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Jennifer L. Petzold-Maxwell  
*Iowa State University*, [jennifer.maxwell@wartburg.edu](mailto:jennifer.maxwell@wartburg.edu)

Lance J. Meinke  
*University of Nebraska-Lincoln*

Michael E. Gray  
*University of Illinois at Urbana-Champaign*

Ronald E. Estes  
*University of Illinois at Urbana-Champaign*

Aaron J. Gassmann  
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*Iowa State University*, [aaronjg@iastate.edu](mailto:aaronjg@iastate.edu)

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Author(s): Jennifer L. Petzold-Maxwell, Lance J. Meinke, Michael E. Gray, Ronald E. Estes, and Aaron J. Gassmann

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# Effect of Bt Maize and Soil Insecticides on Yield, Injury, and Rootworm Survival: Implications for Resistance Management

JENNIFER L. PETZOLD-MAXWELL,<sup>1,2</sup> LANCE J. MEINKE,<sup>3</sup> MICHAEL E. GRAY,<sup>4</sup>  
RONALD E. ESTES,<sup>4</sup> AND AARON J. GASSMANN<sup>1</sup>

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**ABSTRACT** A 2-yr field experiment was conducted to determine the effects on *Diabrotica* spp. (Coleoptera: Chrysomelidae) of an insecticidal seed treatment (Poncho 1250, (AI) / clothianidin) and a granular insecticide (Aztec 2.1G, (AI) / tebufospyr and cyfluthrin) alone and in combination with maize producing the insecticidal toxin Cry3Bb1 derived from the bacterium *Bacillus thuringiensis* (Bt). Yields for Bt maize plots were significantly greater than for non-Bt maize; however, insecticides did not significantly affect yield. Insecticides significantly decreased root injury in non-Bt maize plots, but there were no significant differences in root injury between Bt maize with or without either insecticide. Maize producing the Bt toxin Cry3Bb1 and the soil-applied insecticide Aztec significantly decreased survival of western corn rootworm (*Diabrotica virgifera virgifera* LeConte), while only Bt maize significantly decreased survival of the northern corn rootworm (*Diabrotica barberi* Smith & Lawrence). For both species, Bt maize and each of the insecticides delayed emergence. In the absence of density-dependent mortality, Bt maize imposed 71 and 80% reduction in survival on the western corn rootworm and the northern corn rootworm, respectively. The data from this study do not support combining insecticide with Bt maize because the addition of insecticide did not increase yield or reduce root injury for Bt maize, and the level of rootworm mortality achieved with conventional insecticide was likely too low to delay the evolution of Bt resistance. In addition, delays in emergence from Bt maize combined with insecticides could promote assortative mating among Bt-selected individuals, which may hasten resistance evolution.

**KEY WORDS** *Diabrotica barberi*, *Diabrotica virgifera virgifera*, pesticide mixture, pyramid, resistance management

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and the northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, (Coleoptera: Chrysomelidae) are major pests of maize (*Zea mays* L.) in the United States (Gray et al. 2009). Larval injury to maize roots can result in substantial reductions in yield (Dun et al. 2010, Tinsley et al. 2012). In 2003, genetically modified maize producing insecticidal toxins derived from the bacterium *Bacillus thuringiensis* Berliner (Bt) was commercialized for management of rootworm species (Environmental Protection Agency [EPA] 2003).

Recently, field-evolved resistance to Bt maize producing the Cry3Bb1 toxin was documented in Iowa (Gassmann et al. 2011, 2012; Gassmann 2012). For the western corn rootworm, widespread field-evolved resistance to Bt maize is a potential threat, given this insect's history of rapid adaptation to numerous man-

agement practices, including crop rotation and some conventional insecticides (Meinke et al. 1998, Wright et al. 2000, Levine et al. 2002). In addition, evidence from recent studies suggests a lack of substantial fitness costs of resistance (Oswald et al. 2011, Meihls et al. 2012, Petzold-Maxwell et al. 2012a), and nonrecessive inheritance of resistance (Meihls et al. 2008, Petzold-Maxwell et al. 2012a), both of which increase the risk of resistance evolution. Thus, sustainable insect resistance management (IRM) strategies are essential for prolonging the effectiveness of Bt maize for management of western corn rootworm.

Currently, the United States EPA mandates resistance management for all registered Bt crops, which includes the refuge strategy (Gould 1998, Tabashnik et al. 2003). Under the refuge strategy, non-Bt plants serve as a refuge for Bt-susceptible genotypes, providing a pool of susceptible individuals to mate with resistant individuals that survive exposure to a Bt crop (Gould 1998). The evolution of resistance will be delayed when the heterozygous progeny that result from these matings have lower fitness on the Bt crop than their homozygous resistant parent. Delays in resistance are greatest when Bt crops achieve a high dose, killing 99.99% of susceptible pests, and rendering

<sup>1</sup> Department of Entomology, Iowa State University, 13 Insectary, Ames, IA 50011.

<sup>2</sup> Corresponding author, e-mail: [jpetzold@iastate.edu](mailto:jpetzold@iastate.edu).

<sup>3</sup> Department of Entomology, University of Nebraska, 103 Entomology Hall, Lincoln, NE 68583.

<sup>4</sup> Department of Crop Sciences, University of Illinois, 1102 S. Goodwin Ave., Urbana, IL 61801.

resistance a functionally recessive trait (Tabashnik 1994, Gould 1998). None of the currently commercialized Bt toxins targeting western corn rootworm achieve a high dose (Gassmann 2012).

