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Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants

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Abstract

Insecticidal proteins produced by various subspecies (*kurstaki, tenebrionis*, and *israelensis*) of *Bacillus thuringiensis* (*Bt*) bound rapidly and tightly on clays, both pure mined clay minerals and soil clays, on humic acids extracted from soil, and on complexes of clay and humic acids. Binding reduced susceptibility of the proteins to microbial degradation. However, bound proteins retained biological activity. Purified Cry1Ab protein and protein 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 root exudates of *Bt* potato (Cry3A protein) and rice (Cry1Ab protein) but not in root exudates of *Bt* canola, cotton, and tobacco (Cry1Ac protein). Vertical movement of CrylAb protein, either purified or in root exudates or biomass of *Bt* corn, decreased as the concentration of the clay minerals, kaolinite or montmorillonite, in soil increased. Biomass of transgenic *Bt* corn decomposed less in soil than biomass of near-isogenic non-*Bt* corn, possibly because biomass of *Bt* corn had a significantly higher content of lignin than biomass of non-*Bt* corn. Biomass of *Bt* canola, cotton, potato, rice, and tobacco also decomposed less than biomass of the respective 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, although it was consistently higher. The Cry1Ab protein had no consistent effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or *in vitro*. The Cry1Ab protein was 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.

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). 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 mostly 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.,

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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 40 years (see Vettori et al., 2004). With a few exceptions, no unexpected toxicities from such sprays have been recorded, probably because *Bt* does not survive or grow well in 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) amount 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; Hilbeck et al., 1998a,b, 1999; Losey et al., 1999; Obrycki et al., 2001), and other animal classes. 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; Saxena and Stotzky, 2003) (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. 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).

Adsorption and binding of Bt toxins on clays and humic acids: effects on persistence and insecticidal activity

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. *morrisoni* (strain *tenebrionis*) (*Btt*; 68 kDa; active against Coleoptera), and subsp. *israelensis* (*Bti*: 28 to 130 kDa; active against some 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., 2003), 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 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 toxins

released *in situ* from transgenic plants or commercial spray preparations are adsorbed and bound on such surface-active particles and persist and retain insecticidal activity in soil. The results of these studies are summarized in Table 1.

One result that needs emphasis is that when free toxin from *Btk* was added to nonsterile soils maintained at the −33-kPa water tension and 24 ± 2 °C (i.e., under optimal conditions for microbial activity), insecticidal activity was still detected after 234 days, the longest time evaluated, albeit with a reduction in activity during the incubation (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 (Palm et al., 1994, 1996; Sims and Holden, 1996; Sims and Ream, 1997). It is doubtful whether the half-life concept, developed for the decay of radionuclides, is applicable to the degradation of proteins, which do not decay at a constant rate for all atoms as with radionuclides but, rather, at different rates, especially between free and bound proteins. Moreover, the half-life concept requires first-order kinetics, which were not always achieved in these studies.

Biodegradation of Bt *corn and other* Bt *plants in soil*

The addition of biomass from transgenic corn (*Zea mays* L.) expressing the Cry1Ab protein from *Btk* resulted in a significantly lower gross metabolic activity (i.e., CO_2 evolution) of soil than did the addition of near-isogenic nontransgenic biomass (i.e., same variety but without the *cry1Ab* gene) (Stotzky, 2000, 2002; Saxena and Stotzky, 2003; Flores et al., 2004). 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 of C 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. This difference occurred with stems and leaves from different hybrids of corn, 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 enzymes (i.e., proteases, dehydrogenases, alkaline and acid phosphatases, and arylsulfatases) and the numbers of culturable bacteria and fungi fluctuated throughout the incubations and sometimes 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. All soil samples amended with biomass of Bt corn were lethal to the larvae of the tobacco hornworm (*Menduca 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 significant 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, which was also reported by Zwahlen et al. (2003a), 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 hybrid 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 near-isogenic non-*Bt* corn. 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 *Bt* corn, cotton, and potato 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 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 (Flores et al., 2004).

Because these observations suggested some differences in the chemical composition between *Bt* and

Table 1. Summary of interactions of purified *Bt* toxins with surface-active soil particles and with soil: effects on persistence and larvicidal 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 hydroxy-polymers; 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 the toxin from *Btk* was detected 234 days after addition to nonsterile soils (longest time studied).
- Persistence of larvicidal activity of the toxin from *Btk* 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 in soil 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.

