Differential Accumulation and Distribution of Antifungal Sesquiterpenoids in Cotton Stems Inoculated with *Verticillium dahliae*

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ABSTRACT

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Stems of four cotton cultivars representing *Gossypium barbadense* and *G. hirsutum* were inoculated in the greenhouse with conidial suspensions of mild (SS-4) and severe (T-1) strains of *Verticillium dahliae*. Vascular tissues of the infected, tolerant cultivar Acala SJC-1 contained sufficient amounts of antifungal substances on the third day after inoculation to completely inhibit mycelial growth of the SS-4 strain. Four sesquiterpenoid phytoalexins, desoxyhemigossypol (DHG), hemigossypol (HG), desoxy-6-methoxyhemigossypol (DMHG), and 6-methoxyhemigossypol (MHG), concurrently accumulated for several days in the vascular tissues of all inoculated plants. No sesquiterpenoids or antifungal activity were detected in stele tissues of injured, uninoculated plants. The highest levels of the methylated sesquiterpenoids (DMHG and MHG) were present in the vascular tissues of the most resistant cotton cultivar (Seabrook Sea Island) soon after inoculation and for several days thereafter, and the lowest levels were present in the most susceptible cotton (70-110). Intermediate amounts of both compounds were detected in the two Acala cottons, which are tolerant only to the mild pathotype of the fungus. The accumulation of HG and DHG was almost always lower in the most resistant cotton line than in all of the other cottons tested. Inoculation with the mild strain (SS-4) induced slightly higher levels of total sesquiterpenoids than did inoculation with the severe T-1 strain. Results demonstrate that the level of cotton resistance to Verticillium wilt is directly related to the amount of methylated sesquiterpenoids produced by the infected vascular tissues. Furthermore, these results suggest that the severity of symptoms induced by the T-1 strain is probably due to its capacity to overcome the toxicity of these compounds.

Additional key words: bioassay for fungicides, HPLC analaysis of sesquiterpenoids, phytoalexins.

Phytoalexin production and accumulation is a well-documented response of many plant species to infection with fungi and bacteria (2). Early studies by Bell (3,4) on induced antifungal compounds in cotton plants (*Gossypium* spp.) inoculated with *Verticillium albo-atrum* (microsclerotial form of *V. dahliae* Kleb.) suggested a possible role for gossypol and/or gossypol-related compounds in disease resistance. Zaki et al (30) failed to detect gossypol in extracts from cotton stems inoculated with *Verticillium* but isolated two other induced antifungal compounds, which were given the trivial names hemigossypol (HG) and vergosin (29). Vergosin has since been correctly identified as desoxy-6-methoxyhemigossypol (DMHG) (25). Two additional major sesquiterpenoid stress metabolites, desoxyhemigossypol (DHG) (25) and 6-methoxyhemigossypol (MHG) (7), were isolated and identified in stele tissues of cotton plants infected by *V. dahliae*. In addition to these four major sesquiterpenoids, three minor closely related compounds were also found to occur in cotton stems infected with *Verticillium* (7,14,23). Isohemigossypol (23) has since been shown to be identical to HG (27).

Although the involvement of these compounds in wilt resistance of cotton has been suggested (6,18,29), their role has not been established. This was due in part to the lack of quantitative knowledge of their accumulation and distribution within infected plant tissues. Their presence has been determined colorimetrically (3), histochemically (19), and by thin-layer chromatography (29). Unfortunately, the use of these techniques did not provide sufficient quantitative information to establish the relationship between individual sesquiterpenoids and the level of resistance of different cotton cultivars and species to infection with *V. dahliae*.

The formation of gums and tyloses and the eventual sealing-off of the infected vessels have been reported (1,17) to be part of the host responses that physically restrict *V. dahliae* in infected cotton vessels to the area immediately above primary infection sites (12). The results presented in the accompanying paper (11), however, indicate that resistance to Verticillium wilt in cotton appears to be due to activation of a chemical host response, because after inoculation, few if any differences were observed in the upward distribution of the pathogen in stems of resistant and susceptible cotton cultivars.

We used a high-performance liquid chromatographic (HPLC) method (16) to determine the quantitative and qualitative differences in the accumulation of sesquiterpenoids in cotton cultivars with varying levels of resistance and susceptibility to infection with two strains (SS-4 and T-1) of *V. dahliae*. A preliminary report of this work has been published (10).

MATERIALS AND METHODS

Pathogen and host. The two strains of *V. dahliae* employed in this study were the ones used in the accompanying investigation (11). Cultivars were maintained on potato-dextrose agar (PDA), and conidial suspensions were prepared from 4- to 5-day-old cultures. Suspensions were filtered through two layers of cheesecloth, and the spore concentration was determined with a hemacytometer or by measuring the optical density of the suspension (24).

Four cotton cultivars, representing two species of cultivated cottons (*Gossypium barbadense* L. and *G. hirsutum* L.), were selected because of their resistance, tolerance, or susceptibility to

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the two strains of *V. dahliae*. The reactions of these four cultivars to the two strains of *V. dahliae* are considered in detail in the accompanying paper (11). Cotton plants were grown in a greenhouse in 10-cm pots (11) and were used 5–7 wk after planting.

