

Western corn rootworm and Bt maize

Challenges of pest resistance in the field

Aaron J. Gassmann,* Jennifer L. Petzold-Maxwell, Ryan S. Keweshan and Mike W. Dunbar

Department of Entomology; Iowa State University; Ames, IA USA

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Crops genetically engineered to produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) manage many key insect pests while reducing the use of conventional insecticides. One of the primary pests targeted by Bt maize in the United States is the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Beginning in 2009, populations of western corn rootworm were identified in Iowa, USA that imposed severe root injury to Cry3Bb1 maize. Subsequent laboratory bioassays revealed that these populations were resistant to Cry3Bb1 maize, with survival on Cry3Bb1 maize that was three times higher than populations not associated with such injury. Here we report the results of research that began in 2010 when western corn rootworm were sampled from 14 fields in Iowa, half of which had root injury to Cry3Bb1 maize of greater than 1 node. Of these samples, sufficient eggs were collected to conduct bioassays on seven populations. Laboratory bioassays revealed that these 2010 populations had survival on Cry3Bb1 maize that was 11 times higher and significantly greater than that of control populations, which were brought into the laboratory prior to the commercialization of Bt maize for control of corn rootworm. Additionally, the developmental delays observed for control populations on Cry3Bb1 maize were greatly diminished for 2010 populations. All 2010 populations evaluated in bioassays came from fields with a history of continuous maize production and between 3 and 7 y of Cry3Bb1 maize cultivation. Resistance to Cry34/35Ab1 maize was not detected and there was no correlation between survival on Cry3Bb1 maize and Cry34/35Ab1 maize, suggesting a lack of cross resistance between these Bt toxins. Effectively dealing with the challenge of field-evolved resistance to Bt maize by western corn rootworm will require better adherence to the principles of integrated pest management.

Introduction

The area planted to transgenic crops that produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) has grown rapidly since their commercialization in 1996.¹ Between 2004 and 2010, the area planted to Bt crops more than doubled, increasing from 23 million hectares to 58 million hectares.^{1,2} Bt crops provide effective control of many key insect pests, and have additional benefits that include reductions in the use of conventional insecticides and regional suppression of pests.³⁻⁷ The widespread planting of Bt crops imposes intense selective pressure for pests to evolve resistance, and the evolution of Bt resistance could diminish the benefits these crops provide. Although the majority of insects targeted by Bt crops remain susceptible, several cases of field-evolved resistance to Bt toxins have been documented.⁸⁻¹⁴ Insect resistance management strategies, including the use of refuges and high-dose events, can act to delay resistance.^{9,15-18}

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is among the most serious pests of maize in the United States and has repeatedly demonstrated its ability to invade new habitats and to develop resistance to pest management practices.^{19,20} The larvae of this univoltine pest feed on the roots of

maize causing structural damage and interfering with the acquisition of water and nitrogen.^{21,22} Maize plants that are heavily injured by western corn rootworm often lodge (i.e., list) within their rows, which complicates harvest.²² Although native to the United States, the western corn rootworm was restricted to a small portion of the western United States until the 1940s when it expanded its range eastward, reaching the East Coast in the 1990s.¹⁹ In the 1990s this pest also became established in Europe and has since colonized maize fields in several countries.^{19,23,24}

Various practices have been applied to manage western corn rootworm including crop rotation (i.e., rotating fields out of maize production for at least one season), insecticide application to the soil to manage larvae, and aerial application of insecticides to manage adults.²² Importantly, cases exist in which the western corn rootworm has developed resistance to all of these tactics.²⁵⁻²⁸ For example, western corn rootworm colonized eastern Illinois in the mid-1960s and by the mid-1980s resistance to crop rotation was reported in this area.^{19,25} In Nebraska, western corn rootworm was managed through aerial application of the insecticide methyl-parathion, which was a favored management tactic due in part to resistance of western corn rootworm to soil-applied insecticides.²⁷ Adult management with methyl-parathion subsequently

*Correspondence to: Aaron Gassmann; Email: aaronjg@iastate.edu
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Table 1. Fields visited in Iowa in 2010

Mean Root Injury	Total Fields Visited	Total Populations in Bioassay
Cry3Bb1 maize		
< 0.5	2	1
0.5 to 1.0	3	0
> 1.0	7	4
Cry34/35Ab1 maize		
< 0.5	1	1
Pyramided maize^a		
< 0.1	1	1
Total	14	7

^aPyramided maize produces both Cry34/35Ab1 and Cry3Bb1.

caused the evolution of esterase-mediated resistance in some populations.^{27,29} Cases of resistance by western corn rootworm to crop rotation and to conventional insecticides raise concerns about resistance to other management tactics including Bt maize.

Evolution of resistance is not unique to western corn rootworm, but rather the western corn rootworm exemplifies a broader pattern among arthropods in which pests respond to the application of insecticides, and other management practices, by developing resistance.³⁰ Any pest management practice that imposes mortality on a pest population will select for resistant genotypes, and in some cases pest susceptibility may be viewed as a non-renewable resource that is expended in the process of managing the pest population. However, the application of insect resistance management can delay, and in some cases may prevent, the evolution of pest resistance.^{31,32}

For Bt crops, pest resistance is managed through the refuge strategy.^{15,33} Refuges are especially effective when they are coupled with either high-dose events or pyramided events.^{18,34} The theory behind the refuge strategy predates the development of Bt crops.^{16,17} However, Bt crops provide options that were not possible with conventional insecticides, such as a consistent high dose of toxin.³⁵ The refuge strategy uses refuges of non-Bt host plants as a source of Bt-susceptible pests that can mate with any resistant pests that develop on Bt crops. The heterozygous progeny of such matings are expected to have lower fitness on the Bt crop than their resistant parent, and it is this reduction in fitness that helps to delay resistance.

