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ORIGINAL PAPER

Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil

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Abstract The Cry3Bb1 protein, insecticidal to the corn rootworm complex (*Diabrotica* spp.), of *Bacillus thuringiensis* (*Bt*) subsp. *kumamotoensis* was released in root exudates of transgenic *Bt* corn (event MON863) in sterile hydroponic culture (7.5 ± 1.12 ng/ml after 28 days of growth) and in nonsterile soil throughout growth of the plants (2.2 ± 0.62 ng/g after 63 days of growth). Kitchawan soil, which contains predominantly kaolinite (K) but not montmorillonite (M), was amended to 3 or 6% (vol./vol.) with K (3K and 6K soils) or M (3M and 6M soils) and with 1, 3, 5, or 10% (wt./wt.) of ground biomass of *Bt* corn expressing the Cry3Bb1 protein and incubated at 25 ± 2°C at the –33-kPa water tension for 60 days. Soils were analyzed for the presence of the protein every 7 to 10 days with a western blot assay (ImmunoStrip) and verified by ELISA. Persistence of the protein varied with the type and amount of clay mineral and the pH of the soils and increased as the concentration of K was increased but decreased as the concentration of M was increased. Persistence decreased when the pH of the K-amended soils was increased from *ca*. 5 to *ca*. 7 with $CaCO_3$: the protein was not detected after 14 and 21 days in the pH-adjusted 3K and 6K soils, respectively, whereas it was detected after 40 days in the 3K and 6K soils not adjusted to pH 7. The protein was detected for only 21 days in the 3M soil and for 14 days in the 6M soil, which were not adjusted in pH. These results indicate that the Cry3Bb1 protein does not persist or accumulate in soil and is degraded rapidly.

Keywords *Bacillus thuringiensis* · Transgenic corn · Cry3Bb1 protein · Corn rootworm · Root exudates · Clay minerals · pH · Persistence

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Introduction

Bacillus thuringiensis (*Bt*) is a bacterium present in various environments, including soil. During sporulation, *Bt* produces crystals composed of proteins toxic to insects and other organisms, and the proteins produced by different strains of the bacterium are toxic to different species of insects, including various pests. Thus, the genes that encode these toxic proteins are important in agriculture, as the genes introduced into crop plants can render the plants resistant to its pests, thereby decreasing the need for chemical insecticides (Hoffmann et al. 1992; Bolin et al. 1996; Mascarenhas and Luttrell 1997; Orr and Landis 1997; Schuler et al. 1999; Reed et al. 2001). Many transgenic crops (e.g., corn, cotton, rice, canola, potato) are now protected against lepidopteran and coleopteran pests because they express the insecticidal toxins from *Bt*. However, there is concern that *Bt* crops may pose risks to natural and agricultural

ecosystems (e.g., Rissler and Mellon 1996; Conway 2000; Hails 2000; Stotzky 2000, 2002, 2004).

Bt corn releases the Cry1Ab protein to soil in root exudates throughout the growth of the plant (Saxena et al. 1999, 2002; Saxena and Stotzky 2000), in pollen released during tasseling (Losey et al. 1999; Hansen-Jesse and Obrycki 2000), and from crop residues after harvest (Zwahlen et al. 2003; Stotzky 2004). Insecticidal Cry proteins from *B. thuringiensis* subsp. *kurstaki* (active against Lepidoptera), subsp. *morrisoni*, strain *tenebrionis* (active against Coleoptera), and subsp. *israelensis* (active against Diptera) are rapidly adsorbed and bound on clay minerals and humic substances, which renders them less available for biodegradation, but their insecticidal activity is retained (e.g., Venkateswerlu and Stotzky 1990, 1992; Tapp et al. 1994; Tapp and Stotzky 1995a, b, 1998; Koskella and Stotzky 1997; Crecchio and Stotzky 1998a, 2001; Lee et al. 2003; Stotzky 2004). Other proteins, peptides, amino acids, DNA, and viruses are also protected against microbial degradation and inactivation when bound on such surface-active particles (e.g., Lipson and Stotzky 1985, 1986; Stotzky 1986, 2004; Dashman and Stotzky 1986; Khanna and Stotzky 1992; Gallori et al. 1994; Vettori et al. 1996, 1999; Crecchio and Stotzky 1998b; Calamai et al. 2000; Lozzi et al. 2001; Crecchio et al. 2005). Moreover, repeated and large-scale use of transgenic *Bt* plants could result in the accumulation and persistence of plant-produced *Bt* proteins in soil (e.g., Tapp and Stotzky 1995a, 1998; Crecchio and Stotzky 1998a). The Cry1Ab protein released in root exudates of *Bt* corn persisted in soil for at least 180 days and from biomass for at least 3 years, the longest times studied (Saxena and Stotzky 2002; Stotzky 2002, 2004). Purified Cry1Ab protein remained larvicidal in nonsterile soil for at least 234 days (Tapp and Stotzky 1998), and the protein was detected in residues of *Bt* corn in soil in the field, with little or no degradation in residues in litter bags during the first 2 months and with only a small amount of protein remaining until late Spring of the following year (Zwahlen et al. 2003).

