Fate and Effects in Soil of the Insecticidal Toxins from *Bacillus thuringiensis* in transgenic Plants.

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Abstract

One of the potential problems raised by the large-scale cultivation of transgenic *Bt*-corn, is the amount of insecticidal toxin produced by the crop and released into the soil through the root exudates and the buried biomass. This problem requires a careful evaluation of the mechanisms responsible for the removal of the protein from the soil and of the effects of the toxin on the soil biology.

Insecticidal proteins produced by various subspecies of *Bacillus thuringiensis* bind rapidly and tightly on clays, both pure mined clay minerals and soil clays, and on humic acids extracted from soil. This binding reduces the susceptibility of these proteins to microbial degradation, and the bound proteins retain their biological activity. Both purified proteins and proteins released from the biomass of transgenic *Bt* corn and in root exudates of growing *Bt* corn exhibited binding and persistence in soil. Insecticidal protein was also released in the root exudates of *Bt* potato but not in root exudates *Bt* cotton, canola, and tobacco. The vertical movement of protein, either purified or in root exudates and biomass of *Bt* corn, decreased as the concentration of kaolinite or montmorillonite in the soil increased. The biomass of transgenic *Bt* corn decomposed less in soil than did the biomass of isogenic non-*Bt* corn, possibly because the biomass of *Bt* corn had a significantly higher content of lignin than did the biomass of non-*Bt* corn. The toxins did not appear to have any consistent effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or *in vitro*. The toxins were not taken up from soil by non-*Bt* corn, carrot, radish, or turnip grown in soil in which *Bt* corn had been grown or into which biomass of *Bt* corn had been incorporated.

Riassunto

Uno dei possibili problemi legati alla coltivazione su larga scala del mais Bt-*transgenico è dato dalla quantità di tossina insetticida prodotta dalla pianta e successivamente rilasciata nel suolo, attraverso gli essudati radicali o la biomassa interrata a fine coltura. Questo richiede una valutazione attenta dei meccanismi che portano alla rimozione della tossina dal suolo e degli effetti della tossina sugli equilibri del suolo stesso.*

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Le proteine ad attività insetticida prodotte da varie sottospecie di Bacillus thuringiensis *si legano rapidamente e fortemente alle argille ed agli acidi umici estratti dal suolo. Questo legame riduce la suscettibilità di queste proteine alla degradazione microbica, consentendogli di mantenere la loro attività biologica.*

Sia le proteine purificate, che quelle rilasciate dalla biomassa del mais Bt-*transgenico e negli essudati delle radici dello stesso mais, hanno evidenziato questa capacità di legarsi all'argilla e di persistere nel terreno. La proteina insetticida viene anche rilasciata attraverso gli essudati della radice della patata* Bt-*transgenica, ma non in quelli del cotone, della colza e del tabacco* Bt-*transgenici. Il movimento verticale della proteina, sia essa purificata o presente negli essudati e nella biomassa del* Bt-*mais, diminuisce all'aumentare della concentrazione di caolinite o montmorillonite nel suolo.*

La biomassa del mais Bt-*transgenico si decompone più lentamente nel suolo rispetto a quella del mais non* Bt-*transgenico, probabilmente a causa di un contenuto in lignina significativamente più alto rispetto alla biomassa del mais non* Bt-*transgenico.*

*Le tossine non sembrano avere effetti rilevanti su specifici organismi (lombrichi, nematodi, protozoi, batteri o funghi), sia nel suolo che in vitro. Non e' stato rilevato alcun prelievo o assorbimento di tossine dal suolo da parte di altre piante, quali mais non-*Bt, *carote, ravanelli o rape cresciute su terreno in precedenza coltivato con* Bt-*mais o in cui e' stato incorporata biomassa originata da* Bt-*mais.*

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Abstract

Insecticidal proteins produced by various subspecies (*kurstaki, tenebrionis*, and *israelensis*) of *Bacillus thuringiensis* bound rapidly and tightly on clays, both pure mined clay minerals and soil clays, on humic acids extracted from soil, and on clay-humic acid complexes. Binding reduced the susceptibility of the proteins to microbial degradation. The bound proteins retained their biological activity. Purified proteins and proteins released from biomass of transgenic *Bt* corn and in root exudates of growing *Bt* corn (13 hybrids representing three transformation events) exhibited binding and persistence in soil. Insecticidal protein was also released in the root exudates of *Bt* rice and potato but not in root exudates of *Bt* cotton, canola, and tobacco. The vertical movement of protein, either purified or in root exudates or biomass of *Bt* corn, decreased as the concentration of kaolinite or montmorillonite in the soil increased. The biomass of transgenic *Bt* corn decomposed less in soil than did the biomass of near-isogenic non-*Bt* corn, possibly because the biomass of *Bt* corn had a significantly higher content of lignin than did the biomass of non-*Bt* corn. The biomass of *Bt* canola, cotton, potato, rice, and tobacco also decomposed less than the biomass of near-isogenic non-*Bt* plants. However, the lignin content of these *Bt* plants, which was significantly less than that of *Bt* corn, was not significantly different from that of their near-isogenic non-*Bt* counterparts. The toxins did not have any consistent

effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or *in vitro*. The toxins were not taken up from soil by non-*Bt* corn, carrot, radish, or turnip grown in soil in which *Bt* corn had been grown or into which biomass of *Bt* corn had been incorporated.

1. Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive, aerobic, spore-forming, rod-shaped bacterium that produces a parasporal, proteinaceous, crystalline inclusion during sporulation. This inclusion, which may contain more than one type of insecticidal crystal protein (ICP), is solubilized and hydrolyzed in the midgut of larvae of susceptible insects when ingested, releasing polypeptide toxins that eventually cause death of the larvae (*see* Höfte and Whiteley, 1989; Schnepf *et al.*,1998). More than 3000 isolates of *Bt* from 50 countries have been collected (*see* Feitelson *et al.*, 1992; Crickmore *et al.*, 1998; Schnepf *et al.*, 1998). The ICPs have been classified on the bases of their structure, encoding genes, and host range and on the flagellar H-antigens of the bacteria that produce them (Höfte and Whiteley, 1989; Crickmore *et al.*, 1998). Numerous distinct crystal protein *(cry)* genes have been identified that code for insecticidal proteins (Cry proteins): CryI and CryIIB proteins are specifically toxic to Lepidoptera; CryIIA proteins to Lepidoptera and Diptera; CryIII proteins to Coleoptera; and four CryIV proteins to Diptera. In addition, two genes *(cytA, cytB)* that code for cytolytic proteins (CytA, CytB) are present with the CryIV proteins. This nomenclature has been revised (Crickmore *et al.*, 1998) but will be retained here, as many of the published studies discussed in this chapter were done while the old nomenclature was used. Some ICPs also exhibit activity against other orders of insects (e.g., Homoptera, Hymenoptera, Orthoptera, Mallophaga), as well as against nematodes, mites, *Collembola*, protozoa, and other organisms (Feitelson *et al.*, 1992; Addison, 1993; Crickmore *et al.*, 1998; Schnepf *et al.*, 1998).

Preparations of *Bt*, usually as sprays that contain a mixture of cells, spores, and parasporal crystals, have been used as insecticides for more than 30 years. Until recently, no unexpected toxicities from such sprays have been recorded, probably because *Bt* does not survive or grow well in natural habitats such as soil (e.g., Saleh *et al.*, 1970; West, 1984; West and Burges, 1985; West *et al.*, 1984a,b, 1985; Petras and Casida, 1985), and its spores are rapidly inactivated by UV radiation (Griego and Spence, 1978; Ignoffo and Garcia, 1978). Consequently, there is probably little production of toxins in soil, and the persistence of introduced toxins is a function primarily of the: 1) concentration added; 2) rate of consumption and inactivation by insect larvae; 3) rate of degradation by microorganisms; and

4) rate of abiotic inactivation. However, when the genes that code for these toxins are genetically engineered into plants, the toxins continue to be synthesized during growth of the plants. If production exceeds consumption, inactivation, and degradation, the toxins could accumulate to concentrations that may enhance the control of target pests or constitute a hazard to nontarget organisms, such as the soil microbiota (*see* below), beneficial insects (e.g., pollinators, predators and parasites of insect pests) (e.g., 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 syriaca)* contaminated with pollen from transgenic *Bt* corn *(Zea mays)* (Losey *et al.*, 1999; Obrycki *et al.*, 2001)] 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 *(Ostrinia nubilalis)* raised on transgenic *Bt* corn expressing Cry1Ab toxin (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 (e.g., Van Rie *et al.*, 1990; McGaughey and Whalon, 1992; Entwistle *et al.*, 1993; Tabashnik, 1994; Bauer, 1995; Ferré *et al.*, 1995; Tabashnik *et al.*, 1997). Persistence is enhanced when the toxins are bound on surface-active particles in the environment (e.g., clays and humic substances) and, thereby, rendered less accessible for microbial degradation but still retentive of toxic activity (Stotzky, 2000, 2002) (*see* below).

These potential hazards and benefits are affected by modifications (e.g., truncation and rearrangement of codons; *see* Schnepf *et al.*, 1998) of the introduced toxin genes to code only for the synthesis of active toxins, or a portion of the toxins, rather than of nontoxic crystalline protoxins (Fig. 1). Consequently, it will not be necessary for an organism that ingests the active toxins to have a high midgut pH (*ca.* 10.5) for solubilization of the IPCs and specific proteolytic enzymes to cleave the protoxins into toxic subunits. Therefore, nontarget insects and organisms in higher and lower trophic levels could be susceptible to the toxins, even if they do not have an alkaline gut pH and appropriate proteolytic enzymes. This leaves only the third of the three barriers that appear to be responsible for the host specificity of the ICPs: i.e., specific receptors for the toxins on the midgut epithelium that are often, but not always, present in larger numbers in susceptible larvae (e.g., Van Rie *et al.*, 1990; Wolfersberger, 1990; Garczynski *et al.*, 1991).

2. Adsorption and Binding of *Bt* Toxins on Clays and Humic Acids: Effects on Persistence and Insecticidal Activity

Surface-active particles in soil and other natural habitats (e.g., sediments in aquatic systems) are important in the persistence of organic molecules that, in the absence of such particles, would be rapidly degraded by the indigenous microbiota. These surface-active particles are primarily clay minerals and humic substances. Sand- and silt-size particles do not generally appear to be involved in the persistence of these molecules, as these particles are not surface-active (i.e., they do not have a significant surface charge, because they are composed mainly of primary minerals), and they have a smaller specific surface area (Stotzky, 1986). Many organic molecules are important in the ecology, activity, biodiversity, and evolution of microbes (e.g., as substrates, growth factors, siderophores), as well as in environmental protection and biological control of pests (e.g., Stotzky, 1974, 1986, 1997).

The insecticidal proteins produced by various subspecies of *Bt* are discussed as examples of the resistance to biodegradation of biomolecules when bound on clays and humic substances, as well as of the retention of the biological activity of the bound molecules. Because of the large differences in the chemical composition and structure of the surface-active particles, these systems can serve as models for the study of the fate and effects of other biomolecules (e.g., products of transgenic microbes, plants, and animals, including antibodies, vaccines, and other bioactive compounds; toxins produced by fungi) that are also chemically and structurally diverse and that will eventually reach soil and other natural habitats in biomass, root exudates, feces, urine, and other forms. Surface-active particles are also involved in mediating the effects of anthropogenic pollution (e.g., acid precipitation, heavy metals, pesticides) (Babich and Stotzky, 1983, 1986; Collins and Stotzky, 1989; Stotzky, 1986, 1997).

We have studied the equilibrium adsorption and binding of the purified toxins produced by *B. thuringiensis* subsp. *kurstaki* (*Btk*; 66 kDa; active against Lepidoptera), subsp. *tenebrionis* (*Btt*; 68 kDa; active against Coleoptera), and subsp. israelensis (Bti: 28 to 140 kDa; active against Diptera) on the clay minerals, montmorillonite and kaolinite, and on the clay-, silt-, and sand-size fractions of soil (Venkateswerlu and Stotzky, 1990, 1992; Tapp and Stotzky, 1995a,b, 1997, 1998; Tapp *et al.*, 1994; Koskella and Stotzky, 1997; Lee *et al.*, 2002), as well as the adsorption and binding of the toxin from *Btk* on humic acids from different soils (Crecchio and Stotzky, 1998) and on complexes of clay-humic acids-Al hydroxypolymers (Crecchio and Stotzky, 2001). Montmorillonite and kaolinite are the predominant clay minerals in many soils, and these clays differ in structure and numerous

physicochemical characteristics (e.g., cation-exchange capacity, specific surface area) and in their effects on biological activity in soil (see Stotzky, 1986). The toxins and protoxins were purified from pure cultures and commercial sources of the subspecies of *Bt*, and the clay minerals, various size fractions of soil, and humic acids were prepared as described in the references above. The availability to microbes of free and bound toxins as sources of carbon and/or nitrogen and the comparative insecticidal activity of free and bound toxins have also been studied (e.g., Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001). The purpose of these *in vitro* studies with purified toxins and relatively defined clays and humic acids was to determine whether the toxins released *in situ* from transgenic plants or commercial spray preparations have the potential to be adsorbed and bound on such surface-active particles and to persist and retain insecticidal activity in soil. The results of these studies are summarized in Table 1.

The toxins and protoxins adsorbed rapidly (in <30 min, the shortest time studied) (Fig. 2) on both "clean" and "dirty" montmorillonite and kaolinite and on the clay-size fraction of soil, indicating that toxins released in root exudates and upon disintegration of transgenic plant cells in soil would be only briefly in a free state susceptible to rapid biodegradation. The negative charges on the clean clays were compensated by monomeric cations, either mono- or polyvalent and either homoionic or mixed, whereas the dirty clays were "coated" with two types of polymeric oxyhydroxides of iron and are probably more representative of clays in soil *in situ*. The greater adsorption of the toxin than of the protoxin from *Btk* was probably a result of differences in their molecular mass (M_r) (toxin = 66 kDa; protoxin = 132 kDa) and, possibly, in their conformation. Inasmuch as toxins, rather than protoxins, are expressed in most transgenic plants, this review will discuss essentially only the results of studies obtained with toxins.

Adsorption of the toxins increased with their concentration and then plateaued, indicating that the clays became saturated with the toxins, as observed with other proteins and other organic compounds (Stotzky, 1986, 2000); was maximal between pH 6 and 8 on clean clays and between pH 5 and 9 on dirty clays; was affected by the type of cations on the exchange complex of the clays [adsorption generally decreased as the valency of the cations increased, as has also been observed with other proteins (*see* Stotzky, 1986)]; and was significantly lower on kaolinite than on montmorillonite. Larger amounts of the toxin from *Btk* than that from *Btt* were adsorbed. Only about 10 and 30% of the toxin from *Btk* and *Btt*, respectively, adsorbed at equilibrium were desorbed by one or two washes

with distilled water (Fig. 3). Additional washings desorbed no more toxins, indicating that the toxins were tightly bound on the clays and suggesting that they would not be easily desorbed *in situ* and leached into ground water by rain, irrigation, snow melts, etc. Interaction of the toxins with the clays did not alter significantly their structure, as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Tapp *et al.*, 1994) and enzyme-linked immunosorbent assays (ELISA) (Tapp and Stotzky, 1995a) of the equilibrium supernatants and desorption washes and by Fourier-transform infrared analyses (Table 2) and insect bioassays (Tables 3, 4) of the bound toxins. The toxins partially intercalated montmorillonite, as determined by X-ray diffraction analyses; there was more intercalation by the toxin from *Btt* than by the toxin from *Btk*, but the entire protein from either *Btk* or *Btt* did not penetrate (Table 5). There was no intercalation of kaolinite, a nonexpanding clay mineral. Even though the M_r of the toxins from *Btk* and *Btt* was similar, the toxin from *Btt* intercalated montmorillonite more but adsorbed and bound less on montmorillonite and kaolinite than did the toxin from *Btk*, indicating that the structure of these proteins differs sufficiently to result in different characteristics of adsorption on clays.

The toxins from *Bti* also bound on montmorillonite and kaolinite. Only 2 to 12% of the toxins, depending on the homoionic clay, adsorbed at equilibrium was desorbed by two washes with water. Additional washes desorbed no more toxins. Bound toxins retained activity against the larvae of mosquito (*Culex pipiens*) longer than free toxins in nonsterile water (Lee *et al.*, 2002). Further studies with the toxins from *Bti* are in progress, as the larvae of mosquitoes, as well as of some other dipterans, are filter-feeders. Hence, toxins bound on clays and humic substances may be more effective for the control of mosquitoes and other undesirable dipterans and pose less risk to the environment than the use of transgenic *Bt* cyanobacteria, which could transfer the toxin genes to other bacteria (see Stotzky, 1989; Entwistle et al., 1993).

A dot-blot ELISA method was developed that detects the toxins from *Btk* and *Btt* in soil, with a lower limit of detection of *ca.* 3 ng/g of soil (Tapp and Stotzky, 1995a). Toxins added to nonsterile soil, unamended or amended with montmorillonite or kaolinite, were detected by this method on the clay-size fraction but not on the silt- and sand-size fractions on which the toxins do not appear to adsorb. The use of flow cytometry as a rapid and sensitive method with which to detect the toxins in soil was also developed (Tapp and Stotzky, 1997). Currently, Lateral Flow Quickstix (EnviroLogix, Portland, ME), which are rapid (<10 min) Western blot detection systems with a lower detection limit of <10 parts per 10^9, are being used to detect

the toxin from *Btk*. The immunological results are confirmed by larvicidal assays (Saxena *et al.*, 1999; Saxena and Stotzky, 2000).

The bound toxins retained insecticidal activity. The toxin from *Btk* or *Btt* bound on montmorillonite, kaolinite, and the clay-size fraction of soil was insecticidal to the larvae of the tobacco hornworm (*Manduca sexta*) (Table 3) and the Colorado potato beetle (*Leptinotarsa decemlineata*) (Table 4), respectively, in standard larvicidal assays wherein comparable amounts of purified adsorbed, bound, or free toxins were added to appropriate larval media (Tapp and Stotzky, 1995b). Bound toxin from *Btk*, but not from *Btt*, had a higher toxicity (i.e., had lower LC_{50} values) than free toxin, possibly as the result of the toxin from *Btk* being concentrated on the clays, and the larvae ingested more clay-bound toxin than free toxin spread over the surface of the larval growth medium. The data are expressed as the apparent LC_{50} values (lethal concentration of toxin to kill 50% of a population of the assay larvae). Although the LC_{50} for a particular species of larva should theoretically not vary significantly, the modality of the actual assays reflects the amount of toxin that is present in a particular clay or soil system, which will vary with different clays and soils as well as with different batches of larvae and purified toxin. Consequently, the LC_{50} values in these studies indicate the amount of toxin present under specific conditions rather than the amount of toxin necessary to kill 50% of a larval population under standard assay conditions; e.g., with time, more toxin-containing soil was needed to kill 50% of the larvae, indicating a loss of toxicity, perhaps as the result of degradation of the toxin.

