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Abstract

A 2 yr field study was conducted to determine how a blend of entomopathogens interacted with Bt maize to affect mortality of *Diabrotica* spp. (Coleoptera: Chrysomelidae), root injury to maize (*Zea maize* L.) and yield. The blend of entomopathogens included two entomopathogenic nematodes, *Steinernema carpocapsae* Weiser and *Heterorhabditis bacteriophora* Poinar, and one entomopathogenic fungus, *Metarhizium brunneum*(Metschnikoff) Sorokin. Bt maize (event DAS59122–7, which produces Bt toxin Cry34/35Ab1) decreased root injury and survival of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and northern corn rootworm (*Diabrotica barberi* Smith & Lawrence) but did not affect yield. During year 1 of the study, when rootworm abundance was high, entomopathogens in combination with Bt maize led to a significant reduction in root injury. In year 2 of the study, when rootworm abundance was lower, entomopathogens significantly decreased injury to non-Bt maize roots, but had no effect on Bt maize roots. Yield was significantly increased by the addition of entomopathogens to the soil. Entomopathogens did not decrease survival of corn rootworm species. The results suggest that soil-borne entomopathogens can complement Bt maize by protecting roots from feeding injury from corn rootworm when pest abundance is high, and can decrease root injury to non-Bt maize when rootworm abundance is low. In addition, this study also showed that the addition of entomopathogens to soil contributed to an overall increase in yield.

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Interactions Among Bt Maize, Entomopathogens, and Rootworm Species (Coleoptera: Chrysomelidae) in the Field: Effects on Survival, Yield, and Root Injury

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ABSTRACT   A 2 yr field study was conducted to determine how a blend of entomopathogens interacted with Bt maize to affect mortality of *Diabrotica* spp. (Coleoptera: Chrysomelidae), root injury to maize (*Zea maize* L.) and yield. The blend of entomopathogens included two entomopathogenic nematodes, *Steinernema carpocapsae* Weiser and *Heterorhabditis bacteriophora* Poinar, and one entomopathogenic fungus, *Metarhizium brunneum* (Metschnikoff) Sorokin. Bt maize (event DAS-59122–7, which produces Bt toxin Cry34/35Ab1) decreased root injury and survival of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and northern corn rootworm (*Diabrotica barberi* Smith & Lawrence) but did not affect yield. During year 1 of the study, when rootworm abundance was high, entomopathogens in combination with Bt maize led to a significant reduction in root injury. In year 2 of the study, when rootworm abundance was lower, entomopathogens significantly decreased injury to non-Bt maize roots, but had no effect on Bt maize roots. Yield was significantly increased by the addition of entomopathogens to the soil. Entomopathogens did not decrease survival of corn rootworm species. The results suggest that soil-borne entomopathogens can complement Bt maize by protecting roots from feeding injury from corn rootworm when pest abundance is high, and can decrease root injury to non-Bt maize when rootworm abundance is low. In addition, this study also showed that the addition of entomopathogens to soil contributed to an overall increase in yield.

KEY WORDS   *Diabrotica barberi*, *Diabrotica virgifera virgifera*, *Heterorhabditis*, *Metarhizium*, *Steinernema*

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and the northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, (Coleoptera: Chrysomelidae) are serious pests of maize (*Zea maize* L.) in the United States (Gray et al. 2009). Larval northern corn rootworm and western corn rootworm cause injury to maize predominantly through root feeding, which can result in substantial reductions in yield (Dun et al. 2010, Tinsley et al. 2012). Genetically modified maize producing insecticidal toxins derived from the bacterium *Bacillus thuringiensis* Berliner (Bt) was commercialized for management of rootworm species in 2003 (EPA 2003). Although Bt maize reduces survival of western corn rootworm to adulthood, pest survival and associated crop injury are still observed (Gassmann 2012). In the case of western

corn rootworm, management efforts have been complicated by the repeated development of resistance to management approaches including conventional insecticides, the cultural practice of crop rotation, and Bt maize (reviewed by Gassmann 2012). One 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.

Natural enemies and host plant resistance are two important biotic factors that can affect pest abundance, and numerous studies have tested the compatibility of these bottom-up and top-down factors in regulating pest populations in agro-ecosystems (Cortesero et al. 2000, Hare 2002, Ode 2006). Bt maize offers a very effective form of host plant resistance. Additionally, the soil harbors a rich community of entomopathogens that kill western corn rootworm and northern corn rootworm larvae, and these natural enemies may be added to the soil to achieve augmentative and inundative biological control (Lacey et al. 2001, Pilz et al. 2009, Toepfer et al. 2009). Natural enemies that cause mortality of larval rootworm in-

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clude entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis*, and entomopathogenic fungi in the genera *Metarhizium* and *Beauveria* (Toepfer et al. 2009). Additionally, these entomopathogens occur naturally in maize fields (Pilz et al. 2008).

