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Prediction of Cotton Resistance to *Helicoverpa armigera* Based on the (+)-Gossypol Content in Mature Seed

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ABSTRACT

Various Uzbek commercial cotton varieties were grown in the field and these were exposed to cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) larvae. A significant negative correlation coefficient ($r = -0.89$) and linear regression ($y = 109.88-5.27x$) was observed between the concentration of (+)-gossypol in cotton seed and boll damage. Significant regressions were not observed in similar comparisons with (-)-gossypol or with total gossypol concentrations. This characteristic was used as a phenotypic predictor to guide breeding efforts to increase resistance to *H. armigera*.

Keywords: correlation analysis, cotton, gossypol enantiomers, HPLC, insect resistance
Abbreviations: HPLC, High Performance Liquid Chromatography; **MeCN**, acetonitrile (methylcyanide); **r.h.**, relative humidity (the amount of atmospheric moisture present relative to the amount that would be present if the air were saturated)

INTRODUCTION

The cotton bollworm (*Helicoverpa armigera*) is the primary boll-damaging pest of conventional cotton in Uzbekistan, where it emerges as a moth in early to mid May and completes two generations. It then moves to cotton, which blooms in late July or early August. Regular, systematic scouting for bollworm eggs and bollworm larvae is essential, particularly when the major moth flights are under way. Compensation by the plant for boll damage at this time of year is minimal, and caterpillar feeding, especially on bolls, can dramatically reduce yields. Thus, development of cotton cultivars with resistance to this pest will benefit cotton producers. In many parts of the world, the use of genetically modified cottons containing *Bt* genes derived from the soil bacterium, *Bacillus thurengensis*, are extensively utilized. An alternative to genetic-engineering techniques is to focus on selecting plants that exhibit natural resistance to insects. Plant characteristics such as glabrousness (Lukefahr *et al.* 1971), nectariless (Meredith *et al.* 1973), high concentrations of gossypol and related terpenoid aldehydes including heliocides and their biosynthetic precursors, as well as condensed tannins (Bell and Stipanovic 1977; Stipanovic *et al.* 1977; Bell *et al.* 1978; Chan *et al.* 1978), are known to affect resistance against insects and diseases. Indeed, gossypol [1,1.,6,6.,7,7.-hexahydroxy-5,5.-diisopropyl3,3.-dimethyl-(2,2.-binaphthlene)-8,8.-dicarboxaldehyde] is a natural dimeric sesquiterpenoid found in cotton plants (*Gossypium* sp., *Malvaceae*), and is reported to play a role as a natural defense against insect pests (Bottger *et al.* 1964; Bell and Stipanovic 1977; Hedin *et al.* 1992). Glandless plants, which were first described by McMichael (1954) and do not contain gossypol, were completely defoliated in the field whereas nearby normal glanded plants showed little or no damage (Bottger *et al.* 1964). Thus, there is little doubt that gossypol is an important component in the cotton plant's defense against insect herbivores. However, because of restricted rotation about the compound's binaphthyl bond, gossypol exhibits atropisomerism and exists in two enantiomeric forms, so called (+)- and (-)-gossypol. The toxicity of (+)- and (-)-gossypol to insect pests and pathogens have not been extensively investigated, especially under field conditions. Yang *et al.* (1999) report a *Helicoverpa armigera* study in which larvae were raised on artificial diets containing either (+)- or (-)-gossypol from the 3rd instar through pupation and to emergence of the moth. The larvae raised on the (+)-gossypol diet matured more slowly and percent survival to the adult was lower. However, in a relatively recent artificial feeding study Stipanovic *et al.* (2006, 2008) showed that racemic, (+)- and (-)-gossypol were equally effective at reducing days-to-pupation, pupal weights and survival of *Helicoverpa zea* and *Heliothis virescens* larvae. Recently, Namazov *et al.* (2011) reported no correlation between the percent (+)-gossypol and resistance to *H. armigera*.

