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Evaluating Protective Terpenoid Aldehyde Compounds in Cotton (*Gossypium hirsutum* L.) Roots

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Abstract

Cotton (*Gossypium hirsutum* L.) has epidermal glands containing terpenoid aldehyde (TA) compounds that protect the plant from pests and diseases. One TA, gossypol, has two forms (+) and (−) that are present in varying amounts. This study evaluated the variation in roots for TA compounds and how environment affected the levels of these compounds. Similar to seed, gossypol was the predominant TA in roots. No heliocides were detected with only trace amounts of other TAs, such as hemigossypolone or hemigossypol, detected in a few lines. Among the glanded lines, there were significant differences in gossypol content. Percent plus gossypol was consistently 4% - 10% higher in roots than seed. One line, "Mac7", had 12 - 14 ug/mg gossypol in roots and 18 ug/mg in seed as well as >90% (+) gossypol in both roots and seed. Unlike other tissues in glandless cotton, the roots of glandless lines consistently produced detectable amounts of gossypol with 77% to 82% in the (+) form. Multi-year field tests showed that although gossypol levels in the roots were more affected by insect pressure or other environmental conditions than seed, there was selectable variation in upland cotton for root gossypol content. Unlike other parts of a glandless plant, the roots retained a functioning biochemical pathway for gossypol production.

Keywords

Cotton, Gossypol, Host Plant Resistance, Roots, Cotton Seed, Terpenoid Aldehyde

1. Introduction

Cotton (*Gossypium hirsutum* L.) has a number of traits that enhance the plant's self-protection mechanisms and

provide host plant resistance (HPR). For example, cotton produces a number of terpenoid aldehyde (TA) compounds that are normally contained in epidermal glands found throughout the plant [1]. These compounds help protect the plant from pests and diseases [2] [3]. While gossypol is reported to be the predominant TA compound in seeds, petals and roots, other TAs predominate in "green" tissues such as leaves, bracts, calyces and boll hulls (carpel wall). The two most common are hemigossypolone (HGQ) and a group of related TAs often referred to as heliocides [2] [4]. All the compounds are derived from the terpenoid desoxyhemigossypol (dHG). Gossypol is derived from the conversion of dHG to hemigossypol (HG) and then two HG molecules are joined to form gossypol. The bond joining the two molecules can be either in a plus (+) or minus (−) isomericorientation. Hemigossypolone (HGQ) is an oxidized form of dHG and the heliocide compounds are formed by adding myrcene or *β*-ocimene to the HGQ molecule (**Figure 1**).

TAs in seed have been well studied and numerous reports have shown significant natural variation for total seed gossypol within *G. hirsutum* and *G. barbadense* L. species. Among *G. hirsutum* entriesin the 2012 National Cotton Variety Test, percent total gossypol ranged from 0.55% to 1.89% (www.ars.usda.gov/SP2UserFiles/Place/64021500/2012NCVT.pdf). Gossypol exists as enantiomers (isomers) referred to as (+)- and (−)-gossypol [5] [6] with the (−) enantiomer reported to be more toxic than the (+)-enantiomer. For example, the (−)-enantiomer exhibited a greater ability to inhibit the growth of cancer cell lines [7] [8]. By adding pure (+)- or (−)-gossypol to broiler chicken diets, Lordelo *et al*. [9] found that (−)-gossypol inhibited growth in broilers to a greater extent than (+)-gossypol. The ratio of (+) to (−) gossypol in seeds was variable among species and cultivars. Stipanovic *et al*. [4] analyzed the seeds, leaves, stems and roots from four photo-period sensitive accessions of the *G. hirsutum* race stock *marie galante* (moco cotton) for gossypol and related TAs. Two of the accessions had >90% (+)-gossypol in the seed, but both had lower percentages of (+)-gossypol in the leaves (65%) and in roots with 56% in the first and 64% in the second accession. This indicated that it was possible to have lines with a large excess of (+)-gossypol in the seed and a normal ratio of (+)-gossypol to (−)-gossypol (*i.e*., 3:2) in the leaves and roots. A number of studies had demonstrated the efficacy of gossypol against diseases and insects. One study indicated that the (+) and (−) forms were equally effective against black scurf, caused by the plant pathogen *Rhizoctonia solani* [10]. A second study reported similar results for cotton bollworm (*Helicoverpa zea* Boddie) larvae [11]. Tests to assay antibiosis and weight gain for cotton bollworm and tobacco budworm (*Heliothis virescens* F.) showed less weight gain when fed on cotton lines having higher levels of the leaf TA hemigossypolone (HGQ) [12].

