Implication of Vergosin and Hemigossypol in the Resistance of Cotton to Verticillium albo-atrum

A. I. Zaki, N. T. Keen, and D. C. Erwin

Research Associate, Associate Professor, and Professor, respectively, University of California, Riverside 92502.

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ABSTRACT

Antifungal compounds accumulated in the stele of cotton plants inoculated with *Verticillium albo-atrum*, but none was detected in extracts from noninoculated plants. The major antifungal compounds isolated from inoculated plants were the gossypol-related naphthaldehydes, vergosin and hemigossypol. Experiments with infiltrated stem internode sections did not show a consistent correlation between varietal resistance to Verticillium wilt and the accumulation of phloroglucinol reactive compounds ("gossypol equivalents"), but antifungal activity was related to resistance. A purified phytotoxic protein-lipopolysaccharide from *V. albo-atrum* elicited production of the gossypol-related compounds, and elicited vergosin accumulation that was related to varietal resistance. Tolerant cotton plants inoculated with a mild isolate of *V. albo-atrum* accumulated phloroglucinol reactive compounds and antifungal activity more rapidly than plants inoculated with a severe, defoliating isolate. Vergosin and hemigossypol were antifungal to *V. albo-atrum* and were more active than gossypol.

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Additional key words: phytoalexins, protein-lipopolysaccharide complex, cross protection.

Schnathorst & Mathre (13) first demonstrated that inoculation of cotton (*Gossypium hirsutum* L.) cultivars tolerant to Verticillium wilt with mild isolates of *Verticillium albo-atrum* Reinke & Berth. before subsequent inoculation with severe isolates markedly reduced symptoms. This cross protection indicated that a degree of resistance in cotton to *Verticillium* is "inducible" (12, 14), and suggested that resistance might be chemically based. Extensive work by Bell (1, 2, 3) and Bell & Presley (4, 5) indicated that differential rates of production of gossypol and/or certain gossypol-related chemicals after infection are the basis for cross protection and varietal resistance. Most of this research is reviewed by Bell (3). Zaki et al. (16) failed to detect gossypol in extracts from *Verticillium*-inoculated cotton stems, but isolated two postinfectional inhibitors and identified them as hemigossypol (8-carboxaldehyde,1,6,7-trihydroxy,5-isopropyl,3-methyl naphthalene) and vergosin (8-carboxaldehyde, 1-hydroxy,5-isopropyl,7-methoxy, 3-methyl napthalene) (Fig. 1).

Because the role of postinfectional inhibitors and gossypol-related compounds in the resistance of cotton to Verticillium wilt was unresolved, we obtained data relative to this question.

MATERIALS AND METHODS.—*Culture methods.*—Cotton plants were grown in the greenhouse in 4-inch pots as previously described (6). Cultivars used were the highly susceptible Deltapine Smooth Leaf (DPL), the tolerant varieties Acala 4-42 and SJ-1, and the highly resistant Seabrook Sea Island 12B2 (Seabrook). Spores of *V. albo-atrum* were produced on a glucose-ammonium nitrate synthetic medium (10) and washed with water before use in plant inoculations or bioassays. Plants were inoculated with spore suspensions (generally 10^7/ml) of a severe defoliating isolate [V3H (6)] or a mild, nondefoliating isolate [SS-4(15)] of *V. albo-atrum* by the stem puncture method (6). In some experiments, plants were inoculated with the SS-4 isolate, and reinoculated with the V3H isolate after various intervals of time.

A protein-lipopolysaccharide (PLP) was isolated from culture fluids of the V3H isolate as previously described (7, 8) by diethylaminoethyl (DEAE) cellulose chromatography. Solutions of the protein-lipopolysaccharide (PLP) in 1 mM potassium phosphate, pH 6.8, the buffer alone, or *V. albo-atrum* spore suspensions were introduced into excised cotton stem internode sections following Bell's techniques (1). The sections were incubated at 25 C in petri dish moist chambers, and harvested after 48-72 hr.

Extraction of compounds and analytical methods.—We extracted antifungal compounds from stems of inoculated cotton plants or internode sections by stripping the cortical tissues, cutting the stems into ca. 0.5-cm pieces, and grinding for 30 sec at full speed in a Sorvall Omni-Mixer with 10 ml/g fresh wt of cold 95% ethanol. The extracts were concentrated at 45 C in vacuo and extracted 2-3 times with equal volumes of ethyl acetate. The pooled ethyl acetate fractions were dried with $MgSO_4$ and reduced in volume to 0.1 ml/g fresh wt tissue extracted. Care was taken to avoid taking extracts to dryness during evaporation. The ethyl acetate solutions were referred to as "crude extracts". The water fractions from the ethyl acetate extractions were also retained in early work.

