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Tritrophic interactions among Bt maize, an insect pest and entomopathogens: effects on development and survival of western corn rootworm

Jennifer L. Petzold-Maxwell
Iowa State University, jennifer.maxwell@wartburg.edu

Stefan T. Jaronski
United States Department of Agriculture

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

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Abstract
Agricultural systems often provide a model for testing ecological hypotheses, while ecological theory can enable more effective pest management. One of the best examples of this is the interaction between host-plant resistance and natural enemies. With the advent of crops that are genetically modified to produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt), a new form of host-plant resistance has been introduced to agroecosystems. How Bt crops interact with natural enemies, especially insect pathogens in below-ground systems, is not well understood, but provides a unique opportunity to study below-ground tritrophic interactions. In this study, we used two species of entomopathogenic fungi and three species of entomopathogenic nematodes to determine how this community of soil-borne natural enemies might interact with Bt maize (event 59122, expressing the insecticidal protein Cry34/35Ab1) to affect survival and development of western corn rootworm (*Diabrotica virgifera virgifera*), which is an obligate root feeder and a serious pest of maize. We ran two experiments, one in a greenhouse and one in a growth chamber. Both experiments consisted of a fully crossed design with two maize treatments (Bt maize and non-Bt maize) and two entomopathogen treatments (present or absent). The community of entomopathogens significantly increased mortality of western corn rootworm, and Bt maize increased larval developmental time and mortality. Entomopathogens and Bt maize acted in an independent and additive manner, with both factors increasing the mortality of western corn rootworm. Results from this study suggest that entomopathogens may complement host-plant resistance from Bt crops.

Keywords
Entomopathogens, host-plant resistance, transgenic corn, western corn rootworm

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RESEARCH ARTICLE

Tritrophic interactions among Bt maize, an insect pest and entomopathogens: effects on development and survival of western corn rootworm

J.L. Petzold-Maxwell[1], S.T. Jaronski[2] & A.J. Gassmann[1]

1 Department of Entomology, Iowa State University, Ames, IA, USA
2 The Northern Plains Agricultural Research Laboratory, USDA Agricultural Research Service, Sidney, MT, USA

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Correspondence
J.L. Petzold-Maxwell, Department of Entomology, Iowa State University, Ames, IA 50011, USA. Email: jpetzold@iastate.edu

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Abstract

Agricultural systems often provide a model for testing ecological hypotheses, while ecological theory can enable more effective pest management. One of the best examples of this is the interaction between host-plant resistance and natural enemies. With the advent of crops that are genetically modified to produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt), a new form of host-plant resistance has been introduced to agroecosystems. How Bt crops interact with natural enemies, especially insect pathogens in below-ground systems, is not well understood, but provides a unique opportunity to study below-ground tritrophic interactions. In this study, we used two species of entomopathogenic fungi and three species of entomopathogenic nematodes to determine how this community of soil-borne natural enemies might interact with Bt maize (event 59122, expressing the insecticidal protein Cry34/35Ab1) to affect survival and development of western corn rootworm (*Diabrotica virgifera virgifera*), which is an obligate root feeder and a serious pest of maize. We ran two experiments, one in a greenhouse and one in a growth chamber. Both experiments consisted of a fully crossed design with two maize treatments (Bt maize and non-Bt maize) and two entomopathogen treatments (present or absent). The community of entomopathogens significantly increased mortality of western corn rootworm, and Bt maize increased larval developmental time and mortality. Entomopathogens and Bt maize acted in an independent and additive manner, with both factors increasing the mortality of western corn rootworm. Results from this study suggest that entomopathogens may complement host-plant resistance from Bt crops.

Introduction

The philosophy of integrated pest management includes the use of multiple ecological factors to aid in the control of pest insects (Pedigo & Rice, 2009). Two biotic factors that can affect insect abundance are host-plant resistance and natural enemies. Numerous studies have tested the compatibility of these bottom-up and top-down factors in regulating pest populations in agroecosystems (Bottrell *et al.*, 1998; Cortesero *et al.*, 2000; Hare, 2002; Ode, 2006). The development of transgenic crops that produce insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) has introduced a novel form of host-plant resistance into agricultural systems. To date, relatively little is known about how this host-plant resistance factor will interact with natural enemies, in particular insect pathogens, to affect populations of pest insects (Lundgren *et al.*, 2009).

Host-plant resistance and biological control may affect pest populations in a variety of ways, by interacting in a synergistic or antagonistic manner or having independent effects (Bottrell *et al.*, 1998; Hare, 2002). Plant resistance traits that alter pest behaviour or physiology (i.e. slower developmental rate or increased searching time) can lead to enhanced attack by natural

enemies, resulting in synergism (Johnson & Gould, 1992). Conversely, antagonistic interactions can arise when host-plant resistance traits have direct negative effects on enemies by increasing mortality (Ponsard *et al.*, 2002) or reducing access of prey (Gassmann & Hare, 2005). Finally, natural enemies and host-plant resistance factors can act independently on insect populations, resulting in either complementary or opposing effects (Meissle *et al.*, 2009).

Although such interactions have been well studied for arthropods, fewer studies have investigated interactions with entomopathogens (Cory & Ericsson, 2010). Moreover, an extensive gap in our knowledge exists for below-ground tritrophic interactions (Rasmann & Turlings, 2008; Bruck, 2010). Knowledge of below-ground interactions among host-plant resistance, pest species and entomopathogens has important implications for integrated pest management strategies and tritrophic interactions in natural systems. Entomopathogens are important natural sources of mortality for many species of insects (Kaya & Gaugler, 1993; Meyling & Eilenberg, 2007), and there is a significant potential for the use of entomopathogens in controlling root-feeding pest species (Rasmann & Turlings, 2008; Bruck, 2010). Furthermore, recent experiments show that host-plant factors can influence herbivore–entomopathogen interactions (Rasmann *et al.*, 2005; Cory & Hoover, 2006).

