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Survival of Corn Earworm (Lepidoptera: Noctuidae) on Bt Maize and Cross-Pollinated Refuge Ears From Seed Blends

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Abstract

Refuge is mandated in the United States where genetically modified maize (*Zea mays* L.) expressing insecticidal proteins derived from *Bacillus thuringiensis* Berliner (Bt) are cultivated. Currently, refuge is deployed in different ways including blocks, field strips, or seed blends containing Bt and non-Bt maize. Seed blends provide practical advantages for refuge implementation. However, concerns related to the movement of insect larvae, potential differential survival of heterozygous resistant larvae, reduction in insect production, and cross-pollination of ears resulting in sublethal selection, have delayed seed blend use for Lepidoptera in the southern United States, where maize plantings are used as refuge for *Helicoverpa zea* (Boddie). In this study, we evaluated the relative survival of *H. zea* in Bt events and in seed blends compared with pure stand refuge and the relative survival of *H. zea* on the individual components of the pyramid 1507xMON810xMIR162. The results showed variation on the production of *H. zea* in refuge plants from seed blends compared with pure stand refuge plants. The relative survival of *H. zea* on the events 1507, MON810, MIR162, and 1507xMON810xMIR162 ranked similarly across the three locations tested. These results can be used in computer simulation modeling efforts to evaluate the feasibility of seed blends as a refuge deployment strategy with the pyramid 1507xMON810xMIR162. Because the reduction on survival of *H. zea* due to blending was variable, a sensitivity analysis that includes all possible scenarios of reduction in survival should be considered.

Key words: refuge in a bag, seed blend, cross-pollination, *Helicoverpa zea*

Insect resistance management for lepidopteran pests in the United States relies on the high dose plus refuge strategy. The Environmental Protection Agency mandates growers to adopt refuge in fields cultivated with genetically modified maize (*Zea mays* L.) expressing insecticidal proteins derived from *Bacillus thuringiensis* Berliner (Bt) (USEPA 1998). According to this strategy, resistance development can be delayed if plants express a dose high enough to kill heterozygous resistant individuals, and a refuge with non-Bt maize is available to produce susceptible insects to mate with resistant insects. Currently in the United States, refuge is deployed in three different ways including blocks, field strips, or seed blends containing Bt and non-Bt maize. Seed blends are advantageous for refuge implementation for technical and practical reasons (Davis and Onstad 2000, Carroll et al. 2012). Seed blends can promote more effective random mating of resistant and susceptible insects (Davis and Onstad 2000). Additionally, seed blends are easier for growers to implement than blocks or field strips and eliminate the issue of grower noncompliance with refuge requirements (Davis and Onstad 2000, Carroll et al. 2012). However, concerns related with the movement of insect larvae, potential differential survival of heterozygous resistant larvae, reduction in insect production, and cross-pollination of ears resulting in sublethal selection (Mallet and Porter 1992, Davis and Onstad 2000, Wangila et al. 2013, Yang et al. 2014b) have delayed seed blend use for Lepidoptera in the southern United States. Many of these concerns involve increased resistance risk for corn earworm, *Helicoverpa zea* (Boddie), an ear feeding pest also present in Bt cotton, *Gossypium hirsutum* L. (Yang et al. 2014b).

Significant insect movement in a seed blend introduces the potential for insects to feed on both non-Bt and Bt plant tissues, which could make resistance less recessive via a reduction in overall dose (Davis and Onstad 2000, Carroll et al. 2012). In this scenario, heterozygotes may survive at a higher rate in seed blends than in a pure stand of Bt plants, potentially accelerating resistance evolution (Mallet and Porter 1992). Larval movement may also reduce the number of susceptible refuge insects if they leave the non-Bt plant and feed upon adjacent Bt plants (Davis and Onstad 2000, Carroll et al. 2012). Cross-pollination between Bt and non-Bt plants can increase insect exposure to the Bt toxins via pollen and kernels expressing Bt proteins in blended refuge ears. This may negatively affect survival, growth, development, and fitness of refuge insects feeding on the plants (Burkness et al. 2011, Wangila et al. 2013, Yang et al. 2014b). On a blended refuge ear, insects can escape Bt protein exposure by feeding on silks, husks, and the cob, which are maternal tissues without Bt protein. But later in development, insects may switch to feeding on kernels that express moderate doses (Chilcutt and Tabashnik 2004).

Despite these challenges, computer simulation modeling efforts have shown that blending can be an effective insect resistance management strategy for certain pest species when used with pyramided Bt maize (Carroll et al. 2012). For modeling the relative durability of a pyramid with refuge deployed as seed blends compared with a block refuge, it is important to obtain estimates of survival on the Bt events from the pyramid and to understand the relative survival of the insects on the refuge when refuge plants are deployed as blends and pure stands. The primary objective of this study was to investigate if a blended refuge with transgenic maize event containing 1507xMON810xMIR162 caused changes in survival and development of the ear-feeding pest *H. zea*. A second objective was to estimate the relative survival of *H. zea* on Bt events. Two different field experiments were performed. Both experiments deployed refuge plants in various cluster arrangements to simulate the multiple types of clusters that may randomly appear in a field planted with blended refuge. The first experiment utilized natural infestations of *H. zea* and for the second experiment the ears were artificially infested with *H. zea* neonates.

Materials and Methods

Source of Bt and Non-Bt Maize and Experimental Conditions

Bt and non-Bt maize hybrids were produced at a DuPont Pioneer production facility. In all locations, the blended refuge was tested using a DuPont Pioneer Bt maize hybrid containing the events 1507, MON810, and MIR162, referred to as 1507xMON810xMIR162; which contains the insecticidal genes Cry1F, Cry1Ab, and Vip3Aa (respectively) derived from Bt. A non-Bt hybrid (33W80) of closely related genetics was provided to serve as blended refuge with 1507xMON810xMIR162. The blend components were compared with pure stands of the non-Bt maize, single events of 1507, MON810, and MIR162, and the pyramid 1507xMON810x MIR162. All hybrids used in the study were characterized for trait purity using either polymerase chain reaction (PCR) or qualitative enzyme-linked immunosorbent assay (ELISA) (Envirologix Inc., Portland, ME; Romer Labs Inc. Union, MO). In total, 200 seeds from each entry were tested with PCR and entries identified with unacceptable off-type rates in the PCR analysis were sampled in the field after seedling emergence. Leaf punches were taken and analyzed via PCR or qualitative ELISA. All seeds were treated with thiamethoxam, 0.25 mg of active ingredient per seed (Cruiser 250, insecticidal seed treatment, low rate for secondary pest protection, Syngenta Crop Protection, Greensboro, NC). All plots were machine planted. The refuge component of seed blends in the experiment with artificial infestations was hand planted.

