

Effect of Bt Broccoli or Plants Treated With Insecticides on Ovipositional Preference and Larval Survival of *Plutella xylostella* (Lepidoptera: Plutellidae)

XIAOXIA LIU,^{1,2} MAO CHEN,² DAVID ONSTAD,³ RICK ROUSH,⁴ HILDA L. COLLINS,²
ELIZABETH D. EARLE,⁵ AND ANTHONY M. SHELTON^{2,6}

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ABSTRACT The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), a major pest of cruciferous crops throughout the world, has demonstrated an ability to develop resistance to many different classes of insecticides, including proteins from *Bacillus thuringiensis* that are expressed in plants (Bt plants). The ovipositional preferences and larval survival of strains (resistant strain, RR; heterozygous strain, RS; susceptible strain, SS) of *P. xylostella* to Cry1Ac-expressing broccoli or broccoli plants treated with lambda-cyhalothrin or spinosad were studied under greenhouse condition. Numbers of eggs per plant did not differ between Bt broccoli and non-Bt broccoli for Bt-RR, Bt-RS, and Bt-SS adults. Ovipositing adults (spinosad-RR, spinosad-RS, and spinosad-SS) also could not discriminate between spinosad-treated and untreated plants, and oviposition did not increase over the 13 d after spinosad treatment. For broccoli treated with lambda-cyhalothrin at the diagnostic dose of 20 ppm, all three insect strains (lc-RR, lc-RS, and lc-SS) had constant oviposition over time based on linear regressions. At the field dose of 80 ppm, the lc-RR strain had constant oviposition over time. The lc-SS susceptible strain had increasing oviposition over time, but the oviposition pattern on the nonsprayed broccoli also increased over time. Susceptible females laid fewer eggs on plants sprayed with lambda-cyhalothrin than on unsprayed plants. A residue-persistence test showed that spinosad and lambda-cyhalothrin could effectively control SS *P. xylostella* larvae for 7–9 d after application. These results are discussed in relation to their potential impact on insecticide resistance management strategies.

KEY WORDS insecticide residue, transgenic plants, oviposition preference, resistance management, *Plutella xylostella*

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is the most destructive insect pest of *Brassica* crops in many parts of the world (Talekar and Shelton 1993, Grzywacz et al. 2010). The estimated annual cost for controlling this insect two decades ago was estimated to be \$1 billion (Talekar and Shelton 1993). Synthetic insecticides have been widely used to control *P. xylostella* but strains resistant to almost every insecticide applied in the field have developed (Yu and Nguyen 1992, Sarfraz and Keddie 2005). These include resistance to new chemistries such as spinosyns, avermectins, neonicotinoids, pyrazoles, and oxadiazines in many crucifer-producing re-

gions (Zhao et al. 2006, Grzywacz et al. 2010). Several *Brassica* species expressing insecticidal proteins from the soil bacterium, *Bacillus thuringiensis* (Bt) have been engineered to provide protection against *P. xylostella* and other lepidopteran pests (Shelton et al. 2008a). These Bt *Brassica* crops can effectively control *P. xylostella* (Metz et al. 1995; Tang et al. 1999; Cao et al. 1999, 2002; Shelton et al. 2000, 2002; Zhao et al. 2000, 2003, 2005), but there is concern that resistance may develop to Bt crucifers, because resistance has evolved to foliar sprays of Bt (Tabashnik et al. 1990; Shelton et al. 1993, 2007).

An accepted cornerstone of resistance management is a refuge consisting of plants free of the toxin that allow susceptible insects to survive in the population (Bates et al. 2005). The refuge concept applies equally well to foliar sprays and Bt plants, although it has been required only for the latter. Refuges work best if resistance to the toxin is a recessive characteristic (Onstad and Knolhoff 2008), and strains of insects that have evolved resistance to Bt proteins have generally shown recessive resistance (Ferré et al. 2008). Refuges should provide sufficient susceptible adults to mate

¹ Department of Entomology, China Agricultural University, Beijing, China, 100193.

² Department of Entomology, Cornell University, NYSAES, 630 W. North Street, Geneva, NY 14456.

³ Department of Crop Sciences, University of Illinois, Urbana, IL 61801.

