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ORIGINAL ARTICLE

Performance of Bt-susceptible and -heterozygous dual-gene resistant genotypes of Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) in seed blends of non-Bt and pyramided Bt maize

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Abstract A seed blend refuge has been implemented in the U.S. Corn Belt for Bt maize resistance management. The fall armyworm, Spodoptera frugiperda (J.E. Smith), is a target pest of Bt maize in the Americas. The larvae of this pest are mobile, which may affect the efficacy of seed blend refuges. In this study, field and greenhouse trials were conducted to determine the performance of Bt-susceptible (aabb) and -heterozygous dual-gene-resistant (AaBb) genotypes of S. frugiperda in seed blends of non-Bt and pyramided Bt maize. Three field trials evaluated larval survival, larval growth, and plant injury with aabb in seed blends of Bt maize expressing Cry1A.105/Cry2Ab2/Vip3A with 0–30% non-Bt seeds. Greenhouse tests investigated the performance of aabb and AaBb in seed blends of Cry1A.105/Cry2Ab2 with 0–30% non-Bt seeds. In pure non-Bt maize plots, after 9–13 d of neonates being released on the plants, 0.39 and 0.65 larvae/plant survived with leaf injury ratings of 4.7 and 5.9 (Davis’s 1–9 scale) in the field and greenhouse, respectively. In contrast, live larvae and plant injury were virtually not observed on Bt plants across all planting patterns. Larval occurrence and plant injury by aabb on non-Bt plants were similar between seed blends and pure non-Bt plantings, suggesting that the blended refuges could provide an equivalent susceptible population as structured refuge under the test conditions. In the greenhouse, the two insect genotypes in seed blends performed similarly, indicating that the seed blends did not provide more favorable conditions for AaBb over aabb. The information generated from this study should be useful in managing S. frugiperda and evaluating if seed blends could be suitable refuge options for Bt resistance management in the regions where the insect is a primary target pest.

Key words Bt maize; dual-gene resistance; fall armyworm; resistance management; seed blend

Introduction

An insecticide resistance management (IRM) program, named “high dose/refuge” strategy (HDRS), has been implemented since the first year of Bt crop adoption in the United States to delay resistance development (Matten et al., 2012). This strategy involves planting a portion of
the maize seeds with high dose Bt hybrids that can kill resistant heterozygotes of the target pests, and planting the remaining seeds with non-Bt hybrids to function as refuges for susceptible pest populations. There are two ways in the U.S. Corn Belt to plant the necessary non-Bt refuge maize for IRM: (i) structured refuge and (ii) seed blend refuge. However, only the structured refuge is approved in the southern region of the United States where Bt cotton is also planted (Matten et al., 2012; Yang et al., 2015).

Before 2010, “structured refuge” was the only refuge method that was recommended in the United States for Bt maize IRM. For the structured refuge, non-Bt maize is planted as blocks or strips in or near Bt maize (Ostlie et al., 1997). Global data analysis has shown that the HDRS with the structured refuge is likely to be successful if its key assumptions are met (Huang et al., 2011; Tabashnik & Carriere, 2017). One problem involving the adoption of structured refuges is that the success of the method largely depends on farmer compliance with planting and appropriately managing the refuge. Several surveys have reported that farmer compliance with the structured refuge in the United States decreased over time (Smith & Smith, 2014; US-EPA, 2018). Because of the compliance issue, the Environmental Protection Agency of the United States in 2010 conditionally allowed a seed blend method as another refuge option for planting pyramided Bt maize traits that contain two or multiple Bt genes and the appropriate percentage of Bt and non-Bt seeds is blended before seeds are commercialized, thereby eliminating the compliance issue associated with the structured refuge. The current seed blend rate in the U.S. Corn Belt is a blend of 5% non-Bt and 95% Bt seeds for pyramided maize traits (Matten et al., 2012). Currently, seed blend refuge has not been used in the southern United States, largely due to the similar proteins expressed in Bt maize and Bt cotton, as well as lack of necessary data to determine if seed blends are suitable refuge options for the target pests in this region (Matten et al., 2012; Wangila et al., 2013).