Inoculation. Plants were inoculated by the stem-puncture method (8,11) with suspensions of *V. dahliae* spores containing 2–3 $\times 10^6$ conidia per milliliter. Puncture inoculations, starting 3 cm above the soil line, were made 1 cm apart along the entire length of the stem. Each wound site was estimated to have received 2 μl of the conidial suspension. Control plants were punctured but not inoculated (water was used instead of condidial suspension). In some experiments, plants were inoculated in the hypocotyl section only, with three punctures 0.5 cm apart. Just before inoculation, the greenhouse temperature was dropped from about 25 to about 21 C (optimum for the development of Verticillium wilt in cotton). The inoculated plants were kept in the greenhouse under otherwise normal conditions until collected for chemical analysis or bioassay for antifungal activity.

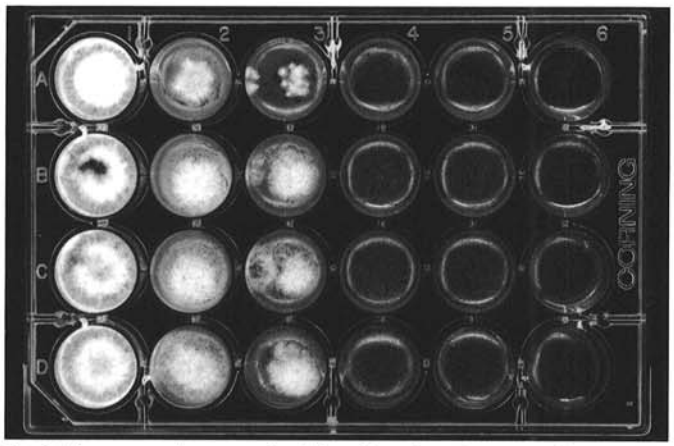

Fig. 1. Representative example of the effect of crude extracts from cotton stele tissues infected by *Verticillium dahliae* on mycelial growth of the mild (SS-4) strain. Extracts were incorporated in potato-dextrose agar (2 ml/well) in 50 μl of acetonitrile. Left to right, in groups of four wells (one lane), are extracts made from Acala SJC-1 cotton stele 0, 1, 2, 3, 4, and 5 days after inoculation with SS-4 strain of the fungus. All extracts are tested at 0.4 g fresh weight per well. A 5-μl aliquot of spore suspension (2×10^5 conidia per milliliter) was pipetted into the center of each well. The plates were incubated at 24–25 C for 6 days before photography.

Extraction of plant tissue and bioassay for presence of compounds with antifungal activity. Compounds present in the stele tissues of the infected stems were extracted by a method modified from Zaki et al (29). Inoculated stems were harvested after various periods of time and were stripped of cortical tissues to obtain the vascular (stele) tissue free of any glands. The stele tissues were cut into 0.5-cm pieces and stored at −20 C or were extracted immediately by homogenization for 2 min with a Polytron homogenizer (Brinkmann Instrument Co., Westburgy, NY) in cold (4 C) ethyl acetate containing 0.1% acetic acid (20 ml of solvent per 1 g fresh weight). The acidified ethyl acetate extracts were dried in vacuo at 35 C. Care was taken to remove any remaining traces of acetic acid by drying samples under a stream of dry nitrogen gas. The dried sample was then dissolved in acetonitrile (0.1 ml/1 g fresh weight of tissues extracted). One gram fresh weight of vascular tissue would have received an average of 12–15 puncture inoculations.

Crude extracts were bioassayed for their antifungal activity against *V. dahliae* by a technique devised for this investigation that uses multiwell plates (Corning Glass Works, Corning, NY). These 24-well tissue-culture plates are available sterile and individually wrapped. The well dimensions are: diameter 16 mm, volume 3.5 ml, and surface area 1.89 cm^2 (for radial mycelial growth). Two milliliters of PDA medium per well was adequate for assessment of conidial germination and inhibition of mycelial growth. A sterilized B-D Cornwall syringe pipette (Becton-Dickinson & Co., Inc., Rutherford, NJ) was used to dispense the autoclaved media aseptically into each well. Each plate provided enough wells to test six different plant extracts simultaneously, with four replicates of each. The medium was allowed to solidify and reach room temperature before 50 μl of the crude cotton stele extracts dissolved in acetonitrile at the appropriate dilution was added. Controls consisted of solvents alone. The solvents were allowed to evaporate to complete dryness under a transfer hood before the test fungus was added to the wells. A 5-μl portion of spore suspension