Thus, there is limited information on how the ratio of (+)- and (-)-gossypol correlates with insect and disease resistance under field conditions, but casual observations in the field indicated that there might be a correlation between the (+)-gossypol content and resistance to herbivorous insects. Thus, based on Yang's finding that (+)-gossypol is more toxic to insect pests in artificial diets than either (-)-gossypol or the racemate (Yang *et al.* 1999), and our own field observations, we hypothesized that the dependence of cotton insect resistance might correlate with the absolute content (not the ratio) of (+)-gossypol in cottonseed. If true, plants with a low total content of (+)-gossypol, but a high concentration of total gossypol will be more susceptible than those with a high total content of (+)-gossypol but low total gossypol content. To test this hypothesis, we measured the relative damage of fruiting bodies compared to total bolls on a plant. Simultaneously, we determined the amount of (+)- and (-)-gossypol present in mature seed; the sum of these provides the total gossypol. Using this data, we determined correlations for resistance among eight cotton lines.

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Short Communication

MATERIALS AND METHODS

Cotton plants

Eight Uzbek cotton lines that are the products of complex interspecific hybrid combinations were studied. These were selected because of the differences in resistance to the cotton bollworm and their complex parentage. These lines were: Namangan-77, and S-6524, both of which are hybrids of *G. hirsutum* and *G. hirsutum* ssp. *punctatum* [Uzbek Cotton Breeding and Seed Production Scientific Research Institute's Collection Cat. No. 05152]; S-2609 [L-1541 (S-2602 x Tashkent-6) x "Duplex"]; S-2610 [L-435 x Acala SJ 08822]; and lines, Mg-01 {Sultan; [F_4(F_1 *G. thurberi* x *G. raimondii*) x *G. hirsutum* L.]}; Mg-02 {Jarkoorgan; [F_4 (F_1*G. thurberi* x *G. raimondii*) x *G. hirsutum L.*]}; and Mg-03[F_4 (F_1*G. thurberi* x *G. raimondii*) x *G. hirsutum L.*] ("Mg" is a Russian abbreviation and means "Interspecific Hybrid") and finally a "Glandless Line" {i.e., gossypol free; Acala SJ-1 x F5 [F4B (C-5619) x (F1C-5619 x 39750) x K-620 x Acala - 1517 x *G. hirsutum* ssp. *marie galante*}. These varieties were grown under field conditions in Tashkent City Region (41°N Latitude).

Field assay of damage to cotton plant fruits by cotton bollworm larvae

Cotton bollworm moths (*H. armigera*) were derived from *H. armigera* larvae provided by the Uzbek Research Institute of Plant Protection. The larvae were reared in a controlled laboratory environment (25°C, 70% r.h., and a LD 14:10 h regime) on a standard artificial diet for *Helicoverpa* species (Teakle 1991) to minimize the possibility of alterations in adult behavior due to host plant experience in the larval stage. This artificial diet contained: soybean seed powder (250 g), wheat germ (30 g), yeast (35 g), sorbic acid (1.1 g), ascorbic acid (3.5 g), sunflower oil (5 ml), agar (14 g), methyl-*p*-hydroxy benzoate (2.2 g), formaldehyde 37% (2.5 ml) and distilled water (up to 650 ml). This colony was raised on this artificial diet for a few generations under these conditions before they were used in the field. Six male and female moths per 100 m^2 were artificially introduced into isolated cotton fields. The plants were not treated with any insecticides before or after the introduction of the insects. After egg laying and larval development, the number of plants with damaged fruit were counted and evaluated as a % of the total number of fruit. The samples consisted of squares, small bolls, and large bolls in the proportion that they occurred in the field. To determine this ratio, we sampled and retained 10 squares and 10 bolls, and then evaluated the fruit to determine the percent damage. This process was repeated 10 times for a total of 100 squares and 100 bolls.