However, Muchaonyerwa et al. (2004) reported that the Cry1Ab protein from *Bt* corn persisted in tropical soils for only several weeks without losing insecticidal activity, and other studies with *Bt* corn and *Bt* cotton (Cry1Ab and Cry1Ac proteins, respectively) indicated that these proteins do not persist and are generally degraded in soil in *ca*. 20 days (Ream et al. 1994; Sims and Holden 1996; Palm et al. 1996; Hopkins and Gregorich 2003). Head et al. (2002) reported that no Cry1Ac protein was detected by both insect bioassay and ELISA in soil samples collected from fields with 3–6 years of continuous cultivation of *Bt* cotton. Dubelman et al. (2005) found that cultivation of corn expressing the Cry1Ab protein for more than three consecutive growing seasons did not result in accumulation or persistence of the protein in soil. The reasons for these contradictory results are not known, and additional studies appear to be necessary.

Transgenic *Bt* corn transformed with event MON863, which expresses the *cry3Bb1* gene from *B. thuringiensis* subsp. *kumamotoensis* to control the corn rootworm complex (*Diabrotica* spp.), was released for commercial use in 2003. The concentration of the Cry3Bb1 protein in tissues of *Bt* corn was significantly higher (81 μg/g of leaves, 41 μg/g of roots) than in other transgenic *Bt* corn lines (i.e., events 176, Bt11, and MON810) expressing the Cry1Ab protein (3–10 μg/g of leaves, negligible in roots) (EcoStrat 2002), and, therefore, the Cry3Bb1 may pose ecological and environmental risks. Ahmad et al. (2005) reported that the Cry3Bb1 protein in root exudates and decaying plant residues of transgenic *Bt* corn (event MON863) did not persist in soil for three consecutive seasons under field conditions and was rapidly degraded. The major objectives of this study were to determine: (1) whether the Cry3Bb1 protein was released in root exudates of *Bt* corn, similar to the Cry1Ab protein; and (2) the reasons for its relatively short persistence in soil. Here, we confirm that the Cry3Bb1 protein introduced to soil in root exudates and biomass of *Bt* corn (MON863) does not persist in soil.

Materials and methods

Release of Cry3Bb1 protein in sterile hydroponic culture and nonsterile soil

To determine whether the Cry3Bb1 protein is released in root exudates of *Bt* corn, two hybrids of corn (*Zea mays* L.), DeKalk DKC46-23 (event MON863) containing a truncated, synthetic version of a gene from *B. thuringiensis* subsp. *kumamotoensis*

coding for the expression of the anticoleopteran δ-endotoxin, Cry3Bb1, and its near-isogenic nongenetically modified variety, DKC46-26, were grown in sterile hydroponic culture and in nonsterile soil. Kernels of corn were surface sterilized with concentrated H_2SO_4 for 5 s, 95% ethanol for 5 min, and 35% H_2O_2 for 10 min and then washed repeatedly with sterile distilled water for 15 min (Saxena et al. 1999). Seeds were placed on nutrient agar (Difco) in Petri dishes at 25 ± 2°C to check for sterility and germinated for 3–4 days. Seedlings were transferred aseptically to sterile test tubes (3.5 cm diameter, 30 cm tall) containing plastic screening (6-mm mesh) 1 cm from the bottom and 12 ml of sterile Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture; Sigma) or to nonsterile soil in pots (18 cm diameter, 21 cm deep; 4 kg of soil/pot). Plants were grown at 25 ± 2°C under a 16-h light and 8-h dark cycle. The hydroponic medium was replenished aseptically as needed. When the corn plants reached the top of the tubes (after approximately 28 days), the Hoagland's solution was analyzed for the Cry3Bb1 protein immunologically by western blot (Immuno-Strip) and by enzyme-linked immunosorbent assay (ELISA) (Agdia, Elkhart, IN) (see below). Plants in soil were harvested at the flowering stage (after approximately 63 days) for biomass, and rhizosphere soil (RS) was collected by gently shaking the roots to remove adhering soil particles, sieved (0.85-mm sieve) to remove any root debris, and the soil incubated without any added *Bt* biomass.

Persistence of Cry3Bb1 protein from *Bt* corn biomass in soil

Biomass of *Bt* corn (mixture of roots, stems, and leaves) was dried at 50°C for 24 h, ground in a Sorval Omni mixer (particle size distribution of the ground material was *ca.* 80% < 0.5 mm and 20% < 2 mm), and incorporated at a concentration of 1, 3, 5, or 10% (wt./wt.) into Kitchawan soil (50 g of soil/jar), in which kaolinite is the predominant clay mineral, either unamended (C) or amended to 3 or 6% (vol./vol.) with montmorillonite (M; 3M, and 6M soils) or kaolinite (K; 3K and 6K soils). M and K were used, as they are the predominant clay minerals in many soils, and they differ markedly in structure and numerous physicochemical characteristics, as well as in their effects on biological activity in soil (see Stotzky 1986). These soil-clay mixtures have been used extensively in this laboratory for a spectrum of studies, and, therefore, there is a large data base available on them (e.g., Babich and Stotzky 1977; Stotzky 1986; Tapp and Stotzky 1995a, b, 1998). Table 1 presents some of the relevant physicochemical properties of these soil-clay mixtures.

