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Ratios of (+)- and (−)-Gossypol in Leaves, Stems, and Roots of Selected Accessions of *Gossypium hirsutum* Var. *marie galante* (Watt) Hutchinson

ROBERT D. STIPANOVIC,* LORRAINE S. PUCKHABER, AND ALOIS A. BELL

Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2765 F & B Road, College Station, Texas 77845

Gossypol is an allelochemical that occurs naturally throughout the cotton plant as an enantiomeric mixture. Gossypol and related terpenoids protect the plant from some insect herbivores. Cottonseed has a high protein content, but it is underutilized because (−)-gossypol, which is toxic to nonruminants, occurs in the seed along with (+)-gossypol. Commercial Upland cottons usually have an approximate 3:2 (+)- to (−)-gossypol ratio in the seed, but plants can be bred with <8% (−)-gossypol using accessions of *Gossypium hirsutum* var. *marie galante* as parents. We report the (+)- and (−)-gossypol ratios and the concentration of related terpenoids in the stems, leaves, and roots of four accessions of *marie galante* that show high, moderate, and near normal levels of (+)-gossypol in the seed; we compare these values to the commercial cultivar Stoneville 474, which has 62% (+)-gossypol in the seed. In the *marie galante* accessions 2452 and 2425 that have the highest levels of (+)-gossypol in the seed, the percent (+)-gossypol and the concentration of gossypol and the related terpenoids were significantly higher ($P = 0.05$) in the stems and leaves as compared to Stoneville 474. Our analysis indicates that progeny from accessions 2452 and 2425 that retain these traits should not be overly susceptible to herbivorous insects.

KEYWORDS: Cotton; (+)-gossypol; (−)-gossypol; *Gossypium hirsutum* var. *marie-galante*

INTRODUCTION

In the cotton plant, the bimolecular coupling of hemigossypol produces gossypol (**Figure 1**) (*1*). Gossypol possesses a chiral axis due to restricted rotation around the binaphthyl bond (atropisomerism) (*2*). This bimolecular coupling usually produces one enantiomer in preference to the other. Gossypol occurs in the seed; it and biosynthetically related terpenoids (**Figure 1**) also occur in the stems, leaves, bolls, and roots. They protect the plant from pathogens and insect herbivores (*3*−*9*). Gossypol is toxic to nonruminant animals; therefore, its presence in the seed decreases the marketability and utilization of this otherwise nutritious byproduct of cotton fiber production. However, recent studies show that feed containing cottonseed with high levels of (+)-gossypol does not adversely affect the growth of chickens and, by extension, other nonruminant animals (*10*−*12*). *Gossypium hirsutum* var. *marie galante* is unique among *G. hirsutum* species in that some accessions exhibit high levels of (+)-gossypol in the seed (*13, 14*). Seeds of most commercial Upland cottons (*G. hirsutum*) have an approximate 3:2 ratio of (+)- to (−)-gossypol. In an effort to understand how plants that exhibit high levels of (+)-gossypol in the seed might be affected by pathogens and insects, we report an analysis of the (+)- to (−)-gossypol ratios and total terpenoids in the leaves, stems, and roots of selected accessions of *G. hirsutum* var. *marie galante* as compared to the commercial cotton cultivar Stoneville 474 (ST 474).

MATERIALS AND METHODS

Equipment and Reagents. High-performance liquid chromatography (HPLC) analyses were performed on a Hewlett/Packard 1090 equipped with a diode array detector and operated under computer control. Solvents were all HPLC grade. (*R*)-(−)-2-Amino-1-propanol (D-alaninol) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Terpenoid standards were obtained from cotton tissues as described elsewhere (*15, 16*).

Plant Material. Seeds of *G. hirsutum* var. *marie galante* accession #2425, #2443, #2452, and #2472 were obtained from the U.S. Cotton Germplasm Collection at College Station (TX). Seeds of ST 474 were obtained from a commercial source. Plants were grown in the greenhouse in College Station. Roots were prepared for analysis by carefully removing the plant from the soil and washing the roots free of debris. The feeder roots were removed from the main taproot. Feeder and taproot tissues, leaves with a diameter of 5−8 cm, and stems (with the bark) were freeze-dried and stored at −20 °C.

Statistical Analysis. Means and standard errors (SE) were calculated using Excel. For roots and foliage, nine samples were analyzed. Statistical differences between means of the accessions and the means of ST 474 (analysis of variance; one-way, $\alpha = 0.05$) were determined using SAS version 8.2 software (SAS Institute, Cary, NC, 1999−2001). Terpenoid concentrations were the result of nine independent replica-

* To whom correspondence should be addressed. Tel: 979-260-9232. Fax: 979-260-9313. E-mail: rdstip@cpru.usda.gov.