Field Trials With Natural Infestations

Experimental Design and Treatments. In total, three trials with natural insect infestations of *H. zea* were performed in the southern United States during 2012. The trials were located in Plains, GA, Starkville, MS, and Stoneville, MS. The trials consisted of eight treatments with six replicates per planting arranged in a randomized complete block design. Planting patterns consisted of pure stands of refuge (Trt 1), MON810 (Trt 2), 1507 (Trt 3), MIR162 (Trt 4), and 1507xMON810xMIR162 (Trt 5). In addition, 1507x MON810xMIR162 was planted as a seed blend, using 5, 10, and 20% refuge seed blending rates. Because no significant differences were detected for mean instar and total *H. zea* across refuge plants in the blending rates (Supp Table 1 [online only]), two cluster types with Bt (b) and refuge (R) plants were identified for comparisons: bRR (Trt 6) and bRb (Trt 7). The cluster type bRR was identified only on plots with blend rates 10 and 20%. The cluster type bRb was common across the three blend rates. Each experimental plot was approximately 12–13 m long and 4 rows wide. Row spacing across all locations was approximately 90–100 cm and plant population was approximately 79,000 plants/ha. The data were collected on all four rows of each plot. Blocks were separated by two empty rows of the same length. Treatments were randomly assigned to the experimental plots. Experiments were planted either with isolation distance of approximately 48 m within a field or were temporally isolated from other maize fields. Late plantings were used to improve the chances to obtain a natural infestation of *H. zea*. Usually, mature larvae only leave one exit hole per ear (Horner et al. 2003), and there is a corresponding feeding cavity on the ear. Thus larval densities may be estimated by counting ears with exit hole. However, not all *H. zea* larvae leave holes in ears when they leave to pupate, so a subsample of ears within the pure stand treatments were bagged to estimate the total number of pupating *H. zea* per treatment. Ears were not bagged in blended refuge entries to allow for larval movement among plants.

Planting. All plots were machine planted. Refuge plants were identified in blended refuge plots using glufosinate painting and refuge plants were marked. The location of Bt (b) and refuge plants (R) were recorded to create cluster maps. During quality control evaluations of the maps, plant location mismatches with the data were identified for one location, Stoneville, MS. The seed blend data for this location were not analyzed; thus, only data from bagged ears in pure stands were analyzed from this location.

Ear Bagging and Corn Earworm Survival to Pupation. Because ears in pure stand entries were bagged, it was possible to assess survival to pupation. In each plot, after silking stage and eggs or larvae were detected on plants, approximately 30 plants were randomly marked, and the ear was covered with a mesh bag. When the *H. zea* larvae started to pupate on refuge plants, the bags were removed and the following data were collected for each ear: number of larvae and number of pupae. Each exit hole in the bag, if present, was counted as one larva reaching pupation, as cited in Yang et al. (2014a), mature larvae of *H. zea* usually drop from the ears to pupate in the soil.

Corn Earworm Larval Survival and Development. Larval survival and development were assessed on clusters of maize plants in each plot on nonbagged ears. Each treatment contained up to ten 3-plant clusters per replicate. Clusters consisted of pure stand Bt maize (bbb), blended refuge (bRb or bRR), or pure stand refuge maize (RRR). The three-plant clusters were identified by marking the center plant. At approximately the reproductive stage R2–R4 (Ritchie et al. 1992), all ears in the clusters were removed from the plants, husks and silks stripped, and all larvae were collected and placed in labeled vials (one vial per plant) containing 70% ethanol. Larval stage was determined based on head capsule width. Head capsule widths used were 0.3–0.4, 0.5–0.7, 0.8–1.2, 1.3–2.0, 2.1–3.0, and $\geq$ 3.1 mm, respectively, for instars 1–6. The number of larvae and larval instar were recorded for each ear as well as the number of exit holes.

Field Trials With Artificial Infestations

Experimental Design and Treatments. In total, two trials with artificial insect infestations were performed in Dallas Center and Johnston Iowa, USA in 2012. The trials consisted of nine treatments with six replicates arranged in a randomized complete block design. Planting patterns consisted of pure stands of refuge (Trt 1), MON810 (Trt 2), 1507 (Trt 3), MIR162 (Trt 4), and 1507xMON810xMIR162 (Trt 5), and four different seed blend clusters made of 1507xMON810xMIR162 plants (Trt 6-9) in combination with refuge plants. Planting patterns with seed blends were used to create four different 5-plant cluster types using Bt (b) and refuge (R) plants: bbRbb (Trt6), bRbRb (Trt7), bbRRb (Trt8), and bRRRb (Trt9). Each experimental unit consisted of approximately a 5 m long by 6 m wide row plot. Row spacing for both locations was approximately 75 cm and plant population was approximately 89,000 plants/ha. The data were collected only from the four center rows of each plot (rows 2–5) where eight clusters were formed. The two external rows (rows 1 and 6) served as border rows. Blocks were separated by two rows of the same length planted with a hybrid containing MIR162. Border refuge rows were used to assess larval development.

Planting, Plant Thinning, and Marking Clusters. Refuge seed in the blended refuge treatments were hand planted within 48 hr of machine planting. Refuge seeds were placed within 2 inches of the machine planted row to establish different cluster types. Extra seeds were planted to ensure that enough plants would be available to form the different cluster treatments. When plants were between the V2 and V3 stages, plots were thinned to achieve a uniform plant stand and the desired clusters. This step was performed prior to insect infestations. Plants that were too close to each other (double plants) or that did not reflect the average condition of the plot (i.e., unhealthy stunted plants, plants delayed in development, etc.) were removed from the plot. For selecting clusters in pure stand treatments (Trt 1-5), two 5-plant clusters were marked per row. The plot was divided approximately in the middle and five consecutive plants were selected on each half. Stakes were used to mark the first and the last plant that composed the cluster. The machine-planted plants that were replaced by the hand-planted plants were also removed. If the quality of the refuge plant left in the plot after thinning was still questionable, the cluster was eliminated from the study. The 1507 (Trt3) was not planted correctly in the Johnston location, and this entry was removed from the comparison.

