High-Performance Liquid Chromatographic Determination of Sesquiterpenoid Stress Metabolites in *Verticillium dahliae* Infected Cotton Stele

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A high-performance liquid chromatographic (HPLC) procedure is described for the determination of four sesquiterpenoid stress metabolites, hemigossypol (**1a**), 6-methoxyhemigossypol (**1b**), desoxyhemigossypol (**2a**), and 6-methoxydesoxyhemigossypol (**2b**), in cotton stele infected with *Verticillium dahliae*. Stress metabolites were quantitatively recovered from crude ethyl acetate extracts of infected stele tissues by a SEP-PAK C18 cartridge. Recovered samples were chromatographed on a Radial-PAK reversed-phase C8 column, eluted with an 0.1% aqueous phosphoric acid–methanol gradient, and monitored at 254 nm. The quantity of each compound was based on calibration with 4,4′-bis(*N*,*N*-dimethylamino)-benzophenone as an internal standard (ISTD). Variation coefficients of 3.87% (**1a**), 3.19% (**1b**), 3.66% (**2a**), 3.59% (**2b**), 4.57% (ISTD), and 4.4% (gossypol) were obtained for the molar responses of each. The retention time coefficients of variation were 2.8% (**1a**), 1.0% (**1b**), 2.0% (**2a**), 2.9% (**2b**), 0.3% (ISTD), and 0.2% (gossypol).

Hemigossypol (**1a**), desoxyhemigossypol (**2a**), and their methyl ether derivatives 6-methoxyhemigossypol (**1b**) and 6-methoxydesoxyhemigossypol (**2b**) were evaluated and identified as antifungal sesquiterpenoid stress metabolites in *Verticillium dahliae* infected cotton stele (Zaki et al., 1972a; Stipanovic et al., 1975b; Bell et al., 1975) (Figure 1). The resistance of different cotton cultivars to *V. dahliae* was attributed to the higher rate of accumulation of these compounds in the infected vascular tissues (Bell, 1967; Zaki et al., 1972a). Although the rapid accumulation of these compounds in diseased cotton tissues was noted and the role of these compounds was suggested as phytoalexins (Bell, 1969; Mace et al., 1976), the detailed disease physiological study of host plant, cotton (*Gossypium spp.*), and fungus (*V. dahliae*) with respect to these sesquiterpenoid stress metabolites was not available. A sensitive method capable of both quantitative and qualitative analyses of these sesquiterpenoids was needed in order to examine, in detail, the mechanism of resistance in cotton to *V. dahliae*. Pons (1977) pointed out that HPLC, in conjunction with column chromatographic isolation of these compounds, is the best approach to study the host plant resistance in cotton against *V. dahliae* infection.

Earlier studies using TLC with phloroglucinol (Zaki et al., 1972a,b; Stipanovic et al., 1975a,b; Bell et al., 1975) and colorimetric analysis with aniline (Hanny et al., 1978) as the developing reagents were limited in both quantitative and qualitative analyses. Waiss et al. (1978) developed a ^{1}H NMR analytical method that offered both quantitative and qualitative analyses of gossypol and its related sesquiterpenoid, but the method lacks the sensitivity required for small-size samples.

Abou-Donia et al. (1981) and Nomeir and Abou-Donia (1982) described an HPLC method for the analysis of gossypol, a preformed triterpenoid aldehyde in cotton. Their method utilizes an isocratic mobile phase to analyze gossypol and is not suitable for the analysis of the low levels of induced sesquiterpenoid stress metabolites in cotton. In this paper we report a rapid, precise, and sensitive HPLC method using a SEP-PAK C18 cartridge as a precolumn purification step, for the quantification of **1a**, **1b**, **2a**, and **2b** as well as gossypol in infected cotton stele. We also describe a simple preparative isolation method for these compounds. Complete quantitative and qualitative experimental data regarding the accumulation and activity of these compounds on several cotton varieties after infection with *V. dahliae* are covered in details in a separate publication.

