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Soil Biology & Biochemistry 40 (2008) 559–586

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Review

Fate and effects of insect-resistant *Bt* crops in soil ecosystems

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Received 29 March 2007; received in revised form 25 October 2007; accepted 2 November 2007

Abstract

Recent applications of biotechnology, especially genetic engineering, have revolutionized crop improvement and increased the availability of valuable new traits. A current example is the use of the insecticidal Cry proteins from the bacterium, *Bacillus thuringiensis* (*Bt*), to improve crops, known as *Bt* crops, by reducing injury from various crop pests. The adoption of genetically modified (GM) crops has increased dramatically in the last 11 years. However, the introduction of GM plants into agricultural ecosystems has raised a number of questions, including the ecological impact of these plants on soil ecosystems. Crop residues are the primary source of carbon in soil, and root exudates govern which organisms reside in the rhizosphere. Therefore, any change to the quality of crop residues and rhizosphere inputs could modify the dynamics of the composition and activity of organisms in soil. Insect-resistant *Bt* crops have the potential to change the microbial dynamics, biodiversity, and essential ecosystem functions in soil, because they usually produce insecticidal Cry proteins through all parts of the plant. It is crucial that risk assessment studies on the commercial use of *Bt* crops consider the impacts on organisms in soil. In general, few or no toxic effects of Cry proteins on woodlice, collembolans, mites, earthworms, nematodes, protozoa, and the activity of various enzymes in soil have been reported. Although some effects, ranging from no effect to minor and significant effects, of *Bt* plants on microbial communities in soil have been reported, using both culturing and molecular techniques, they were mostly the result of differences in geography, temperature, plant variety, and soil type and, in general, were transient and not related to the presence of the Cry proteins. The respiration (i.e., CO_2 evolution) of soils cultivated with *Bt* maize or amended with biomass of *Bt* maize and other *Bt* crops was generally lower than from soils cultivated with or amended with biomass of the respective non-*Bt* isolines, which may have been a result of differences in chemical composition (e.g., the content of starch, soluble N, proteins, carbohydrates, lignin) between *Bt* plants and their near-isogenic counterparts. Laboratory and field studies have shown differences in the persistence of the Cry proteins in soil, which appear to be the result primarily of differences in microbial activity, which, in turn, is dependent on soil type (e.g., pH, clay mineral composition, other physicochemical characteristics), season (e.g., temperature, water tension), crop species (e.g., chemical composition, C:N ratio, plant part), crop management practices (e.g., till vs. no-till), and other environmental factors that vary with location and climate zones. This review discusses the available data on the effects of Cry proteins on below-ground organisms, the fate of these proteins in soil, the techniques and indicators that are available to study these aspects, and future directions.

Keywords: *Bacillus thuringiensis*; *Bt* crops; Biotechnology; Soil microorganisms; Invertebrates; Genetically modified plants; Insect resistance; Nontarget effects; Soil ecosystem functions

1. Introduction

Genetically modified (GM) plants possess a gene or genes that have been transferred from a different species. GM plants have been deliberately developed for a variety of reasons: e.g., longer shelf life, disease resistance, pest resistance, herbicide tolerance, nutritional improvement, resistance to such abiological stresses as drought or nitrogen starvation. The first GM crop approved for use in the USA was the FlavrSavr tomato in 1994, which was developed to have a longer shelf life. Since GM crops were first commercialized in 1996, the planting of GM crops has consistently increased by 10% or more each year worldwide. It is generally expected that commercial cultivation of GM crops will further increase over the coming years

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doi:10.1016/j.soilbio.2007.11.002

(Sanvido et al., 2006). The global area of GM crops increased approximately 60-fold during the 11-year period from 1996 to 2006: from 1.7 million hectares to 102 million hectares (James, 2006).

GM crops are currently grown by more than 10.3 million farmers in 22 countries. In 2006, the USA, followed by Argentina, Brazil, Canada, India, and China, were the six principal users of GM crops globally, with 54.6 million hectares planted in the USA (53% of global GM crop area) of which approximately 28% contained stacked genes with two or three traits. Herbicide tolerance has consistently been the dominant GM trait followed by insect resistance and stacked genes for the two traits. In 2006, herbicide tolerance, used in soybean, maize, canola, cotton, and alfalfa, occupied 68% (69.9 million hectares) of the total global GM crop area, and 19% (19.0 million hectares) was planted with insect-resistant *Bt* crops and 13% (13.1 million hectares) with crops with stacked traits of insect resistance and herbicide tolerance (James, 2006). Soybean and cotton with herbicide-tolerant traits have been the most widely and rapidly adopted GM crops in the USA, followed by insect-resistant cotton and corn. Herbicide-tolerant soybean was the principal GM crop in 2006, occupying 58.6 million hectares (57% of the global GM crop area), followed by maize (25.2 million hectares at 25%), cotton (13.4 million hectares at 13%), and canola (4.8 million hectares at 5%). Herbicide-tolerant alfalfa, the first perennial GM crop to be introduced globally in 2006, was planted on 80,000 hectares in the USA, and RR® Flex herbicide-tolerant cotton was introduced on over 800,000 hectares in the USA and Australia. Insect-resistance based on *Bacillus thuringiensis* (*Bt*) is the second major trait used in commercial GM crops, and *Bt* maize occupied 11.3 million hectares, equivalent to 13% of the global GM crop area (James, 2005). The use of GM crops has been greatest in the USA, where there has been a 33-fold increase in the area of GM crops planted during the last 10 years (1.5 million hectares in 1996 to 49.8 million hectares in 2005). Over the last 11 years, 1996–2006, farmers have consistently increased their plantings of GM crops by double-digit growth rates every single year since GM crops were first commercialized in 1996 (James, 2006).

However, there are concerns that the commercial cultivation of GM crops could result in adverse effects on the environment. One of the potential adverse environmental effects of GM crops is a nontarget effect on soil organisms and a change in microbe-mediated processes and functions in soil, which could be affected by the presence of, for example, insecticidal Cry proteins derived from insect-resistant *Bt* crops in soils through cultivation of *Bt* crops (e.g., Masoero et al., 1999; Escher et al., 2000; Saxena and Stotzky, 2001a; Dinel et al., 2003; Manachini et al., 2004; Höss et al., 2004; Turrini et al., 2004; Gupta and Watson, 2004; Xue et al., 2005; Rui et al., 2005; Griffiths et al., 2005). Interactions between plants and soil ecosystems indicate that, similar to other agricultural crops, GM crops will influence processes and functions in soil. Plants have a major influence on communities of micro- and other organisms in soil, which are fundamental to many functions of soil systems, such as nitrogen cycling, decomposition of wastes, and mobilization of nutrients. The type and amount of nutrients released will affect both the numbers of organisms and their diversity. The major carbon supply to soil systems is from plant litter incorporated after harvest and from root exudation. Saxena et al. (2004) showed that insect-resistant GM crops, such as *Bt* maize, potato, and rice, contributed to the presence and persistence of Cry proteins in soil via root exudation, whereas *Bt* cotton, canola, and tobacco did not. There appeared to be no significant differences in exudation from 12 different *Bt* maize hybrids, which included three transformation events (Bt11, MON810, and 176), expressing the Cry1Ab protein (Saxena et al., 2002). A 3-year field study with *Bt* maize (event MON810) confirmed that the release of Cry protein in root exudates continued throughout growth, and levels of the protein in soil did not correlate with a specific period of plant growth (Nguyen Thu, 2004; Baumgarte and Tebbe, 2005). The continuous release, via root exudates, leads to higher concentrations of Cry protein in rhizosphere than in bulk soil. Moreover, repeated and large-scale use of *Bt* crop plants and their residues after harvest could lead to accumulation and persistence of plant-produced Cry proteins in soil, as a result of their binding on soil components (e.g., Tabashnik, 1994; Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998; Saxena and Stotzky, 2001a, b; Saxena et al., 2002; Zwahlen et al., 2003a; Muchaonyerwa et al., 2004; Stotzky, 2000, 2002, 2004).

This review, which supplements previous reviews of aspects discussed herein (e.g., Stotzky, 2000, 2002, 2004; Federici, 2002; Marvier, 2002; Shelton et al., 2002; Bruinsma et al., 2003; Kowalchuk et al., 2003; Benedict and Ring, 2004; De Maagd, 2004; Gupta and Watson, 2004; Motavalli et al., 2004; O'Callaghan et al., 2005; Liu et al., 2005; Lilley et al., 2006), summarizes the results of numerous studies conducted to determine the: (i) effects of insecticidal Cry proteins derived from GM *Bt* crops on soil ecosystems, including soil microorganisms, microbe-mediated processes and functions, and soil-dwelling invertebrates; and (ii) persistence and fate of Cry proteins in soil. The review does not discuss the environmental effects of herbicide-tolerant plants, primarily because herbicide-tolerant crops are considered not to have direct toxic effects on nontarget organisms, as the enzymes conferring herbicide tolerance are normally present in plants and are not known to have any toxic properties (APHIS-USDA, 1994; Carpenter, 2001). The use of herbicide-tolerant crops could, however, result in indirect environmental effects caused by changes in agricultural practices (e.g., Germida et al., 1998; Siciliano et al., 1998; Siciliano and Germida, 1999; Dunfield and Germida, 2003, 2004).

2. *Bt* and mode of action

Some strains, or subspecies, of the bacterium, *Bacillus thuringiensis*, commonly known as *Bt*, are pathogens of insects and some other organisms and are distributed worldwide in many habitats, including soil. Some strains of *Bt* produce proteins that kill certain insects. These proteins are the active ingredients of commercial *Bt* insecticidal sprays that have been used for many years (e.g., Dipel®, Xentari®, Javlin®, Foray®, M-One®, VIP®). *Bt* insecticides are classified as biopesticides because some bacteria produce the Cry proteins. The toxins of *Bt* are highly selective, and different strains of the bacterium kill different insects and only those insects (Höfte and Whiteley, 1989; Schnepf et al., 1998). There are numerous strains of *Bt*, each with different Cry proteins, and more than 60 Cry proteins have been identified. Most *Bt* maize hybrids express the Cry1Ab protein, and a few express the Cry1Ac or the Cry9C protein, all of which are targeted against the European corn borer (*Ostrinia nubilalis* Hübner) (Lepidoptera), a major pest of maize in North America and Europe. Some recent maize hybrids express the Cry3Bb1 protein, which is targeted against the corn rootworm complex (*Diabrotica* spp.) (Coleoptera), also a major pest of maize, especially in North America. Cotton expressing the Cry1Ac protein is targeted against the cotton bollworm (*Helicoverpa zea* Boddie) (Lepidoptera), which is a major pest of cotton; potato expressing the Cry3A or Cry3C is targeted against the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera), which is a major pest of potato; and Cry4 proteins are targeted against some Diptera, such as certain flies (e.g., *Lycoriella castanescens* Lengersdorf) and mosquitoes (e.g., *Culex pipiens* L.). Some Cry proteins exhibit activity against other orders of insects (e.g., Homoptera, Hymenoptera, Orthoptera, Mallophaga), as well as against nematodes, mites, collembolans, and protozoa (Höfte and Whiteley, 1989; Schnepf et al., 1998; Federici, 2002; Stotzky, 2002; Lee et al., 2003). Some strains of *Bt* are also specifically active against human cancer cells (e.g., Prasad and Shethna, 1974; Seki et al., 1978; Mizuki et al., 1999, 2000; Kim et al., 2000; Lee et al., 2000, 2001; Ito et al., 2004; Okumura et al., 2004; Katayama et al., 2005).

