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The phytoalexins desoxyhemigossypol and hemigossypol are elicited by *Xanthomonas* in *Gossypium* cotyledons

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Abstract

Gossypium (cotton) spp. produce an array of sesquiterpenoid defense compounds, some of which accumulate in pigment glands and in root epidermis of healthy plants, and others which function as phytoalexins. Of the phytoalexins which accumulate in stem stele of Verticillium wilt-resistant cotton in response to *Verticillium dahliae* infection, Mace, Stipanovic and Bell (1985) *Physiological Plant Pathology, 26*, 209, have shown that desoxyhemigossypol (dHG) has the highest antifungal activity. Of the phytoalexins previously observed in foliar tissue of bacterial blight-resistant *Gossypoium hirsutum* in response to *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) infection [2,7-dihydroxycadalene (DHC), lacinilene C, lacinilene C 7-methyl ether, and 2-hydroxy-7-methoxycadalene], DHC has the highest antibacterial activity. Both groups of phytoalexins have cadinane carbon skeletons, but they differ in the positions of oxygen-containing functional groups. dHG and its oxidation product hemigossypol (HG) have now been identified as part of the foliar resistance response to *Xcm*. A time course study showed that the bacterial blight-resistant, pigment-glandless *G. hirsutum* line WbM*gl* accumulated dHG and HG more quickly than the cadalene and lacinilene phytoalexins and to similar peak amounts (1–5 μmol/g fr. wt). Bioassays on logarithmically growing cultures of *Xcm* in defined liquid medium in the dark revealed that both dHG and HG have phytoalexin activity toward this pathogen, but are less potent than DHC. Whether dHG and HG contribute to resistance toward the infection by *Xcm* or play a different role depends on where these phytoalexins accumulate in inoculated cotyledons, which has yet to be determined.

Keywords: *Gossypium hirsutum*; Malvaceae; Upland cotton; *Xanthomonas campestris* pv. *malvacearum*; Ecological biochemistry; Phytoalexin activity; Sesquiterpenes; Desoxyhemigossypol; 2,7-dihydroxycadalene; Hemigossypol

1. Introduction

Gossypium spp. produce a richly diverse array of terpenoid secondary metabolites (Bell, 1986), the largest group of which are cadinanes. Regulation of terpenoid production has also evolved to a high level of complexity. Unstressed *Gossypium* plants accumulate many of these compounds in subepidermal glands of all green tissues, which deter herbivores, and in epidermal cells of roots, probably as defense against soilborne pathogens. Various sesquiterpenoid cadinanes are also elicited by fungal and bacterial infection, by toxic chemicals, and by cold stress (Bell, 1986; Essenberg, Grover & Cover, 1990).

The fungal pathogens *V. dahliae, Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *vasinfectum* infect vascular tissues and elicit production of the 8-hydroxylated cadinanes hemigossypol (HG), desoxyhemigossypol (dHG), and their 3-methyl ethers (Bell, 1969; Hunter, Halloin, Veech & Carter, 1978; Zhang, Mace, Stipanovic & Bell, 1993). Of these, dHG has the highest antifungal activity (Mace, Stipanovic & Bell, 1985).

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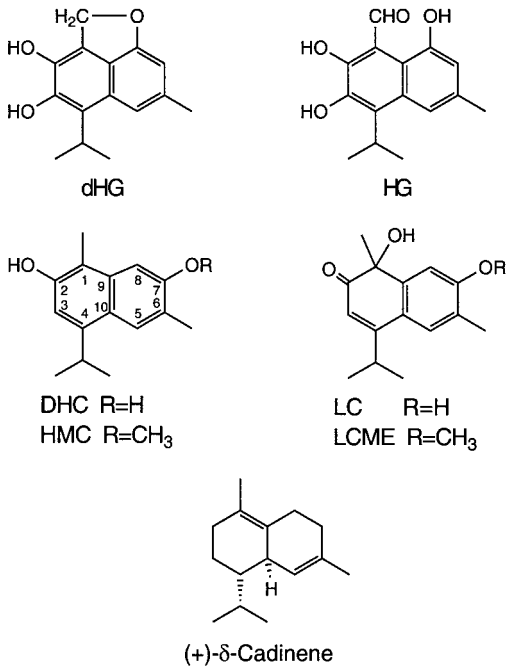

