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Occurrence and larval movement of *Diatraea saccharalis* (Lepidoptera: Crambidae) in seed mixes of non-*Bt* and *Bt* pyramid corn

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Abstract

Background: Larval movement of target pest populations among *Bt* and non-*Bt* plants is a major concern in the use of a seed mixture refuge strategy for *Bt* resistance management. In this study, occurrence and larval movement of the sugarcane borer, *Diatraea saccharalis* (F.), were evaluated in four planting patterns of non-*Bt* and *Bt* plants containing Genuity® SmartStax™ traits in 2009–2011. The four planting patterns were: (1) a pure stand of 27 *Bt* plants; (2) one non-*Bt* plant in the center, surrounded by 26 *Bt* plants; (3) a pure stand of 27 non-*Bt* plants; (4) one *Bt* plant in the center, surrounded by 26 non-*Bt* plants. Studies were conducted under four conditions: (1) open field with natural infestation; (2) greenhouse with artificial infestations; open field with artificial infestations (3) on the center plants only and (4) on every plant. The major objective of this study was to determine whether refuge plants in a seed mixture strategy could provide a comparable refuge population of *D. saccharalis* to a “structured refuge” planting.

Results: Larvae of *D. saccharalis* showed the ability to move from infested plants to at least four plants away, as well as to adjacent rows, but the majority remained within the infested row. However, the number of larvae found on the non-*Bt* plants in the mixture plantings was not significantly reduced compared with the pure stand of non-*Bt* corn.

Conclusion: The results of this study show that refuge plants in a seed mixture may be able to provide a comparable refuge population of *D. saccharalis* to a structured refuge planting.

Keywords: gene pyramiding, transgenic crops, resistance management, *Bacillus thuringiensis*, larval movement, seed mixture strategy, corn borers

1 Introduction

Over the years, a “high-dose/structured refuge” strategy has been the primary insect resistance management (IRM) strategy for planting *Bt* corn in the United States and Canada. This strategy involves planting high-dose *Bt* corn that can kill resistant heterozygotes of a target species on a portion of a grower’s farm.¹ The remaining area is planted to non-*Bt* varieties that serve as a refuge for *Bt*-susceptible insects. The strategy takes advantage of insect movement between *Bt* and non-*Bt* refuge fields, such that the rare resistant survivors from *Bt* plants and susceptible insects from the non-*Bt* refuge plants can mate randomly. Therefore, the majority of their offspring carrying resistance alleles should be heterozygous and thus should be killed by ‘high-dose’ *Bt* corn plants. As a result, resistance allele fre-

quency in field populations of the target species can be maintained at low levels for a long period of time.^{1–3} The high-dose/structured refuge strategy has been successfully implemented in the United States and Canada for *Bt* resistance management for several major lepidopteran targets of *Bt* corn and *Bt* cotton.⁴

In the case of “structured refuge” planting of *Bt* corn targeting above-ground lepidopteran pests, in the United States, outside the cotton-producing regions, the requirements call for planting 20% (for single-gene expressed *Bt* corn) or 5% (for pyramid *Bt* corn) non-*Bt* refuge corn on every farm that plants *Bt* corn. In corn–cotton overlapping regions, a minimum of 50% (for single-gene *Bt* corn) or 20% (for pyramid *Bt* corn) non-*Bt* refuge corn is required.^{5,6} Refuge plants in the structured refuge strategy are to be within 800 m of the *Bt* corn field on each farm.^{1,6}

However, growers' compliance with these structured refuge requirements has been an issue. During the early years of commercialization of *Bt* crops, a relatively high rate of compliance (e.g. 86–92%) with the structured refuge requirements was reported for US and Canadian *Bt* corn growers,^{7,8} but compliance rates dropped to 74–80% in 2007 and 2008 in the United States. A similar declining trend in structured refuge planting was also reported in Canada; compliance with structured refuge requirements slipped from 85% in 2003 to 61% in 2009.⁹

During the 2010–2011 crop seasons, transgenic corn technologies (e.g. Genuity® SmartStax™, Agrisure® Viptera™ 3111) expressing more than one dissimilar pyramid *Bt* protein targeting lepidopteran pests were first commercially planted in the United States and Canada. The use of pyramid *Bt* corn hybrids is expected to delay resistance evolution in target insect populations compared with the use of single-gene *Bt* corn. Because of compliance issues with the use of the structured refuge IRM strategy, the US EPA also recently approved a seed mixture refuge strategy (also called “refuge-in-the-bag” or “RIB”) for planting certain pyramid *Bt* corn hybrids in the northern US corn belt where no cotton is planted.⁶ For the RIB strategy, a defined percentage of non-*Bt* corn seeds is mixed with *Bt* corn seeds in each bag by seed companies prior to being sold to farmers. Farmers simply buy the premixed seeds and plant the mixture in their fields.⁶ In this case, compliance with refuge requirements by farmers will no longer be an issue. With structured refuge the dispersal behavior of adults is important,^{2,3,10–12} but with the RIB strategy the major concern is that larval movement among *Bt* and non-*Bt* plants may hasten resistance evolution in target pest populations. For example, movement of susceptible larvae from non-*Bt* refuge plants to *Bt* plants in an RIB field could cause greater mortality to susceptible insects than in a structured refuge planting and thus result in a lower refuge population.¹³ In addition, differential susceptibility among instars^{14–16} and larval movement among *Bt* and non-*Bt* plants could also create sublethal exposure and promote buildup of resistance in target pest populations by increasing the survival of the resistant heterozygotes or individuals carrying minor resistance alleles. Furthermore, pollen contamination from *Bt* to non-*Bt* plants may also create sublethal exposure to some ear kernels in fields having non-*Bt* corn planted in close proximity to *Bt* plants, leading to cross-pollination.¹⁷ For these reasons, the RIB was not considered to be an appropriate IRM strategy for single-gene *Bt* corn, although it was also discussed as a potential strategy prior to the commercial use of *Bt* corn.¹ A few models have shown that RIB could be an effective IRM strategy for planting pyramid *Bt* corn.¹⁸ However, published field data to support the RIB strategy for pyramid *Bt* corn are limited.^{19,20}

The sugarcane borer, *Diatraea saccharalis* (F.), is a dominant corn stalk borer species in the mid-southern United States, the Caribbean, Central America and the warmer parts of South America to Argentina.²¹ Since 1999, use of *Bt* corn has been the primary tool for managing this species in field corn in the US mid-southern region.^{16,22} To date, the RIB strategy has not been approved in the US southern regions, where cotton is also planted. The objectives of this study were to investigate the occurrence and larval movement of *D. saccharalis* in different planting patterns of non-*Bt* and *Bt* plants containing pyramid *Bt* genes and thus to determine whether refuge plants in the RIB strategy could provide a comparable refuge population of *D. saccharalis* to a structured refuge planting. The results should provide valuable information for assessing whether seed mixtures could be an appropriate refuge strategy for management of *D. saccharalis* with pyramid *Bt* corn technologies.

