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Effects of Entomopathogens on Mortality of Western Corn Rootworm (Coleoptera: Chrysomelidae) and Fitness Costs of Resistance to Cry3Bb1 Maize

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ABSTRACT Fitness costs can delay pest resistance to crops that produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt), and past research has found that entomopathogens impose fitness costs of Bt resistance. In addition, entomopathogens can be used for integrated pest management by providing biological control of pests. The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a major pest of maize and is currently managed by planting of Bt maize. We tested whether entomopathogenic nematodes and fungi increased mortality of western corn rootworm and whether these entomopathogens increased fitness costs of resistance to Cry3Bb1 maize. We exposed western corn rootworm larvae to two species of nematodes, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), and to two species of fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (strain GHA) and *Metarhizium brunneum* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (strain F52) in two assay types, namely, seedling mat and small cup. Larval mortality increased with the concentration of *H. bacteriophora* and *S. feltiae* in the small cup assay, and with the exception of *S. feltiae* and *B. bassiana* in the seedling mat assay, mortality from entomopathogens was significantly greater than zero for the remaining entomopathogens in both assays. However, no fitness costs were observed in either assay type for any entomopathogen. Increased mortality of western corn rootworm larvae caused by these entomopathogens supports their potential use in biological control; however, the lack of fitness costs suggests that entomopathogens will not delay the evolution of Bt resistance in western corn rootworm.

KEY WORDS biological control, *Diabrotica virgifera virgifera*, fungi, nematode, refuge strategy

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is one of the most economically significant pests of maize in the United States (Gray et al. 2009). This pest has repeatedly developed resistance to management strategies, including insecticides, crop rotation, and, recently, to the insecticidal toxin Cry3Bb1, which is derived from the bacterium *Bacillus thuringiensis* (Bt) and is produced by transgenic maize (Levine and Oloumi-Sadeghi 1991, Meinke et al. 1998, Gassmann et al. 2011). In 2003, the United States Environmental Protection Agency registered genetically modified maize (*Zea mays* L.) that produces Cry3Bb1 for the management of western corn rootworm (Environmental Protection Agency [EPA] 2010). In 2012, 67% of maize planted in the United States produced at least one Bt toxin and this widespread planting places intense selection on pest populations to develop resistance (Economic Research Service [ERS] 2012).

In the United States and elsewhere, the refuge strategy is used to delay the development of resistance to Bt crops (Gould 1998). The refuge strategy uses non-Bt host plants that allow the survival of Bt-susceptible insects so they may mate with Bt-resistant insects. To the extent that heterozygous progeny have lower fitness on a Bt crop than their homozygous resistant parents, and therefore resistance is expected to be delayed, with delays becoming greater as the genetic dominance of resistance decreases (Tabashnik et al. 2004). In addition, as the amount of refuge increases, delays in resistance become greater (Carriére and Tabashnik 2001). Furthermore, fitness costs will delay the evolution of resistance and these delays become greater as fitness costs become larger (Carriére et al. 2010).

A fitness cost of Bt resistance occurs when individuals with resistance alleles have lower fitness than Bt-susceptible individuals in the absence of Bt toxin (Gassmann et al. 2009a). Fitness costs can delay re-

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sistance evolution by reducing the frequency of resistance alleles present in the refuge (Carriére and Tabashnik 2001, Gassmann et al. 2009a). Thus, the rate of resistance evolution is affected not only by production of susceptible insects from the refuge, but also by the presence of fitness costs in refuges. Importantly, ecological factors, including entomopathogens, can magnify fitness costs of Bt resistance (Gassmann et al. 2006, Raymond et al. 2007, Hannon et al. 2010).

Several species of entomopathogenic fungi and nematodes have been shown to infect and kill western corn rootworm larvae and have potential for use in biological control (Meyling and Eilenberg 2007, Pilz et al. 2007, Toepfer et al. 2009). Furthermore, entomopathogenic nematodes and fungi have been found to occur naturally in maize fields (Pilz et al. 2008, Rudeen et al. 2013). Fungal conidia contact the insect cuticle, germinate, and subsequently penetrate the hemocoel, where they grow and produce insecticidal compounds, leading to death of the host (Shah and Pell 2003, Lewis et al. 2006). Following death of the host, conidia are subsequently produced on the cuticle and then disperse to infect additional insects (Hajek and St. Leger 1994). Infective juveniles of entomopathogenic nematodes are motile and free-living, and enter the hemocoel through natural openings and release symbiotic bacteria that kills the host within 24–48 h. Nematodes then feed and reproduce inside the cadaver, and a subsequent generation of infective juveniles disperses into the environment (Kaya and Gaugler 1993, Grewal et al. 1994).

