Characterization of Soybean Looper (Lepidoptera: Noctuidae) Tolerance to Bollgard® Cotton: Implications for Resistance Management[1]

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Abstract The soybean looper, *Pseudoplusia includens* (Walker), is an occasional pest of cotton and an annual pest of soybean in the southern United States. The development of resistance by soybean looper to the *Bacillus thuringiensis* Berliner Cry1Ac protein in Bollgard® cotton could potentially influence the efficacy of foliar *B. thuringiensis* products in soybean. Soybean looper larvae and pupae collected from plots of Bollgard cotton weighed less than larvae and pupae collected from non-Bollgard cotton. Soybean loopers collected from non-Bollgard and Bollgard cotton were maintained separately in the laboratory. No differences were observed in the susceptibility of the subsequent generation (F_1) of soybean looper larvae from non-Bollgard and Bollgard cottons to Cry1Ac based on concentration-mortality data. Neonates from each of these colonies were allowed to complete development on non-treated and Cry1Ac-treated (1.0 µg/ml) meridic diet. Larval weights at 9 d and pupal weights were lower on Cry1Ac-treated diet than on non-treated diet. There were no apparent vigor differences in the two colonies based on development on non-treated diet. In addition, developmental times of larvae from both colonies were longer on Cry1Ac diet than on non-treated diet. These data indicate that development of soybean looper on Bollgard cotton has no effect on the tolerance of subsequent soybean looper generations to Cry1Ac.

Key Words *Pseudoplusia includens,* Bollgard, resistance management, *Bacillus thuringiensis*

The soybean looper, *Pseudoplusia includens* (Walker), occurs on late-season cotton, *Gossypium hirsutum* (L.), annually in the mid-southern and southeastern U.S., but populations rarely exceed economic levels. During the 2002 growing season, approximately 3% of the total cotton hectarage in the U.S. was treated for soybean looper, with Mississippi (10%) and Louisiana (42%) having the highest percentage of hectares treated (Williams 2003). Although it is a relatively minor pest of cotton, the soybean looper can be a significant pest of soybeans, *Glycine max* (Merrill), and vegetables in the southern U.S. (Martin et al. 1976). In Louisiana and Mississippi, foliar *Bacillus thuringiensis* Berliner products are recommended for control of soybean looper on soybean (Baldwin et al. 2003, Blaine et al. 2003). During the past 3 yrs, approximately 80% of the cotton in those states consisted of Bollgard® varieties that produce the Cry1Ac protein from *B. thuringiensis.* Expression of Cry1Ac in current Bollgard varieties is not high enough to provide acceptable control of soybean loopers

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(Stewart et al. 2001). Therefore, mid- to late-season populations of soybean loopers feeding on Bollgard cotton are exposed to sub-lethal concentrations of the Cry1Ac protein. Assuming that adequate genetic variation is present, exposure to these sub-lethal concentrations in Bollgard cotton could potentially result in subsequent populations of soybean loopers being more tolerant to Cry1Ac and other *B. thuringiensis* proteins, thus reducing the efficacy of foliar *B. thuringiensis* products in other crops. This, in turn, may increase the likelihood of soybean loopers developing resistance to Cry1Ac and other *B. thuringiensis* proteins. Foliar *B. thuringiensis* products historically have been an important component of integrated pest management in soybeans by effectively controlling economic infestations of soybean loopers without disturbing natural enemy complexes (Ignoffo et al. 1977), and are currently listed in the control guides for soybean looper control in Louisiana (Baldwin et al. 2003) and Mississippi (Blaine et al. 2003). Therefore, information about how Bollgard cotton influences the development of resistance to Cry1Ac in soybean looper is needed. This paper summarizes a series of field and laboratory experiments designed to investigate the effects of sub-lethal concentrations of the Cry1Ac protein from *B. thuringiensis* on subsequent generations of soybean looper.

