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Transgenic *Bt* plants decompose less in soil than non-*Bt* plants

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Abstract

Bt plants are plants that have been genetically modified to express the insecticidal proteins (e.g. Cry1Ab, Cry1Ac, Cry3A) from subspecies of the bacterium, *Bacillus thuringiensis* (*Bt*), to kill lepidopteran pests that feed on corn, rice, tobacco, canola, and cotton and coleopteran pests that feed on potato. The biomass of these transgenic *Bt* plants (*Bt*+) was decomposed less in soil than the biomass of their near-isogenic non-*Bt* plant counterparts (*Bt*−). Soil was amended with 0.5, 1, or 2% (wt wt^{-1}) ground, dried (50 °C) leaves or stems of *Bt* corn plants; with 0.5% (wt wt^{-1}) ground, dried biomass of *Bt* rice, tobacco, canola, cotton, and potato plants; with biomass of the near-isogenic plants without the respective *cry* genes; or not amended. The gross metabolic activity of the soil was determined by CO_2 evolution. The amounts of C evolved as CO_2 were significantly lower from soil microcosms amended with biomass of *Bt* plants than of non-*Bt* plants. This difference occurred with stems and leaves from two hybrids of *Bt* corn, one of which had a higher C:N ratio than its near-isogenic non-*Bt* counterpart and the other which had essentially the same C:N ratio, even when glucose, nitrogen (NH_4NO_3), or glucose plus nitrogen were added with the biomass. The C:N ratios of the other *Bt* plants (including two other hybrids of *Bt* corn) and their near-isogenic non-*Bt* counterparts were also not related to their relative biodegradation. *Bt* corn had a significantly higher lignin content than near-isogenic non-*Bt* corn. However, the lignin content of the other *Bt* plants, which was significantly lower than that of both *Bt* and non-*Bt* corn, was generally not statistically significantly different, although 10–66% higher, from that of their respective non-*Bt* near-isolines. The numbers of culturable bacteria and fungi and the activity of representative enzymes involved in the degradation of plant biomass were not significantly different between soil amended with biomass of *Bt* or non-*Bt* corn. The degradation of the biomass of all *Bt* plants in the absence of soil but inoculated with a microbial suspension from the same soil was also significantly less than that of their respective inoculated non-*Bt* plants. The addition of streptomycin, cycloheximide, or both to the soil suspension did not alter the relative degradation of *Bt*+ and *Bt*− biomass, suggesting that differences in the soil microbiota were not responsible for the differential decomposition of *Bt*+ and *Bt*− biomass. All samples of soil amended with biomass of *Bt* plants were immunologically positive for the respective Cry proteins and toxic to the larvae of the tobacco hornworm (*Manduca sexta*), which was used as a representative lepidopteran in insect bioassays (no insecticidal assay was done for the Cry3A protein from potato). The ecological and environmental relevance of these findings is not clear.

Keywords: *Bacillus thuringiensis*; Cry proteins; Bt plants; Antibiotics; Lignin; Biomass decomposition; C:N ratio; Microbial counts; Enzyme activity; Immunological and insecticidal assays

1. Introduction

Bacillus thuringiensis (*Bt*), a gram-positive, spore-forming bacterium, produces a variety of insecticidal crystal proteins (ICPs) toxic to lepidopteran, dipteran, and coleopteran larvae (Höfte and Whiteley, 1989; Crickmore et al., 1998; Schnepf et al., 1998). This characteristic has made the genes encoding ICPs attractive for genetic improvement of crops to provide protection against insect pests. The incorporation into plants of insecticidal genes from *Bt* has reduced many problems associated with the use of broad-spectrum chemical pesticides, as the toxins are produced continuously within these plants and exhibit relatively high specificity for insect pests. However, there is some concern that genetically engineered *Bt* crops may pose risks to natural and agricultural ecosystems (e.g. Rissler and Mellon, 1996; Conway, 2000; Hails, 2000; Stotzky, 2000, 2002). The

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toxins enter soil by incorporation of plant residues after harvest of a *Bt* crop (Tapp and Stotzky, 1998; Stotzky, 2000, 2002) and in root exudates from some *Bt* plants (Saxena et al., 1999, 2002a,b, 2004; Saxena and Stotzky, 2000, 2001a, 2003), with probably some input from pollen (Losey et al., 1999; Obrycki et al., 2001). The toxins adsorb and bind rapidly on surface-active particles (e.g. clays and humic substances) in soil and, thereby, persist but remain larvicidal (Stotzky, 2000, 2002). When purified Cry1Ab protein from *B. thuringiensis* subsp. *kurstaki* was added to non-sterile soils, activity against the larva of the tobacco hornworm (*Manduca sexta*), the assay larva, was still detected after 234 days (Tapp and Stotzky, 1998), and the toxin was detected in soil for 180 days from root exudates after growth of *Bt* corn (Saxena and Stotzky, 2002) and from biomass of *Bt* corn 3 years after incorporation into soil (Saxena and Stotzky, 2003), the longest times evaluated in all cases.