The mode of action and the structure of the *Bt* insecticidal proteins are two major factors in understanding the safety and risks of *Bt* insecticides or *Bt* transgenic crops (Federici, 2002). *Bt* and its insecticidal proteins have a high degree of specificity, which accounts for their relative safety to most nontarget organisms. Cry proteins produced by the bacterium are usually crystalline (called insecticidal crystal proteins—ICPs) and are protoxins with a molecular mass (M_r) of about 130–140 kDa that require cleavage by proteases to produce the biologically active form (toxins) with a M_r of 60–70 kDa (Höfte and Whiteley, 1989; Schnepf et al., 1998). Therefore, ICPs must be ingested to have an effect and require alkaline conditions, typically in the range of pH 8–11, in the insect midgut, to be solubilized to a form conducive to activation by midgut proteases. Activation appears to require the presence of active indigenous bacteria in the midgut (Broderick et al., 2006). After ingestion, the protein molecules pass through the peritrophic membrane and bind to specific receptors. Binding is an essential step in the intoxication process, and in susceptible insects, the toxicity of a particular Cry protein is correlated with the number of specific binding sites (i.e., receptors) for it on microvilli, as well as its affinity for these sites (Schnepf et al., 1998; Pigott and Ellar, 2007). Many chewing insects that ingest ICPs do not have the appropriate receptors, even if they have alkaline midguts, and, thus, are not sensitive to the toxins. In highly sensitive insect species, the microvilli lose their characteristic structure within minutes of toxin insertion, and the cells become vacuolated and begin to swell. This swelling continues until the cells lyse, resulting in extensive damage to the midgut wall and in paralysis and eventually death of the insect (Luthy and Ebersold, 1981).

However, *cry* genes inserted into most *Bt* plants are in a truncated form, and when expressed in plants, truncated active Cry proteins do not form crystals, and they are already solubilized and activated (i.e., no enzymatic cleavage is required) (Gill et al., 1992; Aronson and Shai, 2001; Stotzky, 2002). Therefore, most of the specificity that accounts for the safety of Cry proteins in commercial bacterial insecticides (i.e., ICPs) does not apply to these same proteins when expressed in *Bt* crops to make them resistant to specific insects.

3. *Bt* crops

The use of Cry proteins in commercial spray applications has been limited as the result of their relatively high cost, rapid environmental inactivation, poor crop coverage, and less than desired levels of pest control, especially when compared with many less expensive conventional chemical insecticides (Benedict and Altman, 2001). Sunlight breaks down the proteins, and rain washes them from plants. Therefore, sprays of *Bt* must be applied exactly where and when the target insects are feeding, and insects must consume the toxins quickly before they disappear. To date, *cry* genes from *Bt* have been inserted into numerous plant species, such as maize, cotton, potato, tomato, rice, eggplant, and canola, to produce their own Cry proteins (Shelton et al., 2002; De Maagd, 2004; Benedict and Ring, 2004). However, genetically modified *Bt* maize and cotton are the major commercial *Bt* crops currently grown in the USA (US Environmental Protection Agency (EPA), 2007).

3.1. Maize

Bt maize is grown on about 11.3 million hectares worldwide and is, by far, the most widely grown *Bt* crop in the world; *cry* genes have been inserted into field, flint, pop, and sweet maize (James, 2005). The "first-generation" Cry proteins engineered into maize were Cry1Ab and

Cry9C. Monsanto's YieldGard® corn, transformation event MON810, expressing Cry1Ab, is the most widely grown *Bt* crop today (Benedict and Ring, 2004).

Current *Bt* maize hybrids are targeted to larval pests collectively classified as "corn borers". Worldwide, the European corn borer (ECB), *Ostrinia nubilalis* Hübner, is the most damaging insect attacking maize. ECB and other borers damage maize by tunneling into stalks and ears, feeding on the silks and tassels, causing plants to lodge, and reducing the flow of sap and nutrients, all of which results in reduction in yield (Metcalf and Metcalf, 1993). *Bt* maize expressing Cry1Ab was initially developed to control ECB, but it has been shown to be effective also against various other lepidopteran pests, such as the corn stalk borer (*Sesamia nonagrioides* Lefebvre), Egyptian cotton leafworm (*Spodoptera littoralis* Boisduval), and corn earworm (*Helicoverpa zea* Boddie) (Gonzales-Nunez et al., 2000; Dutton et al., 2005). The level of suppression of target insects provided by current *Bt* maize hybrids varies according to the pest species attacking the crop, transformation event, hybrid background, and environmental factors that influence the concentration of Cry proteins in plant tissues that need protection (Clark et al., 2000). The levels of the Cry1Ab protein in events Bt11 and MON810 in fresh leaf tissue range from 3 to 10 $\mu g\,g^{-1}$ and from 0.2 to 1.4 $\mu g\,g^{-1}$ in grain, with event MON810 being at the lower end and event Bt11 at the upper end of the range (USEPA, 2007).

The newest *Bt* maize event is MON863, known under the Monsanto trade name of YieldGard® Rootworm Corn (USEPA, 2007). This event was approved in 2003 in the USA for control of beetle pests of the genus *Diabrotica*, collectively called corn rootworms (western, *Diabrotica virgifera virgifera* LeConte; northern, *Diabrotica barberi* Smith and Lawrence; southern, *Diabrotica undecimpunctata howardi* Barber; and Mexican, *D. virgifera zeae* Krysan and Smith) (Metcalf and Metcalf, 1993). Western and northern corn rootworms are considered to be the most economically important pests of corn in much of the Corn Belt of the USA, costing growers an estimated one billion dollars in crop losses and control costs. Event MON863 contains the *Bt* transgene, *cry3Bb1*, and expresses the Cry3Bb1 protein constitutively throughout the plant, including leaf, grain, pollen, and root, at concentrations ranging between 3.2 and 93 $\mu g\,g^{-1}$ of fresh plant tissue, depending on the tissue and its age (USEPA, 2007).

3.2. Cotton

Cotton is the crop most heavily treated with insecticides in the USA. *Bt* cotton was first grown, in 1996, in Australia as Ingard® and in the USA as Bollgard® (Benedict and Altman, 2001; James, 2002a, b). These first-generation *Bt* cotton varieties were developed by Monsanto to express the Cry1Ac protein. Worldwide, several different Cry1Ac events are now grown commercially. In the USA and some other countries, Bollgard® event MON531 was used in breeding programs to develop the first commercial *Bt* cotton varieties. In Australia and other countries, Ingard® event MON757 was used. More than 15 species of caterpillar pests attack cotton worldwide, and each country has three or more species that cause serious crop losses. These pest species are commonly called bollworms (*Earias* spp., *Helicoverpa* spp., *Heliothis* spp., and *Pectinophora* spp.). In the USA, the major target pests of *Bt* cotton are cotton bollworm (*Helicoverpa zea* Boddie) (which is also a damaging pest of maize, called the corn earworm when attacking maize), pink bollworm (*Pectinophora gossypiella* Saunders), and tobacco budworm (*Heliothis virescens* Fabricius). The tobacco budworm and pink bollworm are effectively controlled by Bollgard® cotton, whereas the cotton bollworm is controlled satisfactorily, except during the bloom stage, when it feeds on the reproductive parts of flowers (Benedict and Altman, 2001; James, 2002a, b; Benedict and Ring, 2004).

Bt cotton (events MON531, MON757, and MON1076) expresses the Cry1Ac protein at about 1.56, 12.6, and 12.2 $\mu g\,g^{-1}$ of fresh leaf tissue, respectively, and at about 0.86, 9.9, and 12.7 $\mu g\,g^{-1}$ of fresh seed tissue, respectively (AGBIOS, 2005). Little Cry protein is detected in pollen and none in nectar. Concentration of Cry1Ac protein can vary as much as 3- to 5-fold during the season in the same plant organ. A second-generation *Bt* cotton with stacked *cry* genes, Bollgard II®, was created by inserting the *cry2Ab* gene from *B. thuringiensis* that expresses the Cry2Ab protein into the Bollgard® cotton variety, DP50B (event MON531), which already expresses the Cry1Ac protein, thereby creating the new stacked gene event 15985 (Monsanto Company, 2002). Bollgard II® is significantly more effective than Bollgard® in controlling the caterpillar pests of cotton worldwide. This high level of pest control is the result, in part, of multiple Cry proteins (each with a different range of target insects) and to the increased levels of the total Cry proteins for those insect pests that are susceptible to both proteins, especially the higher concentration of Cry2Ab protein in Bollgard II® than of Cry1Ac protein in Bollgard® (James, 2002a, b; Benedict and Ring, 2004).

3.3. Potato

The major limiting factor in growing potato in many areas of the world is the Colorado Potato Beetle (*L. decemlineata* Say), which is resistant to most classes of chemical pesticides (Georghiou and Lagunes-Tejada, 1991; Metcalf and Metcalf, 1993). The first commercial fields of *Bt* potato, with a number of different Monsanto transformation events expressing the Cry3A protein under the name of New Leaf Potato®, were grown in the USA in 1996 (Shelton et al., 2002). The Cry3A protein, similar to the new Cry3Bb1 protein in Yieldgard Rootworm® maize, is active against certain species of beetle (Coleoptera), and it provides not only excellent control of the larvae—there are essentially no survivors—but it also inhibits reproduction

in the adult beetles. However, in response to marketing and political pressures by the public, many food producers have chosen not to use *Bt* potatoes in their products. As a result, since 2001, *Bt* potatoes are no longer available (Shelton et al., 2002).

4. Effects of insect-resistant *Bt* crops on soil ecosystems

4.1. Soil-dwelling invertebrates

Soil-borne communities are dominated by microorganisms, which account for more than 80% of the total biomass in soil (Kowalchuk et al., 2003). The microbial communities are part of complex food webs, together with numerous and varied soil-dwelling invertebrates (e.g., earthworms, collembolans, mites, woodlice, nematodes). Together, these communities carry out processes in soil ecosystems, such as nutrient cycling and decomposition of organic matter, that have major ecological and agricultural significance, and, hence, they are important mediators of the stability of food webs (Moore et al., 1988). The importance of soil invertebrates as vital components of soils and as potential indicators of soil quality is being increasingly recognized (Blair et al., 1996). Soil-dwelling invertebrates are an essential link in food webs as decomposers, and the health and quality of soil are directly related to the number and diversity of invertebrates present. Therefore, the evaluation of the impacts of *Bt* crops on soil organisms, including invertebrates, is essential in assessing the environmental risks of *Bt* plants. Most studies on lethal or sublethal effects of Cry proteins on invertebrates in soil have, in general, shown only a few or no effects, which are briefly summarized (Table 1).

Earthworms are among the most important components of soil that physically transform above-ground plant litter in soil. They are important in the decomposition of plant litter and responsible for numerous physical and chemical changes that affect the biological properties and processes in soil (Brussaard, 1998). Impacts of *Bt* maize expressing the Cry1Ab protein on the earthworm, *Lumbricus terrestris* L., have been studied in soil microcosms in the laboratory and under field conditions (e.g., Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Lang et al., 2006). No significant differences in percent mortality and weight of earthworms after 40 day in soil planted with *Bt* (variety NK4640Bt) or near-isogenic non-*Bt* maize or after 45 day in soil amended with biomass of *Bt* or non-*Bt* maize were observed, despite the uptake of the Cry protein by the worms, as shown by its presence in the casts and guts of the earthworms (Saxena and Stotzky, 2001b). Zwahlen et al. (2003b) showed that mortality and weight of adult and juvenile earthworms were not significantly different when fed *Bt* or non-*Bt* maize residues over 160 day, with the exception that after 200 day, adults fed *Bt* maize residues had a significant reduction in weight compared with those fed non-*Bt* maize. Lang et al. (2006) found no significant differences in the population density or the biomass of *Lumbricidae* between soils with *Bt* and non-*Bt* maize and between soils with maize treated with or without insecticide (Baythroid®) at five sites during 4 years of maize cultivation in field. However, both the site and the sampling years had a greater significant influence on the population density and the biomass of *Lumbricidae* than the presence of the Cry protein. Ahl Goy et al. (1995) did not find an acute effect of *Bt* maize on mortality or weight gain of the earthworm, *Eisenia fetida* Savigny, during 14 day, even though the Cry1Ab protein was detected in the gut and feces of the earthworms. Clark and Coats (2006) found no deleterious effects on survival and reproduction of *E. fetida* fed leaves from two *Bt* maize varieties (event Bt11 and MON810) expressing the Cry1Ab protein compared with those fed leaves from non-*Bt* isolines. In a laboratory soil microcosm, the Cry1Ab protein from ground *Bt* maize leaves or from root exudates of *Bt* maize had no deleterious effects on survival, growth, development, or reproduction of the earthworm, *Aporrectodea caliginosa* var *tubeculata* Savigny, which is probably the most abundant species in agricultural soils in the temperate climate zone (Vercesi et al., 2006) (Table 1).