Materials and Methods

Field plots. Two Bollgard (Monsanto Co., St. Louis, MO) cotton cultivars (Stoneville 4691B and Stoneville 4892BR, Stoneville Pedigreed Seed, Stoneville, MS) and two non-Bollgard cultivars (Stoneville 474 and Stoneville 4793R) were planted in large blocks near Stoneville, MS on 14 May 2002. Blocks consisted of 16 rows (1.0 m centers) x 30.5 m planted in a completely randomized design. Fertilization and other agronomic practices followed current Mississippi State University Cooperative Extension Service recommendations (McCarty 2002). To insure that the Bollgard varieties were expressing Cry1Ac, the amount of Cry1Ac in leaves from the field plots was quantified using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Envirologix, Inc., No. AP003, Portland, ME) as described in Adamczyk and Sumerford (2001). Leaves that contained slight feeding injury were sampled from the mid-section of plants where soybean loopers were actively feeding.

Field study. Soybean looper larvae were collected from plots of all cotton cultivars on three dates (6 to 8 September) using a standard (1 m^2) drop cloth. Larvae collected from each plot were placed into paper bags and transported to the laboratory. In the laboratory, 50 individuals were randomly selected from each bag and weighed using an analytical balance. In addition, pupae were collected from the abaxial surface of leaves from all cotton cultivars on three dates (11 to 13 September 2002). Pupae from each cultivar were transported to the laboratory and weighed. Preliminary analyses using analysis of variance indicated that there were no differences in larval weights ($F = 0.12$; df = 1, 98; $P = 0.73$) or pupal weights ($F = 2.65$; df = 1, 131; $P = 0.11$) on the two non-Bollgard cultivars (data not shown). Also, no differences were observed in larval weights ($F = 0.09$; df = 1, 98; $P = 0.76$) or pupal weights ($F = 0.46$; df = 1, 102; $P = 0.50$) on the two Bollgard cultivars (data not shown). Therefore, soybean loopers were combined into two colonies ("Non-Bollgard Colony" n = 133 and "Bollgard Colony" n = 104) depending on the type of cotton they were collected from. Mean weights of larvae and pupae collected from non-Bollgard and Bollgard cottons were compared with paired t-tests (PROC TTEST, SAS Institute 1998).

Laboratory studies. To determine if exposure of soybean looper to Bollgard cotton affected the subsequent generation, dose-mortality bioassays were conducted with neonates from each colony. A series of concentrations ranging from 1.0 to 5.0 ppm was used. The desired concentrations were derived by incorporating lyophilized powder of MVP II containing 19.7% Cry1Ac by weight into meridic diet (Thomas and Boethel 1993). Larval mortality was determined at 120 h. Dose-mortality curves were generated with probit analysis (PROC PROBIT, SAS Institute 1998) and LC_{50} values were compared based on overlap of 95% fiducial limits.

In addition to dose-mortality bioassays, two cohorts of neonates from each colony (non-Bollgard and Bollgard) were placed on Cry1Ac-treated (MVP II) meridic diet. A sub-lethal concentration (LC_{10}) of 1.0 µg Cry1Ac/mL of diet was selected based on dose-mortality response data. Also, this concentration resulted in similar development of soybean looper larvae to that observed with Bollgard cotton leaf tissue. To determine if health/vigor differences existed among the two colonies, each cohort of individuals was reared on non-Cry1Ac diet as well. Pupae were then weighed (minimum of 85/colony) and differences among colonies and diet were analyzed using analysis of variance (PROC MIXED, Littell et al. 1996).

To further investigate the effects of exposure to sub-lethal concentrations of Cry1Ac on the development of subsequent soybean looper generations, larvae originally collected from non-Bollgard cotton were reared on Cry1Ac-treated (1 µg/mL, MVP II) meridic diet until pupation. Pupae were separated into two cohorts based on developmental times to determine if their offspring would develop at different rates on Cry1Ac-treated diet. Soybean loopers that completed larval development in 15 to 16 d (n = 150) were termed the "SBL 1 Colony" and soybean loopers that completed larval development in 19 to 22 d (n = 148) were termed the "SBL 2 Colony." Soybean loopers that completed larval development in 17 to 18 d (n = 149) were excluded. Neonates from each colony were placed on non-treated and Cry1Ac-treated (1µg/mL) diet on each of 3-d (replications). Sixty larvae from each colony were placed on each dose per replicate. Larvae were allowed to complete development and time to pupation was recorded. Mean time to pupation was compared between the colonies and diet types with analysis of variance (PROC MIXED, Littell et al. 1996).