Both the inheritance of resistance and the availability of individuals from refuges are critical factors in determining how quickly populations will develop resistance.^{9,36-38} As the recessive nature of a resistance trait increases, the delays in resistance expected under the refuge strategy become greater, with the longest delays expected for resistance traits that are completely recessive.¹⁸ The genetic inheritance of a resistance trait can be predicted based on mortality of susceptible genotypes on a transgenic crop.^{15,18} In cases where Bt crops produce 25 times more toxin than is needed to kill a susceptible pest and the Bt crop kills greater than 99.99% of susceptible individuals, the crop is termed “high dose” and resistance is expected to be recessive.³⁹ As the

survival of susceptible pests on a Bt crop increases, resistance is expected to be more additive in nature, and at a sufficiently high level of survival, resistance may be dominant. In the case of western corn rootworm, experiments in the laboratory and field found that Bt maize kills less than 99.99% of susceptible western corn rootworm, and thus, resistance to Bt maize by the western corn rootworm is not expected to be a recessive trait.⁴⁰⁻⁴⁶ Furthermore, genetic analysis of laboratory strains that were resistant and susceptible to Cry3Bb1 maize revealed that resistance was not recessive.⁴⁵ The lack of recessive inheritance for resistance traits is expected to increase the risk of resistance in the field.

A second factor that will affect resistance evolution is the availability of insects from refuges to mate with insects that experience selection on a Bt crop. For western corn rootworm, both the willingness of farmers to plant refuges and the patterns of pest movement increase the risk of this pest developing resistance to Bt maize. A survey of farmers found that approximately 25% were not in compliance with EPA regulations in terms of either the size or location of refuges.⁴⁷ Furthermore, the western corn rootworm has low rates of in-field dispersal. Measurements of in-field movement suggest that adult western corn rootworm typically disperse less than 40 m per day.^{21,48} In cases where large fields are planted to Bt maize, the dispersal of insects from a block (i.e., structured) refuge is likely to be uneven with a higher density of insects near the refuge, thus diminishing the effectiveness of a block refuge.

In 2009, Iowa populations of western corn rootworm were found that had evolved resistance to Cry3Bb1 maize in the field.¹³ Western corn rootworm were sampled from fields that had severe root injury to Cry3Bb1 maize. Furthermore, these populations were sampled from fields that had a history of continuous maize cultivation, and a history of cultivation of Cry3Bb1 maize. In all fields sampled, the type of Bt maize planted produced Cry3Bb1 and fields had been planted to this type of Bt maize for at least three, and as many as six, consecutive seasons. Rootworm populations from these problem fields had three times higher survival on Cry3Bb1 maize in laboratory bioassays compared with control fields, which were not associated with management problems.¹³ Subsequent field experiments in two of these problem fields found no difference in survival of western corn rootworm between Cry3Bb1 maize and non-Bt maize, and higher root injury to Cry3Bb1 maize than any other treatment tested except maize that had no protection against larval rootworm.⁴⁹

Here we report the results of field visits conducted in Iowa during 2010 and of subsequent laboratory bioassays. Similar to data from 2009, we visited fields in response to notification from growers, assessed the type of maize and level of root injury, and sampled western corn rootworm adults from these fields to generate eggs for laboratory bioassays. The results of this study highlight the need to incorporate Bt maize into a broader integrated pest management strategy in order to preserve the efficacy of this technology and its associated benefits.

Results

In 2010, we visited 14 fields (Table 1). With the exception of S3, we visited these fields in response to notifications from

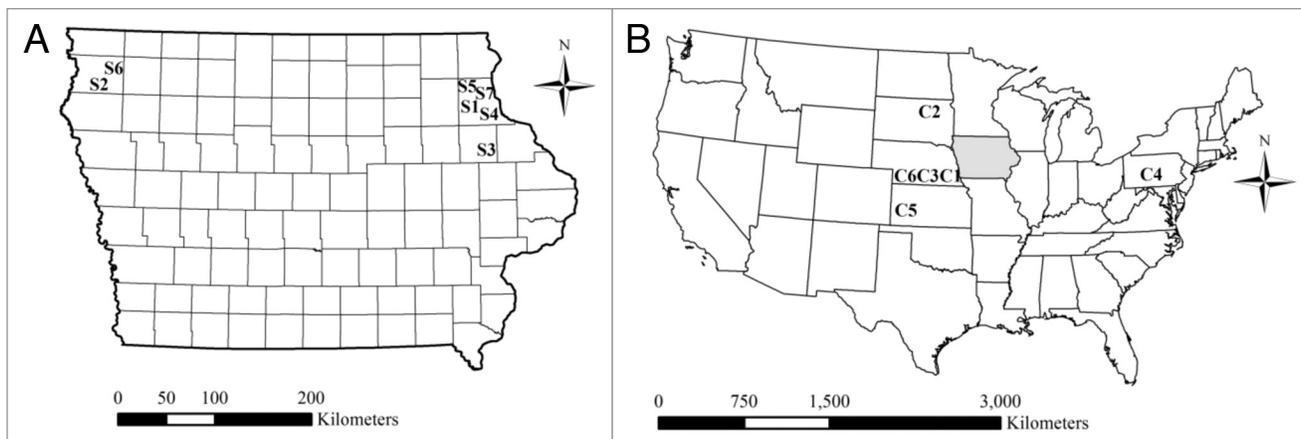


Figure 1. Distribution of (A) fields sampled in Iowa for use in bioassays and (B) control sites within the United States. For (B), Iowa is highlighted in gray.

