Field Development of Resistance to *Bacillus thuringiensis* in Diamondback Moth (Lepidoptera: Plutellidae)

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ABSTRACT Foliar applications of commercial formulations of the insecticidal spore-crystal protein complex of *Bacillus thuringiensis* subsp. *kurstaki* caused development of resistance in field populations of a major lepidopteran pest of vegetables, diamondback moth, *Plutella xylostella* (L.). Laboratory bioassays of larvae showed that the LC_{50} and LC_{95} for a field population of diamondback moth treated repeatedly with *B. thuringiensis* were 25 to 33 times greater than the respective LC_{50}'s and LC_{95}'s for two susceptible laboratory colonies. Mortality at the field rate of *B. thuringiensis* was 34–35% in two resistant populations compared with 90–100% in two susceptible laboratory colonies. The results suggest that the potential for resistance development in pest populations is an important consideration for deployment of *B. thuringiensis* toxin genes in genetically-engineered crop plants and use of *B. thuringiensis* in related tactics.

KEY WORDS Insecta, *Bacillus thuringiensis*, insecticide resistance, microbial pesticide

MORE THAN $3 BILLION is spent worldwide each year to control insect pests (Wilcox et al. 1986). Microbial insecticide sales account for <1% of this total but have been projected to increase to as much as 50% by the year 2000 (Klausner 1984). The most widely used microbial insecticide is a mixture of spores and protein crystals from the lepidopteran pathogen *Bacillus thuringiensis* subsp. *kurstaki* (*B. thuringiensis*) (Flexner et al. 1986, Wilcox et al. 1986). Because *B. thuringiensis* is not toxic to humans, most beneficial insects, and other nontarget organisms, it does not cause the serious environmental and safety problems associated with conventional synthetic insecticides (Flexner et al. 1986, Wilcox et al. 1986). Use of *B. thuringiensis* is expected to increase dramatically because of recent advances in genetic engineering, including insertion and expression of *B. thuringiensis* toxin genes in several major crop plants such as cotton, tobacco, and tomato (Gasser & Fraley 1989). *B. thuringiensis* genes have been transferred also into plant-colonizing bacteria to enhance toxin delivery and persistence (Lindow et al. 1989). Development of new strains of *B. thuringiensis* with enhanced potency and altered host ranges will also increase its usefulness (Ferro & Gelernter 1989, Zehnder & Gelernter 1989).

Pest resistance to conventional insecticides is widespread (National Research Council 1986, Roush & Tabashnik in press); similar resistance to *B. thuringiensis* is a potential threat to the future of insect pest control (Gould 1988a,b; Raffa 1989). Despite its use for 20 yr, the lack of reports of resistance to *B. thuringiensis* led to the presumption that resistance was unlikely, perhaps because of its unique mode of action (Bowman 1981, Briese 1981, Wilcox et al. 1986, Wilding 1986, de Barjac 1987).

In previous studies, laboratory selection increased resistance to *B. thuringiensis* in Indianmeal moth, *Plodia interpunctella* (Hübner) (McGaughey 1985, McGaughey & Beeman 1988), and tobacco budworm, *Heliothis virescens* (F.) (Stone et al. 1989). Colonies of *P. interpunctella* established from populations in grain bins treated with *B. thuringiensis* were significantly more tolerant than colonies from populations in untreated bins, but the mean LC_{50} for treated bins was only 1.2 times higher than the mean for untreated bins (McGaughey 1985).

Based on field trials done at one site, Kirsch & Schmutterer (1988) reported low efficacy of *B. thuringiensis* against diamondback moth, *Plutella xylostella* (L.), a worldwide pest of cole crops (Talekar 1986). Although development of resistance is one possible explanation for their results, alternative hypotheses cannot be excluded. To our knowledge, development of substantial resistance to *B. thuringiensis* in open field populations has not previously been documented.