Isopods, another important component of soil ecosystems and present throughout the world in all terrestrial habitats, are macroarthropods whose main ecological role appears to be the decomposition, especially the fragmentation, of dead plant material (Warburg, 1993). The woodlouse, *Porcellio scaber* Latreille, considered a model decomposer organism, has been a subject of a few studies on the effects of Cry proteins on isopods (Sims, 1997; Escher et al., 2000; Pont and Nentwig, 2005). Sims (1997) observed no effect of purified Cry2A protein on mortality and growth of *P. scaber*. Escher et al. (2000) found no adverse effect of *Bt* maize expressing the Cry1Ab protein on *P. scaber* in a laboratory feeding experiment. *P. scaber* did not differ between *Bt* and non-*Bt* maize in its food preference, and the number of offspring did not differ between the two types of maize. Initial increases in weight of the offspring were significantly higher with non-*Bt* maize, but adult *P. scaber* showed a greater increase in weight when feeding on *Bt* maize leaves. The lower mortality of *P. scaber* offspring and the faster weight gain of adult *P. scaber* on the *Bt* diet was probably a result of differences in the nutritional quality of the *Bt* and non-*Bt* maize leaves (Escher et al., 2000). When consumption of eight maize varieties (two of *Bt* maize and six of non-*Bt* maize varieties) was compared, consumption depended primarily on the variety of maize during a feeding period of 20 d. The consumption of *Bt* maize varieties (N4640-Bt and Max88) expressing the Cry1Ab protein by *P. scaber* was less than consumption of non-*Bt* maize varieties (Wandeler et al., 2002; Pont and Nentwig, 2005). The Cry1Ab protein was detected in the body and feces of *P. scaber*, showing that the woodlouse ingested and excreted the protein, but there were no negative effects of the protein on survival and growth of *P. scaber*. No adverse effects of purified Cry1Ab protein and leaves of transgenic maize expressing

Table 1
Summary of the effects of Cry proteins from *Bacillus thuringiensis* on soil-dwelling invertebrates

Organism	Species	Study location	Experimental variable	Protein expressed	Effect of Cry proteins on organism	References
Earthworm	*Eisenia fetida*	Laboratory	Soil amended with biomass of *Bt* and non-*Bt* maize	Cry1Ab	No effect on mortality and weight	Ahl Goy et al. (1995)
Earthworm	*Lumbricus terrestris*	Laboratory	Soil amended with biomass of *Bt* and non-*Bt* maize; soil planted to *Bt* and non-*Bt* maize	Cry1Ab	No significant differences in mortality and weight	Saxena and Stotzky (2001b)
Earthworm	*Lumbricus terrestris*	Laboratory	Fed residues of *Bt* and non-*Bt* maize	Cry1Ab	No differences in mortality and weight	Zwahlen et al. (2003b)
Earthworm	*Lumbricidae* community	Field	Cultivation of *Bt* and non-*Bt* maize; cultivation of maize treated with commercial insecticide (Baythroidis®) and of untreated maize	Cry1Ab	No effect of Cry protein on numbers in soil planted with *Bt* and non-*Bt* maize and in soil planted with non-*Bt* maize treated with commercial insecticide and with noninsecticide	Lang et al. (2006)
Earthworm	*Eisenia fetida*	Laboratory	Fed leaves of *Bt* and non-*Bt* maize	Cry1Ab	No deleterious effects on survival and reproduction	Clark and Coats (2006)
Earthworm	*Aporrectodea caliginosa*	Laboratory	Fed leaves of *Bt* and non-*Bt* maize	Cry1Ab	No effect on mortality and weight	Vercesi et al. (2006)
Woodlouse	*Porcellio scaber*	Laboratory	Fed biomass of *Bt* and non-*Bt* maize	Cry1Ab	No negative impact of *Bt* maize litter on consumption, reproduction, and growth	Escher et al. (2000)
Woodlouse	*Porcellio scaber*	Laboratory	Fed purified protein	Cry2A	No toxic effect	Sims (1997)
Woodlouse	*Porcellio scaber*	Laboratory	Fed biomass of *Bt* and non-*Bt* maize	Cry1Ab	Fed significantly less on *Bt* maize than on non-*Bt* maize during a feeding period of 20 d	Wandeler et al. (2002)
Woodlouse	*Porcellio scaber*	Laboratory	Fed leaves of *Bt* and non-*Bt* maize	Cry1Ab	No toxic effect	Pont and Nentwig (2005)
Pillbug Sowbug	*Armadillidium nasatum* and *Trachelipus rathkii*	Laboratory	Fed purified protein or leaves of *Bt* and non-*Bt* maize	Cry1Ab	No adverse effects on survival and growth	Clark et al. (2006)
Collembola	*Folsomia candida* *Xenylla grisea*	Laboratory	Addition of four purified proteins to diet	Cry1Ab Cry1Ac Cry2A Cry3A	No effect on survival or reproduction during 21 d	Sims and Martin (1997)
Collembola and Mite	*Folsomia candida* *Oppia nitens*	Laboratory	Fed leaves of *Bt* and non-*Bt* cotton or leaves of *Bt* and non-*Bt* potato	Cry1Ab Cry1Ac	No significant effects on oviposition, numbers of eggs, and body length	Yu et al. (1997)
Collembola	*Folsomia candida*	Field	Cultivation of *Bt* and non-*Bt* maize or *Bt* and non-*Bt* cotton	Cry1Ab Cry1Ac	No effects on numbers	USEPA (2001)
Collembola and Mites	Natural populations	Field	Cultivation of *Bt* and non-*Bt* maize	Cry3Bb1	No deleterious effects on numbers of collembolans and mites in soil	Al-Deeb et al. (2003)
Collembola and Mites	Natural populations	Laboratory	Cultivation of *Bt* and non-*Bt* maize in pots	Cry1Ab	Lower collembolan abundance and higher mite populations under *Bt* maize	Griffiths et al. (2006)
Collembola	*Folsomia candida*	Laboratory	Fed leaves of *Bt* and non-*Bt* maize	Cry1Ab	No effect on survival and reproduction	Clark and Coats (2006)

Table 1 (*continued*)

Organism	Species	Study location	Experimental variable	Protein expressed	Effect of Cry proteins on organism	References
Collembola	*Folsomia candida* *Heteromurus nitidus* *Sinella coeca*	Laboratory	Fed dried leaves of *Bt* and non-*Bt* maize	Cry1Ab	No effect on survival Species specific effects in distributions and feeding preference	Bakonyi et al. (2006)
Collembola	Natural populations	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	No significant differences in population density	Lang et al. (2006)
Collembola	*Protaphorura armata*	Laboratory	Fed purified protein or biomass of *Bt* and non-*Bt* maize	Cry1Ab	No effect of purified or *Bt* maize-Cry1Ab protein on growth and reproduction	Heckmann et al. (2006)
Nematodes	*Caenorhabditis elegans*	Field	Cultivation of *Bt* and non-*Bt* maize	Cry3Bb1	No deleterious effects on numbers in soil with *Bt* maize compared with non-*Bt* soil	Al-Deeb et al. (2003)
Nematodes	Natural populations	Laboratory	Soil planted to *Bt* and non-*Bt* maize	Cry1Ab	No significant differences in numbers between soils with *Bt* and non-*Bt* maize	Saxena and Stotzky (2001b)
Nematodes	Natural populations	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	No significant effect on communities and biodiversity between soils planted with *Bt* and non-*Bt* maize	Manachini and Lozzia (2002)
Nematodes	*Caenorhabditis elegans*	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	Lower abundance in soils with *Bt* maize than with non-*Bt* maize	Manachini and Lozzia (2003)
Nematodes	Natural populations	Field	Cultivation of *Bt* and non-*Bt* eggplant	Cry3Bb1	No effect on community structure	Manachini et al. (2003)
Nematodes	Natural populations	Field	Cultivation of *Bt* and non-*Bt* canola	Cry1Ac	A distinct shift in community structure with *Bt* canola when compared with non-*Bt* isoline	Manachini et al. (2004)
Nematodes	Natural populations	Laboratory	Fed purified protein	Cry1Ab	Negative correlation between the reproduction of the nematodes and the protein	Höss et al. (2004)
Nematodes	Natural populations	Laboratory	Fed purified protein	Cry1Ab	Negative effects on eggs and juveniles	Meadows et al. (1990)
Nematodes	Natural populations	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	Significantly lower abundance in soils with *Bt* maize than with non-*Bt* maize	Griffiths et al. (2005)
Nematodes	*Acrobeloides* spp. *Pratylenchus* spp.	Laboratory	Cultivation of *Bt* and non-*Bt* maize in pots	Cry1Ab	Significantly higher populations under *Bt* maize than non-*Bt* maize	Griffiths et al. (2006)
Nematodes	*Caenorhabditis elegans*	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	Negative effect of Cry1Ab protein on growth, number of eggs, and reproduction	Lang et al. (2006)
	Pratylenchus spp.	Field	Cultivation of *Bt* and non-*Bt* maize	Cry1Ab	No effects	

the Cry1Ab protein were observed under laboratory conditions on the survival and growth of the pillbug, *Armadillidium nasatum* Budde-Lund, and the sowbug, *Trachelipus rathkii* Brandt, which are abundant isopods in soil, especially in the maize-growing region of the central USA (Clark et al., 2006) (Table 1).

Collembolans are key indicator species of soil fertility and health, as they are important in the breakdown and recycling of crop residues, and abundant populations of these microarthropods are generally present in well-managed agricultural soils. They are often present in the root zone of plants and, therefore, can be exposed to Cry proteins exuded into the rhizosphere by *Bt* crops, and as they are active in the decomposition of organic matter, they will also be exposed to Cry proteins in crop residues (O'Callaghan et al., 2005). In general, no negative effects of Cry proteins on collembolans have been observed (e.g., Sims and Martin, 1997; Yu et al., 1997; Al-Deeb et al., 2003; Clark and Coats, 2006). The addition of four purified *Bt* insecticidal proteins (Cry1Ab, Cry1Ac, Cry2A, and Cry3A) at concentrations of $200\,\mu g\,g^{-1}$ to the diet of the collembolans, *Folsomia candida* Willem and *Xenylla grisea* Axelson, for 21 d did not affect their survival or reproduction compared with the unamended diet (Sims and Martin, 1997). In soils in the field, concentrations of Cry proteins in plant material exposed to soil organisms are usually lower and are estimated to be less than $30\,\mu g\,g^{-1}$ of fresh weight, suggesting that the low concentrations of insecticidal Cry proteins present in the tissues of *Bt* crop plants do not pose a risk to soil collembolans (Sims and Martin, 1997). Premarket risk assessment studies submitted for regulatory approval of several *Bt* maize and *Bt* cotton varieties have also not shown any toxic effect of Cry1A proteins on *F. candida* (US Environmental Protection Agency (EPA), 2001). No deleterious effects on survival and reproduction of *F. candida* were observed when fed leaves of *Bt* maize expressing the Cry1Ab protein compared with leaves of non-*Bt* isolines (Clark and Coats, 2006). Bakonyi et al. (2006) showed that *Bt* maize was less preferred as food by *F. candida* than near-isogenic non-*Bt* maize. However, this was not the case for other species of Collembola, i.e., *Heteromurus nitidus* Templeton and *Sinella coeca* Schött. Heckmann et al. (2006) reported that the growth and reproduction of the collembolan, *Protaphorura armata* Tullberg, reared on ground roots of *Bt* maize expressing the Cry1Ab protein were not significantly different from those reared on ground roots of non-*Bt* maize for 4 weeks. *P. armata* performed significantly better on a diet of yeast amended with purified Cry1Ab protein than on ground root tissue of *Bt* and non-*Bt* maize. No significant differences in the population density of collembolans were found in soils cultivated with *Bt* and non-*Bt* maize and between the application of an insecticide (Baythroid®) and no insecticide (Lang et al., 2006) (Table 1).