Table 2. Detailed field history for populations used in bioassays in 2010^{ab}

Site	Year							
	2003	2004	2005	2006	2007	2008	2009	2010
S1	4 or 5 (?)	4	4	4	1	1	1	1 & 3
S2	1 or 4 (?)	1 or 4 (?)	1 or 4 (?)	1 & 2	1 & 2	1 & 2	2	2
S3	4	1	1	1	1	1	1	1 & 3
S4	unknown	unknown	unknown	unknown	1	1	1	1
S5	4	4	1	1	4	4	1	1
S6	4	1	1	1	1	1	1	1 & 3
S7	unknown	unknown	unknown	5 (?)	1	1	1	1

^aField history indicates the crop planted in a field each year. 1 = Cry3Bb1 maize, 2 = Cry34/35Ab1 maize, 3 = maize producing both Cry3Bb1 and Cry34/35Ab1, 4 = maize lacking rootworm active Bt, 5 = soybean. A field was only coded as “4 non-Bt maize” or “5 soybean” if the entire field was planted to non-Bt maize or soybean, respectively. ^bunknown = no data are available for the type of crop planted; (?) with “or” means there was uncertainty about which of two crops was planted; (?) alone means there was uncertainty about whether or not a specific crop was planted.

farmers and crop consultants of rootworm injury to Bt maize in 2010. Seven of the 2010 populations had evidence of severe rootworm feeding, with greater than 1 node of roots injured per plant, while the other seven fields had lower levels of root injury (Table 1).

From the 14 fields visited in 2010, we were able to obtain sufficient eggs to run bioassays for seven populations (Fig. 1A and Tables 2 and 3). All seven of these fields had a history of continuous maize cultivation, with only two of the seven fields planted to soybeans since 2003, and at most only 1 y of soybeans grown in either of these two fields (Tables 2 and 3). Fields sampled in 2010 had been planted to Cry3Bb1 maize for at least 3 y and up to 7 y (Tables 2 and 3). Six of the seven fields were planted to Cry3Bb1 maize in 2010 (Table 2) and injury to Cry3Bb1 maize among these fields ranged from 0.48 to 2.57 nodes (Table 3). Field S2 was planted to Cry34/35Ab1 maize in 2010 and had an average node injury of 0.45; this field also had a history of 5 y of Cry34/35Ab1 maize along with 3 to 6 y of Cry3Bb1 maize (Table 2 and 3). For field S3, which corresponded to field P1 in Gassmann et al.¹³ we sampled roots of maize pyramided with

Cry3Bb1 and Cry34/35Ab1, and observed an average root injury score of 0.02.

For survival on Cry3Bb1 maize, there was a significant interaction between maize type and population type ($f = 44.83$; $df = 1.11$; $p < 0.0001$) (Fig. 2A). No difference in survival was present between control populations and 2010 populations on non-Bt maize. However, on Cry3Bb1 maize, survival was 11 times higher and significantly greater for 2010 populations than control populations (survival on Cry3Bb1 maize for 2010 populations \div survival on Cry3Bb1 maize for control populations = $0.2680 \div 0.0234 = 11.4$) (Fig. 2A). Additionally, we did not detect a significant difference for survival of 2010 populations on non-Bt maize and Cry3Bb1 maize (Fig. 2A).

A significant interaction also was present for survival on Cry34/35Ab1 ($f = 5.15$; $df = 1.11$; $p = 0.04$). However, the pattern of survival was substantially different from Cry3Bb1 maize (Fig. 2B). Survival on Cry34/35Ab1 maize did not differ between 2010 populations and control populations, and for both population types, survival was significantly lower on Cry34/35Ab1 maize than on non-Bt maize. On non-Bt maize survival was

Table 3. Date sampled, root injury, corrected survival and field history for 2010 populations

Site	Date Sampled	Maize Type ^a	Root Injury ^b	Corrected Survival ^b		Field History ^{c,d}				
				Cry3Bb1	Cry34/35Ab1	1	2	3	4	5
S1 ^e	03 Aug	1	1.21 ± 0.60	0.96 ± 0.11	0.13 ± 0.09	4	0	1	3–4	0–1
S2 ^e	10 Aug	2	0.45 ± 0.06	0.89 ± 0.14	0.00 ± NA	3–6	5	0	0–3	0
S3	03 Aug	3	0.02 ± 0.01	0.83 ± 0.11	0.12 ± 0.06	7	0	1	1	0
S4 ^f	03 Aug	1	0.48 ± 0.21	0.79 ± 0.10	0.08 ± 0.03	4	0	0	0	0
S5	03 Aug	1	1.10 ± 0.26	0.65 ± 0.12	0.12 ± 0.05	4	0	0	4	0
S6	10 Aug	1	2.57 ± 0.09	0.60 ± 0.13	0.00 ± NA	7	0	1	1	0
S7 ^g	03 Aug	1	1.22 ± 0.23	0.51 ± 0.12	0.04 ± 0.03	4	0	0	0	0–1
Mean ± SE				0.74 ± 0.06	0.07 ± 0.02					

^aType of maize evaluated for root injury. 1 = Cry3Bb1 maize, 2 = Cry34/35Ab1 maize, 3 = maize producing both Cry3Bb1 and Cry34/35Ab1. ^bMean ± Standard Error. ^cField history indicates the number of field years a certain crop was planted. In some years more than one type of Bt maize was planted and both types were scored as being in the field for one year. 1 = Cry3Bb1 maize, 2 = Cry34/35Ab1 maize, 3 = maize producing both Cry3Bb1 and Cry34/35Ab1, 4 = maize lacking rootworm active Bt, 5 = soybean. ^dA field was only coded as "4 non-Bt maize" or "5 soybean" if the entire field was planted to non-Bt maize or soybean, respectively. ^eThere was some uncertainty regarding field history and this is reflected in the range of values. ^fField history was only available from 2007 to 2010. ^gField history was only available from 2006 to 2010.