This study examined whether entomopathogenic nematodes and fungi cause mortality of larvae of western corn rootworm and whether these entomopathogens can magnify fitness costs of resistance to Cry3Bb1 maize. These results are relevant both to the application of entomopathogens in biological control of western corn rootworm and to insect resistance management for Bt crops. By testing which ecological factors magnify fitness costs of Bt resistance, it may be possible to design non-Bt refuges that enhance fitness costs, thereby delaying Bt resistance more effectively (Carriére and Tabashnik 2001, Pittendrigh et al. 2004).

Materials and Methods

Insect Strains. Field-collected adult males of western corn rootworm from four locations (Hamilton County, OH; Moody/Lake County, SD; Phillips County, CO; and Will County, IL) were crossed with females from a nondiapause strain at the North Central Agricultural Research Laboratory (NCARL; Oswald et al. 2011). From this cross, the following two strains were developed: 1) a susceptible strain not exposed to Bt toxin and 2) a resistant strain (the moderately selected strain in Oswald et al. 2011) that was fed Cry3Bb1 maize for increasing durations over 11 generations (F_0 to F_{10}). Strains were sent from NCARL to Iowa State University at the F_{13}, where they were reared on maize seedling mats according to the methods of Jackson (1986) and Oswald et al. (2011), and maintained at a population size of >1,200 adults. The F_{13}, F_{15}, and F_{17} of the resistant strain were reared on Cry3Bb1 maize (hybrid DKC 61-69, Monsanto Company, Saint Louis, MO) and the susceptible strain was reared on non-Bt maize (hybrid 34M94, DuPont Pioneer, Johnston, IA), both of which were free of seed treatments. The susceptible strain was maintained concurrently with, but independently from, the resistant strain. To increase genetic similarity between strains, the resistant strain was backcrossed to the susceptible strain at a 1:1 ratio during the F_{13} and F_{15}. During the F_{14}, F_{16}, and F_{18} through F_{24}, the resistant and susceptible strains were reared on non-Bt maize (hybrid 34M94). In the F_{24}, survival to adulthood on seedling mats of Cry3Bb1 maize was more than twice as high for the resistant strain compared with the susceptible strain (Hoffmann 2013).

Entomopathogens. The entomopathogens used in this experiment were selected because past research found that they could infect and kill western corn rootworm larvae (Pilz et al. 2007; Toepfer et al. 2008, 2009). The entomopathogenic nematodes *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae) were received from Becker-Underwood (Ames, IA) and reared in *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae as described by Kaya and Stock (1997). Infective juvenile nematodes were used in experiments within 2 wk of emerging from *G. mellonella* cadavers. The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (strain GHA) and *Metarhizium brunneum* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (strain F52) were received from the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Northern Plains Agricultural Research Laboratory, and stored at 6°C until they were used in experiments.

Two assays were used to test of effects of entomopathogens on western corn rootworm larvae. One assay was the small cup assay described by Petzold-Maxwell et al. (2012a). The second assay used a seedling mat, which is a standard medium for rearing western corn rootworm larvae in the laboratory (Oswald et al. 2012). Applying two bioassay methods enabled a more complete understanding of potential interactions between western corn rootworm larvae and entomopathogens. This is because these methods differed in the quantity of corn roots, density of roots, and the spatial distribution of corn roots and soil, all of which could influence interactions between western corn rootworm larvae and entomopathogens, and subsequent larval mortality.

Seedling Mat Assay. This experiment was conducted during February and March 2012 using the F_{22} of the resistant and susceptible strains. For each entomopathogen, a separate experiment was conducted using a fully crossed block design with two insect strains (resistant and susceptible) and four concentrations of entomopathogen. For each of the four species of entomopathogen, eight blocks were run. Both the resistant and susceptible strains were tested in each block using one seedling mat for each of the four

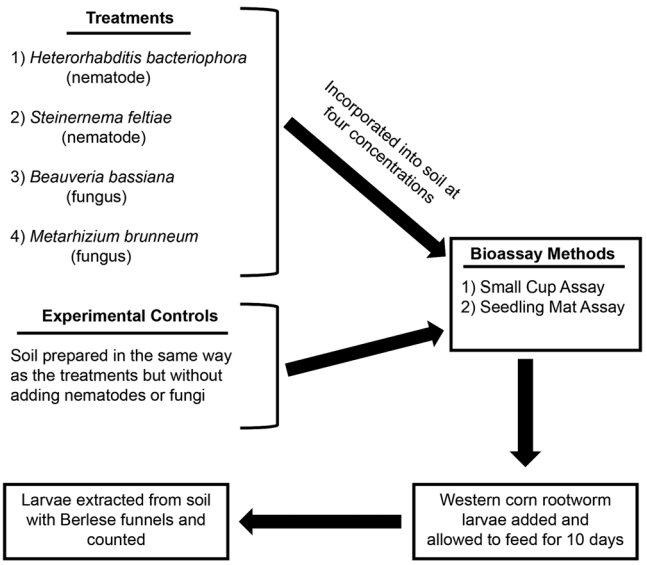

Fig. 1. A diagrammatic representation of methods applied to conduct bioassays.

entomopathogen concentrations and three control seedling mats, for a total of 14 seedling mats per block. Control seedling mats were not treated with entomopathogens but were otherwise identical to seedling mats with entomopathogens. Thus, for each of the four entomopathogen species, 112 seedling mats were tested, and for the entire experiment, total 448 seedling mats were tested.