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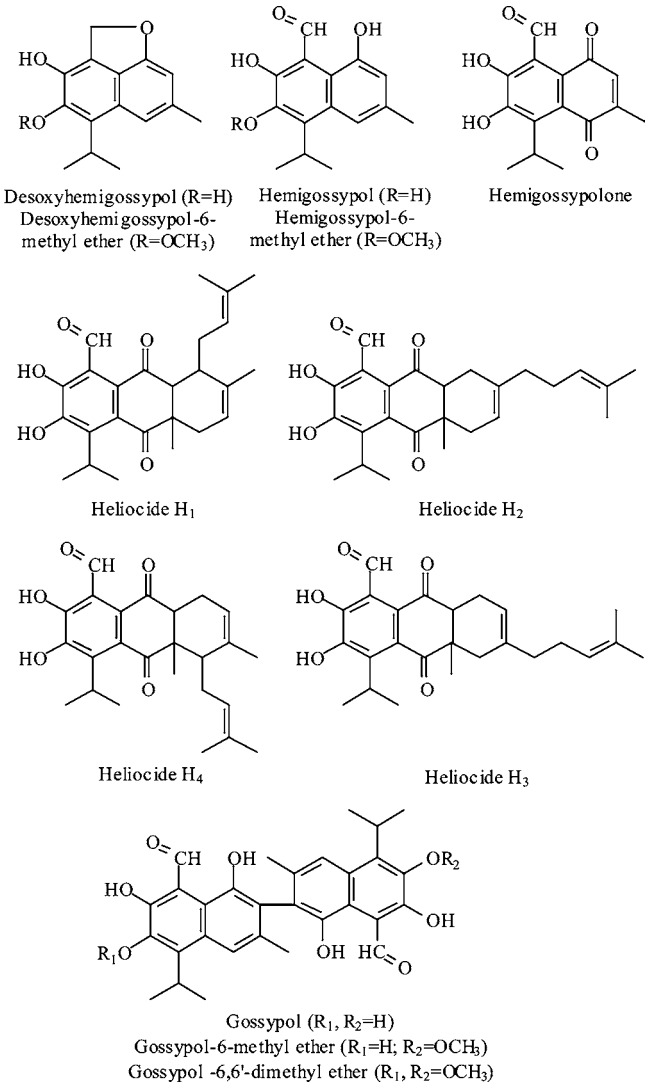

Figure 1. Gossypol and related terpenoids found in cotton plant tissues.

tions. Percentages of (+)- and (−)-gossypol for seed were based on four replications; data for all other tissue were based on nine replications.

Analysis of Total Terpenoids in Leaves, Stems, and Roots. Plant tissue, prepared as described above, was extracted and analyzed by HPLC using the method described by Benson et al. (*17*) for leaves and stems and the method described by Bianchini et al. (*16*) for root tissues.

Analysis of Gossypol Enantiomers. Seeds were dried under vacuum for 48 h, dehulled, and ground using an agate mortar and pestle. Leaves, stems, taproots, and feeder roots were harvested, freeze-dried, and ground in a Waring blender. All ground samples were stored at −20 °C, if HPLC analysis could not be performed immediately. In preparation for HPLC analysis, a ground sample was weighed into a tube, covered with a derivatizing reagent [88% acetonitrile, 10% glacial acetic acid, and 2% (*R*)-(−)-2-amino-1-propanol], and gently mixed. The tubes were sealed and placed in a 70 °C water bath. After 30 min, the tubes were vortexed and centrifuged. A portion of the clear supernatant was subjected to HPLC analysis. For all tissue extracts, the same HPLC method was used but with one of two mobile phases. Specifically, the HPLC instrument (described above) was operated with a 150 mm × 3.0 mm i.d., 5 μm Inertsil ODS-3 column (GLSciences, Tokyo, Japan) maintained at 40 °C. An isocratic mobile phase of either solvent A (80:20 CH_3CN:10 mM KH_2PO_4, the last adjusted to pH 3.0 with concentrated H_3PO_4) run at 0.6 mL/min or solvent B (43:37:20 CH_3CN:MeOH:10 mM KH_2PO_4, the last adjusted to pH 3.0 with concentrated H_3PO_4) run at 0.8 mL/min was used. The chromatogram signal was monitored at 254 nm (bandwidth, 20 nm; ref, 550 nm; and band, 100 nm), and the UV spectra were stored over 220−400 nm. The enantiomeric ratio was obtained directly from the (+)- and (−)-gossypol Schiff base peaks.