No negative effects of Cry proteins on mites have been observed. Yu et al. (1997) fed the soil mite, *Oppia nitens* Acari, fresh and old *Bt* cotton and *Bt* potato leaves expressing the Cry1Ab/Ac and Cry3A protein, respectively, as well as leaves of isogenic non-*Bt* plants, for 7 weeks and observed no significant effects on oviposition, the number of eggs produced per female, or final body length. In a 2-year field study, Al-Deeb et al. (2003) reported that *Bt* maize expressing the Cry3Bb1 protein for control of the corn rootworm did not have any deleterious effects on the numbers of soil mites, collembolans, and nematodes (Table 1).

Nematodes are present in almost all soils with a high population density and a large numbers of species. In addition to the plant-parasitic nematodes that cause considerable economic damage worldwide to many types of crops (Lang et al., 2006), other trophic groups, particularly bacteriophagous and mycophagous nematodes, are important in the metabolic activity of soil, such as mineralizing nutrients to forms that are available to plants. Nematodes are useful indicators of soil quality because of their great diversity and participation in many functions at different levels of food webs in soil, and they are good indicators because their populations, in contrast to those of bacteria, are relatively stable in response to changes in moisture and temperature in soil (Blair et al., 1996).

Studies on the effects of Cry proteins on soil nematodes have shown different results (Table 1). Saxena and Stotzky (2001b) found no significant differences in the number of nematodes in rhizosphere soil of *Bt* and non-*Bt* maize grown in a plant-growth room. No significant effects on communities and biodiversity of nematodes were found in a field experiment comparing *Bt* maize expressing the Cry1Ab protein with near-isogenic non-*Bt* maize (Manachini and Lozzia, 2002). However, nematodes feeding on fungi were more abundant in fields planted with *Bt* maize, whereas nematodes feeding on bacteria were more abundant in non-*Bt* maize fields. No effect on nematode community structure was observed in soil planted to *Bt* eggplant expressing the Cry3Bb1 protein. However, a distinct shift in community structure, i.e., a significantly higher proportion of mycophagous nematodes and a lower proportion of phytophagous nematodes, was observed in soil planted with *Bt* canola expressing the Cry1Ac protein when compared with the respective non-*Bt* isoline (Manachini et al., 2003, 2004). Griffiths et al. (2005) found a significant, but transient, decrease in the numbers of nematodes, as well as of protozoa, in soil under *Bt* maize expressing the Cry1Ab protein at three different field sites when compared with non-*Bt* maize, whereas studies in the greenhouse showed no toxic effect of the Cry1Ab protein on populations of nematodes and protozoa but, rather, significantly higher populations in soils under *Bt* than non-*Bt* maize (Griffiths et al., 2006). The reasons for the differences between the two studies are unclear, but they may have been the result of different environmental conditions in the greenhouse and the field, which could affect interactions between plants and soil organisms. However, only a few studies have investigated individual

nematode species, but these studies, in general, have indicated a negative effect of the Cry proteins (Wei et al., 2003). Höss et al. (2004) observed a negative correlation between the reproduction of the bacteriophagous nematode, *Caenorhabditis elegans,* and the concentration of Cry1Ab protein in a soil bioassay. *C. elegans* also showed a possible sensitivity to the Cry protein in the field, especially in rhizosphere soil of the *Bt* maize variety Novelis transformed by event MON810 (Manachini and Lozzia, 2003). Rhizosphere soil of *Bt* maize expressing the Cry1Ab protein (events MON810 and 176) negatively affected the growth, number of eggs, and reproduction rate of *C. elegans*, whereas no effects of the Cry1Ab protein were detected on the population density of the plant-parasitic nematode, *Pratylenchus* spp. (Lang et al., 2006) (Table 1).

4.2. Soil microorganisms

Microorganisms are the dominant organisms, both in terms of biomass and activity, in soil, and they are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens, decomposition of agricultural chemicals, and improvement of soil structure (e.g., Gupta and Yeates, 1997; Bruinsma et al., 2003). However, the close interaction between crop cultivation and microbe-mediated soil processes inadvertently leads to contact of soil organisms with Cry proteins released from *Bt* crops. The rhizosphere (the zone of soil directly surrounding and influenced by plant roots) contains the majority of the microbiota in soil (>10-fold more than the microbiota in bulk soil), and plant-microbe interactions in the rhizosphere are among the major factors that regulate the health and growth of plants. It is widely acknowledged that root exudates govern which organisms reside in the rhizosphere (e.g., Lynch, 1994; Bardgett et al., 1999). Therefore, any change in the quality and quantity of root exudates could potentially modify the composition (biodiversity) and activity of the soil microbiota and may cause changes in both deleterious and beneficial microorganisms. For example, a decrease in specific microbial populations could lead to a decrease in decomposition processes, alter the level and composition of soil organic matter, and have secondary effects on the survival of plant pathogens (Termorshuizen and Lotz, 2002). Similarly, loss of particular trophic groups of the mesofauna could cause a loss of specific pathways within nutrient cycling processes, thus affecting important biogeochemical pathways.

There have been numerous studies, with different methods (e.g., functional and structural composition of soil microbial communities) and different *Bt* plants (e.g., maize, cotton, rice, canola, potato, tobacco), on the effects of *Bt* crops on soil microbial communities. Different effects, ranging from no effect to minor and significant effects, of these plants on microbial communities (e.g., Stotzky, 2002, 2004; Turrini et al., 2004; Gupta and Watson, 2004; Castaldini et al., 2005; Xue et al., 2005; Rui et al., 2005; Icoz et al., 2007) and activities of some enzymes (Wu et al., 2004b; Flores et al., 2005; Shen et al., 2006; Sun et al., 2007; Icoz et al., 2007) in soil have been reported (Tables 2 and 4). Turrini et al. (2004) found that root exudates of *Bt* maize (event 176) significantly reduced presymbiotic hyphal growth of the arbuscular mycorrhizal fungus, *Glomus mosseae*, compared with root exudates of another *Bt* maize hybrid (event Bt11) and non-*Bt* maize. The microbiota (e.g., bacteria, actinomycetes, fungi) associated with residues of *Bt* cotton were significantly different from those associated with residues of herbicide-tolerant cotton (Roundup Ready®) (Gupta and Watson, 2004). Significant differences were also observed in the composition of the microbiota in Australian soils associated with residues of *Bt* cotton and non-*Bt* cotton by both bright-field and scanning electron microscopy. More extensive colonization by fungi (spores and hyphae) was observed in residues of *Bt* cotton than in residues of non-*Bt* cotton. Using a selective inhibition-SIR (substrate-induced respiration) technique, higher ratios of fungi to bacteria were found in *Bt* cotton residues than in the residues of non-*Bt* cotton (Gupta et al., 2002; Gupta and Watson, 2004). In addition, the types of fungi that colonized the two types of residues were different by microscopic observations. Castaldini et al. (2005) reported consistent significant differences between *Bt* maize expressing the Cry1Ab protein and near-isogenic non-*Bt* maize in both total and metabolically-active 16S rRNA fractions of culturable rhizosphere heterotrophic bacteria by denaturing gradient gel electrophoresis (DGGE) and in mycorrhizal colonization by microscopy (e.g., a significantly lower level of *G. mosseae* was detected in roots of *Bt* maize). Xue et al. (2005) found that the ratio of gram-positive to gram-negative bacteria was lower in soil with *Bt* maize than in soil with near-isogenic non-*Bt* maize, but the ratio was higher in soil with *Bt* potato than with near-isogenic non-*Bt* potato. However, there was no difference in the ratio of fungi to bacteria between soil with *Bt* and non-*Bt* maize and between soil with *Bt* and non-*Bt* potato. Rui et al. (2005) found increased numbers of culturable functional groups of bacteria (potassium-dissolving bacteria, inorganic phosphate-dissolving bacteria, and nitrogen-fixing bacteria) in rhizosphere soil of non-*Bt* cotton than in rhizosphere soil of *Bt* cotton in the early and middle stages of growth of cotton. However, they found no significant differences in numbers after the growing season, and there was no effect of purified Cry1Ac protein on the numbers of these groups.

By contrast, most studies have indicated that *Bt* plants cause no or only minor changes in microbial community structure that are often transient in duration (Table 2). For example, when numbers and species of indigenous soil bacteria and fungi were monitored using culture-based methods and a DNA fingerprinting method, a significant, but transient, increase in the populations of culturable bacteria and fungi was found in soil microcosms with decomposing leaves of *Bt* cotton expressing the Cry1Ac

Table 2
Summary of direct effects of Cry proteins from *Bacillus thuringiensis* on the diversity of microbes and other organisms in soil

Organism	Experimental variable	Protein	Effect on biodiversity	References
Culturable bacteria and fungi	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	A significant, but transient, increase in numbers in soil with *Bt* cotton	Donegan et al. (1995)
Culturable bacteria, fungi, and protozoa	Soil amended with purified protein versus unamended soil	Cry1Ab Cry1Ac	No effect on bacteria, fungi, or protozoa when compared with control soil	Donegan et al. (1995)
Culturable aerobic bacteria and fungi	Soil with *Bt* and non-*Bt* potato	Cry3A	Minimal differences in numbers	Donegan et al. (1996)
Culturable bacteria, fungi, protozoa, nematodes, and earthworms	Soil with *Bt* and non-*Bt* maize and soil amended with biomass of *Bt* and non-*Bt* maize	Cry1Ab	No significant differences in numbers between soils amended with biomass of *Bt* and non-*Bt* maize or in rhizosphere soil of *Bt* and non-*Bt* maize grown in a plant-growth room	Saxena and Stotzky (2001b)
Bacteria, fungi, and algae	Purified protein added to pure and mixed cultures	Cry1Ab Cry3A Cry4	No effect on growth of bacteria, fungi, and algae	Koskella and Stotzky (2002)
Heterotrophic bacterial and saprophytic fungal populations and carbon-cycling microorganisms (cellulolytic, amylolytic, proteolytic) and arbuscular mycorrhizae	Inoculation of soybean with spores of *Btk*, and its purified ICP protein was added	Cry1Ab	No effect on the populations when compared with uninoculated soil Some transient differences in numbers when compared with uninoculated soil No effect on arbuscular mycorrhizae when inoculated with ICP protein but colonization by fungi inhibited when inoculated with spores of *Btk*	Ferreira et al. (2003)
Microbial activity and bacterial community structure	Soil with *Bt* and non-*Bt* maize	Cry3Bb1	No effects on microbial activity (N-mineralization, short-term nitrification rate, and soil respiration) or bacterial community structure	Devare et al. (2004)
Rhizosphere bacterial community	Soil with *Bt* and non-*Bt* maize	Cry1Ab	No differences in community structure between *Bt* and non-*Bt* maize by CLCP, but differences in community structure by ARISA, dependent on plant age and type	Brusetti et al. (2004)
Rhizosphere bacterial community	Soil with *Bt* and non-*Bt* maize	Cry1Ab Cry1F	No significant effects on bacterial and fungal PLFA profiles. However, small significant effect of Cry proteins on community structure observed by CLPP	Blackwood and Buyer (2004)
Total aerobic culturable bacteria, actinomycetes, and fungi	Flooded soils amended with *Bt* and non-*Bt* rice straw	Cry1Ab	No toxic effects on the numbers of total aerobic culturable bacteria, actinomycetes, and fungi	Wu et al. (2004a)
Arbuscular mycorrhizal fungus	Rhizosphere soil of *Bt* and non-*Bt* maize	Cry1Ab	Significantly lower presymbiotic hyphal growth of mycorrhizae in soil with *Bt* maize than with non-*Bt* maize	Turrini et al. (2004)
Composition of soil microbiota	Rhizosphere soils of *Bt* cotton versus herbicide-tolerance (Roundup Ready®) cotton	Cry1Ac	Significantly different microflora in soil with *Bt* cotton residues than in soil with herbicide-tolerant cotton (by microscopic observations)	Gupta and Watson (2004)
Composition of soil microbiota	Rhizosphere soils of *Bt* and non-*Bt* cotton	Cry1Ac	More extensive fungal colonization in soil with *Bt* cotton than in soil with non-*Bt* cotton; higher ratios of fungi to bacteria in soil with *Bt* cotton than in soil with non-*Bt* cotton; different types of fungal spores in soil with *Bt* cotton than in soil with non-*Bt* cotton	Gupta and Watson (2004), Gupta et al. (2002)