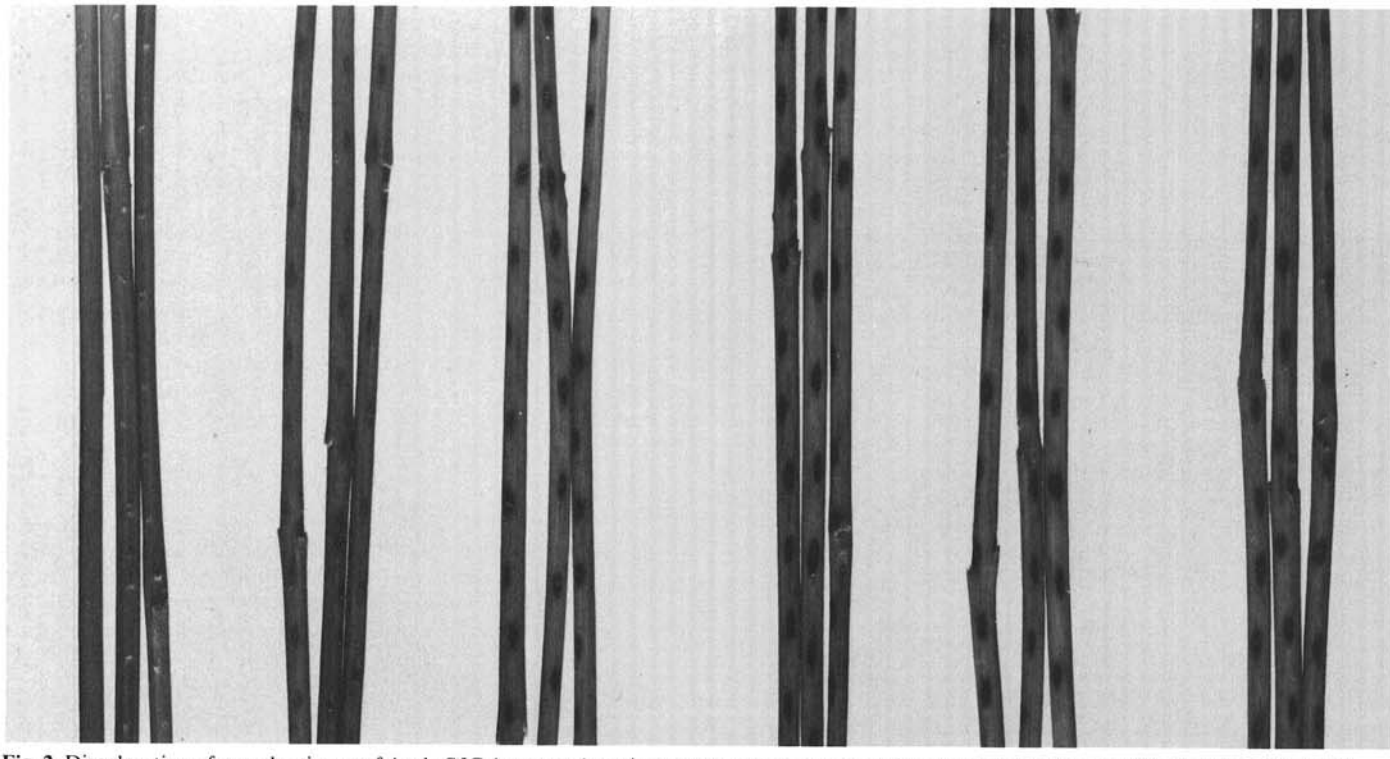

Fig. 2. Discoloration of vascular tissues of Acala SJC-1 cotton plants in response to stem-puncture inoculation with the mild (SS-4) strain of *Verticillium dahliae* ($2–3 \times 10^6$ conidia per milliliter). Left to right, in groups of three stele sections, are samples taken 0, 1, 2, 3, 4 and 5 days after inoculation.

(2×10^5 conidia per milliter) prepared from 4-day-old cultures of *V. dahliae* strain SS-4 was pipetted into the center of each well. The plates were covered and incubated at 24–25 C. For experiments requiring more than 4 days of incubation, the plates were sealed on the third day with a strip of Parafilm (American Can Co., Greenwich, CT) to prevent drying. After 3–4 days, the growth of colonies was determined either by measuring the radial growth or by photographing the entire plate. We found that these procedures clearly illustrate and record the effect of a series of plant extracts on the growth of the fungus.

HPLC determination of antifungal sesquiterpenoids. The HPLC method for quantitative analysis of sesquiterpenoids in infected cotton stele tissues has been described elsewhere (16). The crude stele tissue extracts were prepared for HPLC analysis as follows: A 250-μl aliquot of the extract solution (1 ml acetonitrile solution contains extract from 2 g of fresh tissues) was diluted with 3 ml of distilled water, and a known amount of the internal standard [4,4′ *bis*(*N,N*′-dimethylamino)benzophenone, 10 μg in 200 μl of acetonitrile] was added. The sample mixture was then loaded under minimum pressure on a SEP-PAK C 18 cartridge (Waters Associates, Milford, MA) prewetted with 5 ml of MeOH (0.1% HOAC) followed by 3 ml of H_2O. The cartridge was then washed with 1 ml of H_2O and then 3 ml of MeOH:H_2O (1:1, v/v) containing 0.1% acetic acid. The sequiterpenoids were eluted from the cartridge with 2.5 ml of MeOH containing 0.1% acetic acid. The eluate was dried under nitrogen gas and dissolved in 200 μl of acetonitrile. The sample was filtered and then chromatographed on a Radial PAK C8 reverse-phase column (16).

