CRY1AC LEVELS IN CANDIDATE COMMERCIAL BOLLGARD® VARIETIES AS INFLUENCED BY ENVIRONMENT, VARIETY, AND PLANT AGE: 1999 GENE EQUIVALENCY FIELD STUDIES

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Abstract

A field study evaluating 35 candidate commercial Bollgard® varieties at 11 different field sites showed that Cry1Ac levels (as measured in a sensitive quantitative bioassay) in various tissue types were influenced by field site, sampling time and variety. There was no measurable site by variety contribution to variability in Cry1Ac levels, suggesting that relative varietal differences were independent of site or environment. Based upon the reported data, it was suggested that differences in field efficacy for less sensitive species like fall armyworm and cotton bollworm, if they appear, are likely functions of differences in terminal/foliar levels of Cry1Ac as influenced by plant age, field site or varietal background. Fruiting structures (squares and bolls), the most attractive targets for attack by key pests, generally contained lower levels of Cry1Ac than did terminals. For fruiting structures, field site was a much larger contributor to overall variability than varietal background. For squares, specifically, plant age (tissue sampling time) was also a much larger contributor to Cry1Ac variability than varietal background. Significant differences in field efficacy due to varietal differences in fruiting structure Cry1Ac levels were considered unlikely. Current testing protocols require that all candidate Bollgard® varieties meet minimum Cry1Ac concentration standards in multi-site field studies as measured against square Cry1Ac levels found in Monsanto's original EPA-approved 531 event in the Coker 312 background. Insect resistance management implications were discussed.

Experimental Protocol

During the 1999 growing season, 35 candidate commercial Bollgard® varieties were tested at 11 field sites for levels of Cry1Ac over time. A quantitative bioassay (Greenplate, 1999) was used to estimate Cry1Ac levels in specific cotton tissues (terminals, pre-candle squares; young bolls). At each site, samples were collected beginning at 2 weeks post pinhead square; the first sampling time was determined by the development of the latest maturing variety. From that point, tissue samples were collected at 2-week intervals until 10 weeks post pinhead square. Analyses of variance were performed; the JMP® (version 3.1) statistical software (SAS Institute, Cary NC) was used to perform the statistical evaluations on Cry1Ac levels. Previous work (Greenplate, 1999) showed that tissue type was a significant contributor to variability, so, for the evaluation of 1999 data, a separate ANOVA was performed for each tissue type. Based upon initial data analysis, certain *a posteriori* mean comparisons were made using the Tukey-Kramer HSD test at P = 0.05 (Kramer, 1956).

Results

For terminals and squares, the main effects of field site, sampling time, and variety contributed significantly to variability. For terminals (Table 1), sampling time was the largest source of variability with site and variety as lesser sources. When squares were considered (Table 2), both sampling time and site were larger sources of variability than variety. The ANOVA for bolls (Table 3) did not include sampling time as a variable since missing samples (due to differential maturation rates at various sites) caused missing degrees of freedom. A boll ANOVA evaluating site and variety (Table 3) showed field site to be a larger source of variability for Cry1Ac levels than was variety. Figure 1 and Figure 2 illustrate the differences among sites and sampling times, respectively, for mean Cry1Ac levels in the individual tissue types. Figure 3 shows the relationship among varieties for levels of Cry1Ac in squares. Even though varietal background contributed significantly to variability among square Cry1Ac concentrations (Table 2), the seasonal square means for all varieties were statistically equivalent to or higher than values for the original EPA-approved standard Coker 312/531 (Tukey-Kramer HSD; P = 0.05). An interesting result for all three tissue types was that there were no apparent site-by-variety interactions (Table1; Table 2; Table 3). These results strongly suggest that varietal differences, when they do occur, remain somewhat constant across different environments.

