Subacute Effects of Cry1Ab *Bt* Corn Litter on the Earthworm *Eisenia fetida* and the Springtail *Folsomia candida*

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ABSTRACT Laboratory toxicity studies were conducted to determine the subacute effects of *Bt* Cry1Ab corn leaf material on nontarget soil organisms. Survival and growth were measured for an earthworm, *Eisenia fetida* Savigny, and survival and reproduction were measured for a springtail, *Folsomia candida* Willem. The organisms were provided leaf material of two Bt11 corn varieties, two Mon810 corn varieties, and the isolines of each, in a soil system and monitored for 28 d. An assay control treatment of an optimal food and a reference control treatment, using the herbicide pendimethalin, were used to provide a context for the observed results. Basic nutritional data of protein, fat, and sugar content were analyzed for each food type. Greater growth was observed for *E. fetida* in two *Bt* varieties, Bt11 90-d and Mon810 108-d, compared with their isolines. *F. candida* receiving Bt11 90-d isoline material had more offspring compared with those in the corresponding Bt line, but no other pairs were different. Time to reproduction of *F. candida* was only affected by the reference control treatment. Both protein and sugar content were found to correlate significantly with growth for *E. fetida*, but the nutritional parameters were not found to correlate with the effects observed for *F. candida*. These results indicate that there is little direct hazard from *Bt* corn leaf material to *E. fetida* and *F. candida* but that differences in nutritional parameters of the *Bt* lines and the isolines may lead to differences in the effects on nontarget organisms.

KEY WORDS transgenic corn, nontargets, *Folsomia candida*, *Eisenia fetida*

The use of *Bacillus thuringiensis* Berliner (*Bt*) protein toxins through the expression of genes encoding for their production in transgenic plants has increased substantially in agroecosystems. For example, 2.8 million ha of *Bt* corn (*Zea mays* L.) were planted in the United States in 1998 compared with 9.7 million ha 1 yr later and ≈12 million ha by 2002 (Obrycki et al. 2001, James 2002). Worldwide, the area on which *Bt* corn was grown increased from around 3 million ha in 1998 to 15.5 million ha in 2003 (James 2002, 2003).

Much of the research studying potential nontarget effects of *Bt* proteins has focused on exposure to *Bt*-containing pollen or exposure of predators to prey that has consumed *Bt* proteins. Relatively little work has studied the possible impacts of *Bt* proteins in the soil environment, a portion of the agroecosystem with great potential for exposure to the protein. There are a variety of ways that transgenic *Bt* proteins can enter the environment after planting. *Bt* protein is incorporated into soil by sloughing of root cells, through the release of exudates from roots, and with plant tissue postharvest, so its soil fate is a key parameter governing exposure of nontarget organisms in the environment. Saxena and Stotzky (2000) reported the presence of the protein in secretions from roots. This can occur throughout the growing season, because the *Bt* protein is expressed continuously during the vegetative stages of growth. Additionally, Stotzky and colleagues showed that *Bt* proteins are adsorbed to soil components, especially clays, and are protected from degradation but retain insecticidal activity (Venkateswerlu and Stotzky 1992, Tapp et al. 1994, Tapp and Stotzky 1995, 1998, Koskella and Stotzky 1997). Conflicting results have been reported in assessing *Bt* protein persistence in soil. However, previous studies generally indicate that the dissipation is biphasic, with the concentration of protein rapidly declining initially and then the remaining portion dissipating at a much slower rate (Palm et al. 1994, 1996, Sims and Ream 1997, Herman et al. 2002). In a series of microcosm experiments, Palm et al. (1996) found that 10–40% of the protein remained at the end of a 28-d period. Sims and Ream (1997) determined that ≈20% of the initial Cry2A bioactivity in an insect assay remained after 120 d of incubation in soil. Similarly, Tapp and Stotzky (1998) observed that the insecticidal activity of *Bt* subsp. *kurstaki* protein in soil was retained for >6 mo. In a recent study using corn tissue within field-buried litter bags, it was observed that degradation of Cry1Ab protein in corn tissue is slow, and a low amount of the protein remained until spring (Zwahlen et al. 2003a). Based on these studies and the fact that the *Bt* protein

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Table 1. Specific *Bt* event or hybrid used for each treatment and concentration of *Bt* protein in the leaf material of each *Bt* line

Treatment description	Event (or isoline)	Maturity (d)	Variety (hybrid)	Brand	*Bt* concentration (μg/g)[a]
Bt11–90 (*Bt*+)	Bt11	90	N2555Bt	NK (Syngenta)	2.40 ± 0.07
Bt11–90 (*Bt*−)	Isoline Bt11	90	N2555-F95	NK (Syngenta)	—
Bt11–108 (*Bt*+)	Bt11	108	N58-D1	NK (Syngenta)	2.69 ± 0.08
Bt11–108 (*Bt*−)	Isoline Bt11	108	N58-F4	NK (Syngenta)	—
Mon810–90 (*Bt*+)	Mon810	90	38A25	Pioneer (DuPont)	2.19 ± 0.07
Mon810–90 (*Bt*−)	Isoline Mon810	90	38A24	Pioneer (DuPont)	—
Mon810–108 (*Bt*+)	Mon810	108	34M95	Pioneer (DuPont)	2.20 ± 0.06
Mon810–108 (*Bt*−)	Isoline Mon810	108	34M94	Pioneer (DuPont)	—

[a] *Bt* concentration calculated as mass of protein per wet weight of leaf material.

is expressed throughout the growing season of the plant, it is conceivable that soil organisms are subject to long-term exposure to *Bt* proteins. It is therefore necessary to establish whether there is a hazard to soil biota from long-term exposure to *Bt* proteins.