Another method for delaying resistance is the application of integrated pest management (IPM), which uses multiple methods to reduce pest abundance and preserve yield (Pedigo and Rice 2009). Because IPM relies on multiple methods, selection on pest populations for resistance to any single method is reduced. Insecticides, whether produced by the plant (i.e., Bt crops) or applied to agricultural fields, can be an essential IPM tool. Furthermore, pyramiding of multiple insecticides can delay resistance compared with when insecticides are used separately (Roush 1998). This occurs because each insecticide kills insects that are susceptible to that toxin, and by doing so, also kills insects harboring resistance alleles for the second insecticide (Gould 1998, Roush 1998). To date, few data exist on the potential IRM benefit of pyramiding Bt maize with conventional insecticides targeting western corn rootworm.

The planting of crops genetically engineered to produce two or more Bt toxins targeting a single pest is becoming a more common IRM strategy (James 2012). Simulation models have shown that pyramiding insecticidal Bt toxins in a plant can be more effective at delaying resistance than single toxins (Roush 1998, Zhao et al. 2003, Ives et al. 2011). For western corn rootworm, models predict that plants pyramided with two Bt toxins delay resistance longer than single-toxin plants (Onstad and Meinke 2010). For example, with a 20% block refuge, resistance evolved in 60–64 yr for plants pyramided with Cry3Bb1 and Cry34/34Ab1, whereas resistance evolved much faster for single-traited maize hybrids (3 yr for Cry3Bb1 maize and 12 yr for Cry34/35Ab1 maize) (Onstad and Meinke 2010). Another potential IRM strategy is pyramiding two or more conventional chemical insecticides (Curtis 1985, Mani 1985). Models predict delays in resistance evolution when two or more insecticides are pyramided compared with sequential use, but this is dependent on a number of factors including low initial resistance allele frequency, high proportion mortality caused by both insecticides, adequate refuges, lack of cross-resistance between the two insecticides, and recessive inheritance of resistance for both toxins (Curtis 1985, Mani 1985, Tabashnik 1989, Caprio 1998).

A potential alternative IRM strategy would be to pyramid a Bt crop with a conventional insecticide. Gray and Steffey (2007) showed that plots with Bt maize producing Cry3Bb1 used in combination with the insecticide Counter 15G ((AI)/terbufos) had a mean node-injury score of 0.07 nodes for early root ratings, which was significantly lower than Cry3Bb1 maize alone (0.84 nodes) (Gray and Steffey 2007). However, this difference in node injury scores between Bt maize and Bt maize plus Counter 15G only occurred in one of the three study locations (Gray and Steffey 2007). A study in Iowa showed that the granular insecticides Force 3G ((AI)/tefluthrin) and Aztec 2.1 G used in combination with Bt maize producing

Cry3Bb1 significantly reduced node injury scores compared with Bt maize alone in fields with populations of western corn rootworm that were resistant to Cry3Bb1 maize (Gassmann 2012). Model simulations have shown that combining Bt maize with a conventional insecticidal seed treatment could delay rootworm resistance if refuges also are present (Pan et al. 2011). Although these data suggest a potential benefit of pyramiding soil insecticides or insecticides applied as seed treatments with Bt maize, effects on pest mortality in the field are largely unknown.

Thus, more studies are necessary to understand possible benefits and drawbacks of combining Bt maize with either a soil-applied insecticide or an insecticidal seed treatment targeting rootworm species. By combining these tactics, insects that are resistant to Bt maize could be killed by the insecticide, thereby delaying evolution of Bt resistance. However, the cost to farmers of using an insecticide may not be offset by increased yield, and pyramiding Bt crops with chemical insecticides would diminish the environmental benefit of reduced use of chemical insecticides that often accompanies the planting of Bt crops (Carpenter and Gianessi 2001, Shelton et al. 2002, Naranjo 2009). In addition, it is possible that pyramiding an insecticide with Bt maize may hasten resistance evolution. For instance, Gray et al. (1992) found that in certain years, greater western corn rootworm adults emerged from insecticide-treated plots as compared with control plots in which no soil insecticides were used during planting. The authors pointed out that soil insecticides applied at time of planting were not population-management tools, again indicating that additional research is required before these insecticides are considered resistance management tools. Here, we examine the effects of conventional insecticides and Cry3Bb1 maize on root injury and yield, and on survival, size, and timing of emergence of western corn rootworm and northern corn rootworm.

## Materials and Methods

**Experimental Design.** An insecticidal seed treatment and a planting-time granular soil insecticide, used alone and in combination with either Bt or non-Bt maize, were tested for effects on 1) rootworm survival, timing of emergence, mass, and size; and 2) maize root injury and yield. Field studies were conducted in 2008 and 2009 at three locations: 1) Iowa (Sutherland [2008] and Ames [2009]), 2) Nebraska (Mead), and 3) Illinois (Urbana). In 2009, the Illinois site was flooded from heavy rain, and those data were excluded. The Bt maize used in this study produced the Cry3Bb1 toxin, which is event MON88017 in DeKalb hybrid DKC 61–69, relative maturity = 111 d (Monsanto, Saint Louis, MO). Non-Bt maize was the near-isoline to the Bt maize hybrid but lacked any rootworm active Bt toxins (DKC: 61–72, relative maturity = 111 d). Insecticide treatments were 1) the seed treatment Poncho 1250 (clothianidin, 1.25 mg (AI)/seed, Bayer Crop Science, Research Triangle Park, NC), hereafter referred to as P1250; 2) the gran-

ular soil insecticide Aztec 2.1G (tebupirimphos + cyfluthrin, owned by Bayer CropSciences at the time of the study and now owned by AMVAC, Newport Beach, CA), applied in furrow at a rate of 8.18 kg/ha, hereafter referred to as Aztec; and 3) the control treatment, which did not receive a granular insecticide but received the seed treatment Cruiser 250 (thiamethoxam, 0.25 mg (AI)/seed, Syngenta, Basel, Switzerland) to control secondary pests of maize seedlings. The study was a split-plot design, with maize type (Bt vs. non-Bt) as the whole-plot factor and insecticide treatment (P1250, Aztec, and control) as the split-plot factor. Each block was 21 m long and contained a total of six eight-row plots (2 hybrids  $\times$  3 insecticide treatments), with 0.76 m between rows. Each row contained  $\approx$ 120 plants, with 15 cm between plants. There were four blocks at each location, during each year of the study. The study site was surrounded by eight rows of maize on all sides. Study sites had been planted to a trap crop during the previous year. A trap crop is late-planted maize that attracts adult western corn rootworm and northern corn rootworm that oviposit in the soil. Fields were cultivated and fertilized following standard regional agricultural practices used in maize production.

