Terpenoid Accumulation in Hypocotyls of Cotton Seedlings During Aging and After Infection by Rhizoctonia solani

R. E. Hunter, J. M. Halloin, J. A. Veech, and W. W. Carter

Research Plant Pathologist, Research Plant Physiologists, and Research Plant Pathologist, respectively, Agricultural Research Service, U. S. Department of Agriculture, National Cotton Pathology Research Laboratory, College Station, TX 77840.

Present address of senior author: Southeastern Fruit and Tree Nut Laboratory, P. O. Box 87, Byron, GA 31008.

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ABSTRACT

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The resistance of cotton seedlings to *Rhizoctonia solani* increased between 5 and 12 days after planting. Concentrations of the terpenoids desoxyhemigossypol, desoxy-6-methoxyhemigossypol, hemigossypol, 6-methoxyhemigossypol, gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol were higher in extracts of healthy hypocotyls of 12-day-old cotton seedlings than in those of 5-day-old seedlings. The concentration of each compound in hypocotyls of either age increased even more for the first 48 hr after inoculation with *R. solani*. However, rates of postinfection increases in concentrations of the compounds were not significantly different between 5- and 12-day-old seedlings. Terpenoid compounds may be involved in the age-related increased resistance of cotton seedlings to *R. solani* because of differences in concentrations occurring before infection or in total concentrations present after infection. However, the toxicity of the terpenoid compounds to *R. solani* must be thoroughly tested before it is known whether they play an active part in the age-related resistance.

Additional key words: *Gossypium*, seedling disease, phytoalexins, disease resistance, hemigossypol, gossypol, soreshin.

Gossypol (G) and the related terpenoid compounds, which accumulate in lysigenous glands of healthy cotton plants (*Gossypium* sp.), also may behave as phytoalexins in other tissues of the plant. Bell (1) demonstrated that infection with *Verticillium dahliae* Reinke and Berth, or chemical irritation of boll and stele tissues of *Gossypium hirsutum* L. and *G. barbadense* L. induced new G synthesis. He also found that G inhibited spore germination of certain fungi, and that the concentration of G required for inhibition depended on the fungal species. Bell (2) later showed that the rates of synthesis of gossypol-related compounds in cotton stems and intact plants shortly after inoculation with *V. dahliae* were related directly to host resistance. Zaki et al. (10) were not able to show a consistent relationship between cultivar resistance of cotton to Verticillium wilt and the presence of phloroglucinol-reactive compounds but were able to relate antifungal activity of extracts to resistance. They also demonstrated the fungistatic properties of hemigossypol (HG) and of a compound identified as the terpenoid aldehyde, vergosin. Stipanovic et al. (8) subsequently showed that the proposed structure of vergosin was incorrect, and identified the compound as 3-hydroxy -5-isopropyl -4-methoxy -7-methyl -2*H*-naptho [1,8-bc] furan [trivial name, desoxy-6-methoxyhemigossypol (desMHG)].

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Because of the fungistatic properties of gossypol-related compounds, Hunter attempted to relate the synthesis of total phloroglucinol-reactive compounds in cotton seedlings inoculated with *Rhizoctonia solani* Kühn to increased resistance associated with seedling age (5). Although he found a definite increase in the total phloroglucinol-reactive compounds after inoculation with *R. solani*, no apparent differences occurred between the rates of accumulation in 5- and 12-day-old seedlings.

Recently, a number of fungitoxic gossypol-related compounds from cotton plants were identified and methods for their quantitation were devised (3, 4, 8, 9). With these techniques, we determined the concentrations of seven terpenoid compounds in hypocotyls at different time intervals after inoculation of 5- and 12-day-old seedlings with *R. solani*. A preliminary report of this work was published (6).

MATERIALS AND METHODS

Production of diseased tissue.—Seeds of a glandless cultivar of *Gossypium hirsutum* L. 'Acala G 9098', were germinated in paper towels in the dark at 30 C. Two groups of seed were germinated 1 wk apart. After 27 hr, seedlings were reset in the germination towels so that the cotyledons were above the top of each rolled towel. The rolls were placed upright in 2 cm of water in a beaker and incubated in a growth chamber with a light (26,000 lux) period of 14 hr at 28 C and a dark period of 10 hr at 18 C.

