

# Field tests on managing resistance to *Bt*-engineered plants

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Received 15 July 1999; accepted 2 January 2000

Several important crops have been engineered to express toxins of *Bacillus thuringiensis* (*Bt*) for insect control. In 1999, US farmers planted nearly 8 million hectares (nearly 20 million acres) of transgenic *Bt* crops approved by the EPA. *Bt*-transgenic plants can greatly reduce the use of broader spectrum insecticides, but insect resistance may hinder this technology. Present resistance management strategies rely on a “refuge” composed of non-*Bt* plants to conserve susceptible alleles. We have used *Bt*-transgenic broccoli plants and the diamondback moth as a model system to examine resistance management strategies. The higher number of larvae on refuge plants in our field tests indicate that a “separate refuge” will be more effective at conserving susceptible larvae than a “mixed refuge” and would thereby reduce the number of homozygous resistant (RR) offspring. Our field tests also examined the strategy of spraying the refuge to prevent economic loss to the crop while maintaining susceptible alleles in the population. Results indicate that great care must be taken to ensure that refuges, particularly those sprayed with efficacious insecticides, produce adequate numbers of susceptible alleles. Each insect/*Bt* crop system may have unique management requirements because of the biology of the insect, but our studies validate the need for a refuge. As we learn more about how to refine our present resistance management strategies, it is important to also develop the next generation of technology and implementation strategies.

Key words: Insecta, *Plutella xylostella*, *Bacillus thuringiensis*, resistance, transgenic plants

Expression of proteins produced by a common bacterium, *Bacillus thuringiensis* (*Bt*), in transgenic plants to protect them from insect attack is revolutionizing agriculture<sup>1</sup>. The insecticidal proteins produced by *Bt* are toxic to major pests of many of the world's most important crops such as cotton, rice, and corn. Of the \$US 8.1 billion spent annually on insecticides worldwide, it is estimated that nearly \$2.7 billion could be substituted with *Bt* biotechnology applications<sup>2</sup>. At least 16 companies are presently developing transgenic crops with *Bt* genes, and at least 18 *Bt*-transgenic crops have been approved by the US Department of Agriculture (USDA) for field testing<sup>3</sup>.

When incorporated into plants, *Bt* proteins are made much more persistent and effective, even against insects that feed at sites difficult or impossible to reach with sprays<sup>4</sup>. *Bt* cotton was one of the first insecticidal plants to be approved for commercial use in 1995, and since then the adoption of this technology has been rapid not only in the United States but also in Australia and China. The reasons for the rapid adoption of this new technology are compelling. For example, *Bt* cotton required three or fewer insecticide treatments, compared with historical averages of 5–12 insecticide sprays per year for cotton in the United States<sup>5</sup>. Despite the considerable advantages of *Bt*-transgenic crops, both to the environment and to farmworker safety, concern is widespread that these gains will be short-lived because of evolution of resistance in the pests.

Various deployment strategies have been proposed to delay the onset of resistance<sup>6</sup>, and modeling studies have examined the effect of different deployment strategies<sup>7–10</sup>; however, few empirical data exist. The only commercially available strategy is use of a high dose of a single gene (>LC<sub>90</sub> of heterozygous RS insects) in combination with a refuge. The refuge is composed of nontransgenic plants that

will generate enough SS (homozygous susceptible) individuals to outnumber RR (homozygous resistant) individuals during mating, so that the majority of the population will remain either RS or SS. For cotton in the United States, “expert opinion” in 1994 and a marketing strategy have resulted in the nontransgenic plants being deployed as either a separate refuge in which 20% of the field is planted in nontransgenic plants that can be treated with a non-*Bt* foliar insecticide, or a 4% refuge of nontransgenic plants that are left untreated. The concept is that the refuge will generate enough susceptible insects to dilute resistant alleles while at the same time allowing the nontransgenic plants to generate high yields.

