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QUANTIFICATION OF TOXIN LEVELS IN COTTONS EXPRESSING ONE AND TWO INSECTICIDAL PROTEINS OF *BACILLUS THURINGIENSIS*

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Abstract

Laboratory studies were conducted using ELISA techniques to quantify toxin levels among plant parts of cotton expressing one and two insecticidal proteins *of Bacillus thuringiensis*. For Cry1Ac, consistent patterns were seen for both 2000 and 2001 among all plant parts. The highest concentrations were seen in lower canopy leaves, at 3.6 ppm. Terminal leaves contained 2.4 ppm for Cry1Ac. The lowest concentration of Cry1Ac in both single and dual-toxin varieties was found in internal boll contents, at 1.5 ppm. Pink flower parts contained less toxin than white flower parts, indicating a dramatic reduction in toxin levels over a period of 1-2 d. For Cry2Ab, a different pattern of expression among plant parts was noted between 2000 and 2001. For tissue collected in 2000, Cry2 toxin levels were higher in old leaves compared to terminal leaves (as with Cry1Ac), but the highest concentration of all plant parts was seen in white stamens (26.2 ppm). The lowest concentration of Cry2 in 2000 was found in 10-14 day-old boll walls (10.0 ppm). As in 2000, lower canopy leaves contained more Cry2 toxin in 2001 tissue than did terminal leaf tissue. Additionally, lower canopy leaves contained the highest concentration of toxin among all plant parts in 2001. The lowest concentration of Cry2 in 2001 plant tissue was seen in pink petals (5.4 ppm). Except for petal tissue collected in 2000, flower parts exhibited the same pattern for Cry2Ab as with Cry1Ac, regarding the reduction of toxin expression during the transformation period from fresh white flower to aging pink flower.

Introduction

Since the introduction of Bollgard™ cotton (Monsanto Co., St. Louis, MO) in 1996, growers have benefited from the use of this technology for the season-long control of certain lepidopteran pests. This transgenic cotton, genetically modified to express the Cry1Ac toxin from *B. thuringiensis* (*Bt*), has been advantageous to the grower by reducing foliar insecticide treatments, increasing crop yields, and preserving beneficial arthropod populations. Although it is effective against certain noteworthy pests (e.g.-- tobacco budworm, pink bollworm), some caterpillars are not fully controlled with this technology; thus supplemental insecticide applications have been necessary to control these occasional pests (e.g.--armyworms, loopers) and bollworms under conditions of high population densities.

As of late, cotton expressing two insecticidal proteins of *B. thuringiensis* has been developed (Bollgard™ II--Monsanto Co., St. Louis, MO). This transgenic cotton expresses not only the Cry1Ac toxin in the original *Bt* cotton, but also an additional toxin, resulting from the insertion of a second gene (*cry2Ab*). This novel dual-toxin technology will likely serve as a tool for resistance management and provide better control of armyworms and loopers, as well as additional control of bollworms.

The bollworm is generally less sensitive to *Bt* toxins than is the budworm (Stone and Sims 1993). Adamczyk et al. (2001) reported differential *Bt* toxin levels between various plant parts in Bollgard cotton. Non-uniform Cry1Ac expression in blooms (particularly pollen) of Bollgard cotton has also been noted (Greenplate et al. 1998). Furthermore, bollworms have shown the ability to detect *Bt* toxins in plant tissue and move to seek out a lower toxin-expressing food source (e.g., *Bt* cotton blooms, another plant) (Akin et al. 2000).

Quantifying levels of both Cry1Ac and Cry2Ab in Bollgard II cotton will enable us to determine possible advantages stemming from the addition of the second toxin. Although Cry1Ac may be expressed at low levels in blooms of Bollgard II cotton, significant levels of Cry2 may be adequate for control of bollworms.

