CROP ECOLOGY, MANAGEMENT & QUALITY

Development of a Visual Method to Quantify the Gossypol Content in Cotton Seeds

H. Benbouza, G. Lognay, R. Palm, JP. Baudoin, and G. Mergeai*

ABSTRACT

Twelve *Gossypium* genotypes (species, fertile interspecific allotetraploid and allohexaploid hybrids, backcross progeny of interspecific hybrids) characterized by drastic differences in their seed gossypol content were used to design a visual quantification method of the gossypol concentration in cotton seeds. After developing an high performance liquid chromatography (HPLC) method that allowed measurement of gossypol content in single seed samples, the results of 123 HPLC analyses carried out seed-by-seed were compared with different parameters characterizing the level of gossypol glands in the seed. A significant correlation was found between the gossypol content $\%G$ determined on single seeds by HPLC and the number N of gossypol glands per seed section area (S, expressed in mm^2), leading to the establishment of the following model: $\%G = b_i\ (N/S)$, where the regression coefficient b_i depends on the cotton genotype considered. The estimation of $\%G$ directly from N/S is a rapid and accurate method which can be used in breeding programs to screen the progeny of cotton genotypes showing a high segregation for their seed gossypol content.

One of the main traits of *Gossypium* L. is the presence of pigment glands throughout the plant (Altman et al., 1990). Gossypol, a triterpenoid aldehyde, and its derivatives are predominant secondary metabolites in cotton glands. These compounds have insecticidal, antimicrobial, antifertility and toxic properties (Stipanovic et al., 1984, Fisher et al., 1988, Percy et al., 1996).

Gossypol is the main terpenoid aldehyde found in cotton seeds. Its concentration varies from zero in the seed kernel of some Australian wild diploid species (Brubaker, 1996) to more than 9% in *G. davidsonii* Kell. (Carter et al., 1966). Upland cotton seeds usually contain from 0.6 to 2% gossypol (Lusas and Jividin, 1987). As this triterpenoid is very toxic to humans and monogastric animals (Lusas and Jividin, 1987; Alford et al., 1996), its rate in all food and feed products produced with cotton flour must be very low and has to be systematically controlled. To reach this goal, the most frequently used analytical procedures include spectrophotometry and HPLC (Abou-Donia et al., 1981; Stipanovic et al., 1988; Hron et al., 1990; Tchatchueng et al., 1992). The standardized spectrophotometric techniques requiring the formation of a gossypol-aniline complex lead to overestimated results because of some interferences (Marquié and Bourrély, 1991). Because of its sensitivity and repeatability, HPLC is the method of choice for the measurements of low gossypol concentrations (Abou-Donia et al., 1981; Marquié and Bourély, 1991). It is, however, tedious to apply chemical measurements on a large number of seed samples. In this paper, we present a new quantification method of seed gossypol content based on visual observations of seed characteristics. This new technique is rapid and accurate and is particularly valuable in breeding programs to screen the progeny of cotton genotypes showing a high degree of segregation in the gossypol content of their seeds.

H. Benbouza, JP. Baudoin, and G. Mergeai, Unité de Phytotechnie tropicale et d'Horticulture, Faculté Universitaire des Sciences Agronomiques, 2 Passage des Déportés, B-5030 Gembloux, Belgium; G. Lognay, Unité de Chimie générale et organique, Faculté Universitaire des Sciences Agronomiques, 2 Passage des Déportés, B-5030 Gembloux, Belgium; R. Palm, Unité de Statistique et Informatique, Faculté Universitaire des Sciences Agronomiques, 2 Passage des Déportés, B-5030 Gembloux, Belgium. Received 14 June 2001. *Corresponding author (Mergeai.g@fsagx.ac.be).

