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Effect of Racemic and (+)- and (−)-Gossypol on the Survival and Development of *Helicoverpa zea* Larvae

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Abstract Gossypol is a sesquiterpene that occurs naturally in seed and other parts of the cotton plant. Because of restricted rotation around the binaphthyl bond, it occurs naturally as enantiomeric mixtures with (+)-gossypol to (−)-gossypol ratios that vary between 97:3 and 31:69. Commercial cotton varieties (*Gossypium hirsutum*) normally exhibit an approximate 3:2 ratio. (+)-Gossypol is significantly less toxic than (−)-gossypol to nonruminant animals; thus, cottonseed containing high levels of (+)-gossypol might be safely fed to nonruminants. Gossypol, however, is an important component in the cotton plant's defense against insect herbivores, but it is not known how cotton plants that exhibit high levels of (+)-gossypol in the foliage might be affected by insect herbivory. To address this question, 1-d-old *Helicoverpa zea* larvae were fed diets with 0.16, 0.20, and 0.24% racemic, (+)-, and (−)-gossypol. Larval pupal weights, days-to-pupation, and survival were adversely affected by all gossypol diets compared with the control diet. Statistical differences were determined by comparing the compounds among themselves at the three levels and between the three compounds at the same level. When the compounds were compared among themselves, no large differences were observed in pupal weights or in days-to-pupation among any of the diets. Among the three compounds, at the 0.16% level, the diet containing racemic gossypol was the most effective at reducing survival. At the 0.20 and 0.24% levels of racemic (+)- and (−)-gossypol, survival was not statistically different. The overall results indicate that (+)-gossypol is as inhibitory to *H. zea* larvae as racemic or (−)-gossypol, and thus, cotton plants containing predominantly the (+)-enantiomer in foliage may maintain significant defense against insect herbivory.

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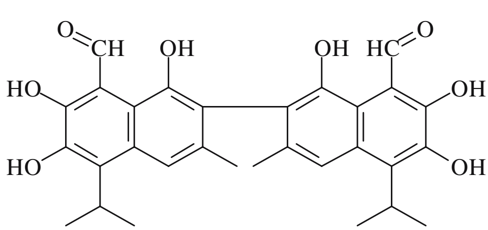
Introduction

World cottonseed production for 2004/2005 is estimated to be 42.9 million metric tons. Cottonseed is a highly nutritious source of protein; however, it is considered a by-product of fiber production because it contains gossypol (Fig. 1), a naturally occurring toxin. Complete elimination of gossypol from the plant is not practical because plants lacking gossypol are highly susceptible to a host of insect herbivores (Jenkins et al., 1966; Lukefahr et al., 1966; Oliver et al., 1971; Meisner et al., 1977; Zur et al., 1979). Moreover, high levels of gossypol in the foliage are associated with resistance to insects.

Gossypol exhibits optical activity because of restricted rotation around the binaphthyl bond (Jaroszewski et al., 1992a). Of the two possible enantiomers, (−)-gossypol is significantly more toxic to nonruminant animals. In commercial *Gossypium hirsutum* cottonseed, the ratio of (+)- to (−)-gossypol is usually about 3:2. However, in some cultivars such as Texas Marker 1, the ratio is approximately 1:1 (actual 53:47). In contrast, some moco cotton varieties that are native to Brazil have (+)- to (−)-gossypol ratios as high as 96:4. Feeding experiments have demonstrated that (+)-gossypol had little effect on mean cumulative weight gain and feed conversion ratios of chickens (Bailey et al., 2000; Stipanovic et al., 2001; Lordelo et al., 2005). This implies that increasing the (+)- to (−)-gossypol ratio in commercial cottonseeds could overcome the impediment to utilizing this high-quality protein as a nonruminant feed source.

Through a traditional backcross breeding program that used these native varieties, the percent (+)-gossypol in the commercial seed was increased to over 90% (Bell et al., 2000). However, little is known concerning how high levels of (+)-gossypol in the foliage might affect resistance to herbivorous insects. It is known that the total concentration of gossypol varies significantly in different plant tissues depending on environmental conditions (Stipanovic et al., 1988). For example, the mean concentration of gossypol in various plant tissues for 14 *G. hirsutum* varieties grown at five locations from south Texas to north Texas (~1000 km) varied as follows: leaves, 0.04–0.10%; flower buds, 0.8–1.0%; seed 0.9–1.1%. Yang et al. (1999) reported that *Helicoverpa armigera* larvae raised on artificial diets containing (+)-gossypol matured more slowly, and the percentage of larvae surviving to the adult stage was lower as compared to those raised on (−)-gossypol. Herein, we report the results on racemic gossypol [a 1:1 mixture of (+)- and (−)-gossypol, designated as (±)-] and (+)- and (−)-gossypol in artificial diet feeding studies on the

