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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

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Abstract

There were no significant differences in the percent mortality and weight of earthworms (*Lumbricus terrestris*) after 40 days in soil planted with Bt (NK4640Bt) or non-Bt corn or after 45 days in soil amended with biomass of Bt or non-Bt corn. The toxin was present in the guts and casts of earthworms in soil planted with Bt corn or amended with biomass of Bt corn, but it was cleared within 2–3 days from the guts after placing in fresh soil. There were no significant differences in the colony-forming units of culturable bacteria (including actinomycetes) and fungi and in the numbers of protozoa and nematodes between rhizosphere soil of Bt and non-Bt corn or between soil amended with biomass of Bt and non-Bt corn. The Cry1Ab protein in root exudates and biomass of Bt corn appears not to be toxic to earthworms, nematodes, protozoa, bacteria, and fungi. The presence of the toxin in the guts and casts of earthworms confirmed that the toxin released in root exudates and from transgenic biomass was bound on surface-active particles in soil, which protected the toxin from biodegradation, as has been observed in this laboratory with purified toxin.

Keywords: Bt corn; Root exudates; Biomass of Bt corn; Earthworms; Nematodes; Microorganisms; Cry1Ab toxin; *Bacillus thuringiensis*

1. Introduction

Bt corn is maize (*Zea mays* L.) that has been genetically modified to express the *cry1Ab* gene from *Bacillus thuringiensis* (Bt) and produce an insecticidal toxin to kill lepidopteran pests, especially the European corn borer (*Ostrinia nubilalis*), which can reduce the yield of corn by 3–7% borer^{-1} plant^{-1} (Lynch, 1980). The toxin from Bt corn is introduced into soil primarily in root exudates (Saxena et al., 1999; Saxena and Stotzky, 2000) and by incorporation of plant residues after harvest of the crop (Tapp and Stotzky, 1998; S. Flores, D. Saxena and G. Stotzky, unpub. data), with probably some input from pollen during tasseling (Losey et al., 1999). In vitro and in situ studies indicated that the toxin released in root exudates and from the biomass of Bt corn adsorbs and binds rapidly on surface-active particles in soil and remains larvicidal for at least 180 days (percentage mortality: 56 ± 11.9 and 68 ± 11.9 for exudates and biomass, respectively), the longest time studied (D. Saxena and G. Stotzky, unpub. data). The toxin was also present in the rhizosphere soil of field-grown Bt corn plants throughout their growth and several months after their death and subsequent frost (Saxena and Stotzky, 2000). When purified toxin from *B. thuringiensis* subsp. *kurstaki* (Btk) was added to non-sterile soils, activity against the larvae of the tobacco hornworm (*Manduca sexta*) was still detected after 234 days, the longest time evaluated (Tapp and Stotzky, 1998). This persistence was considerably longer than persistences estimated in the literature, which ranged in 'half-life' from ca. 8–17 days for purified toxin and 2–41 days for biomass of transgenic corn, cotton, and potato (Palm et al., 1994, 1996; Sims and Holden, 1996; Sims and Ream, 1997).

The toxins produced by Btk (66 kDa; active against Lepidoptera) and *B. thuringiensis* subsp. *tenebrionis* (Btt; 68 kDa; active against Coleoptera) adsorbed and bound rapidly (in $<$30 min, the shortest time studied) on clay minerals [montmorillonite (M) and kaolinite (K)], on the clay-size fraction of soil, on humic acids, and on complexes of M-humic acids-Al hydroxypolymers. These results indicated that the toxins released in root exudates and upon disintegration of transgenic plant cells in soil would be in a free state susceptible to rapid biodegradation only briefly (Venkateswerlu and Stotzky, 1990, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995a,b, 1997; Koskella and Stotzky, 1997; Crecchio and

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Stotzky, 1998, 2001; Stotzky, 2000). The binding of the toxins on these surface-active particles reduced their availability to microbes, which is probably responsible for the persistence of the toxins in soil (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998). The free toxins were readily utilized as sources of carbon and nitrogen by pure and mixed cultures of microbes, including soil suspensions, whereas the bound toxins were not utilized as a source of carbon, slightly as a source of nitrogen, but they did not support growth in the absence of exogenous sources of both available carbon and nitrogen. After exposure of the bound toxins to microbes, both in vitro and in soil, the toxins retained insecticidal activity, even after alternately freezing and thawing or wetting and drying the soil for 40 days. The toxins, free or bound, had no effect on the growth in vitro of a spectrum of bacteria (both Gram-positive and Gram-negative), fungi (both yeasts and filamentous forms—e.g. Zygomycetes, Ascomycetes, Deuteromycetes), and algae (primarily green and diatoms) (J. Koskella and G. Stotzky, unpub. data), in agreement with in situ observations with transgenic Bt plants that generally showed no consistent and lasting effects on microorganisms (Donegan et al., 1995, 1996).