2 Materials and Methods

2.1 Source of *Bt* and non-*Bt* corn, planting patterns and experimental conditions

A *Bt* corn line containing Genuity® SmartStax™ traits and a genetically closely related non-*Bt* corn line were provided by Monsanto Company (St. Louis, MO). The Genuity® SmartStax™ corn contained six *Bt* genes, including Cry1A.105, Cry2Ab2, and Cry1F for controlling above-ground lepidopteran pests and Cry3Bb1, Cry34Ab1, and Cry35Ab1 for managing below-ground corn rootworms, as well as two herbicide tolerance traits: glyphosate (*Roundup*) and glufosinate-ammonium (*Liberty*) tolerance.^{6,23} The non-*Bt* corn expressed both herbicide tolerance traits but contained none of the *Bt* proteins. Expression of Cry proteins in the corn lines was confirmed using an ELISA-based technique (Quantiplate™ kits; EnviroLogix, Portland, ME).

Larval occurrence, larval movement and plant injury of *D. saccharalis* were evaluated in four different planting patterns of *Bt* and non-*Bt* plants under both greenhouse and open field conditions. Each planting pattern consisted of three rows, with nine plants in each row (a total of 27 plants). The four different planting patterns (treatments) were: Trt 1, a pure stand of 27 *Bt* plants (all *Bt*); Trt 2, one non-*Bt* plant in the center, surrounded by 26 *Bt* plants (RIB); Trt 3, a pure stand of 27 non-*Bt* plants (all non-*Bt*); Trt 4, one *Bt* plant in the center, surrounded by 26 non-*Bt* plants (C-*Bt*). The planting pattern of Trt 2 was designed to simulate a 96:4% RIB, which is close to the currently used 95:5% RIB for planting Genuity® SmartStax™ corn in the United States, while Trt 3 was used to simulate a structured refuge planting. A total of five trials were conducted under four different conditions: (1) two trials in the greenhouse with artificial infestation of eggs in the center plants; (2) one trial in open field with natural infestations; (3) one trial in open field with artificial infestation of eggs in the center plants; (4) one trial in open field with artificial infestation of neonates on every plant. A randomized complete block design was used for all five trials.

2.2 Greenhouse trials with artificial infestation

Two trials were conducted in 2010 and 2011 in the greenhouse to investigate larval movement and plant injury of *D. saccharalis* in the four planting patterns of *Bt* and non-*Bt* corn mentioned above. In each trial, seeds of Genuity® SmartStax™ and the non-*Bt* corn were planted in 5 gal plastic pots containing ~5 kg of standard potting soil mixture (Perfect Mix™; Expert Gardener Products, St. Louis, MO) in a greenhouse at the Louisiana State University Agricultural Center's greenhouse in Baton Rouge, Louisiana, as described elsewhere.²⁴ The planting/spacing in the greenhouse was similar to that used in farmer's fields. Two seeds were planted in each pot approximately 20 cm apart and ~60 cm from one row to the next row. There was an approximately 1 m alley from plot to plot. A mixture of southern turf builder, lawn fertilizer (2% iron, 32N-0P-10K; Scotts Company, OH) and lawn and garden plant food (13N-13P-1K; Meherrin Fertilizer, Inc., NC) was applied at the V2 and V8 plant growth stages.²⁵ Irrigation, fertilization and other management practices were used as needed to ensure optimum growth.

Egg infestations were performed at the V11–V13 plant stages for the trial in 2010, and at the VT stage for the trial in 2011. In each trial, the center plant in each treatment plot was infested with 50 (for the trial in 2010) or 70 (for the trial 2011) eggs of a known Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* by stapling a piece of wax paper containing the eggs on the abaxial (underside) of the ninth or tenth leaf from the base

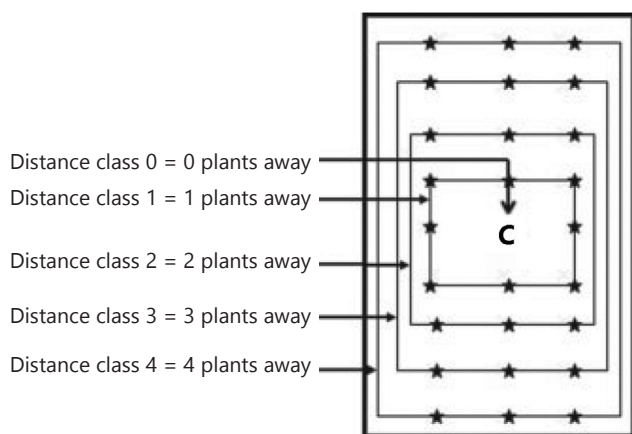


Figure 1. Organization of data on larval occurrence and plant injury of *Diatraea saccharalis* for statistical analysis. Distance class 0 refers to the center plant that was initially infested with eggs of *D. saccharalis*; distance class 1 refers to all eight plants that were one plant away from the center plant; distance class 2 refers to all six plants that were two plants away from the center plant; distance class 3 refers to all six plants that were three plants away from the center plant; distance class 4 refers to all six peripheral plants that were four plants away from the center plant.

with a visible collar. In the trial conducted in 2010, two-day-old eggs (yellow color) were used, and the egg hatching rates were checked after 3 days of infestation. The average egg hatch rate was 50.3% across the four planting patterns. To increase the egg hatchability for the trial conducted in 2011, only black ready-to-hatch eggs were used in the greenhouse infestation, and the hatch rate improved to 86.2%. The Cry1Ab-SS strain of *D. saccharalis* was established from larvae collected from a corn field (32° 8' 6" N, 91° 41' 18" W) near Winnsboro in Franklin Parish in Louisiana during 2009, and it was susceptible to Cry1Ab corn plants and purified Cry1Ab protein.^{26,27} Larvae of the Cry1Ab-SS strain were reared individually in 30 mL plastic cups (Fill-Rite, Newark, NJ) containing a meridic diet (Bio-Serv, Frenchtown, NJ) until the pupal stage, as described elsewhere.¹⁶ Pupae were then transferred from the plastic cups to 3.785 L cardboard cartons (Neptune Paper Products, Newark, NJ) containing approximately 100 g of vermiculite (Sun Gro, Pine Bluff, AR) to allow adults to mate and oviposit eggs. Each container was lined with a wax paper (Reynolds Consumer Products, Richmond, VA) for egg laying. Eggs collected from the wax paper were used in the greenhouse infestations.

