2015

Susceptibility of Nebraska Western Corn Rootworm (Coleoptera: Chrysomelidae) Populations to Bt Corn Events

David S. Wangila
University of Nebraska-Lincoln, dwangila2@unl.edu

Aaron J. Gassmann
Iowa State University, aaronjg@iastate.edu

Jennifer L. Petzold-Maxwell
Wartburg College

B. Wade French
USDA-ARS, Brookings, SD, Wade.French@ars.usda.gov

Lance J. Meinke
University of Nebraska-Lincoln, lmeinke1@unl.edu

Susceptibility of Nebraska Western Corn Rootworm (Coleoptera: Chrysomelidae) Populations to Bt Corn Events

DAVID S. WANGILA,[1] AARON J. GASSMANN,[2] JENNIFER L. PETZOLD-MAXWELL,[2,3] B. WADE FRENCH,[4] and LANCE J. MEINKE[1,5]

J. Econ. Entomol. 108(2): 742–751 (2015); DOI: 10.1093/jee/tou063

ABSTRACT Transgenic plants have been widely adopted by growers to manage the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in field corn. Because of reduced efficacy in some Nebraska fields after repeated use of Cry3Bb1-expressing hybrids, single plant bioassays were conducted in 2012 and 2013 to characterize the susceptibility of western corn rootworm populations to the rootworm-active proteins Cry3Bb1, mCry3A, and Cry34/35Ab1. Results demonstrate that there are heritable differences in susceptibility of Nebraska western corn rootworm populations to rootworm-active Bt traits. Proportional survival and corrected survival data coupled with field histories collectively support the conclusion that a level of field resistance to Cry3Bb1 has evolved in some Nebraska populations in response to selection pressure and that cross-resistance exists between Cry3Bb1 and mCry3A. There was no apparent cross-resistance between Cry34/35Ab1 and either Cry3Bb1 or mCry3A. The potential implications of these results on current and future corn rootworm management strategies are discussed.

KEY WORDS genetically modified crop, evolution, resistance management, *Bacillus thuringiensis*, *Diabrotica virgifera virgifera*

Introduction

Corn rootworms (*Diabrotica* spp.) are economically important pests of field corn in the U.S. Corn Belt. The economic impact of this pest complex to U.S. farmers has been estimated at US$1 billion in yield loss and treatment costs annually (Rice 2004, Sappington et al. 2006). The most significant *Diabrotica* species in the Corn Belt region is the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Gray et al. 2009). Larval feeding on corn roots by this insect pest can lead to root injury, decreased plant growth, and reduced yield (Godfrey et al. 1993, Gray and Steffey 1998, Urías-López and Meinke 2001). The western corn rootworm was not considered a serious pest of corn until the late 1920s–1940s, when it became a problem in northwestern Kansas and southwestern Nebraska (Bare 1930, Tate and Bare 1946, Bryson et al. 1953). Contributing factors included planting of continuous corn, made possible by development of irrigation systems and the introduction of synthetic fertilizer (Meinke et al. 2009). This facilitated an increase in western corn rootworm densities and the initiation of range expansion that now covers many corn growing areas east of the Rocky Mountain region of North America (Gray et al. 2009, Meinke et al. 2009). From the 1950s to 1990s, a large market developed around the manufacture and the subsequent use of soil-applied or foliar-applied insecticides as standard western corn rootworm management tactics in continuous corn (Levine and Oloumi-Sadeghi 1991).

Recent advancements in biotechnology led to the development of transgenic crops that produce insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt). The rootworm-active Bt toxin Cry3Bb1 was registered and introduced commercially in corn hybrids during 2003. Since 2003, the Bt toxins Cry34/35Ab1 (2005), mCry3A (2006), and eCry3.1Ab (2012), which target *Diabrotica* species, were also registered in the United States (EPA 2003, 2005, 2007, 2012a,b, Vaughn et al. 2005, Gatehouse 2008, Tabashnik et al. 2009). Transgenic Bt corn hybrids have been widely adopted by growers and largely replaced soil and foliar insecticides as the primary tactics used in continuous corn for management of corn rootworms. Rootworm-active Bt pyramids were granted registrations more recently in the United States (i.e., Cry3Bb1 and Cry34/35Ab1 [2009], mCry3A and Cry34/35Ab1 [2011], mCry3A and eCry3.1Ab [2013]; Environmental Protection Agency [EPA] 2012a, 2013) and are gradually replacing single Bt trait market share in the U.S. Corn Belt.

All registered Bt events that are active against the western corn rootworm are considered less than high dose, as defined by the EPA (i.e., these Bt events fail to

[1] Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE 68583.
[2] Department of Entomology, Iowa State University, 18 Insectary, Ames, IA 50011.
[3] Current address: Department of Biology, 100 Wartburg Blvd., Wartburg College, Waverly, IA 50677.
[4] North Central Agricultural Research Laboratory, USDA-ARS, Brookings, SD 57006.
[5] Corresponding author, e-mail: lmeinke1@unl.edu.

produce 25 times the concentration of Bt toxins needed to kill 99% of susceptible insects; Storer 2003; Tabashnik 2008, Tabashnik et al. 2008). As a result, root injury and some survival to the adult stage are expected in the field for currently registered rootworm-active proteins expressed in commercial hybrids (Storer et al. 2006, Hibbard et al. 2010, 2011, Clark et al. 2012).

In recent years, greater than expected injury (i.e., more than one node of injury) by the western corn rootworm to corn hybrids expressing Cry3Bb1 has been observed in some areas of the Corn Belt (Gassmann et al. 2011, 2012, 2014, Potter and Ostlie 2011, Wright et al. 2011, Gray 2012, 2014). Problem fields where this phenomenon was observed were typically planted to single Cry3Bb1 trait hybrids for three or more consecutive years (Gassmann et al. 2011, 2012, 2014). Because the western corn rootworm has demonstrated a great capacity to adapt to pest management tactics (Ball and Weekman 1962, Meinke et al. 1998, Levine et al. 2002), the potential evolution of resistance to Bt traits is of primary concern. Field histories and single plant bioassays directly comparing larval survival among Cry proteins were used to confirm that a level of western corn rootworm resistance to Cry3Bb1 evolved in some fields in Iowa and Illinois (Gassmann et al. 2011, 2012, 2014, Gray 2012, 2014). In Nebraska, greater than expected rootworm injury has also been observed in some fields with a history of Cry3Bb1 corn cultivation. However, formal characterization of the susceptibility of Nebraska western corn rootworm populations to rootworm-active Bt corn events has not been conducted. Therefore, during 2012–2013, field and laboratory studies were conducted to provide such data as part of a larger project to develop sustainable corn rootworm management strategies in Nebraska. The laboratory component of this project is presented in this article. The Gassmann single plant diagnostic bioassay technique (Gassmann et al. 2014) was used to compare western corn rootworm susceptibility with Cry3Bb1, Cry34/35Ab1, and mCry3A proteins. Populations from various Cry3Bb1 problem fields were compared with multiple control populations.

Materials and Methods

Insect Populations. In 2011 and 2012, farmers and crop consultants notified us about rootworm control issues in some fields planted to Cry3Bb1 corn. Control issues were usually characterized by moderate to severe root injury and plant lodging (plants not perpendicular to ground, leaning), which can be a characteristic of extensive rootworm feeding. After notification, these fields were visited and seven to eight plants were dug from the problem areas of most fields to evaluate root injury using the 0–3 node injury scale (Oleson et al. 2005). Lateral flow strips (Envirologix Inc., Portland, ME) were used to confirm the expression of Cry3Bb1 proteins in each plant. Fields with an average root injury rating >1.00, which met the EPA criteria for greater than expected injury (EPA 2011), were called Cry3Bb1 problem fields in this study (Table 1).