The 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*; 27, 65, 128, and 135 kDa; active against some Diptera) adsorbed and bound rapidly (in <30 min, the shortest time studied) on clay minerals (montmorillonite and kaolinite), on the clay-size fraction of soil, on humic acids, and on complexes of montmorillonite–humic acids–Al hydroxy-polymers (Tapp et al., 1994; Tapp and Stotzky, 1995a,b; Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Stotzky, 2000, 2002; Lee et al., 2003). The binding of the toxins on these surface-active particles reduced their availability to microbes, which is probably responsible for the persistence of the toxins in soil (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Stotzky, 2000, 2002; Saxena and Stotzky, 2003). These results indicated that the 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. As the result of the binding of the toxins on surface-active particles, the toxins could accumulate in the environment to concentrations that may increase the control of target pests; constitute a hazard to non-target organisms, such as the soil microbiota, 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), and other animal classes; and/or enhance the selection and enrichment of toxin-resistant target insects (e.g. Van Rie et al., 1990; McGaughey and Whalon, 1992; Bauer, 1995; Tabashnik et al., 1997).

The toxin released to soil from *Bt* corn in root exudates or biomass had no significant effects on earthworms, nematodes, and numbers of culturable protozoa, fungi, and bacteria (Saxena and Stotzky, 2001c). The toxin was not taken up from soil by radish, carrot, turnip, and non-*Bt* corn (Saxena and Stotzky, 2001a, 2002).

The greatest input of the toxins into soil will result from post-harvest incorporation of the voluminous biomass of *Bt* crops. The major objective of this study was to compare the decomposition of *Bt* and non-*Bt* plant biomass in soil. Here, we show that biomass of various transgenic *Bt* plants is decomposed less in soil than biomass of near-isogenic non-*Bt* plants.

2. Materials and methods

2.1. Soils

A freshly collected soil (0–10 cm; classified as Riverhead sandy loam) from a farm in East Marion, Long Island, New York, USA, was sieved through a broad-mesh screen (15 mm), to remove stones and plant debris and to disrupt large soil aggregates, and then sieved through a 5-mm sieve. The sieved soil was mixed thoroughly and maintained moist (ca. field capacity) at 24 ± 2 °C. Some physicochemical characteristics of the soil are: pH 5.2; 0.92 and 0.07% carbon and nitrogen; 58, 41, and 1% sand, silt, and clay.

Kitchawan soil, a sandy loam that naturally contains predominantly kaolinite, was collected at the Kitchawan Research Laboratory of the Brooklyn Botanical Garden, Ossining, New York, USA. The soil was amended to 9% (vol vol^{-1}) with montmorillonite. Stable soil–clay mixtures of this soil have been used extensively in this laboratory in studies on the effects of the physicochemical and biological characteristics 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 insecticidal proteins from *Btk* and *Btt* in soil (see Stotzky, 2002; Yin and Stotzky, 1997; Tapp and Stotzky, 1998; Saxena and Stotzky, 2003). Therefore, there is a large data base available on these mixtures.

2.2. Plant material

Trangenic plants genetically modified to express various *cry* genes (Harper et al., 1999; Shu et al., 2000; USEPA, 2001) from different subspecies of *B. thuringiensis* and their near-isogenic non-*Bt* counterparts were used (Table 1). Seeds of corn, rice, canola, tobacco, and cotton and 'eyes' of potato were planted (four pot^{-1} and three pots plant species^{-1} for each *Bt* and non-*Bt* counterpart) in plastic pots (18 cm diameter, 21 cm deep) containing ca. 4.5 kg of the Long Island soil, and the plants were grown in a plant-growth room (26 ± 2 °C, 12-h light–dark cycle; soil water content was maintained at ca. field capacity, and no water stress was apparent in the plants) until flowering and, with the exception of rice and cotton, production of seeds. The age of transgenic and near-isogenic non-*Bt* plants of each species were the same when harvested. The plant biomass was dried at 50 °C to constant weight and ground with

Table 1
Total amount of carbon evolved as CO_2 from soil amended with 0.5% (wt wt^{-1}) ground, dried (50 °C) biomass of different transgenic *Bt* plants (*Bt*+) or their near-isogenic non-*Bt* counterparts (*Bt*−)

Plant	Plant line	Gene transformed	Metabolic activity (mg C 100 g^{-1} soil ± SEM)	Immunological assay	Mortality (% ± SEM)	Larval weight (g ± SEM)
Corn						
Bt−						
	NK4640	–	73.1 ± 2.36	−	6 ± 6.3	0.9 ± 0.04
	PrimePlus	–	76.3 ± 3.42	−	6 ± 6.3	0.8 ± 0.03
	DK647	–	75.3 ± 3.01	−	0 ± 00	1.1 ± 0.06
Bt+						
Bt11	NK4640Bt	*cry1Ab*	52.3 ± 3.62	+	50 ± 10.2	0.09 ± 0.03
Bt11 (sweet corn)	0966	*cry1Ab*	55.2 ± 3.20	+	43 ± 6.3	0.06 ± 0.01
MON810	DK647Bty	*cry1Ab*	58.3 ± 2.83	+	37 ± 7.2	0.08 ± 0.01
Rice						
Bt−	Xiu shu 11	–	55.6 ± 1.38	−	6 ± 6.3	1.0 ± 0.05
Bt+	KMD2; cv. japonica	*cry1Ab*	38.0 ± 1.25	+	50 ± 14.2	0.06 ± 0.01
Potato						
Bt−	R-Burbank	–	59.2 ± 1.20	−	Not determined	
Bt+	Newleaf Plus 350	*cry3A*	36.4 ± 0.50	+	Not determined	
Cotton						
Bt−	SG747; Maris 25107	–	21.1 ± 1.20	−	6 ± 6.3	0.9 ± 0.06
Bt+	Coker 312 531; Maris 31090	*cry1Ac*	15.9 ± 0.25	+	50 ± 14.2	0.09 ± 0.01
Canola						
Bt−	Binapus; cv. westar	–	31.9 ± 1.30	−	6 ± 6.3	1.1 ± 0.08
Bt+	W45; cv. westar	*cry1Ac*	22.2 ± 0.80	+	37 ± 6.3	0.08 ± 0.02
GFP+	W45; cv. westar	*GFP*	29.7 ± 0.00	−	0 ± 0.0	0.9 ± 0.06
GFP*Bt*+	W45; cv. westar	*GFP* and *cry1Ac*	19.9 ± 0.00	+	37 ± 11.9	0.09 ± 0.02
Tobacco						
Bt−	Xanthi	–	34.9 ± 1.30	−	0 ± 0.0	1.1 ± 0.06
Bt+	Bt9	*cry1Ac*	23.3 ± 1.00	+	50 ± 0.0	0.06 ± 0.05
GFP+	–	*GFP*	35.4 ± 0.70	−	6 ± 6.3	0.9 ± 0.03
GFP*Bt*+	–	*GFP* and *cry1Ac*	27.5 ± 1.38	+	43 ± 6.3	0.08 ± 0.01