**Root Injury and Yield.** For each treatment within each block (i.e., each plot), five maize plants were excavated to determine root injury by rootworm larvae. Roots were taken from the penultimate row along either edge of the plot, with a total of 20 plants sampled for each combination of hybrid by insecticide treatment at each location within each year. In total, 120 plants were sampled at each location during each year (5 plants per eight-row treatment plot  $\times$  2 maize hybrids  $\times$  3 insecticide treatments  $\times$  4 blocks). Injury was scored based on a node-injury scale (Oleson et al. 2005) that ranges from zero (no feeding injury) to three nodes of root tissue injured (heavy feeding injury). The center four rows of each eight-row plot were harvested, and mass of grain was converted to metric tons per ha at 15.5% moisture (United States no. 2 shelled corn).

**Rootworm Survival, Emergence Time, Mass, and Size.** Rootworm survival was measured by placing three Illinois-style emergence cages in the penultimate row along either edge of the plot, for a total of 72 emergence cages at each location within each year (3 cages per plot  $\times$  2 maize hybrids  $\times$  3 insecticide treatments  $\times$  4 blocks). Emergence cages were constructed based on a modified design of Fisher (1980), enclosing the base of the plant such that beetles were trapped but the plant continued to grow. Cages were placed in the field before the emergence of rootworm adults from the soil, and rootworm were collected from cages three times per week until adult emergence did not occur for at least one full week. Rootworm adults collected in Nebraska and Illinois in both years were shipped to Iowa State University where they were separated by species then by sex following Hammack and French (2007). For each day rootworm were collected from emergence cages, data were recorded on the number of males and females of each

species per treatment per block from each location. At each location during each year, the day of emergence was calculated for each individual, with day 1 defined as the day the first adult rootworm was collected. Head capsule width (measured as the distance between the outer edges of the eyes) was measured for each insect to the nearest 0.04 mm using a stage micrometer. Insects were then dried in an oven at 60°C for 48 h and weighed to the nearest 0.1 mg (XS205 analytical balance, Mettler-Toledo, Columbus, OH).

**Proportional Reduction in Adult Survival.** The proportional reduction in adult survival of western corn rootworm and northern corn rootworm was calculated for P1250, Aztec, Cry3Bb1 maize, P1250 + Cry3Bb1 maize, and Aztec + Cry3Bb1 maize as the compliment of the number of adults collected from each of these treatments divided by the number of adults collected from non-Bt maize without insecticide. First, at each location within a year, the average number of insects per emergence cage from non-Bt plots with no insecticide was calculated by block. Then, for each block within a location and year, the number of insects per emergence cage for a given rootworm treatment was divided by the average number of insects per emergence cage for non-Bt maize without insecticide. Density-dependent mortality is an important factor to consider when measuring survival of rootworm in the field (Hibbard et al. 2010b). Hibbard et al. (2010b) determined that density-dependent mortality can occur when average root injury exceeds 1 node on the 0–3 node injury scale (Oleson et al. 2005). Injury over 1 node occurred in all locations with the exception of Iowa in 2009 and Nebraska in 2008 (see Results); therefore, only these two locations were used to calculate the reduction in adult emergence imposed by Bt maize, insecticides, and the combination of Bt maize with insecticides. Thus, proportional reduction in survival for each of the five rootworm treatments was calculated based on eight blocks (2 locations  $\times$  4 blocks).

**Data Analysis.** All analyses were conducted in SAS Enterprise Guide 4.2 (SAS Institute 2009) and used a mixed-model analysis of variance (ANOVA) that was run in PROC MIXED. For all models, the significance of random effects was tested based on a log-likelihood ratio statistic ( $-2$  RES Log Likelihood), which follows a  $\chi^2$  distribution with a  $P$  value equal to one-half the probability of obtaining a greater  $\chi^2$  value assuming 1 degree of freedom (Littell et al. 1996). When significant fixed factors were present, pairwise comparisons were conducted based on the Tukey–Kramer method (PDIFF option in PROC MIXED).

For yield and root injury, data were analyzed based on a split-plot design. Fixed effects in the model were maize hybrid (Bt and non-Bt), insecticide (control, P1250, and Aztec), and their interaction. Hybrid was the whole plot factor, and insecticide was the split-plot factor. Random factors included block nested within location and year, and the interaction of block (nested within location and year) with hybrid, and with hybrid by insecticide. Root injury data were transformed by the square root function to ensure normality of the

residuals (untransformed data are presented in graphs).

For survival, day of adult emergence, mass and size, data were analyzed with an ANOVA that included the fixed effects of maize hybrid (Bt and non-Bt), insecticide (control, P1250, and Aztec), sex of an insect, and all possible interactions among these three factors, where hybrid was analyzed as the whole plot factor, and insecticide as the split-plot factor. Random factors included block (nested within location and year), and the interaction of block (nested within location and year) with sex, hybrid, insecticide by hybrid, sex by hybrid, and sex by insecticide by hybrid. To ensure normality of the residuals, survival data for northern and western corn rootworm were transformed by the function  $\log(n + 1)$ , and day of adult emergence for both species as well as mass of western corn rootworm were transformed with the square-root function (untransformed data are presented in graphs).