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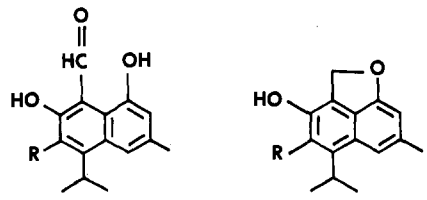
EXPERIMENTAL SECTION

Plant Tissue and Inoculation. Cotton plants of Acala SJC-1 (*Gossypium hirsutum*) or Seabrook Sea Island (*Gossypium barbadense*) cultivars were grown in 10-cm pots under greenhouse conditions. Plants (5–6 weeks old) were inoculated with a mild nondefoliating (SS-4) strain of *V. dahliae* Kleb. by the stem puncture method described by Erwin et al. (1965). Puncture inoculation with suspensions of 2×10^6 conidia/mL was made 1 cm apart, starting 3 cm above the soil line. Inoculated stems were harvested after 3–5 days. Vascular tissues (stele) free of any glands were obtained by stripping the cortical tissues. Steles were stored at −20 °C until extracted.

Extraction. During isolation of stress metabolites from cotton stele, extracts were protected from direct exposure to light, high temperature, active surfaces (silica gel, alumina), and basic conditions to avoid any autooxidation. Terpenoids present in the stele tissues of infected stems were extracted by a method modified from Zaki et al. (1972a). Inoculated steles were diced into small pieces and homogenized for 4 min in cold ethyl acetate containing 0.1% acetic acid (10 mL/g) with a 20-mm Polytron (Brinkman) generator at maximum speed. The homogenate was filtered through a coarse-grade sintered-glass funnel, and the residue was reextracted twice with an equal volume of the same solvent. Pooled ethyl acetate extracts were concentrated to dryness on a rotary evaporator at room temperature and dissolved in acetonitrile (2.0 g of fresh weight/mL). The crude extract was sonicated to enhance the solubilization of extracts in acetonitrile. Insoluble materials were removed either with membrane filters (0.45 μm, Waters) for analytical HPLC analysis or with Whatman No. 3 filters for the preparative LC.

Sample Preparation and Recovery. An aliquot of crude extract (100 μL) was diluted with distilled water (3.0 mL) and the resultant mixture loaded on a prewetted SEP-PAK C18 cartridge (Waters) with minimum applied pressure. The SEP-PAK cartridge was wetted first with 5.0 mL of methanol containing 0.1% acetic acid followed by 3.0 mL of distilled water. The sample was allowed to drip through the cartridge without any applied pressure



(1a) R = OH (2a) R = OH
(1b) R = OCH_3 (2b) R = OCH_3

Figure 1. Structure of hemigossypol (**1a**), 6-methoxyhemigossypol (**1b**), desoxyhemigossypol (**2a**), and 6-methoxydesoxyhemigossypol (**2b**).

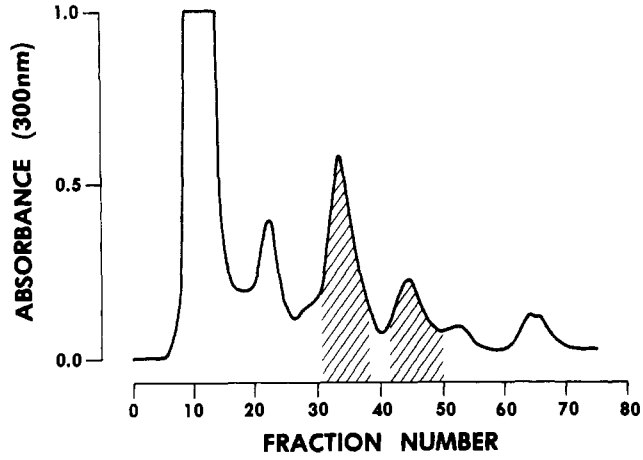


Figure 2. Preparative reversed-phase LC separation of crude extract from Seabrook Sea Island cotton stele tissues after 5 days of inoculation with the SS-4 strain of *V. dahliae*. The LC conditions are described under methods. Tubes 31–39 contained **2a** and **1a** and tubes 42–50 contained **2b** and **1b**.