We included tests for susceptibility to *B. thuringiensis* as part of a long-term investigation of insecticide resistance in diamondback moth (Tabashnik 1986; Tabashnik et al. 1987, 1988; Tabashnik & Cushing 1989). Our initial objectives in the study reported here were to determine if populations of diamondback moth in Hawaii vary significantly in susceptibility to *B. thuringiensis* and to establish baseline data on susceptibility. In particular, we tested the hypothesis that a population from a farm that had been treated repeatedly

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with *B. thuringiensis* was less susceptible to *B. thuringiensis* compared with other populations. Additional tests were done 2 yr after the initial survey to determine if use of *B. thuringiensis* during the intervening period had increased resistance to *B. thuringiensis* in the treated population. Here we report evidence that commercial foliar applications of *B. thuringiensis* caused development of resistance in field populations of diamondback moth.

Materials and Methods

Field Sites and Sampling of Insects. In our initial survey during November 1986–May 1987, we sampled 50–300 individuals from each of six field populations in Hawaii. One population (referred to as SO) was sampled from a commercial watercress, *Nasturtium offinicale* (R. Br.), farm that had been treated with *B. thuringiensis* 50–100 times during 1978–1982. At the time of this survey, *B. thuringiensis* had not been used at this 4-ha farm since 1982 because the grower suspected that *P. xylostella* was becoming resistant to it. The five other field sites sampled in the initial survey were commercial farms (KM, cabbage; KH, broccoli) and cabbage plots at University of Hawaii Experiment Stations (WO, PO, LH). Based on interviews with management personnel at each site, we estimated that each of these five populations had received fewer than 10 treatments of *B. thuringiensis* before our initial sample. SO, WO, and PO are located on the island of Oahu; KH and LH are on the island of Hawaii; KM is on Maui. Precise locations and background information are given by Tabashnik et al. (1987). Two laboratory colonies, LAB-P and LAB-L, also were included in the initial survey. The LAB-P and LAB-L colonies were founded with individuals from Pulehu, Maui, and Leeward, Oahu, respectively. Before the 1986–1987 bioassays with *B. thuringiensis* (see below), LAB-P had been maintained for about 60 generations and LAB-L had been maintained for 13 generations without exposure to insecticides.

During 1988–1989, the grower at the SO site applied Javelin (Sandoz, Des Plaines, Ill.), a new commercial formulation of *B. thuringiensis* derived from the NRD-12 strain of *B. thuringiensis* subsp. *kurstaki*, 15 times to control diamondback moth. Applications of 1.9 liters per ha were made on the following dates: 4 March, 8 May, 26 May, 13 June, 24 June, 22 July, 25 July, 10 August, 19 August, 11 November, 23 November, 1 December, and 9 December in 1988 and 3 May and 12 May in 1989. The grower reported an apparent decline in efficacy from the initial to the latter treatments.

To check for the possibility of increased resistance to *B. thuringiensis* between the initial survey (1986–1987) and 1989, we collected about 140 individuals from SO on 18 May 1989 and tested their offspring for susceptibility to *B. thuringiensis* as in previous bioassays (see below). During 1989 we also retested both untreated laboratory colonies (LAB-L and LAB-P). We also resampled and retested the minimally treated WO population. On 17 August 1989, we sampled approximately 80 individuals from a second commercial watercress farm (NO) where *B. thuringiensis* had been used extensively. The NO farm is approximately 4 km from SO. Detailed spray records were not kept at NO. The grower reported use of various *B. thuringiensis* formulations for at least 8 yr, as often as two to four times monthly. A rough estimate of *B. thuringiensis* use at NO from 1982 to 1989 is 50–400 treatments. The grower at NO also reported an apparent decline in efficacy through time. Several other small (<2 ha) watercress farms are within 5 km of SO and NO. Growers' reports of *B. thuringiensis* use at these other farms varied from none to frequent treatments.

Larvae were reared in the laboratory on untreated cabbage at approximately 28°C with a photoperiod of 14:10 (L:D). With the exception of the two laboratory colonies, larvae used in bioassays were F_1, F_2, or F_3 offspring of field-collected individuals.

