Terpenoid Aldehydes in Root-Knot Nematode Susceptible and Resistant Cottonseeds As Determined by HPLC and Aniline Methods[†]

Nazrin Khoshkhoo,[‡] Paul A. Hedin,*[,§] and Jack C. McCarty, Jr.[§]

Department of Chemistry, Mississippi State University, Drawer-CH, Mississippi State, Mississippi 39762, and Crop Science Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Mississippi State, Mississippi 39762-5367

As part of an effort to identify lines or cultivars of cotton *Gossypium* spp. with resistance to the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN), seeds were analyzed for gossypol and other terpenoid aldehydes (TA's), rather than roots where the RKN lives. If a correlation between plant resistance to RKN and TA's could be demonstrated, the time and costs associated with propagating seedlings could be avoided. Some spectrophotometric and high-pressure liquid chromatography procedures were evaluated. Total terpenoid aldehydes obtained by the aniline analysis were higher than those obtained with the HPLC methods. Gossypol was virtually the only terpenoid aldehyde found in cottonseed, but small amounts of hemigossypolone were also found to be present. No statistically significant correlation was found between seed gossypol concentrations and plant resistance to RKN.

INTRODUCTION

Several lines of research are being carried out to identify sources of resistance in cotton *Gossypium hirsutum* L. plants to the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN). This nematode lives in the soil and enters the young root upon germination of the seed. Some of these investigations have involved gossypol and other terpenoid aldehydes (TA's) which have been shown to be toxic to the RKN (Veech, 1978; Hedin et al., 1984; Bell et al., 1984). TA's increase in roots of resistant plants faster than in susceptible plants after inoculation with RKN (Veech, 1978; Hedin et al., 1984).

Cotton plants contain lysigeneous glands below the epidermis. These glands contain as many as 15 TA's and over 20 volatile terpenes, the major constituents of which are gossypol, hemigossypol, hemigossypolone, and the heliocides (Stipanovic et al., 1988). There are also high concentrations of anthocyanins in the epithelial cells surrounding the gland cavity. However, gossypol is virtually the only TA in cottonseed (Stipanovic et al., 1988).

Most quantitative estimates of gossypol and other TA's are based on their reaction with aniline to form a yellow chromophore. Gossypol also forms chromophores with phloroglucinol, antimony trichloride, and (2,4-dinitrophenyl)hydrazine (Stipanovic et al., 1975). All of these techniques only give information about the total TA's. However, HPLC can provide both qualitative and quantitative information about individual TA's.

The objectives of this study were to quantify gossypol in the seeds of a number of lines or varieties that were known to be susceptible or resistant to the RKN, to determine whether some of the high gossypol or RKN-resistant lines contained significant quantities of other TA's, and to determine whether there was a correlation between seed TA content and resistance to the RKN. This study included several lines or varieties whose seeds have apparently not been analyzed for gossypol. It was also of interest to determine whether either aniline or HPLC analyses were helpful in identifying resistance. The establishment of a correlation between seed TA content and RKN resistance could expedite screening efforts to select and propagate RKN-resistant varieties because the time and costs associated with growing seedlings in the greenhouse could be avoided.

MATERIALS AND METHODS

Procurement and Preparation of Cottonseeds. Test cottonseed entries and their sources are listed in Table 1. On the basis of the *M. incognita* index and the number of *M. incognita* eggs per gram of seedling roots, cottonseed has been classified as either RKN resistant or susceptible (Shepherd et al., 1988). Three lines of particular interest include Auburn-634 RNR, which is a highly RKN-resistant germplasm line that was developed and released jointly by the USDA-ARS and the Alabama Agricultural Experiment Station. Entry 83-315 (M-315) is a resistant experimental line developed by the USDA-ARS Crop Science Research Unit at Mississippi State. The susceptible genotype, M-8, is a double-haploid line, and the remaining entries are commercial cultivars. The seeds were delinted with concentrated sulfuric acid and rinsed with distilled water until they were neutral to pH paper. They were then dried in air at room temperature and ground in a Wiley mill (40-mesh screen).

Preparation of Extraction and HPLC Solvents. Extracting solvent 1: EtOH/water/ether/HOAc (v/v) (59:24:17:0.2) (Smith, 1962). Extracting solvent 2: EtOH/water/EtOAc/HOAc (v/v) (68:32:20:0.2) (Stipanovic et al., 1988). Extracting solvent 3: hexane/EtOAc/HOAc (v/v) (45:15:0.02) (Stipanovic et al., 1988). Solvent 4: This solvent was used to redissolve TA's after extraction with solvents 1, 2, or 3 and subsequent evaporation; IPA/ACN/water/EtOAc (v/v) (35:21:39:5) (Stipanovic et al., 1988). Solvent 5: HPLC mobile phase; ethanol/methanol/isopropyl alcohol (IPA)/acetonitrile (ACN)/water/ethyl acetate (EtOAc)/dimethylformamide (DMF)/phosphoric acid (v/v) (16.7:6:6.5:12.1:23.8:32.0:3.8:5.1:0.1).