As the result of the binding of the toxins on surface-active particles, the toxins could accumulate in the environment to concentrations that may enhance the control of target pests or constitute a hazard to non-target organisms, such as the soil microbiota, beneficial insects (e.g. pollinators, predators and parasites of insect pests) (Flexner et al., 1986; Goldburg and Tjaden, 1990; Addison, 1993; James et al., 1993; Johnson et al., 1995), and other animal classes. This hazard can be direct [e.g. larvae of the monarch butterfly (*Danaus plexippus*) killed by feeding on milkweed (*Asclepias curassavica*) contaminated with pollen from transgenic Bt corn (Losey et al., 1999)] or indirect in tritrophic interactions [e.g. mortality and delayed development of the green lacewing (*Chrysoperla carnea*), a predator of insect pests, when fed larvae of the European corn borer raised on transgenic Bt corn (Hilbeck et al., 1998a,b, 1999)]. The accumulation and persistence of the toxins could also result in the selection and enrichment of toxin-resistant target insects (Van Rie et al., 1990; McGaughey and Whalon, 1992; Entwistle et al., 1993; Tabashnik, 1994; Tabashnik et al., 1997; Bauer, 1995; Ferré et al., 1995).

Therefore, the toxin released into soil from the roots and biomass of Bt corn may affect organisms in soil. Here we show that toxin released from roots and biomass of Bt corn appeared to have no deleterious effect on earthworms, nematodes, and microorganisms.

2. Materials and methods

2.1. Soil

A freshly collected soil from a farm in East Marion, Long Island, New York, was sieved through a broad-mesh screen (15 mm), to remove stones and plant debris and to disrupt large soil aggregates, and then sieved through a 5-mm sieve. The sieved soil was mixed thoroughly and maintained moist at 24 ± 2°C. Some physicochemical characteristics of the soil are: pH 5.2; 0.92 and 0.07% carbon and nitrogen; 58, 41, and 1% sand, silt, and clay.

2.2. Earthworms

Fifteen plastic pots (18 cm dia., 21 cm deep; five each for Bt corn, non-Bt corn, and with no plants) were each filled with ca. 4.5 kg of soil, and 20 medium-size earthworms (*Lumbricus terrestris*), with well developed clitellum, purchased from Carolina Biological Supply Company (Burlington, North Carolina), were placed in each pot and kept overnight in the dark at 24 ± 2°C when the worms entered the soil. Before the earthworms were introduced, the colony-forming units (CFU) of culturable bacteria and fungi, as well as the numbers of protozoa and nematodes, in the soil were determined. Seeds of Bt corn (NK4640Bt) and of isogenic non-Bt corn were planted (three seeds per pot), and after 40 days of growth in a plant-growth room (26 ± 2°C, 12 h light–dark cycle; soil water content maintained at ca. field capacity, and no water stress was apparent in the plants), the plants were gently removed and rhizosphere soil was collected by gently shaking the roots to dislodge adhering small clumps of soil. To determine the effects of biomass from Bt and non-Bt corn on earthworms and the other organisms, 500 g of soil amended with 1% (w w^{-1}) of ground, air-dried biomass of Bt corn (NK4640Bt) or isogenic non-Bt corn (leaves, stems, and roots) was placed into each of five jars (10 cm dia., 16 cm deep), and five medium-size earthworms were added to each jar. Two control jars received no biomass. All jars were kept in the plant-growth room for 45 days with the soil maintained at ca. field capacity. Casts produced by the earthworms were collected from all pots and jars. After incubation, the earthworms in the pots and jars were counted and their weight determined. Three representative worms from each pot and two from each jar were dissected, and soil from the guts was analyzed for the presence of Cry1Ab protein by immunological and larvicidal assays.

To determine the time required to clear the toxin from the guts of earthworms, worms from pots of Bt corn and from jars amended with biomass of Bt corn were gently removed and transferred to pots containing fresh soil that had not been exposed to Bt toxin. After 1, 2, and 3 days, representative earthworms were dissected, and the soil in their guts was analyzed for the presence of the toxin.