Biomass of canola and tobacco containing the gene for green fluorescent protein (GFP) and the genes for both GFP and Cry1Ac protein was also evaluated. Studies were conducted for 32 days. Control soil was not amended with any plant biomass (total carbon evolved: 14.7 ± 1.83 mg C 100 g^{-1} soil). Immunological assay for Cry1Ab and Cry1Ac proteins was with EnviroLogix Lateral Flow Quickstix and for Cry3A protein with Agdia DAS ELISA Kit: −, no toxin detected; +, toxin detected. Mortality determined with the larvae of the tobacco hornworm (*Manduca sexta*) and expressed as mean % mortality ± standard error of the mean (SEM); mean weights, in g, of a single surviving larva ± SEM are also presented.

a Sorvall Omni mixer. Corn plants were separated into leaves and stems and ground separately. The particle size distribution of the ground material was 70% < 0.5 mm and 30% < 1 mm. The carbon and nitrogen content of each plant species was determined with an EA 1108 CHN Analyzer (Fisons Instruments, Lucino di Radano, Italy), and the C:N ratio was calculated.

2.3. Decomposition experiments

Kitchawan soil was amended with 0.5, 1, or 2% (wt wt^{-1}) ground, dried (50 °C) leaves or stems of *Bt* corn (NK6800Bt or NK4640Bt, both with transformation event Bt11) or of the near-isogenic hybrids without the *cry1Ab* gene (NK6800 and NK4640). Some studies were also done with corn hybrids DK647Bty (event MON810) and Prime Plus (event Bt11; sweet corn). Subsamples of the amended or unamended soil (25 or 50 g, oven-dry equivalent, depending on the experiment) at the −33-kPa water tension were placed into small jars (90-ml capacity), and 8–10 jars were placed into individual 1-L 'master' jars, which were attached to a respiratory train that continuously flushed respired CO_2 with water-saturated CO_2-free air into external containers of NaOH and incubated at 25 ± 2 °C (Stotzky et al., 1993).

The Long Island soil was amended with 0.5% (wt wt^{-1}) ground, dried (50 °C) biomass (stems plus leaves) of the various transgenic *Bt* plants or their near-isogenic non-*Bt* counterparts, and 100 g of amended soil (oven-dry equivalent) at the −33-kPa water tension was placed directly into individual 1-L master jars, which were attached to a respiratory train for collection of CO_2 and incubated at 25 ± 2 °C.

Pieces of dried (50 °C) biomass (2 g, oven-dry equivalent; 10–15 mm in size) of the various transgenic *Bt* plants and their non-*Bt* near-isolines were individually placed into jars (230-ml capacity) and inoculated with 10 ml of a soil suspension (100 g of the Long Island soil, 12 h after

collection in the field, was vortexed with 100 ml of sterile tap water, and the larger particles of soil were allowed to settle). Four sets of jars were prepared in duplicate for each biomass, and streptomycin (3 μg ml^{-1}) to inhibit bacteria, cycloheximide (20 μg ml^{-1}) to inhibit fungi, streptomycin plus cycloheximide, or no antibiotics were added to each set. The jars were attached to a respiratory train and incubated at 25±2 °C.

The gross metabolic activity of the soils, with and without added biomass, and of the biomass without soil was determined by CO_2 evolution: CO_2 was trapped in NaOH, precipitated with $BaCl_2$, and the unneutralized NaOH titrated with HCl with an automatic titrator (Stotzky et al., 1993). In some experiments, 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 total culturable bacteria and fungi, were measured (Stotzky et al., 1993). In other experiments, soil samples were analyzed only at the end of the incubation for enzymes and bacterial and fungal counts.

In all experiments, the presence of the toxins in soil and in biomass was determined by immunological assay and by bioassay using the larvae of *M. sexta* (see below).

2.4. Lignin analysis

Samples of ground, dried (50 °C) biomass were weighed into glass tubes (16×150 mm), 2.5 ml of freshly prepared acetyl bromide reagent (25%, vol vol^{-1}, acetyl bromide in glacial acetic acid) was added, and the tubes were capped immediately with Teflon-lined screw caps and heated at 50 °C for 3–4 h. The samples were then quantitatively transferred to 50-ml volumetric flasks that contained 10 ml of 2 M NaOH and 12 ml of glacial acetic acid, diluted to 50 ml with glacial acetic acid, and absorbance was determined at 280 nm (Hatfield et al., 1999; Saxena and Stotzky, 2001b).