Table 1. Root injury ratings and cropping sequence of Cry3Bb1 history fields exhibiting greater than expected injury that were sampled during 2011 and 2012

Field	N[a]	Root rating (NIS) ± SE	Field history[b]					
			07	08	09	10	11	12
2011								
PA	–	–	1	1	1	1	1	–
PB	7	1.67 ± 0.38	1	1	2	1	1	–
2012								
P1	7	1.60 ± 0.14	1	1	1	1	1	4
P2	7	1.23 ± 0.02	1	1	1	1	1	1
P3	7	1.56 ± 0.11	1	1	1	1	1	1
P4	7	1.24 ± 0.13	2	1	2	1	1	1
P5	7	2.00 ± 0.07	2	1	2	1	1	1
P6	8	2.49 ± 0.16	3	1	1	1	1	1
IA-R	12	3.00 ± 0.00	1	1/2	1	1	1	1

[a] Number of roots rated for root injury using 0–3 node injury scale. Roots were not rated from field PA during 2011. Root injury rating at site P1 was recorded in 2011, the Cry34/35Ab1 expressing hybrid was not evaluated in 2012.

[b] Field history indicates the crop that was planted in each field from 2007 to 2012: 1, Cry3Bb1-expressing corn; 2, either soybeans or sugar beets; 3, corn hybrid unknown; 4, primarily Cry34/35Ab1-expressing corn plus small multitrait trial. For IA-R in 2008, part of the field was planted to Cry3Bb1 corn and the other portion was planted to soybeans.

Table 2. Adult western corn rootworm collection date (2011) and mean corrected proportional larval survival in 2012 western corn rootworm bioassays

Site[a]	Date collected[b]	Mean corrected survival ± SE	
		Cry3Bb1	Cry34/35Ab1
Problem field (PA)	23 August	0.67 ± 0.03	0.12 ± 0.01
Problem field (PB)	29 August	0.25 ± 0.02	0.09 ± 0.01
Laboratory control (L1)	1996	0.05 ± 0.01	0.18 ± 0.02

[a] Problem field, Cry3Bb1 history fields with greater than expected injury; Laboratory control, laboratory colony with no previous exposure to rootworm Bt corn.

[b] L1 population, the year field collection was brought into the laboratory and a colony was established.

Two western corn rootworm populations were collected from Cry3Bb1 problem fields during August 2011 and six additional populations were collected from problem fields between 6 July and 15 August 2012 (Tables 1 and 2; Fig. 1). The populations from Cry3Bb1 problem fields were collected from six counties in Nebraska (Fig. 1). The field histories dating back to 2007 were obtained from growers for these Cry3Bb1 problem fields (Table 1).

The only exceptions to the root injury characterization–adult collection procedure described above were fields PA and P1,which had greater than expected injury to Cry3Bb1 corn the year before western corn rootworm beetle collections were made. In each case, root injury was not evaluated the year adults were collected (see Table 1).

Three additional Nebraska field populations were collected in 2012 from fields that had not exhibited unexpected injury to any rootworm Bt hybrid. These populations were used as field control populations in bioassays. Also, one laboratory population in 2012 and

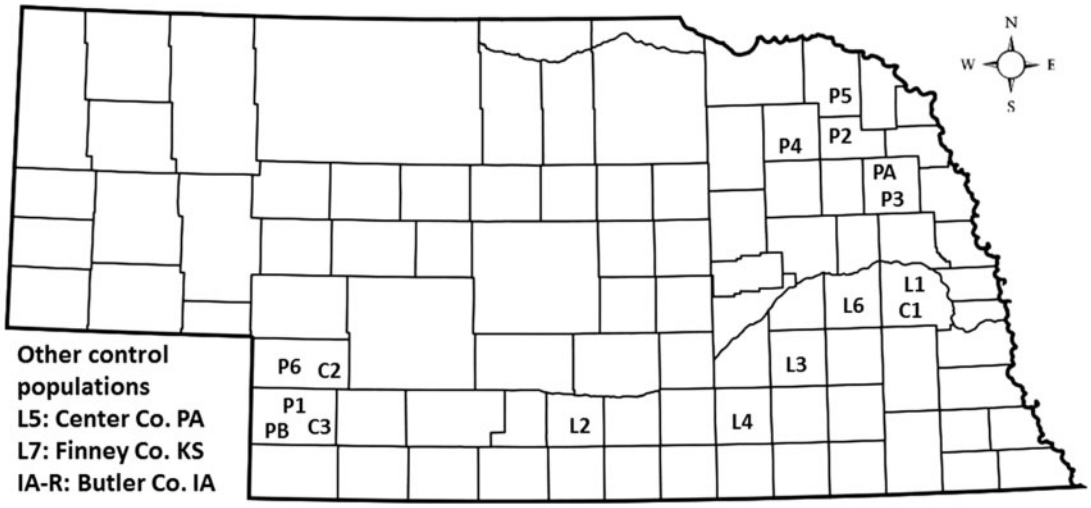

Fig. 1. A map of the state of Nebraska showing the distribution of sites sampled in 2012. Sites beginning with P were Cry3Bb1 history fields exhibiting greater than expected injury (problem fields), while the sites beginning with C were control fields not experiencing unexpected injury to any rootworm Bt toxin. The map also shows the county where the laboratory control populations (L) were collected prior to commercial release of rootworm-active Bt corn events.

seven populations in 2013 from U.S. Department of Agriculture–Agricultural Research Services North Central Agricultural Research Laboratory, Brookings, SD, were concurrently used as laboratory control populations during the bioassays. The laboratory populations had not been exposed to Cry toxins and were collected in the field prior to commercialization of rootworm-active Bt traits (Table 3; Fig. 1). Furthermore, a Cry3Bb1 problem field population from Iowa was used as a positive control in the 2013 bioassays. The Iowa population (IA-R) was collected during 2012, and was associated with a history of Cry3Bb1 corn cultivation and greater than expected feeding injury to Cry3Bb1 corn in 2012 (Table 1; Fig. 1).

The western corn rootworm adults collected from the field were brought to the laboratory at the University of Nebraska–Lincoln, and held for at least 1 mo. Approximately 500–1,200 western corn rootworm adults were collected from each field. The adults were maintained in 28 cm^3 Plexiglass boxes and were provided with a diet of milk stage non-Bt corn ear tissue. Food was replaced regularly every 2–3 d. Autoclaved (Market Forge Sterilmatic, Everett, MA) silty clay loam soil presifted through a U.S. standard 60-mesh sieve (Cospheric, Santa Barbara, CA) was moistened (ca. 30% moisture by volume) and provided in small round plastic containers (300 ml; 11.5 cm in diameter) as oviposition substrate for gravid females. After oviposition, soil was moistened and lids were placed on containers before storage in Ziploc bags. To facilitate diapause development and termination, eggs were held at 25°C for 1–2 mo after oviposition, 7°C for ~5–6 mo, and 22°C until egg hatch (Fisher 1989). Because of obligatory western corn rootworm egg diapause, bioassays were always conducted during the year following beetle collection.

