The *Journal of Cotton Science 12:202–209 (2008)*
http://journal.cotton.org, © The Cotton Foundation 2008

BREEDING AND GENETICS

Modifying Gossypol in Cotton (*Gossypium hirsutum* L.): A Cost Effective Method for Small Seed Samples

Jodi A. Scheffler* and Gabriela B. Romano

ABSTRACT

Cotton (*Gossypium hirsutum* L.) and related species all contain gossypol, a polyphenolic compound that is an integral part of the cotton plant's self-defense system against insect pests and possibly some diseases. Gossypol also has been reported to have antitumor activity, medicinal effects, and contraceptive properties. However, the compound can be toxic to animals, which limits the usefulness of cottonseed as animal feed. Breeding projects to manipulate seed gossypol levels usually require the analysis of numerous samples and often small amounts of material. Our objective was to use a scaled-down version of the American Oil Chemists' Society (AOCS) Official Methods to measure gossypol and develop an easy, low-cost method for preparing small amounts of seed for analysis. Results show that gossypol analysis can be conducted on ground dehulled seed samples as small as 50 mg without significant loss in reproducibility. Comparison of dehulling methods used to obtain seed kernel tissue for analysis showed that wet dehulling of seed results in slightly higher estimates of gossypol levels. Preliminary results for a range of cotton lines, analyzed each year over a 3-y period, indicated that although the percent of total gossypol measured varied slightly across years, these values were generally consistent for the cultivars or genetic stocks tested. These modified methods give consistent results and allow for handling many small seed samples.

Cotton (*Gossypium hirsutum* L.) and related species have pigment glands located throughout the plant, and these glands contain a polyphenolic compound called gossypol (Adams et al., 1960). Gossypol, and other related compounds, are an integral part of cotton's self-defense mechanism and protect the plants from pests and possibly some diseases (Bell and Stipanovic, 1977; Hedin et al., 1992; Jenkins and Wilson, 1996). This compound also has been reported to have antitumor activity (Blackstaffe et al., 1997) and possess contraceptive properties (Matlin, 1994).

Unfortunately, gossypol also has a detrimental effect on humans as well as other monogastric animals. Gossypol is known to have antinutritional effects on animals fed cottonseed products (Blom et al., 2001; Eisele, 1986), and its presence in cottonseed has limited its use in feeding rations (Berardi and Goldblatt, 1980). The seed contains 23% high-quality protein, but is not widely used because of potential gossypol toxicity. At present, cottonseed meal is mostly fed to adult ruminants in limited quantities to prevent negative effects (Kim et al., 1996; Santos et al., 2003). A reduction in seed gossypol content would allow an increase in the proportion of cottonseed meal in ruminant rations and perhaps allow the expansion of its use to other animals.

There are a number of ways to reduce seed gossypol, including mechanical processes to remove gossypol from cottonseed products (Damaty and Hudson, 1975; Gardner et al., 1976; Mayorga et al., 1975). However, these treatments add cost to the products and reduce the nutritional value of the resulting cottonseed meal (Lusas and Jividen, 1987). Another strategy is to eliminate the gossypol containing glands of the plant. McMichael (1959, 1960) developed a glandless genetic stock, and extensive efforts were made to develop glandless cotton cultivars, but these cultivars were susceptible to pests and were not commercially successful (Hess, 1977; Lusas and Jividen, 1987).

Although completely eliminating gossypol producing glands and/or gossypol has not been commercially viable, more moderate strategies have shown potential (Sunilkumar et al., 2006; Vroh Bi et al., 1999). The development of semiglanded lines with 0.3% total seed gossypol (Romano and Scheffler, 2008) represented a step forward in the development of low seed gossypol cultivars; further improvements are possible by exploiting the considerable natural variation for total seed gossypol content within the *G.*