Seedling mats were made using 40 ml of maize seed (≈65 kernels of the non-Bt hybrid 34M94) that was presoaked for 12 h in deionized (DI) water and then placed in a 0.95-liter container (Pactiv Showcase, Johnson Paper and Supply Company, Minneapolis, MN) with a lid that contained six holes (diameter = 1 cm) for ventilation. Seeds were covered with a moist paper towel (23504, GA Pacific, Atlanta, GA), and placed in a growth chamber (25°C, 65% relative humidity [RH], and a photoperiod of 16:8 [L:D] h) for 3–4 d. The paper towel was then removed and seeds covered with 150 g (dry mass) of soil that was a mixture of 40%, by volume, of field-collected soil and 60% Sunshine Sun Gro LC1 potting soil mix (Sun Gro Horticulture Canada Ltd., Vancouver, BC, Canada). Before being placed in containers, the soil was moistened with either 30 ml of entomopathogen solution, or in the case of control mats, 30 ml of a control solution that lacked entomopathogens (Fig. 1).

Concentrations of live infective juvenile nematodes were measured with a microscope (MZ6, Leica Microsystems, Wetzlar, Germany) and a Sedgewick-Rafter counting cell (Pyser-SGI, Edenbridge, Kent, United Kingdom). Nematode solutions were made using DI water and added to soil to achieve the following four concentrations of nematodes within the soil: 50, 100, 150, and 200 nematodes per gram of dry soil. Concentrations were selected based on the results of Petzold-Maxwell et al. (2012a). Each seedling mat with nematodes received 30 ml of nematode solution and each control seedling mat received 30 ml of DI water. Thirty milliliters of liquid moistened the soil to 25% of water holding capacity. Solutions were incorporated into the soil by hand, and the moistened soil was then placed on top of the seedling mats.

Solutions of fungal entomopathogens were made by combining conidia with an autoclaved solution of 0.10% sorbitan monooleate (Tween 80, Acros Organics, Morris Plains, NJ). Concentration of conidia was determined using a hemocytometer (3520, Fisher, Waltham, MA) and microscope (Eclipse E200, Nikon, Melville, NY) with viability measured 24 h before application as described by Goettel and Inglis (1997). Four concentrations of conidia were tested: 1.0×10^4, 1.0×10^5, 1.0×10^6, and 1.0×10^7 viable conidia per g of dry soil. These concentrations were selected based on the results of Petzold-Maxwell et al. (2012b). Soil for seedling mats received either 30 ml of solution with fungal conidia, or in the case of control mats, 30 ml of 0.10% sorbitan monooleate solution that did not contain fungal conidia. Each solution was mixed into the soil by hand before soil was placed on top of and underneath maize seeds. This modification from the seedling mat assay with nematodes was made to increase contact of the soil with maize roots.

For all assays, seedling mats with soil were returned to a growth chamber and allowed to grow for an additional 3–4 d, after which time 25 neonate larvae (<1 d old) of the appropriate strain of western corn rootworm were placed on the surface of the soil with a fine-hair paintbrush. Fine-mesh fabric (25 by 25 cm, 194811 Poly Chiffon, Hobby Lobby Stores Inc., Oklahoma City, OK) covered the underside of the plastic lid to prevent larvae from escaping. Containers were placed in a growth chamber (25°C, 65% RH, and a photoperiod of 16:8 [L:D] h) for 10 d, with seedling mats receiving 30 ml of DI water 7 d after neonates were added. After 10 d, seedling mat, soil, and larvae

from each bioassay container were placed individually on Berlese funnels for 3 d to extract live larvae into vials containing 85% ethanol. The total number of larvae extracted per bioassay container was counted.

Small Cup Assay. This experiment was conducted at the same time as the seedling mat assay. Each of the four species of entomopathogens was tested in a separate set of assays. For each species of entomopathogen, a fully crossed block design was used with two insect strains (susceptible and resistant) and four concentrations of entomopathogen. For each species of entomopathogen, eight blocks were run. Within a block, both the resistant and susceptible strains were tested in two containers for each of the four entomopathogen concentrations and in four control containers that did not receive entomopathogens, for a total of 24 containers per block. For each species of entomopathogen, total 192 bioassay containers were evaluated, and in the entire experiment, total 768 bioassay containers were evaluated.