Field trials have shown that entomopathogens can reduce rootworm survival and injury to maize roots, and in some cases, increase yield (Wright et al. 1993, Krueger and Roberts 1997, Journey and Ostlie 2000, Toepfer et al. 2008, Pilz et al. 2009). For example, the nematode *Heterorhabditis bacteriophora* Poinar significantly reduced root injury caused by western corn rootworm in a field study by 14–54% (Toepfer et al. 2010). In another field experiment, *H. bacteriophora*, *Heterorhabditis megidis* Poinar and *Steinernema feltiae* Filipjev decreased rootworm survival and root injury (Toepfer et al. 2008). The nematode *Steinernema carpocapsae* Weiser also has been shown to decrease western corn rootworm survival and root injury to maize in the field (Wright et al. 1993, Journey and Ostlie 2000). Similarly, field trials assessing the efficacy of the *Metarhizium anisopliae* s.l. on rootworm management have shown a reduction in survival and root injury (Krueger and Roberts 1997, Pilz et al. 2009). Persistence, in addition to efficacy, is an important factor that can influence overall suitability of an organism for management of pest populations (Jaronski 2010). Importantly, once released in maize fields, entomopathogens can persist (i.e., remain present in a viable form). Kurtz et al. (2007) measured the persistence of several nematode species and found persistence of 2–5 mo, and *M. anisopliae* s.l. was found to persist for at least 15 mo in the soil of maize fields (Pilz et al. 2011).

Natural enemy diversity can contribute to increased pest suppression (Snyder 2009). However, few studies have examined how blends of various species of entomopathogens, which can exist together in maize agroecosystems (Pilz et al. 2008), affect rootworm survival and injury to Bt maize in the field. In the few studies examining the effects Bt maize and entomopathogens on western corn rootworm mortality, these two factors were shown to increase mortality in an additive rather than synergistic manner (Meissle et al. 2009, Petzold–Maxwell et al. 2012). Here, we report on results from a 2 yr field study examining the effects of Bt maize producing Cry34/35Ab1 and a community of soil-dwelling entomopathogens (*S. carpocapsae*, *H. bacteriophora*, and *M. brunneum* [formerly *M. anisopliae*]) on root injury, yield, and survival of western and northern corn rootworm.

Materials and Methods

Experimental Design. This study was conducted in 2010 and 2011, and consisted of a randomized compete block design. Treatments included maize type (Bt vs. non-Bt) and entomopathogens (added to the soil vs. not added) in a fully crossed 2 × 2 factorial design, yielding a total of four treatments per block. There were six blocks, with each block containing one rep-

licate (plot) of each treatment (four treatments × 6 replicates = 24 plots). Plots were 6.1 m long and eight rows wide, with 0.76 m spacing between rows. Each row contained ≈35 plants, with 15 cm between plants. The Bt maize used in this study contained the event DAS-59122-7, which produces the rootworm-active Bt binary toxin Cry34Ab1/Cry35Ab1 (hybrid P1395XR, relative maturity = 113 d). The non-Bt maize used in this study was a near-isoline to the Bt maize hybrid but lacked any rootworm active toxins (hybrid P1395HR, relative maturity = 113 d). Maize seeds were planted on 10 May 2010 and 11 May 2011. For treatments in which pathogens were added to the soil, we applied two nematode species (*H. bacteriophora*, strain BU and *S. carpocapsae*, strain BU), and one fungus (*M. brunneum*, strain F52).

Agronomic Information. The study was conducted at Iowa State University's Johnson Research Farm in Ames, IA. Each year, the study took place in a field that was planted to a trap crop the previous year, which is late-planted maize that attracts adult western corn rootworm and northern corn rootworm, which then oviposit in the soil. In both years of the study, fields were tilled with a field cultivator, fertilized with nitrogen in the fall of the previous year and again before planting, and treated with herbicide, in accordance with standard regional agricultural practices used in maize production.

Entomopathogens. Microsclerotia (compact hyphal aggregates) of *M. brunneum* F52 were applied to the plots. Briefly, microsclerotial granules were produced by combining microsclerotial-containing whole cultures of *M. brunneum*, produced in liquid fermentation as described by Jackson and Jaronski (2009), with diatomaceous earth, then dewatered with vacuum filtration. The filter cake of microsclerotia and diatomaceous earth produced from this process was broken into small pieces and air dried to a moisture content of ≤5%, (yielding 900–1,000 granules/g). Granules were applied at a rate of 45 kg/ha, which is approximately double typical rates that are used for granular insecticides in maize systems (i.e., Lance and Sutter 1990, Kakar et al. 2003, Gunewardena and Madugalla 2011). We applied the granules at this higher rate because the procedure for producing microsclerotial granules is relatively new (Jackson and Jaronski 2009), and baseline rates for the use of *M. brunneum* microsclerotial granules in the field have not yet been established for management of rootworm species. Microsclerotial granules of *M. brunneum* were manually dispersed onto the ground, and the entire study site was tilled immediately to a depth of 15 cm with a 1.5 m wide three-point tiller. Maize seeds were then immediately planted into the study area using a mechanical planter (7100 rigid frame max-emerge planter, John Deere, Moline, IL).

All nematode strains used were the proprietary, commercially available strains of Becker–Underwood (Ames, IA), and were in the infective juvenile (IJ) stage. Nematode concentrations were determined using a compound microscope (Nikon Eclipse E200) set at 40× magnification and a Sedgewick–Rafter count-

ing cell (Pyser-SGI, Edenbridge, United Kingdom). Only live nematodes were counted, and suspensions were diluted to the desired concentration with deionized water. Nematode suspensions for each of the two species were stored overnight at 7°C and the following day they were brought to the field in plastic containers held in a cooler. Nematode suspensions were poured into a 5 cm deep furrow created 15–22 cm from one side of each maize row, with a total of 118,080 IJs of each nematode species applied per meter of cropping row (236,160 total nematodes per row meter, which is equal to 16 nematodes/cm^2 and 1.6×10^9 nematodes/ha). This concentration is similar to what has been used in other studies (Thurston and Yule 1990, Toepfer et al. 2005, Kurtz et al. 2009). Furrows were immediately covered with soil after nematode application. Plots not receiving nematodes were treated in the same manner except that we added water without nematodes to the furrows. Nematodes were applied on three and 4 June, 2010 and on 8 June 2011, at which time maize plants had 3–4 fully expanded leaves (V3 to V4 growth stage) (Abendroth et al. 2011).