Analysis of proportional reduction in adult emergence by both of the insecticides (P1250 and Aztec), Bt maize, and Bt maize in combination with each of the insecticides used data from only Iowa in 2009 and Nebraska in 2008 to avoid confounding effects of density-dependent mortality. Separate analyses were run for western corn rootworm and northern corn rootworm. The fixed factor in the model was rootworm treatment (Aztec, P1250, Bt maize, Bt maize plus Aztec, and Bt maize plus P1250), and random factors in the model were block (nested within location and year), and the interaction of block (nested within location and year) with rootworm treatment. Least-squares means for each rootworm treatment were calculated using the LSMEANS statement and tested against 0 to determine if reductions in survival were significantly different from zero based on a two-tailed *t*-test (default output for LSMEANS in PROC MIXED). Pairwise comparisons among treatments were conducted based on the Tukey-Kramer method (PDIFF in PROC MIXED).

**Results**

**Yield and Root Injury.** Yield was significantly affected by maize hybrid, with greater yield for Bt maize than non-Bt maize (Table 1; Fig. 1A). No other factors were significant. For root injury, there was a significant interaction between hybrid and insecticide (Table 1; Fig. 1B). Root injury did not differ significantly among treatments for Bt maize. However, for non-Bt maize roots, injury was significantly lower for both P1250 and Aztec compared with the control, and for Aztec compared with P1250 (Fig. 1B).

**Survival and Emergence Timing.** Survival of western corn rootworm was significantly lower on Bt maize than on non-Bt maize (Table 2; Fig. 2A). Insecticide treatment was also a significant effect in the model (Table 2). Significantly fewer beetles survived in the Aztec treatment compared with the control ( $P = 0.0003$ ) and P1250 treatment ( $P = 0.004$ ), but there was no significant difference between the control and the P1250 treatment ( $P = 0.72$ ) (Fig. 2A). Survival of

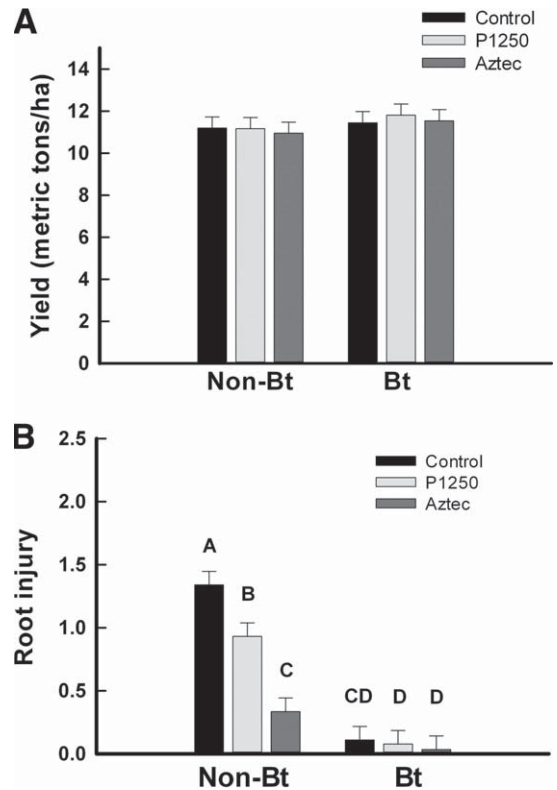
**Table 1.** Analysis of variance for yield and root injury

Source	Fixed effects			
	Yield		Root Injury	
	df <sup>a</sup>	F value	df	F value
Hybrid	1,19	4.48*	1,19	80.41***
Insecticide	2,73	0.62	2,76	30.45***
Hybrid × insecticide	2,73	0.46	2,76	13.98***
Random effects				
Source	df	Yield $\chi^2$	Root Injury $\chi^2$	
Block (site × year)	1	115.2***	282.5***	
Hybrid × block (site × year)	1	2.3	145.9***	
Insecticide × hybrid × block (site × year)	1	0.0	255.0***	

<sup>a</sup> df, numerator degrees of freedom, denominator degrees of freedom.

\*  $P < 0.05$ ; \*\*\*  $P < 0.0001$ .

western corn rootworm was lowest on Bt maize plus Aztec (Fig. 2A), which was also significantly lower than on Bt maize alone ( $P = 0.02$ ). Female survival was significantly greater than male survival (Table 2; Fig. 2A). No other factors in the model were significant.



**Fig. 1.** (A) Yield and (B) root injury for Bt and non-Bt maize with and without soil-applied insecticides. Root injury scores are based on a 0–3 scale (Oleson et al. 2005), with each integer indicating the number of nodes pruned, up to three nodes. Bar heights represent sample means and error bars are the SEM. For (B) root injury, letters represent pairwise differences among means.

**Table 2.** Analysis of variance for survival, emergence time, mass, and size of western corn rootworm

Source	Fixed effects							
	Survival		Emergence		Mass		Size	
	df <sup>a</sup>	F value	df	F value	df	F value	df	F value
Hybrid	1,19	64.62***	1,18	87.28***	1,17	0.00	1,15	22.80**
Insecticide	2,74	9.58***	2,60	12.69***	2,57	0.95	2,56	0.62
Sex	1,19	19.47***	1,18	46.99***	1,17	7.92*	1,15	5.64*
Hybrid × insecticide	2,74	1.35	2,60	1.34	2,57	0.26	2,56	0.26
Hybrid × sex	1,19	0.19	1,18	1.55	1,17	5.04*	1,15	0.35
Insecticide × sex	2,74	0.46	2,60	0.07	2,57	1.37	2,56	0.40
Hybrid × insecticide × sex	2,74	0.65	2,60	0.17	2,57	0.98	2,56	1.30
Random effects								
Source	df	Survival $\chi^2$	Emergence $\chi^2$	Mass $\chi^2$	Size $\chi^2$			
Block (site × year)	1	213.6***	243.7***	33.4***	190.9***			
Hybrid × block (site × year)	1	31.6***	52.1***	19.9***	4.3*			
Sex × block (site × year)	1	0.0	23.1***	1.6	3.8*			
Hybrid × sex × block (site × year)	1	0.0	0.0	0.0	1.4			
Hybrid × insecticide × block (site × year)	1	57.2***	102.4***	6.9**	65.4***			
Hybrid × Insecticide × sex × block (site × year)	1	0.0	37.9***	72.6***	1.7			