2.5. Bacteria, including actinomycetes, and fungi

Colony-forming units (CFU) of culturable aerobic bacteria were estimated on soil extract agar, and CFU of fungi were estimated on Rose Bengal-streptomycin agar (see Stotzky et al., 1993; Saxena and Stotzky, 2001c). Soil (1 g) from the various treatments was suspended in 10 ml of sterile tap water, 10-fold serially diluted, and 0.1 ml of the diluted samples was spread on agar plates that were incubated at 24±2 °C for 5–7 days. The CFU of bacteria, actinomycetes, and fungi were determined on duplicate samples of soil from each pot, vial, and jar.

2.6. Immunological assays

Soil (0.5 g) was vortexed with 0.5 ml of extraction buffer (buffer for Cry1Ab and Cry1Ac proteins from EnviroLogix, Portland, ME, and for Cry3A protein from Agdia, Elkhart, IN), centrifuged, and the supernatants analyzed by Western blot using Lateral Flow Quickstix for Cry1Ab and Cry1Ac proteins (EnviroLogix; detection limit <10 parts 10^{-9}) (Saxena et al., 1999; Saxena and Stotzky, 2000) and the DAS ELISA Kit for Cry3A protein (Agdia; detection limit <20 parts 10^{-9}).

2.7. Larvicidal assays

The larvicidal activity of soils amended with biomass containing Cry1Ab and Cry1Ac proteins, of the transgenic *Bt* biomass, and of control soils and biomass was determined with the larvae of *M. sexta* (Tapp and Stotzky, 1998). Eggs of *M. sexta* and food medium were obtained from Carolina Biological Supply Company (Burlington, NC). The eggs, placed on solidified medium in Petri plates, were incubated at 29±1 °C under a 40 W lamp for 2–3 days, when the eggs hatched. The medium was dispensed, after microwaving for 1 min, in 5-ml amounts into vials (3 cm diameter and 6 cm tall), and 0.1 ml of freshly vortexed suspensions of soil or plant biomass was uniformly distributed over the surface of the solidified medium (8.55 cm^2) with disposable pipette tips (200-μl capacity) that had been cut ca. 1.5 cm from the tip, to ensure that all suspended particles were transferred. After air-drying for 2 h, 4 second-instar larvae were added to each of duplicate vials prepared from duplicate containers, resulting in 16 larvae for each soil or plant sample. Mortality was determined after 3 and 7 days, and percent mortality was based on mortality after 7 days, when all surviving larvae were weighed, to estimate sublethal effects of the toxins. No larvicidal assay was done with soil or plant samples from *Bt* potato.

2.8. Statistics

There were at least three replicates of each treatment, and experiments were repeated at least twice. The data are expressed as the means±the standard errors of the means. Significance among the data was determined by the paired Student's *t*-test using SigmaPlot computer software (Jandel Scientific Corporation).

3. Results

The amounts of C evolved as CO_2 increased as the concentration of biomass added increased when compared with the amounts evolved from the unamended control soil. However, the amounts evolved were significantly lower from soil amended with biomass of *Bt* plants than with their near-isogenic non-*Bt* counterparts. As an example, data obtained with leaves of *Bt* and non-*Bt* corn are presented in Fig. 1.

Similar results were obtained when soil was amended with biomass of *Bt* canola, cotton, potato, rice, tobacco,

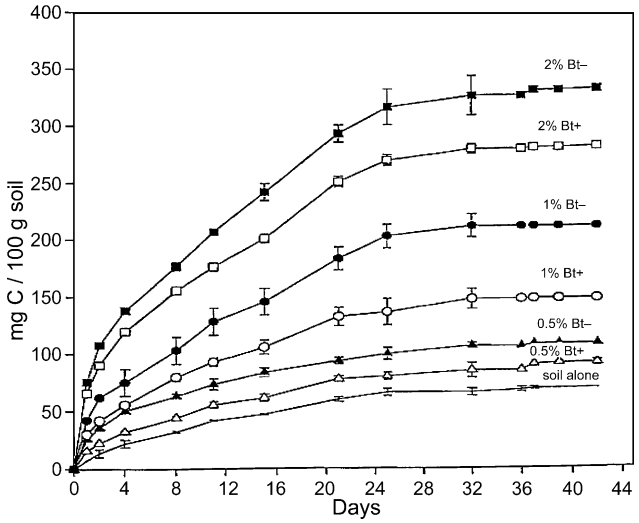

Fig. 1. Gross metabolic activity (cumulative CO_2 evolution) of soil amended with 0.5, 1, or 2% (wt wt^{-1}) ground, dried (50 °C) leaves of *Bt* corn (*Bt*+) (Hybrid 6800Bt) or near-isogenic non-*Bt* corn (*Bt*−) (Hybrid 6800). The standard errors of the means are within the dimensions of the symbols except where indicated by vertical lines.

and other hybrids of corn: the amounts of C evolved as CO_2 were significantly lower (20–39%) from soil amended with biomass of *Bt* plants than of their near-isogenic counterparts without the *cry* genes (Table 1 and Fig. 2A and B; only representative data are shown in Fig. 2). Moreover, biomass of GFP*Bt*+ tobacco and canola, genetically modified to express both green fluorescent protein (GFP) and the Cry1Ac protein, decomposed 13–32% less than GFP+ tobacco and canola (Table 1). Similar results were obtained when plant biomass was incubated without soil but inoculated with a soil suspension (e.g. Fig. 3), with and without antibiotics (e.g. Fig. 4).