When free toxin from *Btk* was added to nonsterile soils in test tubes that were maintained at the –33-kPa water tension and 24 ± 2°C (i.e., under optimal conditions for microbial activity), insecticidal activity was detected after 234 days, the longest time evaluated, albeit with a reduction in activity during the incubation period (Table 6) (Tapp and Stotzky, 1998). This persistence was considerably longer than persistences reported in the literature, which ranged in half-life from *ca.* 8 to 17 days for purified toxins and from *ca.* 2 to 41 days for biomass of transgenic corn, cotton, and potato (e.g., Palm *et al.*, 1994, 1996; Sims and Holden, 1996; Sims and Ream, 1997). Insecticidal activity was greater and persisted longer in soil naturally containing or amended with kaolinite than in soils naturally containing or amended with montmorillonite (Table 6), possibly because montmorillonite-containing soils had a higher pH (5.8 to 7.3) and, therefore, more bacterial activity (Stotzky, 1986), which resulted in greater biodegradation of the toxin. A decrease in the persistence of the toxin in kaolinite-containing soils adjusted to *ca.* pH 7 from pH 4.9 with $CaCO_3$ further indicated that pH is a factor in the persistence of this toxin in soil.

The importance of pH and other physicochemical characteristics, as well as of biological characteristics, of soil in the persistence of the toxins in soil needs to be determined, especially to explain the reported differences in the persistence of the toxins.

The toxins from *Btk*, *Btt*, and *Bti*, free or bound on clays, had no effect on the growth *in vitro* [as measured by standard disk-diffusion assays and dilution tests for bacteria (Harley and Prescott, 1999) and colony diameter and sporulation for fungi (Babich and Stotzky, 1977, 1986)] of pure and mixed cultures of bacteria [both gram-positive (*Bacillus subtilis*, *B. cereus*, *Btk*, *Bti*, *Arthrobacter globiformis*) and gram-negative (*Agrobacterium radiobacter*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *P. mirabilis*, *Escherichia coli*, *Enterobacter aerogenes*, *E. cloacae*, *Oscillatoria* sp.)], fungi [both yeasts (*Saccharomyces cerevisiae*, *Candida albicans*) and filamentous forms (*Rhizopus nigricans*, *Cunninghamella elegans*, *Aspergillus niger*, *Fusarium solani*, *Penicillium* sp.)], and algae [primarily green (*Chlamydomonas* sp., *Oedogonium* sp., *Euglena* sp.) and diatoms] (Koskella and Stotzky, 2002). Although these results were not unexpected, as these organisms do not have receptors for the toxins, they differed from those indicated by Yudina and coworkers (1990, 1996, 1997), who suggested that the toxins from *Bt* have antibiotic properties.

The binding of the toxins from *Btk* and *Btt* on clays reduced their availability to microbes, which is probably responsible for their persistence in soil (Koskella and Stotzky, 1997). The free toxins were readily utilized as sources of carbon (Fig. 4) and/or nitrogen by pure and mixed cultures of microbes, including soil suspensions, especially after the microbes had been "conditioned" (i.e., induction of proteases) on the toxins or other proteins. In contrast, the bound toxins were not utilized as a source of carbon, utilized slightly as a source of nitrogen, and did not support microbial growth in the absence of exogenous sources of both available carbon and nitrogen (Table 7). The utilization of the toxins was determined turbidimetrically (i.e., by measuring increases in protoplasm and cell numbers) and respirometrically (i.e., by measuring uptake of O_2, which reflects metabolic activity). After exposure of the bound toxins to microbes, *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 (albeit wetting and drying somewhat reduced the activity). It was anticipated that such treatments might have released toxins from surface-active particles and rendered them more susceptible to biodegradation (Table 8).

Results similar to those obtained with clays were observed when the toxin from *Btk* was reacted with humic acids prepared from four different soils

and differing in total acidity (5.0 to 7.4 cmol/kg), in the content of carboxyl and phenolic groups (3.2 to 4.2 and 1.0 to 3.4 cmol/kg, respectively), and in the degree of polymerization, as evaluated by the ratio of absorbance at 464 and 665 nm (E4:E6; 5.5 to 7.0): 75 to 80% of the toxin adsorbed at equilibrium was strongly bound, with the exception of humic acids from soil on which sugar beets had been grown and which bound only *ca.* 43% of adsorbed toxin (Fig. 5); the bound toxin was toxic to the larvae of *M. sexta*, with a LC_{50} lower than that of free toxin (Table 9); and the bound toxin did not support the growth of a mixed microbial culture from soil, although the free toxin was readily utilized as a source of carbon (Fig. 6) (Crecchio and Stotzky, 1998). When the toxin was bound on complexes of montmorillonite-humic acids-Al hydroxypolymers, which more closely approximate the presumed composition of surface-active particles in soil, similar results were obtained (Crecchio and Stotzky, 2001).

3. Biodegradation of *Bt* Corn in Soil

The addition of biomass from transgenic corn (*Zea mays* L.) expressing the Cry1Ab toxin from *Btk* resulted in a significantly lower gross metabolic activity (i.e., CO_2 evolution) of soil than did the addition of nontransgenic biomass (i.e., same variety but without the toxin gene) (Fig. 7) (Flores, Saxena, and Stotzky, unpublished data). Soil from the Kitchawan Research Laboratory of the Brooklyn Botanic Garden augmented to 9% (vol/vol) with montmorillonite (*see* below) was amended to 0.5, 1, or 2% (wt/wt) with ground, air-dried leaves or stems of *Bt* corn (NK6800Bt) or of the near-isogenic variety without the *cry1Ab* gene (NK6800) (the particle size distribution of the ground material was 70% <0.5 mm and 30% <1 mm). Subsamples of amended or unamended soil (25 or 50 g oven-dry equivalent, depending on the experiment) at the –33-kPa water tension were placed in small jars (90-ml capacity), and 8 to 10 jars containing soil of each treatment were placed in individual 1-L "master" jars that were attached to a respiratory train and incubated at 25 ± 2°C (Stotzky *et al.*, 1993). The gross metabolic activity of the soil was determined by CO_2 evolution: CO_2 was trapped in NaOH, precipitated with $BaCl_2$, and the unneutralized NaOH titrated with HCl using an automatic titrator. Subsamples of soil, in the small jars, were removed periodically from the master jars, and the activities of proteases, dehydrogenases, alkaline and acid phosphatases, and arylsulfatases, as well as the numbers of culturable bacteria and fungi, were measured (Stotzky *et al.*, 1993). At the end of each incubation, the insecticidal activity of the soil was determined by bioassay using the larvae of *M. sexta* (Tapp and Stotzky, 1998).

There were three replicates of each treatment per experiment, and

experiments were often repeated.

The amounts of C evolved as CO_2 increased in proportion to the amounts of biomass added when compared with the amounts evolved from the unamended control soil. However, the amounts evolved were significantly lower throughout the incubations from soil amended with biomass of *Bt* corn than from soil amended with biomass of near-isogenic non-*Bt* corn (Fig. 7). This difference occurred with stems and leaves from two separate batches of corn tissue, even when glucose was added with the tissue. Changes in the C:N ratio of the soil-biomass systems by the addition of glucose, NH_4NO_3, or glucose plus NH_4NO_3 did not alter the relative differences in CO_2 evolution between soil amended with biomass of *Bt* corn and soil amended with biomass of non-*Bt* corn.

The activities of the enzymes and the numbers of culturable bacteria and fungi fluctuated throughout the incubations and differed with the various treatments, but there were no consistent statistically significant differences in activities and numbers between soil amended with biomass of *Bt* corn and soil amended with biomass of non-*Bt* corn (Table 10). All soil samples amended with biomass of Bt corn were lethal to the larvae of *M. sexta* (LC_{50} values ranged from 0.27 to 0.59 mg of biomass, with confidence intervals of 0.144 to 0.495 and 0.351 to 1.070 mg of biomass, respectively), whereas there was no mortality with soil amended with biomass of non-*Bt* corn and with soil that was not amended.

The reasons for the lower biodegradation of the biomass of *Bt* corn than of the biomass of non-*Bt* corn are not known. It was not the result of differences in the C:N ratios of the biomass, as leaf and stem tissue of one batch of both *Bt* corn and non-*Bt* corn had similar C:N ratios, and changes in the ratios, as well as the addition of an available carbon and energy source in the form of glucose, did not significantly alter the relative differences in biodegradation between biomasses. The lower biodegradation was apparently not the result of the inhibition of the activity of the soil microbiota by the biomass of *Bt* corn, as the numbers of culturable bacteria and fungi and the activity of enzymes representative of those involved in the degradation of plant biomass were not significantly different between soil amended with biomass of *Bt* corn and soil amended with biomass of non-*Bt* corn (Table 10). These results confirmed *in vitro* observations that the toxins from *Btk*, *Btt*, and *Bti* were not toxic to pure and mixed cultures of microbes (Koskella and Stotzky, 2002) and *in situ* observations with transgenic plants that showed no consistent and lasting effects of these plants on the soil microbiota (Donegan *et al.*, 1995, 1996). Although it is tempting to suggest that the insertion of the *cry1Ab* gene into the plant genome affected the susceptibility of *Bt* corn to

biodegradation, there are no data to support this other than the observation that tissues of *Bt* corn show greater resistance to breakage and maceration (Flores, Saxena, and Stotzky, unpublished data) and anecdotal reports that *Bt* corn has greater standability (i.e., less lodging) and is preferred less as feed by cattle than is non-*Bt* corn (Pat Porter, personal communication).

Because these observations suggested some differences in the chemical composition between *Bt* and non-*Bt* corn, the lignin content of 10 different *Bt* corn hybrids, representing three transformation events (Bt11, MON810, and 176), and of their respective non-*Bt* near-isolines, grown in both a plant-growth room and the field, was evaluated. Uniform free-hand sections of fresh stems of corn, harvested after tasseling and ear production and of the same age, from between the third and fourth node (thickness ~11 mm) were examined for lignin by fluorescence microscopy at 400 nm (Hu *et al.*, 1999). A higher content of lignin was observed in the vascular bundle sheath and in the sclerenchyma cells surrounding the vascular bundle of all *Bt* corn hybrids than of their respective non-*Bt* near-isolines, which was confirmed by staining the sections with toluidine blue (Sylvester and Ruzin 1994). The average diameter of the vascular bundle and surrounding lignified cells in *Bt* corn was 21.5 ± 0.84 mm, whereas that of non-*Bt* corn was 12.4 ± 1.14 mm (Fig. 8) (Saxena and Stotzky, 2001c).

The content of lignin of the same portion of the stems (oven-dried, ground, and passed through an 80-mesh sieve) was determined chemically by the acetyl bromide method (Hatfield *et al.*, 1999). The lignin content of all hybrids of *Bt* corn, whether grown in the plant-growth room (Table 11) or in the field (Table 12), was significantly higher (33 to 97% higher) than that of their respective non-*Bt* near-isolines. The lignin content of field-grown plants was higher than that of plants grown in the plant-growth room, which were smaller in size. There was a significantly higher lignin content ($P < 0.002$) in plants transformed by event Bt11 (7.4 ± 0.10 and 6.7 ± 0.12% for field- and growth room-grown plants, respectively) than by event MON810 (6.9 ± 0.07 and 6.2 ± 0.10% for field- and growth room-grown plants, respectively). There were no significant differences in the lignin content of near-isogenic non-*Bt* plants ($P > 0.67$ for field-grown plants and $P > 0.30$ for growth room-grown plants). The lignin content of the only available hybrid transformed by event 176 (5.9 ± 0.13 and 4.0 ± 0.15% for field- and growth room-grown plants, respectively) was lower than that of hybrids transformed by events Bt11 and MON810. These results differ from those reported by Faust (1999), which indicated no significant differences in lignin content between the dried biomass of whole plants of *Bt* (event MON810) and non-*Bt* corn but which indicated that *Bt* corn had a higher

moisture content and a lower level of ammonia than non-*Bt* corn ($P < 0.05$). However, Masoero *et al.* (1999) reported a 16% higher lignin content in *Bt* than in non-*Bt* corn.

Lignin is a major structural component of plant cells that confers strength, rigidity, and impermeability to water. Any modifications in lignin content could result in effects that may have ecological implications (Halpin *et al.*, 1994). For example, the increase in lignin content in *Bt* corn may be beneficial, as it can provide greater resistance to attack by second-generation European corn borer (Ostrander and Coors, 1997), reduce susceptibility to molds (Masoero *et al.*, 1999), and retard litter degradation and decomposition by microbes (Reddy, 1984; Tovar-Gomez *et al.*, 1997), as also indicated in our studies by the lower evolution of CO_2 from soils amended with biomass of *Bt* corn than with biomass of non-*Bt* corn (Flores, Saxena, and Stotzky, unpublished data).

The biodegradation of biomass of *Bt* canola, cotton, potato, rice, and tobacco was also significantly lower than that of the biomass of near-isogenic non-*Bt* plants. The lower biodegradation was apparent both when ground biomass was incorporated into soil and when pieces of biomass were inoculated with a 1:10 soil:water suspension and incubated in the absence of soil, indicating that the lower degradation was a function of the *Bt* biomass and not of the soils. However, the lignin contents of canola, cotton, potato, rice, and tobacco, which were significantly lower than that of corn, were not significantly different between *Bt* and non-*Bt* biomass (Flores, Saxena, and Stotzky, unpublished data).

The lower degradation of the biomass of *Bt* plants may be beneficial, as the organic matter derived from such plants may persist and accumulate longer and at higher levels in soil, thereby improving soil structure and reducing erosion. By contrast, the longer persistence of the biomass of *Bt* plants may extend the time that toxin is present in soil and, thereby, enhance the hazard to nontarget organisms and the selection of toxin-resistant target insects. Additional studies are necessary to clarify the environmental impacts of the lower degradation of the biomass of *Bt* plants, especially as about 8.1 million hectares of *Bt* corn (26% of total corn acreage), 2.4 million hectares of *Bt* cotton (45% of total cotton acreage), and 0.02 million hectares of *Bt* potato (3.5% of total potato acreage) were planted in the United States alone in 2000 (USEPA, 2001).

4. Release, Binding, Persistence, and Insecticidal Activity of *Bt* Toxins in Root Exudates of *Bt* Corn, Rice, Cotton, Canola, Tobacco, and Potato

The Cry1Ab protein was present in root exudates from transgenic *Bt* corn (NK4640Bt) grown in sterile hydroponic culture and in sterile and nonsterile

soil in a plant-growth room (Saxena *et al.*, 1999) (Table 13). The presence of the toxin was indicated by a major band migrating on SDS-PAGE to a position corresponding to a M_r of 66 kDa, the same as that of the Cry1Ab protein, and confirmed by immunological and larvicidal assays. After 25 days, when the hydroponic culture was no longer sterile, the band at 66 kDa was not detected (there were several new protein bands of smaller M_r) and the immunological and larvicidal assays were negative, indicating that microbial proteases had hydrolyzed the toxin. By contrast, the toxin was detected after 25 days in both sterile and nonsterile soil, indicating that the released toxin was bound on surface-active particles in rhizosphere soil, which protected the toxin from hydrolysis, similar to results observed with purified toxins (*see* above).

To verify these results and to estimate the importance of the clay mineralogy and other physicochemical characteristics, which influence the activity and ecology of microbes in soil (Stotzky, 1974, 1986, 1997), on the persistence of the toxin released in root exudates from *Bt* corn, studies were done in soil amended with various concentrations of montmorillonite or kaolinite in a plant-growth room. Kitchawan soil, a sandy loam that naturally contains predominantly kaolinite, was collected at the Kitchawan Research Laboratory of the Brooklyn Botanical Garden, Ossining, NY, and either not amended [control (C)] or amended to 3, 6, 9, or 12% (vol/vol) with montmorillonite (3, 6, 9, and 12M soil) or kaolinite (3, 6, 9, and 12K soil). These stable soil-clay mixtures have been used extensively in studies on the effects of physicochemical and biological factors of soil on the activity, ecology, and population dynamics of microbes and viruses, on gene transfer among bacteria, on mediating the toxicity of heavy metals and other pollutants, and on the persistence of the toxins from *Btk* and *Btt* in soil. Consequently, there is a large database on these mixtures (e.g., Babich and Stotzky, 1977; Stotzky *et al.*, 1993; Tapp and Stotzky, 1995, 1997).

Seeds of *Bt* corn (NK4640Bt) and of the near-isogenic variety without the *cry1Ab* gene were planted in tubes containing nonsterile soil amended or not amended with montmorillonite or kaolinite and in a sandy loam soil in the field. After 10, 20, 30, and 40 days of growth in a plant-growth room (26 ± 2°C, 12-h light-dark cycle), soil from randomly selected tubes (2 tubes each of *Bt* corn and non-*Bt* corn for each soil-clay mixture) was analyzed by immunological and larvicidal assays. Rhizosphere soil from the field-grown plants of *Bt* corn and non-*Bt* corn was similarly analyzed after the production of ears of corn and several months after the death of the plants and frost.

All samples of rhizosphere soil from plants of *Bt* corn grown in the plant-growth room were positive 10, 20, 30, and 40 days after germination for the

presence of the toxin when assayed immunologically with Lateral Flow Quickstix (Saxena and Stotzky, 2000). No toxin was detected in any soil with plants of non-*Bt* corn or without plants. All samples of soil in which *Bt* corn was grown were toxic to the larvae of *M. sexta*, with mortality ranging from 25 to 100% on day 10 and increasing to 88 to 100% on day 40, whereas there was no mortality with any soil from non-*Bt* plants or without plants. In addition, the size and weight of surviving larvae exposed to soils from *Bt* corn were significantly lower (*ca.* 50 to 92% lower) than those exposed to soil from non-*Bt* corn or without plants, and these larvae usually died 2 to 3 days after weighing (Table 14). The larvicidal activity was generally higher in soil amended with montmorillonite than with kaolinite, probably because montmorillonite, a swelling 2:1, Si:Al, clay mineral with a significantly higher cation-exchange capacity and specific surface area than kaolinite, a nonswelling 1:1, Si:Al, clay, bound more toxin in the root exudates than did kaolinite, as has been observed with pure toxin (e.g., Venkateswerlu and Stotzky, 1992; Tapp *et al.*, 1994; see above). Nevertheless, mortality in the montmorillonite and kaolinite soils was essentially the same after 40 days (Table 14), indicating that over a longer time, the persistence of larvicidal activity appeared to be independent of the clay mineralogy and other physicochemical characteristics of the soils. However, the increase in larvicidal activity between day 10 and day 40 indicated that the toxin in the root exudates was concentrated when adsorbed on surface-active components of the soils.

The immunological and larvicidal assays of soil from the rhizosphere of *Bt* corn grown in the field were also positive, even in soil collected after frost from plants that had been dead for several months, whereas they were negative for non-*Bt* corn (Table 15). Although larval mortality in rhizosphere soil from some plants of field-grown *Bt* corn was only 38% and the coefficients of variation were large, the size and weight of the surviving larvae were reduced by 40 to 50% when compared with soil from non-*Bt* corn or without plants. Moreover, most larvae died a few days after being weighed.