Preparation of Stock Solutions for Aniline Analysis. Solvent 6: reference solution; isopropyl alcohol (IPA)/hexane (v/v) (60:40) (Pons et al., 1958). Solvent 7: complexing reagent; pipet 2 mL of 3-amino-1-propanol and 10 mL of glacial acetic acid (reagent grade) into a 100-mL volumetric flask, cool to room temperature, and then dilute to volume with *N,N*-dimethylformamide (DMF); the reagent is stable for 1 week after preparation. Solvent 8: aniline was redistilled over zinc dust and stored in the refrigerator for the aniline test (Pons et al., 1958).

Determination of Free Terpenoid Aldehydes by HPLC Method. Depending on availability and approximate TA content,

* Author to whom correspondence should be addressed.

[†] Taken in part from a Ph.D. thesis by N.K., Department of Chemistry, Mississippi State University.

[‡] Mississippi State University.

[§] U.S. Department of Agriculture.

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Table 1. Gossypol Contents of RKN-Susceptible and -Resistant Cottonseeds As Determined by HPLC and Aniline Methods

	RKN eggs/plant[a]		seed gossypol, %			
cultivar	line[b]	% of M-8	aniline	HPLC-1	HPLC-2	HPLC-3
M-8	GL	100.0	1.2	0.3	0.7	0.3
ST-453	GL	80.3	1.2	0.4	1.1	0.5
ST-69132	GL	77.0	1.4	0.6	1.0	0.6
DPL-5415	GL	76.6	1.1	0.6	1.1	0.5
DPL-90	GL	76.2	1.9	0.6	1.4	0.7
DES-119	GL	59.8	1.2	0.4	0.9	0.4
ST-825	GL	54.4	1.2	0.4	0.8	0.4
Aub-201	gl	54.2	0.9	0.1	0.1	0.0
Coker-201	GL	53.8	1.2	0.4	0.9	0.4
LA-HG	GL	46.4	2.0	0.4	1.8	0.4
ST-213	gl	40.2	0.7	0.1	0.1	0.0
ST-213	GL	39.9	1.2	0.4	0.8	0.3
M-120	GL	1.7	1.2	0.2	0.8	0.3
M-315	GL	1.0	1.8	0.4	1.0	0.4
Aub-634	GL	0.9	1.4	0.3	0.8	0.3
Aub-623	GL	0.7				
lsd 0.05[c]			0.3	0.1	0.2	0.1
av[d]			1.3	0.3	0.9	0.3

[a] Data and description of sources from Shepherd (1986), Shepherd et al. (1988), and Jenkins et al. (1993); M-8 contained (128–239) × 10^3 eggs/plant. [b] GL = glanded, gl = glandless. [c] Lsd 0.05 values were calculated to compare cultivars but not HPLC extracting solvents. [d] Average of 13 glanded and 2 glandless entries.

between 0.2–0.7 g of the pulverized seed samples were mixed with 5 mL of petroleum ether and stirred for 1 h at room temperature. The mixture was then centrifuged for 10 min at 2500*g*. The resultant oil-laden supernatant was discarded, and the remaining pellet was used as starting material for both HPLC and aniline analysis.

The pellets together with 15 mL of glass beads (Regular 140/170) were mixed with 15 mL of solvents 1, 2, or 3 for tests 1, 2, and 3, respectively. The samples were then shaken for 30 min in amber-capped specimen bottles and then filtered through Whatman No. 1 filter paper into 50-mL round-bottom flasks.

The residue was rinsed three times with the appropriate solvent for the respective tests and added to the initial filtrate. The solvent was then evaporated, the TA's were redissolved with 15 mL of solvent 4, and then 25-μL aliquots were analyzed by HPLC using the mobile phase, solvent 5 (Stipanovic et al., 1988).

Preparation of TA Standards for HPLC. Gossypol was provided by U.S. Department of Agriculture (USDA), Southern Regional Research Center, New Orleans, LA, and hemigossypolone was provided by Dr. Robert Stipanovic (USDA, College Station, TX). Stock solutions were prepared by dissolving 6.9 mg of gossypol and 3 mg of hemigossypolone together in 1 mL of methanol, which was then diluted to 50 mL with solvent 4. A series of dilutions were made from the stock solution for the calibration curve. Aliquots of 100 μL were analyzed by HPLC (Stipanovic et al., 1988).