Fig. 1 Structure of gossypol



generalist herbivore *Helicoverpa zea* (Boddie). Because the (+)- to (−)-gossypol ratio varies between approximately 3:2 and 2:3 in commercial cotton cultivars (Cass et al., 1991; Jaroszewski et al., 1992b; Percy et al., 1996), we included (±)-gossypol as a check to approximate commercial cottons.

Methods and Materials

Preparation of (+)-, (−)-, and (±)-Gossypol

A crystallization process to separate the gossypol enantiomers as gossypol–acetone (1:3) followed by storage of the products under vacuum for 3 d to remove essentially all of the acetone was used to obtain chemically pure chiral gossypol (Dowd, 2003). Purified racemic gossypol acetic acid (1:1) was obtained by repeated recrystallization of gossypol from acetone and acetic acid. The gossypol samples were analyzed by high-performance liquid chromatography as previously described (Puckhaber et al., 2002) to confirm enantiomeric content. The racemic gossypol was 49.9% (−)-gossypol and 50.1% (+)-gossypol, whereas the enantiomer samples were >99.5% optically pure.

Preparation of Diets

Five diet sets were prepared. Four contained Alphacel (ICN Nutritional Biochemicals), which is a nonnutritive polysaccharide that was used as a carrier, and one control diet set contained no Alphacel. Each gossypol sample was dissolved in 35 ml of ethyl acetate and was quantitatively added to 15 g of Alphacel. The suspensions were evaporated to dryness. Hexane (~50 ml) was added to the dry samples and evaporated to remove traces of ethyl acetate. The samples were left under vacuum for 36 hr. Six gossypol/Alphacel preparations were made with 800, 1000, or 1200 mg of either (+)- or (−)-gossypol; 893, 1116, and 1339 mg of (±)-gossypol–acetic acid (1:1), which is 89.62% gossypol by weight, was used to make the three (±)-gossypol/Alphacel preparations. To account for the acetic acid in the (±)-gossypol/Alphacel preparations, an equivalent amount of acetic acid was added to the Alphacel/chiral gossypol and Alphacel control (i.e., Alphacel with no gossypol) preparations.

Diets were prepared from a dry soy bean–wheat germ premix (Instant Soybean–Wheat Germ Insect Diet; Stonefly Industries, Inc., Bryan, TX, USA) with a slight modification of the manufacturer's instructions. Specifically, a dilute vinegar solution was prepared from water (75%) and 5% vinegar (Albertson's, white vinegar; 25%). This dilute vinegar solution (390 ml) was added to 15 g of Alphacel with or without gossypol and 100 g of the diet premix. This provided 505 g of finished diet. One additional control diet was prepared according to the manufacturer's instructions (i.e., 100-g dry diet, 284 ml water, and 16 ml 5% vinegar) with no Alphacel. The final levels of gossypol were 0.16, 0.20, or 0.24%.

Larvae

Larvae were reared from *H. zea* (Boddie) moths collected from the wild and raised in rearing chambers at College Station, TX. Newly hatched larvae were allowed to feed on the artificial diet with neither Alphacel nor gossypol for 1 d before transfer to the test diets. This delay assured their fitness and more closely mimics their behavior in nature. That is, other researchers have noted that newly hatched *Heliothis virescens* larvae carefully avoid consumption of cotton glands (Hedin et al., 1992).

Feeding Trials

Larvae were raised on the 11 diets [i.e., soybean–wheat germ diet, soybean–wheat germ diet plus Alphacel, and the latter diet with 0.16, 0.20, or 0.24% of (+)-gossypol, (−)-gossypol, or racemic gossypol]. A single 1-d-old *H. zea* (Boddie) larva was placed in a 22-ml plastic cup that contained 4–5 g of a specific diet. Each treatment consisted of 40 larvae, which were raised in incubators at 27°C on a 12-hr light/12-hr dark regime. The following parameters were measured: number of survivors, days-to-pupation, and pupal weight. After 10 d, cups were inspected daily to check for larval survival and pupation. Pupae were allowed to harden for 1 d and then weighed.