Bt crops are planted on an increasing area each year and covered over 58 million ha globally in 2010, with maize being the most widely cultivated Bt crop (James, 2010). The western corn rootworm is one of the most economically important pests of maize in North America, and has recently spread to Europe (Gray *et al.*, 2009). Bt maize can control several insect pests, including rootworm species. Several entomopathogenic nematodes and fungi have been shown to kill western corn rootworm (Toepfer *et al.*, 2009), although very little is known about how these entomopathogens interact with Bt maize to influence western corn rootworm survival. Furthermore, entomopathogenic nematodes and fungi often co-exist in soil communities (Molina-Ochoa *et al.*, 2003; Shapiro-Ilan *et al.*, 2003). Here, we examine below-ground tritrophic interactions among several pathogens that form a realistic assemblage of a soil-dwelling entomopathogen community, Bt maize expressing the Cry34/35Ab1 insecticidal protein and western corn rootworm.

Species studied

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (Coleoptera: Chrysomelidae) is a univoltine species that feeds primarily on maize roots and is a serious pest of maize (Levine & Oloumisadeghi, 1991). The eggs overwinter in the soil, and larvae feed on roots, completing three instars below ground before pupating in the soil. Adults, which live above ground, feed on maize pollen and silk, and lay eggs in the soil (Levine & Oloumisadeghi, 1991). Unlike pests of some other Bt crops, western corn rootworm displays some survival on Bt maize (Binning *et al.*, 2010).

Maize transformed with rootworm-active Bt toxin has been grown commercially in the USA since 2003 (Vaughn *et al.*, 2005). The Bt maize used in this experiment contained the event DAS-59122-7, which produces the rootworm-active Bt binary toxin Cry34/Cry35Ab1. The non-Bt maize hybrid used was the near isoline to the Bt maize hybrid, but lacked Cry34/35Ab1.

Metarhizium and *Beauveria* are two genera of entomopathogenic fungi that occur in the soil and are natural enemies of a diverse range of insect species, including western corn rootworm (Meyling & Eilenberg, 2007). Upon contact of conidia (spores) with an insect's cuticle, these fungi initiate infection by germinating and subsequently penetrating the haemocoel. After killing the insect, fungi produce new conidia on the surface of the insect's cuticle (Hajek & St. Leger, 1994).

Entomopathogenic nematodes infect a broad range of insect species (Kaya & Gaugler, 1993). These nematodes have a motile, free-living stage (infective juvenile) during which they seek out hosts and enter the host's haemocoel through natural openings, and release symbiotic bacteria that kill the host in 24–48 h, after which the nematodes feed and reproduce inside the cadaver (Kaya & Gaugler, 1993). Entomopathogenic nematodes exhibit different types of host-foraging strategies (Campbell *et al.*, 2003). *Steinernema carpocapsae* is an ambush forager, and primarily searches for hosts at or near the soil surface (Kaya & Gaugler, 1993; Neumann & Shields, 2006), while *Steinernema glaseri* is a highly mobile cruiser (Campbell *et al.*, 2003). *Heterorhabditis bacteriophora* is also a cruiser that exhibits long-distance ranging behaviour to locate hosts and tends to infect hosts lower in the soil profile compared to *S. carpocapsae* (Gaugler *et al.*, 1997). *Metarhizium, Beauveria, Heterorhabditis* and *Steinernema* species are natural pathogens of *Diabroticina* species (Toepfer *et al.*, 2009), and have all been found to occur in maize fields (Pilz *et al.*, 2008).

Materials and methods

Greenhouse experiment

Maize type (Bt versus non-Bt) and entomopathogen community (present versus absent) were tested in a fully crossed design, yielding a total of four treatments.

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The community of entomopathogens consisted of two fungi, *Beauveria bassiana* (strain GHA) and *Metarhizium brunneum* (strain F52), and three nematode species: *S. carpocapsae* (strain BU), *S. glaseri* (strain BU) and *H. bacteriophora* (strain BU). All nematode strains used were the proprietary and commercially available strains of Becker-Underwood (Ames, IA, USA), and were in the infective juvenile stage.

Entomopathogens were incorporated into potting medium prior to planting of maize. Potting medium consisted of 40% field soil (collected from the top 15–20 cm of agricultural fields in Ames, IA), 30% potting soil (Sunshine SB 300; Sun Gro Horticulture, Vancouver, British Columbia, Canada), 15% sieved sand (Quikrete, Atlanta, GA, USA) and 15% coarse perlite (Sunshine; Sun Gro Horticulture, Vancouver, British Columbia, Canada). Fungi were incorporated at a concentration of 1×10^5 viable conidia cm^{-3} dry potting medium for each of the two fungal species, and each nematode species was incorporated at a concentration of 30 infective juveniles cm^{-3} dry potting medium. Conidia of *B. bassiana* (strain GHA) were obtained as dry powder from a manufacturer (Laverlam International, Butte, MT, USA). Conidia of *M. brunneum* (strain F52) were produced in a biphasic liquid–solid substrate fermentation system using methods outlined in Leland *et al.* (2005).

Nematode concentrations were determined using a compound microscope (Nikon Eclipse E200) set at 40× magnification and a Sedgewick-Rafter counting cell (Pyser-SGI, Edenbridge, Kent, UK). Only live nematodes were counted, and solutions were diluted to the desired concentration with deionised water. Fungal conidia were added to a 0.10% solution of surfactant (Tween 80®; Acros Organics, Morris Plains, NJ, USA), suspended using a vortex and sonicator and the concentration determined with a haemocytometer (Hausser, Horsham, PA, USA) and a microscope (Nikon Eclipse E200) at 400× magnification. Viability counts of suspensions were taken by counting the number of germinated conidia obtained from plating 20 µL of the suspension on Sabouraud dextrose agarose and incubating at 25°C for 16–20 h (Goettel & Inglis, 1997). All pathogen suspensions were prepared 1–4 days before they were incorporated into the potting mixture, and prior to their use, each species was stored separately at 7°C. Each treatment pot received all five of the entomopathogen species at concentrations listed above; however, each entomopathogen species, in solution, was added to the potting medium individually. Control pots received the same amount of 0.10% Tween 80 solution as treatment pots but did not receive pathogens. All pots were then saturated to 25% water holding capacity with additional deionised water. Moistened potting medium ($3000\ cm^3$) was dispensed into 4-L plastic pots, which contained a single drainage hole that was covered with a layer of mesh to prevent escape of rootworm larvae.