All plants were cut after 21 days, when the majority of the larvae had developed to fourth-instar and pupal stages on non-*Bt* plants, by a destructive sampling method, and the number of live insects and the tunnel length inside stalks were recorded. Data on the number of live insects recovered after 21 days were organized into distance classes (Figure 1). Distance class 0 refers to the center plants that were initially infested with 50 or 70 eggs; distance class 1 refers to all eight plants that were one plant away from the center plant; distance class 2 refers to all six plants that were two plants away from the center plant; distance class 3 refers to all six plants that were three plants away from the center plant; distance class 4 refers to all six peripheral plants that were four plants away from the center plant.

Larval distributions in the five distance classes for each planting pattern were compared with other planting patterns using a multinomial logistic regression (multinomial logit) model.²⁸ The input data used by log-linear models were arranged in a 5

by 4 contingency table format. The number of insects was categorically distributed over distance classes.²⁹ The multinomial logit analysis was done using the SAS PROC LOGISTIC procedure.³⁰ In addition, the number of insects and the tunnel length were also analyzed using one-way analysis of variance (ANOVA) to examine the difference among the four planting patterns at each distance class. Stalk tunnel length is represented as tunnel length (cm) per plant. Data on number of live larvae in a distance class and tunnel length per stalk for ANOVA were first transformed to $\ln(x + 1)$ scale. Treatment means were separated using LSD tests at $\alpha = 0.05$ level. Untransformed data are presented in the figures.

2.3 Open field trials with artificial infestation of eggs on the center plants

During 2011, larval movement and plant injury of *D. saccharalis* in the four planting patterns were investigated under open field conditions with artificial infestation of eggs on the center plants. The field plots were located at the Louisiana State University Agricultural Center's Macon Ridge Research Station near Winnsboro in Franklin Parish, Louisiana. To limit the effect of the natural insect population, corn seeds were planted on 28 February, which was approximately 3 weeks ahead of a farmer's normal planting date. There was a 2m alley between each plot. At the VT-R1 plant stage,²⁵ 50 ready-to-hatch eggs of *D. saccharalis* were infested on the center plant of each plot, as described in the greenhouse studies. There were seven replications for each planting pattern. To document the natural occurrence of *D. saccharalis* at the trial site, an additional four plots of non-*Bt* plants were planted in the trial field. Artificial infestations were not performed for these four plots. Heavy rain and unexpectedly low temperatures after infestation might have led to a low egg-hatching rate on the infested plants. The hatchability was estimated to be only 35–50%. Larval occurrence and stalk tunnel length were checked 21 days after egg infestation. Data on larval distribution, insect occurrence, and tunnel length were analyzed using the methods described for the greenhouse studies.

2.4 Open field trials with natural infestation of *D. saccharalis*

Field plots with the four planting patterns of *Bt* and non-*Bt* corn were planted at three different times during 2009 at the Macon Ridge Research Station. The natural population of *D. saccharalis* in corn fields was high at the trial site in 2009, and thus no artificial insect infestations were performed. To determine a proper sampling time, occurrence of *D. saccharalis* on non-*Bt* plants at the trial site was closely monitored. Field sampling was started once significant plant damage was observed on non-*Bt* plants and the majority of the larvae were at least at the fourth-instar stage. At each sampling time, all plants of each plot were examined, and the number of insects (including larvae, pupae and pupal cases) per plant was recorded. There were nine replications for each treatment. Sampling was done at the R1–R3 plant stages for all the replicates.²⁵

For statistical analysis, data on the number of insects collected from the center non-*Bt* plants (refuge) in Trt 2 were separated from those recorded from the *Bt* plants. Similarly, data recorded on the center *Bt* plants in Trt 4 were separated from those recorded on the surrounding non-*Bt* plants. Data on the number of insects per plant were first transformed to $\ln(x + 1)$ scale and then subjected to a one-way ANOVA.³⁰ Treatment means were separated using LSD tests at an $\alpha = 0.05$ level of significance. Untransformed data are presented in Table 1.

Table 1. Occurrence (mean±SEM) of *Diatraea saccharalis* in different planting patterns in open field tests with natural infestation – 2009

Planting pattern		Number of larvae per plant*
Trt 1: pure stand of <i>Bt</i> plants		0.17±0.03 a
Trt 2: one non- <i>Bt</i> plant in the center, surrounded by 26 <i>Bt</i> plants (RIB)	<i>Bt</i> plants	0.63±0.50 a
	Non- <i>Bt</i> plant	3.33±0.97 bc
Trt 3: Pure stand of non- <i>Bt</i> plants (structured refuge)		3.79±0.26 c
Trt 4: One <i>Bt</i> plant in the center, surrounded by 26 non- <i>Bt</i> plants	<i>Bt</i> plant	1.56±0.44 b
	Non- <i>Bt</i> plant	3.53±0.21 c

* Means followed by the same letter are not significantly different (LSD, $P > 0.05$).

2.5 Open field trials with artificial infestation of neonates of *D. saccharalis* on all plants

In 2011, one field trial was conducted to examine the occurrence and plant injury of *D. saccharalis* in the four planting patterns of *Bt* and non-*Bt* plants. The trial was planted very late on July 5, 2011, and plants were artificially infested with 10 neonates plant⁻¹ on September 28, 2011. The late planting was originally designed to attract natural insect populations. However, the natural occurrence of *D. saccharalis* was very low in 2011, and thus artificial infestations were employed in the field tests. Three weeks after infestation, plants were checked, and the number of live insects and the tunnel length inside the stalks were recorded as described above. There were five replications for each treatment combination. Data on the number of live larvae per plant and tunnel length per stalk were first transformed to $\ln(x + 1)$ scale and then subjected to a one-way ANOVA,³⁰ as described for the open field trials with natural infestation.