Cotton lines exist with no epidermal glands (glandless), and when glands are not present in the above ground parts of the cotton plant, TA compounds are not produced [13]-[15]. Because of this relationship, TA content is associated with the number of glands present [16] [17]. While glandless lines do not produce TAs in the above ground parts of the plant, Smith [18] reported gossypol being produced by excised root tips from a glandless line,

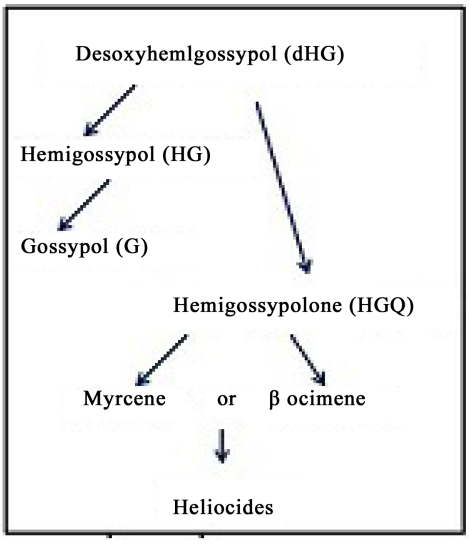

Figure 1. Pathway for biosynthesis of the terpenoid compounds.

indicating that glandless roots may have the intact biochemical pathways necessary to produce gossypol. While much research to date has concentrated on eliminating seed glands and gossypol to improve the seed's value as livestock, poultry or fish feed, it is important to remember that the major function of the TA containing glands is to protect the vegetative and reproductive tissues of the plant. Hedin *et al*. [3] used cotton lines with glanding on the calyx crown (high glanded) as resistant lines and those with no glanding on the calyx crown as susceptible lines to study the relationship between TAs in cotton bracts or floral parts and larval growth of tobacco budworm. They found that HGQ and heliocides were higher in resistant lines with glanding on the calyx crown compared to the susceptible lines. Stipanovic *et al*. [4] evaluated TAs including gossypol, HG, HGQ, heliocides and related derivatives in seeds, stems, leaves and roots of four *marie galante* cotton race stocks and the cultivar "Stoneville 474" (STV474). Two of the four *marie galante* lines had leaf and stem TAs significantly higher than STV474. All the *marie galante* lines had root gossypol levels above 20 ug/mg compared to 2.2 ug/mg for STV474. Several studies have indicated that although TAs can be found in the plant at any stage, the levels can be affected by pest pressure. Hunter *et al*. [19] reported that the TAs gossypol, hemigossypol and their methoxy derivatives increased in 5 or 12 day old hypocotyls inoculated with *Rhizoctonia solani* compared to mock inoculated control plants. Khoshkhoo *et al*. [20] evaluated gossypol, HGQ and heliocide levels in cotton lines resistant and susceptible to root-knot nematode (RKN) before and after infestation with RKN. They found that the levels of TAs increased within four days after inoculation, but were not associated with resistance as a susceptible glanded line had the highest TA levels. A greenhouse study using a diploid relative of Upland cotton, *G. herbaceum*, indicated that both the above ground foliage and below ground root system exhibited increased TA levels when infested with either *Spodoptera exigua* (armyworm) or *Agriotes lineatus* (wireworm) larvae [21].

Variation for TA levels in root and green tissues has not been as extensively studied as seed. One reason has been the lack of assay methods to easily separate and quantify the TAs gossypol, HGQ and heliocides which are predominant in "green" tissues such as leaves, bracts, bolls, calyces and stems. High performance liquid chromatography (HPLC) based methods have been developed to separate and quantify TAs in "green" tissues and "white" tissues such as petals or roots [4] [22], and recent modifications to the original methods currently allow economical large scale screening for TAs in all plant tissues [12] [23].

With the advent of *bt* cotton, boll weevil eradication in the U.S. and improved fungicide treatments, interest in host plant resistance (HPR) declined; however, growing concern over development of insect resistance to the *bt* toxin and increased damage from other plant pests, has renewed interest in enhancing HPR by increasing levels of plant compounds toxic to plant pests. This study focused on roots and evaluated the type, variation and consistency of TAs produced, to assess the possibility of improving root chemical defenses. The tests conducted over four years, concentrated on assessing the levels of gossypol and related TAs in roots among a diverse set of cotton lines. Additional tests evaluated how gland density affected the level of gossypol in roots and seeds, in field as well as greenhouse grown plants. The effect of environment on TA levels was assessed in multi-year yield trials as well as in two field tests measuring the effect of insect pressure on levels of TAs in roots and seed.

2. Materials and Methods

2.1. Plant Material

A range of cotton lines and cultivars (**Table 1**) were used to evaluate the production of TAs in roots under greenhouse and field conditions. Seeds were used as a comparison for each test. To further evaluate the observation by Smith [18] that detached glandless root tips were capable of producing gossypol, sets of glanded and glandless near isogenic lines (NILs) were included in the study. Lines with varying gland densities (GVS2, GVS3, HPR03ne, H1220) were also included to evaluate the association of gland density with root TA levels.