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MATERIAL AND METHODS

Plant Materials

The seeds used in our investigations were produced by selfing 12 distinct genotypes (of different genetic backgrounds) maintained in the cotton collection of the Gembloux Agricultural University: one cultivar of *G. hirsutum* L. $2(A_hD_h)_1$ (cv. Stamf) originating from West Africa; one accession of *G. thurberi* Tod. ($2D_1$); one accession of *G. raimondii* Ulbr. ($2D_5$); one accession of *G. sturtianum* Will. ($2C_1$); one synthetic allohexaploid: *G. hirsutum* × *G. sturtianum* ($2[A_hD_hC_1]$); three synthetic allotetraploids: *G. arboreum* L. × *G. sturtianum* ($2[A_2C_1]$), *G. thurberi* × *G. sturtianum* ($2[D_1C_1]$), *G. australe* F. Muel. × *G. davidsonii* Kell. ($2[G_1D_{3\text{-}2}]$); four plants obtained by backcrossing the HRS (*G. hirsutum* × *G. raimondii* × *G. sturtianum*, $[A_hD_hD_5C_1]$) trispecific hybrid to *G. hirsutum*: HRS BC_2S_1/09, HRS BC_2S_1/14, HRS BC_3/09, and HRS BC_3/13. All these genotypes were characterized by very different levels of seed gland gossypol and the BC_2S_1 and BC_3 plants issued from the HRS hybrid were chosen for their ability to produce segregating progenies for this trait (Vroh Bi et al., 1999).

Seed Glands Counting Technique and Surface Evaluation

Before being analyzed by HPLC, each seed was cut in two longitudinal sections after removal of the teguments to assess its total number of glands, N, per section, its section area, S in mm^2, and the size of the glands. These operations were carried out with a Nikon Eclipse E800 light and fluorescent microscope (Nikon, Tokyo, Japan) using a JVC-3-CCD color video camera (JVC, Tokyo, Japan) and the Archive Plus program of Sony (Sony Electronics, Park Ridge, NJ) to capture and analyze the images. The gossypol glands were distributed in three classes according to their size: small glands ($N1$: size $<2700\ \mu m^2$), medium glands ($N2$: from 2700–7549 μm^2) and large glands ($N3$: $>7549\ \mu m^2$). The total numbers of glands

Abbreviations: G, gossypol; HPLC, high performance liquid chromatography.

of each class ($N1$, $N2$, and $N3$) were counted and the mean surface of a gossypol gland (MGS, expressed in μm^2) was calculated by the following formula: MGS = ($N1 \times 1350 + N2 \times 5125 + N3 \times 10\,000$)/($N1 + N2 + N3$), where 1350, 5125, and 10 000 are considered as the central points of the three gland classes.

HPLC Determination of Gossypol

The HPLC procedure optimized by Marquié and Bourrély (1991) was used to analyze the gossypol content in seed bulks. After being peeled, cut, and weighed, the seeds were ground and sieved (30 mesh). Samples of ground seeds (100 mg each) were hydrolyzed for 10 min in boiling water bath at 100°C with 20 mL of glacial acetic acid. At the same time two samples (1–2 mg) of standard gossypol (Sigma ref. G-8761, St. Louis, MO) were treated similarly. The solutions were filtered through silanized glass wool into 50-mL volumetric flasks. The residues were rinsed three times with 2 to 3 mL of a water/acetonitrile (50:50; v/v) mixture, the recovered solutions were diluted up to 50 mL and homogenized carefully. The samples were then left at room temperature for 3 h before being filtered through a 0.20-μm nylon membrane (MSI).

The samples were directly analyzed on a Merck Hitachi L 6200 chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with a Merck Hitachi L 4000 UV. The chromatographic signals were integrated on a Hewlett Packard HP 1000 integrator (Hewlett Packard, USA). Other analytical conditions were fixed as follows: (i) column—Inertsil 5 μm ODS-3 from Chrompack (the Netherlands); (100 × 3 mm); (ii) mobile phase—acetonitrile/water (acidified to pH = 2.6 with phosphoric acid) 88: 12 (v/v) at flow rate of 0.5 mL min^{-1}; (iii) UV detection at 272 nm; and (iv) duplicates of 20-μL injections were made for all samples. Standard curve for gossypol was constructed from triplicate determinations each for gossypol quantities of 0.5 mg, 1 mg, 1.5 mg, and 2 mg.