Two important groups of organisms within the soil ecosystem that are commonly used for toxicity testing are the earthworms and the springtails (Collembola). Both are generally regarded as decomposers and detritivores and therefore are highly associated with decomposing corn tissue. Earthworms are often considered to be one of the most important groups of soil invertebrates, because of their role in decomposing plant litter and determining soil structure. They can be considered a "keystone" organism in the regulation of nutrient cycling processes and are highly involved in increasing soil fertility (Edwards et al. 1995, Parmelee et al. 1998). Earthworms are important in establishing soil structure through their burrowing, consumption of litter, and promotion of microbial activity (Tomlin et al. 1995). Additionally, earthworms are an important part of the diet of many other organisms, both vertebrate and invertebrate, and are hosts for a variety of pathogens and parasites (Edwards and Bohlen 1996). Springtails are also an important group of soil invertebrates; they fill a wide variety of functional roles in agroecosystems and have been observed at densities of up to several million individuals per square meter (Rusek 1998). They have been documented as consumers of bacteria, yeasts, and fungi, but they can also be predators, phytophages, detritivores, or omniphages (Rusek 1998). In general, springtails play an important role in the population dynamics of fungi, bacteria, actinomycetes, and algae (Parkinson 1983). They are important disseminators of the propagules of soil microbiota, thus indirectly aiding in decomposition processes. In addition, springtails are food sources for many other organisms (Rusek 1998).

At this point, very few published studies have examined the effect of transgenic *Bt* corn on earthworms (Ahl Goy et al. 1995, Saxena and Stotzky 2001a, Zwahlen et al. 2003b). Ahl Goy et al. (1995) found no effects of Cry1Ab corn on mortality or weight of *Eisenia fetida* Savigny in a 14-d test. Saxena and Stotzky (2001a) observed no effect on mortality and weight of *Lumbricus terrestris* L. after 40 d in soil planted with *Bt* corn or after 45 d in soil amended with *Bt* corn biomass. Zwahlen et al. (2003b) observed a significant reduction in weight for adult *L. terrestris* fed *Bt* corn compared with those fed an isoline after a 200-d laboratory exposure. In a field study with immature *L. terrestris,* no negative effects of *Bt* were found.

In the only published study on the effects of plant-produced *Bt* protein on springtails, Yu et al. (1997) observed no negative effects on *Folsomia candida* Willem fed transgenic plant material containing either Cry1Ab, Cry1Ac, or Cry3A protein compared with their isolines. Sims and Martin (1997) also saw no deleterious effects when the springtail species *F. candida* and *Xenylla grisea* Axelson were was given purified Cry1Ab, Cry1Ac, Cry2A, or Cry3A protein.

Because of the potential for long-term exposure of nontarget soil invertebrates to *Bt* proteins from transgenic crops, it is important to assess the long-term impact on these organisms. The objectives of the current studies were to (1) examine the subacute effects of *Bt* corn material on *F. candida* and *E. fetida,* using multiple *Bt* corn varieties for a broad comparison; (2) improve and adapt existing earthworm and springtail nontarget testing methodologies for use with transgenic crops; and (3) investigate if there are nutritional differences between the corn types that affect the response of *F. candida* and *E. fetida.* This information will be useful in better characterizing the effect of *Bt* crops on these soil invertebrates and in providing methodology that can be used to evaluate future *Bt* proteins and future transgenic crops.

Materials and Methods

Corn Material. Eight seeds each of the varieties of corn listed in Table 1 were planted in 5-gal plastic buckets filled with potting soil. The varieties planted consisted of two types each of Bt11 (Syngenta) and Mon810 (Monsanto). Each of these varieties produces Cry1Ab Bt proteins. The two maturities are bred for a 90- or 108-d growing season. In addition, the nearest genetic equivalent, or near-isoline, of each *Bt* line was used. The plants were grown in the greenhouse on a 16:8 light cycle at 27 (light) and 20°C (dark). Plants were watered two to three times weekly, in a manner that ensured adequate moisture. Plants were fertilized twice during the first 2 mo of the growing period with MiracleGro (The Scotts Co., Marysville, OH) at the rate directed on the label. When the plants had tassels (VT stage), the top three groups of leaves were cut from the main stem. The plants were harvested at

tasseling, and the newest leaves were used because this is the stage and location on the plant that should have the highest concentration of *Bt* protein (Fearing et al. 1997). Once leaf material from all of the plants in a treatment was collected, the leaves were cut into smaller pieces of ~1 cm^2 to be ground. Ground plant material was prepared by grinding the leaf material with a mortar and pestle under liquid nitrogen. The corn material was not freeze-dried or lyophilized; the liquid nitrogen was only used to simplify grinding and the corn material was still green and moist after processing. The corn material was ground to make it more useable for *E. fetida* and *F. candida,* both of which prefer material at a later stage of breakdown. Samples were stored at −15°C until use in the bioassays.

Organisms. Earthworms (*E. fetida*) were acquired from a local vender and cultured for >4 yr before this study. *E. fetida* is a litter-dwelling earthworm species and a common laboratory test organism. Earthworms were cultured by adding 10 adults each to a 3-liter rectangular container filled with a 50:50 mixture of potting soil and horse manure, collected from a horse known to be free of medications, and wetted with distilled water. As containers became populated with earthworms, new containers were started. The chambers were stored in a growth chamber under constant darkness at 23°C. Five weeks before starting the earthworm bioassay, ≈80 cocoons (egg cases) were removed from five different culturing containers and placed in potting soil in a fresh container. Young worms, 7–20 mg in weight, were collected from this container at test time. *E. fetida* have a long hatch time, so a 3-wk difference in hatch time was possible; however, this difference was minimized by using only the juvenile earthworms of the mean size range.

Springtail (*F. candida*) cultures were originally obtained from Oklahoma State University, Ecotoxicology and Water Quality Laboratory (Stillwater, OK). The organisms originated from Dr. Renata Snyder, Michigan State University (Lansing, MI). They were cultured in our laboratory for 3 yr before toxicity testing. Culturing was performed on a mixture of hydrated $CaSO_4$ and activated charcoal (Wiles and Krogh 1998). Culture chambers were 946-ml mason jars kept at 20°C. Baker's yeast was provided as a food source. Five weeks before the start of the study, 50 adults were placed in a fresh rearing chamber and allowed to lay eggs. After 4 d, the adults were removed. The test organisms were ≈20 d old at the beginning of the study, the approximate age of reproductive maturity. This parthenogenic organism can live up to 290 d, continuing to reproduce their entire adult life (Wiles and Krogh 1998).