The lignin content of *Bt* corn was significantly higher than of non-*Bt* near-isoline corn (Saxena and Stotzky, 2001b). In contrast, the lignin content of *Bt* canola, cotton, potato, rice, and tobacco was not significantly different from that of their respective non-*Bt* near-isolines, although it was consistently higher in all *Bt* plants (Table 2). The numbers of culturable bacteria and fungi and the activities of representative enzymes (proteases, acid and alkaline phosphatases, arylsulfatases, and dehydrogenases) involved in degradation of plant biomass were not consistently statistically different between soil unamended or amended with biomass of *Bt* or non-*Bt* plants (data not shown).

Soil amended with biomass of *Bt* plants, but not of non-*Bt* plants, was immunologically positive for the presence of the Cry proteins and lethal to the larvae of *M. sexta* (no bioassays were done for the Cry3A protein) (Table 1). There was no significant mortality with soil amended with biomass of non-*Bt* plants or not amended. Similar results were obtained with biomass of all *Bt* plants and their near-isogenic non-*Bt* counterparts in the absence of soil (data not shown).

4. Discussion

The reasons for the lower biodegradation of the biomass of *Bt* than of non-*Bt* plants are not known. It was not the result of differences in the C:N ratios of the biomass, as leaf and stem tissue of some hybrids of *Bt* corn and near-isogenic non-*Bt* corn (e.g. hybrid NK4640) had similar C:N ratios

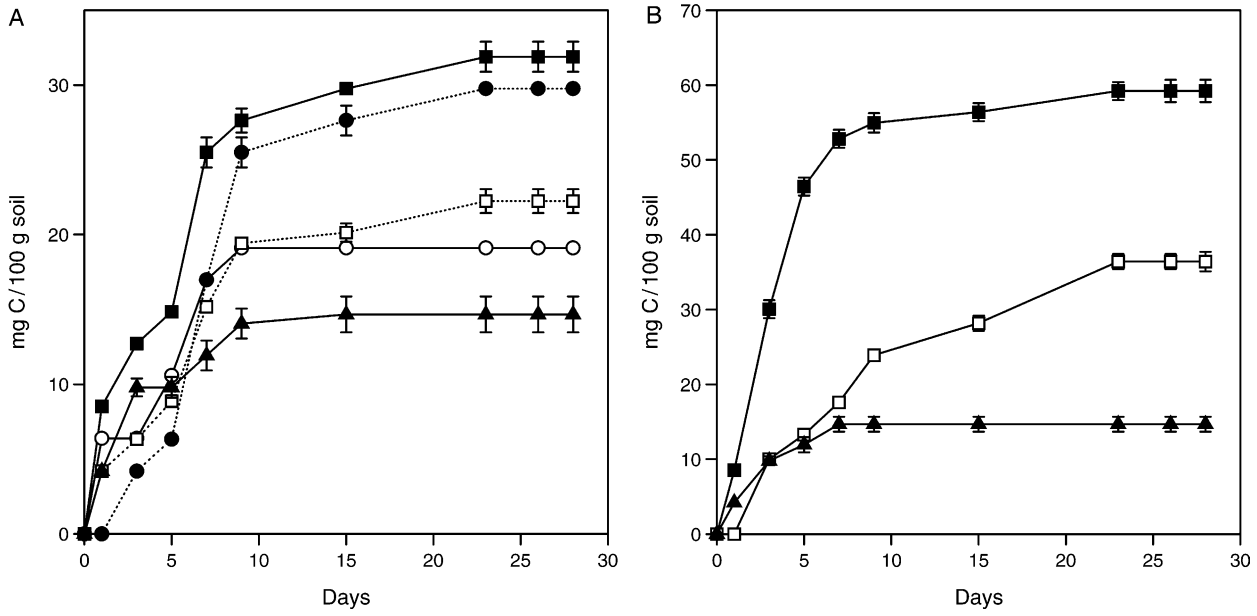

Fig. 2. Gross metabolic activity (cumulative CO_2 evolution) of soil amended with 0.5% (wt wt^{-1}) ground, dried (50 °C) biomass of *Bt* plants (*Bt*+), near-isogenic non-*Bt* plants (*Bt*−), or canola containing the gene for green fluorescent protein (GFP) and the genes for both GFP and Cry1Ac protein: (A) Canola: *Bt* + (□), *Bt*− (■), GFP (●), GFP and *Bt* (○), soil (▲); (B) Potato: *Bt* + (□), *Bt*− (■), soil (▲). The standard errors of the means are within the dimensions of the symbols except where indicated by vertical lines.