Bioassays. We used a leaf residue bioassay (Tabashnik & Cushing 1987, Tabashnik et al. 1987) to test larvae approximately 5 d old (third instar). Disks (6 cm diameter) were cut from fully expanded leaves of cabbage grown from seed ('C-G Cross,' Takii and Company, Kyoto, Japan) in the greenhouse. Disks were dipped for 5 s in distilled water dilutions of wettable powder formulations of the HD-1 strain of *Bacillus thuringiensis* subsp. *kurstaki* and hung vertically to air dry at about 23°C for 2 h. Each disk was then placed in a plastic Petri dish. Five to 11 (usually 10) larvae were placed on each disk (one replicate) and allowed to feed for 48 h at 28°C before they were checked for mortality.

Tests done in 1986–1987 used Dipel (Abbott Laboratories, North Chicago, Ill.) containing 16,000 IU of potency per mg; tests in 1989 used Dipel 2X (Abbott) with 32,000 IU of potency per mg. All concentrations were based on mg active ingredient (AI)/liter, which adjusted the difference in percentage of active ingredient between Dipel (3.2%) and Dipel 2X (6.4%). A logarithmic series of five concentrations ranging from 0.256 to 2,560 mg (AI)/liter plus a distilled water control were used in all tests during 1986–1987 and initial tests done in 1989. Because the lowest and highest concentrations (0.256 and 2,560 mg [AI]/liter) generally caused close to 0 or 100% mortality, respectively, they were replaced with two intermediate concentrations (51.2 and 128 mg [AI]/liter) in many of the 1989 tests. All tests included concentrations of 2.56, 25.6, and 256 mg (AI)/liter, which are equivalent to the recommended field rate times 0.1, 1.0, and 10, respectively.

Each test was replicated 4–16 times. Overall control mortality was 1.7% (16/949). The 1986–1987

Table 1. Concentration–mortality responses of *P. xylostella* larvae to *B. thuringiensis*

	Date	*n*	Slope ± SE	LC_{50} (95% FL),[a] mg (AI)/liter	RR[b] at LC_{50}	LC_{95} (95% FL),[a] mg (AI)/liter	RR[b] at LC_{95}
Untreated laboratory colonies							
LAB-P	1986–1987	240	1.24 ± 0.17	1.76 (1.05–2.89)	1.0	37.6 (17.7–126)	1.0
LAB-P	1989	957	1.43 ± 0.11	2.51 (1.89–3.22)	1.4	35.8 (25.7–54.3)	1.0
LAB-L	1986–1987	242	2.13 ± 0.54	2.42 (1.34–3.60)	1.4	14.2 (7.84–69.6)	0.4
LAB-L	1989	240	1.26 ± 0.18	2.57 (1.48–4.28)	1.5	30.3 (15.3–90.3)	0.8
Heavily treated field populations							
SO	1986–1987	479	1.20 ± 0.11	10.2 (5.70–16.9)	5.8	236 (112–782)	6.3
SO	1989	952	1.12 ± 0.09	24.1 (17.7–32.3)	13.7	707 (422–1,400)	18.8
NO	1989	475	1.38 ± 0.18	63.9 (46.1–89.0)	36.3	998 (518–2,980)	26.5
Minimally treated field populations							
WO	1986–1987	240	1.08 ± 0.21	3.67 (1.25–8.81)	2.1	124 (39.0–1,320)	3.3
WO	1989	953	1.60 ± 0.10	6.33 (5.03–7.76)	3.6	68.0 (53.0–91.7)	1.8
PO	1986–1987	238	1.58 ± 0.21	6.72 (4.34–10.5)	3.8	73.3 (38.7–200)	1.9
KH	1986–1987	210	1.44 ± 0.22	6.58 (3.82–11.2)	3.7	91.2 (43.2–322)	2.4
LH	1986–1987	240	1.15 ± 0.13	11.9 (7.16–20.0)	6.8	321 (148–1,020)	8.5
KM	1986–1987	240	1.30 ± 0.19	1.56 (0.89–2.57)	0.9	28.9 (14.1–92.6)	0.8

[a] 95% fiducial limits.
[b] RR is the resistance ratio determined by dividing the LC_{50} or LC_{95} for a population by the respective LC_{50} or LC_{95} for the susceptible LAB-P colony 1986–1987.

test of SO and all tests in 1989 (except LAB-L) were replicated across at least two different days.