3 Results

3.1 Greenhouse trials with artificial infestation: trial 1 – 2010

3.1.1 Larval distribution of *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

There were no significant differences in the larval distribution of *D. saccharalis* among the three planting patterns (Trts 2, 3 and 4) that had live larvae 21 days after egg infestation ($\chi_1^2 = 2.0509$, $P = 0.1521$). However, the *F*-tests showed that there were significant differences in the number of live larvae found among planting patterns for distance class 0 (center plant) and distance classes 1 and 3 ($F_{3,9} \geq 5.71$, $P \leq 0.0181$). Genuity® SmartStax™ *Bt* corn essentially had complete control of *D. saccharalis*; no live larvae were observed in the pure stand of *Bt* corn (Figure 2A). In contrast, an average of 15.5 larvae were recovered from the center non-*Bt* plants (distance class 0) in the RIB planting, which was significantly ($P < 0.05$) greater than the number of larvae (7.5) found in the center plants in the pure stand of non-*Bt* plants (Figure 2A). There were also no live larvae in the center *Bt* plants or any other *Bt* plants of the trials. In the pure stand of non-*Bt* plants, a few larvae (0.5 larvae per distance class) moved one plant away and survived 21 days after egg infestation, while no live larvae were found in the plants at least two plants away from the initially infested center plants. In contrast, for the planting pattern with a center *Bt* plant surrounded by 26 non-*Bt* plants (Trt 4), a significant number of larvae moved away from the center *Bt* plants to other non-*Bt* plants and survived after 21 days. The furthest larvae in Trt 4 were located in plants at distance class 3, but the number was not significantly different from zero

($F_{3,9} = 2.72$, $P = 0.1068$). No live larvae were found at distance class 4 in any of the four planting patterns.

3.1.2 Plant injury caused by *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

Plant injury by *D. saccharalis* 21 days after egg infestation in the 2010 greenhouse study was light, even in non-*Bt* corn plants, probably owing to the relatively low temperatures in the greenhouse. The study was conducted during winter. No tunnels were observed in the pure stand of *Bt* corn (Figure 2B). The center non-*Bt* plant in the RIB had 5.5 cm of tunnel stalk, while the center non-*Bt* plant in the structured refuge had a mean of 1 cm of tunnel stalk. Nevertheless, the overall *F*-test showed that the number of live larvae in the center plants was not significantly different among the four planting patterns ($F_{3,9} = 2.95$, $P = 0.0907$). Considering distance classes 1 to 4, only a few very short tunnels (≤ 0.4 cm) were observed in the non-*Bt* corn plants, and this was not significantly different ($F_{3,9} \leq 1.15$, $P \geq 0.3803$) among planting patterns.

3.2 Greenhouse trials with artificial infestation: trial 2 – 2011

3.2.1 Larval distribution of *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

There was a significant difference in larval distribution between the pure stand of non-*Bt* plants and the RIB planting ($\chi_1^2 = 4.4104$, $P = 0.0357$). In the pure stand of non-*Bt* corn, 90.1% of live larvae moved away from the center plants and survived on the plants at distance classes 1 to 4, and the plants that hosted the most insect individuals were in distance class 1 (18.0 larvae) (Figure 3A). In contrast, for the RIB planting, the center non-*Bt* plants harbored the most individuals (6.3 individuals), which accounted for 33.8% of the total larvae recovered. For the other pairwise comparisons, the larval distribution at the five distance classes was not significantly different among the four planting patterns ($\chi_1^2 = 3.7256$, $P = 0.0536$).

ANOVA showed that there were also significant differences in the number of live insects recovered from the center plants among the four planting patterns ($F_{3,9} = 39.22$, $P < 0.0001$). As observed in the greenhouse study in 2010, SmartStax™ provided essentially complete control of *D. saccharalis*. Across all distance classes, only one live larva was recovered in the pure stand of *Bt* corn (Figure 3A). An average of five live insects were found on the center plants in the pure stand of non-*Bt* plants, which was similar ($P > 0.05$) to the number (6.3 insects) recovered from the center non-*Bt* plants in the RIB planting (Figure 3A). No insects remained in the center plants and survived in the other two planting patterns. Significant differences in the number of live insects