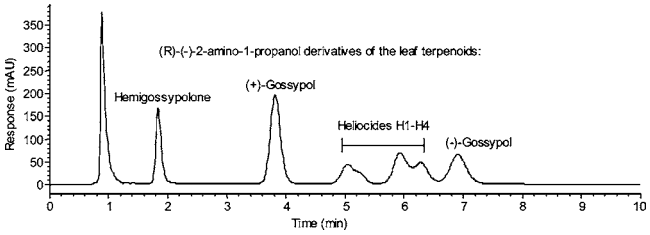

Figure 2. HPLC chromatogram of the terpenoid derivatives of (*R*)-(−)-2-amino-1-propanol from leaves of *G. hirsutum* var. *marie galante* accession 2452.

Seeds were individually reacted with 2.0 mL of derivatizing reagent, and solvent A was used in the HPLC analysis, a protocol used previously by Stipanovic et al. (*14*). For leaves, ∼100 mg of tissue was reacted with 4.0 mL of derivatizing reagent and HPLC solvent B was employed. For feeder roots, ∼100 mg of tissue, 2.0 mL of derivatizing reagent, and solvent B were used, while for taproots, the specifics were ∼50 mg of tissue, 4.0 mL of reagent, and solvent B. Last, for stems, ∼500 mg of tissue was reacted with 5.0 mL of derivatizing reagent and solvent A was used for the HPLC.

RESULTS AND DISCUSSION

Four *marie galante* accessions that exhibited consistently high, moderate, and normal levels of (+)-gossypol in the seeds were selected for analysis. Specifically, seeds from accessions #2452 and #2425 have >94% (+)-gossypol, seeds from accession #2472 have an intermediate level [∼80% (+)-gossypol], and accession #2443 [∼65% (+)-gossypol] is near that of the commercial cultivar ST 474 [∼60% (+)-gossypol] (*14*). Leaves, stems, taproots, and feeder roots were analyzed. The percent (+)-gossypol and the concentration of individual terpenoids were determined.

Previous work has shown that environmental conditions can affect seed weight and total terpenoid levels but not the ratio of (+)- to (−)-gossypol (*18*). Thus, the ratios of (+)- to (−)-gossypol observed in various tissue from plants in this study that were grown in the greenhouse should apply equally to field-grown plants. Despite variations that are expected for field vs greenhouse plants, we measured the total concentration of terpenoids in the stems, leaves, taproots, and feeder roots. For comparison, the terpenoid concentrations and the ratios of (+)- to (−)-gossypol also were determined in these tissues in the commercial cotton cultivar ST 474. The *marie galante* accessions are photoperiodic and do not flower until the second year after planting. Thus, analytical studies on bolls were not possible.

Table 1 gives the percent (+)-gossypol, and **Table 2** gives the concentration of the terpenoids (mean, SE, and range) for the four *marie galante* accessions and ST 474 found in the seeds, leaves, stems, and roots.

(+)-Gossypol Percentages. A typical chromatogram for a leaf extract from accession 2452 after reaction with the derivatizing reagent is shown in **Figure 2**. The (+)- and (−)-gossypol-aminopropanol peaks appear at 3.8 and 7.0 min, respectively. Additionally, the hemigossypolone adduct elutes at 1.8 min and the heliocide adducts appear as multiple adjacent peaks over 4.8−6.7 min. The accession with the highest percent (+)-gossypol in the seeds (i.e., 2452) had higher (+)-gossypol levels in the leaves, stems, and taproots as compared to ST 474. The feeder roots had less. Accession 2425, with the second highest level of (+)-gossypol in the seeds, had a higher percentage of (+)-gossypol in the leaves and stems than ST

