Effect of MIR604 Transgenic Maize at Different Stages of Development on Western Corn Rootworm (Coleoptera: Chrysomelidae) in a Central Missouri Field Environment

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ABSTRACT The establishment and survival of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, was evaluated on transgenic *Bacillus thuringiensis* Berliner maize, *Zea mays* L., expressing the mCry3A protein (MIR604) and non-Bt maize with the same genetic background (isoline maize) at different stages of development in 2007 and 2008. Overall, western corn rootworm larval recovery, root damage, and adult emergence were significantly higher on isoline maize compared with MIR604. The number of larvae and adults collected from MIR604 did not significantly differ among egg hatch dates from each maize developmental stage evaluated in either year. In 2007, damage to isoline maize roots was lower than expected and never exceeded 0.24 nodes of damage. In 2008, over 0.60 nodes of damage occurred on isoline maize roots. The mean weight and head capsule width of larvae and adults recovered from MIR604 and isoline maize were generally not significantly different. Results are discussed in relation to insect resistance management of western corn rootworm.

KEY WORDS *Diabrotica virgifera virgifera*, phenology, mCry3A, insect resistance management

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is an important pest of maize in the United States, costing growers over $1 billion per year in yield losses and control costs (Metcalf 1986, Sappington et al. 2006). Larvae are the most economically damaging life stage, feeding predominately within the cortex of maize roots (Riedell and Kim 1990). Damage from larval feeding can reduce the uptake of water and nutrients by maize plants (Kahler et al. 1985, Godfrey et al. 1993) and increase the susceptibility of plants to lodging because of reduced brace root support (Levine and Oloumi-Sadeghi 1991).

Historically, management strategies for western corn rootworm larvae relied on crop rotation with a nonhost crop, the application of soil insecticides at planting, or adult control measures to prevent egg-laying. However, the behavioral and genetic plasticity of this insect has made the long-term viability of these management options uncertain. For example, in certain regions of the Corn Belt a rotation-resistant variant has developed where females oviposit outside of maize fields (Levine et al. 2002, Gray et al. 2009). In addition, western corn rootworm has developed resistance to several primary insecticides used for its control (Meinke et al. 1998, Wright 2000). Transgenic maize that express *Bacillus thuringiensis* Berliner (Bt) proteins toxic to western corn rootworm have been developed by several seed companies as an additional management tactic to limit larval rootworm damage (Moellenbeck et al. 2001, Ellis et al. 2002, Vaughn et al. 2005, Walters et al. 2008). Since 2003, several Bt products have been registered for commercial sale; three competing Bt maize proteins (Cry3Bb1, Cry34/35Ab1, mCry3A), the pyramid of Cry3Bb1 and Cry34/35Ab1, and recently, the pyramid of mCry3A and Cry34/35Ab1.

Current maize events that express Bt proteins specifically targeting rootworms are considered low-to-moderate dose (EPA 2002, Storer et al. 2006, Hibbard et al. 2010 a, b) with expression generally concentrated somewhat in root tips and younger root tissue (Meissle et al. 2009). Research conducted with maize expressing event MON863 showed that the concentration of Cry3Bb1 protein decreased significantly as plants matured from the vegetative (V)4 to V9 developmental stage (Vaughn et al. 2005). Gray et al. (2007) reported that the average damage rating (Oleson et al. 2005) of eight commercial YieldGard Rootworm hybrids increased from an average rating of 0.17 on 24 July to 0.40 on 8 August 2006 in Monmouth, IL, and from 0.31 on 20

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July to 0.80 on 7 August 2006 in Urbana, IL. Gray and Steffey (2005) suggested that diminished levels of Cry3Bb1 compromised root protection in July and August. Despite the potential for decreasing Cry3Bb1 expression levels over the growing season, MON863 provided equivalent root protection for western corn rootworm eggs that hatched at maize developmental stages ranging from V3 to reproductive (R) 3 (Hibbard et al. 2009). The characterization of mCry3A protein expression levels in MIR604 across varying developmental stages has not been documented, but similar to Gray et al. (2007) for MON863, damage to MIR604 can also increase late in the season (B. E. Hibbard, unpublished data). The goal of this project was to evaluate the relative effectiveness of mCry3A on western corn rootworm across a wide range of maize developmental stages.