To determine whether the release of the Cry1Ab protein is a common phenomenon with transgenic *Bt* corn and is not restricted to the NK4640Bt hybrid, the release of the protein in the exudates of 12 additional *Bt* hybrids, representing three different transformation events, and of their near-isogenic nontransgenic counterparts was studied with plants grown in the plant-growth room and in the field. In addition, the persistence of the protein released from all *Bt* hybrids in rhizosphere soil was evaluated.

All samples of rhizosphere soil from the 12 hybrids grown in the plant-growth room were positive 40 days after germination for the presence of the toxin when assayed immunologically with Quickstix, and all samples

were toxic to the larvae of *M. sexta*, with mortality ranging from 38 to 100% (Table 16). No toxin was detected immunologically or by larvicidal assay in any soil in which plants of non-*Bt* corn or no plants had been grown (Saxena *et al.*, 2002b). In addition, the weight of surviving larvae exposed to soils from *Bt* corn was significantly lower (80 to 90%) than those exposed to soils from non-*Bt* corn or without plants, and these larvae usually died after an additional 2 to 3 days.

The immunological and larvicidal assays of soil from the rhizosphere of all *Bt* hybrids grown in the field and harvested after the production of ears of corn were also positive, whereas they were negative for all non-*Bt* corn near-isolines (Table 17). Although the larval mortality in rhizosphere soil from some plants of field-grown *Bt* corn was only 37%, the weight of the surviving larvae was reduced by 85 to 98% when compared with soil from non-*Bt* corn or without plants, and most of these larvae died after a few more days. There were no discernable or consistent differences in exudation of the toxin (as evaluated by mortality, weight of surviving larvae, or immunologically) between plants derived from different transformation events, regardless of whether they were grown in the plant-growth room or in the field.

These results indicated that the toxin released in exudates from roots of *Bt* corn could accumulate in soil and retain insecticidal activity, especially when the toxin is bound on surface-active soil particles and, thus, becomes resistant to degradation by microorganisms. Although some toxin was probably released from sloughed and damaged root cells, the major portion was derived from exudates, as there was no discernable root debris after centrifugation of the Hoagland's solution when plants were grown in hydroponic culture.

In addition to the large amount of toxin that will be introduced to soil in plant biomass after harvest and some that will be introduced in pollen released during tasseling (Losey *et al.*, 1999; Obrycki *et al.*, 2001), these results indicated that toxin will also be released to soil from roots during the entire growth of a *Bt* corn crop. The presence of the toxin in soil could improve the control of insect pests, or the persistence of the toxin in soil could enhance the selection of toxin-resistant target insects and constitute a hazard to nontarget organisms. Because *Bt* corn contains truncated genes that encode toxins rather than the nontoxic crystalline protoxins produced by *Bt*, potential hazards are exacerbated, as it is not necessary for an organism ingesting the toxins to have a high gut pH (*ca.* 10.5) for solubilization of the protoxins and for specific proteases to cleave the protoxins into toxins (Fig. 1). Moreover, receptors for the toxins are present in both target and nontarget insects (Höfte and Whiteley, 1989).

Consequently, nontarget insects and organisms in higher and, perhaps also, in lower trophic levels could be susceptible to the toxins.

The Cry1Ac protein was not released in the root exudates of *Bt* canola, cotton, and tobacco containing the *cry1Ac* gene, whereas the Cry1Ab protein was released in the root exudates of *Bt* rice containing the *cry1Ab* gene, and the Cry3A protein was released in the root exudates of *Bt* potato containing the *cry3A* gene. Immunological assays of soil from the rhizosphere and of the hydroponic solution of *Bt* canola, cotton, and tobacco were negative, and the soil and solution were not toxic to the larvae of *M. sexta*. In contrast, the soil and solution of *Bt* rice were immunologically positive for the Cry1Ab protein and toxic to the larvae of *M. sexta*, and those of *Bt* potato were immunologically positive for the Cry3A protein and toxic to larvae of the Colorado potato beetle (*L. decemlineata*). The percent mortality of soil and hydroponic solution from *Bt* potato was 34.9 and 37.9 % respectively, and the size of the surviving larvae was reduced by 25 to 40% when compared with soil from non-*Bt* potato or from soil without plants (Saxena and Stotzky, unpublished data) (Table 18).

The absence of the Cry1Ac protein in the root exudates of *Bt* canola, cotton, and tobacco, both in rhizosphere soil and hydroponic culture, indicated again that the presence of the Cry1Ab protein in the root exudates of *Bt* corn and rice and of the Cry3A protein in the root exudates of *Bt* potato were not the result of sloughing of or damage to root cells, as the proteins were not present in the root exudates of other *Bt* plants, even when grown in soil. It is not clear how a 66-kDa protein, such as the Cry1Ab protein, is release intact from roots, as the release of molecules with such a high M_r from roots usually requires the presence of a "signal peptide" (Borisjuk *et al.*, 1999). The endoplasmic reticulum is presumably close to or associated with the plasma membrane in the roots of corn and, apparently, of rice and potato, but this does not appear to be the situation in the roots of canola, cotton, and tobacco.

5. Vertical Movement of *Bt* Toxin Released to Soil as Purified Protein or in Root Exudates or from Biomass of *Bt* Corn

When 0.8, 1.6, or 3.2 *µg*/g of soil, oven-dry weight equivalent, of purified Cry1Ab protein was added to the top of plastic columns containing 50 g of soil, the toxin was detected in leachates from all columns 1 and 3 h after addition, with the largest amount (*ca.* 75%) detected from columns containing soil not amended with clay (C soil) and the lowest amount (*ca.* 16%) detected from columns containing soil amended to 12% with montmorillonite or kaolinite; intermediate amounts of protein were

leached from soils amended to 3, 6, or 9% with the clays (Tables 19 and 20) (Saxena *et al.*, 2002a). Larvicidal activity against *M. sexta* was higher with leachates from soil not amended or amended to 3 or 6% with montmorillonite or kaolinite (mortality ranged from 12 ± 6.3 to 68 ± 11.9%, and the weight of a single larva ranged from 0.03 ± 0.01 to 0.3 ± 0.03 g) than from soil amended to 9 or 12% with the clays (mortality ranged from 12 ± 6.3 to 37 ± 12.5%, and the weight of a single larva ranged from 0.05 ± 0.01 to 0.6 ± 0.03 g), indicating that the protein moved less through soil as the clay concentration was increased (Tables 21, 22, and 23). However, after 12 and 24 h, no protein was detected in any of the leachates, even by the immunological assay with a lower detection limit of 10 parts per 10^9, indicating that the protein bound on the soils and was no longer susceptible to desorption (Tables 21, 22, and 23). The presence of the Cry1Ab protein in the leachates was confirmed by SDS-PAGE.

The vertical distribution in the columns of protein that was not recovered in the leachates confirmed that the protein moved less through soil amended with the higher concentrations of clays (Table 24). For example, when soil at 2 to 4, 70 to 80, and 140 to 150 mm from the top of randomly-selected columns of C, 3K, 12K, 3M, and 12M soil to which 0.8 or 3.2 µg of protein/g of soil had been added was analyzed after periodic leaching over 24 h for the presence of the protein, larvicidal activity at 2 to 4 mm of soil amended to 12% with montmorillonite or kaolinite was higher (mortality ranged from 37 ± 7.3 to 100%, and the weight of a single larva ranged from 0.04 ± 0.01 to 0.06 ± 0.03 g) than that of soil not amended or amended to 3% with montmorillonite or kaolinite (mortality ranged from 12 ± 7.3 to 81 ± 11.9%, and the weight of a single larva ranged from 0.05 ± 0.03 to 0.4 ± 0.03 g). Mortality generally decreased with depth in the columns as the clay concentration was increased (Table 24).

The Cry1Ab protein was present in the leachates from soil columns in which hybrids of *Bt* corn were grown, whereas it was absent in the leachates from columns in which their respective non-*Bt* near-isolines were grown (Table 25). Hybrids of three transformation events (Bt11: NK4640Bt, N7590Bt; MON810: DK647Bty; and 176: Maximizer) and their near-isolines [NK4640, N7590, and DK647 (isoline for Maximizer 176 was not available)] were grown in glass tubes for 40 days in a plant-growth room (26 ± 2°C, 12 h light-dark cycle) when the tubes were leached with 10 ml of water, the leachates centrifuged, and the supernatants analyzed for the presence of the protein. Although the mortality of the leachates was only 12 ± 7.3 to 20 ±10.5%, there was a *ca.* 62% reduction in larval weight (0.2 ± 0.06 to 0.5 ± 0.07) compared with larvae exposed to leachates from columns in which non-*Bt* corn or no plants were grown (0.8 ± 0.04 to 1.1 ± 0.07), indicating

vertical movement of the protein from the rhizosphere (Table 25).
The protein was also present in leachates from columns of soil amended 3 y earlier with biomass of *Bt* corn, whereas it was absent in leachates from columns of soil amended with biomass of non-*Bt* corn (Table 25). Mortality was 43 ± 6.3%, and the weight of a single larva was 0.08 ± 0.02, indicating that as the biomass degraded, the protein was released and some bound on soil particles and some dissolved in soil water and moved down with excess water.
The movement of the Cry1Ab protein through soil was influenced by its tendency to stick to surface-active particles, particularly to clay and organic matter (Venkateswerlu and Stotzky, 1992; Tapp *et al.*, 1994; Tapp and Stotzky, 1998; Stotzky, 2000; Crecchio and Stotzky, 1998, 2001). The protein exhibited both strong binding and high persistence in soils that contained the higher clay concentrations (e.g., 9M, 12M, 9K, and 12K soils), and it remained near the soil surface, increasing its probability of being transported to surface waters via erosion and runoff. In contrast, in soils with lower clay concentrations (e.g., C, 3M, 6M, 3K, and 6K soils), the protein was leached more through the soils, and it may more likely contaminate groundwater. The possibility of contamination of surface or groundwater, which depends greatly on the desorption of the protein and on the amount of water impacting on the soil as rain, irrigation, snow melts, etc., may pose a hazard to nontarget aquatic Lepidoptera, which are more plentiful in waters (e.g., 279 species in North America alone) than in soil (Lange, 1984; Williams and Feltmate, 1994). Without an adequate input of water, the protein is more likely to remain within the biologically-active root zone, where some protein, especially that not bound on surface-active particles, will be mineralized.

6. Effect of *Bt* Toxin from Root Exudates and Biomass of *Bt* Corn on Earthworms, Nematodes, Protozoa, Bacteria, and Fungi in Soil

To determine the effects of the Cry1Ab toxin released in root exudates from *Bt* corn on various organisms in soil, *ca.* 4.5 kg of a loam soil from a farm in East Marion, Long Island, NY, was placed in plastic pots, and 20 medium-size (3.5 ± 0.32 g) earthworms (*Lumbricus terrestris*), purchased from Carolina Biological Supply Company, were placed in each pot. Seeds (3 per pot) of *Bt* corn (NK4640Bt) and near-isogenic non-*Bt* corn were planted, and some pots remained unplanted. After 40 days of growth in a plant-growth room (26 ± 2° C, 12-h light-dark cycle), the plants were gently removed and rhizosphere soil was collected. To determine the effects of biomass from *Bt* corn on the organisms, 500 g of soil amended with 1% (wt/wt) of ground, air-dried biomass of *Bt* (NK4640Bt) or non-*Bt* corn

(NK4640) (leaves, stems, and roots) was placed in glass containers, and five medium-size earthworms were added to each container. Control containers received no biomass. The containers were kept in the plant-growth room for 45 days. Before introduction of the worms, the numbers of nematodes (Van Gundy, 1982) and of culturable protozoa, fungi, and bacteria, including actinomycetes, (Stotzky *et al.*, 1993) in the soil in the pots and containers were determined.

In both experiments, casts produced by the worms were collected. After incubation, the numbers of earthworms were counted and their weight determined. Three representative worms from each pot with plants and two worms from each container with biomass were dissected, and soil from the guts, as well as from the casts, was analyzed for the presence of the Cry1Ab protein by immunological and larvicidal assays.

There were no significant differences in the percent mortality and weight of earthworms after 40 days in soil planted with *Bt* or non-*Bt* corn or not planted or after 45 days in soil amended with biomass of *Bt* or non-*Bt* corn or not amended (Table 26) (Saxena and Stotzky, 2001b). However, the toxin was present in both casts and guts of worms in soil planted with *Bt* corn or amended with biomass of *Bt* corn (Table 27), whereas it was absent in casts and guts of worms in soil planted with non-*Bt* corn or not planted and in soil amended with biomass of non-*Bt* corn or not amended. When worms from pots of *Bt* corn or from containers of soil amended with biomass of *Bt* corn were transferred to pots containing fresh soil, the toxin was cleared from the guts in 1 to 2 days. All samples of soil amended with biomass of *Bt* corn and from the rhizosphere of *Bt* corn were positive for the presence of the toxin and were lethal to the larvae of *M. sexta*, even after 45 and 40 days, respectively, whereas 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, which were also negative in the immunological assays (Table 27).

There were no statistically significant differences ($P>0.5$) in the numbers of nematodes and culturable protozoa, bacteria (including actinomycetes), and fungi between rhizosphere soil of *Bt* and non-*Bt* corn or between soil amended with *Bt* or non-*Bt* biomass (Table 28).

These results suggested that the toxin released in root exudates of *Bt* corn or from the degradation of the biomass of *Bt* corn is not toxic to a variety of organisms in soil. However, the toxin was detected in the guts and casts of earthworms grown with *Bt* corn and in soil amended with biomass of *Bt* corn (Table 27), indicating again that the released toxin bound on surface-active particles in soil, which protected the toxin from biodegradation, similar to what has been observed with purified toxins. Because only one

species of earthworms and only culturable microorganisms and nematodes were evaluated, more detailed studies on the composition and diversity of these groups of organisms are necessary, including studies using techniques of molecular biology (e.g., denaturing gradient and temperature gradient gel electrophoresis, single strand conformational polymorphism), the BIOLOG or equivalent system for bacteria, speciation of fungi, and nutritional groups of protozoa and nematodes, to confirm the absence of effects of the Cry1Ab toxin on biodiversity in soil.

7. Uptake of *Bt* Toxin from Soil by Plants

Because of public concern that plants will take up *Bt* toxins from soil [e.g., a supermarket chain in the United Kingdom will not sell produce from plants that have been grown on soils previously planted with *Bt* crops (Nuttall, 2000)], non-*Bt* corn, carrot, radish, and turnip were grown in soil in which *Bt* corn had previously been planted or which was amended with ground biomass of *Bt* corn. Non-*Bt* corn and turnip were harvested after 20, 30, 50, and 120 days, and carrot and radish were harvested after 30, 50, 120, and 180 days. The leaves, stems, and roots were analyzed immunologically and by bioassay with the larvae of *M. sexta*. No Cry1Ab protein was detected in the tissues of any of the plants, whereas the protein was present in all samples of soil (Tables 29 and 30). Only representative data are shown here; for more details see Saxena and Stotzky (2001a, 2002).

No toxin was detected, either immunologically or by larvicidal assay, in the tissues of non-*Bt* corn, carrot, radish, and turnip grown for 90 days in soil amended with 3 or 9% kaolinite or montmorillonite and to which purified Cry1Ab protein (3.2 *μg*/g soil, oven-dry equivalent) had been added (mortality with all plant tissues from clay-amended soils without toxin ranged from 0 to 12.5 ± 6.25%, and the weight of a single larva ranged from 0.6 ± 0.05 to 1.2 ± 0.10 g, which reflected the normal mortality and weight of the larvae). By contrast, all soil samples were both immunologically positive for the Cry1Ab protein and lethal to the larvae 90 days after addition of the purified protein (mortality ranged from 31 ± 12.5 to 81 ± 12.5 %, and the weight of a single larva ranged from 0.01 ± 0.01 to 0.1 ± 0.06 g). Soil amended with 9% kaolinite or montmorillonite was more toxic (56 ± 6.3 and 62 ± 12.5% mortality, respectively, and the weight of a single larva was 0.09 ± 0.03 and 0.08 ± 0.03 g, respectively) than soil amended with 3% kaolinite or montmorillonite (31 ± 12.5 and 37 ± 7.5%, mortality, respectively, and the weight of a single larva was 0.2 ± 0.08 and 0.1 ± 0.05 g, respectively) or not amended (20 ± 10.5% mortality, and the weight of a single larva was 0.4 ± 0.09).

To determine whether the Cry1Ab protein is taken up in hydroponic culture, *Bt* corn was grown aseptically in Hoagland's solution for 15 days in the plant-growth room, and two-day old seedlings derived from surface-sterilized seeds of non-*Bt* corn that had been germinated on agar plates were transferred aseptically and grown in the same solution for 7 and 15 days. The tissues of three representative plants and the solution were analyzed after 7 and 15 days for the toxin by immunological and larvicidal assays. No Cry1Ab protein was detected in the tissues of non-*Bt* corn, whereas the toxin was easily detected in the hydroponic solution (Table 31). These results indicated that the lack of uptake of the toxin from soil was not the result of the binding of the protein on surface-active particles, as no such surfaces were present in the hydroponic solution.

These results of these studies indicated that the toxin released to soil in root exudates of *Bt* corn, from the degradation of the biomass of *Bt* corn, or as purified toxin is not taken up by non-*Bt* corn, carrot, radish, and turnip from soil, where the toxin is bound on surface-active particles, or by non-*Bt* corn from hydroponic culture, where the toxin is not bound on particles. The persistence of the toxin in soil for 180 days after its release in root exudates or from biomass of *Bt* corn or 90 days after its addition in purified form, the longest times evaluated, indicated again that the toxin was bound on surface-active particles in soil, which protected the toxin from biodegradation. These results were not unexpected, as it is doubtful that plants can take up molecules as large as 66 kDa.

The results of studies with transgenic biomass are summarized in Table 32.

8. Conclusions

These studies on the interaction of insecticidal proteins with two types of surface-active particles that differ greatly in composition and structure demonstrate further the importance of surface-active particles to the biology of natural habitats. These studies also confirm and extend previous observations on the influence of clays and other surface-active particles on the activity, ecology, and population dynamics of microbes and viruses in soil and other natural habitats, as well as on the transfer of genetic information among bacteria by conjugation, transduction, and transformation (e.g., Stotzky, 1986, 1989, 2000; Vettori *et al.*, 1999; Yin and Stotzky, 1997).

Moreover, the results obtained with these insecticidal proteins indicate their potential environmental importance when bound on surfaces in soil. For example, the persistence of the bound toxins from *Bt* could pose a potential hazard to nontarget organisms and result in the selection of toxin-resistant target insects and, thereby, negate the benefits of using a

biological, rather than a synthetic chemical, insecticide. However, the persistence of the bound toxins could also enhance the control of target pests. These aspects require more study, especially a case-by-case evaluation of each toxin.