Because both Bt and non-Bt maize seeds were treated with the fungicides Apron XL® and Maxim XL® (Syngenta Crop Protection, Greensboro, NC, USA), they were washed with a 10% bleach solution for 1 h, after which they were rinsed thoroughly and dried overnight. Two maize seeds were planted in each pot, and thinned to one seedling per pot following germination. Plastic catch basins were placed below each pot, and plants were watered by filling these basins and by moistening the top several centimetres of soil with water every 2–3 days. This method of watering prevented entomopathogens from being flushed from the soil. Position of plants on greenhouse benches was randomised weekly, and each plant was given 500 mL of fertilizer solution (4 mL fertilizer powder L^{-1} water) (Miracle-Gro, Scotts Company, Marysville, OH, USA) every 2 weeks for the first 10 weeks, and then monthly thereafter. When plants were 12 weeks old, they were trimmed to approximately 75 cm to avoid contact with greenhouse lights. Greenhouse temperature throughout the experiment was 26.5°C ± 2.6°C (mean ± SD); average relative humidity (RH) was 28.3% ± 6.4%. Supplemental lighting was supplied with 400-W high-pressure sodium bulbs (Ruud Lighting Inc., Racine, WI, USA) set to 16/8 L/D.

A total of eight blocks were established, with 24 plants per block, for a total of 192 plants in the experiment. Each block consisted of six plants in each of the four treatments: two maize types (Bt versus non-Bt) crossed with two pathogen treatments (present versus absent). Individual blocks were set up on the same day, and the entire experiment was set up over a 2-week period.

There were three data collection periods during the experiment: two time points to assess larval abundance and development, and a third time point to measure survival to adulthood. Two of the six plants in each block by treatment combination were sampled at each of the three time points. This resulted in 16 replicates (eight blocks × two plants) for each of the 12 combinations of treatment by time point (three time points × two corn hybrids × two pathogen treatments). In addition, six pots containing Bt plants were used to monitor larval development to determine the timing of the second time point, as described below.

The eggs used in this experiment were collected from a non-diapausing, laboratory-reared colony maintained at a population size of 2400 adults per generation in an environmental chamber (25°C, 65% RH, 16/8 L/D) at Iowa State University. When maize plants reached

the V4 stage (four fully formed leaves), approximately 4 weeks after planting, 250 western corn rootworm eggs suspended in a 0.15% water agar solution were added to a 2-cm deep furrow that surrounded each seedling. In addition, eggs were added to two Petri dishes containing moist soil to monitor hatching of eggs.

To determine persistence of the community of entomopathogens in treatment pots, soil samples were taken from both treatment and control pots at 1 and 28 days after pathogens were initially added, and western corn rootworm larvae were then exposed to this soil following the methods of Kaya & Stock (1997). After 6 days of exposure to the soil, mortality was measured for larvae exposed to soil from pathogen-treated pots and control pots. Rootworm mortality was measured as mortality imposed by the entire community of entomopathogens in the soil and we did not measure the mortality caused by individual species of pathogen. Abbott's correction was used to adjust larval mortality from soil treated with pathogens by mortality from soil in control pots [(average control survival − treatment survival] \ average control survival = corrected treatment mortality) (Abbott, 1925). Corrected mortality for larvae at 1 and 28 days was 76.4% ± 5.9% and 25.17% ± 22.16% (mean ± SE), respectively, indicating that the level of mortality imposed by the entomopathogen community had decreased by threefold 28 days after the pathogens had been mixed into soil. This likely occurred because the survival and viability of the pathogens decreased over time. As such, more entomopathogens were added to treatment pots 5 days after obtaining these results, and again 12 days thereafter (39 and 51 days after pathogens were initially added to the potting medium). These additions occurred 6 days before each of the larval sampling time points (described below). Pathogen suspensions were prepared as previously described and the same quantity of pathogens per pot was added as before. Pathogens were added by pouring a total of 40 mL of each entomopathogen species solution into six 10-cm holes per pot. Control pots received the same amount of 0.10% Tween 80 solution and water as the treatment pots but no entomopathogens were added.

Larval abundance was sampled at two time points, and survival to adulthood was measured at a third time point, for a total of three time points. An equal number of pots from all four treatments was sampled at each time point. The first time point occurred 17 days after initial egg hatch (45 days after initial pot set-up), which is when the fastest developing larvae fed non-Bt maize are expected to reach the third instar (Nowatzki *et al.*, 2008). Because larval development is slower on Bt plants compared to non-Bt plants (Nowatzki *et al.*, 2008), we included a second time point, which occurred when larvae fed Bt maize reached the third instar. To determine this, we monitored larval development on Bt maize and found that approximately 50% of the larvae had reached the third instar 26 days after initial egg hatch. Thus, our second time point was 29 days after initial egg hatch (57 days after initial pot set-up). Therefore, the two time points were 17 and 29 days after eggs hatched. At both time points, we placed the soil from each pot on separate Berlese funnels for 7 days to extract larvae, although most larvae were extracted within 48 h. It is possible that some larvae were still developing on the corn while soil was on the Berlese funnels; however, because all treatments within each replicate were placed on funnels at the same time, no bias would be introduced to the data. The total number of larvae extracted from each pot was recorded and larval instar was scored. We did not assess the number of pupae in the soil for this experiment. Instar was determined from a random subsample of 50 larvae per pot and was determined using head capsule width following Hammack *et al.* (2003). Head capsule width was measured using a Lieca MZ6 dissecting microscope and accompanying image analysis software (Motic Images Inc., British Columbia, Canada).