Table 2 (*continued*)

Organism	Experimental variable	Protein	Effect on biodiversity	References
Culturable heterotrophic bacteria and mycorrhizae	Soil with *Bt* and non-*Bt* maize	Cry1Ab	Differences between soil with *Bt* and non-*Bt* maize: significantly lower level of mycorrhizal infection by *Glomus mosseae* in roots of *Bt* maize	Castaldini et al. (2005)
Culturable bacteria and fungi	Soil with *Bt* and non-*Bt* maize, and soil with *Bt* and non-*Bt* potato	Cry1Ab	Lower ratio of gram-positive to gram-negative bacteria in soil with *Bt* maize than in soil with non-*Bt* maize, and no differences in the ratio of fungi to bacteria	Xue et al. (2005)
		Cry3A	Higher ratio of gram-positive to gram-negative in soil with *Bt* potato than non-*Bt* potato, and no differences in the ratio of fungi to bacteria	
Culturable bacteria and fungi; activities of some enzymes	Soil amended with biomass of *Bt* and non *Bt*-maize	Cry1Ab	No significant differences	Flores et al. (2005)
Culturable functional bacteria (potassium-dissolving bacteria, inorganic phosphate-dissolving bacteria, and nitrogen-fixing bacteria)	Rhizosphere soils of *Bt* and non-*Bt* cotton	Cry1Ac	Increased numbers of culturable functional bacteria in soil of non-*Bt* cotton than in soil of *Bt* cotton in early and middle growth stages of cotton; no significant differences in numbers after the growing season	Rui et al. (2005)
	Soils amended with purified protein versus unamended soil		No effects on numbers of culturable functional bacteria	
Protozoa	Soil with *Bt* and non-*Bt* maize in the field	Cry1Ab	Significantly lower protozoa population in soils with *Bt* maize than with non-*Bt* maize.	Griffiths et al. (2005)
	Soils with *Bt* and non-*Bt* maize in pots in greenhouse		Significantly higher protozoa populations in soils with *Bt* maize than with non-*Bt* maize.	Griffiths et al. (2006)
Microbial biomass and activity of enzymes	Soil with *Bt* and non-*Bt* maize	Cry1Ab	No significant differences	Lang et al. (2006)
Functional diversity of microbial communities and activity of some enzymes	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	No adverse effects of *Bt* cotton on soil ecosystem	Shen et al. (2006)
Microbial populations and activity of enzymes	Soil with *Bt* and non-*Bt* maize	Cry1Ab Cry3Bb1	No consistent significant effects on numbers of culturable bacteria, gram-negative bacteria, chitin- and cellulose-utilizing bacteria, nitrifyers, denitrifiers, protozoa, and fungi by plate and MPN counts; no differences on microbial community structure by DGGE; and no consistent significant effects on activities of some enzymes (arylsulfatases, acid and alkaline phosphatases, dehydrogenases, proteases)	Icoz et al. (2007)
Microbial biomass	Soil with *Bt* and non-*Bt* maize	Cry3Bb1	No adverse effects of *Bt* maize on microbial biomass	Devare et al. (2007)

protein (Donegan et al., 1995). Minimal differences were observed in the populations of culturable, aerobic bacteria and fungi in soil with transgenic *Bt* potato expressing the Cry3A protein (Donegan et al., 1996). Other studies have also indicated only some minor changes in microbial community structure in soils with *Bt* plants (e.g., Brusetti et al., 2004; Blackwood and Buyer, 2004; Griffiths et al., 2006) or no apparent deleterious effects of transgenic Cry proteins released by *Bt* plants in root exudates or from biomass incorporated into soil on soil microbial communities (e.g., Donegan et al., 1995; Saxena and Stotzky, 2001b; Devare et al., 2004, 2007; Wu et al., 2004a; Flores

et al., 2005; Shen et al., 2006; Naef et al., 2006; Icoz et al., 2007) and some enzymes (Flores et al., 2005; Shen et al., 2006; Icoz et al., 2007). The addition of purified Cry1Ab or Cry1Ac protein to soil did not result in any detectable effects on bacteria, fungi, or protozoa when compared with the control soil (Donegan et al., 1995), and the purified insecticidal proteins from *B. thuringiensis* subsp. *kurstaki*, *morrisoni* (strain *tenebrionis*), and *israelensis* did not affect the growth of a variety of bacteria, fungi, and algae in either pure or mixed culture (Koskella and Stotzky, 2002). Similarly, Saxena and Stotzky (2001b) found no significant differences in the number of colony-forming units of culturable bacteria (including actinomycetes) and fungi and in the numbers of earthworms, protozoa, and nematodes between rhizosphere soil of *Bt* and non-*Bt* maize or between soil amended with biomass of *Bt* and non-*Bt* maize. Ferreira et al. (2003) showed that inoculation of soybean with *B. thuringiensis* subsp. *kurstaki* (*Btk*) or its ICP did not affect the bacterial or fungal populations of rhizosphere soil, but a transient increase in some functional groups of carbon-cycling microorganisms, such as cellulolytic, amylolytic, and proteolytic, was observed in the beginning of the incubation. The ICP did not show any effect on colonization by arbuscular mycorrhizae (AM), whereas colonization by AM fungi of soybeans inoculated with spores of *Btk* was inhibited when compared with uninoculated plants. No deleterious effects of growing *Bt* maize expressing the Cry3Bb1 protein from *B. thuringiensis* subsp. *kumamotoensis*, active against the corn rootworm complex (*Diabrotica* spp.), for two consecutive seasons in the field were detected on microbial biomass and activity or on bacterial community structure, as determined by terminal restriction fragment length polymorphism (T-RFLP) analysis (Devare et al., 2004). Brusetti et al. (2004) compared the rhizosphere bacterial community associated with *Bt* maize expressing the Cry1Ab protein and near-isogenic non-*Bt* maize using several techniques, including viable counts, community-level catabolic profiling (CLCP), and PCR-based automated ribosomal intergenic spacer analysis (ARISA), and found that the viable counts and CLCP did not show any differences between *Bt* and non-*Bt* maize, but ARISA showed that the community structure differed with the age of the plants and between *Bt* and non-*Bt* maize, suggesting that root exudates could select different bacterial communities. No significant effects of the Cry1Ab and Cry1F proteins from *Bt* maize on bacterial and fungal phospholipids fatty acid (PLFA) profiles and only a few significant effect of the Cry proteins on microbial community level physiological profiles (CLPP) were reported by Blackwood and Buyer (2004), and these effects were the result primarily of differences in soil type. Wu et al. (2004a) found that decomposing transgenic *Bt* rice straw (Cry1Ab protein) was not toxic to culturable bacteria, actinomycetes, and fungi in a flooded paddy soil under laboratory conditions. Some apparent increases were observed in the populations of anaerobic fermentative bacteria, denitrifying bacteria, hydrogen-producing bacteria, and methanogenic bacteria in soil with *Bt* rice straw in the beginning of the incubation, but they were transient. Flores et al. (2005) found no significant effects of the Cry1Ab protein released to soil from *Bt* maize in root exudates or biomass on culturable bacteria, fungi, and protozoa. No significant differences were found in microbial biomass of *Bt* and non-*Bt* maize over 4 consecutive years of maize cultivation (Lang et al., 2006), and no significant differences between soils with *Bt* and non-*Bt* cotton were found in functional diversity of microbial communities, suggesting that there were no adverse effects of *Bt* cotton on the soil ecosystem (Shen et al., 2006). Similarly, the Cry1Ab and Cry3Bb1 proteins in root exudates and decaying plant residues of *Bt* maize had no consistent significant effects on a variety of culturable microorganisms and their enzymatic activities in soil during 4 consecutive years of maize cultivation (Icoz et al., 2007). Some differences between soils with *Bt* and non-*Bt* maize in the numbers of the various groups of microorganisms were observed at some sampling times, but these differences were not consistent from one season to the next and were independent of the presence of the Cry proteins. The results from microbial plate counts were confirmed by DGGE, which also showed no differences in the diversity of bacteria between soils with *Bt* or non-*Bt* maize but showed differences in bacterial patterns with season (Icoz et al., 2007) (Table 2).

Plants alter the composition and diversity of soil microbial communities in a selective manner (Nehl et al., 1997). The microbial community resulting from plant-selective pressure varies between plant species (Germida et al., 1998; Grayston et al., 1998; Smalla et al., 2001), indicating that plant type influences which microorganisms colonize their rhizosphere. Moreover, Rengel et al. (1998) suggested that the selective effect of plants on the rhizosphere community can occur even at the cultivar level. Experiments in which root growth solutions, collected from *Bt* and non-*Bt* maize grown in hydroponic culture, were added to soil indicated that root exudates of *Bt* maize led to the development of bacterial communities in soil that differed from those associated with exudates of near-isogenic non-*Bt* maize. It was suggested that the exudates of *Bt* maize differed from those of non-*Bt* maize in several ways and not only in the content of the Cry protein (Brusetti et al., 2004). Studies, using culture-independent methods, showed some minor or no *Bt*-specific effects on soil microorganisms, and plant age and type appeared to be the major factors affecting bacterial diversity (Donegan et al., 1995; Griffiths et al., 2005, 2006; Naef et al., 2006). For example, Donegan et al. (1995) found that the endotoxin from *B. thuringiensis* subsp. *kurstaki*, both purified and produced in transgenic plants, did not have a direct effect on soil microorganisms and that the effects observed, which were related to the plant varieties used, may have been caused by unexpected changes in plant characteristics that resulted from genetic manipulation or tissue culturing of the engineered plants. Field and

greenhouse trials with maize expressing the Cry1Ab protein showed that changes in microbial, protozoan, and nematode communities as a result of the *Bt* trait were small and less than changes resulting from different non-*Bt* maize cultivars, different crops, soil type, and stage of plant growth (Griffiths et al., 2005, 2006). Naef et al. (2006) reported no direct effect of the Cry1Ab protein in maize residues on the pathogen, *Fusarium graminearum*, and on the biocontrol agent, *Trichoderma atroviride*, and showed that some *Bt* and their near-isogenic non-*Bt* maize counterparts differed more in the chemical composition of the maize tissue as a result of different environmental conditions, such as drought-stress, than from the Cry protein content alone and which can affect the saprophytic growth of fungi on crop residues (Table 3). Similarly, Siciliano et al. (1998) and Siciliano and Germida (1999) reported that the composition of the root-associated microbial community differed between canola cultivars (transgenic glyphosate-resistant canola and near-isogenic non-transgenic canola cultivars), as determined by CLPP and fatty acid methyl ester (FAME) analyses, and suggested that these effects could have been caused by altered chemical composition of the plant tissue as a result of the insertion of genes. Differences in the composition of crop residues as the result of the introduction of a transgenic trait have been observed in transgenic *Bt* crops (e.g., Masoero et al., 1999; Escher et al., 2000; Saxena and Stotzky, 2001a; Folmer et al., 2002; Jung and Sheaffer, 2004; Mungai et al., 2005; Poerschmann et al., 2005).