Recently the debate on the appropriate strategies for controlling insects through the use of *Bt* plants has focused on the size of the refuge needed<sup>11</sup>, or indeed whether refuges that are large enough can be economically acceptable to the users or sellers of *Bt* crops. In cotton, for example, some workers have called for a dramatic increase in refuge sizes over the Environmental Protection Agency (EPA) requirements, such as refuges as large as 50%, if farmers are allowed to spray them<sup>12</sup>. The use of current transgenic cultivars thus faces the following dilemma. The maximum benefits to crop production, farm profitability, and reduction of pesticide use may come from larger proportions of transgenic crops, but long-term enjoyment of these benefits may be feasible only by limiting the percentage of the crops that are transgenic. Careful modeling studies and empirical data are needed to address this question.

Testing a resistance management strategy is inherently difficult because it requires both a *Bt*-expressing plant and an insect that has developed resistance to the *Bt* toxin expressed in the plant. For this study, we have used the diamondback moth, *Plutella xylostella*, the only

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insect that has developed resistance to *Bt* toxins in the field<sup>13</sup>, in combination with crucifers engineered to express a Cry1A(c) toxin<sup>14</sup> to study factors that influence the development of resistance. Resistance in this population of diamondback moth was due to a single autosomal recessive gene<sup>15</sup>, and the plants expressed high levels of the toxin<sup>14</sup>.

In greenhouse trials<sup>16</sup> we introduced diamondback moths that had an initially low Cry1A(c) resistance gene frequency into cages with various ratios of *Bt* broccoli and non-*Bt* broccoli plants. The insect populations were allowed to cycle and, after a set number of generations, the larvae were tested for resistance. We found that pure stands of *Bt*-expressing plants (0% refuge) resulted in rapid development of highly resistant diamondback moth populations, and increasing the size of the refuge delayed the development of resistance. Furthermore, the placement of the refuge plants significantly affected the development of resistance. When both plant types were mixed in a random spatial arrangement ("mixed seedling model"), larvae were able to move between plant types. As they moved from refuge plants to *Bt*-expressing plants, they died and caused an overall decline in the number of susceptible alleles. This resulted in a more rapid development of resistance than when plants were separated by a distance that limited the movement of larvae. Additional greenhouse and laboratory data demonstrated that resistant diamondback moths display similar levels of weight gain, growth, and survival on *Bt* plants as they do on non-*Bt* plants<sup>17</sup>.

These studies have documented that *Bt*-resistant insects can survive on *Bt* plants and that different management strategies will influence the durability of resistance. Although these studies provided some insight into variables that could be manipulated to delay the onset of resistance, the present field study was performed to provide further data to help identify variables that may influence resistance management in the field.