Materials and Methods

Study Site and Design. Field studies were conducted in 2007 and 2008 at the University of Missouri Bradford Research and Extension Center (MU-BREC) located in Boone Co., MO, using methods similar to Hibbard et al. (2009). The soil type at this site was Mexico silt loam comprised of 2% sand, 70% silt, and 28% clay (MU Soils Testing Lab). Cultural practices (tillage, fertilization, herbicide application, etc.) were typical of agricultural procedures for the area. Each year, the field site selected for research had been planted with soybean, *Glycine max* L., the previous year. Central Missouri does not have the rotation-resistant strain of the western corn rootworm that oviposit outside of maize in soybeans and other crops, so we assumed that any western corn rootworm recovered in our plots were from eggs that we infested.

Field sites were planted with MIR604 (GarstX75905) and its isoline (GarstX73295) in plots that were 7.6 m long and four rows wide with a 0.91 m row spacing and 21.6 cm plant spacing using a mechanical planter (Wintersteiger/Hege, Salt Lake City, UT). The experiment was set up as a randomized complete block split-plot design with a 10 × 2 (infestation date × maize pedigree) factorial treatment arrangement. Infestation date served as the main plot factor and maize pedigree as the subplot factor in five replicate blocks. Infestation date was randomly assigned to one of 10 main plots within each of the five replicates. Within each main plot and replication, maize pedigree was randomly assigned to one of two, two-row subplots (two isoline and two MIR604 rows). Five plants, at least 1 m apart, were infested in the first row in each subplot. The second row served as a buffer to prevent across-row larval movement (Hibbard et al. 2003, 2004). Gene check strips (Envirologix Inc., Portland, ME) were run on all infested MIR604 plants and 5% of the infested isoline plants to ensure that the mCry3A protein was expressed in the appropriate subplots. The five infested plants were randomly assigned to one of five sample types described below. During the study, temperature and rainfall data were collected from a weather station located at the MU-BREC. In 2007, the daily mean temperature was 23.4°C (range, 17.7–29.3°C) and total rainfall was 32.2 cm. In 2008, the daily mean temperature was 21.6°C (range, 16.4–26.9°C) and total rainfall was 89.9 cm.

Insects and Infestation. Feral western corn rootworm eggs obtained from French Agricultural Research, Inc., Lamberton, MN, were infested at a rate of 800 viable eggs per plant. Eggs were deposited into two small holes (≈1 cm wide and 10 cm deep) located ≈7 cm from the base of the plant on each row side, which were then covered with soil. For both years of the study the first infestation date was at planting and the second was at VE (Ritchie et al. 1992). Infestations continued for each of the eight additional infestation dates at approximately weekly intervals, so as to encompass a range of maize developmental stages at egg hatch (V4-R3). Infestation dates in 2007 were on 20 April; 7, 14, 22, 29 May; 5, 12, 18, 25 June; and 2 July. Infestation dates in 2008 were on 6, 16, 29 May; 4, 11, 17, 25 June; and 2, 8, 16 July. Because actual dates may be less useful than maize phenology, future reference to egg infestation dates will be in terms of maize developmental stage at egg hatch (≈295 degree days [DD] after infestation under Central Missouri conditions). The egg level of 800 viable eggs per plant was chosen so that egg levels were as high as possible with minimal density-dependent mortality (Hibbard et al. 2004, 2010b). A subsample of eggs was removed from cold storage before infestation to determine viability and the actual number of eggs infested was adjusted accordingly each year.

Data Collection. Each year, five sample types were collected: larval recovery at ≈370 DD after infestation (most larvae at early second instar stage), larval recovery at ≈430 DD after infestation (most larvae at early third instar stage), root damage at 600 and 800 DD after infestation (when ≈50 and 100% of the larvae had pupated, respectively), and adult emergence. DD accumulation began on the day of infestation and was calculated by subtracting the developmental threshold of 11.1°C (Wilde 1971, Levine et al. 1992) from the average 24 h bare soil temperature at a depth of 5 cm. Soil temperature data were obtained from the field using a HOBO (model U23–003, Bourne, MA).