At the third time point, we measured survival to adulthood for two plants in each block by treatment combination for a total of 16 plants per treatment. A piece of mesh cloth was secured to the base of each maize plant and to the top of each pot. Adults were collected from the cages with a hand aspirator three times per week. Collection stopped after two consecutive weeks had elapsed with no adult emergence.

Growth chamber experiment

This experiment also tested the effects of a community of entomopathogens, Bt maize and their interaction on the survival and development of western corn rootworm. This experiment differed from the greenhouse experiment in that a seedling mat in a small container was used instead of a single plant in a flower pot. Seedling mats are useful because they usually enable higher larval and adult survival and provide a more consistent and uniform source of food for larvae (Nowatzki *et al.*, 2008). We used the same entomopathogens [*B. bassiana* (strain GHA), *M. brunneum* (strain F52), *S. carpocapsae* (strain BU), *S. glaseri* (strain BU) and *H. bacteriophora* (strain BU)] and maize (Cry34/35Ab1 maize and a non-Bt near isogenic hybrid) as in the greenhouse experiment; however, seeds used in this experiment had not received any seed treatment.

Seedling mats were grown in plastic rectangular containers (21 × 27 × 10 cm L × W × H; Rubbermaid, Fairlawn, OH, USA). Each container consisted of 180 mL of pre-germinated maize seed (either Bt or non-Bt),

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100 mL of water and 1300 *g* of the potting medium (containing or lacking entomopathogens) moistened to 25% water holding capacity as described for the greenhouse experiment. Control containers received soil moistened with the same amount of Tween 80 solution used for entomopathogen-treated soil but did not receive pathogens. A total of 48 containers were prepared, with 12 containers for each of the four treatments (pathogens present versus absent crossed with Bt versus non-Bt maize).

Fifteen hundred western corn rootworm eggs suspended in 0.15% agar solution were added to each container, and additional eggs were added to two Petri dishes with moistened soil to monitor hatching. Eggs used in this experiment were from the same strain used in the greenhouse experiment. Containers were held in a growth chamber for the duration of the experiment (25°C; 16/8 L/D; approximately 65% RH). Survival and development of larvae in each of the treatments were measured at two time points, in order to account for the difference in western corn rootworm development on Bt and non-Bt maize as previously described. Because containers were clear plastic, we monitored larval development through direct observation.

The first time point occurred when numerous third instar larvae were present in trays with non-Bt maize and the second time point occurred when third instar larvae were present in trays with Bt maize. Time points were 14 and 24 days after the first eggs hatched (23 and 33 days after containers were assembled). As with the greenhouse experiment, all treatments were sampled equally at each of the two time points. At the first time point, all containers were divided into three equal sections. One section was placed on a Berlese funnel to extract larvae, after which a subsample of soil was checked carefully for pupae and the total number of pupae in the entire section then estimated. The remaining two sections were each placed in containers with a fresh seedling mat, potting medium and pathogens in accordance with their respective treatments (i.e. seedling mats with Bt maize and soil containing entomopathogens were placed in containers with Bt maize and entomopathogens). For each pair of seedling mats, one was used to sample western corn rootworm at the second time point, while the other was used to measure survival to adulthood. In addition, any adults that emerged throughout the experiment from seedling mats sampled at the second time point were captured and counted.

Seedling mats used to measure survival to adulthood were covered with a piece of cotton mesh and checked three times per week for adult emergence. Seedling mats were watered as needed. Collection of adults ended after two consecutive weeks with no adult emergence (approximately 8 weeks after initial egg hatch).

Statistical analysis

The greenhouse and growth chamber experiments were analysed separately. All analyses were performed in SAS 9.2 (SAS, 2009).

Greenhouse experiment

Because larval stage is not easily defined as a dependent or independent variable, the number of insects in each larval instar at the first and second time points was analysed with a test of independence (PROC CATMOD in SAS) based on a log-linear model that included the factors of instar, pathogen treatment and maize type. Within each instar, a Student's *t*-test was used to determine significant differences in abundance between maize type and entomopathogen treatments (Sokal & Rohlf, 1995).

Total survival of western corn rootworm at both time points and survival to adulthood were each analysed with a mixed-model analysis of variance (ANOVA) (PROC MIXED). Fixed factors in the analysis included pathogen (present versus absent) and maize type (Bt versus non-Bt), and random factors were block and its interactions with the fixed factors. Random effects were tested with 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). Block and its interactions were removed from the model to increase statistical power when these factors were not significant at a level of $\alpha < 0.25$ (Quinn & Keough, 2002). However, lower order terms were always retained if their higher order interactions were significant. There was a significant interaction between maize type and entomopathogens at the first time point, thus pairwise comparisons were made between maize types within each of the two pathogen treatments (PDIFF option in PROC MIXED).

Growth chamber experiment

Insect stadium at each time point was analysed using a test of independence (PROC CATMOD) based on a log-linear model that included the factor of stadium (first, second and third instar, pupa and adult), maize type (Bt versus non-Bt) and pathogen treatment (present versus absent). Data were transformed by the $x + 1$ function to enable analysis of zeros in the log-linear model. Within each stadium, Student's *t*-test was used to determine significant differences in abundance between maize types or entomopathogen treatments.

Data on survival of western corn rootworm to adulthood for the third time point were analysed with a pure model I ANOVA (PROC GLM) that included the factors of maize type (Bt versus non-Bt) and pathogen treatment (present versus absent). Because we did not include a blocking factor in this experiment, we did not use a mixed-model analysis. Data were log transformed to ensure normality of the residuals.

Because seedling mats sampled at time point 2 and at adult emergence were generated by subdividing seedling mats sampled at time point 1 into multiple sections, data were analysed with a repeated measures ANOVA (PROC MIXED) based on a split-plot design (Quinn & Keough, 2002). Fixed factors in the analysis included maize type, pathogen, time and all interactions among these three factors. Random factors in the analysis were seedling mat nested with maize type by pathogen treatment, and the interaction of time with seedling mat, which was nested with maize type by pathogen treatment.