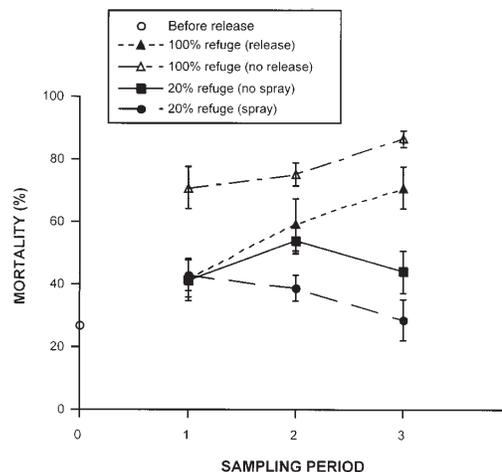
### Results and discussion

Our 1996 field experiment examined the effect of refuge size and refuge placement (mixed vs. separate refuges) on the distribution of the larvae within the plots as well as the level of resistance in diamondback moths at the end of the season. Our results demonstrated that the cumulative number of larvae per plant on refuge plants through the season in the 20% mixed refuge was significantly lower (6.4 vs. 14.6) than the 20% separate refuge (Table 1). This finding indicates that, as in our previous greenhouse experiments, a separate refuge is more effective at conserving the number of susceptible alleles because larvae on these refuge plants will be more likely to survive to adults (either SS or RS) that can mate with RR individuals and thereby reduce the number of RR offspring. This finding provides evidence to support the use of a separate refuge for *Bt*-transgenic crops that are attacked by insects that can move between plants as larvae. On the *Bt*-expressing plants over the season, an average of  $\leq 0.3$  larva was found in any of the treatments, indicating that the diamondback moth population was being controlled by the *Bt*-expressing plants (Table 1). This was also confirmed by the absence of any larvae on the *Bt*-expressing plants at the end of the season. In leaf-dip assays taken through the season, no differences in suscepti-

**Table 1. Sum of average number of larvae per plant from all counts taken in field study, 1996<sup>a</sup>**

Treatment	Number of larvae per plant	
	On refuge	On <i>Bt</i> plant
0% Refuge	–	0.1c
20% Mixed refuge	6.4b	0.3c
20% Separate refuge	14.6a	0.1c
100% Refuge	11.1a	–

<sup>a</sup>Numbers followed by different letters are significantly different ( $p \leq 0.05$ ).



**Figure 1. Mortality of diamondback moth larvae at 10 p.p.m. Javelin in a leaf dip bioassay.**

bility were detected between diamondback moths taken from any of the treatments (Table 2). Furthermore, comparing the level of resistance at the beginning of the test to the level at the end, it appears that the insects actually became more susceptible. This was the result of immigration of native susceptible diamondback moths into our field plots, which diluted the frequency of resistant alleles of the released insects and prevented the establishment of resistance even when R allele frequencies of released larvae were as high as 0.12. This result was not seen in our previous greenhouse studies in which we had a closed system prohibiting immigration. Despite the differences in the number of larvae on refuge plants in the mixed and separate refuges in this field study (Table 1), we were not able to document differences in mortality (Table 2) over the relatively short period of this experiment. However, the differences in larval populations on the refuge plants in these treatments do lay the groundwork for differences in susceptibility to occur given a longer time period.

Our results from this field study might be taken as justification for not needing any refuge within a planting because of the presence of immigrating susceptible alleles. However, such an approach would only be justified if immigration patterns of susceptible insects were well known and had been shown to be consistent. Usually one does not know a priori whether such immigration of susceptible alleles will occur. Under conditions in which there is no such immigration, high levels of resistance and crop damage can occur<sup>16</sup>.

Growers may be unwilling to sacrifice large numbers of refuge plants to delay the onset of resistance. Thus, current recommendations allow the management of insects on these refuge plants through the use of insecticides with a different mode of action than the *Bt*-transgenic plants. The critical question in such a strategy is

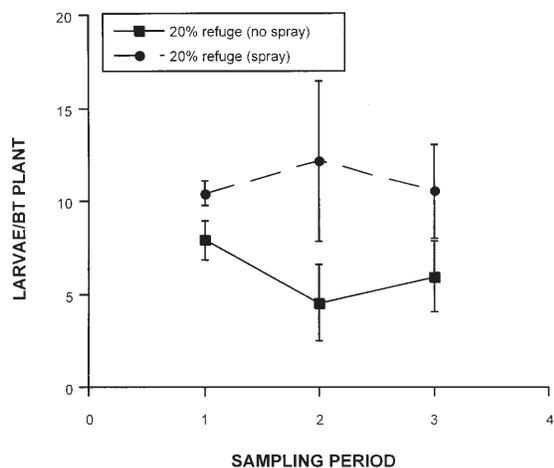
**Table 2. Mortality of larvae to Javelin at the beginning and end of field study, 1996<sup>a</sup>**

Treatment <sup>b</sup>	Percent mortality to Javelin WG (10 p.p.m.)
Time zero larvae	10 <sup>c</sup>
0% Refuge	91a
20% Mixed refuge	–
20% Separate refuge	97b
100% Refuge	98b
	95b

<sup>a</sup>Numbers followed by different letters are significantly different ( $p \leq 0.05$ ).

