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Keywords

Disciplines

Agronomy and Crop Sciences | Ecology and Evolutionary Biology | Entomology | Plant Breeding and Genetics

Comments

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Abstract

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a serious pest of corn and is managed with corn that produces insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt). Beginning in 2009, resistance to Cry3Bb1 corn, and severe injury to Cry3Bb1 corn in the field, was observed in Iowa. However, few data exist on how Cry3Bb1-resistant western corn rootworm interact with various management practices in the field. Using a field experiment, we measured adult emergence and feeding injury to corn roots for both Cry3Bb1-resistant and Cry3Bb1-susceptible populations of western corn rootworm when tested against various Bt corn hybrids and a soil-applied insecticide. Between 2012 and 2013, we evaluated five fields that were associated with greater than one node of feeding injury to Cry3Bb1 corn by western corn rootworm (i.e., problem-field populations), and a laboratory strain that had never been exposed to Bt corn (i.e., control population). Adult emergence for western corn rootworm and root injury to corn were significantly higher in problem-field populations than control populations for both Cry3Bb1 corn and mCry3A corn. By contrast, corn with Cry34/35Ab1, either alone or pyramided with Cry3Bb1, significantly reduced adult emergence and root injury in both problem fields and control fields. In problem fields, application of the soil-applied insecticide to Cry3Bb1 corn significantly reduced root injury, but not adult emergence. Our results are discussed in terms of developing strategies for managing western corn rootworm with resistance to Cry3Bb1 and mCry3A, and delaying the additional evolution of Bt resistance by this pest.

Key words: *Diabrotica virgifera virgifera*, Bt corn, cross-resistance, insect resistance management

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a key pest of corn in the United States (Ward et al. 2005, Gray et al. 2009). Western corn rootworm larvae feed on corn roots and can cause lodging and reduce yield (Kahler et al. 1985). One node of pruned roots (i.e., 10–20 roots) causes an average yield loss of 17%, and large infestations of western corn rootworm often remove multiple nodes of roots from unprotected corn plants (Dun et al. 2010). Corn hybrids producing four types of Bt toxins (eCry3.1Ab, Cry3Bb1, mCry3A, and Cry34/35Ab1), either singly or as a pyramid, are commonly used for management of western corn rootworm (Al-Deeb and Wilde 2005, Storer et al. 2006, Hibbard et al. 2011). Pyramided Bt hybrids provide better protection of corn roots from rootworm injury than the single-trait Bt corn (Prasifka et al. 2013, Head et al. 2014). Because Bt corn hybrids provide protection against insect pests, they can reduce the use of conventional insecticides (Phipps and Park 2002, Shelton et al. 2002, Naranjo 2009).

Management of western corn rootworm has been a challenge to corn growers because of resistance to multiple pest management practices, including the use of insecticides (Meinke et al. 1998, Zhu et al. 2001), crop rotation (Levine et al. 2002), and Bt corn (Gassmann 2012, Gassmann et al. 2014). Tabashnik et al. (2014) has defined resistance as, "a genetically based decrease in susceptibility to a pesticide" and field-evolved resistance as "a genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field." Cry3Bb1 corn and mCry3A corn were registered with the United States Environmental Protection Agency (US-EPA) in 2003 and 2005, respectively (US EPA 2003, 2010a), and the area planted to Bt corn hybrids has increased over time. In response to multiple generations of selection imposed by Bt corn, some populations of western corn rootworm in Iowa and Nebraska, have evolved resistance to Cry3Bb1 and mCry3A corn (Gassmann et. al 2011, 2012, 2014; Gassmann 2012; Wangila et al. 2015). Previous experiments indicated that western corn rootworm have potential to develop resistance to individual Bt toxins (Cry3Bb1, mCry3A, Cry34/35Ab1, and eCry3.1Ab) after three to seven generations of selection in the laboratory (Lefko et al. 2008; Meihls et al. 2008, 2011; Oswald et al. 2011; Deitloff et al. 2016; Frank et al. 2013). Five insect

species [western corn rootworm, *Diabrotica virgifera virgifera* LeConte; African stem borer, *Busseola fusca* (Fuller); fall armyworm, *Spodoptera frugiperda* (J. E. Smith); bollworm, *Helicoverpa zea* (Boddie); and pink bollworm, *Pectinophora gossypiella* (Saunders)] have already developed field-evolved resistance to either Bt corn or Bt cotton (Van Rensburg 2007; Tabashnik et al. 2008, 2013; Storer et al. 2010; Dhurua and Gujar 2011; Gassmann et al. 2011). Field-evolved Bt resistance by western corn rootworm can increase the dependence of farmers on conventional insecticides, increasing the amount of insecticide released into the environment. More broadly, field-evolved Bt resistance presents a threat to the continued success of Bt crops (Tabashnik 1994, Gassmann et al. 2011, 2014).

Non-Bt refuges and pyramided hybrids are two strategies used to delay the rate at which western corn rootworm evolves Bt resistance. Refuges of non-Bt crops enable the survival of Bt-susceptible pests that may mate with Bt-resistant individuals, thus reducing the proportion of homozygous resistant individuals in a population and delaying the evolution of resistance (Gould 1998). Additionally, the high-dose-refuge strategy has been successful in managing Bt resistance in some lepidopteran pests (Huang et al. 2011). A Bt crop is defined as "high dose" if it produces either 25 times more Bt toxin than is required to kill susceptible pests or is capable of killing >99.99% of the susceptible individual in the field (US EPA 1998). If a Bt crop produces a high dose of toxin, resistance is expected to be a functionally recessive trait (Gassmann et al. 2009). However, Bt corn hybrids developed for management of western corn rootworm produce less than a high dose of Bt toxin, which increases the risk of resistance evolution (Tabashnik and Gould 2012, Devos et al. 2013, Gassmann et al. 2014). The pyramid strategy for delaying the evolution of Bt resistance in pest populations targets the same pest species with multiple Bt toxins that have different modes of action (Roush 1998). Pyramided Bt crops delay the evolution of resistance by redundant killing because individuals that are resistant to one toxin are killed by the other toxin in the pyramid (Storer et al. 2012). Currently, the US EPA has registered multiple types of pyramided corn targeting western corn rootworm. Examples include Cry34/35Ab1 pyramided with either Cry3Bb1 or mCry3A (US EPA 2011a, b).