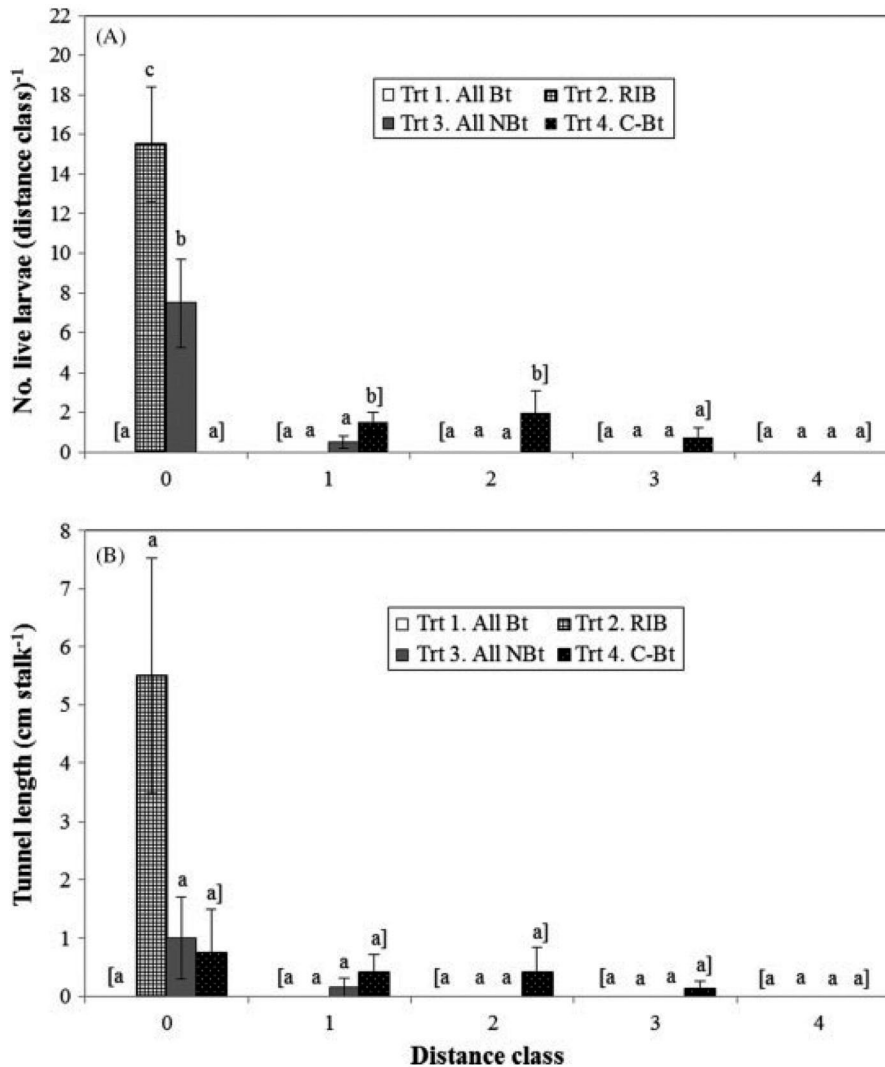


Figure 2. Larval occurrence (A: mean number of larvae per distance class \pm SEM) of and tunnel length (B: cm stalk⁻¹, mean \pm SEM) caused by *Diatraea saccharalis* in four planting patterns of *Bt* and non-*Bt* corn (greenhouse trial 1 – 2010). Mean values followed by the same letter within the same distance class in brackets are not significantly different ($P > 0.05$; LSD test).

were also observed among the four planting patterns at each of distance classes 1 to 4 ($F_{3,9} \geq 4.13$, $P \leq 0.0426$). The number of live insects recorded at each of distance classes 1 to 4 in the pure stand of non-*Bt* plants was significantly greater ($P < 0.05$) than the number found in any other planting patterns. The number of live insects was not significantly different between the RIB and Trt 4 at any of distance classes 1 to 3. No insects survived at these three distance classes in the pure stand of *Bt* plants.

3.2.2 Plant injury caused by *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

Tunnel length in plant stalks was highly correlated with the occurrence of live larvae recovered from the plants in the 2011 greenhouse study. Virtually no tunnels were observed in the pure stand of *Bt* corn (Figure 3B). Tunnel length was significantly different among the four planting patterns at each of the five distance classes ($F_{3,9} \geq 7.12$, $P \leq 0.0095$). On the center plants, an average of 71.5 cm of tunnel was observed in the pure stand of non-*Bt* plants, which was not significantly different from that (60 cm) recorded in the RIB planting (Figure 3B). At distance classes 1 to 4, tunnel length in the pure stand of non-*Bt* plants was significantly greater ($P < 0.05$) than that in the other three plant-

ing patterns. At distance class 1, the tunnel length in Trt 4 was also significantly longer ($P < 0.05$) than that of the RIB planting.

3.3 Open field trial with artificial infestation of eggs on the center plants

3.3.1 Larval distribution of *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

At the time the data were collected for this trial, no individuals of *D. saccharalis* had been found in the four non-*Bt* plant plots that were not artificially infested with *D. saccharalis*. The results indicated that natural infestation of *D. saccharalis* at the trial site was low and thus should not confound the artificial infestations. There were no significant differences ($\chi_1^2 = 0.14037$, $P = 0.2361$) in larval distribution of *D. saccharalis* among the four planting patterns. However, the number of live insects recovered was significantly different among the four planting patterns for the center plants ($F_{3,18} = 14.72$, $P \leq 0.0004$) and plants at distance class 1 ($F_{3,18} = 13.06$, $P \leq 0.0001$), but not at the greater distances ($F_{3,18} \leq 1.50$, $P \geq 0.2484$). No larvae survived after 21 days in the pure stand of *Bt* plants (Figure 4A). On the center plants, an average of 2.4 live insects were found in the pure stand of non-*Bt* plants, which was similar to the number (2.3 insects) observed