To quantify the gossypol content on single seed samples, *G. hirsutum* cv. Stamf was used and the following parameters were reinvestigated and optimized: (i) the preparation of the gossypol standard solutions (direct weighing of the pure reference or preparation of gossypol of known concentration from a 1000 μg g^{-1} stock solution in acetone); (ii) grinding conditions of the seeds: direct grinding of a single seed, cryo-grinding at −196°C with liquid nitrogen as such or in the presence of glacial acetic acid; (iii) hydrolysis duration: 5, 10, and 20 min.

RESULTS AND DISCUSSIONS

Development of a Seed-by-Seed Gossypol HPLC Method

The retention times of the standard gossypol provided by Sigma and of the gossypol extracted from cotton seeds of *G. hirsutum* cv. Stamf were in close agreement: 5.70 min and 5.71 min, respectively. However, chromatograms obtained from the Stamf seed extracts also contained additional unidentified peaks with much shorter retention times (between 2 and 3 min), which did not interfere with the quantification of gossypol.

The first modification we brought to the method proposed by Marquié and Bourrély (1991) involved the establishment of the standard curves. Indeed, as it was impossible to obtain reproducible results by direct weighing of small gossypol quantities as suggested in the original method, we considered the solubilization of the molecule in acetone as an alternative. This solvent was chosen on the basis of Nomeir and Abou-Dounia (1982) results which showed that, when the compound was solubilized in organic solvents (acetone, acetonitrile, chloroform, ethanol, and methanol), its decomposition rate was lowest in acetone. Under these conditions, the calibration was linear for a wide array of concentrations (0.02–200 μg gossypol mL^{-1}).

In spite of good repeatability when grinding the seed one by one without special treatment, the values obtained for gossypol concentrations were systematically lower than values recorded using bulk seed (0.49% ±

Table 1. Influence of the grinding method on the assessment of single cotton seed gossypol content by HPLC.

Method	Simple dry grinding	Cryo-grinding with liquid nitrogen	Cryo-grinding with liquid nitrogen and glacial acetic acid
Number of seeds evaluated	16	5	16
Max gossypol content (%)	0.57	0.66	1.40
Mean gossypol content (%)	0.49	0.61	0.97
Min gossypol content (%)	0.36	0.54	0.67
Standard deviation (%)	0.06	0.05	0.18
Coefficient of variation (%)	11.4	8.4	18.4

Table 2. Comparison of the gossypol contents (%) of different *Gossypium* genotypes assessed according to the *seed-by-seed* and to the *bulk seed* HPLC analysis methods.

	Seed-by-seed analysis method					Bulk seed analysis method				
		Gossypol content (%)					Gossypol content (%)			
Genotypes	*n*	*min*	*mean*	*max*	*s.d.*	*r*	*min*	*mean*	*max*	*s.d.*
G. hirsutum Stamf	16	0.67	0.97	1.40	0.18	6	1.02	1.06	1.08	0.02
G. raimondii	6	1.91	2.33	2.69	0.34	5	1.10	1.15	1.24	0.07
G. thurberi	7	1.64	2.58	3.28	0.58	4	2.14	2.20	2.31	0.08
2(*G. hirsutum* × *G. sturtianum*)	9	0.007	0.03	0.057	0.01	4	0.04	0.04	0.05	0.00
2(*G. thurberi* × *G. sturtianum*)	7	0.005	0.01	0.023	0.01	4	0.01	0.01	0.01	0.00
2(*G. arboreum* × *G. sturtianum*)	9	0.004	0.01	0.010	0.00	4	0.01	0.01	0.01	0.00
2(*G. australe* × *G. davidsonii*)	6	0.199	0.26	0.310	0.05	4	0.29	0.31	0.32	0.01
HRS BC_2S_1/09 (segregating genotype)	16	0.048	0.33	0.873	0.22					
HRS BC_3/09 (segregating genotype)	19	0.150	0.54	0.932	0.19					
HRS BC_2S_1/14 (segregating genotype)	11	0.264	0.43	0.637	0.15					
HRS BC_3/13 (segregating genotype)	17	0.452	0.71	0.987	0.18					

n: number of seeds, *r*: number of replicates, *s.d.*: standard deviation.