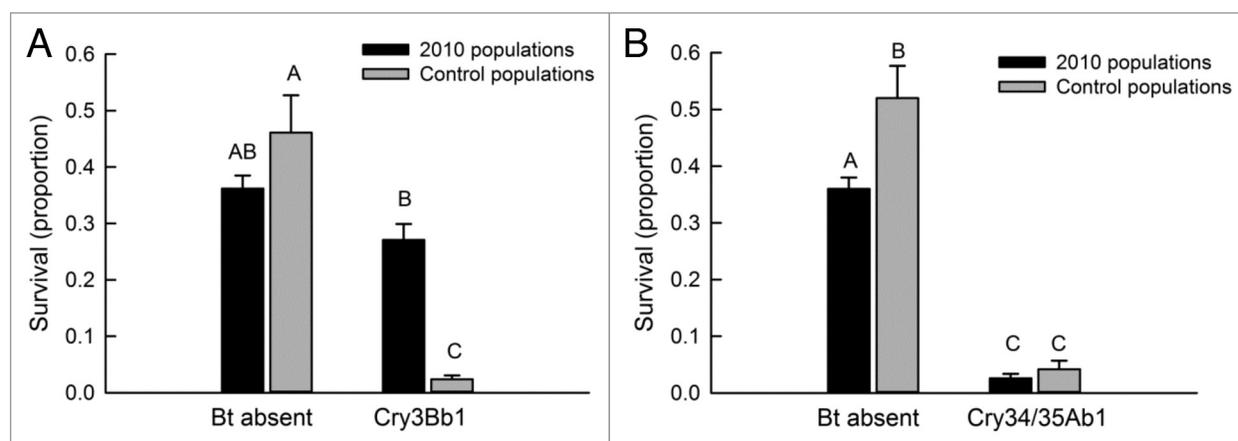


Figure 2. Survival of western corn rootworm on (A) Cry3Bb1 maize and (B) Cry34/35Ab1 maize. Bar heights represent sample means and error bars are the standard error of the mean. Letters indicate pairwise differences among means.

significantly higher for control populations than 2010 populations (Fig. 2B).

A second set of complementary analyses were conducted using survival on Bt maize that was corrected for survival on the accompanying non-Bt near isolate (Tables 3 and 4). Corrected survival on Cry3Bb1 maize was significantly greater for 2010 populations than control populations ($f = 102.61$; $df = 1.11$; $p < 0.0001$). By contrast, corrected survival did not differ between control populations and 2010 populations on Cry34/35Ab1 maize ($f = 0.04$; $df = 1.11$; $p = 0.85$). Additionally, there was no significant correlation between survival on Cry34/35Ab1 maize and survival on Cry3Bb1 maize ($r = 0.05$; $df = 11$; $p = 0.87$), indicating a lack of cross resistance between these Bt toxins (Fig. 3). Corrected survival on Cry3Bb1 maize was significantly greater for S2 than control populations ($t = 37.6$; $df = 5$; $p < 0.001$).

For bioassays with Cry3Bb1 maize vs. non-Bt maize a significant interaction between population type and hybrid type was present for the proportion of first and third instar larvae

(Table 5). Larval development was delayed on Cry3Bb1 maize relative to non-Bt maize for control populations, however, developmental delays were minimal for 2010 populations (Table 5). For control populations, there was a significantly higher proportion of first instar larvae and a significantly lower proportion of third instar larvae on Cry3Bb1 maize compared with non-Bt maize, but these differences were not present for 2010 populations. The pattern of larval development on Cry34/35Ab1 maize did not differ between larvae from control populations and 2010 populations, and development was significantly delayed on Cry34/35Ab1 maize (Table 5). For both control populations and 2010 populations, there was a significantly higher proportion of first and second instar larvae and a significantly lower proportion of third instar larvae on Cry34/35Ab1 maize compared with non-Bt maize. Of the 72 bioassay cups checked for pupae at the completion of the bioassay, none yielded any pupae, indicating that larvae were not pupating in the bioassay containers.

Table 4. Location, corrected survival and date taken into culture for control populations

Site	Location	Year in Culture	Corrected Survival ^a	
			Cry3Bb1	Cry34/35Ab1
C1	Butler Co., Nebraska	1999	0.08 ± 0.04	0.13 ± 0.07
C2	Potter Co., South Dakota	1995	0.07 ± 0.05	0.11 ± 0.05
C3	York Co., Nebraska	1999	0.07 ± 0.05	0.14 ± 0.04
C4	Centre Co., Pennsylvania	2001	0.05 ± 0.02	0.06 ± 0.03
C5	Finney Co., Kansas	2000	0.05 ± 0.03	0.00 ± NA
C6	Phelps Co., Nebraska	1995	0.00 ± NA	0.04 ± 0.02
Mean			0.05 ± 0.01	0.08 ± 0.02

^aMean ± Standard Error.