Insect Infestation. The four center rows of each six-row plot were infested. All plants were artificially infested. *H. zea* eggs were supplied by Chesapeake-PERL, Inc. (Newark, DE). Artificial infestation occurred when 50% of the plants in the experiment were shedding pollen (growth stage R1). Eggs were incubated in the laboratory. Once hatch was nearly complete, *H. zea* neonates were mixed with commercially available corn cob grits and applied with a mechanical dispensing device (Wiseman et al. 1980). The device was precalibrated to deliver approximately 25 larvae per plant onto the silks of the primary ear. All plants in the four inner rows (rows 2–5) were infested.

Corn Earworm Survival and Development. To evaluate larval survival, the ears of the five-plant clusters were stripped and checked for presence of larvae when most larvae had reached 4th and 5th instars. All living larvae were collected and placed in labeled vials (one vial per plant) containing 70% ethanol. Larval samples were sent to the laboratory in Johnston, IA for staging based on the width of their head capsules.

Statistical Analysis. Statistical analyses were conducted using SAS software, Version 9.3 (SAS Institute Inc., Cary, NC). In locations with artificial infestations, pairwise comparisons were made for mean number of larvae and mean instar across treatments. Data from locations with natural infestations and nonbagged ears were analyzed with the same procedures to compare mean number of exit holes, number of larvae, number of exit holes + larvae, and instar across treatments. Likewise, data from locations with natural infestations and bagged ears were analyzed to compare mean number of larvae, pupae and total corn earworms (number of larvae + pupae). Treatments with a small number of larvae were excluded from comparisons of mean instar.

On a per location basis, the following groups of comparisons were made: 1) pairwise comparisons among refuge plants in blended 1507xMON810xMIR162 treatments, Bt plants in blended 1507x MON810xMIR162 treatments, pure stand 1507xMON810x MIR162, pure stand 1507, pure stand MON810, pure stand MIR162, and pure stand refuge for plants with nonbagged ears from artificially and naturally infested trials; 2) pairwise comparisons among refuge plants in cluster types in blended plots with 1507xMON810xMIR162 plants; 3) pairwise comparisons among Bt plants in cluster types in blended plots with 1507x MON810xMIR162 plants; 4) pairwise comparisons among pure stand 1507xMON810xMIR162, pure stand 1507, pure stand MON810, pure stand MIR162, and pure stand refuge for bagged ears from naturally infested trials.

For comparisons in 1) and 4), linear mixed models were used with treatment as the fixed effect, replication as the random effect and each plot as experimental unit with clusters within each plot and plants within each cluster as subsamples. Residual variances were modeled as heterogeneous for treatments with or without MIR162 trait. For comparisons in 2) and 3), different cluster types were compared. Linear mixed models were used with cluster type and interaction of treatment and cluster type being the fixed effects, replication, and interaction of replication, treatment and cluster type as the random effects, and covariance between plants within cluster was modeled using a compound symmetry variance structure.

Comparisons were also made between refuge plants in blended 1507xMON810xMIR162 treatments and pure stand refuge across the two locations with natural infestation and across the two locations with artificial infestation. Linear mixed models were used with planting type (pure stand refuge or refuge from blended treatments), location, interaction of planting type and location, blend rate nested within planting type, and interaction of location and blend rate

Table 1. Mean larvae, mean pupae, and mean total *H. zea* recovered from bagged ears in Plains, GA, Starkville, MS, and Stoneville, MS, USA (2012)

Maize type	Mean (95%CI)[a]		
	Larvae	Pupae	Total *H. zea*[b]
Plains, GA			
Refuge	0.14 (0.07–0.22)B $n = 180$	0.46 (0.39–0.53)A $n = 180$	0.61 (0.50–0.71)A $n = 180$
1507	0.25 (0.18–0.32)A $n = 176$	0.12 (0.05–0.19)B $n = 176$	0.36 (0.26–0.47)B $n = 176$
MON810	0.12 (0.05–0.20)B $n = 178$	0.04 (0.00–0.11)BC $n = 178$	0.16 (0.06–0.27)C $n = 178$
MIR162	0.00 (0.00–0.00)C $n = 177$	0.01 (0.00–0.03)C $n = 177$	0.01 (0.00–0.03)D $n = 177$
1507xMON810xMIR162	0.00 (0.00–0.00)C $n = 179$	0.00 (0.00–0.02)C $n = 179$	0.00 (0.00–0.02)D $n = 179$
Starkville, MS			
Refuge	0.29 (0.19–0.39)B $n = 180$	0.21 (0.15–0.27)A $n = 180$	1.26 (1.04–1.47)A $n = 180$
1507	0.46 (0.36–0.55)A $n = 180$	0.09 (0.04–0.15)B $n = 180$	1.02 (0.80–1.23)AB $n = 180$
MON810	0.51 (0.41–0.61)A $n = 179$	0.15 (0.09–0.20)AB $n = 179$	0.93 (0.71–1.14)B $n = 179$
MIR162	0.03 (0.00–0.13)C $n = 150$	0.00 (0.00–0.06)C $n = 150$	0.03 (0.00–0.27)C $n = 150$
1507xMON810xMIR162	0.01 (0.00–0.01)C $n = 180$	0.00 (0.00–0.00)C $n = 180$	0.01 (0.00–0.02)C $n = 180$
Stoneville, MS			
Refuge	0.22 (0.09–0.34)A $n = 180$	0.18 (0.09–0.26)A $n = 180$	0.39 (0.24–0.55)A $n = 180$
1507	0.32 (0.20–0.44)A $n = 176$	0.05 (0.00–0.13)B $n = 176$	0.37 (0.21–0.52)A $n = n = 176$
MON810	0.22 (0.10–0.35)A $n = 175$	0.02 (0.00–0.11)B $n = 175$	0.24 (0.08–0.40)A $n = 175$
MIR162	0.00 (0.00–0.00)B $n = 186$	0.00 (0.00–0.01)B $n = 186$	0.00 (0.00–0.00)B $n = 186$
1507xMON810xMIR162	0.00 (0.00–0.00)B $n = 190$	0.00 (0.00–0.01)B $n = 190$	0.00 (0.00–0.00)B $n = 190$

The number of data points is represented in tables by the letter *n* (*n* refers to number of plants).

[a]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

[b]Total *H. zea* was calculated adding number of larvae, pupae, and bag exit holes if present.

(nested within planting type) as the fixed effects, replication nested within location as the random effect. Each plot was treated as experimental unit with clusters within each plot and plants within each cluster as subsamples. For a number of exit holes, number of larvae, and number of exit holes + larvae, residual variances were modeled as heterogeneous by location.