Further evaluation of varietal differences in Cry1Ac suggested an influence of cotton maturity type. For the purposes of this unplanned, or *a posteriori,* comparison, varieties were divided into short-season (early maturing) and long-season (late maturing) based upon seed company designation. Seasonal means for short-season and long-season varieties were compared within each tissue type using the Tukey-Kramer HSD (P = 0.05). Long-season varieties tended to show higher seasonal mean levels of Cry1Ac than short-season varieties; means were significantly higher for long-season varieties in terminals and squares; means were statistically similar in bolls (Figure 4). Figure 5 shows mean Cry1Ac levels for long- and short-season terminals over time. Logarithmic trend-lines provided the best fit to the seasonal Cry1Ac level decline. Short- and long-season terminal means exhibited similar rates of decline in Cry1Ac levels (lines were parallel), but the long-season mean Cry1Ac level was always higher. Considering the differing phenologies of long- and short-season varieties, it is possible that the observed trend simply reflected the same decline in long-season varieties as in short-season varieties, but that the decline was delayed by 2-4 weeks because of the more rapid maturation and earlier cut-out (cessation of fruit production) of short-season varieties. Figure 6 shows the best-fit logarithmic trend-lines for terminals of individual long-and short-season varieties over time. Long season varieties were more variable and contained varieties that looked very much like short-season varieties, even though long-season means were higher (Figure 5). Figures 7 and 7 show values for Cry1Ac in squares of long- and short-season varieties; Figure 7 shows mean values for all varieties, while Figure 8 illustrates individual varieties. A similar relationship to that found in terminals was also seen in squares, even though long-season and short-season differences were smaller. When bolls were evaluated in a similar manner, it was difficult to separate long- and short-season varieties (Figure 9; Figure 10).

Discussion

In spite of the presence of statistically measurable differences in Cry1Ac levels among varieties, there are little data to suggest that measurable varietal differences in field efficacy against target pests are likely to occur. In a laboratory study, Adamczyck *et al* (2000) reported differential weights and survival of fall armyworm and cotton bollworm larvae when fed leaves from two different Bollgard® varieties with measurable (*via* ELISA) differences in Cry1Ac levels. Differences in boll or square efficacy were not reported. Absent has been solid field evidence supporting varietal differences in terms of efficacy against target pests. Both the work reported here and that of Adamczyck *et al* (2000) support the possibility of differences among varieties when it comes to attack by lepidopteran species whose sensitivity to Cry1Ac is significantly less than that of key target pests such as tobacco budworm and pink bollworm. In the present study, varietal differences in Cry1Ac levels were greatest in terminal foliar tissue. Furthermore, fall armyworm is relatively insensitive to Cry1Ac and not considered a target pest of Bollgard®. Cotton bollworm populations, although generally well controlled by Bollgard®, can potentially vary some 300-fold in sensitivity to Cry1Ac (Stone and Sims, 1993; Luttrel, *et al* 1999); survival of this species in Bollgard® is reported on a regular basis (Greenplate *et al* 1998).

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Levels of Cry1Ac in squares and bolls have been found to be lower and less variable than those in terminal foliage (Greenplate, 1999, Adamczyck *et al* 2000) and the present study showed them to be less influenced by variety than by either sampling time or site. In the evaluation of candidate Bollgard® varieties, levels of Cry1Ac must meet minimum standards as measured against the original EPA-approved genetic transformation event 531 in Monsanto's initial transformed variety, Coker 312. Current commercial varieties, derived from Coker 312/531, have been suggested by Gould (1998) as meeting high-dose IRM (insect resistance management) standards against tobacco budworm and pink bollworm; this assumes full control of heterozygotes where the resistance allele is at least partially recessive (Gould *et al*, 1997). In commercial gene equivalency evaluations, seasonal mean Cry1Ac levels of squares (the most vulnerable tissue type and also indicative of boll Cry1Ac production) in candidate varieties must be equivalent or higher than those found in concurrently grown Coker 312/531. This ensures that Cry1Ac levels in commercial Bollgard® varieties, while potentially variable and certainly influenced by factors such as environment (field site) and age of the plant, will meet established standards. Since current testing protocols require that minimum high-dose standards are met, it is not likely that the proliferation of Bollgard® varieties will significantly influence IRM considerations for key target pests.

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References

Adamczyck, J. J., Jr., Adams, L. C., and D. D. Hardee. 2000. Quantification of Cry1Ac δ-endotoxin in transgenic Bt cotton: correlating insect survival to different protein levels among plant parts and varieties. *In* Proceedings of the Beltwide Cotton Conference Volume 2: 929-932 (2000).

Greenplate, J. T. 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard® cotton fruit and terminals. J. Econ. Entomol. 92: 1377-1383.