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 (26 ± 2 °C, 12-h light-dark cycle) and the field, was evaluated. Uniform free-hand sections between the third and fourth node of fresh stems of corn, harvested after tasseling and ear production and of the same age, 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 (Saxena and Stotzky, 2001c).

The content of lignin of the same portion of the stems was also 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 or in the field, was significantly higher (33 to 97% higher) than that of their respective non-*Bt* near-isolines. There was a significantly higher lignin content ($P < 0.002$) in plants transformed by event Bt11 than by event MON810. There were no significant differences in lignin content among near-isogenic non-*Bt* corn hybrids. The lignin content of the only available hybrid transformed by event 176 was lower than that of hybrids transformed by events Bt11 and MON810. These results differed 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 and protects more labile components, such as carbohydrates and proteins, against biodegradation. Hence, 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 et al., 2004).

The biodegradation of biomass of *Bt* canola, cotton, potato, rice, and tobacco was also significantly lower than that of the biomass of the respective 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, although they were consistently higher in *Bt* biomass (Flores et al., 2004).

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).

Release, binding, persistence, and insecticidal activity of Bt *toxins in root exudates of* Bt *canola, corn, cotton, potato, rice, and tobacco*

The Cry1Ab protein was present in root exudates from transgenic *Bt* corn grown in sterile hydroponic culture and in sterile and nonsterile soil in a plant-growth room (Saxena et al., 1999). The presence of the toxin was indicated by a major band migrating on SDS-PAGE to a position corresponding to a molecular mass (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, as has been observed with purified toxins.

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 (3 to 12%) of montmorillonite or kaolinite (e.g., Babich and Stotzky, 1977; Stotzky et al., 1993; Tapp and Stotzky, 1995b, 1997) in a plant-growth room. All samples of rhizosphere soil from plants of *Bt* corn were positive 10, 20, 30, and 40 days after germination for the presence of the toxin when assayed immunologically with Lateral Flow Quickstix (EnviroLogix, Portland, ME), which are rapid (<10 min) Western blot detection systems with a lower detection limit of ca. 10 parts per 10^9 (Saxena and Stotzky, 2000). No toxin was detected in any soil in which plants of near-isogenic non-*Bt* corn or no plants had been grown. All samples of soil in which *Bt* corn was grown were also 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* corn or without plants. In addition, the size and weight of surviving larvae exposed for 7 days 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 later. 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). However, mortality in soil amended with montmorillonite or kaolinite was essentially the same after 40 days, 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. The increase in larvicidal activity between day 10 and day 40 indicated that the toxin in the root exudates accumulated 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 (Saxena and Stotzky, 2000). Although larval mortality in rhizosphere soil from plants of field-grown *Bt* corn ranged

from 38 to 100% 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 later.

To determine whether the release of the Cry1Ab protein in root exudates is a common phenomenon with transgenic *Bt* corn and not restricted to the hybrid studied originally, the release of the protein in the exudates of 12 additional *Bt* corn hybrids, representing three different transformation events (B11, MON810, and 176), and of their near-isogenic nontransgenic counterparts was studied with plants grown in nonsterile soil in the plant-growth room and in the field. 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%. 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 soil from *Bt* corn was significantly lower (80 to 90%) than that of larvae exposed to soil from non-*Bt* corn or without plants.

The immunological and larvicidal assays of soil from the rhizosphere of all *Bt* hybrids grown to maturity in the field were also positive, whereas they were negative for soil from all non-*Bt* corn near-isolines. The larval mortality in rhizosphere soil from plants of field-grown *Bt* corn ranged from 37 to 100%, and the weight of the surviving larvae was reduced by 85 to 98% when compared with soil from non-*Bt* corn or without plants. There were no 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 again indicated that the toxin released in exudates from roots of *Bt* corn could accumulate in soil and retain insecticidal activity, especially when the toxin bound on surface-active soil particles becomes resistant to degradation by microorganisms. Although some toxin was probably released from damaged and sloughed 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 biomass from *Bt* corn after harvest and some that will be introduced in pollen released during tasseling (e.g., 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 persistence of the toxin in soil could improve the control of insect pests, enhance the selection of toxin-resistant target insects, and/or constitute a hazard to nontarget organisms. Because *Bt* corn contains truncated genes that encode toxin rather than the nontoxic crystalline protoxin produced by *Bt*, potential hazards are exacerbated, as it is not necessary for an organism ingesting the toxin to have a high gut pH (ca. 10.5) for solubilization of the protoxin and specific proteases to cleave the protoxin into toxin. Moreover, receptors for the toxin 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 toxin.

