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Performance of Cry1Ab-susceptible and -heterozygous resistant populations of sugarcane borer in sequential feedings on non-Bt and Bt maize plant tissue

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Abstract

A seed mix strategy has been used to provide refuge to susceptible insects for resistance management in planting transgenic maize expressing *Bacillus thuringiensis* Berliner (Bt) proteins. To determine whether larval movement in a seed mix planting creates favorable conditions for resistant heterozygotes of a target pest, performance of Cry1Ab-susceptible (SS) and -heterozygous resistant (RS) populations of the sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), was evaluated in sequential feeding on non-Bt and Cry1Ab (event MON 810) maize plant tissue. For each insect population, nine feeding sequences were employed. SS and RS feeding on non-Bt plants for their entire larval stages survived well and >60% of the adult pairs produced viable eggs, with an average of 269 progeny per female, whereas none of the two populations on Bt maize plants survived to the pupal stage. SS larvae could not develop to adults if the larvae fed on non-Bt plants for ≤15 days and then moved to Bt plants. In contrast, 4.2–29.2% of RS larvae that fed on non-Bt plants for ≥9 days and then moved to Bt plants developed to adults, and 63.6% of pairs of these adults produced viable eggs, with an average of 185 progeny per female. For SS larvae that fed on Bt plants for 1 or 2 days and then moved to non-Bt plants, few larvae developed to adults with varied emergence times, whereas 28.1 and 13.5% RS larvae feeding on Bt plants for 1 and 2 days, respectively, successfully developed to adults; 43.8% of pairs of these adults produced viable eggs, with an average of 220 progeny per female. For the case of the single Bt gene maize plants (event MON 810), the results suggest that RS insects may have advantages in survival and reproduction over SS if RS larvae hatch and feed on Bt plants during the first 1 or 2 days and then move to non-Bt plants. This advantage is less for RS larvae that hatch and feed on non-Bt plants first and then move to Bt plants, unless the larval movement occurs in the later stages (e.g., fourth or fifth instars).

Introduction

A seed mix strategy has been used to provide refuge to susceptible insects for resistance management in planting transgenic maize containing two or more pyramided *Bacillus thuringiensis* Berliner (Bt) genes in the USA (Matten et al., 2012). For the seed mix strategy, a defined percentage of non-Bt maize seeds is mixed with Bt maize seeds in each bag by seed companies prior to being sold to growers (Wangila et al., 2013). With the structured refuge method (Ostlie et al., 1997) that has been used for providing refuge to susceptible insects since 1996, the adult dispersal behavior of the target pest populations is important in resistance development, but with the seed mix, the major concern is that larval movement of the target insects among non-Bt and Bt plants may accelerate resistance evolution (Wangila et al., 2013). For example, larval movement between non-Bt and Bt plants in seed mix plantings may create a non-high dose environment that can be more

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favorable for survival of the resistant heterozygotes compared to the susceptible insects and thus hasten resistance development in the field (Wangila et al., 2013). However, empirical data that address this important issue are lacking, largely due to lack of suitable resistant insect strains to work with and the difficulty in conducting the studies.

The sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), is a target species of Bt maize in South America and the mid-southern region of the USA (Castro et al., 2004; Huang et al., 2012). A Cry1Ab-resistant strain of *D. saccharalis* was isolated using F_2 screen of a two-parent family collected from non-Bt maize fields near Winnsboro, Franklin Parish, LA, USA in 2004 (Huang et al., 2007, 2012). The Bt-resistant strain was highly resistant to both purified Cry1Ab protein and transgenic maize hybrids that contain a single Bt gene Cry1Ab (e.g., MON 810) (Huang et al., 2007; Wu et al., 2007; Ghimire et al., 2011; Wangila et al., 2012). Larvae of the Cry1Ab-resistant strain are also highly resistant to Cry1Aa and Cry1Ac, but show a low level of cross-resistance to Cry1A.105 and Cry1F, and are not cross-resistant to Cry2Ab2 (Wu et al., 2009a; Zhang et al., 2013). The Cry1Ab resistance in *D. saccharalis* is inherited as a single autosomal gene (Wu et al., 2009b). The resistance is partially recessive to partially dominant depending on the Bt concentrations or plant growth stages. The resistance is not associated with any fitness costs (Wu et al., 2009c; Zhang et al., 2014). In maize fields, *D. saccharalis* larvae have similar feeding and movement behavior to other common maize borers, such as the European corn borer, *Ostrinia nubilalis* (Hübner), and the southwestern corn borer, *Diatraea grandiosella* Dyar. The availability of the well-documented Cry1Ab-resistant strain of *D. saccharalis* provided an opportunity to investigate whether heterozygous Bt-resistant maize borer larvae have advantages over their susceptible counterparts in seed mix plantings of non-Bt and Bt maize. The objective of this study was to compare the performance of Cry1Ab-susceptible and Cry1Ab-heterozygous resistant populations of *D. saccharalis* in sequential feedings on non-Bt and Bt maize plant tissue. Data generated from this study should provide valuable information for evaluating resistance management strategies for Bt maize against maize stalk borers.