In addition to suggesting potential hazards and benefits of bound toxins from *Bt*, the results of these studies indicate that caution must be exercised before transgenic plants and animals genetically modified to function as "factories" ("pharms") for the production of vaccines, hormones, antibodies, blood substitutes, toxins, pharmaceuticals, and other bioactive compounds are released to the environment (Table 33). Because of the large differences in the chemical composition and structure of clays and humic acids, these studies can serve as models for the potential fate and effects of other biomolecules, which are also chemically and structurally diverse, that will be introduced to soil from such factories. As with *Bt* plants, where only a portion of the plants is harvested (e.g., ears of corn, bolls of cotton, kernels of rice, tubers of potato) and the remainder of the biomass is incorporated into soil wherein the toxins released from disintegrating biomass are rapidly bound on surface-active particles, some of the biomass of these plant factories will also be incorporated into soil. With transgenic animal factories, feces, urine, and subsequently even carcasses containing bioactive compounds will eventually reach soil and other natural habitats (e.g., surface and groundwaters). If these bioactive compounds bind on clays and humic substances - and as many of these compounds are proteinaceous, they most likely will - they may also persist in natural habitats. If they retain their bioactivity, they could affect the biology of these habitats. Consequently, before the use of such plant and animal factories (and, probably, also microbial factories), the persistence of their products and the potential effects of the products on the inhabitants of soil and other habitats must be thoroughly evaluated.

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Table 1. Summary of interaction of purified *Bt* toxin with surface-active particles: effects on persistence and insecticidal activity

- Larvicidal proteins from *Bacillus thuringiensis* subspp. *kurstaki* (*Btk*; antilepidopteran), *tenebrionis* (*Btt*; anticoleopteran), and *israelensis* (*Bti*; antidipteran) bound rapidly and tightly on clays, humic acids, and complexes of clay-humic acid-Al hydroxypolymers; binding was pH dependent and greatest near the isoelectric point (pI) of the proteins; binding of the toxin from *Btk* was greater than binding of the toxin from *Btt*, even though the M_r of both was similar (66 and 68 kDa, respectively).
- Bound toxins retained their structure, antigenicity, and insecticidal activity.
- Intercalation of clays by the toxins was minimal.
- Biodegradation of the toxins was reduced when bound; microbial utilization of the toxins as a source of carbon was reduced significantly more than use as a source of nitrogen.
- Larvicidal activity of bound toxins was retained.
- Larvicidal activity of the toxin from *Btk* was detected 234 days after addition to nonsterile soils (longest time studied).
- Persistence of larvicidal activity was greater in acidic soils, probably, in part, because microbial activity was lower than in less acid soils; persistence was reduced when the pH of acidic soils was raised to *ca.* 7.0 with $CaCO_3$.
- Persistence was similar under aerobic and anaerobic conditions and when soil was alternately wetted and dried or frozen and thawed, which indicated tight binding.
- Persistence in soil was demonstrated by dot-blot ELISA, flow cytometry, Western blots, and larvicidal assays.
- Toxins from *Btk*, *Btt*, and *Bti* had no microbiostatic or microbicidal effect against a spectrum of bacteria (gram positive and negative), fungi (filamentous and yeast), and algae, neither in pure nor in mixed cultures.

Table 2. Frequencies (cm^{-1}) of the infrared bands (Amide I and II) of the protoxin and toxin from *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and of the toxin from *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*) alone or bound on montmorillonite (M) or kaolinite (K) homoionic to different cations. Difference spectra obtained by Fourier-transform infrared analyses.

Sample	Amide I	Amide
Btk protoxin alone	1651	1538
M-Na	1658	1542
M-Ca	1651	1542
K-Na	1657	1546
K-Ca	1651	1543
Btk toxin alone	1651	1544
M-H	1641	1544
M-Ca	1650	1546
M-Al	1651	1540
Btt toxin alone	1651	1543
M-Na	1651	1536
M-H	1651	1536
M-K	1657	1536
M-Ca	1658	1541
M-Mg	1651	1537
M-La	1651	1537
M-Al	1646	1537
K-Na	1651	1543
K-Ca	1651	1543

(From Tapp, H., Calamai, L., and Stotzky, G., Soil Biol. Biochem. 26, 663, 1994. With permission.)

Table 3. Lethal concentration (LC_{50}) and 95% confidence interval (CI) of the toxin from *Bacillus thuringiensis* subsp. *kurstaki*, free, adsorbed, or bound on clay minerals or the clay-size fraction from soil, for larvae of the tobacco hornworm (*Manduca sexta*) after 7 days of exposure.

Clay[b]	Mortality with clay alone[c]	Clay-toxin complex[a]			
		Adsorbed		Bound	
		LC_{50} (ng/100 ml)[d]	*95% CI*	LC_{50} (ng/100 ml)[d]	95% CI
M-mix	0	22.0	16.42-27.80	30.7	20.10-50.43
K-mix	0	18.0	11.33-27.55	23.0	14.01-40.32
K-soil	0	21.9	18.67-25.77	21.4	15.82-29.12
K6M-soil	0	23.3	18.21-29.12	22.5	18.71-27.00

[a] *The toxin was evaluated after both equilibrium adsorption and binding on the clays.*
[b] *The clays were montmorillonite (M) and kaolinite (K) with a mixed-cation complement (M-mix and K-mix) and the clay-size fraction separated from Kitchawan soil, unamended (K-soil) or amended to 6% (v/v) with montmorillonite (K6M-soil).*
[c] *Mortality at each clay concentration was not significantly different from the natural mortality of the controls.*
[d] *LC_{50} of free toxin = 90.4 ng/100 ml with a 95% CI of 58.58 to 144.06 ng/100 ml.*

(From Tapp, H. and Stotzky, G., Appl. Environ. Microbiol. 61, 1786, 1995. With permission.)

Table 4 Lethal concentration (LC_{50}), 95% confidence interval (CI), and relative potency of the toxin from *Bacillus thuringiensis* subsp. *tenebrionis*, free, adsorbed, or bound on clay minerals or the clay-size fraction from soil, for larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*) after 3 days of exposure.

[a] *The toxin was evaluated after both equilibrium adsorption and binding on the clays. Two different batches of* B. thuringiensis *subsp.* tenebrionis *toxin(purified from M-One®) were used.*
[b] *The clays were montmorillonite (M) and kaolinite (K) with a mixed-cation complement (M-mix or K-mix) or homoionic to the indicated cations and the clay-size fraction separated from Kitchawan soil, unamended (K-soil) or amended to 6% (v/v) with montmorillonite (K6M-soil) or to 6% with kaolinite (K6K-soil).*
[c] *Mortality at each clay concentration was not significantly different from the natural mortality of the controls.*
[d] *Potency of adsorbed or bound toxin relative to the potency of free toxin at all concentrations.*
[e] *LC_{50} of free toxin = 1.3 µg/50 µl with a 95% CI of 1.02 to 1.62 µg/50 µl.*
[f] *LC_{50} of free toxin = 0.2 µg/50 µl with a 95% CI of 0.10 to 0.28 µg/50 µl.*

	Clay[b]	Mortality with clay alone[c]	Adsorbed	
			LC_{50} (µg/50 µl)	95% CI
Batch 1[e]	M-Na	0	1.5	1.07-2.05
	M-K	0	1.7	1.13-2.52
	M-K	0	1.2	0.81-1.71
	M-La	0	2.6	1.80-3.56
	M-mix	0	1.3	0.75-2.13
	K-mix	0	4.5	3.05-6.61
	K6M-soil	0	0.5	0.33-0.79
Batch 2[f]	K-K	0	0.4	0.15-0.75
	K-soil	0	0.3	0.16-0.38
	K6K-soil	0	0.2	0.10-0.27

(From Tapp, H. and Stotzky, G., Appl. Environ. Microbiol. 61, 1786, 1995. With permission.)

Table 5 Interlayer spacings (d_{001} value in nm) of montmorillonite (M) or kaolinite (K) homoionic to different cations and of their complexes with the protoxin or toxin from *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and of the toxin from *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*).

[a] *Values in brackets give d0001 spacings after heating at 110°C for monovalent cations or at 150°C for polyvalent cations.*
[b] *ND = not determined.*
[c] *Asymmetrical broad peak, suggesting the masking of peaks at higher spacings.*
[d] *Broad peak ranging from ca. 1.00 to 1.51 nm and indicating random interstratification.*
[e] *RI = random interstratification; tracings show a typical pattern of an interstratified clay, suggesting a random intercalation of the protein.*

(From Tapp, H., Calamai, L., and Stotzky, G., Soil Biol. Biochem. 26, 663, 1994. With permission.)

Clay-toxin complex[a]				
			Bound	
	Relative potency[d]	LC_{50} (µg/50 µl)	95% CI	Relative potency[d]
	0.86	17.8	12.93-24.57	0.07
	0.77	13.4	8.95-20.18	0.10
	1.05	9.5	6.51-13.43	0.13
	0.52	96.1	40.69-264.32	0.01
	1.05	14.1	9.45-21.05	0.10
	0.28	1.4	.95 - 1.91	0.91
	2.38	1.8	1.16 - 2.64	0.70
	0.47	0.2	0.08 - 0.34	0.95
	0.88	2.3	1.30 - 4.40	0.10
	1.16	0.4	0.25 - 0.63	0.51

Clay	Clay alone	Clay-*Btt* complex	Clay-*Btk* complex	
			Toxin	Protoxin
M-H	1.26 (1.19)[a]	2.63 (2.40)	ND[b]	ND
M-Na	1.25 (0.97)	2.57 (2.39)	1.28[c] (1.50)[d]	1.77 (1.69)
M-K	1.20 (1.01)	2.05 (1.94)	1.28 (1.22)	ND
M-Mg	1.50 (1.35)	2.05 (2.00)	RI[e] (RI)	ND
M-Ca	1.50 (0.99)	1.70 (1.63)	1.54 (1.44)	1.31 (1.26)
M-La	1.55 (1.24)	2.17 (2.08)	ND	ND
M-Al	1.51 (1.24)	1.51	1.78 (1.60)	ND
K-Na	0.72	0.71	0.72	0.72
M-H	0.72	0.71	0.71	0.72

Table 6 Lethal concentration (LC_{50}) and 95% confidence interval (CI) of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* after incubation for 234 days in soil for larvae of *Manduca sexta*.
The soils were Mopala (MOP, a tropical soil in which montmorillonite is the dominant clay mineral) and Kitchawan (K, a soil from New York in which kaolinite is the dominant clay mineral), either unamended or amended to 6% (v/v) with montmorillonite (K6M) or to 3, 6, or 9% (v/v) with kaolinite (K3K, K6K, K9K). Values in brackets indicate the LC_{50} and CI when the pH of

Day of incubation	MOP		K6K		K
	LC_{50}	95% CI	LC_{50}	95% CI	LC_{50}
0	53	42.6-65.4	71	NA[b]	34
0					55
40	416	288.8-680.0	126	96.1-157.4	29
47					44
71	677	517.9-940.0	494	NA	53
90					47
112	ND[c]	ND	ND	ND	48
134					451
146	ND	ND	ND	ND	39
184					264
195	ND	ND	ND	ND	63
234					232

the K and K6K soils was adjusted from 4.9 to approximately 7.0 with $CaCO_3$.

[a] *LC_{50} of free toxin = 91 ng/100 µl with a 95% CI of 62.0-132.4 ng/100 µl. There was no mortality with the soils alone. Two replicate tubes and 36 larvae per toxin concentration.*
[b] *NA = no data available; the 95% CI could not be calculated from the data.*
[c] *ND = not determined.*
(Modified from Tapp, H. and Stotzky, G., Soil Biol. Biochem. 30, 471, 1998. With permission.)

Soil						
	K3K		K6K		K9K	
95% CI	LC_{50}	95% CI	LC_{50}	95% CI	LC_{50}	95% CI
25.7-43.3	26	19.9-34.3	16	11.6-21.5	17	11.6-24.0
31.4-97.0			10	4.0-19.1		
22.0-37.9	77	52.8-109.4	53	32.4-84.9	42	27.6-62.5
25.0-76.7			43	23.0-80.0		
35.0-78.8	90	62.4-127.8	43	29.1-59.4	77	52.5-111.7
27.7-80.0			79	36.5-182.8		
33.7-60.5	46	33.2-60.5	72	50.6-101.0	46	30.6-66.6
NA			101	55.0-201.5		
26.4-47.8	58	31.5-77.6	47	36.6-57.1	37	22.5-55.3
109.1-684.9			312	121.4-939.4		
NA	61	35.4-85.1	69	34.0-100.1	111	70.5-181.1
126.0-500.1			195	100.9-439.1		

Table 7 Utilization of the toxin from *Bacillus thuringiensis* subsp. *kurstaki*, free or bound on homoionic clay minerals or the clay-size fraction from soil, as a source of carbon, nitrogen, or carbon plus nitrogen by various microbial cultures.

Source of organisms	Clay or soil fraction bound [a]
Mixed culture from a protein-enriched soil slurry[c]	M-Na
	M-Ca
	M-Al
	M-Na
	K-Ca
	K-soil
	K6K-soil
	K6M-soil
Soil slurry[d]	M-Na
	K-Na
	K-soil
	K6K-soil

[a] *K (kaolinite) or M (montmorillonite) homoionic to Na, Ca, or Al. See Table 4 for description of K, K6K, and K6M soils.*
[b] *+ = utilization; - = no utilization (determined by measurement of O_2 uptake by the direct Warburg method).*
[c] *Mixed culture from a protein-enriched soil slurry incubated at 37°C in Davis citrate minimal medium for 50 h.*
[d] *Mixed culture from a garden soil (1:4 [wt/vol] soil:tap water) amended with free pepsin or with free or bound toxin from* B. thuringiensis *subsp.* kurstaki *and incubated at 25°C for 7 h.*
[e] *Utilization of bound toxin as a source of nitrogen was* ca. *30% of the utilization of the free toxin.*
[f] *ND = not determined.*

(From Koskella, J. and Stotzky, G. Appl. Environ. Microbiol. 63, 3561, 1997. With permission.)

Table 8 Lethal concentration (LC_{50}) and relative potency (RP) of the toxin from *Bacillus thuringiensis* subsp. *kurstaki*, free or bound on

Utilization of toxin as source of[b]					
Carbon		Nitrogen		Carbon + nitrogen	
Free	Bound	Free	Bound	Free	Bound
+	-	+	+[e]	+	-
+	-	ND[f]	ND	ND	ND
+	-	ND	ND	ND	ND
+	-	+	+	+	-
+	-	+	+	+	-
+	-	+	+	+	-
+	-	+	+	+	-
+	-	+	+	+	-
ND	ND	ND	ND	+	-
ND	ND	ND	ND	+	-
ND	ND	ND	ND	+	-
ND	ND	ND	ND	+	-

montmorillonite (M-Na) or kaolinite (K-Na) homoionic to Na, for larvae of *Manduca sexta* after 40 days of incubation in soil in test tubes maintained at room temperature and the –33-kPa water tension, alternately wetted and dried, or alternately frozen and thawed. Data are expressed as the mean lethal concentration (LC_{50}) ± the standard errors of the means (SEM).

[a] *LC_{50} was measured after incubation of the toxin, free or bound, for 40 days in K-soil (100 µg toxin/g of soil) continuously at the –33-kPa water tension and room temperature, which was then alternately air dried and rewetted or alternately frozen and thawed.*

Treatment	LC_{50} (µg/100µl) ± SEM[a]				
		Toxin bound on[b]		RP of toxin bound on[c]	
	Free toxin	M-Na	K-Na	M-Na	K-Na
Stock[d]	0.7 ± 0.01	0.7 ± 0.01	0.5 ± 0.00	1.0	1.4
Room temperature[e]	5.5 ± 1.55	3.0 ± 0.90	3.9 ± 1.00	1.8	1.4
Air dried and rewetted[f]	11.2 ± 3.30	17.2 ± 5.36	17.0 ± 1.54	0.7	0.7
Frozen and thawed[g]	24.0 ± 2.25	13.4 ± 0.34	7.6 ± 0.70	1.8	3.2

[b] *M-Na, montmorillonite homoionic to sodium; K-Na, kaolinite homoionic to sodium.*
[c] *RP = relative potency: potency of bound toxin relative to potency of free toxin.*
[d] *Free toxin was stored as aliquots at –20°C, and bound toxin was stored as a clay-toxin pellet at 4°C; both were assayed for their LC_{50} immediately after purification or binding on clay minerals.*
[e] *Maintained at room temperature at the –33-kPa water tension.*
[f] *Maintained at room temperature at the –33-kPa water tension for 7 days, air dried for 7 days, and rewetted and maintained at the –33-kPa water tension for 7 days. This cycle was repeated twice.*
[g] *Maintained at room temperature at the –33-kPa water tension for 7 days and then at –20°C for 7 days. This cycle was repeated three times.*
(From Koskella, J. and Stotzky, G., Appl. Environ. Microbiol. 63, 3561, 1997. With permission.)

Table 9 Lethal concentration (LC_{50}) and 95% confidence interval (CI) of the toxin from *Bacillus thuringiensis* subsp. *kurstaki*, free or bound on humic acids from four soils, for the larvae of *Manduca sexta*.

Source of humic acids (Soil)	Mortality with humic acids alone[a]	Humic acid-toxin complex	
		LC_{50} (ng /100 µl)[b]	95% CI[c]
Sardegna	0	233	192.1 - 280.8
Foresta Umbra	0	254	209.4 - 307.5
Hop	0	272	225.6 - 326.5
Sugar beet	0	215	177.4 - 259.7

a Concentration of humic acids at 10 times the concentration present in the humic acid-toxin complexes.
b LC_{50} of free toxin = 304 ng/100 µl with a 95% CI of 251.1 to 367.5 ng/100 µl.
c CI = confidence interval.
(From Crecchio, C. and Stotzky, G., Soil Biol. Biochem. 30, 463, 1998. With permission.)

Table 10 Summary of effects on some microbe-mediated processes in soil of adding 0.5, 1.0, or 2.0% (w/w) ground, air-dried leaves or stems of corn containing (*Bt+*) (NK6800Bt) or not containing (*Bt-*) (NK6800) the *cry1Ab* gene from *Bacillus thuringiensis* subsp. *kurstaki*.

Process	Among leaves	Among stems	Between leaves & stems
CO_2 evolution	Lower with *Bt+*	Lower with *Bt+*	Higher with stems
Alkaline phosphatase	Lower with *Bt+*	NCD[a]	Higher with stems
Acid phosphatase	Higher with *Bt+*	NCD	NCD
Protease	NCD	NCD	NCD
Dehydrogenase	NCD	NCD	NCD
Bacteria[b]	NCD	NCD	NCD
Fungi[c]	NCD	NCD	NCD

[a] *NCD = no consistent differences over a 42-day incubation.*
[b] *Soil dilutions plated on soil extract agar containing cycloheximide.*
[c] *Soil dilutions plated on rose bengal-streptomycin agar.*

(From Flores, S., Saxena, D., and Stotzky, G., unpublished data.)