Four major sesquiterpenoid stress metabolites were detected in extracts from infected steles: DHG, HG, DMHG, and MHG. The identity of each sesquiterpenoid was confirmed (16) by mass-spectrometry, NMR spectroscopy, and comparison with authentic samples provided by R. D. Stipanovic, USDA, National Cotton Pathology Research Laboratory, College Station, TX.

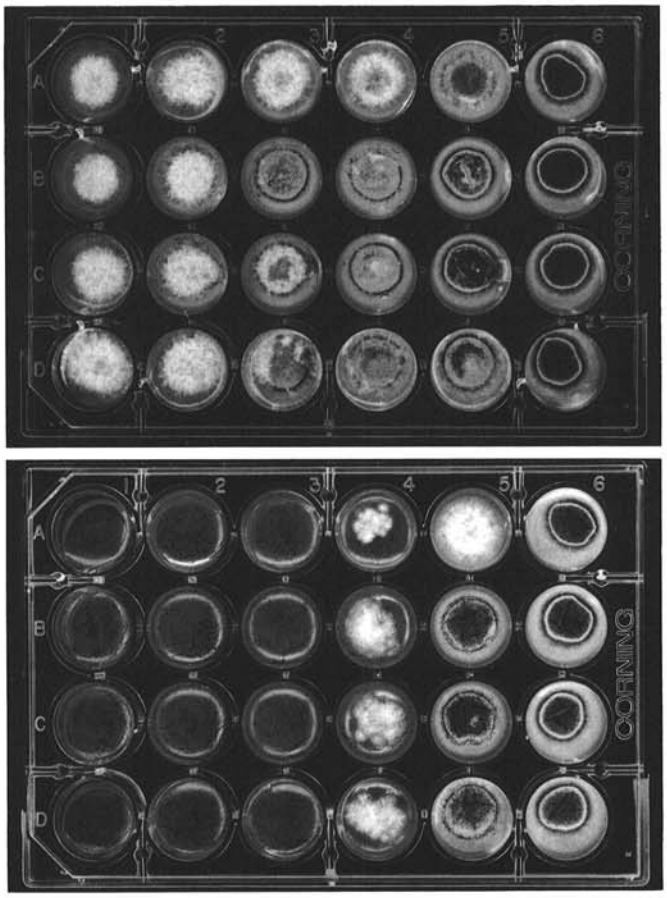

Fig. 3. Effect of extracts from **(top)** puncture-uninoculated and **(bottom)** *Verticillium dahliae*-inoculated steles of Acala SJC-1 cotton stems on the mycelial growth of SS-4 strain of the fungus. Extracts were made 3 days after inoculation. Left to right: lanes 1–5 are crude extracts at 0.5, 0.4, 0.3, 0.2, and 0.1 g fresh weight per well; lane 6 is control, potato-dextrose agar (received acetonitrile but no extracts).

RESULTS

Antifungal activity in cotton steles infected with *V. dahliae*. The multiwell plate technique was useful for following the accumulation of antifungal compounds in stele extracts of cotton stems after inoculation. Considerable antifungal activity was detected in crude extracts, thus confirming earlier reports (4,5,29). A representative multiwell plate assay is shown in Figure 1, which demonstrates the progressive increase of antifungal activity in extracts from Acala SJC-1 cotton stele tissue after inoculation with the SS-4 strain of *V. dahliae*. Under the conditions used in this bioassay, the activity was detectable in cotton steles 2 days after inoculation and complete inhibition of mycelial growth was observed 3 days after inoculation. During the first 5 days of infection, there was a direct correlation between the level of fungitoxicity of the extracts and the degree of discoloration in the discrete area surrounding the inoculated sites (Fig. 2). Bioassay of stele extracts from 3-day-postinfection plants revealed that extracts equivalent to 0.3 g fresh weight per well (representing four or five inoculation sites) was sufficient to completely inhibit the growth of SS-4 strain of *V. dahliae* (Fig. 3B). In contrast, extracts from punctured but uninoculated stems showed no antifungal activity at any of the concentrations tested (Fig. 3A).

Relationship between resistance and accumulation of methylated sesquiterpenoids. No sesquiterpenoids were detected in healthy or injured cotton stele tissues at any time an analysis was made. On the other hand, four sesquiterpenoids were always present in the stele tissues of all cultivars after inoculation with *V. dahliae*. These four sesquiterpenoids are DHG, HG, DMHG, and MHG (16). The concentrations of the two methylated derivatives were about four times greater in the resistant Seabrook Sea Island (SBSI) stele than in the susceptible 70-110. Figure 4 demonstrates