Greenplate, J. T., Head, G. P., Penn, S. R., and V. T. Kabuye. 1998. Factors potentially influencing the survival of *Helicoverpa zea* on Bollgard® cotton. *In* Proceedings of the Beltwide Cotton Conference Volume 2: 1030-1033 (1998).

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Ann. Rev. Entomol. 42: 701-726.

Gould, F., Anderson, A., Jones, A., Sumerford, D., Heckel, G. G., Lopez, J., Micinski, S., Leonard, R., and M. Laster. 1997. Initial frequency of alleles for resistance to Bacillus thuringiensis toxins in field populations of Heliothis virescens. Proc. Natl. Acad. Sci. USA 94: 3519-3523.

Kramer, C. Y. 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12: 309-310.

Luttrel, R. G., Wan, L., and K. Knighten. 1999. Variation in susceptibility of Noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol. 92: 21-32.

Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J. Econ. Entomol. 86: 989-994.

Table 1. ANOVA effects table showing contribution from various sources to variability for Cry1Ac levels in terminals.

Summary of Fit	
RSquare	0.645889
RSquare Adj	0.518477
Root Mean Square Error	11.82801
Mean of Response	25.91818
Observations (or Sum Wgts)	1713

Effect Test					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
Site	10	10	18882.77	13.4972	<.0001
Sampling time	1	1	52468.411	375.0376	<.0001
Site*Sampling time	10	10	20303.68	14.5128	<.0001
Variety	36	36	75034.237	14.8982	<.0001
Site*Variety	360	360	46330.705	0.9199	0.8325
Sampling time*Variety	36	36	10270.315	2.0392	0.0003

Table 2. ANOVA effects table showing contribution from various sources to variability for Cry1Ac levels in squares.

Summary of Fit	
RSquare	0.588182
RSquare Adj	0.415447
Root Mean Square Error	9.669687
Mean of Response	17.87251
Observations (or Sum Wgts)	1534

Effect Test					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
Site	10	10	37638.463	40.2538	<.0001
Sampling time	1	1	23450.216	250.7968	<.0001
Site*Sampling time	10	10	15468.671	16.5435	<.0001
Variety	36	36	14712.901	4.3709	<.0001
Site*Variety	360	360	23226.704	0.69	1
Sampling time*Variety	36	36	3051.145	0.9064	0.6286

Table 3- ANOVA effects table showing contribution from various sources to variability for Cry1Ac levels in bolls.

Summary of Fit	
RSquare	0.491873
RSquare Adj	0.184507
Root Mean Square Error	9.124432
Mean of Response	16.66102
Observations (or Sum Wgts)	1049

Effect Test					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
Site	10	10	9770.159	11.7352	<.0001
Variety	35	35	15439.589	5.2985	<.0001
Site*Variety	350	350	24788.873	0.8507	0.9555

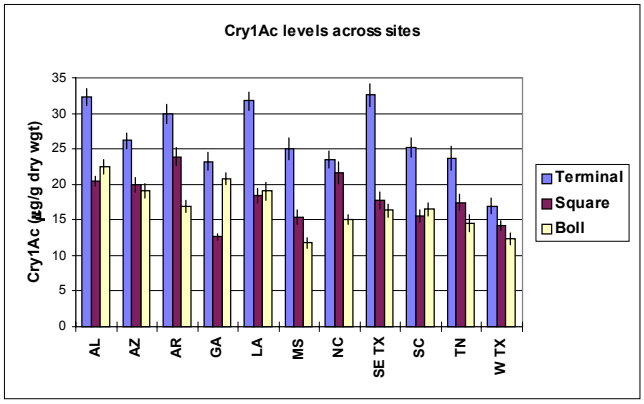


Figure 1. Seasonal means for Cry1Ac levels in individual tissue types at specific field sites.

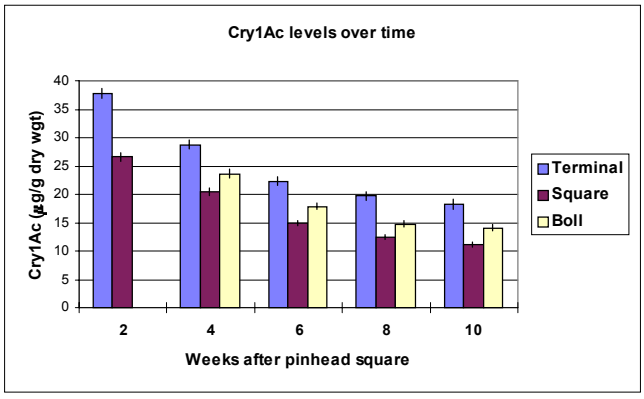


Figure 2. Seasonal means for Cry1Ac levels in individual tissue types at specific sampling times.