Least squares means (LS–Means) and 95% confidence intervals for treatment were estimated from the mixed model and the prespecified statistical comparisons were made. A statistically significant difference was identified if the *P*-value was less than 0.05. Alphabetical letters were assigned to each treatment, and treatments with a common letter were not statistically different from each other at the significance level of 0.05. The LS-Means, the 95% confidence limits and the letters are reported (Tables 1–8).

Results

Field Trials With Natural Infestations

Total Corn Earworm Survival on Pure Stands. The total number of *H. zea* estimated using numbers obtained from plants with bagged ears is shown in Table 1. In Plains, GA survival rate estimates for *H. zea* on Bt plants relative to survival on refuge plants reached values of 59, 26, 2, and 0% in 1507, MON810, MIR162, and 1507xMON810xMIR162, respectively. In Starkville, MS survival rate estimates for *H. zea* on Bt plants relative to survival on refuge plants reached values of 81, 74, 2, and 1% in 1507, MON810, MIR162, and 1507xMON810xMIR162, respectively. In Stoneville, MS survival rate estimates for *H. zea* on Bt plants relative to survival on refuge plants reached values of 95, 62, 0, and 0% in 1507, MON810, MIR162, and 1507xMON810xMIR162, respectively. In all three locations, there was no significant difference in survival between MIR162 and 1507xMON810xMIR162. However, differences between 1507, MON810, and the refuge varied by location.

Recovery and Development of Corn Earworm Larvae on Pure Stands and Blends. Larval production on plants expressing single events and the pyramid traits were estimated by adding the exit holes and larvae found on nonbagged ears. Evaluations in naturally infested ears revealed that mean exit holes + larvae per ear from pure stand refuge plants were 1.17 (Plains, GA) and 2.22 (Starkville,

Table 2. Mean number of exit holes, mean larvae, mean total *H. zea* produced (exit holes + larvae) and mean instar of larvae recovered from ears in Plains, GA and Starkville, MS, USA (2012)

Planting	Maize type	Mean (95%CI)[a]			
		Exit holes	Larvae	Exit holes + larvae	Instar
Plains, GA					
Pure stand	Refuge	0.27 (0.10–0.43)A $n = 180$	0.90 (0.73 – 1.07)AB $n = 180$	1.17 (0.91–1.42)A $n = 180$	3.7 (3.4–4.0)A $n = 162$
	1507	0.11 (0.00–0.28)ABC $n = 180$	1.07 (0.90–1.24)A $n = 180$	1.18 (0.93–1.44)A $n = 180$	3.1 (2.7–3.4)B $n = 193$
	MON810	0.00 (0.00–0.16)BC $n = 180$	0.69 (0.52–0.87)B $n = 180$	0.69 (0.44–0.95)B $n = 180$	2.5 (2.2–2.9)C $n = 125$
	MIR162	0.00 (0.00–0.02)C $n = 174$	0.04 (0.00–0.07)C $n = 174$	0.04 (0.00–0.08)C $n = 174$	1.0 $n = 6$
	1507xMON810xMIR162	0.00 (0.00–0.02)C $n = 180$	0.01 (0.00–0.04)C $n = 180$	0.01 (0.00–0.05)C $n = 180$	1.0 $n = 2$
Blend	Refuge	0.17 (0.08–0.27)AB $n = 219$	0.91 (0.81–1.00)A $n = 219$	1.08 (0.93–1.23)A $n = 219$	3.3 (3.0–3.6)A $n = 204$
	1507xMON810xMIR162	0.01 (0.00–0.02)C $n = 345$	0.02 (0.00–0.04)C $n = 345$	0.03 (0.00–0.05)C $n = 345$	2.6 $n = 8$
Starkville, MS					
Pure stand	Refuge	1.80 (1.50–2.10)A $n = 171$	0.42 (0.29–0.54)B $n = 171$	2.22 (1.88–2.56)A $n = 171$	4.8 (4.6–5.0)A $n = 71$
	1507	0.85 (0.55–1.14)B $n = 177$	0.78 (0.66–0.91)A $n = 177$	1.63 (1.28–1.97)B $n = 177$	4.4 (4.2–4.6)BC $n = 138$
	MON810	0.84 (0.54–1.14)B $n = 174$	0.72 (0.60–0.84)A $n = 174$	1.56 (1.22–1.91)B $n = 174$	4.5 (4.3–4.7)ABC $n = 126$
	MIR162	0.07 (0.00–0.15)CD $n = 108$	0.28 (0.20–0.36)BC $n = 108$	0.36 (0.23–0.48)C $n = 108$	3.1 (2.7–3.5)DE $n = 33$
	1507xMON810xMIR162	0.00 (0.00–0.07)D $n = 177$	0.07 (0.00–0.14)D $n = 177$	0.07 (0.00–0.18)D $n = 177$	2.5 (1.9–3.0)E $n = 12$
Blend	Refuge	1.08 (0.91–1.25)B $n = 263$	0.22 (0.15–0.29)C $n = 263$	1.30 (1.10–1.50)B $n = 263$	4.6 (4.3–5.0)AB $n = 62$
	1507xMON810xMIR162	0.10 (0.06–0.14)C $n = 388$	0.04 (0.00–0.09)D $n = 388$	0.14 (0.07–0.21)D $n = 388$	3.6 (2.7–4.5)CD $n = 17$

The number of data points is represented in tables by the letter *n* (for exit holes, larvae, and exit holes + larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured).

[a]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

Table 3. Mean number of exit holes, mean larvae, mean total *H. zea* produced (exit holes + larvae) and mean instar of larvae recovered from refuge ears in Plains, GA and Starkville, MS, USA combined (2012)

Planting	Mean (95%CI)[a]			
	Exit holes	Larvae	Exit holes + larvae	Instar
Pure Stand	1.03 (0.83–1.24)A $n = 351$	0.66 (0.56–0.76)A $n = 351$	1.69 (1.46–1.93)A $n = 351$	4.3 (4.0–4.5)A $n = 233$
Blend	0.63 (0.51–0.74)B $n = 482$	0.56 (0.50–0.62)A $n = 482$	1.19 (1.05–1.32)B $n = 482$	4.0 (3.7–4.2)A $n = 266$

The number of data points is represented in tables by the letter *n* (for exit holes, larvae, and exit holes + larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured).