⁴ Melbourne School of Land and Environment, Parkville Campus, University of Melbourne, Victoria 3010, Australia.

⁵ Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853.

⁶ Corresponding author, e-mail: ams5@cornell.edu.

with resistant adults emerging from the Bt field or sprayed field to produce heterozygous susceptible offspring. However, if different insect genotypes prefer to lay their eggs on one plant type over another, this could disrupt one of the underlying requirements of the refuge strategy. For example, if susceptible adults prefer to lay their eggs on plants producing toxins, this would effectively reduce the size of the refuge and numbers of susceptible alleles in the population. Therefore, different behaviors by the host can affect resistance evolution (Hoy et al. 1998, Onstad 2008).

Changes of plant volatiles in either concentrations or components (or both) might alter host plant finding behaviors and thus the diversity of insect pests in agroecosystems. Yan et al. (2004) reported that two compounds found in Bt cotton were absent in a non-Bt variety. Because of our interest in deploying Bt crucifers against *P. xylostella* (Shelton et al. 2008a, Grzywacz et al. 2010), we wish to enhance our general understanding of resistance evolution to Bt plants, as well as conventional insecticides. The purpose of our current study was to investigate whether there are differences in oviposition and larval survival by the different genotypes (homozygous resistant, heterozygous resistant, homozygous susceptible) of *P. xylostella* to Bt plants or plants treated with lambda-cyhalothrin or spinosad, two commonly used foliar insecticides.

Materials and Methods

Insects. Seven strains of *P. xylostella* were used: 1) Cry1Ac-RR strain, which can survive if fed on Cry1Ac broccoli plants (Zhao et al. 2005, Liu et al. 2011); 2) Pearl-RR strain, which has a high level of resistance to spinosad (Zhao et al. 2006); 3) Waipio-RR strain, which has a high level of resistance to lambda-cyhalothrin (Zhao et al. 2006); 4) susceptible strain (SS-Geneva 88), which has been maintained on a wheat germ-casein artificial diet for over 500 generations (Shelton et al. 1991); 5) Cry1Ac-RS, which was developed by crossing Cry1Ac-resistant individuals with G88; 6) Pearl-RS, which was developed by crossing Pearl-RR with G88; and 7) Waipio-RS, which was developed by crossing Waipio-RR with G88. All insect strains were reared on artificial diet and maintained in a climatic chamber at $27 \pm 1^\circ\text{C}$, $50 \pm 10\%$ RH, and a photoperiod of 16:8 (L:D) h.

Plants and Insecticides. Transgenic 'Green Comet' broccoli (*Brassica oleracea* L.) plants producing a high level of Cry1Ac on which susceptible *P. xylostella* larvae could not survive were used in this study (Metz et al. 1995). The expression of Cry1Ac in the broccoli plants was verified when plants were 4–5 wks old by screening them with susceptible *P. xylostella* neonates. Non-Bt Green Comet broccoli was used as the control.

Commercial formulations of two insecticides were used: spinosad (SpinTor 2 SC) supplied by Dow AgroSciences (Indianapolis, IN) and lambda-cyhalothrin (Warrior T) supplied by Syngenta Crop Protection (Greensboro, NC). The concentrations we used were the diagnostic doses (diagnostic doses was the least

concentration that could kill all susceptible larvae, including SS and RS larva, but resistant strain could survive on this dose condition) of 10 ppm for spinosad (Zhao et al. 2002) and 20 ppm for lambda-cyhalothrin (L.X.X., unpublished), and the field doses listed on the insecticide labels of 90 ppm for spinosad and 80 ppm for lambda-cyhalothrin. The surfactant Dyne-Amic (Helena Chemical Company, Collierville, TN) at 1% vol:vol was added to all sprays. Potted non-Bt Green Comet broccoli plants with eight true leaves were sprayed with a small hand-held sprayer to simulate a field application.

