Greenhouse Tests on Resistance Management of Bt Transgenic Plants Using Refuge Strategies

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ABSTRACT Experimental evaluation of the effectiveness of resistance management tactics is vital to help provide guidelines for the deployment of transgenic insecticidal crops. Transgenic broccoli expressing a Cry1Ac gene of *Bacillus thuringiensis* (Bt) and the diamondback moth, *Plutella xylostella* (L.), were used in greenhouse tests to evaluate the influence of size and placement of nontransgenic refuge plants on changes in resistance allele frequency and pest population growth. In the first test with an initial Cry1Ac-resistance (R) allele frequency of 0.007, *P. xylostella* were introduced into cages with the following treatments: 0, 3.3, 10, 20, and 100% refuge plants. Results after four generations showed that resistance could be delayed by increasing the proportion of refuge plants in the cage. Population growth was also influenced by refuge size with the highest populations occurring in treatments that had either no refuge plants or all refuge plants. In the second test, we evaluated the effect of refuge placement by comparing 20% separate and 20% mixed refuges. *P. xylostella* with an initial frequency of resistant alleles at 0.0125 were introduced into cages and allowed to cycle; later generations were evaluated for resistance and population growth. Separating the refuge had a pronounced effect on delaying resistance and slowing establishment of resistant larvae on Bt plants. Combining information from both trials, we found a strong negative correlation between the number of larvae on Bt plants and the mortality of the population in leaf dip bioassays. Results from larval movement studies showed that separate refuges delayed resistance better than mixed refuges because they conserved relatively more susceptible alleles than R alleles and did not increase the effective dominance of resistance.

KEY WORDS *Plutella xylostella*, *Bacillus thuringiensis*, resistance, transgenic plants

PLANTINGS OF TRANSGENIC crops expressing insecticidal proteins of *Bacillus thuringiensis* (Bt) increased rapidly after their first commercialization in 1996. In the United States the percent of total acreage in 1996 planted in Bt crops was <1% for Bt corn (≈16,000 ha), 14% for Bt cotton (≈700,000 ha), and <1% for Bt potato (≈4,000 ha). By 1999, U.S. farmers had planted nearly 8 million hectares of transgenic Bt crops (USDA-EPA 1999). Reductions in insecticide use have been documented with the introduction of Bt plants, especially cotton (USDA-EPA 1999). However, there is concern that these gains will be short-lived due to the evolution of resistance to Bt in the pests (Gould 1998, Mellon and Rissler 1998). Various deployment strategies have been proposed to delay the onset of resistance. These fall into four tactics, some of which are not mutually exclusive (McGaughey and Whalon 1992). The strategy most widely adopted is use of a high dose of a single gene with a refuge. The key assumptions for this strategy include a high dose expression of Bt toxin to kill essentially all SS and most SR insects, recessive inheritance of resistance, a low initial resistance gene frequency, and random mating of adults. The refuge is composed of nontransgenic plants, which will generate enough SS individuals to outnumber RR individuals during mating so that the majority of the population will remain either RS or SS. For example, in cotton the nontransgenic plants are deployed as a separate refuge in which either 4% of the field is planted with nontransgenic plants which will be left unsprayed or 20% of the field is planted in nontransgenic plants which can be treated with a non-Bt foliar insecticide. The concept is that either refuge will still generate enough susceptible insects to dilute resistant alleles while at the same time allowing the nontransgenic plants to be marketable.

Various modeling studies have examined the effect of different deployment strategies (Tabashnik 1994b; Alstad and Andow 1995; Roush 1996, 1997a, 1997b); however, no empirical data exist on the effects of refuge size. To gather empirical data one must have both a resistant insect and a Bt transformed plant. The diamondback moth, *Plutella xylostella* (L.), is the only insect pest that has developed resistance to Bt insec-

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ticides under field conditions (Shelton et al. 1993, Tabashnik 1994a, Tang et al. 1996). It is also one of only three insects (the others being the tobacco budworm and the pink bollworm) that have populations that have been able to survive and reproduce on Bt transgenic crops (Gould et al. 1997, Ramachandran et al. 1998, Tang et al. 1999, Liu et al. 1999). In the current study we used *P. xylostella* in combination with broccoli engineered to express a Cry1Ac toxin of Bt as a model system in greenhouse tests to examine factors that influence the development of resistance. The inheritance of Bt resistance in the filed population for this study was incompletely recessive and probably controlled by a single allele (Tang et al. 1997).