J.A. Scheffler*, and G.B. Romano, Crop Genetics and Production Research Unit, USDA-ARS, 141 Experiment Station Road, Stoneville, Mississippi, 38776
*Corresponding Author: Jodi.Scheffler@ars.usda.gov

hirsutum and *G. barbadense* (L.) species. Lee (1973) noted that a group of *G. hirsutum* and *G. barbadense* lines with "normal" glanding varied from 0.97% to 2.47% total seed gossypol. Percy et al. (1996) and Stipanovic et al. (2005) reported similar results. Among the *G. hirsutum* entries in the 2005 National Cotton Variety Test (NCVT), the percent total gossypol ranged from 0.68% to 1.70% (http://www.cottoninc.com/AgriculturalResearch/StateVarietyTrialData/).

To be successful commercially, any variety with low seed gossypol needs to exhibit consistently low seed gossypol from year to year. Pons et al. (1953) evaluated eight commercial varieties at 13 locations over three years. While analysis of variance for percent gossypol was significant for year and variety effects, rankings of the variety means did not change over years. Although the actual values for percent gossypol changed slightly across locations and years, the varieties with low levels remained low and those with high values remained high.

Gossypol exists in two enantiomeric (isomeric) forms designated plus (+) and minus (–) and *Gossypium* species produce both forms in varying proportions. Gossypol's toxicity is dependent upon the enantiomeric form of which the (+) form is the least toxic (Joseph et al., 1986; Lordelo et al., 2005; Yu, 1987). Because of this reported difference in toxicity there is interest in separating and measuring the two forms. Although it is important to decrease the toxicity of seed gossypol, "high (+)" cultivars may not be successful if the (+) form is not an effective defense against pests and/or diseases. Two studies indicate that the (+) and (–) forms are equally effective against the plant pathogen *Rhizoctonia solani* (Puckhaber et al., 2002) and corn earworm (*Helicoverpa zea*) larvae (Stipanovic et al., 2006).

The goal of our program is to develop elite lines with low total gossypol levels in the seed and an increased proportion of the (+) gossypol enantiomer, which offers pest protection but has reduced toxicity in animal feed. This requires a quick, simple, and economical method to categorize and measure gossypol type and concentration that uses few seeds and allows many samples to be evaluated. The American Oil Chemists' Society (AOCS) has two methods for measuring total seed gossypol (AOCS, 1998). The first of these methods (Official Method Ba 8-78) uses a spectrophotometric approach that extracts and measures gossypol as a di-aniline Schiff's base complex by UV-light absorbance. Because the absorbance is nonspecific, this method is believed to overestimate the gossypol concentration by as much as 15% (Hron et al., 1999). In addition, the method is unable to distinguish between the two gossypol enantiomers. The second and newer method (Recommended Practice Ba 8a-99) separates the gossypol from contaminating compounds by high performance liquid chromatography (HPLC). In this method, the Schiff's base complex is formed with chiral (R)-(-)-2-amino-1-propanol, which allows the gossypol enantiomers to be separated and detected as individual peaks. Both methods use 200-mg samples of ground kernels that are obtained by cracking a 50-g sample of dry seed, removing the hull pieces, and taking a representative subsample of ground kernels.

Although the second AOCS method gives reliable results and can be used in advanced stages of a variety development programs, it is not practical for use with the small number of seed available in early generations of a breeding program, for genetic studies that evaluate individual plants, or for screening germplasm collections (Stipanovic et al., 2005; Sunilkumar et al., 2006; Vroh Bi et al., 1999). A 50-g sample requires a minimum of 500 seeds and it is difficult to obtain even 50 extra seeds in early generations. To support our program to modify gossypol in cottonseed, we needed to conduct gossypol analysis on small amounts of seed (e.g., seeds from individual plants, single bolls, or even individual seeds). Consequently, we used a modified version of the AOCS HPLC method, developed by Michael Dowd (USDA-ARS, New Orleans, Louisiana, personal communication) for use with small samples and developed a simplified protocol for preparing the seed for analysis that uses only small quantities of seed (1–20 seeds).