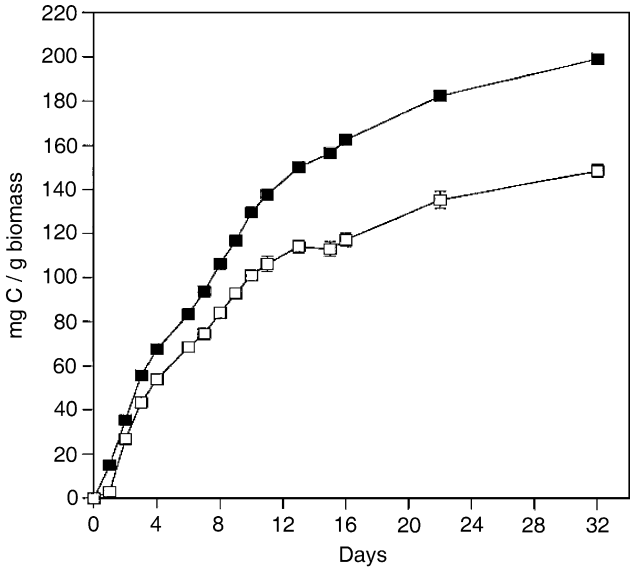

Fig. 3. Gross metabolic activity (cumulative CO_2 evolution) of 2 g of pieces (10–15 mm) of dried (50 °C) biomass (leaves plus stems) of *Bt* corn (*Bt*+) (Hybrid NK4640Bt) or near-isogenic non-*Bt* corn (*Bt*−) (Hybrid NK4640) incubated without soil but with 10 ml of a non-sterile soil suspension: *Bt* + (□), *Bt*− (■). The standard errors of the means are within the dimensions of the symbols except where indicated by vertical lines.

(Table 3); the addition of an available carbon and energy source in the form of glucose with biomass of *Bt* or non-*Bt* corn, as well as changes in the C:N ratios of the soil-biomass systems by the addition of glucose and/or NH_4NO_3, did not

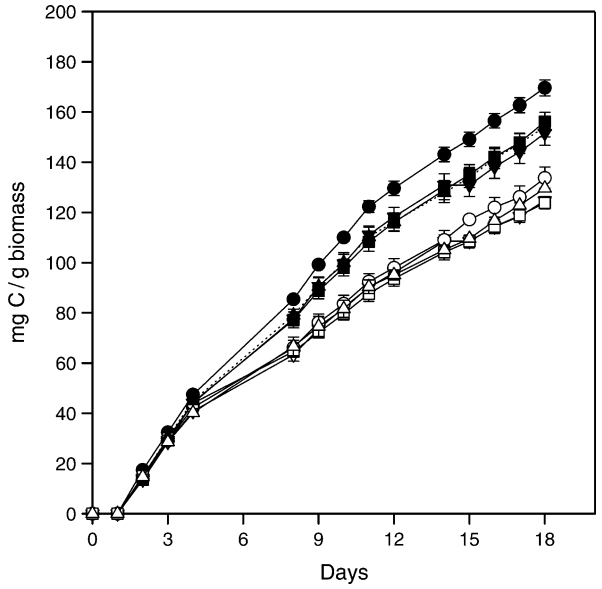

Fig. 4. Effects of antibiotics on gross metabolic activity (cumulative CO_2 evolution) of 2 g of pieces (10–15 mm) of dried (50 °C) biomass of *Bt* corn (*Bt*+) (Hybrid NK4640Bt) or near-isogenic non-*Bt* corn (*Bt*−) (Hybrid NK4640) incubated without soil but with 10 ml of a non-sterile soil suspension and antibiotics: no antibiotics (○); 3 μg ml^{-1} streptomycin (□); 20 μg ml^{-1} cycloheximide (△); 3 μg ml^{-1} streptomycin plus 20 μg ml^{-1} cycloheximide (▽). Closed symbols are *Bt*− and open symbols are *Bt*+ biomass. The standard errors of the means are within the dimensions of the symbols except where indicated by vertical lines.

Table 2
Lignin content of transgenic *Bt* plants (*Bt*+) and their near-isogenic non-*Bt* counterparts (*Bt*−)

Plant	Gene transformed	Lignin (%±SEM)	*p*
Corn (stems)			
Bt−	–	3.2±0.12	0.0001
Bt+	*cry1Ab*	6.3±0.14	
Rice			
Bt−	–	2.3±0.32	0.3744
Bt+	*cry1Ab*	2.8±0.41	
Potato			
Bt−	–	0.9±0.16	0.0728
Bt+	*cry3A*	1.1±0.24	
Cotton			
Bt−	–	2.0±0.44	0.8269
Bt+	*cry1Ac*	2.2±0.58	
Canola			
Bt−	–	0.8±0.19	0.2671
Bt+	*cry1Ac*	1.1±0.32	
GFP+	*GFP*	0.7±0.22	0.1762
GFP*Bt*+	*GFP* and *cry1Ac*	1.2±0.32	
Tobacco			
Bt−	–	0.6±0.21	0.1577
Bt+	*cry1Ac*	0.9±0.26	
GFP+	*GFP*	0.6±0.30	0.2142
GFP*Bt*+	*GFP* and *cry1Ac*	1.0±0.35	

Canola and tobacco containing the gene for green fluorescent protein (GFP) and the genes for both GFP and Cry1Ac protein were also evaluated. Mean±standard errors of the means and *p* values.

significantly alter the relative differences in biodegradation between biomasses (Table 4); and there was no consistent relation between C:N ratios and the amounts of C evolved as CO_2 with the biomass of the other plants (Tables 1 and 5). It was apparently not the result of the inhibition of the activity of the soil microbiota by the biomass of *Bt* plants, as the numbers of culturable bacteria and fungi and the activity of enzymes representative of those involved in the degradation of plant biomass did not differ consistently or significantly between *Bt* and near-isogenic non-*Bt* plants, confirming in vitro observations that the Cry proteins were not toxic to a spectrum of pure and mixed cultures of microbes (Koskella and Stotzky, 2002). *Bt* corn had a significantly higher lignin content than non-*Bt* corn, but the lignin content of the other *Bt* plants, although 10–66% higher, was not statistically significantly different from that of their respective non-*Bt* near-isolines (Table 2). Changes in the amount of lignin or in its composition or conformation could alter the amount of protection offered to associated polysaccharides, proteins, and other plant components more susceptible to biodegradation and could influence the rates of decomposition of transgenic plant biomass (e.g. Reddy, 1984; Tovar-Gomez et al., 1997; Hopkins et al., 2001).

