2006 Beltwide Cotton Conferences, San Antonio, Texas - January 3 - 6, 2006

THE EFFECTS ON LARVAL DEVELOPMENT AND MORTALITY, PUPATION AND TOLERANCE TO TOXIC PROTEIN IN COTTON BOLLS, POLLEN, AND ARTIFICIAL DIET BIOASSAYS OF PINK BOLLWORM (PBW) FEEDING ON 'NUCOTN 33B®' COTTON BOLLS

Thomas J. Henneberry
USDA-ARS-WCRL
Phoenix, AZ
Lynn Forlow Jech
USDA-ARS-WCRL
Phoenix, AZ
Jamie Maurer
USDA-ARS-WCRL
Phoenix, AZ

Abstract

Cottons that have a gene or genes from *Bacillus thuringiensis* Kurstaki (Berliner) that produce insect toxins have been grown in Arizona since 1996. The threat of pink bollworm (PBW), *Pectinophora gossypiella* (Saunders) resistance development to the toxin has been of continuing concern. The increasing need for information on PBW interactions in transgenic cotton cultures to provide information to supplement resistant management programs prompted us to conduct laboratory studies from 2001 to 2005 to determine the effect of PBW feeding on Bt bolls on larval development, mortality, pupation, and tolerance to the Cry1Ac toxin. Initially, higher larval mortality occurred and more days were required for pupal development following four day feeding periods on 'NuCOTN 33B®'[Bt(4) strain] bolls compared with similar feeding periods on 'Delta and Pineland (DPL) 5415' [DPL(4) strain] non-toxin containing bolls. Mortality and larval development time decreased as the number of larval feeding generations increased suggesting adaptation to the toxin in bolls. Small numbers of larvae of the Bt(4) PBW strain developed by feeding for 35 of 40 generations for four days in each generation on NuCOTN 33B® bolls, survived on Bt pollen during a 14-day test period and survived to adult emergence on artificial diet containing Cry1Ac toxin (10 μg/ml), but not on Bt bolls. No larvae of a susceptible DPL(4) 5415-PBW control strain developed to maturity when feeding on Bt pollen or bolls or high concentrations (10 μg/ml) of Cry1Ac protein in artificial diet.

Introduction

Extensive pink bollworm (PBW), *Pectinophora gossypiella* (Saunders), cotton boll monitoring in Arizona has demonstrated continuing commercial field efficacy of Bt cottons from 1997 through 2005 resulting in averages of less than one percent seasonal boll infestations. Under laboratory conditions, tolerance to Bt toxin in various formulations has been demonstrated (Bartlett 1995, Liu et al. 2001a, Tabashnik et al. 2002). The difference between field and laboratory resistance development remains unexplained. However, the threat of resistance and loss of the new technology has been of concern since the first transgenic cotton crop successes were reported (Mellon and Rissler 1998). The large acreage of transgenic crop production, overlapping growing seasons by sequentially planted genetically engineered crops, and other scenarios were visualized as potentially disasterous situations with high probabilities of resistance development. The resistance concern was further heightened with the knowledge that insect resistance to *B. thuringiensis* in conventional spray applied insect control formulations had been demonstrated (Tabashnik 1994). The need for additional information on PBW transgenic cotton interactions prompted us to determine the effects of PBW larval feeding on 'NuCOTN 33B®' (Bt) (Monsanto Co., St. Louis, MO) cotton bolls on larval developmental rates, mortalities and tolerance to Cry1Ac toxin. Studies were conducted during 2001 to 2005 and controls were 'Delta and Pineland (DPL) 5415' (Delta and Pineland Co, Scott, MS) non-toxin containing cotton bolls. In this paper, the Bt cotton cultivar used contained only the BG® gene that results in production of the Cry1Ac toxin.

Materials and Methods

Effects of Larval feeding on Bt Cotton on Mortality and Development. All cottons were grown in the greenhouse from seed. PBW larvae used in the study were from a laboratory culture that has been reared on artificial diet (Bartlett and Wolf 1985) for more than 300 generations. For the first generation of the current study,

four-to five-day-old PBW larvae were placed on the surfaces of 80 to 150, 21 to 28-day old immature green Bt or DPL 5415 cotton bolls. Larvae were confined on the bolls for four [Bt(4) and DPL(4) strains] days following studies that showed that four-day feeding of PBW larvae on Bt bolls resulted in 30 to 40% PBW larval mortality. After the feeding periods, mortality was recorded and surviving larvae were held in capped cylindrical plastic containers with two to three pieces of artificial diet (~ 2g) until pupation. Larvae in the containers were checked daily for mortality. Dates of pupation were also recorded.