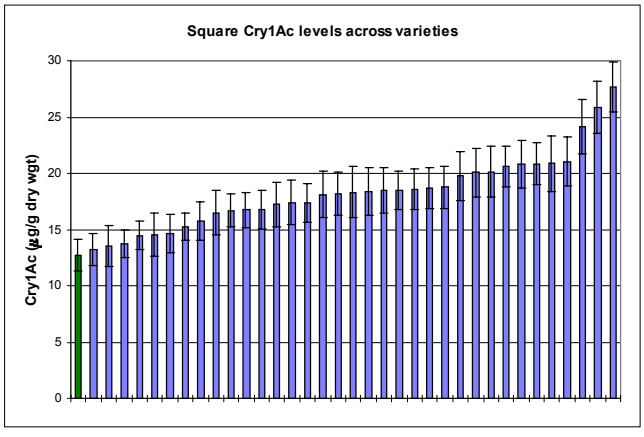


Figure 3. Season means for Cry1Ac levels in squares for specific varieties. Coker 312/531 (control variety) is located at far left. Seasonal means for all other varieties were statistically similar or higher than Coker 312/531 as measured by Tukey-Kramer HSD at P = 0.05.

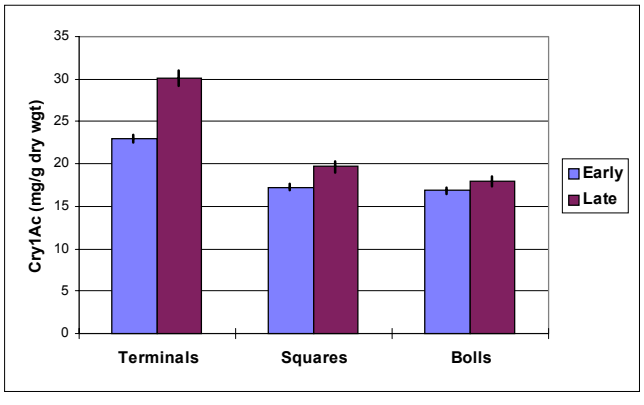


Figure 4. Seasonal mean Cry1Ac levels for specific tissues of long-season (Late) and short-season (Early) Bollgard® varieties. Means for short- and long-season varieties were statistically different in terminals and squares, but similar in bolls as evaluated by Tukey-Kramer HSD (P = 0.05).

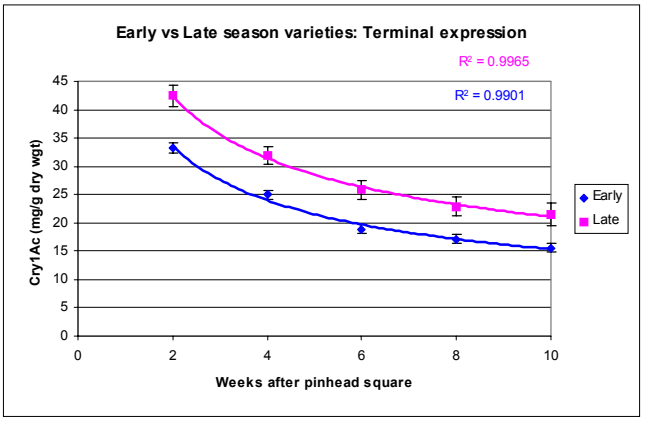
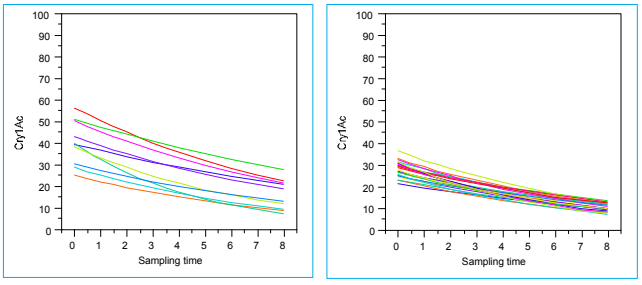


Figure 5. Cry1Ac levels in terminals over time for long-season (Late) and short-season (Early) varieties.