For larval recovery samples, we used the technique described by Hibbard et al. (2004). Briefly, root balls with associated soil were removed from the field in plastic mesh "onion" bags (Sacramento Bag Manufacturing Co., Woodland, CA) and hung over water pans in a greenhouse bay with the cooling system turned off. Under Missouri summer conditions, temperatures in the greenhouse routinely exceeded 50°C during the day. This technique was similar to a Tullgren funnel in that corn rootworm larvae fell from the drying samples into the water pans placed below. Larvae were collected from the water pans at least twice daily for a minimum of 7 d and were stored in scintillation vials containing 95% ethanol. In the laboratory, each larva was examined under a microscope for the presence of urogomphi to differentiate southern corn rootworm larvae, *Diabrotica undecimpunctata howardi* Barber, (urogomphi present) from western corn rootworm

larvae (urogomphi absent) (Krysan 1986). Total number, dry weight, and head capsule width were recorded for all western corn rootworm larvae recovered. At 600 and 800 DD plants selected for root damage evaluations were removed from the soil, washed, and rated using the 0–3 node injury scale (Oleson et al. 2005). For adult emergence, cages similar in design to Hein et al. (1985) with further modifications to keep plants alive by Pierce and Gray (2007) were placed over plants during the whorl stage. Cages were set up to collect insects (i.e., stalk sealed and collecting jars on) at ≈550–600 DD. Adult insects were collected three times per week and were stored in scintillation vials containing 95% ethanol. In the laboratory, total number, gender, dry weight, and head capsule width were recorded for all western corn rootworm beetles collected. The number of southern corn rootworm beetles collected was also recorded.

Statistical Analysis. Data were analyzed by year using the generalized linear mixed model of the SAS statistical package (PROC GLIMMIX; SAS Institute 2008). The statistical model contained the main plot effect of infestation date, the subplot effect of maize pedigree (MIR604 and isoline) and their interaction. Replication within infestation date was included as the random statement, all other variables were fixed. Least squares means (LSMEANS) of fixed effects were calculated separately for each year and comparisons were performed using the *t*-test output of the SAS model. Data for larval and adult dry weights and head capsule width were considered as missing values in the analysis when no larvae or adults were recovered from a particular plant. Differences in the number of larvae recovered at 370 and 430 DD after infestation were not significant, so data were averaged by recovery time and then analyzed as average larval number. Root damage at 600 and 800 DD after infestation were also not significantly different, so these data were also averaged by recovery time and then analyzed as average damage rating. Results from all tests were considered statistically different at $P < 0.05$.

Results

Larval Recovery. Infestation date, maize pedigree, and their interaction had a significant effect on the number of western corn rootworm larvae recovered from maize roots for each year of the study (Table 1; Fig. 1). Significantly more larvae were recovered from isoline maize compared with MIR604 when all sample dates were combined for both years (Table 1; Fig. 1). In both 2007 and 2008, larval numbers from isoline maize peaked when egg hatch occurred at ≈V7 developmental stage (Fig. 1). There was no significant difference in the number of larvae recovered from MIR604 roots among the widely varying egg hatch dates in either year of the study (Fig. 1).

There was no significant difference in the mean (±SE) dry weight of larvae recovered ≈370 DD after infestation from MIR604 (0.40 ± 0.20 mg) and isoline maize (0.40 ± 0.09 mg) in 2007 ($F = 1.97$; df = 1,2; $P = 0.2954$). Although the dry weight of larvae recovered ≈370 DD after infestation in 2008 was significantly affected by maize pedigree ($F = 5.36$; df = 1,12; $P = 0.0391$), weight was greater for those larvae recovered from MIR604 roots (0.58 ± 0.21 mg) compared with isoline roots (0.26 ± 0.05 mg), though an estimate of least squares means was not possible because of the large number of missing values (larvae were not always recovered, especially from MIR604). In 2007 and 2008, the mean (±SE) head capsule width of western corn rootworm larvae recovered ≈370 DD after infestation from MIR604 (0.39 ± 0.02 and 0.34 ± 0.03 mm, respectively) and isoline maize (0.42 ± 0.02 and 0.35 ± 0.02 mm, respectively) were not significantly different ($F = 2.48$; df = 1,2; $P = 0.2557$ and $F = 0.31$; df = 1,12; $P = 0.5860$, respectively).

In 2007 and 2008, the mean (±SE) dry weight of western corn rootworm larvae recovered ≈430 DD after infestation from MIR604 (0.65 ± 0.19 and 1.09 ± 0.14 mg, respectively) and isoline maize (0.91 ± 0.11 and 0.72 ± 0.07 mg, respectively) were not significantly different ($F = 2.68$; df = 1,7; $P = 0.1455$ and $F = 3.91$; df = 1,17; $P = 0.0646$, respectively). Although the mean (±SE) head capsule width of larvae recovered ≈430 DD after infestation in 2007 was significantly affected by maize pedigree ($F = 5.64$; df = 1,7; $P = 0.0492$), head capsule width was greater for those larvae recovered from isoline roots (0.51 ± 0.01 mm) compared with MIR604 roots (0.47 ± 0.03 mm), though an estimate of least squares means was not possible because of the large number of missing values. In 2008, there was no significant difference in the head capsule width of larvae recovered ≈430 DD after infestation from MIR604 (0.47 ± 0.01 mm) and isoline maize (0.45 ± 0.01 mm) ($F = 0.59$; df = 1,17; $P = 0.4539$).