2.3. Nematodes

Nematodes were extracted from soil by the Baermann technique (Van Gundy, 1982). Duplicate samples of soil (50 g) from each pot of Bt and non-Bt corn or without plants and from each jar amended or not amended with biomass of Bt or non-Bt corn were uniformly placed over tissue paper

supported by a screen in a funnel, which was connected to a rubber collection tube and secured with a pinch clamp. Sufficient tap water was added to the funnel to cover the surface of the soil. The samples were incubated at 24 ± 2°C for 36 h. To estimate the total numbers of nematodes, the content of the collection tube was transferred to a flat-bottom partitioned counting dish (36 squares), and the numbers of nematodes were counted in 10 random squares with a stereoscopic dissecting microscope (10 to 100×). Total numbers of nematodes in the samples were calculated by multiplying the average count in the 10 squares by 36.

2.4. Protozoa

Total numbers of culturable protozoa were estimated by a most-probable number (MPN) method using agar plates (Stotzky et al., 1993). The medium (5 g NaCl, 10 g agar, and 1 l tap water; autoclaved for 15 min at 121°C) was aseptically dispensed into sterile Petri plates and allowed to solidify and dry overnight. The plates were then cored with the open end of a flamed test tube (25-mm OD), so that islands of agar, 25 mm dia., were physically separated from the remaining agar. A loopful of a suspension of mixed bacteria isolated from the soil and grown on soil extract agar (SEA; 0.2 g KH_2PO_4, 1 g dextrose, 15 g agar, 100 ml soil extract, and 900 ml tap water) was spread over each island to serve as a food source for the protozoa. Soil was serially diluted, and 100 μl each of three dilutions (10^{-2}, 10^{-3}, 10^{-4}) was added to the center of five replicate islands, and the plates were incubated in the dark at 24 ± 2°C. After 5–7 days, each island was examined under low-power magnification (100×) for the presence of protozoa. Total numbers of protozoa were calculated from MPN tables.

2.5. Bacteria, including actinomycetes, and fungi

The CFU of culturable bacteria were estimated on SEA (Stotzky et al., 1993). Soil (1 g) from the various treatments was suspended in 10 ml of sterile tap water and 10-fold serially diluted. The CFU of bacteria were determined by spreading 100 μl of diluted samples on agar plates and incubating at 24 ± 2°C for 5 days. The CFU of actinomycetes were determined by probing colonies that developed with a dissecting needle: if the colony remained as a discrete, small mass, it was considered to be an actinomycete, whereas if the colony smeared and lost its periphery, it was considered to be a bacterium other than an actinomycete. These empirical observations were confirmed by microscopic examination of the colonies.

The CFU of fungi were estimated on Rose Bengal-streptomycin agar (Stotzky et al., 1993) (10 g dextrose, 5 g peptone, 1 g KH_2PO_4,0.5 g MgSO4.7H2O, 33 mg Rose Bengal, 2.4 ml of a stock solution of 1.25 g streptomycin l^{-1}, 20 g agar, and 1 l tap water) on which 100 μl of 10-fold serially diluted soil samples was spread. The CFU of fungi were counted after incubation for 5–7 days at 24 ± 2°C. The CFU of bacteria and fungi were determined on duplicate samples of soil from each pot and jar.

Table 1
Effect on earthworms (*Lumbricus terrestris*) of CrylAb toxin released in root exudates and from biomass of corn plants with (Bt +) or without (Bt −) the *crylAb* gene, in soil amended with ground residues of Bt + or Bt − corn, or in the absence of plants or biomass in soil (expressed as percentage mortality and weight of a single earthworm ± standard error of the mean)

Soil	Percentage mortality	Weight (g)
Root exudates[a]		
No plants	8 ± 2.5	3.6 ± 0.10
Bt − plants	14 ± 1.0	3.6 ± 0.03
Bt + plants	10 ± 1.5	3.9 ± 0.27
Biomass[b]		
No biomass	30 ± 10.0	3.9 ± 0.06
Bt − biomass	28 ± 4.80	4.3 ± 0.15
Bt + biomass	24 ± 7.48	4.0 ± 0.19

[a] Mortality and weight of earthworms were determined in soil with no plants or with Bt + or Bt − corn after 40 days of plant growth ($n = 20$ worms × 5 replications treatment^{-1}).

[b] Mortality and weight of earthworms were determined after 45 days of incubation with ground residues of Bt + or Bt − corn or with no residues ($n = 5$ worms × 5 replications treatment^{-}).