Determining the contents of gossypol enantiomers and total gossypol in cottonseed

The ratio of (+)- to (-)-gossypol and their absolute content in cottonseed were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection as earlier described for ovine tissues (Kim *et al*. 1996). For this purpose, 2 to 3 cottonseed were dehulled and the kernels were cooled with liquid nitrogen and ground to a powder; approximately 20 mg of this powder was weighed and reacted with 0.2 ml of derivatizing reagent {d-alaninol [(+)-2-amino-1-propanol)], glacial acetic acid and MeCN (from, Sigma-Aldrich, DE) 2:10:88 (v/v/v)} and heated at 70°C for 30 min and diluted to a final volume of 0.5 ml after centrifugation. The supernatant was separated on a reversed-phase Zorbax Eclipse XDB C18 (3.0 × 100 mm, 3.5 um) column (Agilent Technologies, Inc, USA) with 75% of MeCN in 0.01M KH_2PO_4 pH 3.0 as the mobile phase with a flow rate of 0.6 ml/min. The elution of analytes was monitored at 254 nm. The absolute content of (+) or (-) gossypol was determined based on a calibration utilizing racemic gossypol (99% pure, Sigma-Aldrich, enantiomer ratio 1:1). The ratio of (+)- to (-)-gossypol was calculated based on the respective peak areas of the (+)- and (-)-gossypol peaks. Kim *et al.* (1996) have established that (+)-gossypol elutes first. The total gossypol is calculated as the sum of (+)- and (-)-gossypol.

Statistical analysis

The field test was a completely randomized design. Five different rows of plants were used for each cotton line and the experiment was replicated 3 times. HPLC measurements also were repeated 3 times. Data were subjected to analysis of deviation or means, and standard errors were calculated. Least significant differences (P = 0.05) were calculated from the data analyzed by MS Excel 2003 (MS Office 2003 for Windows, Microsoft Corp., USA). The statistical procedures such as calculation means, standard errors and the correlation coefficients were also performed with MS Excel 2003.

RESULTS AND DISCUSSION

As indicated above, gossypol is an important component in the cotton plant's defense against herbivorous insects. In more recent work, Anilkumar *et al.* (2009) demonstrated that gossypol largely accounts for the inability of *Helicoverpa zea* larvae that are resistant to the Cry1 Ac toxin to complete development on cottons expressing this Bt toxin. Thus, the level of total gossypol in the seed might be expected to affect insect resistance to the cotton boll worm, but the correlation between (+)- or (-)-gossypol levels and resistance to this pest have not been documented. **Table 1** shows the results of our work. The total content of gossypol and the concentration of (+)- and (-)-gossypol in mature cotton seed from 8 cotton lines along with the percent damage to these lines in the field is shown. Thus, Mg-02 was found to exhibit an intermediate concentration of total gossypol (29.4 mg/g), but the highest concentration of (+)-gossypol and it sustained the lowest level of damage. In contrast, S-6524 has an intermediate concentration of (+)-gossypol and the highest concentration of total gossypol, but it sustained a relative high level of damage.

The correlations between gossypol content in seed with the percent damage is shown at the bottom of **Table 1**. And the correlation for (+)-gossypol with percent damage is shown in **Fig. 1**. This provides a calibration curve that may be useful in predicting what plants will be most resistant to *H. armigera* based on the content of (+)-gossypol in mature seed. Thus, as we observed experimentally, if the (+)-gossypol content is $C_{(+)G} < 10$ mg/g, then the line will be susceptible, if the (+)-gossypol content is $10 \leq C_{(+)G} \leq 15$ mg/g then the line will be resistant, if the (+)-gossypol content is greater than 15 mg/g then the line will be highly resistant.

Table 1 Content of total, (+)- and (-)-gossypol in mature cottonseed and the percent damage by *Helicoverpa armigera* observed in the field and the correlation between content of gossypol and percent damage.

