

Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*

C. ZWAHLEN,*† A. HILBECK,† R. HOWALD* and W. NENTWIG*

*Zoological Institute, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland, †Geobotanical Institute, Swiss Federal Institute of Technology Zurich (ETH), Zürichbergstrasse 38, CH-8044 Zurich, Switzerland

Abstract

A 200-day study was carried out to investigate the impact of transgenic *Bacillus thuringiensis* (Bt) corn on immature and adult *Lumbricus terrestris* in the field and in the laboratory. Another objective of this study was to develop test methods that could be used for standard testing of the impact of transgenic plants on different earthworm species in the field and in the laboratory. For this purpose two different experiments were involved, a laboratory experiment with adult *L. terrestris* and a field experiment with immature *L. terrestris*. No lethal effects of transgenic Bt corn on immature and adult earthworms were observed. Immature *L. terrestris* in the field had a very similar growth pattern when fed either (Bt+) or (Bt-) corn litter. No significant differences in relative weights of (Bt+) and (Bt-) corn-fed adult *L. terrestris* were observed during the first 160 days of the laboratory trial, but after 200 days adult *L. terrestris* had a significant weight loss of 18% of their initial weight when fed (Bt+) corn litter compared to a weight gain of 4% of the initial weight of (Bt-) corn-fed earthworms. Further studies are necessary to see whether or not this difference in relative weight was due to the Bt toxin or other factors discussed in the study. Degradation of Cry1Ab toxin in corn residues was significantly slower in the field than at 10 °C in the laboratory. Enzyme-linked immunosorbent assay results indicated that earthworms in both experiments were exposed to the Bt toxin throughout the whole experimental time.

Keywords: *Bacillus thuringiensis*, *Lumbricus terrestris*, risk assessment, soil, testing systems, transgenic plants

Received 13 June 2002; revision received 7 January 2003; accepted 7 January 2003

Introduction

Bacillus thuringiensis (Berliner) (Bt) toxins from transgenic corn (*Zea mays* L.) expressing the δ -endotoxin Cry1Ab enter the soil in three different ways: (1) by pollen during tasselling; (2) by root exudates (Saxena *et al.* 1999; Saxena & Stotzky 2000; Saxena & Stotzky 2001a,c); and (3) by plant residues (Zwahlen *et al.* 2003). As the toxin is released continuously into the soil by root exudates, it can be adsorbed on surface-active particles and thereby retain its insecticidal activity throughout the growing season (Saxena *et al.* 1999; Saxena & Stotzky 2000; Saxena & Stotzky 2001a). However, large amounts of Bt toxin enter the soil via plant residues during harvest of the Bt crop plants. A field study investigating the degradation of Bt

toxin in corn material, remaining in the field after harvest in autumn, indicate that the degradation of the toxin in corn plant residues is slow, with a low amount of toxin remaining until late spring (Zwahlen *et al.* 2003). Based on these studies, long-term exposure of nontarget soil organisms is conceivable (Palm *et al.* 1994; Palm *et al.* 1996; Sims & Ream 1997; Saxena & Stotzky 2001a; Saxena *et al.* 2002). For risk assessment of the long-term ecological consequences, it therefore needs to be established whether the Bt toxin expressed in transgenic corn is a hazard for nontarget soil organisms.

Because Bt genes in transgenic plants originate from soil-living *B. thuringiensis*, it has been proposed that *B. thuringiensis* toxin production might have evolved as a defence against bacterial-feeding invertebrates or potential microbial competitors (Addison 1993). However, to date, the functional role of *B. thuringiensis* in the soil is still unclear. In his review, Addison (1993) presented evidence that several taxa with representatives in the soil fauna are

Correspondence: ‡Present address: C. Zwahlen, Department of Entomology, 219 Hodson Hall, University of Minnesota, St Paul, MN55108, USA. E-mail: claudia.zwahlen@gmx.net

indeed susceptible to *B. thuringiensis*, although most studies have focused on organisms of economic importance.

In temperate regions, earthworms are considered to be the most important group of soil invertebrates due to their efficiency in decomposing plant litter and their influence on soil structure (Dunger & Fiedler 1997). *Lumbricus terrestris* (L.) feeds mainly on leaves with roots as a minor component of the diet (Lee 1985). As *L. terrestris* also ingest some soil, individuals will probably be exposed to Bt toxins from root exudates and plant biomass bound to clay minerals (Saxena *et al.* 2002).

To date, two studies (Ahl Goy *et al.* 1995; Saxena & Stotzky 2001a) considered the effect of transgenic Bt corn on earthworms. In a 14-day toxicity study, Ahl Goy *et al.* (1995) did not find any significant effects of transgenic Bt corn expressing the Cry1Ab toxin on mortality or weight gain of the epigeic species *Eisenia foetida* (Savigny). Similarly, in a laboratory experiment, Saxena & Stotzky (2001a) did not find any significant differences in percentage mortality and weight of earthworms after 40 days in soil planted or not planted either with Bt or non-Bt corn or after 45 days in soil amended or unamended with biomass of Bt or non-Bt corn.

Because in temperate regions the Bt toxin persists in the corn residues for at least 240 days when litter is incorporated into the soil (Zwahlen *et al.* 2003), we wanted to investigate whether or not Bt corn residues may pose a hazard to one of the most common earthworm species in Europe, and perhaps worldwide, when exposed to Bt corn residues during a long period. We used both immature and adult *L. terrestris* to examine the influence of Bt corn on different life stages.

The objectives of our study were to: (1) assess the effects of transgenic Bt corn residues on immature and adult *L. terrestris*; (2) develop test systems for the field and the laboratory that could be used for standard testing of the impact of transgenic Bt plants on different earthworm species; and (3) compare the degradation of the Bt toxin in corn residues in the field and the in laboratory.

Materials and methods

Plants

Two corn hybrids were used in the experiments. One was genetically modified corn from Syngenta (N4640Bt, transformation event Bt11) (referred to as Bt+) containing a truncated, synthetic version of a gene from *B. thuringiensis* ssp. *kurstaki* coding for the expression of the insecticidal *B. thuringiensis*- δ -endotoxin Cry1Ab. The other corn hybrid was the near isoline of the previously described one, which was not genetically modified (N4640) (referred to as Bt- or control). The plants were cultivated in plastic pots in a plant-growth chamber at an average temperature of 23.3 °C

(20 °C for 8 h in dark and 25 °C for 16 h in light). Three weeks after pollen was shed, leaves were cut into approximately 2 × 2 cm² pieces and were dried at 40 °C for 72 h. The plant material was stored frozen at -20 °C until it was used for the experiments.