<sup>b</sup>At the end of the experiment, no larvae were present on any of the Cry1Ac-expressing plants so mortality data were based on progeny of larvae collected off refuge plants only.

<sup>c</sup>Based on genetic studies, 10 p.p.m. was the diagnostic concentration that killed 100% of susceptible and F<sub>1</sub> larvae and 0% of resistant larvae<sup>15</sup>.



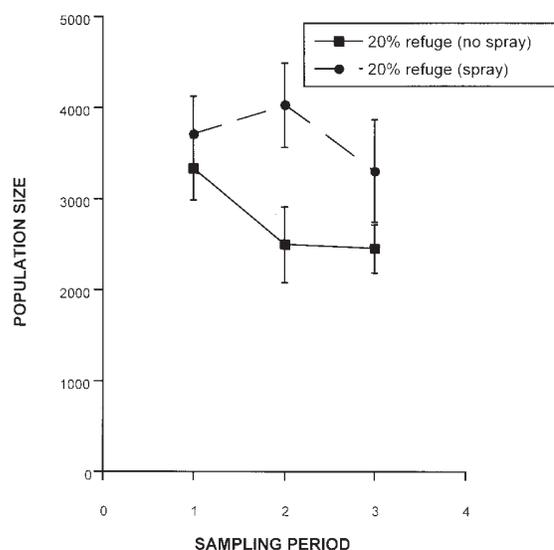
**Figure 2.** Number of diamondback moth larvae per *Bt* broccoli plant.

whether enough susceptible insects will survive in the refuge to provide an effective source of susceptible alleles.

Our 1997 field experiments examined the results of spraying the plants in the 20% separate refuge. Because there is no documented cross-resistance between Cry1C and Cry 1A *Bt* toxins<sup>18,19</sup>, we examined how spraying the refuge with M-C (Mycogen, encapsulated Cry1C) affected DBM larval density and resistance on Cry1Ac broccoli. Our results indicate that in both 100% refuge treatments (where insects were released or where insects were not released), susceptibility increased significantly over time (Fig. 1). With a discriminating dose of 10 p.p.m., the population had a rate of 27% mortality before release into the treatments, but in both 100% refuge treatments the mortality at 10 p.p.m. increased to >70% by the third count. The similar increase in susceptibility in both treatments is indicative of immigration of susceptible insects into those plots, as was also seen in the 1996 field studies. However, despite high rates of immigration of susceptible insects, when resistance allele frequencies in the plot were high, spraying the refuge resulted in progressively higher levels of resistance over the course of the season than when the refuge was not sprayed (Fig. 1). In both the second and third counts, the insect population in the sprayed refuge had a significant and >15% lower average mortality at the diagnostic dose for resistance (10 p.p.m.), compared with the insects in the unsprayed refuge. Insects collected from the *Bt* plants would have a RR genotype for *Bt* var. *kurstaki* resistance, and we consistently found significantly higher numbers of *Bt* var. *kurstaki*-resistant larvae on the *Bt* plants when the refuge was sprayed than when it was not sprayed (Fig. 2). This is the opposite of what should occur if resistant alleles are to be maintained in the refuge for an effective resistance management strategy.

To illustrate this further, we examined the overall diamondback moth population within our experimental plots of 300 broccoli plants. Because each 20% refuge plot had 240 *Bt* plants and 60 refuge plants, a higher number of larvae per *Bt* plant translated to a significantly higher overall population in the plot in the second and third counts when the refuge was sprayed than when not sprayed (Fig. 3). The important point demonstrated here is that spraying the refuge reduces its potential to dilute resistance. By leaving the refuge unsprayed and giving more susceptible insects a chance to survive, short-term sacrifices of relatively more insects in the refuge may translate to seasonal reductions in resistance and reductions in the total number of larvae per plot. The critical question is whether such populations would result in unacceptable crop losses.