The concentration of Cry3Bb1 protein in the biomass was estimated to be 1.6 ± 0.20 μg/g of dry biomass by ELISA, which was lower than reported concentrations of the protein, which ranged from 13 to 54 μg/g of fresh weight in above-ground plant material and 3.2 to 66 μg/g of fresh weight in roots (USEPA 2003; Vaughn et al. 2005). The reasons for this marked discrepancy in the concentration of the Cry3Bb1 protein in plant tissues are not known: they could be a result of differences between field- and laboratory-grown plants; source and age of the seeds; and methods of extraction and analysis. However, Wander and Gunapala (2004) reported similar low concentrations of the Cry3Bb1 protein in plant biomass in a field study: the concentration of the protein ranged from 1.7 to 2.5 μg/g of fresh weight in leaves, 0.4–1.0 μg/g of fresh weight in stems, and 0.1–0.4 μg/g of fresh weight in roots.

The biomass-amended soil–clay mixtures were incubated at 25 ± 2°C for 60 days at their –33-kPa

Table 1 Some physicochemical properties of the soil–clay mixtures used

Property	Soil–clay mixture				
	C	3K	6K	3M	6M
pH	4.9	4.8	4.9	5.4	5.8
Organic matter (%)	2.99	3.04	3.05	2.92	2.81
Total nitrogen (%)	0.13	0.13	0.13	0.13	0.13
CEC (cmol/kg)[a]	11.9	12.4	11.2	15.1	20.5
Sand (%)	56.8	58.2	54.8	57.8	52.2
Silt (%)	33.8	31.2	33.6	30.6	35.8
Clay (%)	9.4	10.6	11.6	11.6	12.0
Dominant clay minerals[b]	K, i	K, i	K, i	K, M, i	K, M, i

The Kitchawan soil was either unamended (C) or amended to 3 or 6% (vol./vol.) with kaolinite (3K, 6K) or montmorillonite (3M, 6M)

[a] CEC: Cation-exchange capacity

[b] K: kaolinite, M: montmorillonite, i: illite (minor component) (Tapp and Stotzky 1998)

water tension. Every 7–10 days, soils in individual jars were mixed with a glass rod, and two replicates of 0.5 g of soil each were analyzed for the presence of the Cry3Bb1 protein by western blot (ImmunoStrip test), and 2 g of soil from each jar was analyzed in duplicate for pH on a 1:5 soil:water suspension. For ELISA, 2 g of soil was removed from each jar, stored at –20°C, and soil samples that gave a positive results in the western blot assay were analyzed for the presence of the Cry3Bb1 protein by ELISA (some soils negative by western blot were also tested by ELISA, and they were always negative). The amount of the Cry3Bb1 protein on day 1 was estimated after 2 h of incubation of the soils at their –33-kPa water tension at room temperature after the addition of biomass.

To determine the effect of soil pH on the persistence of the Cry3Bb1 protein, the pH of the 3K and 6K soils, in which there was longer persistence of the protein than in the M soils, was adjusted to *ca*. pH 7 by the addition of $CaCO_3$, based on previous studies with the Cry1Ab protein (Tapp and Stotzky 1998). The soils were incubated and assayed for the presence of the protein as above.

Immunological assay

The presence of the Cry3Bb1 protein in root exudates and soil was determined immunologically with ImmunoStrips (lower limit of detection is 0.4 ng of protein/ml), which are essentially western blot assays (Agdia, Elkhart, IN). Root exudates were analyzed directly in the Hoagland's solution. Samples of soils (0.5 g) were vortexed for 1–2 min with 0.5 ml of an extraction buffer (SEB4 buffer; Agdia) in 1.5-ml Eppendorf tubes, centrifuged at 10,500 × *g* for 5 min, and the supernatants were analyzed with ImmunoStrips following the recommended protocol of the manufacturer.

ELISA

The relative concentration of the Cry3Bb1 protein in root exudates, plant biomass, rhizosphere soil, and in the soil samples from the incubation experiment was determined with an ELISA kit (*Bt*-Cry3Bbl ELISA; Agdia) (lower limit of detection is 1 ng of protein/ml of extracted sample). Root exudates were analyzed directly in the Hoagland's solution; leaves plus stems and roots of individual plants from hydroponic culture and 0.1 g of dry (50°C) ground plant biomass were homogenized in 1 ml of extraction buffer (PBST; phosphate buffered saline–Tween 20), provided in the kit, with a mortar and pestle; and 0.5 g of soil was vortexed for 1–2 min with 0.5 ml of PBST. The NaCl, KCl, and Tween 20 in the PBST buffer solution (pH 7.6) presumably maximized the extraction efficiency by minimizing ionic and hydrophobic interactions between soil particles and the Cry3Bb1 protein (Palm et al. 1994). Extraction buffers containing a high content of salts and Tween 20 at a neutral or higher pH have been used in numerous studies (e.g., Hopkins and Gregorich 2003; Ferreira et al. 2003; Muchaonyerwa et al. 2004; Vadakattu and Watson 2004; Baumgarte and Tebbe 2005; Douville et al. 2005) and have resulted in *ca*. 60–80% recovery of Cry proteins from soil. After incubation at room temperature for 30 min, samples were centrifuged at 10,500 × *g* for 5 min, and the supernatants (100 µl) (plant extracts were diluted to be within the range of the Cry3Bb1 ELISA standards) were added to each well of an ELISA plate coated with the antibody to the Cry3Bb1 protein and containing 100 µl of an antibody-peroxidase conjugate. After incubation for 2 h at 27°C, the wells were washed six times with diluted PBST buffer, 100 µl of the tetramethyl benzidine substrate solution was added, the plates incubated for 15 min at room temperature, and the optical density at 650 nm was measured in a Microplate reader (Bio-Rad, Model 550) using Microplate Manager 4 Software. A standard curve, using a purified Cry3Bb1 protein solution (7.6 mg of total protein/ml) kindly provided by the Monsanto Company, was used for estimating the concentration of the Cry3Bb1 protein in plant and soil samples.