Long Season Short Season

Figure 6. Cry1Ac levels in terminals over time for individual long-season and short-season varieties. Sampling time was measured in weeks (0 sampling time was 2 weeks post pinhead square).

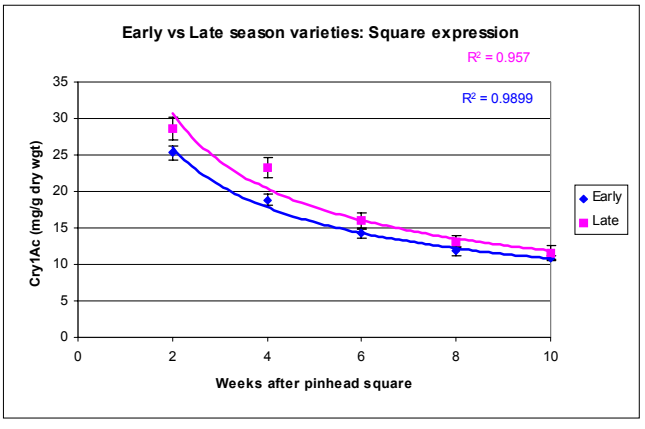
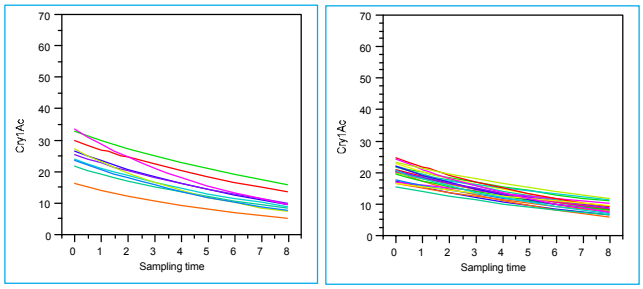


Figure 7. Cry1Ac levels in squares over time for long-season (Late) and short-season (Early) varieties.



Long Season Short Season

Figure 8. Cry1Ac levels in squares over time for individual long-season and short-season varieties. Sampling time was measured in weeks (0 sampling time was 2 weeks post pinhead square).

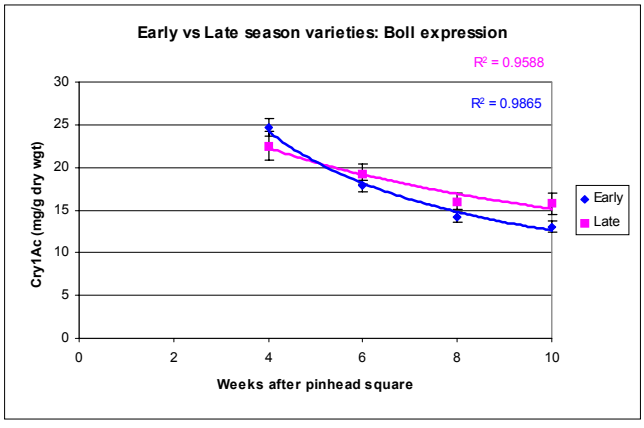
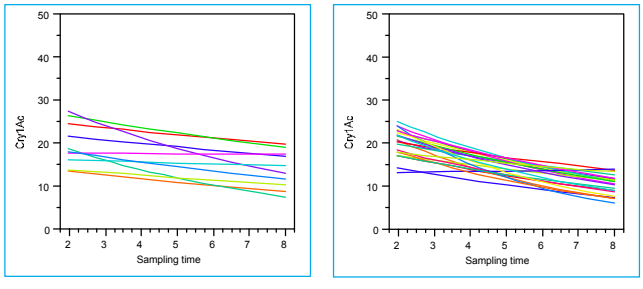


Figure 9. Cry1Ac levels in bolls over time for long-season (Late) and short-season (Early) varieties.



Long Season Short Season

Figure 10. Cry1Ac levels in bolls over time for individual long-season and short-season varieties. Sampling time was measured in weeks (0 sampling time was 2 weeks post pinhead square).