Results

Greenhouse experiment

Larval abundance at time points 1 and 2 was affected by a significant maize type by instar interaction, which indicates that larvae developed at a different rate on the two types of maize (Table 1, Fig. 1). There was also a significant pathogen by instar interaction indicating that some instars suffered higher mortality from pathogens than did others (Table 1, Fig. 1).

At the first time point (17 days after hatch), there were significantly more first and second instar larvae and fewer third instar larvae on Bt maize compared to non-Bt maize ($P < 0.014$ for all comparisons) (Fig. 1A). Pathogens significantly decreased abundance of third instars on both types of maize ($P < 0.029$ in both cases), and second instars on non-Bt maize ($P = 0.027$), but did not affect abundance of first instars for either maize type ($P > 0.45$ in both cases) (Fig. 1A).

For total survival at the first time point, there was a significant interaction between maize type and pathogens (Table 2, Fig. 1B). Entomopathogens significantly decreased survival on non-Bt maize ($t_{53} = 5.38$, $P < 0.0001$), but did not on Bt maize ($t_{53} = 1.39$, $P = 0.169$) (Fig. 1B). This likely arose because most larvae on Bt maize were in the first and second instars, and these stages were less affected by entomopathogens compared to later instars.

At the second time point (29 days after hatch), there were significantly more first and second instar larvae on Bt maize than non-Bt maize ($P < 0.005$ in both cases), and no difference in abundance of third instars (Fig. 1C). Entomopathogens significantly decreased survival for second and third instar larvae ($P < 0.029$ for all), but did not affect first instar larvae ($P > 0.311$ in both cases) (Fig. 1C).

For total survival at the second time point, the interaction between maize type and pathogens was not significant; entomopathogens significantly decreased survival on both maize types (Table 2, Fig. 1D). The effect of corn type was also significant. Survival was

Table 1 Test of independence for abundance of western corn rootworm at several developmental stages

	Time Point 1[a]			Time Point 2[a]		
Factor[b]	d.f.	χ^2	*P*	d.f.	χ^2	*P*
Greenhouse						
Maize type	1	0.20	0.6548	1	66.37	<0.0001
Instar	2	432.04	<0.0001	2	169.75	<0.0001
Pathogen	1	69.33	<0.0001	1	41.95	<0.0001
Maize type × pathogen	1	2.32	0.1274	1	2.00	0.1570
Maize type × instar	2	1338.87	<0.0001	2	129.96	<0.0001
Pathogen × instar	2	22.01	<0.0001	2	14.90	0.0006
Pathogen × instar × maize type	2	3.98	0.1367	2	3.19	0.2027
Growth chamber						
Maize type	1	1.75	0.1859	1	2.22	0.1366
Stage	3	627.76	<0.0001	4	307.58	<0.0001
Pathogen	1	21.41	<0.0001	1	18.58	<0.0001
Maize type × pathogen	1	1.89	0.1689	1	0.29	0.5887
Maize type × stage	3	1119.48	<0.0001	4	534.06	<0.0001
Pathogen × stage	3	6.71	0.0819	4	14.47	0.0059
Pathogen × stage × maize type	3	19.84	0.0002	4	15.81	0.0033

[a]Seventeen and 29 days after initial egg hatch in the greenhouse experiment, and 14 and 24 days after initial egg hatch in the growth chamber experiment.
[b]Instar: larval instars, stage: larval instars, pupae and adults, maize type: Bt or non-Bt maize, pathogen: soil containing or lacking pathogens.

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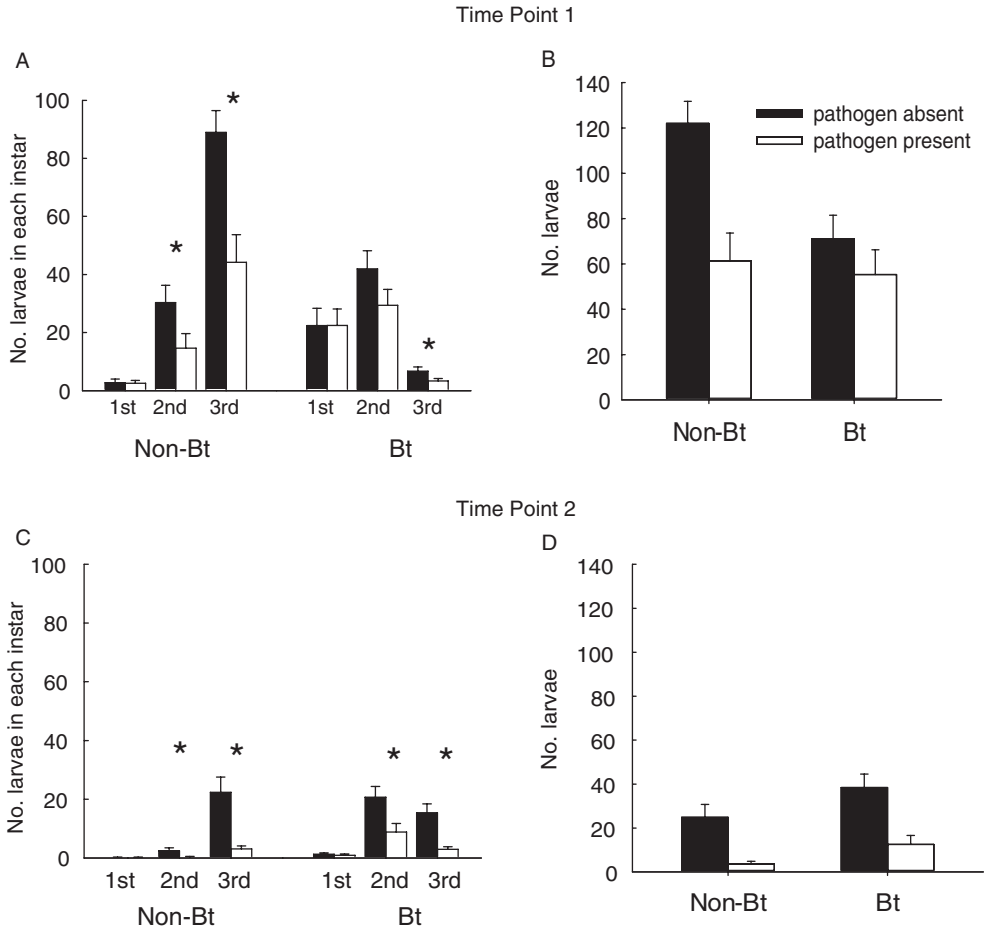