Bioassays used 44-ml containers with lids (Translucent Plastic Souffle Cup, Solo Cup Company, Highland Park, IL). Maize seed was the same as in the seedling mat assay. Seed was soaked for 12 h and then placed on moistened paper towels for 3 d, after which time there was ≈2 cm of a radical root per seed. Three germinated seedlings were placed at the bottom of each bioassay container and covered with soil that contained pathogens, or in the case of control containers, lacked pathogens. Solutions of nematodes and fungi were prepared in the same manner as the seedling mat assay. For each bioassay container, 30 g of sieved field soil (<600 μm) was combined with 4.5 ml of either the appropriate entomopathogen solution or a control solution that lacked pathogens, and then placed on top of the maize seedlings. Adding 4.5 ml of liquid moistened the soil to 25% of water holding capacity. Finally, six neonate larvae from either the resistant or susceptible strain were placed on the surface of the soil in each bioassay container. Small holes in the lid provided ventilation and mesh fabric under the lid prevented larvae from escaping. Containers were placed in a single layer between two plastic trays (CT1216, Carlisle Foodservice Products, Oklahoma City, OK) that were lined with moistened paper towels. Plastic trays containing cups were then placed inside a large plastic bag (Hefty EasyFlaps 13 Gallon Tall Kitchen Bags, Reynolds Consumer Products, Lake Forest, IL) to retain humidity and provide a dark environment. Trays were placed inside a growth chamber (25°C, 65% RH) for 10 d. Soil in each container received 1 ml of DI water at Day 7. On Day 10, soil with seedlings and larvae were placed on Berlese funnels for 3 d to extract live larvae into a vial with 85% ethanol. The total number of larvae extracted in each bioassay container was recorded (Fig. 1).

Data Analysis. Analyses were conducted using SAS Enterprise Guide 5.1 (SAS Institute 2012). For each bioassay, data on larval mortality in the experimental controls was compared between resistant and susceptible strains with an analysis of variance (ANOVA) using the MIXED procedure. Larval mortality in the presence of entomopathogens was first adjusted for control mortality using Abbott's correction (Abbott 1925), and then analyzed separately for each combination of entomopathogen species and assay type using a mixed model ANOVA and analysis of covariance (ANCOVA) based on the MIXED procedure described by Hannon et al. (2010). In addition, a *t*-test was used to test whether corrected mortality was significantly greater than zero (PROC TTEST).

For corrected mortality in each combination of entomopathogen species and assay type, an ANOVA was used first to test whether a difference in the regression slope of rootworm mortality onto entomopathogen concentration was present between strains. Fixed factors in this ANOVA were the continuous variable of entomopathogen concentration, the categorical variable of insect strain, and their interaction. If the slopes did not differ, data were then analyzed within an ANCOVA that included the categorical variable of insect strain and the continuous covariate of entomopathogen concentration. In both the ANCOVA and ANOVA, block and its interactions with fixed factors were coded as random factors and were tested for significance with a log-likelihood ratio statistic (−2 RES log likelihood in PROC MIXED) based on a one-tailed chi-square test with one degree of freedom (Littell et al. 1996). If a random factor was not significant at $P < 0.25$, it was removed from the model to increase the statistical power (Quinn and Keough 2002). Lower order terms were retained if their higher order interactions were significant.

For each entomopathogen, *t*-tests were conducted as described by Sokal and Rohlf (2003) to test whether 1) average corrected mortality for larvae in the small cup assay differed from average corrected mortality in the seedling mat assay, this was also conducted for the controls, and 2) whether average corrected mortality differed between species of nematodes and fungi in the small cup assay.

Results

In the seedling mat assay, larval mortality in the experimental controls, which lacked entomopathogens, ranged from 17 to 39% (Table 1). Control mortality did not differ significantly between western corn rootworm strains, indicating that fitness costs were not present in the absence of pathogens. No significant difference in mortality between strains was found for *H. bacteriophora* (Fig. 2A), *S. feltiae* (Fig. 2B), or *M. brunneum* (Fig. 2C), which suggests that the presence of these pathogens did not impose fitness costs of Bt resistance (Table 2). In the presence of *B. bassiana* (Fig. 2D), larval mortality was significantly lower for the resistant strain at the two lower concentrations when compared with the susceptible strain, again indicating the absence of any fitness cost associated with Bt resistance. Corrected mortality of western corn rootworm was significantly greater than zero in the presence of *H. bacteriophora* and *M. brunneum*, providing evidence that these entomopathogens increased mortality of western corn rootworm in the

Table 1. Percent larval mortality for experimental control containers that did not receive entomopathogens in the seedling mat assay and small cup assay, and accompanying analysis of variance comparing larval mortality between strains