or vacuum. After the sample was loaded completely, the cartridge was washed once with 50% aqueous methanol containing 0.1% acetic acid (5.0 mL). Sesquiterpenoids were then eluted with 2.0 mL of methanol containing 0.1% acetic acid. The methanolic eluate was dried under N_2, dissolved in acetonitrile to give a final volume of 400 μL (0.50 mg of fresh weight/μL), and analyzed by HPLC. The percent recovery of the SEP-PAK C18 purification step was determined by a triplicate run of crude extract (from 500 mg of fresh weight) spiked with gossypol (100 μg), or 4,4′-bis(*N*,*N*-dimethylamino)benzophenone (100 μg; J. T. Baker). A known amount of the latter compound was added routinely as an internal standard (ISTD) to determine the percent recovery for each unknown sample.

Quantitative HPLC Analysis of Sesquiterpenoids. Twenty microliters of the sample solution was chromatographed on a Radial PAK C8, reversed phase column (8.0 mm × 10 cm, 10 μm RCM housed in a Z-module Waters), under the following conditions: mobile phase, a gradient of 0.1% aqueous phosphoric acid–methanol (20–100% methanol for 30 min) delivered with two pumps (Waters, Model 6000 A) at a rate of 2.3 mL/min according to a concave gradient #8 of Model 660 Waters solvent programmer. The sesquiterpenoids were detected with a fixed-wavelength detector (Waters, Model 440): wavelength 254 nm; sensitivity 0.05 aufs or other setting appropriate for the concentration of sesquiterpenoids in the individual unknown sample. Under the conditions described for HPLC, the retention time for ISTD was 29.1 min, and the relative retention times for **2a**, **1a**, **2b**, and **1b** were 0.12, 0.31, 0.39, and 0.47 min, respectively. Sesquiterpenoids were quantitated by measuring the area under the peak relative to the ISTD. A standard solution containing known amounts of the sesquiterpenoids and the ISTD was chromatographed to calculate the mean molar response for each compound and was used for subsequent quantitation of samples. Sesquiterpenoid standards, except **2a**, were provided by R. Stipanovic, U.S. Department of Agriculture, National Cotton Pathology Research Laboratory, College Station, TX. Additional standards were purified from stele tissues of cotton plants inoculated with SS-4 strain of *V. dahliae*.

Isolation of Large Quantities of Sesquiterpenoids. Large quantities of purified sesquiterpenoids were required to confirm the identity of each compound by NMR and mass spectrometry and to determine the retention time and the molar response of detection in the analytical HPLC method. A 130-g fresh weight of infected stele tissues was extracted with acidified cold ethyl acetate as described previously. The filtered extract was loaded on a preparative column packed with C18 Bondapak (37–50 μm, 100 g, 4 × 21 cm). The column was preequilibrated with acetonitrile–H_2O (3:2, v/v), and chromatography was performed with the same solvent mixture delivered at the rate of 2.7 mL/min. The effluent was monitored at 300 nm and collected at the rate of 16.0 mL/tube. Two sesquiterpenoid-containing fractions (tubes 31–39 and tubes 42–50) were pooled and concentrated by partitioning with ethyl acetate containing 0.1% acetic acid. The organic phase was subsequently evaporated to dryness in a rotary evaporator at 30 °C and the residue dissolved in acetonitrile. These two partially purified fractions were further chromatographed on a semipreparative C18 column (Rsil, 10 μm, 10 mm × 25 cm Alltech) using a mobile phase of methanol–0.1% aqueous phosphoric acid (3:1, v/v), delivered at 1.7 mL/min with an Altex 110A pump. Sesquiterpenoids were detected with a Tracor 390 variable-wavelength detector (300 nm, sensitivity 2 aufs). Repeated injections of 200 μL from each of the previous two fractions were made, and compounds **2a** and **1a** were collected between 31.8 and 33.5 min and between 36.0 and 37.5 min, respectively, after the injections of the material contained in tubes 31–39. Compounds **2b** and **1b** were collected between 42.2 and 43.9 min and between 45.5 and 47.5 min, respectively, after the injection of the fraction-containing tubes 42–50. The compounds were recovered from the collected eluents by partitioning against ethyl acetate containing 0.1% acetic acid, and the subsequent organic layers were evaporated to dryness in a rotary evaporator at 30 °C. The purity of the collected compounds was determined with the analytical HPLC system described previously.