<sup>a</sup> df, numerator degrees of freedom, denominator degrees of freedom.  
 \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

Day of emergence for western corn rootworm was significantly affected by hybrid, the sex of a beetle, and the type of insecticide (Table 2; Fig. 2B). Emergence was significantly later for beetles in Bt plots versus non-Bt plots, and for females. Insects emerged significantly later from Aztec plots compared with control plots ( $P < 0.0001$ ) and P1250 plots ( $P = 0.02$ ), and from P1250 plots compared with control plots ( $P = 0.05$ ).

At the Illinois site, all beetles collected were western corn rootworm; however, northern corn rootworm were present in Iowa and Nebraska. Survival of northern corn rootworm was significantly reduced in Bt maize plots (Table 3; Fig. 2C); no other factors significantly affected survival. Insecticide treatment had a significant effect on day of emergence for northern corn rootworm (Table 3; Fig. 2D). Adults from control plots emerged significantly earlier than adults from Aztec plots ( $P = 0.04$ ) and P1250 plots ( $P = 0.05$ ), but no difference was detected between Aztec and P1250 plots ( $P = 0.97$ ). There was also a significant interaction between hybrid and sex; females emerged from non-Bt plants significantly later than males ( $P = 0.003$ ), but no difference was detected between males and females in Bt plots ( $P = 0.99$ ). Overall, insects emerged significantly later from Bt plots than from non-Bt plots (Table 3; Fig. 2D).

**Mass and Size.** Insecticide treatment did not significantly affect mass or size of western corn rootworm (Tables 2 and 4). For mass, there was a significant hybrid by sex interaction (Table 2). Mass was significantly greater for females than males on Bt maize ( $P = 0.003$ ), but did not differ between males and females on non-Bt maize ( $P = 0.41$ ). Size, as measured by head capsule width, was significantly affected by hybrid and sex (Table 2). Females were significantly larger than males, and insects from non-Bt plots were significantly larger than insects from Bt plots (Table 4).

For northern corn rootworm, mass was not significantly affected by any of the factors (Table 3), but for size there was a significant insecticide by sex interaction (Table 3). Males and females did not significantly differ in size for P1250 ( $P = 0.521$ ) or Aztec plots ( $P = 0.904$ ); however, males were significantly larger than females in control plots ( $P = 0.001$ ) (Table 4). No other factors significantly affected size (Table 3).

**Proportional Reduction in Adult Survival.** Average root ratings were below one node for Iowa in 2009 (mean: 0.20; SD: 0.25) and for Nebraska in 2008 (mean: 0.82; SD: 0.25) (Fig. 3). Because density-dependent mortality can occur when mean root ratings exceed 1.0 on the 0–3 scale (Hibbard et al. 2010b), only these two locations were used to calculate reduction in survival imposed by each of the rootworm treatments: Aztec, P1250, Bt maize, Bt maize plus Aztec, and Bt maize plus P1250 (Table 5). For western corn rootworm, there was a significant effect of rootworm treatment ( $F_{4,28} = 2.83$ ;  $P = 0.04$ ). The proportional reduction in adult survival imposed by Bt maize in combination with Aztec was significantly greater than for P1250 alone (Table 5). No other significant differences existed among treatments. The reduction in survival to adulthood was significantly greater than zero for all rootworm treatments with the exception of P1250 alone (Table 5).

There was also a significant effect of rootworm treatment for northern corn rootworm ( $F_{4,28} = 5.32$ ;  $P = 0.003$ ). When either insecticide was combined with Bt maize, the reduction in survival was significantly greater than for that insecticide alone (Table 5). In addition, proportional reduction in survival for Bt maize alone did not differ from Bt maize combined with either insecticide (Table 5). The reduction in survival of northern corn rootworm was significantly greater than zero for all rootworm treatments (Table 5).