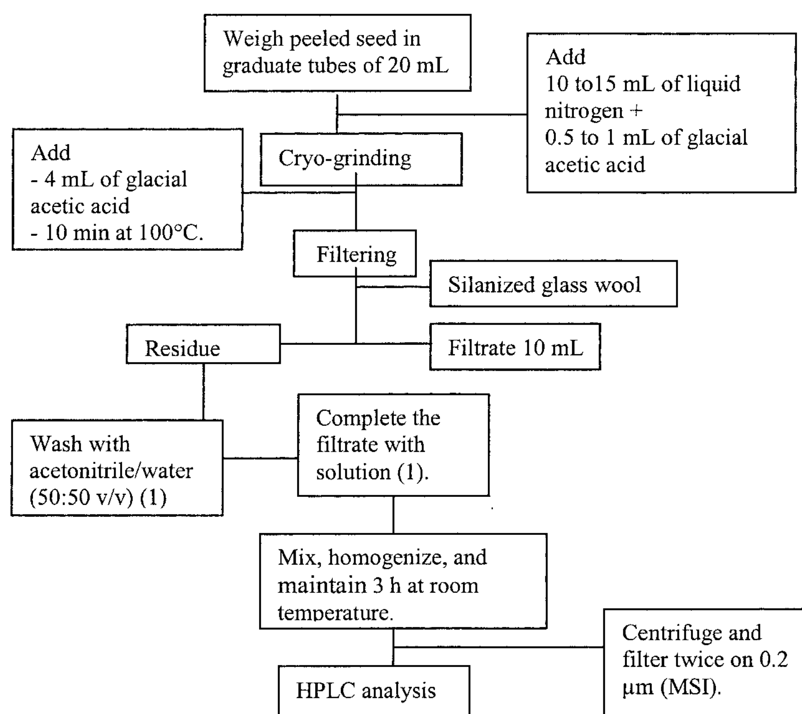

Fig. 1. Protocol of seed-by-seed gossypol analysis.

0.06, $n = 16$ instead of 1.06% ± 0.02, $n = 6$) of the same cultivar (Stamf). We tested the effect of adding liquid nitrogen alone (about 15 mL per seed) or in combination with glacial acetic acid (0.5–1 mL per seed) on the HPLC assessment of single seed gossypol content. The results obtained for these different treatments are shown in Table 1.

Compared with the two other treatments, the addition of liquid nitrogen and glacial acetic acid released much more of the gossypol contained in the seed glands. This improvement was clear from the intense yellow color of the seed extracts. According to Yatsu et al. (1986), one can assume that this treatment burst the walls of cells protecting the gossypol glands. The gossypol contents measured after cryo-grinding with acetic acid were significantly higher ($P < 0.001$) than those obtained when no treatment was applied.

Among the three hydrolysis times tested (5, 10, and 20 min), the best compromise was reached, as stated by Marquié and Bourrély (1991), with the 10-min hydroly-

Table 3. Results of gossypol gland counting and seed section area evaluation.

		Total glands number				Section area (mm²)			
Genotypes	n†	*min*	*mean*	*max*	*s.d.*	*min*	*mean*	*max*	*s.d.*
G. hirsutum var. Stamf	16	71	113.7	148	14.8	17.3	21.2	23.2	1.7
G. raimondii	6	87	135.2	157	26.5	12.4	13.9	14.6	0.8
G. thurberi	7	89	114.6	138	6.1	8.1	9.3	11.0	1.1
2(*G. hirsutum* × *G. sturtianum*)	9	22	41.6	51	8.7	18.9	23.7	28.7	3.7
2(*G. thurberi* × *G. sturtianum*)	7	9	21.9	44	11.6	9.5	11.1	12.1	1.1
2(*G. arboreum* × *G. sturtianum*)	9	12	25.6	51	14.3	13.3	16.3	19.3	2.0
2(*G. australe* × *G. davidsonii*)	6	45	85.8	77	12.1	10	14.2	21.0	4.7
BC_2S_1/09	16	29	62.3	92	21.1	10.4	22.5	26.5	5.0
BC_3/09	19	53	86.8	128	21.5	16.8	26.5	33.2	3.7
BC_2S_1/14	11	58	96.6	133	22.3	18.4	27.8	33.1	5.9
BC_3/13	17	54	103.4	124	18.1	14.3	23.9	29.9	4.9

† *n*: seed number.