Results and Discussion

Field study. Soybean looper larvae collected from Bollgard cotton weighed less than larvae collected from non-Bollgard cotton ($t = -2.95$, df = 198, $P < 0.01$) (Fig. 1). Larval weights averaged (±SEM) 137.4 ± 9.2 mg on Bollgard cotton and 176.2 ± 9.3 mg on non-Bollgard cotton. These results corroborate previous data showing differences in weights of soybean looper larvae collected from Bollgard and non-Bollgard cottons (Sumerford and Solomon 2000). In that study, soybean looper larvae collected from Bollgard weighed 21.2 mg and larvae collected from non-Bollgard cotton weighed 189.6 mg. Although larval weights on non-Bollgard cotton are similar between the two studies, there appears to be some discrepancy in mean weights of larvae collected from Bollgard cotton between the two studies. There are several possible explanations for these differences. Expression of the Cry1Ac protein varies among different cotton varieties (Adamczyk and Sumerford 2001). The Bollgard variety (Deltapine NuCOTN 33B) used by Sumerford and Solomon (2000) produces approximately 2X more of the Cry1Ac protein than the Bollgard varieties (Stoneville 4691B and Stoneville 4892BR) used in the current study (Adamczyk and Sumerford

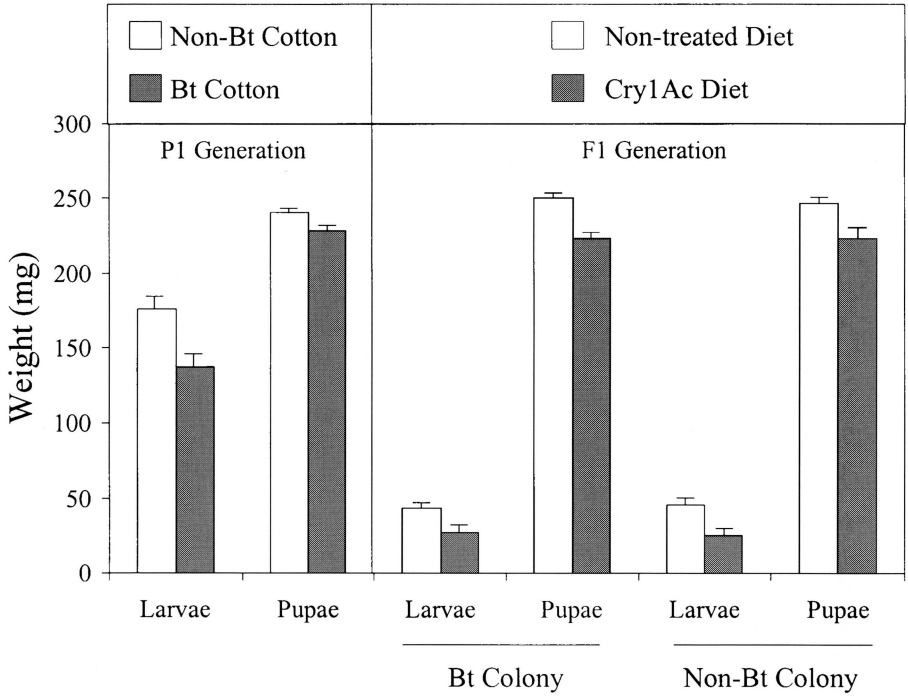

Fig. 1. Larval and pupal weights (+SEM) of soybean looper, *P. includens,* on non-Bollgard cotton, Bollgard cotton, non-treated diet, and Cry1Ac-treated diet during the P1 (field) and F1 (laboratory) generations.

2001). Therefore, the differences in larval weights between the two studies may be partially explained by differences in expression among the different varieties. Furthermore, previous research has shown that soybean looper mortality and time to pupation varies among commercial Bollgard cottons (Clemens 2000). Also, in the study reported by Sumerford and Solomon (2000), soybean looper larvae were collected from the Bollgard plots during early July; whereas, larvae were collected during early September in the current study. This also may have influenced soybean looper development because overall expression of the Cry1Ac protein decreases in plants as the season progresses and insect performance is negatively correlated with protein expression (Greenplate 1999, Adamczyk et al. 2001). Based on ELISA results, Cry1Ac expression averaged (±SEM) 1.6 ± 0.3 ppm for Stoneville 4691B and 1.7 ± 0.2 ppm on Stoneville 4892BR in the current study. Another possible explanation for differences in the two studies is that previous selection of soybean loopers on Bollgard cotton has led to increased tolerance to Cry1Ac since Sumerford and Solomon (2000). However, these data do not provide adequate information to support this. Future experiments should be designed to determine if there has been a shift in soybean looper tolerance to Cry1Ac.