Soil Description. The soil used for all assays was a field-collected agronomic soil from a reference field at the Iowa State University Ag Engineering/Agronomy Farm, ~4 mi west of Ames, IA. The field is known to have had no pesticide application for >30 yr. Soil physiochemical properties were analyzed by Midwest Laboratories (Omaha, NE) using standard protocols. The soil was classified as a composite of Nicollett and Webster with a sandy loam texture. It was 60% sand, 22% silt, and 18% clay. The organic matter content was 2.7%, and field capacity was determined to be 17% (water/dry soil). After collection, the soil was sieved (2.83 mm mesh size) and stored at 4°C until use in the bioassays. Before testing, the soil was allowed to air-dry to >97% solid, moistened to near field capacity, and then allowed to air-dry again. This procedure was intended to reduce soil fauna and prevent competition or predation on the test organisms.

Enzyme-linked Immunosorbent Assay Measurements of Soil and Corn Leaf Material. The soil from the reference field was tested by enzyme-linked immunosorbent assay (ELISA) before use in the assays to ensure that there was no background level of *Bt* protein from previous use of *Bt* insecticide or from naturally occurring *B. thuringiensis.* The soil was extracted using a high pH and high-salt buffer as described by Palm et al. (1994), and the concentration of *Bt* protein was confirmed to be below detection limits by use of an ELISA kit provided by Envirologix (Portland, ME). The procedure described in the kit was followed in conducting the ELISA measurements, except the extraction buffer previously described was used rather than the buffer provided with the kit. Samples of the ground corn leaf material were also measured at both the beginning and end of the test periods for each assay, using the same kits.

Plant Material Nutritional Measurements. Nutritional measurements of fat by gas chromatography (GC), sugar profile by high-performance liquid chromatography (HPLC), and total protein for all eight corn types and a mixture of ground cereal leaves, called cerophyl (Fisher, Chicago, IL), were conducted by Medallion Labs (Minneapolis, MN), using standard methods.

Earthworm Survival and Growth. The earthworm test was conducted following some of the guidelines from standardized methods (ASTM 1998), but significant modifications were made. In general, the standard methods measure acute toxicity in adults, adult weight loss, or adult reproductive success (Kula and Larink 1998). In this study, juvenile weight gain was chosen as a subacute endpoint because it is likely to be more sensitive than adult weight loss and it is less time and labor intensive than an adult reproduction assay. The test design used 10 treatments, consisting of the eight corn varieties and two controls, with 20 replicates per treatment. In addition to the isoline control for each *Bt* corn line, assay and reference controls were used that incorporated an optimal food. Assay control treatments were intended to show an optimal growth and reference control treatments were intended to show toxicant-inhibited growth. These controls were designed to provide a frame of reference for any results observed in the corn treatments. Both of the control treatments received the optimal food cerophyl, a mixture of ground, dried cereal leaves. This food was found to yield excellent growth in preliminary studies (data not shown). For each replicate, 25 g of soil were placed in a 3.5-cm (diameter) by 4-cm glass jar with two 2-mm holes in the lid. Soil moisture was adjusted to 120% of field capacity. Two grams of

the appropriate plant material (corn or cerophyl) were incorporated into the soil, and the units were aged for 21 d at 23°C to begin decomposition. The soil for the reference control treatment was fortified to 180 mg/kg with the herbicide pendimethalin. Pendimethalin was used as the reference control because its effects on *E. fetida* were previously characterized in this assay (Belden et al. 2005). Juvenile earthworms were cleaned in distilled water, blotted dry, and weighed. Each individual was randomly assigned to a treatment, and the units were placed onto a tray in a randomized complete block design. The tray was kept in an incubator at 24°C in the dark for 28 d. Although standard methods for earthworm testing (ASTM 1998) suggest 24-h light, this is partly to ensure that the individuals remain in the treated soil. However, in this test it was desirable that the more natural dark setting be used, allowing the worms to feed on the decomposing plant material at the top of the soil surface. As it was, no worms were observed out of the soil, even in the reference control treatment. Each week, the individuals were fed 100 mg of the appropriate plant material (corn or cerophyl). At this time, the disturbance of the soil surface was estimated as a percentage of the total surface area. This observation was intended to study potential behavioral alterations arising from any treatment. The test unit soil pH was monitored during the 28-d period, with individual test unit pHs ranging from 6.91 to 7.63. After 28 d, the earthworms were washed clean of soil in distilled water and weighed.

Springtail Survival, Reproduction, and Population Growth. The springtail assay was conducted similarly to standardized methods (ISO 1999). The same test design described for the earthworm test was used; there were eight corn treatments, two controls, and 20 replicates per treatment. For each replicate, 25 g of soil were placed in a 3.5-cm (diameter) by 4-cm glass jar, and the moisture level was adjusted to field capacity. One gram of the appropriate food type (corn material or cerophyl) was added, and the food material and soil aged for 21 d at 20°C to begin decomposition. After 21 d, three kernels (≈1 mg) of baker's yeast were added to the control units. Soil for the test units in the reference control treatment was fortified to 90 mg/kg with pendimethalin. As with the earthworm test, the effects of pendimethalin on *F. candida* population growth in this assay have been characterized previously (Belden et al. 2005). One springtail was randomly assigned to each jar. The jars were capped loosely, arranged on a tray in a randomized complete block design, and placed in an incubator on a 16:8 light cycle at 20°C for 28 d. To reduce direct light exposure, the test units were separated from the light source with one layer of brown paper. Food and moisture were checked every 72 h, and moisture was maintained at the pretest level based on the initial mass of the test units. In the controls, yeast was added if needed and replaced if uneaten. Beginning on day 7, test units were examined every 3–4 d under a dissecting scope for the presence of eggs or offspring, and for the survival of the adult. After 28 d, the containers were flooded with a saturated sucrose solution to float the springtails. The container was then exposed to ethyl acetate fumes to anesthetize the springtails, and the number of springtails was determined by total visual count using a magnifying glass or dissecting scope.