Table 4. Values of the correlation coefficients calculated† between the total gossypol content, %*G*, and seven variables characterizing gossypol-glands distribution on the embryos.

N/S‡	*N1/N3*	*N1/S*	*N2/S*	*N3/S*	*MGS*	*MGS*N/S*
0.906***	0.066	0.246**	0.892***	0.287**	0.147	0.907***

* $P < 0.05$.
** $P < 0.01$.
***$P < 0.001$.
† Number of observations: 123.
‡ *N/S*: number of glands divided by the section area in mm², *N1/N2*: number of glands of class 1 divided by number of glands of class 3; *N1/S*, *N2/S* and *N3/S*: number of glands of class 1, 2, or 3 divided by the section area in mm²; *MGS*: mean surface (μm²) of gossypol glands; *MGS*N/S* portion of gland surface.

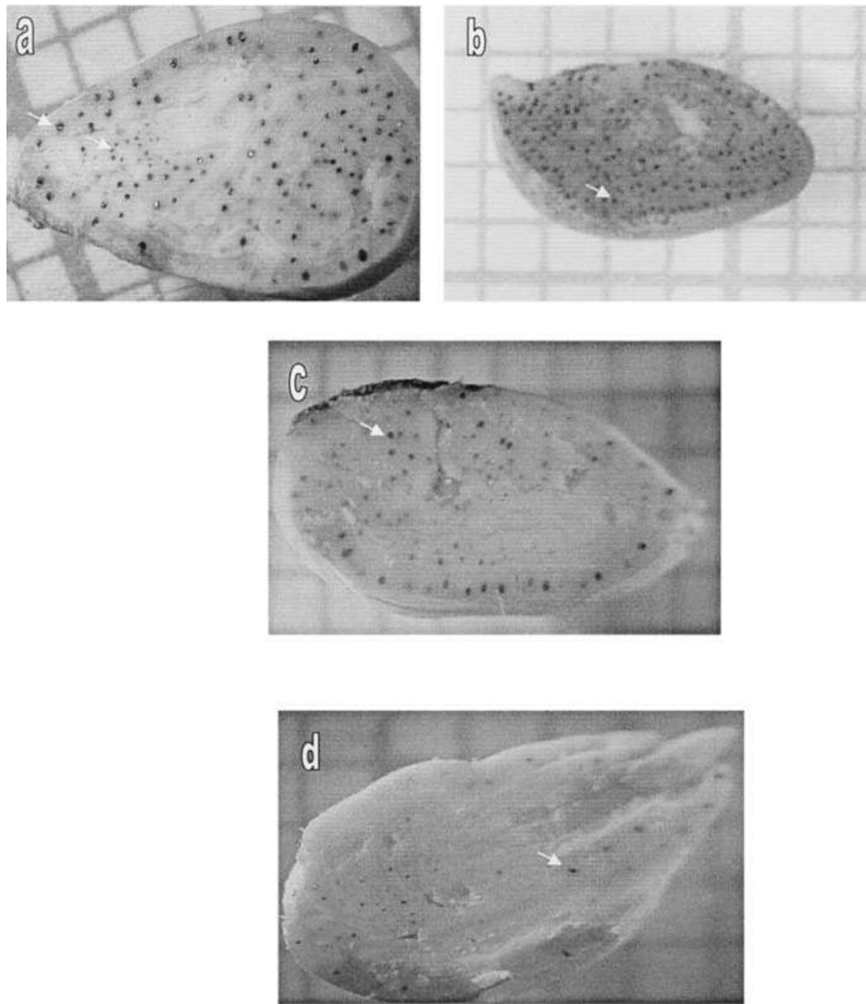

Fig. 2. Distribution of gossypol glands on the seed sections. (a) *G. hirsutum*, (b) *G. raimondii*, (c) seed BC_3, and (d) seed BC_2S_1. The arrows indicate gossypol glands.

