Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis*

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SUMMARY

Theoretical projections suggest that refuges from exposure can delay insect adaptation to environmentally benign insecticides derived from *Bacillus thuringiensis*, but experimental tests of this approach have been limited. We tested the refuge tactic by selecting two sets of two colonies of diamondback moth (*Plutella xylostella*) for resistance to *B. thuringiensis* subsp. *aizawai* in the laboratory. In each set, one colony was selected with no refuge and the other with a 10% refuge from exposure to *B. thuringiensis* subsp. *aizawai*. Bioassays conducted after nine selections were completed show that mortality caused by *B. thuringiensis* subsp. *aizawai* was significantly greater in the refuge colonies than in the no-refuge colonies. These results demonstrate that the refuges delayed the evolution of resistance. Relative to a susceptible colony, final resistance ratios were 19 and eight for the two no-refuge colonies compared to six and five for the refuge colonies. The mean realized heritability of resistance to *B. thuringiensis* subsp. *aizawai* was 0.046 for colonies without refuges, and −0.002 for colonies with refuges. Selection with *B. thuringiensis* subsp. *aizawai* decreased susceptibility to *B. thuringiensis* toxin Cry1Ab, but not to Cry1C or *B. thuringiensis* subsp. *kurstaki*. Although the ultimate test of refuges will occur in the field, the experimental evidence reported here confirms modelling results indicating that refuges can slow the evolution of insect resistance to *B. thuringiensis*.

1. INTRODUCTION

Use of insecticidal proteins from *Bacillus thuringiensis* for pest control is increasing rapidly because of their environmental safety (Entwistle *et al*. 1993) and widespread pest resistance to synthetic insecticides (National Research Council 1986; Roush & Tabashnik 1990). As *B. thuringiensis* is used more extensively in conventional sprays and genetically engineered crops, insect adaptation becomes more likely (Gould 1988; McGaughey & Whalon 1992; Tabashnik 1994*a*). So far, the only documented cases of field-evolved resistance to *B. thuringiensis* are in the diamondback moth (*Plutella xylostella*), a global pest of cruciferous plants (Talekar & Shelton 1993; Tabashnik 1994*a*).

Resistance to *B. thuringiensis* in the diamondback moth has been reported in populations in Hawaii, Florida, New York, China, Japan, Malaysia, the Philippines, and Thailand (Tabashnik *et al*. 1990; Ferré *et al*. 1991; Tanaka & Kimura 1991; Syed 1992; Shelton *et al*. 1993; Zhao *et al*. 1993). Previous studies have documented high levels of resistance to *B. thuringiensis* subsp. *kurstaki* in field and laboratory populations of the diamondback moth, but only low levels of resistance to *B. thuringiensis* subsp. *aizawai* (Fongsmut 1990; Syed 1992; Leibee & Savage 1993; Shelton *et al*. 1993; Tabashnik *et al*. 1993; Liu *et al*. 1996). This pattern may result from the presence of the insecticidal crystal proteins Cry1C and Cry1D in *B. thuringiensis* subsp. *aizawai*, but not in *B. thuringiensis* subsp. *kurstaki* (Höfte & Whiteley 1989; Ferré *et al*. 1991; Tabashnik *et al*. 1993, 1996; Tang *et al*. 1996).

Of the many strategies proposed to delay evolution of resistance to insecticides, refuges from exposure appear to be among the most promising (Comins 1977; Georghiou & Taylor 1977 ; Curtis *et al*. 1978; Curtis 1985) and have received the greatest attention for delaying pest resistance to *B. thuringiensis* (Gould 1988; Mallet & Porter 1992; McGaughey & Whalon 1992; Tabashnik 1994*a*, *b*; Alstad & Andow 1995). Georghiou & Taylor (1977) defined refuges (or refugia) as untreated areas that leave portions of the pest population unexposed to insecticide. In principle, refuges enable the survival of susceptible individuals, which decreases the intensity of selection and slows the evolution of resistance. Under ideal conditions, relatively large numbers of susceptible individuals from refuges survive and mate with few resistant survivors from treated areas. Projections from computer simulations suggest that if resistance is recessive and mating is random, refuges can greatly delay insect adaptation to synthetic insecticides (Comins 1977; Georghiou & Taylor 1977; Curtis *et al*. 1978, 1993; Curtis 1985) or to *B. thuringiensis* (Gould 1988; Mallet & Porter 1992; McGaughey & Whalon 1992; Tabashnik 1994*a*, *b*; Alstad & Andow 1995;). It has also been suggested that naturally-occurring refuges, such as alternate hosts that are not treated with insecticide, have delayed insect adaptation to insecticides (Suckling *et al*. 1985; Tabashnik & Croft 1985; Roush & Daly 1990).