The Cry1Ac protein was not released in hydroponic culture or in soil in the root exudates of *Bt* canola, cotton, and tobacco containing the *cry1Ac* gene, whereas the Cry1Ab protein was released in root exudates of *Bt* rice containing the *cry1Ab* gene, and the Cry3A protein was released in root exudates of *Bt* potato containing the *cry3A* gene. Immunological assays of soil from the rhizosphere and hydroponic solutions of *Bt* canola, cotton, and tobacco were negative, and the soil and solutions were not toxic to larvae of *M. sexta*. In contrast, the soil and solution of *Bt* rice were immunologically positive for the Cry1Ab protein and toxic to larvae of *M. sexta*, and those of *Bt* potato were immunologically positive for the Cry3A protein (DAS ELISA Kit; Agdia, Elkhart, IN; lower detection limit of ca. 20 parts per 10^9) and toxic to larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*). The percent mortality of soil and hydroponic solution in which *Bt* potato was grown was 35 and 38%, respectively, and the size of the surviving larvae was reduced by 25 to 40% when compared with soil or solution in which near-isogenic non-*Bt* potato or no potato were grown (Saxena and Stotzky, 2004). Recent studies have shown that the Cry3Bb1 protein, active against corn rootworm beetles, is also released in root exudates from corn containing the *cry3Bb1* gene (Stotzky, unpublished data).

The absence of the Cry1Ac protein in the root exudates of *Bt* canola, cotton, and tobacco, in both 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 was not the result of damage to or sloughing of root cells, as the proteins were not present in the root exudates of the other *Bt* plants, even when grown in soil where damage to roots would be expected. It is not clear how a 66-kDa protein, such as the Cry1Ab protein, is released 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 based on our studies also, apparently, of rice and potato, but this does not appear to be the situation in the roots of canola, cotton, and tobacco. Obviously, further studies on the physiology and morphology of transgenic *Bt* plants are necessary.

Vertical movement in soil of Bt *toxin as purified protein, in root exudates, or from biomass of* Bt *corn*

When 0.8, 1.6, or 3.2 μg of purified Cry1Ab protein/g of soil, oven-dry weight equivalent, was added to the top of columns (3 cm diameter and 15 cm long) 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 and the lowest amount (ca. 16%) detected from columns containing soil amended to 12% (vol/vol) with montmorillonite or kaolinite; intermediate amounts of protein were leached from soils amended to 3, 6, or 9% with the clays (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 $\pm$ 6.3 to 68 $\pm$ 11.9%, and the weight of a single larva ranged from 0.03 $\pm$ 0.01 to 0.3 $\pm$ 0.03 g) than from soil amended to 9 or 12% with the clays (mortality ranged from 12 $\pm$ 6.3 to 37 $\pm$ 12.5%, and the weight of a single larva ranged from 0.05 $\pm$ 0.01 to 0.6 $\pm$ 0.03 g), indicating that the protein moved less through soil as the clay concentration was increased. However, after 12 and 24 h, no protein was detected in any leachates, even by the immunological assay with a lower detection limit of 10 parts per 10^9, indicating that the toxin bound on the soils and that its desorption was reduced. The presence of Cry1Ab protein in the leachates was confirmed by SDS-PAGE.

The vertical distribution in the columns of the Cry1Ab protein that was not recovered in the leachates confirmed that the protein moved less through soil amended with the higher concentrations of the clays. For example, when soil at 2 to 4, 70 to 80, and 140 to 150 mm from the top of columns of soil unamended or amended to 3 or 12% with montmorillonite or kaolinite and 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 the clays was higher (mortality ranged from 37 $\pm$ 7.3 to 100%, and the weight of a single larva ranged from 0.04 $\pm$ 0.01 to 0.06 $\pm$ 0.03 g) than that of soil not amended or amended to 3% with the clays (mortality ranged from 12 $\pm$ 7.3 to 81 $\pm$ 11.9%, and the weight of a single larva ranged from 0.05 $\pm$ 0.03 to 0.4 $\pm$ 0.03 g). Mortality generally decreased with depth in the columns as the clay concentration was increased.