A major concern in the use of seed blends is that larval dispersal of insects among non-Bt and Bt plants could impact the efficacy of the refuge (Mallet & Porter, 1992; Razze & Mason, 2012). The movement of susceptible larvae from non-Bt refuge plants to surrounding Bt plants in seed blends may result in higher mortalities compared to the structured refuge (Davis & Onstad, 2000). In addition, heterozygous-resistant individuals in seed blends may have a fitness advantage relative to the homozygous susceptible insects, which may occur due to the dilution of Bt proteins when insects feed on non-Bt maize followed by feeding on Bt maize, and vice versa (Alyokhin, 2011; Onstad et al., 2011). Some studies have assessed such possible advantages of the heterozygous insect genotypes in seed blends for the systems involving single-gene Bt resistance to single-gene Bt crops (Brévault et al., 2015; Yang et al., 2017). For example, Brévault et al. (2015) reported that a seed blend of two non-Bt and seven Cry1Ac-cotton plants increased the dominance of a single-gene Cry1Ac resistance in the fall armyworm, Helicoverpa zea (Boddie). In addition, Yang et al. (2017) also conducted a laboratory study using detached ears removed from seed blends of single-gene Cry1F maize with 5% and 20% refuges, and found that the seed blends also significantly elevated the dominance of a single-gene Cry1F resistance in the fall armyworm, Spodoptera frugiperda (J.E. Smith). However, the potential fitness advantages associated with seed blends in systems involving dual-/multiple-gene Bt resistance to dual-/multiple-gene Bt crops have not been investigated yet. As mentioned above, the seed blend refuge was approved for planting pyramided Bt maize traits that contain two or multiple Bt genes, rather than for single-gene Bt maize traits (Matten et al., 2012). Currently, the single-gene Bt maize traits have almost completely been replaced by the more advanced dual- or multiple-Bt gene maize traits (Huang, 2021). The resistance dominance in seed blends could be different between the two biological systems: single-gene resistance-to-single-gene Bt plants and dual-/multiple-gene resistance-to-dual-/multiple-gene Bt crops.

In North and South America, S. frugiperda is a major pest of field maize. Recently, this polyphagous pest has invaded Africa and Asia, and has become a significant threat to the food security in these regions (Goergen et al., 2016; Day et al., 2017; Wang et al., 2019). To date, S. frugiperda is actually the only pest that has evolved practical resistance with field control failures of Bt maize across multiple countries (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014, 2020; Omoto et al., 2016; Chandrasena et al., 2018). Recently, a dual-gene-resistant strain of S. frugiperda that was highly resistant to maize plants containing pyramided Cry1A.105/Cry2Ab2 proteins has been established by crossing a known single-gene Cry1A.105-resistant and a single gene Cry2Ab2-resistant strains in the laboratory (Niu et al., 2019; Zhu et al., 2019). The availability of the dual-gene Bt-resistant strain and the larval mobile behavior of S. frugiperda (Pannuti et al., 2016) provided an opportunity to test the influence of seed blends on the performance of susceptible and dual-gene-resistant heterozygous genotypes. In this study, field trials were conducted to generate
necessary information to determine if seed blends could produce comparable numbers of susceptible (aabb) *S. frugiperda* as structured refuges, while greenhouse tests were employed to assess whether seed blends provide a fitness advantage for the Cry1A.105/Cry2Ab2-dual-gene-resistant heterozygous genotype (AaBb) over aabb. Data generated from this study could be useful to analyze if seed blend refuges are suitable for Bt maize IRM in the areas where *S. frugiperda* is a primary target pest.

**Materials and methods**

**Maize hybrids**

Two pyramided Bt maize hybrids with Genuity® VT Double Pro (VT-2P, DKC 66–87) and Genuity® Trecepta™ (Trecepta, DKC 66-26), and one non-Bt maize hybrid (DKC 66–94) were provided by Bayer Crop Science (St. Louis, MO, USA). VT-2P, which expresses the pyramided Cry1A.105 and Cry2Ab2 proteins, has been commercially available in the United States since 2010 (Ghimire *et al*., 2011). Trecepta is a relatively new Bt maize trait that expresses three Bt proteins: Cry1A.105, Cry2Ab2, and Vip3A (Zhu *et al*., 2019). Both VT-2P and Trecepta control above-ground lepidopteran pests, including *S. frugiperda*. The non-Bt hybrid, DKC 66–94, is a genetically closely related hybrid to the two Bt maize hybrids.

**Insect sources**

Two insect genotypes, a susceptible (aabb) and a dual-gene Cry1A.105/Cry2Ab2-resistant heterozygous (AaBb) genotypes of *S. frugiperda* were used in the study. Genotype aabb was derived from two-parental families collected from non-Bt maize fields near Winnsboro, Louisiana, in 2016. This genotype has been documented to be susceptible to Cry1A.105, Cry2Ab2, Cry1F, and Vip3A proteins, as well as to maize plants expressing one or more of these proteins (Yang *et al*., 2018, 2019a; Niu *et al*., 2019; Zhu *et al*., 2019). AaBb was developed from reciprocal hybrids between aabb and a dual-gene Cry1A.105/Cry2Ab2-resistant genotype (AABB) of *S. frugiperda*, as described in Zhu *et al.* (2019). AABB has demonstrated to be able to complete its life cycle on pyramided Cry1A.105/Cry2Ab2 maize plants (e.g., VT-2P), and the adults derived from the larvae feeding on VT-2P plants produced normal progeny (Niu *et al*., 2019; Zhu *et al*., 2019).