Identification of Sesquiterpenoids. Purity of individual sesquiterpenoid standards was determined by TLC, using several solvent systems, and by HPLC. Identity of each sesquiterpenoid was confirmed by ^1H NMR (200 MHz, Nicolet), ^{13}C NMR (JEOL, PFT 100), and mass spectrometry (EI and/or CI, VG 7070F, VG Analytical) as well as by peak enrichment, showing coincident retention time with the authentic standards (courtesy of R. Stipanovic) in the described analytical HPLC system. Spectra were in agreement with those reported by Stipanovic et al. (1975b) for **2a**, Zaki et al. (1972 a) for **1a**, Stipanovic et al. (1975b) for **2b**, and Bell et al. (1975) for **1b**.

Sample Decomposition. Seabrook Sea Island (*Gossypium barbadense*) plants were infected with *V. dahliae* (SS-4) in the same manner described earlier and harvested at 5 days after the inoculation. Infected steles were prepared for HPLC analysis as described previously. Samples were stored for 7 days under the following conditions: (a)

in an amber vial at −20 °C; (b) in an amber vial at 4 °C; (c) in an amber vial at room temperature; (d) in a clear vial at room temperature. These samples were examined periodically by HPLC to determine the decomposition patterns under different storage conditions. No attempt was made to identify the breakdown products.

RESULTS AND DISCUSSION

Sesquiterpenoid stress metabolites from infected cotton are reported to be relatively unstable during isolation and purification (Zaki et al. 1972a; Bell et al., 1975; Stipanovic et al., 1975b). One of the difficulties in isolating these compounds in large quantities was possibly due to rapid autooxidation under certain conditions. When infected steles were extracted directly with cold ethyl acetate containing 0.1% acetic acid instead of 95% ethanol (Bell, 1967; Zaki et al., 1972a,b), the extraction of polar constituents such as sugars and amino acids was minimized. The crude extract was concentrated to dryness and resuspended immediately in acetonitrile. Acetonitrile-insoluble impurities were precipitated and filtered off before the crude extract was chromatographed on HPLC. No sesquiterpenoids were detected in the acetonitrile-insoluble materials.

Sesquiterpenoid stress metabolites were recovered quantitatively from SEP-PAK C18 cartridges that were loaded with crude extracts from infected steles. Crude extracts in acetonitrile were diluted with water to increase the polarity of loading solvent. Polar impurities of crude extract were removed from the loaded cartridge with 5.0 mL of methanol and 0.1% acetic acid (1:1, v/v). Sesquiterpenoid stress metabolites were subsequently eluted by decreasing the polarity of eluting solvent to methanol containing 0.1% acetic acid. It was important that the initial loading solvent was allowed to drip through the cartridge at a slow rate. This was done to enhance the binding of more polar stress metabolites such as **1a** and **2a**. A typical HPLC chromatogram of SEP-PAK C18 purified extract of cotton stele is shown in Figure 3a. The percent recovery was measured by fortifying cotton stele (500 mg) with gossypol and ISTD prior to extraction, filtration, and preparation with SEP-PAK C18 cartridge. Samples were quantitatively analyzed with HPLC using external standards of gossypol and ISTD. The recovery ranged between 75.82 and 91.90% for gossypol and between 93.06 and 101.40% for ISTD. The mean percent recovery for gossypol was 85.91 ± 8.79% and for ISTD 97.34 ± 4.17%. For all subsequent analysis, known amounts of ISTD were added to the crude extracts of the unknown samples before the SEP-PAK C18 purification step.