The abundance of entomopathogens in the soil was measured twice during each year of the study. Nematode abundance in the soil was measured by baiting with *Galleria mellonella* L. larvae (Lacey and Kaya 2012). Individual larvae were caged in $2.5 \times 5 \times 1$ cm mesh screen cages, and a piece of wire was used to tie three cages together. Each group of three caged larvae was buried 7 to 12 cm deep and placed randomly along the furrow where nematodes were originally applied. The cages were covered with firmly packed soil. After 5 d, larvae were removed from plots and from their cages. Dead larvae were placed on moistened filter paper in a petri dish, which was placed inside of another larger petri dish filled with water (i.e., a modified White trap) (White 1927, Kaya and Stock 1997). This enabled us to capture any nematodes that emerged from *G. mellonella* cadavers. The number of *G. mellonella* larvae infected with nematodes was recorded. In some cases, *Metarhizium* spp. were also detected on these cadavers, and identified using the methods of Lacey and Kaya (2012). This baiting procedure was carried out on 15 June and 21 July 2010, and 27 June and 1 Aug 2011. Individual plots received three groups of the three larvae (nine larvae total), with each group placed in a separate row. In the first assessment of 2010 (15 June), data were collected from all of the 12 pathogen-treated plots and four of the nonpathogen-treated plots. For all other assessments, data were collected from all 24 plots (12 treated with pathogens and 12 not treated with pathogens).

We measured the abundance of *Metarhizium* spp. by taking soil samples and quantifying the density of fungal spores with serial dilutions following the methodology of Goettel and Inglis (1997). Samples were taken on two dates in 2010 (23 July and 27 August), and one date in 2011 (20 July). A hand trowel was used to remove the top 7 cm and a sample of ≈300 ml was then collected from a depth of 7–12 cm. In each plot, three rows were selected at random and sampled. Samples from within a single plot were combined in a

1 liter plastic bag and mixed thoroughly, resulting in one sample per plot. On 23 July 2010, three control plots (two non-Bt and one Bt) and three pathogen plots (two Bt and one non-Bt) were sampled. On 27 August 2010 and 20 July 2011, each of the 24 plots was sampled. Three serial dilutions were made (1:10, 1:100, and 1:1000) per sample, and three replicates of each dilution were plated on oatmeal dodine media modified from Chase et al. (1986). Dodine concentration was adjusted to 0.39 g/L to accommodate greater sensitivity of *Metarhizium* spp., and the original antibiotics substituted with 0.25 g/L chloramphenicol. This selective medium allows *Metarhizium* to grow but inhibits the growth of other fungi and soil microorganisms. Petri dishes were placed in an incubator held at 27°C, and checked for individual colony growth after 11 d, with each colony scored as a single colony forming unit (CFU). The number of CFUs per gram of dry soil was calculated after adjusting for moisture content of the soil (Goettel and Inglis 1997).

Digital soil sensors were buried horizontally at a depth of 13 cm and connected to a data logger (EM50, Decagon Devices, Pullman, WA) to measure soil moisture and temperature in the field plots from 14 June (2010) and 16 June (2011), to just before when maize was harvested. Each year, two sensors were used for both temperature and moisture (four sensors total), and sensors were randomly placed in the research site and separated by 15.2 m. Sensors recorded data hourly, and daily averages were calculated.

Rootworm Abundance, Root Injury, and Yield. Rootworm survival was measured by placing Illinois-style emergence cages in rows two and seven of each eight-row plot. These corresponded to the two rows between the center four rows, and the outermost row on each side of the plot. In total, five cages were placed in each plot, for a total of 120 emergence cages for the entire study (5 cages per plot × 4 treatments × 6 replications). Cages were placed in the field on 1 July 2010 and on 30 June 2011, which was before the emergence of rootworm adults from the soil. Emergence cages were constructed based on a modified design of Fisher (1980) and captured adult corn rootworm when they emerged from the soil. Rootworm were collected from cages three times per week for 11 wk in 2010 and 12 wk in 2011 (data collection ended on 15 Sept 2010 and 21 Sept 2011), which spans the period of adult emergence for western and northern corn rootworm. Each time rootworm were collected from emergence cages, we recorded the total number of male and female western and northern corn rootworm adults per cage, and the day of collection. For both northern corn rootworm and western corn rootworm, percentage mortality on Bt maize was calculated as the complement of percentage survival on Bt maize (1-[total number of insects per Bt plant/total number per non-Bt plant]) within each block, then averaged over blocks, for each combination of year by pathogen treatment.