**Experimental design**

This study consisted of three field trials (Trials I, II, and III) and one greenhouse test. The field trials were designed to investigate larval survival, larval growth, and plant injury by aabb in seed blends of non-Bt and Trecepta maize to determine if seed blends with the triple-gene Bt maize could produce a comparable number of susceptible insects as the structured refuge. The greenhouse study was designed to assess the performance of aabb and AaBb genotypes in seed blends of non-Bt and VT-2P maize to determine if the Cry1A.105/Cry2Ab2-dual-gene-resistant heterozygous genotype of *S. frugiperda* had survival and fitness advantages over the susceptible insects in the seed blends. The reason for the use of the dual-gene VT-2P maize trait in the greenhouse tests was because we had the corresponding dual-gene Bt-resistant (resistance to both Cry1A.105 and Cry2Ab2) *S. frugiperda* population (AABB) available for the tests. Therefore, the heterozygous insect genotype AaBb was truly “heterozygous” corresponding to the Cry1A.105/Cry2Ab2 dual-gene Bt maize trait used in the study. The Trecepta maize trait was used in the field trials because it was a new triple-gene Bt maize that was recently commercialized.

**Field trials**

Trials I and II of the three field trials were conducted in 2018 and 2019, respectively, in East Baton Rouge Parish, USA, LA. Trial III was conducted in 2019 in Rapides Parish, Louisiana, USA. Each trial contained six planting patterns of Trecepta and non-Bt maize with four rows (1 m row spacing) and 25 plants spaced ∼15 cm apart in each row (4 × 25 = 100 plants/plot). These six planting patterns were: (1) all non-Bt plants (100% non-Bt), (2) all Bt plants (100% Bt), (3) a seed blend of 5% non-Bt and 95% Bt plants (5:95RIB); (4) a seed blend of 10% non-Bt and 90% Bt plants (10:90RIB), (5) a seed blend of 20% non-Bt and 80% Bt plants (20:80RIB), and (6) a seed blend of 30% non-Bt and 70% Bt plants (30:70RIB). The 5:95RIB is the currently adopted rate of the seed blend in the U.S. Corn Belt for pyramided Bt maize IRM. The pure non-Bt planting was included to simulate the structured refuge planting as described in Ostlie *et al.* (1997). The other three refuge rates (10%, 20%, and 30%) evaluated in this study are considered as possible seed blend rates for the U.S. southern region (Guo *et al*., 2019; Yang *et al*., 2020).

Microsoft Excel random number generator was used to determine the locations where non-Bt refuge seeds were
planted in each seed blend plot. To reduce the possible effects of natural S. frugiperda infestation, trials were intentionally planted earlier than the normal planting period. At planting, two non-Bt seeds were hand-planted at each refuge location and marked with wood sticks, and 20% extra Bt seeds were hand-planted in each row. After plant emergence, plants were thinned to configure the designed plant densities. No foliar insecticides were applied in the trial plots; while irrigation, fertilization, and herbicide application were used to meet the agronomical needs of maize. For each trial, treatment plots were arranged in a randomized complete block design (RCBD) with four replications. There was a 2-m space between plots and a 3-m distance between blocks. Bt protein expression/nonexpression was confirmed by testing leaf tissue using the EnviroLogix’s ELISA kits (ME, USA).

In maize fields, S. frugiperda larvae mainly feed on leaf tissues during vegetative plant stages. To ensure sufficient data collections for insect infestations, two (for Trial I), three (Trial II), or five (Trial III) neonates of the aabb genotype, depending on the total number of neonates available for each trial, were released on the newest fully expanded leaf of each plant at V4–V5 plant stages. After manual infestations, larval survival was carefully monitored. When most live larvae on non-Bt maize plants in each trial reached the 4th instar, larval survival, larval growth stage, and plant injury were recorded for all plants (usually 9–11 d after neonate release, depending on the weather conditions). The growth stage of a larva was estimated based on its body length as described in Capinera (2017a), while plant injuries by S. frugiperda was rated using Davis’ 1–9 rating scale, in which a rating of 1 means no injury, while a rating of 9 denotes severe foliar injury (Davis et al., 1992).