Materials and Methods

Plants. Cytoplasmic male sterile broccoli, *Brassica oleracea* L. subsp. *italica*, were transformed (Metz et al. 1995) using *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pMON10517-1 (Monsanto, St. Louis, MO). The latter carried the neomycin phosphotransferase gene and a full-length, synthetic, *B. thuringiensis* Cry1Ac-like gene, derived from HD-73. Progeny were produced by pollinating transformed plants with Green Comet hybrid broccoli. Toxin expression in the progeny was verified by screening the plants with *P. xylostella* neonates when plants were 4–5 wk old. About 30 eggs from the susceptible colony (see below) were placed onto each plant and, after 144 h in the greenhouse (27 ± 2°C), the number of hatched eggs and the number of live larvae were counted. Only those plants that showed 0% survival (average hatch rate was 67.2%) were categorized as high expressing and were used as Bt plants in the greenhouse tests. These Bt plants could also kill 100% of the neonates of RS F_1 heterozygotes (susceptible × resistant strain) (Shelton et al. 1998), indicating that the Bt toxin reached a "high dose" for resistance management. Non-Bt Green Comet hybrid broccoli were used as refuge plants.

Insects. Our susceptible insects were from a colony of *P. xylostella* collected in 1988 from cabbage at the New York State Agricultural Experiment Station, Robbins Farm, Geneva, NY, and had been maintained on a wheat germ-casein artificial diet (Shelton et al. 1991) from 109 to 127 generations. Before tests on plants, eggs from the Geneva colony were reared on oilseed rape plants, *Brassica napus* L. subsp. *oleifera* ('Dwarf Essex'), in the greenhouse. The susceptible colony exhibited 100% mortality on our high-expressing Bt broccoli (Metz et al. 1995). During the screening test, eggs placed on non-Bt broccoli (Green Comet hybrid) were used as controls and showed an average of 71.8% survival (of those that hatched) with an average hatch rate of 74.0%.

Our resistant insects were collected from Loxahatchee, FL, in 1992 and 1994. Insects from the first collection were used in trials examining refuge size and the latter in trials examining refuge placement. Previous studies of *P. xylostella* from Loxahatchee indicated that resistance was due to a single autosomal recessive gene (Tang et al. 1997). Both colonies were selected with a *Bacillus thuringiensis* subsp. *kurstaki* product (see below) then reared for >1 generation on high-expressing Bt broccoli to ensure that only resistant individuals were used for genetic crosses. Crosses were then performed between susceptible and resistant colonies so that the genetic background of the insects carrying the resistance genes would be as similar as possible to the susceptible insects, the latter being the bulk of the insects used for the tests. F_1 insects were produced by reciprocal crosses of Geneva × Loxahatchee, and F_2 insects by mass mating F_1 insects.

Insecticide. A commercial formulation of *Bacillus thuringiensis* subsp. *kurstaki* (Javelin WG, Novartis, Greensboro, NC, lot no. 7300960) was used in these trials. This product contains spores and the protoxin proteins of Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B (Koziel et al. 1993). Javelin was used to evaluate resistance levels, as described below, and a foliar application of this product was used as one of the treatments described below.

Greenhouse Cage Studies. All tests were conducted in greenhouses at the New York State Agricultural Experiment Station in Geneva, NY. Broccoli in 15 cm diameter pots were six to 12 wk old when they were placed into cages. Plants were fertilized with a controlled released fertilizer, Osmocote (N:P:K, 14:14:14, Scotts, Marysville, OH). Cloth cages measured 1.83 m long by 0.91 m wide by 1.83 m high, had three large sleeve openings, held 30 plants (six rows of five plants per row), and were suspended by a PVC exoskeleton. Cage placement within the greenhouse was randomly assigned and two greenhouses, either adjoining or in the same wing, were used for each experiment. Greenhouse temperature ranged from 26 to 33°C, with a photoperiod of 16:8 (L:D) h, and uncontrolled relative humidity. Assuming that Bt resistance is under monogenic control (Tang et al. 1997) and that the Geneva and Loxahatchee colonies are fixed for susceptibility and resistance, then based on the number and genotype of individuals released we were able to estimate the resistance allele frequency at the start of each greenhouse study.