Table 2. Total protein, fat, and sugar of each corn line and control food

Treatment	Total protein (%)	Total fat (%)	Total sugar (%)
Bt11–90 (*Bt*+)	3.22 ± 0.003	0.13 ± 0.01	0.0
Bt11–90 (*Bt*−)	2.36 ± 0.002	0.64 ± 0.03	0.0
Bt11–108 (*Bt*+)	2.18 ± 0.002	0.56 ± 0.02	0.0
Bt11–108 (*Bt*−)	2.97 ± 0.003	0.60 ± 0.02	0.0
Mon810–90 (*Bt*+)	2.23 ± 0.002	0.54 ± 0.02	0.49 ± 0.02
Mon810–90 (*Bt*−)	1.85 ± 0.002	0.50 ± 0.02	0.78 ± 0.02
Mon810–108 (*Bt*+)	1.99 ± 0.002	0.54 ± 0.02	0.74 ± 0.02
Mon810–108 (*Bt*−)	1.74 ± 0.002	0.35 ± 0.01	0.0
Control (Cerophyl)	27.0 ± 0.027	1.59 ± 0.06	5.28 ± 0.16

All values are expressed as dry weight:weight percentages.

Statistical Analysis and Design. Each test was conducted as a randomized complete block design. Individuals were randomly assigned to a treatment and each treatment was represented once within a block. For the earthworm change in mass and the springtail total number analyses, analysis of variance (ANOVA) using the general linear model (GLM) was performed. For comparison of corn lines to the control treatments, Dunnett's adjustment was used. Because some of the individuals in the springtail reproduction assay did not reproduce before the end of the 28-d test period, the data are considered to be right-censored; i.e., the reporting value for time to reproduction for some individuals was >28 d. Therefore, these data were analyzed by the rank-order Kruskall-Wallis test. Differences in springtail percentage reproducing were analyzed using a χ^2 comparison in the frequency procedure. For analysis of the effects of nutritional parameters, the GLM procedure was performed, and the model statement included all three parameters (protein, sugar, and fat) and their interactions. All analyses were performed using SAS, Version 8.2 (SAS Institute 1999).

Results

ELISA Measurements of Corn and Soil. The ELISA measurements of the soil confirmed that there was no background level of *Bt* protein present in the soils used in the test. The level of *Bt* protein measured in the leaf material of each corn variety is shown in Table 1. The leaf material concentration of protein ranged from 2.19 to 2.68 μg/g.

Plant Material Nutritional Measurements. The material from the different corn varieties had relatively low amounts of the nutrients for which analyses were performed (Table 2). The control food had higher amounts of total protein, total fats, and total sugars. In three of four *Bt*-isoline pairs, the *Bt* line had a higher percentage total protein than its isoline. Most of the

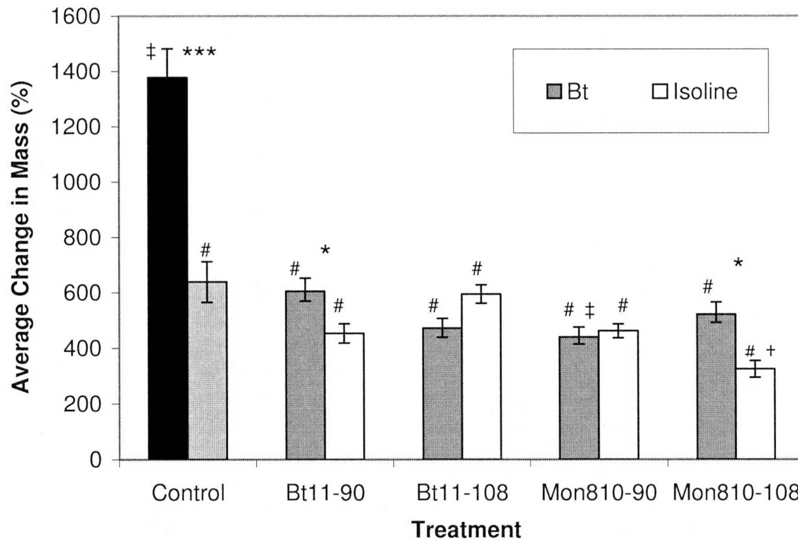

Fig. 1. Average percentage change in mass for *E. fetida* reared on Bt11 corn, Mon810 corn, non-*Bt* corn, or two controls. Black bar, assay control; hatched bar, reference control; gray bars, *Bt* corn lines; white bars, isolines. Statistical differences in means are marked by $^{*}P < 0.05$; $^{***}P < 0.0001$. #Treatment differs from assay control ($P < 0.05$, Dunnett's adjustment). ‡Treatment differs from reference control ($P < 0.05$, Dunnett's adjustment).

corn material had very little to no sugars. The nutritional measurements are presented in Table 2.

Earthworm Survival and Growth. None of the treatments resulted in mortality of the earthworms (data not shown). Only one individual died during the duration of the test. All of the surviving individuals gained weight. Individuals fed the assay control food achieved a significantly greater percentage weight gain ($t = 10.69, P < 0.0001$) than those in the reference control (Fig. 1). The reference control worked as intended, resulting in percentage weight gain that was 745 ± 138% lower than in the assay control. Earthworms in all corn leaf treatments exhibited significantly less weight gain than those in the assay control ($P < 0.0001$, Dunnett's adjustment). Earthworms in the corn treatments gained a similar amount of weight to those in the reference control treatment. Growth was significantly lower than that in the reference control treatment for individuals in the Mon810–90 (*Bt*+) treatment ($P = 0.0299$, Dunnett's adjustment) and Mon810–108 (*Bt*−) treatment, but growth for individuals in the remaining six corn treatments was not statistically different from that in the reference control ($P > 0.05$, Dunnett's adjustment).