The high-dose/refuge strategy is the current foundation for managing pest resistance to *Bt* plants. Whereas the consensus is that the efficiency of this strategy depends on early implementation before the frequency of resistance alleles is high, evaluation under field con-



**Figure 3.** Total number of diamondback moth larvae per plot.

ditions with this criterion is inherently difficult. We can approach such an evaluation by increasing the R allele frequency, as we did with multiple releases, and then assess changes in susceptibility and effectiveness of the refuge in conserving susceptible alleles within a field. Our results indicate that the use of refuges can be a sound strategy. However, this strategy will also depend on our ability to effectively monitor and manage susceptible alleles on an individual field or farm basis as well as on an areawide basis. Within an individual field or farm, treating the refuge with a highly effective insecticide may dilute the abundance of susceptible alleles to such an extent that the refuge is rendered ineffective unless there is substantial immigration of susceptible alleles from wild hosts or from surrounding non-*Bt* crops. On the other hand, growers may be reluctant to sacrifice a large number of refuge plants to insects just to maintain susceptible alleles. An alternative to the strategy of having a 20% refuge that can be sprayed (the requirement for cotton) is the EPA-approved strategy (also in cotton) of having a 4% refuge that remains unsprayed. Critical experiments need to be performed to assess which approach, as well as which refuge size, would be more effective in conserving susceptible alleles while providing acceptable crop yields, and such tests need to be performed in the specific insect/*Bt* crop system.

As we refine resistance management strategies for the currently available *Bt* crops, it is also imperative that other strategies for managing overall resistance to *Bt* be developed and implemented in the near future. Having *Bt* expressed in plants so that the insect population is subjected to selection pressure for particular periods of time (e.g., through an inducible promoter) or in particular plant parts (e.g., through tissue-specific promoters) may provide larger refuges for susceptible alleles both within the field and within a region while at the same time minimizing crop loss<sup>10</sup>. Although this appears to be technically difficult at present, it may be an approach that merits further development. Other options may be more feasible in the near future. Theoretical models suggest that pyramiding two dissimilar toxin genes in the same plant has the potential to delay the onset of resistance much more effectively than single-toxin plants released spatially or temporally<sup>10,20</sup>, and may require smaller refuges. Other non-*Bt* genes may also aid in managing resistance to *Bt* crops. Currently the most promising ones being evaluated in transgenic plants include vegetative insecticidal proteins (vips), as well as various genes from other insects, animals, plants, and bacteria that act as inhibitors of insect digestive enzymes (e.g., protease inhibitors,  $\alpha$ -amylase inhibitors, and cholesterol oxidase<sup>21</sup>).

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The development and implementation of engineered insecticidal plants is currently in its infancy and the only available technology is that of *Bt*-transgenic plants. Using the diamondback moth/*Bt* broccoli system as a model, we have investigated aspects important to the long-term deployment of this novel technology. Although the diamondback moth/*Bt* broccoli system may not exactly duplicate the currently available insect/*Bt* crop systems such as cotton, corn, and potatoes, it can help identify areas for further work. Concurrently with more field studies conducted to refine the presently utilized recommendations, industry, public sector scientists, and farmers must work together to develop a second generation of technology and implementation strategies to ensure the even longer term durability of *Bt*-transgenic plants.

## Experimental protocol

**The diamondback moth.** Using field-collected diamondback moth populations and our laboratory susceptible population (G88) we were able to make synthetic populations of diamondback moth with the desired resistance allele frequency for Cry1A toxins. Field-collected diamondback moth populations that showed resistance to Cry1A were collected in 1996 and 1997. The year the population was collected it was mated with G88 to create the synthetic population, then reared on plant material and re-released in the field. Although these synthetic populations were manipulated for field studies in isolated research plots, there was no carryover through the winter, since the diamondback moth does not overwinter in upstate New York where these tests were conducted<sup>22</sup>.