The Cry1Ab protein was present in leachates from soil columns in which hybrids of *Bt* corn were grown, whereas it was absent in leachates from columns in which their respective non-*Bt* near-isolines were grown. 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 columns for 40 days in a plant-growth room when the soils were leached with water, the leachates centrifuged, and the supernatants analyzed for the presence of the protein. Although the mortality of the leachates was only 12 $\pm$ 7.3 to 20 $\pm$ 10.5%, there was a ca. 62% reduction in larval weight (0.2 $\pm$ 0.06 to 0.5 $\pm$ 0.07) compared with larvae exposed to leachates from soils in which non-*Bt* corn or no plants were grown (0.8 $\pm$ 0.04 to 1.1 $\pm$ 0.07), indicating some vertical movement of the protein from the rhizosphere.

The protein was also present in leachates from columns of soil amended 3 years earlier with biomass of *Bt* corn, whereas it was absent in leachates from soil amended with biomass of near-isogenic non-*Bt* corn. Mortality was 43 $\pm$ 6.3%, and the weight of a single larva was 0.08 $\pm$ 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 (Saxena and Stotzky, 2003).

The movement of the Cry1Ab protein through soil was influenced by its tendency to bind on surface-

active particles, particularly to clay and organic matter (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1998; Crecchio and Stotzky, 1998, 2001; Stotzky, 2000, 2002). The protein exhibited both strong binding and high persistence in soils that contained the higher clay concentrations (i.e., 9 and 12% montmorillonite or kaolinite), and it remained near the soil surface, increasing its probability of being transported to surface waters via erosion and runoff. By contrast, in soils with lower clay concentrations (i.e., unamended or amended to 3 or 6% with the clays), 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 on the desorption of the protein and on the amount of water impinging 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.

Effect of Bt *toxin from root exudates and biomass of* Bt *corn on earthworms, nematodes, protozoa, bacteria, and fungi in soil*

There were no significant differences in mortality and weight of earthworms (*Lumbricus terrestris*) after 40 days in soil planted with *Bt* or near-isogenic non-*Bt* corn or not planted or after 45 days in soil amended with 1% of ground, air-dried biomass of *Bt* or near-isogenic non-*Bt* corn or not amended (Saxena and Stotzky, 2001b). Similar results were reported in fields planted with *Bt* corn (Zwahlen et al., 2003b). The Cry1Ab protein was present in casts and guts of worms in soil planted with *Bt* corn or amended with biomass of *Bt* corn. When worms from these soils were transferred to fresh soil, the toxin was cleared from the guts in 1 to 2 days. Soil amended with biomass of *Bt* corn and from the rhizosphere of *Bt* corn was immunologically positive for the presence of the toxin and lethal to the larvae of *M. sexta* after 45 and 40 days, respectively, whereas there was no mortality in soil amended with biomass of near-isogenic 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. There were no statistically significant differences ($P > 0.5$) in the numbers of nematodes [determined by the method of Van Gundy (1982)] and culturable protozoa, bacteria (including actinomycetes), and fungi [determined by the methods described in Stotzky et al. (1993)] between rhizosphere soil of *Bt* and non-*Bt* corn or between soil amended with *Bt* or non-*Bt* biomass.

These results suggested that the Cry1Ab protein released in root exudates of *Bt* corn or from the degradation of the biomass of *Bt* corn is not toxic to a variety of nontarget organisms in soil. The presence of the toxin in the guts and casts of earthworms grown with *Bt* corn and in soil amended with biomass of *Bt* corn demonstrated again that the released protein bound on surface-active particles in soil, which protected it from biodegradation by procaryotes and eucaryotes (e.g., gut enzymes of earthworms), similar to what has been observed with purified proteins. Because only one species of earthworms and only culturable microorganisms 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 or temperature gradient gel electrophoresis, single strand conformational polymorphism), the BIOLOG or similar system for bacteria, speciation of fungi, and nutritional groups of protozoa and nematodes, to confirm the absence of effects of the Cry1Ab protein on biodiversity in soil. Moreover, studies on the effects of other Cry proteins on these and other organisms in soil are necessary.

