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ARTHROPOD MANAGEMENT

Efficacy of Seed Mixes of Transgenic *Bt* and Nontransgenic Cotton Against Bollworm, *Helicoverpa zea* Boddie

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INTERPRETIVE SUMMARY

The 1996 advent of Monsanto's transgenic *Bt* cotton, commercially available as "NuCOTN" with the Bollgard *Bt* gene, provided growers with a new tool for controlling caterpillar pests in cotton. A delta-endotoxin protein (*cry*1Ac) from *Bacillus thuringiensis* Berliner var. *kurstaki* (*Bt*) expressed in these transgenic cotton plants is toxic to many lepidopteran larval pests of cotton. Monsanto initially claimed that Bollgard cotton would meet the needs of a high-dose resistance-management strategy by providing an adequately high toxin concentration. Studies confirmed the high-dose toxin level for tobacco budworm, *Heliothis virescens* F.; however, bollworm, *Helicoverpa zea* Boddie, was observed to be much less susceptible than tobacco budworm to the *cry*1Ac endotoxin. Because a proportion of bollworm larvae were found to survive on Bollgard cotton, the prospect for rapid development of bollworm resistance to *cry*1Ac was a general concern. Therefore, development of deployment strategies was necessary to delay resistance.

A management system that incorporated nontransgenic cotton refuges to produce nonexposed bollworms was suggested. A refuge in time or space would serve to decrease selection pressure to the *Bt* toxin. Early testing of transgenic *Bt* cotton included studies of seed blends offering a refuge for a resistance-management strategy. These blends contained a higher percentage of transgenic *Bt* cottonseed mixed with a smaller percentage of non-*Bt* cottonseed. By placing a blend of the two cottonseed types in the same bag, Monsanto could ensure that a refuge would be planted by growers with *Bt* and non-*Bt* plants interspersed in the same rows within a field.

Experiments conducted in 1994 and 1995 examined the effects of plantings of pure and blended genotypes of Bollgard *Bt* cotton and non-*Bt* cotton on (i) the larval population development of bollworm (*H. zea*), (ii) fruit damage, and (iii) yield. Mean percent larval infestation and mean percent damaged fruit increased in both years as the percentage of *Bt* seed in the blends decreased. Conversely, seed cotton yields decreased as the percentage of *Bt* seed in the blends decreased.

Other studies have shown bollworm and/or tobacco budworm larvae become increasingly tolerant of the *Bt* toxin with size, or instar, and are typically able to feed on and survive in *Bt* cotton once they reach the third instar. In mixed plantings of *Bt* and non-*Bt* cotton, larvae can develop on non-*Bt* plants and move to *Bt* plants to feed and cause injury, thereby increasing yield loss. Studies indicate that caterpillars that develop on non-*Bt* plants and move to *Bt* plants to feed will be selected for resistance to the *Bt* cotton (Mallet and Porter, 1992; Gould, 1996; Halcomb et al., 1996). Caterpillar pests that move from plant to plant within a mixed planting may not mate at random due to differential development rates, and the difference in the fitness of pest genotypes in the mixture may not be directly related to the ratio of *Bt*:non-*Bt* seed in the mixture. Our data suggest that the treatments incorporating blends of *Bt* and non-*Bt* seed sustained too much fruit damage and yield loss for the blended seed concept to be practical in areas where bollworm populations occur at high levels. Therefore, seed mixtures appear to be an unlikely candidate for resistance-management purposes.

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Abbreviations: *Bt*, *Bacillus thuringiensis*

After review of this and other studies, Monsanto and the EPA provided growers with two refuge options in 1996. For every 100 acres of Bollgard cotton planted, (i) 4 acres had to be planted to non-*Bt* cotton cultivars that could not be treated with insecticides for caterpillar control, or (ii) 25 acres had to be planted to non-*Bt* cotton cultivars that could be treated with any conventional insecticides except *Bt* product formulations. These refuge options have persisted, even with growing concern about the lack of science behind the size of the refuge.