The 7-hydroxylated cadinanes 2,7-dihydroxycadalene (DHC), lacinilene C (LC), and their respective 7-methyl ethers (HMC and LCME) are elicited in leaves and cotyledons by bacterial pathogens, *X. campestris* pathovars (Essenberg et al., 1990). Of these 7-hydroxylated compounds, DHC has the most potent antibacterial activity, LC and LCME are also active, and HMC at the limit of its solubility in 30° water is practically inactive (Essenberg et al., 1990).[1] Determination of the cellular concentrations of these phytoalexins after bacterial infection of bacterial blight-resistant leaves indicated that DHC, LC, and LCME are adequate to account for the observed resistance (Pierce, Cover, Richardson, Scholes & Essenberg, 1996).

Only the 8-hydroxylated cadinanes have been detected in *Gossypium* roots and stems (Bell, 1986). Although only 7-hydroxylated cadinanes have been carefully studied in foliar tissues (Essenberg, 1990, Górski, Vickstrom, Pierce & Essenberg, 1995, Pierce et al., 1996), the occurrence of 8-hydroxylated cadinanes has been observed in fungus-infected (Halloin & Bell, 1979) and bacteria-infected (Davis & Essenberg, 1995) cotyledons.

This study was motivated by curiosity concerning whether dHG and HG function as phytoalexins in foliar resistance of *Gossypium* to *Xanthomonas* infection. Accumulation of these sesquiterpenes was confirmed, the time course of their accumulation and that of the 7-hydroxylated cadinanes following infection were compared, and antibacterial activities of dHG and HG were bioassayed. In order to avoid complication of the analyses by the terpenoids present in pigment glands of healthy leaves, a glandless, bacterial blight-resistant line of *G. hirsutum* was used.

[1] Despite the questionable antibacterial activity of HMC, for simplicity we use the term phytoalexin for all six bacteria-elicited sesquiterpenoids: dHG, HG, DHC, LC, HMC and LCME.

2. Results and discussion

2.1. Isolation and identification of dHG and HG

Bacterial blight-resistant glandless cotton cotyledons were analyzed to determine whether HG and dHG are induced after infiltration of the intercellular spaces with a suspension of *Xcm*. The cotyledons were harvested and frozen 44–60 h post-inoculation. All work with the sesquiterpenoids was under subdued lighting, since the lacinilenes and cadalenes are sensitive to photooxidation (Sun, Essenberg & Melcher, 1989). Extraction and isolation methods were based on earlier methods (Davila-Huerta et al., 1995; Greenblatt & Stipanovic, 1984; Lee, Garas & Waiss, 1986). Acid (0.1% H_3PO_4) was included in all HPLC mobile phases to decrease peak tailing due to ionization of the terpenoid phenolic groups (Greenblatt & Stipanovic, 1984). Initial separation of sesquiterpenoids was by repeated C_{18} reversed-phase HPLC with a semi-preparative column. HG eluted slightly earlier than dHG; however the two compounds were not resolved and were collected as a single fraction. The final separation method was adapted from that of Lee et al. (1986) employing C_8 reversed-phase HPLC, which resolves dHG and HG, with dHG eluting earlier.

When each of the isolated sesquiterpenes was co-chromatographed with an approximately equal amount of authentic standard on reversed-phase C_8 HPLC, peak width at half height was no greater than for the standard alone or isolated sesquiterpene alone. Since their UV absorption spectra also matched those of authentic standards, the isolated sesquiterpenes were identified as dHG and HG.