Protein assay and SDS-PAGE

The amount of protein in root exudates was determined directly in the Hoagland's solution by the Lowry assay, using bovine serum albumin as the standard (Lowry et al. 1951). The proteins were separated by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels (9.0 cm × 9.0 cm)

(SDS-PAGE) at room temperature with a voltage of 10 V/cm (10 mA). After the front of the stacking dye (Bromophenol Blue) moved into the resolving gel, the voltage was increased to 15 V/cm, and electrophoresis was continued for 3–4 h until the dye reached the bottom of the resolving gel (Laemmli 1970). The gels were stained for 2 h with a 0.25% (wt./vol.) Brilliant Blue R 250 dye solution (45% methanol and 10% glacial acetic acid) and destained for 24 h with four changes of the destaining solution (30% methanol and 10% glacial acetic acid). Proteins in the Hoagland's solution were precipitated with 20% trichloroacetic acid (TCA) before SDS-PAGE: 1 ml of TCA was added to 1 ml of the Hoagland's solution, vortexed, incubated for 60 min on ice, centrifuged at 10,500 × *g* for 10 min, and the protein pellet washed 3–4 times with 300 µl of cold acetone by vortexing and centrifugation. The pellet was air-dried at room temperature and resuspended in SDS-PAGE loading buffer by heating at 100°C for 10 min.

Statistics

At least 10 replicate samples of root exudates, plant biomass (grown in Hoagland's solution and soil), and rhizosphere soils were evaluated by western blots, and five replicate samples were used for ELISA, the data for which are expressed as the means ± the standard errors of the means (SEM). Western blots and ELISA of incubated soils amended with biomass of *Bt* corn were done with two replicates and expressed as the means ± SEM. Analysis of variance (ANOVA) was used to determine significance among the data, and $p < 0.05$ was considered significant.

Results

Release of Cry3Bb1 protein in root exudates in hydroponic culture and nonsterile soil

The Cry3Bb1 protein was detected by western blot in root exudates of transgenic *Bt* corn (DKC46-23, event MON863) in sterile hydroponic culture. The concentration of the Cry3Bb1 protein released in root exudates was 7.5 ± 1.12 ng/ml, as determined by ELISA. The content of the Cry3Bb1 protein in leaves plus stems and in roots was 725 ± 17.9 and 449 ± 19.2 ng/plant, respectively, after 28 days of growth in hydroponic culture. No Cry3Bb1 protein was detected in either root exudates or plant biomass of non-*Bt* corn (DKC46-26). The concentration of total protein in root exudates of *Bt* corn, determined by the Lowry assay, was 119 ± 6.8 µg/ml. No band on SDS-PAGE corresponding to a molecular mass (M_r) of 74 kDa, the M_r of the Cry3Bb1 protein, was detected in root exudates of *Bt* corn, even after concentration of the proteins by precipitation with 20% TCA. The concentration of the Cry3Bb1 protein in the Hoagland's solution was apparently too low to be detected by SDS-PAGE. However, several bands of smaller M_r (37 and 45 kDa) were detected by SDS-PAGE, suggesting that the Cry3Bb1 protein was degraded during preparation of the samples.

The Cry3Bb1 protein was also detected by western blot in nonsterile rhizosphere soil of *Bt* corn after 63 days of growth; the concentration was 2.2 ± 0.62 ng/g of soil, as determined by ELISA.

Persistence of Cry3Bb1 protein in soil

Persistence varied with the type of clay mineral present. In general, the protein persisted, as determined by western blots, in the Kitchawan soil (C) unamended with clay, in which K is the predominant clay mineral, and in the C soil amended to 3 or 6% with K (3K and 6K soils) for *ca*. 40 days of incubation, whereas the protein persisted in the C soil amended to 3 or 6% with M (3M and 6M soils) for only *ca*. 21 days of incubation (Figs. 1 and 2).

The Cry3Bb1 protein was detected in the C soil amended with 1% of *Bt* biomass after 21 days and in the C soil amended with 3, 5, or 10% of biomass after 40 days by western blots. The amount of the protein in the C soil amended with 1% of biomass was estimated by ELISA to be 1.8 ng/g of soil initially (i.e., after 2 h of incubation at room temperature), and there were no significant differences ($F = 3.15$, $df = 5$, $p = 0.098$) in the amount of protein during the following 21 days, but no protein was detected after 30 days (Fig. 1). The initial protein concentration in the C soil amended with 3, 5, or 10% of biomass was 7.8 ± 0.45, 40.4 ± 7.56, and 90.4 ± 6.08 ng/g of soil, respectively, and it decreased significantly to 0.7 ± 0.67 ($F = 12.01$, $df = 5$, $p < 0.01$), 1.8 ± 0.47