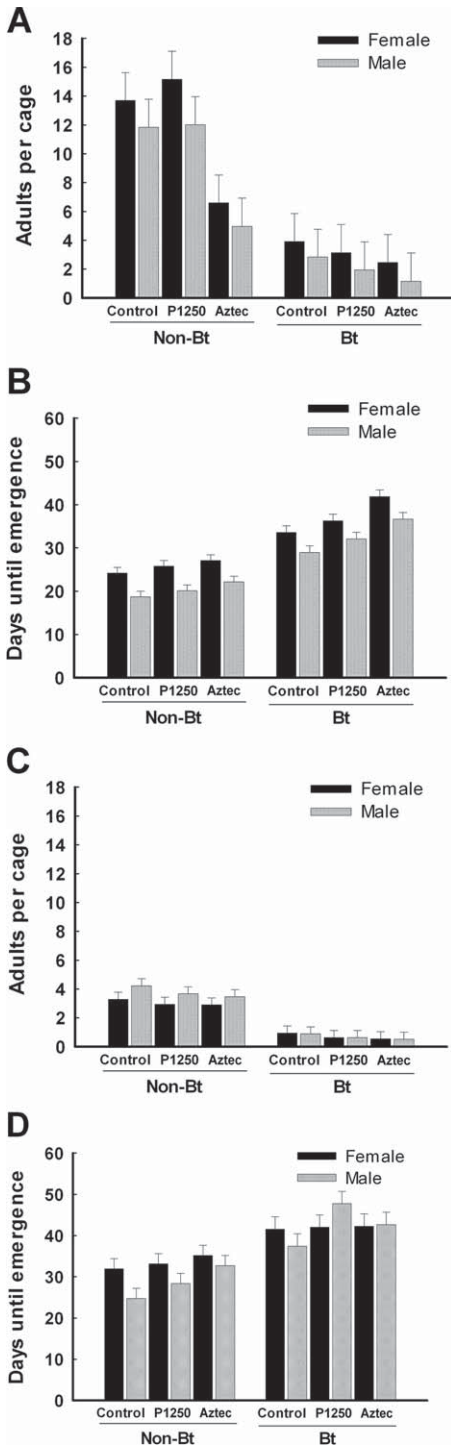


Fig. 2. (A) Survival and (B) time of emergence of western corn rootworm and (C) survival and (D) emergence time of northern corn rootworm, on Bt and non-Bt maize with and without soil-applied insecticides. Bar heights represent sample means and error bars are the SEM.

Discussion

Considering data among all locations in both years, Bt maize and the soil-applied insecticide Aztec significantly decreased survival of western corn rootworm (Fig. 2A; Table 2) but only Bt maize significantly decreased survival of northern corn rootworm (Fig. 2C; Table 3). Using data from study sites where average node injury was less than one node, and density dependent mortality was expected to be absent (see Materials and Methods), Bt maize alone imposed a 71 and 80% reduction in survival for western corn rootworm and northern corn rootworm, respectively (Table 5). For Bt crops, a high-dose event is defined as an event that produces 25 times more toxin than is needed to kill a susceptible pest and kills at least 99.99% of susceptible insects (EPA 1998). High-dose events result in resistance that is functionally recessive, thereby decreasing the risk of resistance evolution (Tabashnik et al. 2004). No studies have demonstrated mortality of western corn rootworm from Bt maize reaching 99.99% (Gassmann 2012), and mortality in this study was lower than previously reported for Cry3Bb1 maize in field studies, which ranged from 89 to 99.79% (Oyediran et al. 2007, Clark et al. 2012).

A pyramid of two insecticides that target the same pest can delay the evolution of resistance to either insecticide (Roush 1998). This is achieved by redundant killing, in which insects with resistance alleles for one toxin are killed by the second toxin, and homozygous susceptible insects are killed by both insecticides (Curtis 1985, Comins 1986). In the absence of cross-resistance, resistance to both toxins would require resistance alleles at two loci. In this study, the reduction in survival achieved by Bt maize was 71 and 80% for western and northern corn rootworm, respectively. For both pests, reductions in survival were greater for P1250, and were 53 and 36% for western and northern corn rootworm, respectively (Table 5). In mathematical models, Roush (1998) showed that resistance evolution can be greatly delayed by pyramids if mortality of susceptible insects is >95% from each toxin. As the level of mortality decreases, pyramids achieve less of a benefit in delaying resistance compared with separate and sequential release of two insecticides (Roush 1998). For example, if initial resistance allele frequency in the population is  $10^{-5}$ , and there is a 20% refuge, the pyramid strategy delays the evolution of resistance by  $\approx 1$ , 10, and >20 additional generations compared with a sequential release strategy when mortality of susceptible homozygotes is 80, 85, and 90%. Thus, pyramiding Aztec and Bt maize could potentially delay resistance of rootworm longer than separate and sequential use of these insecticides; however, mortality achieved by soil-applied insecticides may be too low to have a meaningful effect on frequency of alleles for resistance to Bt maize.

There are other factors that may diminish the potential delays in resistance achieved by pyramiding Cry3Bb1 with Aztec. Pyramiding insecticides is considerably more effective when resistance allele fre-



**Table 3. Analysis of variance for survival, emergence time, mass, and size of northern corn rootworm**

Source	Fixed effects							
	Survival		Emergence		Mass		Size	
	df <sup>a</sup>	F value	df	F value	df	F value	df	F value
Hybrid	1,15	59.35***	1,13	47.15***	1,12	2.11	1,12	3.55
Insecticide	2,60	2.70	2,38	4.42*	2,39	0.62	2,38	0.60
Sex	1,15	1.46	1,13	4.21	1,12	1.29	1,12	3.83*
Hybrid × insecticide	2,60	0.03	2,38	0.85	2,39	0.72	2,38	0.51
Hybrid × sex	1,15	3.67	1,13	5.23*	1,12	0.08	1,12	2.94
Insecticide × sex	2,60	0.04	2,38	2.46	2,39	0.02	2,38	3.73*
Hybrid × insecticide × Sex	2,60	0.20	2,38	0.62	2,39	0.88	2,38	0.57
Random effects								
Source	df	Survival $\chi^2$	Emergence $\chi^2$	Mass $\chi^2$	Size $\chi^2$			
Block (site × year)	1	70.5***	146.5***	1.3	38.4***			
Hybrid × block (site × year)	1	30.4***	5.0*	0.4	0.5			
Sex × block (site × year)	1	0.7	5.0*	0.0	1.8			
Hybrid × sex × block (site × year)	1	1.5	0.6	0.0	0.0			
Hybrid × insecticide × block (site × year)	1	7.8**	4.9*	0.5	0.6			
Hybrid × insecticide × sex × block (site × year)	1	0.0	1.5	0.0	0.2			