2.2. Experimental Plan

Field experiments were conducted at Stoneville, Mississippi, arranged in randomized complete block designs (RCBD) with two replications (**Table 2**). Experimental plots were either two or four 6 m rows with 1 m between rows. All tests except the untreated "No TRT" plots were conducted within the same 50 m × 320 m area at Stoneville (Stoneville Field). The "No TRT" tests were grown at another farm (Livingston Farm) 3 km away. The Livingston Farm area was managed without any insecticide treatments to allow experiments assessing a crop's natural ability to protect itself. All the plots were fertilized, irrigated and weeds controlled following

Table 1. Information for entries used in the study.

Entry	Glands	Pedigree Information
STV GL	normal	near isogenic line (NIL) [12]
STV gl	no glands	near isogenic line (NIL) [12]
MAXXA GL	normal	near isogenic line (NIL) [12]
MAXXA gl	no glands	near isogenic line (NIL) [12]
JACO GL	normal	near isogenic line (NIL) [12]
JACO gl	no glands	near isogenic line (NIL) [12]
A6 GL	normal	near isogenic line (NIL) [12]
A6 gl	no glands	near isogenic line (NIL) [12]
GVS5069	3/4 normal	F11 progeny of cross A1006 (ACSI elite line) x STV 7A gl, has high levels of leaf TA
GVS2	1/2 normal	gland density 1/2 of STV GL [17]
GVS3	1/4 normal	gland density 1/4 of STV GL [17]
HPR03ne	on calyx crown	(MD51ne x H1220) x A1006ne nectariless okra elite line
H1220	on calyx crown	PI 578226
Mac7	normal	ARS Release P.0063.14 >90% (+) gossypol
GVS6	normal	elite line >90% (+) gossypol [27]
GVS7	normal	elite line >90% (+) gossypol [27]
U3 UEL	normal	ultra early line <110 days to maturity (UEL) Uzbekistan origin
U9 UEL	normal	ultra early line <110 days to maturity (UEL) Uzbekistan origin
Acala 1517-99	normal	CV-115, PI 612326
DP432 RR	normal	PVP 200400047
FM832	normal	FIBERMAX 832 PVP 9800259
MD51ne	normal	CV-103, PI 566941
MD26ne	normal	PI 666042
MD87	normal	PI 666044
Phy72	normal	PHY72 Acala PVP 200100115
Phy800	normal	PHY 800 Pima type Patent No. 8,319.043
PIMA S7	normal	PI 560140 Pima type
SG747	normal	Sure-Grow 747 PVP 9800118
STV474	normal	ST 474 PVP 9400152
UA48	normal	CV-129, PI 660508

Table 2. Summary of field and greenhouse tests designed in a randomized complete block design with two replications.

Study	Year	Nr. of Entries	Root	Seed
Yield Trial 2008	2008	11	X	X
TRT vs No TRT Test 2009	2009	13	X	X
TA Variation Study (TAVS)	2009	17	X	X
TAVS Greenhouse 2009/10	2010	17	X	
Yield Trial 2011	2011	18	X	X
TRT vs No TRT Test 2012	2012	17	X	X

recommended management practices. With the exception of the untreated (No TRT) tests, all the field trials received in furrow insecticide and fungicide. In 2009, the tests were also sprayed for early season insects. The “No

TRT" tests received no in furrow insecticide or spray treatments.

From each plot, 20 plants were sampled at stage V5 [24]. Stage V5 was defined as when the cotyledons and four primary leaves are present and the next true leaf's mid-vein had expanded to 2.5 cm. V6 is the stage when the sixth true leaf's mid-vein length is 2.5 cm and so forth until the plant transitions to the reproductive stages with the appearance of the first visible square. The greenhouse experiment was conducted using seed harvested from the 2009 Terpenoid Aldehyde Variation Study (TAVS) field test (**Table 2**). The plants were sown into 36 L plastic pots filled with a well-drained potting mix (Metro Mix 360, Sun-Gro, Agawam, MA) that had been pre-moistened for 24 hours. Forty seed per pot were sown at a 1 cm depth and thinned at seedling emergence to 25 plants per pot. Greenhouse growing conditions were 16 hours light at 32˚C and 8 hours dark at 21˚C. At stage V5, the roots were harvested, processed, quick frozen and stored at −80˚C prior to analysis.