2.2. Quantitation of phytoalexins

A method was developed for quantitation of the six sesquiterpenoid phytoalexins now known to be elicited by *X. campestris* pv. *malvacearum* in cotton cotyledons, DHC, LC, HMC, LCME, dHG, and HG. Spiking experiments with dHG showed that substantial losses occurred during the isolation procedure which had been used for preparative isolation. It is known that dHG autoxidizes to HG in aqueous solution (Stipanovic, Mace, Bell & Beier, 1992). Therefore, the following modifications were made in the procedure to improve recoveries. To decrease the rate of autoxida-

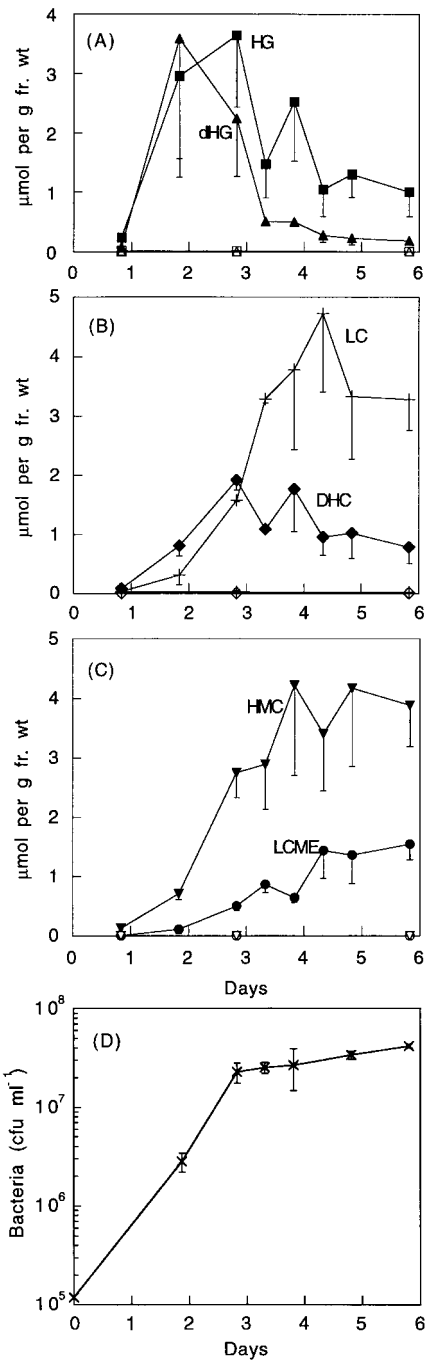

Fig. 1. Accumulation of phytoalexins and multiplication of *Xcm* in cotyledons of bacterial blight-resistant *G. hirsutum* WbM*gl*. Both cotyledons of 15 plants (7.5 plants designated for each of sets I and II) were entirely infiltrated with a suspension of 7×10^6 cfu ml^{-1} of *Xcm* (closed symbols and +), and 6 plants were infiltrated with sterile satd $CaCO_3$ soln (3 plants per set; open symbols and |). (A) dHG (▲, △) and HG (■, □); (B) DHC (◆, ◇) and LC (+, |); (C) HMC (▼, ▽) and LCME (●, ○); (D) *Xcm* population densities in *Xcm*-inoculated cotyledons (x) (Essenberg et al., 1979). Phytoalexins were determined from pooled 0.33 cm^2 disks, one from each cotyledon in a set, and *Xcm* populations were determined from pooled 0.033 cm^2 disks. Phytoalexins were determined by the analytical extraction, isolation, and quantitation method. All panels: points and error bars represent mean and s.e.

tion, ascorbic acid was added to the extracting solvents, which were chilled and degassed with argon (Stipanovic et al., 1992). The slowest step in the preparative procedure had been evaporation of the combined supernatant fractions to reduce the MeOH content to 10% (so that sesquiterpenoids would partition onto the C_{18} cartridge). This step was replaced by addition of H_2O (containing ascorbic acid) to dilute the MeOH to 10%.

Percent recoveries of phytoalexins were estimated by duplicate experiments in which known amounts of the compounds were added to frozen, mock-inoculated cotyledon tissue immediately before extraction. After correction for endogenous amounts in the mock-inoculated cotyledon tissue, average recoveries were: dHG, 33%; HG, 45%; LC, 68%; HMC, 100%. Recovery of DHC by a similar procedure had been determined by Miranda to be 98% (Miranda, 1993). Recovery of LCME by the current procedure was not determined. With earlier procedures, it has been within the same range as recoveries of LC and DHC (Pierce et al., 1996). Hunter et al. (1978) reported recoveries of 34–43% and 60% for dHG and HG, respectively, with higher recoveries of dHG at higher concentrations. With the exception of LCME values, data presented below were corrected for these losses that occur during extraction.

