Terpenoid Aldehydes in Root-Knot Nematode Susceptible and Resistant Cotton Plants†

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High-pressure liquid chromatography (HPLC) with a reversed-phased column was used to determine whether a correlation existed between the concentration of terpenoid aldehydes (TAs) in roots and leaves of cotton (*Gossypium hirsutum* L.) plants and the level of host plant resistance to the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN). Several susceptible and resistant lines of glanded and glandless cotton plants were examined. Root TA contents of three resistant lines increased by the fourth day after inoculation. However, two of the resistant lines, one glanded and the other glandless, had very low intrinsic TA contents. While they increased after inoculation, they were still much lower than those of a susceptible glanded line. Thus, increases in TAs apparently cannot be correlated with, or explain resistance to, the RKN in all lines. Analyses of TAs in leaves did not prove helpful in identifying trends that could be correlated with resistance.

INTRODUCTION

The root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN) is a sedentary endoparasite that retards growth and development of cotton *Gossypium hirsutum* L. by attacking the root system, causing galling, stunting, and other adverse effects. Shepherd et al. (1988) reported that the most RKN resistant cultivars (Aub-634 and M-120 RNR, glanded) and 89-8275 (glandless) contained from 1200 to 5000 eggs per plant, whereas the susceptible lines Coker-201 (glanded), Aub-201 (glandless), and M-8 (glanded) contained from 6000 to more than 100 000 eggs per plant at 40 days after inoculation. Production of a large number of RKN eggs in susceptible roots in a relatively short time is associated with a tremendous amount of damage inflicted upon the young cotton seedlings by the nematode. As the galls increase in size, the root cortex surrounding the galls splits, exposing a relatively large area of the central cylinder (Mace et al., 1978). RKN also increases the incidence and severity of other soil-borne diseases such as fusarium wilt caused by *Fusarium oxysporum* Schlect f. sp. vasinfectum (Atl.c.) Snyd. and Hans (Bell, 1986).

Gossypol and related terpenoid aldehydes (TAs) including hemigossypolone and the heliocides H_1, H_2, H_3, and H_4 are toxic compounds that occur naturally in upland (*G. hirsutum*) cotton (Stipanovic et al., 1988). They have been reported to contribute to the resistance of cotton to the bollworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.) (Giebel, 1974; Hedin et al., 1988). In earlier studies, RKN infection was shown to induce the biosynthesis of TAs that may contribute to the resistance of cotton against this nematode (Hedin et al., 1984; Shepherd et al., 1988). Other cottons, notably *Gossypium barbadense*, biosynthesize several methoxylated TAs in addition to those listed. Veech (1978) found a correlation between changes of the concentrations of the methoxylated TAs after infection and RKN resistance. Garas and Waiss (1992) found a correlation between accumulation of these methoxylated TAs after inoculation with *Verticillium dahliae* (Kleb) and *G. barbadense* lines known to be resistant to this fungus.

With regard to location in the plant, gossypol is the major TA in the roots (Bell et al., 1986); however, the leaves contain up to 90% of the C_{25} TA heliocides H_1, H_2, H_3, and H_4, with lesser amounts of gossypol and hemigossypolone (Stipanovic et al., 1988). Heliocides H_2 and H_3 (less toxic than H_1 and H_4) are biosynthesized from the Diels–Alder reaction of hemigossypolone with *trans*-β-ocimene, and their formation leads to intermediate levels of insecticidal activity. Heliocides H_1 and H_4, the more toxic of the heliocides, are biosynthesized by a Diels–Alder reaction of hemigossypolone with myrcene (Bell et al., 1986).

The objective of this study was to investigate whether there was a relationship between the TA concentration in the roots and/or leaves of several RKN resistant and susceptible lines of cotton cultivars and the level of host plant resistance to the RKN.

MATERIALS AND METHODS

Cultivars and Race Stocks. RKN susceptible cotton lines were selected and grown in field plots by Shepherd (1974, 1979, and 1982) at Auburn University and Mississippi State University. RKN susceptible lines were Aub-201 (glandless) and M-8 and Coker-201 (glanded). RKN resistant lines were 89-8275 (glandless) and Aub-634 and M-120 (glanded) (Table 1).