A number of compounds present in plants, including cotton, increase in response to pest attack. To test the response of roots under field conditions, plants were grown under conditions where soil-borne insects and pathogens were not controlled and no insecticides were used. The objective of the "No TRT" tests was to determine if insect pressure or soil borne pathogens would increase the TA levels in the roots of young cotton plants. As a control, the same lines were grown in a test where the plants were treated with in-furrow insecticide and fungicide (**Table 2**). In 2009, the treated test was also sprayed with an insecticide to control early season insects. In 2012, early season insects were well below the integrated pest management (IPM) recommended limit for insecticide application. At stage V5, roots were sampled from both field tests and analyzed for the TAs gossypol, HGQ and heliocides, HQ and methoxy compounds.

2.3. Sampling Root and Seed Tissue

Twenty stage V5 plants were sampled from each plot or greenhouse pot, placed on ice and taken to the lab for processing. Fifteen plants were processed, with the roots cut from the plant just below soil level where the red/green stem intersects the white root tissue. Any root hairs were trimmed from the main taproot, then rinsed thoroughly to remove any soil particles, drained and cut into 5 mm sections. The root sections were placed in a 50 ml centrifuge tube and frozen at −80˚C. The samples were subsequently freeze-dried and stored at −20˚C. Freeze-dried samples were first ground in a Cyclotec 1093 Sample Mill (FOSS, Eden Prairie, MN) with a 1-mm mesh screen and then ground to a fine powder with a KLECO metal ball grinder (Garcia Machine, Visalia, CA). For seed preparation, 20 seed from each plot were placed in a 50 ml centrifuge tube and soaked in water overnight. The seed kernel was then gently pressed from the hull, placed back in the 50 ml tube and frozen at −80˚C. Seeds were freeze-dried and ground to a fine powder with the KLECO grinder. All ground tissue samples were stored at −20˚C prior to HPLC analysis [23].

2.4. Terpenoid Aldehyde Analysis

Determination of total seed gossypol and the (+)- and (−)-enantiomeric ratios, was performed according to a method originally described by Hron *et al*. [25] and modified to accommodate large numbers of samples [23]. Briefly, a 100 mg ground sample was extracted with 3 mL of complexing reagent [by volume 2:10:88, R-(−)2-amino-1-propanol, acetic acid, N, N-dimethylformamide] incubated in a 100˚C heater block for 30 minutes, cooled to room temperature, vortexed for 30 seconds, and diluted with 8 mL mobile phase (by volume 85:15; acetonitrile: 10 mM potassium phosphate pH = 3). An aliquot was centrifuged at 6000 rpm for 2 min for pellet formation. The liquid was poured into an HPLC vial for gossypol enantiomer quantification by HPLC. The analytical method used in determining TA concentrations was high performance liquid chromatography (HPLC) performed on a Waters 717 Autosampler/600 Pump, coupled to a Waters 2998 Photodiode Array Detector (PDA) set at 254 nm. A 20 µL injection was made on an Intersil ODS-2 column (5 µm, 4.6 mm × 100 mm i.d.) connected to a MetaGuard pre-column (4.6 mm) in which the flow rate was set to 1 mL/min for 5 minutes with isocratic conditions, acetonitrile:10mM potassium phosphate pH = 3.0 (85:15). Root samples were extracted using a method originally reported by Stipanovic *et al*. [4] with modifications. The extraction consisted of 100 mg ground tissue per 3 mL complexing reagent agent [by volume 2:10:88, R-(−)2-amino-1-propanol: acetic acid: acetonitrile]. The sample was heated in a 70˚C heater block for 30 minutes, cooled to room temperature, and vortexed for 30 seconds. An aliquot was centrifuged at 6000 rpm for 2 min for pellet formation then the liquid was poured into an HPLC vial for TA quantitation. The HPLC analysis was performed on a Waters 717 Autosampler/600 Pump, coupled to a Waters 2998 Photodiode Array Detector (PDA) set at 272 nm. A 20 µL injec-

tion was made on an Intersil ODS-3 column (5 µm, 3.0 mm × 150 mm i.d.) connected to a MetaGuard pre-column (2.0 mm) in which the flow rate was set to 0.8 mL/min for 12 minutes with isocratic conditions, acetonitrile:methanol:10 mM potassium phosphate pH = 3.0 (by volume 43:37:20). Gossypol, hemigossypolone (HGQ) and heliocides were quantified using known standards. The presence of hemigossypol (HG) and methoxy derivatives was estimated based on peak areas at known elution times.

2.5. Statistical Analyses

Tissue and seed data were analyzed in a randomized complete block design using the GLIMMIX procedure SAS 9.3 (SAS Inst., Inc., Cary, NC). Fixed variables included entry, HGQ, heliocides, gossypol, (+)- to (−)-gossypol ratio, and location (treated and untreated) whereas block was treated as a random effect. The Gaussian distribution and identity function model specifications were used for the analysis. Standard errors for block were examined to determine if differences between blocks existed. p values for fixed effects were used to determine if differences ($p \leq 0.05$) existed. Conservative Least-Square means at the $p = 0.05$ level were used to test for differences between entries. Pearson product moment correlations were calculated using JMP 10.0.0 (SAS Inst., Inc., Cary, NC).