Soil preparation

The soil used for both experiments was a Cambisol loam (50% sand, 33% silt, 17% clay, pH 5.7) which was collected from the field where the field investigations were carried out. The soil was sieved (2 mm mesh size), homogenized, brought to a water content of 18% and was stored in covered plastic pots at 4 °C until it was used for both, the laboratory and field experiments.

Lumbricus terrestris

Sampling. Locally adapted juvenile and adult *Lumbricus terrestris* (Oligochaeta: Lumbricidae) were collected from the soil of an apple orchard near the field where the field trial was performed. In approximately three 10-min intervals 10 litres (i.e. a total of 30 L) of 0.33% (v/v) mustard flour suspension (Pakoba AG, Stettlen, Switzerland) were poured into a square rack (0.25 m²) to extract the earthworms from the soil. The irritant substance mustard flour brings earthworms to the soil surface, where they can be collected. This method has the advantage that mustard flour is less toxic to earthworms compared to formalin, an alternatively used extraction solution, which often kills the earthworms (Gunn 1992). Immediately after collection, the earthworms were washed thoroughly with water to remove the mustard flour suspension. The earthworms were kept in a climate-controlled chamber (10 °C, 24 h darkness) in the same soil used for the experiments until the beginning of the experiment. They were fed with dried conventional field-grown corn until the initiation of the experiment.

Biology. *L. terrestris* is one of the most frequent species in Switzerland and is ubiquitous in agricultural soils (BUWAL 1997). Species of the genera *Lumbricus* are native in Eurasia and North America and have been transported throughout the whole world by humans (Lee 1985; Edwards & Bohlen 1996). The lifespan of *L. terrestris* is approximately 5–9 years in culture (Satchell 1967; Lakhani & Satchell 1970; Edwards & Bohlen 1996) but will probably be somewhat less in the field. Sexual reproductivity is usually reached within 1 year (Evans & Guild 1948; Wilcke 1952; Satchell 1967), but the duration of the prereproductive phase is strongly influenced by environmental factors (Lee 1985). Adult and immature earthworms can be distinguished by the development of a clitellum when reaching sexual reproductivity. *L. terrestris* grow rapidly for approximately

3 years, with short seasonal pauses in midsummer and midwinter, and reach an average weight of approximately 9.5–11 g in culture and 5–6.25 g in field populations (Satchell 1967; Lakhani & Satchell 1970). After 3 years, the average weight of the earthworms begins to decrease, possibly because of a weight decrease shortly before death. Often their weight does not change greatly for the next 4 years, although not many earthworms survive 7 years in the field. Based on data and conclusions by Lakhani & Satchell (1970) we suggest that most of the adult *L. terrestris* used in our experiments were still in their growth phase (weight data see below).

Experimental design

Two different experiments were carried out: a laboratory trial with 120 adult *L. terrestris* and a field trial with 140 immature *L. terrestris*. The methods used were according to Helling (1997) and were modified for our experiments.

Mean weight (\pm standard error SE) of adult *L. terrestris* was 3.38 ± 0.08 g (range: 1.95–4.96 g) for the (Bt $-$) group and 3.36 ± 0.08 g for the (Bt $+$) group (range: 1.92–4.68 g). Mean weight (\pm SE) of immature *L. terrestris* was 1.40 ± 0.03 g (range: 0.97–1.98 g) for the (Bt $-$) group and 1.41 ± 0.03 g (range: 0.98–1.98 g) for the (Bt $+$) group. The weight variability of earthworms was distributed equally over the different laboratory and field sample dates in the experiment. Moreover, weight development was recorded individually in the experiments to account for the variation in individual weight.

Laboratory experiment with adult *L. terrestris*. Glass tubes (50 cm height, 2.6 cm inner diameter) (Tewis Laborbedarf AG, Bern, Switzerland) were used for the laboratory trial (Fig. 1). They were filled with 253 g soil that was compressed to get a soil height of approximately 35 cm. Adult *L. terrestris* were placed individually into the tubes and were allowed to burrow into the soil before the corn leaf material (either Bt $+$ or Bt $-$) was put onto the surface of the soil. The top and bottom of the glass tubes were sealed with plastic caps (2.6 cm diameter) with a hole (1.1 cm diameter) that was covered with fine mesh netting (1 mm mesh size) to allow for air circulation. The experiment was carried out in a climate-controlled chamber at 10 °C and darkness. The tubes were put randomly into a large plastic container, which was filled (8 cm height) with the same soil used in the experiments. The soil in the glass tubes and the container was sprayed every 2 weeks with water to keep the soil moist. During the following 200 days from mid-November 2000 to early June 2001, in intervals of 40 days, 12 glass tubes per treatment were removed. The entire amount of corn leaf material necessary for feeding the earthworms for the entire trial was added at the beginning of the experiment. The added leaf material ranged from 0.29 to 1.45 g per cage depending on the earthworm assigned

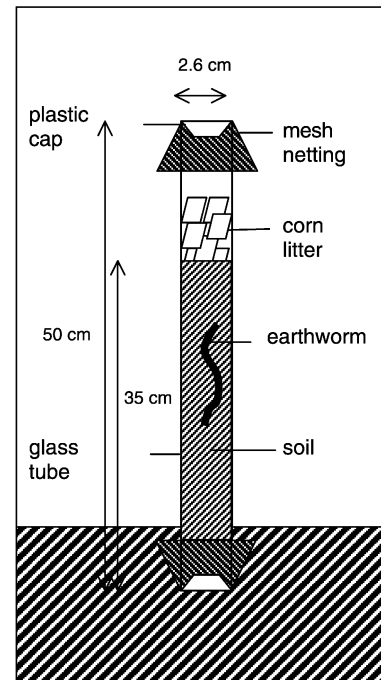


Fig. 1 Glass tubes in the laboratory experiment with adult *L. terrestris*. Tubes are filled with soil (dark upward diagonal) and corn litter (Bt $+$ /Bt $-$) and are closed with caps (white) and mesh netting (dark downward diagonal).

experiment duration (i.e. 0.65 g for earthworms sampled after 40 days, 1.3 g for 80 days, 1.95 g for 120 days, 2.6 g for 160 days 3.25 g for 200 days).

