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HEREDITY OF GOSSYPOL GLANDS ON THE BOLL SURFACE IN GOSSYPIUM HIRSUTUM L.

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Gossypols confer a good degree of resistance to bollworm and tobacco budworm. Most of the commercially grown cotton varieties have normal gossypol glanding. The objective of the study was to determine the inheritance of the glanding trait with both of the high glanded and glandless genotypes into the genetic background of normally glanded genotype. The information of the inheritance pattern for gossypol glands is very important for breeding high foliage glanding cotton genotypes. For this purpose two cross combinations were made involving a common normal glanding parent (HRVO-1) along with two other parents i.e; high glanding (HG-142) and zero glanding or glandless (Acala 63-74). The non-significant chi square values in F_2 for these two crosses showed incomplete dominance for the glanding trait, which was further confirmed by the values of BC_2 of both the crosses. Previous studies conducted on the genetics of gossypols were based on visual observations. The gossypol quantification reported herein is more reliable and can be judged more accurately to that of the previous findings; which could not differentiate between the segregated classes in F_2 of high glanding and intermediate high glanding in a cross of normal glanding × high glanding and glandless and intermediate glandless in a cross of normal glanding × glandless

Keywords: Cotton, inheritance, gossypol glands, insect non-preference, quantification, spectrophotometry

Abbreviations: (GOP) Government of Pakistan; (ICAC) International Cotton Advisory Committee; (WTO) World Trade Organization; (AOCS) American Official of Chemical Society; (OD) Optical Density; (mg) Milligram; (NG) Normal Glanding; (HG) High Glanding.

INTRODUCTION

Before the introduction of Bt cotton, insect pest control was totally based on the use of chemical insecticides. By 1970's and 80's the use of insecticides increased tremendously in almost all cotton producing countries of the world including Pakistan. According to an estimate, in Pakistan about 122.3 million US dollars were spent on the import of insecticides during 2010 to 2011 (GOP, 2010 & 2011). The environmental concerns demand cotton production free from insecticides. For minimizing the losses to cotton production in Pakistan by the insect pests, the use of synthetic insecticides has become necessary for the last many years to control pests. This has increased the cost of cotton production in the country, which has exceeded to 40% of the total cost of growing cotton. The latest studies by International Cotton Advisory Committee (ICAC) have shown that the cost of production of cotton ranges from less than 50 cent to over US$ 2.5 per kg lint (ICAC, 2006). The use of insecticide could be a factor to raise cost of cotton production. The indiscriminate and incessant use of synthetic insecticides is not only developing resistance in the insect/pests but also posing threat to the ecosystem (Renou *et al*., 2001).

The WTO regulations demand for an insecticide free cotton production. In nature, there are some traits that confer non-preference to the insect pests infestation and gossypols are among them. Gossypols are the pigment glands distributed on the plant body covering the stem, leaf, bract, calyx and carpel walls. Gossypium species are characterized by their lysigenous glands containing terpenoid aldehydes, important secondary phytoalexins consisting mainly of gossypol, which constitute one of the important plant defense systems against pests and diseases (Yingfan *et al*., 2010). Karavina *et al*. (2012) reported the deleterious effects of high gossypol content on bollworm/spotted bollworms. Density of glands had an influence on Heliothis larval growth. The relationship of gossypol gland density with bollworm incidence suggested lowest incidence of bollworm with highest gossypol gland density on the ovary (Mohan *et al*., 1995). Similarly, the lowest in gossypol glanding had high rate of insect infestation (Al-Ameer *et al*., 2010).

Generally increasing gland density in cotton plant results in increasing concentration of the toxic compounds. The principal determinants of gland density are Gl_1, Gl_2 and Gl_3 alleles. Gl_1 is responsible for gland formation only on stem, petioles and carpel walls, whereas the Gl_2 and Gl_3 affect gland formation in cotyledons and leaves/carpel walls respectively, as well as the organs affected by Gl_1. In other words it can be said that Gl_2 and Gl_3 mask the effect of Gl_1