3. Results and Discussion

3.1. Assessing Levels of Gossypol and Related TAs in Seeds and Roots

Results from the lines that were tested in all four trials conducted over the four years were summarized and compared. All the tests were conducted in the same field under the same management conditions. The results showed that gossypol was the only detectable TA in the seed. In the roots, gossypol was the predominant TA with HGQ below the detectable limit (0.01 ug/mg) (**Table 3**). Heliocides were not detected and HG was detected as small peaks (<0.1 ug/mg) in two of the samples. Roots ranged from 4.7 to 13.8 ug/mg for lines with normal glanding, while seed ranged from 12.3 to 21.4 ug/mg gossypol. The three glandless lines had no seed gossypol or other TAs, but averaged 3.3 ug/mg gossypol in the roots. No other TAs were detected in the glandless roots. The GVS2 and GVS3 lines with reduced glanding ranged from 2.9 to 5.8 ug/mg root gossypol depending on the year. Seed contained from 2.8 to 5.9 ug/mg gossypol. There was a clear difference in the percent of (+)-gossypol between roots and seed, with roots from 60% to 82% and seeds ranging from 49% to 66%.

Table 3. Summary of total gossypol (ug/mg) and percent plus (+) gossypol for stage V5 roots and mature seed from multi-year field tests. Values are the mean of two field replications.

Entry	ROOT								SEED							
	YLD Trial 2008	% (+)	aTRT Test 2009	% (+)	YLD Trial 2011	% (+)	TRT Test 2012	% (+)	YLD Trial 2008	% (+)	TRT Test 2009	% (+)	YLD Trial 2011	% (+)	TRT Test 2012	% (+)
STV GL	6.7	72	13.4	68	10.4	69	9.3	68	15.4	56	13.9	60	16.3	61	17.5	58
MAXXA GL	6.8	71	9.6	69	4.7	65	7.3	69	17.7	58	12.3	63	14.1	62	16.4	59
JACO GL	8.6	70	8.8	72	8.3	69	10.3	69	17.1	59	17.7	62	18.8	63	20.4	60
DP432RR	bNT	NT	13.8	71	9.4	67	8.9	69	NT	NT	15.5	63	17.8	63	17.9	61
H1220	8.7	71	12.3	74	8.4	68	11.9	70	21.4	65	16.2	68	17.3	69	19.5	66
GVS5069	13.5	60	13.5	66	8.0	65	9.7	67	13.2	56	10.0	58	13.9	60	15.7	58
GVS2	2.9	77	5.1	75	3.2	72	3.1	70	5.8	52	2.8	57	4.6	56	5.2	56
GVS2	3.9	73	5.8	72	3.3	75	4.9	69	4.5	51	3.3	49	4.1	52	5.9	54
STV gl	2.3	81	5.8	78	3.6	77	2.8	79	0.0	0	0.0	0	0.0	0	0.0	0
MAXXA gl	2.5	82	5.1	77	1.9	76	1.9	78	0.0	0	0.0	0	0.0	0	0.0	0
JACO gl	2.4	81	5.0	79	2.5	79	NT	NT	0.0	0	0.0	0	0.0	0	NT	NT

[a]TRT Test = Indicates it was the TRT part of the TRT (treated) vs NoTRT test. All tests in the table were conducted in the same field at Stoneville Mississippi, but in different years. [b]NT = Not tested.

In 2011, roots and seeds were sampled from a set of cotton lines with a known broad range of seed gossypol (Table 4). Root gossypol ranged from 1.9 to 3.6 ug/mg for the glandless lines and they were 35% lower than their glanded NILs. The two high plus gossypol lines (GVS6, GVS7) each had Mac7 as one parent and had higher plus (+) gossypol in both seeds and roots ranging from 89% to 93%. Again HGQ and heliocides were below 0.01ug/mg and there were trace amounts (<0.1 ug/mg) of HG in some samples.

3.2. Effect of Environment and Insect Pressure on TA Levels

To determine whether root and seed values would remain consistent over environments, a TA Variation Study (TAVS) was conducted with a second set of lines that were grown and sampled in the field. The seeds from those plots were subsequently planted in the TAVS greenhouse test (Table 2) with roots sampled at stage V5 (Table 5).