The Effect of Refuge Size. The first greenhouse cage study used only mixed refuges and examined the effect of increasing refuge size on insect population development and resistance levels. The five treatments were as follows: 0, 3.3, 10, 20, and 100% refuge that received a single foliar application of Javelin when second-generation larvae were present. The spray was applied with a single cone nozzle (TXVS 12, R&D Sprayers, Opelousas, LA) at 3.15 kg/cm^2 pressure from a CO_2 tank. The area of the cage and the delivery rate of the nozzle were used to make the calculations so that the dose applied to plants in the cage was equivalent to 0.6 kg/ha. A spreader-sticker (Bond, Loveland Industries, Loveland, CO) was added at 0.1% vol:vol to enhance spray deposition. Two cage replicates were set up for each treatment. In the mixed refuge treatments, the position of the refuge plants was randomly assigned except in the 20% refuge treatment where

each row contained one refuge plant and the position within the row was randomly assigned.

The insect populations were started in each cage by releasing 3,288 susceptible and 56 F_2 eggs. Taking into account the average percent hatch (75%) from batches of F_2 eggs that were set aside, there should have been 10.5 susceptible larvae, 10.5 resistant larvae, and 21 heterozygous larvae eclosing from the F_2 eggs. The hatch rate for the susceptible colony was 90.0%. Using these calculations, we estimated there were 42 resistant alleles out of a total of 6,002, resulting in an initial resistance allele frequency of 0.007. All eggs were laid on aluminum foil (Shelton et al. 1991), which enabled us to count and cut the foil into appropriate portions. The susceptible eggs were divided into 30 roughly equal portions and pinned onto one leaf of each plant in a cage. Because the number of F_2 eggs released was small, we decided it would be less injurious to the eggs if we did not cut the foil up to release on all 30 plants. Instead, for cages that contained only one type of plant, the F_2 eggs were divided into six roughly equal portions and released onto one randomly chosen plant per row. For cages that contained both refuge and Bt plants, the proportion refuge was used to determine the number of eggs that would be released on one refuge plant and the remainder of the eggs were divided into five roughly equal parts and released onto one Bt plant in each of the other five rows.

After this initial introduction of insects into the cage, no further introductions were made and insect populations were allowed to cycle within the cage. If a plant became severely defoliated ($\approx$70% of the leaf area lost), it was replaced with another plant of the same type. The defoliated plant was cut at its base and placed onto the new replacement plant so that larvae would not be lost. Once larvae had abandoned the defoliated plant, it was removed. When the majority of the larvae were in their third generation, the number of insects on every plant was counted. Fourth generation adults were then collected from each cage and their progeny were evaluated for resistance to Javelin.

The Effect of Refuge Placement. A separate study compared mixed versus separate refuges, where the latter was designed to limit the movement of larvae between the refuge and the Bt broccoli. The three treatments were as follows: 20% mixed refuge (five cage replicates), 20% separate refuge (five cage replicates), and 100% refuge (two cage replicates) as a control. Plant placement in the cages was as described above. For the 20% separate refuge treatments, the refuge was placed in either the first two or the last two rows and a gap of at least 12 cm separated the refuge from the Bt plants, which occupied the remaining four rows. Into each cage we introduced 390 susceptible pupae and 10 F_1 pupae. The starting resistance allele frequency was 0.0125. To ensure that resistance alleles were not lost, one resistant pupa was added to each cage as each next insect generation pupated. For each generation, larvae on each Cry1Ac or refuge plant were counted and the percent larvae on Cry1Ac plants in each cage were calculated. The first adult collection was made in the second generation and the second collection was made at the end of the study in the fifth or sixth generation. Their progeny were evaluated for resistance to Javelin. The numbers of larvae on Bt or non-Bt broccoli in each cage were counted and percent larvae on Cry1Ac plants was calculated for each generation. Data were analyzed with analysis of variance (ANOVA) using the SAS program (SAS Institute 1985). Data were transformed using arcsine (square root [p]) for percent larvae on Cry1Ac plants, and using log(x) for total larvae per cage before each ANOVA was performed.