Table 1. Percent (+)-Gossypol in Seeds, Leaves, Stems, and Roots of Accessions of *G. hirsutum* Var. *marie galante* and in the Commercial Cotton Cultivar Stoneville 474

	accession no. [percent (+)-gossypol][a]									
	2452		2425		2472		2443		ST 474	
tissue	mean (SE)	range	mean (SE)	range	mean (SE)	range	mean (SE)	range	mean (SE)	range
seeds	**96.7** (0.5)	95.5–97.6	**94.5** (0.1)	94.3–94.8	**81.3** (0.5)	80.0–82.1	**65.6** (0.8)	63.3–67.1	61.5 (1.6)	58.5–65.8
leaves	**64.0** (5.3)	59.6–68.8	**65.0** (4.4)	59.0–67.5	**63.4** (3.2)	60.4–66.3	50.6 (2.3)	48.5–52.2	59.4 (0.8)	55.6–63.5
stems	**79.1** (8.3)	68.6–92.3	**75.1** (6.7)	63.4–80.6	70.3 (0.5)	68.5–73.0	59.3 (4.3)	54.9–62.6	68.2 (0.9)	64.7–72.4
taproots	**83.7** (10.9)	64.6–90.7	74.0 (5.4)	67.6–78.8	**90.2** (3.0)	84.1–92.5	67.1 (1.7)	65.3–69.1	71.1 (0.4)	69.0–73.2
feeder roots	56.2 (3.7)	52.8–59.2	64.3 (9.4)	51.2–70.6	60.8 (12.2)	51.5–72.6	56.9 (7.1)	52.6–66.4	63.9 (0.9)	60.7–67.9

[a] Bold numbers indicate that the mean is significantly greater than the ST 474 mean ($P = 0.05$).

Table 2. Concentration (μg/mg) of Terpenoids in Seeds, Foliage, and Roots from Four Accessions of *G. hirsutum* Var. *marie galante* and Commercial Cotton Cultivar Stoneville 474