Field experiment with juvenile *L. terrestris*. The field experiment with juvenile *L. terrestris* was carried out in a field near Bern, Switzerland. In this field, cob corn had been grown until late autumn, and the experiment was initiated at the beginning of December 2000, shortly after plowing and sowing of oat (*Avena sativa* L.). Tubular soil cages made of curtain cloth (1 mm mesh size) (Loeb AG, Bern, Switzerland) were filled with 453 g soil to a height of approximately 60 cm (Fig. 2) and were placed into 60 cm deep column-shaped boreholes in the field. Nine soil cages were evenly located along one field row of 8 m length (distance between each location was 1 m). This was repeated 15 times, i.e. 15 field rows (distance between each row was also 1 m). The last row had five soil cages (resulting in 140 burrows within a field area of 8×15 m²). Juvenile *L. terrestris* were placed individually into the soil cages and the corn material (either Bt $+$ or Bt $-$) was put on the soil surface inside the soil cages before they were subsequently tied up closed with a piece of string. During the following 200 days from early December 2000 to late June 2001, in intervals of 40 days, 14 soil cages per treatment were randomly removed from the field. The entire amount of corn leaf material necessary for feeding the earthworms

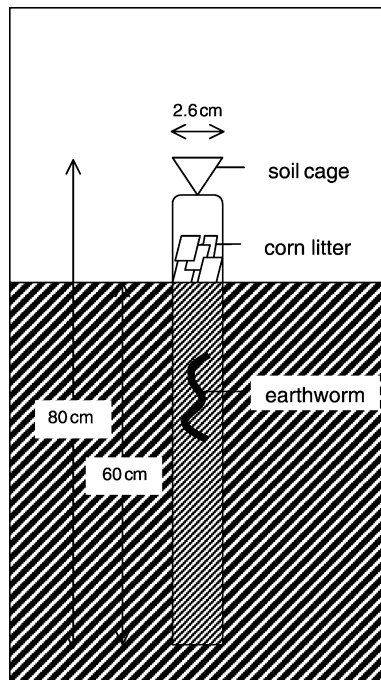


Fig. 2 Soil cages in the field experiment with immature *L. terrestris*. Soil cages are filled with soil (dark upward diagonal) and corn litter (Bt+/Bt-) and are burrowed 60 cm into the soil (wide upward diagonal).

for the entire trial was added at the beginning of the experiment. The added leaf material ranged from 0.29 to 1.45 g per cage depending on the earthworm assigned experiment duration (i.e. 0.29 g for earthworms sampled after 40 days, 0.58 g for 80 days, 0.87 g for 120 days, 1.16 g for 160 days, 1.45 g for 200 days).

Mortality and individual weight of adult and immature earthworms was recorded. Percentage of individual weight gain or loss, respectively, from the initial weight was calculated. Weight data from one immature individual that reached the adult stage during the experiment was excluded. The remaining corn material in the glass tubes (laboratory) and soil cages (field) was frozen immediately at -20°C until it was analysed by enzyme-linked immunosorbent assay (ELISA) and used in the herbivore bioassays.

Data analysis of mortality. Mortality of the two treatments (Bt+/Bt-) of each sample date and total mortality were analysed using Fisher's exact test, which accounts for the binomial probability distribution of the mortality data (GraphPad Software Inc. 2000).

Data analysis of weight. Individual weight as percentage weight gain/loss of the initial weight, of the two treatments (Bt+/Bt-) was compared for each sample date using the Mann-Whitney *U*-test (GraphPad Software Inc. 2000).

All observations were independent, i.e. each individual accounts for one data point.

ELISA of plant material

B. thuringiensis toxin was quantified by ELISA as described by Gugerli (1979, 1986) and Zwaalen *et al.* (2003). At each sample date, from the initial corn material, 10 leaves (each from a different sample) per treatment were analysed for the presence of the Cry1Ab toxin. Thawed leaf pieces were cut into two parts and weighed. One part was used for the ELISA analyses, the other was dried at 40°C to determine the dry weight of the recovered plant residues. The test material was placed into universal bags (Bioreba AG) and homogenized with a hand model homogenizer (Bioreba AG) in 5 mL extraction buffer to extract the Bt toxin. Homogenized samples were put into 14 mL tubes (Sarstedt AG) and were centrifuged with a Universal 30 RF centrifuge (Hettich AG) at 5000 r.p.m. for 5 min. The supernatants were used for the analyses.

To determine the calibration curve, reference samples of purified Cry1Ab toxin were suspended in pooled extracts of control leaves at concentration of 1000, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.01 ng Cry1Ab toxin/mL. All samples were prepared in duplicate. Microtitre immunoassay plates Immunolon®4 (Dynatech Laboratories Inc.) were analysed with a MRX microplate reader operated with the Revel software package, version G 3.2. (Dynex Technologies Inc.). Optical density (OD) was measured at 405 nm. Polyclonal coating IgG and alkaline phosphatase-conjugated IgG against the Cry1Ab toxin were produced by Bioreba AG. Diethanolamine and 4-nitrophenylphosphate for the substrate buffer were obtained from Merck SA.

Detection level. The threshold values of detectable toxin were defined as OD-mean plus three times the standard deviation (SD) of the OD of the control leaf samples. Samples were considered as positive when both duplicates were above the threshold and as negative when at least one of the duplicate samples was negative. Detection levels of the Cry1Ab toxin were at or below 0.4 ppb Cry1Ab toxin.

Quantitative analysis. The means of the duplicates were used for the quantitative analyses. Data were log-transformed before a linear regression was carried out to calculate the concentrations of the Cry1Ab toxin in the samples. Toxin concentrations of samples from the field and the laboratory experiment from the same sample date were compared using the Mann-Whitney *U*-test (GraphPad Software Inc. 2000).

Temperature and rainfall measurement

Rainfall and temperature in a soil depth of 20 cm was measured at a gauging station in Bern, Switzerland (data

provided by MeteoSchweiz, Zurich, Switzerland), which was about 7 km away from our field site.

Herbivore bioassays

Bioassays with highly susceptible neonate larvae *Ostrinia nubilalis* (Hübner) (eggs obtained from French Agricultural Research Inc.) (Koziel *et al.* 1993) were carried out to determine the insecticidal activity of the Bt toxin in the remaining corn plant residues. Approximately 50 mg of the remaining sample material was mixed with 250 µL extraction buffer (EnviroLogix) with a mortar and pestle before it was mixed with approximately 300 mg meridic diet. The diet was fed to 10 larvae that were placed into one vial. This was repeated 10 times (including initial corn material) with corn material from 10 different glass tubes or soil cages, respectively, per treatment and sample date, resulting in a total of 2200 larvae examined. The vials were closed with a plastic lid. The larvae were kept in a climate-controlled chamber at 25 °C (16 h light and 8 h dark). After 5 days, mortality and weight of surviving larvae was recorded (except mortality and weight of larvae fed with corn from the laboratory trial after 40 days was recorded after 6 days).