There did not seem to be a consistent effect of the *Bt* protein. However, earthworms reared on leaf material from two *Bt*-producing varieties grew by a significantly larger percentage than those in the corresponding isoline. The percentage weight change for individuals fed Bt11–90 (*Bt*+) was 152 ± 136% greater than for individuals fed its isoline ($t = 2.21, P = 0.0286$). The percentage weight change for individuals fed Mon810–108 (*Bt*+) was 197.04 ± 135.56% greater than for individuals fed its isoline ($t = 2.87, P = 0.0046$).

Estimates of differences between each corn variety were made by ignoring *Bt* protein presence and analyzing the data from each *Bt*/isoline corn variety pairing. Earthworm growth was significantly lower for individuals fed Mon810–108 (*Bt*±) compared with both Bt11–90 (*Bt*±) ($t = 2.18, P = 0.0304$) and Bt11–108 (*Bt*±) ($t = 2.27, P = 0.0247$) treatments. There were no statistical differences observed for comparisons between any other corn variety pairings ($P > 0.05$).

There were no differences in the behavioral observations detected between treatments (data not shown). Most individuals had turned over the surface of their test unit within the first week, and all of the individuals had done so by the third week.

Springtail Survival, Reproduction, and Population Growth. None of the treatments resulted in statistically significant mortality of the springtails (data not shown). There were seven individuals that were either found dead or were not observed again and were presumed dead. The largest number dead in any one treatment was two individuals. With the exception of the reference control, all of the means for total number of springtails per treatment were >15. However, a number of individuals throughout the treatments did not achieve reproduction. Individuals in the assay control treatment produced significantly more offspring ($t = 4.78, P < 0.0001$) than those in the reference control (Fig. 2). The reference control showed a negative effect, resulting in 27.1 ± 11.2 fewer springtails than the assay control treatment. Total number of springtails was not different from the assay control ($P > 0.05$, Dunnett's adjustment) for any of the corn treatments (Fig. 2). Individuals reared on five of the corn varieties produced significantly greater offspring

Fig. 2. Average number of *F. candida* offspring produced by individuals reared on Bt11 corn, Mon810 corn, non-*Bt* corn, or two controls. Black bar, assay control; hatched bar, reference control; gray bars, *Bt* corn lines; white bars, isolines. Statistical differences in means are marked by $^{*}P < 0.05$; $^{***}P < 0.0001$. $^{\#}$Treatment differs from assay control ($P < 0.05$, Dunnett's adjustment). ‡Treatment differs from reference control ($P < 0.05$, Dunnett's adjustment).

than those reared on the reference control (Bt11–90 (*Bt*−): $P < 0.0001$; Bt11–108 (*Bt*+): $P = 0.0474$; Mon810–90 (*Bt*−): $P = 0.0083$; Mon810–108 (*Bt*+): $P = 0.0004$; Mon810–108 (*Bt*−): $P = 0.0024$; Dunnett's adjustment). Total number of springtails was not significantly different from the reference control for the Bt11–90 (*Bt*+), Bt11–108 (*Bt*−), and Mon810–90 (Bt+) treatments ($P > 0.05$, Dunnett's adjustment).

There was not a consistent effect of the *Bt* protein across the corn varieties; only the Bt11–90 (*Bt*±) pairing exhibited a statistical difference in total number of offspring produced. Individuals reared on Bt11–90 (*Bt*−) produced 13.7 ± 11.7 more total springtails than those reared on the Bt-containing line, Bt11–90 (*Bt*+) ($t = 4.78$, $P < 0.0001$). This corresponds to a 44.9 ± 33.8% reduction in total number of springtails produced. As with the earthworm analysis, estimates of the differences between corn varieties were made by ignoring the presence of *Bt* and analyzing the data from each *Bt*/isoline corn variety pairing. There were no statistical differences for any of these comparisons ($P > 0.05$).

The number of springtails that reproduced after 28 d was >75% in all treatments except for the reference control (Table 3). In the reference control, only 25% of the individuals had reproduced by day 28, significantly fewer than in the assay control ($\chi^2 = 25.0$, $P < 0.0001$). Percentage reproduction at 28 d was not statistically different from the assay control treatment for any of the corn varieties ($P > 0.05$). Similarly, time to reproduction was only affected by the reference control treatment. The average time to first reproduction event for most treatments was around 20 d. The time to reproduction in the reference control treatment was significantly longer than that in the assay control, at >28 d (Table 2, $\chi^2 = 24.63$, $P < 0.0001$, Kruskall-Wallis test).

Effects of Nutritional Parameters. A statistical model developed using the nutritional parameters of protein, sugar, and fat content showed that protein, sugar, and the interaction of the two had a significant effect on the growth of earthworms ($P = 0.0005$, $P = 0.0384$, and $P = 0.0024$, respectively). Both of the *Bt* varieties that resulted in higher growth than their isoline have higher protein and/or sugar content: Bt11–90, 3.22% versus 2.36% (protein); Mon810–108, 1.99% versus 1.74% (protein) and 0.74% versus 0.0% (sugar). No correlation was observed between the

Table 3. Reproduction of *F. candida* fed Bt11 corn, Mon810 corn, non-*Bt* corn, or two controls

Treatment	Percent reproducing	Time to reproduction (d)
Assay control	95.0a	20.15 ± 1.16a
Bt11–90 (*Bt*+)	84.2a	21.85 ± 1.43a
Bt11–90 (*Bt*−)	88.9a	20.85 ± 1.14a
Bt11–108 (*Bt*+)	89.5a	21.05 ± 1.25a
Bt11–108 (*Bt*−)	85.0a	21.90 ± 1.42a
Mon810–90 (*Bt*+)	100.0a	19.85 ± 1.23a
Mon810–90 (*Bt*−)	88.9a	21.00 ± 1.22a
Mon810–108 (*Bt*+)	89.5a	21.20 ± 1.64a
Mon810–108 (*Bt*−)	75.0a	20.9 ± 1.22a
Reference control	25b	>28b

Percentage reproducing is based on no. reproducing at 8 wk. Time to reproduction is no. of days to first observed offspring, expressed as mean ± SE. Means within a column followed by different letters are significantly different at $P < 0.05$ (Kruskall-Wallis).

studied nutritional parameters and any of the endpoints measured in the springtail assay.