Table 3. Adult western corn rootworm collection date (2012) and mean corrected proportional larval survival in 2013 bioassays for populations from Cry3Bb1 problem fields and control fields

Site[a]	Date collected[b]	Mean corrected survival (±SE)[c]		
		Cry3Bb1	mCry3A	Cry34/35Ab1
P1	6 July	0.73 ± 0.04	0.70 ± 0.06	0.17 ± 0.02
P2	12 July	0.79 ± 0.06	0.59 ± 0.06	0.22 ± 0.02
P3	26 July	0.63 ± 0.04	1.00 ± NA	0.37 ± 0.03
P4	26 July	0.90 ± 0.06	0.91 ± 0.06	0.27 ± 0.03
P5	26 July	0.61 ± 0.04	0.96 ± 0.04	0.14 ± 0.02
P6	15 Aug.	0.79 ± 0.05	0.94 ± 0.05	0.20 ± 0.02
P mean		0.74 ± 0.04a	0.85 ± 0.07a	0.23 ± 0.03a
C1	9 Aug.	0.09 ± 0.02	0.13 ± 0.03	0.00 ± NA
C2	14 Aug.	0.07 ± 0.01	0.29 ± 0.02	0.00 ± NA
C3	15 Aug.	0.14 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
C mean		0.10 ± 0.02b	0.15 ± 0.07b	0.02 ± 0.02b
L1	1996	0.06 ± 0.02	–	0.16 ± 0.03
L2	1995	0.02 ± 0.01	0.33 ± 0.04	0.34 ± 0.03
L3	1996	0.00 ± NA	0.42 ± 02	0.05 ± 0.01
L4	1994	0.15 ± 0.02	–	0.04 ± 0.01
L5	2000	0.00 ± NA	0.15 ± 0.03	0.03 ± 0.03
L6	1999	0.04 ± 0.01	0.24 ± 0.02	0.20 ± 0.02
L7	2000	0.00 ± NA	0.03 ± 0.01	0.00 ± NA
L mean		0.04 ± 0.02b	0.23 ± 0.07b	0.12 ± 0.05ab
IA-R	10 July	0.87 ± 0.05	0.41 ± 0.08	0.19 ± 0.04

[a] P1–P6, Cry3Bb1 history fields with greater than expected injury; C1–C3, field populations from sites not experiencing unexpected injury to rootworm Bt hybrids; L1–L7, laboratory colonies that had not been exposed to rootworm-active Cry toxins.

[b] L1–L7 populations: The date collected refers to the year each colony was collected from the field to initiate a laboratory colony.

[c] Overall means within columns with the same letter are not significantly different ($P > 0.05$, one-way ANOVA, LSMEANS test).

Bioassays. Similar procedures and whole-plant bioassay techniques described in Gassmann et al. (2011) were used in this study. Bioassays were conducted at Iowa State University in 2012, and followed the same

methods, as presented in Gassmann et al. (2014). All bioassays from 2013 were conducted at the University of Nebraska–Lincoln. In 2012, Cry3Bb1 (DeKalb DKC 6169) and Cry34/35Ab1 (Mycogen 2T789)-expressing hybrids plus their respective non-Bt near isolines (DeKalb DKC 6172, Mycogen 2T777) that did not express any rootworm-active Bt toxins were used in bioassays. In 2013, Cry3Bb1 (Stone 6021VT3), mCry3A (Syngenta N68B-3000 GT), and Cry34/35Ab1 (Pioneer Brand P1151XR)-expressing hybrids and their respective non-Bt near isolines (Stone 6021RR2, Syngenta N68B- GT, Pioneer Brand P1151HR) that did not express rootworm-active Bt toxins were used in bioassays. No seed treatments were applied to seed used in 2012 but all seeds used in 2013 were initially treated with a neonicotinoid insecticide at 0.25 mg (AI) per kernel. Seed treatments were removed in 2013, using the methods described in Gassmann et al. (2011) before use in bioassays.

In 2013, a mixture of two types of potting soils, Metro-mix professional growing mix (F1153Sun Gro Horticulture Distr. Bellevue, WA) and LC1 Nix Professional growing mix (F1153 Sun Gro Horticulture Distr. Bellevue, WA) were mixed in a 1:1 ratio, according to Gassmann et al. (2011) procedures, and used as the soil for all bioassays. Clear 1-liter plastic pots (Johnson Paper & Supply Co. Minneapolis, MN) were filled with moistened soil (~three-fourth full), leaving some space at the top of the pots for water application. One seed was planted in each pot and the pots were kept on benches in the greenhouse. An optimal temperature was maintained between 13.8 and 27.8°C (average daily low and high temperatures).

In 2013, supplemental lighting was provided as needed in the greenhouse by high-pressure sodium lights (400 W 208 V Jasond light bulbs, P. L Light Systems,Beamsville, ON, Canada) to maintain a photoperiod of 14:10 (L:D) h. Water was provided on a regular basis every 2–3 d in equal amounts to each pot. A 60-ml hypodermic syringe (Becton, Dickinson and company, Franklin Lakes, NJ) was used to draw and apply 50 ml of water to each potted plant. Vigoro water soluble, all-purpose plant food fertilizer (Swiss Farms Products, Howard Hughes Parkway, NV) was applied to all potted plants at the V2 growth stage (Abendroth et al. 2011). The fertilizer was dissolved in water in a ratio of 4 mg of fertilizer to 1 ml of water before application to each pot using a 60-ml syringe. Each potted plant received exactly 100 ml of fertilizer solution. Lateral flow strips (Envirologix Inc., Portland, ME) were used to confirm the expression of Bt proteins in Bt corn hybrids. A randomized complete block design was used to arrange the hybrids in plastic pots on greenhouse benches.

In 2013, potted plants with fully expanded leaf tissue at the V5 stage (Abendroth et al. 2011) were used in the bioassays according to Gassmann et al. (2011). Twelve western corn rootworm neonates were counted and placed immediately on exposed root tissues of each potted corn plant using a soft hair brush size 10/0 (Dalere Rowney Ltd., Bracknell, England) by moving soil slightly to expose the roots. After infestation, exposed roots were lightly covered with soil taking care not to injure the larvae. The infested plants in their respective pots were placed in growth chambers (Percival Scientific, Boone, IA) maintained at 24.0°C, a photoperiod of 14:10 (L:D) h and 65% humidity for 17 d. Preliminary experiments indicated that 17 d under these conditions would enable fastest developing larvae to reach third instar but not the pre-pupal stage. The bioassay pots were arranged in a randomized complete block design within the growth chamber. Within each population tested, there were 12 replications for each corn hybrid and four and six corn hybrids assayed in 2012 and 2013, respectively (i.e., 2012: $n = 48$; 2013: $n = 72$ total plants per population). An exception to this occurred in 2013, where bioassays could only be conducted with Cry3Bb1 and Cry34/35Ab1 for laboratory control populations L1 and L4. Water was provided as needed (<50 ml per pot) to avoid drowning larvae and to enable corn plants to grow optimally. At 17 d postinfestation, each corn plant was cut above the soil level to remove the stalk and the above-soil plant tissues before all contents in the pots were transferred and spread evenly into Berlese funnels to extract larvae. Light intensity was modified to set the Berlese funnel temperature at 40°C using 40 W, 120 V soft white light bulbs (Philips Lighting Company, Worcester, MA) for 4 d. Larvae were collected in 70% ethyl alcohol inside clear glass jars (Solo Cup Company, Lake Forest, IL) attached to Berlese funnels.