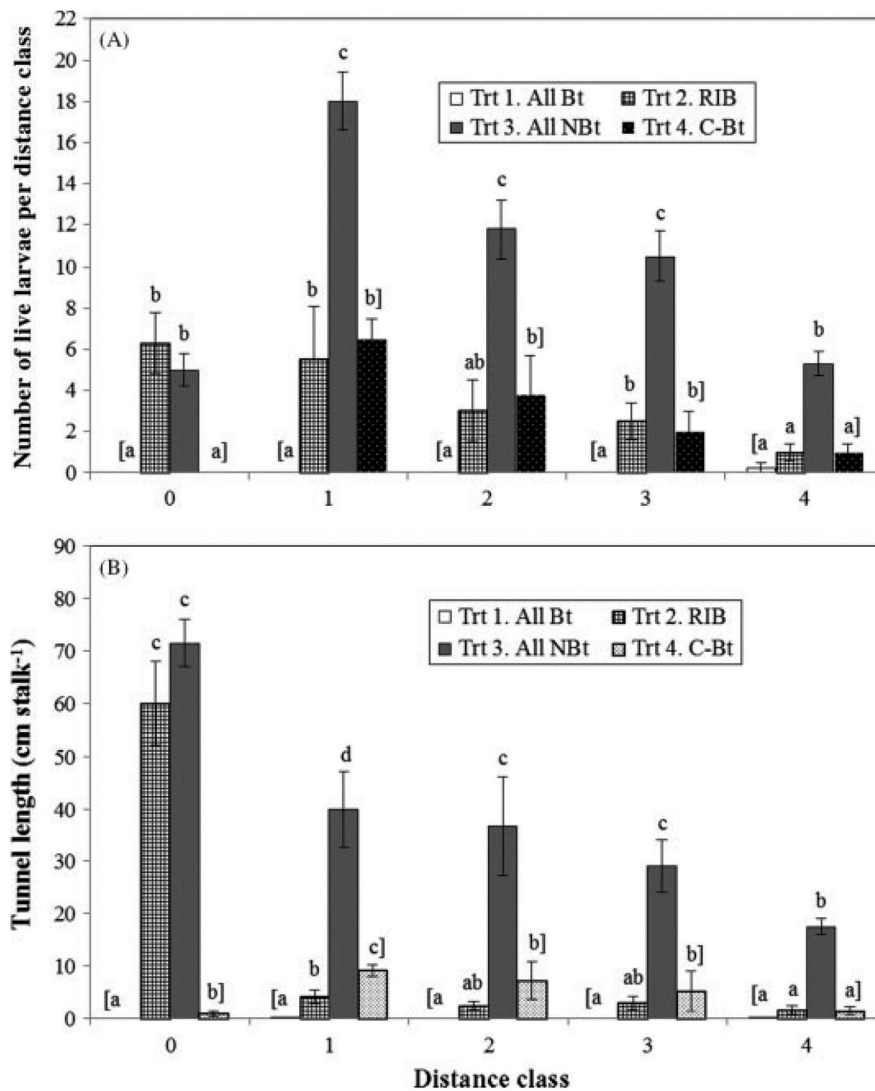


Figure 3. Larval occurrence (A: mean number of larvae per distance class \pm SEM) of and tunnel length (B: cm stalk⁻¹, mean \pm SEM) caused by *Diatraea saccharalis* in four planting patterns of *Bt* and non-*Bt* corn (greenhouse trial 2 – 2011). Mean values followed by the same letter within the same distance class in brackets are not significantly different ($P > 0.05$; LSD test).

in the RIB planting, while no insects were recovered in the center plants in the pure stand of *Bt* plants and Trt 4. At distance class 1, significantly more insects were found in the pure stand of non-*Bt* plants than the number observed in any of the other three planting patterns. Some live insects were also located at distance classes 2 to 4, but generally the number was small (≤ 0.7 larvae per distance class).

3.3.2 Plant injury caused by *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

Tunnel length was also highly correlated with the number of live insects recovered at each distance class in the four planting patterns (Figure 4B). Tunnel length in plants at distance classes 0, 1 and 2 was significantly different among planting patterns ($F_{3,18} \geq 3.53$, $P \leq 0.0354$), but not at greater distances ($F_{3,18} \leq 1.00$, $P \geq 0.4155$). No tunnels were observed in the pure stand of *Bt* corn plants. In the center plants, an average tunnel length of 12.7 cm plant⁻¹ was observed in the pure stand of non-*Bt* plants, which was not significantly different from that (9.71 cm) of the center plants in the RIB planting, while no tunnels were found in the other two planting patterns. At distance classes 1 and 2, an average tunnel length of 1.5 and 1.3 cm plant⁻¹ was recorded, re-

spectively, in the pure stand of non-*Bt* plants, which was significantly greater than that (0–0.05 cm) of the other three planting patterns. Only a few very short tunnels (≤ 0.31 cm plant⁻¹) were found at distance classes 3 and 4 across the four planting patterns.

3.4 Open field trials with natural infestation of *D. saccharalis*

Occurrence of *D. saccharalis* in the field trial conducted in 2009 was significantly different among planting patterns ($F_{5,40} = 15.38$, $P < 0.0001$). An average of 3.79 insects plant⁻¹ were found in the pure stand of non-*Bt* plants (Table 1), which was not significantly different ($P > 0.05$) from the number (3.33) on the center non-*Bt* plants in the RIB planting or from the number (3.53) on the non-*Bt* plants in Trt 4. Live insects were also observed in the pure stand of *Bt* corn, and the number (average of 0.17) was similar ($P > 0.05$) to that recorded on the *Bt* plants in the RIB planting, but the number was significantly smaller ($P < 0.05$) than that on the non-*Bt* plants. In addition, an average of 1.56 insects plant⁻¹ were observed in the center *Bt* plants that were surrounded by 26 non-*Bt* plants (Trt 4), which was significantly greater ($P < 0.05$) than the number of insects that survived in the pure stand of *Bt* corn or the *Bt* plants in the RIB planting.