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However, few experimental tests of refuges have been reported.

In the present study, we tested the refuge tactic by selecting colonies of diamondback moth for resistance to *B. thuringiensis* subsp. *aizawai* either with or without 10% refuges. We also examined the effects of selection with *B. thuringiensis* subsp. *aizawai* on responses to *B. thuringiensis* subsp. *kurstaki*, and to the single *B. thuringiensis* toxins, Cry1C and Cry1Ab. The results showed that 10% refuges delayed the evolution of diamondback moth resistance to *B. thuringiensis* subsp. *aizawai*.

2. MATERIALS AND METHODS

(*a*) *Origins and rearing of insect colonies*

We started with two colonies of diamondback moth: LAB-P and NO-93. LAB-P was a susceptible colony that had been reared in the laboratory for more than 130 generations without exposure to insecticides. NO-93 was established from approximately 120 moths derived from a field population that had evolved substantial resistance to *B. thuringiensis* subsp. *kurstaki* and toxin Cry1Ab (Tabashnik *et al*. 1990; Liu *et al*. 1995), moderate resistance to toxin Cry1C, but only slight resistance to *B. thuringiensis* subsp. *aizawai* (Liu *et al*. 1996). NO-93 was reared in the laboratory without exposure to insecticides. All rearing (Tabashnik *et al*. 1990) and experiments were conducted in environmental chambers at 28 °C and a 14 h light:10 h dark photoperiod.

(*b*) **B. thuringiensis** *subspecies and toxins*

We used wettable powder formulations of *B. thuringiensis* subsp. *aizawai* (XenTari) and *B. thuringiensis* subsp. *kurstaki* (Dipel 2X) from Abbott Laboratories (Chicago, IL). Insecticidal proteins in XenTari include Cry1Aa, CryAb, Cry1C, Cry1D, and Cry2B. Dipel contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B (Abbott Laboratories 1992). We used liquid formulations of Cry1C and Cry1Ab toxins (Mycogen, San Diego, CA). Insecticides were diluted with distilled water containing 0.2% of Triton AG-98 (a surfactant, Rohm & Haas Co., Philadelphia, PA).

(*c*) *Selections and bioassays*

Two sets of selection experiments were started with colonies derived from NO-93. Each set had two colonies: one selected with no refuge (SN1 for set one and SN2 for set two), and one selected with a 10% refuge (SR1 for set one and SR2 for set two). Sets one (SN1 and SR1) and two (SN2 and SR2) were derived from NO-93 at the second and third laboratory-reared generations, respectively. We conducted selections in nine of the next ten generations. When one of the two colonies of a set was too small for selection, both the no-refuge and refuge colonies of that set were reared for one generation without selection.

Procedures for exposing larvae to *B. thuringiensis* subsp. *aizawai* in selection experiments were similar to the bioassay methods described previously (Tabashnik *et al*. 1990; Liu *et al*. 1995). Leaf disks (6 cm in diameter) were cut from cabbage grown in the greenhouse, dipped in dilutions of *B. thuringiensis* subsp. *aizawai* for 5 s, allowed to dry, and placed in Petri dishes (9.5 cm in diameter) with moist filter paper disks. A group of 10–15 five-day-old larvae was placed in each Petri dish.