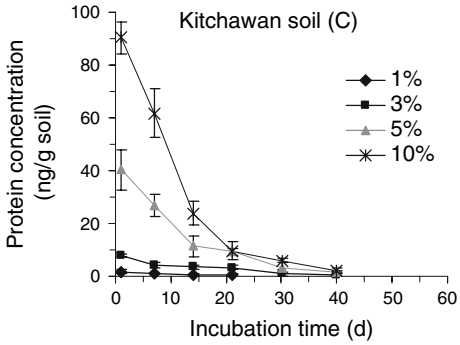

Fig. 1 Persistence of Cry3Bb1 protein, as determined by ELISA, in Kitchawan soil (C) amended with 1, 3, 5, or 10% (wt./wt.) of ground biomass of *Bt* corn (event MON863). The concentration of the Cry3Bb1 protein in ground plant biomass was 1.6 ± 0.20 μg/g. The initial amounts of the Cry3Bb1 protein in the soils were estimated after 2 h of incubation at room temperature at their –33-kPa water tension after the addition of biomass. The data are expressed as the means ± the standard errors of the means

($F = 14.19$, $df = 5$, $p < 0.01$), and 2.1 ± 1.01 ($F = 50.55$, $df = 5$, $p < 0.01$) ng/g of soil, respectively, in 40 days; no protein was detected after 50 days (Fig. 1).

The protein was detected by western blots in the 3K soil after 14 days when amended with 1% of biomass, after 40 days when amended with 3 or 5% of biomass, and after 30 days when amended with 10% of biomass. The initial amount of the protein in the 3K soil amended with 1 or 3% of biomass was estimated by ELISA to be 0.4 ± 0.23 and 2.4 ± 0.22 ng/g of soil, respectively, and there were no significant differences ($F = 1.34$, $df = 2$, $p = 0.373$; $F = 2.73$, $df = 5$, $p = 0.126$, respectively) in the amount of the protein (0.5–3.2 ng/g of soil) detected during the following 14 days and 40 days, respectively. However, in the 3K soil amended with 5% of biomass, the Cry3Bb1 protein decreased significantly ($F = 9.96$, $df = 5$, $p < 0.01$) in 40 days from an estimated initial amount of 28.1 ± 2.05 to 0.8 ± 0.31 ng/g of soil, and in the 3K soil amended with 10% of biomass, it decreased significantly ($F = 36.54$, $df = 5$, $p < 0.01$) from 96.5 ± 7.17 to 3.4 ± 1.80 ng/g of soil in 30 days; no protein was detected after 50 days (Fig. 2a).

The protein was detected by western blots after 30 days in the 6K soil amended with 1% of biomass,

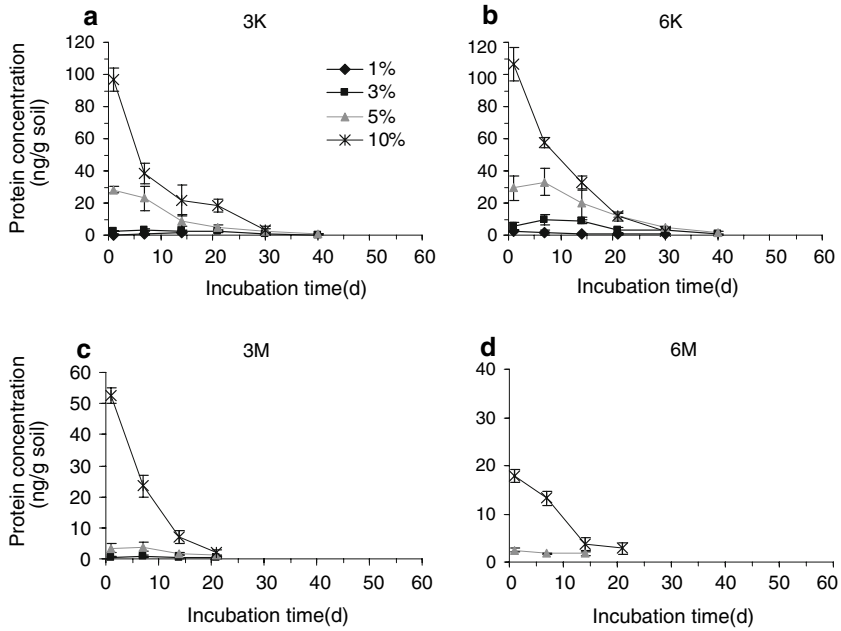

Fig. 2 Persistence of Cry3Bb1 protein, as determined by ELISA, in Kitchawan soil amended to 3 or 6% (vol./vol.) with kaolinite (3K and 6K soils) or amended to 3 or 6% (vol./vol.) with montmorillonite (3M and 6M soils) and with 1, 3, 5, or 10% (wt./wt.) of ground biomass of *Bt* corn (event MON863). The concentration of the Cry3Bb1 protein in ground plant biomass was 1.6 ± 0.20 μg/g. The initial amounts of the Cry3Bb1 protein in the soils were estimated after 2 h of incubation at room temperature at their –33-kPa water tension after the addition of biomass. The data are expressed as the means ± the standard errors of the means

after 40 days when amended with 3 or 5% of biomass, and after 30 days when amended with 10% of biomass. The initial amount of the protein in the 6K soil amended with 1 or 3% of biomass was estimated by ELISA to be 2.6 ± 0.28 and 5.7 ± 2.30 ng/g of soil, respectively, and the amount did not differ significantly ($F = 2.15$, $df = 5$, $p = 0.190$; $F = 3.29$, $df = 5$, $p = 0.089$, respectively) during the subsequent 30 days and 40 days, respectively. In the 6K soil amended with 5 or 10% of biomass, the Cry3Bb1 protein decreased significantly from 29.4 ± 7.53 to 1.6 ± 1.18 ng/g of soil ($F = 4.95$, $df = 5$, $p < 0.05$) after 40 days and from 106.2 ± 14.57 to 2.3 ± 1.65 ng/g of soil ($F = 40.31$, $df = 5$, $p < 0.01$) after 30 days, respectively; no protein was detected after 50 days (Fig. 2b).