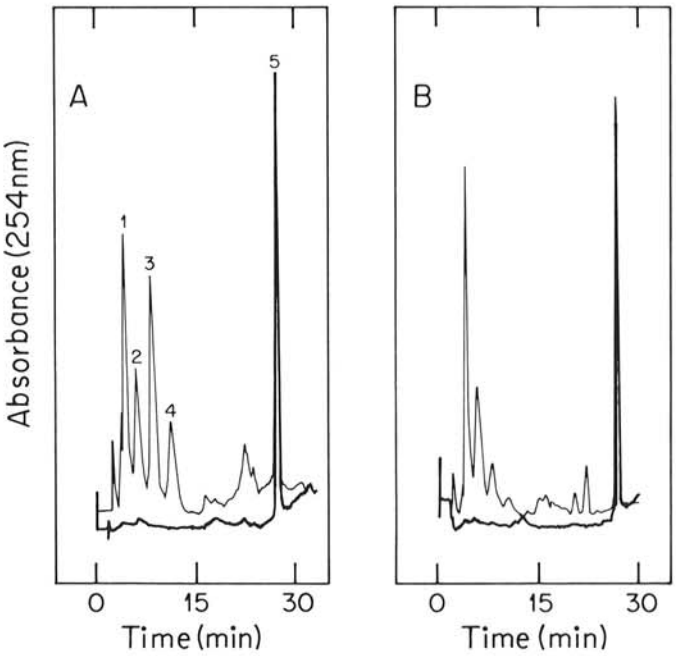

Fig. 4. High-performance liquid chromatogram (HPLC) demonstrating the accumulation of sesquiterpenoids in **A,** resistant Seabrook Sea Island and in **B,** susceptible 70-110 cotton steles 4 days after inoculation with the mild SS-4 strain of *Verticillium dahliae*. HPLC profiles of extracts from the infected tissues are superimposed on those of extracts from injured but uninoculated control tissues. Stele tissues were extracted separately and chromatographed on a reverse-phase Radial PAK C 8 column. Compounds: 1 = desoxyhemigossypol, 2 = hemigossypol, 3 = desoxy-6-methoxyhemigossypol, 4 = 6-methoxyhemigossypol, and 5 = 4,4′ *bis*(*N,N*′dimethylamino)benzophenone (as internal standard).