In most selections, ≈ 30 groups of larvae from the no-refuge colony (total $n = 268 \sim 400$) were exposed to leaf discs treated with *B. thuringiensis* subsp. *aizawai*. To estimate control mortality, three groups from the no-refuge colony (total $n \approx 30$) were exposed to untreated leaf discs (dipped in water with 0.2% Triton AG-98). For the refuge colony, 90% of larvae (≈ 27 groups) were exposed to treated discs and 10% (three groups) were exposed to untreated discs. The 10% of larvae from the refuge colony exposed to untreated discs provided an estimate of control mortality as well as a refuge from *B. thuringiensis* subsp. *aizawai*. In each set of selected colonies, the concentration of *B. thuringiensis* subsp. *aizawai* in a particular generation was the same for the no-refuge and refuge colonies. The initial concentration for selection was 62 mg AI l^{-1} (AI stands for 'active ingredient'), which is comparable to half of the recommended maximum field application rate on the product label. We increased the concentration, as necessary, to achieve $\approx$ 60–90% mortality. For the no-refuge colony, moths emerging from untreated groups were discarded, and moths emerging from treated groups were pooled for mating and oviposition to begin the next generation. For the refuge colony, moths emerging from untreated and treated groups were pooled into a single container to produce progeny to begin the next generation.

We used leaf residue bioassays (Liu *et al*. 1995) to evaluate susceptibility to *B. thuringiensis*. Groups of larvae were exposed to treated leaf discs as described above. Mortality was recorded after 5 d. In all bioassays, each concentration of *B. thuringiensis* tested was replicated at least four times. Bioassays with *B. thuringiensis* subspp. *aizawai* and *kurstaki* included four or five concentrations and an untreated control. For each set of colonies, we conducted simultaneous bioassays of the no-refuge colony, the refuge colony, and NO-93 after nine selections had been completed.

To further characterize the resistance of colony SN1, we tested SN1, NO-93, and LAB-P simultaneously with *B. thuringiensis* subsp. *kurstaki*, Cry1C, and Cry1Ab after all selections had been completed. We used four concentrations (0.01, 0.1, 1, and 10 ml l^{-1}) of Cry1C and a single concentration of Cry1Ab (10 ml l^{-1}). We did not include lower concentrations of Cry1Ab because they caused little or no mortality to larvae from the NO-93 and SN1 colonies.

(*d*) *Data analysis*

We used analysis of variance (ANOVA) (Proc GLM, SAS Institute 1985) to test for effects of refuges on mortality of larvae exposed to two diagnostic concentrations of *B. thuringiensis* subsp. *aizawai* after all selections had been completed. Mortality was adjusted for control mortality and transformed by arcsin $\sqrt{p}$ (p = proportion of mortality). We used the same analysis to test for effects of concentration, differences between the two sets of selections, and interactions among factors. Compared with conventional probit analysis, ANOVA of responses at diagnostic concentrations is a more powerful approach because (i) it enables an overall test of the effect of refuges, and (ii) it is more sensitive to changes in genotype frequency (Roush & Miller 1986; Tabashnik *et al*. 1987).

We used probit analysis (Proc PROBIT, SAS Institute 1985) to estimate the concentrations of *B. thuringiensis* needed to kill 50% of larvae (LC_{50}s) and the slopes of concentration–mortality lines. LC_{50}s were considered significantly different if their 95% fiducial limits (FL) did not overlap. Resistance ratios were calculated as the LC_{50} of a particular colony divided by the LC_{50} of the susceptible LAB-P colony. Realized heritability (h^2) of resistance was calculated for each selected colony using the method described by Tabashnik (1992). Selection intensities for colonies selected with 10% refuges were weighted by 0.9 to adjust for the effect of refuges in reducing selection pressure.

The percentages of mortality caused by Cry1C and

Cry1Ab to larvae from SN1, NO-93, and LAB-P were transformed by arcsin $\sqrt{p}$ and analysed using ANOVA and the Ryan–Einot–Gabriel–Welsch multiple-range test (Proc GLM, SAS Institute 1985) to check for differences among colonies after selection had been completed.