2.3. Time course of phytoalexin accumulation

A time course study of the bacterial-blight-resistant, glandless cotton line WbM*gl* showed that all six phytoalexins accumulated to at least 1 µmol/g fresh weight (average tissue concentrations of ≥1 mM, Fig. 1). Phytoalexins appeared during the second day post-inoculation. dHG and HG levels rose the most quickly, followed by DHC and HMC and then by LC and LCME. dHG was the least persistent of the phytoalexins, decreasing in amount after 2 days. Amounts of HG and DHC went through somewhat later maxima and decreased gradually thereafter. HMC, LC, and LCME were the most persistent, and were still abundant in the cotyledons at 6 days.

Multiplication of *Xcm* in the cotyledons was logarithmic for about 3 days and then was inhibited, as is characteristic of *Xcm* growth in bacterial blight-resistant cotton cotyledons (Górski et al., 1995; Pierce, Essenberg & Mort, 1993). The inhibition occurred as DHC reached its maximum level. These patterns are similar to those observed in cotyledons of OK1.2, a cotton line whose level of bacterial blight resistance is very similar to that of WbM*gl* (Górski et al., 1995).

The rising and falling levels of phytoalexins result from the competing processes of biosynthesis and metabolism/degradation, both of which may be occurring simultaneously. A ^{13}C-pulse-labeling study of

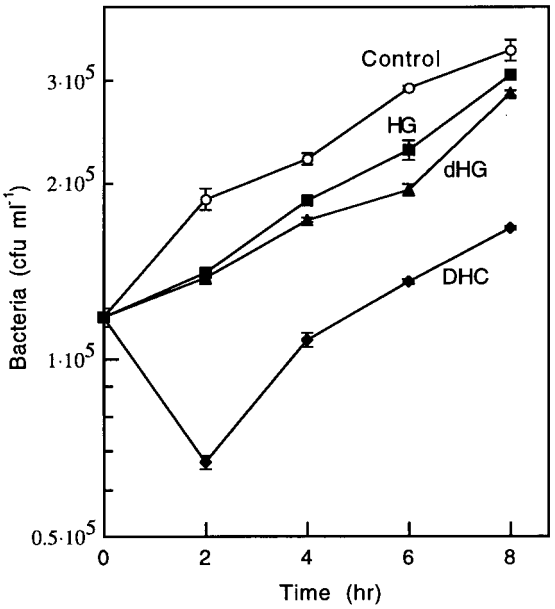

Fig. 2. Effects of 70 μM phytoalexins on multiplication of *Xcm* in liquid culture in the dark. Seventy micromolar dHG (▲), HG (■), and DHC (◆); control (○). Each point represents the mean bacterial population of two replicate cultures. Error bars indicate s.e. for each data point. One-tailed, paired-sample t-tests indicated that bacterial populations in each of the phytoalexin-containing cultures from 2–8 h were lower than those in the control cultures (HG and dHG, $P < 0.05$; DHC, $P < 0.0125$) and populations in the DHC cultures were lower than those in the HG ($P < 0.025$) and dHG ($P < 0.05$) cultures. Populations in the HG and dHG cultures were not different ($0.1 < P < 0.2$).

DHC and HMC biosynthesis in cotyledons of OK1.2 indicated high biosynthetic incorporation rates into both compounds 2 and 3 days post-inoculation, followed by several-fold lower rates at 4 and 6 days (Górski et al., 1995). The overall tissue content of DHC fell after 3 days, despite that continued synthesis. Although precursor-product relationships have not been demonstrated *in planta*, it is likely that dHG is a precursor to HG; likewise DHC to LC, and HMC to LCME, since all of these autoxidations occur nonenzymatically under aerobic conditions (Stipanovic, Bell & Howell, 1975; Stipanovic, Greenblatt, Beier & Bell, 1981).