Discussion

We found that western corn rootworm from continuous maize fields with a history of cultivation of Cry3Bb1 maize had significantly higher survival and faster development on Cry3Bb1 maize in laboratory bioassays than did control populations that did not experience selection for Cry3Bb1 resistance in the field (Fig. 2A). By contrast, no difference in survival or developmental rate on Cry34/35Ab1 maize was detected between 2010 populations and control populations. In some cases, resistance to Cry3Bb1 maize was associated with severe root injury in the field (Tables 1 and 3). Additionally, we did not detect any correlation among populations for survival on Cry3Bb1 maize and Cry34/35Ab1 maize, indicating a lack of cross resistance between these Bt toxins. These data are consistent with Gassmann et al.¹³ in which a history of selection on Cry3Bb1 maize in the field resulted in resistance to Cry3Bb1 maize but did not confer cross resistance to Cry34/35Ab1 maize.

Of all fields visited in 2010, seven of 14 fields were planted to Cry3Bb1 maize and had an average node injury rating of greater than 1.0 (Table 1). This level of injury is higher than other published accounts of injury for commercialized Cry3Bb1 maize hybrids.⁵⁵⁻⁵⁷ Averaging across several studies, Dun et al.⁵⁸ found a 17.9% reduction in yield for each node of roots lost to feeding by western corn rootworm, indicating that in seven of the fields visited in 2010 farmers may suffer substantial reductions in yield.

Fields sampled in 2010 had the common feature of having been planted to Cry3Bb1 maize for at least 3 y and as many as 7 y (Table 3). The appearance of resistance after three to seven generations of selection is consistent with laboratory selection using Cry3Bb1 maize. Oswald et al.⁵⁹ found that western corn rootworm strains exposed to incremental selection (increasing by 12 or 24 h of exposure to Cry3Bb1 maize per generation) displayed approximately double the survival on Cry3Bb1 maize after four generations of selection and approximately four times greater survival after eight generations of selection. For intensely selected strains (those exposed to Cry3Bb1 maize for at least 14 d per generation), resistance to Cry3Bb1 maize was measured after seven generations of selection and was found to be approximately four times higher than unselected strains. Meihls et al.⁴⁵ also found a rapid response to selection on Cry3Bb1 maize. After three generations in which larvae were continuously exposed to

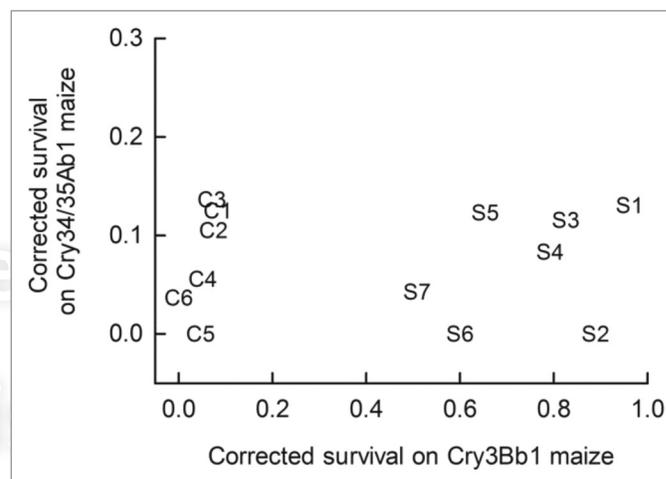


Figure 3. Correlation analysis for survival of populations on Cry3Bb1 maize and Cry34/35Ab1 maize. Symbols correspond to Tables 3 and 4.

Cry3Bb1 maize, larval survival did not differ on Cry3Bb1 maize and non-Bt maize. By contrast, the unselected control colony had significantly lower survival on Cry3Bb1 maize than non-Bt maize.⁴⁵ After six generations of selection, larval survival and survival to adulthood did not differ between non-Bt maize and Cry3Bb1 maize for the selected strain but was significantly lower on Cry3Bb1 maize for the unselected strain.⁴⁵

Because of the similarity between laboratory selection experiments with Cry3Bb1 maize and results observed in the field here and in Gassmann et al.,^{13,49} data from laboratory selection experiments with other Bt traits targeting western corn rootworm may be helpful in predicting durability in the field. Lefko et al.⁶⁰ found that under continuous selection on Cry34/35Ab1 maize, resistance levels increased but fluctuated considerably for the initial four to six generations of selection. However, after five to seven generations of selection, resistance remained stable in two replicated lines and selected lines displayed survival that was at least 10 times greater than observed during the first generation of selection.⁶⁰ Simulation modeling also predicted a plateau in the response to selection following six generations of selection.⁶⁰ Under laboratory selection on mCry3a maize, Meihls et al.⁶¹ found significantly greater survival after four generations

Table 5. Proportion of insects in each larval instar by population type and maize hybrid