Materials and methods

Sources of insects and maize hybrids

In this study, two populations of *D. saccharalis* were tested: a Cry1Ab-susceptible strain (SS) and a heterozygous resistant population (RS). SS was established from larvae collected from non-Bt plants near Winnsboro in Franklin Parish in northeast Louisiana in 2009. SS is susceptible to purified Cry1Aa, Cry1Ab, Cry1Ac, Cry1A.105, and Cry2Ab2 proteins (Huang et al., 2012), as well as to Bt maize plants expressing Cry1Ab, Cry1A.105, and Cry2Ab2. RS was developed by crossing SS with a Cry1Ab-resistant strain (RR-2004) that was established using an F_2 screen in 2004 (Huang et al., 2007). RR-2004 can survive and complete its entire larval development (from neonate to pupa) on commercial Cry1Ab maize plants (Huang et al., 2007; Wu et al., 2007; Ghimire et al., 2011; Wangila et al., 2012). Before RR-2004 was used for this study, it had been backcrossed to SS 3× and reselected for Cry1Ab resistance with Cry1Ab maize leaf tissue at the F_2 generation of each backcross. RR adults of the fifth generation after the last backcross (or first generation after the last resistance reselection) were used in the crosses with SS to produce the RS population for this study.

A Bt maize hybrid, DKC69-70 (Monsanto, St. Louis, MO, USA), and a closely related non-Bt maize hybrid, DKC62-95, were used as the maize plant materials for the study. DKC69-70 produces a single Bt protein, Cry1Ab (MON 810). The seeds of each maize hybrid were planted in 18.9-l plastic pots each containing ca. 5 kg of standard potting soil mixture (Perfect Mix; Expert Gardener Products, St. Louis, MO, USA) in a greenhouse located in Baton Rouge, LA, USA. Plants were thinned to two plants per pot 1 week after planting as described in Wangila et al. (2012). Expression/nonexpression of the Cry1Ab protein for the Bt and non-Bt maize hybrids was confirmed using an enzyme-linked immunosorbent assay (ELISA) (QuantiPlate kits; EnviroLogix, Portland, ME, USA).

Treatments

Survival, development, and progeny production of SS and RS in sequential feedings on non-Bt and Bt plant tissue were evaluated in the laboratory as shown in Table 1. For each insect population, there were nine feeding sequences (named Feed-1 to Feed-9) which were conducted simultaneously. In Feed-1, which served as a negative control, newly hatched larvae (<24 h old) were placed on non-Bt maize plant tissue where they fed until pupation. In Feed-2, which served as a positive control, larvae were fed on Bt plants for their entire larval stage.

In seed mix plantings, maize borer larvae can feed and move among Bt and non-Bt (refuge) plants (Ross & Ostlie, 1990; Bell et al., 2005; Goldstein et al., 2010; Wangila et al., 2013). Feed-3 to 7 were designed to simulate the sequential feeding behavior of maize borer larvae moving from non-Bt to Bt plants in seed mix plantings, whereas Feed-8 and 9 were conducted to mimic larvae moving from Bt to non-Bt plants. A preliminary test showed that survival rates of neonates feeding on Bt maize leaf tissue after 3 days was low,

Table 1 Feeding sequences of Cry1Ab-susceptible and -heterozygous resistant populations of *Diatraea saccharalis* on non-Bt and Cry1Ab maize leaf and stalk tissue