[a]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

MS; Table 2). In Plains GA, there was no difference between the mean exit holes +larvae recovered from refuge plants and 1507. Mean instar [3.1 (2.7–3.4)] from ears of 1507 was lower and significantly different than the mean value estimated from pure stand refuge plants [3.7 (3.4–4.0)]. Other single events and the pyramid exhibited significantly ($P < 0.05$) fewer larvae compared with the refuge. Relative production estimates (%) based on exit holes + larvae on Bt plants relative to refuge plants reached values of 59, 3, and 1% for MON810, MIR162, and 1507xMON810xMIR162, respectively. Virtually no larvae were recovered from MIR162, 1507xMON810xMIR162, or blended 1507xMON810xMIR162. No statistical differences in the mean exit holes + larvae recovered from ears of blended (1.08) and pure stand (1.17) refuge plants were found. Mean instar [2.5 (2.2–3.4)] from ears of MON810 was significantly different than the mean value estimated from pure stand refuge plants [3.7 (3.4–4.0)]. There were very few exit holes + larvae

Table 4. Mean number of exit holes, mean larvae, mean total *H. zea* produced (exit holes + larvae) and mean instar of larvae recovered from ears of refuge in two plant cluster configurations in Plains, GA and Starkville, MS, USA (2012)

Cluster design[a]	Mean (95%CI)[b]			
	Exit holes	Larvae	Exit holes + larvae	Instar
Plains, GA				
b**RR**	0.31 (0.13–0.48)A $n = 62$	1.08 (0.88–1.28)A $n = 62$	1.39 (1.15–1.63)A $n = 62$	3.4 (2.8–3.9)A $n = 66$
b**R**b	0.16 (0.03–0.29)A $n = 157$	0.88 (0.75–1.02)A $n = 157$	1.05 (0.88–1.21)B $n = 157$	3.3 (2.9–3.6)A $n = 138$
Starkville, MS				
b**RR**	1.05 (0.70–1.39)A $n = 74$	0.31 (0.21–0.42)A $n = 74$	1.35 (1.00–1.71)A $n = 74$	4.7 (4.0–5.3)A $n = 23$
b**R**b	1.10 (0.85–1.34)A $n = 171$	0.22 (0.14–0.29)A $n = 171$	1.31 (1.06–1.57)A $n = 171$	4.6 (4.2–5.1)A $n = 37$

The number of data points is represented in tables by the letter n (for exit holes, larvae, and exit holes + larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured). Other treatments are presented in Table 1.

[a] R indicates refuge plant and b indicates Bt plant. Bold letters represent the number and type of plants that were used to calculate the mean value.

[b]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

Table 5. Mean number of above exit holes, mean larvae, mean total *H. zea* produced (exit holes + larvae) and mean instar of larvae recovered from ears of Bt plants in two plant cluster configurations in Plains, GA and Starkville, MS, USA (2012)

Cluster design[a]	Mean (95%CI)[b]			
	Exit holes	Larvae	Exit holes + larvae	Instar
Plains, GA				
bRR	0.00 (0.00–0.03)A $n = 31$	0.03 (0.00–0.09)A $n = 31$	0.03 (0.00–0.11)A $n = 31$	4.0 $n = 1$
bRb	0.01 (0.00–0.02)A $n = 314$	0.02 (0.00–0.05)A $n = 314$	0.03 (0.00–0.06)A $n = 314$	2.6 $n = 7$
Starkville, MS				
bRR	0.13 (0.00–0.25)A $n = 37$	0.07 (0.00–0.15)A $n = 37$	0.19 (0.03—0.35)A $n = 37$	4.0 $n = 3$
bRb	0.10 (0.05–0.15)A $n = 342$	0.04 (0.01–0.07)A $n = 342$	0.13 (0.07–0.20)A $n = 342$	4.2 $n = 13$

The number of data points is represented in tables by the letter *n* (for exit holes, larvae, and exit holes + larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured).

Other treatments are presented in Table 1.

[a]R indicates refuge plant and b indicates Bt plant. Bold letters represent the number and type of plants that were used to calculate the mean value.

[b]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

recovered from 1507xMON810xMIR162 to make statistical comparisons.

In Starkville, MS the single events and the pyramid exhibited significantly fewer exit holes + larvae compared with the refuge ($P < 0.05$, Table 2). Relative production estimates (%) based on exit holes + larvae on Bt plants relative to refuge plants reached values of 73, 70, 16, and 3% for 1507, MON810, MIR162, and 1507x MON810xMIR162, respectively. Very few larvae were recovered from MIR162, 1507xMON810xMIR162, or blended 1507x MON810xMIR162. There were statistically significant differences in the mean exit holes + larvae recovered from ears of blended (1.30) and pure stand (2.22) refuge plants, with pure stand plants producing around 70% more larvae than blended refuge plants. The mean instar of larvae recovered from pure stand refuge plants (4.8) and blended refuge plants (4.6) was not significantly different at $P < 0.05$. When the number of exit holes, larvae, exit holes + larvae, and mean instar were combined across the two locations (Table 3), there were statistically significant differences in the mean number of exit holes and exit holes + larvae recovered from ears of blended and pure stand refuge plants. Pure stand refuge plants produced around 63% more exit roles and 42% more exit holes + larvae than blended refuge plants.

In Plains, GA the cluster configuration with two contiguous refuge plants (bRR) blended with 1507xMON810xMIR162 exhibited more exit holes + larvae (1.39) than the cluster configuration with single refuge plant [bRb, (1.05), Table 4] and the difference was statistically significant ($P < 0.05$). No statistical differences between cluster configurations were detected in Starkville, MS. Mean instar of *H. zea* on refuge plants blended with 1507xMON810xMIR162 at different cluster configurations were similar within locations and varied between 3.4 and 3.3 (Plains, GA) and 4.7 and 4.6 (Starkville, MS) (Table 4). Very few larvae were found on blended 1507xMON810xMIR162 plants (Table 5). There were too few larvae recovered to analyze instar.

Table 6. Mean number of *H. zea* larvae and mean instar of larvae recovered from ears in Dallas Center and Johnston, IA, USA (2012)

Planting	Maize type	Mean (95%CI)[a]	
		Larvae	Instar
Dallas Center, IA			
Pure stand	Refuge	1.50 (1.27–1.74)BC $n = 119$	4.5 (4.4–4.7)A $n = 179$
	1507	2.18 (1.94–2.41)A $n = 120$	3.8 (3.7–3.9)B $n = 255$
	MON810	1.60 (1.37–1.83)B $n = 120$	2.8 (2.6–2.9)C $n = 189$
	MIR162	0.07 (0.03–0.11)D $n = 120$	3.2 $n = 8$
	1507xMON810xMIR162	0.01 (0.00–0.05)E $n = 120$	1.0 $n = 1$
Blend	Refuge	1.24 (1.01–1.47)C $n = 184$	4.6 (4.4–4.7)A $n = 224$
	1507xMON810xMIR162	0.00 (0.00–0.04)E $n = 286$	–
Johnston, IA[b]			
Pure stand	Refuge	1.19 (0.74–1.64)A $n = 119$	4.4 (4.3–4.5)A $n = 142$
	MON810	1.49 (1.04–1.94)A $n = 120$	2.9 (2.8–3.0)B $n = 177$
	MIR162	0.14 (0.10–0.18)B $n = 120$	2.4 $n = 17$
	1507xMON810xMIR162	0.01 (0.00–0.05)C $n = 120$	3.0 $n = 1$
Blend	Refuge	1.02 (0.57–1.47)A $n = 191$	4.3 (4.2–4.4)A $n = 193$
	1507xMON810xMIR162	0.02 (0.00–0.06)C $n = 288$	4.7 $n = 5$

The number of data points is represented in tables by the letter *n* (for larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured).