3. RESULTS

Refuges delayed the evolution of resistance to *B. thuringiensis* subsp. *aizawai* by diamondback moth (figure 1). After completion of nine rounds of selection, mortality at two diagnostic concentrations of *B. thuringiensis* subsp. *aizawai* was significantly higher in the refuge colonies (SR1 and SR2) than in the no-refuge colonies ($p < 0.01$). ANOVA also showed that, as expected, effects of concentration on mortality were significant ($p < 0.001$). Differences between the two sets of colonies and interactions among factors were not significant ($p > 0.1$) (figure 1).

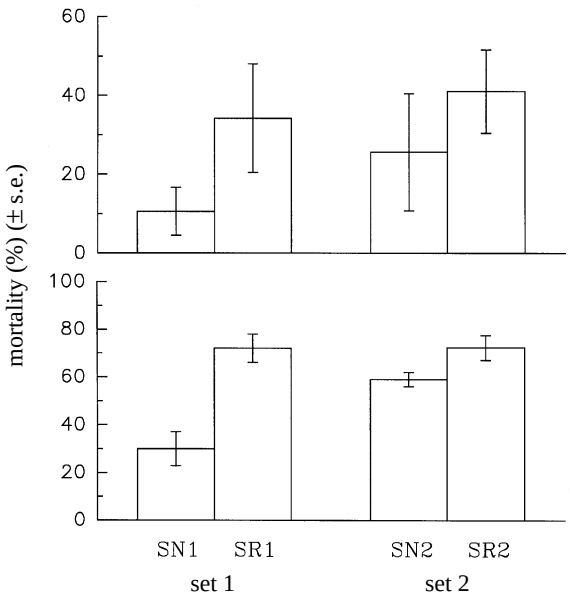

Figure 1. Responses of diamondback moth larvae to *B. thuringiensis* subsp. *aizawai* at 7.7 mg AI l^{-1} (top) or 30.9 mg AI l^{-1} (bottom) after nine generations of selection either without a refuge (SN1 and SN2) or with a 10% refuge (SR1 and SR2). Mortality data represented in the figure were transformed by arcsin $\sqrt{p}$ (p = proportion of mortality) and analysed by three-way analysis of variance (SAS Institute 1985), which revealed significant effects of refuge (d.f. = 1, 24, $F = 12.08$, $p < 0.01$) and concentration of *B. thuringiensis* subsp. *aizawai* (d.f. = 1, 24, $F = 19.36$, $p < 0.001$). Effects of set and interactions were not significant.

The trends in LC_{50}s of *B. thuringiensis* subsp. *aizawai* were similar to the patterns seen with mortality at diagnostic concentrations. Final LC_{50}s (in mg AI l^{-1}) were 44 and 20 for the no-refuge colonies compared with 14 and 11 for the refuge colonies (table 1). However, the LC_{50} of the no-refuge colony was significantly greater than the LC_{50} of the refuge colony in set one, but not in set two (table 1). The final LC_{50}s of *B. thuringiensis* subsp. *aizawai* of the refuge colonies in each set were nearly identical to the respective LC_{50}s of the parental colony (NO-93) that was reared without selection (table 1). Relative to the susceptible colony (LAB-P), final resistance ratios were 19 and eight for the no-refuge colonies compared to six and five for the refuge colonies (table 1).

Estimates of realized heritability (h^2) also reflect the effect of refuges in slowing the evolution of resistance to *B. thuringiensis* subsp. *aizawai*. The mean realized heritability of resistance was close to 0 (-0.001 and -0.002) for the two refuge colonies compared with 0.046 (0.069 and 0.022) for the two no-refuge colonies (table 2).