Feeding sequence	Insect rearing	Insect bioassay
Feed-1: non-Bt day 0 to pupa	Not applicable	Neonates were assayed on non-Bt leaf tissue for 9 days (day 0–9); those surviving to day 9 were transferred to jars containing non-Bt stalk tissue until pupal stage.
Feed-2: Bt day 0 to pupa	Not applicable	Neonates were assayed on Bt leaf tissue for 9 days (day 0–9); those surviving to day 9 (if any) were transferred to jars containing Bt stalk tissue until pupal stage.
Feed-3: non-Bt day 0–3; Bt day 4 to pupa	Neonates were reared on non-Bt leaf tissue for 3 days (day 0–3).	3-day-old larvae from insect rearing were assayed on Bt leaf tissue for 6 days (day 4–9); survivors (if any) were then transferred to jars containing Bt stalk tissue until pupal stage.
Feed-4: non-Bt day 0–6; Bt day 7 to pupa	Neonates were reared on non-Bt leaf tissue for 6 days (day 0–6).	6-day-old larvae from insect rearing were assayed on Bt leaf tissue for 3 days (day 7–9); survivors (if any) were then transferred to jars containing Bt stalk tissue until pupal stage.
Feed-5: non-Bt day 0–9; Bt day 10 to pupa	Neonates were reared on non-Bt leaf tissue for 9 days (day 0–9).	9-day-old larvae from insect rearing were assayed in jars containing Bt stalk tissue until pupal stage.
Feed-6: non-Bt day 0–12; Bt day 13 to pupa	Neonates were reared on non-Bt leaf tissue for 9 days (day 0–9), and then transferred into jars containing non-Bt stalk tissue and reared for three more days (day 9–12).	12-day-old larvae from insect rearing were assayed in jars containing Bt stalk tissue until pupal stage.
Feed-7: non-Bt day 0–15; Bt day 16 to pupa	Neonates were reared on non-Bt leaf tissue for 9 days (day 0–9), and then transferred into jars containing non-Bt stalk tissue and reared for six more days (day 9–15).	15-day-old larvae from insect rearing were assayed in jars containing Bt stalk tissue until pupal stage.
Feed-8: Bt day 0–1; non-Bt day 2 to pupa	Neonates were reared on Bt leaf tissue for the 1st day (day 0–1).	1-day-old larvae from Bt leaf tissue were assayed on non-Bt leaf tissue for 8 days (day 2–9); survivors (if any) were then transferred to jars containing non-Bt stalk tissue until pupal stage.
Feed-9: Bt day 0–2; non-Bt day 3 to pupa	Neonates were reared on Bt leaf tissue for the first 2 days (day 0–2).	2-day-old larvae from Bt leaf tissue were assayed on non-Bt leaf tissue for 7 days (day 3–9); survivors (if any) were then transferred to jars containing non-Bt stalk tissue until pupal stage.

only 17–30% (data not shown). Thus, evaluating larvae moving from Bt to non-Bt plants was performed only for the cases that larvae fed on Bt plants for 1 or 2 days and then moved to non-Bt plants. Feed-8 evaluated the larvae that fed on Bt leaf tissue for the 1st day and then moved to non-Bt maize plants, whereas Feed-9 examined the larvae that fed on Bt leaf tissue for the first 2 days and then moved to non-Bt maize plants. Therefore, there were 18 treatment combinations of insect populations and feeding sequences for the entire study.

Insect rearing and bioassay

Each feeding sequence (except Feed-1 and 2) was divided into two periods: the first period was for insect rearing and the second was for bioassay. Insects were

reared to provide a sufficient number of larvae to conduct the bioassays listed in Table 1. Insect rearing started with neonates feeding on non-Bt (Feed-3 to 7) or Bt maize plant tissue (Feed-8 and 9) until they were used in the bioassays. Neonates were used directly in the bioassays for Feed-1 and 2 and thus there was no 'insect rearing period' for these two feeding sequences. In the field, newly hatched maize borer larvae usually feed on leaf tissue before the third instar and later they bore into and feed inside the stalk (Mason et al., 1996). To simulate this feeding behavior, two types of plant tissue (leaf tissue and stalk tissue) were used in the insect rearing and bioassays.