Surviving pupae were held in waxed cardboard PBW moth mating-oviposition cages. Holes in the cage lids were screened for ventilation and oviposition. Paper towel pieces were placed over the screens and weighted down with metal washers. Oviposition substrates with eggs were placed in the same type of cardboard containers filled to one-quarter capacity with artificial diet. Containers were checked daily and 4-5 days following egg hatch, five larvae were placed on each of 80 to 150 Bt bolls to begin the second and thereafter subsequent generations as described for the first PBW generation. DPL 5415 cotton bolls were controls and procedures in each generation were identical to those described for Bt bolls.

Larval Mortality Following Feeding on Bt Bolls, Pollen, and Cry1Ac toxin in Artificial Diet. Following generations 12, 18, 38 and 40, first instar PBW larvae of the Bt(4) strain were placed on Bt bolls to identify a possible increase in tolerance to the Cry1Ac toxin in the bolls. Bolls with larvae were examined daily and mortality recorded.

Cotton pollen bioassays for the same purpose were conducted following completion of generation 37 of the Bt(4) strain. Pollen was collected from Bt and DPL 5415 cotton flowers picked from greenhouse grown cottons. Flowers with pollen were shaken over paper sheets for collection and the pollen placed into vials and frozen in the refrigerator until used. Small amounts (~8 mg) of Bt or DPL 5415 pollen were placed on moist, filter papers in the bottoms of 10.0 cm diameter plastic petri dishes with lids. Pollen bioassays were developed because of the report by Sims (1995) that cotton pollen from Bt contained low Cry1Ac levels compared to amounts in other cotton plant parts. The lower level toxin sources were used in an effort to detect low level PBW larval resistance that might be missed in boll bioassays with higher Bt toxin levels. First instar PBW larvae (five) were introduced into each of five containers with Bt or DPL 5415 pollen. Larvae in the dishes were examined on day 14 after initiation of the experiment and living and dead larvae recorded. Pollen was replenished when completely consumed or moldy. Larvae were from the DPL(4) and Bt(4) strain colonies.

A modification of the artificial diet bioassay described by Patin et al. (1999) also was used to determine tolerances of the Bt(4) and DPL(4) strains to Cry1Ac toxic protein. Cry1Ac toxin (MVP-II® Bioinsecticide, Mycogen, San Diego, CA) in stock solution was mixed into artificial diet in amounts necessary to create final concentrations of 0 and 10 µg Cry1Ac/ml of diet. The Cry1Ac-fortified diet was supplied by the Extension Arthropod Resistance Management Laboratory of the University of Arizona, Tucson, AZ. Neonate larvae (5) were transferred with a fine brush into 10 bioassay cups for each concentration. Cups with larvae were examined on day 21 following infestation using the survival bioassay criteria of Tabashnik et al. (2002). Living and dead larvae were recorded. Studies selecting for resistance to Cry1Ac toxin have shown that the 10µg/ml concentration in artificial diet is a diagnostic dose for identification of PBW larvae homozygous for resistance to the toxin (Tabashnik et al. 2002)

Cry1Ac toxin in bolls and pollen was determined using commercial enzyme-linked immunosorbent assay kits (ELISA Envirologix, Inc., Cry1Ab/Cry1Ac plate kit Envirologix, Portland, ME). Materials, sample preparations, solutions, extractions, dilutions, and assays were exactly as described in the kits. The amounts of Cry1Ac protein were determined in 0.6-cm boll piece samples from each of 15 to 20 cotton bolls in each PBW generation. Samples were excised from the same bolls larvae fed on. Sample pieces were weighed and placed in 1.5-cm microcentrifuge tubes. Boll pieces were homogenized in extraction buffer with a fitted pestle and processed thereafter using the Envirologix procedures. Pollen samples (~8 mg) were analyzed using the same procedures.

Data were analyzed using student "*t*" -tests for paired comparisons between the Bt(4) and DPL(4) strain mortalities and days to pupation. ANOVA or regressions was used for statistical analysis of the effects of PBW larval feeding on bolls, pollen and Cry1Ac-fortified diet. Mortality percentages were corrected for control mortalities using Abbott's formula (Abbot 1925). All percentages were arcsine transformed before statistical analysis.