Data analysis

Mean mortality and average individual weight of the two treatments for each experiment and sample date were compared using the Mann–Whitney *U*-test (GraphPad Software Inc. 2000).

Results

Laboratory experiment with adult *L. terrestris*

Mortality. No significant differences in mortality between the (Bt+) and the (Bt–) treatments were observed (Table 1).

Table 1 Number of dead (and total number) adult and immature *L. terrestris* fed either with (Bt+) or (Bt–) corn after 40, 80, 120, 160 and 200 days. Differences between (Bt+) and (Bt–) treatments were not statistically significant ($P > 0.05$, Fisher's exact test)

After	Adult <i>L. terrestris</i>			Immature <i>L. terrestris</i>		
	Bt+	Bt–	<i>P</i>	Bt+	Bt–	<i>P</i>
40 days	0 (12)	0 (12)	> 0.05	0 (13)	0 (14)	> 0.05
80 days	0 (12)	1 (12)	> 0.05	0 (14)	0 (14)	> 0.05
120 days	0 (12)	1 (12)	> 0.05	0 (13)	0 (14)	> 0.05
160 days	0 (12)	0 (12)	> 0.05	1 (14)	0 (13)	> 0.05
200 days	0 (11)	0 (12)	> 0.05	1 (14)	1 (14)	> 0.05
Total mortality	0 (59)	2 (60)	> 0.05	2 (68)	1 (69)	> 0.05

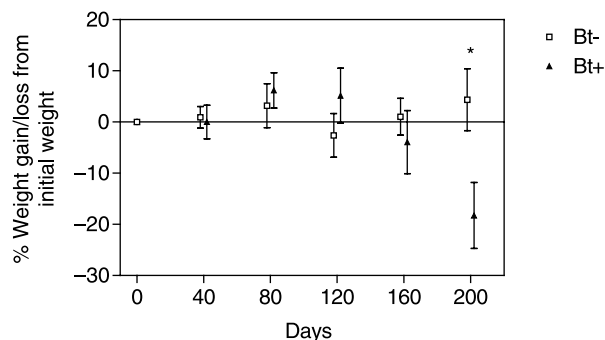


Fig. 3 Mean (\pm SE) weight gain, respectively, weight loss (in percentage) compared to the initial weight of the adult (Bt–) corn-fed (white rectangles) and (Bt+) corn-fed (black triangles) *L. terrestris* in the laboratory trial after 40, 80, 120, 160 and 200 days. Columns with * represent significant differences at $P = 0.05$ (Mann–Whitney *U*-test).

Total mortality of earthworms was low with 3.3% when fed (Bt–) corn and 0% when fed with (Bt+) corn.

Development. During the first 160 days of the experiment, mean weight of adult *L. terrestris* neither changed much nor differed significantly between (Bt+) and (Bt–) groups (Fig. 3). In contrast to the first 160 days, at the end of the experiment after 200 days (Bt+) corn-fed *L. terrestris* had lost $18.3 \pm 6.5\%$ [mean \pm standard error (SE)] of their initial weight, whereas (Bt–) corn-fed *L. terrestris* had gained $4.4 \pm 6.1\%$ weight compared to their initial weight. This difference was statistically significant ($U = 30.0, P = 0.0289$).

Field experiment with immature *L. terrestris*

Mortality. No significant differences in mortality between earthworms in the (Bt+) and (Bt–) treatments were observed (Table 1). Total mortality of *L. terrestris* was 1.4% when fed (Bt–) corn litter and 2.9%, when fed (Bt+) corn litter.

Development. The mean relative weight of (Bt+) and (Bt–) corn-fed earthworms during the whole experiment was similar and there were no statistically significant differences between the two treatments (Fig. 4). Forty days after the beginning of the experiment, mean (\pm SE) weight of (Bt–) corn-fed *L. terrestris* was slightly higher than initially ($+1.2 \pm 2.3\%$ of the initial weight), whereas (Bt+) corn-fed earthworms had lost $4.1 \pm 2.4\%$ of their initial weight. During the next 80 days, mean relative individual weight of earthworms developed fairly similar. In both groups (Bt+ and Bt–), weight gain increased and reached a maximum 120 days after the beginning of the experiment. After that, mean relative weight decreased compared to the maximum weight after 120 days but was still higher [$10.4 \pm 4.0\%$ in the (Bt+) treatment and $6.2 \pm 2.9\%$ in the (Bt–) treatment] at the end of the experiment than initially.

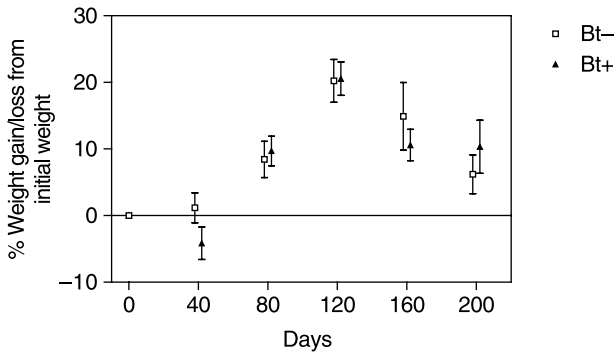


Fig. 4 Mean (\pm SE) weight gain, respectively, weight loss (in percentage) compared to the initial weight of the immature (Bt-) corn-fed (white rectangles) and (Bt+) corn-fed (black triangles) *L. terrestris* in the field trial after 40, 80, 120, 160 and 200 days. No significant differences were observed ($P > 0.05$, Mann-Whitney *U*-test).

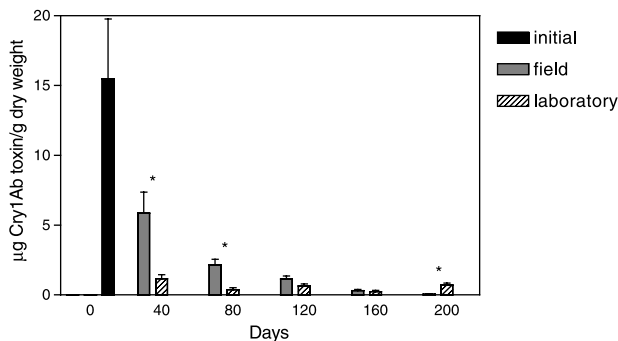


Fig. 5 Mean (\pm SE) of the Bt toxin concentration (μg Cry1Ab toxin/g dry weight) in leaves initially (black bar), from the field trial (grey bars) and from the laboratory trial (hatched bars). The total number of samples analysed per treatment was 110. Columns with * represent significant differences at $P = 0.05$ (Mann-Whitney *U*-test).