Survival of Larvae on the Insecticide-Treated Broccoli. We evaluated the survival of second instar Pearl-RR, Waipio-RR, Pearl-RS, Waipio-RS, and SS (resistant strain, RR; heterozygous strain, RS; susceptible strain, SS) larvae exposed to the insecticide residues at several times after application. We placed a different cohort of larvae on plants on the day of the spray and at 3, 5, 7, 9, and 11 d after spraying with either spinosad at 90 ppm or lambda-cyhalothrin at 80 ppm. Control plants were sprayed with the surfactant only. All plants were kept in the greenhouse. For each strain, a total of 50 larvae in five replications for each day were tested against both insecticides and 50 larvae on a surfactant-sprayed control. Immediately after the larvae were placed on the plants, the plants were moved into a climatic chamber at $27 \pm 1^\circ\text{C}$, $50 \pm 10\%$ RH, and a photoperiod of 16:8 (L:D) h. Survival was checked after 3 d.

Oviposition of *P. xylostella* on Cry1Ac Broccoli or Broccoli Sprayed With Insecticides. Oviposition of *P. xylostella* was assessed at 1, 4, 7, 10, and 13 d post-treatment. In addition to the insecticide-treated plants, Bt broccoli with eight true leaves was evaluated. In the greenhouse, we placed two control plants (untreated non-Bt broccoli or surfactant-sprayed non-Bt broccoli) and two plants of a single treatment (either Bt plants, or the insecticide-treated plants of a particular day after treatment) into a 1m*1m*1m screened cage (Shelton et al. 2008b). Sprayed plants were kept in the greenhouse for 4 h before they were placed into the cage. We introduced into each cage two mated *P. xylostella* females of a particular genotype (described above). After 2 d (before the eggs would hatch) we assessed oviposition by removing all leaves and counting the number of eggs per plant. All treatments were replicated four times. We used these methods to explore the following issues: whether there are differences in oviposition on Bt and non-Bt plants; whether sprays affect oviposition; whether oviposition changes over time since the spray application.

Data Analysis. Survival rates of *P. xylostella* larvae were transformed using arcsine square root to fit a normal distribution, then were analyzed using one-way analysis of variance (ANOVA) within the same strain between different days after treatment. The means of survival rates between different sprayed days within the same strain were compared with the Tukey honestly significant difference (HSD) test at a 5% level of significance. For each insecticide, the numbers of eggs collected over the residue period were

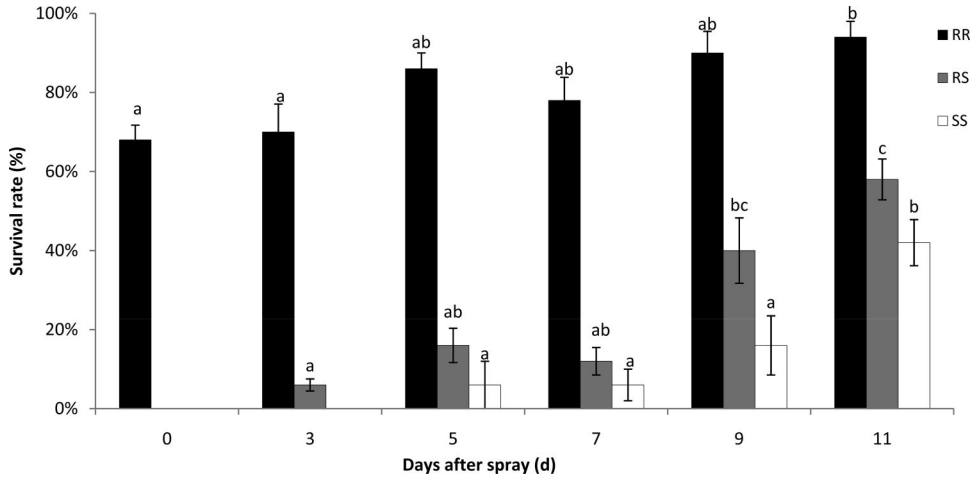


Fig. 1. Survival of second instars of different genotypes of *P. xylostella* at days after spraying spinosad with 90 ppm. The RR strain is resistant to spinosad. Means (\pm SE) ($n = 5$) marked with different letters are significantly different between days after spray within the same genotype of *P. xylostella* based on Tukey's test ($P < 0.05$).

averaged within the sprayed or nonsprayed plants, and were analyzed using a *t*-test. For each of the 12 combinations of two insecticides, two doses, and three insect strains and the corresponding 12 nonsprayed controls, we performed linear regression on the numbers of eggs per plant against the days since treatment and determined whether the slope was significantly different from 0 at the 5% level. The numbers of eggs per plant between Bt and non-Bt plants and between plants treated with insecticides or without insecticides were analyzed using *t*-tests. Statistical analyses were conducted using SPSS 17.0 Windows (SPSS, Chicago, IL) and Matlab 7.0.4 (Mathworks, Natick, MA).