Seedlings were inoculated at 5 or 12 days after planting and were harvested at 0, 12, 24, 48, and 72 hr after inoculation.

A single isolate (63 SD 2) of *R. solani* was maintained on potato-dextrose agar (PDA) at 25 C by weekly transfer. Inoculum was produced by growing the fungus in petri dishes, each containing 10 ml of a liquid medium (5). After 5 days of incubation at 25 C, the medium was decanted and the fungal mat was rinsed in distilled water and cut into strips 3 × 9 mm. Plants were inoculated by placing one strip across each hypocotyl just below the top of the germination towel.

Extraction and quantitation of terpenes.—Plants were removed from the germination towels, rinsed with distilled water, blotted dry, and placed on ice. Hypocotyl segments containing lesions were cut crosswise just above and below the lesion, weighed, and placed in a cold solution of 0.25% $NaHSO_3$ in 95% ethanol. Comparable segments from healthy plants were harvested at the same time as those from diseased plants. The tissues were triturated in a Sorvall Omni-Mixer at top speed for 3 min. The resulting homogenate was centrifuged at 15,000 *g* for 15 min. The supernatant fluid was saved, and the pellet was resuspended in fresh cold $NaHSO_3$-ethanol solution and recentrifuged. The supernatant fluids were combined and stored in amber bottles at −20 C.

Two volumes of 50%-saturated, aqueous sodium chloride solution and one volume of ethyl acetate (EA) were added to each ethanolic extract (one volume) and mixed in a separatory funnel. This, and all subsequent steps were done in a dimly-lighted room. The EA was separated and another volume of EA was mixed with the aqueous ethanol fraction. The combined EA fractions were washed twice with one volume of 50% saturated aqueous sodium chloride solution and dried over anhydrous sodium sulfate, and then the EA was removed in vacuo at 30 C. The residue was dissolved in a small amount of EA (100 μliters EA/g fresh tissue); hereafter this fraction is designated the crude terpenoid sample.

The terpenoid aldehydes, HG, G, 6-methoxyhemigossypol (MHG), 6-methoxygossypol (MG), and 6,6′-dimethoxygossypol (DMG), were separated on thin layers of polyamide powder developed with benzene:chloroform:methanol:acetic acid (150:50:3:2,v/v). From each crude terpenoid sample, 200-300 μliters were streaked half-way across the bottom of each of two plates, and the plates were developed immediately. After development, the plates were air-dried for 3 min and sprayed with phloroglucinol reagent (equal parts of concentrated hydrochloric acid and 5% phloroglucinol in ethanol). The five individual colored bands were scraped from the plates into separate beakers and left overnight for full color development. The next day, 7 ml of 90% ethanol were added to each beaker. The mixture was stirred for 15 min, centrifuged at 10,000 *g* for 15 min, and the colored liquid was decanted. The absorbance of each phloroglucinol derivative then was determined at its λ max, and the concentrations of terpenoid aldehydes were calculated from ε values given by Bell et al. (3). Blanks used in the spectrophotometric determinations were prepared by treating a sample of 95% ethanol containing 0.25% $NaHSO_3$ in the same manner as the extracts from plant tissues.

The concentrations of terpenoids were corrected with percentage of recovery values obtained by adding known quantities of the pure terpenoids to triturated healthy plant tissue and determining the amounts recovered by the procedure.

The concentrations of the two desoxy terpenoids, desoxyhemigossypol (desHG) and desMHG, were determined after first separating them from the other terpenoids on silica gel 7GF plates. Thirty to 300 μliters of the crude terpenoid samples were streaked on plates, which were developed with either ethyl ether:naphtha (30:70,v/v) for desHG or with benzene for desMHG. The band of desHG was located under shortwave UV (254 nm) according to its R_f value (8) and the desMHG was located under longwave UV (365 nm) as a dull orange fluorescent band after a few seconds of exposure (8). The bands were scraped into micro-funnels containing sintered glass disks and the compounds were eluted with 95% ethanol until 3 ml of ethanol was collected in a test tube under vacuum. The absorbance was read immediately and the concentrations were calculated according to ε values given by Stipanovic et al. (8). The concentrations of the desoxy compounds then were corrected with percentage of recovery values obtained as for the other terpenoids. However, the source of the desoxy compounds was from thin-layer plates because purified samples of these two compounds were not available. These recovery values and those for the other terpenoids were obtained from two replications of each of three concentrations that extended over the range of concentrations found in the plant extracts.