MATERIALS AND METHODS

Plant material. Gossypol content in the genus *Gossypium* can range from 0% to 10% of the dry seed weight. The plant types used to test this method were selected based on their gossypol content to cover the range of values found in normal cotton cultivars and wild unadapted lines. The glanded cotton (*G. hirsutum*) cultivars used were: Acala 1517, Coker 312, DES 119, FM 832, H1220, MAXXA, MD51ne, PIMA S7, SG 747, STV 474 and STV 7A. Unadapted glanded experimental lines were also included. PI 163604 and PI 196458 are unadapted *G. hirsutum* accessions. Mac 7 is a primitive genetic stock and *G. h.* var. *punctatum* (Schum. & Thonn.) Roberty is a race stock of *G. hirsutum*. STV 7A glandless is a glandless form of the cultivar STV 7A. Plants without glands have negligible

amounts of seed gossypol and STV 7A glandless was used as a "no gossypol" control (Hess, 1977).

Seed preparation. For wet dehulling, 16 seeds per sample were placed in 50-ml centrifuge tubes with ~15 ml of warm water (below 40 ºC). The tubes were incubated at 27 ºC for 16 h. After the seeds were imbibed, the chalazal (rounded) ends of the seeds were compressed by hand to extrude the kernel (embryo with cotyledons) from the seed coat (hull) through the micropylar (pointed) end (Fig. 1). Twenty 16-seed samples could consistently be completed per hour. The hulls were discarded (except as noted below) and the dehulled seeds were stored frozen at -80 ºC until used. Ginned fuzzy seed or acid delinted seed were used with equal effectiveness. "Hard-seeded" unadapted lines were also analyzed, but sometimes needed to be nicked to allow imbibition of water. When testing seed for the first time, the seeds were checked after 16 h. Those that were still hard were nicked with a razorblade on the chalazal end and left for an additional 16 h. For seed that was known to be hard-seeded, the seed was nicked before addition of the water.

Figure 1. Hull being removed from the kernel by pressing on the chalazal end of the seed. a. fuzzy seed b. delinted seed c. dehulled seed and empty fuzzy hull (seedcoat) d. dehulled seed and empty delinted hull.

The 50-ml tubes with frozen dehulled samples were uncapped and placed in a prechilled freeze dryer (Model 2400, Freeze Dry Company Inc., Nisswa, MN) for 5 d at -20 ºC. After freeze-drying, a steel grinding ball (3/8" [1cm], McMaster-Carr, Atlanta, GA) was added to each tube, and the samples were ground (1 min) to a fine powder in a tissue pulverizer (Kleco 8200-50 ml, Visalia, CA). In our lab, we routinely ground 160 samples per hour. Ground seeds were stored at -20 ºC until analyzed for gossypol. Care was taken to ensure the dry tissue did not take up moisture. For hull versus kernel analysis, the hulls were saved and processed using the same procedure described for the kernels.

For dry dehulling, a procedure similar to that described in the AOCS Official Methods (AOCS, 1998) was used; samples were first cracked with a Waring blender (Waring, Torrington, CT). After sieving to separate the coarse hull pieces and fines, whole kernels and large kernel pieces were recovered. These were then freeze-dried under the same conditions as the wet dehulled seed. The dried samples were then ground in a Wiley Mill (Thomas Scientific, Swedsboro, NJ) fitted with 2-mm screens resulting in particles that would pass a 20-mesh sieve. Ground samples were stored at -20 °C until analyzed.