Results

Survival of Larvae on Insecticide-Treated Broccoli.

Larvae of the SS strain of *P. xylostella* could not survive on spinosad-treated plants on the day of spray and the third day after spray. The survival rate was 6% at the fifth day, but it increased significantly with increasing days after spray: 6, 16, and 40% at the 7th, 9th, and 11th days, respectively (Fig. 1; $F = 8.141$, $df = 3, 16$, $P = 0.002$). For the Pearl-RS *P. xylostella* strain, no larvae could survive on the spray day, but the survival rates were 6, 16, 12, 40, and 58% at 3, 5, 7, 9, and 11 d after the spray ($F = 8.507$; $df = 4, 20$; $P < 0.001$), respectively. For the Pearl-RR strain, 68% of larvae could survive on the spray day, and >90% of larvae survived on the 9th and 11th days after spray ($F = 4.305$; $df = 5, 24$; $P = 0.006$). More than 90% of all Pearl-RR, Pearl-RS, and SS strains survived on control plants sprayed with surfactant.

More than 80% of the Waipio-RR strain survived on treated plants, and there were no significant differences between days after spray ($F = 2.064$; $df = 5, 24$; $P = 0.105$) (Fig. 2). For the Waipio-RS strain, the larvae could not survive on the spray day or at 3 and 5 d postspray. The survival rates were 6, 28, and 48%

on the 7th, 9th, and 11th day after spray ($F = 6.825$; $df = 2, 12$; $P = 0.010$), respectively. No SS *P. xylostella* survived on plants-treated with lambda-cyhalothrin until 9 d after spray, and only 15% of larvae survived at 11 d after spray. More than 90% of all Waipio-RR, Waipio-RS, and SS strains survived on control plants sprayed with surfactant.

Oviposition of *P. xylostella* on Untreated, Non-Bt Broccoli. On conventional, unsprayed broccoli, nine cases showed constant oviposition over time (slopes not statistically different from zero) and three cases had increasing oviposition based on positive slopes being significantly different from zero in the linear regression (data not shown). Thus, oviposition on untreated broccoli was usually constant over time when measured as discrete 2-d bouts by different sets of females.

Oviposition of *P. xylostella* on Bt or Non-Bt Broccoli. The three genotypes of *P. xylostella* (Cry1Ac-RR, Cry1Ac-RS, and SS) did not show any ovipositional preference between Bt plants and non-Bt plants. For the Cry1Ac-RR strain, the numbers of eggs laid by two mated females over 2 d were 31.9 ± 4.6 (mean \pm SEM) and 30.9 ± 7.3 (mean \pm SEM) per Bt plant and non-Bt plant ($t = -0.405$, $P = 0.686$), respectively. For the Cry1Ac-RS strain, the numbers of eggs were 26.5 ± 1.0 (mean \pm SEM) and 21.5 ± 5.3 (mean \pm SEM) per Bt plant and non-Bt plant ($t = -1.095$, $P = 0.273$), respectively. For the SS strain, the numbers of eggs were 45.3 ± 6.8 and 34.6 ± 3.0 per Bt plant and non-Bt plant ($t = -1.214$, $P = 0.225$), respectively.