Degradation of the Cry1Ab toxin

Initial Cry1Ab toxin concentration (mean \pm SE) in the transgenic Bt leaves was $15.5 \pm 4.3 \mu\text{g/g}$ dry weight (dw) (Fig. 5).

Laboratory experiment with adult *L. terrestris*. In the laboratory trial, the Cry1Ab toxin concentration in corn leaves decreased drastically during the first 40 days to $1.2 \pm 0.3 \mu\text{g/g}$ dry weight, i.e. about 8% of the initial toxin amount (Fig. 5). During the remaining 160 days of the experiment, mean toxin concentrations remained at a low level ranging between 0.2 and $0.7 \mu\text{g}$ toxin/g dry weight (80 days: $0.4 \pm 0.1 \mu\text{g/g}$ dw (2% of initial concentration); 120 days: $0.6 \pm 0.1 \mu\text{g/g}$ dw (4%); 160 days: $0.2 \pm 0.1 \mu\text{g/g}$ dw (2%); 200 days: $0.7 \pm 0.1 \mu\text{g/g}$ dw (5%)). These results show that adult earthworms were exposed continuously to low Cry1Ab concentrations

during the whole experiment. However, in some leaves the Bt toxin was not detectable anymore (in four leaves after 80 days; in three leaves after 120 and 200 days).

Field experiment with immature *L. terrestris*. In the field trial, the Cry1Ab toxin concentration in corn leaves decreased during the first 40 days, from December to mid-January, to $5.9 \pm 1.5 \mu\text{g}$ toxin/g dry weight, i.e. 38% of the initial toxin amount (Fig. 5). Eighty days after the beginning of the trial, at the end of February, the concentration of the Cry1Ab toxin had decreased to $2.2 \pm 0.4 \mu\text{g/g}$ dry weight, i.e. 14% of the initial toxin amount. After 120 days, in early March, another 7.0% of the initial amount had degraded, and concentration in the Bt corn leaves was $1.1 \pm 0.2 \mu\text{g/g}$ dry weight. By mid-May, after 160 days, the Cry1Ab toxin amount in leaves had dropped to 2% of the initial toxin concentration ($0.3 \pm 0.1 \mu\text{g/g}$ dry weight). At the end of the trial after 200 days, $0.05 \pm 0.02 \mu\text{g}$ Cry1Ab toxin/g dry weight was left in the residues. These data indicate that immature earthworms were exposed to the Cry1Ab toxin throughout the whole experiment from December until June, and although the amount of toxin was low at the end of the trial, it was still above the detection limit in eight of 10 leaf samples analysed.

Comparison of Cry1Ab toxin concentration in laboratory and field. When comparing field and laboratory trials, degradation curves of the Cry1Ab toxin in corn leaves were significantly different during the first 80 days and at the end of the trial after 200 days (Fig. 5). Toxin concentrations in transgenic corn leaves from the field degraded significantly slower than in leaves from the laboratory during the first 80 days (40 days: $U = 4.0$, $P = 0.0001$; 80 days: $U = 2.0$, $P = 0.001$). Cry1Ab toxin concentrations were roughly five to six times higher in the field-degrading leaves than in the laboratory-degrading leaves during this time. Although after 120 days Cry1Ab concentration in corn residues from the field was still almost twice as high as in the laboratory, this difference was not statistically significant. After 160 days, toxin degradation in the field and the laboratory had reached similar levels. Interestingly, at the end of the experiment after 200 days, the concentration in transgenic Bt corn leaf samples from the laboratory trial was significantly higher than in those from the field trial ($U = 0.0$, $P = 0.0003$). Apparently, degradation in the field continued while it appeared to stagnate in the laboratory.

Temperature

Average soil temperature (\pm SE) from December 2000 to June 2001 was $8.5 \pm 0.4 \text{ }^\circ\text{C}$ (Fig. 6). Maximum and minimum temperatures during this time were 20.4 and $1.5 \text{ }^\circ\text{C}$, respectively. Compared to the laboratory experiment with

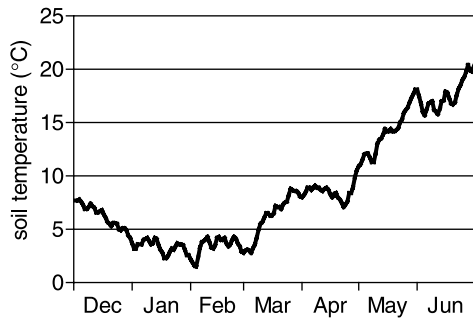


Fig. 6 Average daily soil temperature (°C) at 20 cm depth during the field experiment from early December 2000 to late June 2001.

constant 10 °C, mean daily temperature in the field was always below this temperature until late April 2001 and always above this temperature during May and June until the end of the field experiment.

Herbivore bioassays

Mean mortality (\pm SE) of *O. nubilalis* larvae fed initial corn material (Bt+) was $100 \pm 0.0\%$, whereas mortality of larvae fed (Bt-) diet was significantly lower ($3.6 \pm 1.8\%$) (Table 2).

Laboratory experiment. The mean mortality of larvae fed the (Bt+) diet was significantly higher than in these with the (Bt-) diet until 40 days after the beginning of the laboratory experiment (Table 2). After that, mean mortality continued to be lower in the (Bt-) treatment than in the (Bt+) treatment, except after 160 days when mortality in both treatments was similar. However, the differences were no longer statistically significant. In contrast, weight of larvae

in the (Bt+) treatment was significantly lower than in the (Bt-) treatment until 120 days after the beginning of the laboratory experiment, but no statistically significant differences between the two treatments were observed after 160 and 200 days (Table 2).

Field experiment. The mean mortality of (Bt+) diet-fed larvae was significantly higher than of larvae in the (Bt-) treatment until 80 days after the beginning of the field experiment (Table 2). After that, a statistically significant lethal effect was no longer detected although the mean mortality in the (Bt-) treatment was only half of that in the (Bt+) treatment until 160 days. Also, no consistent sublethal effect was observed after 80 days. After 200 days, *O. nubilalis* larvae raised on (Bt-) diet were significantly heavier than larvae raised on (Bt+) diet (Table 2).