Other studies have shown that the effects of GM plants on microbial communities are subject more to seasonal variations or to other environmental factors, such as soil type and agricultural practices, than to expression of Cry or other proteins in plants (e.g., Lottmann et al., 1999, 2000; Heuer et al., 2002; Dunfield and Germida, 2003; Blackwood and Buyer, 2004; Griffiths et al., 2000, 2005; Baumgarte and Tebbe, 2005; Fang et al., 2005). For example, Lottmann et al. (1999, 2000) found that although transgenic potato expressing T4 lysozyme influenced the composition of root-associated bacterial antagonists, such as *Pseudomonas putida* (which inhibits the pathogen, *Erwinia carotovora*, that causes soft rot and blackleg in potato) and *Serratia grimesii* (which inhibits the fungus, *Verticillium dahliae*), this was dependent on both the year and the time of sampling. The rhizosphere microbial community associated with two transgenic potato lines that produced the lectins, *Galanthus nivalis* agglutinin (GNA) and concanavalin A (con A), had different CLPP than the control line, but the profiles were also subject to seasonal variation, and differences did not persist from one season to the next (Griffiths et al., 2000). Heuer et al. (2002) found significant differences between the community structure of the rhizosphere of transgenic and non-transgenic potatoes that were dependent on environmental factors but independent of the expression of T4 lysozyme by the transgenic plants. Dunfield and Germida (2003) found significant seasonal differences in the microbial community associated with the rhizosphere of a

Table 3
Summary of indirect effects of Cry proteins from *Bacillus thuringiensis* on the diversity of microbes and other organisms in soil

Organism	Experimental variable	Protein	Effect on biodiversity	References
Microbial populations	Soil with *Bt* and non-*Bt* maize and purified protein	Cry1Ab	No direct effects of Cry protein, but effects of plant characteristics	Donegan et al. (1995)
Rhizosphere bacterial community	Soil with *Bt* and non-*Bt* maize	Cry1Ab	Soil type was most important factor affecting the composition of community	Blackwood and Buyer (2004)
Rhizosphere bacterial community	Soil with *Bt* and non-*Bt* maize	Cry1Ab	Bacterial community structure in soil of *Bt* maize was less affected by the Cry protein than by other environmental factors	Baumgarte and Tebbe (2005)
Rhizosphere bacterial diversity	Soil with *Bt* and non-*Bt* maize	Cry1Ab	Rhizosphere bacterial diversity affected more by soil texture than by cultivation of *Bt* varieties	Fang et al. (2005)
Microbial community, protozoa, and nematodes	Soil with *Bt* and non-*Bt* maize	Cry1Ab	Small changes in microbial and macrofaunal communities depended more on cultivars, soil type, and plant growth stage than on presence of the Cry protein	Griffiths et al. (2005, 2006)
Fusarium graminearum and *Trichoderma atroviride*	Soil with *Bt* and non-*Bt* maize	Cry1Ab	No direct effect of Cry1Ab protein. Differences between soil with *Bt* and non-*Bt* maize depended more on the chemical composition of the maize tissue	Naef et al. (2006)
Microbial populations and activities of some enzymes	Soil with *Bt* and non-*Bt* maize	Cry1Ab Cry3Bb1	Differences between *Bt* and non-*Bt* maize were affected more by plant varieties and season (sampling date), and they were transient	Icoz et al. (2007)

herbicide-tolerant (glyphosate-resistant) transgenic variety of canola, Quest, and a conventional variety, Excel, but these differences also did not persist into the next season. The bacterial community structure in the rhizosphere of *Bt* maize expressing the Cry1Ab protein was less affected by the protein than by environmental factors, such as the age of the plant or heterogeneities in the field (Baumgarte and Tebbe, 2005). Blackwood and Buyer (2004), using PLFP and CLPP, reported that *Bt* maize did not result in large differences in soil microbial communities and that soil type was the most important factor affecting the composition of the communities. Fang et al. (2005) reported that bacterial diversity in the rhizosphere of transgenic and non-transgenic maize was affected more by soil texture than by cultivation of transgenic varieties. Similarly, Icoz et al. (2007) found seasonal variations in the numbers of microorganisms and enzyme activities, probably as the result of differences in the water content of the soils, ambient temperatures, and stage of growth of the plants at the times of sampling. These seasonal differences were independent of the presence of the Cry proteins in the plants (Table 3).

4.3. Effects of methodology

The reasons for the observed differences of the effects of *Bt* plants on soil microbial community structure, as well as their implications, are usually not clear. Knowledge of the complex diversity of soil microorganisms is limited, as only a small portion of soil microbial populations can be cultured and identified using standard analytical methods (e.g., Motavalli et al., 2004). Because of this limited knowledge, the importance and the functional consequences of differences in soil microbial populations are difficult to determine. Microbial diversity is measured by various techniques, such as traditional plate, most probable number (MPN), and direct microscopic counts, as well as by molecular-based procedures and fatty acid analyses. However, the approach of determining microbial diversity from the number of isolates has become less popular, not only because a limited number of microorganisms can be cultured, but also because the procedures are laborious. As a consequence, an increasing number of molecular techniques are now used because they do not rely on isolation and cultivation of microorganisms (e.g., Johnsen et al., 2001). Culture-independent studies based on obtaining genes of 16S rRNA directly from the environment by broad-specificity primer PCR and cloning have greatly improved the understanding of microbial diversity (e.g., Pace, 1997; Hugenholtz et al., 1998). However, techniques based on PCR, such as DGGE and TGGE, also have several drawbacks (e.g., Von Wintzingerode et al., 1997), such as the formation of chimeric or heteroduplex DNA molecules during PCR amplification. Moreover, amplification by PCR can be inhibited if contaminants are not removed by the purification process, only the dominant populations are revealed, and bands from more than one species may be hidden behind a single band, resulting in an underestimation of the bacterial diversity (Heuer et al., 2001; *see* Nannipieri et al., 2003).

Another approach for evaluating the effects of transgenic plants on microorganisms is to monitor microbial processes rather than population levels or taxonomic groups. One method of monitoring microbial processes is to measure enzymatic activity, as such measurements can assist in the prediction of the effects of GM plants on nutrient cycling. However, a single method should not be relied upon to give an accurate assessment of the impacts on microorganisms of the use of transgenic plants. By combining several methods, more aspects of the microbial community can be examined, and significant effects detected with one method can potentially be confirmed using additional methods. For example, Landi et al. (2000) have suggested that calculating the ratio between the activity of an enzyme and the microbial biomass would provide more meaningful information than either measurement alone. The ratio between two measured parameters represents an easy combination of two different measurements in a single criterion, which can give some indications of changes occurring in microbial activity. However, any change in the ratio does not depend exclusively on variations in the extracellular enzyme activity because the intracellular enzyme activity can also increase or decrease without any change in microbial biomass (Nannipieri et al., 2002). Moreover, it is necessary to measure the activities of a large number of enzymes and to combine these measured activities into a single index to provide meaningful information about microbial activity in soil. It is conceptually wrong to assume a simple relationship between the activity of a single enzyme and microbiological activity in soil (Nannipieri et al., 2002).

4.4. Microbe-mediated processes and functions

Soil organisms regulate many processes in soil that are essential for plant growth, soil health, and sustained productivity. Crop residues are the primary source of carbon for microbial populations in soils. A unique balance between the major components of soil ecosystems, i.e., physical, chemical, and biological components, is necessary for long-term sustainability of crop production and soil quality. Hence, the composition of the biota associated with residues is of great significance in regulating the essential biological functions in this ecosystem. Among the many essential functions of the soil microbiota are decomposition of soil organic matter and mineralization and immobilization of nutrients (e.g., Gupta and Watson, 2004). However, there has been relatively little research on the influence of *Bt* crops and Cry proteins on these microbe-mediated processes in soil (O'Callaghan et al., 2005). Concerns have been raised that *Bt* crops may influence nutrient cycling in soil by causing changes in soil microbial activity as the result of differences in the amount and composition of root exudates and crop residues,

changes in microbial functions resulting from gene transfer from the transgenic crop, and alteration in microbial populations because of the effects of management practices used for transgenic crops (Motavalli et al., 2004).

Microbial activity, a term used to indicate a vast range of activities carried out by microorganisms in soil, has been reported to be affected by the presence of *Bt* crops. Various methods have been used to determine microbiological activity: e.g., respiration, such as the evolution of CO_2, reflects the rate of catabolism; nitrification reflects the rate of oxidation of ammonium to nitrate; dehydrogenase activity reflects the intracellular flux of electrons to O_2 and is the result of the activity of intracellular enzymes catalyzing the transfer of hydrogen and electrons from one compound to another (Nannipieri et al., 1990). For example, soil respiration did not show any changes during growth of *Bt* maize, whereas there was a lower evolution of CO_2 from soils amended with residues of *Bt* maize (Castaldini et al., 2005). By contrast, Dinel et al. (2003) reported a 30.5% lower evolution of CO_2 from soils planted with *Bt* maize than from soils planted with near-isogenic non-*Bt* maize. Flores et al. (2005) reported a significantly lower (20–39%) evolution of CO_2 from soils in microcosms amended with biomass of *Bt* maize, canola, cotton, potato, rice, and tobacco than from soils amended with their near-isogenic non-*Bt* counterparts, which they attributed to a higher lignin content in the biomass of the *Bt* plants. However, Hopkins and Gregorich (2003) did not observe any differences in the decomposition of plant material from *Bt* and non-*Bt* maize, as determined by CO_2 evolution, and no differences in the evolution of CO_2 were found in soils amended with biomass of *Bt* maize or with equivalent amounts of biomass of non-*Bt* maize (Devare et al., 2004) (Table 4).

Wu et al. (2004a, b) found increased activities of phosphatases and dehydrogenases, as well as an increase in methanogenesis, after the addition of transgenic *Bt* rice straw to flooded soil. Sun et al. (2007) found that the addition of leaves and stems of two *Bt* cotton varieties to soil stimulated the activities of soil ureases, acid phosphomonoesterases, invertases, and cellulases, whereas the activity of soil arylsulfatases was inhibited. In contrast, Flores et al. (2005) reported that the activities of these enzymes were not consistently different in soil amended or unamended with biomass of *Bt* or non-*Bt* maize. Similarly, Shen et al. (2006) found no consistent significant difference in the activities of some enzymes (ureases, alkaline phosphatases, dehydrogenases, phenol oxidases, and proteases) between soils of *Bt* and non-*Bt* cotton, and no significant differences were found in the activity of some enzymes (catalase, dimethylsulphoxide reductase, β-glucosidase, and arginine deaminase) at four trial sites of *Bt* and non-*Bt* maize after 11 sampling dates over 4 consecutive years of maize cultivation (Lang et al., 2006). These results were in agreement with those of Icoz et al. (2007), who also found no consistent significant differences in the activities of representative enzymes involved in the biodegradation of plant biomass between soils with *Bt* maize and with their near-isogenic non-*Bt* maize counterparts during 4 consecutive years of maize cultivation (Table 4).