Oviposition of *P. xylostella* on Broccoli Sprayed With Spinosad. At the diagnostic dose of 10 ppm, the Pearl-RS and SS strains produced constant oviposition over time according to the linear regressions. However, the Pearl-RR strain had declining oviposition over time (negative slope significantly different from zero) (data not shown). At the field dose of 90 ppm, the Pearl-RR strain had constant oviposition over

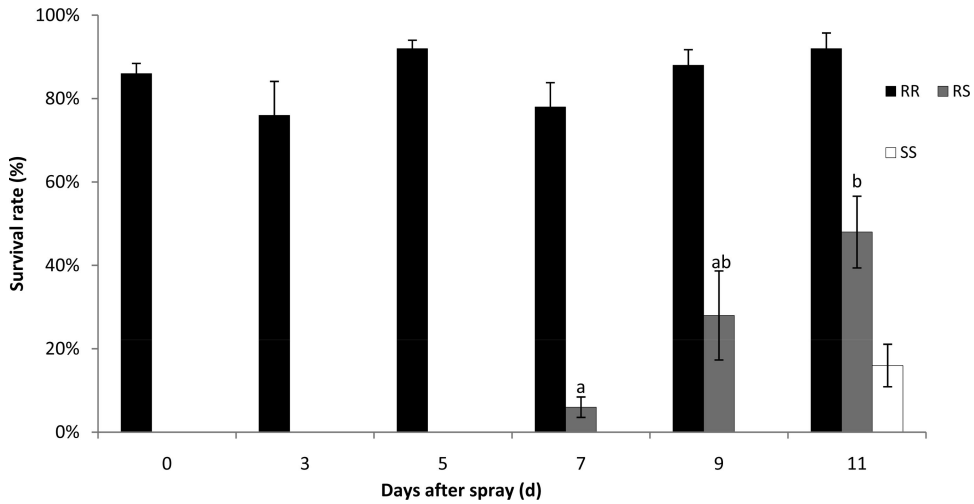


Fig. 2. Survival of different genotypes of *P. xylostella* at days after spraying lambda-cyhalothrin with 80 ppm. The RR strain is resistant to lambda-cyhalothrin. Means (\pm SE) ($n = 5$) marked with different letters are significantly different between days after spray within the RS genotype *P. xylostella* based on Tukey's test ($P < 0.05$). No difference for RR genotype ($P > 0.05$). No larvae of SS strain could survive at the spray day, 3rd, 5th, 7th, and 9th days.

time, the SS strain had increasing oviposition and the Pearl-RS strain had declining oviposition, according to the linear regressions.

With regard to nonsprayed broccoli, the oviposition by the SS strain also increased over time; therefore, no conclusion can be drawn about the influence of insecticide residue in this case. Nevertheless, we can state that oviposition by the Pearl-RS or Pearl-RR strains did not increase over the 13 d period after the spinosad treatment.

We combined the data collected over the insecticide residue period and present the means and statistics in Table 1. In no case is oviposition different on spinosad-treated and untreated broccoli. Oviposition is highest by SS females in cages with only 10 ppm spinosad: 48.1 ± 4.29 to 51.5 ± 4.56 eggs per plant (Table 1). There were significant differences between strains in three out of four scenarios ($df = 59$): plants

with 90 ppm ($F = 2.67$; $P = 0.076$) and untreated broccoli in the same cage ($F = 3.73$; $P = 0.030$); plants with 10 ppm ($F = 11.33$; $P < 0.001$) and untreated broccoli in same cage ($F = 17.32$; $P < 0.001$). The lowest oviposition was consistently produced by the spinosad-resistant Pearl-RR strain on both kinds of plants, suggesting a possible fitness cost.

Oviposition of *P. xylostella* on Broccoli Sprayed With Lambda-Cyhalothrin. At the diagnostic dose of 20 ppm, all three insect strains had constant oviposition over time based on linear regressions. At the field dose of 90 ppm, the Waipio-RR strain had constant oviposition over time. The SS susceptible strain had increasing oviposition over time, but the oviposition pattern on the nonsprayed broccoli also increased over time; therefore, no conclusion can be drawn about influence of insecticide residue in this case. The linear regression produced the following equation for the Waipio-RS strain: $E = 5.16 + 1.31D$, where E is eggs per plant and D is days since treatment. The standard error of the slope is 0.507, $t = 2.59$, and $P = 0.019$ indicating that the slope is significantly different from zero. The same strain had constant oviposition on the nonsprayed broccoli. Thus, this is the only case of the 12 that suggests that a declining insecticide residue could have caused an increasing oviposition rate over time. Therefore, we conclude that oviposition generally does not increase over a 13-d residue period for either insecticide (data not shown).