The lower degradation of *Bt* biomass did not appear to be the result of differences in the microbiological characteristics of the soils, as the degradation of the biomass of all *Bt* plants in the absence of soil but inoculated with a microbial suspension from the same soil was significantly less than

Table 3
Total amount of carbon evolved as CO_2 from soil amended with various amounts of ground, dried (50 °C) leaves or stems of two hybrids of *Bt* corn (*Bt*+) or of near-isogenic non-*Bt* corn (*Bt*−)

Source (and C:N ratio) of tissue	Concentration of tissue (%)	mg C 100 g^{-1} soil ± SEM	
		Bt corn	non-*Bt* corn
Hybrid NK6800			
Leaves	0.5	91 ± 2.4	108 ± 0.0
(*Bt*+, 38.4; *Bt*−, 26.6)	1	148 ± 1.3	211 ± 1.2
	2	281 ± 1.3	331 ± 2.3
Stems	0.5	112 ± 0.1	181 ± 0.3
(*Bt*+, 26.0; *Bt*−, 17.5)	1	213 ± 0.0	236 ± 0.0
	2	266 ± 0.1	378 ± 0.5
Control, (12.5)		38 ± 2.0	
Hybrid NK4640			
Leaves	1	116 ± 0.3	121 ± 0.6
(*Bt*+, 79.0; *Bt*−, 80.3)	1 and glucose	188 ± 1.7	195 ± 1.2
	2	170 ± 1.6	183 ± 1.0
Stems	1	87 ± 1.0	108 ± 1.8
(*Bt*+, 149.9; *Bt*−, 171.8)	1 and glucose	146 ± 1.3	157 ± 0.5
	2	186 ± 0.2	216 ± 0.5
Control, (12.5)		32 ± 4.2	
Control and glucose		108 ± 2.0	

Studies with hybrid NK6800 were conducted for 42 days and with hybrid NK4640 for 32 days. Glucose (1%, wt wt^{-1}; 0.4% C) was added as indicated with hybrid NK4640. 'Control' is soil not amended with corn tissue. Data have been normalized to the means ± standard error of the means (SEM) 100 g^{-1} soil, oven-dry equivalent.

that of near-isogenic non-*Bt* counterparts. This was confirmed by the lack of significant differences in the degradation of *Bt* and non-*Bt* biomass by the addition to the soil suspension of streptomycin, cycloheximide, or both to reduce the growth of bacteria, fungi, or both, respectively. Hence, the differences appeared to be primarily the result of the presence of the *cry* genes. This was further indicated by the similarity in decomposition, both in the presence and absence of soil, of (1) non-*Bt* plants and plants transformed to express GFP, and (2) plants expressing only the Cry1Ac protein and both the Cry1Ac protein and GFP.

These results differed from those reported by Hopkins and Gregorich (2003), who found no difference in the decomposition of leaves of *Bt* corn (variety Pioneer 38W36) and non-*Bt* corn (variety Pioneer 3893). The reason for this difference in results is not clear. Hopkins and Gregorich (2003) studied only one variety of *Bt* corn, whereas the current study evaluated four varieties of *Bt* corn (both leaves and stems separately and together) and five other species of *Bt* plants. Although differences in decomposition among the varieties and between leaves and stems of corn were observed, decomposition of all *Bt* plants was significantly less than that of their near-isogenic non-*Bt* counterparts. In the current study, a relation between a higher lignin content in and lower decomposition of biomass of *Bt* plants was apparent, especially with *Bt* corn. The importance of

Table 4
Total amount of carbon evolved as CO_2 during 32 days from soil amended with 0.5% (wt wt^{-1}) ground, dried (50 °C) leaves or stems of *Bt* corn (Hybrid NK4640Bt) or of near-isogenic non-*Bt* corn (Hybrid NK4640) and 1% carbon (C) as glucose, 1% nitrogen (N) as NH_4NO_3, 1% C as glucose plus 1% N as NH_4NO_3 (C and N), or no additions of C or N (Control)

Source of tissue	Treatment	mg C 100 g^{-1} soil ± SEM	
		Bt corn	non-*Bt* corn
Leaves	Control	54 ± 1.0 (56.9)	65 ± 4.0 (57.5)
	+C	463 ± 2.0 (313.3)	490 ± 3.0 (317.2)
	+N	79 ± 1.5 (0.2)	84 ± 0.6 (0.2)
	+C and N	484 ± 2.0 (1.2)	486 ± 2.0 (1.2)
Stems	Control	43 ± 0.5 (81.2)	65 ± 0.8 (85.6)
	+C	448 ± 2.5 (465.9)	480 ± 3.0 (485.6)
	+N	64 ± 2.0 (0.2)	76 ± 1.0 (0.2)
	+C and N	471 ± 1.5 (1.2)	482 ± 1.2 (1.2)
None	Control	32 ± 4.2 (12.5)	
	+C	437 ± 6.0 (781.8)	
	+N	55 ± 4.0 (0.02)	
	+C and N	460 ± 4.0 (1.0)	

The final C:N ratios of the soil-biomass systems are shown in parentheses. In 'None', no corn tissue was added. Data have been normalized to the means ± standard error of the means 100 g^{-1} soil, oven-dry equivalent.