On 29 July 2010 and 11 Aug 2011, six maize plants from each plot were dug from rows two and seven (the two rows between the center four rows and the out-

Table 1. Analysis of variance for root injury and yield

	Root injury			Yield		
	df[a]	F	P	df	F	P
Year	1, 10	52.9	<0.0001	1, 10	237.2	<0.0001
Hybrid[b]	1, 10	42.8	<0.0001	1, 30	2.3	0.142
Pathogen[c]	1, 10	2.5	0.148	1, 30	6.2	0.019
Year × hybrid	1, 10	7.3	0.022	1, 30	1.7	0.199
Year × pathogen	1, 10	0.1	0.740	1, 30	0.0	0.835
Hybrid × pathogen	1, 10	0.1	0.808	1, 30	0.0	0.992
Year × hybrid × pathogen	1, 10	1.4	0.262	1, 30	1.8	0.193

[a] df: numerator degrees of freedom, denominator degrees of freedom.
[b] Hybrid: Bt vs non-Bt maize.
[c] Pathogen: entomopathogens added vs entomopathogens not added.

ermost rows of the eight row plot) to determine root injury by rootworm larvae, resulting in a total of 36 plants per treatment (6 plants per plot × 6 plots per treatment). Injury was scored based on the node-injury scale (Oleson et al. 2005), which ranges from zero (no feeding injury) to three nodes of root tissue injured (heavy feeding injury). On 13 October of both years, the middle four rows in each plot were harvested, and weights were converted to kilogram per hectare at 15.5% moisture (United States No. 2 shelled corn).

Data Analysis. Analysis of variance (ANOVA) was performed in SAS Enterprise Guide 4.2 (SAS Institute 2009). All ANOVAs were mixed models (PROC MIXED), with random effects tested using a log-likelihood ratio statistic (-2 RES log likelihood in PROC MIXED) based on a one-tailed χ^2 test assuming one degree of freedom (Littell et al. 1996), with random factors removed from the model to increase statistical power when these factors were not significant at a level of $\alpha < 0.25$ (Quinn and Keough 2002). However, lower order terms were always retained if their higher order interactions were significant. When a significant interaction was present pairwise comparisons were made using the PDIFF statement in PROC MIXED.

Rootworm Survival, Timing of Emergence, Root Injury, and Yield. A mixed-model ANOVA was used to measure yield and root injury. Fixed factors were entomopathogen treatment (added to the soil vs. not added to the soil), hybrid (non-Bt vs. Bt), year (2010 vs. 2011), and all possible interactions of these three factors. Block nested within year, and all interactions of hybrid and pathogen treatment with block nested within year were random factors in the models. Root injury data were transformed with the square root function to ensure normality of the residuals.

Data on rootworm survival and Julian day of emergence were analyzed with a mixed-model ANOVA. For both models, fixed factors were hybrid, entomopathogen treatment, year, sex, and all possible interactions of these factors. Block within year, and all possible interactions of block within year with hybrid, entomopathogen treatment, and sex were random factors in the model. To ensure normality of the residuals, data for survival of western corn rootworm and northern corn rootworm were transformed with the square root function and log (x + 0.5) function, respectively.

Because past research has found that entomopathogens can increase mortality of pests, decrease pest injury to crops, and increase crop yield, we tested three a priori hypotheses. Our a priori hypotheses (H_a) were 1) survival of rootworm will be lower on pathogen-treated maize than maize not treated with pathogens, 2) root injury will be lower for pathogen-treated maize than maize not treated with pathogens, and 3) yield will be higher for pathogen-treated maize than maize not treated with pathogens. The null hypotheses (H_o), respectively, were 1) survival of rootworm will not differ between pathogen-treated maize and maize not treated with pathogens, 2) root injury will not differ between pathogen-treated maize and maize not treated with pathogens, and 3) yield will not differ between pathogen-treated maize and maize not treated with pathogens. Thus, for data on survival, root injury and yield, treatments with and without pathogens were compared for each combination of hybrid by year (e.g., non-Bt corn in 2010) with a one-tailed *t*-test (Sokal and Rohlf 1995).

Entomopathogen Abundance. Data on the percentage of *Galleria mellonella* (L.) infected with nematodes and on the number of CFUs of *Metarhizium* spp. per gram of dry soil were analyzed with a *t*-test (one tailed, assuming unequal variances) that compared plots treated and untreated with entomopathogens (Sokal and Rohlf 1995). The null hypothesis (H_o) was that pathogen-treated plots would not differ from untreated maize in the abundance of pathogens, and the alternative hypothesis (H_a) was that pathogen treated maize would have a higher abundance of pathogens than the untreated plots.

Results

Root Injury. Overall, root injury was significantly reduced by Bt maize, and significantly higher in 2010 than 2011. There was a significant year by hybrid interaction for root injury, with a larger decrease in root injury by Bt maize plants compared with non-Bt maize plants in 2010 ($P = <0.0001$) than in 2011 ($P = 0.02$) (Table 1; Fig. 1a). In 2010, when root injury was greater, entomopathogens did not affect root injury for non-Bt plants ($t_{70} = 0.18$; $P = 0.43$; one-tailed test), however, they significantly decreased injury for Bt plants ($t_{70} = 3.68$; $P = 0.0002$; one-tailed test) (Fig. 1a).
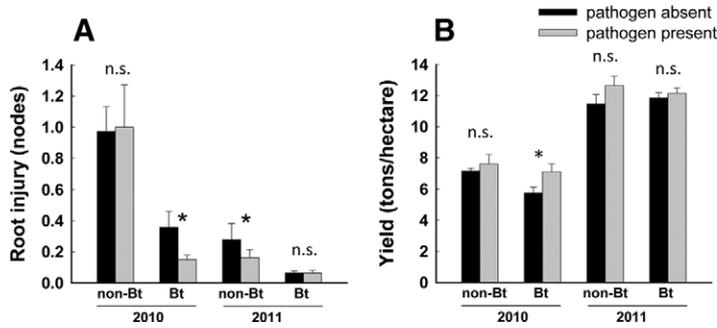
Fig. 1. Average (A) root injury and (B) yield. Bar heights represent sample means and error bars are the SEM. Asterisks represent a significant difference between maize plants treated and not treated with entomopathogens ($P < 0.05$), based on a one-tailed t-test; nonsignificant differences are indicated with n.s. (not significant).