Table 1. Summary statistics from an ANOVA for effects impacting number of western corn rootworm larvae recovered, root damage rating, and number of adults collected from field sites in 2007 and 2008

Year	Analysis	Effect	df	*F* value	*P*
2007	Larvae	Infestation date	9, 40	17.58	<0.0001
		Maize pedigree	1, 40	78.09	<0.0001
		Date × pedigree	9, 40	12.97	<0.0001
	Damage	Infestation date	9, 40	4.81	0.0002
		Maize pedigree	1, 40	13.66	0.0007
		Date × pedigree	9, 40	3.38	0.0036
	Adults	Infestation date	9, 40	21.48	<0.0001
		Maize pedigree	1, 40	43.33	<0.0001
		Date × pedigree	9, 40	21.73	<0.0001
2008	Larvae	Infestation date	9, 40	4.18	0.0007
		Maize pedigree	1, 40	46.34	<0.0001
		Date × pedigree	9, 40	3.25	0.0047
	Damage	Infestation date	9, 40	5.27	<0.0001
		Maize pedigree	1, 40	39.11	<0.0001
		Date × pedigree	9, 40	4.29	0.0006
	Adults	Infestation date	9, 40	3.31	0.0042
		Maize pedigree	1, 40	17.23	0.0002
		Date × pedigree	9, 40	3.29	0.0044

Randomized complete block split-plot design with infestation date as main plot effect and maize pedigree as subplot effect.

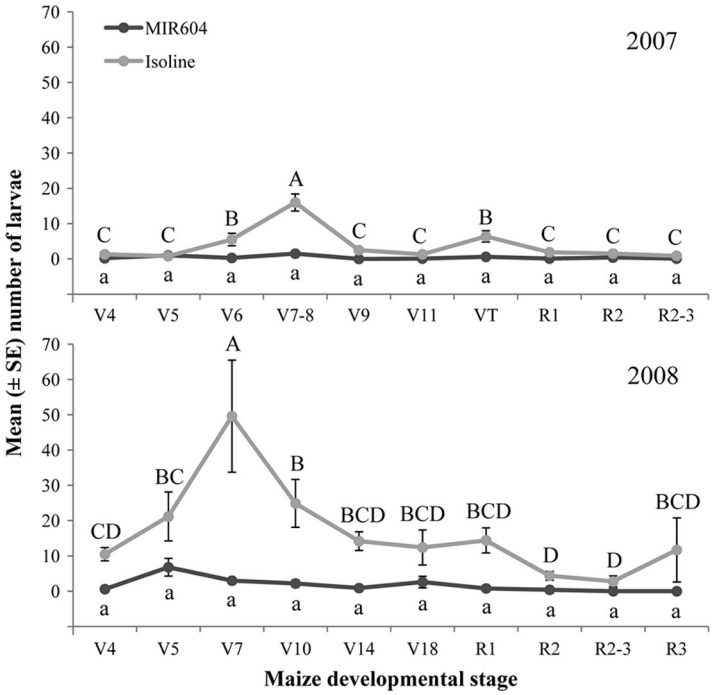

Fig. 1. Mean (±SE) number of western corn rootworm larvae recovered from MIR604 and isoline maize in 2007 and 2008 when infested with 800 viable western corn rootworm eggs hatching at various maize developmental stages. Means across each line followed by the same uppercase (isoline) or lowercase (MIR604) letter are not significantly different ($P > 0.05$).

Root Damage. Damage to maize roots was significantly impacted by infestation date, maize pedigree, and their interaction for each year of the study (Table 1; Fig. 2). Significantly more damage was found on isoline maize compared with MIR604 when all sample dates were combined for both years (Table 1; Fig. 2). Similar to larval recovery data, damage on isoline maize was generally greatest when egg hatch occurred at the ≈V7–8 developmental stages in both years of the study (Fig. 2). There was no significant difference in damage to MIR604 roots among the widely varying egg hatch dates in either year of the study (Fig. 2).