Instar	Significant Factors ^a			Population Type and Maize Hybrid ^{b,c}			
				2010 Populations		Control Populations	
				Cry3Bb1	Non-Bt ^d	Cry3Bb1	Non-Bt ^d
First	H	P	I	0.02 ± 0.01 ^A	0.00 ± 0.00 ^A	0.16 ± 0.07 ^B	0.02 ± 0.01 ^A
Second	H	-	-	0.40 ± 0.06 ^A	0.26 ± 0.09 ^B	0.52 ± 0.16 ^A	0.17 ± 0.06 ^B
Third	H	-	I	0.58 ± 0.06 ^{AB}	0.74 ± 0.09 ^A	0.32 ± 0.12 ^B	0.81 ± 0.07 ^A
Instar	Significant Factors ^a			Population Type and Maize Hybrid ^{b,c}			
				2010 Populations		Control Populations	
				Cry34/35Ab1	Non-Bt ^d	Cry34/35Ab1	Non-Bt ^d
First	H	-	-	0.12 ± 0.07 ^A	0.01 ± 0.00 ^B	0.26 ± 0.14 ^A	0.01 ± 0.01 ^B
Second	H	-	-	0.76 ± 0.12 ^A	0.38 ± 0.08 ^B	0.59 ± 0.18 ^A	0.23 ± 0.06 ^B
Third	H	-	-	0.12 ± 0.05 ^A	0.61 ± 0.08 ^B	0.15 ± 0.08 ^A	0.76 ± 0.07 ^B

^aEffects in the ANOVA that were significant. H = maize hybrid (Bt vs. non-Bt), P = population type (2010 population vs. control population), I = the interaction between maize hybrid and population type. ^bCapital letters indicate significant differences among means within a row, which were based on significant main factors (H or P) in the ANOVA or on pairwise comparisons in cases where a significant interaction (I) was present. ^cMean ± Standard Error. ^dRepresents non-Bt near isoline of the companion Bt hybrid.

of selection, a pattern that remained after populations were exposed to an additional three and seven generations of selection. Because these selection experiments were conducted without an infusion of unselected (i.e., refuge) insects, the data may be viewed as a worst case scenario. However, given the low rate of pest dispersal and lack of compliance in refuge planting, this worst case scenario will likely be realized in some fields.^{21,47,48} Thus, for Bt maize with only one rootworm active Bt toxin that is not a high dose event, continuous use of the same toxin for three to five growing seasons may lead to resistance in some cases.

Currently, the mechanism of field-evolved resistance to Cry3Bb1 maize in western corn rootworm is unknown. Analysis of resistance ratios (LC₅₀ of resistant ÷ LC₅₀ of susceptible) for laboratory selected strains of western corn rootworm found a resistance ratio of 22 for a Cry3Bb1-selected strain and of 15.4 for a mCry3A-selected strain.^{45,61} These values are lower than those found for some Bt-resistant Lepidoptera. For example, resistance ratios between 520 and 1,700 were found for strains of pink bollworm, *Pectinophora gossypiella*, with resistance to Cry1Ac cotton.⁶² Because Cry3Bb1 maize is not a high dose event, and some susceptible insects can survive on Bt plants, relatively small resistance ratios (e.g., 10 to 20) may substantially enhance survival on Bt maize. This hypothesis is consistent with the bioassay data reported here, in which field-selected populations had 11 times higher survival on Cry3Bb1 maize than control populations.

Survival on non-Bt maize in bioassays suggests that fitness costs may be associated with field-evolved resistance to Cry3Bb1 maize, because in one of two cases tested, 2010 populations displayed significantly lower survival on non-Bt maize than control populations (Fig. 2B). However, this effect also may have resulted from control populations undergoing selection for survival in laboratory conditions, because they had been kept in laboratory culture for an average of 13 generations (Table 4). Fitness costs often accompany Bt resistance, although few data are currently available for western corn rootworm.^{13,63} Analysis of data on Bt resistance across insect taxa by Gassmann et al.⁶³ found that the magnitude of fitness costs was positively correlated

with resistance ratio, with more resistant strains suffering greater fitness costs. As such, if relatively small resistance ratios are associated with Bt resistance in western corn rootworm, then fitness costs may be less than those observed for insects with high levels of Bt resistance, such as pink bollworm.

While many fields studied here were planted at least in part to Cry3Bb1 maize in 2010, field S2 was planted to Cry34/35Ab1 maize (Table 2). Although resistance to Cry3Bb1 was found in S2 (see Results), that field had not been planted to Cry3Bb1 maize since 2008, which raises the possibility that once selected, Bt resistance might persist in a population. In a laboratory-selected strain, resistance was found to remain at a similar level after six generations without selection.⁴⁵ Parallels may be present with resistance to the insecticide aldrin, which took several generations to build within populations of western corn rootworm but has since persisted for more than 20 y in the absence of selection.⁶⁴

While resistance to Cry3Bb1 was associated with three or more years of cultivation for Cry3Bb1 maize, not all fields experience severe rootworm injury to Cry3Bb1 maize (Table 3). This is to be expected because root injury in the field will be a function of resistance and pest abundance.⁶⁵ Levels of severe root injury to Bt maize are expected only when resistant pests reach a sufficiently high abundance in the field. Additionally, survival of western corn rootworm larvae, and subsequent root injury, may be affected by field conditions such as soil type and degree of soil saturation.⁶⁶ This too will influence whether or not a Bt-resistant population imposes severe root injury in the field.

Because Bt toxins targeting corn rootworm are not present in high-dose events, pyramiding multiple Bt toxins is likely the optimal insect resistance management strategy.³⁴ However, for fields in which resistance is present to one Bt toxin, the benefits of pyramiding likely will be diminished. Computer modeling suggests that for Bt crops containing events that are less than high dose, the durability of pyramids is contingent on the frequency of resistance alleles at the time the pyramid is deployed. For example, Roush³⁴ found that sequential deployment vs. pyramiding

provided similar delays in resistance once resistance allele frequencies exceeded ca. 0.01, but at lower frequencies of resistance alleles, pyramiding Bt events provided longer delays in resistance than sequentially releasing Bt events. Similarly, Gould et al.⁶⁷ reported that an order of magnitude increase in resistance allele frequency (i.e., from 0.001 to 0.01) to an order of magnitude decrease in the time until a pest population developed resistance to a pyramid. Thus, for Bt events that are less than high dose, releasing events initially as pyramids rather than single events followed by pyramids is a better resistance management strategy.