[a]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

[b]The 1507 (Trt3) was not planted correctly in the Johnston location and this entry was removed from the comparisons.

Table 7. Mean number of *H. zea* larvae and mean instar of larvae recovered from refuge ears from Dallas Center and Johnston, IA, USA combined (2012)

Planting	Mean (95%CI)[a]	
	Larvae	Instar
Pure stand	1.35 (1.11–1.59)A $n = 238$	4.5 (4.4–4.5)A $n = 321$
Blend	1.13 (0.96–1.30)B $n = 375$	4.4 (4.4–4.5)A $n = 417$

The number of data points is represented in tables by the letter *n* (for larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured).

[a]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

Field Trials With Artificial Infestations

Recovery and Development of Corn Earworm Larvae on Pure Stands and Blends. Evaluations targeting survival of *H. zea* larvae before they started to exit ears (4th and 5th instars) in artificially infested ears revealed that mean larvae per ear from pure stand refuge plants were 1.50 (Dallas Center, IA) and 1.19 (Johnston, IA) (Table 6). The mean number of larvae found in ears of blended refuge plants was not significantly different from pure stand refuge plants. Events 1507 and MON810 did not exhibit reduced numbers of larvae when compared with larval numbers obtained from pure stand refuge plants. Very few larvae were recovered from pure stands of MIR162, 1507xMON810xMIR162, or blended 1507xMON810xMIR162. Mean instar of *H. zea* recovered per ear from pure stand refuge plants was 4.5 (Dallas Center, IA) and 4.4 (Johnston, IA), respectively. The mean instar recovered from 1507, MON810, and 1507xMON810 was significantly lower than the means estimated for pure stand refuge plants indicating that insects were stunted (Table 6). At both locations, the mean instar recovered from blended refuge plants was not significantly different from the mean instar of larvae recovered from pure stand refuge (Table 6). When the number of larvae and mean instar were combined across the two locations (Table 7), there were statistically significant differences in the mean number of larvae recovered from ears of blended and pure stand refuge plants. Pure stand plants produced around 19% more larvae than blended refuge plants. Mean instar of *H. zea* on refuge plants blended with 1507xMON810xMIR162 at different cluster configurations varied between 4.4 and 4.7 (Dallas Center, IA) and 4.2 and 4.3 (Johnston, IA) and were similar regardless of cluster configuration (Table 8). Larvae were found on blended 1507xMON810xMIR162 plants in one of the two locations (Johnston, IA) and only in two of the four blend configurations (bRbRb and bbRRb) for a total of five larvae recovered from

Table 8. Mean number of *H. zea* larvae and mean instar of larvae recovered from ears of refuge in four plant cluster configurations in Dallas Center and Johnston, IA, USA (2012)

Cluster design[a]	Mean (95%CI)[b]	
	Larvae	Instar
Dallas Center, IA		
bb**R**bb	1.33 (1.13–1.54)A $n = 24$	4.7 (4.4–4.9)A $n = 32$
b**R**b**R**b	1.27 (1.07–1.47)A $n = 47$	4.4 (4.2–4.6)A $n = 58$
bb**RR**b	1.13 (0.92–1.33)A $n = 48$	4.6 (4.4–4.8)A $n = 54$
b**RRR**b	1.22 (1.02–1.43)A $n = 65$	4.5 (4.4–4.7)A $n = 80$
Johnston, IA		
bb**R**bb	0.96 (0.49–1.43)A $n = 24$	4.2 (4.0–4.5)A $n = 23$
b**R**b**R**b	1.00 (0.53–1.47)A $n = 48$	4.3 (4.1–4.5)A $n = 46$
bb**RR**b	1.10 (0.63–1.58)A $n = 48$	4.3 (4.1–4.5)A $n = 53$
b**RRR**b	1.01 (0.54–1.49)A $n = 71$	4.3 (4.2–4.5)A $n = 71$

The number of data points is represented in tables by the letter n (for Larvae, n refers to number of plants; for instar, n refers to number of larvae measured). Other treatments are presented in Table 6.

[a]R indicates refuge plant and b indicates Bt plant. Bold letters represent the number and type of plants that were used to calculate the mean value.

[b]LS Means and 95% confidence intervals for treatments were estimated from a mixed model. Treatments with a common letter were not statistically different from each other at the significance level of 0.05.

Table 9. Mean number of *H. zea* larvae and mean instar of larvae recovered from ears of Bt plants in four plant cluster configurations in Dallas Center and Johnston, IA, USA (2012)

Cluster design[a]	Mean	
	Larvae	Larvae
Dallas Center, IA		
bbR**bb**	0.00 $n = 96$	–
bR**b**R**b**	0.00 $n = 72$	–
bbRR**b**	0.00 $n = 72$	–
bRRR**b**	0.00 $n = 46$	–
Johnston, IA		
bbR**bb**	0.00 $n = 96$	–
bR**b**R**b**	0.06 $n = 72$	4.5 $n = 4$
bbRR**b**	0.01 $n = 72$	5.0 $n = 1$
bRRR**b**	0.00 $n = 48$	–

The number of data points is represented in tables by the letter *n* (for larvae, *n* refers to number of plants; for instar, *n* refers to number of larvae measured). Other treatments are presented in Table 6.

[a]R indicates refuge plant and b indicates Bt plant. Bold letters represent the number and type of plants that were used to calculate the mean value.

blended 1507xMON810xMIR162 plants (Table 9). There were not enough larvae to perform statistical analysis.