Greenhouse tests

Greenhouse tests were used to assess the performance of AaBb and aabb genotypes in five planting patterns of non-Bt and VT-2P maize. Each planting pattern consisted of four rows with 14 plants in a row (56 plants per plot). To simulate seed blends, five planting patterns were created: (i) all non-Bt plants (100% non-Bt), (ii) all Bt plants (100% Bt), (iii) a seed blend of six randomly planted non-Bt plants and 50 Bt plants (10:90RIB), (iv) a seed blend of 11 randomly planted non-Bt plants and 45 Bt plants (20:80RIB), and (v) a seed blend of 17 randomly planted non-Bt plants and 39 Bt plants (30:70 RIB). The five planting patterns were arranged in each of four greenhouse rooms in a RCBD with four replications, and the greenhouse room was considered a block factor. In the greenhouse study, four maize seeds were planted in each 18.9-L plastic pot containing Perfect Mix™ soil (Expert Gardener products, St. Louis, MO, USA), as described in Wangila et al. (2013). The locations where non-Bt refuge seeds were planted in a RIB configuration were marked with wood sticks. After 1 week, plants were thinned to two plants per pot. Maize plants were 20 cm apart in a row and 60 cm from adjacent rows. There was a 1-m distance between plots. Irrigation, fertilization, and hand weeding were used as needed to ensure optimum plant growth. As described in the field trials, the expression of Bt proteins in maize was confirmed with the ELISA kits (EnviroLogix, ME, USA). When plants reached V4–V7, three neonates (<24 h) of aabb or AaBb genotype were released on each plant as previously described. When most larvae on non-Bt maize plants reached the 4th instar (usually after 10–13 d of neonate release), the number of live larvae, larval growth, and plant injury ratings (Davis’s 1–9 scale) were recorded as previously described.

Data analysis

To facilitate data analysis, larval growth stages recorded in field trials and greenhouse tests were converted to a larval growth index: a value of 1 = 1st instar, 2 = 2nd instar, …, and 6 = 6th (Yang et al., 2014a). Also, data (e.g., number of live larvae per plant, larval growth index, and plant injury rating) recorded from non-Bt plants in seed blend plots were separated from those data observed from Bt plants in the same plot as described in Wangila et al. (2013). Little to no plant injury and larval survival occurred on all Bt plants in both field trials and greenhouse tests across all planting patterns. Thus, data recorded from Bt plants were excluded in the analysis of variance (ANOVA). Data on the number of live larvae per plant, larval growth index, and plant injury ratings observed from non-Bt plants were transformed to log (x +1) scale for normality and then subjected to one-way ANOVA with planting pattern as the main factor for each of the three field trials, and two-way ANOVA with planting pattern and insect genotype as the two main factors for the greenhouse tests (SAS Institute, 2010). Replication was considered a random effect in both models. As the results of the three individual field trials were generally consistent (see results), to make a more concrete conclusion across all trials, data generated from the three field trials were also analyzed together in a mixed model (hereafter referred as “combined analysis”) using one-way ANOVA with both trial and replication as random factors, and planting pattern as the treatment factor (Kaur...
et al., 2019). Tukey’s HSD tests at $\alpha = 0.05$ were used to separate the treatment means. Untransformed data are presented in the tables.

Results

Larval survival of aabb in different planting patterns of non-Bt and Trecepta maize in open field trials

Across all three trials, only one live larva (4th instar) was observed on the Bt plants. Thus, data collected from Bt plants were not included in the statistical analysis. Analysis of the data from the non-Bt plants showed that the effect of planting pattern on larval survival of aabb was not significant for each of the three trials ($F_{4,10-12} < 2.72, P > 0.0852$) or for the combined data ($F_{4,41} = 1.34; P = 0.2717$) (Table 1). Based on combined data, an average of 0.39 ± 0.11 (mean ± SEM) larvae/plant was found in 100% non-Bt planting, and the number observed on non-Bt refuge plants in the four seed blends ranged from 0.26 ± 0.10 to 0.51 ± 0.13 (Table 1).

Larval growth index of aabb in different planting patterns of non-Bt and Trecepta maize in open field trials

The effect of planting patterns on larval growth index recorded from non-Bt plants also was not significant across each of the three trials ($F_{4,8-10} < 2.84, P > 0.0978$) and for combined data ($F_{4,34} = 0.99; P = 0.4258$) (Table 1). The growth index of larvae recovered from pure non-Bt was 3.72 ± 0.13 in combined data, and the corresponding index for non-Bt refuge plants in the four seed blends ranged from 3.40 ± 0.28 to 3.76 ± 0.10 (Table 1).