In 2011, when root injury was lower, entomopathogens significantly decreased root injury for non-Bt plants ($t_{70} = 1.89$; $P = 0.03$; one-tailed test), but not for Bt plants ($t_{70} = -6.8 \times 10^{-16}$; $P = 0.50$; one-tailed test) (Fig. 1a).

Yield. Across both years and hybrids, entomopathogens significantly increased yield, and yield was significantly greater in 2011 than 2010 (Table 1; Fig. 1b). No other factors significantly affected yield. For each year by hybrid combination, yield was numerically greater in entomopathogen-treated plots, and this affect was significant for Bt maize in 2010 ($t_{10} = 2.15$; $P = 0.03$; one-tailed test).

Rootworm Survival. Abundance of western corn rootworm was higher in 2010 (Table 2; Fig. 2a). Bt maize significantly reduced survival for western corn rootworm both years (Tables 2 and 3; Fig. 2a), although the difference in survival between Bt maize and non-Bt maize was greater in 2010 compared with 2011, accounting for the significant year by hybrid interaction (Table 2; Fig. 2a). There was a significant effect of sex (Table 2); adults collected per plant was greater for females (mean per plant: 2.0; SE: 0.35) than males (mean per plant: 1.15; SE: 0.25). Entomopatho-

gens did not have an effect on survival (Table 2; Fig. 2a).

For northern corn rootworm, survival was decreased by Bt maize relative to non-Bt maize (Table 2; Fig. 2b). There was a significant hybrid by sex interaction (Table 2). Although survival did not differ between males and females on Bt maize (mean males per plant: 0.24; SE: 0.06; mean females: 0.23; SE: 0.05; $P = 0.95$), on non-Bt maize survival was significantly higher for males than females (males: 1.39; SE: 0.18; females: 1.0; SE: 0.15; $P = 0.02$). There was a significant interaction between year and pathogen treatment: in 2010, more insects emerged from the pathogen-treated plots ($P = 0.01$), whereas in 2011 there was no difference in emergence in pathogen-treated and untreated plots ($P = 0.30$) (Fig. 2b). There was also a significant interaction between year and sex (Table 2). In 2010, more males emerged than females (males: 1.13; SE: 0.21; females: 0.61; SE: 0.14; $P = 0.01$), and in 2011 there was no significant difference (males: 0.50; SE: 0.11; females: 0.63; SE: 0.14; $P = 0.36$). Across years and hybrids, entomopathogens did not significantly affect survival (Table 2).

Table 2. Analysis of variance for survival of western corn rootworm and northern corn rootworm

	Western corn rootworm			Northern corn rootworm		
	df[a]	F	P	df	F	P
Year	1, 10	59.7	<0.0001	1, 10	3.9	0.076
Hybrid[b]	1, 10	126.6	<0.0001	1, 10	76.4	<0.0001
Path[c]	1, 10	0.7	0.416	1, 10	2.6	0.137
Sex	1, 10	60.2	<0.0001	1, 10	3.1	0.110
Year × hybrid	1, 10	19.5	0.001	1, 10	0.2	0.696
Year × path	1, 10	0.0	0.901	1, 10	10.1	0.010
Year × sex	1, 10	0.1	0.805	1, 10	9.8	0.011
Hybrid × path	1, 10	0.5	0.502	1, 10	1.3	0.277
Hybrid × sex	1, 30	1.5	0.224	1, 10	5.6	0.039
Path × sex	1, 30	0.5	0.506	1, 20	0.1	0.747
Year × hybrid × path	1, 10	0.9	0.368	1, 10	0.7	0.410
Year × hybrid × sex	1, 30	0.1	0.804	1, 10	1.3	0.284
Year × path × sex	1, 30	0.0	0.896	1, 20	0.5	0.500
Hybrid × path × sex	1, 30	0.3	0.613	1, 20	0.1	0.759
Year × hybrid × path × sex	1, 30	0.7	0.416	1, 20	3.2	0.091

[a] df: numerator degrees of freedom, denominator degrees of freedom.
[b] Hybrid: Bt vs non-Bt maize.
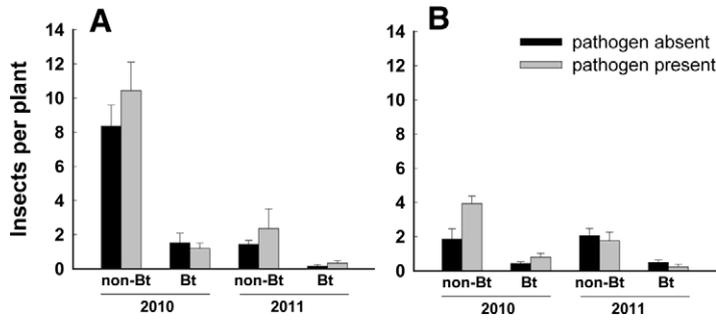[c] Path: entomopathogens added vs entomopathogens not added.