Greenhouse Methods and Harvesting Samples for the Analyses. Inoculum was obtained from previously RKN infested susceptible cotton. Procedures used were those of Shepherd (1979) or variations thereof. When the plants were 40 days old, the roots were harvested and cleaned thoroughly with a low-pressure water spray. The roots were placed in sealable plastic containers and shaken for 3 min in 25 mL of 20% Clorox. The Clorox–egg solution was then poured onto a two-screen assembly (200-mesh over 500-mesh screen), and the Clorox was washed from the eggs with water. The number of RKN eggs per root had previously been determined by counting the eggs under a light microscope (Shepherd, 1986; Shepherd et al., 1988; Jenkins et al., 1993).

Cotton seeds of lines listed above were planted in a greenhouse in 250-cm^3 pots of autoclaved soil that either had been inoculated

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Table 1. Root Gossypol Concentration of RKN Susceptible and Resistant Cotton Plants (Milligrams per Gram of Dry Weight)[a]

		RKN eggs/plant (as % of M-8)					
days after inoc	inoc[b]	M-8 GL,[c] 100.0	Aub-201 gl, 54.2	Coker-201 GL, 53.8	89-8275 gl, 2.2	M-120 GL, 1.7	Aub-634 GL, 0.9
4	-	2.09	0.16	0.40	0.14	0.16	1.80
	+	1.97	0.23	0.38	0.18	0.41	4.25
		-0.12[d]	+0.07	+0.02	+0.04	+0.25	+2.45
8	-	3.20	0.08	0.08	0.23	0.36	1.75
	+	4.85	0.22	0.40	0.25	0.38	4.77
		+1.65	+0.14	+0.32	+0.02	+0.02	+3.02
12	-	3.93	0.08	0.07	0.13	0.18	1.87
	+	5.89	0.15	0.43	0.11	0.04	5.58
		+1.96	+0.07	+0.36	-0.02	-0.14	+3.71
16	-	1.47	0.16	0.57	0.11	0.23	2.40
	+	4.15	0.26	0.39	0.22	0.27	5.00
		+2.68	+0.10	-0.18	+0.11	+0.04	+2.60
20	-	0.83	0.11	0.18	0.19	0.19	2.58
	+	5.06	0.18	0.18	0.98	1.03	6.15
		+4.23	+0.07	0.00	+0.79	+0.84	+3.57
25	-	0.87					4.06
	+	4.12					7.15
		+3.25					+3.09
av over 25 days	-	2.07	0.12	0.26	0.16	0.22	2.41
	+	4.34	0.21	0.36	0.35	0.43	5.48
av change		+2.27	+0.09	+0.10	+0.19	+0.21	+3.07

[a] LSD 0.05; line/genotype (comparison among lines, only) = 0.09, treatment (comparison between treatments, i.e., inoculation, only) = 0.09. [b] (+), inoculated; (-), noninoculated. [c] GL, glanded; gl, nonglanded. [d] Change in TA concentration after inoculation with RKN.

with 10 000 *M. incognita* eggs or had not been inoculated 7 days prior to planting. The seeds were planted in 250-cm^3 pots in triplicate with four plants in each replicate. Four days after emergence, roots of Aub-201, Coker-201, 89-8275, and M-120 and roots and leaves of Aub-634 and M-8 cultivars were harvested, cleaned thoroughly with water, and dried by blotting with tissue, and their approximate weights were determined. The roots and the leaves were freeze-dried separately.

They were then ground in a Wiley mill (40-mesh screen) to a very fine powder and stored at -20 °C in sealed plastic bags. The roots and leaves were harvested on five or six dates during a 3-week period.