2.6. Immunological assay

Samples of soil (0.5 g) from the guts and casts of earthworms in soil planted with Bt or non-Bt corn or in soil amended with biomass of Bt or non-Bt corn, as well as in soils without plants or biomass, were vortexed with 500 μl of extraction buffer (EnviroLogix, Portland, ME), centrifuged, and the supernatants analyzed by Western blot using Lateral Flow Quickstix (EnviroLogix; detection limit <10 parts 10^{-9}) (Saxena et al., 1999; Saxena and Stotzky, 2000).

2.7. Larvicidal assay

The larvicidal activity of the soils was determined with the larvae of the tobacco hornworm (*M. sexta*) (Tapp and Stotzky, 1998). Eggs of *M. sexta* and food medium were obtained from Carolina Biological Supply Company. The eggs, placed on solidified medium in Petri plates, were incubated at 29 ± 1°C under a 40 W lamp for 2–3 days, when the eggs hatched. The medium was dispensed, after microwaving, in 5-ml amounts into vials (3 cm dia. and 6 cm tall) and allowed to solidify. Aliquots (100 μl) of freshly vortexed soil suspensions were uniformly distributed over the surface of the medium (8.55 cm^2) with disposable pipette tips (200-μl capacity; Fisher Scientific) that had been cut ca. 1.5 cm from the tip, to ensure that all suspended particles of soil were transferred. After air-drying, four second-instar larvae were added to each of duplicate vials prepared from duplicate soil tubes, resulting in 16 larvae for each soil sample. Mortality was determined after 3 and 7 days, and percent mortality was based on mortality after 7 days.

Table 2
Cry1Ab toxin in casts and soil in the guts of earthworms (*Lumbricus terrestris*) in the presence of corn plants with (Bt +) or without (Bt −) the *cryIAb* gene, in soil amended with ground residues of Bt + or Bt − corn, or in the absence of plants or biomass

Sample	No plants		Root exudates[a]				No biomass		Biomass[b]			
			Bt −		Bt +				Bt −		Bt +	
	Immuno. test[c]	Percentage mortality[d]	Immuno. test	Percentage mortality	Immuno. test	Percentage mortality	Immuno. test	Percentage mortality	Immuno. test	Percentage mortality	Immuno. test	Percentage mortality
Soil	–	0	–	5 ± 5.0	+	92.5 ± 3.06	–	6.3 ± 6.25	–	2.5 ± 2.5	+	97.5 ± 2.5
		(1.0 ± 0.10)		(1.0 ± 0.09)		(0.02 ± 0.00)		(0.9 ± 0.07)		(0.9 ± 0.07)		(0.01 ± 0.00)
Cast	–	4.2 ± 4.16	–	2.5 ± 2.50	+	100	–	6.3 ± 6.25	–	5 ± 5.0	+	90 ± 6.12
		(0.9 ± 0.07)		(0.8 ± 0.04)				(0.9 ± 0.05)		(0.8 ± 0.04)		(0.02 ± 0.00)
Gut	–	0	–	0	+	97.5 ± 2.50	–	6.3 ± 6.25	–	2.5 ± 2.50	+	92.5 ± 3.06
		(0.8 ± 0.10)		(0.8 ± 0.08)		(0.05 ± 0.00)		(0.8 ± 0.10)		(0.9 ± 0.08)		(0.01 ± 0.00)

[a] Samples from soil with no plants or with Bt + or Bt − corn were evaluated after 40 days of plant growth.
[b] Samples from soil with ground residues of Bt + or Bt − corn or with no residues were evaluated after 45 days of incubation.
[c] Determined with Lateral Flow Quickstix: – = no toxin detected; + = toxin detected.
[d] Determined with the larvae of the tobacco hornworm (*Manduca sexta*); 8–16 larvae assay^{-1}, expressed as percentage mortality and mean weight (g), of a single larva ± standard error of the mean (in parentheses).

2.8. Statistics

The data are expressed as the means ± the standard errors of the means. The significance among the data was determined by the paired Student's *t*-test using SigmaPlot computer software (Jandel Scientific Corporation).

3. Results

There were no significant differences ($P > 0.5$) in the percent mortality and weight of earthworms after 40 days in soil planted or not planted with Bt or non-Bt corn or after 45 days in soil amended or not amended with biomass of Bt or non-Bt corn (Table 1). The toxin was present in the guts and casts of earthworms in soil planted with Bt corn or amended with biomass from Bt corn, whereas it was absent from the guts and casts of worms in soil planted with non-Bt corn, amended with biomass of non-Bt corn, or not planted or amended (Table 2). The toxin was cleared from the guts within 2–3 days after the worms were placed into fresh soil not amended with biomass of Bt corn or in which Bt corn had not been grown. All samples of soil amended with biomass of Bt corn and from the rhizosphere of Bt plants were positive for the presence of the toxin and were lethal to the larvae of *M. sexta*, after 45 and 40 days, respectively. In contrast, no toxin was detected and there was no mortality in soil amended with biomass of non-Bt corn, in rhizosphere soil of non-Bt corn, or in soil with no plants or not amended (Table 2).