Chemical analysis. The protocol used to extract and quantify total gossypol, its (+) and (–) isomers, and to prepare the standards, was a modified method based on Hron et al. (1999) and Dowd and Pelitire (2001). For each extraction, the ground, dried sample was weighed and placed in a tube of sufficient size to accommodate added reagents. Complexing reagent was added to each tube, and the tubes were heated at 95–100 ºC for 30 min. The complexing reagent consisted of 2% (R)-(-)-2-amino-1-propanol, 10% glacial acetic acid, and 88% *N*,*N* dimethylformamide. The ratio of complexing reagent-to-sample was 20:1 (v/w), equivalent to the ratio used in the AOCS method. After allowing the samples to cool to room temperature, mobile phase [85:15 (v/v) acetonitrile/10 mM KH_2PO_4 buffer (pH 3.0)] at four times the volume of the complexing reagent was added to the tube. The tube was then shaken to fully mix the mobile phase. Solids were allowed to settle, and ~1.5 ml of supernatant was transferred to a 2-ml microfuge tube and centrifuged for 2 min at 12,000 rpm to settle any remaining particles. The particle-free supernatant was transferred into an HPLC vial. Samples are analyzed on an HPLC fitted with an SGE Inertsil ODS-2 reverse phase column

(5 µm, 100 mm x 4.0 mm i.d.) (Varian, Lake Forest, CA) and a diode array detector that was set at 254 nm. Mobile phase flow rate was 1ml/min, and injection volume was 20 µl, unless the sample concentration was above the range of the standard curve, in which case the injection volume was halved and the calculations were adjusted accordingly. Under these conditions, the analysis time for each sample was 5 min. Gossypol-acetic acid [89.62% racemic gossypol] was used to prepare standard response curves. Standard solutions with 50:50 (+) and (–) enantiomers were used to construct standard curves. Total gossypol was calculated as the sum of the two gossypol forms.

RESULTS AND DISCUSSION

The AOCS Official Methods protocol (AOCS, 1998) uses a plate mill on the whole seed to crack the seed hulls. The hulls are then separated from the kernel fraction by screening through a mesh sieve. This procedure does not recover a measurable amount of seed kernel. To ensure a representative sample is obtained with this "dry dehulling" method, relatively large seed samples are required. In addition, dry dehulling requires some hand cleaning of the samples to ensure the removal of hull particles. Because the hull contains only a small amount of gossypol, any contamination of the analysis sample with hulls will bias the percent gossypol estimate downward. To decrease the amount of seed material required and eliminate some of the cleaning steps, a "wet dehulling" method was developed for preparing small seed samples. The process requires that the seed be imbibed overnight, which loosens the hull, and then the hull is pressed gently to extrude the kernel (Fig. 1). Although additional time might be needed to dry the wet kernel after extraction, the process is advantageous because many samples can be freeze-dried simultaneously in 48 to 72 h. In addition, the entire kernel is recovered and all hull material is removed. The soaking period is kept to the minimum needed to achieve the separation (~16 h). To compare the effect of the dehulling method on the percent total gossypol, 10 cotton lines were wet and dry dehulled and analyzed (Table 1). Three 100-mg subsamples were analyzed for each line and dehulling method combination. The gossypol values obtained for the two methods were highly correlated, but the wet dehulled samples were, in general, slightly higher in gossypol content than the dry dehulled samples. The lower gossypol level in the dry dehulled samples might be due to failure to remove all the hull material from the ground sample or moisture being taken up during the Wiley Mill grinding process following freeze-drying. The goal of our program was to develop breeding lines with lower seed gossypol. Using the wet dehulling procedure provided a consistently higher estimate of gossypol content than would be obtained by the typical dry dehulling approach. Therefore, plants selected using values obtained with the wet dehulling method would be more conservative estimates of the actual seed gossypol content.

To confirm that the total gossypol content of the hull was negligible, 16 seeds were dehulled using

Table 1. Comparison of gossypol content using a dry dehulling versus a wet dehulling method. Each value is the mean of three subsamples from the same powder source