The protein was detected in the 3M soil for only 21 days, except in the 3M soil amended with 1% of biomass, where no protein was detected by western blots after addition. The initial amount of the protein in the 3M soil amended with 3 or 5% of biomass was estimated by ELISA to be 0.5 ± 0.18 and 3.6 ± 1.45 ng/g of soil, respectively, and the amount did not change significantly ($F = 0.21$, $df = 3$, $p = 0.882$; $F = 1.50$, $df = 3$, $p = 0.343$, respectively) during 21 days, whereas in the 3M soil amended with 10% of biomass, the amount of the protein decreased significantly ($F = 87.12$, $df = 3$, $p < 0.01$) from an initial amount of 52.5 ± 2.58 to 1.9 ± 1.01 ng/g of soil after 21 days; no protein was detected after 30 days (Fig. 2c).

The Cry3Bb1 protein was not detected by western blots in the 6M soil amended with 1 or 3% of biomass throughout the 60 days of incubation. However, the protein was detected in the 6M soil amended with 5 or 10% of biomass after 14 and 21 days, respectively. The amount of the protein in the 6M soil amended with 5% of biomass did not differ significantly ($F = 0.72$, $df = 3$, $p = 0.626$) during 14 days from an initial estimated amount of 2.5 ± 0.37 ng/g of soil, whereas in the 6M soil amended with 10% of biomass, the amount of the protein decreased significantly ($F = 27.89$, $df = 3$, $p < 0.01$) from an initial estimated amount of 17.8 ± 1.38 to 2.9 ± 1.22 ng/g of soil in 21 days; no protein was detected after 30 days (Fig. 2d).

The persistence of the Cry3Bb1 protein in the soils also varied with the amount of clay mineral added. As the concentration of K was increased from 3 to 6%, persistence of the protein increased, whereas persistence decreased with an increase in the amount of M added from 3 to 6% (Figs. 1 and 2). For example, there was no significant difference ($F = 0.82$, $df = 1$, $p = 0.393$) in the estimated initial amount of the protein between the 6K and 3K soils, but after 7 days, there was a significant ($F = 6.87$, $df = 1$, $p < 0.05$) difference between the 6K and 3K soils: the amount of the protein in the 6K soils was significantly ($p < 0.05$) higher than in the 3K soils. However, no significant differences ($F = 3.71$, $df = 1$, $p = 0.090$) in the amount of the protein between the 6K and 3K soils were observed after 14 days (Fig. 2a, b). There was also a significant difference in the amount of the protein between the 3M and 6M soils: in contrast to the K soils, the amount of protein in the 3M soils was significantly ($F = 120.34$, $df = 1$, $p < 0.01$) higher than in the 6M soils initially and after 7 days ($F = 10.16$, $df = 1$, $p < 0.05$). No significant differences in the amount of the protein between the 3M and 6M soils were observed after 14 days ($F = 2.19$, df = 1, $p = 0.177$) (Fig. 2c, d).

The concentration of the Cry3Bb1 protein in the soils varied with the amount of *Bt* biomass added. As the amount of biomass added was increased from 1 to 10%, the initial concentration of the protein increased in all soils (Figs. 1–3). However, the increases were not correlated with the persistence of the protein. For example, in the C, 3K, and 6K soils, regardless of the concentration of *Bt* biomass added initially, the protein was detected for only 40 days and only at a low level (<2.2 ng/g of soil), with no significant differences ($F = 2.56$, $df = 2$, $p = 0.119$) between the soils. No protein was detected in any soil after 50 and 60 days of incubation.

The addition of *Bt* biomass increased significantly ($F = 32.17$, $df = 1$, $p < 0.01$) the pH of the soils, especially after day 7. For example, there were no significant differences in the initial pH between soils amended with 1, 3, 5, and 10% of biomass, whereas after 7 days, the pH of the C, 3K, 6K, 3M, and 6M soils amended with 1% of biomass was 4.7, 4.5, 5.1, 5.8, and 5.1, respectively, and when amended with 10% of biomass, the pH was 7.5, 6.1, 7.0, 7.5, and 6.8, respectively. These differences in pH were maintained during the 60 days of incubation. However, after 30 days, in general, the pH of all soils decreased slightly (data not shown).