Table 11 Lignin content of different hybrids of corn with (*Bt+*) and without (*Bt-*) the *cry1Ab* gene grown in a plant growth room.

Company	*Bt+*		
	Hybrid	Event	% Lignin
Novartis	N7590Bt	Bt11	7.2 ± 0.10
Novartis	N67-T4	Bt11	6.3 ± 0.25
Novartis	N3030Bt	Bt11	7.0 ± 0.22
Novartis	NC4990Bt	Bt11	6.6 ± 0.18
Novartis	NK4640Bt	Bt11	6.3 ± 0.14
Novartis	Maximizer[a]	176	4.0 ± 0.15
Pioneer	P31B13	MON810	6.0 ± 0.24
DeKalb	DK647Bty	MON810	6.2 ± 0.25
DeKalb	DK679Bty	MON810	6.6 ± 0.11
DeKalb	DK626Bty	MON810	6.1 ± 0.20

Table 12 Lignin content of different hybrids of corn with (*Bt+*) and without (*Bt-*) the *cry1Ab* gene grown in the field.

Company	*Bt+*		
	Hybrid	Event	% Lignin
Novartis	N7590Bt	Bt11	7.4 ± 0.15
Novartis	N67-T4	Bt11	7.1 ± 0.22
Novartis	NC4990Bt	Bt11	7.7 ± 0.06
Novartis	NK4640Bt	Bt11	7.9 ± 0.10
Novartis	Maximizer[a]	176	5.9 ± 0.13
Pioneer	P32P76	MON810	6.8 ± 0.14
Pioneer	P31B13	MON810	7.0 ± 0.04
DeKalb	DK626Bty	MON810	6.8 ± 0.15

[a] *Isoline of* Bt- *Maximizer not available.*
(From Saxena, D. and Stotzky, G., Am. J. Bot., 88,1704, 2001).

Hybrid	*Bt-* % Lignin	P
N7590	4.8 ± 0.14	0.00001
N67-H6	3.9 ± 0.15	0.00175
N3030	4.4 ± 0.22	0.00003
NC4880	3.4 ± 0.27	0.00020
NK4640	3.2 ± 0.12	0.00001
--	--	--
P3223	3.2 ± 0.18	0.00032
DK647	4.4 ± 0.22	0.00174
DK679	3.8 ± 0.10	0.00005
DK626	3.2 ± 0.18	0.00006

[a] *Isoline of* Bt- *Maximizer not available.*
(From Saxena, D. and Stotzky, G., Am. J. Bot., 88,1704, 2001)

Hybrid	*Bt-* % Lignin	P
N7590	5.2 ± 0.11	0.00007
N67-H6	4.5 ± 0.14	0.00082
NC4880	4.8 ± 0.22	0.00033
NK4640	4.9 ± 0.09	0.00001
--	--	--
P32P75	4.8 ± 0.22	0.00016
P3223	4.9 ± 0.13	0.00007
DK626	5.1 ± 0.12	0.00085

Table 13 Presence of toxin in root exudates from corn with (*Bt+*) (NK4640Bt) and without (*Bt-*) (NK4640) the *cry1Ab* gene.

[a] *Assayed immunologically and by larvicidal assay* (Manduca sexta) *on indicated days after germination of seeds, which was usually 3 days after planting.*

[b] *Determined with Lateral Flow Quickstix: __ = no toxin detected; + = toxin detected.*

Growth conditions	Day assayed				
	7				
	Immuno. test[b]		Toxicity (LC_{50})[c]		Immuno.
	Bt-	*Bt+*	*Bt-*	*Bt+*	*Bt-*
Hoagland's solution	-	+	NT[d]	5.6 µg (2.61-7.82)	-
Soil	-	+	NT	2.3 µg (1.32-3.86)	-

[c] *Toxicity [concentration necessary to kill 50% of larvae (LC_{50})] expressed in µg of total protein/bioassay vial and 95% confidence interval (in parentheses).*
[d] *NT= No toxicity.*
(Modified from Saxena, D., Flores, S., and Stotzky, G., Nature 402,480, 1999. With permission.)

after germination of seed[a]

15					25	
est	**Toxicity (LC_{50})**		**Immuno. test**		**Toxicity (LC_{50})**	
Bt+	*Bt-*	*Bt+*	*Bt-*	*Bt+*	*Bt-*	*Bt+*
+	NT	5.2 µg (2.81-8.21)	-	-	NT	NT
+	NT	1.8 µg (0.96-3.40)	-	+	NT	1.6 µg (0.86-2.62)

Table 14 Larvicidal activity, expressed as % mortality and mean weight (in g) of a single larva ± standard error of the mean (in parentheses), of rhizosphere soil from corn with (Bt+) (NK4640Bt) and without (Bt-) (NK4640) the *cry1Ab* gene grown in a plant-growth room[a].

[a] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; weight data normalized to one larva/treatment.*

Soil[c]	Day assayed			
	10		20	
	Bt-	*Bt+*	*Bt-*	*Bt+*
C	0 (1.0 ± 0.05)	50 ±10.2 (0.4 ± 0.08)	0 (1.1 ± 0.02)	50 ±10.2 (0.2 ± 0.02)
3K	0 (1.0 ± 0.04)	25 ±10.2 (0.5 ± 0.07)	0 (1.1 ± 0.03)	38 ± 7.2 (0.2 ± 0.01)
6K	0 (1.0 ± 0.03)	25 ±10.2 (0.4 ± 0.04)	0 (1.1 ± 0.08)	38 ± 7.2 (0.2 ± 0.02)
9K	0 (1.0 ± 0.04)	75 ±10.2 (0.1 ± 0.04)	0 (1.0 ± 0.02)	75 ±10.2 (0.2 ± 0.03)
12K	0 (0.8 ± 0.06)	50 ±10.2 (0.3 ± 0.01)	0 (1.1 ± 0.05)	63 ± 12.5 (0.2 ± 0.02)
3M	0 (1.0 ± 0.04)	38 ± 7.2 (0.5 ± 0.02)	0 (1.0 ± 0.04)	50 ±10.2 (0.2 ± 0.04)
6M	0 (0.8 ± 0.01)	100 ± 0.0	0 (1.0 ± 0.04)	100 ± 0.0
9M	0 (1.0 ± 0.02)	100 ± 0.0	0 (1.0 ± 0.03)	100 ± 0.0
12M	0 (1.0 ± 0.01)	50 ±10.2 (0.8 ± 0.04)	0 (1.0 ± 0.05)	100 ± 0.0

No mortality with soils without plants (weight of a single larva: 0.8 to 1.3 ± 0.02 g).
[b] Usually 3 days after planting.
[c] Soil amended to 3,6,9 or 12% (vol/vol) with kaolinite (K) or montmorillonite (M) or not amended with clay (C).
(From Saxena, D. and Stotzky, G., FEMS Microbiol. Ecol. 33, 35, 2000. With permission)

after germination of seed[a]

30		40	
Bt-	*Bt+*	*Bt-*	*Bt+*
0 (1.2 ± 0.02)	63 ± 7.2 (0.1 ± 0.01)	0 (0.9 ± 0.06)	100 ± 0.0
0 (1.2 ± 0.08)	38 ± 7.2 (0.1 ± 0.01)	0 (1.0± 0.02)	88 ± 7.2 (0.1 ± .02)
0 (1.1 ± 0.03)	50 ±10.2 (0.1 ± 0.02)	0 (0.8 ± .06)	88 ± 7.2 (0.1 ± .01)
0 (1.2 ± 0.04)	88 ± 7.2 (0.1 ± 0.01)	0 (0.9 ± 0.02)	100 ± 0.0
0 (1.2 ± 0.03)	88 ± 7.2 (0.1 ± 0.03)	0 (0.9 ± 0.05)	100 ± 0.0
0 (1.2 ± 0.02)	63 ± 12.5 (0.1 ± 0.01)	0 (0.9 ± 0.05)	100 ± 0.0
0 (1.1 ± 0.05)	100 ± 0.0	0 (0.9 ± 0.03)	100 ± 0.0
0 (1.2 ± 0.01)	100 ± 0.0	0 (1.0 ± 0.04)	100 ± 0.0
0 (1.0 ± 0.01)	100 ± 0.0	0 (1.0 ± 0.02)	100 ± 0.0

Table 15 Larvicidal activity, expressed as % mortality and mean weight (in g) of a single larva ± standard error of the mean (in parentheses), of rhizosphere soil from corn (*Bt+*) (NK4640Bt) and without (*Bt-*) (NK4640) the *cry1Ab* gene grown *in situ* under field conditionsa.

Plant	% Mortality[b]	
	***Bt-* Corn**	***Bt+* Corn**
Before frost		
F1	0 (0.9 ± 0.01)	38 ± 7.2 (0.3 ± 0.02)
F2	0 (0.9 ± 0.03)	100 ± 0.0
F3	0 (1.1 ± 0.03)	38 ± 12.5 (0.5 ± 0.09)
F4	0 (1.0 ± 0.02)	100 ± 0.0
F5	0 (1.0 ± 0.03)	38 ± 7.2 (0.6 ± 0.01)
F6	0 (1.0 ± 0.02)	38 ± 7.2 (0.5 ± 0.02)
After frost		
F7	0 (1.0 ± 0.07)	75 ± 10.2 (0.1 ± 0.01)
F8	ND[c]	88 ± 12.5 (0.03 ± 0.00)

[a] *Immunological tests with Lateral Flow Quickstix showed the presence of toxin in the rhizosphere soil of all* Bt+ *plants but not of* Bt- *plants.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; weight data normalized to one larva/treatment.*
No mortality with soil without plants (weight of a single larva: 0.8 to 1.3 ± 0.02 g.)
[c] *Not determined*
(From Saxena, D. and Stotzky, G., FEMS Microbial Ecol. 33, 35, 2000. With permission.)

Table 16 Presence of the Cry1Ab toxin in rhizosphere soil of different hybrids of corn with (Bt+) and without (Bt-) the *cry1Ab* gene grown in a plant-growth room.

Company	Bt+				Bt-		
	Hybrid	Event	Imm. test[a]	% Mortality[b]	Hybrid	Imm. test	% Mortality
Novartis	N7590Bt	Bt11	+	93 ± 6.3 (0.03 ± 0.01)	N7590	-	0 (0.8 ± 0.03)
Novartis	N67-T4	Bt11	+	81 + 6.3 (0.05 ± 0.02)	N67-H6	-	0 (0.8 ± 0.02)
Novartis	N3030Bt	Bt11	+	38 ± 7.2 (0.15 ± 0.02)	N3030	-	6.3 ± 6.25 (0.8 ± 0.03)
Novartis	NC4990Bt	Bt11	+	81 ± 6.3 (0.11 ±0.09)	NC4880	-	0 (0.9 ± 0.04)
Novartis	NK4640Bt	Bt11	+	100 ± 0.0 (-)	NK4640	-	0 (1.1 ± 0.05)
Novartis	Maximizer[c]	176	+	43 ± 11.9 (0.08 ± 0.02)	-- --		-- --
Pioneer	P32P76	MON 810	+	81 ± 6.3 (0.07 ± 0.05)	P32P75	-	0 (0.8 ± 0.02)
Pioneer	P33B51	MON 810	+	93 ± 6.3 (0.02 ± 0.01)	P33B50	-	0 (0.9 ± 0.06)
Pioneer	P31B13	MON 810	+	50 ± 10.2 (0.07 ± 0.02)	P3223	-	0 (0.9 ± 0.04)
DeKalb	DK647Bty	MON 810	+	56 ± 11.9 (0.10 ± 0.07)	DK647	-	6.3 ± 6.25 (0.9 ± 0.04)
DeKalb	DK679Bty	MON 810	+	68 ± 18.8 (0.14 ± 0.02)	DK679	-	6.3 ± 6.25 (0.8 ± 0.02)
DeKalb	DK626Bty	MON 810	+	68 ± 6.3 (0.08 ± 0.08)	DK626	-	0 (0.7 ± 0.09)

[a] *Determined with Lateral Flow Quickstix; - = no toxin detected; + = toxin detected.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; weight data normalized to one larva/ treatment ± standard error of the mean (in parentheses). No mortality with soils without plants (weight of a single larva: 0.7 to 1.2 ± 0.02 g).*
[c] *Isoline of* Bt- *Maximizer not available*
(Modified from Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 133, 2002).

Table 17 Presence of the Cry1Ab toxin in rhizosphere soil of different hybrids of corn with (*Bt+*) and without (*Bt-*) the *cry1Ab* gene grown in the field.

Company	Bt+				Bt-		
	Hybrid	Event	Imm. test[a]	% Mortality[b]	Hybrid	Imm. test	% Mortality
Novartis	N7590Bt	Bt11	+	75 ± 10.2 (0.05 ± 0.02)	N7590	-	6.3 ± 6.25 (0.9 ± 0.06)
Novartis	N67-T4	Bt11	+	68± 11.9 (0.02 ± 0.01)	N67-H6	-	0 (1.2 ± 0.08)
Novartis	N3030Bt	Bt11	+	37 ± 7.2 (0.09 ± 0.04)	N3030	-	0 (0.9 ± 0.03)
Novartis	NC4990Bt	Bt11	+	62 ± 12.5 (0.07 ±0.02)	NC4880	-	6.3 ± 6.25 (1.1 ± 0.07)
Novartis	NK4640Bt	Bt11	+	81 ± 6.25 (0.09 ±0.01)	NK4640	-	0 (1.1 ± 0.09)
Novartis	0966 (Supersweet)[c]	Bt11	+	63 ± 6.5 (0.09 ± 0.05)	PrimePlus (Supersweet)	-	6.3 ± 6.25 (0.8 ± 0.02)
Novartis	Maximizer[c]	176	+	37 ± 7.2 (0.18 ± 0.03)	-- --		-- --
Pioneer	P32P76	MON 810	+	68 ± 15.7 (0.08 ± 0.02)	P32P75	-	0 (0.9 ± 0.06)
Pioneer	P33B51	MON 810	+	68 ± 15.7 (0.09 ± 0.01)	P33B50	-	0 (0.9 ± 0.04)
Pioneer	P31B13	MON 810	+	43 ± 6.5 (0.06 ± 0.03)	P3223	-	6.3 ± 6.25 (0.9 ± 0.05)
DeKalb	DK647Bty	MON 810	+	37 ± 12.5 (0.08 ± 0.04)	DK647	-	0 (1.2 ± 0.08)
DeKalb	DK679Bty	MON 810	+	56 ± 11.9 (0.04 ± 0.01)	DK679	-	12.5 ± 6.25 (0.8 ± 0.03)
DeKalb	DK626Bty	MON 810	+	50 ± 10.2 (0.09 ± 0.03)	DK626	-	6.3 ± 6.25 (0.9 ± 0.01)

[a] *Determined with Lateral Flow Quickstix; - = no toxin detected; + = toxin detected.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; weight data normalized to one larva/ treatment ± standard error of the mean (in parentheses). No mortality with soils without plants (weight of a single larva: 0.8 to 1.2 ± 0.04 g).*
[c] *Soil kindly provided by Dr. David Andow.*
[d] *Isoline of* Bt- *Maximizer not available.*
(Modified from Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 133, 2002).

Table 18 Presence of Cry1Ab (corn and rice), Cry1Ac (canola, cotton, and tobacco), and Cry3A (potato) protein from *Bacillus thuringiensis* (*Bt*) in rhizosphere soil and hydroponic solution from plants with (*Bt+)* and without (*Bt-*) the *cry1Ab*, *cry1Ac*, and *cry3A* gene, respectively.

Plant	Soil[a]			Hydroponic solution[a]		
	Immuno test[b]	% Mortality[c]	Larval weight (mg)[c]	Immuno test	% Mortality	Larval weight (mg)
Corn						
Bt-	-	0	1170 ± 42	-	6.3 ± 6.30	1030 ± 32
Bt+	+	100 ± 0.0	0.0	+	68.7 ± 11.90	120 ± 12
Rice						
Bt-	-	6.3 ± 6.30	1200 ± 38	-	0	1130 ± 45
Bt+	+	63.3 ± 6.30	220 ± 18	+	43.5 ± 7.25	120 ± 30
Cotton						
Bt-	-	6.3 ± 6.30	990 ± 42	-	6.3 ± 6.30	920 ± 31
Bt+	-	6.3 ± 6.30	920 ± 57	-	0	930 ± 50
Canola						
Bt-	-	6.3 ± 6.30	980 ± 24	-	6.3 ± 6.30	940 ± 31
GFP[d]	-	0	860 ± 26	-	0	1,100 ± 70
Bt+	-	12.5 ± 7.25	780 ± 36	-	6.3± 6.30	850 ± 33
GFP*Bt+*	-	12.5 ± 7.25	740 ± 21	-	12.5± 7.25	840 ± 38
Tobacco						
Bt-	-	6.3 ± 6.30	1,260 ± 93	-	0	1,140 ± 92
GFP	-	6.3 ± 6.30	960 ± 36	-	0	930 ± 50
Bt+	-	0	860 ± 43	-	12.5± 7.25	870 ± 34
GFP*Bt+*	-	12.5 ± 7.25	810 ± 53	-	12.5± 7.25	780 ± 56
Potato						
Bt-	-	27.9 ± 3.63	ND[e]	-	39.5± 2.75	ND
Bt+	+	66.3±10.21	ND	+	77.0± 2.75	ND

[a] *Days of growth after planting: corn, soil: 40, solution: 35; rice, soil: 50, solution: 60; canola, soil: 55 days, solution: 40; tobacco, soil: 65, solution: 55; cotton, soil: 55, solution: 40; potato, soil: 65, solution: 35.*

[b] *Cry1Ab and Cry1Ac proteins were determined with EnviroLogix Lateral Flow Quickstix, and Cry3A protein was determined with Agdia ELISA Kit; - = no toxin detected; + = toxin detected.*

[c] *Larvicidal activity of Cry1Ab and Cry1Ac proteins was determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; expressed as % mortality and mean weight, in mg, of a single larva ± standard error of the mean. Larvicidal activity of Cry3A protein was determined with the larvae of the Colorado potato beetle* (Leptinotarsa decemlineata)*; at least 80 larvae/assay; expressed as % mortality. No significant mortality in soil without plants.*

[d] *Green fluorescent protein (canola and tobacco were engineered to contain the*

genes for Bt *protein, GFP, or both).*
[e] *Not determined.*
(From Saxena, D. and Stotzky, G., unpublished data.)

Table 19 Proteins in leachates from soil columns at different times after adding 0.8 µg of Cry1Ab protein/g of soil (oven-dry weight equivalent).