Field-evolved Cry3Bb1 resistance by western corn rootworm was first reported in Iowa in 2011 from fields that were sampled during 2009 (Gassmann et al. 2011). Since then, the number of Cry3Bb1-resistant western corn rootworm populations in Iowa has increased over time (Gassmann et al. 2011, 2012, 2014). Additionally, cross-resistance has been documented between Cry3Bb1 corn and mCry3A corn for western corn rootworm (Gassmann et al. 2014, Wangila et al. 2015). The association between Cry3Bb1-resistant populations of western corn rootworm and severe injury to Cry3Bb1 corn in the field has been well established. However, less research has tested how Cry3Bb1-resistant populations might interact with other management strategies, and how farmers might best manage these populations. To test how Cry3Bb-resistant western corn rootworm populations interact with various approaches for larval management, we conducted a two-year field experiment comparing problem-field populations (i.e., fields associated with Cry3Bb1 resistance and more than one node of root injury to Cry3Bb1 corn from western corn rootworm) against a Bt-susceptible control populations of western corn rootworm. We measured root injury to corn and adult emergence for western corn rootworm when challenged with several management approaches including Bt corn and soil-applied insecticide. Knowledge generated from this study will be useful in characterizing

the effect of Cry3Bb1-resistance in field populations of western corn rootworm and increasing the effectiveness of management practices targeting Cry3Bb1-resistant populations.

Materials and Methods

A two-year field study was conducted during the summers of 2012 and 2013. During each year, western corn rootworm in two types of fields were evaluated: 1) problem fields, which were associated with a history of continuous corn cultivation, use of Cry3Bb1 corn, greater than one node of feeding injury to Cry3Bb1 corn, and resistance to Cry3Bb1 corn and 2) control fields, which were planted to soybean during the previous year and then artificially infested with a diapausing Bt-susceptible laboratory strain of western corn rootworm that had never been exposed to Bt corn. The Bt susceptible strain of western corn rootworm had been reared on non-Bt corn at USDA-ARS North Central Agricultural Research Laboratory (Brookings, SD) since 1987 (Kim et al. 2007). Control fields were not checked for rotation-resistant western corn rootworm because past research indicates that this rootworm variant is extremely rare in central Iowa (Dunbar and Gassmann 2013). In 2012, three problem-field populations and one control-field population were evaluated, and in 2013, two problem-field populations, and one control-field population were evaluated (Fig. 1) .

Problem fields were selected for this study based on the occurrence of greater than one node of feeding injury to Cry3Bb1 corn in a year other than the year this study was conducted. Injury to Cry3Bb1 corn was measured using methods described in Gassmann et al. (2011, 2014). Additionally, the western corn rootworm population in each problem field was tested for resistance to Cry3Bb1 corn using the single-single plant bioassay described in Gassmann et al. (2014). For each problem field population, a resistance ratio (RR) was calculated following Gassmann et al. (2014) as: corrected survival on Cry3Bb1 corn for a problem field population ÷ corrected survival on Cry3Bb1 corn for control populations. Resistance ratios greater than one indicate elevated survival on Cry3Bb1 corn for field populations compared to control populations, with severe injury to Cry3Bb1 corn in the field typically associated with resistance ratios of three or greater for on-plant survival (Andow et al. 2016).
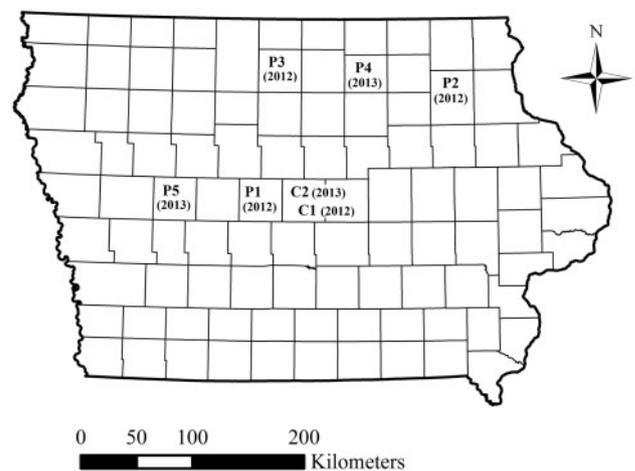

Fig. 1. Location of study sites in Iowa. P1 to P5 were problem fields, and C1 and C2 were control fields. The location of sites is accurate to the level of an individual county.

Site P1 was located on an Iowa State University research farm (Bruner Farm) and was originally intended to serve as an additional Bt-susceptible population (i.e., control field). However, during 2013 (the year after this site was studied) the northern two-thirds of the field (which was adjacent to the study site), was planted to Cry3Bb1 corn that suffered > 2.0 nodes of root injury ($N = 12$), on average, from western corn rootworm (RR = 22.7). The portion of the field adjacent to site P1 had been planted to Cry3Bb1 corn for six consecutive years at the time of this study. Consequently, site P1 was reclassified as a problem field. Problem fields P2 and P3, in this study, were the same fields studied in Gassmann et al. (2014) as sites P4 (RR = 4.5) and P1 (RR = 8.1), respectively. Site P4, in this study, was located on an Iowa State University research farm (Northeast Research and Demonstration Farm), and was the same field studied in Gassmann et al. (2011) as site P4 (RR = 2.4) and was one of two fields studied in Gassmann 2012. Site P5 had been planted to Cry3Bb1 corn for seven years prior to this study and suffered > 2.0 nodes of root injury ($N = 12$ roots) to Cry3Bb1 corn in 2012 (the year prior to this study; RR = 27.8).

At each field location, a total of nine management options (i.e., treatments) were evaluated in a randomized complete block design with four replications. The treatments tested were: 1) Cry3Bb1 corn (DKC 59-88, Monsanto, St. Louis, MO), 2) Cry3Bb1 corn with the soil-applied insecticide, tebupirimphos plus cyfluthrin, applied at a rate of 0.62 g per row meter (Aztec 2.1 G, AMVAC, New Port Beach, CA), 3) non-Bt near-isoline to Cry3Bb1 corn (DKC59-89), 4) non-Bt near-isoline to Cry3Bb1 corn with soil-applied insecticide (Aztec 2.1 G), 5) Cry34/35Ab1 corn (Mycogen 2K592, Dow Agrosciences, Indianapolis, IN), 6) corn pyramided with Cry3Bb1 and Cry34/35Ab1 (Mycogen 2K594), 7) non-Bt near-isoline to Cry34/35Ab1 corn and pyramided corn (Mycogen 2K591), 8) mCry3A corn (Agrisure H8211 3000 GT, Golden Harvest Brand, Syngenta, Research Triangle Park, NC), and 9) non-Bt near-isoline to mCry3A corn (Agrisure H-8211 GT). Soil-insecticide was only applied to Cry3Bb1 corn and its non-Bt near isoline because we were interested in testing how the application of soil insecticide to Cry3Bb1 corn affected the interaction between Cry3Bb1-resistant western corn rootworm and Cry3Bb1 corn.