Resistance Evaluation. For the bioassays we used a cabbage leaf dip method similar to that previously described (Tang et al. 1996, Perez et al. 1997). For each replicate of a bioassay, the highest concentration was prepared by adding the appropriate amount of insecticide to water. Lower concentrations were obtained by performing a dilution series. For LC_{50} determinations, a 3.16$\times$ dilution series was used and seven to eight concentrations were chosen so that upper and lower boundaries of mortality were represented. Five leaf discs (32 mm diameter, dipped for 10 s and allowed to dry for 1.5 h) were prepared for each test concentration and for each control. The level of resistance to Javelin was measured in the larval progeny of adults taken from the cages, at the times noted above, by using a 10$\times$ dilution series. Ten leaf discs were prepared for each test concentration and five for each control. For all bioassays performed, Bond spreader/sticker (Loveland Industries) was added at 0.1% to all test concentrations and to the water control. Leaf discs were placed into 30-ml clear plastic cups (one disc per cup) with 6 second instars, which were reared on rape in the greenhouse before the bioassay. Mortality was determined after 72 h at 27°C. Bioassays were replicated on two different days. To estimate parameters of concentration-mortality regression, data from the two replicates were pooled and analyzed using the POLO probit program (Russell et al. 1977).

Regression. Correlation and linear regression coefficients were calculated (Kaleidagraph, Abelbeck Software 1994) for percentage of the total larvae on Cry1Ac plants versus percentage mortality at 10 ppm Javelin for all treatments in cage studies that included Cry1Ac plants in the treatment. If G equaled the generation that was counted, then percent mortality was always evaluated for the G + 1 or the G + 2 generation.

Insect Behavior on Bt and non-Bt Plants. Differences in the rate of resistance development in mixed versus separate refuges could be either due to adults discriminating between plants for oviposition or larvae moving between plant types. Our previous studies on oviposition (Tang et al. 1999) indicated that both susceptible and Cry1Ac-resistant *P. xylostella* were unable to discriminate between Cry1Ac and normal broccoli and laid similar numbers of eggs on each. To determine how insect genotype, plant type, and plant position could influence larval movement, we set up a factorial experiment in which one Bt-expressing and one normal plant were put in a cage, and 25 third-

Table 1. The susceptibility to Javelin (6.4% WG of Bt) for the colonies of *P. xylostella* that served as sources for inoculating cages[a]

Insect colony	n	LC_{50} (ppm)	95% CL[b]	Slope (SE)	χ^2 (df)	Resistance ratio	% Survived at 10 ppm[c]
Geneva 88	196	0.2	0.1–0.3	1.33 (0.15)	7.08 (4)	1.0	1.3
Loxahatchee 92	314	513	323–963	1.29 (0.29)	2.05 (5)	2,565	—
Loxahatchee 94	225	907	331–3760	0.88 (0.11)	12.36 (6)	4,535	85.3

[a] LC_{50} values were estimated by feeding 2nd instars on cabbage leaf discs dipped in a dilution series of Javelin for 10 s. Mortality was read after 72 h at 27°C.
[b] CL, confidence limits.
[c] Mean survival of F_1 and F_2 larvae at 10 ppm were 9.9% and 17.3%, respectively.

instar susceptible (Geneva) or F_1 larvae (reciprocal crosses of Geneva × Cry1Ac-resistant) were released onto one of the two plants (five larvae per leaf × five leaves). The plants were arranged in either a touching position, i.e., their leaves touched at a single point, or in a nontouching position where there was a minimum of 10 cm separating the leaves of the two plants. At 24, 48, and 72 h, numbers of larvae (dead and alive) on each plant were counted. N equaled two for each treatment combination using susceptible larvae and N equaled six for each treatment combination using F_1 larvae. A total of 56 cages was set up for the test.

Data were analyzed by ANOVA (PROC GLM, SAS Institute, 1985) using a three factorial model (insect genotype, plant type, and plant position) within each time period.

Results and Discussion

Baseline Resistance Levels. The LC_{50} value of Javelin for the susceptible Geneva colony was 0.2 ppm, and the resistance ratios of the Loxahatchee colonies were >2,500-fold compared with the susceptible colony (Table 1). Mortality of the different types of larvae that were used to inoculate cages for each greenhouse study was also examined using a discriminating concentration. Ten parts per million Javelin was chosen because it could adequately separate SS and RR genotypes (Tang et al. 1997). At 10 ppm Javelin, Geneva larvae showed 97–100% mortality, Loxahatchee larvae showed 15% mortality, F_1 larvae showed 89–91% mortality, and F_2 larvae showed 82–84% mortality (Table 1).

The Effect of Refuge Size. The effect of varying the size of a mixed refuge on insect population is shown in Table 2. In all cages, except for one cage of the 0% refuge treatment, insect populations cycled after the initial inoculation and we were able to obtain data from larval counts and resistance levels. In the one cage with 0% refuge, one adult was observed during the first insect generation, but no adults were observed in later generations and the population went extinct in this cage. This contrasted with the other replicate of the 0% refuge treatment, where resistance became rapidly established once a few resistant adults survived the first generation of selection.