The reversed-phase C8 analytical column that was used (Radial-PAK C8, 10 μm, Waters) required an extensive preconditioning in order to achieve an adequate chromatographic separation of all four sesquiterpenoids. This was performed by eluting a new column for 48 h with methanol–0.1% aqueous phosphoric acid (1:4, v/v) at 2.3 mL/min. While there was no detectable separation of **1a**, **1b**, **2a**, and **2b** without the preconditioning step, a complete resolution was achieved after the preconditioning of the column. The column was stable for more than 1 year of routine daily use after the preconditioning. As the column deteriorated, shorter retention times and the loss of resolution were observed. The stability of the preconditioned column was not examined in detail.

The retention times and the molar responses (expressed in units of peak area per nanomole) of **1a**, **1b**, **2a**, and **2b** are presented in Table I. For the retention time, coefficients of variations of 2.8% (**1a**), 1.0% (**1b**), 2.0% (**2a**), 2.9% (**2b**), 0.3% (ISTD), and 0.2% (gossypol) were obtained. The coefficients of variation of the molar responses were determined as 3.8% (**1a**), 3.19% (**1b**), 3.66% (**2a**), 3.59% (**2b**), 4.57% (ISTD), and 4.4% (gossypol). Therefore, this method has relatively good precision. The limit

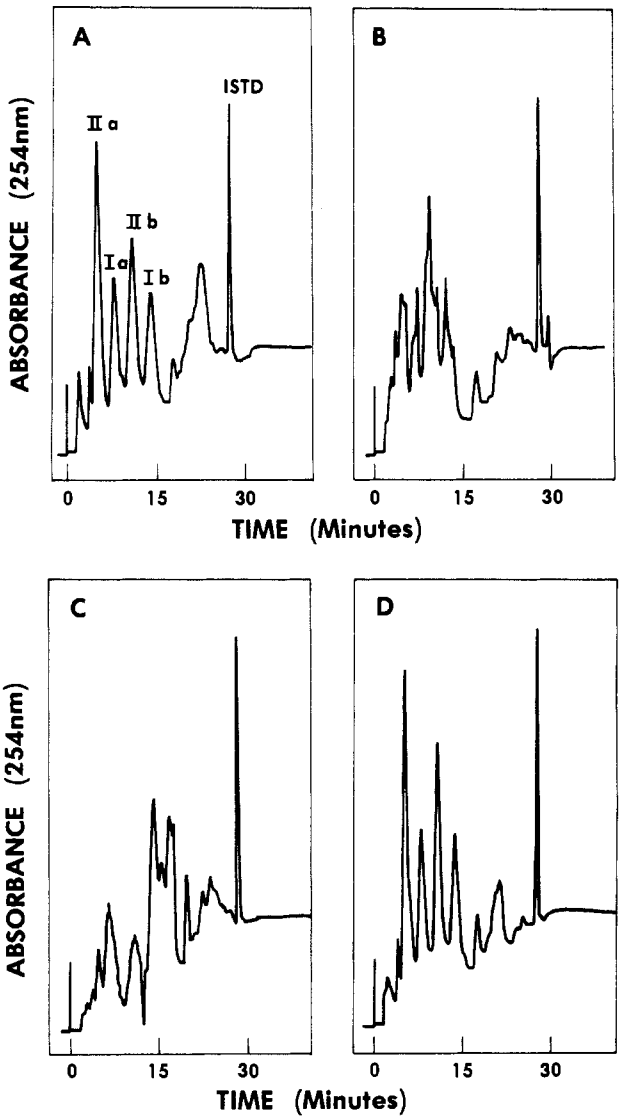


Figure 3. HPLC chromatograms of SEP-PAK C18 purified crude extracts from *V. dahliae* (SS-4) infected cotton stele (SBSI, 5 days after inoculation): (A) sample of 0 days after extraction and purification; (B) sample stored at room temperature in the presence of light for 24 h; (C) sample stored at room temperature in an amber vial for 48 h; (D) sample stored at −20 °C in an amber vial for 7 days.