Better use of integrated pest management in conjunction with robust insect resistance management will be essential for maintaining the viability of Cry3Bb1 maize and likely all types of Bt maize. A common pattern observed among fields in this study and in 2009 was continuous maize cultivation and continuous use of Cry3Bb1 maize.¹³ Pest susceptibility to management tactics is often a non-renewable resource that is expended in the process of managing a pest. However, the rate at which this occurs will be driven, in part, by how frequently a management practice is applied. By applying a greater diversity of practices such as crop rotation, cultivation of different Bt events and use of non-Bt maize with soil insecticides, selection for resistance to any single Bt toxin will be diminished. Bt maize for management of western corn rootworm is a valuable tool, but both laboratory and field data show that there are limits to the durability of this technology. Better incorporation of Bt maize into integrated pest management for western corn rootworm is likely the best management option to deal with future challenges.

Methods

During the summer of 2010, fields were visited in Iowa in response to grower reports of rootworm injury to Bt maize targeting corn rootworm (Table 1). One of the fields (Field S3 in Tables 2 and 3, and Fig. 1A) was visited to follow-up on a field that had been sampled in 2009 and was reported as field P1 in Gassmann et al.¹³ In 2010 S3 was planted to a combination of Cry3Bb1 maize and pyramided maize with Cry3Bb1 and Cry34/35Ab1. Three additional problem fields described in Gassmann et al.¹³ were not included in this study. The reason for these omissions was that one had been rotated to soybeans (field P2 in ref. 13), and consequently did not allow for survival of larval western corn rootworm. A second field could not be visited for logistical reasons (field P3 in ref. 13). A maize field that was ca. 0.75 Km from P4 in Gassmann et al.¹³ was sampled (root injury to Cry3Bb1 maize = 0.52 ± 0.15 nodes, mean \pm standard deviation, $n = 5$ roots), but we obtained only 400 eggs and could not run bioassays.

Upon visiting a field, samples of the predominant rootworm species were collected, which was western corn rootworm in all cases. For each population used in bioassays, we sampled between 91 and 429 adult western corn rootworm per field (mean = 200; standard deviation = 115). Data on the history of crops grown in the field was gathered by interviewing farmers, crop consultants, and local extension personnel.

We sampled five roots from each field. Roots were not sampled at random but were sampled to confirm the presence of rootworm feeding. When possible roots were sampled from a portion of the field in which plants displayed rootworm injury, which included plants that were lodged (i.e., listing) and goosenecked (i.e., plants that were bent at base in an attempt to grow upright after becoming lodged). Our sampling methodology was to walk into a field at least 10 m and scan a row for approximately 5 sec. If a plant was noticeably goose necked or lodged, that plant was sampled. If all plants appeared the same, a plant was selected haphazardly. Any plants that displayed stunted or lacked a full sized ear were not sampled. After digging a root, we moved approximately 10 m toward the interior of the field, and sampled another plant following the same methodology. For all plants sampled, the Bt toxin that the farmer thought was present in the maize (i.e., Cry3Bb1, Cry34/35Ab1 or a pyramid of the two) was confirmed by ELISA with a kit (Envirologix, Portland, Maine).

Western corn rootworm adults were brought to the laboratory and each population was held in an individual cage (18 cm \times 18 cm \times 18 cm L \times W \times H) (Megaview Science, Taiwan) in a growth chamber (25°C; 16/8 L/D). Cages contained maize leaf tissue and an artificial diet (western corn rootworm diet, Bio-Serv, Frenchtown, New Jersey), as food for the rootworm adults, and a water source provided by a 1.5% agar solid. Adults were provided with an oviposition substrate that consisted of moist, finely sieved soil ($< 180 \mu\text{m}$) placed in a Petri dish (diameter = 10 cm). Eggs obtained from each population were placed separately in 45 mL plastic cups containing moistened sieved soil, sealed in a plastic bag, and placed in a cold room at 6°C for at least 5 mo to break diapause. Eggs were held in a cold room until their removal for bioassays. For fields visited in 2010 (Table 1), we conducted bioassays on all populations from which we obtained at least 3,000 eggs, which was the minimum number needed to ensure sufficient replication of the bioassay across all maize hybrids evaluated.

For control populations, we obtained eggs from the United States Department of Agriculture's North Central Agricultural Research Laboratory (NCARL) in Brookings, South Dakota (Fig. 1B and Table 4). All control populations were diapausing strains of western corn rootworm that were brought into the laboratory prior to 2003, which is the year that Bt maize was commercialized for management of western corn rootworm. Thus, control populations represent field populations that never experienced selection for resistance to Bt maize. Eggs of control populations were sent from NCARL to Iowa State University in diapause and upon arrival at Iowa State University were placed in a cold room at 6°C for later use in bioassays.

For both 2010 populations and control populations, neonate larvae were obtained for bioassays by removing eggs from 6°C after at least 5 mo had passed. Following exposure to cold, eggs were stored for 1 week at 25°C. Eggs were then washed from the soil using a screen with 250 μm openings and then placed atop moistened sieved soil held in a 10 cm Petri dish. Neonate larvae began hatching approximately 1 week thereafter.