Data Analysis. Proportional survival was calculated as the number of larvae recovered after 17 d divided by the 12 neonates that had initially been placed in each bioassay pot. Because bioassay data for 2012 were from only two Cry3Bb1 field populations and one laboratory control population, data were analyzed with a one-way analysis of variance (ANOVA; PROC GLIMMIX in SAS) to compare mean proportion larval survival among six combinations of hybrid by population with data from each type of Bt corn and non-Bt near isoline analyzed separately (e.g., three populations each on Cry3Bb1 and isoline).

The mean proportion larval survival from the 2013 bioassays was analyzed with a two-way, mixed-model ANOVA, using PROC GLIMMIX in SAS (SAS Institute 2010, Cary, NC). Data for the three types of Bt corn (Cry3Bb1, mCry3A, and Cry34/35Ab1 and their respective non-Bt near isolines) were analyzed separately. Each ANOVA included the fixed factors population type (six Cry3Bb1 problem field populations, three non-problem-field control populations, and seven laboratory control populations [exception: only five laboratory populations for mCry3A]), corn hybrid (Bt hybrid and the respective non-Bt near isoline), and their interaction. The random factors in the analysis were population nested within population type, and the interaction between corn hybrid and the population nested within population type. The arcsine square root transformation was used to improve normality of the residuals prior to analysis (Snedecor and Cochran 1989). For all analyses, the treatment differences were determined by the LSMEANS test at the $P = 0.05$ level

of significance (SAS Institute 2010). Nontransformed data are reported in this article.

Corrected mortality was also calculated for each population and Cry protein using the correction of Abbott (1925). Corrected survival was then calculated as a complement of corrected mortality. A one-way ANOVA, using PROC GLIMMIX in SAS (SAS Institute 2010), was used to compare the mean corrected survival among each population type for each type of Bt corn. For all analyses, the treatment differences were determined by the LSMEANS test at the $P = 0.05$ level of significance (SAS Institute 2010).

Correlation analyses were used to measure the strength of association between the corrected survival of problem field and laboratory control populations on Cry3Bb1and mCry3A corn, Cry3Bb1 and Cry34/35Ab1 corn, and mCry3A and Cry34/35Ab1 corn. A Pearson's correlation coefficient was used to test for significance against the null hypothesis of $P = 0$ (PROC CORR in SAS).

Results

Proportion Survival on Bt and Non-Bt Near Isoline Hybrids. *2012 Bioassays.* Proportional larval survival was significantly different among treatments included in the one-way ANOVA ($F = 7.23$; df = 5, 54; $P < 0.0001$; Fig. 2A). Mean survival of the three populations (PA, PB, and laboratory control) was not significantly different on non-Bt near isoline corn. When reared on Cry3Bb1, the mean proportional survival of the two problem field populations was not significantly different, but only PA survival was significantly greater than the control population (Fig. 2A). Survival of population PB and the laboratory control were significantly lower than survival on their respective non-Bt near isoline hybrids. However, survival of PA on Cry3Bb1 was not significantly different than survival of PA on non-Bt near isoline (Fig. 2A).

A significant treatment effect also occurred in the one-way ANOVA of proportional survival when populations were reared on Cry34/35Ab1 and non-Bt near isoline hybrids ($F = 6.13$; df = 5, 64; $P = 0.0001$). However, the patterns among treatments were different than those observed in the Cry3Bb1 analysis (Fig. 2A and B). Similar to the Cry3Bb1 analysis, there were no significant differences in mean survival among populations when reared on non-Bt corn (Fig. 2B). In contrast to the Cry3Bb1 analysis, mean survival on Cry34/35Ab1 was not significantly different among populations, and mean survival of each population on Cry34/35Ab1 was significantly lower than survival of each population on non-Bt near isoline (Fig. 2B).

2013 Bioassays. A similar pattern was apparent when proportional survival on Cry3Bb1 and mCry3A was analyzed. A significant interaction between population type and hybrid occurred for the populations reared on Cry3Bb1 and non-Bt near isoline corn ($F = 16.74$, df = 2, 13, $P = 0.0003$) and mCry3A and non-Bt near isoline corn ($F = 8.86$; df = 2, 12; $P = 0.0043$). In both Cry3Bb1 and mCry3A analyses, mean survival on the non-Bt corn hybrid was not significantly different among population types (Fig. 3A and B). In contrast, mean survivorship of Cry3Bb1 problem field populations on Cry3Bb1 and mCry3A corn was not significantly different than mean survivorship of any population type reared on non-Bt corn, but was significantly greater than mean survival of either control population (Fig. 3A and B).

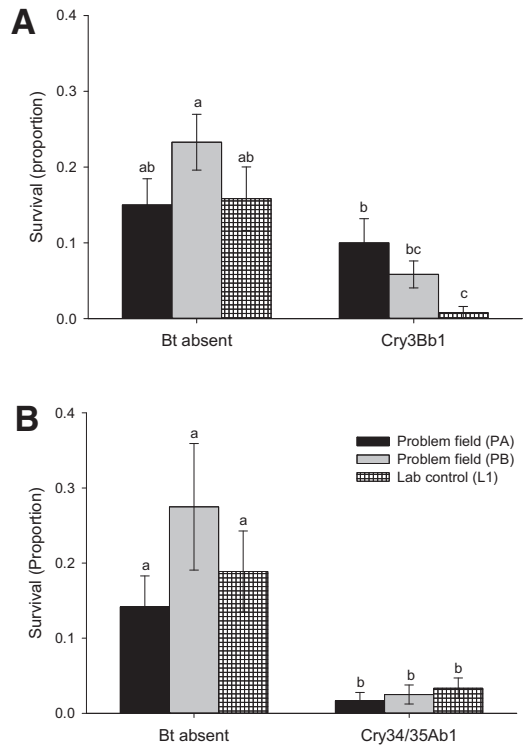

Fig. 2. Mean (±SE) larval proportional survival on (A) Cry3Bb1 corn and (B) Cry34/35Ab1 corn for the two western corn rootworm populations collected in 2011 from Cry3Bb1 history fields exhibiting greater than expected injury (PA, PB), and a laboratory population that had never been exposed to rootworm-active Cry proteins (L1). "Bt absent" refers to the non-Bt near isoline hybrid in each figure. Mean values followed by a same letter are not significantly different (one-way ANOVA, $P > 0.05$; LSMEANS test).

A different pattern was observed in bioassays with Cry34/35Ab1 and non-Bt near isoline corn. The interaction between population type and corn hybrid for the populations reared on Cry34/35Ab1 was not significant ($F = 0.51$; df = 2, 13; $P = 0.6136$). The main effect hybrid was significant ($F = 293.05$; df = 1, 13; $P < 0.0001$), as fewer larvae survived on plants expressing Cry34/35Ab1 than on the non-Bt near isoline hybrids (Fig. 3C). However, mean survival of the three population types was not significantly different on Cry34/35Ab1 corn ($F = 2.29$; df = 2, 13; $P = 0.1409$).