Adult Emergence. The number of western corn rootworm beetles that were collected from maize plants varied significantly among infestation date, maize pedigree, and their interaction for each year of the study (Table 1). Significantly more beetles were recovered from isoline maize compared with MIR604 when all sample dates were combined for both years (Table 1; Fig. 3). There was no significant difference in adult emergence from MIR604 roots among the widely varying egg hatch dates in either year of the study (Fig. 3). In 2007 and 2008, the mean (±SE) number of southern corn rootworm beetles that emerged from MIR604 (0.16 ± 0.09 and 0.84 ± 0.26, respectively) and isoline maize (0.30 ± 0.13 and 1.04 ± 0.28, respectively) was not significantly different in either year ($F = 1.18$; df = 1,40; $P = 0.2837$ and $F = 0.72$; df = 1,40; $P = 0.3997$, respectively).

Total emergence of western corn rootworm beetles from MIR604 and isoline maize was 1 and 84 beetles, respectively, in 2007 and 6 and 149 beetles, respectively, in 2008. Overall, there was a female bias in the percentage of beetles recovered from MIR604 and isoline maize. For each year of the study, all beetles that emerged from MIR604 roots were female. The percentage of female beetles that emerged from isoline roots was 71 and 60% for 2007 and 2008, respectively. Because only one western corn rootworm beetle emerged from MIR604 roots in 2007, dry weight and head capsule width were not analyzed. In 2008, the mean (±SE) dry weight and head capsule width of beetles that emerged from MIR604 (1.83 ± 0.30 mg and 1.08 ± 0.04 mm, respectively) and isoline maize (1.71 ± 0.13 mg and 1.03 ± 0.01 mm, respectively) was not significantly different ($F = 0.47$; df = 1,1; $P = 0.6180$ and $F = 22.67$; df = 1,1; $P = 0.1318$, respectively).

Discussion

Larval recovery, root damage (with the exception of 2007 data), and adult emergence from MIR604 did not significantly differ among egg hatch dates at all maize developmental stages evaluated, which suggests that the concentration of the mCry3A protein in MIR604 roots does not decline over the season to the point where western corn rootworm mortality is impacted. Although root damage ratings differed significantly for MIR604 plants across egg hatch dates in 2007, roots

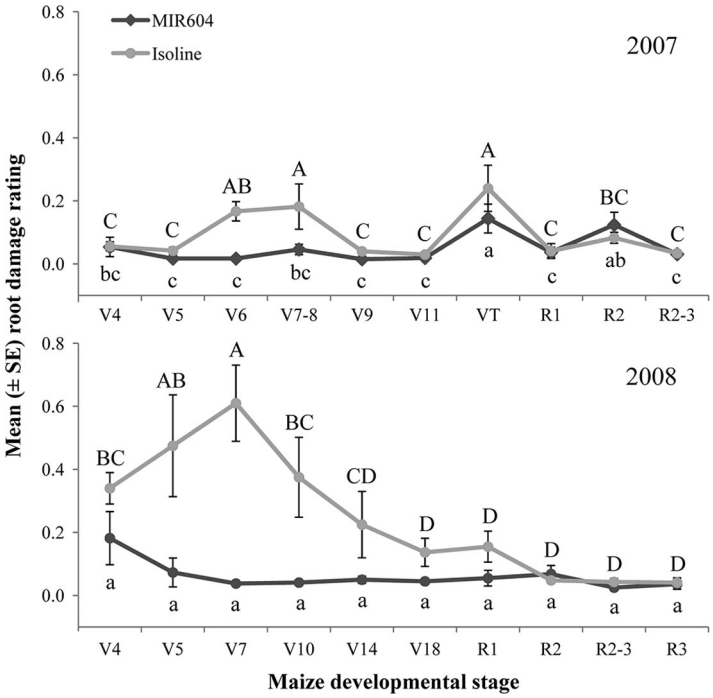

Fig. 2. Mean (±SE) root damage rating of MIR604 and isoline maize in 2007 and 2008 when infested with 800 viable western corn rootworm eggs hatching at various maize developmental stages. Means across each line followed by the same uppercase (isoline) or lowercase (MIR604) letter are not significantly different ($P > 0.05$).