Materials and Methods

Plant tissue was collected from 16-18 node cotton near peak bloom at three locations in Mississippi – Starkville (Plant Science Research Center) and Stoneville (USDA Southern Insect Management Research Unit) for both 2000 and 2001, and an additional location in Shelby (Rives Neblett Farm) in 2001 (Figure 1). Single-toxin cultivars used were DP50B and NuCOTN33B in 2000 and 2001, respectively. For dual-toxin varieties, MON15985 and NuCOTN 33BII were used in 2000

and 2001, respectively. In 2000, planting dates were May 16 for Starkville and 23 May for Stoneville. Dates of tissue collection were July 19 and August 4 for Stoneville and Starkville, respectively. In 2001, planting dates were April 26 for Stoneville and Starkville, and April 27 for Shelby. Collection dates for these locations were July 13 for Stoneville, July 18 for Starkville, and August 2 for Shelby. Plant parts were collected equally from all replicates of field plots, and grouped by location across replicates. Plant parts collected were terminal leaves, lower canopy leaves, young squares (ca. 1/3 grown), white flower petals, stamens from white flowers, pink flower petals (pink coloration indicates that flowers are 1 d old), stamens from pink flowers, 1-3 day-old bolls, and 10-14 day-old bolls. For 10-14 d-old bolls, boll walls were separated from internal boll contents. Plant tissue except for internal boll contents and stamens were washed and all parts were stored in -80 C until lyophilized. All plant parts were freeze-dried using a Genesis 25LL lyophilizer (Virtis Company, Inc., Gardiner, NY 12525).

Plant tissue was homogenized, crushed into a fine powder using mortar and pestle, and stored in –20 C, until ELISA assays were performed. Tissue was then subjected to a "sandwich" enzyme-linked immunosorbent assay (Envirologix, Inc., Portland, ME 04103), as described by Adamczyk et al. (2000). This kit uses a color development step to which intensity of color is proportional to toxin concentration in the sample extract. 20 mg tissue was used for each sample. Absorbences were read using a Thermomax®microplate reader (Molecular Devices, Inc., Sunnyvale, CA, 94089), then converted to concentrations with SOFTmax Pro®2.1.1 computer software (Molecular Devices, Inc.).

Experiments were analyzed as completely randomized design, with three replications per plant part per location, and a Proc GLM mean separation using Fisher's protected LSD (SAS Institute, 1998).

Results and Discussion

For Cry1Ac (in both single and dual-toxin varieties), results were similar for both 2000 and 2001 plant tissue collected. Old leaves contained higher levels of toxin than all other plant parts (3.6 parts per million, ppm) (Figure 2). Internal boll contents of 10-14 d-old bolls had lower levels of Cry1Ac than all other plant parts (1.45 ppm). Comparison of petal tissue shows that white petals had higher concentrations of Cry1Ac than did pink petals (3.06 vs. 2.36 ppm). Similar results were noted with white stamens (2.67 ppm) and pink stamens (2.19 ppm). The apparent reduction of toxin levels in flower parts over a short period of time may provide a low-toxin food source that may allow survival of lepidopteran pests.

For dual toxin varieties, Cry2Ab levels differed among plant parts for both 2000 and 2001 (Figure 3). As with Cry1, lower canopy leaves expressed toxin at higher levels than terminal leaves. In 2000, petal tissue did not exhibit a significant change in toxin levels during aging from white to pink flowers, but toxin levels were different between pink and white petals in 2001. Toxin levels in white and pink stamenal tissue also differed, as was the case with Cry1 toxin levels.

Cry1Ac levels between single and dual-toxin varieties were also compared across all plant parts (Table 1). Cry1Ac concentration was 2.57 $\pm$0.10 ppm for Bollgard cotton varieties and 2.33 $\pm$0.09 ppm for Bollgard II plant tissue ($p<0.05$). This indicates that the insertion of the *cry*2 gene had little effect on Cry1 toxin expression.

Comparing levels of Cry2 to Cry1 across all plant parts, Cry2 levels were ca. five-fold higher than Cry1 across both years (Table 3). A regression analysis was performed to determine if there was correlation between Cry1 levels and Cry2 levels across all plant parts, and found that there was little relationship between Cry1 and Cry2 concentration ($r^2 = 0.0044$, $p=0.4252$) (Proc REG; SAS Institute, 1998). For tissue collected in 2000, Cry2Ab levels were nine-fold more than that of Cry1Ac (Table 4), but were only three-fold more in tissue collected in 2001 (Table 4).