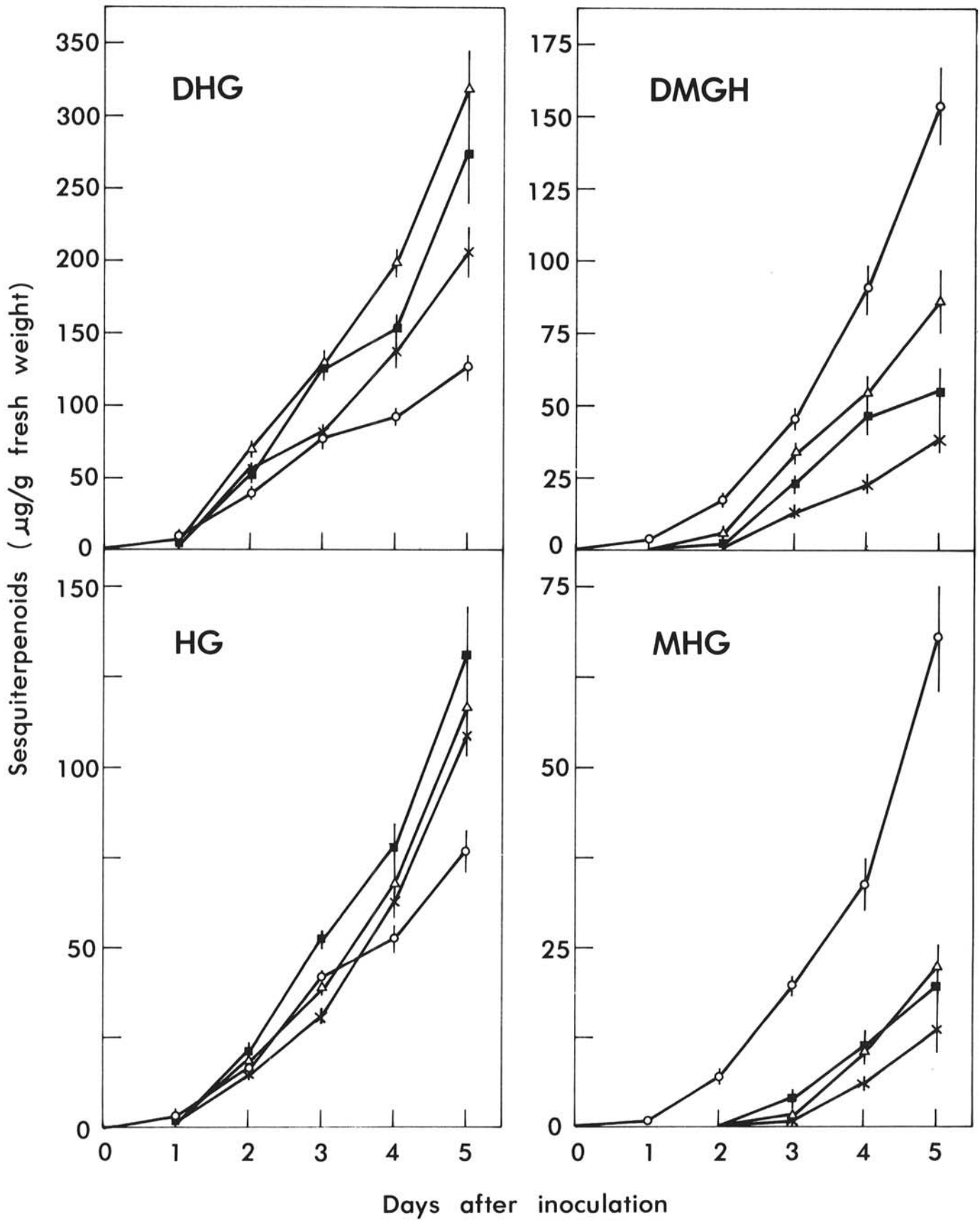

Fig. 5. Time-course for the accumulation of sesquiterpenoid stress metabolites in stele tissues of four cotton cultivars after inoculation with the mild SS-4 strain of *Verticillium dahliae*. Cotton cultivars: o = Seabrook Sea Island, □ = Acala SJC-1, Δ = Acala 4-42, and × = 70-110. Compounds: DHG = desoxyhemigossypol, HG = hemigossypol; DMHG = desoxy-6-methoxyhemigossypol, and MHG = 6-methoxyhemigossypol. Each point and vertical bar is the mean and standard error from 10 plants per cultivar per sampling date in four experiments.

the marked differences in regard to the relative amount of methylated sesquiterpenoids that accumulated in the steles of these two cotton cultivars after inoculation with *V. dahliae* (SS-4).

Time-course of sesquiterpenoid metabolism in infected cotton steles. Inoculation of cotton plants with either mild (SS-4) or severe defoliating (T-1) strains of *V. dahliae* induces discoloration (Fig. 2) and accumulation of sesquiterpenoid stress metabolites in the steles of infected stems. The mild strain (SS-4) induced the accumulation of slightly higher levels of total sesquiterpenoids than the severe defoliating strain (T-1). The sesquiterpenoids were detected as early as 24 hr after inoculation with either strain of the fungus. Although for 5 days after inoculation there was a steady increase in the total amount of these compounds, there were no significant differences in rates of accumulation in three of the cotton cultivars (SBSI, Acala SJC-1, and Acala 4-42). In the most susceptible cultivar (70-110), however, the total quantities of sesquiterpenoids were as much as 31% less than in the other cotton cultivars tested (Figs. 5 and 6).

Although the total amounts of induced sesquiterpenoids did not show a direct correlation with the level of cultivar resistance to Verticillium wilt, the individual sesquiterpenoids showed a markedly different rate of accumulation in the four cultivars.