2.4. Antibacterial activities in vitro

Bioassays on logarithmically growing cultures of *Xcm* in defined liquid medium in the dark were performed to observe the inhibitory activities of dHG and HG in comparison with the inhibitory activity of DHC (Fig. 2). The concentration used in this bioassay, 70 μM, is at or slightly above the solubility of HG under these conditions. Both dHG and HG partially inhibited the growth of *Xcm*, but were not nearly so active as DHC, which achieved about 50% killing. For all three compounds, inhibition or killing occurred during the first 2 h of exposure to the phytoalexins. Thereafter, growth rates were comparable to those of the control cultures. This multiplication of *Xcm* in the bioassays was probably made possible by decomposition of the sesquiterpenes. DHC has a half-life of less than 1 h in this defined bacterial growth medium in the dark; its principal decomposition product is LC (Steidl, 1988). Of the 8-hydroxylated cadinane phytoalexins, dHG is the least stable. It decomposes to HG in a reaction which, like the decomposition of DHC to LC, is accelerated by iron salts and inhibited by the metal chelator diethylenetriaminepentaacetic acid and by anaerobic conditions (Stipanovic et al., 1992). Our relatively low recoveries of dHG and HG from cotyledon tissue suggest that HG is also autoxidizable. The effects of the sesquiterpenes on bacterial populations shown in Fig. 2 are a result of both the intrinsic antibacterial activities of these compounds and their stabilities. Although the stabilities of dHG and HG relative to DHC and LC are not known, since they have not been measured under a uniform set of conditions, bioassays like that of Fig. 2 are probably a fair indication of the relative effectiveness of these compounds as phytoalexins, since the same two properties, antibacterial activity and stability, presumably influence their effectiveness against the bacterial pathogen *in planta*.

In another test (data not shown), activities of DHC, HG, and LC at 80 μM concentrations were compared. DHC was bacteriocidal, HG was partially inhibitory, and LC was slightly stimulatory. This positive effect of predominantly *S*-LC (the more anti-*Xcm* of the two enantiomers) was previously observed at 200 μM, although at higher concentrations it is inhibitory, exhibiting an ED_{90} of 0.5 mM in the dark (Essenberg et al., 1982).

Thus DHC remains the most potent phytoalexin discovered in cotton plants against *Xcm*. HG and dHG, like LC and LCME (Essenberg et al., 1990), are more weakly anti-*Xcm* phytoalexins which are also induced by this pathogen. In contrast, relative fungicidal activities toward the cotton pathogen *V. dahliae* are dHG > HG > DHC (Mace et al., 1985; Mace, Stipanovic & Bell, 1987). The antimicrobial activities of DHC and dHG appear to be due at least in part to their ability to form free radicals and generate toxic forms of reduced oxygen as they are being oxidized (Mace & Stipanovic, 1995; Steidl, 1988; Stipanovic et al., 1992; Sun et al., 1989). The toxicities of DHC, LC, and LCME are enhanced by light (Samad and Essenberg, unpublished results; Steidl, 1988; Sun et al., 1989); the effects of light on antimicrobial activities of dHG and HG are not known.

HG and dHG meet the definition of phytoalexins (Paxton, 1981) against *Xcm*, since they accumulate in

cotton cotyledons in response to *Xcm* infection and have growth-inhibitory activity against it. The earliness of their accumulation (Fig. 1) also suggests that they may contribute to the observed resistance to *Xcm*. However, whether *Xcm* is actually exposed to dHG and HG depends on the location of their accumulation, which is not yet known. DHC, LC, LCME, and HMC have been shown to accumulate precisely in the hypersensitively necrotic mesophyll cells (Pierce & Essenberg, 1987), which are the cells closest to the bacterial colonies (Essenberg et al., 1979; Essenberg et al., 1992). Concentrations of DHC, LC, and LCME in those cells have been determined for three cotton lines during their resistant responses to *Xcm* and found to be high enough to account for resistance (Pierce et al., 1996). Whether HG and dHG also accumulate in the hypersensitively necrotic cells is a very open question. Histochemical studies with antimony trichloride, which gives a red product from HG and its methyl ether derivative and a green product from dHG, have shown all of these phytoalexins accumulate within *V. dahliae*-infected xylem vessels and in specialized paravascular parenchyma cells of cotton stems (Mace, 1978, 1983; Mace, Stipanovic & Bell, 1989), cell types in which cadalenes and lacinilenes have not been detected. That both vascular and mesophyll cells of cotyledons can accumulate the 8-hydroxylated cadinane phytoalexins was shown with the antimony trichloride stain on cotyledons inoculated with the vascular pathogen *V. dahliae* or the nonpathogen *Colletotrichum dematium*, respectively (Halloin & Bell, 1979).