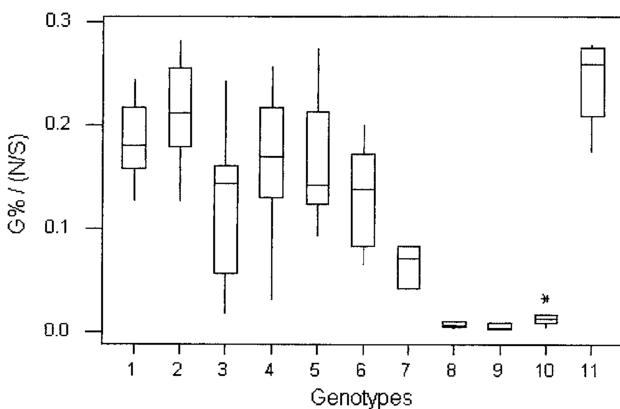

Fig. 3. Variation of the mean estimated seed gossypol content of the evaluated genotypes. Genotypes are as follows: 1 = *G. hirsutum*, 2 = *G. thurberi*, 3 = BC_2S_1/09, 4 = BC_3/09, 5 = BC_3/13, 6 = BC_2S_1/14,7 = 2(*G. australe* × *G. davidsonii*), 8 = 2(*G. thurberi* × *G. sturtianum*), 9 = 2(*G.arboreum* × *G. sturtianum*), 10 = 2(*G. hirsutum* × *G. sturtianum*), 11 = *G. raimondii.*

sis of the seed kernel meal with glacial acetic acid at 100°C in a boiling-water bath. After a 20-min. hydrolysis, the chromatograms contained additional major unidentified peaks. This phenomenon is probably linked to a release of unidentified molecules in the reactive medium or to gossypol decomposition or polymerization.

The centrifugation of the samples at 3300 *g* for 10 min followed by a double filtration on nylon filter membranes of 0.20 μm (MSI) to eliminate micro suspended particles avoided excessive back-pressure in the analytical columns. For each series of analyses, analytical drift was avoided during a working period by injecting external standards, at least three reference solutions with

Table 5. Analysis of variance of the ratios %*G*/(*N*/*S*).

Source	Degree of freedom	Sum of squares	Mean squares	*F*	*P*
Genotypes	10	0.6094	0.06094	26.9	0.000
Errors	112	0.2541	0.00227		
Total	122	0.8636			

Table 6. Values of the averages, the standard deviation, and the standard error of the %*G*/(*N/S*) ratio per genotype.

Genotypes	n_i seed number	$\overline{r}_i$ ratio mean	$\hat{\sigma}_i$ standard deviation	s.e.$_i$ standard error
G. hirsutum var. Stam f	16	0.18312	0.03616	0.03727
G. thurberi	7	0.2105	0.05211	0.05571
G. raimondii	6	0.24451	0.04049	0.04373
2(*G. hirsutum* × *G. sturtianum*)	9	0.01492	0.00823	0.00868
2(*G. thurberi* × *G. sturtianum*)	7	0.00783	0.00283	0.00303
2(*G. arboreum* × *G. sturtianum*)	9	0.00618	0.00345	0.00364
2(*G. australe* × *G. davidsonii*)	6	0.06619	0.01938	0.02093
BC_2S_1/09	16	0.12175	0.06623	0.06827
BC_3/09	19	0.17010	0.06129	0.06288
BC_2 S_1/14	11	0.12701	0.04858	0.05074
BC_3/13	17	0.16631	0.05616	0.05779

known gossypol contents close to those of the samples. The improved protocol presented schematically in Fig. 1 was finally adopted.