(Niles 1980). *Gl*$_2$ and *Gl*$_3$ are the major gland production loci in cotton (Calhoun, 1997). *Gl*$_2$ mainly expressed the gossypol gland distribution on the cotyledons (Barrow and Davis, 1974). Lee (1974) reported the effects of *Gl*$_3$ on the carpel wall. Most of the cultivated upland cottons are free of gossypol glands on the sepal margins, such phenotypes were referred as normal glanded and those expressing gossypols on the sepal margins were designated as high glanded (Calhoun *et al*., 1997). Crosses between high glanding (HG) and normal glanding (NG) parents (Calhoun, 1997) produced high glanding plants in F_1 whereas, a ratio of 3 HG : 1 NG appeared in F_2 and ratios of 1 HG: 2 segregating: 1 NG amongst $F_{2:3}$ progeny. This information defining the genetics involved for the inheritance of this glanding trait was based upon visual observations considering Calhoun (1997).

Quantification of the gossypol levels is therefore, required to confirm the inheritance of the glanding trait. The reliability/authenticity of the results reported herein can be judged more accurately from the BC_2 progenies of the normal glanding × high glanding and normal glanding × glandless; the explanation for which was not reported in the earlier visual based studies. Most of the cotton commercial cotton varieties have normal gossypol glands (Calhoun *et al*., 1997). The objective of this study was to determine the inheritance of the glanding trait with both of the high glanded and glandless genotypes into the genetic background of normally glanded genotype; to aid breeders in developing improved high glanding cultivars against insect pests.

MATERIALS AND METHODS

Three cotton genotypes: HRVO-1, Acala 63-74 and HG-142 were selected out of 43 germplasm entries on the basis of gossypol quantification. The genotype HRVO-1 was normal glanded (*Gl*$_2$*Gl*$_2$), while the genotypes Acala 63-74 and HG-142 were glandless (*gl*$_3$*gl*$_3$) and high glanded (*Gl*$_3$*Gl*$_3$), respectively (Calhoun, 1997; Nawab *et al*., 2014).

***Generation developed in glasshouse*:** The selected parents were planted in 30 cm (dia) × 30 cm (depth) earthen pots, containing a mixture of equivalent proportion of sand, soil and farmyard manure during November 2004 in a glasshouse. Temperature in the glasshouse was maintained at 30±2°C during the day and 25±2°C at night by using built-in steam heaters. The plants were exposed to natural sunlight supplemented with artificial lighting for a photoperiod of 16 hours (ICAC, 2007). Two crosses (HRVO-1 × HG-142 and HRVO-1 × Acala 63-74) comprising of a high glanding (HG-142) and glandless (Acala 63-74) parent were attempted to obtain F_0 seed during February through March, 2005. The selfed seed of the parents was obtained by covering their floral buds with butter paper bags.

***Generation development in field*:** The F_1 and their parents were planted during the normal crop season of 2005-06. The seed for the F_0, F_2, BC_1 and BC_2 generations was produced for each of the two combinations through manual selfing and crossing. The F_1 plants of each cross were divided in three groups for developing BC_1, BC_2 and F_2 for each combination.

***Field sowing and planting geometry*:** The experiment in the field was laid out in a randomised complete block design with three replications of each of the six generations of the two crosses. A single plot (4.5 m × 0.75 m) per replication accommodating approximately 16 plants spaced 30 cm apart was assigned to each of the parents and their respective F_1. Four plots per replication were assigned to each of the backcrosses and eight plots per replication were assigned to raise the F_2 population of each cross.

***Selection of plants at maturity*:** Five plants were selected randomly for the parents and their F_1 while 50 and 30 plants in each replication were selected in F_2 and backcross generations respectively to record the gossypol quantification data during 2006-07.

Gossypol extraction: The gossypol glands present on the surface of the unopened bolls were quantified on a spectrophotometer (Cecil CE-2021) at 440 nm wavelength (AOCS Official Method, 1989).

Unopened bolls were excised and washed with distilled water to remove the dust particles. The outer surface of the bolls containing the gossypol glands was peeled off and weighed on a digital balance. About 1.0 g sample was crushed in a mortar and pestle using one drop of glacial acetic acid and one drop of 70% aqueous acetone. Optical density (OD) of the sample aliquots reacted with aniline was determined in comparison to the reference solution without aniline. The difference in the OD readings of reagent blank and that of the OD value of the sample aliquot reacted with aniline gave the corrected absorbance at the wavelength of 440 nm.