Discussion

Overall mortality in the earthworm assay was near zero, which agrees with the results of previous studies using *Bt* from transgenic plants (Ahl Goy et al. 1995; Saxena and Stotzky 2001b; Zwahlen et al. 2003b). Smirnoff and Heimpel (1961) did show 100% mortality of *L. terrestris* exposed to the *Bt* bacterial preparation Thuricide. However, the concentration used was $\approx 10^4$–10^5 times greater than those that would occur in normal usage, and there seem to be confounding factors with formulation. Furthermore, several studies showed a lack of mortality of earthworms using bacterial formulations of *Bt* at up to 1,000 times the field application rates (Benz and Altwegg 1975, Addison and Holmes 1996). Although it is possible that the aging in soil reduced the *Bt* concentration in the plants, Zwahlen et al. (2003a) showed retention of *Bt* protein in buried litter over a much longer time period than studied in this experiment.

Earthworms in the assay control treatment exhibited ≈50% more growth than those in the reference control treatment. This result is comparable with that reported by Belden et al. (2005), who reported a >1,500% increase in mass for earthworms in the assay control treatment. The food used for both control treatments was an optimal food and had higher amounts of protein, fat, and sugar than any of the corn types (Table 2). The reference control worked in a manner that showed how a potential effect of a toxicant would appear in the assay if the nutritive value of the foods was equal. Individuals in all of the corn treatments actually responded in a similar fashion to the reference control, indicating that the corn was probably not the optimal food for *E. fetida.* This is not entirely unexpected, considering that *E. fetida* prefers a very rich food source rather than fresh plant residue (Kula and Larink 1998). Because of this, another earthworm, such as *L. terrestris*, might be valuable to test in this assay. However, *E. fetida* is still a good choice for toxicity testing because it is easily cultured, and there is extensive knowledge of its response to many chemicals. Ideally, future tests would be run with both *E. fetida* and *L. terrestris* (or another anecic species) to examine earthworms from groups with different ecological strategies.

As stated previously, there did not seem to be a consistent effect of Cry1Ab protein on earthworm growth. Again, this result seems to concur with those of previous studies (Ahl Goy et al. 1995, Saxena and Stotzky 2001a, Zwahlen et al. 2003b). Unlike any previous work, change in weight was greater for individuals fed corn material from two Cry1Ab Bt varieties, Bt11–90 (*Bt*+) and Mon810–108 (*Bt*+), in comparison with those fed their isolines. These differences seem to be at least partially explained by the differences in nutritive quality of the treatments, as shown with the model described previously. Furthermore, for those *Bt*/isoline pairings that were not statistically different, the trends in growth also followed protein and sugar content. Similar to the comparisons of *Bt* lines to their isolines, the differences in growth described previously that resulted from comparison of the varieties (ignoring *Bt* presence or absence) also seem to follow the nutritive value of the varieties. Individuals fed Mon810–108 (*Bt*±) grew less than individuals fed either Bt11–90 (*Bt*±) or Bt11–108 (*Bt*±), and Mon810–108 (*Bt*±) had lower protein content than both Bt-11 maturities.

The design of this study differs significantly from previous work. A broader selection of corn varieties was tested, allowing for better comparison than just one *Bt* line to its isoline. All three of the other studies used Cry1Ab producing corn lines, but only examined one variety and one type. Additionally, this study examined juvenile earthworms, a stage in which there is a rapid rate of growth and potential for sensitivity to changes in growth rate. Ahl Goy et al. (1995) conducted a 14-d test and found no effects of *Bt* corn, but do not state if they used adult or juvenile *E. fetida.* Saxena and Stotzky (2001a) used a different earthworm, *L. terrestris*, in a very similar test and found no effects of Cry1Ab corn on weight. However, their study used sexually mature earthworms, which are less likely to be growing quickly and may be less sensitive to effects on growth. Zwahlen et al. (2003b) saw no effects of Cry1Ab corn in a field study with immature *L. terrestris* but did see a decrease in weight, associated with *Bt* corn, for adult *L. terrestris* in a 200-d laboratory study.

The lack of mortality observed in this springtail assay also agrees with the limited published literature. In a study with Cry1Ab cotton, Cry1Ac potatoes, and Cry3A potatoes, no mortality of *F. candida* was reported (Yu et al. 1997). Further studies using purified proteins or the bacterial formulation Dipel also found no mortality (Addison and Holmes 1996, Sims and Martin 1997). The dose of protein in the study by Sims and Martin (200 μg/g in food) was much higher than that tested in this study. However, it is uncertain if the springtails were truly exposed by mixing the purified *Bt* protein into a yeast diet.

As stated previously, the individuals in the reference control treatment produced ≈90% fewer offspring than those in the assay control. This reduction is similar to that reported previously (Belden et al. 2005). However, control reproduction was lower overall in this study. This may be caused by a slight difference in age of the springtails before the start of the test, allowing those tested by Belden et al. (2005) to have an extra clutch and produce more offspring. The reference control again aptly shows the subacute effect of a toxicant because of its ability to reduce total number of offspring without adult mortality.

Because all of the individuals in corn treatments produced a total number of offspring that was not statistically different from the control, it can be assumed that these corn varieties were acceptable substrates for *F. candida.* This may not have been the case if the initial corn material had not been aged in the soil to begin decomposition, because it is known that these

springtails feed on fungi and other microorganisms more than on the decomposing plant matter itself. Probably because of the variability in the data, three corn varieties, Bt11–90 (*Bt*+), Bt11–108 (*Bt*−), and Mon810–90 (*Bt*+), were not statistically different from the reference control. However, individuals in all three of these treatments produced >300% more offspring than those in the reference control.