Uptake of Bt *toxin from soil by plants*

Because of some 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 had been amended with ground biomass of *Bt* corn or purified Cry1Ab protein. No Cry1Ab protein was detected immunologically and by bioassay, even after 120 or 180 days of growth, in the tissues (leaves, stems, and roots) of any of the plants, whereas the protein was present in all samples of soil (Saxena and Stotzky, 2001a, 2002).

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

Table 2. Summary of fate and effects in soil of *Bt* toxins in root exudates and biomass of transgenic plants

- Biodegradation of biomass of transgenic *Bt* corn, measured by CO_2 evolution, was significantly lower in soil and *in vitro* (biomass inoculated with a soil suspension) than that of near-isogenic non-*Bt* corn.
- No consistent statistically significant differences in numbers of culturable bacteria and fungi and in activity of representative enzymes between soil amended with Bt or non-*Bt* corn or not amended.
- Lower metabolic activity of soil amended with *Bt* corn may have been result of significantly higher lignin content in *Bt* than in near-isogenic non-*Bt* corn.
- Biodegradation of biomass of *Bt* canola, cotton, potato, rice, and tobacco was also significantly lower than that of biomass of near-isogenic non-*Bt* plants, but lignin content of these plant species, which was considerably lower than that of corn, was not significantly different between *Bt* and non-*Bt* biomass.
- Cry1Ab 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.
- Cry1Ab 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.
- Cry1Ab protein was also released in root exudates of rice, Cry3A protein was released in root exudates of *Bt* potato, and Cry3Bb1 was released in root exudates of *Bt* corn. Cry1Ac protein was not released in root exudates of *Bt* canola, cotton, and tobacco.
- Cry1Ab protein, purified or 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 released in root exudates persisted for at least 180 days and that from biomass for at least 3 years in soil (the longest times studied).
- Cry1Ab protein – purified, in root exudates, and from biomass of *Bt* corn – moved slightly through soil leached 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* apparently persist, accumulate, and remain insecticidal in soil as the result of binding on clays and humic substances and, therefore, could enhance control of insect pests, enhance selection of toxin-resistant target species, and/or pose a hazard to nontarget organisms.

germinated on agar were transferred aseptically and grown in the same solution. No Cry1Ab protein was detected in the tissues of non-*Bt* corn after 7 and 15 days by immunological and larvicidal assays, whereas it was easily detected in the hydroponic solution. Consequently, the apparent lack of uptake of the toxin from soil was not the result of its binding on surface-active particles, as no such surfaces were present in the hydroponic solution. These results were not unexpected, as it is doubtful that plants can take up molecules as large as 66 kDa. However, the possibility that the protein was taken up and then degraded within the plants cannot be excluded based on these studies. These results also demonstrated again the persistence of the protein in soil for at least 120 to 180 days after its release in root exudates or from biomass of *Bt* corn and for 90 days after its addition in purified form, the longest times evaluated in all cases, indicating again that the toxin was bound on surface-active particles in soil, which protected it from biodegradation.

The results of studies with transgenic plants and their biomass are summarized in Table 2.

Conclusions

These studies on the interaction of insecticidal proteins with surface-active particles demonstrate further the importance of such particles to the biology of soil and other natural habitats (e.g., sediments). 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, as well as on the transfer of genetic information among bacteria by conjugation, transduction, and transformation, in soil and other natural habitats (e.g., Stotzky, 1986, 1989, 2000; Vettori et al., 1999; Yin and Stotzky, 1997).

Moreover, these results indicate the potential environmental importance of insecticidal proteins 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 could 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 insecticidal protein.

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, and other pharmaceuticals, as well as other bioactive compounds, are released to the environment. 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, a substantial portion of the biomass of these plant factories containing the products of introduced novel genes will also be incorporated into soil. With factories of transgenic animals, feces, urine, and subsequently even carcasses containing bioactive compounds will eventually reach soil and other natural habitats. If these bioactive compounds (including prions from diseased animal carcasses) 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 large-scale use in the field of such plant and animal 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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