Discussion

Overall mortality in both experiments, the field experiment with immature *L. terrestris* and the laboratory experiment with adult *L. terrestris*, was low (0–3.3%) and did not differ significantly between (Bt+) and (Bt-) corn-fed earthworms. These results are in agreement with the results of Saxena & Stotzky (2001a). Saxena & Stotzky (2001a) did not find any significant differences in mortality and weight of earthworms exposed to root exudates or plant biomass of transgenic Bt corn or the near isogenic control line after 40 or 45 days, respectively. These results indicate that older immature *L. terrestris* and adult *L. terrestris* are not affected lethally by the ingestion of Bt corn litter after 45 and 200 days, either in the field or in the laboratory. However, because earthworms are resistant

Table 2 Mean (\pm SE) mortality (%) and weight (mg/individual) of *O. nubilalis* larvae when either fed (Bt+) or (Bt-) leaf residues from different sample dates (leaf residues mixed with meridic diet)

	Mortality (%)				Weight (mg/individual)			
	Bt+	Bt-	P	U	Bt+	Bt-	P	U
Initially	100.0 \pm 0.0	3.6 \pm 1.8	< 0.0001	0.0	—	0.25 \pm 0.08	—	—
Field experiment								
40 days	92.0 \pm 8.0	9.3 \pm 3.7	< 0.0001	2.0	—	0.15 \pm 0.02	—	—
80 days	50.7 \pm 14.2	2.0 \pm 2.0	< 0.01	16.5	0.29 \pm 0.09	0.30 \pm 0.12	> 0.05	24.0
120 days	29.9 \pm 12.0	12.2 \pm 2.9	> 0.05	38.0	0.26 \pm 0.04	0.16 \pm 0.02	> 0.05	18.5
160 days	53.7 \pm 12.1	25.1 \pm 7.4	> 0.05	29.5	0.20 \pm 0.04	0.28 \pm 0.05	> 0.05	24.0
200 days	0.0 \pm 0.0	2.1 \pm 1.4	> 0.05	36.0	0.15 \pm 0.02	0.26 \pm 0.04	< 0.01	11.0
Laboratory experiment								
40 days	14.2 \pm 3.8	3.1 \pm 2.2	< 0.05	23.0	0.64 \pm 0.06	1.62 \pm 0.26	< 0.01	5.0
80 days	9.1 \pm 3.2	1.1 \pm 1.1	> 0.05	25.5	0.13 \pm 0.04	0.16 \pm 0.02	< 0.05	21.0
120 days	35.4 \pm 14.4	5.9 \pm 2.6	> 0.05	33.0	0.17 \pm 0.02	0.26 \pm 0.03	< 0.05	14.5
160 days	4.2 \pm 1.7	4.5 \pm 2.6	> 0.05	45.0	0.55 \pm 0.03	0.46 \pm 0.03	> 0.05	24.0
200 days	4.4 \pm 2.0	0.0 \pm 0.0	> 0.05	36.0	0.41 \pm 0.05	0.54 \pm 0.06	> 0.05	25.5

animals, records of their survival alone may not give a true measure of the toxicity of pesticides or other toxins (Atlavinyté *et al.* 1982).

To assess whether earthworms were affected sublethally by the ingestion of Bt corn, we compared the relative weight of *L. terrestris* on both treatments.

In the field, immature *L. terrestris* showed a similar growth pattern in both treatments (Bt+/Bt-), with few statistically insignificant differences between the two groups. Forty days after the beginning of the experiment, in mid-January, relative weight was more or less unchanged. This was due probably to a stagnation in growth during midwinter as described by Satchell (1967) and Lakhani & Satchell (1970) or to the raw corn plant material first undergoing a degradation process, as decomposition reportedly improves food acceptability (Edwards & Bohlen 1996). From mid-January to early April, i.e. 120 days after the beginning of the experiment, the relative weight of *L. terrestris* increased. After that, relative weight decreased somewhat until the end of the experiment in late June but was still higher at the end of the experiment than initially.

In the laboratory, the mean relative weight of adult *L. terrestris* changed little during the whole experiment for the (Bt-) and (Bt+) corn litter-fed earthworms and weights did not differ significantly until and including 160 days after the beginning of the experiment. However, for the last sample date, i.e. 200 days after the beginning of the experiment, mean weight loss of approximately 18% of earthworms that had fed (Bt+) corn litter was detected. One explanation for the basically unchanged weight of the adult earthworms in the (Bt-) treatment and until 160 days in the (Bt+) treatment could be that several adult earthworms produced cocoons during the course of the experiment, which possibly led to a lower growth rate of these earthworms as energy was invested into reproduction. Another reason for the unchanged weights could be the temperature used in the laboratory. Although *L. terrestris* prefer a temperature of about 10 °C as used in our laboratory experiment, and show the highest activity, this temperature is not necessarily the temperature at which they grow fastest (Edwards & Bohlen 1996). Compared to the temperature in the field, 10 °C was above the average soil temperature in the field until the end of April and below it until the end of the experiment in June.

However, the relative weight of (Bt+) corn-fed earthworms dropped significantly below that of (Bt-) corn-fed earthworms after 200 days. Further experiments, lasting longer than 200 days, are necessary to confirm this effect. As well as a potentially adverse effect of the Bt toxin, another reason could also be unanticipated changes in the plant quality due to the genetic transformation. Donegan *et al.* (1995), for instance, reported transient increases in total bacterial species composition within two of three transgenic Bt cotton lines producing the Cry1Ac toxin

they examined. These authors suggest that the genetic manipulation or the tissue culturing of the plants may have resulted in a change in plant characteristics. As microorganisms are essential for earthworm nutrition (Shipitalo *et al.* 1988), a change in plant compound composition that leads to a different colonization of leaves by microorganisms might have an effect on the nutritional quality of the plant material ingested by earthworms. Saxena & Stotzky (2001b) reported significantly higher lignin contents (33–97% higher) in all the tested transgenic Bt corn varieties expressing the Cry1Ab toxin than in their near isogenic control lines. For example, lignin content in the Bt corn variety used in our experiments was 61% (field) and 97% (plant growth room) higher than in the near isogenic control line (Saxena & Stotzky 2001b). As lignin is relatively indigestible, a higher lignin content could lead to a delayed litter degradation and decomposition by microbes or to a lower nutritional quality for earthworms in the long term (Escher *et al.* 2000; Saxena & Stotzky 2001b).