Aniline Method for Determination of Total TA's. Depending on availability, approximately 0.02-g samples of the listed cottonseed varieties were weighed in 50-mL volumetric flasks, 10 mL of solvent 7 was added to each, and they were then mixed and heated at 100 °C for 30 min. Two milliliters of the cooled solutions was transferred into 25-mL volumetric flasks, and 2 mL of aniline (solvent 8) was added. The flasks were then heated at 100 °C for 30 min, cooled to room temperature, and made up to volume with solvent 6. At the same time, 2 mL of each sample was transferred into another 25-mL volumetric flask and made up to volume with solvent 6 to be reserved as a reference solution. The absorbency of each sample was determined at 440 nm (Pons et al., 1958).

Preparation of the Gossypol Standard for Anthrone Analysis. Pure gossypol (31.3 mg) was dissolved in 50 mL of solvent 7 (Pons et al., 1958). Then, 2-, 4-, 6-, 8-, and 10-mL quantities of standard gossypol solution were transferred into 50-mL volumetric flasks, and sufficient solvent 7 was added to a total volume of 10 mL. The flasks were then heated at 100 °C for 30 min at room temperature, and 2-mL aliquots of each diluted standard solution were transferred into a 25-mL volumetric flask. Two milliliters of solvent 7 was added to each flask, and they were then heated in a boiling water bath for 30 min, after which time they were cooled and made up to the volume with solvent 6. The flasks were then allowed to stand in the dark at room temperature for 1 h. At the same time, 2 mL of the diluted gossypol standard solution was transferred into another 25-mL volumetric flask and made up to volume with solvent 6 and reserved as a reference solution. The absorbency of each solution was determined at 440 nm.

Statistical Analysis. Chemical analyses were performed in triplicate. Data obtained from the various tests and measurements were subjected to the analysis of variance, and the least significant difference (lsd) was calculated using SAS (Spatz and Johnston, 1984; SAS, 1985; DiIorio, 1991).

RESULTS AND DISCUSSION

Gossypol was found by HPLC methods to be virtually the only TA present in RKN-resistant and -susceptible seeds from several lines of cotton, although very small amounts of hemigossypolone were found using extracting solvents 1 and 2. This confirms the report of Stipanovic et al. (1988) with regard to seeds.

Data obtained from HPLC analyses using the three different extracting solvents (see Materials and Methods) were compared with those obtained on the same seeds using the aniline method of analysis (Pons et al., 1958). These data and LSD 0.05 values that separate cultivars but not HPLC methods are presented in Table 1.

The listing of the lines and commercial varieties in Table 1 is in descending order according to susceptibility as expressed by RKN eggs found per plant (Shepherd, 1986; Shepherd et al., 1988; Jenkins et al., 1993). The numbers are normalized as percent of eggs/plant found in the very susceptible line M-8. The average seed gossypol content for the 13 glanded entries as determined by the aniline procedure was 1.3%, while the average gossypol content by HPLC using extracting solvent HPLC-2 was 1.0%. These values are roughly comparable to those of Stipanovic et al. (1988) in which the average seed gossypol content of 14 glanded unidentified *G. hirsutum* varieties collected at five locations was 1.13% by the aniline method and 1.06% by the HPLC method using extracting solvent HPLC-2.

The TA concentrations obtained by aniline analysis were higher than those obtained by the HPLC method. This may have occurred (1) because the solvents used in the aniline procedure may have extracted more terpenoid aldehydes from the cottonseed than those used in HPLC techniques, (2) because heating the extracts in the presence of aniline may also have produced color products other than TA's, and (3) because of the presence or production of oxygenated lipids that contributed to the chromophore measured by the aniline method (Stipanovic et al., 1984).

The higher gossypol analysis obtained after extraction with HPLC-2 (HPLC-1; 0.3%, HPLC-2; 0.9%, HPLC-3; 0.3%, 13 glanded and 2 glandless entries) is apparently a function of polarity of the extraction solvent. The rationale for the use of the less polar solvents HPLC-1 and HPLC-3 was to determine if there might be a better correlation of less bound gossypol and RKN resistance. However, no such trends were evident.

For cottonseed, there seems to be no direct relationship between the gossypol concentration and resistance to RKN, at least not for the cultivars studied above. However, this is not very surprising because induced biosynthesis of gossypol occurs after infection with RKN (a phytoalexin residue), and it appears to be more correlated with host plant resistance to RKN than intrinsic TA's (Stipanovic et al., 1975; Veech, 1978; Hedin et al.,

1984). Therefore, to study the relationship between resistance to RKN and TA content accurately, resistant and susceptible cultivars which are very similar or of the same type should be selected, and the cultivars must be inoculated with RKN so that their relative TA responses can be measured.

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