Soil[a]	Time (in h) when columns were leached after addition of Cry1Ab protein				% of added Cry1Ab protein recovered in leachates
	0.1[c]	1	3	9	
	Protein (µg)[b]				
C	11 ± 1.5	10 ± 0.8	6 ± 0.8	3 ± 0.2	75± 6.3
3K	13 ± 1.4	6 ± 0.5	5 ± 0.5	1 ± 0.4	63 ± 6.3
6K	10 ± 0.6	8 ± 0.5	5 ± 0.6	1 ± 0.3	60± 5.6
9K	0	9 ± 1.0	6 ± 0.4	0	38 ± 5.3
12K	0	8 ± 0.4	3 ± 0.4	0	28 ± 1.7
3M	11 ± 0.4	8 ± 0.8	4 ± 0.5	2 ± 0.3	63 ± 5.1
6M	9 ± 0.6	9 ± 1.0	5 ± 0.7	0	58 ± 6.3
9M	0	9 ± 1.0	4 ± 0.4	0	33 ± 4.6
12M	0	6 ± 1.1	2 ± 0.3	0	20 ± 1.1

[a] *The soil was either not amended [Control (C)] or amended to 3, 6, 9, or 12% (vol/vol) with montmorillonite (M; 3M, 6M, 9M, or 12M soil) or kaolinite (K; 3K, 6K, 9K, or 12K soil).*
[b] *Protein estimated by the Lowry method; mean ± standard error of the mean. Total protein from columns with Cry 1Ab protein – total protein from control columns without Cry1Ab protein.*
[c] *Times (in h) after adding Cry1Ab protein to the top of soil columns containing 50 g of soil (oven-dry weight equivalent). The columns were leached with 5 ml of tap water at each indicated time.*
(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 20 Proteins in leachates from soil columns at different times after adding 1.6 µg of Cry1Ab protein/ g of soil (oven-dry weight equivalent).

Soil[a]	Time (in h) when columns were leached after addition of Cry1Ab protein				% of added Cry1Ab protein recovered in leachates
	0.1 h[c]	1h	3h	9h	
	Protein (µg)[b]				
C	28 ± 1.2	20 ± 0.8	7 ± 0.7	3 ± 0.2	73 ± 5.3
3K	21 ± 0.7	15 ± 0.4	5 ± 0.1	3 ± 0.1	55 ± 3.4
6K	22 ± 1.0	13 ± 0.2	5 ± 0.3	0	50 ± 3.4
9K	0	20 ± 0.5	5 ± 0.4	0	31 ± 6.1
12K	0	13 ± 0.1	5 ± 0.2	0	23 ± 2.1
3M	23 ± 0.8	16 ± 0.4	6 ± 0.4	3 ± 0.2	60 ± 5.5
6M	22 ± 0.7	12 ± 0.5	6 ± 0.4	0	50 ± 4.2
9M	0	10 ± 0.4	5 ± 0.1	0	19 ± 3.6
12M	0	10 ± 0.5	3 ± 0.1	0	16 ± 2.2

[a] *The soil was either not amended [Control (C)] or amended to 3, 6, 9, or 12% (vol vol^{-1}) with montmorillonite (M; 3M, 6M, 9M, or 12M soil) or kaolinite (K; 3K, 6K, 9K, or 12K soil).*

[b] *Protein estimated by the Lowry method; mean ± standard error of the mean. Total protein from columns with Cry 1Ab protein – total protein from control columns without Cry 1Ab protein.*

[c] *Times (in h) after adding Cry1Ab protein to the top of soil columns containing 50 g of soil (oven-dry weight equivalent). The columns were leached with 5 ml of tap water at each indicated time.*

(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 21 Detection and larvicidal activity of the Cry1Ab protein in leachates from soil columns to which purified Cry1Ab protein (0.8 µg/ g soil) had been added[a].

Soil[d]	Time (in h) when columns were				
	0.1		1		
	IT[c]	% Mortality[d]	IT	% Mortality	IT
C	+	56 ± 6.3 (0.1 ± 0.02)	+	37 ± 12.5 (0.06 ± 0.04)	+
3K	+	12 ± 7.3 (0.1 ± 0.03)	+	25 ± 14.4 (0.2 ± 0.04)	+
6K	+	18 ± 6.3 (0.07 ± 0.04)	+	18 ± 6.3 (0.1 ± 0.04)	+
9K	-	0 (0.9 ± 0.08)	+	18 ± 6.3 (0.6 ± 0.03)	+
12K	-	0 (1.2 ± 0.09)	+	25 ± 14.4 (0.4 ± 0.04)	+
3M	+	37 ± 7.2 (0.08 ± 0.03)	+	25 ± 14.4 (0.08 ± 0.01)	+
6M	+	31 ± 6.3 (0.2 ± 0.08)	+	25 ± 14.4 (0.6 ± 0.07)	+
9M	-	6.3 ± 6.3 (0.9 ± 0.08)	+	31 ± 12.2 (0.08 ± 0.01)	+
12M	-	0 (0.8 ± 0.04)	+	18 ± 11.9 (0.2 ± 0.07)	+

[a] *Columns containing 50 g of soil (oven-dry weight equivalent) to which 0.8 µg/ g soil (oven-dry weight equivalent) of purified Cry1Ab protein had been added on the top were leached with 5 ml of tap water at each indicated time.*

[b] *The soil was either not amended [Control (C)] or amended to 3, 6, 9, or 12% (vol/ vol) with montmorillonite (M; 3M, 6M, 9M, or 12M soil) or kaolinite (K; 3K, 6K, 9K, or 12K soil).*

[c] *Immunological test (IT) determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*

[d] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva ±*

standard error of the mean (in parentheses). Larvicidal activity of leachates collected after 6, 12, and 24 h was not determined. Mortality with leachates from soil columns to which no Cry1Ab protein had been added ranged from 0 to 13 ± 12.5%, and the

leached after addition of Cry1Ab protein

3	6	9		12	24
% Mortality	IT	IT	% Mortality	IT	IT
37 ± 11.9 (0.1 ± 0.01)	+	+	18 ± 11.9 (0.4 ± 0.02)	-	-
43 ± 6.3 (0.1 ± 0.03)	+	+	0 (0.2 ± 0.07)	-	-
25 ± 6.3 (0.08 ± 0.02)	+	+	6.3 ± 6.3 (0.7 ± 0.06)	-	-
18 ± 6.3 (0.08 ± 0.04)	-	-	0 (1.1 ± 0.08)	-	-
12 ± 6.3 (0.1 ± 0.04)	-	-	6.3 ± 6.3 (0.9 ± 0.01)	-	-
31 ± 12.5 (0.1 ± 0.02)	+	+	12 ± 7.2 (0.8 ± 0.04)	-	-
31 ± 12.5 (0.1 ± 0.02)	-	-	0 (0.8 ± 0.03)	-	-
12 ± 6.3 (0.3 ± 0.05)	-	-	0 (0.9 ± 0.05)	-	-
12 ± 6.3 (0.1 ± 0.02)	-	-	12 ± 12.5 (0.7 ± 0.01)	-	-

weight of a single larva ranged from 0.7 ± 0.01 to 1.3 ± 0.03 g.
(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 22 Detection and larvicidal activity of the Cry1Ab protein in leachates from soil columns to which purified Cry1Ab protein (1.6 µg/ g soil) had been added[a].

Soil[b]	Time (in h) when columns were				
	0.1		1		
	IT[c]	% Mortality[d]	IT	% Mortality	IT
C	+	62 ± 12.5 (0.06 ± 0.01)	+	43 ± 6.3 (0.05 0.01)	+
3K	+	37 ± 7.2 (0.1 ± 0.03)	+	37 ± 12.5 (0.1 ± 0.03)	+
6K	+	25 ± 14.4 (0.03 ± 0.01)	+	25 ± 0.0 (0.05 ± 0.02)	+
9K	-	0 (0.9 ± 0.08)	+	31 ± 12.5 (0.08 ± 0.01)	+
12K	-	0 (0.9 ± 0.01)	+	12 ± 12.5 (0.4 ± 0.04)	+
3M	+	43 ± 6.3 (0.06 ± 0.01)	+	25 ± 14.4 (0.06 ± 0.03)	+
6M	+	37 ± 12.5 (0.3 ± 0.03)	+	43 ± 11.9 (0.5 ± 0.02)	+
9M	-	0 (1.2 ± 0.03)	+	25 ± 14.4 (0.3 ± 0.07)	+
12M	-	0 (0.9 ± 0.03)	+	12 ± 7.2 (0.08 ± 0.03)	+

[a] *Columns containing 50 g of soil (oven-dry weight equivalent) to which 1.6 µg/ g soil (oven-dry weight equivalent) of purified Cry1Ab protein had been added on the top were leached with 5 ml of tap water at each indicated time.*

[b] *The soil was either not amended [Control (C)] or amended to 3, 6, 9, or 12% (vol/ vol) with montmorillonite (M; 3M, 6M, 9M, or 12M soil) or kaolinite (K; 3K, 6K, 9K, or 12K soil).*

[c] *Immunological test (IT) determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*

[d] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva ±*

standard error of the mean (in parentheses). Larvicidal activity of leachates collected at 6, 12, and 24 h was not determined. Mortality with leachates from soil columns to which no Cry1Ab protein had been added ranged from 0 to 13 ± 12.5%, and the

leached after addition of Cry1Ab protein

3	6	9		12	24
% Mortality	IT	IT	% Mortality	IT	IT
43 ± 11.9 (0.08 ± 0.03)	+	+	12 ± 12.5 (0.5 ± 0.09)	-	-
31 ± 12.5 (0.1 ± 0.04)	+	+	0 (0.3 ± 0.02)	-	-
25 ± 14.4 (0.07 ± 0.03)	+	-	6.3 ± 6.3 (0.8 ± 0.02)	-	-
18 ± 6.3 (0.09 ± 0.06)	+	-	0 (1.2 ± 0.05)	-	-
18 ± 6.3 (0.09 ± 0.04)	-	-	6.3 ± 6.3 (1.3 ± 0.03)	-	-
31 ± 12.5 (0.1 ± 0.03)	+	+	20 ± 10.5 (0.8 ± 0.04)	-	-
37 ± 12.5 (0.08 ± 0.04)	+	-	0 (0.9 ± 0.04)	-	-
20 ± 10.2 (0.2 ± 0.04)	-	-	0 (1.1 ± 0.06)	-	-
18 ± 6.3 (0.1 ± 0.06)	-	-	6.3 ± 6.3 (0.8 ± 0.05)	-	-

weight of a single larva ranged from 0.7 ± 0.01 to 1.3 ± 0.03 g.
(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 23 Detection and larvicidal activity of the Cry1Ab protein in leachates from soil columns to which purified Cry1Ab protein (3.2 µg/ g soil) had been added[a].

Soil[d]	Time (in h) when columns were				
	0.1		1		
	IT[c]	% Mortality[d]	IT	% Mortality	IT
C	+	68 ± 11.9 (0.05 ± 0.01)	+	43 ± 11.9 (0.08 ± 0.01)	+
3K	+	50 ± 10.2 (0.2 ± 0.03)	+	31 ± 12.5 (0.2 ± 0.03)	+
6K	+	43 ± 6.3 (0.08 ± 0.02)	+	25 ± 14.4 (0.06 ± 0.03)	+
9K	-	0 (1.3 ± 0.04)	+	18 ± 6.3 (0.3 ± 0.02)	+
12K	-	0 (1.1 ± 0.08)	+	18 ± 6.3 (0.3 ± 0.06)	+
3M	+	56 ± 11.9 (0.08 ± 0.03)	+	43 ± 6.5 (0.08 ± 0.01)	+
6M	+	43 ± 11.9 (0.1 ± 0.02)	+	25 ± 0.0 (0.4 ± 0.07)	+
9M	-	0 (1.3 ± 0.02)	+	37 ± 12.5 (0.08 ± 0.01)	+
12M	-	0 (1.1 ± 0.02)	+	25 ± 0.0 (0.1 ± 0.06)	+

[a] *Columns containing 50 g of soil (oven-dry weight equivalent) to which 3.2 µg/ g soil (oven-dry weight equivalent) of purified Cry1Ab protein had been added on the top were leached with 5 ml of tap water at each indicated time.*

[b] *The soil was either not amended [Control (C)] or amended to 3, 6, 9, or 12% (vol/ vol) with montmorillonite (M; 3M, 6M, 9M, or 12M soil) or kaolinite (K; 3K, 6K, 9K, or 12K soil).*

[c] *Immunological test (IT) determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*

[d] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva ±*

standard error of the mean (in parentheses). Larvicidal activity of leachates collected at 6, 12, and 24 h was not determined. Mortality with leachates from soil columns to which no Cry1Ab protein had been added ranged from 0 to 13 ± 12.5%, and the

leached after addition of Cry1Ab protein

3	6	9		12	24
% Mortality	IT	IT	% Mortality	IT	IT
37 ± 12.5 (0.09 ± 0.05)	+	+	18 ± 6.3 (0.3 ± 0.04)	-	-
25 ± 0.0 (0.09 ± 0.07)	+	+	12 ± 7.2 (0.3 ± 0.02)	-	-
25 ± 14.4 (0.07 ± 0.02)	+	-	6.3 ± 6.3 (0.8 ± 0.07)	-	-
25 ± 14.4 (0.09 ± 0.05)	+	-	6.3 ± 6.3 (1.0 ± 0.03)	-	-
18 ± 6.3 (0.2 ± 0.08)	+	-	0 (0.8 ± 0.04)	-	-
43 ± 11.9 (0.2 ± 0.03)	+	+	12 ± 7.2 (0.1 ± 0.01)	-	-
18 ± 6.3 (0.08 ± 0.01)	+	-	0 (0.9 ± 0.02)	-	-
18 ± 6.3 (0.1 ± 0.06)	-	-	0 (1.1 ± 0.04)	-	-
12 ± 6.3 (0.09 ± 0.01)	-	-	0 (0.9 ± 0.02)	-	-

weight of a single larva ranged from 0.7 ± 0.01 to 1.3 ± 0.03 g.
(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 24 Retention of Cry1Ab protein at different depths in columns of different soils, as determined by mortality and reduction in growth of *Manduca sexta*[a].

Soil[d]	Cry1Ab protein added (µg/g			
	0.8			
				Depth at which
	2-4	70-80	140-150	2-4
C	12 ± 7.2 (0.2 ± 0.07)	25 ± 14.4 (0.2 0.06)	18 ± 6.3 (0.4 ± 0.06)	37 ± 7.2 (0.08 ± 0.03)
3K	18 ± 6.3 (0.1 ± 0.03)	37 ± 12.5 (0.05 ± 0.01)	0 (0.3 ± 0.05)	68 ± 11.9 (0.4 ± 0.01)
12K	37 ± 7.2 (0.06 ± 0.03)	12 ± 7.2 (0.1 ± 0.05)	6.3 ± 6.3 (0.5 ± 0.05)	100 (-)
3M	31 ± 6.3 (0.04 ± 0.01)	25 ± 14.4 (0.07 ± 0.03)	12 ± 7.2 (0.4 ± 0.07)	81 ± 11.9 (0.05 ± 0.03)
12M	50 ± 10.2 (0.04 ± 0.01)	18 ± 11.9 (0.2 ± 0.07)	12 ± 12.5 (0.4 ± 0.03)	100 (-)

[a] *Columns containing 50 g of soil (oven-dry weight equivalent) to which purified Cry1Ab protein was added on the top were leached six times with 5 ml of tap water/ leaching over 24 h.*

[b] *The soil was either not amended [Control (C)] or amended to 3 or 12% (vol/ vol) with montmorillonite (M; 3M or 12M soil) or kaolinite (K; 3K or 12K soil).*

[c] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16*

soil, oven-dry weight equivalent)				
3.2			None	
soil was sampled (mm)				
70-80	140-150	2-4	70-80	140-150
% Mortality[c]				
31 ± 6.3 (0.04 ± 0.03)	31 ± 6.3 (0.1 ± 0.06)	0 (0.9 ± 0.06)	0 (1.2 0.02)	6.3 ± 6.3 (1.2 ± 0.05)
62 ± 12.5 (0.06 ± 0.01)	18 ± 11.9 (0.2 ± 0.03)	6.3 ± 6.3 (0.9 ± 0.02)	0 (1.0 ± 0.07)	0 (1.0 ± 0.02)
43 ± 11.9 (0.1 ± 0.06)	0 (0.6 ± 0.02)	6.3 ± 6.3 (1.2 ± 0.09)	0 (0.9 ± 0.07)	0 (0.8 ± 0.09)
50 ± 10.2 (0.06 ± 0.03)	12 ± 7.2 (0.5 ± 0.02)	0 (0.9 ± 0.08)	0 (0.9 ± 0.03)	0 (1.1 ± 0.06)
37 ± 7.2 (0.09 ± 0.03)	6.3 ± 6.3 (0.5 ± 0.03)	6.3 ± 6.3 (0.7 ± 0.08)	0 (0.8 ± 0.09)	6.3 ± 6.3 (0.8 ± 0.06)

larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva ± standard error of the mean (in parentheses).

(From Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem., 34 111, 2002).

Table 25 Detection and larvicidal activity of Cry1Ab protein in leachates from soil columns in which plants of different hybrids of *Bt* corn or their non-*Bt* near-isolines were grown and from soil columns in which *Bt* biomass and near-isogenic non-*Bt* biomass had been incorporated.

Hybrid (*Bt* corn)	Transformation event	Immuno. test[a]	% Mortality[b]	Hybrid (non-*Bt* corn)	Immuno. test	% Mortality
			Plants[c]			
N7590Bt	Bt11	+	12 ± 7.2 (0.4 ± 0.06)	N7590	-	0 (0.9 ± 0.01)
NK4640Bt	Bt11	+	20 ± 10.2 (0.2 ± 0.06)	NK4640	-	6.3 ± 6.3 (0.8 ± 0.04)
DK647Bty	MON810	+	18 ± 11.9 (0.3 ± 0.07)	DK647	-	0 (1.1 ± 0.07)
Maximizer[d]	176	+	12 ± 7.2 (0.5 ± 0.07)	--	--	--
			Biomass[e]			
NK4640Bt 30 days	Bt11	+	25 ± 0.0 (0.2 ± 0.03)	NK4640 30 days	-	6.3 ± 6.3 (0.9 ± 0.06)
NK4640Bt 3 years	Bt11	+	43 ± 6.3 (0.08 ± 0.02)	NK4640 3 years	-	6.3 ± 6.3 (0.8 ± 0.03)

[a] *Determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*

[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva ± standard error of the mean (in parentheses).*

[c] *Corn plants were grown in soil amended to 6% (vol/ vol) with montmorillonite (6M soil) for 40 d, and then the soil was leached 3 times with 10 ml of tap water.*

[d] *Isoline for* Bt- *Maximizer not available.*

[e] *1% (wt/ wt) ground biomass of corn was incorporated into 6M soil and kept at* ca. *field capacity (–33-kPa water tension) and room temperature for 30 d or 3 y in plastic containers; Soil (50 g, oven- dry weight equivalent) was then placed in glass tubes and leached 3 times with 10 ml of tap water.*

(Modified from Saxena, D., Flores, S., and Stotzky, G., Soil Biol. Biochem. 34, 111, 2002).