Statistical Analyses

Logistic regression and frequency analysis (Proc Logistic and Proc Freq, respectively) were performed to determine the comparative probability of survival on different diets. Results of logistic regression are the ratio of survival odds for larvae consuming two different diets. For example, the odds of survival $[p/(1-p)]$ on the 0.16% (−)-gossypol diet is $[0.70/(1-0.70)]$ (see Table 1), whereas the odds of survival in the 0.20% (−)-gossypol diet is $[0.40/(1-0.40)]$. To compare the survival on these diets, the ratio of these two odds was calculated (Hosmer and Lemeshow, 2000; Agresti, 2002). This provides a measure of the likelihood of survival between these two groups. Thus, the odds ratio of larval survival on the 0.16% (−)-gossypol diet compared to the 0.20% (−)-gossypol diet shown in Table 1 is calculated as follows:

$$\left(0.7/(1-0.7)\right)\Big/\left(0.4/(1-0.4)\right) = \left(0.7/0.3\right)\Big/\left(0.4/0.6\right) = 3.5$$

Table 1 Odds ratios of survival and probability of significance for survival of *Helicoverpa zea* larvae fed with racemic, (+)-, or (−)-gossypol at different concentrations

Gossypol form	Odds ratio comparison between gossypol concentrations		
	0.16/0.20	0.16/0.24	0.20/0.24
(±)-[a]	nsd[b] (0.470)[c]	nsd (0.352)	4.8 (0.036)
(−)-	3.5 (0.008)	16.3 (<0.001)	nsd (0.742)
(+)-	10.5 (<0.001)	22.7 (<0.001)	2.2 (0.075)

[a] (±)- denotes gossypol racemate.

[b] nsd denotes not statistically different (logistic regression analysis with a 95% Wald confidence level).

[c] Value in parentheses is the probability that the difference within the survival comparison is statically significant.

Statistical differences between days-to-pupation on different diets and least-square mean weights of pupae on different diets were determined using Proc Mixed in SAS version 9.1 (SAS Institute, Cary, NC, USA, 2003) software computations. Survival odds and differences in days-to-pupation and least-square mean pupae weights were made between the control diet with no Alphacel and the control diet with Alphacel, but no gossypol. Similarly, all gossypol diets were compared to the two control diets. In addition, in designing the experiment, an *a priori* decision was made to evaluate the gossypol diet data by dividing the study into 18 groups. Nine groups considered how a concentration of each enantiomer or the racemate affected the larvae compared to other concentrations of the same enantiomer or racemate. The other nine groups considered how each enantiomer or racemate at a specific concentration affected the larvae compared to the other enantiomer or racemate at the same concentration. These were the only pairwise comparisons evaluated among the gossypol diets.

Results

We found a clear dose response to gossypol for survival and, to a lesser extent, pupal weight. Furthermore, overall survival, days-to-pupation, and pupal weights were not different when the larvae were fed with racemic, (+)-, and (−)-gossypol at the same concentration. Specific analyses of the data are given below.

Survival

The survival results of the feeding experiment are shown in Fig 2. Contingency table analysis indicated no significant difference in survival between the diet and the diet with Alphacel. The same analysis showed that larval survival on all of the 0.20% and 0.24% gossypol diets was lower than the diet with Alphacel without gossypol. For the 0.16% diet, this was only true for the (±)-gossypol diet.

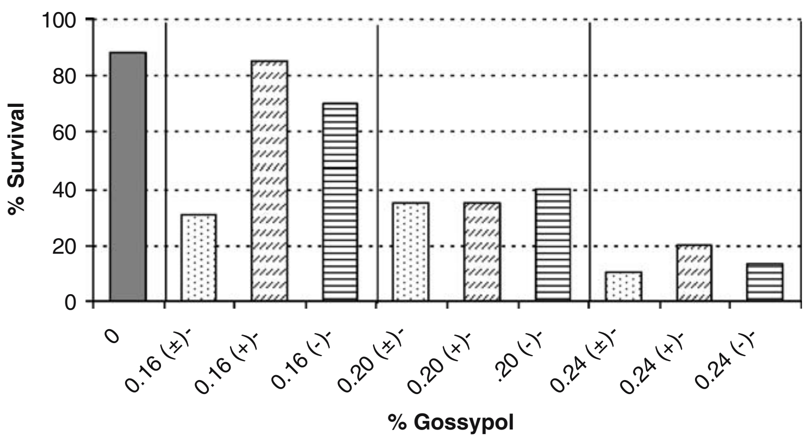


Fig. 2 Percentage survival of *Helicoverpa zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (−)-gossypol