Cultivation of *Bt* crops may also have several indirect effects on microbe-mediated processes in soil ecosystems. The primary indirect effects of *Bt* crops on such processes are the result of changes in the amount and composition of crop residues from *Bt* crops. Differences in the chemical composition of crop residues have been observed in *Bt* crops (Table 4). For example, Masoero et al. (1999) found that two *Bt* maize hybrids had higher starch and lignin and lower protein and soluble N contents than their near-isogenic non-*Bt* maize counterparts. Saxena and Stotzky (2001a) found a 33–97% higher lignin content in 10 *Bt* maize hybrids, representing three different transformation events (Bt11, MON810, and 176), than in their respective non-*Bt* isolines by fluorescence microscopy, staining, and the acetyl bromide method. These results were confirmed by Poerschmann et al. (2005), who also found higher concentrations of total lignin in the leaves and stems of *Bt* maize (events MON810 and 176) than in the near-isogenic non-*Bt* lines by molecular-level based thermochemolysis using tetramethylammonium hydroxide (TMAH) in combination with gas chromatography-mass spectrometry (GC-MS) and by cupric oxide oxidation. By contrast, Escher et al. (2000) found a lower lignin content, as well as a lower C:N ratio, and a higher content of soluble carbohydrates in leaves of *Bt* maize (event Bt11) than in its corresponding near-isogenic non-*Bt* variety by sequential lignin analysis using the methods of Scehovic (1975). Other studies on the chemical composition of *Bt* crops residues have shown no significant differences. For example, Folmer et al. (2002) observed no differences in the chemical composition between *Bt* (event Bt11) and non-*Bt* maize tissues. Jung and Sheaffer (2004) found no consistent significant differences in the lignin content between six *Bt* maize hybrids expressing the Cy1Ab protein (events MON810 and Bt11) and their six non-*Bt* isolines by the acetyl bromide and Klason methods. Similarly, no differences were observed in the chemical composition of 2-year-old field-grown *Bt* and non-*Bt* maize residues of five different varieties or in their rates of nitrogen mineralization under laboratory conditions (Mungai et al., 2005). Using near infrared spectroscopy, Lang et al. (2006) found differences in lignin content only between stalk and root but not between *Bt* maize varieties (events MON810 and 176) and their near-isogenic counterparts. Lignin is a major structural component of plant cells that confers strength, rigidity, and impermeability to water. Any modifications in lignin content could result in effects that may have ecological implications (Halpin et al., 1994). For example, the increase in lignin content in *Bt* maize 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).

Table 4
Summary of the effects of *Bt* crops on microbe-mediated processes and functions in soil

Process/function	Experimental variable	Protein	Effect	References
Phosphatases, dehydrogenases, and methanogenesis	Soils amended with *Bt* and non-*Bt* rice straw	Cry1Ab	Increased activities in soil of phosphatases and dehydrogenases, as well as an increase in methanogenesis, after the addition of transgenic *Bt* rice straw to flooded soil	Wu et al. (2004b)
Proteases, neutral phosphatases, urease, and anaerobic respiration			No significant differences in activities	Wu et al. (2004b)
Arylsulfatases, phosphatases, dehydrogenases, and proteases	Soils amended with *Bt* and non-*Bt* maize	Cry1Ab	No differences in the activities of enzymes between soil with *Bt* and near-isogenic non-*Bt* maize	Flores et al. (2005)
Catalase, dimethylsulphoxide reductase, β-glucosidase, and arginine deaminase	Soils with *Bt* and non-*Bt* maize	Cry1Ab	No significant differences in the activities of enzymes between soils with *Bt* and non-*Bt* maize during 4 years of maize cultivation	Lang et al. (2006)
Ureases, alkaline phosphatases, dehydrogenases, phenol oxidases, and proteases	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	No differences in the activities of enzymes between soil with *Bt* and near-isogenic non-*Bt* cotton	Shen et al. (2006)
Arylsulfatases, phosphatases, dehydrogenases, and proteases	Soils with *Bt* and non-*Bt* maize	Cry1Ab Cry3Bb1	No consistent, significant differences in the activities between soil with *Bt* and non-*Bt* maize during 4 years of maize cultivation	Icoz et al. (2007)
Ureases, acid phosphomonoesterases, invertases, cellulases, and arylsulfatases	Soils amended with *Bt* and non-*Bt* cotton	Cry1Ac	The addition of biomass of *Bt* cotton to soil stimulated the activities of all enzymes, except that of arylsulfatase, which was inhibited	Sun et al. (2007)
CO_2 evolution	Soils with *Bt* and non-*Bt* maize	Cry1Ab	Lower evolution of CO_2 in soils planted to *Bt* maize than to non-*Bt* maize	Dinel et al. (2003)
CO_2 evolution	Soils amended with biomass of *Bt* and non-*Bt* maize	Cry1Ab	Lower evolution of CO_2 from soils amended with biomass of *Bt* maize than with biomass of non-*Bt* maize	Devare et al., 2004
CO_2 evolution	Soils amended with biomass of various *Bt* and non-*Bt* plants	Cry1Ab Cry1Ac Cry3A	Lower evolution of CO_2 from soils amended with biomass of *Bt* maize, rice, potato, cotton, canola, and tobacco than with biomass of near-isogenic non-*Bt* counterparts	Flores et al. (2005)
CO_2 evolution	Soils amended with biomass of *Bt* and non-*Bt* maize	Cry1Ab	No differences between soils with *Bt* and non-*Bt* maize during growth. Lower evolution of CO_2 from soils amended with biomass of *Bt* maize than of non-*Bt* maize	Castaldini et al. (2005)
Decomposition rate (CO_2 production)	Soils with *Bt* and non-*Bt* maize	Cry1Ab	No differences in the decomposition of *Bt* and non-*Bt* maize	Hopkins and Gregorich (2003)
Decomposition rate	Soils with *Bt* and non-*Bt* maize	Cry1Ab	Very small differences in the rate of decomposition of *Bt* and non-*Bt* maize	Gupta and Watson (2004)
N-mineralization	Soils with *Bt* and non-*Bt* maize	Cry3Bb1	No differences in potential aerobic N-mineralization	Devare et al. (2004)
N-mineralization	Soils amended with *Bt* and non-*Bt* maize residues	Cry1Ab	No consistent differences in N-mineralization	Mungai et al. (2005)
N-mineralization	Soils amended with biomass of *Bt* and non-*Bt* maize	Cry1Ab	No effect on decomposition processes	Cortet et al. (2006)
N mineralization potential and nitrification and respiration rates	Soil with *Bt* and non-*Bt* maize	Cry3Bb1	No adverse effects of *Bt* maize on N mineralization potential and on nitrification and respiration rates	Devare et al. (2007)
Chemical composition	*Bt* and non-*Bt* maize	Cry1Ab	*Bt* maize had higher content of starch and lignin and lower content of protein and soluble N than non-*Bt* maize	Masoero et al. (1999)
Chemical composition	*Bt* and non-*Bt* maize	Cry1Ab	No differences in chemical composition of *Bt* (event Bt11) and isogenic non-*Bt* maize tissues	Folmer et al. (2002)

Table 4 (*continued*)

Process/function	Experimental variable	Protein	Effect	References
Chemical composition	*Bt* and non-*Bt* maize	Cry1Ab	No consistent differences	Mungai et al. (2005)
C:N ratio, lignin, carbohydrates	*Bt* and non-*Bt* maize	Cry1Ab	Lower C:N ratio and lignin content and higher content of soluble carbohydrates in *Bt* maize (event Bt11) than in non-*Bt* maize	Escher et al. (2000)
Lignin content	*Bt* and non-Bt maize	Cry1Ab	Higher lignin content in *Bt* maize (events Bt11, MON810, 176) than in near-isogenic non-*Bt* maize	Saxena and Stotzky (2001a)
Lignin content	*Bt* and non-*Bt* maize	Cry1Ab	No differences in lignin content of 12 *Bt* maize hybrids (events MON810 and Bt11) and isogenic non-*Bt* maize	Jung and Sheaffer (2004)
Lignin content	*Bt* and non-*Bt* maize	Cry1Ab	Higher lignin content in *Bt* maize than in non-*Bt* maize; *Bt* maize decomposed less in soil than non-*Bt* maize	Flores et al. (2005)
Lignin content	*Bt* and non-*Bt* maize	Cry1Ab	Higher content of lignin in *Bt* maize (events MON810 and 176) than in near-isogenic non-*Bt* maize	Poerschmann et al. (2005)
Lignin content	*Bt* and non-*Bt* maize	Cry1Ab	No significant differences between *Bt* (events MON810 and 176) and near-isogenic non-*Bt* maize	Lang et al. (2006)

The variations in plant composition reported may be a result of differences in the analytic methodologies used (Escher et al., 2000; Saxena and Stotzky, 2001a; Folmer et al., 2002; Jung and Sheaffer, 2004; Poerschmann et al., 2005; Lang et al., 2006), the age of the maize material at the time of analysis, and the *Bt* transformation event in the transgenic maize varieties tested. In general, high lignin content decreases the rate of decomposition of crop residues (Parton et al., 1996; Hopkins et al., 2001) and, thus, may affect the residue-associated microbial populations. Moreover, net N mineralization occurs if the C:N ratio is less than 30, and net N immobilization occurs if the ratio is greater than 30 (Wagner and Wolf, 1999; Trinsoutrot et al., 2000). Therefore, the lower or higher lignin content observed in some *Bt* crops may act to increase or reduce rates of residue decomposition and mineralization of organic N contained in the residues. In addition, composition of the residues and properties of the soil may interact with the effects of tillage in soil. However, incorporation of *Bt* residues did not significantly affect the level of inorganic N in soil under either till or no-till conditions, and *Bt* and non-*Bt* maize residues did not differ significantly in their effects on N dynamics in both laboratory and field studies (Mungai et al., 2005). Devare et al. (2004) reported no differences in the potential for anaerobic N-mineralization in soils collected from fields planted with either *Bt* maize expressing the Cry3Bb1 protein or near-isogenic non-*Bt* maize. In a continuation of this study, a 3-year field assessment of *Bt* maize expressing the Cry3Bb1 protein vs. near-isogenic non-*Bt* maize grown with and without the insecticide, Tefluthrin, demonstrated that neither the *Bt* maize nor the insecticide had adverse effects on microbial biomass, N mineralization potential, or rates of nitrification and respiration. Rhizosphere effects on and seasonal changes in the measured parameters were consistently observed throughout the study, indicating that their influence on microbial biomass and activity was probably greater than any subtle effects resulting from crop variety and treatment (Devare et al., 2007). There was only a small difference in the rate of decomposition, determined using the litterbag technique, between leaf residues of *Bt* and non-*Bt* cotton varieties during an 8-week incubation experiment (Gupta and Watson, 2004). Cortet et al. (2006) reported that decomposition and mineralization were mainly influenced by climatic conditions with no negative effect of the Cry1Ab protein on decomposition processes after 4 months of incubation in the field (Table 4).

Soil properties also influence N mineralization directly or indirectly. For example, different clays can alter the mineralization of N by binding organic matter to form soil aggregates that physically protect soil N from heterotrophic soil organisms (e.g., Stotzky, 1986; Christensen, 1996). Soil texture may indirectly alter factors, such as the availability of soil water, pore size distribution, nutrient availability, and specific surface area that influence conditions for organisms involved in N mineralization. Management practices, such as tillage, can cause a substantial decrease in soil organic matter content and N mineralization, leading to a lower soil content of inorganic N (Six et al., 1999). Tillage tends to promote faster release of N from plant residues by mixing and incorporating crop residues in soil compared with surface-placed residues in no-till systems (Varco et al., 1993). No results have been presented that *Bt* crops significantly stimulate or suppress transformations of soil nutrients in field environments (Motavalli et al., 2004).