In treatments that had refuge plants mixed with the Cry1Ac plants, we found that increases in percent refuge resulted in increases in the number of larvae per refuge plant and increases in the total number of insects per cage in the third generation (Table 2). The number of larvae per Cry1Ac plant, however, were lower and less variable.

Another indication of the population size was obtained in the fourth generation when adults were collected so that their progeny could be evaluated for resistance (Table 2). By this time, in the remaining 0% refuge cage, resistant insects were abundant on the Cry1Ac plants. Plant damage levels were extreme on all plants in the cage as evidenced by the need to replace 16 of 30 of the 1Ac plants at least once because they were defoliated, and the final adult population was >600 adults (Table 2). It appeared that, in this replicate, selection in the absence of a refuge rapidly eliminated most of the susceptible alleles from the population, probably during the first generation. The larval population surviving at 10 ppm Javelin was 77% (Table 2), which was similar to the parent resistant colony (85.3%, Table 1). Therefore, resistance was nearly fixed in this cage.

Table 2. Effects of refuge proportion in the cage on the population and resistance development of *P. xylostella* after 4 generations

Treatment	Larvae/plant of 3rd generation		Insects/cage in 3rd generation	Adults/cage of 4th generation	% Resistant individuals[a]	Plant replaced/total plants[b]	
	Cry1Ac	Refuge				Cry1Ac	Refuge
0% refuge	2.6	—	159	≫600	77	16/30	—
3.3% refuge	0.1	8.0	12	168	51	19/58	1/2
10% refuge	1.8	13.5	90	222	28	13/54	5/6
20% refuge	1.3	33.0	213	325	8	3/48	11/12
100% refuge[c]	—	4.0	119	≫600	0	—	40/60

[a] Percentage of larvae survived at discriminating concentration of Javelin (10 ppm) after 72 h at 27°C.
[b] Summed over both cages except in the 0% refuge treatment where the population in one replicate went extinct.
[c] Received a single foliar spray of Javelin when 2nd generation larvae were present.

In the 3.3% refuge treatment, there was only an average of 168 adults collected in the fourth generation, which was the smallest population recorded for all treatments (Table 2). The nature of the population on the whole, however, was resistant and six and 13 out of 29 Cry1Ac plants were defoliated and had to be replaced in the cages (Table 2). The 51% survival of resistant larvae was less than that of the 0% refuge treatment (77%), indicating that even a small refuge could preserve some susceptible alleles in the population. Faced with the intensity of selection, however, the 3.3% refuge was probably too small to delay resistance long enough to prevent early crop damage particularly in populations that have already developed low levels of resistance. Even greater delays in resistance were achieved, however, by increasing the refuge to 10 or 20%. The larvae that survived 10 ppm Javelin in these two treatments were 28 and 8%, respectively, (Table 2) at the time of adult collection. By installing these larger refuges, we also found that fewer Cry1Ac plants needed replacement due to defoliation. In the 10% refuge treatment, we replaced eight and five of 27 Cry1Ac plants in each cage. For the 20% refuge treatment, three and 0 of 24 Cry1Ac plants were replaced. Fewer Cry1Ac plant replacements was another indication that, despite multiple generations of selection with the Cry1Ac plants, enough susceptible alleles were maintained in the population to prevent extensive establishment of resistant insects. There was a trade-off, though, in that almost all refuge plants needed replacement in both treatments.

The last treatment to consider was the 100% refuge plus one spray. One spray application of Javelin at half the recommended field rate was applied in the second generation to knock down the population that was exploding after the initial egg release. Larval counts in the third generation showed that the spray was effective and there was an average of 119 larvae per cage (Table 2). The percentage of the fifth-generation larvae that survived 10 ppm Javelin was 0%, indicating that the resistant allele frequency was below our limits of detection, ≈0.1.