At each study site, there were a total of 36 plots and each plot was 30 m long and 27 m wide. Plots consisted of four rows of corn, with plants spaced 15 cm apart within rows, and rows spaced 76 cm apart (86,487 plants per ha). Corn plants in control fields received western corn rootworm eggs from the laboratory-reared Bt-susceptible strain, Diapausing Laboratory Strain (DLS), provided by USDA-ARS North Central Agricultural Research Laboratory (Brookings, SD). When corn reached the V2 to V3 stage, western corn rootworm eggs were applied to control fields at the rate of 3,030 viable eggs per meter in 2011 and 3,411 viable eggs per meter in 2012. Egg viability was determined in the laboratory by randomly sampling 30–80 eggs and placing them on a 1.5% agar solid, held within a Petri dish (diameter = 10 cm), which was then sealed inside a plastic bag and incubated at 25°C. Petri dishes ($N = 4$) were checked daily for newly hatched larvae, which were counted and removed from the Petri dish. Egg viability was calculated as the number of neonates recovered divided by the number of eggs placed in a Petri dish. Western corn rootworm in all problem fields were naturally occurring field populations.

In each plot, Illinois-style emergence cages (Fisher 1980) were used to collect adult western corn rootworm that emerged from plants. An emergence cage covered an area of 0.48 m^2 around a single corn plant and allowed the corn plant to grow normally through an opening in the center of the cage. Cages were placed in the center two rows of each plot in June when corn plants reach the V5-V9 stage, which was before the western corn rootworm adults began emerging from the soil. In 2012, two emergence cages were placed in each plot. In 2013, three cages were placed per plot except for two plots, which only received two cages. Over the course of the study, western corn rootworm adults were collected from a total of 606 plants using Illinois-style emergence cages.

The presence or absence of rootworm-active Bt traits was confirmed for some caged plants with ELISA using a kit (EnviroLogix, Portland, ME). Adult beetles that emerged from caged plants were trapped by inverted funnels in collection cups attached to each cage. Emergence cups were collected and replaced with new cups weekly for 10–12 wks. Emergence cups containing adult beetles were brought into the laboratory. Beetles were killed by freezing at −20°C. Beetles from an individual emergence cup were then placed into a 1.5 ml microcentrifuge tube with 1 ml of 85% ethanol. Sex of western corn rootworm adults was determined by examining their basal tarsal pad under a dissecting microscope (MZ6, Leica, Microsystems, Wetzlar, Germany) following Hammack and French (2007). The number of adult male and female western corn rootworm collected over the entire season from a single caged plant were summed to provide a measure of total adult emergence for western corn rootworm per plant.

For each study site, the date of the first adult emergence was recorded as day one. In subsequent weeks, the number of days from the date of first adult emergence was used to calculate the day of emergence for each individual adult beetle. Twenty-five adult male and 25 adult female western corn rootworm were selected at random from each treatment at a study site and measured for head capsule width following Keweshan et al. (2015). When there were less than 25 adults in a treatment, all available adults were measured. A total of 2,467 adults (1,379 females and 1,088 males) were measured. Five corn roots per plot were sampled haphazardly (i.e., by chance and without bias) between 8 to 29 August and rated for rootworm feeding injury using the 0–3 node-injury scale (Oleson et al. 2005).

Data Analysis

Data on the total number of adults emerging, day of emergence, head capsule width, and root injury were analyzed with mixed model analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (SAS 2009). Data on root injury were transformed with the square root function and were analyzed with an ANOVA that included the fixed factors of population types (problem field vs. control field), management options, and their interaction, and the random factors of 1) site nested within population type, 2) block nested within site nested within population type, 3) management option crossed within site nested within population type, and 4) management option crossed with block nested within site nested within population type.

Data on adult emergence and day of adult emergence were transformed with the Log$_{10}$ (x+1) function, whereas adult size (head capsule width) data were transformed using square root function. All three variables were analyzed with mixed-model ANOVA that included the fixed factors of population type, management option, sex and all possible interactions among these three factors. Random factors included: 1) site nested within population type, 2) block nested within site nested within population types, 3) management option crossed with site nested within population types, 4) management option crossed with block nested within site nested within population types, 5) sex crossed with site nested within population types, 6) sex

crossed with block nested within site nested within population type, 7) sex by management option by site nested within population type, and 8) sex by management option by block nested within site nested within population type.

The statistical significance of random factors was tested based on the absolute difference between the -2 residual log likelihood statistic with and without a given random factor in the model, with the associated probability calculated based on a one-tail Chi-square test with one degree of freedom (Littell et al. 1996). Only random factors with a significant contribution, at alpha ≤ 0.25, were retained in the mixed-model ANOVA while nonsignificant factors were pooled to increase the statistical power (Quinn and Keough 2002). All lower order effects were retained in the model if their higher order interactions were significant.

For both root injury and adult emergence, a significant interaction was present between population type and management option. Means were compared based on least-squares means (LSMEAN) using the PDIFF option (SAS 2009). Comparison among management options were made within each population type, and these comparisons were restricted to each hybrid family (i.e., DeKalb, Mycogen and Golden Harvest) for a total of 10 pairwise comparisons within each population type. Within each population type, six pairwise mean comparisons were made among the four treatments with Dekalb hybrids (Cry3Bb1 corn with insecticide, Cry3Bb1 corn without insecticide, non-Bt corn with insecticide, and non-Bt corn without insecticide; Fig. 2A), one pairwise comparison was made between two treatments Golden Harvest hybrids (mCry3A and non-Bt

corn; Fig. 2B), and three pairwise comparisons were made among the three treatments with Mycogen hybrids (Cry34/35Ab1 corn, corn with Cry34/35Ab1 plus Cry3Bb1, and non-Bt corn; Fig. 2C). Within each population, a critical value of $P \leq 0.005$ was used based on a Bonferroni correction for 10 comparisons. Comparisons were not made between problem field and control field populations, in the ANOVA, because factors other than resistance, such as pest density, may have affected differences between field classes.