The Cry3Bb1 protein was detected by western blots in the rhizosphere soil (RS) to which no exogenous *Bt* biomass was added only until day 14

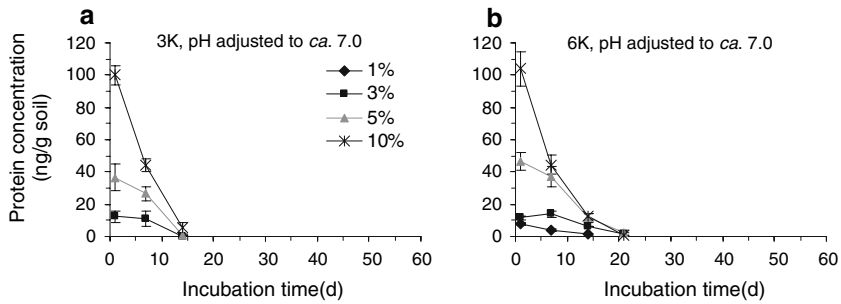

Fig. 3 Persistence of Cry3Bb1 protein, as determined by ELISA, in Kitchawan soil amended to 3 or 6% (vol./vol.) with kaolinite (3K and 6K soils), adjusted to *ca*. pH 7 with $CaCO_3$, and 1, 3, 5, or 10% (wt./wt.) of ground biomass of *Bt* corn (event MON863) was added. The concentration of the Cry3Bb1 protein in ground plant biomass was 1.6 ± 0.20 μg/g. The initial amounts of the Cry3Bb1 protein in the soils were estimated after 2 h of incubation at room temperature at their –33-kPa water tension after the addition of biomass. The data are expressed as the means ± the standard errors of the means

of the incubation, indicating that the protein released in root exudates was degraded quickly. The concentration of the protein was estimated by ELISA to be 2.2 ± 0.62, 2.0 ± 0.17, and 0.7 ± 0.11 ng/g of soil initially and after 7 and 14 days, respectively. There were no significant changes in the pH of the unamended RS during the 60 days of incubation (data not shown).

To determine whether the relatively rapid decrease in the amount of the Cry3Bb1 protein in the M soils was the result their higher pH, which enhanced the biodegradation of the protein, as has been shown for the Cry1Ab protein (Tapp and Stotzky 1998), the pH of the 3K and 6K soils, in which the protein was detected for 40 days, was adjusted to *ca*. pH 7 by the addition of $CaCO_3$. When the pH of the 3K and 6K soils amended with 0, 1, 3, 5, or 10% of *Bt* biomass was adjusted to *ca*. pH 7, there was a decrease in the persistence of the protein (Fig. 3). For example, the protein was detected by western blots and ELISA in the pH-adjusted 3K soil amended with *Bt* biomass for only 14 days (Fig. 3a), whereas it was detected in the pH-unadjusted 3K soil amended with biomass after 40 days (Fig. 2a). The protein was detected for only 21 days in the pH-adjusted 6K soil amended with biomass (Fig. 3b), whereas it was detected in the pH-unadjusted 6K soil amended with biomass after 40 days of incubation (Fig. 2b).

Discussion

The Cry3Bb1 protein was released in root exudates of transgenic *Bt* corn (event MON863) and was present in rhizosphere soil throughout growth of the plants, as determined by western blots and ELISA, similar to the Cry1Ab (in *Bt* corn and rice) and Cry3A (in *Bt* potato) proteins (Saxena et al. 1999, 2002, 2004; Saxena and Stotzky 2000; Stotzky 2004), albeit at lower levels. However, the Cry3Bb1 protein was detected in rhizosphere soil unamended with *Bt* corn biomass (i.e., only released in root exudates) for only 14 days. The persistence of the protein in soil amended with biomass of *Bt* corn was dependent on the type and amount of clay mineral present and on the pH of the soils. In general, the protein persisted in the C, 3K, and 6K soils for *ca*. 40 days, whereas it persisted in the 3M and 6M soils for only 21 days, regardless of the amount of *Bt* biomass added (Figs. 1 and 2). Although the addition of increasing amounts of *Bt* biomass significantly increased the amount of the protein detected initially and during the first 7 and 14 days of incubation, the differences in the amount of the protein detected in soils to which different concentrations of biomass had been added were generally not significant after 21 days. The addition of *Bt* biomass also significantly increased the pH of the soils, which apparently enhanced the biodegradation of the protein.

The degradation of *Bt* proteins, as well as of other proteins, peptides, amino acids, DNA, and other biomolecules, in soil depends largely on the level of microbial activity (e.g., Lipson and Stotzky 1985, 1986; Dashman and Stotzky 1986; Stotzky 1986; Khanna and Stotzky 1992; Gallori et al. 1994; Vettori et al. 1996, 1999; Palm et al. 1996; Koskella and Stotzky 1997; Crecchio and Stotzky 1998a; Tapp and Stotzky 1998). Many properties of soil, including

microbial activity, depend on the type and amount of clay minerals present (Stotzky 1986). The growth and metabolic activity (as measured respirometrically) of bacteria are higher in soils that contain M, either naturally or amended with mined M, as soils containing M are generally better buffered against decreases in pH than soils not containing M (Stotzky 1986; Chenu and Stotzky 2002). Hence, the more rapid decrease in the concentration of the Cry3Bb1 protein in the M soils was probably the result of more rapid microbial utilization of the protein, as has been shown with the Cry1Ab protein (Tapp and Stotzky 1998).