Figure 1 Number of larvae in each instar (A and C), and total survival (B and C) for western corn rootworm at the first and second time points in the greenhouse experiment. Time point 1 was 17 days after eggs hatched and time point 2 was 29 days after eggs hatched. Bar heights represent sample means and error bars are the standard error of the mean. Asterisks (*) indicate significant difference between entomopathogen treatments within a stadium and maize treatment (*t*-test, $P < 0.05$).

significantly higher for larvae on Bt maize compared to non-Bt maize (Table 2, Fig. 1D), which can be attributed to an experimental artefact of insects on non-Bt maize reaching the pupal stadium, which was not measured in this experiment.

The presence of entomopathogens in the soil significantly reduced survival to adulthood ($F_{1,49} = 52.69$, $P < 0.0001$) (Fig. 2A). There was no significant effect of maize type on survival to adulthood and no significant interaction between maize type and pathogens ($P > 0.05$ in both cases) (Fig. 2A).

Growth chamber experiment

There was a significant three-way interaction between pathogen, stadium and maize type for both the first and second time points (Table 1). At the first time point (14 days after hatch), there were significantly more first and second instar larvae, and fewer third instar larvae and pupae on Bt maize compared to non-Bt maize ($P < 0.047$ in all cases), again indicating a developmental delay on Bt maize (Fig. 3A). Entomopathogens significantly decreased abundance of third instar larvae on Bt maize ($P = 0.032$), but did not significantly affect abundance for other stadia on either maize type at this time point (Fig. 3A).

At the second time point (24 days after egg hatch), there were significantly more third instar larvae and fewer adults on Bt maize compared to non-Bt maize for both pathogen treatments ($P < 0.004$ for all comparisons) (Fig. 3C). Entomopathogens significantly decreased abundance of third instar larvae and adults on non-Bt maize ($P < 0.021$ for both comparisons); there were no insects in any other growth stages at this time point. On Bt maize, there were less than three insects in the first instar and adult stages; entomopathogens significantly

Table 2 Mixed model analysis of variance for survival of western corn rootworm in the greenhouse experiment at 17 days (time point 1) and 29 days (time point 2)

	Time Point 1			Time Point 2		
Fixed Effect[a]	d.f.	*F*	*P*	d.f.	*F*	*P*
Maize type	1,53	12.75	0.0008	1,51	6.9	0.0114
Pathogen	1,53	22.95	< 0.0001	1,51	31.33	<0.0001
Maize type × pathogen	1,53	7.94	0.0068	1,51	0.57	0.4536
Random Effect	d.f.	χ^2	*P*	d.f.	χ^2	*P*
Block	1	24.3	< 0.0001	1	6.8	0.0046

[a]Maize type: Bt or non-Bt maize, pathogen: soil containing or lacking pathogens.

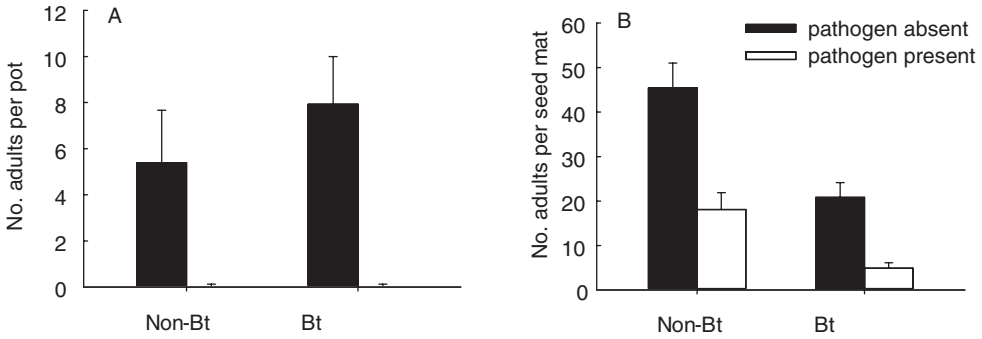

Figure 2 Survival to adulthood for western corn rootworm in the greenhouse experiment (A) and the growth chamber experiment (B). Bar heights represent sample means and error bars are the standard error of the mean.

decreased the number of third instar larvae ($P = 0.031$), but did not affect second instar larvae or pupae ($P > 0.169$ for both) (Fig. 3C).

Entomopathogens ($F_{1,47} = 41.64, P < 0.0001$) and maize type ($F_{1,47} = 24.28, P < 0.0001$) significantly affected survival to adulthood, with Bt maize and the presence of pathogens resulting in lower survival (Fig. 2B). There was no interaction between maize type and the pathogen treatments ($F_{1,47} = 1.67, P = 0.2025$).

In the repeated measures analysis, maize type, pathogen treatment and time were significant but there were no significant interactions (Table 3). Both Bt maize and the presence of pathogens significantly decreased survival of western corn rootworm, and survival decreased over time (Figs 2B, 3B and 3D).

Discussion

Host-plant resistance and biological control by natural enemies can act together to decrease pest populations (Bottrell *et al.*, 1998; Cortesero *et al.*, 2000; Hare, 2002; Ode, 2006), although little is known about how host-plant resistance, herbivorous insects and natural enemies affect below-ground tritrophic interactions or how Bt crops will interact with insect pathogens (Rasmann & Turlings, 2008; Lundgren *et al.*, 2009; Bruck, 2010). The community of entomopathogens used in this study increased mortality of western corn rootworm, while Bt maize slowed development and increased mortality. In combination, we found a complex interaction between Bt maize and insect pathogens that arose because Bt maize acted to delay larval development, and insect pathogens killed later instar larvae. These factors resulted in interactive effects on survival when insects were sampled during larval development. However, for survival to adulthood, the effects of Bt maize and insect pathogens were independent of each other and were largely additive in nature.