New, somewhat modified refuge options are available for the 2001 growing season. Two options were added: (i) the 5% embedded refuge in which 5% of the cotton planted can be conventional cotton embedded in the field of *Bt* cotton and treated with insecticides as the *Bt* cotton is treated; (ii) the community refuge in which growers can work together to meet the refuge requirements on a community scale instead of on an individual basis. Scientists and others concerned with preserving this technology have asked the EPA for larger refuges and more studies of resistance-management strategies for transgenic *Bt* cotton. Although much discussion about a change in the refuge strategy has occurred, studies in North Carolina in 1994 and 1995 indicated that seed mixtures would not be a practical, economic option for the grower and would not provide the necessary refuge for susceptible Heliothine larvae.

ABSTRACT

Field trials conducted in northeastern North Carolina in 1994 and 1995 examined the effects of plantings of pure and blended genotypes of Bollgard cotton on (i) the larval population development of caterpillar pests, (ii) fruit damage, and (iii) yield. Treatments in 1994 included (i) 100% *Bt* seed:0% non-*Bt* seed; (ii) 0% *Bt*:100% non-*Bt*; (iii) 90% *Bt*:10% non-*Bt*; (iv) 85% *Bt*:15% non-*Bt*; (v) 80% *Bt*:20% non-*Bt*; and (vi) 75% *Bt*:25% non-*Bt*. The same treatments were tested again in 1995, with the omission of the 90% *Bt*:10% non-*Bt* and 80% *Bt*:20% non-*Bt* seed blends. Bollworms made up 95 to 98% of larval pest populations in 1994 and 1995. The 0% *Bt*:100% non-*Bt* plots had significantly higher larval numbers and damaged fruit and significantly lower yields than all other seed treatments both years. The treatment containing 100% *Bt* seed had lower percent larval infestation and percent damaged fruit and higher yields than all other seed blends in both years. In general, mean percent larval infestation and mean percent damaged fruit increased in both years as the percentage of *Bt* seed in the blends decreased. Conversely, seed cotton yields decreased as the percentage of *Bt* seed in the blends decreased. In these experiments, the treatments incorporating blends of *Bt* and non-*Bt* seed sustained too much fruit damage and yield loss for the blended seed concept to be practical.

A revolutionary insect-management technology was provided to the cotton industry in 1996 when Monsanto commercialized Bollgard cotton. Transgenic Bollgard cottons express the delta-endotoxin protein *cry*1Ac from *Bacillus thuringiensis* Berliner var. *kurstaki* (*Bt*), which is toxic to several of the most important lepidopteran larval pests of cotton. A concern associated with the widespread planting of Bollgard cottons is that lepidopteran pests may develop resistance to the *cry*1Ac endotoxin as they have to many traditional insecticides (Graves et al., 1991). Although studies with Bollgard demonstrated excellent control of tobacco budworm, *Heliothis virescens* F., several studies confirmed that bollworm, *Helicoverpa zea* Boddie, is much less susceptible to the *cry*1Ac endotoxin than tobacco budworm is (Stone and Sims, 1993; Sims, 1995). Documentation that a proportion of bollworm larvae could survive on Bollgard cotton confirmed that *cry*1Ac was not expressed at a concentration high enough to provide a “high-dose strategy” approach to resistance management (Hardee et al., 2001). Bollworm survival on Bollgard cotton was shown to be of such magnitude that deployment of resistance-management strategies other than the high-dose refuge strategy was necessary. One approach would be to increase the refuge size and/or refuge efficiency.