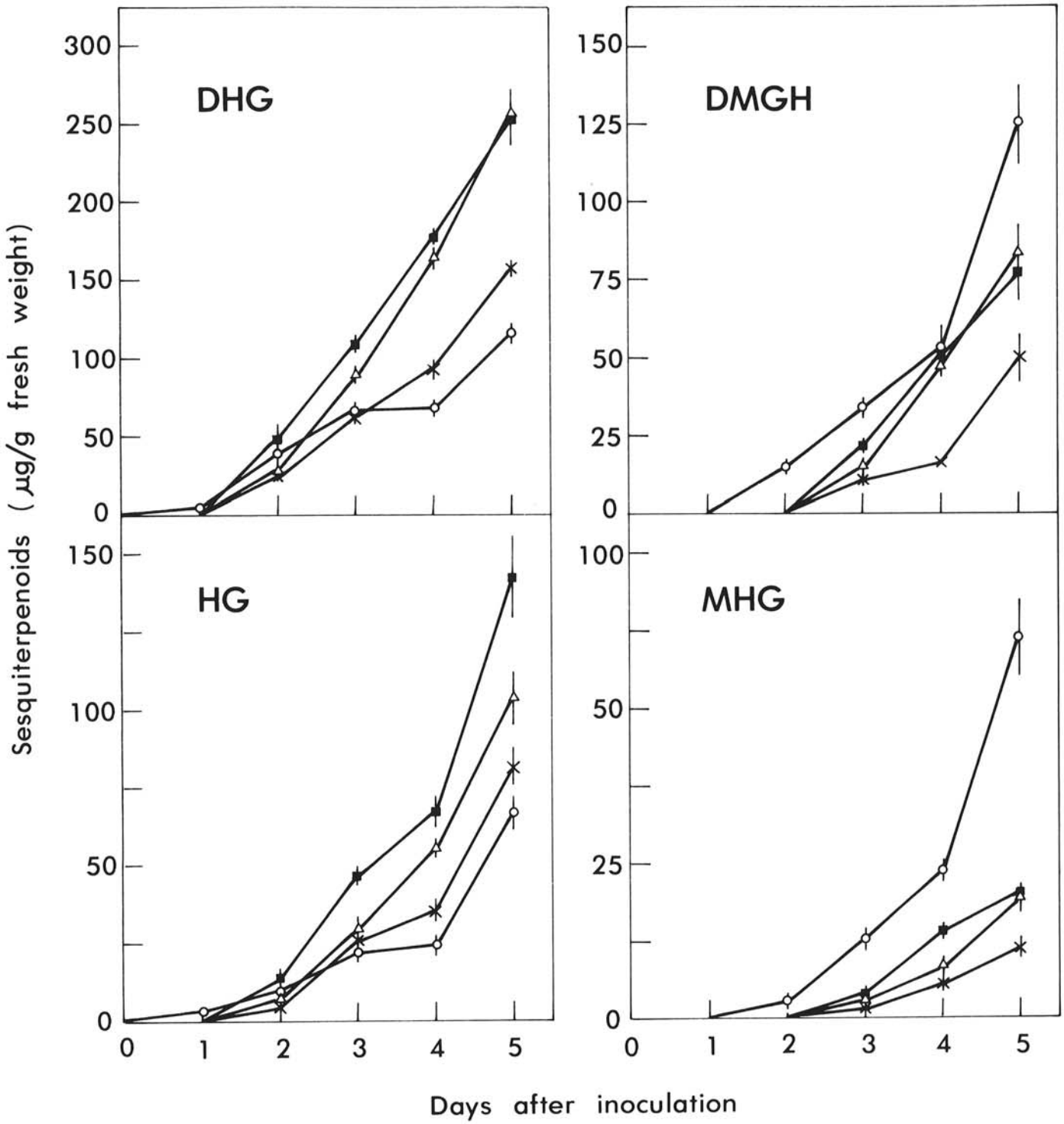

Fig. 6. Time-course for the accumulation of sesquiterpenoid stress metabolites in stele tissues of four cotton cultivars after inoculation with the severe defoliating (T-1) strain of *Verticillium dahliae*. Cotton cultivars: o = Seabrook Sea Island, □ = Acala SJC-1, Δ = Acala 4-42, and × = 70-110. Compounds: DHG = desoxyhemigossypol, HG = hemigossypol; DMHG = desoxy-6-methoxyhemigossypol, and MHG = 6-methoxyhemigossypol. Each point and vertical bar is the mean and standard error from 10 plants per cultivar per sampling date in three experiments.

Regardless of whether T-1 or SS-4 strain was used for inoculation, the most resistant cotton cultivar (SBSI) consistently contained significantly higher levels of MHG and DMHG. The lowest levels of both compounds were detected in the most susceptible cotton (70-110). The two Acala cottons (SJC-1 and 4-42), however, contained an intermediate amount of these two methylated derivatives. These amounts were significantly lower than those in the most resistant cotton (Figs. 5 and 6). This chemical response of Acala cultivars correlates positively with their level of resistance to Verticillium wilt. Essentially, both are tolerant to the mild SS-4 strain and susceptible to the severe T-1 strain. It was apparent from this study that the response of resistant cotton cultivars to infection with *V. dahliae* consists of the formation of two stress metabolites (DHG and HG), both of which are apparently converted more readily to their methylated derivatives, DMHG and MHG, than in the susceptible cultivars.

Vertical distribution of sesquiterpenoids. The distribution of sesquiterpenoids in cotton vascular tissues after hypocotyl puncture inoculation was determined by dividing the stems above the inoculation site into six sections. The sesquiterpenoids were quantitatively determined in comparable sections from SBSI and Acala SJC-1 plants 10 days after inoculation with the SS-4 strain of *V. dahliae*. In both cultivars, the greatest accumulation of sesquiterpenoids occurred in the inoculated sites of the stele tissues. The concentration of stress metabolites dropped sharply in the area 5 cm above the inoculation site, then gradually declined to reach its lowest level in the uppermost section of the stem (20–25 cm above inoculation). In the resistant SBSI cotton, 60–77% of the total sesquiterpenoids were the methylated derivatives (DMHG and MHG), whereas in Acala SJC-1 (tolerant to SS-4 strain), fewer than 17% of the accumulated sesquiterpenoids were methylated (Table 1).

After the decrease in the accumulation of sesquiterpenoids up to 10 cm from the infection site in the Acala SJC-1 stele, there was a significant increase in the rate and extent of their accumulation. These increases occurred in the area 10–20 cm above the inoculation site. However, at the uppermost part of the stem, the concentration was lower (45 μg/g fresh weight). This pattern of sesquiterpenoid distribution in Acala SJC-1 inoculated with the SS-4 strain is probably due to the response of tissues to secondary infection resulting from the formation of new conidia in the upper vascular tissues of the infected plants.

DISCUSSION

Results reported in this paper are consistent with those reported by other workers (7,20,29) indicating that several antifungal sesquiterpenoid stress metabolites accumulate in the cotton vascular tissues in response to infection with *V. dahliae*. We found that the antifungal activity of the crude extracts from these tissues (Figs. 1 and 3) was associated with the accumulation of four major sesquiterpenoids: DHG, HG, DMHG, and MHG (Figs. 4 and 5). The degree of resistance in cotton to Verticillium wilt, however, appears to be directly related to the amounts of DMHG and MHG produced by the infected vascular tissues. These two methylated compounds are derivatives of DHG and HG. It is now apparent that the nonmethylated sesquiterpenoids, DHG and HG, are produced by all infected cotton cultivars regardless of their level of disease resistance. Furthermore, we concluded, in contrast to others (12), that the total content of all four major sesquiterpenoids present in the infected tissues is not directly correlated to the varietal resistance.