Sample Preparation for HPLC Analyses. The extracting and HPLC solvents were of ACS grade and were filtered through a 0.45-μm Millipore filter. Extractions were conducted in subdued light (Stipanovic et al., 1988). Samples were analyzed with a Waters HPLC system which included a 6000A pump, a variable autoinjector Model 712, and a UV-vis 490E detector. Separation of TAs was performed with a 4.6 mm × 25 cm Hypersil ODS column (Alltech Associates, Inc., Deerfield, IL). The mobile phase of ethanol-methanol-isopropyl alcohol (IPA)-acetonitrile (ACN)-water-ethyl acetate (EtOAc)-dimethylformamide-phosphoric acid (16.7:6.5:12.1:23.75:31.95:3.8:5.1:0.1) (Stipanovic et al., 1988) was monitored at 272 nm at a flow rate of 1 mL/min. Samples were analyzed as replicates of two or more injections of 100 μL.

Depending on availability and estimated content of TAs in the samples, 0.05-0.1 g of ground roots and leaves was shaken in capped amber specimen bottles with 15 mL of glass beads (Regular, 140/170), 10 mL of hexane-EtOAc (3:1, solvent 1), and 100 μL of 10% acetic acid for 1 h (Stipanovic et al., 1988). The solutions were filtered through Whatman No. 1 filter paper into 50-mL round-bottom flasks, and the residue was rinsed three times with solvent 1. The solvent was evaporated and the flask washed with solvent 1 (5 × 1 mL) and transferred onto a silica Sep-pak (Fisher Scientific, Fair Lawn, NJ). The Sep-Pak was dried with nitrogen gas, and the TAs were eluted with 5 mL of IPA-ACN-H_2O-EtOAc (35:21:39:5) (Stipanovic et al., 1988); 100-μL aliquots were then analyzed by HPLC.

Procurement and Preparation of the Standards. Gossypol was provided by the U.S. Department of Agriculture (USDA), Southern Regional Research Center, New Orleans, LA, and hemigossypolone and the heliocides H_1 and H_2 were provided by Dr. Robert Stipanovic (USDA, College Station, TX). For HPLC analysis, standard curves were obtained for gossypol, hemigossypolone, and heliocides H_1 and H_2. The standard curve for hemigossypolone was also used to quantify hemigossypol, while standard curves for H_1 and H_2 were also used to quantify H_3 and H_4, respectively. The identities of H_3 and H_4 were established by their elution times relative to those of H_1 and H_2 (Stipanovic et al., 1988). Stock solutions were prepared by dissolving 6.1 mg of gossypol, 3.0 mg of hemigossypolone, 2.9 mg of H_1, and 2.0 mg of H_2 in 1 mL of methanol, which were then diluted to 50 mL with the mobile phase. A series of dilutions were made from the stock solution for the calibration curve. Aliquots of 100 μL were analyzed by HPLC. They were also added to test samples to confirm recovery.

Statistical Analysis. Six cultivars of upland cotton *G. hirsutum* were planted in the greenhouse in a randomized complete block design with three replicates. Data obtained from various analyses and measurements were subjected to the analysis of variance using SAS (Spatz and Johnston, 1984; SAS, 1985; DiIorio, 1991).

RESULTS AND DISCUSSION

The mean numbers of RKN eggs per root expressed as percent of eggs found on the very susceptible M-8 line of 40-day-old plants of five lines are listed in Table 1. These and a number of other experimental lines and commercial cultivars have been classified in order of relative resistance to RKN on the basis of the number of eggs present in the

Table 2. Root Hemigossypolone Concentration of RKN Susceptible and Resistant Cotton Plants (Milligrams per Gram of Dry Weight)[a]