Plant injury by aabb in different planting patterns of non-Bt and Trecepta maize in open field trials

Plant injury ratings in the field trials were highly correlated to the number of larvae that occurred on the plants. Little or no plant injury was observed on Bt plants across all trials and planting patterns with a plant injury rating of ≤1.3 in the combined data (Table 1). Results from non-Bt plants showed that the effect of planting pattern on plant injury ratings was not significant in Trial I ($F_{4,12} = 0.70, P = 0.6055$) and Trial III ($F_{4,10} = 1.11, P = 0.4029$), but significant in Trial II ($F_{4,11} = 7.70, P = 0.0033$) and for combined data ($F_{4,41} = 2.94, P = 0.0317$) (Table 1). Based on combined data, the injury rating (3.8 ± 0.6) observed on non-Bt refuge plants in 5:95RIB was significantly ($P < 0.05$) less than that (5.0 ± 0.5) observed in 20:80RIB, but not significantly different ($P > 0.05$) from those in 100% non-Bt (4.7 ± 0.5) or the other two seed blends (4.2 ± 0.5 in 10:90RIB and 5.0 ± 0.5 in 30:70RIB) (Table 1).

Larval survival of aabb and AaBb in different planting patterns of non-Bt and VT-2P maize in greenhouse tests

No live larvae were observed on VT-2P plants across all planting patterns and for both aabb and AaBb insect genotypes (Table 2). Results from non-Bt plants showed that the effect of treatment on larval survival was not significant for insect genotype ($F_{1,20} = 3.07, P = 0.0949$), planting pattern ($F_{3,20} = 1.45, P = 0.2570$) and their interaction ($F_{3,20} = 0.88, P = 0.4682$) (Table 2). An average of 0.62 ± 0.10 and 0.41 ± 0.15 larvae per plant were recovered from 100% non-Bt plants infested with aabb and AaBb, respectively. The corresponding values on the refuge plants in the three seed blends ranged from 0.38 ± 0.08 to 0.45 ± 0.12 for aabb and from 0.27 ± 0.14 to 0.39 ± 0.10 for AaBb (Table 2).

Larval growth index of aabb and AaBb in different planting patterns of non-Bt and VT-2P maize in greenhouse tests

The effect on larval growth index was significant for planting pattern ($F_{3,20} = 3.58, P = 0.0321$), but not significant for insect genotype ($F_{1,20} = 0.09, P = 0.7689$) and their interaction ($F_{3,20} = 0.81, P = 0.5058$) (Table 2). Larval growth indices on non-Bt refuge plants in 30:70RIB (3.06 ± 0.45 for aabb and 3.08 ± 0.20 for AaBb) appeared to be smaller ($P < 0.05$) than those recorded in 100% non-Bt (3.78 ± 0.29 for aabb and 3.40 ± 0.22 for AaBb) and the other two seed blends (3.67 ± 0.39 with aabb and 4.00 ± 0.36 with AaBb in 10:90RIB, and 3.79 ± 0.34 with aabb and 3.50 ± 0.76 with AaBb in 20:80RIB). The differences in larval growth index for all other comparisons were not significant ($P > 0.05$) (Table 2).

Plant injury rating by aabb and AaBb in different planting patterns of non-Bt and VT-2P maize in greenhouse tests

As described in the field trials, plant injury ratings by S. frugiperda in the greenhouse tests were closely related to the larval survival. No notable plant injuries on Bt plants were observed for both aabb and AaBb across all planting patterns.

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Table 1 Larval survival, growth, and plant injury rating (Davis 1–9 scale) (mean ± SEM) of *S. frugiperda* in seed blends of non-Bt and pyramided maize containing Trecepta® trait.

| Planting pattern | Trial I | Trial II | Trial III | Combined |
|------------------|---------|----------|-----------|----------|
|                  | No. larvae per plant | Larval growth index | Plant injury rating | No. larvae per plant | Larval growth index | Plant injury rating | No. larvae per plant | Larval growth index | Plant injury rating | No. larvae per plant | Larval growth index | Plant injury rating | No. larvae per plant | Larval growth index | Plant injury rating |
| 100% non-Bt plants | 0.20 ± a | 3.90 ± a | 5.7 ± a | 0.80 ± a | 3.41 ± a | 5.4 ± a | 0.24 ± a | 3.77 ± a | 2.5 ± a | 0.39 ± a | 3.72 ± b | 4.7 ± b |
| 100% Bt plants    | 0.00 ± | N/A | 1.3 ± | 0.00 ± | N/A | 1.0 ± | 0.00 ± | N/A | 1.0 ± | 0.00 ± | N/A | 1.1 ± |
| 5:95RIB Non-Bt plant | 0.25 ± a | 4.25 ± a | 5.1 ± a | 0.37 ± a | 2.56 ± a | 3.0 ± b | 0.37 ± a | 4.06 ± a | 3.0 ± a | 0.33 ± a | 3.62 ± a | 3.8 ± a |
| 5:95RIB Bt plant  | 0.00 ± b | N/A | 1.4 ± | 0.00 ± b | N/A | 1.0 ± | 0.00 ± b | N/A | 1.1 ± | 0.00 ± b | N/A | 1.1 ± |
| 10:90RIB Non-Bt plant | 0.09 ± a | 3.67 ± a | 6.1 ± | 0.18 ± a | 2.58 ± a | 3.3 ± a | 0.50 ± a | 3.74 ± a | 3.2 ± b | 0.26 ± b | 3.40 ± a | 4.2 ± b |
| 10:90RIB Bt plant | 0.00 ± a | N/A | 1.6 ± | 0.00 ± a | N/A | 1.0 ± | 0.00 ± a | N/A | 1.0 ± | 0.00 ± a | N/A | 1.2 ± |