Discussion

In this study, dissection of bagged ears quantifying larval recovery was used to compare the relative production of insects in pure stand Bt to pure stand refuge. The trends were similar across the three locations tested, with mean relative survival estimates for *H. zea* on Bt plants relative to refuge plants of 78, 54, 1.3, and 0.3 % for 1507, MON810, MIR162, and 1507xMON810xMIR162, respectively. Other studies with Bt11, MON810, and MIR162 revealed similar (Burkness et al. 2010) and dissimilar (Storer et al. 2001, Horner et al. 2003) results. One study from 2001 (Storer et al. 2001) showed that Bt maize expressing Cry1Ab (single events Bt11 and MON810) produced 65–95% fewer *H. zea* adults than non-Bt maize. Another study (Horner et al. 2003) from 2003 showed that Bt maize expressing Cry1Ab (MON810) suppressed the establishment and development of *H. zea* to late instars by at least 75%. Burkness et al. (2010) found a few *H. zea* larvae surviving exposure to MIR162 but no survivors in the pyramid Bt11xMIR162. Burkness et al. (2010) concluded that MIR162 approaches high-dose efficacy against *H. zea*. In this study, only a few larvae and two pupae were recovered from MIR162 which supports the concept that MIR162 approaches high-dose against *H. zea* under field conditions. It is important to remember that for single events such as MIR162, due to open pollination and gene segregation in the ear, approximately 75% of the kernels per ear will express the trait (50% hemizygous and 25% homozygous) while 25% of kernels will not inherit the gene (Chilcutt and Tabashnik 2004). Also the maize hybrid used in this study does not have good husk coverage and longer tighter silk channels allowing easier access of insects on kernels. Therefore, it is possible that some *H. zea* larvae may have escaped exposure to silks as neonates and fed on the kernels without the Bt protein.

Overall, the data on larvae + exit holes obtained with nonbagged ears showed similar trends as the data obtained with bagged ears, except in Plains, GA where a numerical difference in favor of slightly more larvae + exit holes in 1507 than on refuge plants was found. However, the mean values were not significantly different. These results are not unexpected considering that corn hybrids containing the event 1507 only suppresses *H. zea* (Siebert et al. 2012). Mean instar values indicated that larvae recovered from 1507 were significantly stunted compared to larvae recovered from refuge plants. The relative production of *H. zea* on refuge plants deployed as blends and pure stands for our investigation was done by comparing the number of larvae plus exit holes found on ears. These results based on exit holes + larvae recovered on nonbagged ears should be interpreted with caution considering that not all *H. zea* larvae create exit holes on ears. Also the relative survival rates obtained with nonbagged ears were different from the estimates obtained with bagged ears because ear dissections occurred earlier for nonbagged ears than for bagged ears and insects were not contained on the nonbagged ears. Dissections of refuge ears targeting late instars revealed variable results across the four trials. In three trials, no significant differences in the number of *H. zea* were detected. In addition, the development of larvae feeding on blended refuge plants was not significantly delayed compared with the development of larvae on pure stand refuge plants. At one location, the number of *H. zea* on blended refuge plants was significantly reduced when compared with pure stand refuge plants. However, when data from the two

locations under natural infestations were combined (Table 3), significant differences in favor of more *H. zea* on pure stands were detected. Pure stand plants produced around 42% more exit holes + larvae than blended refuge plants. Similarly, when data from the two locations under artificial infestations were combined (Table 7), significant differences in favor of more *H. zea* on pure stands were detected. Pure stand refuge plants produced around 19% more larvae than blended refuge plants in trials with artificial infestations. It is important to mention that when the data from natural infestation trials were analyzed considering the blend rates of 5, 10, and 20%, no significant differences on insect numbers or development were detected (Supp Table 1 [online only]). It is possible that the size of plots selected were too small to detect differences among blend rates because cross-pollination occurred across the plots. Regardless of the blend rate or type of cluster compared in natural infestation trials, the main comparison was the number of larvae plus exit holes in the pure stand refuge plants versus in the blended refuge plants.

Several factors may have contributed to the variability of the results including Bt pollen exposure, degree of cross-pollination in kernels, feeding behavior of *H. zea*, environment, hybrid background and maturity, insect density, time of egg hatch, variability on insect susceptibility to Bt proteins, and potential interactions among these variables. Previous results on relative survival obtained with cross-pollinated ears are variable (Burkness et al. 2011, Babu 2013, Yang et al. 2014a,b). In experiments that created 100% cross-pollinated ears (Burkness et al. 2011), non-Bt plants were cross-pollinated with Bt plants expressing Cry1Ab, survival was intermediate, averaging 43% and 63% for *Ostrinia nubilalis* and *H. zea*, respectively. In experiments with 5% blended refuge (Yang et al. 2014b), larval occurrence (3rd and 5th instars) and ear damage on the refuge ears cross-pollinated with pollen containing Cry1A.105, Cry2Ab2, and Cry1F genes were similar to or greater than ears from pure stand non-Bt refuge. However, in a second experiment with the same pyramid conducted by the same laboratory (Yang et al. 2014a), cross-pollinated refuge greatly impacted the survival of *H. zea*. Cross-pollination in blends caused the majority of refuge kernels to express at least one Bt protein, and a reduction in survival from neonate to adult *H. zea* of 88.1%. However, in this study, which showed the most significant reduction in survival on blended refuge, the ears were removed from the plants and held in the laboratory (Yang et al. 2014a) for evaluation. In another experiment with 100% cross-pollinated ears (Babu 2013), with Bt plants expressing Cry1A.105 and Cry2Ab2, survival was intermediate, averaging 67% and 64% for *H. zea* in 2011 and 2012, respectively.

The results reported in this set of experiments were obtained in the field with naturally occurring cross-pollinated ears. The studies measured the relative survival of *H. zea* on refuge plants deployed as seed blends and pure stands, with the assumption that differences in survival may be due to the impact of Bt pollen or cross-pollinated kernels. As we did not measure the amount of cross-pollination, it is possible that lower than expected rates of cross-pollination in these experiments may have influenced the survival rates in these and at least one other study relying on natural cross-pollination (Yang et al. 2014b). A review of studies investigating degree of cross-pollination (Burkness and Hutchison 2012) revealed that the levels of cross-pollinated kernels in the first row of non-Bt planted adjacent to Bt maize varied greatly from 3 to 82% (Byrne and Fromherz 2003, Ma et al. 2004, Bannert et al. 2008, Burkness and Hutchison 2012). If less cross-pollination has occurred in a particular field and kernel feeding has occurred, a lower impact on *H. zea* mortality could be expected and vice versa. For this reason, it is important to interpret all results from these cross-pollination studies with caution as results can be as variable as the actual cross-pollination rates. In addition, studies that manipulate ears may impact the results as these manipulations may influence insect behavior and ultimately survival. For example, other studies have obtained dissimilar results indicating either no impact of cross-pollination with natural infestations (Yang et al. 2014b) or negative impact with artificial infestations (Yang et al. 2014a). It is possible that the manipulation of the ears for removing natural infestations (Yang et al. 2014a) and removing the ears from the plant for laboratory observations may have altered the natural feeding site of *H. zea* on the ear contributing to the differences between the two studies (Yang et al. 2014a,b). A recent study with 100% cross-pollinated ears (Babu 2013) indicated a negative impact from cross-pollination, but this experiment represents a worse-case scenario, since in natural conditions the cross-pollination rarely will reach 100% (Byrne and Fromherz 2003, Ma et al. 2004, Bannert et al. 2008, Burkness and Hutchison 2012).