Root gossypol content was similar in the field and greenhouse ($r = 0.77$, $p < 0.0001$) and increased to $r = 0.86$ when the exceptional line, STV GL, was omitted from the analysis. Seed gossypol was consistently higher than root gossypol among the normally glanded lines with the average difference 8.1 ug/mg (field roots) or 8.2 ug/mg (greenhouse roots) between seed and roots. Differences between seed and root gossypol in the reduced gland lines (GVS2, GVS3) were 0.4 ug/mg for field grown roots and 2.6 ug/mg for greenhouse grown roots. It was also notable that the glandless line STV gl had 4 ug/mg gossypol in the roots under both field and greenhouse conditions. Differences in percent (+)-gossypol were similar to those observed in the previous test with the exception of the high (+)-gossypol line Mac7 which had 90% or 91% in both seed and roots. HGQ and heliocides were below 0.01ug/mg. The two Pima lines, PIMA S7 and Phytogen 800 had methoxy derivatives in both seed and root (data not shown).

The 2009 and 2012 treated (TRT) versus no treatment (No TRT) tests gave different results. As predicted, the test conducted in 2009 showed a consistently higher level of gossypol in the roots of the No TRT test plots compared to the TRT test plots (Table 6). Differences between the TRT and No TRT plots were less for seed

Table 4. Comparison of total gossypol (ug/mg) and percent plus (+) gossypol in stage V5 roots and mature seed from the 2011 Yield Trial. Values are the mean of two field replications.

Entry	Root (ug/mg)		% (+)	Seed (ug/mg)		% (+)
STV GL	[a]**10.4**	bcde	69	**16.3**	bcd	61
STV gl	**3.6**	fgh	77	**0.0**	k	0
MAXXA GL	**4.7**	efgh	65	**14.1**	efg	62
MAXXA gl	**1.9**	h	76	**0.0**	k	0
JACO GL	**8.3**	cdefg	69	**18.8**	a	63
JACO gl	**2.5**	gh	79	**0.0**	k	0
GVS5069	**8.0**	defg	65	**13.9**	fg	60
GVS2	**3.2**	gh	72	**4.6**	j	56
GVS3	**3.3**	gh	75	**4.1**	j	52
DP432RR	**9.4**	cdef	67	**17.8**	ab	63
H1220	**8.4**	cdef	68	**17.3**	abc	69
SG747	**10.7**	bcd	64	**12.9**	g	55
MD87	**12.9**	abc	65	**15.3**	def	54
MD26ne	**9.9**	cde	64	**15.7**	cde	57
U3 UEL	**5.8**	efgh	70	**8.5**	hi	66
U9 UEL	**6.8**	defg	67	**12.5**	g	59
GVS6	**14.7**	ab	89	**17.8**	ab	93
GVS7	**16.3**	a	90	**10.2**	h	91

[a]Means followed by a common letter are not significantly different at $p = 0.05$.

Table 5. Comparison of total gossypol (ug/mg) and percent plus (+) gossypol in stage V5 roots grown in the field 2009 and greenhouse 2009/2010. Total and (+) gossypol from seed harvested from the same plots as the 2009 field root samples and used for the greenhouse study. Values are the mean of two field replications.

Entry	Seed Field 2009		% (+)	Root Field 2009		% (+)	Root Green-House 2009/10		% (+)
STV GL	[a]**16.6**	abc	60	**10.2**	b	72	**5.2**	cde	70
STV gl	**0.0**	h	0	**4.3**	efg	75	**4.2**	cde	78
GVS2	**4.7**	g	53	**4.1**	efg	73	**1.8**	f	72
GVS3	**4.8**	g	50	**4.8**	efg	74	**3.1**	ef	76
DP432RR	**18.7**	a	64	**6.6**	cde	73	**6.4**	bcd	72
H1220	**18.0**	ab	66	**8.0**	bc	74	**8.9**	b	71
SG747	**13.7**	de	57	**7.9**	bc	69	**8.4**	b	69
FM832	**10.8**	f	55	**7.8**	bc	70	**6.5**	bcd	68
STV474	**16.5**	bc	65	**5.7**	cdef	74	**6.9**	bc	70
Phy72	**12.1**	ef	62	**5.5**	cdef	73	**5.2**	cde	72
MD51ne	**16.2**	bc	65	**5.7**	cdef	73	**4.2**	cde	72
Acala 1517-99	**11.8**	ef	57	**5.0**	def	74	**4.5**	cde	73
Mac7	**17.8**	ab	90	**13.7**	a	91	**12.0**	a	91
U3 UEL	**15.6**	cd	62	**4.3**	efg	75	**4.3**	cde	73
U9 UEL	**12.0**	ef	60	**3.8**	fg	75	**4.1**	cde	71
PIMA S7	**12.1**	ef	46	**3.8**	fg	73	**4.3**	cde	72
Phy800	**11.9**	ef	49	**4.3**	g	73	**4.0**	de	78

[a]Means followed by a common letter are not significantly different at p = 0.05.