(to be continued)
| Planting pattern | Trial I | Trial II | Trial III | Combined |
|-----------------|---------|----------|-----------|-----------|
|                 | No. larvae per plant † | Larval growth index † | Plant injury rating † | No. larvae per plant † | Larval growth index † | Plant injury rating † | No. larvae per plant † | Larval growth index † | Plant injury rating † | No. larvae per plant † | Larval growth index † | Plant injury rating † |
| 20:80RIB Non-Bt plant | 0.20 ± 0.07 a 0.16 ± 0.04 a | 4.00 a 3.58 ± 0.21 a | 5.6 ± 0.3 | 0.93 ± 0.20 a 0.83 ± 0.20 a | 6.1 ± 0.7 | 0.39 ± 0.20 a 0.33 ± 0.10 a | 3.4 ± 0.5 | 0.51 ± 0.20 a 0.44 ± 0.10 a | 5.0 ± 0.5 b |
| 20:80RIB Bt plant | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± 0.00 a | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.1 |
| 30:70RIB Non-Bt plant | 0.16 ± 0.04 a | 3.58 ± 0.21 a | 6.3 ± 0.3 a | 0.83 ± 0.20 a 0.7 b | 5.7 ± 0.8 | 0.33 ± 0.10 a 0.11 a | 3.0 ± 0.5 | 0.44 ± 0.12 a 0.11 a | 5.0 ± 0.5 ab |
| 30:70RIB Bt plant | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± 0.00 a | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 0.00 ± N/A | 1.3 ± 1.0 |
| ANOVA | $F_{4,12} = 0.67$ | $F_{4,8} = 1.62$ | $F_{4,12} = 0.70$ | $F_{4,11} = 2.72$ | $F_{4,8} = 2.84$ | $F_{4,11} = 7.70$ | $F_{4,10} = 0.94$ | $F_{4,10} = 0.89$ | $F_{4,10} = 1.11$ | $F_{4,10} = 1.34$ | $F_{4,10} = 0.99$ | $F_{4,10} = 2.94$ |
| P | P | P | P | P | P | P | P | P | P | P | P | P |
| 0.06249 | 0.2600 | 0.6055 | 0.0852 | 0.0978 | 0.0033 | 0.4733 | 0.5069 | 0.4029 | 0.2717 | 0.4258 | 0.0317 |

Note: Virtually no larvae and plant injury were observed on all Bt plants. Thus, data recorded from Bt plants were excluded in ANOVA. † Means followed a same letter within a column were not significantly different (Tukey’s HSD at $\alpha = 0.05$).

1 † N/A, data are not available.
patterns with a plant injury rating of 1.0 ± 0.0 (Table 2). The effect of treatment on plant injury rating of non-Bt plants was not significant for insect genotype (\(F_{1,20} = 0.00, P = 0.9501\)), planting pattern (\(P_{3,20} = 1.51, P = 0.2418\)), and their interaction (\(F_{1,20} = 0.35, P = 0.7930\)). Plant injury ratings observed in 100% non-Bt were 5.9 ± 0.6 and 5.4 ± 0.8 for plants infested with aabb and AaBb, respectively. The corresponding injury ratings in the three seed blends ranged from 4.4 ± 0.2 to 5.1 ± 0.6 for aabb and from 4.7 ± 0.4 to 5.3 ± 0.3 for AaBb.