Host-plant resistance conferred by Bt maize expressing the insecticidal protein Cry34/35Ab1 resulted in an overall decrease in western corn rootworm survival in the growth chamber experiment (Table 3, Figs 2B, 3B and 3D). In the greenhouse experiment, the number of larvae in the soil was significantly lower on Bt maize compared to non-Bt maize at the first time point (Fig. 1B), but significantly higher at the second time point (Fig. 1D). The greater abundance of larvae on Bt maize at the second time point likely arose because most insects on non-Bt maize had pupated due to faster development than on Bt maize, and pupal abundance was not assessed in this

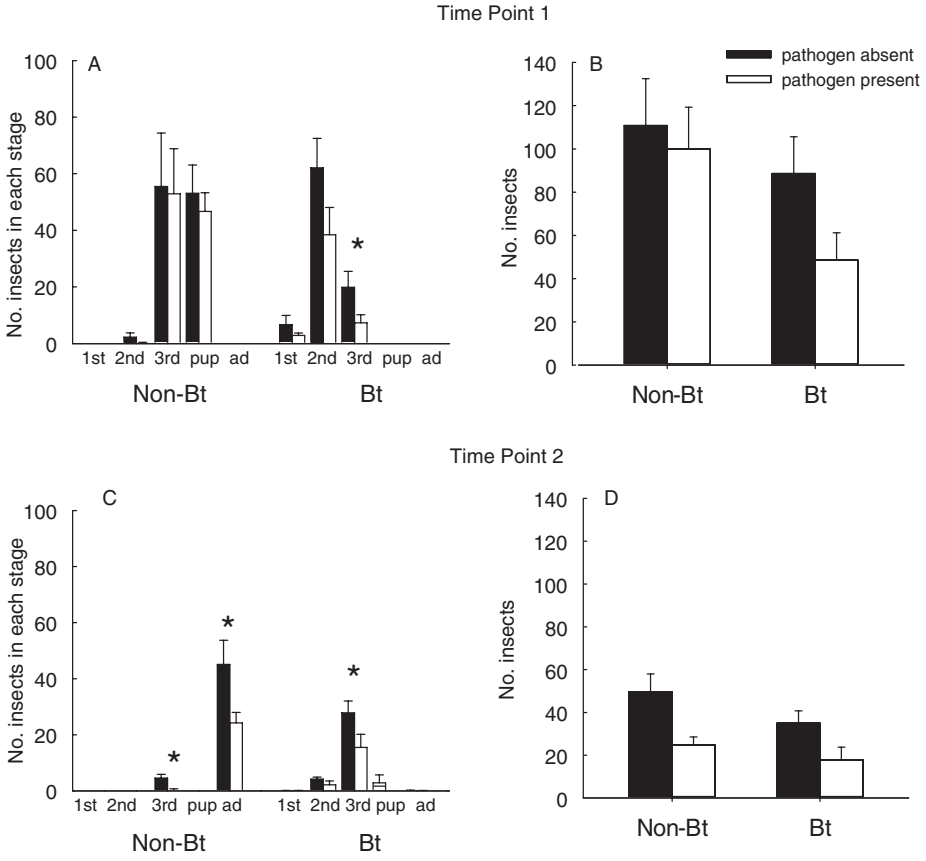

Figure 3 Number of larvae in each stadium (A and C), and total survival (B and C) for western corn rootworm at the first and second time points in the growth chamber experiment. Time point 1 was 14 days after eggs hatched and time point 2 was 24 days after eggs hatched. Bar heights represent sample means and error bars are the standard error of the mean. Asterisks (*) indicate significant difference between entomopathogens present versus absent treatment, within a stadium (*t*-test, $P < 0.05$).

experiment. Bt maize under field conditions, including Bt maize that expresses Cry34/35Ab1, reduces survival of western corn rootworm to adulthood, but some survival is still observed (Storer *et al.*, 2006; Hibbard *et al.*, 2009). We observed a consistent effect of Bt maize in delaying larval development, which was present in both experiments (Figs 1A, 1C, 3A and 3C). Other studies have found similar effects (Nowatzki *et al.*, 2008; Meissle *et al.*, 2009; Binning *et al.*, 2010). In addition, a significant reduction in survival to adulthood on Bt maize was observed in the growth chamber experiment.

The entomopathogen species studied here have all been shown to kill western corn rootworm (reviewed by Toepfer *et al.*, 2009). In both the greenhouse and growth chamber experiments, this pathogen community reduced survival of western corn rootworm (Figs 1–3). Entomopathogens imposed more mortality on third and second instars than first instars (Figs 1A, 1C, 3A and 3C). Second and third instar western corn rootworm are more susceptible to *S. carpocapsae* and *H. bacteriophora* compared to first instars (Jackson & Brooks, 1995; Kurtz *et al.*, 2009), which was observed in this study. Because it can take up to 7 days for mortality to occur from fungal infection (Shah & Pell, 2003), it could be the case that earlier instars were infected with conidia, but mortality was

Table 3 Repeated measures analysis of variance for survival of western corn rootworm in the growth chamber experiment

Effect[a]	d.f.	*F*	*P*
Maize type	1,44	9.17	0.0041
Pathogen	1,44	9.71	0.0032
Maize type × pathogen	1,44	0.05	0.8205
Time	2,88	45.25	<0.0001
Maize type × time	2,88	1.64	0.2006
Pathogen × time	2,88	0.05	0.9466
Maize type × pathogen × time	2,88	1.16	0.3176

[a]Maize type: Bt or non-Bt maize, pathogen: soil containing or lacking pathogens and time: survival at 14 days, 24 days and to adulthood.

not imposed until insects reached later instars. Mortality from entomopathogens primarily observed in later instars may explain why an effect of entomopathogens was not observed for larvae fed Bt maize at the first time point of the greenhouse experiment (when most larvae were in the first and second instars).