Table 5
Content of carbon and nitrogen and C:N ratio of transgenic *Bt* (*Bt*+) and near-isogenic non-*Bt* (*Bt*−) plants, as well as of canola and tobacco containing the gene for green fluorescent protein (GFP) and the genes for both GFP and Cry1Ac protein

Plant	Carbon (%)	Nitrogen (%)	C:N ratio	*p*
Corn				
Bt+	41.8±0.29	1.4±0.01	29.0±0.11	0.0004
Bt−	39.7±0.28	1.1±0.01	35.9±0.14	
Rice				
Bt+	40.2±0.26	2.4±0.03	16.8±0.13	0.002
Bt−	39.7±0.18	2.0±0.01	19.9±0.09	
Potato				
Bt+	38.8±0.28	4.1±0.01	9.4±0.01	0.0010
Bt−	39.7±0.20	3.2±0.01	12.1±0.01	
Cotton				
Bt+	41.8±0.27	1.6±0.01	25.6±0.01	0.00005
Bt−	37.8±0.17	2.7±0.01	13.7±0.02	
Canola				
Bt+	34.5±0.12	2.9±0.01	11.8±0.05	0.0032
Bt−	36.1±0.09	3.2±0.01	11.0±0.01	
GFP	36.9±0.19	2.4±0.01	15.4±0.11	0.0025
GFP*Bt*+	35.9±0.23	2.8±0.02	12.5±0.05	
Tobacco				
Bt+	37.5±0.50	1.6±0.01	22.2±0.18	0.0004
Bt−	35.3±0.30	3.3±0.02	10.6±0.02	
GFP	33.6±0.16	4.0±0.02	8.3±0.01	0.00001
GFP*Bt*+	35.4±0.12	1.8±0.01	18.9±0.03	

Means ± standard error of the means and *p* values for C:N ratio. See Table 1 for details on the plants.

the relative contents of lignin in the *Bt*+ and *Bt*− corn biomass studied by Hopkins and Gregorich (2003) is not known, as the lignin content of this biomass was not determined. The study of Hopkins and Gregorich (2003) was done at 50% of the water-holding capacity of their soil, whereas the current study was done at the −33-kPa water tension of the soils used. Microbial activity in soil is optimal at the −33-kPa water tension (Stotzky, 1974). Differences in the methods of collecting CO_2 evolved from soil (continuous flushing of CO_2 with water-saturated CO_2-free air into external containers of NaOH in the current study and batch collection of CO_2 in containers of NaOH placed within closed incubation chambers of soil in the study by Hopkins and Gregorich (2003)) may have also influenced the results, as the latter method removes water from soil and necessitates periodic replacement of water, which perturbs soil and affects CO_2 evolution (Stotzky, 1960). Moreover, a lower decomposition of the biomass of *Bt* corn than of non-*Bt* corn was also observed in field studies with litter bags.

The ecological and environmental relevance of these observations is also not clear. If the lower decomposition of the biomass of *Bt* plants continues for extended time, it may be beneficial, as the organic matter derived from *Bt* plants would persist longer and accumulate at higher levels in soil, thereby improving soil structure and reducing erosion. By contrast, the longer persistence of the biomass of *Bt* plants would extend the time that the toxins are present in soil and, thereby, could enhance the hazard to non-target organisms and the selection of toxin-resistant target insects (Ferré et al., 1995). Toxin released in root exudates of *Bt* corn persisted in rhizosphere soil for at least 180 days (Saxena and Stotzky, 2002), and purified Cry1Ab protein added to non-sterile soil was still detected after 234 days (Tapp and Stotzky, 1998), the longest times studied. However, the greatest input of the toxins into soil will result from post-harvest incorporation of plant biomass. The Cry1Ab protein was detected immunologically in soil amended 3 years earlier (the longest time studied) with *Bt* corn biomass and incubated under optimal conditions of temperature and soil water tension in the laboratory (Saxena and Stotzky, 2003). This persistence was considerably longer than persistences estimated in the literature based on 'half-life' values, which ranged from ca. 8 to 17 days for purified toxin and 2–41 days for biomass of transgenic corn, cotton, and potato (Palm et al., 1994, 1996; Sims and Ream, 1997; Hopkins and Gregorich, 2003).

Additional studies are necessary to clarify the environmental impacts of the lower degradation of the biomass of *Bt* plants, especially as 8.1 million hectares of *Bt* corn or 26% of total corn acreage, 2.4 million hectares of *Bt* cotton or 45% of total cotton acreage, and 0.02 million hectares of *Bt* potato or 3.5% of total potato acreage were planted in 2000 in the United States alone (USEPA, 2001). Moreover, at least 26 plant species, including corn, cotton, canola, potato, rice, broccoli, peanut, eggplant, and other crop species, have been modified to express Cry proteins, and plants into which the *cry1Aa*, *cry1Ba*, *cry1Ca*, *cry1H*, and *cry2Aa* genes, encoding proteins that target lepidopteran larvae, and the *cry6A* gene, which targets coleopteran larvae, have been introduced are in the developmental stage (Kuiper et al., 2001).

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