Crude extracts were bioassayed for antifungal activity or were analyzed for phloroglucinol reactive

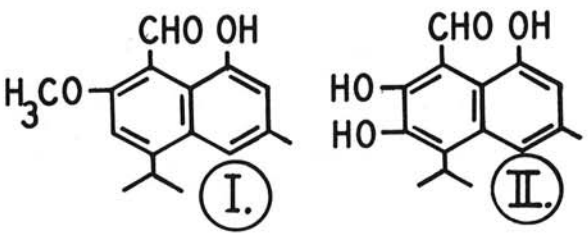

Fig. 1. Proposed structures of vergosin (I) and hemigossypol (II).

compounds by Bell's colorimetric technique (1). Results of the phloroglucinol analysis were reported as "gossypol equivalents" (1), as gossypol was used as a standard. Crude extracts were also chromatographed on thin-layer chromatography (TLC) plates as described elsewhere (16), and sprayed with phloroglucinol in 2 N HCl according to Bell (1).

In some experiments, the antifungal naphthaldehyde vergosin (16) was determined in the extracts by: (i) separation on GF_{254} TLC plates; (ii) elution of substances in the absorbing vergosin band with methanol; (iii) quantitation by colorimetric phloroglucinol analysis with standard purified (16) vergosin.

Antifungal bioassays.—Crude extracts or purified compounds were bioassayed against *V. albo-atrum* by a germ tube elongation method. Extracts were placed into microscope depression slides, and organic solvents were expelled under a stream of nitrogen. Then 0.2 ml of glucose-ammonium nitrate synthetic medium (10) at half strength supplemented with 5% ethanol and 0.25% Tween 20 (polyoxyethylene sorbitol monolaurate) and containing ca. 5×10^5 spores of the V3H isolate of *V. albo-atrum* were added to the slides, and the residues in the bottom of the slide were thoroughly agitated with a wooden toothpick, or aqueous crude extracts (0.1 ml) were similarly placed into the slide wells and 0.1 ml of normal strength synthetic medium containing 10% ethanol and 0.5% Tween 20 in addition to fungus spores were added and mixed thoroughly. Slides were incubated in a moist chamber at 25 C in the dark. The length of germ tubes was measured with an ocular micrometer at a magnification of X80. At least 25 measurements were made per treatment, and means were calculated.

A mycelial growth bioassay using *V. albo-atrum* was conducted in depression slides as above, except that after addition of the extract and evaporation of the solvent, 0.2 ml of molten potato-dextrose agar was added, thoroughly mixed, and allowed to solidify. A small drop (ca. 5 μliters) of V3H spores in water was added to the center of the slides, and the slides were incubated at 25 C in petri dish moist chambers. After 2-3 days, colony diameters were measured, colony areas computed, and ED_{50} values determined when pure compounds were bioassayed.

The presence of antifungal chemicals on thin-layer chromatography plates was determined by the previously described method (9) using *Cladosporium cucumerinum*.