For insect rearing, leaf tissue was cut into ca. 7-cm-long sections from fully expanded leaves at the V5-V9 plant stages. Four to six pieces of the leaf tissue were placed into the wells of 8-well trays (Bio-Smart-8; C-D International, Pitman, NJ, USA). In each well, 20–25 neonates of an insect population were reared on the leaf tissue for 1–9 days, depending on the treatment. For Feed-6 and 7 in which 12- (Feed-6) or 15-day-old (Feed-7) larvae were needed in the bioassay, larvae that had been reared on the leaf tissue for 9 days were transferred to 1-l plastic jars (model S-14511B; Uline, Coppell, TX, USA) containing maize stalk tissue for continued rearing. In each plastic jar, there were three 8-cm-long sections of maize stalk tissue sliced from VT-R1 maize plants and each jar contained 12 larvae. The larvae in the jar were reared on the stalk tissue for an additional 3 and 6 days to provide the insect source for assaying Feed-6 and 7, respectively. The stalk sections in the jars were split halfway to expose the inner tissue to the larvae (Li et al., 2007). Larval rearing trays and jars were held in growth chambers maintained at 28 °C, L16:D8 cycle, and ca. 50% r.h. Leaf tissue was replaced every 3 days and stalk tissue was replaced every 3–5 days.

Neonates (Feed-1 and 2) or larvae of appropriate ages (Feed-3 to 9) collected from the larval rearing trays or jars were used in the bioassays as listed in Table 1. For each bioassay, there were four replications each containing 24 larvae ($n = 4 \times 24 = 96$ larvae for a bioassay). For Feed-1 to 4, 8, and 9, three neonates (<24 h old, for Feed-1 and 2) or three larvae of 1, 2, 3, or 6 days old (for Feed-8, 9, 3, and 4, respectively), collected from the insect rearing trays, were assayed in the wells of 32-well trays (Bio-Ba-32; C-D International) containing maize leaf tissue. After 3–9 days in the leaf tissue assay when the larvae were 9 days old (the total time for rearing and bioassay), live larvae were transferred into the 1-l jars containing maize stalk tissue and the assay continued until the pupal stage. As described for insect rearing, each jar in the insect assay contained three sections of maize stalk tissue. There was one jar for each replication if the number of total survivors after the leaf tissue assay was ≤12 in a replication or two jars if the number of total survivors from the leaf tissue assay was >12. For Feed-5, 6, and 7, 12 larvae that were 9 (for Feed-5), 12 (Feed-6), or 15 (Feed-7) days old collected from the insect rearing were assayed in each of the 1-l jars containing maize stalk tissue until the pupal stage using the method described above. There were two jars for each replication (24 larvae per replication). Bioassay trays and jars were placed in growth chambers maintained at the same conditions as for insect rearing.

Insect survival and pupation for each bioassay were checked on the 3rd day after the assay was started and every 3 days thereafter. For each replication of an assay, pupae collected were separated by sex and maintained under the same environmental conditions as the larval assay. Adult emergence was checked daily. Newly emerged (<24 h old) virgin male and female adults collected within an assay were paired in 2-l paper containers (one pair per container) (Huhtamaki Foodservice, De Soto, KS, USA). Number of pairs for each bioassay varied from 0 to 16, depending on number of moths that emerged, time of emergence, and sex ratio. Adult containers were placed in a growth chamber maintained at 26 °C, L14:D10 cycle, and >90% r.h. (Zhang et al., 2014). The number of progeny (neonates) produced per pair was recorded by counting the neonates that hatched.

Data analysis

Larval survivorship (%) was calculated based on the days used for the assays (excluding the time for insect rearing). Larval survivorship for a bioassay was measured every 3 days until the first pupa was observed. Neonate-to-pupa development time of an individual was measured starting at the neonate stage (1st day) to the day when it pupated. For Feed-1 and 2, development time was the time that the insect spent in the assay, whereas for Feed-3 to 9, development time included the time that the insect spent in both insect rearing and assay. For progeny production, a pair that successfully produced progeny was considered a replication for a bioassay in the data analysis. Data were analyzed using two-way ANOVA with insect population and feeding sequence as the two main factors (SAS Institute, 2010). For ANOVA, original data on the percentage of larval survival were transformed using arcsine $\sqrt{x}$, whereas neonate-to-pupa development time and progeny production were transformed using log (x+1), to meet the assumptions of normality. For each

parameter measured, treatment means were separated using the LSMEAMS test at $\alpha = 0.05$ (SAS Institute, 2010).

Results

Survivorship of SS and RS populations of *Diatraea saccharalis* in sequential feedings on non-Bt and Cry1Ab maize plant tissue

The effects of insect population, feeding sequence, and the interaction between the two factors on insect survivorship were all significant at each observation time, as were the effects on the survival from larval transfer to pupa and from larval transfer to the adult stage (Table 2). On non-Bt maize plant tissue, both SS and RS populations survived well, but, in general, the RS population had higher survival than SS at all observation times except for the 3rd day after larval release. In contrast, larvae of the SS and RS populations fed on Cry1Ab maize plant tissue (Feed-2) performed similarly and, though a portion of the larvae survived after 3 days (17.7% SS and 30.2% RS), all larvae died after 6 (SS) or 18 days (RS).