Cotton variety (in order of descending insect resistance)	% Bolls damaged by *Helicoverpa armigera*	Content of total gossypol in seed (mg/g)	Content of (+)-gossypol in seed (mg/g)	Content of (-)-gossypol in seed (mg/g)
Mg02	2.3 ± 0.3	29.4	20.05	9.35
Namangan-77	11 ± 2	27.9	18.97	8.93
Mg01	15 ± 2	24.6	15.33	9.27
S2609	31 ± 3	29.2	17.23	11.97
S6524	40 ± 2	24.1	14.25	9.85
S2610	42 ± 3	37.5	11.81	25.69
Mg03	45 ± 3	18.4	9.68	8.72
"Gossypol-free" Line	80 ± 2	14.1	8.94	5.16
Correlation coefficients		-0.55	-0.89	-0.049

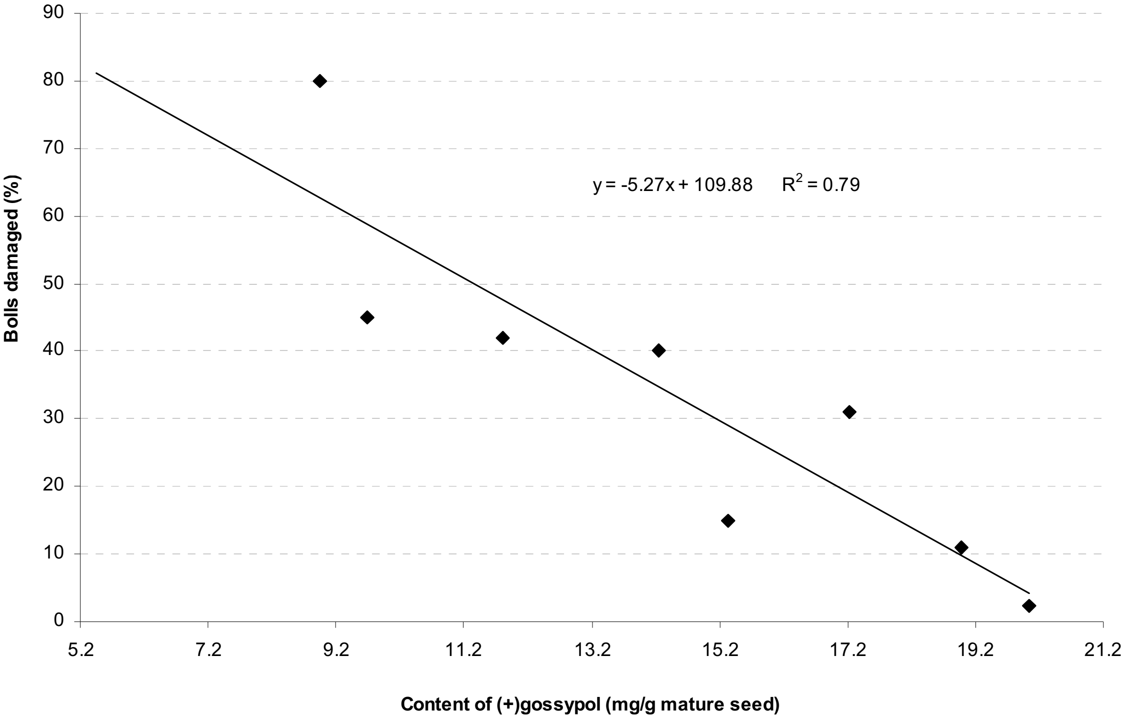

Fig. 1 Correlation between the content of (+)-gossypol in mature seed and percent plant damage by *Helicoverpa armigera*.

Thus, it may be possible to identify lines that are more resistant to *H. armigera* by determining the ratio of (+)- to (-)-gossypol and total gossypol in mature seed.

Our present findings support the hypothesis that the resistance factor may be on an allele closely associated with the high (+)-gossypol seed trait, and during the breeding process it is introduced along with this trait. This investigation requires addition study with more lines, but these field observations combined with chemical and statistical methods may help accelerate development of insect resistance cotton varieties, an important goal in cotton genetics and cotton breeding programs.

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