<sup>a</sup> df, numerator degrees of freedom, denominator degrees of freedom.  
 \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

quencies are low at the time of deployment (Roush 1998). However, for western corn rootworm, the frequency of alleles for resistance to Cry3Bb1 may not be low, given the occurrence of several populations for which field-evolved resistance to Cry3Bb1 has been documented (Gassmann et al. 2011, 2012; Gassmann 2012). Initial resistance allele frequency estimated from a greenhouse selection experiment with western corn rootworm collected before the registration of Cry3Bb1 maize (Meihls et al. 2008) was 0.2 (Onstad and Meinke 2010), much greater than estimates of 0.001–0.0001 commonly used in western corn rootworm resistance management models (Crowder et al. 2006, Onstad and Meinke 2010). Another important factor affecting resistance evolution is random mating between insects from the refuge and selected insects (e.g., insects from Bt maize), with the rate of resistance evolution positively associated with assortative mating among Bt-selected individuals (Gould 1998). In this study, Bt maize delayed adult emergence by an average of 12 d for both western corn rootworm and

northern corn rootworm relative to non-Bt maize (Fig. 2B and D). Delays in emergence because of development on Bt maize relative to non-Bt maize is a commonly observed phenomenon for rootworm species (Binning et al. 2010, Hibbard et al. 2010a, Petzold-Maxwell et al. 2012b). In this study, the addition of insecticides exacerbated delays in emergence compared with Bt maize alone. For western corn rootworm, time of emergence was latest in plots with Bt maize and Aztec, and for northern corn rootworm delays were greatest in plots with Bt maize and either P1250 or Aztec (Fig. 2B and D). This increase in temporal asynchrony of emergence for insects from non-Bt maize and insects from Bt maize combined with an insecticide could hinder random mating of susceptible and resistant insects, thereby increasing the rate of resistance evolution.

Nonrandom mating could also occur if insects emerging from plots with Bt maize, soil insecticides, or both, were in some way less attractive to individuals emerging from non-Bt maize plants. In this study,

**Table 4. Mean mass and head capsule width of western corn rootworm and northern corn rootworm**

Insecticide	Hybrid	Sex	Western corn rootworm <sup>a</sup>		Northern corn rootworm <sup>a</sup>	
			Mass (mg) <sup>b</sup>	Head capsule width (mm)	Mass (mg) <sup>b</sup>	Head capsule width (mm)
Control	Non-Bt	F	1.74 (0.08)	1.13 (0.006)	1.41 (0.09)	1.05 (0.009)
Control	Non-Bt	M	1.71 (0.08)	1.12 (0.006)	1.46 (0.09)	1.09 (0.008)
P1250	Non-Bt	F	1.66 (0.08)	1.13 (0.006)	1.54 (0.09)	1.06 (0.008)
P1250	Non-Bt	M	1.65 (0.08)	1.12 (0.006)	1.50 (0.09)	1.08 (0.008)
Aztec	Non-Bt	F	1.77 (0.08)	1.13 (0.006)	1.60 (0.09)	1.06 (0.009)
Aztec	Non-Bt	M	1.76 (0.09)	1.12 (0.006)	1.45 (0.09)	1.08 (0.008)
Control	Bt	F	1.82 (0.09)	1.11 (0.007)	1.68 (0.10)	1.03 (0.013)
Control	Bt	M	1.52 (0.09)	1.09 (0.009)	1.52 (0.12)	1.06 (0.014)
P1250	Bt	F	1.76 (0.09)	1.11 (0.009)	1.55 (0.10)	1.06 (0.020)
P1250	Bt	M	1.64 (0.09)	1.10 (0.010)	1.49 (0.12)	1.07 (0.020)
Aztec	Bt	F	1.89 (0.09)	1.11 (0.008)	1.65 (0.11)	1.07 (0.013)
Aztec	Bt	M	1.76 (0.09)	1.11 (0.010)	1.65 (0.11)	1.04 (0.014)

<sup>a</sup> Values show sample mean; 1 SEM is shown in parentheses.  
<sup>b</sup> Data for mass were transformed for analysis; untransformed means are shown.

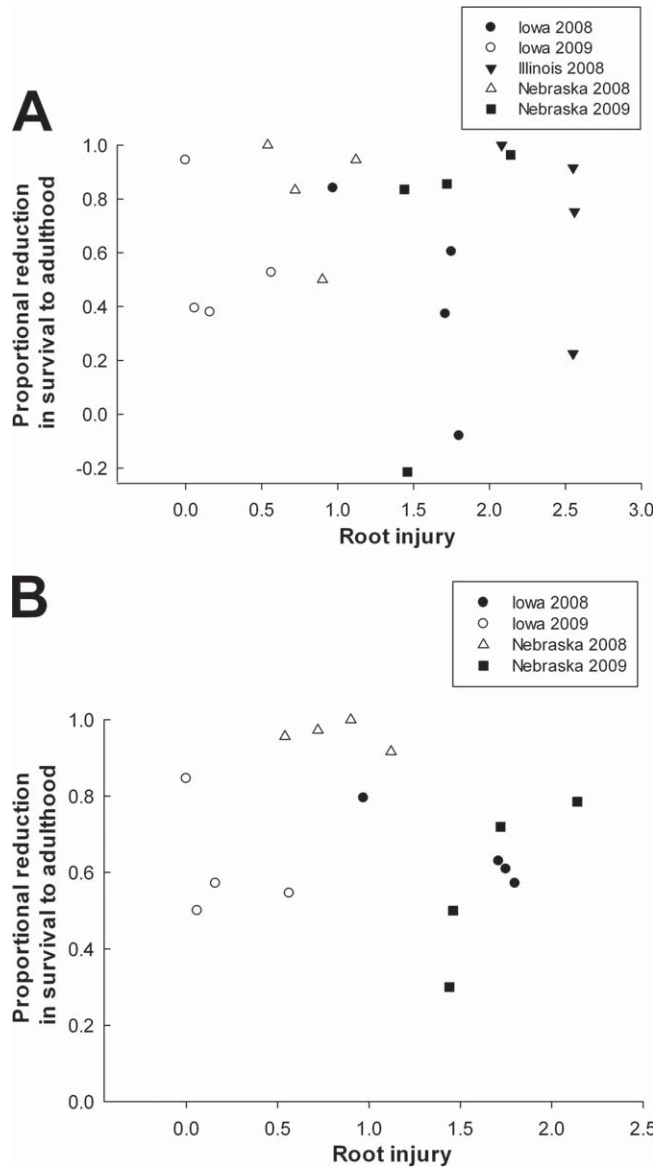


Fig. 3. Root injury and proportional reduction in survival on Bt maize for (A) western corn rootworm and (B) northern corn rootworm. Root injury scores are based on a 0–3 scale (Oleson et al. 2005), with each integer indicating the number of nodes pruned up to three nodes. Symbols show sample means for each block within a location. See Materials and Methods for details on how proportional reduction in survival was calculated.