There were no statistically significant differences ($P > 0.5$) in the CFU of culturable bacteria (including actinomycetes) and fungi and in the numbers of protozoa and nematodes between rhizosphere soil of Bt and non-Bt corn; between soil amended with Bt and non-Bt biomass; and between soil with plants or amended with biomass and soil with no plants or not amended, with the exception of bacteria (Table 3). The CFU of culturable bacteria in soil with no plants or with no biomass were significantly higher than in soil with plants or biomass, both Bt and non-Bt. The reasons for these differences are being investigated.

4. Discussion

The results of these studies indicated that the toxin released in root exudates of Bt corn or from the degradation of biomass of Bt corn is not toxic to earthworms, nematodes, protozoa, bacteria, and fungi. Even though the toxin was present in the guts and casts of earthworms grown with Bt corn and in soil amended with biomass of Bt corn, there was no apparent toxicity to earthworms. However, these results should be considered as being preliminary, as only one species of earthworm and only total numbers of culturable bacteria, fungi, protozoa, and nematodes were evaluated. More detailed studies on the composition and diversity of these groups of organisms are necessary (e.g. denaturing gradient and temperature gradient

Table 3
Effect on various groups of organisms of CrylAb toxin released in root exudates and from biomass of corn plants with (Bt +) or without (Bt −) the *crylAb* gene (mean ± standard error of the mean)

Organism	No plants	Root exudates[a]		No biomass	Biomass[b]	
		Bt−[c]	Bt+		Bt−	Bt+
Bacteria ($\times 10^7$)[d]	14.0 ± 1.62	7.6 ± 0.14	5.8 ± 0.23	11.2 ± 0.16	8.1 ± 0.20	6.3 ± 0.10
Actinomycetes ($\times 10^5$)[d]	4.2 ± 0.45	2.3 ± 0.20	2.3 ± 0.36	2.4 ± 0.22	2.8 ± 0.53	2.5 ± 0.34
Fungi ($\times 10^6$)[d]	3.3 ± 0.41	2.7 ± 0.56	1.8 ± 0.21	3.8 ± 0.56	3.6 ± 0.53	3.4 ± 0.72
Protozoa ($\times 10^4$)[e]	1.3 ± 0.05	1.8 ± 0.02	1.4 ± 0.02	1.1 ± 0.07	1.1 ± 0.02	1.0 ± 0.08
Nematodes ($\times 10^3$)[f]	1.0 ± 0.03	1.3 ± 0.04	1.3 ± 0.08	1.4 ± 0.01	1.5 ± 0.05	1.4 ± 0.06

[a] Soil with no plants or with Bt + or Bt − corn was evaluated after 40 days of plant growth.
[b] Soil was evaluated after 45 days of incubation with ground residues of Bt + or Bt − corn or with no residues.
[c] $P > 0.5$ for all comparisons between Bt + and Bt − corn.
[d] Colony-forming units g^{-1} soil, oven-dry equivalent; serial dilution plate method.
[e] Numbers g^{-1} soil, oven-dry equivalent; most-probable number method.
[f] Numbers 50 g^{-1} soil, oven-dry equivalent; Baermann method.

gel electrophoresis, single-strand conformation polymorphism, and the BIOLOG system for bacteria; speciation of fungi; nutritional groups of protozoa and nematodes) to confirm the absence of the effect of the Cry1Ab toxin on biodiversity in soil.

The persistence of the toxin confirmed that the released toxin bound on surface-active particles in soil, which protected the toxin from biodegradation, as has been observed with purified toxin (Tapp et al., 1994; Tapp and Stotzky, 1995a,b, 1998; Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Stotzky, 2000). The persistence of the toxin in soil could improve the control of insect pests, enhance the selection of toxin-resistant target insects, and constitute a hazard to non-target organisms. The potential hazard could be exacerbated, as most Bt corn contains genes that encode toxins rather than the non-toxic crystalline protoxins produced by Bt, and, therefore, it is not necessary for an organism that ingests the toxins to have high gut pH (ca. 10.5) for solubilization of the protoxins and specific proteases to cleave the protoxins into toxins (Höfte and Whiteley, 1989). More studied are obviously necessary to resolve these possibilities.

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