***Chemicals*:** The laboratory grade Isopropyl alcohol (2-propanol), n-hexane (boiling range 68-69°C), gossypol acetic acid (standard), dimethylformamide, 3-amino-1-propanol, glacial acetic acid and aniline were purchased from SIGMA suppliers.

***Statistical analyses*:** Chi-squared values and probabilities of goodness of fit of the segregation ratios of F_2 and backcross generations were tested against theoretical ratio (Harris, 1912; Snedecor and Cochran, 1980). The data were analysed using analysis of variance technique (Steel *et al*., 1996) using MSTATC (1989) version 1.5.

RESULTS AND DISCUSSION

Increasing gland density in cotton plant results in increasing concentration of toxic compounds. F_1 populations of the two crosses, normal glanding × glandless and normal glanding ×

high glanding showed incomplete dominance of glanding. The F_1 of normal glanding × glandless (HRVO-1 × Acala 63-74) cross was intermediate glandless (Fig. 1) while the F_1 of normal glanding × high glanding (HRVO-1 × HG-142) cross was intermediately high glanding (Fig. 2). Calhoun (1997) also observed intermediate high glanding state in F_1 of normal glanding × high glanding cross. For glanding trait the F_2 showed a typical monohybrid genotypic as well as phenotypic ratio of 1:2:1 for incomplete dominance (Table 1). In the cross (HRVO-1 × Acala 63-74) the gossypol content in the glandless parent (Acala 63-74) and its F_1 was 0.04 mg/g and 0.140 mg/g (Table 2), respectively.

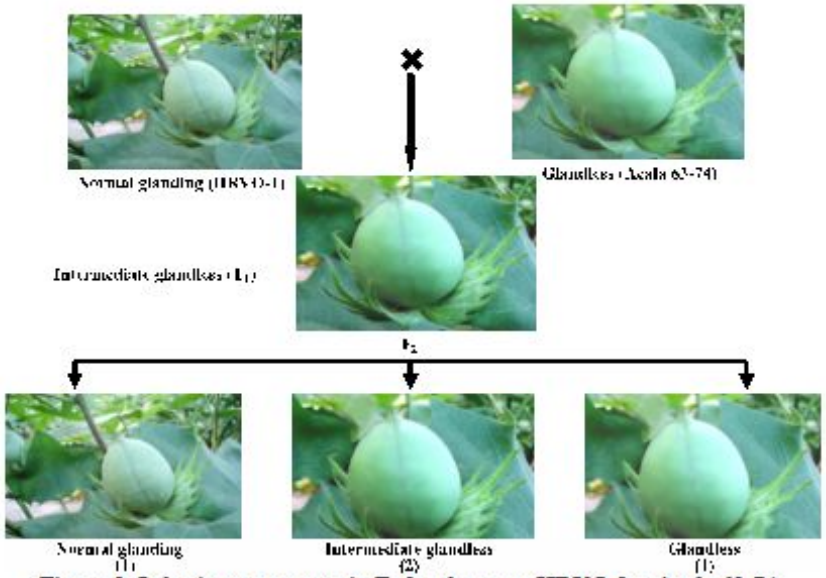

Figure 1. Inheritance pattern in F_2 for the cross HRVO-1 × Acala 63-74.