The Effect of Refuge Placement. Between the treatment of the 20% mixed and 20% separate refuge, the total number of larvae per cage was significantly different only for the first generation between the treatments of the 20% mixed and 20% separate refuge (Table 3). This indicates that for generations 2–5, differences in the percent of larvae located on either plant type could not be attributed to population size. Although considerable variation of replicates within a treatment did occur, there was a trend for fewer larvae to be on Cry1Ac plants in the separate refuge. The percentage of larvae on Cry1Ac plants in the separate refuge was significantly less than in the mixed refuge for generations 1, 4, and 5 (Table 3). Higher percentage of larvae on Cry1Ac plants correlated to higher percentage of resistant individuals in the cage, as shown by lower mortality with the discriminating concentration of 10 ppm Javelin (see Fig. 1). In the separate refuge treatment for the first generation, no insects survived on the Cry1Ac plants. In the mixed refuge during the first generation, 17.4% of the larvae were on Cry1Ac plants. These must have originated from insects which completed earlier stages of the development on refuge plants because the starting resistance allele frequency was 0.0125 making the homozygous resistance frequency 1.56×10^{-4}. This suggests that separating the refuge has a pronounced effect on delaying resistance development for an insect like *P. xylostella* which can move between plants in its larval stage.

Table 3. Population and resistance development of *P. xylostella* after 5 generations under 20% mixed or separate refuge treatment in the cage

Generation	Treatment[a]	
	20% Mixed refuge	20% Separate refuge
	Total larvae per cage (±SEM)	
1	286 (27)a	588 (89)b
2	181 (43)a	146 (21)a
3	113 (27)a	101 (52)a
4	256 (57)a	238 (238)a
5	461 (209)a	289 (131)a
	% Larvae on Cry1Ac plants (±SEM)	
1	17.4 (3)a	0 (0)b
2	3.8 (2)a	1.1 (1)a
3	18.6 (9)a	5.0 (5)a
4	38.5 (9)a	7.2 (3)b
5	39.1 (12)a	8.6 (5)b

[a] Values in a row followed by different letters are significantly different ($P < 0.05$, HSD).

Regression. In all the tests we performed, the percentage of *P. xylostella* larvae on Bt plants was significantly negatively correlated with mortality at the discriminating concentration of Javelin (10 ppm) (Fig. 1). Data from all studies where mixed or separate refuges were present in combination with Cry1Ac plants were included. The negative linear relationship was described by the equation:

$$y = 95.8 - 0.91x \ (r^2 = 0.76, P < 0.01),$$

where y is the percentage of *P. xylostella* larvae on Cry1Ac broccoli and x is percent mortality at the discriminating concentration of Javelin (10 ppm). This type of regression serves several purposes. First,

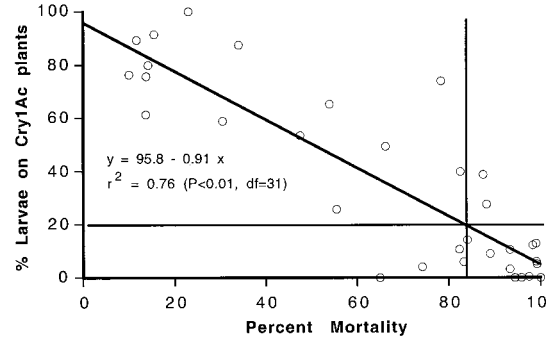

Fig. 1. Linear correlation between percent of the total larvae on Cry1Ac plants versus percent mortality at 10 ppm Javelin for treatments in all greenhouse studies.

it relates percent mortality in a laboratory assay of a Bt spray formulation with percent larvae found on Bt plants in a model system where resistance alleles are present and are under selection by the plants. In this particular case, 10 ppm Javelin is a discriminating concentration and so estimates of the resistance allele frequency can be related to percent larvae on Bt plants. Regardless of the number of generations it took, a threshold of 20% larvae on 1Ac plants, or 84% mortality at 10 ppm Javelin, could be used to identify a population that was on the brink of turning into a population in which resistance could no longer be controlled, i.e., continued selection would favor the spread of resistance alleles beyond which the refuge could effectively manage. Second, it demonstrates one method for determining when developing levels of resistance pose a serious enough threat to a Bt crop to warrant its removal or rotation out of the seed market. During the early years when a Bt crop is introduced into the environment, insects may primarily be found on the refuge plants but rarely on the Bt plants. However, after several years of use of Bt plants, one may slowly find that the percent of the total larvae found on the Bt plants is starting to increase noticeably and this will be an early warning of potential problems with resistance.

Insect Behavior on Bt and non-Bt Plants. Insect genotype, susceptible or F_1 from either reciprocal cross, had no effect on movement of third instars off ($F = 1.41$, 1.37, and 1.12 for 24, 48, and 72 h, respectively; df = 2, 51; $P > 0.05$) or onto plants ($F = 0.34$, 0.04, and 0.29, for 24, 48, and 72 h, respectively; df = 2, 51; $P > 0.05$). Therefore, when reporting results from analyses of the other factors in the model, data from all three genotypes were combined.