		accession no. [concentration of terpenoids (μg/mg)][a]									
		2452		2425		2472		2443		ST 474	
tissue	compd[b]	mean (SE)	range	mean (SE)	range	mean (SE)	range	mean (SE)	range	mean (SE)	range
seeds	Goss	4.96 (1.13)	2.24–7.77	2.40 (0.30)	1.91–3.28	4.43 (1.36)	1.38–7.98	3.27 (0.41)	2.45–3.99	3.46 (0.62)	2.11–5.08
leaves	HGQ	**2.04** (0.20)	0.95–2.79	**2.39** (0.21)	1.26–3.22	0.93 (0.10)	0.58–1.42	0.84 (0.17)	0.37–1.64	1.00 (0.08)	0.70–1.40
	HH1	**2.60** (0.41)	1.15–4.97	**1.01** (0.08)	0.55–1.50	**1.78** (0.15)	1.33–2.51	**4.23** (0.59)	1.69–6.10	0.22 (0.02)	0.17–0.32
	HH2	**5.29** (0.58)	3.06–7.96	**2.60** (0.19)	2.08–3.86	**3.05** (0.16)	2.60–3.97	**3.32** (0.43)	1.40–4.58	0.90 (0.07)	0.57–1.17
	HH3	**1.69** (0.17)	1.01–2.52	**0.87** (0.06)	0.69–1.24	**0.98**(0.05)	0.85–1.27	**1.08** (0.13)	0.53–1.45	0.35 (0.02)	0.24–0.43
	HH4	**1.10** (0.16)	0.51–2.03	**0.47** (0.04)	0.29–0.68	**0.71** (0.05)	0.55–1.01	**1.66** (0.22)	0.76–2.31	0.15 (0.01)	0.13–0.19
	Goss	**3.50** (0.29)	2.09–5.21	**1.97** (0.08)	1.44–2.21	**2.94** (0.26)	2.24–4.59	**2.23** (0.29)	0.95–3.14	0.85 (0.13)	0.31–1.45
stems	HGQ	**0.09** (0.02)	0.04–0.16	**0.12** (0.03)	0.05–0.36	0.05 (0.01)	0.04–0.08	0.06 (0.01)	0.03–0.09	0.04 (0.01)	0.02–0.07
	HH1	**0.47** (0.08)	0.19–0.80	**0.19** (0.03)	0.11–0.31	**0.19** (0.01)	0.14–0.23	**0.30** (0.05)	0.18–0.62	0.03 (0.01)	0.02–0.06
	HH2	**0.50** (0.08)	0.20–0.83	**0.30** (0.05)	0.14–0.58	**0.14** (0.01)	0.10–0.22	**0.19** (0.03)	0.11–0.40	0.06 (0.01)	0.03–0.10
	HH3	**0.17** (0.02)	0.08–0.27	**0.11** (0.02)	0.06–0.19	**0.06** (0.01)	0.05–0.08	**0.08** (0.01)	0.05–0.14	0.02 (0.01)	0.02–0.04
	HH4	**0.17** (0.03)	0.07–0.29	**0.08** (0.01)	0.05–0.12	**0.08** (0.01)	0.06–0.10	**0.11** (0.02)	0.07–0.22	0.02 (0.01)	0.01–0.02
	Goss	**0.51** (0.09)	0.18–0.98	0.13 (0.02)	0.07–0.25	**0.22** (0.02)	0.15–0.29	**0.21** (0.04)	0.12–0.45	0.05 (0.01)	0.02–0.08
taproots	dHG	0.06 (0.01)	0.00–0.10	0.06 (0.01)	0.00–0.11	0.08 (0.02)	0.00–0.18	**0.12** (0.01)	0.09–0.20	0.09 (0.01)	0.00–0.13
	HG	0.10 (0.02)	0.04–0.22	0.11 (0.04)	0.02–0.35	0.14 (0.04)	0.04–0.33	**0.37** (0.07)	0.17–0.91	0.06 (0.01)	0.03–0.10
	dMHG	**0.03**(0.01)	0.00–0.05	0.02 (0.01)	0.00–0.07	**0.04** (0.01)	0.00–0.08	**0.06** (0.01)	0.03–0.09	ND[c]	ND
	MHG	0.02(0.01)	0.01–0.04	**0.03** (0.01)	0.00–0.06	**0.03** (0.01)	0.02–0.07	**0.07** (0.02)	0.03–0.18	ND	ND
	HGQ	0.10 (0.02)	0.00–0.15	ND	ND	**0.12** (0.03)	0.00–0.25	**0.12** (0.02)	0.00–0.16	0.06 (0.02)	0.00–0.14
	Goss	**25.91** (3.63)	13.45–51.04	**20.79** (1.88)	13.70–30.65	**32.35** (2.03)	24.39–42.68	**26.60** (2.61)	14.05–37.99	2.16 (0.18)	1.57–3.32
	MG	**1.27** (0.06)	1.06–1.59	1.09 (0.16)	0.00–1.73	**1.14** (0.15)	0.00–1.47	**1.46** (0.12)	1.01–2.12	0.79 (0.03)	0.69–1.00
	DMG	0.20 (0.13)	0.00–0.94	ND	ND	0.17 (0.11)	0.00–0.77	0.19 (0.13)	0.00–0.93	ND	ND
feeder roots	dHG	**0.15** (0.03)	0.08–0.34	**0.09** (0.01)	0.07–0.10	**0.13** (0.01)	0.07–0.22	**0.13** (0.02)	0.07–0.28	0.03 (0.02)	0.00–0.12
	HG	**0.39** (0.11)	0.09–1.08	0.21 (0.03)	0.09–0.42	**0.36** (0.06)	0.08–0.55	**0.37** (0.08)	0.12–0.83	0.15 (0.03)	0.02–0.33
	dMHG	**0.07** (0.01)	0.04–0.14	**0.05** (0.01)	0.03–0.07	**0.06** (0.01)	0.03–0.08	**0.06** (0.01)	0.04–0.09	0.01 (0.01)	0.00–0.04
	MHG	0.03 (0.01)	0.01–0.06	0.03 (0.01)	0.01–0.08	0.04 (0.01)	0.00–0.11	0.04 (0.01)	0.01–0.09	0.02 (0.01)	0.00–0.09
	HGQ	**0.13** (0.02)	0.00–0.22	0.05 (0.02)	0.00–0.11	**0.09** (0.02)	0.00–0.16	**0.10** (0.02)	0.00–0.15	ND	ND
	Goss	**10.74** (0.93)	7.26–14.70	5.07 (0.74)	1.96–8.93	**8.14** (1.47)	2.57–16.59	6.12 (1.23)	2.25–14.29	5.88 (0.77)	2.67–9.86
	MG	2.30 (0.22)	1.40–3.40	1.58 (0.19)	0.92–2.63	1.81 (0.31)	0.83–3.89	1.74 (0.37)	0.94–4.46	2.72 (0.32)	1.66–4.29
	DMG	0.88 (0.20)	0.00–1.74	0.28 (0.14)	0.00–1.01	0.38 (0.16)	0.00–1.19	0.60 (0.18)	0.00–1.67	1.14 (0.12)	0.80–2.00

[a] Bold numbers indicate that the mean is significantly greater than the ST 474 mean ($P = 0.05$). [b] Goss, gossypol; HGQ, hemigossypolone; HH1, heliocide H_1; HH2, heliocide H2; HH3, heliocide H3; HH4, heliocide H4; dHG, desoxyhemigossypol; HG, hemigossypol; dMHG, desoxyhemigossypol-6-methyl ether; MHG, hemigossypol-6-methyl ether; MG, gossypol-6-methyl ether; DMG, gossypol-6,6′-dimethyl ether. [c] ND, none detected.