days after inoc	inoc	M-8 GL	Aub-201 gl	Coker-201 GL	89-8275 gl	M-120 GL	Aub-634 GL
4	-	0.12	0.47	0.41	0.20	0.10	0.00
	+	0.02	0.67	0.36	0.53	0.54	0.07
		−0.10[b]	+0.20	−0.05	+0.33	+0.44	+0.07
8	-	0.07	0.24	0.46	0.24	0.19	0.00
	+	0.04	0.55	0.52	0.37	0.53	0.02
		−0.03	+0.31	+0.06	+0.13	+0.34	+0.02
12	-	0.00	0.50	0.10	0.18	0.19	0.02
	+	0.08	0.36	0.28	0.34	0.09	0.01
		+0.08	−0.14	+0.18	+0.16	−0.10	−0.01
16	-	0.00	0.44	0.21	0.20	0.18	0.02
	+	0.02	0.69	0.49	0.39	0.38	0.02
		+0.02	+0.25	+0.28	+0.19	+0.20	0.00
20	-	0.02	0.44	0.33	0.37	0.12	0.03
	+	0.01	0.90	0.13	0.41	0.52	0.21
		−0.01	+0.46	−0.20	+0.04	+0.04	+0.18
25	-	0.07					0.01
	+	0.06					0.07
		−0.01					+0.06
av over 25 days	-	0.05	0.42	0.30	0.24	0.16	0.02
	+	0.04	0.64	0.36	0.41	0.41	0.07
av change		−0.01	+0.22	+0.06	+0.17	+0.25	+0.05

[a] LSD 0.05; line/genotype (comparison among lines, only) = 0.03, treatment (comparison between treatments, i.e., inoculation, only) = 0.09. [b] Change in terpenoid aldehyde concentration after inoculation with RKN.

roots 40 days after infection with 10 000 RKN eggs (Shepherd, 1986; Shepherd et al., 1988; Jenkins et al., 1993). Resistance to the RKN in some selected lines approaches immunity in that these resistant lines contained only about 2% or fewer of the eggs per plant found in the roots of the very susceptible M-8 line (Table 1).

An isocratic reversed-phase high-performance liquid chromatographic (HPLC) technique was used to identify and to quantify those TAs present in roots and leaves by reference to available standards and, in the case of H_3 and H_4, by their elution times relative to those of H_1 and H_2 (Stipanovic et al., 1988). In roots, small amounts of hemigossypolone and hemigossypol were present in addition to gossypol. In leaves, hemigossypolone, gossypol, and heliocides H_1–H_4 were found. Several of the lines possessed two additional peaks at 7.5 and 8.3 min which had been identified in previous work as gossypolone (Phillips and Hedin, 1990) and gossypol lactone (Hedin et al., 1991), respectively.

The mean concentrations (milligrams per gram of dry weight) of gossypol and hemigossypolone in the roots for varieties and dates after inoculation are presented in Tables 1 (gossypol) and 2 (hemigossypolone). Data for contents and changes of a third TA, hemigossypol (not shown), were generally similar to those for hemigossypolone but were lower. The mean concentrations (milligrams per gram of dry weight) of the heliocides H_1, H_2, H_3, and H_4 and gossypol and hemigossypolone in leaves are presented in Tables 3 and 4. Statistical data are listed as footnotes in the tables.

Attempts to correlate the root gossypol content with RKN resistance were largely unsuccessful. The noninoculated resistant lines varied widely in root gossypol content. The glandless line 89-8275 and the glanded line M-120 contained on the average less than 10% as much gossypol as the other glanded line, Aub-634 (0.16, 0.22, and 2.41 mg/g, respectively). Yet they were equally resistant to RKN in terms of eggs per plant (Table 1). In tests where these resistant lines were inoculated, their gossypol contents each increased about 2-fold (0.35, 0.43, and 5.48 mg/g, respectively), but the magnitude of the increase of Aub-634 was much greater. The susceptible lines yielded a similar pattern. (Noninoculated: Aub-201, 0.12; Coker-201, 0.26; and M-8, 2.07 mg/g. Inoculated: Aub-201, 0.21; Coker-201, 0.36; and M-8, 4.34 mg/g).