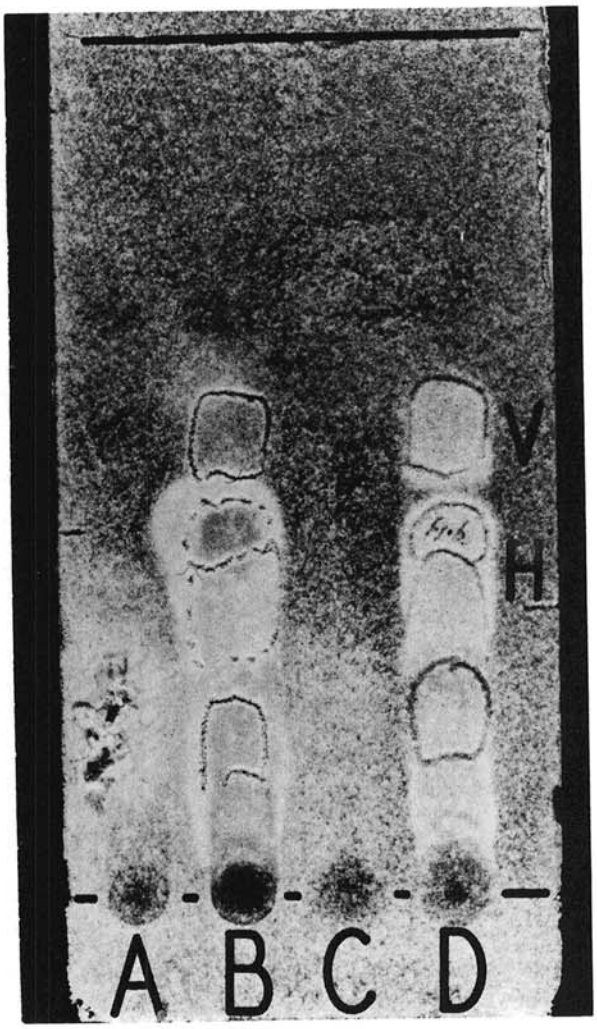

Fig. 2. Thin-layer chromatography bioassay of crude extracts from stems of *Verticillium*-infected or noninoculated cotton plants. Extracts were made at 14 days after inoculation of plants. Five μliters of each crude extract (= 0.05 g fresh wt stem tissue) were spotted on Silica Gel GF_{254} plates (0.375 mm) which were developed with hexane/ethyl acetate/methanol (60/40/1, v/v). Light areas denote antifungal compounds in the bioassay with *Cladosporium cucumerinum*. A = noninoculated SJ-1; B = V3H inoculated SJ-1; C = noninoculated Seabrook; D = V3H inoculated Seabrook. "V" and "H" denote R_F positions of purified vergosin and hemigossypol, respectively; upper and lower horizontal lines denote solvent front and starting lines, respectively.

RESULTS.—We observed considerable accumulation of antifungal compounds in xylem extracts from cotton plants inoculated with *V. albo-atrum*, thus confirming reports by Bell (2, 3) (Fig. 2). Similar extracts from noninoculated plants did not produce inhibitory spots in the TLC bioassay (Fig. 2), and stimulated the growth of *V. albo-atrum*

in the germ tube elongation bioassay relative to control spores on the synthetic medium only. All of the antifungal activity in extracts from inoculated plants apparently partitioned into ethyl acetate, since water fractions had no detectable activity in any of the bioassays.

Although no antifungal compounds were detected in extracts from unchallenged plants, at least two antifungal compounds were detected by the TLC bioassay in extracts from *Verticillium*-inoculated plants (Fig. 2). One of these spots corresponded to hemigossypol [Fig. 1, 2, and (16)]. A spot corresponding to vergosin was observed only in extracts from inoculated Seabrook Sea Island plants (Fig. 2). The bioassay plates indicated that inoculated SJ-1 plants contained more hemigossypol than did Seabrook, but produced little vergosin (Fig. 2). Considerable variation was observed between experiments, but the data in Table 1 are representative in indicating that more antifungal activity was present in stem internode sections of resistant plants inoculated with *V. albo-atrum* than in susceptible plants.

The purified *V. albo-atrum* PLP elicited production of phloroglucinol reactive compounds in stem internode sections of cotton stems (Table 1). However, the PLP elicited greater quantities of phloroglucinol reactive material in susceptible than in resistant cotton varieties. In no case were phloroglucinol reactive compounds observed in control sections incubated with buffer or water only. Amounts of phloroglucinol reactive compounds elicited were proportional to the PLP concentration. In typical experiments with Seabrook internode sections, PLP concentrations of 0.45, 1.12, and 5.0 mg/ml elicited production of 15, 42, and 118 μg gossypol equivalents/g fresh wt tissue.

Analysis of vergosin confirmed the TLC bioassay (Fig. 2) in showing that this compound was present in higher concentration (74 ppm) in PLP-treated Seabrook Sea Island stem segments than in DPL (4.8 ppm) or SJ-1 (4.8 ppm) (Table 1). Bioassays also showed that the crude extracts from PLP-treated plants inhibited fungus growth relative to extracts from unchallenged plants and that more activity was obtained from Seabrook Sea Island.

Inoculation of Acala 4-42 or SJ-1 cotton plants with the nondefoliating SS-4 isolate of *V. albo-atrum* cross protected against subsequent inoculation with the severe defolating V3H isolate. In these experiments, cross protection was first observed at 48 hr after inoculation (Fig. 3).

Inoculated plants began accumulating phloroglucinol reactive material in the xylem at ca. 36 hr after inoculation (Fig. 4), but the rate of accumulation was faster in plants inoculated with the mild SS-4 isolate than with the severe V3H isolate. Bioassays of crude extracts from these plants similarly showed that antifungal activity accumulated more rapidly in plants inoculated with the SS-4 isolate (Fig. 4).