Biosynthetic studies are also yielding information relevant to the localization and biological roles of the cadinane phytoalexins in leaves. All six of the phytoalexins discussed here are biosynthesized from farnesyl diphosphate (FPP) via (+)-δ-cadinene (Davis & Essenberg, 1995). Enzymes which catalyze the cyclization of FPP to δ-cadinene (E.C. 4.6.1.11, CDN1) have been extracted from *V. dahliae*-infected stele of *G. barbadense* (Benedict et al., 1995) and from *Xcm*-infected cotyledons of *G. hirsutum* (Davis & Essenberg, 1995; Davis, Tsuji, Davis, Pierce & Essenberg, 1996) and in both tissues shown to increase dramatically in activity following infections (Alchanati et al., 1998; Davis et al., 1996). Two classes of cDNA coding for (+)-δ-cadinene synthases have been cloned and characterized from *Verticillium*-elicitor-treated suspension cultures of *G. arboreum* (Chen, Chen, Heinstein & Davisson, 1995; Chen, Wang, Davisson & Heinstein, 1996) and from *Xcm*-inoculated *G. hirsutum* cotyledons (Davis, 1998; Davis, Chen, Essenberg & Pierce, 1998). RNA analyses have demonstrated that these genes are induced by pathogens or pathogen-derived elicitors in *G. arboreum* (Chen et al., 1995; Chen et al., 1996), *G. barbadense* (Alchanati et al., 1998), and *G. hirsutum* (Davis, 1998) and are developmentally controlled in maturing seeds of *G. hirsutum* (Meng et al., 1999). We have developed an immunohistochemical stain for (+)-δ-cadinene synthase (with polyclonal antibodies raised against CDN1-C, but probably positive for CDN1-A also) which shows that *Xcm*-inoculation of cotyledons leads to appearance of the enzyme in vascular and epidermal cells, as well as in the hypersensitively responding mesophyll cells at infection centers (Park, 1997). Since the 7-hydroxylated phytoalexins DHC, LC, LCME, and HMC have only been found in the mesophyll-cell infection centers of *Xcm*-inoculated cotyledons (Essenberg et al., 1992; Pierce & Essenberg, 1987), it seems plausible that the (+)-δ-cadinene synthase detected in vascular and epidermal cells is functioning in synthesis of the 8-hydroxylated phytoalexins dHG and HG. Use of the antimony trichloride stain (Mace et al., 1989) on *Xcm*-inoculated cotyledons could reveal whether HG and dHG are co-localized with the cadalenes and lacinilenes in mesophyll cells at bacterial infection centers and/or whether they accumulate in epidermal and vascular cells, as they do in root and stem tissues.

3. Experimental

3.1. Plants, bacteria, and inoculation

Seedlings of Upland cotton (*G. hirsutum* L.) of a bacterial blight-resistant line lacking pigment glands, WbM*gl*, were used in all experiments (Davis & Essenberg, 1995). The seedlings were grown in a growth chamber as previously described (Pierce et al., 1993). *Xcm* strain 3631 (Pierce et al., 1993) was cultured in nutrient broth and diluted in sterile H_2O satd with $CaCO_3$ to an inoculum concentration of ca 5×10^6 cfu ml^{-1}. Entire cotyledons were inoculated 5–7 days after seedling emergence by infiltration from a needleless syringe as previously described (Pierce & Essenberg, 1987). Mock-inoculated control cotyledons were infiltrated with sterile satd $CaCO_3$ soln.