The likely role of DMHG and MHG as important factors in the resistance of cotton to Verticillium wilt was obtained with various host-pathogen combinations, which provided three levels of responses to infection: resistance, tolerance, and susceptibility. The stem-puncture technique used for inoculation induced reproducible symptoms consistent with the wilt reaction expected from each host-pathogen combination. Therefore, variations in the host chemical response to infection allowed us to differentiate the general chemical reaction of all cottons from the response of resistant or tolerant cotton lines.

The highest levels of MHG and DMHG were detected in the most resistant cotton (SBSI), and the lowest, in the most susceptible cotton (70-110) (Figs. 4 and 5). Comparatively, intermediate amounts of both compounds were detected in the two Acala cottons. This observation certainly reflects their level of resistance to Verticillium wilt. Although the accumulation of the methylated derivatives directly correlates with the degree of resistance, the accumulation of HG and DHG was almost always lower in SBSI, the most resistant cotton line, than in all other cottons tested. Zaki et al (29) reported that although HG was the major constituent in crude extracts from inoculated cotton stems, the SBSI plants accumulated considerable DMHG (referred to as vergosin in the paper). They suggested that both compounds relate to varietal resistance. The results obtained in our study are consistent with their observation on DMHG but not on HG. It appears, therefore, that susceptibility of cotton to *Verticillium* is perhaps due to inability of the host to carry out as much methylation of DHG and HG.

The severe pathogenic behavior of the T-1 strain of *V. dahliae* on cotton was apparently not due to host inability to recognize and respond to infection by this strain, because inoculation with either SS-4 (mild) or T-1 (severe) strains of the fungus induced the accumulation of comparable amounts of sesquiterpenoids (Figs. 5

TABLE 1. Vertical distribution of sesquiterpenoids in vascular tissues of cultivars Seabrook Sea Island (SBSI) and Acala (A) SJC-1 cottons 10 days after hypocotyl inoculation with the SS-4 strain of *Verticillium dahliae*

Distance from inoculation site (cm)[a]	Cultivar	Sesquiterpenoids (μg/g fresh weight)[b]					
		DHG[c]	HG	DMHG	MHG	Total	Percentage methylated
Inoculation site	SBSI	151 ± 21	67 ± 12	205 ± 38	124 ± 18	547 ± 90	60
	A-SJC-1	325 ± 7	109 ± 6	49 ± 6	7 ± 6	490 ± 13	11
0–5	SBSI	33 ± 3	28 ± 3	96 ± 15	56 ± 11	213 ± 27	71
	A-SJC-1	86 ± 23	33 ± 11	12 ± 4	0	131 ± 38	9
5–10	SBSI	15 ± 2	11 ± 2	51 ± 1	33 ± 4	110 ± 2	77
	A-SJC-1	47 ± 10	25 ± 3	9 ± 1	0	83 ± 12	11
10–15	SBSI	11 ± 4	8 ± 1	36 ± 1	15 ± 4	70 ± 7	73
	A-SJC-1	101 ± 12	54 ± 11	22 ± 5	0	177 ± 6	12
15–20	SBSI	6 ± 0	5 ± 1	16 ± 1	9 ± 1	36 ± 4	68
	A-SJC-1	83 ± 16	56 ± 5	28 ± 1	0	167 ± 20	17
20–25	SBSI	Trace	Trace	Trace	Trace	Trace	...
	A-SJC-1	30 ± 9	15 ± 2	0	0	45 ± 11	0

[a] Cotton plants were puncture-inoculated at the hypocotyl with a conidial suspension (2-3 × 10^6/ml) of SS-4 strain of *V. dahliae*. Ten days after inoculation, the stems were divided into six sections. These sections were extracted separately for sesquiterpenoid analysis as described in the text.

[b] Mean and standard error of two experiments; each consists of 25 plants per cultivar. Contents of sesquiterpenoids are rounded-off to the whole numbers.

DHG = desoxyhemigossypol, HG = hemigossypol, DMHG = desoxy-6-methoxyhemigossypol, and MHG = 6-methoxyhemigossypol.

and 6). This, however, might be due to the capacity of the severe isolate to detoxify the methylated sesquiterpenoid phytoalexins.

In disease reactions where fungus-mediated detoxification of phytoalexins has been carefully examined, a direct relationship between conversions of phytoalexins to less toxic products and pathogenicity has been found. Usually, these conversions involve the metabolism of lipophilic phytoalexin compounds to more polar products by the introduction of hydroxyl groups in oxidative catabolism (26). A number of pathogenic fungi are known to detoxify phytoalexins through enzymic demethylation that involves monooxygenases (9,13,15,21,22,28). Although our demonstration of the capacity of the severe T-1 isolate of *V. dahliae* to cope with the presence of sesquiterpenoids provides no direct proof of the value of detoxification as a pathogenic attribute, it does provide circumstantial evidence that justifies further studies on probable relationships between the accumulation of methylated sesquiterpenoids, detoxification by demethylation, and pathogenicity in the cotton-*V. dahliae* interactions.

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