For Feed-3 and 4, the two insect populations performed similarly, with 16.7–82.3% of larvae surviving 3 days after larval transfer (Table 2) but virtually all larvae having died 6 days after larval transfer, and none reaching pupation. For Feed-5 and 6, SS could not survive to the pupal stage, but 8.3% (Feed-5) and 5.2% (Feed-6) of RS developed to pupae and 7.3% (Feed-5) and 4.2% (Feed-6) to adults (Table 2). In Feed-7, significantly more RS than SS larvae survived 3 days after larval transfer (84.4 vs. 54.2%) (Table 2). An average of 4.2% SS larvae developed to pupae but no moths emerged from the pupae, whereas 46.9 and 29.2% of RS larvae developed to pupae and adults, respectively.

In Feed-8, RS larvae performed significantly better than SS (Table 2). An average of 2.1% SS larvae survived 3 days after the larvae were transferred to non-Cry1Ab plant tissue, but after 9 days all SS larvae were dead. In contrast, survival of RS in Feed-8 after 3 days of larval transfer to the non-Bt maize plant tissue was 72.9%. An average of 35.4% of RS larvae developed to pupae and 28.1% larvae successfully developed to the adult stage.

In Feed-9, larval survival, pupation, and moth emergence of the RS population were all significantly greater than those of the SS larvae. Both populations started pupation 21 days after larval transfer. After 18 days, 18.8% of RS larvae were still alive compared to only 7.3% of SS. An average of 6.3 and 5.2% of SS larvae developed to the pupal and adult stages, respectively. These values for RS were 17.7 and 13.5%, respectively.

Development and progeny production of SS and RS populations in sequential feedings on non-Bt and Cry1Ab maize plant tissue

The effects of insect population and feeding sequence on neonate-to-pupa development time were significant, but not their interaction (Table 3). Larvae of SS and RS on non-Bt maize plant tissue (Feed-1) took on average 23.9 and 22.9 days, respectively, to become pupae, and the difference between the two insect populations was not significant. In Feed-7, RS larvae developed faster to the pupal stage than SS larvae (19.9 vs. 22 days, on average). In Feed-9, both insect populations had similar development times. The development time of RS in Feed-5, 6, and 8 was 25.3, 21.8, and 21.1 days, respectively. Pupae were not observed for other treatment combinations, therefore larval development time could not be calculated.

Because no or a limited number of moths emerged in some treatment combinations, pairing for SS could be conducted only for Feed-1, and for RS for Feed-1, 5, 7, 8, and 9 (Table 3). The number of pairs in a treatment combination varied from 2 to 16. On average, 38.5-100% of these pairs produced progeny successfully. The effects of insect population and feeding sequence on the number of progeny produced per successful pair were not significant. Because only one treatment with SS was included in the ANOVA, no effect of factor interaction could be analyzed. The number of progeny produced in successful pairs did not differ significantly among the six treatment combinations, with an overall mean ($\pm$ SEM) of 240.2 $\pm$ 19.5 progeny per pair.

Discussion

Both the SS and RS populations of *D. saccharalis* in this study survived well on non-Bt maize plant tissue, though the overall performance of RS appeared to be better than that of SS. Why the heterozygous population performs better than its susceptible counterpart in the absence of selection is unknown. One possible explanation could be a fitness advantage that might be associated with the resistance. Unlike Bt resistance in most other insect species, studies have shown that fitness costs are not associated with Cry1Ab resistance in *D. saccharalis* (Wu et al., 2009c; Zhang et al., 2014). In contrast, a possible fitness advantage has been observed in two other Cry1Ab-resistant strains of *D. saccharalis* (Zhang et al., 2014). Fitness advantages to Bt resistance have rarely been reported for other insect species. The only other notable case is a Cry3Bb1-resistant western corn rootworm, *Diabrotica virgifera virgifera* LeConte, population that was found to emerge 2–3 days earlier than susceptible insects when reared on non-Bt maize plants (Oswald et al., 2012). It was suggested that selection for the Cry3Bb1 resistance