northern corn rootworm head capsule width and mass were not significantly reduced by P1250, Aztec, or Bt maize (Tables 3 and 4). The mass and size of western corn rootworm adults were not affected by either Aztec or P1250, although insects from Bt maize were significantly smaller than insects from non-Bt maize (Tables 2 and 4). Larval feeding on Bt maize has been shown to reduce the size or mass of adults in some studies (Binning et al. 2010, Murphy et al. 2011), but not others (Storer et al. 2006, Frank et al. 2011, Meihls et al. 2011, Zukoff et al. 2012). If insects emerging from Bt maize or insecticide-treated plants incur sublethal

effects on fitness, the spread of resistance genes may be delayed (Tabashnik et al. 2004). However, if these sublethal effects, such as reduced size or mass, lead to assortative mating among selected individuals, the rate of resistance evolution could increase (Gould 1998).

While neither Aztec nor P1250 had an effect on root injury to Bt maize, both of these insecticides reduced injury to non-Bt maize roots (Table 1; Fig. 1B). In general, there is a negative relationship between root injury and yield, with one node of roots lost to feeding by rootworm reducing yield by 15.2% (Tinsley et al. 2012) to 17.9% (Dun et al. 2010). However, the sig-

**Table 5. Proportional reduction in survival to adulthood for western corn rootworm and northern corn rootworm from Bt maize, insecticides, and the combination of Bt maize and insecticides**

Rootworm treatment	Western corn rootworm <sup>a</sup>		Northern corn rootworm <sup>a</sup>	
	Reduction in survival to adulthood (proportion)	Significantly different from zero	Reduction in survival to adulthood (proportion)	Significantly different from zero
P1250	-0.18 (0.50)a	No	0.39 (0.15)ab	Yes
Aztec	0.53 (0.15)ab	Yes	0.36 (0.16)a	Yes
Bt maize	0.71 (0.10)ab	Yes	0.80 (0.07)bc	Yes
Bt maize + P1250	0.75 (0.10)ab	Yes	0.82 (0.05)c	Yes
Bt maize + Aztec	0.83 (0.08)b	Yes	0.80 (0.11)bc	Yes

<sup>a</sup> Different letters within a column indicate significant differences among each of the mortality factors.

nificant root protection afforded by P1250 and Aztec on non-Bt maize did not lessen yield loss in this study, as yield was only significantly protected by Bt maize (Fig. 1A). It is possible that plants in non-Bt plots that did not receive Aztec or P1250 tolerated certain levels of injury, such that this injury did not translate to a decrease in yield compared with non-Bt maize with insecticides (Spike and Tollefson 1989, Gray and Steffey 1998, Urias-Lopez and Meinke 2001). Efficacy of seed treatments and soil insecticides targeting rootworm larvae is often not consistent and depends on a number of biotic and abiotic factors (van Rozen and Ester 2010).

When developing an IPM strategy, a producer must weigh management costs against benefits from preserving yield (Pedigo and Rice 2009). In this study, Bt maize significantly reduced injury, but using an insecticide in combination with Bt maize did not significantly decrease root injury compared with the use of Bt maize alone (Fig. 1B). Similarly, Bt maize resulted in significant yield protection, but the use of insecticides did not result in an additional increase in yield when combined with either Bt or non-Bt maize. Thus, the additional cost of an insecticide may not have offered farmers any economic benefits. Because economic losses in maize systems depend on a number of interacting factors, including level of injury from rootworm species, environmental stress, and hybrid (Gray and Steffey 1998, Urias-Lopez and Meinke 2001), a standard economic injury index has been difficult to develop. However, it is generally agreed that a root injury rating corresponding to 0.5 or more on the 0–3 node injury scale can result in economic losses (Hein and Tollefson 1985, Gray and Steffey 1998, O’Neal et al. 2001). Here, Bt maize alone significantly reduced injury from 1.3 to 0.1 nodes on the 0–3 scale (Oleson et al. 2005), well below the economic injury index, thus attempting to achieve additional reductions in injury from supplemental insecticides may not offer an economic benefit.

Our data suggest that pyramiding an insecticide with Bt maize may offer few benefits from either an IRM or IPM perspective. From an IPM perspective, combining Cry3Bb1 maize with an insecticide did not decrease root injury or increase yield (Fig. 1), and would not have provided a single-season economic benefit to producers. In addition, reductions in the use of chemical insecticides are often associated with the planting of Bt crops (Carpenter and Gianessi 2001,

Shelton et al. 2002, Naranjo 2009), and this benefit would be diminished by pyramiding Bt crops with chemical insecticides. From an IRM perspective, the level of mortality achieved with an insecticide may be too low to provide much, if any, benefit in terms of delaying resistance. In addition, Gray et al. (1992) showed that in some years, more western corn rootworm adults emerged from plots that received insecticides applied at the time of planting compared with control plots. Given the presence of populations of western corn rootworm that have evolved resistance to Cry3Bb1 maize in the field (Gassmann et al. 2011, 2012; Gassmann 2012), it will be important to continue to develop improvements in resistance management, and to promote more long-term (multi-year) integrated approaches to management, such as crop rotation and rotation among other available pest management tactics for corn rootworm.

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