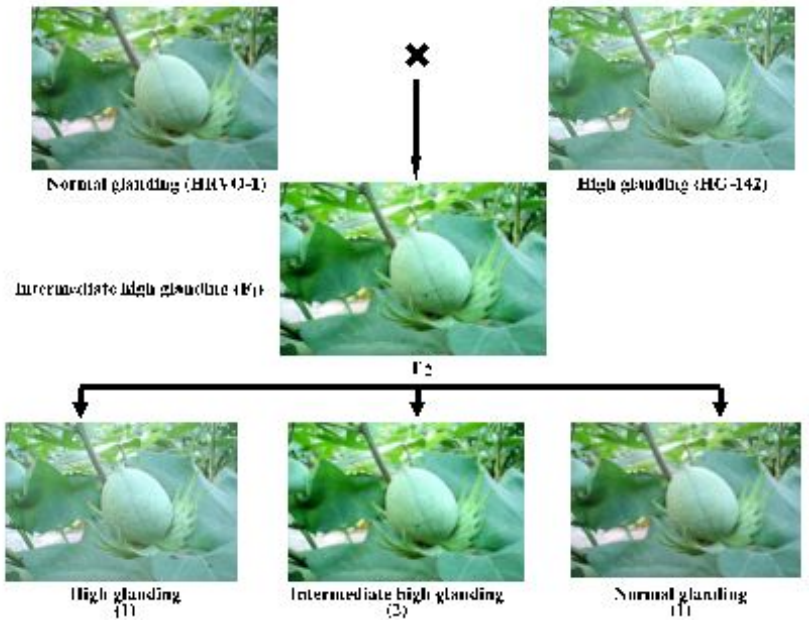

Figure 2. Inheritance pattern in F_2 for the cross HRVO-1 × HG-142.

Table 1. Chi-squared values and probabilities of goodness of fit of segregation ratios of F_2 and backcross generations for gossypol glanding trait on cotton bolls in crosses HRVO-1 × Acala 63-74 and HRVO-1 × HG-142.

Cross	Gene-ration	Expected ratio	Observed value			Expected value			χ^2 value	Prob.
			Normal glanding	Inter-mediate glandless	Glandless	Normal glanding	Inter-mediate glandless	Glandless		
HRVO-1 ×	F_2	1:2:1	48	68	34	37.5	75	37.5	3.91	0.25-0.10
Acala 63-74	BC_2	1:1	-	49	41	-	45	45.0	0.18	0.75-0.50
HRVO-1 ×	F_2	1:2:1	40	71	39	37.5	75	37.5	0.44	0.90-0.75
HG 142	BC_2	1:1	-	42	48	-	45	45.0	0.40	0.75-0.50

Table 2. Generation means for gossypol content (mg/g) and total gossypol (%) in two crosses of cotton.

Generation		HRVO-1 × HG-142		Generation		HRVO-1 × Acala 63-74	
		Gossypol content (mg/g)	Total gossypol (%)			Gossypol content (mg/g)	Total gossypol (%)
P1 (HRVO-1)		0.60	0.240	P1 (HRVO-1)		0.590	0.233
P2 (HG-142)		1.14	0.455	P2 (Acala 63-74)		0.040	0.020
F_1		0.88	0.351	F_1		0.140	0.050
	NG	0.62	0.242		NG	0.566	0.238
	I.HG	0.87	0.353	F_2	I.GL	0.143	0.064
	HG	1.15	0.460		GL	0.056	0.024
F_2	Mean	0.88	0.351		Mean	0.200	0.081
BC_1	NG	0.60	0.240	BC_1	NG	0.580	0.231
	I.HG	0.88	0.350		I.GL	0.139	0.056
	Mean	0.74	0.295		Mean	0.360	0.144
BC_2	HG	1.15	0.460	BC_2	GL	0.056	0.022
	I.HG	0.89	0.350		I.GL	0.143	0.057
	Mean	1.02	0.410		Mean	0.099	0.040
LSD (0.05)		0.018	0.018	LSD (0.05)		0.057	0.018

NG = Normal Glanding; I.HG = Intermediately High Glanding; GL = Glandless; I.GL = Intermediately Glandless

Similarly, in the same cross the total gossypol (%) in the parent (Acala 63-74) and its F_1 were recorded as 2 and 5%, respectively (Table 2). Lee (1973) while conducting direct crosses of normal glanding parent ($Gl_2Gl_2gl_3gl_3$) with four glandless parents ($gl_2gl_2gl_3gl_3$) for seed gossypols , recorded the mean gossypol level ranging from 0.068 mg/g to 0.320 mg/g in F_1 of direct crosses and from 0.064 mg to 0.253 mg in F_1 of the reciprocal crosses with four normal glanding parents. In the cross of glandless with four glandless parents, the gossypol level ranged from 0.004 mg to 0.014 mg in F_1. He termed the gossypol yields ranging from 0.004 mg to 0.320 mg as glandless. In another study the range of gossypol content as determined by Mansour *et al*. (2004) in relation to the bollworm infestation was 20-25 mg/100gram (0.20-0.25 mg/g), which was considered low in relation to the non-significant association with bollworm incidence. From the F_2 data regarding the glanding trait inheritance in HRVO-1 × Acala 63-74 cross, was categorized into three main classes, normal glanding (Gl_2Gl_2) intermediate glandless (Gl_2gl_3): glandless (gl_3gl_3) as evident from Figure 1. There were statistically significant differences between the parents and their F_1 which justified the distinctness of these three classes (Table 2) as appeared in F_2. In contrast, Calhoun (1997) showed two main classes of glandless and normal glanding in the F_2 of the cross of normal glanding and glandless. His studies were based on visual classifications which failed to distinguish between the intermediate and glandless classes obtained in F_2. The appearance of intermediately glandless-ness in F_1 of the cross HRVO-1 × Acala 63-74 was an indication that the single dose of Gl_2 was inadequate for gland production on bolls.