Interactions of host-plant resistance and natural enemies can be independent, synergistic or antagonistic with respect to effects on insect pests (Bottrell *et al.*, 1998; Hare, 2002). In this study, Bt maize decreased developmental rate of western corn rootworm, and either increased mortality (Figs 2B, 3B and 3D) or did not affect mortality (Figs 1D and 2A). Entomopathogens decreased survival of western corn rootworm but did not affect developmental rate. Together, both Bt maize and entomopathogens increased mortality of western corn rootworm, and did so in an independent but complementary manner, with both agents acting to increase mortality. The additive nature of this effect on mortality was most apparent in the growth chamber study (Fig. 2B). For the greenhouse study, only two adults emerged from all pots that received entomopathogens, which made it difficult to evaluate interactions between Bt maize and entomopathogens in that experiment. Meissle *et al.* (2009) also showed that interactions between Bt maize and the entomopathogen *M. brunneum* were additive.

Prolonged insect development can affect susceptibility to natural enemies; however, most of the available information pertains to above-ground systems (Benrey & Denno, 1997; Turlings & Benrey, 1998). Developmental delays caused by host-plant resistance may result in a longer period of exposure to natural enemies, and consequently, increased mortality as described in the slow-growth–high-mortality hypothesis (Benrey & Denno, 1997). In addition, poor quality hosts can increase searching behaviour of insect and thus lead to more encounters with natural enemies (Johnson *et al.*, 1997). By contrast, antagonistic effects can arise if host-plant factors such as allelochemicals make prey less susceptible to natural enemies (Bottrell *et al.*, 1998; Hare, 2002). Additive, independent effects can arise as well (Hare, 2002), as was found in this study. It may be the case that developmental delays caused by Bt maize in this study did not lead to interactive effects with natural enemies, because the probability of encountering pathogens was not highly dependent on larval development time. Western corn rootworm larvae spend a significant amount of their time feeding within roots (Clark *et al.*, 2006). As such, longer periods of feeding throughout development on Bt maize may not have resulted in increased contact with entomopathogens.

Surveys examining the occurrence of entomopathogenic nematodes and fungi have shown that they are widely distributed: *Metarhizium* and *Beauveria* have a cosmopolitan distribution (Roberts & Leger, 2004; Rehner, 2005), and nematodes in the genera *Steinernema* and *Heterorhabditis* also have been isolated from diverse ecosystems globally (reviewed by Hominick *et al.*, 1996). The community of entomopathogens used in this study reflects what has been found in natural and agricultural systems, which can contain both entomopathogenic nematodes and fungi (Chandler *et al.*, 1997; Bruck, 2004; Pilz *et al.*, 2008). Several surveys have recovered *M. brunneum*, *B. bassiana* and nematodes in the genera *Steinernema* and *Heterorhabditis* from soil samples at the same site (Molina-Ochoa *et al.*, 2003; Shapiro-Ilan *et al.*, 2003). Thus, the community of entomopathogens used in this study formed a biologically realistic assemblage of soil-dwelling entomopathogens.

The few studies that examine abundance of entomopathogenic nematodes have shown a high degree of temporal and spatial patchiness, in both natural and agricultural systems (Campbell *et al.*, 1998, and references therein). These studies have documented that nematode density in soils can range from numbers as low as less than 1 to 20 nematodes cm^{-3} (Campbell *et al.*, 1998; McGraw & Koppenhofer, 2009). Densities of entomopathogenic fungi in soil are also highly variable, and can range from 0 to greater than 10^5 viable conidia g^{-1} soil, with average upper levels at approximately 1000 viable conidia g^{-1} soil (Scheepmaker & Butt, 2010), which implies that our initial inoculation rates of entomopathogens were higher than what naturally occurs. However, persistence in our experiment declined sharply over time as evidenced by a large decrease in the number of dead rootworm exposed to pathogen-treated soil 28 days after soil inoculation, compared to mortality from those exposed to pathogen-treated soil 1 day after inoculation (see Materials and methods). Populations of naturally occurring entomopathogenic fungi and nematodes generally are stable and not characterized by rapid rates of decline over time (Stuart *et al.*, 2006; Meyling & Eilenberg, 2007; Sun *et al.*, 2008; Hussein *et al.*, 2010; Scheepmaker & Butt, 2010; Campos-Herrera *et al.*, 2011). Thus, initial inoculation rates in our experiment were likely higher than what is found in nature, but persistence was likely lower than that of naturally occurring entomopathogens. In addition, our results highlight the potential for entomopathogenic nematodes and fungi to be applied to Bt maize fields as biological control agents, where a range of concentrations similar to what was used in this study would be appropriate (Krueger & Roberts, 1997; Scheepmaker & Butt, 2010; Toepfer *et al.*, 2010*a*,*b*; Pilz *et al.*, 2011).

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Naturally occurring entomopathogens in agroecosystems have been shown to provide significant regulation of pest populations (Steinkraus, 2007, and references therein). Results from life-table studies have shown that mean total mortality for western corn rootworm, measured from egg stage to adult, is 99.7% (Toepfer & Kuhlmann, 2006). Although the extent to which naturally occurring entomopathogens regulate western corn rootworm populations is largely unknown, studies have shown that *Metarhizium, Beauveria, Heterorhabditis* and *Steinernema* species are natural pathogens of *Diabroticina* species (Toepfer *et al*., 2009), and have all been found to occur in maize fields (Pilz *et al*., 2008). Thus, naturally occurring entomopathogens, when combined with Bt maize, may be enhancing mortality of western corn rootworm.

In this study, we found that host-plant resistance conferred by genetic engineering of maize to produce Bt toxins acted to increase mortality and delay development; however, these developmental delays did not increase mortality from insect pathogens, which killed corn rootworm in a manner that was independent of maize type. Nonetheless, the independent but complementary effects observed here illustrate the potential compatibility of naturally occurring entomopathogens with Bt crops for regulating pest populations, and the potential for plant defences in roots to benefit from entomopathogens.

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