It is generally understood that the RKN penetrates the roots of both susceptible and resistant plants but that multiplication in the resistant roots does not occur. The mechanisms of the resistant response have not been reported, however. Hedin et al. (1984) demonstrated that gossypol in RKN resistant root tips (Aub-623) increased more quickly during the first 8 days after inoculation than did gossypol in inoculated M-8 root tips. Differences narrowed at later dates. In the present tests, the same tendency was observed at the fourth day, but not beyond, when the glanded lines were compared (fourth day changes: Coker-201, +0.02; M-8, −0.12; M-120, +0.25; and Aub-634, +2.45 mg/g). Changes in the gossypol content after inoculation continued to be greater when Aub-634 at 8 and 12 days (+3.02 and +3.71 mg/g) was compared with M-8 (+1.65 and +1.96 mg/g).

Root hemigossypolone analyses indicated that somewhat of an inverse pattern existed in that the noninoculated lines that were low in gossypol relative to M-8 and Aub-634 were higher than M-8 and Aub-634 in hemigossypolone (see 25-day averages, Table 2). Inoculation again resulted in increased biosynthesis of root hemigossypolone in all

Table 3. Leaf Heliocides in RKN Susceptible and Resistant Cotton Plants (Milligrams per Gram of Dry Weight)[a]

days after inoc	inoc	mg/g heliocide H_1 M-8	heliocide H_1 Aub-634	heliocide H_2 M-8	heliocide H_2 Aub-634	heliocide H_3 M-8	heliocide H_3 Aub-634	heliocide H_4 M-8	heliocide H_4 Aub-634
4	–	4.3	2.5	15.9	10.9	0.2	0.2	0.1	0.1
	+	8.4	3.1	18.0	5.1	1.3	0.3	0.6	0.1
		+4.1[b]	+0.6	+2.1	−5.8	+1.1	+0.1	+0.5	0.0
8	–	2.2	1.6	6.5	5.4	0.1	0.1	0.1	0.0
	+	2.9	2.3	9.0	5.7	0.6	0.1	0.3	0.1
		+0.7	+0.7	+2.5	+0.3	+0.5	0.0	+0.2	+0.1
12	–	4.6	3.1	13.4	11.2	0.1	0.3	0.1	0.1
	+	2.7	2.8	9.0	7.8	0.1	0.1	0.0	0.0
		−1.9	−0.3	−4.4	−3.4	0.0	−0.2	−0.1	−0.1
16	–	2.2	3.4	6.1	10.8	0.1	0.1	0.0	0.1
	+	1.0	4.0	4.2	11.0	0.1	0.2	0.0	0.1
		−1.2	+0.6	−1.9	+0.2	0.0	+0.1	0.0	0.0
20	–	3.0	2.3	8.2	8.1	0.2	0.1	0.1	0.1
	+	2.4	14.4	4.9	8.9	0.1	0.1	0.0	0.7
		−0.6	+12.1	−3.3	+0.8	−0.1	0.0	−0.1	+0.6
25	–	4.6	1.0	12.9	2.3	0.3	0.1	0.2	0.0
	+	1.4	2.2	5.5	5.9	0.2	0.1	0.1	0.1
		−3.2	+1.2	−7.4	+3.6	−0.1	0.0	−0.1	+0.1
av over 25 days	–	0.2	0.2	10.5	8.1	3.5	2.3	0.1	0.1
	+	0.4	0.1	8.4	7.4	3.1	4.8	0.2	0.2
av change		+0.2	−0.1	−2.1	−0.7	−0.4	+2.5	+0.1	+0.1

[a] LSD 0.05; heliocide H_1: line/genotype (comparison among lines, only) = 0.08, treatment (comparison between treatments, i.e., inoculation, only) = 0.09, heliocide H_2: line/genotype = 0.08, treatment = 0.07. [b] Change in terpenoid aldehyde concentration after inoculation with RKN.