In the second cross HRVO-1 × HG-142 (normal glanding × high glanding), three genotypic classes were also appeared in F_2. The data regarding the glanding trait inheritance in HRVO-1 × HG-142 cross was categorized into three main classes of high glanding, intermediate high glanding and normal glanding (Gl_2Gl_2) plants as shown in Figure 2. The intermediate high glanding state which was appeared in the F_1 was again expressed in the F_2 generation. This intermediate high glanding class was similar to that of the F_1 and was significantly different from both of the parents (Table 2) which justified the distinctness of these three classes (both of the parents and F_1 of the cross HRVO-1 ×

HG-142). But the findings of Calhoun (1997) were based on visual observations of gossypol glands and categorized two main classes of high glanding and normal glanding in F_2 from a cross of normal glanding and high glanding parents. The present studies quantified the gossypols and clearly distinguished between the intermediate and high glanding classes. The non-significant χ^2 in F_2 for the inheritance of glanding trait in these two crosses fit well against the expected ratio of 1:2:1. This pattern of segregation in these two crosses was further confirmed from the non-significant χ^2 values obtained in the backcrosses with parent-I (HRVO-1) and parent-II (HG-142) which depicted that the observed values fit well against the expected ratios of 1:1 (Barrow and Davis, 1974). Observations of 1 high glanding: 2 intermediate high glanding: 1 normal glanding in F_2 from the Table 1 in the cross HRVO-1 × HG-142 indicated incomplete dominance.

Calhoun (1997) explained that high glanding was controlled primarily by a single locus but the expression was affected by interaction with recessive (*gl*) alleles at Gl_2 or Gl_3 loci or both. The segregation pattern in F_2 according to the findings of Calhoun (1997) helped in further genotyping. Normal glanding ($Gl_2Gl_2gl_3gl_3$) and glandless (gl_3gl_3) in the cross HRVO-1 × Acala 63-74 and normal glanding (Gl_2Gl_2) and high glanding (Gl_3Gl_3) in the cross HRVO-1 × HG-142 were easily distinguishable. From the data reported here, it is clear that the glanding traits (high glanding and glandless) were inherited simply (incomplete mode of inheritance) and in the crosses between normal glanding and high glanding and between normal glanding and glandless, can be selected in much of the same way as any other incompletely dominant allele. The same inference regarding the gossypol inheritance can also be cited from the research findings of Calhoun (1997) and Nawab *et al*. (2014). However, the environment as well as minor genes may affect the degree of expression of the glanding trait (White *et al*., 1982). The present study is a step forward towards the development of natural resistance against insect pests by reducing the reliance upon chemical insecticides. The long-term goal can be achieved to elucidate the gene network mechanism controlling glands and gossypol while increasing the resistance of cotton to pests. Glandless seed and glanded foliage cotton can bring a revolutionary change in cotton breeding.

***Conclusions*:** In-built mechanism of resistance against insects is a safe and secure method according to the bio-safety requirements. Gossypol is one of the naturally conferring resistant traits against insect pests. The non-significant χ^2 in F_2 for glanding trait in all the crosses fit well against the monohybrid ratio of 1:2:1. The reliability of the results reported herein can be judged more accurately from the segregated classes (high glanding, intermediate high glanding & normal glanding) in a cross of normal glanding × high glanding and (glandless, intermediate glandless & normal glanding) in a cross of normal glanding × glandless, the explanation for which was not reported in the earlier studies. The explanation in the previous studies regarding the genetics of gossypols were on visual observations but the gossypol quantification based results are considered to be more reliable in distinguishing the various classes whose identification can only be possible through quantification technique.

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