Line	Dry Dehulling (% total gossypol)	Std. dev.	Wet Dehulling (% total gossypol)	Std. dev.
MD51ne	1.33	0.03	1.38	0.02
Acala 1517	1.03	0.04	1.10	0.01
Mac7	1.01	0.01	1.01	0.01
DES 119	1.36	0.01	1.50	0.02
FM 832	0.84	0.01	0.88	0.01
SG747	1.07	0.02	1.22	0.01
Coker 312	1.31	0.00	1.39	0.03
H1220	1.48	0.02	1.56	0.05
G. h. var. *punctatum*	1.57	0.02	1.66	0.03
PI 196458	2.07	0.05	2.24	0.01

the wet dehulling method and the two components analyzed separately (Table 2). Two wild plant introductions (PI 163604, PI 196458) and three cultivars were evaluated. STV 7A glandless had negligible gossypol and was used as the "no gossypol" control. The results confirmed that even using 400 mg of hull sample (four times the mass of kernel samples), higher injection volumes, and reduced dilution factor for the hulls compared to kernels, the total gossypol content in hulls was negligible.

To test the effect of decreasing the sample size on the accuracy and repeatability of the HPLC gossypol measurement, analyses were conducted on uniform lots of ground FM 832 and MD51ne cottonseed with 200-, 100-, and 50-mg samples. Each lot of seed was prepared using 200 seeds (approximately 20 g). The seed lots were prepared by wet dehulling, and 10 replicates were analyzed for each sample size and cultivar combination (Table 3). The mean values for the three sample sizes were not statistically different and the coefficient of variability (CV) percentages were also similar. Hron et al. (1999), using a modification of the AOCS official method, reported a CV of 1.7% for a 200-mg sample size. Our results indicated that accurate gossypol analysis could be conducted on a considerably smaller scale than specified by the AOCS methods or the modified method of Hron et al. (1999). Because selection programs to modify the gossypol profile of the cotton plant require large numbers of analyses, we estimated the cost of the analysis method relative to scale. Only direct costs were considered (i.e., operator time and depreciation of the analytical instrument were not included). Sample size was found to significantly influence analysis cost. At the 200-mg sample size, the estimated cost to analyze for the (+) and (–) gossypol enantiomers was $2.87/sample. Decreasing the sample weight to 100 mg lowered the cost by 32% ($1.96) and 50-mg samples were reduced to $1.51/sample. The cost of the chiral propanol complexing reagent represents 45% of the direct cost for a 200-mg sample, but only 32% at the 100-mg sample scale. If only total gossypol is of interest, then these costs can be further reduced by substituting 3-amino-1-propanol for (R)-(-)-2-amino-1-propanol in the method. This change results in the two gossypol enantiomers eluting as a single peak during chromatography. The cost at the 100-mg sample scale with 3-amino-1-propanol

Table 2. Comparison of percent total gossypol in hulls and dehulled kernels. The test included two unadapted plant introductions, three cultivars, and a glandless line as the "no gossypol" control

Plant Identification	Hull Only				Dehulled Kernels			
	Mass (mg)	Dilution Factor	Injection Volume (ul)	% Total Gossypol	Mass (mg)	Dilution Factor	Injection Volume (ul)	% Total Gossypol
PI 163604	399.2	0.5	40	0.011	100.1	4.0	5	3.201
PI 196458	398.8	0.5	40	0.030	100.3	4.0	5	2.020
MD51ne	399.2	0.5	40	0.005	100.5	2.0	10	1.551
H1220	398.2	0.5	40	0.076	99.4	2.0	10	1.430
FM 832	399.1	0.5	40	0.054	99.9	2.0	10	0.849
STV 7A glandless	399.9	0.5	40	0.017	400.1	1.0	20	0.084

Table 3. Effect of sample size on the reproducibility of gossypol analysis values. Each cultivar and weight combination is the mean of 10 replications using the same powder source

Sample mass (mg)[z]	Cultivar	Mean % total gossypol (±sd)[y]	Range	% CV[x]
200	FM832	0.82 (±0.01)	0.80-0.83	0.98
	MD51ne	1.49 (±0.03)	1.46-1.54	1.69
100	FM832	0.81 (±0.01)	0.80-0.84	1.67
	MD51ne	1.50 (±0.02)	1.46-1.53	1.49
50	FM832	0.82 (±0.01)	0.80-0.84	1.61
	MD51ne	1.52 (±0.03)	1.47-1.58	2.27

[z] n=10 for each mass class and variety

[y] Sd = standard deviation

[x] % CV = coefficient of variability

becomes $1.32/sample, a 33% savings over the cost at the same scale with *R*-(-)-2-amino-1-propanol. A summary of the costs for 100-mg samples is provided in Table 4.