Responses of no-refuge colony SN1 at the end of the experiment suggest that selection with *B. thuringiensis* subsp. *aizawai* caused little or no increase in resistance to *B. thuringiensis* subsp. *kurstaki* or to toxin Cry1C, but did increase resistance to toxin Cry1Ab. The LC_{50} of *B. thuringiensis* subsp. *kurstaki* did not differ significantly between SN1 and the parental colony NO-93 (table 1). Relative to LAB-P, resistance ratios for *B. thuringiensis* subsp. *kurstaki* were 151 for SN1 and 80 for NO-93

Table 1. *Effects of selection with* B. thuringiensis *subsp.* aizawai *on responses of diamondback moth larvae to* B. thuringiensis *subspp.* aizawai *and* kurstaki

colony*	generation	n	slope ± s.e.	mg AI l^{-1} LC_{50} (95% FL)	RR†
B. thuringiensis subsp. *aizawai*					
SN1	15	400	1.8 ± 0.2	44 (33–58)	19
SR1	15	200	1.3 ± 0.2	14 (7–22)	6
NO-93‡	15	400	1.9 ± 0.3	14 (10–19)	6
SN2	17	200	1.7 ± 0.4	20 (8–36)	8
SR2	17	200	1.7 ± 0.3	11 (7–17)	5
NO-93‡	17	200	1.4 ± 0.2	12 (6.2–19)	5
LAB-P§	—	720	1.2 ± 0.1	2.4 (1.4–3.4)	1
B. thuringiensis subsp. *kurstaki*					
SN1	15	200	2.5 ± 0.6	136 (85–215)	151
NO-93‡	15	200	1.6 ± 0.3	72 (49–106)	80
LAB-P‡	—	480	1.5 ± 0.2	0.9 (0.7–1.3)	1

* LAB-B, susceptible; NO-93, unselected parental colony; SN1 and SN2, selected with no refuge; SR1 and SR2, selected with a 10% refuge.
† Resistance ratio; LC_{50} of tested colony/LC_{50} of LAB-P.
‡ From Liu *et al.* (1996).
§ Based on results pooled from three bioassays, including two from Liu *et al.* (1996).

Table 2. *Estimation of realized heritability (h^2) of resistance to* B. thuringiensis *subsp.* aizawai *in diamondback moth*

		estimation of mean response per generation			estimation of mean selection differential per generation						
colony*	n†	log(initial LC_{50})‡	log(final LC_{50})	R	p§	i‖	initial slope	final slope	$\sigma\hat{p}$	S	h^2
SN1	9	1.15	1.64	0.0547	17.2	1.48	1.94	1.81	0.53	0.79	0.069
SR1	9	1.15	1.14	−0.0011	11.1	1.54	1.94	1.33	0.61	0.94	−0.001
SN2	9	1.08	1.30	0.0239	11.5	1.69	1.37	1.73	0.65	1.09	0.022
SR2	9	1.08	1.06	−0.0027	7.4	1.70	1.37	1.72	0.65	1.10	−0.002

* SN1 and SN2, selected with no refuge; SR1 and SR2, selected with a 10% refuge.
† Number of generations selected.
‡ LC_{50}s and slopes from table 1.
§ Mean percentage survival to adult emergence of treated individuals adjusted for control mortality using Abbott's method.
‖ Selection intensity; obtained from Appendix A of Falconer (1989).

Table 3. *Responses of diamondback moth larvae to Cry1C and Cry1Ab toxins of* B. thuringiensis

		Cry1C		Cry1Ab (10 ml l^{-1})	
colony	generation	n	% mortality ± s.e.*	n	% mortality ± s.e.
LAB-P	—	120	85.0 ± 5.6 a†	40	100 a
NO-93	17	120	54.3 ± 11.2 b	40	55.0 ± 9.6 b
SN1	17	120	43.2 ± 12.0 b	40	15.0 ± 8.7 c

* Mean mortality after 5 days at the three highest concentrations of Cry1C (0.1, 1.0, 10 ml l^{-1}).
† Data were transformed by arcsin $\sqrt{p}$ (p = proportion of mortality) prior to analysis of variance.
Values in each column followed by different letters were significantly different (Ryan–Einot–Gabriel–Welsch multiple range test, $p < 0.05$).

(table 1). Variation in mortality among colonies was significant in response to either Cry1C (d.f. = 2, 27, $F = 20.1$, $p < 0.0001$) or Cry1Ab (d.f. = 2, 9, $F = 39.7$, $p < 0.0001$). Cry1C caused similar mortality in SN1 and NO-93; both of these colonies had significantly lower mortality than LAB-P (table 3). For Cry1Ab, mortality was significantly lower for SN1 than for NO-93 and significantly lower for NO-93 than for LAB-P (table 3).