Fig. 2. Average number of (A) western corn rootworm and (B) northern corn rootworm surviving per plant. Bar heights represent sample means and error bars are the SEM. For none of the combinations of hybrid by year by pathogen treatment was there significantly greater survival in the absence of pathogens than in the presence of pathogens based on a one-tailed *t*-test.

Timing of Rootworm Emergence. Both species emerged an average of 7 d earlier in 2010. For western corn rootworm, females emerged significantly later than males in both years, and insects emerged an average of 7 d later from Bt maize compared with non-Bt maize (Table 4; Fig. 3a). For northern corn rootworm, insects emerged from Bt maize an average of 5 d later compared with non-Bt maize and this effect was marginally significant (Table 4; Fig. 3b). There was a significant pathogen by sex interaction (Table 4). Pathogens did not affect average Julian day of emergence for females (pathogen: 213.9; SE: 2.0; no pathogen addition: 213.3; SE: 2.3; $P = 0.84$), but males in plots treated with pathogens emerged earlier than those in untreated plots (pathogen: 207.1; SE: 2.1; no pathogen addition: 218.2; SE: 2.1; $P = 0.001$) (Fig. 3b).

Entomopathogen Abundance. Soil temperatures were typical of local conditions and similar between the 2 yr of the study (Supp. Fig. 1 [online only]) (National Oceanic and Atmospheric Administration 2012). Soil conditions were wetter during the first year of the study (2010) (Supp. Fig. 2 [online only]). In 2010, 101 cm of rainfall occurred during the study (June through October), whereas 39 cm of rainfall occurred during the same time in 2011 (National Oceanic and Atmospheric Administration 2012). Thirty year average rainfall for the area during those months is 52 cm (National Oceanic and Atmospheric Administration 2012). However, although soil was drier in 2011, soil matric potential never dropped below −405 kPa (standing water has a matric potential of zero, and permanent wilting point occurs at a matric potential of approximately −1,500 kPa for most plant species [Papendick and Campbell 1981]). Drier soil conditions in 2011 may account for the lower percentage of *G. mellonella* infected with nematodes that year compared with 2010 (Table 5), because nematode virulence can increase with soil moisture content (Grant and Villani 2003).

For each of the two time points that pathogens were sampled, a higher percentage of *G. mellonella* larvae were infected with nematodes from plots treated with pathogens compared with untreated plots (Table 5). This difference was significant in all sampling time points except the last one in 2011. In addition, within pathogen-treated plots three *G. mellonella* larvae were infected with *Metarhizium* spp. in 2011, and five were infected in 2012; however, none of the *G. mellonella* from untreated plots were infected.

Because of excessive growth of contaminant mold on soil dilution plates, we were unable to measure the abundance of *Metarhizium* spp. in 2010. In 2011 there were significantly more *Metarhizium* spp. CFUs per gram of dry soil in plots treated with entomopathogens (mean: 13,954 CFU/g soil; SE: 5,017) than in untreated plots (mean: 2,979 CFU/g soil; SE: 1,991) ($t_{14} = 2.05$; $P = 0.030$).

Discussion

We found that entomopathogenic nematodes and fungi reduced injury to roots of Bt maize when rootworm abundance was high, and to roots of non-Bt maize when abundance was low (Fig. 1a). While entomopathogens did not decrease survival of either northern corn rootworm or western corn rootworm, the presence of entomopathogens in the soil significantly increased yield (Table 1; Fig. 1b).

A number of field studies have found that soil-applied entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis*, and entomopathogenic fungi in the genus *Metarhizium*, reduce rootworm survival and root injury (Wright et al. 1993, Krueger and Roberts 1997, Toepfer et al. 2008, Pilz et al. 2009). In 2010, when rootworm abundance was high, the community of entomopathogens reduced

Table 3. Percent mortality of western corn rootworm and northern corn rootworm from Bt maize in plots treated and untreated with entomopathogens

	% mortality[a]			
	Western corn rootworm		Northern corn rootworm	
	2010	2011	2010	2011
Pathogen addition	85.8 (4.3)	80.1 (7.5)	76.7 (7.3)	86.9 (6.5)
Untreated	82.3 (5.3)	89.1 (5.7)	72.6 (5.4)	68.7 (14.0)

[a] One SEM is shown in parenthesis.