Neonate larvae from 2010 populations and from control populations were evaluated for their survival on transgenic maize

following Gassmann et al.¹³ We used two transgenic maize hybrids, each of which contained a unique Bt toxin that targets western corn rootworm. The hybrid DKC 6169 (DeKalb Brand, Monsanto Company) produced Cry3Bb1 and the hybrid 2T789 (Mycogen Brand, Dow AgroSciences, Indianapolis, Indiana) produced Cry34/35Ab1. For both hybrids, we also measured survival of rootworm on a near isogenic hybrid that lacked a gene for a rootworm active Bt toxin but otherwise was genetically similar to its respective Bt hybrid. In the case of Cry3Bb1 maize, the non-Bt hybrid was DKC 6172 (DeKalb) and for Cry34/35Ab1 maize the non-Bt hybrid was 2T777 (Mycogen). Prior to their use in bioassays, all maize seeds were soaked for one hour in a 10% bleach solution during which they were stirred every 15 min. Seeds were then rinsed 10 times with deionized water and allowed to dry for at least 24 h. Although seeds did not receive any type of seed treatment, they were bleached to remove any traces of insecticide that may have been present.

Maize plants used in bioassays were grown in the greenhouse (16/8 L/D) in 1 L containers following Gassmann et al.¹³ with one plant per container. Once plants had five fully formed leaves, they were used in bioassays. For bioassays, plants were first trimmed to a height of 20 cm with two to three leaves (trimmed to 8 cm long) remaining on the plant. Recently hatched larvae (less than 24 h old) were removed from the soil's surface within their Petri dish using a fine brush and then placed at the base of a maize plant on a root that had been exposed by moving away a small amount of soil. Each plant received 11.85 ± 0.54 larvae (mean \pm standard deviation). Although the goal was to place 12 larvae in each cup, in some cases an insufficient number of larvae was available. Cups containing plants, soil and larvae were placed in an incubator (I41LL with light and humidity control, Percival Scientific, Perry, Iowa) for 17 d (25°C, 65% RH, 16/8 L/D) and plants were watered as needed. A length of 17 d was chosen because it provided sufficient time for the majority (ca. 75%) of larvae on non-Bt maize to reach the third and final instar (see Table 5).⁵⁰ Maize plants remained in their original 1 L containers throughout the bioassay. Bioassays were conducted between February and September, 2011 and bioassays alternated between 2010 populations and control populations during the entire testing period.

After 17 d in an incubator, bioassay cups were removed and the above-ground tissue of the maize plant excised. The soil, containing roots and larvae, was then removed from the 1 L plastic container and placed on a Berlese funnel for 4 d to extract larvae from the soil. Rootworm larvae were collected in 15 mL glass vials containing 10 mL of 85% ethanol. The total number of larvae recovered from each bioassay container was counted. For all larvae, we used a microscope (Leica MZ6) with digital camera and image analysis software (Motic Images Inc.) to measure width of larval head capsules and then determined larval instar based on the scale of Hammack et al.⁵¹ To ensure that larvae were not pupating in bioassay cups, we carefully inspected the soil of three bioassay cups for each of the four maize hybrids evaluated (DCK 6169, DCK 6172, 2T777 and 2T789) for three of 2010

populations and three of control populations (n = 72 bioassay cups). Individual bioassays were randomly selected and the soil carefully checked for pupae immediately after removal from a Berlese funnel.

The average sample sizes per population were 11.00 ± 2.18 (mean \pm standard deviation) bioassay cups for Cry3Bb1 maize and for its non-Bt near isoline, and 11.00 ± 2.18 bioassay cups for Cry34/35Ab1 maize and for its non-Bt near isoline. In all, there were seven 2010 populations and six control populations, and a total of 572 bioassay cups were run for the entire experiment. Rootworm survival per bioassay cup was calculated as the proportion of larvae recovered after 17 d divided by the number of neonate larvae placed into that cup. For larvae recovered from each bioassay cup, we calculated the proportion in each of the three larval instars.

Data analysis. All data analyses were conducted using SAS Enterprise Guide 4.2.⁵² Data on the proportion of rootworm surviving per bioassay cup and the proportion of insects in each larval instar were analyzed with a mixed model analysis of variance (ANOVA) (PROC MIXED in SAS). Data were analyzed separately for Cry34/35Ab1 maize and Cry3Bb1 maize. Data were transformed by the arcsine of the square root to ensure normality of the residuals. Fixed effects in the model included maize type (Bt maize vs. non-Bt maize) and population type (control population vs. 2010 population). Random factors in the analysis were population nested within population type, and the interaction of maize type with population nested within population type. When a significant interaction between maize type and population type was present, pairwise comparisons were made among least-squares means using the PDIFF option in PROC MIXED with an experimentwise error rate of $p < 0.05$.

For each population, rootworm survival on Bt maize was corrected for survival on non-Bt maize. We calculated corrected survival on Bt maize as the complement of corrected mortality based on Abbott's correction.⁵³ For each population, survival on Bt maize in each bioassay cup was adjusted for average survival on the non-Bt near isoline. For Cry3Bb1 maize and Cry34/35Ab1 maize, the average corrected survival for each population was compared between 2010 populations and control populations using a one-way ANOVA (PROC ANOVA). We tested the correlation between corrected survival on Cry3Bb1 maize and Cry34/35Ab1 maize using the mean value for each population (PROC CORR). Corrected survival on Cry3Bb1 maize for S2 vs. control populations was compared with a t-test.⁵⁴

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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