Analysis. LC_{50}, LC_{95}, and slope were estimated for each bioassay with probit analysis (SAS Institute 1985) as described in Tabashnik et al. (1987). Analysis of variance (ANOVA) of arcsine-transformed percentage of mortality was performed to make planned contrasts between bioassays; the GLM procedure (SAS Institute 1985) was used as described by Tabashnik et al. (1987). Two-way ANOVA was done with concentration as one independent variable. The other independent variable was either population or sampling date (i.e., 1986–1987 versus 1989), depending on the contrast. An implicit assumption of these ANOVA tests is that control mortality is similar across bioassays; otherwise mortality factors other than insecticide could cause significant differences between bioassays. This assumption was met in all of the planned contrasts except WO 1986–1987 versus 1989. Control mortality for WO in 1986–1987 was the highest of any bioassay (9.8%), whereas it was low in 1989 (0.6%). Rather than using ANOVA in this case, we calculated mortality corrected for control mortality (Abbott 1925) at each of the three concentrations (2.56, 25.6, and 256 mg [AI]/liter) that were used in 1986–1987 and 1989 tests. As a rough index of relative expected mortality in the field, we calculated the corrected mortality (Abbot 1925) at the field rate for all bioassays.

Results

1986–1987. Tests done during 1986–1987 showed that the heavily treated SO population was significantly more resistant to *B. thuringiensis* than either of the two laboratory strains. The LC_{50} (10.2 mg [AI]/liter) and LC_{95} (236 mg [AI]/liter) were about six times higher for the SO strain compared with the susceptible LAB-P strain (Table 1). The SO strain had significantly higher LC_{50} than either the LAB-P or LAB-L strains, as indicated by nonoverlapping 95% FL (Table 1). The LC_{95} for SO was significantly greater than the LC_{95} for LAB-L, but the 95% FL at the LC_{95} overlapped between SO and LAB-P. ANOVA showed that SO had significantly lower overall mortality than LAB-P ($F = 10.7$; df = 1, 50; $P = 0.002$) or LAB-L ($F = 8.5$; df = 1, 50; $P = 0.005$).

Compared with the most susceptible field population (KM), the SO population was 6.5-fold more resistant at the LC_{50} and 8.3-fold more resistant at the LC_{95} (Table 1). The LC_{50} and LC_{95} for the SO population in 1986–1987 were less than twice the mean LC_{50} and LC_{95} for the five minimally treated field populations (6.1 ± 1.7 SE and 127.5 ± 50.7 SE mg [AI]/liter, respectively).

1989. Bioassay results showed a significant increase in resistance to *B. thuringiensis* in the SO population between 1986–1987 and 1989. The LC_{50} for SO in 1989 (24.1 mg [AI]/liter) was more than twice the LC_{50} for SO in 1986–1987 (Table 1). ANOVA showed that SO had significantly lower overall mortality in 1989 compared with 1986–1987 ($F = 11.1$; df = 1, 94; $P = 0.001$).

To check the possibility that changes in the responses of the SO population were the result of differences in the bioassay rather than development of resistance, we compared the 1989 results for the SO population with 1989 tests of LAB-P, LAB-L, and WO strains using the same batch of *B. thuringiensis*. The LC_{50}'s and LC_{95}'s for the two untreated laboratory colonies and the minimally treated WO population showed no significant change between the initial tests of 1986–1987 and the tests performed in 1989 (Table 1). ANOVA also showed no significant overall change in mortality between 1986–1987 and 1989 for either LAB-P (*F*