Assay type	Larval mortality		df	*F*	*P*
	Resistant susceptible	Strain			
Seedling mat assay					
H. bacteriophora	18%	17%	1,7	0.1	0.76
S. feltiae	18%	17%	1,7	0.04	0.84
M. brunneum	21%	25%	1,7	1.76	0.23
B. bassiana	38%	39%	1,7	0	0.98
Small cup assay					
H. bacteriophora	19%	20%	1,7	0.08	0.78
S. feltiae	9%	15%	1,7	1.57	0.25
M. brunneum	32%	22%	1,7	4.52	0.07
B. bassiana	13%	13%	1,7	0	1.000

seedling mat assay (Table 3). However, no increase in mortality from entomopathogens in the seedling mat assay was detected for *S. feltiae* or *B. bassiana*, as corrected mortality did not differ significantly from zero for either of these pathogens (Table 3). For all pathogens, there was not a significant affect of concentration on mortality (Table 2), with larval mortality remaining at similar values as pathogen concentration increased (Fig. 2).

In the small cup assay, larval mortality in experimental controls that lacked entomopathogens did not differ significantly between strains, suggesting an ab-

Table 2. ANCOVA and ANOVA for larval mortality from four species of entomopathogens in the seedling mat assay

Effect	df	*F*	*P*
H. bacteriophora[a,b]			
Strain	1,47	1.58	0.21
Concn	1,7	2.16	0.18
S. feltiae[c,d]			
Strain	1,7	0.03	0.87
Concn	1,47	0.71	0.40
M. brunneum[d,e]			
Strain	1,7	1.11	0.33
Concn	1,47	0.32	0.58
B. bassiana[f,g]			
Strain	1,7	21.52	0.002
Concn	1,7	1.46	0.27
Strain × Concn	1,7	5.89	0.04

[a] Strain × concentration was not significant ($F = 1.17$; df = 1,7; $P = 0.31$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[b] Random factors included in the model were block and block × concentration.

[c] Strain × concentration was not significant ($F = 1.67$; df = 1,7; $P = 0.24$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[d] Random factors included in the model were block and block × strain.

[e] Strain × concentration was not significant ($F = 1.22$; df = 1,7; $P = 0.31$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[f] Because of the significant strain × concentration interaction, data were analyzed with an ANOVA.

[g] Random factors included in the model were block, block × strain, block × concentration, and block × strain × concentration.

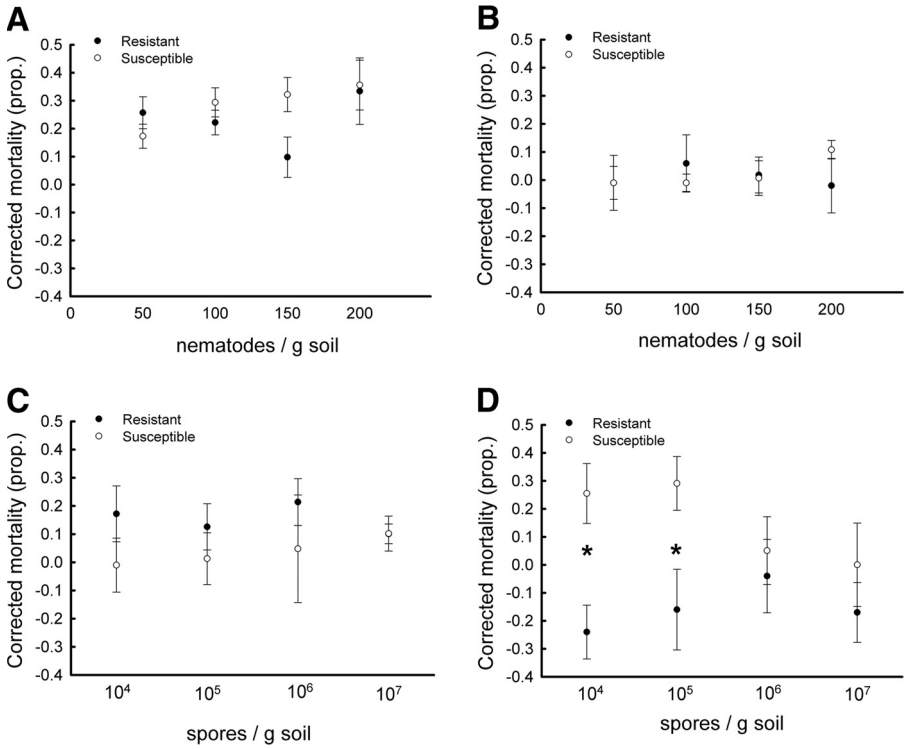

Fig. 2. Corrected mortality (mean ± SE) for western corn rootworm larvae in the seedling mat assay when exposed to entomopathogenic nematodes—(A) *H. bacteriophora* and (B) *S. feltiae*—and entomopathogenic fungi—(C) *M. brunneum* and (D) *B. bassiana*. An asterisk indicates significant difference between strains within a concentration.