To make comparisons between control field and problem fields for adult emergence and root injury, we calculated the reduction in adult emergence and root injury for each treatment in each experimental block as Log_{10}(treatment ÷ control); where $0 =$ no difference between treatment and control; $-1 =$ a 10-fold decrease in response to a treatment; $1 =$ a 10-fold increase in response to a treatment. A t-test was used to compare a particular treatment (e.g., Cry3Bb1 corn) from problem fields ($N = 20$ blocks) against that same treatment in control fields ($N = 8$ blocks) (PROC TTEST). Homogeneity of variance between samples was determined with an F test, and when variances were unequal (1 out of 12 cases) a Satterthwaite correction was applied (PROC TTEST).

For adult size, there was a significant interaction between population type and management option (Table 1). Within both control field and problem fields, means for adult size were compared among management options within each hybrid family (10 pairwise comparisons in total for each population type; Table 2). For the adult emergence time, there was no significant interaction between population type and management option, but a significant effect of

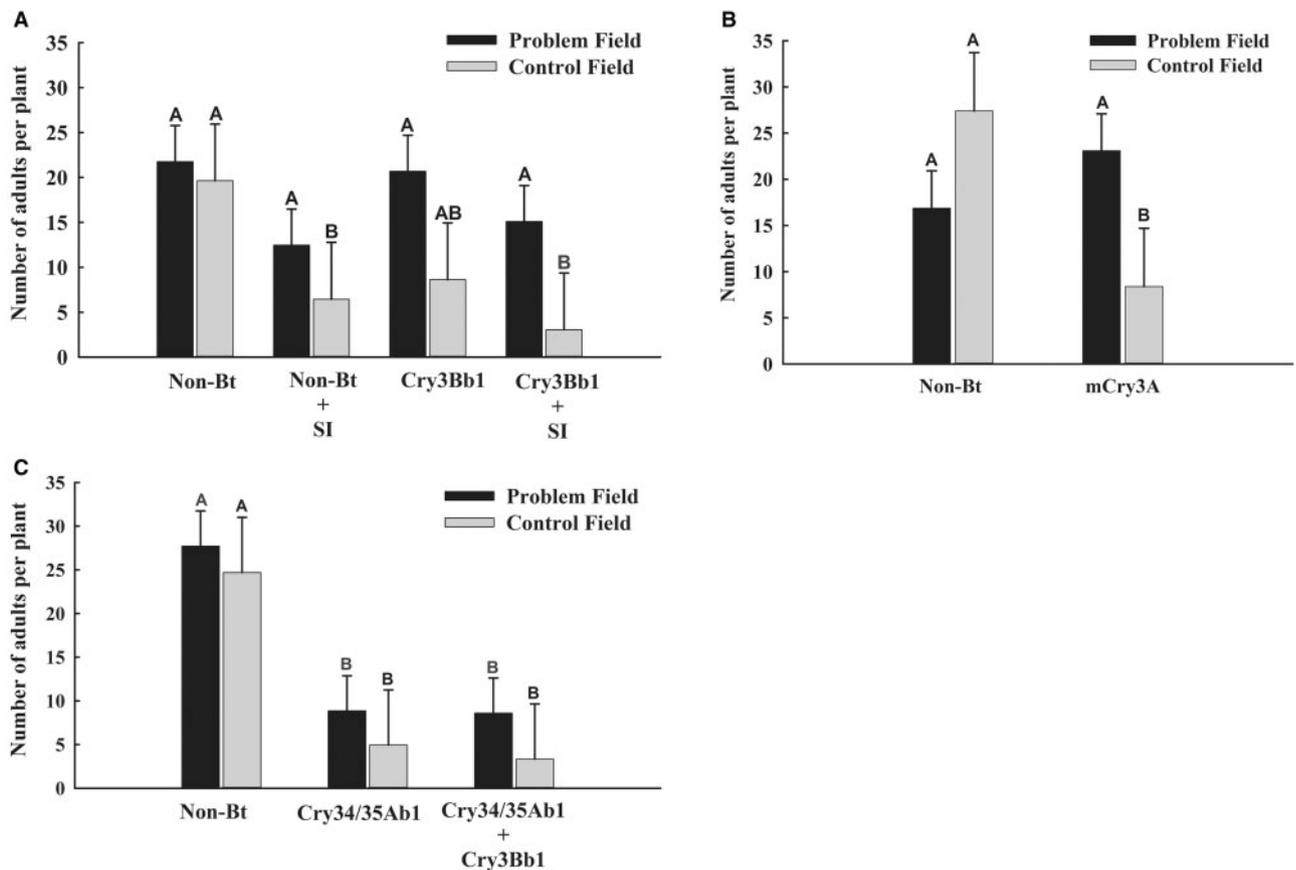**Fig. 2.** Western corn rootworm adult emergence in problem-field and control populations. Data within each graph represent corn from the same hybrid family. The presence of SI on the x-axis in 2A indicates treatments that received soil-applied insecticide at planting. Capital letters indicate significant differences between management options within a combination of population type (problem field vs. control field) by hybrid family.

Table 1. Analysis of variance for adult size and emergence time of western corn rootworm

		Adult size			Emergence time	
Fixed effects	df	F	P	df	F	P
Population type	1, 5	0.40	0.56	1, 5	0.001	0.95
Management	8, 40	2.29	0.04	8, 40	4.39	<0.01
Population type × Management	8, 40	2.33	0.04	8, 40	1.89	0.09
Sex	1, 5	10.90	0.02	1, 5	39.69	<0.01
Population type × Sex	1, 5	0.01	0.91	1, 5	1.09	0.35
Management × Sex	8, 2196	0.35	0.95	8, 40	0.94	0.49
Population type × Management × Sex	8, 2196	0.89	0.53	8, 40	1.91	0.09
Random effects	df	χ^2	P	df	χ^2	P
Site (Population type)	1	1.60	0.21	1	10.50	<0.01
Block (Site [Population type])	1	0.00	1.00	1	7.80	<0.01
Management × Site (Population type)	1	1.30	0.25	1	3.00	0.08
Management × Block (Site [Population type])	1	6.80	<0.01	1	27.40	0.00
Sex × Site (Population type)	1	23.80	0.00	1	8.90	<0.01
Sex × Block (Site [Population type])	–*	–	–	1	0.80	0.37
Sex × Management × Site (Population type)	–	–	–	1	2.60	0.11
Sex × Management × Block (Site [Population type])	–	–	–	1	2.60	0.11

* – Indicates that this random factor was not significant ($P > 0.25$) and was removed from the model.

management option was present (Table 1). Thus, mean comparisons among management options were made irrespective of the population type and were restricted to each hybrid family for a total of 10 pairwise comparisons. For both adult size and for day of adult emergence, a critical value of $P < 0.005$ was used based on a Bonferroni correction for 10 comparisons (Table 2).