Table 26 Effect on earthworms (*Lumbricus terrestris*) of Cry1Ab toxin released in rootexudates and from biomass of corn with (*Bt+*) (NK4640Bt) and without (*Bt-)* (NK4640) the *cry1Ab* gene in soil (expressed as % mortality and mean weight of a single earthworm ± standard error of the mean).

Soil	% 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 ± 4.8	4.3 ± 0.15
Bt+ biomass	24 ± 7.5	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 x 5 replications/treatment).*

[b] *Mortality and weight of earthworms were determined after 45 days of incubation with 1% (wt/wt) ground biomass of* Bt+ *or* Bt- *corn or with no biomass (n = 5 worms x 5 replications/ treatment).*

(From Saxena, D. and Stotzky, G., Soil Biol. Biochem. 33,1225, 2001. With permission.)

Table 27 Cry1Ab toxin in soil, casts, and guts of earthworms (*Lumbricus terrestris*) in the presence of corn plants (root exudates) with (*Bt+*) (NK4640Bt) and without (*Bt-*) (NK4640) the *cry1Ab* gene, in soil amended with ground biomass of *Bt+* or *Bt-* corn, and in the absence of plants or biomass.

Sample	No plants		Root exudates[a]			
			Bt-		*Bt+*	
	Immuno. test[c]	% Mortality[d]	Immuno. test	% Mortality	Immuno. test	% Mortality
Soil	-	0 (1.0 ± 0.10)	-	5 ± 5.0 (1.0 ± 0.09)	+	92.5 ± 3.06 (0.02 ± 0.00)
Cast	-	4.2 ± 4.16 (0.9 ± 0.07)	-	2.5 ± 2.50 (0.8 ± 0.04)	+	100
Gut	-	0 (0.8 + 0.10)	-	0 (0.8 + 0.08)	+	97.5 ± 2.50 (0.05 ± 0.00)

[a] *Samples from soil with no plants or with* Bt+ *or* Bt- *corn were evaluated after 40 days of plant growth.*
[a] *Samples from soil with 1% (wt/wt) ground biomass of* Bt+ *or* Bt- *corn or with no biomass 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 to16 larvae/ assay; expressed as % mortality and mean weight, in g, of a single larva + standard error of the mean (in parentheses).*
(From Saxena, D. and Stotzky, G., Soil Biol. Biochem. 33,1225, 2001. With permission.)

Organism	No plants	Root exudates	
		Bt-	*Bt+*
Bacteria[d]	$1.4 \pm 0.16 \times 10^8$	$7.6 \pm 0.14 \times 10^7$	$5.8 \pm 0.23 \times 10^7$
Actinomycetes[d]	$4.2 \pm 0.45 \times 10^5$	$2.3 \pm 0.20 \times 10^5$	$2.3 \pm 0.36 \times 10^5$
Fungi[d]	$3.3 \pm 0.41 \times 10^6$	$2.7 \pm 0.56 \times 10^6$	$1.8 \pm 0.21 \times 10^6$
Protozoa[e]	$1.3 \pm 0.05 \times 10^4$	$1.8 \pm 0.02 \times 10^4$	$1.4 \pm 0.02 \times 10^4$
Nematodes[f]	$1.0 \pm 0.03 \times 10^3$	$1.3 \pm 0.04 \times 10^3$	$1.3 \pm 0.08 \times 10^3$

Table 28 Effect on various groups of organisms of Cry1Ab toxin released in root exudates[a] and from biomass[b] of *Bt* corn with (*Bt+*) (NK4640Bt) and without (*Bt-*) (NK4640) the *cry1Ab* gene in soil (mean ± standard error of the mean)[c].

No biomass		Biomass[b]			
		Bt-		*Bt+*	
Immuno. test	% Mortality	Immuno. test	% Mortality	Immuno. test	% Mortality
-	6.3 ± 6.25 (0.9 ± 0.07)	-	2.5 ± 2.5 (0.9 ± 0.07)	+	97.5 ± 2.5 (0.01 ± 0.00)
-	6.3 ± 6.25 (0.9 ± 0.05)	-	5 ± 5.0 (0.8 ± 0.04)	+	90 ± 6.12 (0.02 ± 0.00)
-	6.3 ± 6.25 (0.8 ± 0.10)	-	2.5 ± 2.50 (0.9 ± 0.08)	+	92.5 ± 3.06 (0.01 ± 0.00)

[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 1% (wt/wt) ground biomass of* Bt+ *or* Bt- *corn or with no biomass.*
[c] *P > 0.5 for all comparisons between* Bt+ *and* Bt- *corn.*
[d] *Numbers/g soil, oven-dry equivalent; serial dilution plate method.*
[e] *Numbers/g soil, oven-dry equivalent; most-probable number method.*
[f] *Numbers/50 g soil, oven-dry equivalent; Baermann method.*
(From Saxena, D. and Stotzky, G., Soil Biol. Biochem. 33,1225, 2001. With permission).

No biomass	Biomass[b]	
	Bt-	*Bt+*
$1.1 \pm 0.01 \times 10^8$	$8.1 \pm 0.20 \times 10^7$	$6.3 \pm 0.10 \times 10^7$
$2.4 \pm 0.22 \times 10^5$	$2.8 \pm 0.53 \times 10^5$	$2.5 \pm 0.34 \times 10^5$
$3.8 \pm 0.56 \times 10^6$	$3.6 \pm 0.53 \times 10^6$	$3.4 \pm 0.72 \times 10^6$
$1.1 \pm 0.07 \times 10^4$	$1.1 \pm 0.02 \times 10^4$	$1.0 \pm 0.08 \times 10^4$
$1.4 \pm 0.01 \times 10^3$	$1.5 \pm 0.05 \times 10^3$	$1.4 \pm 0.06 \times 10^3$

Table 29 Uptake of Cry1Ab toxin by non-*Bt* (*Bt*-) (NK4640) corn grown in soil containing the Cry1Ab toxin released as root exudates by previously grown *Bt* (*Bt+*) (NK4640Bt) corn and from ground biomass of *Bt* corn.

Days of growth	Soil		Leaves	
	Immuno. test[a]	% Mortality[b]	Immuno. test	% Mortality
20	+	75 ± 10.2 (0.1 ± 0.01)	-	0 (1.0 ± 0.06)
30	+	63 ± 7.2 (0.1 ± 0.02)	-	0 (0.9 ± 0.03)
50	+	75 ± 10.2 (0.04 ± 0.02)	-	0 (0.9 ± 0.08)
120	+	63 ± 7.2 (0.08 ± 0.01)	-	6.3 ± 6.25 (0.9 ± 0.05)
20	+	88 ± 7.2 (0.05 ± 0.01)	-	0 (0.9 ± 0.02)
30	+	88 ± 7.2 (0.06 ± 0.01)	-	6.3 ± 6.25 (0.8 ± 0.03)
50	+	88 ± 7.2 (0.09 ± 0.01)	-	6.3 ± 6.25 (0.8 ± 0.08)
120	+	75 ± 10.2 (0.07 ± 0.01)	-	6.3 ± 6.25 (0.9 ± 0.08)

[a] *Determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; expressed as % mortality and mean weight, in g, of a single larva ±*

standard error of the mean (in parentheses).
[c] Bt- *corn was grown in soil in which* Bt+ *corn had been grown or in which 1% (wt/wt) ground biomass of* Bt+ *corn had been incorporated.*

Stems		Roots	
Immuno. test	% Mortality	Immuno. test	% Mortality
Root exudates[c]			
-	0 (0.6 ± 0.05)	-	0 (0.6 ± 0.06)
-	0 (0.7 ± 0.04)	-	0 (0.6 ± 0.05)
-	6.3 ± 6.25 (0.6 ± 0.01)	-	6.3 ± 6.25 (0.7 ± 0.03)
-	0 (0.6 ± 0.02)	-	6.3 ± 6.25 (0.8 ± 0.05)
Biomass[c]			
-	0 (0.5 ± 0.05)	-	0 (0.5 ± 0.03)
-	0 (0.6 ± 0.04)	-	0 (0.6 ± 0.04)
-	0 (0.7 ± 0.06)	-	6.3 ± 6.25 (0.7 ± 0.04)
-	75 ± 14.4 (0.6 ± 0.08)	-	6.3 ± 6.25 (0.6 ± 0.08)

(Modified from Saxena, D. and Stotzky, G., Nature Biotech. 19, 199, 2001. With permission.)

Table 30 Uptake of Cry1Ab toxin by carrot (*Daucus carota*) grown in soil containing the Cry1Ab toxin released as root exudates by previously grown *Bt* corn (NK4640Bt) and from ground biomass of *Bt* corn.

Days of growth	Soil		Leaves	
	Immuno. test[a]	% Mortality[b]	Immuno. test	% Mortality
30	+	68 ± 11.9 (0.1 ± 0.02)	-	0 (0.8 ± 0.02)
50	+	68 ± 6.3 (0.08 ± 0.03)	-	0 (0.9 ± 0.06)
120	+	62 ± 12.3 (0.06 ± 0.01)	-	0 (0.8 ± 0.08)
180	+	56 ± 11.9 (0.1 ± 0.03)	-	0 (1.1 ± 0.09)
50	+	88 ± 12.5 (0.08 ± 0.06)	-	0 (0.8 ± 0.05)
120	+	75 ± 14.4 (0.04 ± 0.02)	-	0 (0.8 ± 0.01)
180	+	68 ± 11.9 (0.05 ± 0.01)	-	0 (0.9 ± 0.02)

[a] *Determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; expressed as % mortality and mean weight, in g, of a single larva ± standard error of the mean (in parentheses).*

Days of growth	Soil		Leaves	
	Immuno. test[a]	% Mortality[b]	Immuno. test	% Mortality
7	+	87.5 ± 7.21 (0.07 ± 0.01)	-	0 (0.8 ± 0.08)
15	+	93.7 ± 6.25 (0.09 ± 0.00)	-	6.3 ± 6.25 (0.8 ± 0.02)

[c] *Carrots were grown in soil in which* Bt+ *corn had been grown or into which 1% (wt/wt) ground biomass of* Bt+ *corn had been incorporated.*
(Modified from Saxena, D. and Stotzky, G., Plant Soil 239, 165, 2002).

Stems		Roots	
Immuno. test	% Mortality	Immuno. test	% Mortality
Root exudates[c]			
-	0 (0.8 ± 0.09)	-	0 (0.8 ± 0.09)
-	6.3 ± 6.25 (0.8 ± 0.06)	-	0 (0.7 ± 0.02)
-	0 (0.7 ± 0.05)	-	6.3 ± 6.25 (0.6 ± 0.09)
-	6.3 ± 6.25 (0.8 ± 0.02)	-	6.3 ± 6.25 (0.6 ± 0.08)
Biomass[c]			
-	0 (0.7 ± 0.02)	-	0 (0.6 ± 0.05)
-	0 (0.7 ± 0.06)	-	0 (0.7 ± 0.03)
-	0 (0.9 ± 0.06)	-	12.5 ± 12.5 (0.8 ± 0.02)

Table 31 Uptake of Cry1Ab toxin by non-*Bt* corn (NK4640) grown in hydroponic solution containing the Cry1Ab toxin released as root exudates by previously grown *Bt* corn (NK4640Bt).

Stems		Roots	
Immuno. test	% Mortality	Immuno. test	% Mortality
-	6.3 ± 6.25 (0.6 ± 0.02)	-	0 (0.6 ± 0.02)
-	6.3 ± 6.25 (0.6 ± 0.2)	-	12.5 ± 7.21 (0.6 ± 0.02)

[a] *Determined with Lateral Flow Quickstix: - = no toxin detected; + = toxin detected.*
[b] *Determined with the larvae of the tobacco hornworm* (Manduca sexta)*; at least 16 larvae/assay; expressed as % mortality and mean weight, in g, of a single larva ± standard error of the mean (in parentheses).*
(Modified from Saxena, D. and Stotzky, G., Plant Soil 239, 165, 2002).

Table 32 Summary of fate and effects of *Bt* toxin in root exudates and biomass of transgenic plants.

- Biodegradation of biomass of transgenic *Bt* corn, measured by CO_2 evolution, was significantly lower than that of near-isogenic non- *Bt* corn.
- No consistent statistically significant differences in the numbers of culturable bacteria, fungi and the activity of representative enzymes between soil amended with *Bt* or non-*Bt* corn or not amended.
- Reduced metabolic activity of soil amended with *Bt* corn may have been result of significantly higher lignin content in *Bt* than in non-*Bt* corn.
- Biodegradation of biomass of *Bt* rice, cotton, canola, tobacco, and potato was also significantly lower than that of biomass of near-isogenic non-*Bt* plants, but lignin content of these plant species, which was significantly lower than that of corn, was not significantly different between *Bt* and non-*Bt* biomass.
- CrylAb protein was released in root exudates of *Bt* corn (13 hybrids representing three transformation events) and persisted in rhizosphere soil *in vitro* and *in situ*; protein accumulated more in soil amended (3 to 12%) with montmorillonite than with kaolinite.
- CrylAb protein released in root exudates or from biomass of *Bt* corn appeared to have no effect on numbers of earthworms, nematodes, protozoa, bacteria, and fungi in soil.
- CrylAc protein was not released in root exudates of *Bt* canola,

tobacco, and cotton. Cry1Ab protein was released in root exudates of rice, and Cry3A protein was released in root exudates of *Bt* potato.

- CrylAb protein released in root exudates and from biomass of *Bt* corn was not taken up from nonsterile soil or sterile hydroponic culture by non-*Bt* corn, carrot, radish, and turnip, even though the toxin persisted for at least 180 days in soil (the longest time studied).
- CrylAb protein - purified, in root exudates, and from biomass of *Bt* corn - moved through soil during leaching with water; movement was less in soils amended with montmorillonite than with kaolinite and decreased as the concentration of added clays increased.
- Toxins from *Bt* could persist, accumulate, and remain insecticidal in soil as the result of binding on clays and humic substances and, therefore, pose a hazard to nontarget organisms, enhance selection of toxin-resistant target species, or enhance control of insect pests.

Table 33 Potential hazards to the environment from biological "factories" (transgenic plants, animal, and microbes)

Bioactive products: e.g., vaccines, hormones, antibodies, blood substitutes, toxins, enzymes, various other pharmaceuticals, genetically modified foods

Plants (primarily soil)

- Biomass (eventually incorporated into soil)
- Root exudates [e.g., green fluorescent protein, human placental alkaline phosphatase, and xylanase (from

Clostridium thermocellum) from genetically engineered tobacco (signal peptide coding region from the endoplasmic reticulum protein, calreticulin, in hydroponic culture[a]); Cry1Ab and Cry3A insecticidal proteins from *Bt* corn and *Bt* potato, respectively]

- Pollen (e.g., monarch butterflies killed by feeding on milkweed contaminated with pollen from *Bt* corn)
- Tritrophic interactions (predators killed by ingesting prey feeding on genetically engineered plants; e.g., green lacewings on European corn borers fed *Bt* corn; ladybird beetles on aphids fed potato expressing the lectin from snowdrop anemone)[b]

Animals (soil, surface and ground waters)

- Feces
- Urine
- Carcasses

Microbes (soil, surface and ground waters)

- Waste media
- Waste cells
- Fermentor breakdowns

[a] *Borisjuk* et al., *1999*
[b] *Birch* et al., *1999*
(Modified from Stotzky, G., J. Environ. Qual. 29, 691, 2000. With permission.)

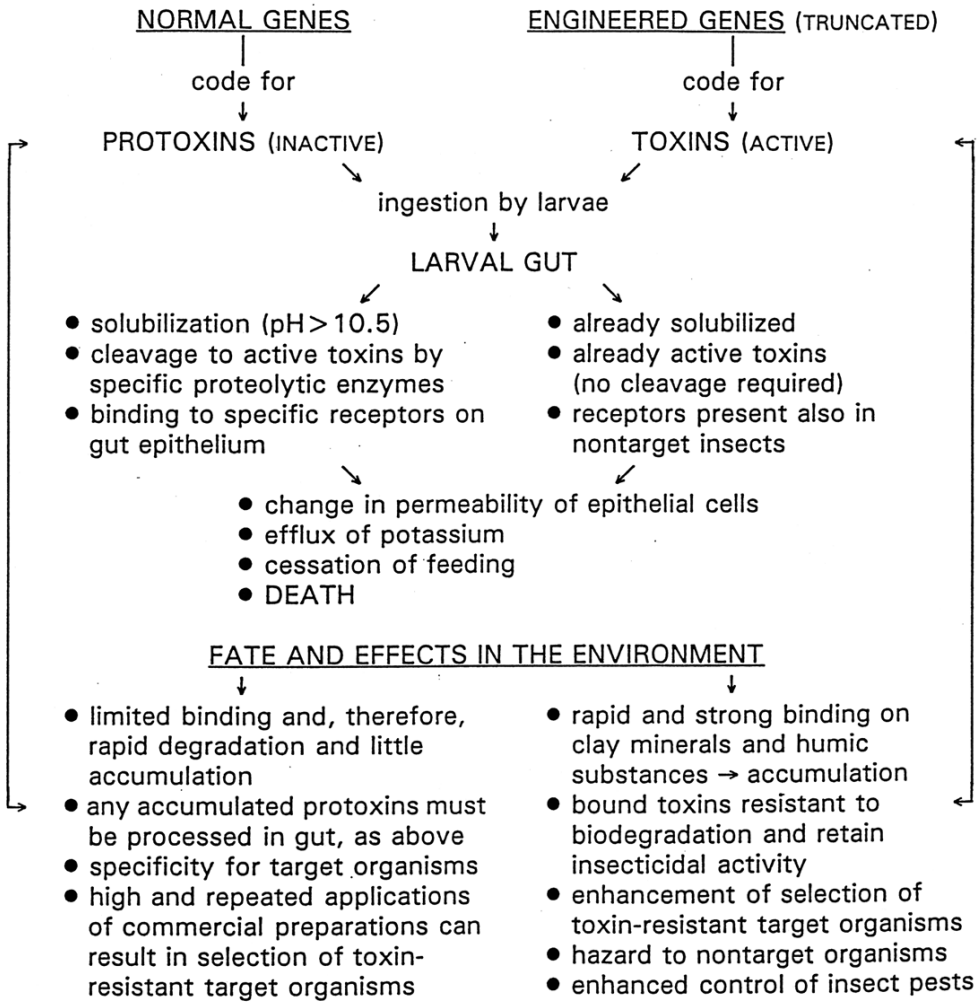

Fig.1: *Environmental aspects of normal and genetically - engineered insecticidal toxins from* ***BACILLUS THURINGIENSIS***