Plant type of the release host greatly influenced the amount of movement off the plant (Fig. 2). Significantly more larvae moved off the release host when it expressed Bt than when it was a normal plant ($F = 85.06$, 295.38, and 271.33 for 24, 48, and 72 h, respectively; df = 1, 51; $P < 0.05$). When the release host was a Bt plant, most larval movement off the Bt plant occurred during the first 48 h with little change of movement between 48 and 72 h. When the release host was a normal plant, all larval movement occurred during the first 24 h with little change of movement between 24 and 72 h. Plant type of the release host also significantly affected the amount of movement onto the second plant in the cage (Fig 2). More larvae were found on the second plant when the release host expressed Bt than when it was a normal plant ($F = 17.92$, 13.77, and 14.10 for 24, 48, and 72 h, respectively; df = 1, 51; $P < 0.05$). For both types of release host, movement to the second plant occurred within the first 24 h with little change of movement between 24 and 72 h.

Plant position (touching at a single point or not touching) had no impact on larval movement off the release host ($F = 0.99$, 0.37, 0.01 for 24, 48, and 72 h, respectively; df = 1, 51; $P > 0.05$) (Fig. 3). Significantly more larvae reached the second plant, however, when plants were touching than when not touching ($F = 17.92$, 9.64, and 12.82 for 24, 48, and 72 h, respectively; df = 1,51; $P < 0.05$) (Fig. 3). Most movement onto the second plant occurred within the first 24 h of the release with little movement occurring afterwards.

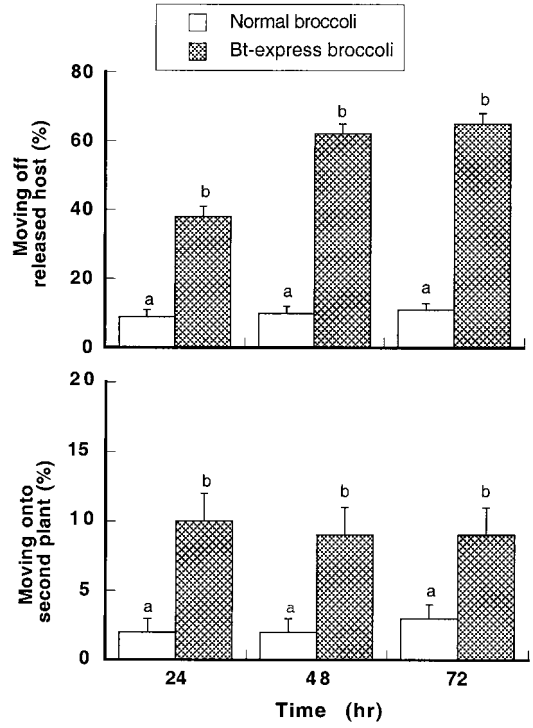

Fig. 2. Effect of release host type on larval movement (mean + SE) off the release host or onto the second plant in the cage. Within each time period, different letters indicate significant differences ($P < 0.05$).

By knowing how larval dispersal differs on Bt plants versus normal plants and what chance a larva has of reaching a second plant, we can then understand why a separate refuge performed better than a mixed refuge at delaying resistance in our greenhouse tests. However, we only studied movement at one density (25 larvae per plant) and one instar (third instar) with plants that touched at only one point. Increases in density and the number of areas that touch could greatly influence the relative difference between loss and preservation of SS and SR genotypes in mixed versus separate refuges.

Movements occurring at low frequencies included susceptible and F_1 larvae moving off refuge plants and onto a second plant. In the mixed refuge, if these larvae reached a second plant, they would typically encounter Bt plants and die, resulting in losses of S and R alleles from the SS and SR genotypes. These larvae also could encounter another refuge plant and live but, due to the low frequency at which larvae move off refuge plants and onto a second plant, it is unlikely that a significant number of S alleles would be conserved in this fashion. In the separate refuge, however, if these larvae reached a second plant, they would encounter refuge plants and live, thereby conserving S and R