Table 4. Leaf Terpenoid Aldehydes of RKN Glanded Susceptible and Resistant Cotton Plants (Milligrams per Gram of Dry Weight)[a]

days after inoc	RKN inoc	gossypol M-8	gossypol Aub-634	hemigossypolone M-8	hemigossypolone Aub-634
4	–	0.76	0.94	1.15	0.80
	+	0.65	0.21	2.81	1.26
		−0.11[b]	−0.73	+1.66	+0.46
8	–	0.09	0.03	0.51	0.28
	+	0.05	0.01	1.05	0.28
		−0.04	−0.02	+0.54	0.00
12	–	0.11	0.14	0.55	0.42
	+	0.00	0.02	0.36	0.22
		−0.11	−0.12	−0.19	−0.20
16	–	0.01	0.00	0.20	0.15
	+	0.03	0.14	0.14	0.24
		+0.02	−0.14	−0.06	+0.09
20	–	0.13	0.00	0.27	0.11
	+	0.01	0.01	0.11	0.15
		−0.12	+0.01	−0.16	−0.04
25	–	0.14	0.00	0.46	0.04
	+	0.00	0.00	0.60	0.06
		−0.14	0.00	+0.14	+0.02
av over 25 days	–	0.21	0.19	0.52	0.30
	+	0.12	0.07	0.85	0.37
av change		−0.09	−0.12	+0.33	+0.07

[a] LSD 0.05; gossypol: line/genotype (comparison between lines, only) = 0.09, treatment (comparison between treatments, i.e., inoculation, only) = 0.09, Hemigossypolone: line/genotype = 0.03, treatment = 0.09. [b] Change in terpenoid aldehyde concentration after inoculation with RKN.

lines by the fourth day except for M-8, but no marked increase in hemigossypolone of the inoculated resistant lines above that of the inoculated susceptible lines at early or later dates was evident. Although evidently not an important factor in resistance to the RKN, hemigossypolone (and heliocides H_1 and H_2) was found to be greatly increased in cotton lines resistant to an insect, the tobacco budworm *H. virescens* (F.) relative to gossypol (Hedin et al., 1991), and presumed to be associated with resistance.

Although the cotton root is the site of RKN action and therefore appropriately of greatest focus, the TA content and changes found in leaves of inoculated and noninoculated plants was also investigated in search of diagnostic leads.

In leaves of noninoculated M-8 (susceptible) and Aub-634 (resistant) plants, hemigossypolone and heliocides H_1 and H_2 were the major TAs, while gossypol, hemigossypolone, and heliocides H_3 and H_4 were present in lesser amounts. About 90% of the TAs contained in leaves were heliocides H_1–H_4 (Table 3), with about 5% each of gossypol and hemigossypolone (Table 4).

The total leaf TA contents as determined by HPLC (sum of H_1, H_2, H_3, H_4, G, HGQ; Tables 3 and 4) averaged over the 25-day period were 1.5% for M-8 and 1.1% for Aub-634. These values are comparable to those of Stipanovic et al. (1988), who reported 0.5–1.4% for *G. hirsutum* leaves of unidentified lines sampled at five locations, also using the HPLC procedures that were also used in this work. However, they were higher than that found by Stipanovic et al. (1988) using the aniline method (0.4–0.6%) and by Hedin et al. (1991) using the phloroglucinol procedure (0.2–0.4%).

The TA contents of the leaves of plants that had been inoculated were not appreciably changed, either with the susceptible or with the resistant lines (Tables 3 and 4) except for hemigossypolone at day 4 (M-8, +16.6 mg/g; Aub-634, +4.6 mg/g). Therefore, it was found that changes in leaf TA content after inoculation are small or nonexistent and therefore do not appear to be helpful for evaluating the response of the plant to invasion by the RKN. Had helpful changes been evident, leaf sampling could have provided a means of monitoring changes that would have been more convenient to harvest and not destructive to the plant.

This study showed that the root TA content of resistant lines increased by the fourth day after inoculation. However, two resistant lines, one glanded and the other glandless, had very low intrinsic TA contents. While they increased after inoculation, they were still much lower than that of a susceptible glanded line (M-8). Two RKN (89-8275 and M-120) lines have low TAs yet have the same genes for resistance to RKN as other lines high in TAs. Thus, increases in TAs or TA level alone cannot explain resistance in all lines. Also, analysis of TAs in leaves did

not prove helpful in identifying trends that could be correlated with resistance.

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