. Plant Pathol. 4:173-185.
17. Kilburn, K. H., McCormick, J. P., Schafer, T. R., Thurston, R. J., and McKenzie, W. H. 1977. Biologically active components in cotton dust. Page 66 in: Proc. Special Session on Cotton Dust. National Cotton Council, Memphis, TN.
18. Klement, Z. 1965. Method of obtaining fluid from the intercellular spaces of foliage and the fluid's merit as substrate for phytobacterial pathogens. Phytopathology 55:1033-1034.
19. Lyon, F. M., and Wood, R. K. S. 1975. Production of phaseollin, coumestrol, and related compounds in bean leaves inoculated with *Pseudomonas* spp. Physiol. Plant Pathol. 6:117-124.
20. Lyon, G. D., and Bayliss, C. E. 1975. The effect of rishitin on *Erwinia carotovora* var. *atroseptica* and other bacteria. Physiol. Plant Pathol. 6:177-186.
21. Ornston, L. N., and Stanier, R. Y. 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. J. Biol. Chem. 241:3776-3786.
22. Rathmell, W. G., and Sequeira, L. 1975. Induced resistance in tobacco leaves: The role of inhibitors of bacterial growth in the intercellular fluid. Physiol. Plant Pathol. 5:65-73.
23. Stall, R. E., and Cook, A. A. 1968. Inhibition of *Xanthomonas vesicatoria* in extracts from hypersensitive and susceptible pepper leaves. Phytopathology 58:1584-1587.
24. Stipanovic, R. D., Bell, A. A., and Howell, C. R. 1975. Naphthofuran precursors of sesquiterpenoid aldehydes in diseased *Gossypium*. Phytochemistry 14:1809-1811.
25. Stipanovic, R. D., Greenblatt, G. A., Beier, R. C., and Bell, A. A. 1981. 2-Hydroxy-7-methoxycadalene. The precursor of lacinilene C 7-methyl ether in *Gossypium*. Phytochemistry 20:729-730.
26. Stipanovic, R. D., and Wakelyn, P. J. 1974. Three water-soluble fluorescent pigments from *Gossypium* bracts. Pages 225–236 in: Cotton Dust. H. Ayer, ed. Proc. Topical Symp., 12–13 November 1974, Atlanta, GA. Am. Conf. of Governmental Industrial Hygienists. Cincinnati, OH.
27. Stipanovic, R. D., Wakelyn, P. J., and Bell, A. A. 1975. Lacinilene C, a revised structure, and lacinilene C 7-methyl ether from *Gossypium* bracts. Phytochemistry 14:1041-1043.
28. Suzuki, H., Yasuda, S., and Hanzawa, M. 1972. The extractives of Ohyonire, *Ulmus lacinata* Mayr. IV. The isolation of lacinilene C from the heartwood and the revised structure of lacinilene B. Mokuzai Gakkaishi 18:617-622.
29. Wyman, J. G., and VanEtten, H. D. 1978. Antibacterial activity of selected isoflavonoids. Phytopathology 68:583-589.
30. Zaki, A. I., Keen, N. T., and Erwin, D. C. 1972. Implication of vergosin and hemigossypol in the resistance of cotton to *Verticillium albo-atrum*. Phytopathology 62:1402-1406.
31. Zaki, A. I., Keen, N. T., Sims, J. J., and Erwin, D. C. 1972. Vergosin and hemigossypol, antifungal compounds produced in cotton plants inoculated with *Verticillium albo-atrum*. Phytopathology 62:1398-1401.