Results

Effects of Larval feeding in Bt Cottons on Mortality and Development. Preliminary PBW larval feeding studies on Bt bolls showed mortalities of 21, 36, 66 and 92% following 2, 4, 6, and 8 day feeding periods, respectively (Table 1). The four-day feeding period was selected for studies to determine long-term toxin effects.

The mean mortality of larvae per generations one to 40 varied but was less for DPL 5415 compared with Bt cotton for generation one through 20. (Table 2). Average mortality for PBW larvae fed for four days on Bt bolls was not significantly different compared with DPL 5415 larvae in generations 30 and 40. However, the differences between development to pupation on Bt and DPL 5415 remained significantly different. Average numbers of days to development to pupation for larvae fed on Bt bolls for four days also decreased with increasing numbers of feeding generations (Table 2).

Cry1Ac toxin ELISA measurements in Bt bolls for all plant samples averaged 0.236 ± 0.006 ppm per gram of wet weight boll tissue and ranged from 0.001 to 1.045 µg/g (n = 839).

Larval Mortality following feeding on Bt Bolls, Pollen and Cry1Ac Toxin in Artificial Diet. For neonate PBW larvae of the Bt(4) strain placed on Bt bolls following generation 38, no surviving Bt(4) larvae were found on day five or later for Bt bolls (Table 3).

For cotton pollen bioassays, no significant PBW larval mortality differences occurred between PBW larval strains on day 3 of feeding on Bt or DPL 5415 pollens (Table 4). All DPL(4) strain larvae were dead on day seven following feeding on Bt pollen. Bt(4) larval strain mortality was 71% when feeding on DPL 5415 pollen for 14 days and 80% when feeding on Bt pollen for 14 days. The results suggest some PBW larval tolerance to toxin in Bt plant pollen. All Bt pollen samples were ELISA positive for Cry1Ac protein. Toxin amounts averaged 0.12 µg/g of pollen.

On day 21 following WCRL colony neonate larval infestation, 100.0% survival occurred on artificial diets without Cry1A protein compared with 0 % survival on diet with 10 µg/ml of Cry1A toxin (Table 5). For the WCRL strain, no larvae developed to maturity on diet containing 10.0 µg/ml of Cry1A toxin. Thirty percent of the Bt(4) PBW larvae developed to maturity on the artificial diet containing 10.0 µg/ml concentrations of Cry1A protein, considered to be the discriminating dose for homozygous resistance (Tabashnik et al. 2002).

Discussion

The first transgenic cottons grown in Arizona and the cultivar used ('NuCOTN 33B®') in these studies produced only Cry1Ac toxin. Several insect species including PBW larvae have been laboratory selected for resistance to Cry1Ac toxin (Gould and Tabashnik 1998). In the field in Arizona, outstanding PBW population suppression in commercial transgenic cotton plantings has occurred since 1996. Less than 1% boll infestations have developed in sampled fields of the 60% or greater acreages of Bt cotton grown in the state each year (Dennehy et al. 2004). The single biggest threat to transgenic cotton efficacy in the field continues to be the potential for resistance development. The reason(s) for the difference in relatively rapid selection for resistance in the laboratory compared with field populations remains undefined. Tabashnik et al. (2002) selected a PBW resistant strain 300 fold to 3100 fold resistant to Cry1Ac on artificial diet compared to a susceptible control colony. The increase in resistance did not increase survival of the resistant strain on Bt bolls. The authors suggested the differences in survival in laboratory diet biossays and Bt bolls assays could be caused by difference in toxin concentration, plant defensive components, nutrients or other factors.

Feeding periods of four- or seven-days on Bt bolls resulted in slower larval development rates similar to results previously reported (Henneberry et al. 2001, 2003, 2004) and those by other authors (Liu et al. 1999, 2001b). PBW larvae adapted over succeeding generations to the factor(s) in the Bt bolls that initially resulted in the slower developmental rates. Similar adaptation occurred for larval mortalities following four day feeding periods on Bt bolls. However, in contrast to the results of Liu et al. (2001a, b) none of the PBW larvae of our strains even after 35 generations of four day feeding periods on Bt bolls could successfully complete larval development on Bt bolls. In our study, some of the Bt(4) larval strain survived on artificial diet containing 10µg/ml of Cry1A toxin suggesting that feeding periods on Bt bolls has selected for tolerance to the toxin.