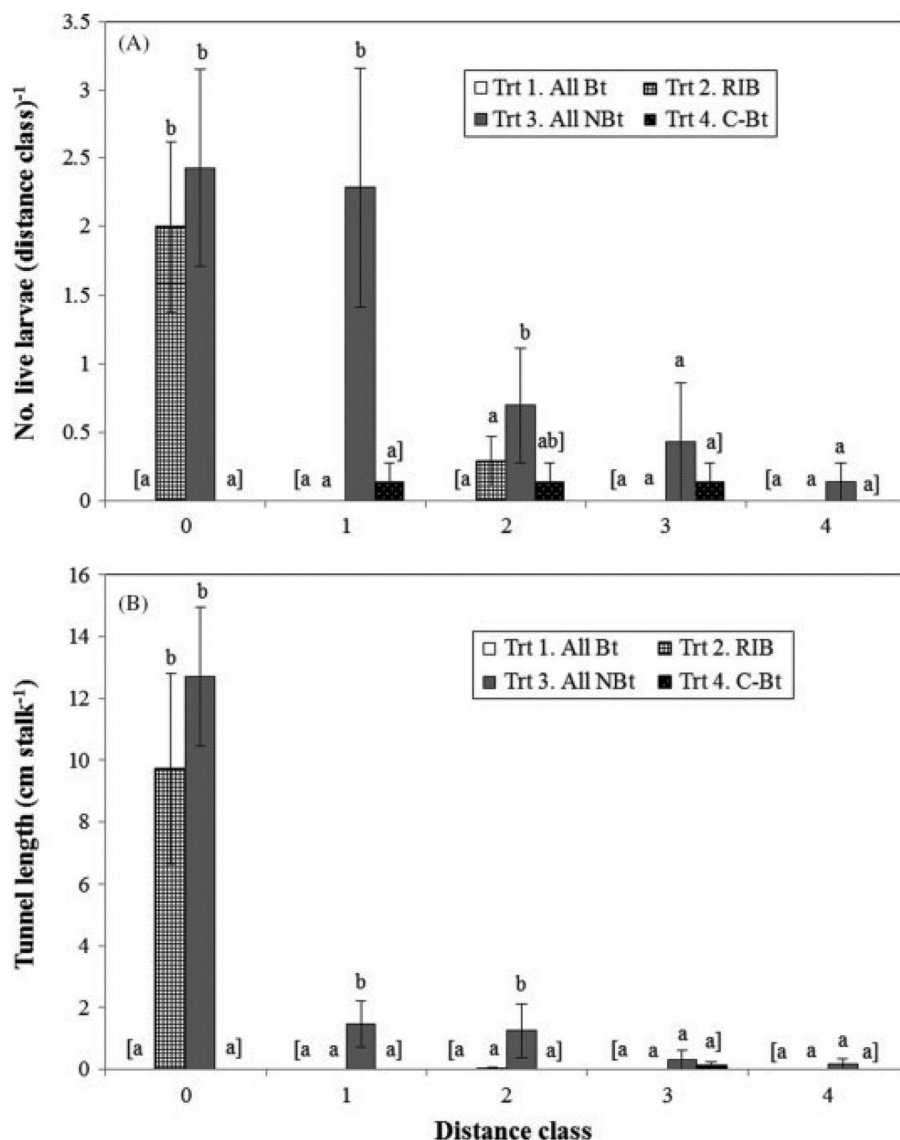


Figure 4. Larval occurrence (A: mean number of larvae per distance class \pm SEM) and tunnel length (B: cm stalk $^{-1}$, mean \pm SEM) caused by *Diatraea saccharalis* in four planting patterns of *Bt* and non-*Bt* corn (open field trial with artificial infestation of 50 eggs on the center plant). Mean values followed by the same letter within the same distance class in brackets are not significantly different ($P > 0.05$; LSD test).

3.5 Open field trials with artificial infestation of neonates of *D. saccharalis* on all plants

3.5.1 Occurrence of *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

The number of insects that survived 21 days after artificial infestation of 10 neonates plant $^{-1}$ was significantly different among the treatments ($F_{5,20} = 2.74$, $P = 0.0483$). No live insects were found in the pure stand of *Bt* plants, and only 0.02 insects plant $^{-1}$ were recorded in the *Bt* plants of the RIB planting (Table 2). An average of 0.84 live insects plant $^{-1}$ was found in the pure stand of non-*Bt* plants, which was not significantly different ($P > 0.05$) from that of the center non-*Bt* plants (0.4 insects plant $^{-1}$) in the RIB planting or on the non-*Bt* plants in Trt 4. In addition, an average of 0.64 insects plant $^{-1}$ was found in the center *Bt* plants in Trt 4, which was significantly greater ($P < 0.05$) than the number observed in the pure stand of *Bt* corn or the *Bt* plants in the RIB planting.

3.5.2 Plant injury caused by *D. saccharalis* with different planting patterns of non-*Bt* and *Bt* plants

Tunnel length inside stalks was highly correlated with the lar-

val occurrence 21 days after release of neonates. Stalk tunnel length was significantly different among treatments ($F_{5,20} = 3.01$, $P = 0.0348$). No tunnels were observed in the pure stand of *Bt* corn (Table 2). Tunnel length (1.0 cm plant $^{-1}$) in the center non-*Bt* plants in the RIB planting was not significantly different ($P > 0.05$) from that observed in other plants. An average tunnel length of 1.49 cm stalk $^{-1}$ was observed in the pure stand of non-*Bt* plants, which was also not significantly different ($P > 0.05$) from that (0.72 cm plant $^{-1}$) found in non-*Bt* plants in Trt 4, but it was significantly greater ($P < 0.05$) than that recorded in *Bt* plants in the pure stand of *Bt* plants or in the RIB planting.

4 Discussion

In the pure stands of *Bt* plants there were virtually no survivors of *D. saccharalis* and no tunnels inside the stalks 21 days after egg/larval infestation in all four tests that involved use of artificial infestation. The open field study with heavy natural infestation of *D. saccharalis* in 2009 also had an occurrence of *D. saccharalis* in the pure stand of *Bt* plants that was considerably lower than that observed on the non-*Bt* plants. Similarly, in the

Table 2. Larval occurrence (mean±SEM) and stalk tunnel length (mean±SEM) caused by *Diatraea saccharalis* in different planting patterns in open field tests with artificial infestation – 2009

Planting pattern		Number of larvae per plant*	Tunnel length per plant*(cm)
Trt 1: pure stand of <i>Bt</i> plants		0.00±0.00 a	0.00±0.00 a
Trt 2: one non- <i>Bt</i> plant in the center, surrounded by 26 <i>Bt</i> plants (RIB)	<i>Bt</i> plants	0.02±0.02 a	0.06±0.06 a
	Non- <i>Bt</i> plant	0.40±0.24 ab	1.00±0.77 abc
Trt 3: pure stand of non- <i>Bt</i> plants (structured refuge)		0.84±0.25 b	1.49±0.53 c
Trt 4: one <i>Bt</i> plant in the center, surrounded by 26 non- <i>Bt</i> plants	<i>Bt</i> plant	0.60±0.60 ab	0.40±0.40 ab
	Non- <i>Bt</i> plant	0.72±0.14 b	0.92±0.26 bc

* Means followed by the same letter are not significantly different (LSD, $P > 0.05$).

RIB planting, few insects survived, and they caused little injury on the *Bt* plants in the five trials. Collectively, the results showed that the transgenic corn containing SmartStax™ traits was effective for controlling *D. saccharalis* and protecting plant injury from the insect. The results of the present study were consistent with the results observed in a previous greenhouse study with artificial infestation of three genotypes of *D. saccharalis*,³¹ which showed that SmartStax™ *Bt* corn was effective against all three genotypes, including Cry1Ab-susceptible, Cry1Ab-resistant, and heterozygous genotypes.