Corrected Survival on Bt and Non-Bt Near Isoline Corn Hybrids. *2012 Bioassays.* When proportional survival on Bt corn was corrected for survival on the non-Bt near isoline corn hybrid (Table 2), survival of population PA and PB was 13.4× and 5× greater, respectively, than the laboratory control

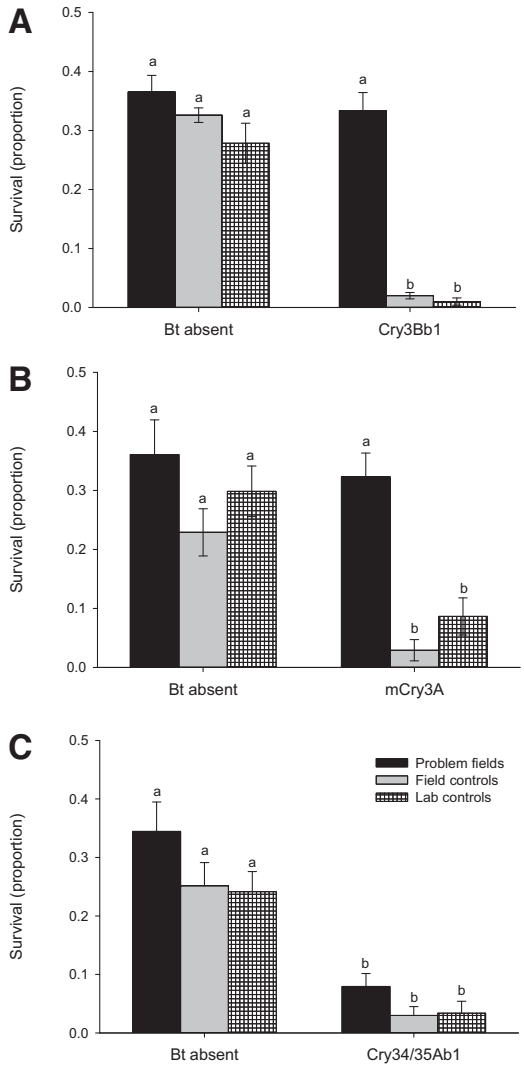

Fig. 3. Mean larval proportional (±SE) survival on (A) Cry3Bb1 corn, (B) mCry3A corn, and (C) Cry34/35Ab1 corn, for western corn rootworm populations collected in 2012 from Cry3Bb1 history fields exhibiting greater than expected injury (Problem fields, $n = 6$), fields not experiencing unexpected control problems (Field controls, $n = 3$), and laboratory populations that had never been exposed to rootworm-active Cry proteins (Lab controls, $n = 7$ except for mCry3A where $n = 5$). "Bt absent" refers to the non-Bt near isoline hybrid in each figure. Mean values followed by the same letter are not significantly different (two-way mixed model ANOVA, $P > 0.05$; LSMEANS test).

population (L1) when reared on Cry3Bb1. However, the corrected survival for each Cry3Bb1 problem field population on Cry34/35Ab1 corn was very similar to the corrected survival of the laboratory control population (Table 2).

2013 Bioassays. The mean corrected survival on Cry3Bb1 was significantly different among population types ($F = 143.53$; df = 2, 13; $P < 0.0001$). Mean corrected survival of Cry3Bb1 problem field populations was significantly higher and 7.4×–18.5× greater than the mean corrected survival of field and laboratory control populations, respectively, when reared on Cry3Bb1 corn (Table 3). There was no significant difference in mean corrected survival among field and laboratory control populations (Table 3).

A similar pattern was observed when populations were reared on mCry3A, as mean corrected survival was significantly different among population types ($F = 30.43$; df = 2, 11; $P < 0.0001$). Mean corrected survival of Cry3Bb1 problem field populations was significantly higher and 3.7×–5.7× greater than mean corrected survival of laboratory and field control populations, respectively (Table 3). Mean corrected survival of laboratory and field control populations was not significantly different (Table 3).

A significant difference in corrected survival also occurred among population types when reared on Cry34/35Ab1 ($F = 4.97$; df = 2, 13; $P = 0.0249$), but a different pattern was obtained than observed for Cry3Bb1 or mCry3A (Table 3). The mean corrected survival of Cry3Bb1 problem field populations was not significantly different than the mean corrected survival of laboratory control populations, but was significantly different than mean corrected survival of field control populations (Table 3). Similar to Cry3Bb1 and mCry3A analyses, mean corrected survival of laboratory and field control populations was not significantly different (Table 3).

Correlation Analyses. *2013 Bioassays.* There was a significant positive correlation between corrected survival of western corn rootworm larvae on Cry3Bb1 corn and mCry3A corn ($r = 0.868$; df = 9; $P = 0.0005$; Fig. 4A). In contrast, no significant correlation was detected between corrected survival on Cry3Bb1 corn and Cry34/35Ab1 corn ($r = 0.482$; df = 11; $P = 0.0952$) or corrected survival on mCry3A corn and Cry34/35Ab1 corn ($r = 0.555$; df = 9; $P = 0.0761$; Fig. 4B and C).

Discussion

Proportional survival and corrected survival data from 2012 and 2013 bioassays (Tables 2 and 3; Figs. 2 and 3) demonstrate that there are heritable differences in susceptibility of Nebraska western corn rootworm populations to rootworm-active Bt proteins. The field histories (Table 1) and bioassay data jointly support the conclusion that a level of field resistance to Cry3Bb1 in some Nebraska populations has evolved in response to selection pressure. The preliminary data obtained from western corn rootworm collections in 2011 suggest that resistance evolution may have been taking place for some time before formal documentation of resistance in this study. Greater than expected root injury and severe lodging in field PA were documented by an agricultural consultant in 2010, but the susceptibility of the 2010 population was not characterized. Remedial actions mitigated the problem resulting in greatly reduced root injury and lower adult densities in 2011. Nonetheless, the corrected survival of larvae on Cry3Bb1 from the field collection (Table 2) was similar to corrected survival reported for some Cry3Bb1-resistant populations collected in Iowa during 2009

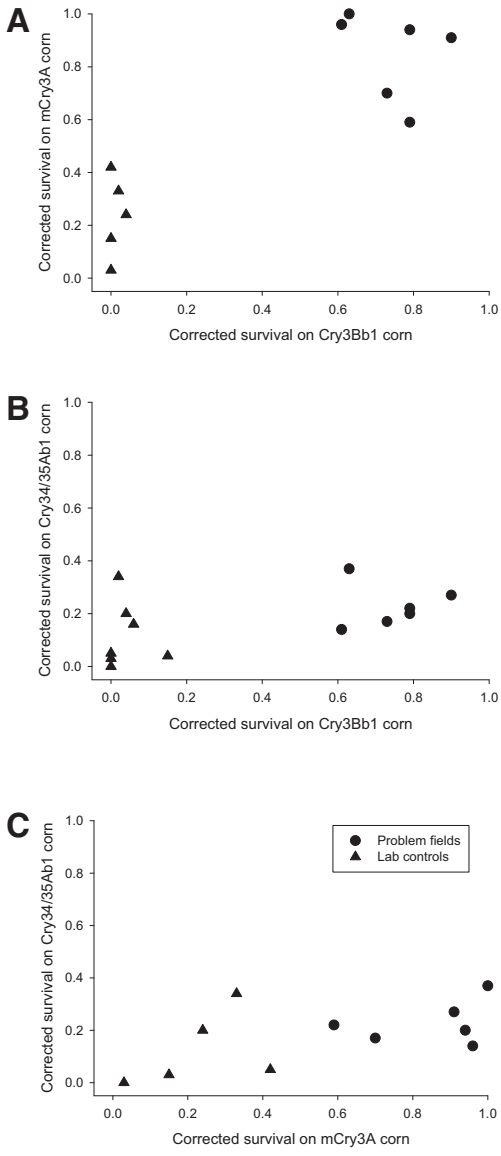

Fig. 4. Correlation among 2012 collected populations for corrected proportional survival of western corn rootworm: (A) Cry3Bb1 and mCry3A corn, (B) Cry3Bb1 and Cry34/35Ab1 corn, and (C) mCry3A and Cry34/35Ab1 corn. Problem fields: Cry3Bb1 history fields exhibiting greater than expected injury ($n = 6$); control populations: laboratory colonies that had never been exposed to rootworm-active Cry proteins ($n = 7$ except for mCry3A where $n = 5$).