For our breeding and genetic studies, we found that using 100-mg samples provided the best combination of reproducibility, ease of handling, and lower cost. We then tested our method on field-grown material to determine if we could use the method to select reliably for gossypol content under field conditions in different environments. We used the 100-mg sample size to test seeds from 15 cultivars and lines grown in the field over 3 y at Stoneville, Mississippi (Table 5). Each year, 16 seeds from each cultivar or line were bulked, prepared and analyzed. We found that using six seeds also gave similar results, however, 16 seeds were easy to process and guaranteed a sufficient representative sample. The results obtained over years showed that although the percentage of total gossypol in the seed varied across years, the percent total for each line was generally consistent. Similar results were reported by Pons et al. (1953).

Table 4. Cost analysis per 100-mg sample for total gossypol only or total and (+) and (–) gossypol. Only direct costs were considered and were based on analysis of a set of 40 samples

Supplies	Total gossypol only		Total and (+)/(-) gossypol	
	Cost per Sample	Percent of total cost	Cost per Sample	Percent of total cost
Chemicals	$0.45	34.0	$1.09	55.6
15-ml test tube	$0.16	12.0	$0.16	8.0
2-ml microfuge tube	$0.03	2.1	$0.03	1.5
autoinjector vial with cap	$0.37	28.0	$0.37	19.0
Inertsil column (1 per 2000 samples)	$0.21	15.9	$0.21	10.4
Guard column (1 per 500 samples)	$0.05	3.8	$0.05	2.5
HPLC syringe (1 per 1000 samples)	$0.06	4.2	$0.06	3.0
Total cost per sample	$1.32	100	$1.96	100

Table 5. Percent total seed gossypol measured in 15 cotton lines grown in the field over 3 y at Stoneville, Mississippi

Cultivar/Line	Percent Total Gossypol			Mean across years	Std dev
	Year Grown in the Field				
	2003	2004	2005		
MD51ne	1.410	1.380	1.351	1.380	0.03
Acala 1517	1.386	1.100	NA[z]	1.243	0.20
Mac7	1.502	1.010	1.554	1.355	0.30
DES 119	1.706	1.500	1.568	1.591	0.10
FM 832	0.845	0.880	0.818	0.848	0.03
SG747	1.072	1.220	1.130	1.141	0.07
Coker 312	1.232	1.390	NA[z]	1.311	0.11
H1220	1.354	1.460	1.324	1.379	0.07
G. h. var. *punctatum*	1.745	1.660	1.669	1.691	0.05
STV 7A glanded	1.253	1.277	1.379	1.303	0.07
STV 7A glandless	BMT[y]	BMT[y]	BMT[y]	0.000	0.00
MAXXA	0.958	0.940	0.968	0.955	0.01
MAXXA glandless	NA[z]	BMT[y]	0.032	0.016	0.00
PIMA S7	0.958	0.943	0.985	0.962	0.02
STV 474	1.343	NA[z]	1.257	1.300	0.06

[z] NA - not analyzed

[y] BMT - below measurable threshold

Our results show that the AOCS Official Method (Ba 8a-99) for measuring cottonseed gossypol can be reduced in scale without significant loss of reproducibility. This reduction in analysis scale decreases the amount of seed needed for reliable determinations of gossypol content and enantiomer identification and the costs associated with seed analyses required for gossypol breeding and genetics projects. The method allows for the handling of many samples more conveniently and gives consistent results over laboratory replications. A preliminary evaluation of gossypol content in seed samples collected and analyzed over 3 y, indicates that it is possible to accurately select for gossypol content in plants grown under field conditions; however, any line being considered for commercialization should be tested for stability in different environments. This method makes it possible to test in early generations for gossypol content and will promote the development of new cotton genetic lines with modified seed gossypol profiles. We are also using this method for genetic studies and evaluation of material from the Cotton Germplasm Collection.