Table 2 Mean (± SEM) insect survivorship (%) of Cry1Ab-susceptibile (SS) and -heterozygous resistant (RS) populations of *Diatraea saccharalis* in sequential feedings on non-Bt and Cry1Ab maize plant tissue

Feeding sequence	Insect population	Days after larvae were transferred from rearing and used for bioassay							Larva-to-pupa (%)	Larva-to-adult (%)
		3	6	9	12	15	18	21		
Feed-1: non-Bt	SS	94.8 ± 1.0hi	87.5 ± 4.8f	76.0 ± 6.2f	54.2 ± 7.2e	51.0 ± 3.1e	50.0 ± 2.4e	46.9 ± 2.0c	37.5 ± 2.4de	30.2 ± 2.0d
day 0 to pupa	RS	98.9 ± 1.0i	97.9 ± 2.1g	90.6 ± 2.0g	74.0 ± 1.0f	71.9 ± 1.0f	70.8 ± 0.0f	66.7 ± 0.0d	59.4 ± 1.0f	50.0 ± 1.7e
Feed-2: Bt	SS	17.7 ± 4.6b	0a	0a	0a	0a	0a	0a	0a	0a
day 0 to pupa	RS	30.2 ± 1.0b	2.1 ± 2.1a	2.1 ± 2.1a	1.0 ± 1.0a	1.0 ± 1.0ab	0a	0a	0a	0a
Feed-3: non-Bt	SS	20.3 ± 2.9b	0a	0a	0a	0a	0a	0a	0a	0a
day 0–3; Bt day 4 to pupa	RS	16.7 ± 7.0b	0a	0a	0a	0a	0a	0a	0a	0a
Feed-4: non-Bt	SS	82.3 ± 7.9fgh	0a	0a	0a	0a	0a	0a	0a	0a
day 0–6; Bt day 7 to pupa	RS	60.4 ± 7.1cd	1.0 ± 1.0a	0a	0a	0a	0a	0a	0a	0a
Feed-5: non-Bt	SS	82.3 ± 2.6efg	49.0 ± 12.9de	19.8 ± 6.0bc	6.3 ± 4.0b	3.1 ± 2.0a	2.1 ± 2.1b	1.0 ± 1.0b	0a	0a
day 0–9; Bt day 10 to pupa	RS	66.7 ± 7.2cde	55.2 ± 9.7de	29.2 ± 1.7cd	16.7 ± 5.9cd	Pupated	n/a	n/a	8.3 ± 3.8b	7.3 ± 3.1b
Feed-6: non-Bt	SS	66.7 ± 7.6cdef	57.3 ± 5.5de	25.0 ± 2.9cd	5.2 ± 1.0b	1.0 ± 1.0ab	0a	0a	0a	0a
day 0–12; Bt day 13 to pupa	RS	83.3 ± 4.8gh	65.6 ± 3.6e	34.4 ± 7.1d	Pupated	n/a	n/a	n/a	5.2 ± 2.0b	4.2 ± 2.4b
Feed-7: non-Bt	SS	54.2 ± 3.4c	41.7 ± 6.6cd	Pupated	n/a	n/a	n/a	n/a	4.2 ± 1.7b	0a
day 0–15; Bt day 16 to pupa	RS	84.4 ± 3.9gh	Pupated	n/a	n/a	n/a	n/a	n/a	46.9 ± 4.9e	29.2 ± 3.8d
Feed-8: Bt	SS	2.1 ± 2.1a	1.0 ± 1.0a	0a	0a	0a	0a	0a	0a	0a
day 0–1; non-Bt day 2 to pupa	RS	72.9 ± 5.5defg	58.3 ± 8.8de	50.0 ± 11.2e	44.8 ± 9.7e	41.7 ± 8.0e	37.9 ± 5.9e	Pupated	35.4 ± 5.2d	28.1 ± 4.3d
Feed-9: Bt	SS	18.8 ± 2.1b	13.5 ± 2.0b	9.4 ± 2.0b	9.4 ± 2.0bc	7.3 ± 2.0c	7.3 ± 2.0c	Pupated	6.3 ± 2.1b	5.2 ± 1.0b
day 0–2; non-Bt day 3 to pupa	RS	50.0 ± 8.8c	30.2 ± 6.9c	24.0 ± 5.2cd	22.9 ± 5.2d	20.8 ± 4.5d	18.8 ± 5.2d	Pupated	17.7 ± 5.4c	13.5 ± 4.3c
ANOVA	Insect	$F_{1,54}$ = 35.42, P<0.0001	$F_{1,51}$ = 34.21, P<0.0001	$F_{1,48}$ = 47.08, P<0.0001	$F_{1,45}$ = 50.68, P<0.0001	$F_{1,42}$ = 67.88, P<0.0001	$F_{1,42}$ = 79.81, P<0.0001	$F_{1,33}$ = 18.31, P = 0.0002	$F_{1,54}$ = 139.55, P<0.0001	$F_{1,54}$ = 140.28, P<0.0001
	Feeding	$F_{8,54}$ = 53.35, P<0.0001	$F_{8,51}$ = 95.74, P<0.0001	$F_{7,48}$ = 106.7, P<0.0001	$F_{1,45}$ = 50.68, P<0.0001	$F_{7,42}$ = 107.91, P<0.0001	$F_{7,42}$ = 159.6, P<0.0001	$F_{6,33}$ = 691.65, P<0.0001	$F_{8,54}$ = 81.94, P<0.0001	$F_{8,54}$ = 74.16, P<0.0001
	Interaction	$F_{8,54}$ = 15.31, P<0.0001	$F_{7,51}$ = 7.87, P<0.0001	$F_{7,48}$ = 11.14, P<0.0001	$F_{6,45}$ = 11.55, P<0.0001	$F_{5,42}$ = 20.92, P<0.0001	$F_{5,42}$ = 28.63, P<0.0001	$F_{3,33}$ = 18.31, P<0.0001	$F_{8,54}$ = 16.6, P<0.0001	$F_{8,54}$ = 18.13, P<0.0001