never exceeded 0.18 nodes of damage for each year of the study. Damage to isoline plants in our study was lower than expected in 2007 from an infestation rate of 800 eggs per plant. Damage exceeding >0.6 nodes was reached on isoline plants in 2008, which was more consistent with the number of eggs infested. Overall, these results are consistent with Hibbard et al. (2009), which showed that maize expressing the Cry3Bb1 protein similarly maintained efficacy across a wide range of maize developmental stages. They found that the number of western corn rootworm larvae recovered, root damage, and adult emergence from MON863 was not significantly different at egg hatch dates ranging from the V3 to R3 stage. Although the mortality of western corn rootworm larvae because of feeding on MON863 was equivalent across maize developmental stages, growth of surviving larvae appeared relatively normal. Data from our study show a similar trend and are consistent on this point with data from Al-Deeb and Wilde (2005), Storer et al. (2006), Oyediran et al. (2007), and El Khishen et al. (2009). There were generally no significant differences between the size of insects (i.e., dry weight and head capsule width) recovered from MIR604 and isoline maize.

When neonate rootworm larvae locate and recognize susceptible maize roots (non-Bt), they typically orient and move toward the root tips (Strnad and Bergman 1987), where they immediately initiate feeding (Clark et al. 2006). Over the course of a growing season larvae then move into the newly developed nodal roots (Strnad and Bergman 1987). Although neonate larvae are able to initially establish on older roots, they are generally unsuitable for complete development to the adult stage (Hibbard et al. 2008). Our data from isoline maize similarly showed that fewer beetles emerged from eggs that hatched at the later developmental stages. Furthermore, root damage was also generally lowest when eggs hatched during the later maize developmental stages.

To slow resistance development of insect pests to Bt crops the U.S. Environmental Protection Agency has mandated that an insect resistance management plan must be in place before registration of any Bt product. Most insect resistance management plans for Bt crops in the United States before that of transgenic rootworm-resistant maize have focused primarily upon the use of a high-dose/refuge plan, and resistance to these crops has not been detected in field populations in the first 9 yr since registration (Tabashnik et al. 2003). For western corn rootworm, resistance to transgenic maize expressing the Cry3Bb1 (Meihls et al. 2008, Oswald et al. 2011), Cry34/35Ab1 (Lefko et al. 2008), and mCry3A (Meihls et al. 2011) proteins has been selected for in the laboratory, and there is also evidence for resistance to Cry3Bb1 that has developed in fields with three or more consecutive years of planting (Gassmann et al. 2011). The reduced levels of extractable Bt protein later in the season (Vaughn et al. 2005) has been proposed as a mechanism that could speed the development of resistance in the field. However,

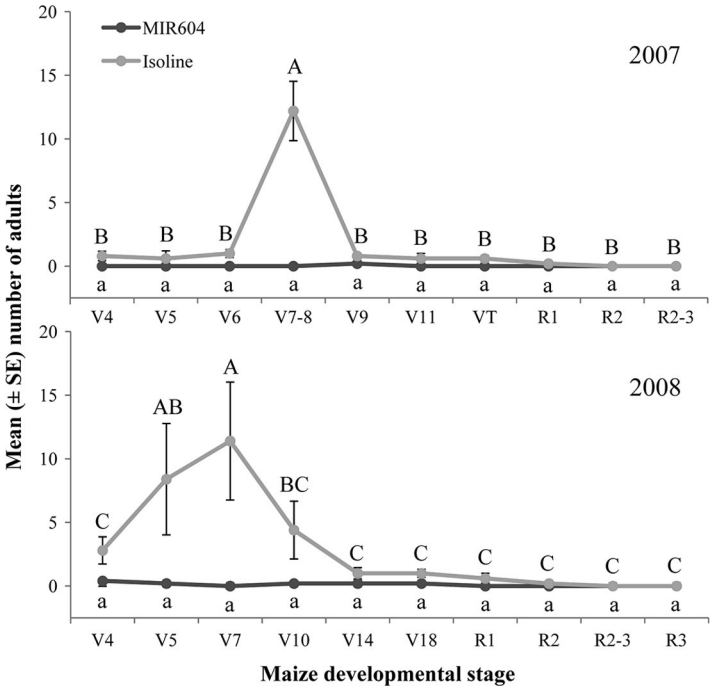

Fig. 3. Mean (±SE) number of western corn rootworm adults collected from MIR604 and isoline maize in 2007 and 2008 when infested with 800 viable western corn rootworm eggs hatching at various maize developmental stages. Means across each line followed by the same uppercase (isoline) or lowercase (MIR604) letter are not significantly different ($P > 0.05$).

because MIR604 had equal activity on western corn rootworm larvae across the wide range of maize developmental stages evaluated in the current study, if reduced levels of mCry3A occur later in the season, they are not reduced enough to affect product performance.

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