Mortality of control populations of western corn rootworm caused by the single Bt traits (i.e., Cry3Bb1, mCry3A, Cry34/35Ab1 corn) and by soil-applied insecticide on non-Bt corn were calculated as $M = 1 - (E \div EI)$ where "M" is the proportional mortality caused by the trait or insecticide, "E" is an average total number of adults that emerged per plant in Bt corn or non-Bt corn with insecticide, and "EI" is the average number of adults that emerged from non-Bt isoline corn without insecticide (Table 3). We corrected for density-dependent mortality of western corn rootworm larva on non-Bt isoline corn following Hibbard et al. (2010a). Calculations of density-dependent mortality were based on a density of 924 eggs per 30.5-cm row in 2012 and 1,041 eggs per 30.5-cm row in 2013.

Results

Adult emergence for western corn rootworm was affected by a significant interaction between population type and management option (Table 4). Significantly more adults emerged from Cry3Bb1 corn in problem fields than control fields, and this effect occurred whether or not Cry3Bb1 corn was treated with soil-applied insecticide (Table 5, Fig. 2A). Adult emergence was also significantly greater on mCry3A corn in problem fields versus control fields (Table 5, Fig. 2B). Additionally, adult emergence did not differ between mCry3A corn and the non-Bt near isoline or problem-field populations, but it was significantly lower on mCry3A corn for control populations (Fig. 2B). These data indicate the presence of resistance to Cry3Bb1 corn and mCry3A corn by populations of western corn rootworm from problem fields.

By contrast, compared to the non-Bt near isoline, adult emergence was significantly lower on Cry34/35Ab1 corn and on corn containing a pyramid of Cry34/35Ab1 and Cry3Bb1 in both problem fields and control fields (Fig 2C). Additionally, there was no

difference in the reduction in adult emergence achieved with Cry34/35Ab1 corn in problem fields versus control fields (Table 5). However, the reduction in adult emergence was greater on corn pyramided with Cry34/35Ab1 and Cry3Bb1 in control fields compared to problem fields (Table 5), an effect that likely arose from resistance to Cry3Bb1 by problem-field populations.

The addition of soil insecticide to Cry3Bb1 corn and to the non-Bt isoline did not significantly reduce adult emergence in problem fields (Fig. 2A). In control fields, the application of the soil insecticide significantly reduced adult emergence from non-Bt corn but not from Cry3Bb1 corn (when compared with Cry3Bb1 corn without insecticide). These results suggest that the addition of soil-insecticide to Cry3Bb1 corn did little to reduce adult emergence. Additionally, there was a significant interaction between sex and management option (Table 4). In general, more females than males were collected, but this difference was more pronounced on Bt corn and corn with soil-applied insecticide than non-Bt corn (Table 2).

Root injury was affected by a significant interaction between population type and management options (Table 6). Cry3Bb1 corn without insecticide had significantly greater root injury in problem fields compared to control fields (Table 5, Fig. 3A). While root injury did not differ between Cry3Bb1 corn and non-Bt corn in problem fields, it was significantly lower on Cry3Bb1 corn than the non-Bt isoline corn in control fields (Fig. 3A). The addition of soil-applied insecticide significantly reduced root injury for both Cry3Bb1 corn and non-Bt corn in problem fields and for non-Bt corn in control fields. The reduction in root injury was greater in control fields than problem fields (Table 5, Fig. 3A). For mCry3A corn, root injury was significantly greater in problem fields compared to control fields (Table 5, Fig. 3B). Additionally, no significant difference in root injury was observed between mCry3A corn and non-Bt corn in problem fields, but root injury was significantly reduced on mCry3A corn in control fields (Fig. 3B).

For Cry34/35Ab1 corn and corn with Cry34/35Ab1 plus Cry3Bb1, no significant difference in root injury was found between problem fields and control fields (Table 5). In both problem fields and control fields, root injury was significantly lower for either Cry34/35Ab1 corn or Cry34/35Ab1 plus Cry3Bb1 corn than the non-Bt near-isoline.

Table 2. Adult emergence, emergence time, and head capsule width of western corn rootworm