The concentrations of terpenoids reported here are the means of corrected concentrations determined in duplicate from each of three replications of plant material.

RESULTS

Disease development.—After 12 hr, a slight discoloration appeared on the hypocotyl areas covered by *R. solani* inoculum. The discoloration on 12-day-old seedlings was not as pronounced as that on 5-day-old seedlings. After 24 hr, definite sunken lesions were visible on seedlings of both ages. At 72 hr after inoculation, 5-day-old seedlings contained lesions in which the stele was destroyed; in 12-day-old seedlings, invasion of the stele had just begun. Lesions on seedlings of both ages had extended up and down the hypocotyl beyond the area of inoculation. Disease development generally progressed about 24 hr more quickly in 5-day-old than in 12-day-old seedlings.

Terpene concentration.—The recovery percentages were different for each terpene and generally were higher at the higher concentrations. The recovery percentages for the desoxy compounds were curvilinear functions of the concentration, but were linear for the other compounds. Examples of recovery percentages for each terpene at 100 and 25 nmoles/g were: desHG 43 and 34, desMHG 88 and 78, HG 60 and 60, MHG 72 and 62, G 31 and 23, MG 43 and 34, and DMG 40 and 33, respectively.

The concentrations of individual terpenoid compounds in hypocotyls of healthy seedlings were higher in 12-day-old seedlings than in 5-day-old seedlings (Table 1). Most

of the terpenoid compounds increased in concentration throughout the 72-hr period following inoculation (Fig. 1). Exceptions were desHG, which decreased in concentration, and desMHG and HG which did not