Table 3. Corrected larval mortality imposed by entomopathogens in the seedling mat and small cup assays

Assay type	Mean	SE	t^a	df	P
Seedling mat assay					
H. bacteriophora	0.26	0.03	9.92	63	<0.0001
S. feltiae	0.02	0.03	0.70	63	0.49
M. brunneum	0.10	0.04	2.71	63	0.01
B. bassiana	−0.003	0.05	0.06	63	0.95
Small cup assay					
H. bacteriophora	0.39	0.03	13.75	127	<0.0001
S. feltiae	0.46	0.03	18.27	127	<0.0001
M. brunneum	0.09	0.03	2.96	127	0.004
B. bassiana	0.11	0.02	4.49	127	<0.0001

[a] The null hypothesis was that the mean equals zero.

Table 4. Analysis of covariance for larval mortality from each of four entomopathogen species in the small cup assay

Effect	df	F	P
H. bacteriophora[a,b]			
Strain	1,7	0.48	0.51
Concn	1,111	23.88	<0.0001
S. feltiae[c,d]			
Strain	1,118	1.11	0.29
Concn	1,118	14.89	0.0002
M. brunneum[e,f]			
Strain	1,111	1.07	0.30
Concn	1,7	1.11	0.33
B. bassiana[g,b]			
Strain	1,7	0.93	0.37
Concn	1,111	1.34	0.25

[a] Strain × concentration was not significant ($F = 1.82$; df = 1,7; $P = 0.22$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[b] Random factors included in the model were block and block × strain.

[c] Strain × concentration was not significant ($F = 0.62$; df = 1,7; $P = 0.46$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[d] Random factor included in the model was block.

[e] Strain × concentration was not significant ($F = 0.10$; df = 1,7; $P = 0.76$) and was removed from the model to allow data to be analyzed with an ANCOVA.

[f] Random factors included in the model were block and block × concentration.

[g] Strain × concentration was not significant ($F = 0.58$; df = 1,7; $P = 0.47$) and was removed from the model to allow data to be analyzed with an ANCOVA.

sence of fitness costs when pathogens were not present (Table 1). Mortality in the experimental controls ranged from 9 to 32%, with a mean value of 17.9%, which was significantly lower than mortality of the experimental controls in the seedling mat assay (mean mortality = 23.0%; $t = 2.7$; df = 443; $P = 0.007$). As with the seedling mat assay, there was no significant difference between strains for corrected mortality in the small cup assay, indicating a lack of fitness costs (Fig. 3; Table 4). Corrected mortality for western corn rootworm larvae was significantly greater than zero in the presence of all four pathogens (Table 3) in the small cup assay, providing evidence for the capacity of all pathogens tested to impose mortality on western corn rootworm larvae. Furthermore, for both species