Much progress has been made leading to an understanding of the genetics of resistance and the impact of transgenic cottons on PBW populations. (Liu et al. 2000, Tabashnik et al. 2000, see literature cited for others). The results of our present studies contribute some information on the effects of the Cry1Ac protein on mortality and development of immature PBW stages. Much additional information will be required to perfect and provide additional tools to develop knowledge on understanding of existing resistance management programs and develop complementary approaches. The overall areawide impact of transgenic cotton on PBW has been demonstrated (Carrière et al. 2003). The regional reduction in PBW populations has encouraged western cotton growers and the National Cotton Council to envision the potential for PBW eradication in the Western United States and possibly Mexico (Anonymous 2001). The key component of the eradication system is transgenic cotton and its efficacy must be protected and extended with dependable resistance management tactics. Continued research on selection of resistance to Bt toxin may provide information on how PBW resistance may occur in the field. The information may suggest new methods of resistance management to extend the efficacy of Bt cottons in the field.

References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265-267, 6 pp.

Anonymous. 2001. Pink bollworm eradication: a window of opportunity. National Cotton Council of Amer., Memphis, TN.

Bartlett, A.C. 1995. Resistance of the pink bollworm to Bt transgenic cotton, pp. 766-768. *In* D.A. Richter and J. Armour [Eds.] Proc. Beltwide Cotton Prod. Res. Conf., Natl. Cotton Council of Amer., Memphis, TN.

Bartlett, A.C. and W.W. Wolf. 1985. *Pectinophora gossypiella*, pp. 415-430. *In* R. F. Moore and P. Singh [Eds.] Handbook of insect rearing, vol. 2. Elsevier Scientific Publishing Co., Amsterdam.

Carrière, Y., C. Ellers-Kirk, M. Sisterson, L. Antilla, M. Whitlow, T.J. Dennehy, and B.E. Tasbashnik. 2003. Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. Agricultural Sciences 100: 1579-1523.

Dennehy, T.J., C. Gopalan, S.A. Brinks, B.D. Wood, Y. Carrière, B.E. Tabashnik, L. Antilla, and M. Whitelow. 2004. Update on pink bollworm resistance to Bt cotton in the southwest, pp. 213-223. *In* J. Silvertooth [Ed.] Cotton, A College of Agriculture and Life Science Report, Series P-138, 2004, Cooperative Extension, Agric, Expt. Stn. The University of Arizona, Tucson, AZ.

Gould, F. and B.E. Tabashnik. 1998. Bt cotton resistance management, pp. 67-105. *In* Now or Never Serious New Plans to Save a Natural Pest Control. M. Melton and J. Rissler [Eds.] Union of Concerned Scientists, Cambridge, MA.

Henneberry, T.J., L. Forlow Jech, and T. de la Torre. 2001. Effects of transgenic cotton on cabbage looper, tobacco budworm and beet armyworm (Lepidoptera: Noctuidae) larval mortality and development and foliage consumption in the laboratory. Southwest. Entomol. 26: 325-338.

Henneberry, T.J., L.J. Forlow Jech, and T.M. de la Torre. 2003. NuCOTN 33B® and Delta and Pineland cottons: Pink Bollworm (Lepidoptera: Gelechiidae) infestations and Cry1Ac toxic protein in overwintered and seeded cottons with bioassay mortalities of other lepidopterous larvae. Southwest Entomol. 28: 281-292.

Henneberry, T.J., L.J. Forlow Jech, and T. de la Torre. 2004. Sublethal pink bollworm larval feeding periods on Bt cotton bolls: Effects on boll entry attempts, larval development and mortality, 2001-2003. Entomological Soc. Amer., Arthropod Management test. 29: M3, 6pp.

Liu, Y.-B., B.E. Tabashnik, S.K. Meyer, Y Carrière, and A.C. Bartlett. 2001a. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94: 248-252.

Liu, Y.-B., B.E. Tabashnik, T.J. Dennehy, A.L. Patin, M.A. Sims, S.K. Meyer, and Y Carrière. 2001b. Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (lepidoptera: Gelechiidae). J. Econ. Entomol. 94: 1237-1242.

Liu, Y.-B., B.E. Tabashnik, S.K. Meyer, Y. Carrière, and A.C. Bartlett. 2000. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94: 248-252

Liu, Y.-B., B.E. Tabashnik, T.J. Dennehy, A.L. Patin, and A.C. Bartlett. 1999. Development time and resistance to Bt crops. Nature (Lond.). 400: 519.

Mellon, M. and J. Rissler [Eds.] 1998. Now or Never Serious New Plans to Save a Natural Pest Control, 149 pp. Union of Concerned Scientists Publications, Cambridge, MA.