(Gassmann et al. 2011). Population PB susceptibility to Cry3Bb1 may have been in transition towards resistance as proportional survival was not significantly different than either PA or the laboratory control (Fig. 2A). The reasons for this are unclear, but collection from only second year corn (Table 1) and immigration from surrounding fields in 2010 following crop rotation were probably contributing factors.

The 2013 bioassays provide a more in-depth understanding of western corn rootworm population susceptibility to rootworm-active Bt traits and confirm resistance to Cry3Bb1 in multiple populations in northeast and southwest Nebraska (Fig. 1; Table 3). Most western corn rootworm populations showing some level of resistance to Cry3Bb1 were planted to a Cry3Bb1-expressing hybrid for at least three and up to six consecutive years (Table 1). It is interesting to note that resistance to Cry3Bb1 was detected in populations from P4 and P5 in only the third consecutive year a Cry3Bb1 hybrid was planted. Because both sites occur in areas where there is a high frequency of annual corn–soybean rotation, it is unlikely that immigration from surrounding resistant populations significantly impacted susceptibility levels. Resistance to Cry3Bb1 has been detected after three generations of selection in previous laboratory studies (Meihls et al. 2008) and under field conditions (Gassmann et al. 2011, 2012). Corrected survival of Cry3Bb1-resistant populations from this study falls within the same range as that reported for Cry3Bb1-resistant populations in Iowa and resistance ratios (i.e., mean corrected survival of Cry3Bb1 problem field populations/mean corrected survival of control populations) were similar or greater than those reported in Iowa (Gassmann et al. 2011, 2012, 2014). Data from this study reconfirm that relatively low Cry3Bb1 resistance ratios can be associated with significant larval root injury by western corn rootworm in commercial cornfields.

The mean proportional survival, corrected survival, and correlation data from 2013 bioassays collectively indicate that a possible cross-resistance relationship exists between Cry3Bb1 and mCry3A (Figs. 3 and 4; Table 3). However, there was no apparent cross-resistance relationship between Cry34/35Ab1 and either Cry3Bb1 or mCry3A corn. This conclusion is also supported by results of recent multitrait field trials conducted at four Cry3Bb1 problem sites in which greater than expected node injury scores (Oleson et al. 2005) were recorded for both Cry3Bb1 and mCry3A treatments (L.J.M., unpublished data). At each site, there was no historical record of mCry3A hybrid use prior to the on-farm trials. Similar results have been previously reported by Gassmann et al. (2014) who documented cross-resistance between Cry3Bb1 and mCry3A and found absence of cross-resistance between Cry3Bb1 and Cry34/35Ab1 or mCry3A and Cry34/35Ab1.

In contrast to results of Cry3Bb1 and mCry3A bioassays, mean proportional survival and corrected survival data from the 2012 and 2013 Cry34/35Ab1 bioassays do not provide evidence of resistance evolution to Cry34/35Ab1. In 2013 Cry34/35Ab1 bioassays, mean corrected survival of problem field populations was significantly greater than the mean survival from field control populations, but the relative survival in all population types was low, especially compared with that recorded from problem field populations in Cry3Bb1 or mCry3A bioassays (Table 3). In addition, the collective range of corrected survival from individual problem field populations and field control populations was very similar to the range exhibited by laboratory control

populations. This is reinforced by the fact that the mean corrected survival of the laboratory control populations was not significantly different than the means of the other two population types (Table 3).

The presence of resistant and susceptible western corn rootworm populations in relatively close proximity (i.e., P1 and C3; P6 and C2) suggests that a mosaic of Cry3Bb1 susceptibility existed in several counties of southwestern Nebraska during 2012. In each county, the Cry3Bb1 problem field and control field were <8 km apart (Fig. 1). A similar situation was reported by Gassmann et al. (2011) in which several Cry3Bb1-resistant populations and a susceptible population co-occurred in the same northeastern Iowa county in 2009. Western corn rootworm resistance to Cry3Bb1 was first documented in northeast Iowa (Gassmann et al. 2011), but the problem is now increasing in geographic scope. This is now a regional issue and covers a large geographical area (Gassmann et al. 2014, Gray 2014). Because the distance between northeast Iowa and southwest Nebraska is extensive and summer prevailing winds are often from the west or southwest in the Corn Belt, it is unlikely that Cry3Bb1 resistance originated in northeast Iowa in 2009 and then spread from one focal point to other areas. Available data collectively suggest that local selection is driving resistance to evolve initially in multiple independent locations following repeated use of Cry3Bb1 corn. Because the mechanism or mechanisms of Cry3Bb1 resistance are currently unknown, it would be interesting to determine if the mechanisms of resistance for populations in southwestern Nebraska are similar to those of populations in northeastern Iowa or central Illinois.

In this study, some field populations were identified that were susceptible to each Bt trait included in bioassays (e.g., population C1). However, susceptible field control populations can be difficult to find especially after a technology has been widely adopted and many target pest populations have had some exposure to the technology. This is especially the case with western corn rootworm because Cry3Bb1 resistance ratios are small. Reduced susceptibility can occur in rootworm field populations used as controls before greater than expected injury occurs, making it difficult to statistically separate survival on the control from survival of a suspected resistant population (Gassmann et al. 2011). The laboratory control populations that were used in this study exhibited a range of susceptibility to rootworm-active Bt traits, especially when challenged with mCry3A and Cry34/35Ab1 (Table 3). By using multiple control populations, more of the natural variability can be captured and reflected in the control. Therefore, use of multiple laboratory control colonies that were established from field collections prior to commercialization of rootworm-active Bt traits arguably provides a more appropriate control than comparison to only one standard susceptible population.

Results presented in this article document that the Gassmann on-plant bioassay technique can be used across laboratories as a diagnostic tool to gain results that are highly correlated with field efficacy. Comparable results were obtained when the same laboratory control colony (denoted as L1) was used in the 2012 and 2013 bioassays conducted in different laboratories at Iowa State University and the University of Nebraska, respectively. The Iowa positive control population used in this study was also bioassayed at Iowa State University in 2013. The very high corrected survival in Iowa State University Cry3Bb1 bioassays and low corrected survival in Cry34/35Ab1 bioassays (i.e., corrected survival: Cry3Bb1: >1.00; Cry34/35Ab1: 0.22 ± 0.02; A.J.G., unpublished data) were similar to the results obtained in Nebraska for IA-R (Table 3). The two laboratories used different corn hybrids in bioassays that expressed the rootworm-active Bt traits of interest, suggesting that specific hybrids are not necessary to obtain reliable bioassay results. It appears that the bioassay technique can be used to generate consistent results regardless of location, time, or hybrid.