Treatments	Sex	Adult emergence per plant	Head capsule width (μm)[a]		Emergence time (d)[b]	
		Mean ± SE (*n*)	Mean ± SE (*n*)		Mean ± SE (*n*)	
Problem fields						
Non-Bt	M	5.4 ± 0.8 (48)	1139.6 ± 6.5 (117)	A	18.7 ± 0.8 (259)	A
	F	16.3 ± 2.4 (48)	1165.3 ± 6.4 (110)		28.7 ± 0.5 (781)	
Non-Bt + SI	M	2.4 ± 0.4 (46)	1125.8 ± 7.4 (83)	A	21.7 ± 1.4 (109)	A
	F	10.2 ± 1.3 (46)	1151.7 ± 6.8 (114)		32.1 ± 0.7 (467)	
Cry3Bb1	M	4.7 ± 0.8 (48)	1129.6 ± 5.7 (115)	A	24.0 ± 0.8 (225)	A
	F	16.4 ± 2.0 (48)	1154.1 ± 6.0 (114)		31.8 ± 0.5 (786)	
Cry3Bb1 + SI	M	3.2 ± 0.6 (47)	1114.0 ± 7.4 (78)	A	24.3 ± 1.1 (158)	A
	F	12.1 ± 1.9 (47)	1159.7 ± 6.6 (116)		32.0 ± 0.6 (589)	
Non-Bt	M	4.7 ± 0.7 (46)	1117.2 ± 6.6 (96)	A	19.1 ± 0.9 (214)	A
	F	11.9 ± 1.4 (46)	1156.2 ± 7.3 (110)		27.8 ± 0.7 (546)	
mCry3A	M	5.5 ± 0.8 (48)	1142.1 ± 5.3 (119)	A	20.2 ± 0.8 (265)	A
	F	17.3 ± 2.0 (48)	1155.0 ± 6.7 (110)		27.2 ± 0.5 (828)	
Non-Bt	M	6.6 ± 0.8 (48)	1132.9 ± 5.4 (114)	A	15.8 ± 0.6 (318)	A
	F	20.3 ± 2.6 (48)	1168.9 ± 6.4 (118)		23.8 ± 0.5 (975)	
Cry34/35 Ab1	M	1.9 ± 0.3 (48)	1088.1 ± 7.2 (83)	B	23.4 ± 1.4 (90)	B
	F	6.8 ± 0.8 (48)	1115.3 ± 7.3 (99)		30.3 ± 0.8 (324)	
Cry34/35Ab1 + Cry3Bb1	M	1.3 ± 0.2 (46)	1100.2 ± 9.7 (49)	B	25.5 ± 1.7 (58)	B
	F	7.0 ± 1.3 (46)	1125.4 ± 6.9 (106)		31.8 ± 0.7 (324)	
Control fields						
Non-Bt	M	6.7 ± 1.6 (20)	1098.7 ± 8.2 (46)	A	19.4 ± 0.6 (134)	A
	F	11.9 ± 1.9 (20)	1141.3 ± 10.9 (48)		25.1 ± 0.8 (238)	
Non-Bt + SI	M	1.0 ± 0.3 (19)	1124.6 ± 18.7 (15)	A	23.4 ± 2.7 (19)	A
	F	5.4 ± 1.0 (19)	1152.5 ± 8.8 (42)		29.5 ± 1.2 (102)	
Cry3Bb1	M	1.1 ± 0.3 (20)	1123.8 ± 15.3 (17)	A	21.1 ± 2.9 (22)	A
	F	7.3 ± 1.6 (20)	1137.6 ± 9.5 (43)		30.7 ± 1.3 (145)	
Cry3Bb1 + SI	M	0.5 ± 0.2 (20)	1108.6 ± 19.5 (8)	A	33.0 ± 2.9 (9)	A
	F	2.5 ± 0.6 (20)	1152.1 ± 13.3 (36)		32.2 ± 2.4 (49)	
Non-Bt	M	7.4 ± 1.3 (20)	1106.8 ± 9.8 (46)	A	19.2 ± 0.8 (147)	A
	F	19.7 ± 3.6 (20)	1118.2 ± 13.5 (44)		23.8 ± 0.6 (393)	
mCry3A	M	2.0 ± 0.5 (20)	1113.5 ± 9.8 (35)	A	25.4 ± 2.2 (40)	A
	F	6.3 ± 1.8 (20)	1147.8 ± 10.7 (45)		35.3 ± 1.2 (126)	
Non-Bt	M	9.7 ± 2.4 (20)	1097.0 ± 8.9 (41)	A	17.5 ± 0.5 (194)	A
	F	14.3 ± 2.9 (20)	1120.6 ± 9.4 (47)		26.0 ± 0.7 (286)	
Cry34/35 Ab1	M	1.1 ± 0.3 (20)	1110.8 ± 16.4 (18)	A	20.7 ± 2.3 (22)	B
	F	3.9 ± 0.7 (20)	1132.1 ± 12.4 (38)		34.1 ± 1.8 (78)	
Cry34/35Ab1 + Cry3Bb1	M	0.7 ± 0.2 (19)	1102.7 ± 34.7 (9)	A	24.4 ± 3.5 (14)	B
	F	2.4 ± 0.5 (19)	1155.3 ± 14.8 (39)		30.5 ± 2.3 (46)	

[a]Capital letters indicate significant differences between management options within a hybrid family for a particular population type irrespective of sex.

[b]Capital letters indicate significant differences between management options within a hybrid family, irrespective of sex or population type.

Table 3. Control population mortality caused by Bt traits and insecticides

Parameters	Traits			
	Cry3Bb1	mCry3A	Cry34/35Ab1	Non-Bt isoline + Aztec
Number of plants caged	20	20	20	19
Adults emerged from trait (per plant)	8.35 ± 1.74	8.30 ± 2.21	5.00 ± 0.85	6.37 ± 1.25
Adults emerged from non-Bt isoline (per plant)	18.60 ± 2.81	27.00 ± 2.93	24.01 ± 3.38	18.52 ± 2.96
Trait mortality (proportion)[a]	0.55	0.69	0.79	0.66
Predicted adult emergence in non-Bt isoline (per plant)[b]	24.55 ± 2.70	32.95 ± 2.90	29.95 ± 3.32	24.44 ± 2.84
Adjusted trait mortality (proportion)[c]	0.66	0.75	0.83	0.74

[a]Proportion mortality = 1 − (adult emergence in a trait ÷ adult emergence in non-Bt isoline).

[b]Number of adults that would have emerged in non-Bt isoline if there was no density-dependent mortality, estimation based on Hibbard et al. 2010; 1.059 and 1.281% more adults would have survived in non-Bt isolines in 2012 and 2013, respectively, if there was no density-dependent mortality.

[c]Adjusted trait mortality, trait mortality adjusted for density-dependent mortality in non-Bt isolines = 1 − (adult emergence in trait ÷ predicted adult emergence in non-Bt isoline).

The timing of adult emergence was affected significantly by sex and management option, but population type did not have an effect on emergence time (Table 1). In general, male beetles emerged significantly earlier (20.2 ± 0.25 d after first adult emergence, mean $\pm$ SE) than females (28.6 ± 0.17 d after first adult emergence). Regardless of sex and population type, western corn rootworm adults showed significantly delayed emergence on plots with Cry34/35Ab1 corn and corn pyramided with Cry34/35Ab1 plus Cry3Bb1 compared to the non-Bt near isoline (Table 2). The day of adult emergence was similar between Cry34/35Ab1 corn and corn with Cry34/35Ab1 plus Cry3Bb1.

There was a significant effect of sex on adult size as measured by adult head capsule width (Table 1). Females were significantly larger (head capsule width = $1,147 \pm 2.0 \, \mu m$) than males ($1,121 \pm 2.0 \, \mu m$). There was no interaction between sex and population type or management options. However, we found a significant interaction between population type and management options for adult size (Table 1). For problem-field populations, the adults that emerged from Cry34/35Ab1 corn and corn pyramided with Cry34/35Ab1

plus Cry3Bb1 were significantly smaller than adults from the non-Bt near isoline. However, in control populations, the size of adults did not differ among non-Bt corn, Cry34/35Ab1 corn, and corn pyramided with Cry34/35Ab1 and Cry3Bb1corn.

For control populations of the western corn rootworm, which had never been exposed to Bt toxin, the mortality from single-trait Bt corn hybrids ranged from 66 to 83%, which was similar to the level of mortality for non-Bt corn treated with soil-applied insecticide (Table 3).