Soybean looper pupae collected from Bollgard cotton weighed less than pupae collected from non-Bollgard cotton (t = −3.44, df = 235, P < 0.01) (Fig. 1). Pupal

weights averaged (±SEM) 228.8 ± 2.4 mg from Bollgard cotton and 240.4 ± 2.3 mg on non-Bollgard cotton. Clemens (2000) reared soybean loopers on foliage from non-Bollgard and Bollgard cottons grown in the greenhouse and found differences in pupal weights between the two cotton types. Although weights of soybean looper pupae were different between Bollgard and non-Bollgard cotton in the current study, the differences in weights were not as great as those observed with larvae. Soybean looper larvae collected from Bollgard cotton weighed 22% less than larvae collected from non-Bollgard cotton; whereas, pupae collected from Bollgard cotton only weighed 5% less than pupae collected from non-Bollgard cotton. Therefore, the differences in larval weights may be partially explained by slower development on Bollgard cotton.

Laboratory studies. Exposure to Bollgard cotton did not have an apparent affect on tolerance of the subsequent generation of soybean loopers to Cry1Ac. Dose-mortality data indicated no differences in tolerance of soybean loopers to Cry1Ac between the Bollgard (LC_{50} = 3.59 [3.02-4.35], χ^2 = 2.41, df = 4) and non-Bollgard (LC_{50} = 3.08 [2.57-3.65], χ^2 = 2.85, df = 4) colonies.

For soybean loopers reared on non-treated and Cry1Ac-treated meridic diet, there was an effect for Cry1Ac concentration on larval weight (F = 40.65; df = 1, 8; P < 0.01) (Fig. 1). However, there was not a significant colony effect (F < 0.01; df = 1, 8; P = 0.95) or a colony x Cry1Ac concentration interaction (F = 0.51; df = 1, 8; P = 0.50) suggesting that the Cry1Ac in the Bollgard cotton did not adversely affect the subsequent generation of soybean loopers. Larval weights averaged (±SEM) 44.7 ± 1.5 mg on non-treated diet and 26.5 ± 2.2 mg on Cry1Ac-treated (1 µg/mL) meridic diet, regardless of colony (non-Bollgard vs Bollgard). Similarly, weights of soybean looper pupae were different between non-treated and Cry1Ac-treated diet regardless of colony (F = 35.21; df = 1, 10; P < 0.01) (Fig. 1). Also, there was not a significant colony effect (F = 0.18; df = 1, 10; P = 0.68) or colony by Cry1Ac concentration interaction (F = 0.16; df = 1, 10; P = 0.70), again suggesting that the Cry1Ac in Bollgard did not affect the subsequent generation. Soybean looper pupae weighed (mean ± SEM) 248.7 ± 2.1 mg on non-treated meridic diet and 223.1 ± 3.3 mg on

Table 1. Developmental times of the F1 generation of soybean loopers; *P. includens,* from larval eclosion to pupation on non-treated and Cry1Ac-treated (MVP II) meridic diet in the laboratory. Means within a row followed by the same lower case letter and within a column followed by the same upper case letter are not significantly different (Fisher's Protected LSD, α = 0.05)

	Days to Pupation (±SEM)			
Concentration (µg/mL)	Bt colony	Non-Bt colony	Mean (±SEM)	$P > F$
0	16.5 (0.3)	16.5 (0.1)	16.5 (0.1)B	—
1	19.4 (0.4)	18.4 (0.6)	18.9 (0.4)A	—
Mean (±SEM)	17.9 (0.6)a	17.4 (0.5)a	—	0.24
$P > F$	—	—	<0.01	

Cry1Ac-treated meridic diet, regardless of colony. Similar to the field study, differences in pupal weights on non-treated and Cry1Ac treated diet were not as great as the differences in larval weights on non-treated and Cry1Ac diet, again suggesting that Cry1Ac may slow soybean looper development. Soybean looper larvae reared on Cry1Ac-treated diet weighed approximately 40% less than those reared on non-treated diet; while, pupae weighed approximately 10% less on Cry1Ac-treated diet.