We combined the data collected over the residue period and present the means and statistics in Table 2. With the Waipio-RR and Waipio-RS strains, oviposition on lambda-cyhalothrin-treated and untreated broccoli is statistically the same. However, oviposition by SS females is significantly lower on treated plants (both doses) than on untreated broccoli (Table 2). The susceptible moths laid few eggs on both the broc-

Table 1. Oviposition (eggs per plant per 2-d period) by two mated female *P. xylostella* on broccoli treated with spinosad or untreated, in the same cage

Insect strain	Dose	Mean	SE	t-value	P
Pearl-RR	90 ppm	27.1	2.14	1.083	0.286
	None	23.8	2.23		
Pearl-RS	90 ppm	37.8	3.79	0.935	0.356
	None	33.1	3.30		
SS	90 ppm	33.2	3.60	-0.304	0.763
	None	34.7	3.49		
Pearl-RR	10 ppm	26.9	3.03	0.742	0.463
	None	24.2	2.01		
Pearl-RS	10 ppm	46.0	2.94	1.675	0.102
	None	39.2	2.77		
SS	10 ppm	48.1	4.29	-0.551	0.585
	None	51.5	4.56		

The Pearl strain is resistant to spinosad. For statistics, $n = 20$, $df = 38$, and P is for a two-tailed test.

Table 2. Oviposition (eggs per plant per 2-d period) by two mated female *P. xylostella* on broccoli treated with lambda-cyhalothrin or untreated, in the same cage

Insect strain	Dose	Mean	SE	t-value	P
Waipio-RR	80 ppm	20.2	2.52	0.554	0.583
	None	18.1	2.83		
Waipio-RS	80 ppm	14.4	2.45	-1.453	0.155
	None	19.9	2.93		
SS	80 ppm	1.1*	0.49	-2.353	0.024
	None	8.3*	3.05		
Waipio-RR	20 ppm	26.4	2.53	0.979	0.334
	None	23.3	2.03		
Waipio-RS	20 ppm	28.9	3.22	-0.086	0.932
	None	29.3	4.12		
SS	20 ppm	22.0*	3.31	-2.072	0.045
	None	34.7*	5.18		

The Waipio strain is resistant to lambda-cyhalothrin. Means marked with "*" are significantly different between sprayed and nonsprayed plants based on a *t*-test ($P < 0.05$). For statistics, $n = 20$, $df = 38$, and *P* is for a two-tailed test.

coli treated with 80 ppm (field dose) and the untreated plants in the same cage. Thus, it is possible that oviposition on untreated plants could have been affected by the insecticide on the nearby treated broccoli.

Discussion

Oviposition by Lepidoptera is influenced by a variety of factors, including phytochemicals, presence of conspecific eggs, and host shape and texture (Thompson and Pellmyr 1991, Renwick 2002, Shelton and Nault 2004, Sarfraz et al. 2006). In this study, we investigated the effect of Bt plants or plants treated with insecticides on ovipositional preference by different strains (RR, RS, and SS) of *P. xylostella*. For Cry1Ac, our results showed that Cry1Ac-RR, Cry1Ac-RS, and SS strains of *P. xylostella* could not discriminate between Bt and non-Bt broccoli plants. The eggs laid on Bt and non-Bt plants were not significantly different when the insects were given a choice. These results were similar to our earlier research in which resistant and susceptible female *P. xylostella* were unable to discriminate between Bt and non-Bt broccoli plants, although the RS genotype was not included in that study (Tang et al. 1999). Ramachandran et al. (1998) reported that resistant *P. xylostella* moths could not discriminate between Cry1Ac canola and normal canola, but they also did not check the RS genotype. Liu et al. (2002) studied the behavior of resistant and susceptible pink bollworm, *Pectinophora gossypiella* (Saunders), to Bt and non-Bt cotton and concluded that oviposition was independent of susceptibility to Cry1Ac, although they also did not include the RS genotype. In contrast to these studies, but still without testing the RS genotype, Men et al. (2005) reported that *Helicoverpa armigera* (Hübner) adults preferred to lay eggs on non-Bt cotton rather than on Cry1Ac-expressing cotton, and the total number of eggs deposited on non-Bt cotton in 3 d was 95% greater than that on Bt plants. Collectively, these cases suggest that