Table 6. Total gossypol (ug/mg) and percent plus (+) gossypol for stage V5 roots and mature seed from a 2009 field test comparing entries with insecticides for early season insects and the same entries not treated. Values are the mean of two field replications.

[a]Entry	[b]TRT Root	% (+)	No TRT Root	% (+)	[c]Difference	TRT Seed	% (+)	No TRT Seed	% (+)	Difference
STV GL	**13.4**	68	**19.1**	63	5.7	**13.9**	60	**15.2**	61	1.3
STV gl	**5.8**	78	**5.5**	77	−0.3	**0.0**	0	**0.0**	0	0.0
MAXXA GL	**9.6**	69	**13.2**	67	3.6	**12.3**	63	**13.9**	63	1.6
MAXXA gl	**5.1**	77	**6.1**	77	1.0	**0.0**	0	**0.0**	0	0.0
JACO GL	**8.8**	72	**15.6**	66	6.8	**17.7**	62	**19.5**	62	1.8
JACO gl	**5.0**	79	**6.1**	78	1.1	**0.0**	0	**0.0**	0	0.0
A6 GL	**8.0**	71	**19.5**	65	11.5	**12.4**	62	**13.4**	61	1.1
A6 gl	**5.0**	77	**6.9**	76	1.9	**0.0**	0	**0.0**	0	0.0
GVS5069	**13.5**	66	**14.7**	63	1.2	**10.0**	58	**12.9**	59	2.9
GVS2	**5.1**	75	**7.7**	71	2.6	**2.8**	57	**3.1**	55	0.3
GVS3	**5.8**	72	**5.9**	69	0.1	**1.3**	49	**2.3**	52	1.0
DP432RR	**13.8**	71	**12.9**	69	−0.9	**15.5**	63	**17.7**	63	2.2
H1220	**12.3**	74	**16.1**	67	3.8	**16.2**	68	**17.7**	68	1.6

[a]There were significant differences among entries p < 0.0001. [b]TRT = Indicates it was part of the TRT (treated) vs NoTRT test. TRT test received in-furrow insecticide and fungicide while the No TRT test did not. The TRT test was sprayed to control early season insects. [c]Difference between TRT and No TRT gossypol (ug/mg) levels.

gossypol, and the percent (+)-gossypol was not significantly different between the TRT and No TRT plots. Correlations for root gossypol between the TRT and No TRT plots were 0.77 and 0.97 for seed gossypol. In contrast, the 2012 TRT plots had consistently higher root gossypol than the No TRT test plots although the levels were not as high (**Table 7**). Seed gossypol was also slightly higher in the TRT plots. Correlations for gossypol between the TRT and No TRT plots were 0.86 for root and 0.97 for seed. Again percent (+)-gossypol was highest in the GVS6 and GVS7 lines and in general higher in the roots than in the seed. HGQ and heliocides were below detectable 0.01 ug/mg limit and there were trace amounts (<0.1 ug/mg) of HG in a few samples.

Eleven of the lines were evaluated over four years in field trials conducted on the Stoneville Field (**Table 3**). Comparing these lines highlighted several general trends that were consistent over years. Among the normally glanded lines, there were significant differences in gossypol level between lines and while the absolute amounts varied across years, the rankings generally remained the same, indicating there is selectable variation in Upland cotton for root gossypol. On average, percent plus gossypol remained consistent and was 4% - 10% higher in roots than seed. Some of the other lines evaluated in the field tests (**Tables 4-7**) exhibited unique profiles. The high (+)-gossypol line Mac7 and the two lines derived from it (GVS6, GVS7) exhibited high levels of gossypol in roots, but GVS7 had low seed gossypol, suggesting that gossypol in the root and seed may be independently inherited. These lines also had 80% - 94% (+)-gossypol in both the roots and seed. This profile is similar to that found in many race stock *mariegalante* lines [4] [26], although there is no known *marie galante* in the ancestry of Mac7, GVS6 or GVS7 [27]. The two Pima lines, PIMA S7 and Phytogen 800, (**Table 4**) had lower than average root gossypol, but did have detectable amounts of methoxy derivative TAs.

As in seed, gossypol was the predominant TA in roots and there were no heliocides or HGQ detected at the 0.01 ug/mg limit. The roots in some cotton lines did have detectable amounts of HG (0.5 - 0.8 ug/mg) as estimated

Table 7. Total gossypol (ug/mg) and percent plus (+) gossypol for stage V5 roots and mature seed from a 2012 field test comparing entries with insecticides for early season insects and the same entries not treated. Values are the mean of two field replications.