Discussion

We found that, on both Cry3Bb1 corn and mCry3A corn, adult emergence for western corn rootworm was significantly greater for problem-field populations than Bt-susceptible control populations (Table 5; Fig. 2). Additionally, in problem fields, adult emergence on Cry3Bb1 corn and mCry3A corn was similar to the corresponding non-Bt isoline corn. These results indicate that problem-field populations were resistant to both Cry3Bb1 corn and mCry3A corn and had developed cross-resistance between Cry3Bb1 and mCry3A. Problem-field populations caused significantly higher levels of root injury than control populations to Cry3Bb1 corn and mCry3A corn, and root injury to Cry3Bb1 corn and mCry3A corn was similar to

Table 4. Analysis of variance for adult emergence by western corn rootworm

Fixed effects	df	F	P
Population type	1, 5	1.38	0.29
Management	8, 40	16.01	<0.01
Sex	1, 5	68.89	<0.01
Population type × Management	8, 40	4.01	<0.01
Population type × Sex	1, 5	1.28	0.31
Management × Sex	8,932	2.09	0.03
Population type × Management × Sex	8, 932	1.14	0.33
Random effects	**df**	**χ^2**	**P**
Site (Population type)	1	3.90	0.05
Block (Site (Population type))	1	6.70	0.01
Management × Site (Population type)	1	1.40	0.24
Management × Block (Site (Population type))	1	86.8	<0.01
Sex × Site (Population type)	1	20.30	<0.01

Table 6. Analysis of variance for corn root injury caused by western corn rootworm

Fixed effects	df	F	P
Population type	1, 5	0.20	0.67
Management	8, 40	32.23	<0.01
Population type × Management	8, 40	7.73	<0.01
Random effects	**df**	**χ^2**	**P**
Site (Population type)	1	17.2	<0.01
Block (Site (Population type))	1	2.2	<0.13
Management × Site (Population type)	1	4.5	<0.03
Management × Block (Site (Population type))	1	126.4	<0.01

Table 5. Effect of management treatments on emergence of adult western corn rootworm and root injury

Treatments	Adult emergence		Root injury	
	Control field	Problem field	Control field	Problem field
Non-Bt+SI	-0.47 ± 0.11 (df = 26, t = 1.30, P = 0.20)	-0.20 ± 0.12	-0.89 ± 0.09 (df = 26, t = 2.21, P = 0.04)	-0.54 ± 0.09
Cry3Bb1	-0.35 ± 0.13 (df = 26, t = 2.81, P < 0.01)	0.06 ± 0.08	-1.15 ± 0.11 (df = 26, t = 6.90, P < 0.01)	-0.10 ± 0.09
Cry3Bb1+SI	-0.80 ± 0.15 (df = 26, t = 3.44, P < 0.01)	-0.19 ± 0.10	-1.23 ± 0.05 (df = 25.6, t = 5.85, P < 0.01)*	-0.57 ± 0.10
Cry34/35Ab1	-0.75 ± 0.12 (df = 26, t = 1.84, P = 0.08)	-0.44 ± 0.09	-0.41 ± 0.08 (df = 26, t = 0.06, P = 0.95)	-0.41 ± 0.09
Cry35/35Ab1+Cry3Bb1	-0.94 ± 0.13 (df = 26, t = 2.36, P = 0.03)	-0.53 ± 0.10	-0.96 ± 0.09 (df = 26, t = 1.47, P = 0.15)	-0.72 ± 0.10
mCry3A	-0.57 ± 0.15 (df = 25, t = 4.78, P < 0.01)	0.12 ± 0.07	-1.05 ± 0.07 (df = 26, t = 6.26, P < 0.01)	-0.10 ± 0.09

Data are presented as mean $\pm$ SE for $\log_{10}$(treatment÷control), with 0 = no difference between treatment and control; -1 = a 10-fold decrease in response to a treatment; 1 = a 10-fold increase in response to a treatment. Each block represents one replicate for each of the treatments. See Materials and Methods for additional details on these calculations.

Comparisons between means for problem fields and control field are based on a two tailed t-test with equal variance, except when indicated by an asterisks (*) where we used Satterthwaite correction due to unequal variances.
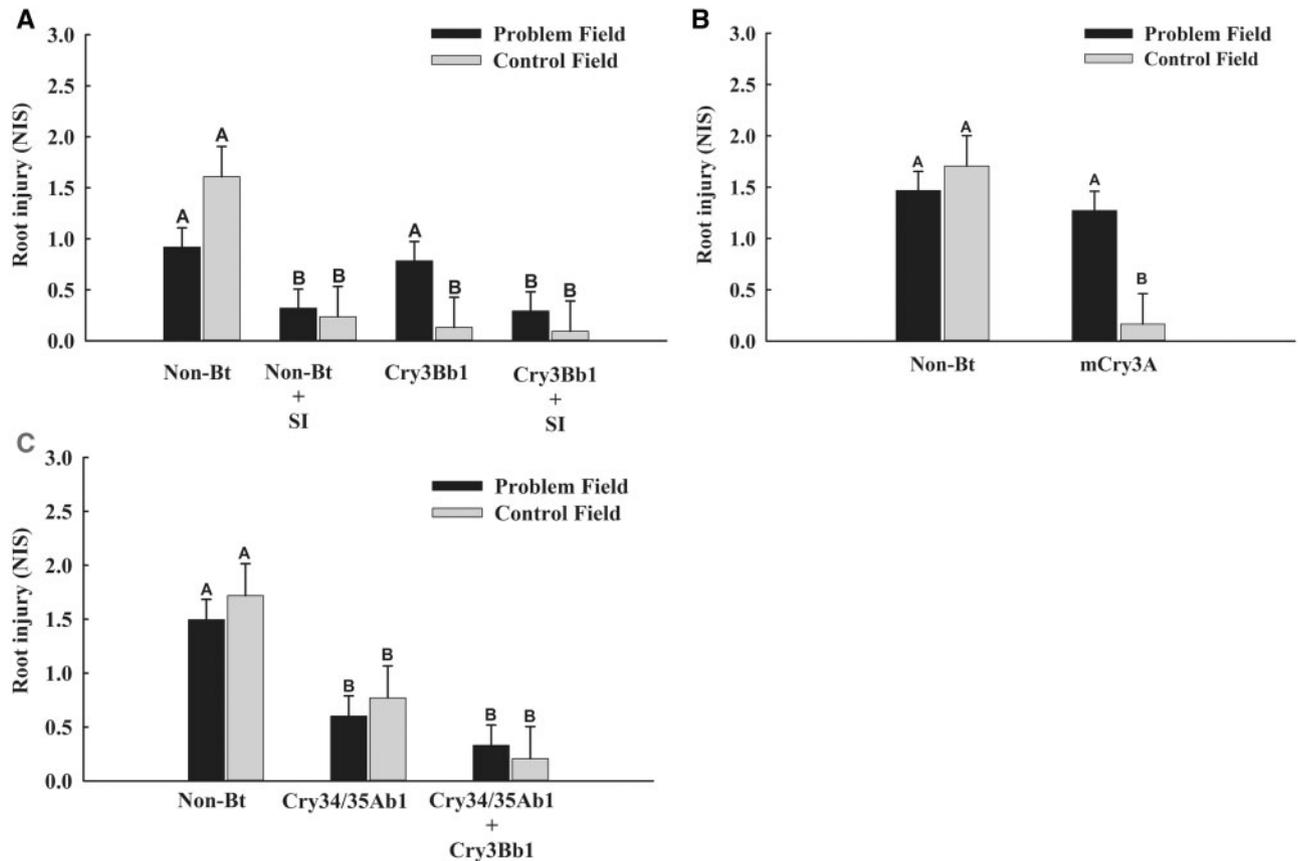
Fig. 3. Root injury caused by problem-field and control populations of western corn rootworm. Data within each graph represent corn hybrids from the same hybrid family. The presence of SI on the x-axis in 3A indicates treatments that received soil-applied insecticide at planting. Capital letters indicate significant differences between management options within a combination of population type (problem field vs. control field) by hybrid family.