ACKNOWLEDGMENTS

The authors are indebted to Michael Dowd for allowing us to use his unpublished HPLC analysis protocol and to Scott Pelitire for analyzing a portion of the samples and successfully transferring the HPLC assay method to our lab. The authors would also like to thank Teheshia Brooks, Gus Eifling, Shenita Harper, Shirley Howard, Pameka Johnson, Robin Jordan, LaJoi McAdory, and Jennifer Tonos for their invaluable technical assistance.

DISCLAIMER

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply approval or recommendation of the product to the exclusion of others that may be suitable.

REFERENCES

Adams, R., T.A. Geissman, and J.D. Edwards. 1960. Gossypol, a pigment of cottonseed. Chem. Rev. 60:555–574.

American Oil Chemists' Society. 1998. AOCS Official Method Ba 8-78 and Ba 8a-99. p. 1–3. *In* D. Firestone (ed.) Official Methods and Recommended Practices of the AOCS. 5th edition. American Oil Chemists' Society, Champaign, IL.

Bell, A.A., and R.D. Stipanovic. 1977. The chemical composition, biological activity, and genetics of pigment glands in cotton. p. 244–258. *In* Proc. Beltwide Cotton Prod. Res. Conf. Atlanta, GA 10-12 Jan. 1977. Natl. Cotton Counc. Am., Memphis, TN.

Berardi, L.C., and L.A. Goldblatt. 1980. Gossypol. pp. 183–237. *In* I.E. Liener (ed.). Toxic Constituents of Plant Foodstuffs. 2nd edition. Academic Press, New York, NY;.

Blackstaffe, L., M.D. Shelley, and R.G. Fish. 1997. Cytotoxicity of gossypol enantiomers and its quinone metabolite gossypolone in melanoma cell lines. Melanoma Res. 7:364–372.

Blom, J.H., K.J. Lee, J. Rinchard, K. Dabrowski, and J. Ottobre. 2001. Reproductive efficiency and maternal-offspring transfer of gossypol in rainbow trout (*Oncorhynchus mykiss*) fed diets containing cottonseed meal. J. Anim. Sci. 79:1533–1539.

Damaty, S.M., and J.F. Hudson. 1975. Preparation of low gossypol cottonseed flour. J. Sci. Food Agric. 26:109–115.

Dowd, M.K., and S.M. Pelitire. 2001. Recovery of gossypol acetic acid from cottonseed soapstock. Industrial Crops Prod. 14:113–123.

Eisele, G.R. 1986. A perspective on gossypol ingestion in swine. Vet. Hum. Toxicol. 28:118–22.

Gardner, H.K., Jr., R.J. Hron, and H.L.E. Vix. 1976. Removal of pigment glands (gossypol) from cottonseed. Cereal Chem. 53:549–560.

Hedin, P.A., W.L Parrott, and J.N. Jenkins. 1992. Relationships of glands, cotton square terpenoid aldehydes, and other allelochemicals to larval growth of *Heliothis virescens* (Lepidoptera: Noctuidae). J. Econ. Entomol. 85:359–364.

Hess, D.C. 1977. Genetic improvement of gossypol-free cotton varieties. Cereal Food World 22:98–103.

Hron, R.J., Sr., H.L. Kim, M.C. Calhoun, and G.S. Fisher. 1999. Determination of (+)-, (-)-, and total gossypol in cottonseed by high-performance liquid chromatography. AOCS 76:1351–1355.

Jenkins, J.N., and F.D. Wilson. 1996. Host plant resistance. p. 563–597. *In* E.G. King, J.R. Phillips, and R. Coleman (eds). Cotton Insects and Mites: Characterization and Management., The Cotton Foundation, Memphis, TN.