The odds ratio of survival at different levels of racemic, (+)-, or (−)-gossypol is given in Table 1. When the effect of feeding racemic gossypol at different levels was compared, the only statistically significant difference in survival was between the larvae fed with the 0.20 and 0.24% diets (Table 1). That is, larvae fed with the diet containing 0.20% racemic gossypol were 4.8 times more likely to survive as compared with larvae fed with the diet containing 0.24% racemic gossypol. Among the (−)-gossypol diets, larvae fed with the 0.16% diet were 3.5 times more likely to survive than larvae fed with the 0.20% diet and 16.3 times more likely to survive than larvae fed with the 0.24% diet. The (+)-gossypol diets showed a similar trend with larvae fed with the 0.16% diet being 10.5 times more likely to survive than larvae fed with the 0.20% diet and 22.7 times more likely to survive than larvae fed with the 0.24% diet, whereas larvae fed with the 0.20% diet were 2.2 times more likely to survive than those on the 0.24% diet. All other comparisons of survival with concentration differences were not statistically different. When survival was compared between the two enantiomers and racemic gossypol treatments at equivalent concentrations, the only significant differences were at the 0.16% level (Table 2). Specifically, larvae fed with the (−)-gossypol diet at the 0.16% level were 6.2 times more likely to survive compared with larvae fed with the racemic diet at this level, and larvae fed with the (+)-gossypol diet at the 0.16% level were 14.9 times more likely to survive than larvae fed with the racemic diet at this level. This same set of comparisons at the 0.20 and 0.24% diets showed no statistical differences in survival. Odds ratios of survival of the (+)-gossypol diets compared to the (−)-gossypol diets at the same levels were not statistically different.

Days-to-Pupation

There was no statistical difference in the time needed to reach pupation between the diets with and without Alphacel (data not shown). However, there were differences between the Alphacel diet without gossypol and the Alphacel diets that contained gossypol (Fig. 3). In all of the latter, the days-to-pupation increased. Among the various gossypol diets, moderately significant different delays in pupation were noted between (−)-gossypol at the 0.16 and 0.24% levels ($P = 0.03$) and between the 0.20 and 0.24% levels ($P = 0.02$). The only other pupation delay was noted for the (+)-gossypol diets between the 0.16 and 0.24% levels ($P = 0.06$), and this difference

Table 2 Odds ratios of survival and probability of significance for survival of *H. zea* larvae fed with racemic, (+)-, or (−)-gossypol at equivalent concentrations

Percent gossypol in diet	Odds ratio comparison between gossypol forms		
	(−)-[a]/(±)-	(+)-/(±)-	(−)-/(+)-
0.16	6.2 (<0.001)[b]	14.9 (<0.001)	nsd (0.298)
0.20	nsd[c] (0.664)	nsd (0.792)	nsd (0.592)
0.24	nsd (0.724)	nsd (0.385)	nsd (0.791)

[a] (±)- denotes gossypol racemate.

[b] Value in parentheses is the probability that the difference within the survival comparison is statically significant.

[c] nsd denotes not statistically different (logistic regression analysis with a 95% Wald confidence level).

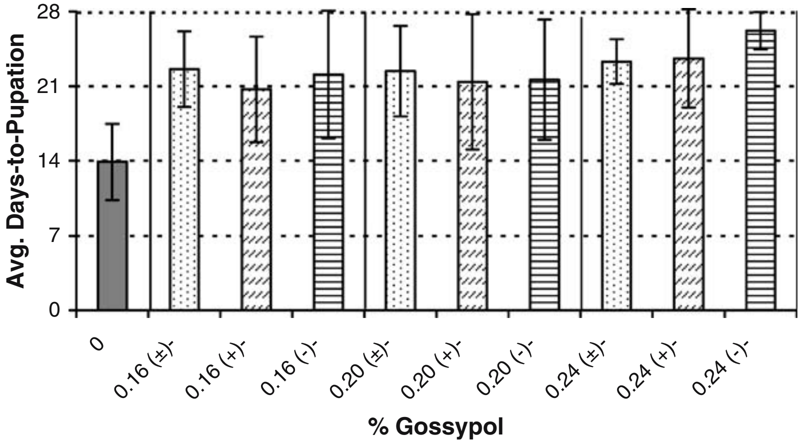


Fig. 3 Average days-to-pupation of *H. zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (−)-gossypol

was also moderate. No significant delays to pupation were noted among the three racemate levels. Furthermore, there were no significant differences in days-to-pupation between the racemate and the (+)-gossypol or (−)-gossypol diets or between the (+)-gossypol and (−)-gossypol diets at the same levels of gossypol.