3.2. Extraction and isolation of phytoalexins

At 44–60 h post-inoculation, the cotyledons were harvested, quick-frozen in liquid N_2 and stored at -80° until extraction. Frozen, inoculated cotyledons (150 g fr. wt) were processed by repetition of the following procedure: 10 g were homogenized in 150 ml of chilled (4°) HPLC-grade MeOH–H_2O (4:1) in an ice bath with a rotary blender at maximum speed (4 × 30 sec). The homogenate was transferred with a 10-ml rinse of the same solvent and centrifuged. The residue was extracted again with half the original vol. of MeOH–H_2O (4:1). The pooled hydro-organic phase was conc. to 40 ml by rotary evaporation at ambient

temp. The crude extract was passed through two C_{18} cartridges (total of 2 g sorbent) connected in series and preconditioned with 2 ml MeOH and 5 ml H_2O. Phytoalexins were eluted with 10 ml MeOH–H_2O (17:3). The MeOH content was adjusted to less than 40% by addition of H_2O. This was followed by extraction with 26 ml of $CHCl_3$ twice. The combined extracts were conc. by rotary evaporation, evaporated to dryness with a stream of argon, and stored at –70° in the dark. To avoid photooxidation of the sesquiterpenoids, all work was carried out under low light.

Preliminary separation of the cotyledon extracts was by HPLC on a C_{18} column (10 μm particle diameter, 250 mm × 10 mm) with detection by UV absorption at 254 nm. Solvents were: A [MeOH–H_2O-H_3PO_4 (100:900:1)] and B [MeOH–H_3PO_4 (1000:1)]. All solvent gradients were linear. Elution program 1 was: 0–72 min: 55.5–63.5% B; flow rate 4.0 ml min^{-1}. Pressure was 3500–4000 psi. Repeated injections of extract from 25 g of cotyledon tissue in MeOH–H_2O (1:1) were performed. A fraction containing incompletely resolved dHG and HG with retention time of 40–46 min was collected. The sesquiterpenoids were recovered by partitioning against $CHCl_3$, followed by evaporation to dryness with an argon stream.

HG and dHG were resolved by HPLC on a C_8 column (5 μm particle diameter, 250 mm × 4.6 mm) preconditioned with MeOH–H_2O (1:1) for 36 h at 1.0 ml min^{-1} (Lee et al., 1986). Elution program 2 with solvents A & B (see above) was: 0–8 min: 30–65% B; 8–30 min: 65–75% B; flow rate 1.0 ml min^{-1}. Pressure was 1800–2000 psi. Fractions containing dHG and HG were collected at 19 and 19.6 min, respectively.

Authentic samples of HG and dHG used as chromatographic standards and for bioassays were a gift of R. D. Stipanovic, USDA, National Cotton Pathology Research Laboratory, College Station, TX, and had been isolated from *V. dahliae*-infected stele tissue of *G. barbadense* and purified as previously described (Bell, Stipanovic, Howell & Fryxell, 1975; Stipanovic et al., 1975). The phytoalexins LC and LCME were extracted from *Xcm*-inoculated *G. hirsutum* WbM*gl* cotyledons by the procedure described above for HG and dHG. DHC and HMC were synthesized in our laboratory by J. Steidl (Stipanovic & Steidl, 1986). LC, LCME, DHC, and HMC were purified by HPLC on C_{18}, essentially as previously described (Essenberg et al., 1990).

3.3. Co-chromatography of dHG and HG with authentic standards

Purified dHG and HG were co-chromatographed by C_8 HPLC alone and mixed with ca equal amounts of the authentic standards. dHG was eluted by program 2. UV absorption maxima after HPLC were: dHG from glandless cotyledons: UV $\lambda_{max}^{72\%\ MeOH}$ nm: 224 (s), 247, 290, A_{247}/A_{290} = 6.1; standard dHG: UV $\lambda_{max}^{72\%\ MeOH}$ nm: 224 (s), 246, 292, A_{246}/A_{292} = 6.2. For HG, elution program 3 with solvents A and B was: 0–30 min: 30–60% B, 30–45 min: 60% B isocratic; flow rate of 1.0 ml min^{-1}. UV absorption maxima after HPLC were: HG from glandless cotyledons: UV$\lambda_{max}^{72\%\ MeOH}$ nm: 228, 278, 377, A_{228}/A_{377} = 6.2; standard HG: UV $\lambda_{max}^{72\%\ MeOH}$ nm: 227, 279, 377, A_{227}/A_{377} = 6.3.