Joseph, A.E., S.A. Matlin, and P. Knox. 1986. Cytotoxicity of enantiomers of gossypol. Br. J. Cancer 54:511–513.

Kim, H.L., M.C. Calhoun, and R. D. Stipanovic. 1996. Accumulation of gossypol enantiomers in ovine tissues. Comp. Biochem. Physiol. 113:417–420.

Lee, J.A. 1973. The inheritance of gossypol level in *Gossypium* II: Inheritance of seed gossypol in two strains of cultivated *Gossypium barbadense* L. Genetics 75:259–264.

Lordelo, M.M., A.J. Davis, M.C. Calhoun, M.K. Dowd, and N.M. Dale. 2005. Relative toxicity of gossypol enantiomers in broilers. Poult. Sci. 84:1376–1382.

Lusas, E.W., and G.M. Jividen. 1987. Glandless cottonseed: a review of the first 25 years of processing and utilization research. J. Am. Oil Chem. Soc. 64:839–854.

Matlin, S.A.1994. Prospects for pharmacological male contraception. Drugs 48:851–863.

Mayorga, H., J. Gonzalez, J.F. Menchu, and C. Rolz. 1975. Preparation of a low free gossypol cottonseed flour by dry and continuous process. J. Food Sci. 40:1270–1274.

McMichael, S.C. 1959. Hopi cotton, a source of cotton-seed free of gossypol pigments. Agron. J. 51: 630.

McMichael, S.C. 1960. Combined effects of glandless genes gl_2 and gl_3 on pigments in the cotton plant. Agron. J. 52**:**385-396.

Percy, R.G., M.C. Calhoun, and H.L. Kim. 1996. Seed gossypol variation within *Gossypium barbadense* L**.** cotton**.** Crop Sci. 36:193–197.

Pons, Jr., W.A., C.L. Hoffpauir, and T.H. Hopper. 1953. Gossypol in cottonseeds. Influence of variety of cottonseed and environment. J. Agric. Res. Food Chem. 1:1115–1118.

Puckhaber, L.S., M.K. Dowd, R.D. Stipanovic, and C.R. Howell. 2002. Toxicity of (+)- and (–)-gossypol to the plant pathogen *Rhizoctonia solani*. J. Agric. Food Chem. 50:7017–7021.

Romano, G.B., and J.A. Scheffler. 2008. Lowering seed gossypol content in glanded cotton (*Gossypium hirsutum* L.) lines. Plant Breeding (doi:10.1111/j.1439-0523.2008.01545.x)

Santos, J.E., M. Villasenor, P.H. Robinson, E.J. DePeters, and C.A. Holmberg. 2003. Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. J. Dairy Sci. 86:892–905.

Stipanovic, R.D., L.S. Puckhaber, A.A. Bell, A.E. Percival, and J. Jacobs. 2005. Occurrence of (+)- and (–)-gossypol in wild species of cotton and in *Gossypium hirsutum* var. *marie-galante* (Watt) Hutchinson. J. Agric. Food Chem. 53:6266–6271.

Stipanovic, R.D., J. Lopez, M.K. Dowd, L.S. Puckhaber, and S.E. Duke. 2006. Effect of racemic and (+) and (–)- gossypol on the survival and development of *Helicoverpa zea* larvae. J. Chem. Ecol. 32:959–968.

Sunilkumar, G., L.M. Campbell, L. Puckhaber, R.D. Stipanovic, and K.S. Rathore. 2006. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proc. Nat. Acad. Sci. 103:18054–18059.

Vroh Bi, I., J.P. Baudoin, B. Hau, and G. Mergeai. 1999. Development of high-gossypol cotton plants with low-gossypol seeds using trispecies bridge crosses and in vitro culture of seed embryos. Euphytica. 106:243–251.

Yu, Y.-W. 1987. Probing into the mechanism of action, metabolism and toxicity of gossypol by studying its (+)- and (–) stereomers. J. Ethnopharmacol. 20:65–78.