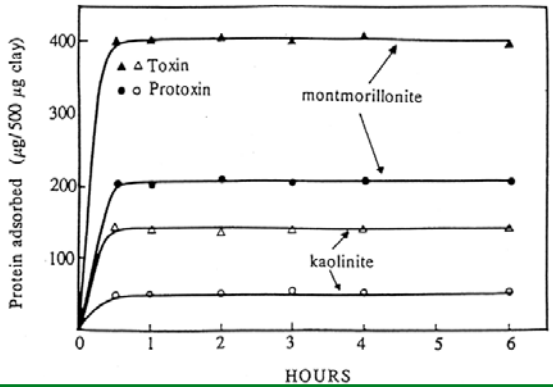

Fig.2

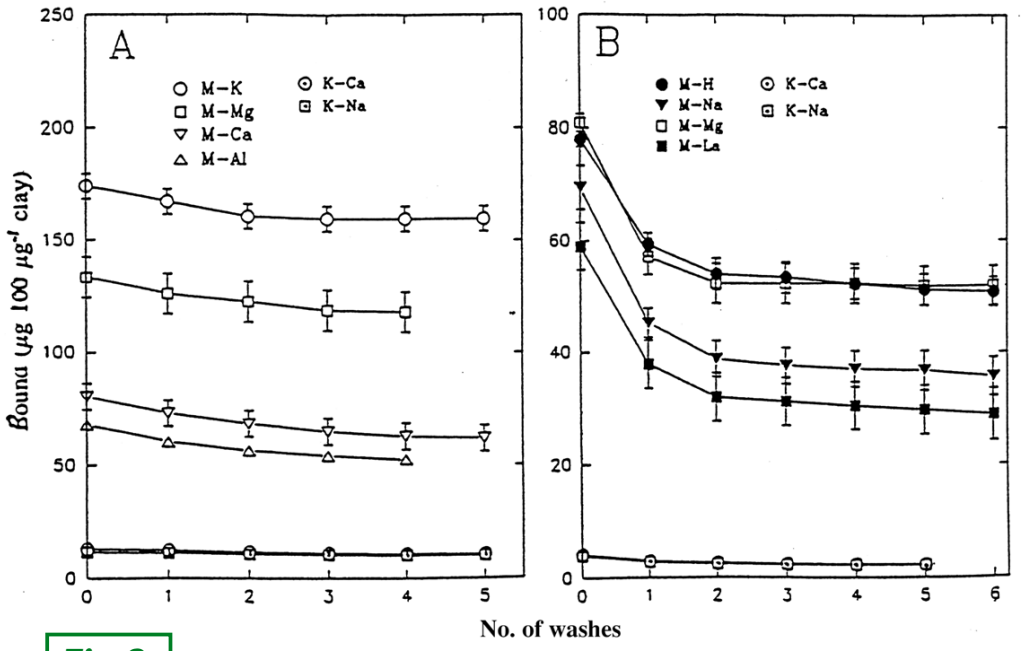
A
B
M–K
M–Mg
M–Ca
M–Al
K–Ca
K–Na
M–H
M–Na
M–Mg
M–La
K–Ca
K–Na
Bound (µg 100 µg⁻¹ clay)
No. of washes

Fig.3

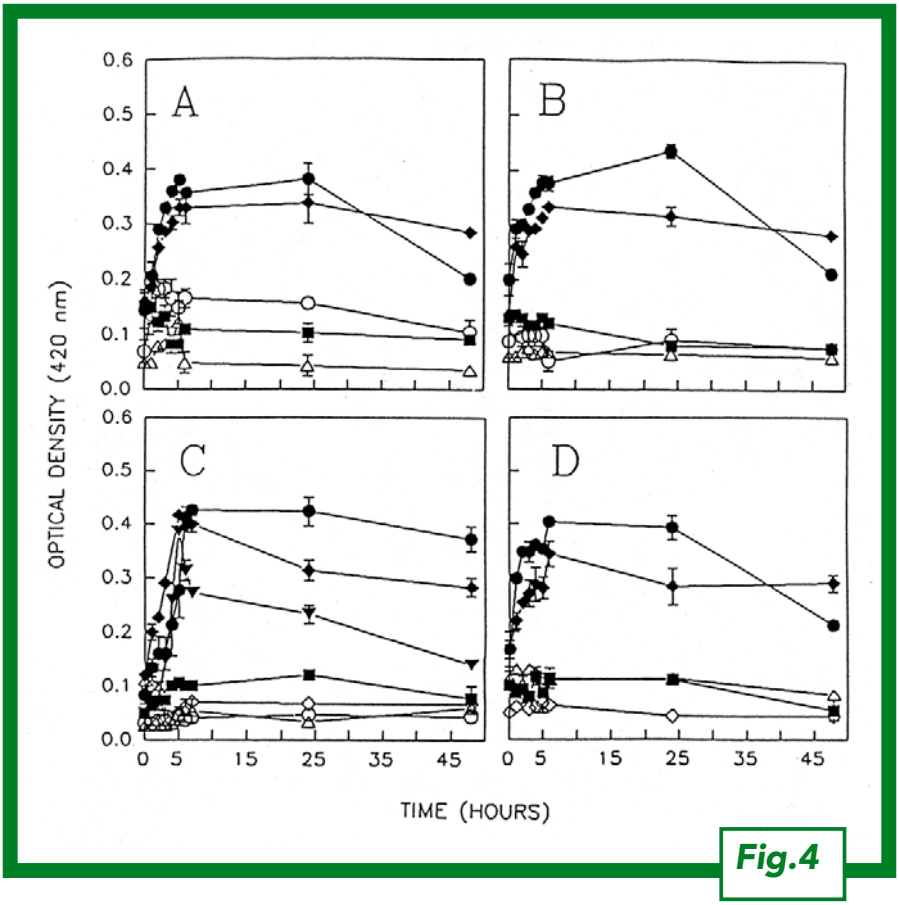
A
B
C
D
OPTICAL DENSITY (420 nm)
TIME (HOURS)

Fig.4

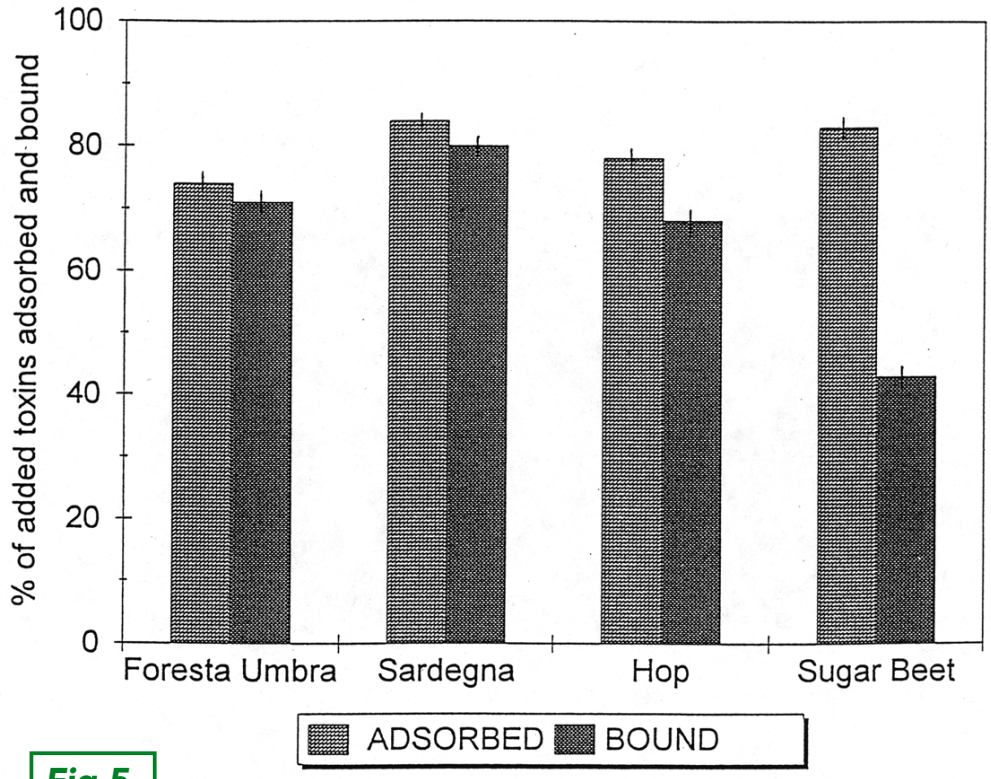
% of added toxins adsorbed and bound
100
80
60
40
20
0
Foresta Umbra
Sardegna
Hop
Sugar Beet
ADSORBED
BOUND

Fig.5

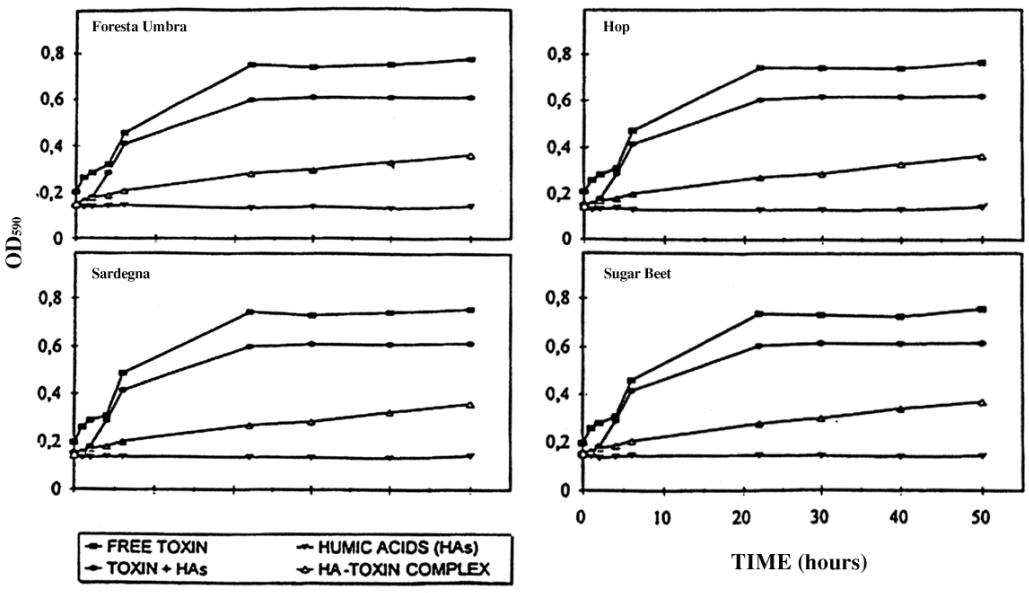
Foresta Umbra
Hop
Sardegna
Sugar Beet
OD590
0,8
0,6
0,4
0,2
0
10
20
30
40
50
TIME (hours)
FREE TOXIN
TOXIN + HAs
HUMIC ACIDS (HAs)
HA-TOXIN COMPLEX

Fig.6

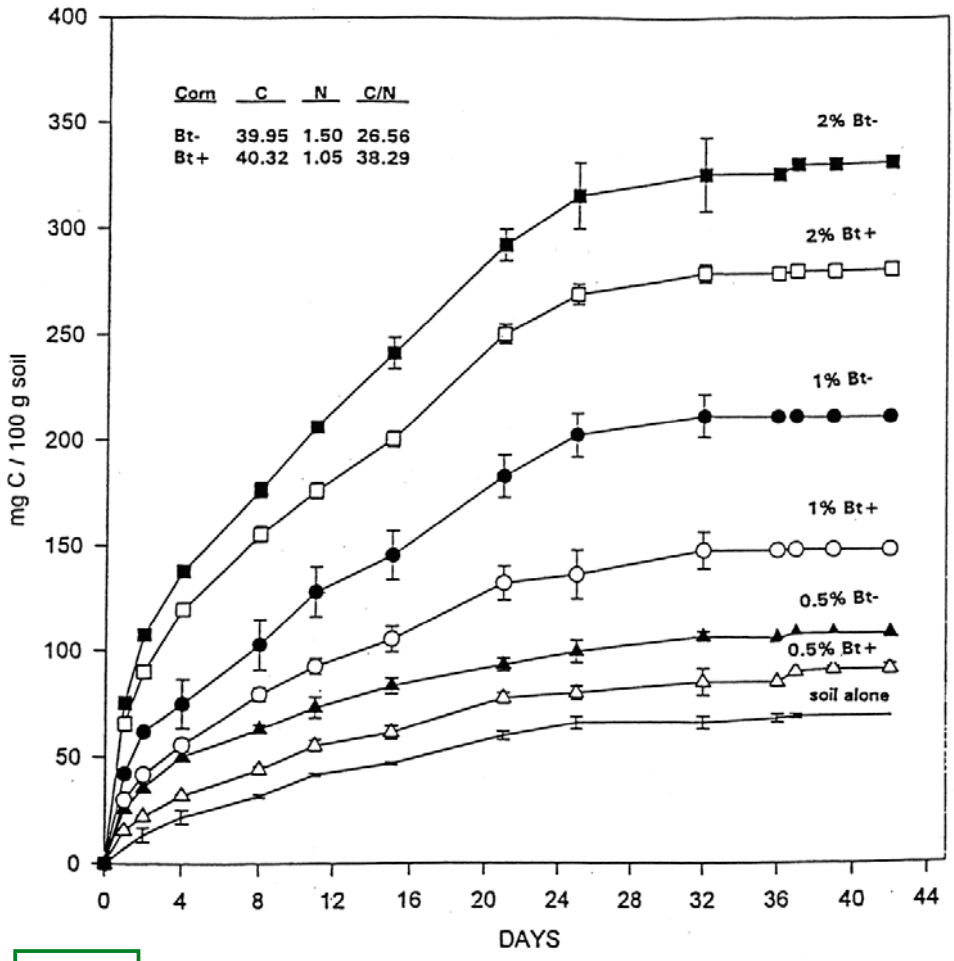
Corn C N C/N
Bt- 39.95 1.50 26.56
Bt+ 40.32 1.05 38.29
2% Bt-
2% Bt+
1% Bt-
1% Bt+
0.5% Bt-
0.5% Bt+
soil alone
mg C / 100 g soil
DAYS
0
50
100
150
200
250
300
350
400
0
4
8
12
16
20
24
28
32
36
40
44

Fig.7

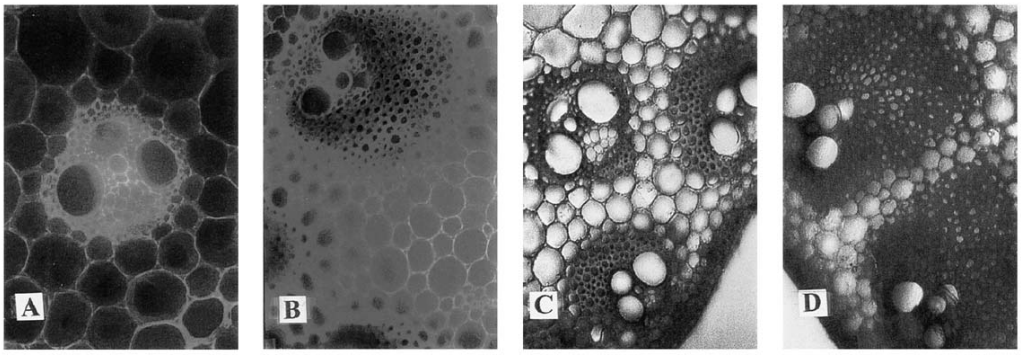
A
B
C
D

Fig.8

12. Figure Legends

1. Environmental aspects of normal and genetically-engineered insecticidal toxins from *Bacillus thuringiensis*. (Modified from Stotzky, G., *J. Environ. Qual.* 29, 691, 2000. With permission.)

2. Rate of adsorption at equilibrium of 1 mg of the protoxin or toxin from *Bacillus thuringiensis* subsp. *kurstaki* on 0.5 mg of montmorillonite or kaolinite with a mixed cation complement. Data are expressed as the means ± the standard errors of the means, which are within the dimensions of the symbols. (From Venkateswerlu, G. and Stotzky, G., *Curr. Microbiol.* 25, 225, 1992. With permission.)

3. Desorption of the toxin from (A) *Bacillus thuringiensis* subsp. *kurstaki* and (B) *Bacillus thuringiensis* subsp. *tenebrionis* from homoionic montmorillonite (M)- and kaolinite (K)-toxin complexes after equilibrium adsorption. Data normalized to 100 µg of clay. Means ± SEMs. Values at 0 number of washes indicate amounts of toxin adsorbed at equilibrium. (From Tapp, H., Calamai, L., and Stotzky G., *Soil Biol. Biochem.* 26, 225, 1992. With permission.)

4. Growth of (A) *Proteus vulgaris*, (B) *Enterobacter aerogenes*, (C) a mixed microbial culture from a protein-enriched soil slurry, or (D) a mixed bacterial culture from a suspension of the protoxin from *Bacillus thuringiensis* subsp. *kurstaki* on pepsin (500 µg/ml) or the toxin from B. thuringiensis subsp. kurstaki (500 µg/ml), free or bound on montmorillonite homoionic to Ca. Means ± SEMs. ◆- pepsin; ▼ - pepsin plus montmorillonite (1 mg/ml); ● - toxin; ■ - toxin bound on montmorillonite (1.4 mg/ml); Δ- control (pepsin, no microbes); **O** - control (1 mg montmorillonite/ml, no microbes); ◊-control [no toxin, no clay, microbes). (From Koskella, J. and Stotzky, G., *Appl. Environ. Microbiol.* 63,3561, 1997. With permission.)

5. Percentage of added toxin from *Bacillus thuringiensis* subsp. *kurstaki* (140 µg) adsorbed or bound (after 5 washings with double distilled water) on humic acids (200 µg) from four soils. Means ± SEMs. (Modified from Crecchio, C. and Stotzky G., *Soil Biol. Biochem.* 30, 463, 1998. With permission.)

6. Utilization of the toxin from *Bacillus thuringiensis* subsp. *kurstaki*, free or bound on humic acids (HAs) from four soils, as a source of carbon and energy by a mixed microbial culture from soil. Means ± SEMs. (From Crecchio, C. and Stotzky G., *Soil Biol. Biochem.* 30, 463, 1998. With permission.)

7. Gross metabolic activity (cumulative CO^2 evolution) of Kitchawan soil amended to 6% (v/v) with montmorillonite and with 0.5, 1, or 2% (w/w) ground, air-dry leaves of corn containing (*Bt+*) (NK6800Bt) or not containing (*Bt-*) (NK6800) the *cry1Ab* gene from *Bacillus thuringiensis* subsp. *kurstaki*. Soil was incubated at the –33-kPa water tension and 25 ± 1°C. Means ± SEMs. (From Flores, S., Saxena D., and Stotzky, G., unpublished data.)

8. Fluorescence microscopy of a transverse stem section of the third internode from the soil surface from non-*Bt* (NK4640) (A) and *Bt* (NK4640Bt) (B) corn grown in a plant growth room. Lignin autofluorescence was visualized following ultraviolet excitation at 400 nm (x250). The same transverse sections from non-*Bt* (C) and *Bt* (D) corn were stained with toluidine blue. More lignified cells and large vascular bundles were observed in *Bt* corn than in non-*Bt* corn (x250). (Saxena D. and Stotzky, G., *Am. J. Bot.* 88, 1704, 2001).