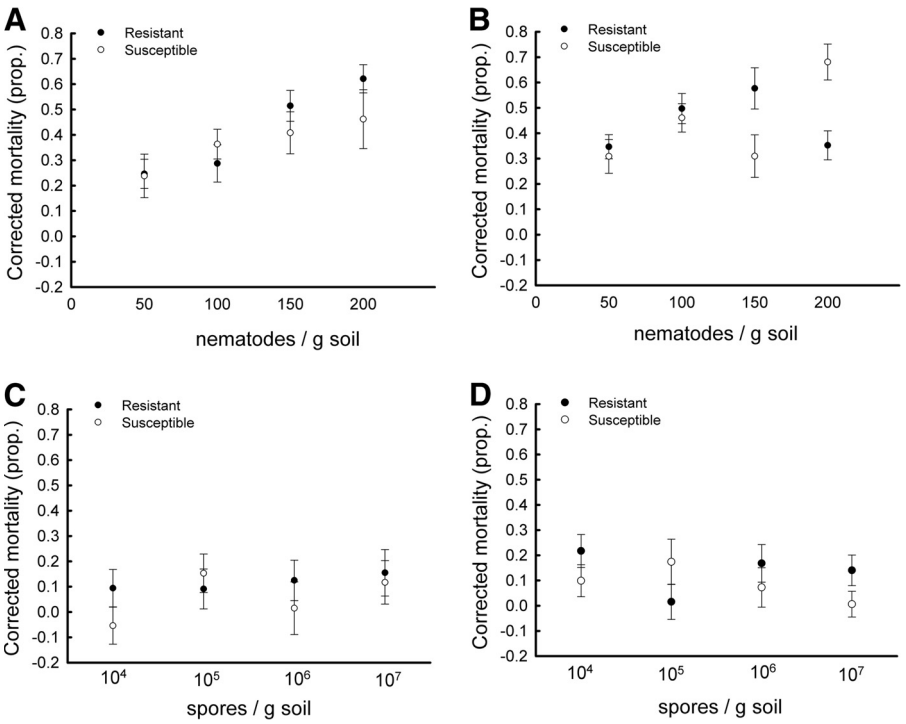

Fig. 3. Corrected mortality (mean ± SE) for western corn rootworm larvae in the small cup assay when exposed to entomopathogenic nematodes—(A) *H. bacteriophora* and (B) *S. feltiae*—and entomopathogenic fungi—(C) *M. brunneum* and (D) *B. bassiana*.

of nematodes in the small cup assay, there was a significant affect of pathogen concentration on larval mortality, with larval mortality increasing as the concentration of pathogens increased (Fig. 3A and B; Table 4). In contrast, for both species of entomopathogenic fungi, no significant effect of concentration was detected (Table 4), and larval mortality displayed similar values as pathogen concentration increased (Fig. 3C and D).

In general, mortality of western corn rootworm larvae from entomopathogens was greater in the small cup assay than in the seedling mat assay (Table 3). Significantly greater corrected mortality in the small cup assay versus the seedling mat assay was observed for *H. bacteriophora* ($t = 3.09$; df = 190; $P = 0.002$), *S. feltiae* ($t = 11.18$; df = 190; $P < 0.0001$), and *B. bassiana* ($t = 2.38$; df = 190; $P = 0.02$). However, no difference between assay methods was found for *M. brunneum* ($t = 0.17$; df = 190; $P = 0.86$). In addition, mortality of western corn rootworm larvae from entomopathogens tended to be greater for nematodes than fungi (Table 3). In the small cup assay, *H. bacteriophora* imposed significantly greater mortality than either *B. bassiana* ($t = 7.4$; df = 254; $P < 0.0001$) or *M. brunneum* ($t = 7.5$; df = 254; $P < 0.0001$), and *S. feltiae* imposed significantly greater mortality than either *B. bassiana* ($t = 9.9$; df = 254; $P < 0.0001$) or *M. brunneum* ($t = 9.7$; df = 254; $P < 0.0001$).

Discussion

Fitness costs can delay the evolution of Bt resistance, and the presence of entomopathogens can magnify fitness costs (Gassmann et al. 2009a). The entomopathogenic nematodes and fungi tested in this study did not increase larval mortality for the Bt-resistant strain when compared with the Bt-susceptible strain (Tables 2 and 4), indicating that fitness costs of Bt resistance were not imposed by these entomopathogens. However, increased mortality of western corn rootworm larvae caused by entomopathogens was detected in six of the eight experiments conducted in this study (Table 3). These results parallel the work of Petzold-Maxwell et al. (2012a), which also found that entomopathogens did not increase fitness costs of resistance to Cry3Bb1 maize for western corn rootworm. Although significantly higher mortality was observed for the susceptible strain compared with resistant strain at some concentration of conidia in the seedling mat assay with *B. bassiana*, mortality in this assay was highly variable and did not differ significantly from zero (Fig. 2D; Table 3). Furthermore, no difference was observed between strains in the small cup assay with *B. bassiana* (Fig. 3D). Higher concentrations of *B. bassiana* would likely need to be used in the seedling mat assay to provide a more complete test of the potential effects of *B. bassiana* on differences in fitness between resistant and susceptible strains.

Effects of entomopathogens on fitness costs of Bt resistance have been tested for pest species other than western corn rootworm. The entomopathogenic nematodes *S. riobrave* and *H. bacteriophora* imposed the fitness cost of higher mortality for Cry1Ac-resistant *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidea; Gassmann et al. 2006, 2008, 2009b), although fitness costs were absent in the presence of *Steinernema carpocapsae* Weiser, *Steinernema* sp. (ML18 strain), and *H. sonorensis* (Hannon et al. 2010). In addition, Cry1Ac-resistant *Plutella xylostella* L. (Lepidoptera: Plutellidae) exposed to an insect virus (Vcal MNPV) experienced a fitness cost of decreased egg viability (Raymond et al. 2007). Although some entomopathogens may increase fitness costs of Bt resistance for some insect species, pathogen-mediated costs appear not to be present in some cases.