McGaughey (1990) suggested alternating insecticides, using multiple toxins, providing untreated refuges, selecting appropriate doses, or treating selected plant parts as a tactic for preventing resistance development to *Bt* toxins. Arpaia and Ricchiuto (1993) examined alternative strategies for potato pest management and suggested using mixtures of plants, including non-*Bt* types, as a refuge. Gould (1991) also suggested the use of untreated pest habitats offering refuge in or near the crop as a means of delaying resistance when multiple toxins were used. One means of providing such refuges would be to mix seed of *Bt* cotton with that of non-*Bt* cotton. These non-*Bt* plants may help maintain susceptible insects in the population so that the gene frequency of resistance is kept low.

The study reported herein examined the efficacy of selected seed mixes of *Bt* and non-*Bt* cotton under conditions known to promote high bollworm larval pest populations (i.e., disruption of arthropod natural enemies and late planting of cotton). The experiments conducted in 1994 and 1995 examined the effects of plantings of pure and blended genotypes of Bollgard cotton and non-*Bt* cotton on (i) the larval population development of *H. zea*, (ii) fruit damage, and (iii) yield.

MATERIALS AND METHODS

Test Description

1994. The trial was planted on the C.A. Martin Farm in Martin County, North Carolina, on 17 May. The soil type was a Norfolk-Ruston sandy loam [fine-loamy, kaolinitic, thermic Typic Kandiudults – fine-loamy siliceous, semiactive, thermic Typic Paleudults]. The experimental design was a randomized complete block with six treatments and six replications. Each plot was eight rows wide (spaced 91.4 cm apart) by 9.1 m long. Treatments included mixtures of *Bt* and non-*Bt* seed and will be expressed herein as a ratio of percent (%) *Bt* seed:% non-*Bt* seed. Seed blends in 1994 were as follows: (i) 100:0; (ii) 0:100; (iii) 90:10; (iv) 85:15; (v) 80:20; and (vi) 75:25.

1995. The test was planted at the Upper Coastal Plain Research Station in Edgecombe County, North Carolina, on 5 and 22 May. The soil type was a Norfolk-Ruston sandy loam. This study of the effects of seed blends on *H. zea* populations, fruit damage, and yield was part of a larger field trial that also examined the effects of arthropod natural enemy conservation versus disruption and early versus late planting dates in Bollgard cotton and non-*Bt* cotton (Lambert et al., 1996). The experiment was a randomized complete split-split block with main plots, subplots, and sub-subplots as natural enemy conservation/disruption, planting date, and seed blend, respectively, replicated four times. Only data for disrupted cotton plots will be reported herein. The two planting dates for 1995 represent early and late planting dates. The data were averaged over planting date in 1995 for the purpose of reporting only the effects of seed blends. The cottonseed blends included (i) 100:0; (ii) 0:100; (iii) 85:15; and (iv) 75:25. Each plot was four rows wide (spaced 91.4 cm apart) by 12.2 m long.

Aldicarb [2-methyl-2-(methylthio) propion-aldehyde O-(methylcarbamoyl) oxime] (Temik 15G, Rhone-Poulenc Ag, Research Triangle Park, NC) was applied at 840 g a.i. ha^{-1} in-furrow at planting for early-season thrip control each year. At appropriate times throughout the seasons, fertility, weed control, plant growth regulation, and defoliation practices as recommended by North Carolina State University (1994) were followed for obtaining maximum cotton yields.

Disruption of arthropod natural enemies was accomplished each year through application of insecticides during mid-season. These insecticide applications were made for predator and parasite suppression in order to achieve maximum bollworm infestations. Acephate [O,S-Dimethyl acetyl-phosphoramidothioate] (Orthene 75S, Valent USA, Walnut Creek, CA) was foliarly applied at 841 g a.i. ha^{-1} to all plots on 14 July 1994. Aldicarb was applied as a side-dress treatment at 1681 g a.i. ha^{-1} on 11 July 1995. All plots in 1995 were further disrupted ~10 d later with a foliar application of acephate applied at 1121 g a.i. ha^{-1}.