4. DISCUSSION

Results from the present study indicate that a 10% refuge helped to maintain susceptibility of diamondback moth to *B. thuringiensis* subsp. *aizawai*. Thus, these data provide experimental evidence supporting the conclusion from mathematical models that refuges can delay insect adaptation to *B. thuringiensis* (Gould 1988; Mallet & Porter 1992; McGaughey & Whalon 1992; Tabashnik 1994*a*, *b*; Alstad & Andow 1995).

Although resistance of diamondback moth to *B. thuringiensis* subsp. *aizawai* evolved faster in colonies selected without refuges than in colonies with refuges, the estimates for realized heritability of resistance in the two colonies selected without refuges were only 0.069 and 0.022. These values are less than the lowest value of realized heritability of resistance to *B. thuringiensis* subsp. *kurstaki* from a previous study of three colonies of diamondback moth ($h^2 = 0.14$, 0.17, 0.18; Tabashnik 1992). Higher heritability of resistance to *B. thuringiensis* subsp. *kurstaki* than to *B. thuringiensis* subsp. *aizawai* could reflect greater exposure to *B. thuringiensis* subsp. *kurstaki* in the field, more difficulty inherent in diamondback moths evolving resistance to *B. thuringiensis* subsp. *aizawai*, or both.

Selection of colony SN1 with *B. thuringiensis* subsp. *aizawai* decreased susceptibility to Cry1Ab but not to Cry1C, which supports the idea that resistance to these two toxins in diamondback moth is controlled by independently-segregating genes (Liu *et al.* 1996). Thus, the diversity of toxins in *B. thuringiensis* subsp. *aizawai* might have slowed evolution of resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth.

Comparisons with related work show that resistance to *B. thuringiensis* subsp. *aizawai* evolved much more readily in Indianmeal moth (*Plodia interpunctella*) than in diamondback moth. The realized heritability of resistance to *B. thuringiensis* subsp. *aizawai* was 0.43 in Indianmeal moth colony 343R, which had been selected previously for resistance to *B. thuringiensis* subsp. *kurstaki* (McGaughey & Johnson 1992; Tabashnik & McGaughey 1994). Two colonies of Indianmeal moth derived from about 100 susceptible adults also had a relatively high realized heritability of resistance to *B. thuringiensis* subsp. *aizawai* (mean = 0.29) (McGaughey & Johnson 1992; Tabashnik & McGaughey 1994).

Laboratory selection experiments such as ours can show the potential of refuges for delaying pest resistance to *B. thuringiensis*, but their correspondence with field outcomes is uncertain. For example, our experiments used relatively small colonies of diamondback moth, which might have lacked rare resistance genes that occur in large field populations. We used a moderate

selection intensity, which would tend to reduce the effect of refuges (Mallet & Porter 1992). In our refuge colonies, we pooled survivors from treated and untreated leaves in mating containers. This approach eliminates the possibility of isolation by distance between susceptible and resistant individuals, and would therefore enhance the effectiveness of refuges (Tabashnik 1994*b*). We used a 10% refuge, which is much smaller than the size suggested by some researchers (see Alstad & Andow 1995), but more than double the minimum refuge size currently required for transgenic cotton in the USA.

Deployment of genetically engineered plants that produce *B. thuringiensis* toxins has generated much interest in refuges for delaying the evolution of pest resistance. Because the first generation of genetically engineered crops express *B. thuringiensis* toxins throughout the growing season, such crops accentuate the need for refuges or other tactics to delay resistance. In principle, however, refuges can be effective whether *B. thuringiensis* toxins are used in transgenic plants, transgenic micro-organisms, or in conventional sprays. Refuges may also help to delay the evolution of resistance to other biological insecticides as well as to synthetic insecticides. The ultimate test of refuges will occur in the field, but in the meantime, available theoretical and empirical results suggest that refuges can delay insect adapatation to *B. thuringiensis*.

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