[a]Entry	[b]TRT Root	% (+)	No TRT Root	% (+)	[c]Difference	TRT Seed	% (+)	No TRT Seed	% (+)	Difference
STV GL	**9.3**	68	**6.9**	68	**−2.4**	**17.5**	58	**17.9**	59	**0.5**
STV gl	**2.8**	79	**1.7**	81	**−1.1**	**0.0**	0	**0.0**	0	**0.0**
MAXXA GL	**7.3**	69	**4.2**	68	**−3.1**	**16.4**	59	**16.0**	60	**−0.4**
MAXXA gl	**1.9**	78	**2.6**	80	**0.7**	**0.0**	0	**0.0**	0	**0.0**
JACO GL	**10.3**	69	**8.0**	69	**−2.3**	**20.4**	60	**18.9**	64	**−1.5**
GVS5069	**9.7**	67	**5.8**	66	**−3.8**	**15.7**	58	**13.8**	60	**−1.9**
GVS2	**3.1**	70	**1.8**	77	**−1.4**	**5.2**	56	**4.7**	60	**−0.5**
GVS3	**4.9**	69	**3.2**	73	**−1.7**	**5.9**	54	**6.4**	54	**0.5**
DP432RR	**8.9**	69	**8.6**	70	**−0.3**	**17.9**	61	**15.6**	64	**−2.3**
H1220	**11.9**	70	**6.2**	70	**−5.7**	**19.5**	66	**18.1**	67	**−1.4**
HPRne3	**5.6**	68	**5.5**	68	**−0.1**	**10.1**	59	**8.7**	61	**−1.3**
MD26ne	**10.4**	64	**8.3**	65	**−2.1**	**17.4**	55	**16.5**	56	**−0.9**
MD87	**10.8**	65	**7.2**	63	**−3.6**	**18.2**	52	**15.1**	55	**−3.1**
UA48	**9.5**	66	**6.4**	68	**−3.1**	**15.0**	56	**14.0**	58	**−1.0**
SG747	**10.7**	63	**9.6**	64	**−1.1**	**12.4**	54	**11.5**	56	**−0.9**
GVS6	**10.9**	91	**8.5**	91	**−2.4**	**18.5**	88	**17.1**	88	**−1.4**
GVS7	**14.2**	92	**9.5**	94	**−4.7**	**11.4**	88	**9.5**	81	**−1.9**

[a]There were significant differences among entries $p < 0.0001$. [b]TRT = indicates it was part of the TRT (treated) vs NoTRT test. TRT test received in-furrow insecticide and fungicide while the No TRT test did not. The TRT test was sprayed to control early season insects. [c]Difference between TRT and No TRT gossypol (ug/mg) levels.

by time of elution, but it was not consistent within lines. Unlike gossypol, HGQ and heliocides, HG was not assayed against a known standard, just elution time and may be an artifact. The glandless lines consistently produced low levels of gossypol (1.9 - 5.0 ug/mg) in the roots with 77% to 82% in the (+)-form. This indicates that glandless roots retain a functioning biochemical pathway for gossypol production, and suggests that the absence of gossypol in the other parts of glandless plants may be regulatory, not a functional loss of biosynthesis pathway components. The observation by others that gossypol and HG were produced by glandless cotyledons inoculated with fungal conidia from *Verticillium dahliae* or *Colletotrichum dematium* [28] supports this conclusion. Further microscopic studies are underway to localize the gossypol in the glandless roots.

Although only the No TRT test in 2009 had higher levels of root gossypol compared to the TRT test, the levels of gossypol were consistent for all normally glanded lines within each test and year, indicating that one or more environmental factors similarly influenced all the lines at a location in a single year. While the 2012 root gossypol levels were higher in the TRT test than the No TRT test, they were still lower than the 2009 levels. The 2009 growing season was below normal with cumulative degree days (DD60) in May and June 30% below normal. There were seedling disease problems and early insect infestations, requiring insecticide applications on the TRT test. In 2012, the DD60 was above normal with low early season insect and disease pressure. Future testing in multiple environments, should further clarify how TA levels in roots respond to biotic and abiotic stresses.

4. Conclusion

With the renewed interest in growing glandless cotton for cold pressed oil, high protein flour and shrimp feed [29], breeders are working to make glandless varieties more tolerant to insects and seedling diseases. The finding that glandless lines consistently produce gossypol in roots indicates that traditional breeding methods can enhance the protective TAs in roots of glandless cultivars, and suggests that extra-glandular TAs may be produced in “green tissues” while keeping the seed free of gossypol. The results of the present study show there is selectable variation in roots for the protective terpenoid aldehyde gossypol and 65% - 94% is found in the (+)-form. While gossypol is consistently present, the roots demonstrate the ability to respond to biotic or abiotic stress. It is interesting that one line Mac7 has > 90% (+)-gossypol in both seed and roots, similar to the *marie galante* (moco) cottons of Brazil. Future tests will determine if high levels of (+)-gossypol can confer better protection against insects or diseases.

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