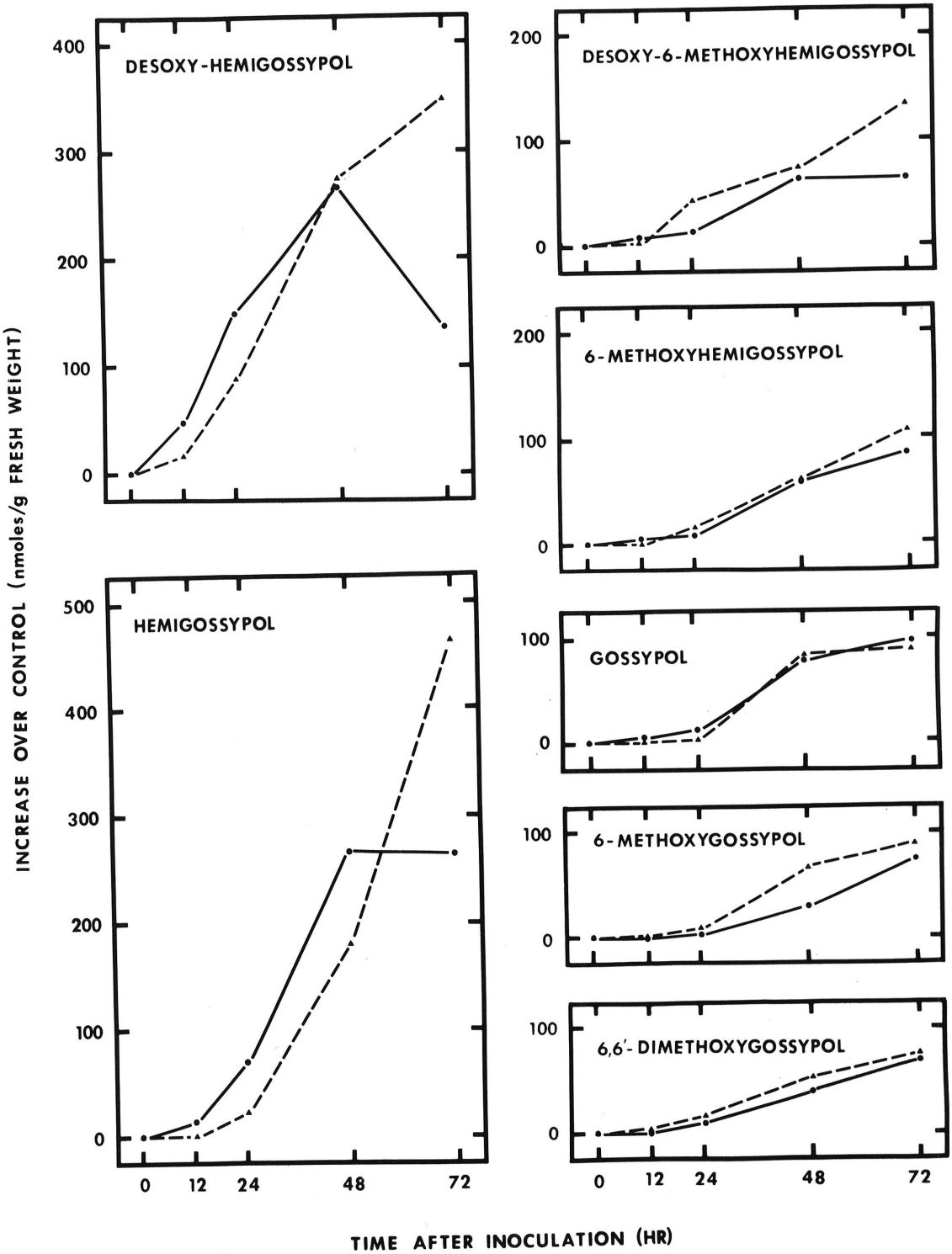

Fig. 1. Increases in concentrations (over healthy control plants) of terpenoid compounds in hypocotyl extracts of seedlings inoculated with *Rhizoctonia solani* when 5 (•——•) and 12 (▲- - -▲) days old.

TABLE 1. Concentrations of terpenoid compounds in hypocotyl extracts of healthy 5- and 12-day-old cotton seedlings

Compound	Terpenoids from fresh seedling hypocotyls at age: 5 days (nmoles/g tissue)	12 days (nmoles/g tissue)
Desoxyhemigossypol	12.2 ± 2.2[a]	25.0 ± 1.0
Desoxy-6-methoxyhemigossypol	0.7 ± 0.7	7.4 ± 2.8
Hemigossypol	2.8 ± 3.3	25.2 ± 5.0
6-Methoxyhemigossypol	5.8 ± 3.0	48.4 ± 4.1
Gossypol	2.6 ± 0.8	30.6 ± 2.7
6-Methoxygossypol	5.8 ± 1.2	48.4 ± 2.4
6,6′-Dimethoxygossypol	0.0	13.5 ± 6.4

[a]Mean and standard deviation based on three replicates.

change in concentration, during 48 to 72 hr after inoculation of 5-day-old seedlings; however, marked increases in each of these sesquiterpenoids occurred during the first 48 hr after inoculation.

DISCUSSION

Earlier, Bell et al. (4) described the isolation of six terpenoid aldehydes from cotton roots. Mace et al. (7) found five of these aldehydes in healthy cotton roots. We isolated these same five aldehydes and the two precursors from the hypocotyls of healthy cotton seedlings and found an increase in the concentrations of all compounds as the seedlings aged or after they were inoculated with *R. solani*.

The decrease in desHG and the failure of HG and desMHG to continue to accumulate between 48 and 72 hr after inoculation of 5-day-old seedlings is consistent with the pathways proposed by Bell et al. (4). Because much of the cortical tissue was destroyed 48 hr after inoculation of 5-day-old seedlings, it would be logical to suppose that the precursors of HG and MHG, (desHG and desMHG, respectively) would cease to be formed but would continue to be autoxidized to HG and MHG (8). These two compounds in turn form G, MG, and DMG by random dimerization (3).

The rate of accumulation of the terpenes in seedlings of both ages following inoculation with *R. solani* did not differ except for desHG, desMHG, and HG between 48 and 72 hr after inoculation of 5- and 12-day-old seedlings. Thus, any resistance in the older seedlings which might be attributable to the presence of terpenes would depend on the higher concentrations present either before inoculation or after infection.

Gossypol-related compounds have been found to be fungistatic and are thought to play a role in the resistance to Verticillium wilt of cotton (1, 2, 7, 10). Because of their fungistatic properties and the higher concentrations we found in older seedlings, there is the possibility that gossypol-related compounds contribute to the age-related increase in resistance to *R. solani*. A more definite role in this disease can be more accurately assessed only after thorough bioassays.

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