When the pH of the 3K and 6K soils was adjusted to *ca*. 7, there was a significant decrease in the persistence of the Cry3Bb1 protein (Fig. 3), indicating that the decrease in the pH-adjusted K soils, as well as in the M soils (Fig. 2c, d), was a result of the more rapid biodegradation of the protein at higher pH values. Tapp and Stotzky (1998) observed a greater reduction in the insecticidal activity of Cry1Ab protein added to nonsterile soils during 234 days of incubation when the pH of the soils was either initially higher or was increased from 4.9 to *ca*. 7 by the addition of $CaCO_3$. Insecticidal activity was greater and persisted longer in soil naturally containing or amended with K than in soils naturally containing or amended with M, presumably because soils containing M had a higher pH (5.8–7.3) and, therefore, more microbial activity (Stotzky 1986), which resulted in more biodegradation of the protein (Tapp and Stotzky 1998). In addition to the increase in microbial activity in soils with a pH near neutrality, adsorption of Cry1Ab, Cry3Aa, and Cry4 proteins on clays decreased with an increase in pH (Tapp et al. 1994; Tapp and Stotzky 1998; Vettori et al. 1999; Crecchio and Stotzky 2001; Lee et al. 2003), which also rendered a portion the proteins more susceptible to biodegradation. Similarly, Wang et al. (2006) reported that the Cry1Ab protein from biomass of *Bt* rice degraded faster in an alkaline soil (half-life of 11.5 days) than in an acidic soil (half-live of 34.3 days). Free Cry1Ab protein (i.e., not bound on clays or humic substances) was readily utilized as a sole source of carbon and/or nitrogen by pure and mixed cultures of microbes, whereas the bound protein was resistant to utilization, especially as a source of carbon (Koskella and Stotzky 1997; Crecchio and Stotzky 1998a, 2001).

The results of this study were similar to those reported by Ahmad et al. (2005), who found no detectable Cry3Bb1 protein in soil planted with *Bt* corn for three consecutive seasons in Manhattan, KS, USA, and concluded that the Cry3Bb1 protein released in root exudates or from decaying plant residues does not persist and is rapidly degraded in soil. In a field study conducted (2003–2006) at the Rosemount Experiment Station of the University of Minnesota, the *Bt* corn variety, DeKalb DKC46-24, that expresses the Cry3Bb1 protein and its near-isogenic non-*Bt* variety, DKC46-28, were evaluated for the persistence of the Cry3Bb1 protein in soil by western blots and ELISA, as well as for their effects on microbial diversity and enzyme activities. This field study also showed no detectable Cry3Bb1 protein in rhizosphere soils of *Bt* corn expressing this protein, indicating again that the Cry3Bb1 protein does not persist long in soil (Icoz et al. 2007).

Variable rates of degradation of Cry proteins in soil have been reported. Head et al. (2002) found no detectable levels of Cry1Ac protein by ELISA and insect bioassay in soils from fields on which transgenic *Bt* cotton had been grown and the biomass incorporated into soil for 3–6 consecutive years. The proteins from Bt corn, Bt cotton, and *Bt* potato (Cry1Ab, Cry1Ac, and Cry3Aa protein, respectively) did not persist and were generally degraded in soil within 20 days (Ream et al. 1994; Palm et al. 1996; Sims and Holden 1996). Sims and Holden (1996) reported a 90% decrease in the insecticidal activity of Cry1Ab protein in 15 days in soil and suggested that the Cry1Ab protein in corn plant tissue is unstable in soil under field conditions and likely to degrade rapidly under normal cultivation practices. Hopkins and Gregorich (2003) reported that much of the *Bt* δ-endotoxin in corn residues is highly labile and quickly decomposes in soils in the field but that a small fraction may be protected from degradation in relatively recalcitrant residues. Dubelman et al. (2005) found no evidence of persistence or accumulation of Cry1Ab protein in soils from fields planted for at least three consecutive years with *Bt* corn.

By contrast, Zwahlen et al. (2003) found that the Cry1Ab protein in corn plant tissue was stable and degraded only as the plant material degraded, and the Cry1Ab toxin could still be detected after 240 and 200 days under tillage and no-tillage, respectively, even when only small amounts of plant material

remained. Saxena and Stotzky (2002) reported that the Cry1Ab protein in root exudates and biomass of *Bt* corn persisted in soil for up to 180 days and 3 years, respectively, the longest times studied. Wang et al. (2006) reported that the Cry1Ab protein from biomass of *Bt* rice was still detectable in acidic soils after 120 days of incubation. Persistence is probably a result of the binding of *Bt* proteins on surface-active particles, such as clay minerals and humic acids (Venkateswerlu and Stotzky 1990, 1992; Tapp et al. 1994; Tapp and Stotzky 1995a, b, 1998; Koskella and Stotzky 1997; Crecchio and Stotzky 1998a, 2001), where the proteins are protected against microbial degradation and yet retain their insecticidal activity.

In conclusion, despite the low level of the Cry3Bb1 protein in the plant biomass used in this study, in contrast to higher concentration reported by others (e.g., Vaughn et al. 2005), the major result of this study was that the protein does not accumulate in soil. The Cry3Bb1 protein is released in root exudates and from decaying plant residues of *Bt* corn, but the protein does not persist in soil and is degraded rapidly, suggesting that it probably poses little ecological or environmental risk. The importance of pH and other physicochemical, as well as biological, characteristics of soil in the persistence of various Cry proteins in soil needs to be determined, especially to explain the reported differences in the persistence of the proteins.

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