The lack of a consistent effect on total number of springtails of the Cry1Ab *Bt* protein is also consistent with previous work (Addison and Holmes 1996, Sims and Martin 1997, Yu et al. 1997). None of the previous studies found differences in production of offspring. In this study, one isoline, Bt11–90 (*Bt*−), did show an increased number of springtails compared with the *Bt* line. It is somewhat difficult to explain this difference, but it is also a good illustration of the importance of testing multiple corn varieties and events. The magnitude of the difference in total number in the Bt11–90 pairing is within the range of the overall variability in production among all the different corn treatments. If only that one hybrid had been tested, the results may suggest a negative effect of the *Bt* protein. However, in the context of the response of *F. candida* to all eight corn varieties, it is less likely that this response is because of a negative effect of the *Bt* protein. This is further shown by the result, reported above, that there were no statistical differences in production for the comparisons of the corn varieties when *Bt* presence was ignored.

Although time to reproduction and number of individuals reproducing at 8 wk were not affected by any of the corn treatments, these parameters were shown to be sensitive in this assay by the response of the reference control. Fewer individuals in the reference control treatment reproduced than in all other treatments and those that did so took longer. This was a good illustration of a subacute effect of a toxicant in the assay, because the reproduction effects occurred without significant mortality in the treatment.

Unlike in the earthworm assay, the studied nutritional parameters were not correlated with the production differences observed in the springtail assay. This is not entirely unexpected, because the springtails were probably not feeding directly on the plant material, but rather on the fungi and other microorganisms that are present on the decomposing matter. Donegan et al. (1995) observed transient changes in populations of bacteria in soil with transgenic cotton leaf residues. The authors suggested that the transformation of the plant may have led to changes in other plant characteristics that affected bacterial growth. It is possible that this effect could have influence on *F. candida* because of differences in the abundance of its preferred food types. Other parameters, such as lignin or cellulose content, might have been useful to measure to explain differences in the springtail response, including the observed difference between the Bt11–90 (*Bt*+) and Bt11–90 (*Bt*−). The relative quantity of lignin could help determine how rapidly the plant material degrades and becomes available to microorganisms. Saxena and Stotzky (2001b) reported that five varieties of Bt11 and four varieties of Mon810 all had higher lignin content than their respective isolines. Masoero et al. (1999) also observed a higher lignin content for one *Bt* corn variety. Additionally, Masoero et al. (1999) suggested that the transgenic corn had a reduced susceptibility to mold attack. These factors could all play a role in the suitability of transgenic or conventional corn leaf material as a substrate for soil organisms.

The assays conducted in this study represent an improvement on some parts of the previous methodology for performing subacute assays with transgenic crops, especially compared with assays using purified *Bt* proteins. The methods used represent a more realistic route of exposure for assessing the effects of transgenic crops. Studies using purified proteins use an unrealistic route of exposure (mixing of purified protein with soil or yeast) that is more applicable to conventional pesticides that are taken up through the outer surface of the organism. One major disadvantage of using actual plant material is that it does not allow for testing of multiple dose levels of the *Bt* protein. Testing multiple corn varieties, with some variability in amount of protein expressed, can help provide a broader view. Additionally, this allows differences not directly caused by the toxin to be placed into the context of variability between different hybrids (i.e., Mon810 versus Bt11). It is important in the development of assays that examine the effects of transgenic crops to include multiple controls as was done here. The use of a *Bt* line and its near isoline allows for a comparison of the effect of *Bt* proteins. The assay control provides a baseline of an optimal response for the organism tested, and the reference control provides a basis for comparing any treatment's relative deviation from that optimum.

These results indicate that the Cry1Ab protein within several commercially important corn lines does not have a direct effect on the survival and fitness of *E. fetida* or *F. candida.* However, analysis of related species, especially *L. terrestris,* may be warranted. There were differences observed between the corn treatments in both tests, indicating that the *Bt* line and its isoline were not always equivalent. Differences in nutritional factors of these transgenic crops and perhaps their suitability for populations of degrader organisms may play a role in how these transgenic crops affect agroecosystems. These types of effects would never be observed in tests with purified proteins, so it is important to consider the plant material as a whole, in conjunction with true dose–response testing.

References Cited

Addison, J. A., and S. B. Holmes. 1996. Effect of two commercial formulations of *Bacillus thuringiensis subsp. kurstaki* on the forest earthworm *Dendrobaena octaedra.* Can. J. Forest Res. 26: 1594–1601.

Ahl Goy, P., G. Warren, J. White, L. Privalle, and P. Fearing. 1995. Interaction of an insect tolerant maize with organisms in the ecosystem. Proceedings of the key biosafety

aspects of genetically modified organisms, 10–11 April 1995. Braunschweig, Germany.

American Society of Testing and Materials [ASTM]. 1998. Standard guide for conducting laboratory soil toxicity or bioaccumulation studies with the Lumbricid earthworm Eisenia fetida. American Society of Testing and Materials, West Conshohocken, PA.

Belden, J. B., T. A. Phillips, B. W. Clark, and J. R. Coats. 2005. Environmental hazard evaluation of pendimethalin contaminated soil. B. Environ. Contam. Tox. 74: 769–776.

Benz, G., and A. Altwegg. 1975. Safety of *Bacillus thuringiensis* for earthworms. J. Invertebr. Pathol. 26: 125–126.

Donegan, K. K., C. J. Palm, V. J. Fieland, L. A. Porteous, L. M. Ganio, D. L. Schaller, L. Q. Bucao, and R. J. Seidler. 1995. Changes in levels, species, and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis var. kurstaki* endotoxin. Appl. Soil Ecol. 2: 111–124.

Edwards, C. A., and P. J. Bohlen (eds.). 1996. Biology and ecology of earthworms. Chapman & Hall, London, UK.

Edwards, C. A., P. J. Bohlen, D. R. Linden, and S. Subler. 1995. Earthworms in agroecosystems, pp. 185–214. In P. F. Hendrix (ed.), Earthworm ecology and biogeography in North America. CRC, Boca Raton, FL.

Fearing, P. L., D. Brown, D. Vlachos, M. Meghji, and L. Privalle. 1997. Quantitative analysis of CryIA(b) expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. Mol. Breeding. 3: 169–176.