Larval movement of corn stalk borers in corn fields appears to be very common.^{13,32} Studies on *O. nubilalis* have shown that 50–56% of the neonates during the first 48 h after hatching abandoned the primary host plants and dispersed to other plants along the infested row, as well as to plants in adjacent rows.³² After this period, approximately 85–94% remained within the infested rows when sampling was done 21 days after infestation.³² For this reason, larval dispersal of *D. saccharalis* in the present study was examined by infesting eggs on plants to simulate the natural occurrence. Overall, both greenhouse and open field trials in the present study showed that larvae of *D. saccharalis* have the ability to move from initially infested plants to at least four plants away, with the majority of larvae staying within three plants away from the release plant. Larvae of *D. saccharalis* also can move from the released row to adjacent rows, but the degree of dispersal varied greatly depending on the test conditions. For example, in the two greenhouse tests, 93.8% of live larvae in the pure stand of non-*Bt* corn in the trial conducted in 2010 were found in the center plants that were initially infested with 50 eggs plant⁻¹, while that number was only 9.9% for the trial in 2011. In addition, all live larvae recovered in the pure stand of non-*Bt* corn in the trial in 2010 were in the center row. In contrast, 56% of larvae moved from the center plants and survived on the two side rows in the pure stand of non-*Bt* corn in the trial conducted in 2011 (data not shown). Notable differences in larval movement/survival in the other three planting patterns were also observed between the two greenhouse trials. The authors believe that the major factor that caused these differences was variation in environmental conditions between the two tests, especially the differences in insect population densities and temperatures in the greenhouse. As mentioned above, more eggs were infested in the trial conducted in 2011 than in 2010, and the eggs used in 2011 were 1–2 days older than those used in 2010, which resulted in a much greater hatching rate than in 2010. Therefore, larval population densities of *D. saccharalis* in the trial in 2011 were much greater than those in the trial in 2010, as shown in Tables 1 and 2, suggesting that larval dispersal of

D. saccharalis was density dependent. The much higher population densities in 2011 likely increased the larval dispersal from the infested plants to other plants. In addition, the trial in 2010 was conducted during the wintertime, while the test in 2011 was performed during the early summer season. Thus, the temperatures in the greenhouse were higher during the trial period in 2011 than in 2010, which probably resulted in more feeding (as shown in Figure 3B), faster growth and more larval movement. The results suggest that studies on larval movement of corn stalk borers should consider the environmental conditions carefully.

Previous studies showed that larval dispersal of *O. nubilalis* was through silking or walking. With silking, neonates of *O. nubilalis* secrete silks that they use to hang from the host plant tissue to reach other tissues of the same host or to come into contact with other plant tissues.³³ In some cases, the silk is laid in strands hanging down the host plant but open to air currents that drag the neonates to adjacent host plants.^{33–35} Preliminary observation showed that neonates of *D. saccharalis* exhibit a similar dispersal behavior in the open corn field. Neonates of *O. nubilalis* also can employ several predispersal behavioral responses. As with other lepidopteran larvae, they display a leaf exploration phase in which they search for palatable surfaces/plant tissues in the leaf whorl or leaf tissues and feed on these preferred tissues. The ability of neonates of *O. nubilalis* to assess the host quality, leading to either acceptance or rejection, is the primary means for feeding and silking on suitable host plants (e.g. *Bt* plants).³⁵ The authors believe such food selection behaviors could also occur in larval movement of *D. saccharalis* in different planting patterns of *Bt* and non-*Bt* corn. In the present study, significant larval movement of *D. saccharalis* was also documented in the planting pattern with a center *Bt* plant surrounded by 26 non-*Bt* plants (Trt 4). In the open field trials with natural infestations in 2009, the occurrence (1.56 insects plant⁻¹) of *D. saccharalis* on the center *Bt* plants in Trt 4 was significantly greater ($P < 0.05$) than that observed in the pure stand of *Bt* plants (0.17 insects plant⁻¹) or on the *Bt* plants in the RIB planting (0.63 insects plant⁻¹) (Table 1). Similar results were also observed in the open field trials with artificial infestation of ten neonates in 2011, although the differences did not reach the $\alpha = 0.05$ level (Table 2). Such differences were most likely caused by a combination of larval movement and differential larval susceptibility to *Bt* proteins. Studies have shown that later instars of corn borers, including *D. saccharalis*, are usually more tolerant to *Bt* proteins than young larvae.^{14–16,36} Thus, young larvae could feed on non-*Bt* plants first and later move to *Bt* plants and survive to adulthood. This kind of feeding/dispersal behavior could be significant for resistance development if the RIB planting creates a

more favorable environment for such behavior to resistant heterozygotes or individual insects carrying minor resistance alleles than their susceptible counterparts.

In spite of the variation in larval movement/survival under different test conditions, the number of larvae of *D. saccharalis* recovered from the center non-*Bt* plants of the RIB planting was not significantly smaller than the number found in the pure stand of non-*Bt* corn (structured refuge) for all three trials with artificial infestation of eggs in the center plants. Plant injury (tunnel length inside the stalks) was also similar in the center plants between the two planting patterns in the three trials. Additionally, in the other two open field trials, one with natural infestation and the other with artificial infestation of neonates on all plants, there were also no significant differences in larval occurrence of *D. saccharalis* on the center non-*Bt* plants in the RIB planting compared with that observed in the pure stand of non-*Bt* plants. Collectively, the results of this study show that refuge plants in a seed mixture may be able to provide a comparable refuge population of *D. saccharalis* to a structured refuge planting. However, additional studies are necessary to determine whether RIB planting could also create a more favorable condition for survival of *Bt*-resistant heterozygotes because of the significant larval movement of *D. saccharalis* in the corn field as demonstrated in this study.

Several earlier studies have discussed the utility of the RIB strategy for IRM.^{13,37,38} Gould and Anderson³⁷ suggested that an RIB strategy could be successful in delaying the evolution of insect resistance to *Bt* crops. RIB was also predicted to enhance random mating between insects within the field if larval movement among *Bt* and non-*Bt* plants was not a significant event.³⁹ Mallet and Porter³⁸ reported that, if insect movement were independent of the presence of toxin inside plants, *Bt* and non-*Bt* seed mixtures could be used to delay resistance evolution for *Bt* crops. The results of the present study suggest that a seed mixture strategy (RIB) may be a suitable IRM strategy for managing the risk of *D. saccharalis* evolving resistance to pyramid *Bt* corn events such as SmartStax™. However, *D. saccharalis* is only one of three major target species of *Bt* corn in the southern region of the United States. The other two major targets of the second-generation pyramid *Bt* corn in this region are the corn earworm, *Helicoverpa zea* (Boddie), and fall armyworm, *Spodoptera frugiperda* (JE Smith).^{40,41} These two species are also among the major pests of cotton, soybean, and other crops in the region. Additional studies are also needed to evaluate the RIB strategy for managing these pests, especially for *H. zea* because of its kernel-feeding nature in corn fields.

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