Table 4. Analysis of variance for date of adult emergence for western corn rootworm and northern corn rootworm

	Western corn rootworm			Northern corn rootworm		
	df[a]	F	P	df	F	P
Year	1, 6	12.6	0.012	1, 7	11.3	0.012
Hybrid[b]	1, 6	9.4	0.022	1, 7	5.1	0.058
Path[c]	1, 6	0.3	0.611	1, 7	5.3	0.054
Sex	1, 10	18.9	0.001	1, 10	0.3	0.618
Year × hybrid	1, 6	2.3	0.180	1, 7	0.3	0.608
Year × path	1, 6	0.0	0.989	1, 7	2.0	0.204
Year × sex	1, 10	0.1	0.737	1, 10	1.6	0.241
Hybrid × path	1, 6	1.5	0.266	1, 7	1.8	0.223
Hybrid × sex	1, 706	0.3	0.602	1, 281	1.4	0.235
Path × sex	1, 706	0.5	0.498	1, 281	8.6	0.004
Year × hybrid × path	1, 6	0.7	0.450	1, 7	1.8	0.228
Year × hybrid × sex	1, 706	2.8	0.096	1, 281	0.2	0.658
Year × path × sex	1, 706	3.4	0.065	1, 281	0.6	0.428
Hybrid × path × sex	1, 706	0.1	0.783	1, 281	3.4	0.068
Year × hybrid × path × sex	1, 706	1.1	0.293	1, 281	1.3	0.247

[a] df: numerator degrees of freedom, denominator degrees of freedom.
[b] Hybrid: Bt maize or non-Bt maize.
[c] Path: entomopathogens present or absent.

root injury to Bt maize but not to non-Bt maize. In 2011, when rootworm abundance was low, entomopathogens reduced root injury to non-Bt maize (Fig. 1a). Root injury was not reduced for Bt maize in 2011, when rootworm abundance was very low and injury was less than one tenth of a node. In a field trial in Hungary, Toepfer et al. (2010b) found a negative correlation between the number of western corn rootworm eggs infested per plant and the efficacy of *H. bacteriophora* at reducing injury to roots of non-Bt maize. Thus, when rootworm abundance is low, entomopathogens alone may be able to reduce root injury to some extent. However, at higher abundance an effect of entomopathogens on root injury may only be achieved when coupled with another management technique, in this case Bt maize.

Entomopathogens numerically increased yield in all year by hybrid comparisons, and there was an overall statistically significant increase in yield caused by the presence of entomopathogens (Fig. 1b; Table 1). Higher yields in entomopathogen-treated plots could

have resulted from decreased root injury; however, decreased injury was not observed for non-Bt plants in 2010 or Bt plants in 2011 (Fig. 1a). In general there is a negative relationship between root injury and yield (Dun et al. 2010). However, it is possible that root injury was reduced in ways not measured by the node injury scale of Oleson (2005), which only measures the extent to which nodal roots are pruned to within ≈4 cm of the plant. For example, decreased feeding injury to roots that grow from nodal roots or a reduction in pruning to nodal roots beyond 4 cm of the plant's base, both of which were not measured with the node injury scale used here, may have contributed to greater yield in the pathogen-treated plots. Another explanation for higher yields in entomopathogen-treated plots is that root systems in these plots were more healthy and vigorous because of interactions with the entomopathogen *M. brunneum* in the rhizosphere (soil/root interphase). *M. anisopliae* s.l. is a rhizosphere competent fungus (Hu and St Leger 2002), and has been shown to stimulate root devel-
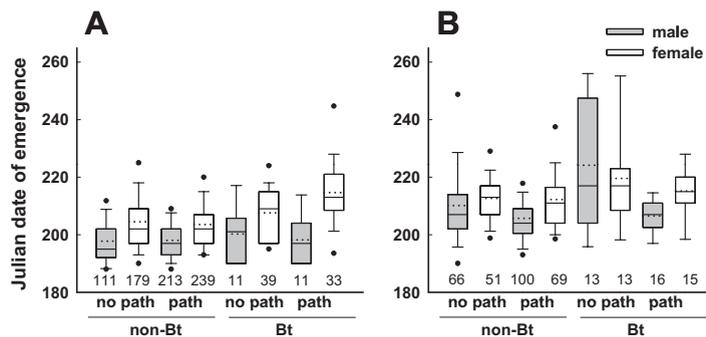

Fig. 3. Average Julian date of adult emergence for (A) western corn rootworm and (B) northern corn rootworm. Solid and dashed lines in box plots show median and mean day of emergence, respectively; boxes show the 25th and 75th percentiles, whiskers show the 10th and 90th percentiles, and dots show the 5th and 95th percentiles. Numbers below box plots show total number of adults that emerged for each treatment. Along the x-axis, "path" = pathogen added to the soil and "no path" = pathogen not added to the soil.

Table 5. Percentage of *G. mellonella* larvae infected with nematodes in field plots treated or untreated with entomopathogens

	% infected			
	2010		2011	
	No pathogens	Pathogens	No pathogens	Pathogens
TP1[a]	13.9 (5.3)	32.4 (5.2)*[b]	0.9 (0.9)	8.3 (3.4)*
TP2	2.8 (1.5)	14.8 (4.4)*	0.1 (0.1)	6.5 (3.7)

[a] TP: sampling time point (15 June and 21 July) in 2010 and (27 June and 1 Aug) in 2011.
[b] Asterisks indicate a significant difference in percent infected, within a time point, and year ($P < 0.05$).

opment in switchgrass (*Panicum virgatum* L.) and haricot beans (*Phaseolus vulgaris* L.) (Sasan and Bidochka 2012). Other studies also have reported increases in maize yield with addition of *M. anisopliae* s.l. to the soil (Maniania et al. 2002, Kabaluk and Ericsson 2007).