the non-Bt near isolines in problem fields (Table 5; Fig. 3). By contrast, Cry34/35Ab1 corn (both as a single trait and pyramided with Cry3Bb1) significantly reduced root injury and adult emergence, compared to non-Bt corn, in both problem fields and control fields. In problem fields, the application of soil insecticide to Cry3Bb1 corn did not reduce the adult emergence, but it significantly reduced root injury.

In this study, we have characterized Bt resistance by western corn rootworm with a field-based comparison of Bt-resistant problem-field populations and known Bt-susceptible control populations. Researchers have previously characterized Bt resistance by using plant-based laboratory bioassays, and these bioassays have documented resistance to Cry3Bb1 corn and mCry3A corn, and cross-resistance between Cry3Bb1 and mCry3A by western corn rootworm in Iowa and Nebraska (Gassmann et al. 2011, 2012, 2014; Wangila et al. 2015). This study provided further evidence of the evolution of resistance and cross-resistance to Cry3Bb1 and mCry3A corn by western corn rootworm.

In a field study with Cry3Bb1-resistant western corn rootworm, Gassmann (2012) documented a significant reduction in western corn rootworm adult emergence and root injury with the application of soil insecticide to Cry3Bb1 corn, whereas in a study of Cry3Bb1-susceptibe western corn rootworm, Petzold-Maxwell et al. (2013a) found no effect of soil insecticide on root injury to Cry3Bb1 corn but significant reduction in adult emergence. In our study, the application of soil insecticide to Cry3Bb1 corn significantly reduced root injury by western corn rootworm; however, it did not reduce the adult emergence in problem fields (Figs. 2 and 3). This result

suggests that the application of soil insecticide on Cry3Bb1 corn in problem fields can serve as a short-term mitigation of root injury, thereby reducing yield loss. However, because adult emergence in problem-field populations was not reduced by the application of soil-applied insecticide to Cry3Bb1 corn, this management strategy will not diminish the population size of western corn rootworm in fields with Cry3Bb1 resistance. Consequently, approaches to manage Cry3Bb1-resistant western corn rootworm should include crop rotation, planting of pyramided corn with Cry34/35Ab1, planting of non-Bt corn with soil-applied insecticide, or adult management with insecticides.

The larval mortality in control fields due to Cry3Bb1 corn, mCry3A corn, and Cry34/35Ab1 corn was less than 84% (Table 3). This level of mortality is less than some previous reports (Clark et al. 2012, Hibbard et al. 2010b, Storer et al. 2006), but similar to other studies (Petzold-Maxwell et al. 2013a b). The level of mortality found here is further evidence that Bt corn hybrids achieve less than the 99.99% mortality that is needed for a Bt trait to be considered high dose against a target pest (US EPA 1998). High-dose traits can kill both homozygotes susceptible and heterozygote resistant individuals, causing resistance to become functionally recessive trait (Tabashnik et al. 2004). Use of high-dose traits along with refuges is one IRM strategy for delaying Bt resistance. The lack of a high dose by Bt corn targeting corn rootworm has likely facilitated the evolution of Bt resistance by this pest (Tabashnik and Gould 2012, Devos et al. 2013, Gassmann et al. 2014). However, it is important to note that during both years of this study Iowa experienced drought conditions (Northey 2013, Daigh et al. 2014), which may have

increased survival of western corn rootworm larvae by reducing mortality of rootworm larvae from saturated soils (Riedell and Sutter 1995), and decreasing the virulence of entomopathogens (Grant and Villani 2003). Drought stress can reduce the amount of Bt toxin produced by plants (Rochester 2006, Martins et al. 2008), which may increase injury by insect pests to Bt plants (Brewer et al. 2014). However Gulli et al. (2014) found no effect of drought stress on the level of expression of Cry1Ab transcript.

The sublethal effects of Bt crops can influence the growth, development, and reproduction of insect pests (Stewart et al. 2001, Binning et al. 2010, Murphy et al. 2011). The presence of sublethal effects of a Bt trait can also delay the evolution of Bt resistance (Tabashnik et al. 2004). We found sublethal effects for adult emergence time and adult size on Cry34/35Ab1, and sublethal effects arose in both problem-field populations and control populations. However, sublethal effects appeared to be absent for Cry3Bb1 corn and mCry3A corn in this study, and this was the case both populations from problem fields and control fields.

In this study, the Bt-resistant problem-field populations and known Bt-susceptible control populations were compared in the experimental field setting. We found evidence for resistance and cross-resistance to Cry3Bb1 corn and mCry3A corn by western corn rootworm in Iowa. Field-evolved Bt resistance by western corn rootworm has posed a significant management challenge to some farmers and threatens the utility of Bt corn as a management tool for western corn rootworm. The development of transgenic Bt crops is an expensive and time-consuming process. Therefore, maintaining the efficacy of currently available Bt traits should be a high priority. Continuous monitoring and characterization of Bt resistance in the field is an important component of an insect resistance management plan. Sustainable management of western corn rootworm should focus on an integrated approach that includes crop rotation, pyramided hybrids that contain Cry34/35Ab1, and the use of non-Bt corn with soil-applied insecticides.

Acknowledgments

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