Herman, R. A., J. D. Wolt, and W. R. Halliday. 2002. Rapid degradation of the Cry1F insecticidal crystal protein in soil. J. Agr. Food Chem. 50: 7076–7078.

International Organization for Standardization [IOS]. 1999. Soil quality—inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. International Organization for Standardization, Geneva, Switzerland.

James, C. 2002. Global status of commercialized transgenic crops: 2002. ISAAA, Ithaca, NY.

James, C. 2003. Global status of commercialized transgenic crops: 2003. ISAAA, Ithaca, NY.

Koskella, J., and G. Stotzky. 1997. Microbial utilization of free and clay-bound insecticidal toxins from Bacillus thuringiensis and their retention of insecticidal activity after incubation with microbes. Appl. Environ. Microb. 63: 3561–3568.

Kula, H., and O. Larink. 1998. Tests on the earthworms *Eisenia fetida* and *Aporrectodea calignosa*, pp. 95–111. *In* H. Lokke and C.A.M. van Gestel (eds.), Handbook of soil invertebrate toxicity tests. Wiley, New York.

Masoero, F., M. Moschini, F. Rossi, A. Prandini, and A. Pietri. 1999. Nutritive value, mycotoxin contamination and in vitro rumen fermentation of normal and genetically modified corn (Cry1A(B)) grown in northern Italy. Maydica 44: 205–209.

Obrycki, J. J., J. E. Losey, O. R. Taylor, and L.C.H. Jesse. 2001. Transgenic insecticidal corn: beyond insecticidal toxicity to ecological complexity. BioScience 51: 353–361.

Palm, C. J., K. Donegan, D. Harris, and R. J. Seidler. 1994. Quantification in soil of *Bacillus thuringiensis var. kurstaki* δ-endotoxin from transgenic plants. Mol. Ecol. 3: 145–151.

Palm, C. J., D. L. Schaller, K. K. Donegan, and R. J. Seidler. 1996. Persistence in soil of transgenic plant produced Bacillus thuringiensis var. kurstaki delta-endotoxin. Can. J. Microbiol. 42: 1258–1262.

Parkinson, D. 1983. Functional relationships between soil organisms, pp. 153–165. *In* P. Lebrun, H. M. Andre, A. De Medts, C. Gregoire, W. Wauthy, and G. Wauthy (eds.), New trends in soil biology. Louvain la-Neuve, Dieu-Brichart, France.

Parmelee, R. W., P. J. Bohlen, and J. M. Blair. 1998. Earthworms and nutrient cycling processes: integrating across the ecological hierarchy, pp. 123–146. *In* C. A. Edwards (ed.), Earthworm ecology. CRC, Boca Raton, FL.

Rusek, J. 1998. Biodiversity of Collembola and their functional role in the ecosystem. Biodivers. Conserv. 7: 1217–1219.

SAS Institute. 1999. The SAS System for Windows, version 8.2. SAS Institute, Cary, NC.

Saxena, D., and G. Stotzky. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn in vitro and in situ. FEMS Microbiol. Ecol. 33: 35–39.

Saxena, D., and G. Stotzky. 2001a. *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol. Biochem. 33: 1225–1230.

Saxena, D., and G. Stotzky. 2001b. Bt corn has a higher lignin content than non-Bt corn. Am. J. Bot. 88: 1704–1706.

Sims, S. R., and J. W. Martin. 1997. Effect of the *Bacillus thuringiensis* insecticidal proteins CryIA(b), CryIA(c), CryIIA, and CryIIIA on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola). Pedobiologia 41: 412–416.

Sims, S. R., and J. E. Ream. 1997. Soil inactivation of the *Bacillus thuringiensis subsp. kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: laboratory and field studies. J. Agr. Food Chem. 45: 1502–1505.

Smirnoff, W. A., and A. M. Heimpel. 1961. Notes on the pathogenicity of Bacillus thuringiensis var. thuringiensis Berliner for the earthworm, *Lumbricus terrestris* Linnaeus. J. Insect Pathol. 3: 403–408.

Tapp, H., and G. Stotzky. 1995. Insecticidal activity of the toxins from *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* adsorbed and bound on pure and soil clays. Appl. Environ. Microbiol. 61(5): 1786–1790.

Tapp, H., and G. Stotzky. 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* from soil. Soil Biol. Biochem. 30: 471–476.

Tapp, H., L. Calamai, and G. Stotzky. 1994. Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* on clay minerals. Soil Biol. Biochem. 26: 663–679.

Tomlin, A. D., M. J. Shipitalo, W. M. Edwards, and R. Protz. 1995. Earthworms and their influence on soil structure and infiltration, pp. 159–184. *In* P. F. Hendrix (ed.), Earthworm ecology and biogeography in North America. CRC, Boca Raton, FL.

Venkateswerlu, G., and G. Stotzky. 1992. Binding of the protoxin and toxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* on clay minerals. Curr. Microbiol. 25: 1–9.

Wiles, J. A., and P. H. Krogh. 1998. Tests with the Collembolans *Isotoma viridis*, *Folsomia candida* and *Folsomia fimetaria*, pp. 131–156. In H. Lokke and C.A.M. van Gestel (eds.), Handbook of soil invertebrate toxicity tests. Wiley, New York.

Yu, L., R. E. Berry, and B. A. Croft. 1997. Effects of *Bacillus thuringiensis* toxins in transgenic cotton and potato on *Folsomia candida* (Collembola: Isotomidae) and *Oppia nitens* (Acari: Oribatidae). J. Econ. Entomol. 90: 113–118.

Zwahlen, C., A. Hilbeck, P. Gugerli, and W. Nentwig. 2003a. Degradation of the Cry1Ab protein within the transgenic *Bacillus thuringiensis* corn tissue in the field. Mol. Ecol. 12: 765–775.

Zwahlen, C., A. Hilbeck, R. Howald, and W. Nentwig. 2003b. Effects of transgenic corn litter on the earthworm *Lumbricus terrestris.* Mol. Ecol. 12: 1077–1086.

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