Results on gossypol content quantification using the seed-by-seed and the bulk seed HPLC analysis methods are presented in Table 2. The results obtained by the bulk seed analysis method are consistent with those obtained by the seed-by-seed HPLC method, the latter, however, being slightly lower. This is notable in the case for the two wild American species (*G. thurberi*, *G. raimondii*). We hypothesize that the cryo-grinding in liquid nitrogen and acetic acid led to a more complete recovery of gossypol.

Quantification of the Internal Gossypol Glands Density

Table 3 presents the results obtained from the observations made on the section of the seeds used to assess the gossypol content with the seed-by-seed HPLC analysis method. For genotypes BC_3S_1/09 and BC_2S_2/09, we noted a reduction of the number of gossypol glands on the external tissues of the whole seed kernel, compared with the gossypol gland density observed in parental species (*G. hirsutum*, *G. raimondii*) of the HRS trispecific hybrid.

The allotetraploids (*G. thurberi* × *G. sturtianum*), 2(*G.* arboreum × *G. sturtianum*), 2(*G. australe* × *G. davidsonii*), and the synthetic allohexaploid 2(*G.* hirsutum × *G. sturtianum*), which include all the chromosomes of the wild Australian species in their genome, also show a significant reduction in the number of gossypol glands. Figure 2 shows different gossypol glands distribution observed on seed longitudinal sections.

Establishment of a Relation between the Seed-by-Seed Evaluation of the Gossypol Content by HPLC, and the Parameters Observed on the Seed Section

To allow the estimation of the gossypol content from visual observations made on seed sections starting from the 123 observations carried out, correlation coefficients were calculated between the gossypol content %*G* and the following variables: *N*/*S*, *N*1/*S*, *N*2/*S*, *N*3/*S*, *N*1/*N*3, *MGS*, and *MGS***N*/*S* (Table 4). With a coefficient of correlation higher than 0.9 (Table 4), *N*/*S* and *MGS* × *N*/*S* are the two variables that best explain the variation of gossypol content %*G*. Among them, variable *N*/*S* is the easiest to use because its determination requires only simple counting of the total number of glands, instead of measuring the gossypol glands area.

Considering two or several variables simultaneously does not improve the relation between the gossypol content %*G* and the variables characterizing the distribution of gossypol glands. The relationship between gossypol content %*G* and *N*/*S* is linear, but the conditional variance of %*G* is proportional to $(N/S)^2$. This justifies the use of weighted regression. For the straight line fitted by weighted least squares, the constant, although significant, is of no importance and can therefore be removed. The simplified model is $\%G = b \times (N/S)$, which means that the %*G*/(*N*/*S*) ratio does not depend on *N*/*S*.

Examination of the box-plots (Fig. 3) and a one-criterion variance analysis of the %*G*/(*N*/*S*) ratios (Table 5) shows that the differences between the studied genotypes are very important. The results can be used to quantify the contents of gossypol in single seeds belonging to one of the 11 genotypes studied (Table 6). The estimate of the seed gossypol content is given by $G\ \% = \overline{r}_i(N/S)$ and the standard error of this estimate is equal to

$$(N/S)\sqrt{\hat{\sigma}_i^2(n_i + 1)/n_i} = (N/S)\ se_i$$

In these relations, $\overline{r}_i$ is the average and $\hat{\sigma}_i$ is the standard deviation of the ratio %*G*/(*N*/*S*) for the genotype *i*; n_i is the number of seeds studied for this genotype *i*, and se_i is the standard error of the estimated %*G*/(*N*/*S*) ratio for a seed of genotype *i*.

For example, the gossypol content of a seed with a *N*/*S* ratio of 5 will vary according to which genotype it belongs, from $0.00618 \times 5 = 0.031\%$ (standard error = $0.00364 \times 5 = 0.02\%$) for *G. arboreum* × *G. sturtianum* hybrid to $0.24451 \times 5 = 1.22\%$ (standard error = $0.04373 \times 5 = 0.22\%$) for *G. raimondii*.

This new method is rapid and accurate and can be used in breeding programs to screen the progeny of cotton genotypes showing a high degree of segregation for their seed gossypol content.

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