In the agar bioassay, purified vergosin gave an ED_{50} value of 60 μg/ml against *V. albo-atrum* as compared to a value for gossypol above 100 μg/ml. Vergosin at 100 μg/ml completely inhibited growth of *V. albo-atrum*. Although purified hemigossypol was toxic to *V. albo-atrum*, no attempts were made to calculate an ED_{50} value because of the extreme reactivity of this compound (16). Hemigossypol appeared to be toxic at approximately the same

TABLE 1. Phloroglucinol reactive compounds (as gossypol equivalents), vergosin, and antifungal activity in crude extracts from cotton stem internode sections infiltrated with *Verticillium albo-atrum* spores or purified *V. albo-atrum* protein-lipopolysaccharide (PLP) complex[a]

Infiltrated	Variety	Gossypol equivalents[b]	Vergosin[c]	Inhibition of germ tube growth[d]
		μg/g fresh wt stems	μg/g fresh wt	%
Fungus spores[e]	DPL (S)[g]	30		75
Fungus spores	SJ-1 (T)	55		91
Fungus spores	Seabrook (R)	31		99
PLP[f]	DPL (S)[g]	42	4.8	40
PLP	SJ-1 (T)	21	4.8	48
PLP	Seabrook (R)	11	74	88

[a] Internode sections prepared according to Bell (1); sections were incubated at 25 C for 48 hr; no phloroglucinol positive material (gossypol equivalents), antifungal activity, or vergosin was detected in extracts from stem segments infiltrated with water or 1 mM potassium phosphate only.

[b] Determined colorimetrically against gossypol standards.

[c] Estimated by phloroglucinal analysis after elution from thin-layer chromatography plates.

[d] Germ tube elongation bioassay with *V. albo-atrum* spores and 10 μliters crude extract. Data are relative to those obtained from spores placed on synthetic medium only.

[e] 10^7 spores/ml of the V3H isolate were infiltrated into segments.

[f] PLP purified by DEAE cellulose chromatography (7, 8); 1.12 mg/ml in 1 mM potassium phosphate, pH 6.8 was infiltrated into segments.

[g] (S) = susceptible variety; (T) = tolerant; (R) = resistant.

concentrations as vergosin, and was toxic in the TLC bioassay at a minimum of 5 μg spotted.

DISCUSSION.—Our data are consistent with those of Bell (1, 2) and Bell & Presley (4, 5) indicating that production of gossypol-related compounds occurs in cotton stems inoculated with *V. albo-atrum* spores. We found that the major antifungal compounds formed are hemigossypol and vergosin [Fig. 1, and (16)], although other unidentified antifungal compounds and phloroglucinol reactive but nonantifungal compounds were also observed by TLC of crude extracts. In contrast to the early reports from Bell's laboratory (1, 2), we have never detected the production of gossypol in fungus-inoculated cotton stems, and our experiments with spore-infiltrated stem segments did not show a consistent correlation between varietal resistance to *Verticillium* and "gossypol equivalents". We did observe, however, a relationship between antifungal activity produced by spore-infiltrated stem segments of cotton varieties and their *Verticillium* resistance (Table 1). Furthermore, the specific antifungal compound, vergosin, was produced in greater quantity in resistant Seabrook Sea Island plants challenged with *V. albo-atrum* or with the purified protein-lipopolysaccharide complex than in susceptible plants (Fig. 2, Table 1). Vergosin is only slightly reactive with phloroglucinol, whereas hemigossypol is strongly reactive (16). In our experience, hemigossypol is the major phloroglucinol reactive constituent in crude extracts from inoculated cotton stems, but Seabrook plants also accumulate considerable vergosin. We conclude, therefore, that phloroglucinol reactivity of crude extracts, although perhaps a useful preliminary indicator for the production of antifungal compounds in cotton stems, is not a reliable quantitative measure of their accumulation.

Although hemigossypol was produced by all cotton cultivars after inoculation with *V. albo-atrum*, vergosin was only detected by TLC bioassay in the resistant Seabrook (Fig. 2). Although the significance of this finding remains unexplored, it represents a marked distinction between the susceptible and resistant varieties used here.

Considerable evidence (7, 8, 11) indicates that a fungus-produced PLP complex may be involved in symptom formation in *Verticillium*-infected cotton plants. The data presented here show that the PLP elicits production of vergosin and other gossypol-related compounds in stem internode sections. It is possible that the PLP functions as an elicitor of hemigossypol and vergosin production during Verticillium wilt. The occurrence of such a mechanism would present the interesting situation in which a pathogen-produced metabolite acted not only to injure host tissue, but also to invoke an apparent plant defense system.