Pupal Weights

There was no large statistical difference in pupal weights between the diet alone and the diet plus Alphacel (data not shown). Pupae from larvae raised on all diets

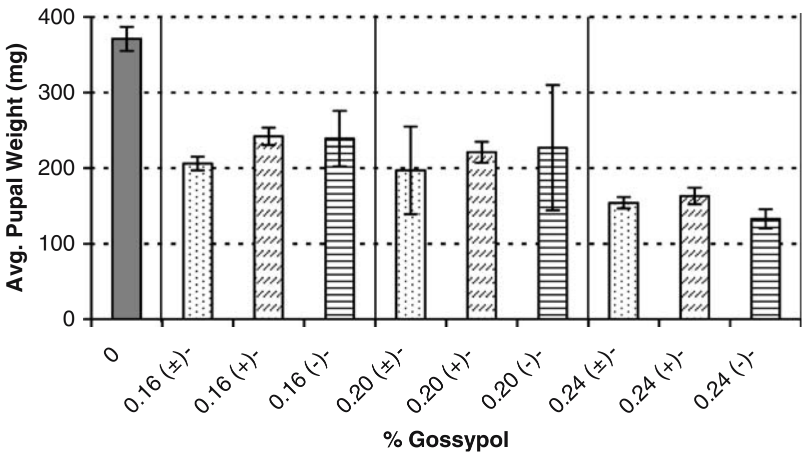


Fig. 4 Average pupal weights (mg) of *H. zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (−)-gossypol

containing gossypol were smaller than those raised on the diet plus Alphacel (Fig. 4). Statistical differences in pupal weights were observed when comparing (±)- or (+)- or (−)-gossypol under our *a priori* criteria. For the (±)-gossypol diets, pupae from larvae raised on the 0.24% diet weighed less than those raised on the 0.16% diet ($P = 0.1$); no difference was found when the other racemate diets were compared. Pupae weighed less for larvae raised on the 0.24% (−)-gossypol diet vs. the 0.20 and 0.16% (−)-gossypol diets ($P < 0.001$). Pupae from larvae raised on the (+)-gossypol diets were different at all three concentrations ($P < 0.1$). There were no significant differences in pupal weights between the racemate and the (+)-gossypol or (−)-gossypol diets at the same levels of gossypol except at the 0.16% level where pupae weights were less for the racemate vs. (+)- or (−)-gossypol ($P = 0.06$ and $P = 0.09$, respectively). Additionally, there were no significant differences between the (+)-gossypol and (−)-gossypol diets at the same levels.

Discussion

These results show that racemic, (+)-, and (−)-gossypol were comparable at reducing survival, days-to-pupation, and pupal weight. One exception was in the survival study where the 0.16% racemic gossypol diet reduced larval survival more effectively than did either of the individual enantiomers at that level. In addition, (−)-gossypol was more effective in extending the days needed to reach pupation at the 0.24% level than either (+)-gossypol or the racemate. No large significant differences in pupal weights were observed when racemic, (+)-, or (−)-gossypol were compared at the same levels.

These results are somewhat different from those reported by Yang et al. (1999) for *H. armigera*, who, in their artificial diet study, found that larvae matured more slowly, and the percent survival was lower when (+)-gossypol was incorporated into the diet as compared with (−)-gossypol. Yang et al. did not include (±)-gossypol in their study.

Others have reported enhanced biological activity of (−)-gossypol in a variety of enzyme, cell, and whole animal studies (Matlin et al., 1985; Lindberg et al., 1987; Benz et al., 1990; Sang et al., 1991; Gonzalez-Garza et al., 1993; Li and He, 1993; Lin et al., 1993; Blackstaffe et al., 1997; Shelley et al., 1999). Therefore, one might anticipate that the (−)-enantiomer would be a more potent inhibitor of insect herbivory. However, we did not find meaningful differences between the enantiomers or between the enantiomers and the racemate in this study. Thus, these results are consistent with the theory that cotton plants with high levels of either (+)- or (−)-gossypol in the foliage will not be substantially more susceptible to generalist insect herbivores than cotton plants with ratios of (+)- to (−)-gossypol that vary between 3:2 and 2:3. However, these results are based on the generalist herbivore *H. zea*, and an additional study on at least one other species that utilizes cotton as a host will be conducted. Ultimately, validation of this hypothesis awaits field studies.

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