Means within a column followed by the same letter are not significantly different (LSMEAMS test: P>0.05).
n/a, not applicable.

Table 3 Mean (± SEM) development time (days) and progeny production of Cry1Ab-susceptibile (SS) and -heterozygous resistant (RS) populations of *Diatraea saccharalis* in sequential feedings of non-Bt and Cry1Ab maize plant tissue

		Neonate-to-pupa		Progeny production (larvae/pair)		
Feeding sequence	Insect population	No. pupae observed	Development time	No. pairs	% pairs produced progeny	No. progeny per successful pair
Feed-1: non-Bt day 0 to pupa	SS	36	23.9 ± 0.8cd	11	63.3	228.3 ± 46.4a
	RS	57	22.9 ± 0.4bc	16	68.8	295.5 ± 35.4a
Feed-2: Bt day 0 to pupa	SS	0	n/a	0	n/a	n/a
	RS	0	n/a	0	n/a	n/a
Feed-3: non-Bt day 0–3;	SS	0	n/a	0	n/a	n/a
Bt day 4 to pupa	RS	0	n/a	0	n/a	n/a
Feed-4: non-Bt day 0–6;	SS	0	n/a	0	n/a	n/a
Bt day 7 to pupa	RS	0	n/a	0	n/a	n/a
Feed-5: non-Bt day 0–9;	SS	0	n/a	0	n/a	n/a
Bt day 10 to pupa	RS	8	25.3 ± 1.5d	2	100	179.5 ± 59.5a
Feed-6: non-Bt day 0–12;	SS	0	n/a	0	n/a	n/a
Bt day 13 to pupa	RS	5	21.8 ± 1.3abc	0	n/a	n/a
Feed-7: non-Bt day 0–15;	SS	4	22.0 ± 0.5bc	0	n/a	n/a
Bt day 16 to pupa	RS	45	19.9 ± 0.5a	9	55.8	187.2 ± 28.0a
Feed-8: Bt day 0–1; non-Bt	SS	0	n/a	0	n/a	n/a
day 2 to pupa	RS	34	21.1 ± 0.6ab	13	38.5	247.2 ± 43.2a
Feed-9: Bt day 0–2; non-Bt	SS	6	22.8 ± 0.6bc	0	n/a	n/a
day 3 to pupa	RS	17	21.5 ± 0.2ab	3	66.7	152.5 ± 94.5a
ANOVA	Insect	n/a	$F_{1,24} = 6.81, P = 0.015$	n/a	n/a	$F_{1,26} = 1.37, P = 0.25$
	Feeding	n/a	$F_{5,24} = 6.38, P = 0.0007$	n/a	n/a	$F_{4,26} = 1.21, P = 0.33$
	Interaction	n/a	$F_{5,24} = 0.40, P = 0.67$	n/a	n/a	n/a