Overall, there is a need for integrated approaches to western corn rootworm management that promote long-term gains from sustainable pest population suppression, balance management costs, and preserve yields (Pedigo and Rice 2009, Cullen et al. 2013, Petzold-Maxwell et al. 2013, Sumerford et al. 2013). Many Cry3Bb1 problem fields described in this article have been in corn production for a long time (some up to 20–30 yr), contributing to high annual western corn rootworm densities coupled with repetitive use of Cry3Bb1-expressing hybrids. Many complex factors contribute in different ways to this cropping pattern but many tie into the economics and tradition of corn and livestock production in Nebraska. Because the western corn rootworm is highly adaptable to management practices (Miller et al. 2009) and laboratory selection with all commercially available rootworm Bt traits has led to resistant populations (Lefko et al. 2008, Meihls et al. 2008, 2011, 2012, Oswald et al. 2011, Frank et al. 2013), good stewardship of current and future products is needed to protect against high selection pressure that may accelerate the evolution of resistance (Tabashnik et al. 2004, 2014, EPA 2007). To manage western corn rootworm densities in a sustainable way over time and prolong the life of Bt traits, it is important to move away from use of Bt technologies as stand-alone tactics and instead incorporate trait use with other tactics (e.g., crop rotation, tactic rotation, etc.) into an integrated pest management framework (Gassmann et al. 2012, Cullen et al. 2013). The move by industry to slowly replace single trait hybrids with pyramided corn hybrids should provide greater durability of rootworm-active Bt technologies (Onstad and Meinke 2010, Prasifka et al. 2013, Head et al. 2014), although it is unclear how pyramids that include a trait that is compromised by some level of resistance will perform over time in the field (Gassmann et al. 2014). As new transgenic technologies are developed for western corn rootworm management, especially if they are less than high dose, it will be important to incorporate those into an integrated pest management framework at the time of commercialization and not wait until mitigation is needed in the field to take a multitactic approach to management.

Acknowledgments

We thank James Brown and summer interns for helping with western corn rootworm collection from the field and laboratory rearing. We also thank Monsanto Company, St. Louis, MO; DuPont-Pioneer, Johnston, IA; and Syngenta Seeds, Minnetonka, MN, for providing seed for this study. This study was funded by Biotechnology Risk Assessment Grant Program competitive grant 2012-33522-20010 from the U.S. Department of Agriculture (USDA) National Institute of Food and Agriculture and by Nebraska Agricultural Experiment Station–Enhanced Hatch-Multi State Project NEB-28-106.

References Cited

(EPA) Environmental Protection Agency. 2003. *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (vector zmir13L) in event MON863 corn (006484) fact sheet. (http://www.epa.gov/oppbppd1/biopesticides/ingredients_keep/factsheets/factsheet_006484.htm) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2005. Biopesticide registration action document, *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in event DAS-59122-7 corn. (http://www.epa.gov/pesticides/chem_search/reg_actions/registration/decision_PC-006490_1-Oct-05.pdf) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2007. Biopesticide registration action document. Modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 Corn SYN-IR604-8. (http://www.epa.gov/opp00001/chem_search/reg_actions/registration/decision_PC-006509_1-Mar-07.pdf) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2011. 2009 resistance monitoring review for Cry3Bb1. (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0922-0003) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2012a. Current & previously registered section 3 PIP registrations. (http://www.epa.gov/pesticides/biopesticides/pips/pip_list.htm) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2012b. Pesticide product registration, 5307 corn. (http://www.epa.gov/pesticides/chem_search/ppls/067979-00022-20120731.pdf) (accessed 19 January 2015).

(EPA) Environmental Protection Agency. 2013. Pesticide product registration, Btl 1 x MIR162 x MIR604 x TCI507 x 5307 5% Refuge Seed Blend Corn. (http://www.epa.gov/pesticides/chem_search/ppls/067979-00026-20130802.pdf) (accessed 19 January 2015).

Abbott, W. S. 1925. A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265–267.

Abendroth, L. J., R. W. Elmore, M. J. Boyer, and S. K. Marlay. 2011. Corn growth and development. Iowa State University, Ames, IA.

Ball, H. J., and G. T. Weekman. 1962. Insecticide resistance in the adult western corn rootworm in Nebraska. J. Econ. Entomol. 55: 439–441.

Bare, O. S. 1930. Corn root-worm does damage in southwestern Nebraska. Annual Report of Cooperative Extention work in Agriculture and Home Economics, Entomology, State of Nebraska, 21.

Bryson, H. R., D. A. Wilbur, and C. C. Burkhardt. 1953. The western corn rootworm *Diabrotica virgifera* Lec. in Kansas. J. Econ. Entomol. 46: 955–959.

Clark, T. L., D. L. Frank, B. W. French, L. J. Meinke, D. Moellenbeck, T. T. Vaughn, and B. E. Hibbard. 2012. Mortality impact of MON863 transgenic maize roots on western corn rootworm larvae in the field. J. Appl. Entomol. 136: 721–729.

Cullen, E. M., M. E. Gray, A. J. Gassmann, and B. E. Hibbard. 2013. Resistance to Bt corn by western corn rootworm (Coleoptera: Chrysomelidae) in the US Corn Belt. J. Int. Pest Manag. 4: D1–D6.

Fisher, J. R. 1989. Hatch of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) eggs exposed to two different overwintering and hatch regimes. J. Kansas Entomol. Soc. 62: 607–610.

Frank, D. L., A. Zukoff, J. Barry, M. L. Higdon, and B. E. Hibbard. 2013. Development of resistance to eCry3.1Ab-expressing transgenic maize in a laboratory-selected population of western corn rootworm (Coleoptera:Chrysomelidae). J. Econ. Entomol. 106: 2503–2513.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS One 6: e22629.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2012. Western corn rootworm and Bt maize: challenges of pest resistance in the field. GMCrops Food 3: 235–244.

Gassmann, A. J., J. L. Petzold-Maxwell, E. H. Clifton, M. W. Dunbar, A. M. Hoffmann, D. A. Ingber, and R. S. Keweshan. 2014. Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize. Proc. Natl. Acad. Sci. 111: 5141–5146.

Gatehouse, J. A. 2008. Biotechnological prospects for engineering insect-resistant plants. Plant Physiol. 146: 881–887.

Godfrey, L. D., L. J. Meinke, and R. J. Wright. 1993. Field corn vegetative and reproductive biomass accumulation: response to western corn rootworm (Coleoptera: Chrysomelidae) root injury. J. Econ. Entomol. 86: 1557–1573.

Gray, M. E. 2012. Continuing evolution confirmed of field resistance to Cry3Bb1 in some Illinois fields by western corn rootworm. The Bulletin, 24 August 2012, Univ. Ill. Ext. (http://bulletin.ipm.illinois.edu/article.php?id=1704) (accessed 17 January 2015).

Gray, M. E. 2014. Field evolved western corn rootworm resistance to Bt (Cry3Bb1) confirmed in three additional Illinois counties. The Bulletin, 3 April 2014, Univ. Ill. Ext. (http://bulletin.ipm.illinois.edu/?p=1913) (accessed 17 January 2015).

Gray, M. E., and K. L. Steffey. 1998. Corn rootworm (Coleoptera: Chrysomelidae) larval injury and root compensation of 12 hybrids: an assessment of the economic injury index. J. Econ. Entomol. 91: 723–740.

Gray, M. E., T. W. Sappington, N. J. Miller, J. Moeser, and M. O. Bohn. 2009. Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annu. Rev. Entomol. 54: 303–321.

Head, G., M. Carroll, T. Clark, T. Galvan, R. M. Huckaba, P. Price, L. Samuel, and N. P. Storer. 2014. Efficacy of SmartStax- insect-protected corn hybrids against corn rootworm: the value of pyramiding the Cry3Bb1 and Cry34/35Ab1 proteins. Crop Prot. 57: 38–47.

Hibbard, B. E., D. L. Frank, R. Kurtz, E. Boudreau, M. R. Ellersieck, and J. F. Odhiambo. 2011. Mortality impact of Bt transgenic maize roots expressing eCry3.1Ab, mCry3A, and eCry3.1Ab plus mCry3A on western corn rootworm larvae in the field. J. Econ. Entomol. 104: 1584–1591.