3.4. Analytical-scale extraction, isolation, and quantitation of phytoalexins

With the exception of $CHCl_3$, all extracting solvents were chilled to 4° and degassed with argon. Frozen cotyledon tissue (80 mg fr. wt) was homogenized in 1.3 ml of 85% aqueous MeOH/0.1% ascorbic acid with a glass homogenizer. The homogenate was centrifuged for one min. The supernatant was degassed gently with argon. The tissue residue was extracted with 1.3 ml of 80% MeOH/0.1% ascorbic acid, and the homogenizer was rinsed with 0.5 ml of the same solvent. The supernatants were combined, and 22.3 ml of H_2O/0.1% ascorbic acid was added to dilute the MeOH content to 10%. The resulting soln was loaded at a rate of ca 4–5 ml min^{-1} onto a small C_{18} cartridge (0.36 g sorbent) preconditioned with 1 ml MeOH and 3 ml H_2O. The phytoalexins were eluted with 2.5 ml of 85% MeOH/0.1% ascorbic acid at a rate of ca 2 ml min^{-1}. H_2O/0.1% ascorbic acid (2.8 ml) was added to the eluate to dilute the MeOH to 40%. The resulting solution was extracted with 2.65 ml of $CHCl_3$ twice. The combined extracts were evaporated to 0.5 ml, passed through a 0.45 μm polypropylene filter which had been prerinsed with 1 ml $CHCl_3$, evaporated under argon, and stored at −70° in the dark.

Analysis of phytoalexins was by HPLC on C_8 at room temperature. Samples were injected in 30 μl MeOH, eluted with program 2, and detected at 254 nm. Retention times were: LC, 14.6 min; DHC, 15.6 min; LCME, 17.5 min; dHG, 19.0 min; HG, 19.6 min; HMC, 22.1 min. Quantities were calculated from the ratios of sample peaks' integrated areas to those obtained by chromatography of known amounts of standards. Phytoalexin recoveries were estimated from analyses after known amounts (10–20 nmol) were added to 80 mg of frozen, mock-inoculated WbM*gl* cotyledon tissue just before extraction.

3.5. Bioassays

Inhibitory activities of phytoalexins toward multiplication of *Xcm* were determined in a defined MOPS-buffered medium based on that developed by Neidhardt et al. (Neidhardt, Bloch & Smith, 1974) as

modified by McNally et al. (McNally, Gabriel & Essenberg, 1984) to include 7 amino acids, but with 2.5% sucrose as carbon source. A logarithmically growing bacterial culture in the defined medium (890 μl) at 30° was introduced aseptically into sterile 1.5-ml microcentrifuge tubes containing 63 nmol phytoalexin in 10.5 μl MeOH and vortex-mixed. To determine whether the phytoalexins remained in solution or adsorbed to the polypropylene tube walls, similar preparations of DHC (the most water-soluble of the phytoalexins tested (Essenberg et al., 1990)) and of HG (the least water-soluble (Mace et al., 1985)) were made without bacteria. Aliquots were withdrawn and diluted in MeOH, and UV spectra were recorded. Results indicated that all of the DHC and 92% of the HG dissolved in the growth medium with 1.2% MeOH. An *Xcm* suspension in defined medium with 1.2% MeOH was prepared as control. All tubes were wrapped with aluminum foil to exclude light and then mounted at a 30° angle from the horizontal and agitated at 100 rpm at 30°. Viable bacterial concentrations were determined by withdrawing aliquots, diluting them in sterile, satd $CaCO_3$ soln, and plating in duplicate on Difco nutrient agar.

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