474 but not in either root tissue. Accession 2472, with an intermediate level of (+)-gossypol in the seeds, had a higher percentage of (+)-gossypol only in the leaves and taproots as compared to ST 474. Other than the seed, all of the plant tissue examined in accession 2443, with a near normal percentage of (+)-gossypol in the seeds, had less (+)-gossypol than ST 474.

Total Terpenoid Concentrations. The total terpenoids were determined by HPLC methods previously described (*15*, *16*). The mean concentrations for the terpenoids that are most critical for disease resistance [i.e., desoxyhemigossypol and hemigossypol (*19*−*21*)] were significantly higher in the feeder roots of accession 2452 than in ST 474. Other terpenoids were either comparable to ST 474 or, in the case of gossypol and hemigossypolone, were significantly greater than in ST 474.

The total gossypol concentrations in the seed of all *marie galante* accessions were not significantly different than those in ST 474 (**Table 2**). The leaves and stems of accessions 2452 and 2425 had mean concentrations of gossypol and the other terpenoids that were from two to 11 times higher than in ST 474 (significantly different, $P = 0.05$). In root tissues, the total terpenoid concentrations were more in line with that in ST 474. However, 2452 was remarkable in having from two to 10 times more gossypol in the root tissues as compared to ST 474.

Anticipated Effects on Disease Resistance. The difference in the (+)- and (−)-gossypol ratio in the taproots of accession 2452 was greater than in ST 474, but in the feeder roots, the opposite was observed. Earlier in vitro studies on the seedling

disease pathogen *Rhizoctonia solani* showed that racemic and (+)- and (−)-gossypol were not very effective at reducing pathogen growth or survival, nor did they differ in their toxicity (*22*). Thus, differences in the (+)- to (−)-gossypol ratios in this tissue should not affect disease susceptibility. The higher constitutive concentrations of desoxyhemigossypol and hemigossypol in 2452 (**Table 2**) may indicate that these plants will be more resistant to soilborne fungal pathogens. However, a quick response to infection as evidenced by a rapid induction of terpenoid synthesis in infected tissue is an essential element in disease resistance (*16*, *23*). Thus, while the higher constitutive concentrations of desoxyhemigossypol and hemigossypol in healthy feeder roots may augment disease resistance, they may not be predictors of resistance.

Anticipated Effects on Insect Resistance. The percentages of (+)-gossypol in the leaves and stem of accessions 2452 and 2425 were higher than in ST 474 (**Table 1**). Because the concentration of the terpenoids in accession 2452 was from two to five times that in ST 474, plants exhibiting these high levels of the terpenoids in foliage may actually be more resistant than some currently used commercial cultivars.

Implications for Breeding. The variation in the ratio of (+)- to (−)-gossypol in the different tissues in these four *G. hirsutum* var. *marie galante* accessions indicates that this trait is under separate genetic regulation in the individual tissues. This high (+)-gossypol trait has been incorporated into commercial cotton cultivars, and a correlation was found to exist between the level of (+)-gossypol in the flower petals with that in the seed (*24*).

Of the four *G. hirsutum* var. *marie galante* accessions tested, accession 2452 appears to be the most suitable as a parent for a breeding program to produce plants with high (+)-gossypol in the seed. That is, accession 2452 consistently gives seed with >95% (+)-gossypol in the seed, but it also provides high levels of terpenoids in the stems, leaves, and roots. Thus, one would predict that plants with these characters would exhibit disease resistance comparable to commercial cotton cultivars. For insect resistance, on the basis of the higher levels of individual terpenoids in the leaves, one can predict that plants with these traits would show superior insect resistance as compared to ST 474. Furthermore, because the total gossypol in the 2452 seed was not statistically different from ST 474, processing seeds of progeny from 2452 that retain this character should be no more difficult than that of the commercial cultivar. Because levels of (+)- and (−)-gossypol and the concentration of the terpenoids in the bolls could not be measured, one cannot positively predict the resistance of these plants to insect herbivores. Actual field trials are required to ultimately answer these questions. Nevertheless, at this time, there are no obvious inheritable traits in *marie galante* accessions exhibiting high (+)-gossypol that would lead to increased susceptibility to pathogens or herbivorous insects.

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