Hibbard, B. E., T. L. Clark, M. R. Ellersieck, L. N. Meihls, A. A. El Khishen, V. Kaster, H. Y. Steiner, and R. Kurtz. 2010. Mortality of western corn rootworm larvae on MIR604 transgenic maize roots: field survivorship has no significant

impact on survivorship of F1 progeny on MIR604. J. Econ. Entomol. 103: 2187–2196.

Lefko, S. A., T. M. Nowatzki, S. D. Thompson, R. R. Binning, M. A. Pascual, M. L. Peters, E. J. Simbro, and B. H. Stanley. 2008. Characterizing laboratory colonies of western corn rootworm (Coleoptera: Chrysomelidae) selected for survival on maize containing event DAS-59122-7. J. Appl. Entomol. 132: 189–204.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of *diabroticite* rootworms in corn. Ann. Rev. Entomol. 36: 229–255.

Levine, E., J. L. Spencer, S. A. Isard, D.W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Meihls, L. N., M. L. Higdon, B. D. Siegfried, N. J. Miller, T. W. Sappington, M. R. Ellersieck, T. A. Spencer, and B. E. Hibbard. 2008. Increased survival of western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. Proc. Natl. Acad. Sci. USA 105: 19177–19182.

Meihls, L. N., M. L. Higdon, M. Ellersieck, and B. E. Hibbard. 2011. Selection for resistance to mCry3A-expressing tansgenic corn in western corn rootworm. J. Econ. Entomol. 104: 1045–1054.

Meihls, L. N., M. L. Higdon, M. R. Ellersieck, and B. E. Hibbard. 2012. Greenhouse-selected resistance to Cry3Bb1-producing corn in three western corn rootworm populations. PloS One 7: e51055.

Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.

Meinke, L. J., T. W. Sappington, D. W. Onstad, T. Guillemaud, N. J. Miller, K. Judith, L. Nora, L. Furlan, K. Jozsef, and T. Ferenc. 2009. Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. J. Agric. Forest Entomol. 11: 29–46.

Miller, N. J., T. Guillemaud, R. Giordano, B. D. Siegfried, M. E. Gray, L. J. Meinke, and T. W. Sappington. 2009. Genes, gene flow and adaptation of *Diabrotica virgifera virgifera*. J. Agric. Forest Entomol. 11: 47–60.

Oleson, J. D., Y. Park, T. M. Nowatzki, and J. J. Tollefson. 2005. Node-injury scale to evaluate root injury by corn rootworms (Coleoptera: Chrysomelidae). J. Econ. Entomol. 98: 1–8.

Onstad, D. W., and L. J. Meinke. 2010. Modeling evolution of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to transgenic corn with two insecticidal traits. J. Econ. Entomol. 103: 849–860.

Oswald, K. J., B. W. French, C. Nielson, and M. Bagley. 2011. Selection for Cry3Bb1 resistance in a genetically diverse population of non diapausing western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 104: 1038–1044.

Pedigo, L. P., and M. E. Rice. 2009. Entomology and pest management. Prentice Hall, Upper Saddle River, NJ.

Petzold-Maxwell, J. L., A. P. Alves, R. E. Estes, M. E. Gray, L. J. Meinke, E. J. Shields, S. D. Thompson, N. A. Tinsley, and A. J. Gassmann. 2013. Applying an integrated refuge to manage western corn rootworm (Coleoptera: Chrysomelidae): effects on survival, fitness, and selection pressure. J. Econ. Entomol. 106: 2195–2207.

Potter, B., and K. Ostlie. 2011. Performance problems with Bt- rootworm corn: trait resistance in Minnesota and corn rootworm management. Cropping systems team. (http://www.nwroc.umn.edu/prod/groups/cfans/@pub/@cfans/@swroc/documents/asset/cfans_asset_404474.pdf) (accessed 17 January 2015).

Prasifka, P.L., D. M. Rule, N. P. Storer, S. P. Nolting, and W. H. Hendrix III. 2013. Evaluation of corn hybrids expressing Cry34Ab1/Cry35Ab1 and Cry3Bb1 against the western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 106: 823–829.

Rice, M. E. 2004. Transgenic rootworm corn: assessing potential agronomic, economic, and environmental benefits. Online. Plant Health Progress. Doi: 10.1094/PHP-2004-0301-01- RV. (https://www.plantmanagementnetwork.org/sub/php/review/2004/rootworm/Rice.pdf) (accessed 19 January 2015).

Sappington, T.W., B. D. Siegfried, and T. Guillemaud. 2006. Coordinated *Diabrotica* genetics research: accelerating progress on an urgent insect pest problem. Am. Entomol. 52: 90–97.

SAS Institute. 2010. SAS/ STAT User's, 3rd ed., SAS Institute Inc, Cary, NC.

Snedecor, W. G., and W. G. Cochran. 1989. Statistical methods, 8th ed. Iowa State University Press, Ames, IA.

Sumerford, D. V., G. P. Head, A. Shelton, J. Greenplate, and W. Moar. 2013. Field-evolved resistance: assessing the problem and moving forward. J. Econ. Entomol. 106: 1525–1534.

Storer, N. P. 2003. A spatially explicit model simulating western corn rootworm (Coleoptera: Chrysomelidae) adaptation to insect-resistant maize. J. Econ. Entomol. 96: 1530–1547.

Storer, N. P., J. M. Babcock, and J. M. Edwards. 2006. Field measures of western corn rootworm (Coleoptera: Chrysomelidae) mortality caused by Cry34/35Ab1 proteins expressed in maize event 59122 and implications for trait durability. J. Econ. Entomol. 99: 1381–1387.

Tabashnik, B. E. 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. USA 105: 19029–19030.

Tabashnik, B. E., A. J. Gassmann, D. W. Crowder, and Y. Carriere. 2008. Insect resistance to Bt crops: evidence versus theory. Nat. Biotechnol. 26: 199–202.

Tabashnik, B. E., F. Gould, and Y. Carriere. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904–912.

Tabashnik, B. E., J.B.J. Van Rensburg, and Y. Carrie're. 2009. Field-evolved insect resistance to Bt crops: definition, theory, and data. J. Econ. Entomol. 102: 2011–2025.

Tabashnik, B. E., D. Mota-Sanchez, M. E. Whalon, R. M. Hollingworth, and Y. Carrie're 2014. Defining terms for proactive management of resistance to Bt crops and pesticides. J. Econ. Entomol. 107: 496–507.

Tate, H. D., and O. S. Bare. 1946. Corn rootworms. Nebraska Agric. Exper. Station Bul. 381: 1–12.

Urías-López, M. A., and L. J. Meinke. 2001. Influence of western corn rootworm (Coleoptera: Chrysomelidae) larval injury on yield of different types of maize. J. Econ. Entomol. 94: 106–111.

Vaughn, T., T. Cavato, G. Brar, T. Coombe, T. DeGooyer, S. Ford, M. Groth, A. Howe, S. Johnson, K. Kolacz, C. Pilcher, J. Purcell, C. Romano, L. English, and J. Pershing. 2005. A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize. Crop Sci. 45: 931–938.

Wright, B., and L. Meinke. 2011. Corn rootworm management update: ensure diversity when selecting seed for 2012. CropWatch, 22 Sept 2011, Univ. Nebr. Ext. (http://cropwatch.unl.edu/archive/-/asset_publisher/VHeSpfv0Agju/content/4651826) (accessed 17 January 2015).

Received 18 July 2014; accepted 3 December 2014.