**The transgenic plants.** Using an *Agrobacterium tumefaciens*-mediated transformation system and a synthetic *cry1A(c)* gene provided by Monsanto, we produced diamondback moth-resistant broccoli plants<sup>23</sup>. The plants allowed survival of about 90% of neonate larvae from the resistant strain but caused 100% mortality of the F<sub>1</sub> neonates heterozygous for resistance<sup>14</sup>. Mortality assays with susceptible larvae using leaves taken from different locations within the plant showed that toxin expression is fairly uniform throughout the plant when the plant is in its vegetative stage<sup>17</sup>. We used cytoplasmic male sterile transgenic plants hemizygous for the *cry1A(c)* gene for field tests.

**Field tests, 1996.** Four treatments with three replicates were arranged in a completely randomized design: 0% refuge, 20% separate refuge, 20% mixed refuge, and 100% refuge (plots were located at the New York State Agricultural Experiment Station in Geneva, NY, three plot replicates per treatment, 300 plants per plot, and 60 m minimum distance separating plots), into which we released second-instar diamondback moth larvae (312 larvae per plot per release) at seven periods over the course of the season, at an approximate R allele frequency of 0.12. Plants were spaced at 46 cm between plants and 90 cm between rows. Plots with a separate refuge had two border rows on one side of the field, and the border rows were separated from the *Bt* plants by one blank row of bare ground. Mixed refuges had non-*Bt* plants randomly assigned within the plot. Releases were initiated on 16 June and continued at one- to three-week intervals until 26 August. We inoculated the plots with insects at various times to ensure high diamondback moth populations, with multiple and overlapping generations and with R alleles. We were simulating what would happen when insect populations immigrate into and cycle within a field, and are challenged by *Bt* plants. Additional treatments were included for reference to reflect native diamondback moth populations and were 0% refuge and 100% refuge with no insect release in either (one replicate each). Counts of larvae (all stages) were taken at five periods over the season beginning on 1 July and ending on 12 September. Leaf-dip bioassays with Javelin WG (*Bt* var. *kurstaki*; Novartis) to evaluate resistance were done with progeny of the released larvae (time zero) and with progeny of larvae counted in the final collection.

**Field studies, 1997.** Four treatments with three replicates were arranged in a completely randomized design: 20% refuge, 20% sprayed refuge (sprays were applied five times at 8- to 13-day intervals throughout the growing season), and 100% refuge into which we released diamondback moth pupae at six periods early in the season at approximate R allele frequencies of 0.8 based on 10 p.p.m. Javelin. The fourth treatment was a 100% refuge with no release. Releases were initiated on 15 May and continued at one- to two-week intervals until 25 June for the same purposes as noted in 1996. Plant and plot spacing were as in 1996. Larval counts (eight plants sampled destructively per plot) were taken at three periods over the season beginning on 22 July and ending on 11 August. In each sample, all larvae counted were taken back to the lab and reared. In each of the 20% refuge plots, larvae were collected from

equal numbers of refuge and *Bt* plants and then combined. Progeny of larval collections and time zero larvae (i.e., representative of what was released into the plots) were used in leaf-dip bioassays with Javelin to evaluate resistance.

Data in Table 1 on the number of larvae per plant were analyzed by ANOVA with the means weighted inversely by their variances. Data in Figures 2 and 3 were analyzed using ANOVA, and nonoverlap of the standard errors indicates significant differences. Because the plots were inoculated with equal numbers of insects, less than normal variation within a treatment probably occurred. The bioassays for insects were conducted using our normal protocol<sup>24</sup>, and the concentration or dose/mortality relationships contained in Table 1 and Figure 1 were estimated assuming a probit model by using POLO<sup>25</sup>.

## Acknowledgments

We thank Melissa Birkett, Hilda Collins, Jennifer Cooley, Bryna Mitchell, Sabrina Siebert, Powell Smith, and Joe Zhao for their assistance in this project. The research was funded by USDA NRI grants 91-37302-6199, 93-01977 and 95-37302-1783.

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