Data Collection. Cotton plants were sampled on three dates in 1994 and one date in 1995 for percent (%) egg deposition. This was measured as the number of terminals per 50 and 25 terminals examined in 1994 and 1995, respectively, on which one or more Heliothine eggs had been deposited. Eggs were counted in all plots in 1994 and in pure *Bt* and non-*Bt* plots only in 1995. Cotton plants were sampled on five dates in 1994 and six dates in 1995 for percent larval infestation and percent damaged fruit. Numbers of live larvae per 25 or 50 squares and/or bolls per plot were recorded to determine percent larval infestation. Fruit damage was quantified as the number of squares and/or bolls observed that were damaged by bollworm or budworm. Squares were considered damaged when sufficient feeding on the anthers had occurred to cause the plant to abort the square. Bolls were considered damaged when the carpel wall had been penetrated.

Heliothine larvae were collected from *Bt* and non-*Bt* cotton plots each year and transported to the laboratory for species identification using methods described by Neunzig (1969). The center two rows of each plot were harvested mechanically on 19 Oct. each year.

Data Analysis. Numbers of eggs, larvae, and damaged fruit per plot were converted to percentages prior to analysis, and yields were reported as pounds of seed cotton per acre. All data were subjected to ANOVA using PROC GLM (SAS Institute, 1990). Means were separated ($P < 0.05$) using the LSMEANS procedure of SAS.

RESULTS

Pest Species

Larval populations collected from non-*Bt* cotton in 1994 were 98.5% *H. zea* (n = 66) and 1.5% *H. virescens* (n = 1). All larvae collected in *Bt* plots (n = 30) in 1994 were identified as *H. zea*. Samples collected from non-*Bt* plots in 1995 were identified as 95.2% *H. zea* (n = 60) and 4.8% *H. virescens* (n = 3). Bollworm and tobacco budworm populations were 97.7% (n = 42) and 2.3% (n = 1), respectively, in *Bt* plots in 1995.

Seed Blends

No significant seed blend effects were observed for mean percent egg deposition in 1994 (F = 1.70; df = 7, 35; P = 0.1405) or in 1995 (F = 0.41; df = 1, 7; P = 0.5415). Mean percent larval infestation and mean percent damaged fruit for each cottonseed blend in 1994 and 1995 are presented in Tables 1 and 2, respectively. Significant cottonseed blend effects were detected in 1994 and 1995 for mean percent larval infestation (F = 33.10; df = 7, 35; P = 0.0001 and F = 54.56; df = 4, 28; P = 0.0001, respectively) and mean percent damaged fruit (F = 60.05; df = 7, 35; P = 0.0001 and F = 82.88; df = 4, 28; P = 0.0001, respectively). The 0:100 cotton plots had significantly higher mean percent larval infestation and mean percent damaged fruit than all other cottonseed blends in 1994 and 1995. The 100:0 plots had the lowest mean percent larval infestation in 1994 and 1995. The plots containing 100% *Bt* seed had significantly lower mean percent damaged fruit than all other cottonseed blends except the 90:10 seed blend in 1994 and significantly lower mean percent damaged fruit than all other cottonseed blends in 1995. Although there were no significant differences in mean percent larval infestation in seed blends containing 75 to 90% and 75 to 85% *Bt* seed in 1994 and 1995, respectively, numbers of larvae generally increased as the percentage of *Bt* seed in the blend decreased in both years. Significant differences in percent damaged fruit were observed among the seed blends in both years; damage was inversely related to the percentage of *Bt* seed in the blend.

Seed treatment effects for yield were significant in 1994 (F = 27.77; df = 7, 35; P = 0.0001) and in 1995 (F = 85.91; df = 4, 28; P = 0.0001). The 0:100 cotton plots had significantly lower yields than all other seed blends in both years (Table 3). Plots containing 100% *Bt* seed had significantly higher yields than all other

Table 1. Mean percent *Helicoverpa zea* larval infestation for cottonseed blends in Martin (1994) and Edgecombe (1995) counties, North Carolina.