Discussion

The number of neonates initially infested in each plant was different among the three field trials largely as a result of varied availabilities of the total number of larvae for each field infestation. It is well known that *S. frugiperda* has a strong cannibalistic behavior, which could result in larval mortalities even during their early larval stages (Raffa, 1987; Chapman et al., 1999). The strong larval cannibalistic behavior could be the major reason why the number of survivors observed in the current study was not necessarily correlated to the number of larvae initially infested in the three trials. For the similar reason, many related field or greenhouse studies associated with the evaluations of the performance of *S. frugiperda* on crop plants measured the percentages of plants that contain live larvae and/or plant injury levels rather than from only a more specific condition. This means that the conclusions are more concrete and can be applied in a broader range. Furthermore, we acknowledge that the natural infestation levels of *S. frugiperda* in the trial areas are normally low in the regular maize growing seasons. This pest usually occurs in late-planted maize fields in the area. In the current study, trials were intentionally planted earlier than the normal planting time to minimize the effects of *S. frugiperda* natural infestation. Thus, the natural infestation at the trial sites, if any, would be low and should not affect the overall results.

### Table 2 Larval survival, growth, and plant injury rating (Davis 1–9 scale) (mean ± SEM) of susceptible and Cry1A.105/Cry2Ab2-dual-gene heterozygous-resistant genotypes of *S. frugiperda* in seed blends of non-Bt and pyramided VT-2P maize.

| Planting pattern | Maize | Insect genotype | No. larvae per plant | Larval growth index*† | Plant injury Rating*† |
|------------------|-------|-----------------|----------------------|-----------------------|-----------------------|
| 100% non-Bt plants | aabb | 0.65 ± 0.11 a | 3.78 ± 0.29 b | 5.9 ± 0.6 a |
|                  | AbBb | 0.41 ± 0.15 a | 3.40 ± 0.22 b | 5.4 ± 0.8 a |
| 100% Bt plants   | aabb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
|                  | AbBb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
| 10:90RIB Non-Bt plants | aabb | 0.38 ± 0.08 a | 3.67 ± 0.39 b | 5.1 ± 0.6 a |
|                  | AbBb | 0.39 ± 0.10 a | 4.00 ± 0.36 b | 5.3 ± 0.3 a |
|                  | aabb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
|                  | AbBb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
| 20:80RIB Non-Bt plants | aabb | 0.45 ± 0.12 a | 3.79 ± 0.34 b | 4.4 ± 0.2 a |
|                  | AbBb | 0.27 ± 0.14 a | 3.50 ± 0.76 b | 4.8 ± 0.3 a |
|                  | aabb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
|                  | AbBb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
| 30:70RIB Non-Bt plants | aabb | 0.43 ± 0.19 a | 3.06 ± 0.45 a | 4.8 ± 0.4 a |
|                  | AbBb | 0.36 ± 0.11 a | 3.08 ± 0.20 a | 4.7 ± 0.4 a |
|                  | aabb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
|                  | AbBb | 0.00 ± 0.00 | N/A | 1.0 ± 0.0 |
| ANOVA            | Insect genotype | \(F_{1,20} = 3.07; P = 0.0949\) | \(F_{1,20} = 0.09; P = 0.7689\) | \(F_{1,20} = 0.00; P = 0.9501\) |
|                  | Planting pattern | \(F_{3,20} = 1.45; P = 0.2570\) | \(F_{3,20} = 3.58; P = 0.0321\) | \(F_{3,20} = 1.51; P = 0.2418\) |
|                  | Interaction     | \(F_{3,20} = 0.88; P = 0.4682\) | \(F_{3,20} = 0.81; P = 0.5058\) | \(F_{3,20} = 0.35; P = 0.7930\) |

Note: Virtually no larvae and plant injury were observed on all Bt plants. Thus, data recorded from Bt plants were excluded in ANOVA.

*Means followed a same letter within a column were not significantly different (Tukey’s HSD at \(\alpha = 0.05\)).

N/A, data are not available.
Previous studies have reported that Bt maize hybrids expressing Cry1A.105/Cry2Ab2 or Cry1A.105/Cry2Ab2/Vip3A are effective against *S. frugiperda* (Santos-Amaya *et al.*, 2015; Horikoshi *et al.*, 2016; Niu *et al.*, 2019; Zhu *et al.*, 2019). In the current study, virtually no live larvae with no or little plant injury were observed on Bt plants across all field trials and greenhouse tests. The results of this study confirmed that maize hybrids containing these pyramided Bt traits were highly effective in controlling this important crop pest in the United States.