The fact that the mild SS-4 isolate of *Verticillium* elicited more rapid accumulation of antifungal activity and phloroglucinol reactive material in tolerant cotton plants (Fig. 4) suggests that the antifungal compounds may be the basis for the cross

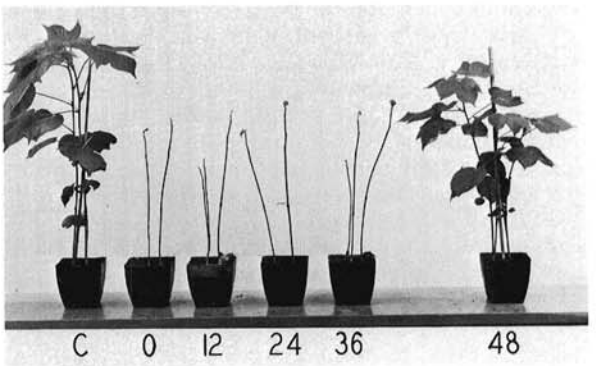

Fig. 3. SJ-1 cotton plants were not inoculated (C) or inoculated with the SS-4 isolate of *Verticillium albo-atrum*, then reinoculated with the V3H isolate after the noted periods of time in hours. Plants were photographed at 14 days after final inoculations were made.

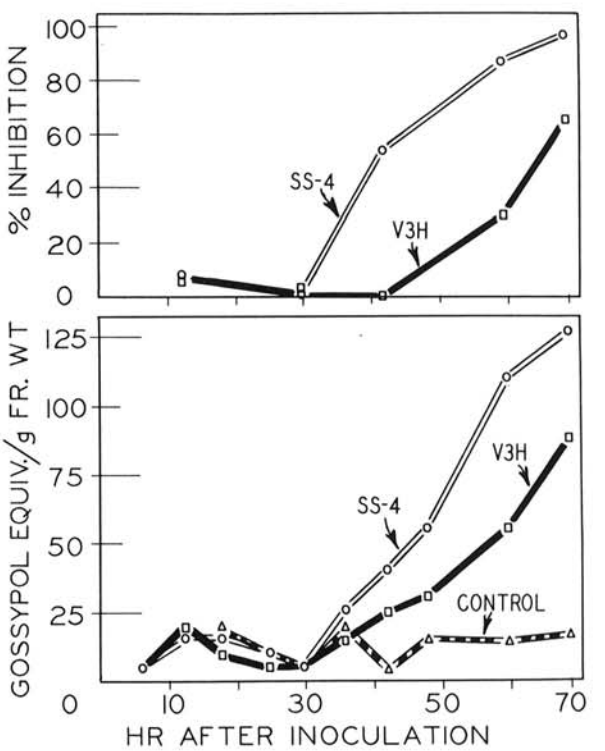

Fig. 4. Antifungal activity (upper) as determined by the germ tube elongation method with phloroglucinol reactive material as μg gossypol equivalents per g fresh wt tissue (lower) in crude extracts from SJ-1 cotton plants that were not inoculated (control) or at various times after inoculation with the SS-4 and V3H isolates of *Verticillium albo-atrum*. Percent inhibition values (upper) were calculated relative to *V. albo-atrum* spores placed in synthetic medium only. Five μliters of crude extract were used per bioassay. Extracts from noninoculated plants stimulated fungus growth at all sampling periods.

protection phenomenon observed when such plants are first inoculated with mild, then with severe, isolates of *V. albo-atrum*. It is of interest that the initial time of appearance of cross protection at ca. 48 hr (Fig. 3) occurs when both phloroglucinol reactive compounds and antifungal activity are much higher in SS-4 inoculated plants than in plants inoculated with the severe V3H isolate (Fig. 4).

On the whole, our data support, but do not prove, the hypothesis (1, 3) that the de novo production of gossypol-related compounds in the xylem of infected plants constitutes a defense mechanism in cotton to *Verticillium*. Before a final conclusion can be made regarding the physiologic role of such a defense mechanism in the determination of resistance and susceptibility, it appears necessary to obtain data relative to: (i) fungitoxic levels of the gossypol-related naphthaldehydes at specific, localized infection sites in the xylem; (ii) quantitation of actual fungus colonization of the vascular system of infected resistant and susceptible plants; (iii) rates of production of the gossypol-related compounds in near-isogenic resistant and susceptible cultivars; (iv) rates of production of these compounds in petioles and leaves, where resistance or susceptibility is most crucial in nature; (v) whether introduction of purified preparations of the antifungal naphthaldehydes into inoculated susceptible plants will produce a more resistant reaction.

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