A similar blend of entomopathogens (*M. brunneum, B. bassiana, H. bacteriophora, S. carpocapsae,* and *S. glaseri*) decreased western corn rootworm abundance on both Bt and non-Bt maize on seedling mat assays in the laboratory (Petzold–Maxwell et al. 2012), but we did not observe a decrease in rootworm survival in this study. If rhizospheric interactions between *M. brunneum* and maize do indeed lead to larger root masses, as discussed above, measures of rootworm abundance may have been better described as insects per unit root mass, because larger root masses likely support more insects. This is one explanation for higher rootworm survival observed in entomopathogen-treated plots in some cases (Fig. 2). Field studies employing the entomopathogen species used in the current study have shown decreased survival of rootworm in a number of cases (Krueger and Roberts 1997; Journey and Ostlie 2000; Toepfer et al. 2008, 2010; Pilz et al. 2009). However, entomopathogen efficacy, particularly with nematodes, has also been shown to be variable and sometimes inconsistent under field conditions, and can depend on many factors including environmental conditions, application method, concentration, and strain (e.g., Wright et al. 1993, Jackson and Brooks 1995, Riga et al. 2001).

In this study, Bt maize decreased the abundance of rootworm (Fig. 2), with mortality ranging from 80.1 to 89.1% for western corn rootworm, and 68.7 to 86.9% for northern corn rootworm (Table 3). One important factor to consider when calculating mortality from Bt maize relative to non-Bt maize is the role of density-dependent mortality (Hibbard et al. 2010). Hibbard et al. (2010) determined that root injury exceeding one node on the 0–3 node injury scale (Oleson et al. 2005) is required for density-dependent mortality to occur. Because injury in this study did not exceed one node, density-dependent mortality likely was not present. Determining pest mortality from a Bt crop is important for insecticide resistance management (Hibbard et al. 2010). 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, or an event that

imposes at least 99.99% mortality on 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 reaching 99.99% by Bt maize (Gassmann 2012), and dose in this study was lower than what has previously been reported for Bt maize producing Cry34/35Ab1 (Storer et al. 2006, Binning et al. 2010).

In addition to decreasing survival, Bt maize also increased developmental time in both northern corn rootworm and western corn rootworm, as emergence was delayed on Bt maize relative to non-Bt maize (Fig. 3), consistent with field data from other studies (Storer et al. 2006, Murphy et al. 2010, Hibbard et al. 2011). Western corn rootworm is protandrous, with females emerging later than males (Branson 1987). The reason for earlier average emergence dates for male northern corn rootworm in pathogen-treated plots is unknown. Differences were more pronounced for insects on Bt maize, but few insects emerged from these plots so additional experiments are needed.

The effectiveness of entomopathogenic fungi and nematodes for managing soil-dwelling pests partly depends on their persistence in the field (O'Callaghan 1998, Inyang et al. 2000, Kurtz et al. 2007). Persistence is defined as the durability of an organism (Onstad et al. 2006), and is a sum of parameters including survival, dispersal, host finding, and reproduction (Susurluk and Ehlers 2008). Results from this study indicate that both the entomopathogenic nematodes and fungi used in this study persisted throughout the larval stages of rootworm, or longer, because they were detected at significantly higher abundance in pathogen-treated plots compared with untreated plots (Table 5 and Results). Mean CFU per gram of soil were over fourfold higher in plots treated with entomopathogens in 2011. The low levels of *Metarhizium* spp. in control plots indicate that this genus occurred naturally in our study site, as has been reported for other maize fields (Pilz et al. 2008), and agricultural fields in general (Scheepmaker and Butt 2010). Percent of *G. mellonella* larvae infected with nematodes was higher for pathogen-treated plots than for control plots (Table 5). Both entomopathogenic nematodes and fungi have been shown to persist in maize fields (Kurtz et al. 2007, Pilz et al. 2011). Entomopathogenic nematodes can persist for several months and up to 2 yr after application to agricultural fields (Ferguson et al. 1995, Kurtz et al. 2007). In general, densities of *Metarhizium* applied in the field show a slow but steady decline over time, with persistence of at least 1 year (reviewed by Scheepmaker and Butt 2010). Thus, entomopathogens applied to the soil before hatch of rootworm eggs may persist in the soil through the period of time during which larval feeding occurs, and reduce root injury from larval feeding.

We examined how natural enemies interacted with Bt maize to affect rootworm survival, injury to maize, and yield by studying a blend of three entomopathogens that can co-occur together in maize fields (Pilz et al. 2008). There is often a positive relationship

between predator diversity and pest suppression (Snyder 2009), with greater species richness of natural enemies reducing pest density and increasing crop yields in some cases (Cardinale et al. 2003). However, because we used multiple species of natural enemies in combination, it is not possible to determine how each species individually affected root injury and yield. It may be the case that the variable environmental conditions between the 2 yr of the study affected the three entomopathogen species in different ways, possibly resulting in one or more entomopathogen species affecting rootworm injury and yield to a greater extent in year 1 compared with year 2. Future experiments aimed at understanding how each species of entomopathogen affects rootworm mortality, root injury, and yield would be beneficial.

While the potential of entomopathogenic nematodes and fungi for managing rootworm species has been recognized in a number of studies (Toepfer et al. 2009), successful management by entomopathogens depends on a number of variables. These include the particular strains used, adaptability to local conditions, soil temperature, and composition, availability of alternative hosts, and application methods (Kaya and Gaugler 1993, Shah and Pell 2003, Jaronski 2007, Cory and Ericsson 2010). The current study highlights the potential of using entomopathogens for management of rootworm species in combination with both Bt and non-Bt maize.

Acknowledgments

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