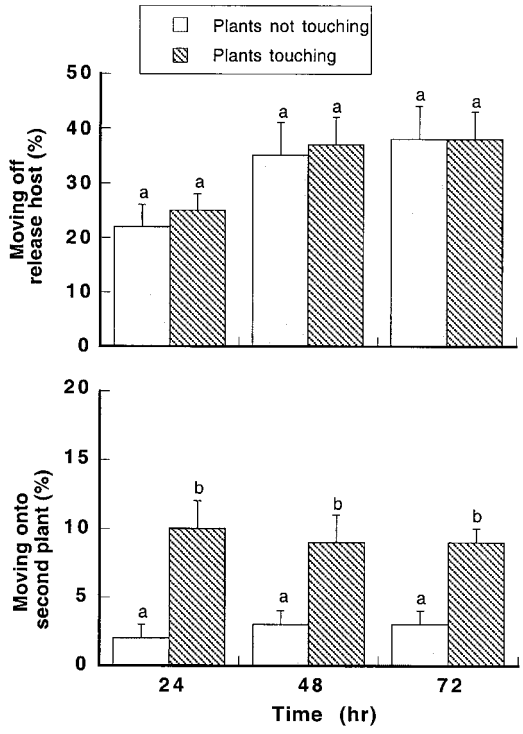

Fig. 3. Effect of plant position on larval movement (mean ± SE) off the release host or onto the second plant in the cage. Within each time period, different letters indicate significant differences ($P < 0.05$).

alleles from the SS and SR genotypes. Thus, for susceptible and F_1 larvae moving off refuge plants, the separate refuge should be better than the mixed refuge at conserving S alleles and slowing the development of resistance There is a trade-off in that some R alleles would also be conserved which would pose more of a problem at higher R allele frequencies, e.g., when detectable numbers of resistant insects were emerging off the Bt plants or when resistance was partially dominant.

Movements that occurred at much higher frequencies were susceptible larvae moving off Bt plants. In both mixed and separate refuges, susceptible larvae moving off Bt plants would always die. They would either encounter another Bt plant and die or, if they reached a refuge plant, as might happen in a mixed refuge, they would still die because a lethal dose of toxin would have already been ingested. (This was what we observed as the eventual demise of susceptible larvae in our study.) For susceptible larvae moving off Bt plants, there appears to be little difference in mixed or separate refuges and the S alleles would always be lost.

F_1 larvae moving off Bt plants also occurred at much higher frequencies. In separate refuges, F_1 larvae moving off Bt plants would die because they would encounter another Bt plant. F_1 larvae moving off Bt plants then results in the loss of S and R alleles from the SR genotype. In mixed refuges, however, there is the possibility that after leaving a Bt plant, an F_1 larva could encounter a refuge plant and live. This implies that only a sublethal dose of toxin was ingested before the larva abandoned the plant. In this case, mixed refuges would conserve the S and R alleles whereas the separate refuge would lose them. This difference means that the development of resistance should be favored more in the mixed refuge than the separate refuge. How much faster resistance evolves depends upon several factors: e.g., the more dominant resistance is, the more likely that the SR genotype would survive after ingesting the Bt plant; and the more points that touch between plants, the more quickly the SR larva could abandon the Bt plant for the preferred host, thereby increasing the effective dominance of the trait.

It should be noted that in our movement studies, F_1 larvae never survived after leaving the Bt plant. Therefore, the superior performance of separate refuges in our greenhouse tests could have been due to combined effects of plant proximity which acted to increase the effective dominance of resistance or to our observation that separate refuges are better at conserving S alleles than mixed refuges when susceptible and F_1 larvae leave refuge plants. In either case, these results are consistent with results of models challenging the use of seed mixtures compared with separation of the refuge from the main crop (e.g., Tabashnik 1994b; Roush 1996).

In summary, overall these trials provide some insight into factors which influence the successful deployment of Bt plants and help explain results from our field trials with the Bt broccoli-*P. xylostella* system (Shelton et al. 2000). Results from the greenhouse and laboratory studies indicate that pure stands of Bt-expressing plants (0% refuge) can result in rapid development of highly resistant *P. xylostella* populations, and increasing the size of the refuge will delay the development of resistance. 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 susceptible and F_1 larvae moved from refuge plants to Bt-expressing plants, they died and caused an overall decline in the number of susceptible alleles. As they moved from Bt plants to refuge plants, F_1 larvae had greater chances of survival, thereby increasing the effective dominance of resistance. Both factors resulted in a more rapid development of resistance than when plants were separated by a distance that limited the movement of larvae. Therefore, refuges can delay resistance but, for insects such as *P. xylostella*, which can disperse as larvae, are best placed outside the transgenic Bt crops. These present results agree with results from our small field studies (Shelton et al. 2000), but further tests with larger field plots should be conducted in the future.

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