Corrected mortality of western corn rootworm was significantly greater than zero when treated with any of the four pathogens in the small cup assay, and when treated with *H. bacteriophora* and *M. brunneum* in the seeding mat assay (Table 3). For *H. bacteriophora*, *S. feltiae*, and *B. bassiana*, significantly higher larval mortality was imposed in the small cup assay than in the seedling mat assay. This may be due to the soil surrounding individual seedlings in the small cup assay, which would increase contact of larvae with pathogens. In contrast, in the seedling mat assay, larvae could move within a mat of maize roots and not contact the soil. Thus, the small cup assay may more closely resemble field conditions where individual nodal maize roots are surrounded by soil. In addition, entomopathogenic nematodes exhibit different foraging strategies, with *H. bacteriophora*, a cruiser, actively searching for a host, whereas *S. feltiae*, an intermediate forager, exhibits both cruiser and ambush strategies (Grewal et al. 1994). The more active foraging of *H. bacteriophora* compared with *S. feltiae* may account for significant mortality imposed by *H. bacteriophora* but not *S. feltiae* in the seedling mat assay.

Data from the small cup assay support the use of entomopathogenic nematodes and fungi as one component of an integrated pest management (IPM) strategy by providing biological control of western corn rootworm. In a field study with western corn rootworm, Toepfer et al. (2008) found that *H. bacteriophora* and *H. megidis* imposed ≈70% mortality and *S. feltiae* imposed 32% mortality when applied at 3.4×10^9 nematodes per hectare. (Toepfer et al. 2008). In the small cup assay, *H. bacteriophora* and *S. feltiae* were applied at an average rate of 4.7×10^9 nematodes per hectare and imposed an average of 39 and 46% mortality, respectively (Table 3). *Metarhizium anisopliae* imposed 31% mortality against western corn rootworm when applied in the field at a range of 4×10^{13} to 7×10^{13} conidia per hectare (Pilz et al. 2009). In the small cup assay reported here, *M. brunneum* and *B. bassiana* were applied at an average rate of 3.5×10^{12} conidia per hectare and imposed an average 9 and 11% mortality, respectively (Table 3). At the application rates studied in the small cup assay, *H. bacteriophora* and *S. feltiae* imposed significantly higher mortality than *M. brunneum* and *B. bassiana*, suggesting that nematodes may be more effective biological control agents for western corn rootworm larvae. However, additional

ecological complexities can arise in the field, including semiochemical-based recruitment of nematodes to injured maize roots (Rasmann et al. 2005) and colonization of the rhizosphere by entomopathogenic fungi (Bruck 2010). Such complexities are likely not captured by the short-duration laboratory bioassays conducted in this study.

The IPM benefit of entomopathogens for management of rootworm has been found in field studies evaluating crop yield and larval feeding injury. Field trials indicate that entomopathogens, both fungi and nematodes, have the ability to decrease injury to maize roots, and in some cases, increase yield (Krueger and Roberts 1997, Journey and Ostlie 2000, Toepfer et al. 2010, Petzold-Maxwell et al. 2013). For example, a combination of two entomopathogenic nematodes, *H. bacteriophora* and *S. carpocapsae*, and one fungus, *M. brunneum*, significantly increased maize yield for both Bt maize and non-Bt maize (Petzold-Maxwell et al. 2013). Furthermore, these pathogens reduced root injury to Bt maize when western corn rootworm abundance in the field was high, and to non-Bt maize when rootworm abundance was low (Petzold-Maxwell et al. 2013). In a field study, *H. bacteriophora* significantly reduced root injury from feeding by larvae of western corn rootworm by 25–79% (Toepfer et al. 2010). Thus, use of entomopathogens in conjunction with other management tactics, such as crop rotation or Bt maize, may help to augment management of western corn rootworm and preserve yield.

Current data suggest that few fitness costs may accompany Cry3Bb1 resistance in western corn rootworm. Both the results of this study and the results of Petzold-Maxwell et al. (2012a), found an absence of fitness costs in the presence of entomopathogens. In a study of five Cry3Bb1-resistant western corn rootworm strains (including the strain used here), Oswald et al. (2012) did not detect fitness costs for survival, fecundity, and egg viability, but did detect increased fitness for resistant strains through faster developmental rate and higher fecundity. In contrast, Meihls et al. (2012) found evidence of fitness costs affecting fecundity and longevity among three Cry3Bb1-resistant strains of western corn rootworm in greenhouse and field studies. In the absence of fitness costs, resistance will evolve more rapidly and it will persist after selection (Carriére and Tabashnik 2001). In the field, Cry3Bb1 resistance has been detected in western corn rootworm populations after as few as three pest generations (Gassmann et al. 2011) and has been found to persist after fields were rotated away from Cry3Bb1 maize (Gassmann et al. 2012). The increased risk of resistance associated with a lack of fitness costs highlights the need for sound IPM for western corn rootworm, and the data presented here illustrate the potential use of entomopathogens as one component of an IPM strategy. Future research on strains of western corn rootworm with field-evolved resistance to Bt corn will be an important next step in understanding fitness costs and other biologically relevant variables that affect the evolution of Bt resistance.

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