Summary

These data indicate that there are differences in toxin levels among plant parts in single and dual-toxin *Bt* cotton varieties. For Cry1Ac, expression patterns were similar for both years. There was no relationship between toxin levels of Cry1Ac and Cry2ab in Bollgard II cotton varieties. However, Cry2Ab levels were higher than those of Cry1Ac in all cases for dual-toxin cotton tissue. Cry2Ab toxin expression patterns were different from 2000 to 2001. This suggests that variety, environment, or even location can play a significant role in Cry2Ab expression patterns among plant parts.

References

Adamczyk, Jr., J. J., D. D. Hardee, L. C. Adams, and D. V. Sumerford. 2000. Correlating differences in larval survival and development of bollworms (Lepidoptera:Noctuidae) and fall armyworms (Lepidoptera:Noctuidae) to differential expression of Cry1A(c) δ-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol.. 94:284-290.

Akin, D. S., S. D. Stewart, J. J. Adamczyk, Jr., and K. S. Knighten. 2001. Behavioral response of lepidopteran pests on cotton expressing insecticidal proteins of *Bacillus thuringiensis*. Proceedings of the Beltwide Cotton Conference. yadayada (2001). National Cotton Council, Memphis, TN.

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

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. Cry1Ac levels (ppm) among plant parts for three locations 2000 and 2001.

Plant part	Concentration [ppm]
Terminal leaf	2.4 ± 0.15 **cd**
Lower canopy leaf	3.6 ± 0.35 **a**
Young square	1.8 ± 0.15 **ef**
White flower petal	3.1 ± 0.23 **b**
White stamen	2.7 ± 0.13 **bc**
Pink petals	2.4 ± 0.11 **cd**
Pink stamen	2.2 ± 0.13 **de**
Young boll	2.1 ± 0.15 **de**
10-14 d-old boll wall	2.9 ± 0.21 **b**
10-14 d-old boll – internal contents	1.5 ± 0.08 **f**

Numbers not followed by a common letter are significantly different ($p<0.05$, Proc GLM; SAS Institute 1998).

Table 2. Cry2Ab levels (ppm) among plant parts for three locations separated by year.

	Concentration [ppm]	
Plant part	**2000**	**2001**
Terminal leaf	13.5 ± 2.07 **f**	5.5 ± 0.80 **ef**
Lower canopy leaf	21.0 ± 1.72 **bc**	14.4 ± 0.34 **a**
Young square	15.5 ± 1.52 **ef**	11.2 ± 1.52 **b**
White flower petal	20.1 ± 2.77 **bcd**	8.4 ± 0.60 **cd**
White stamen	26.2 ± 0.69 **a**	8.8 ± 0.70 **bcd**
Pink petals	17.5 ± 0.24 **de**	5.4 ± 0.48 **f**
Pink stamen	18.8 ± 2.17 **cde**	5.6 ± 0.46 **ef**
Young boll	22.9 ± 1.43 **ab**	8.0 ± 1.76 **cde**
10-14 d-old boll wall	10.0 ± 2.27 **g**	9.0 ± 0.99 **bc**
10-14 d-old boll–internal contents	22.0 ± 0.85 **bc**	6.4 ± 0.32 **def**

Numbers not followed by a common letter are significantly different ($p<0.05$, Proc GLM; SAS Institute 1998).

Table 3. Cry1Ac lev els (ppm) in Bollgard and Bollgard II Varieties 2000 and 2001.

Bollgard	2.57 ± 0.10 **a**
Bollgard II	2.33 ± 0.09 **a**

Numbers not followed by a common letter are significantly different ($p<0.05$, Proc GLM; SAS Institute 1998).

Table 4. Comparison of Cry1Ac and Cry2Ab levels (ppm) 2000 and 2001.

2000/2001	
Cry1Ac	2.44 ± 0.07 **a**
Cry2Ab	12.42 ± 0.59 **b**
2000	
Cry1Ac	2.02 ± 0.09 **a**
Cry2Ab	18.76 ± 0.78 **b**
2001	
Cry1Ac	2.75 ± 0.08 **a**
Cry2Ab	8.05 ± 0.39 **b**

Numbers not followed by a common letter are significantly different ($p<0.05$, Proc GLM; SAS Institute 1998).