Cottonseed blend	Mean percent larval infestation†	
% *Bt*:% non-*Bt*	1994	1995
100: 0	8.1 b	3.1 c
90: 10	9.1 b	n/a
85: 15	9.7 b	5.0 c
80: 20	11.8 b	n/a
75: 25	11.3 b	6.2 b
0: 100	23.8 a	12.8 a

† Means followed by the same letter within each column are not significantly different according to LSMEANS procedure ($P < 0.05$).

Table 2. Mean percent *Helicoverpa zea* damaged fruit for cottonseed blends in Martin (1994) and Edgecombe (1995) counties, North Carolina.

Cottonseed blend	Mean percent damaged fruit†	
% *Bt*:% non-*Bt*	1994	1995
100: 0	21.9 d	12.0 d
90: 10	23.7 cd	n/a
85: 15	27.7 bc	17.6 c
80: 20	31.5 b	n/a
75: 25	32.1 b	23.1 b
0: 100	65.1 a	36.9 a

† Means followed by the same letter within each column are not significantly different according to LSMEANS procedure ($P < 0.05$).

Table 3. Mean yield (kg seed cotton ha^{-1}) for cottonseed blends in Martin (1994) and Edgecombe (1995) counties, North Carolina.

Cottonseed blend	Mean yield†	
	1994	1995
% *Bt*:% non-*Bt*	kg. seed cotton ha^{-1}	
100: 0	3211.8 a	3044.2 a
90: 10	2425.8 bc	n/a
85: 15	2755.5 ab	2777.3 b
80: 20	1816.0 c	n/a
75: 25	2096.1 bc	2698.5 b
0: 100	320.8 d	1542.4 c

† Means followed by the same letter within each column are not significantly different according to LSMEANS procedure ($P < 0.05$).

treatments except the 85:15 seed blend in 1994 and significantly higher yields than all other seed blends in 1995. While there was an overall significant increase in yield from 75% *Bt* seed to 90% *Bt* seed in the blends in 1994, there were few significant differences among blends that differed by 5% *Bt* seed. The 85:15 plots had higher yields than all other cottonseed blends (including 90:10) and significantly higher yields than the 80:20 seed blend. Yields in the 75:25 plots were slightly higher than yields in the 80:20 plots in 1994. The 85:15 cotton had higher yields than the 75:25 blend in 1995.

DISCUSSION

No significant differences in percent egg deposition were detected between seed blends in 1994 and 1995. The only difference between *Bt* and non-*Bt* plants is the presence or absence of the *Bt* toxin within the plants. This toxin apparently is undetectable to ovipositing moths, which indiscriminately deposit their eggs onto plants within the canopy of the cotton crop; therefore, differences in the number of eggs among pure and blended genotypes of *Bt* and non-*Bt* cotton would not be expected.

Numbers of larvae and damaged fruit were higher and yields were lower in seed treatments that did not contain 100% *Bt* seed. Specifically, seed cotton yields in 1994 were 24, 14, 43, and 35% lower in blends containing 90, 85, 80, and 75% *Bt* seed, respectively, than were yields in the seed treatment containing 100% *Bt* seed. Yields in seed blends containing 85 and 75% *Bt* seed in 1995 were 9 and 11% lower, respectively, than yields in the 100:0 treatment. These data suggest that the treatments incorporating blends of *Bt* and non-*Bt* seed sustained too much fruit damage and yield loss for the blended seed concept to be practical in areas where bollworm populations occur at high levels.

Seed blend efficacy trials by Durant (1995) also demonstrated that yields in seed treatments containing 100% *Bt* seed were significantly higher than some seed treatments containing mixtures of *Bt* and non-*Bt* seed. Durant concluded that seed mixes containing less than 90% *Bt* seed may not provide acceptable control of bollworm and tobacco budworm. Similar observations were made in suppression studies by Halcomb et al. (1996) where the numbers of fourth and fifth instar tobacco budworm and bollworm increased with a decrease in the percentage of *Bt* seed in the mixture.