Table I. HPLC Retention Times and Molar Responses of Sesquiterpenoid Stress Metabolites

compd	retention time[a]		molar resp,[a] cm²/nmol
	R_t, min	RRT (ISTD)	
desoxyhemigossypol (**2a**)	3.58 ± 0.07	0.123 ± 0.002	150.4 ± 5.5
hemigossypol (**1a**)	8.93 ± 0.59	0.307 ± 0.021	228.8 ± 8.8
6-methoxydesoxy-hemigossypol (**2b**)	11.30 ± 0.40	0.388 ± 0.013	153.3 ± 5.5
6-methoxy-hemigossypol (**1b**)	13.58 ± 0.30	0.467 ± 0.011	250.4 ± 8.0
gossypol	25.87 ± 0.04	0.889 ± 0.002	262.4 ± 11.66
4,4′-bis(*N*,*N*-dimethylamino)-benzophenone (ISTD)	29.10 ± 0.22	1.000	519.9 ± 23.8

[a] Mean of five determinations.

of detection was 10–20 ng, but the best reproducibility was obtained in the range of 0.5–2.0 μg of each sesquiterpenoid stress metabolite. Detector responses of all sesquiterpenoids tested and ISTD were linear over the range of 0.1–4.0 μg. Linear correlation coefficient (r^2) values were 0.9960 (**1a**), 0.9983 (**1b**), 0.9960 (**2a**), 0.9875 (**2b**), 0.9733 (ISTD), and 0.9999 (gossypol).

Sesquiterpenoid stress metabolites were separated and purified by running successive reversed-phase preparative LC and reversed-phase semipreparative HPLC. Two fractions (31–39, 42–50) containing compounds **2a**, **1a** and **2b**, **1b**, respectively, were collected from the preparative LC column (Figure 2). These fractions were partitioned with ethyl acetate containing 0.1% acetic acid, and the concentrated samples were chromatographed on a semipreparative HPLC. Semipreparative HPLC eluents containing appropriate compound were manually collected, and their purities were determined on the analytical HPLC system. Compounds collected from the semipreparative HPLC were greater than 95% chromatographically pure.

The possible decomposition of crude extract from diseased cotton stele tissues was reported (Zaki et al., 1972a; Bell et al., 1975; Stipanovic et al., 1975b). We have examined the stabilities of crude extracts under the storage conditions described in the Experimental Section. Within 24 h, the sample stored at room temperature in the presence of direct light showed degradation of all four sesquiterpenoids (Figure 3b), while samples stored in other conditions showed no sign of decomposition. After 48 h, the sample stored at room temperature in the presence of light showed complete degradation of **1a**, **1b**, **2a**, and **2b**, while the sample stored at room temperature but in an amber vial started to show some degradation of **1a** and **2a** (Figure 3c). By the fourth day after extraction, samples stored at room temperature had degraded completely regardless of light. Samples stored at +4 and −20 °C showed little or no sign of degradation 8 days after preparation (Figure 3d). Although high temperature and light induced degradation of sesquiterpenoids, we have observed also slow decomposition of these compounds in the samples stored at −20 °C for 3–4 months. This is due presumably to the reaction of the carbonyl group with sulfhydryl and amino groups in the crude extracts. Consequently, samples were prepared under minimal light exposure and kept under cold conditions.

The HPLC method described here provides highly reproducible quantitative and qualitative data on the induced stress metabolites in cotton plant subsequent to inoculation with *V. dahliae*. We believe it is adaptable to studies on other preformed terpenoids and sesquiterpenoids in cotton or similar terpenoids from other plant species. Furthermore, its sensitivity, accuracy, and precision make it appropriate for the physiological and chemical studies involving these compounds. The physiological studies on the host–parasite interaction utilizing the HPLC method described here are discussed elsewhere.

ACKNOWLEDGMENT

We extend our special thanks to Dr. Robert Stipanovic of the National Cotton Pathology Research Laboratory, USDA, SEA-AR, College Station, TX 77841, for supplying sesquiterpenoid standards. We are also grateful to M. Benson, W. Witt, and S. Tillin who performed ^{1}H NMR, ^{13}C NMR, and MS measurements.

Registry No. **1a**, 40817-07-0; **1b**, 50399-95-6; **2a**, 57765-65-8; **2b**, 40817-06-9.

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Received for review August 19, 1985. Accepted January 21, 1986. Reference to a company and/or product named by the department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others that may also be suitable.