refuge (non-Bt) plants will have eggs laid on them in at least equal numbers to Bt plants; therefore, refuges could provide some susceptible adults to mate with resistant adults from Bt fields. However, these studies did not include the RS genotype, which can be an important contributor to the system, because resistant alleles will be most prevalent in this genotype before the evolution of resistance (Roush 1997). We believe that our study is the first to examine this genotype. If susceptibility of the Cry1Ac-RS genotype was between the Cry1Ac-RR and SS genotypes and if the RS genotype had preferred to lay eggs on Bt plants, this would have accelerated resistance evolution, as would have a preference by the Cry1Ac-RR genotype. Fortunately, our present results indicate that the population could not discriminate between Bt and non-Bt broccoli plants.

Spinosad, the first member of the Naturalyte class of insecticides, is classified as a reduced risk insecticide and has been embraced by integrated pest management (IPM) practitioners as a biorational pesticide (Williams et al. 2003). It has been registered for over 180 crops in the United States and in over 35 countries for the control of caterpillars, beetles, leafminers, and thrips (Zhao et al. 2002). *P. xylostella* has developed resistance to the insecticide in some parts of the world (Zhao et al. 2002). Our current study addressed the oviposition preferences by Pearl-RR, Pearl-RS, and SS genotypes and revealed no differences between sprayed plants and unsprayed plants. Our results indicated spinosad could effectively control SS larvae for 9 d, but had low toxicity to the adults. Laboratory studies have reported that spinosad was highly stable and capable of causing high mortality (up to 1 mo) against *Leptomastix dactylopii* (Howard) after being applied to foliage or artificial surfaces (Bernardo and Viggiani 2000). However, in the field, spinosad degrades quickly and generally showed little toxicity at 3–7 d postapplication (Williams et al. 2003).

Pyrethroids have been found to possess significantly longer activity against many economically important agricultural pests, especially Lepidoptera (Liu et al. 1981). We studied the ovipositional preference of moths encountering residues of lambda-cyhalothrin. Our results showed that no susceptible *P. xylostella* larvae could survive on sprayed plants within 9 d after spraying, but resistant *P. xylostella* could. For the ovipositional preference experiment, the resistant *P. xylostella* strain did not avoid oviposition on sprayed plants on any test days. However, no eggs of *P. xylostella* were found on either sprayed or nonsprayed plants because all susceptible *P. xylostella* adults were killed by the insecticide on the day of the spray. More eggs were found on nonsprayed plants at 9 d after spraying at the field dose than on treated plants for susceptible *P. xylostella*. Moths responded to pyrethroids by avoiding deposits on sprayed plants, and preferentially oviposited on untreated surfaces. We are not sure why this occurred but warrants further investigation. Kumar and Chapman (1984) reported similar results, with the mean number of *P. xylostella* eggs laid per leaf disc treated with the LC₅₀ of pyre-

throids being significantly lower than on the untreated control.

We drew the following conclusions concerning our four major hypotheses. First, oviposition is generally constant over time on untreated broccoli. Second, survival of larvae generally increased over the 13-d residue period indicating that the toxicity for both insecticides declined. Third, oviposition for 2 d on untreated broccoli is the same as that on insecticide-treated broccoli, except for oviposition by susceptible moths on plants treated with a field dose of lambda-cyhalothrin. Fourth, oviposition on Bt broccoli is the same as that on conventional, unsprayed broccoli.

The development of insect resistance is a very serious problem worldwide with >400 species of insects and mites now resistant to one or more pesticides, including Bt sprays and Bt plants (Shelton et al. 2002, Chen et al. 2006, Onstad 2008, Zhao et al. 2010, Arthropod Pesticide Resistance Database 2010). Considerable attention has been paid to the biochemical and physiological mechanisms of resistance (Baxter et al. 2010), but the role of behavior may be substantial because adult moths may respond to insecticides by avoiding oviposition. Furthermore, these adult responses may be correlated with larval behavioral responses (Roush and McKenzie 1987, Head et al. 1995). Thus, it is clear that more attention is needed to the role of insect behavior in resistance evolution and management. This will be especially important as more emphasis is placed on using refuges as part of an overall resistance management strategy (Bates et al. 2005).

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