Means within a column followed by the same letter are not significantly different (LSMEAMS test: P>0.05).
n/a, not applicable.

also resulted in accelerated larval development. Another possible explanation for the better performance of the RS *D. saccharalis* population would be 'hybrid vigor'. Possible hybrid vigor of Bt resistance in absence of selection has been reported for two other Cry1Ab-resistant strains of *D. saccharalis* (Zhang et al., 2014) and a Cry1F-resistant population of the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Vélez et al., 2013). Such hybrid vigor can occur when crossing two populations with divergent genetic backgrounds (Gassmann et al., 2009). However, as mentioned above, to alleviate differences in genetic background among the two insect populations, the resistant strain of *D. saccharalis* had been backcrossed 3× with its susceptible counterpart and reselected before it was used in this study. A similar backcross-and-reselection procedure was also used in the studies by Vélez et al. (2013) and Zhang et al. (2014). These results suggest that, if the outperformance of the heterozygotes was indeed due to differences in genetic background, genetic divergence may occur more rapidly than expected (Zhang et al., 2014).

The SS and RS populations of *D. saccharalis* on Cry1Ab maize plant tissue (Feed-2) performed similarly, with all larvae dead after 18 days and no adults produced. Thus, the Cry1Ab resistance was completely recessive when it was measured based on the relative fitness in neonate-to-pupa survival, neonate-to-adult survival, or progeny production on Bt plant tissue; $D_{WT} = 0$, as suggested by Bourguet et al. (2000). In other words, the Bt maize plants used in the study produced a sufficient dose to kill heterozygous resistant RS. However, even with completely recessive resistance, our results indicated that RS insects can have advantages in survival over SS in sequential feedings on non-Bt and Bt plant tissue, especially if RS larvae hatch and feed on Bt plants during the first 1 or 2 days and then move to non-Bt plants. These survival advantages for RS can lead to successful progeny production in the sequential feedings. The advantage is less for the RS larvae that hatch and feed on non-Bt plants first and then move to Bt plants, unless the larval movement occurs in later stages (e.g., fourth or fifth instars).

The advantages of RS in sequential feedings on non-Bt and Bt plant tissue could have important implications for resistance management. In the field, maize borer larvae can feed and move frequently among plants, especially

during the early larval stages (Ross & Ostlie, 1990; Mallet & Porter, 1992; Davis & Onstad, 2000; Bell et al., 2005; Goldstein et al., 2010; Burkness et al., 2011; Wangila et al., 2013; Oyediran et al., 2016). For example, Ross & Ostlie (1990) reported that more than half of *O. nubilalis* neonates could abandon the primary host plants and disperse to other plants during the first 2 days after hatching. Studies also demonstrated that such larval dispersal is greater off Bt maize than off non-Bt maize plants for *O. nubilalis* (Davis & Onstad, 2000; Goldstein et al., 2010; Razze & Mason, 2012). In addition, later stages of maize borer larvae (from third instars on) in the field also can move among plants (Mason et al., 1996). Field observations show that larvae of *D. saccharalis* and *O. nubilalis* have the ability to move across several plants within a row of plants as well as to adjacent rows (Mason et al., 1996; Davis & Onstad, 2000; Walker et al., 2000; Wangila et al., 2013; Oyediran et al., 2016). In either case, our results suggest that the larval dispersal behavior of maize borers could result in survival and successful reproduction of RS populations in seed mix plantings of non-Bt and Bt maize plants even with high dose expression. In a recent greenhouse study, Brévault et al. (2015) also reported that seed mix plantings could increase the dominance of resistance to Cry1Ac cotton in *Helicoverpa zea* (Boddie).

To ensure resistant insects were not released in our work, as well as in the study by Brévault et al. (2015), studies were conducted only in laboratory or greenhouse conditions. More importantly, both the current and Brévault et al.'s studies evaluated only a case of single gene-to-single gene resistance, whereas the seed mix strategy, for controlling above-ground lepidopteran targets, is allowed to be used only for planting pyramided maize hybrids containing two or more dissimilar Bt genes (Matten et al., 2012). Therefore, caution must be taken in extrapolating the information generated under such experimental conditions to the field environments. In addition, the effects observed can be expected to vary with the nature of the Bt resistance and the dose of the Bt plant, reinforcing the need for case by case assessments of the seed mix strategy (Carroll et al., 2012).

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