The similar larval occurrence and plant injury of aabb on non-Bt plants among the six planting patterns observed in the open field trials suggest that seed blends of non-Bt and Trecepta maize did not have significant adverse effects on the performance of the susceptible *S. frugiperda* on the non-Bt refuge plants, at least under the test conditions. Performance of target insects in seed blends has been evaluated in a few studies. Wangila *et al.* (2013) reported that larval abundance of the sugarcane borer, *Diatraea saccharalis* (F.), on non-Bt refuge plants in seed blends was not reduced compared to pure non-Bt maize plantings. Another study by Yang *et al.* (2014b) reported that larval abundance (3rd–5th instars) and ear injury of *H. zea* on seed blend refuges also were not reduced compared to pure non-Bt plantings (Yang *et al.*, 2014b). *D. saccharalis* is a target pest of maize in the mid-south region of the United States (Huang *et al.*, 2007), while *H. zea* is a major target pest of both Bt maize and Bt cotton in the entire southern U.S. region (Yang *et al.*, 2014b). The resistance of *H. zea* to Cry1A/Cry2A maize and cotton has led to field control problems in the United States (Dively *et al.*, 2016; Reisig *et al.*, 2018; Kaur *et al.*, 2019). A limitation of the current study and the study by Yang *et al.* (2014b) was that both experimental designs could not test the effects on the entire insect life cycle. In maize fields, late instars of *S. frugiperda* and *H. zea* larvae move off maize plants and pupate in the soil (Camin, 2017a; 2017b). Thus, both the field and greenhouse trials were terminated when the majority of larvae reached the 4th instar to ensure capturing larval survival data for analysis. To assess the potential impact on the entire insect life cycle, additional studies would need to focus the effect on late instar survival to adulthood and reproduction (Yang *et al.*, 2014a).

In contrast to previous studies with the systems involving a single-gene resistance to a single-gene Bt crop (Brévault *et al.*, 2015; Yang *et al.*, 2017), the comparable performance of aubb and AaBb in the current greenhouse tests across the five planting patterns of non-Bt and VT-2P maize indicates that the seed blends did not provide fitness advantages for the dual-gene-resistant heterozygous *S. frugiperda* over the susceptible counterparts. At this time, this is the first study that has evaluated the performance of a dual-gene heterozygous-resistant population on whole plants of the corresponding dual-gene Bt maize in seed blend plantings. A previous study reported that the AaBb genotype in sequential feedings of non-Bt and VT-2P leaf tissue in a laboratory rearing out-performed aubb in two of eight feeding sequences (Zhou *et al.*, 2018). Additional studies are warranted to elucidate the observed differences between the current greenhouse whole plant tests and the laboratory leaf tissue bioassays by Zhou *et al.* (2018).

The complete control (100% mortality) of AaBb observed on VT-2P plants in both 100% Bt, and seed blend plantings in the current study shows that the Cry1A.105/Cry2Ab double-gene resistance in *S. frugiperda* was functionally recessive on VT-2P maize plants. The recessive resistance observed in the current study was similar to the reports on two Brazilian Cry1A.105/Cry2Ab2-resistant populations of *S. frugiperda*, in which resistance in both populations was found to be functionally recessive on VT-2P hybrids (Santos-Amaya *et al.*, 2015; Horikoshi *et al.*, 2016). However, two other studies by Niu *et al.* (2019) and Zhu *et al.* (2019) reported that the Cry1A.105/Cry2Ab dual-gene resistance was incompletely recessive on plants containing the same VT-2P trait. The different dominance levels of resistance observed between the current study and the past two studies by Niu *et al.* (2019) and Zhu *et al.* (2019) most likely occurred due to the different test methods used among the studies. In Niu *et al.* (2019), neonates were released on maize plants at V7–V9 stages and in Zhu *et al.* (2019) the infestations were performed at V9–V10 stages. In contrast, the neonate infestations were carried out at V4–V7 plant stages in this study. Varied dominance levels of resistance to Bt crops measured at different plant growth stages have been reported in several other target species. For example, resistance of the old world bollworm, *Helicoverpa armigera* (Hübner), to Cry1Ac cotton in Australia was found to be completely recessive on 4-week-old cotton (Bird & Akhurst, 2004), while it was incompletely dominant on 14-week-old cotton (Bird & Akhurst, 2005). The commonly observed different dominance levels for Bt crop resistance among populations of the same species indicate that there can be significant interactions between insect genotypes and test conditions.

In summary, pyramided Bt maize containing VT-2P or Trecepta was very effective against *S. frugiperda*. In this study, seed blends of non-Bt and Trecepta maize did not affect the performance of the susceptible populations on refuge plants. Seed blends of non-Bt
and VT-2P did not provide fitness advantages for the Cry1A.105/Cry2Ab2 dual-gene heterozygous genotype over the susceptible individuals. In greenhouse whole plant tests, the Cry1A.105/Cry2Ab2 dual-gene resistance in S. frugiperda was functionally recessive on VT-2P maize. Data generated from this study should provide useful information for managing S. frugiperda and assessing if seed blends could be suitable for Bt maize IRM in the areas where S. frugiperda is a primary target pest.

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Disclosure

The authors declare no competing financial interests. This paper reports research results only. Mention of a proprietary product name does not constitute an endorsement for its use by Louisiana State University Agricultural Center.

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