The variation of the reduction of *H. zea* in a blended refuge compared with pure stands can also be attributed to variations in the feeding behavior of this pest or natural variability in susceptibility to Bt proteins. *H. zea* may feed only on the ear tips, where cob and silks are maternal and therefore do not express Bt proteins. Later, when they may be far less susceptible to Bts, *H. zea* may move to kernels where the three main components, including the pericarp, germ, and endosperm consist of both maternal and paternal tissues. The reduced susceptibility to Bt proteins in late instars have been reported in other insects (Keller et al. 1996, Wierenga et al. 1996, Huang et al. 1999, Wang et al. 2007). In a cross-pollinated blended refuge ear, the refuge plant may be cross-pollinated by hybrid hemizygous-Bt plants and segregation in kernels is expected (Horner et al. 2003; Chilcutt and Tabashnik 2004; Burkness et al. 2010, 2011; Babu 2013). It is also possible that, if an insect is capable of detecting Bt, it could avoid lethal exposure by feeding exclusively on maternal tissue in the ear tip or selecting kernels to feed upon that do not express Bt proteins. Previous studies have shown that the pattern of kernel damage caused by intoxicated *H. zea* on MON810 Bt maize was characteristically different with spatial patterns of kernels damaged showing scattered, discontinuous patches of partially consumed kernels, which were arranged more linearly than the compact feeding pattern on non-Bt ears (Horner et al. 2003). Bioassays with Cry1Ac and Cry2Ab revealed that *H. zea* selected diet with low concentrations of Cry1Ac compared with diet with higher concentrations of Cry1Ac, but the avoidance of Cry2Ab was not as noticeable as that observed for Cry1Ac (Gore et al. 2005). In addition, *H. zea* from different locations may exhibit variation in susceptibility to Bt proteins (Siegfried et al. 2000). Therefore, the ability of *H. zea* to survive, detect, and avoid Bt proteins may vary depending on the make-up of proteins in a pyramid and susceptibility of the larvae to the toxin that may increase or decrease the negative effects of cross-pollination due to kernel feeding.

Environmental conditions can also affect the feeding behavior of *H. zea* and host suitability. Drought stress accompanied by low relative humidity can also accelerate senescence and dry-down of silks (Bassetti and Westgate 1993, Horner et al. 2003), forcing the insects to move earlier into kernels where they may be more exposed to Bt proteins. Also some hybrids have better husk coverage and longer tighter silk channels that may exclude insects from feeding on kernels (Wiseman and Isenhour 1994, Dowd and White 2002, Burkness et al. 2010). Hybrids with poor husk coverage and shorter opened silk channels may have more kernel damage. Under natural infestations, another important factor is the density of insects within

the ear because the successful establishment of young larvae may be density-dependent and greater when more eggs are laid per ear (Horner et al. 2003). *H. zea* may also hatch later in the crop cycle (Burkness et al. 2010) and eggs may be laid on wilted or brown silks (Horner et al. 2003) forcing insects to feed on kernels.

In this study, larval sampling of nonbagged ears also served the purpose of comparing the relative production of insects in different refuge cluster configurations and to compare the performance of the Bt plants in blends versus pure stands. Most of the cross-pollination in plants occurs between plants in close proximity, and as the distance between plants increases, the rate of cross-pollination decreases (Ma et al. 2004, Bannert et al. 2008, Burkness and Hutchison 2012). By comparing the relative number of individuals on clusters with different numbers of refuge plants, the potential negative effects of cross-pollination on survival of insects can be tested. Sampling of ears targeting late instar *H. zea* larvae in clusters revealed variable results. In three trials, no significant differences in the number of *H. zea* were detected between clusters containing more refuge plants and clusters containing less refuge plants. However, at one location the number of corn earworms in blended refuge plants from clusters with one refuge plants was significantly reduced when compared with clusters with two refuge plants. When the data from locations with natural infestations were combined, significant differences in favor of more insects on pure stands were detected. Similarly, when the data from locations with artificial infestations were combined, significant differences in favor of more insects on pure stands were detected. Also in this study, virtually no insects were found on blended or pure stands of 1507xMON810xMIR162 plants both under artificial and natural infestations indicating that the pyramid was highly efficacious in reducing the numbers of *H. zea*.

Several factors must be considered when evaluating the feasibility of seed blends as a refuge deployment strategy for ear-feeding Lepidoptera. In this study, we evaluated the relative survival of *H. zea* in seed blends compared with pure stand refuge and the relative survival of *H. zea* on the individual components of the pyramid 1507xMON810xMIR162. Very few larvae were recovered from pure stands of MIR162, 1507xMON810xMIR162, or blended 1507xMON810xMIR162. These results indicate that both MIR162 and 1507xMON810xMIR162 were highly efficacious in reducing the number of *H. zea* larvae at all locations. The results showed variation on the production of *H. zea* on refuge plants from seed blends compared with pure stand refuge plants. The relative survival of *H. zea* on the events 1507, MON810, MIR162, and 1507x MON810xMIR162 ranked similarly across the three locations tested. These results can be used in computer simulation models to evaluate the feasibility of seed blends as a refuge deployment strategy with the pyramid 1507xMON810xMIR162. Because the reduction in survival of *H. zea* due to blending was variable, a sensitivity analysis that includes all possible scenarios of reduction in survival should be considered. It is important to consider not only the reduction in numbers of *H. zea* produced in seed blends but also the fitness of the insects that were potentially exposed to sublethal doses. Therefore, more studies are necessary to fully evaluate the impact of blending by investigating the impact of cross-pollination on the fitness of *H. zea* because sublethal doses can lead to extended larval and prepupal development and reduction of pupal weight, fecundity and fertility of adults which may also effect the timing of emergence for refuge insects (Horner et al. 2003, Yang et al. 2014b).

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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