Several reports have investigated the impact of microbial *B. thuringiensis* formulations on earthworms. For example, Smirnof & Heimpel (1961) reported 100% mortality of *L. terrestris* when exposed to different concentrations of the Bt preparation Thuricide® (Bioferm Corporation) containing *B. thuringiensis* var. *kurstaki* spores. Interestingly, earthworm mortality of 100% occurred during a relatively long time period of 2 months when exposed to the lowest dose. Histopathological studies showed that the bacteria had invaded all tissues in the body of their host where they could sporulate and form crystals. Bacteria from the Bt preparation possibly also produced β -exotoxins, which presumably contribute to the overall toxicity of a Bt strain (Lereclus *et al.* 1993). Moreover, Addison (1993) wrote in his review that the formulation used by Smirnof & Heimpel (1961) contained diatomaceous earth as the carrier [a geological deposit made up of the fossilized skeletons and tests of siliceous marine and fresh water organisms, particularly diatoms and other algae (Hill 1986)]. This substance would have an abrasive effect on the gut lining of the earthworms and might therefore make it possible for the bacteria to invade the coelom. In contrast, Benz & Altwegg (1975) did not find any significant effects on earthworm density in plots treated either with Dipel® or Bactospeine®, two preparations containing *B. thuringiensis* spores.

However, in a 14-day toxicity study with transgenic Bt corn expressing the Cry1Ab toxin, Ahl Goy *et al.* (1995) did not find any significant effects on mortality or weight gain of *Eisenia foetida*. Ahl Goy *et al.* (1995) used a corn leaf protein powder mixed into a soil substrate (Ecostrat 2000). As *E. foetida* does not feed through soil, it is unclear whether or not the earthworms ingested any meaningful amounts of the Bt protein equivalent to those ingested when feeding on degrading plant material as they do in the field (Ecostrat

2000). Moreover, because *E. foetida* is an epigeic species which prefers highly enriched organic environments and does not usually survive in agricultural soils with low organic matter (Topp 1981; Edwards & Bohlen 1996), it is unlikely to occur in large populations in fields planted with transgenic corn. *E. foetida* is a standard test species used in acute toxicity trials with pesticides. Conclusions regarding the impact on earthworms in agroecosystems are limited. Therefore, we recommend working with earthworm species that are common in areas where transgenic plants grow or will be grown, such as *L. terrestris*, *L. rubellus* (Hoffmeister), *Nicodrilus longus* (Ude), *N. caliginosus* (Savigny) or *N. nocturnus* (Evans).

ELISA results demonstrated that *L. terrestris* in both experiments were exposed to the Bt toxin in the decaying plant material until the end of the trials after 200 days, although toxin concentrations at the end of the experiments were low. Herbivore bioassays did not always provide conclusive results whether or not the Bt toxin was in its insecticidal active form until the end of the experiments. In the bioassays for the field experiment, there were clear lethal effects of (Bt+) diet-fed *O. nubilalis* until and including 80 days after initiation of the field experiment. After that, mortality was still higher in the (Bt+) treatments, except for the last sample after 200 days. However, this was no longer statistically significant, which could be the result of a lack of statistical power for smaller differences and larger variability. Due to high mortality rates at the beginning and 40 days after the beginning of the experiments weights could not be recorded until 80 days after initiation of the experiment, and subsequent data were inconclusive. We suggest that affected larvae died while those that were not lethally affected developed similarly to those in the control, hence mean weight data were confounded by mortality. In the laboratory trial mean mortality was always higher in the (Bt+) treatments than in the (Bt-) treatments, with the exception for the sample after 160 days when mean Cry1Ab concentration was lowest; but the difference in mortality between (Bt+) and (Bt-) diet-fed larvae was statistically significant only 40 days after initiation of the experiment. Until 120 days after the beginning of the experiment, *O. nubilalis* larvae were affected sublethally by the ingestion of (Bt+) corn-containing diet.

In general, our data show that *O. nubilalis* larvae were affected lethally and/or sublethally by the Bt toxin when the Bt toxin concentrations in leaf residues were above 1.2 µg/g dry weight in the plant material. When toxin concentrations were below 1.2 µg/g dry weight, mortality data continued to be higher in the (Bt+) treatment but mortality rates either dropped or variability increased, and therefore in most cases they were not statistically significant. Weight data did not provide clear results in the field experiment and could have been confounded by mortality. These data suggest that below a certain Bt toxin

concentration, it was no longer possible to determine whether or not the detected Bt toxin was still in its insecticidal state. However, data by Saxena *et al.* (2002) studying the leaching of Cry1Ab toxin from the same transgenic Bt corn line as used in our trials showed that the Bt toxin was still in its insecticidal form after 350 days. In their studies with *L. terrestris*, Saxena & Stotzky (2001a) also showed clear evidence of the Bt toxin in the cast of the earthworms. Results from our field experiment also confirmed previous results of a slow degradation of Bt toxin in transgenic corn litter that was incorporated into the soil (Zwahlen *et al.* 2003).

We also compared the Cry1Ab toxin degradation data from the field with those obtained in the laboratory trial to evaluate to what degree laboratory trials realistically mimic a field situation. Our results showed that the degradation of the Bt toxin in leaves in the laboratory was different from that in the field. This was due probably to the differences in temperature. Temperature in the laboratory trial was higher than in the field trial during the first 140 days, thus probably leading to a faster degradation of the Cry1Ab toxin in the laboratory than in the field. After that, the temperature in the field was higher than in the laboratory and the amount of toxin left in the plant residues from the field was significantly lower than from the laboratory on the last sample date. These differences need to be kept in mind when interpreting laboratory experiments.

In conclusion, our results show that earthworms are exposed to the Bt toxin for long time periods (see also Zwahlen *et al.* 2003). Although the earthworms were not affected lethally by the exposure to Bt corn in our trials, based on our data we cannot rule out sublethal long-term effects. Extended studies lasting more than 200 days are necessary to assess the long-term impact of transgenic Bt plants on earthworms. Further, we recommend that other life history traits and fitness parameters, such as longevity, development time from hatch to sexual maturity, numbers of cocoons produced, fertility and survival of offspring, should be investigated to study the impact of long-term exposure and ingestions of degrading Bt corn on *L. terrestris* populations. Furthermore, newly hatched earthworms should also be tested because their susceptibility to Bt toxins could be higher, if they are susceptible at all, than the susceptibility of adult and older immature earthworms as used in our experiments.

Acknowledgements

We are grateful to J. Simmen, B. Magun and I. Antognini for technical assistance and K. Jost for providing the field. We would also like to thank H. Wandeler, Bern, Switzerland), Prof Dr J. Zettel, Dr L. Kuhn-Nentwig, Dr J.-P. Airoidi (University of Bern, Switzerland) and two unknown reviewers for helpful discussions and important advice. Many thanks to Dr P. Gugerli (Federal Research Station for Plant Production of Changins (RAC), Nyon,

Switzerland) for helpful discussions and providing the antiserum for the production of the antibodies.