= 0.71; df = 1, 88; P = 0.40) or LAB-L (F = 0.09; df = 1, 50; P = 0.76). The 1986–1987 versus 1989 comparison for the WO population, which was not amenable to ANOVA (see *Materials and Methods*), showed that mortality at one-tenth the field rate (2.56 mg [AI]/liter) decreased (56% in 1986–1987, 28% in 1989), whereas mortality was essentially unchanged at the field rate (77% in 1986–1987, 78% in 1989) and 10 times the field rate (97% in 1986–1987, 100% in 1989). The 1989 LC_{50} from the SO population was 9.6, 9.4, and 3.8 times greater than the 1989 LC_{50}'s for the LAB-P, LAB-L, and WO strains, respectively (Table 1). The 1989 LC_{95} for SO was 20, 23, and 10 times greater than the LC_{95} for the LAB-P, LAB-L, and WO strains, respectively (Table 1). The lack of significant change in LC_{50} or LC_{95} of the untreated laboratory colonies or WO and the concurrent, significant increase in resistance in the heavily treated SO population strongly suggest that field applications of *B. thuringiensis* caused evolution of resistance in the SO population.

The heavily treated NO population, sampled only in 1989, had the highest LC_{50} (63.9 mg [AI]/liter) of any population tested. The LC_{50} and LC_{95} of NO were 25 to 33 times greater than the respective LC_{50} and LC_{95} of the susceptible LAB-P and LAB-L colonies (Table 1). The LC_{50} of the NO population was significantly greater than the LC_{50} of all other populations; its LC_{95} was significantly greater than all others except SO and the 1986–1987 LC_{95}'s of WO and LH (Table 1).

Mortality at the Field Rate. Mortality at a concentration of 25.6 mg (AI)/liter, equivalent to the field rate, was 90–100% for the susceptible LAB-P and LAB-L colonies (Table 2). Mortality at the field rate was 60–90% for the minimally treated field populations. In 1986–1987, the SO population had a mortality of 60% at the field rate. The field rate killed only 35% of SO larvae and 34% of NO larvae in the 1989 tests.

Table 2. Mortality of *P. xylostella* larvae at a concentration of *B. thuringiensis* equivalent to the field rate (25.6 mg [AI]/liter)

	Date	Replicates	Dead/total	Mortality[a]
Untreated laboratory colonies				
LAB-P	1986–1987	4	39/41	95%
LAB-P	1989	16	150/160	94%
LAB-L	1986–1987	4	40/40	100%
LAB-L	1989	4	36/40	90%
Heavily treated field populations				
SO	1986–1987	8	51/82	60%
SO	1989	16	56/159	35%
NO	1989	8	27/80	34%
Minimally treated field populations				
WO	1986–1987	4	31/39	77%
WO	1989	16	124/158	78%
PO	1986–1987	4	32/39	82%
KH	1986–1987	4	25/33	74%
LH	1986–1987	4	24/40	60%
KM	1986–1987	4	36/40	90%

[a] Corrected for mortality in water-treated controls (Abbott 1925).

Discussion

Laboratory bioassays showed that two diamondback moth populations that were treated repeatedly with commercial formulations of *B. thuringiensis* developed significant resistance to *B. thuringiensis*. Although the relationship between laboratory bioassays and field efficacy is not always straightforward (ffrench-Constant & Roush in press), responses of larvae to a concentration of *B. thuringiensis* equivalent to the recommended field rate suggest that the levels of resistance attained by the two heavily treated field populations may be sufficiently high to substantially reduce field efficacy. Considered in conjunction with growers' reports of reduced effectiveness, these bioassay data suggest that the observed resistance to *B. thuringiensis* has practical as well as statistical significance.

Significant intraisland variation in diamondback moth resistance to DDT, diazinon, permethrin, and fenvalerate suggested that gene flow was not sufficient to overcome differences in insecticide susceptibility between populations in Hawaii (Tabashnik et al. 1987). Significant differences in resistance to *B. thuringiensis* among diamondback moth populations on the island of Oahu support the hypothesis (Tabashnik et al. 1987) that local variation in resistance is caused by local variation in insecticide use. Preliminary analyses of electrophoretic data (M. Caprio & B.E.T., unpublished data) also suggest that gene flow among diamondback moth populations in Hawaii is too low to counteract selection for resistance.