In addition to causing significant damage to non-*Bt* plants in a mixture, caterpillar pests, which are usually susceptible to the *Bt* toxin, may survive on and damage *Bt* plants. With an intrafield refuge such as mixed seed plantings, *Bt* plants would be planted in the same row as non-*Bt* plants. Bollworm and/or tobacco budworm larvae become increasingly tolerant of the *Bt* toxin with size or instar, and are typically able to feed on and survive in *Bt* cotton, once they reach the third instar. Studies by Durant et al. (1996) indicated that movement of larvae to *Bt* plants from non-*Bt* plants in a mixed planting may result in a significant increase in damage and reduced yields in these plots. Halcomb et al. (1996) also examined inter-plant movement of tobacco budworm in mixed plantings of *Bt* and non-*Bt* cotton and found that larvae can develop on non-*Bt* plants in mixtures and move to *Bt* plants to feed and cause injury. These studies also showed plant-to-plant movement by third and fifth instar tobacco budworms with fifth instars moving greater distances and to a greater number of plants as the proportion of *Bt* seed in the blend increased.

Much of the concern surrounding the intrafield refuge, or mixed seed, strategy for resistance management is that seed mixtures may enhance resistance in mobile insects such as bollworm and tobacco budworm (Mallet and Porter, 1992). This mobility could increase response to selection pressure for resistance. Halcomb et al. (1996) concluded that all bollworms and tobacco budworms that develop on non-*Bt* plants and move to *Bt* plants to feed and cause injury will be selected for resistance to the *Bt* toxin. According to Gould (1996), seed mixtures can be an effective resistance-management strategy if there is random mating and if the difference in fitness of pest genotypes in the mixture is directly related to the ratio of *Bt*:non-*Bt* seed in the mixture. These assumptions do not hold true when caterpillar pests are able to move from plant to plant within a mixed planting. Mallet and Porter (1992) also predicted the failure of seed mixtures for resistance management due to insect mobility, which increases overall effective dominance and can cause more rapid evolution of resistance.

Studies reported herein indicated that the percentages of *Bt* seed in the mixtures tested and the doses of *cry*1Ac expressed in *Bt* cotton plants were not high enough to adequately suppress high numbers of caterpillar pests and prevent subsequent yield loss. Thus, refuges will have to be accomplished through some means other than the blended seed strategy for bollworm and tobacco budworm on cotton. To avoid problems associated with larval movement between *Bt* and non-*Bt* plants, Gould (1996) suggested interfield refuges with mixtures of *Bt* and non-*Bt* plants at the field-to-field level. Hardee et al. (2001) proposed planting intrafield refuges of non-*Bt* cotton strips of adequate width to minimize the "sink" effect of the surrounding *Bt* cotton. Monsanto has used the interfield refuge strategy as part of its resistance-management plan for Bollgard cotton since 1996. For every 40.5 ha of Bollgard cotton planted, (i) 1.62 ha had to be planted to non-*Bt* cotton cultivars that could not be treated with insecticides for caterpillar control, or (ii) 10.1 ha had to be planted to non-*Bt* cotton cultivars that could be treated with any conventional insecticides except *Bt* product formulations. Model assumptions concerning random mating between *Bt* selected and nonselected moths must be considered with the interfield refuge concept (Gould, 1996).

Transgenic *Bt* cotton provides an effective and more environmentally sound means of controlling cotton caterpillar pests. Further studies are necessary to determine the most practical refuge options and to quantify amounts of refuge necessary to delay resistance to transgenic *Bt* cotton in bollworm. The development of an effective resistance-management plan for these insect-resistant cottons will provide growers with another tool in an integrated pest-management scheme for cotton.

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