References

- Addison JA (1993) Persistence and nontarget effects of *Bacillus thuringiensis* in soil: a review. *Canadian Journal of Forest Research*, **23**, 2329–2342.
- Ahl Goy P, Warren G, White J, Privalle L, Fearing P (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*, vol. 309, pp. 50–53. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem, Blackwell, Berlin.
- Atlavinyté O, Galvelis A, Dačulytė J, Lugauskas A (1982) Effects of entobacterin on earthworm activity. *Pedobiologia*, **23**, 372–379.
- Benz G, Altwegg A (1975) Safety of *Bacillus thuringiensis* for earthworms. *Journal of Invertebrate Pathology*, **26**, 125–126.
- Bundesamt für Umwelt Wald und Landschaft (BUWAL) (1997) Die Regenwurmfauna von Dauergrünland des Schweizer Mittellandes. *BUWAL Schriftenreihe Umwelt*, **291**, 1–91.
- Donegan KK, Palm CJ, Fieland VJ *et al.* (1995) Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. *Applied Soil Ecology*, **2**, 111–124.
- Dunger W, Fiedler HJ, eds (1997) *Methoden der Bodenbiologie*. Gustav Fischer Verlag, Jena.
- Ecostrat (2000) *Review on Non-Target Organisms and Bt-Plants* [Report]. Greenpeace International, Amsterdam. Available at: <http://www.greenpeace.org/~geneng/reports/gmo/ecostratbt.pdf>
- Edwards CA, Bohlen PJ (1996) *Biology and Ecology of Earthworms*, 3rd edn. Chapman & Hall, London.
- Escher N, Käch B, Nentwig W (2000) Decomposition of transgenic *Bacillus thuringiensis* maize by microorganisms and woodlice *Porcellio scaber* (Crustacea: Isopoda). *Basic and Applied Ecology*, **1**, 161–169.
- Evans AC, Guild WJ (1948) Studies on the relationships between earthworms and soil fertility. IV. On the life cycles of some British Lumbricidae. *Annals of Applied Biology*, **35**, 471–484.
- GraphPad Software Inc. (2000) *GraphPad Prism 3.02*. GraphPad Software Inc., San Diego, USA.
- Gugerli P (1979) Le test immuno-enzymatique (ELISA) et son application pour le diagnostic rapide des viroses de la pomme de terre. *Revue Suisse d'Agriculture*, **11**, 253–260.
- Gugerli P (1986) Potato viruses. In: *Methods of enzymatic analysis* (ed. Bergmeyer HU.), vol. XI, pp. 430–446. VCH-Verlagsgesellschaft, Weinheim.
- Gunn A (1992) The use of mustard to estimate earthworm populations. *Pedobiologia*, **36**, 65–67.
- Helling B (1997) Einfluss der Regenwürmer auf die Stickstoff-Mineralisation und die bodenbiologische Aktivität landwirtschaftlich genutzter Flächen bei verschiedenen N-Düngern. *Agrarökologie, Bern*, **23**, 1–140.
- Hill SB (1986) *Diatomaceous earth: a non toxic pesticide*. Available at: <http://www.eap.mcgill.ca/Publications/eap4.htm>.
- Koziel MG, Beland GL, Bowman C *et al.* (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Biotechnology*, **11**, 194–200.
- Lakhani KH, Satchell JE (1970) Production by *Lumbricus terrestris* L. *Journal of Animal Ecology*, **39**, 473–492.
- Lee KE (1985) *Earthworms. Their ecology and relationships with soils and land use*. Academic Press, Sydney.
- Lereclus D, Delécluse A, Lecadet MM (1993) Diversity of *Bacillus thuringiensis* toxins and genes. In: *Bacillus thuringiensis, an environmental biopesticide: theory and practice* (eds Entwistle PF, Cory S, Bailey MJ, Higgs S), chapter 2, pp. 37–70. Wiley, Chichester.
- Palm CJ, Donegan KK, Harris D, Seidler RJ (1994) Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ -endotoxin from transgenic plants. *Molecular Ecology*, **3**, 145–151.
- Palm CJ, Schaller DL, Donegan KK, Seidler RJ (1996) Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* δ -endotoxin. *Canadian Journal of Microbiology*, **42**, 1258–1262.
- Satchell JE (1967) Lumbricidae. In: *Soil biology* (eds Burgess A, Raw F), pp. 259–322. Academic Press, London.
- Saxena D, Flores S, Stotzky G (1999) Insecticidal toxin in root exudates from Bt corn. *Nature*, **402**, 480.
- Saxena D, Flores S, Stotzky G (2002) Vertical movement in soil of insecticidal Cry1Ab protein from *Bacillus thuringiensis*. *Soil Biology and Biochemistry*, **34**, 111–120.
- Saxena D, Stotzky G (2000) Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn *in vitro* and *in situ*. *FEMS Microbiology Ecology*, **33**, 35–39.
- Saxena D, Stotzky G (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 Biology and Biochemistry*, **33**, 1225–1230.
- Saxena D, Stotzky G (2001b) Bt corn has a higher lignin content than non-Bt corn. *American Journal of Botany*, **88**, 1704–1706.
- Saxena D, Stotzky G (2001c) Bt toxin uptake from soil by plants. *Nature Biotechnology*, **19**, 199.
- Shipitalo MJ, Protz R, Tomlin AD (1988) Effect of diet on the feeding and casting activity of *Lumbricus terrestris* and *L. rubellus* in laboratory culture. *Soil Biology and Biochemistry*, **20**, 233–237.
- Sims SR, Ream JE (1997) Soil inactivation of the *Bacillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: laboratory microcosm and field studies. *Journal of Agricultural and Food Chemistry*, **45**, 1502–1505.
- Smirnov WA, Heimpel AM (1961) Notes on the pathogenicity of *Bacillus thuringiensis* var. *thuringiensis* Berliner for the earthworm, *Lumbricus terrestris* Linnaeus. *Journal of Insect Pathology*, **3**, 403–408.
- Topp W (1981) *Biologie der Bodenorganismen*. Quelle & Meyer, Heidelberg.
- Wilcke DE (1952) Zur Kenntnis der Lumbricidenfauna Deutschlands. *Zoologischer Anzeiger*, **151**, 315–320.
- Zwahlen C, Hilbeck A, Gugerli P, Nentwig W (2003) Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Molecular Ecology*, **3**, 765–775.

This study is part of Claudia Zwahlen's doctoral research under the supervision of Angelika Hilbeck and Wolfgang Nentwig. The PhD project focuses on the degradation of transgenic *Bacillus thuringiensis* (Bt) corn in soil. A. Hilbeck is an agroecologist specialized in plant and insect ecology and biocontrol. R. Howald is an ecologist working with transgenic Bt corn and Bt rape. W. Nentwig is Professor in Community Ecology.