Patin, A.L., T.J. Dennehy, M.A. Sims, B.E. Tabashnik, Y.-B. Liu, L. Antilla, D. Gouge, T.J. Henneberry, and R. Staten. 1999. Status of pink bollworm susceptibility to Bt in Arizona, pp. 991-996. *In* P. Dugger and D. Richter [eds.] Proc. Beltwide Cotton Res. Conf., Natl. Cotton Council of Amer., Memphis, TN.

Sims, S.R. 1995. *Bacillus thuringiensis* Kurstaki [Cry1Ac] protein expressed in transgenic cotton: Effects on beneficial and non-target insects. Southwestern Entomol. 20: 493-500.

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis.* Annul. Rev. Entomol. 39: 47-79.

Tabashnik, B.E., A.L. Patin, T.J. Dennehy, Y.-B. Liu, Y Carrière, M.A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 97: 12,980-12,984.

Tabashnik, B.E., Y.-B. Liu, T.J. Dennehy, M.A. Sims, M. Sisterson, R.W. Biggs, and Y. Carrière. 2002. Inheritance of resistance to Bt toxin Cry1Ac in a field-derived strain of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 95: 1018-1026.

TABLE 1. Pink Bollworm Larval Mortality After Feeding on DPL 5415 or Bt Greenhouse Grown Cotton Bolls for 2, 4, 6, or 8 Days.

Cultivars	% Mortality After n Days of Infestation:			
	2	4	6	8
DPL5415	4 b	34 a	24 b	31 b
Bt	21 a	36 a	66 a	92 a
t[a]	2.2*	0.1 ns	3.8*	4.9*

[a] Student's *t* value. Means of 6 to 28 paired comparisons.
* significantly different.

TABLE 2. Mean Pink Bollworm Larval Mortality Percentages and Days to Pupation Following Four-Day Feeding Periods in Each of 35 of 40 Generations.

Generation[a]	Percent Mortality		Days to Pupation	
	Bt4	DPL4	Bt4	DPL4
1	37.5 a	15.8 b	12.3 a	6.1 b
10	30.4 a	16.5 b	9.4 a	5.7 b
20	38.7 a	23.3 b	10.3 a	7.4 b
30	24.4 a	23.3 a	7.0 a	5.2 b
40	33.9 a	20.5 a	8.2 a	5.2 b

[a] Means in each of the indicated generations from a total of 40 generations of continuous study.

TABLE 3. Mean Pink Bollworm Larval Mortality Percentages on NuCOTN33B® and Deltapine 5415 Bolls Following 38 Generations of Four-Day Feeding Periods on Transgenic and Non-Transgenic Cotton Bolls.

PBW Strains	Plant Source (Bolls)	% Mortality on Day[a]:	
		5	11
DPL(4) Control	DPL5415	83	83
DPL(4) Control	NuCOTN33B®	100	100
BG®(4)	DPL5415	63	91
BG®(4)	NuCOTN33B®	100	100
F	--	2.33 ns	0.07 ns

[a] Means of 3 replications, ns = not significantly different.

TABLE 4. Mean Percentages of Pink Bollworm Larval Mortality When Fed Pollen From Delta and Pineland 5415 or NuCOTN 33B® Cotton Flowers.

		No. Days Feeding[a]	
PBW Larval Strain	Pollen Source	3	14
DPL(4)	DPL 5415	26 a	47 c
DPL(4)	NuCOTN33B®	69 a	100 a
BG®(4)[b]	DPL 5415	52 a	71 b
BG®(4)	NuCOTN33B®	68 a	80 b
F	- -	2.32 ns[b]	9.95

[a] Means of 4 replications in a column not followed by the same letter are significantly different.
[b] ns = not significantly different.

TABLE 5. Mean Mortality Percentages and Developmental Stage of Pink Bollworm Larvae Fed on Artificial Diet With None or a Resistance Discriminating Concentration of Cry1Ac Toxin.

PBW Strain/	%	Surviving Developmental Instar (No.)[b]			
µg/ml	Survival[a]	1	2	3	4
WCRL Colony					
0.0	100.0 c	0	0	0	39
10.0	0.0 a	0	11	0	0
BG®4					
0.0	100.0 c	0	0	0	31
10.0	67.5 b	0	0	0	15

[a] Means of 10 replications, 5 larvae/replication in the same category not followed by the same letter are significantly different. $P \leq 0.05$ by ANOVA and the Method of Least Significant Differences. Survival based on living and dead larvae counted on day 21 and larvae reaching the fourth instar or pupae considered survivors.
[b] N = 50 in all cases, totals may vary due to missing larvae.