Available data suggest that resistance to *B. thuringiensis* resulted from selection by *B. thuringiensis* rather than cross-resistance from selection by other insecticides. The mode of action of *B. thuringiensis* differs from that of conventional insecticides (Harvey et al. 1986, de Barjac 1987), which would tend to reduce the potential for cross-resistance. Although cross-resistance among conventional insecticides in diamondback moth is common (Tabashnik et al. 1987), evidence from Taiwan suggests that cross-resistance between conventional insecticides and *B. thuringiensis* is unlikely. Compared with a susceptible strain of diamondback moth, the BC strain from Taiwan was 33 to >50,000 times more resistant to 23 insecticides representing all major classes of conventional insecticides, yet its LC_{50} for *B. thuringiensis* was less than twice the LC_{50} of the susceptible strain (Sun et al. 1986). In a similar study, two strains of *Anopheles albimanus* Wiedemann and five strains of *Culex quinquefasciatus* Say representing the principal mechanisms of resistance to conventional insecticides lacked cross-resistance to *Bacillus thuringiensis* subsp. *israelensis* (Sun et al. 1980).

In contrast with the lack of cross-resistance between conventional insecticides and *B. thuringiensis*, McGaughey & Johnson (1987) found that selection with Dipel increased *P. interpunctella* resistance to 36 of 57 *B. thuringiensis* isolates tested. In particular, selection with Dipel (derived from the HD-1 strain of subspecies *kurstaki*), caused cross-resistance to 16 other isolates from subspecies *kurstaki*. Our data suggest that field applications of Javelin (derived from the NRD-12 strain of subspecies *kurstaki*), increased diamondback moth resistance to Dipel in the SO population. These results are consistent with the previously demonstrated trend of cross-resistance between different isolates of subspecies *kurstaki*. We did not address the issue of cross-resistance between different subspecies of *B. thuringiensis*.

The limited comparative data available suggest that results of laboratory selection experiments may not accurately reflect the potential for resistance to *B. thuringiensis* in the field. Resistance to *B. thuringiensis* progressed rapidly in six laboratory selection experiments with *P. interpunctella*, yet only low levels of resistance were found in field populations (McGaughey 1985, McGaughey & Beeman 1988). Conversely, 10 and 30 generations of laboratory selection with diamondback moth failed to significantly increase its resistance to *B. thuringiensis* in two independent experiments (Devriendt & Martouret 1976, Krieg & Langenbruch 1981).

Much additional work is needed to elucidate the mechanistic and genetic basis of field-derived resistance to *B. thuringiensis* and the extent of cross-resistance to different *B. thuringiensis* toxins. Because the basis of field-derived resistance sometimes differs from resistance produced by laboratory selection (Roush and McKenzie 1987), it will be essential to include resistant field strains in such studies.

The lack of previous reports of substantial field resistance to *B. thuringiensis* led many to conclude that such resistance was unlikely, particularly in defoliating crop pests. Results of our study show that resistance to *B. thuringiensis* can develop in the field in response to relatively transitory foliar applications. Expression of *B. thuringiensis* toxin genes in crop plants and related advances in technology are likely to intensify selection for resistance to *B. thuringiensis* (Gould 1988a,b; Raffa 1989). The principles of integrated pest management and pesticide resistance management (National Research Council 1986, Roush & Tabashnik in press) provide a general framework for devising methods to prolong the efficacy of *B. thuringiensis*. Specific tactics that may be useful include tissue-specific and facultative expression of toxin genes in genetically engineered crop cultivars, incorporation of untreated refugia, and spatial and temporal variation in control methods (Gould 1988a,b; Raffa 1989). The efficacy of an extraordinarily specific and environmentally safe microbial insecticide may be rapidly lost to pest resistance if *B. thuringiensis* is not deployed wisely.

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