To further support the previous findings, experiments investigating developmental times of soybean looper on Cry1Ac-treated diet showed no effect on the subsequent generation (Tables 1 and 2). There were no differences in the development of F1 soybean loopers from Bollgard and non-Bollgard cottons ($F = 1.55$; df = 1, 10; $P = 0.24$) (Table 1). For the F2 generation, there was no effect from soybean looper colony (SBL 1 vs SBL 2; $F = 0.24$; df = 1, 6; $P = 0.64$) or no colony by Cry1Ac concentration interaction ($F = 2.82$; df = 1, 6; $P = 0.14$) (Table 2). There was, however, an effect of Cry1Ac concentration on soybean looper development ($F = 12.61$; df = 1, 6; $P = 0.01$). Soybean loopers completed larval development in (mean ± SEM) 17.4 ± 0.4 d on Cry1Ac-treated diet compared to 15.7 ± 0.1 d on non-treated diet regardless of colony (SBL 1 vs SBL 2). In addition, these data support the assumption that differences in developmental times of soybean looper larvae between Bollgard and non-Bollgard cottons partially explains differences in larval weights on Bollgard and non-Bollgard cottons.

Previous research has shown that pink bollworm, *Pectinophora gossypiella* (Saunders), develops slower on Bollgard cotton than on non-Bollgard cotton (Liu et al. 1999). Those authors suggest that delayed development of pink bollworm on Bollgard cotton may accelerate the development of resistance because of asynchronous emergence of moths from non-Bollgard and Bollgard cottons. This may not necessarily be the case for soybean looper, because soybean looper has a wide host range (Turnipseed and Kogan 1976) and moths emerging from other hosts may be synchronous with moths emerging from Bollgard cotton, but no data are available to fully support this theory.

In the study presented by Liu et al. (1999), a laboratory strain of pink bollworm selected for resistance to Cry1Ac was used. Results of our study suggest that al-

Table 2. Developmental times of the F2 generation of soybean loopers, *P. includens,* from larval eclosion to pupation on non-treated and Cry1Ac-treated (MVP II) meridic diet in the laboratory. Means within a row followed by the same lower case letter and within a column followed by the same upper case letter are not significantly different (Fisher's Protected LSD, $\alpha = 0.05$)

	Days to Pupation (±SEM)			
Concentration (µg/mL)	SBL 1 Colony	SBL 2 Colony	Mean (±SEM)	$P > F$
0	16.0 (0.1)	15.6 (0.1)	15.7 (0.1)B	—
1	16.8 (0.7)	17.7 (0.5)	17.4 (0.4)A	—
Mean (±SEM)	16.4 (0.4)a	16.6 (0.5)a	—	0.64
$P > F$	—	—	0.01	

though development of soybean looper is slower on Cry1Ac-treated diet than on non-treated diet, there are no differences in the tolerance of soybean looper collected from non-Bollgard and Bollgard cotton to Cry1Ac. Therefore, based on results of this experiment, asynchronous emergence may not have the same level of effect on the development of soybean looper resistance to Bollgard cotton as would be expected with pink bollworm. Although some Bollgard cultivars produce higher levels of Cry1Ac than others (Adamczyk and Sumerford 2001), soybean loopers are more tolerant to the Cry1Ac protein at the levels in current Bollgard cottons than pink bollworm (Perlak et al. 1990). Therefore, the selection pressure from a single generation in Bollgard cotton is much lower for soybean looper than pink bollworm. However, these results should be interpreted cautiously because of temporal variation in Cry1Ac expression during the season (Adamczyk et al. 2001). During the early to mid parts of the flowering period (July and early August), Bollgard cotton may provide more selection pressure on soybean looper than what was observed with the current study in September.

Finally, the new lines of transgenic cottons are currently available (Bollgard II, Monsanto Co.) or being developed (Widestrike, Dow Agrosciences and VipCot, Syngenta Crop Protection) for commercial production. Those cottons produce two *B. thuringiensis* proteins and provide better control of soybean looper; thus, providing a higher selection pressure for the development of resistance in this pest. Therefore, future studies should begin evaluating the relative susceptibility of soybean loopers emerging from dual-protein transgenic cottons to multiple *B. thuringiensis* proteins.

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