4.5. Persistence and fate of Cry proteins in soil

The increasing use of *Bt* crops could lead to an increase in Cry proteins in soil. *Bt* maize, as well as *Bt* rice and potato, release Cry proteins to soil in root exudates throughout the growth of the plant (Saxena et al., 1999, 2004; Saxena and Stotzky, 2000; Icoz and Stotzky, 2007). Cry proteins are also released in pollen during tasseling (e.g., Losey et al., 1999; Obrycki et al., 2001) and from crop residues after harvest (e.g., Zwahlen et al., 2003a; Stotzky, 2002, 2004). In addition, "remarkable" amounts of Cry protein are found in the gastrointestinal tract of cows and in their feces (Einspanier et al., 2004), as well as in the feces of decomposers, such as woodlice (Pont and Nentwig, 2005) and earthworms (e.g., Saxena and Stotzky, 2001b). Moreover, Cry proteins from *B. thuringiensis* subsp. *kurstaki* (active against Lepidoptera), subsp. *morrisoni* (strain *tenebrionis*) and subsp. *kumamotoensis* (active against Coleoptera), and subsp. *israelensis* (active against Diptera) are rapidly adsorbed and bound on clay minerals and humic substances, which renders the proteins resistant to biodegradation but with retention of larvicidal activity. This results in continual exposure of soil organisms to these proteins (e.g., Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995a, b, 1998; Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Lee et al., 2003; Stotzky, 2000, 2002, 2004; Icoz and Stotzky, 2007; Fiorito et al., 2007). Binding of Cry proteins on soil components indicates that there is the potential for their long-term persistence and, thereby, longer exposure of target and nontarget organisms to them in soil. The Cry1Ab protein released in root exudates of *Bt* maize persisted in soil microcosms for at least 180 day and for at least 3 years from biomass of *Bt* maize, the longest times studied (Saxena and Stotzky, 2002; Stotzky, 2002, 2004). Zwahlen et al. (2003a) reported that the Cry1Ab protein in *Bt* maize litter persisted in soil *in situ* for at least 8 months. However, conflicting results have been reported about the persistence of Cry proteins in soil (Table 5). Several laboratory studies in soil microcosms have suggested that Cry proteins do not persist and degrade rapidly in soil. For example, the proteins from *B*t maize, *Bt* cotton, and *Bt* potato (Cry1Ab, Cry1Ac, and Cry3Aa protein, respectively) did not persist and were generally degraded in soil with a "half-life" of 20 d or less (Ream et al., 1994; Palm et al., 1996; Sims and Ream, 1997). Sims and Holden (1996) reported a 50% decrease (half-life) in the insecticidal activity of the Cry1Ab protein in 1.6 d and a 90% decrease in 15 d in soil and suggested that the Cry1Ab protein in maize plant tissue is unstable under field conditions and likely to degrade rapidly under normal cultivation practices. Donegan et al. (1995) reported that the purified protein from *B. thuringiensis* subsp. *kurstaki* and the Cry1Ac protein from *Bt* cotton persisted at detectable levels for up to 28 and 56 d in soil, respectively, as measured by ELISA. Under field conditions and in a laboratory soil microcosm, Sims and Ream (1997) estimated the 50% decrease of the Cry2A protein from biomass of *Bt* cotton to be 15.5 and 31.7 d for the laboratory and field conditions, respectively, by an insect bioassay. In both environments, approximately 25% of the initial bioactivity of the Cry2A protein remained after 120 d in soil. Palm et al. (1996) found that 10–40% of the Cry1Ac protein remained at the end of a 28-d period. Wang et al. (2006) reported that the Cry1Ab protein from biomass of *Bt* rice degraded with a half-life of 11.5 d in an alkaline soil and a half-life of 34.3 d in an acidic soil. No detectable Cry3Bb1 protein was found under laboratory conditions after 21 d in montmorillonite-amended and after 40 d in kaolinite-amended soil to which different amount of *Bt* maize residues expressing the Cry3Bb1 protein had been added (Icoz and Stotzky, 2007). Field studies on the persistence of Cry proteins released by *Bt* plants were generally in good agreement with laboratory studies and also showed that Cry proteins do not persist and degrade rapidly in soil. For example, Head et al. (2002) found no detectable levels of Cry1Ac protein by ELISA and insect bioassay in soils collected from fields on which transgenic *Bt* cotton that had been grown and the biomass incorporated into soil for 3–6 consecutive years. Hopkins and Gregorich (2003) reported that much of the *Bt* δ-endotoxin in maize 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 the Cry1Ab protein in soils from fields planted for at least 3 consecutive years with *Bt* maize. Ahmad et al. (2005) found no detectable Cry3Bb1 protein in soil planted with *Bt* maize for 3 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. No detectable Cry3Bb1 protein was found in rhizosphere soils of *Bt* maize expressing the Cry3Bb1 protein over 4 consecutive years of *Bt* maize cultivation (Icoz et al., 2007) (Table 5).

However, Tapp and Stotzky (1995a, b, 1998) showed in soil microcosms that the insecticidal activity of Cry proteins was retained as the result of their adsorption and binding on clay particles, which protected the proteins from microbial degradation, for at least 234 d. Zwahlen et al. (2003a) found, in field studies, that the Cry1Ab protein in maize tissue was stable and degraded only as the plant material degraded, and the Cry1Ab protein could still be detected after 240 and 200 d under tillage and no-tillage, respectively, even when only small amounts of plant material remained. Muchaonyerwa et al. (2004) reported that the Cry1Ab protein from biomass of *Bt* maize persisted in Zimbabwean soils for several weeks without losing insecticidal properties. Wang et al. (2006) reported that the Cry1Ab protein from biomass of *Bt* rice was still detectable in acidic soils after 120 d of incubation. In contrast to the Cry3Bb1 protein, Icoz et al. (2007) found that the Cry1Ab protein was still detectable in rhizosphere

Table 5
Summary of persistence of Cry proteins in soil

Protein	Study location	Experimental variable	Persistence of proteins in soil	References
Cry1Ab Cry1Ac Cry3Aa	Laboratory	Soil amended with biomass of *Bt* maize, cotton, and potato	No persistence of proteins in soil; proteins degraded in soil with a half-life of 20 d	Ream et al. (1994)
Cry1Ab Cry1Ac	Laboratory	Soil amended with purified protein or biomass of *Bt* cotton	Purified proteins and Cry proteins from cotton tissue decreased rapidly, with a half-life of approximately 4 and 7 d, respectively, by ELISA	Palm et al. (1996)
Cry1Ab Cry1Ac	Laboratory	Soils amended with purified protein or biomass of *Bt* cotton	Purified protein was detected up to 28 d, and the protein from *Bt* cotton was detected up to 56 d	Donegan et al. (1995)
Cry1Ab	Laboratory	Soil amended with purified protein	Protein still detectable in soil after 234 d by larvicidal assay	Tapp and Stotzky (1998)
Cry1Ab	Laboratory	Soil amended with biomass of *Bt* maize	50% decrease (half-life) in the insecticidal activity of Cry1Ab protein in 1.6 d and a 90% decrease in 15 d	Sims and Holden (1996)
Cry2A	Laboratory	Soil amended with biomass of *Bt* cotton	Half-life of bioactivity was estimated at 15.5 d by insect bioassay	Sims and Ream (1997)
	Field	*Bt* cotton cultivation	Half-life of bioactivity was estimated at 31.7 d by insect bioassay	
Cry1Ab	Laboratory	Soil with *Bt* maize or amended with biomass of *Bt* maize	Cry1Ab protein from root exudates and in plant biomass persisted for at least 180 and 350 d, respectively, in soil	Saxena and Stotzky (2002)
Cry1Ab	Laboratory Field	Soil amended with biomass of *Bt* maize or cultivation of *Bt* maize for 4 y	No persistence of protein in soil	Hopkins and Gregorich (2003)
Cry1Ab	Laboratory	Soils amended with biomass of *Bt* maize	Protein persisted for several weeks	Muchaonyerwa et al. (2004)
Cry3Bb1	Laboratory	Soils amended with biomass of *Bt* maize	Protein was detected only 21 d in soils amended with montmorillonite and 40 d in soils amended with kaolinite (K); after adjustment of pH of the K soils to *ca.* 7, protein was detected for only 21 d	Icoz and Stotzky (2007)
Cry1Ac	Field	*Bt* cotton cultivation	No detectable level of protein in soil for 3–6 consecutive years	Head et al. (2002)
Cry1Ab	Field	*Bt* maize cultivation	No persistence for 3 years	Dubelman et al. (2005)
Cry3Bb1	Field	*Bt* maize cultivation	No detectable level of protein in soil during 3 consecutive years	Ahmad et al. (2005)
Cry1Ab	Field	*Bt* maize cultivation	Protein detected in soils during 4 consecutive years	Icoz et al. (2007)
Cry3Bb1	Field	*Bt* maize cultivation	No protein detected in soils during 4 consecutive years	
Cry1Ab	Field	*Bt* maize cultivation	Protein in *Bt* maize litter persisted at least 8 months	Zwahlen et al. (2003a, b)
Cry1Ab	Field	*Bt* maize cultivation	Protein persisted through winter but no accumulation	Baumgarte and Tebbe (2005)
Cry1Ab	Laboratory Field	Soils amended with biomass of *Bt* rice and *Bt* rice cultivation	The half-lives of the protein in soils amended with *Bt* rice straw (4%, ww^{-1}) was estimated at 11.5 for alkaline soils and 34.3 d for acidic soils	Wang et al. (2006)

soil of *Bt* maize expressing the Cry1Ab protein over 4 consecutive years of *Bt* maize cultivation under field conditions (Table 5).

The variable degradation times for Cry proteins in soil might be the result of the different crops and Cry proteins used and of ecological factors, such as the level of nutrients, temperature, pH, and types and amount of clay minerals and organic matter present in the soil. For example, some of the variations in decomposition rates found by Zwahlen et al. (2003a) and Head et al. (2002) could be explained by differences in the C:N ratio between crop species, which affects decomposition rates and resulted in a two- to three-fold faster rate of degradation of residues of *Bt* cotton, which had a narrower C:N ratio, than of *Bt* maize (Sanvido et al., 2006). However, Flores et al. (2005) found that differences in C:N ratio did not explain the lower biodegradation of *Bt* maize biomass than of isogenic non-*Bt* maize biomass. The variations could

also be explained by the binding of Cry proteins on surface-active particles (Venkateswerlu and Stotzky, 1992; Tapp and Stotzky, 1998). The binding of Cry proteins on clays and humic acids reduced their availability to microbes, which is probably responsible for the persistence of the proteins in soil (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Lee et al., 2003). Temperature also influences biodegradation: e.g., an increase of 10 °C in soil temperature can result in a two- to three-fold increase in microbial activity and, therefore, in higher rates of decomposition (Pont and Nentwig, 2005). Insecticidal activity of the Cry1Ab protein in *Bt* maize residues was detectable in soil for several months after the first frost, which was probably the result of a low rate of microbial degradation at the reduced temperatures (Saxena and Stotzky, 2000).

The persistence and biodegradation of Cry proteins, as well as of other proteins, peptides, amino acids, DNA, viruses, and other biomolecules, in soil depends largely on the level of microbial activity (e.g., Stotzky, 1986; Dashman and Stotzky, 1986; Lipson and Stotzky, 1985, 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, 1998, 2001; Tapp and Stotzky, 1998). Many properties of soil, including microbial activity, depend on the type and amount of clay minerals present and on the pH (*see* Stotzky, 1986, and references therein). The growth and metabolic activity (as measured respirometrically) of bacteria are higher in soils that contain montmorillonite (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). For example, the Cry3Bb1 protein from *Bt* maize was detected in soil amended with kaolinite (K) for *ca.* 40 day, whereas it was detected in soil amended with M for only 21 day, regardless of the amount (1%, 3%, 5%, and 10%) of *Bt* biomass added (Icoz and Stotzky, 2007). 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). Tapp and Stotzky (1998) observed a greater reduction in the insecticidal activity of the Cry1Ab protein added to nonsterile soils during 234 d of incubation when the pH of the soils was either initially near neutrality 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, which resulted in more rapid biodegradation of the protein (Tapp and Stotzky, 1998). When the pH of the K soils in which the Cry3Bb1 protein persisted for 40 d was adjusted to *ca.* 7, the protein persisted for only 21 day, indicating that the decrease in protein in the pH-adjusted K soils, as well as in the M soils, was a result of the more rapid biodegradation of the protein at the higher pH values (Icoz and Stotzky, 2007). In addition to the increase in microbial activity in soils with a pH near neutrality, adsorption of the Cry1Ab, Cry3Aa, and Cry4 proteins on clays decreased with an increase in pH to above their isoelectric point (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1998; Vettori et al., 1999; Crecchio and Stotzky, 2001; Lee et al., 2003), which also rendered the proteins more susceptible to biodegradation. Similarly, Wang et al. (2006) reported that the Cry1Ab protein from biomass of *Bt* rice degraded faster in alkaline soil (half-life of 11.5 d) than in acidic soil (half-life of 34.3 d). 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, 1998, 2001). The importance of pH and other physicochemical, as well as biological, characteristics of soil for the persistence of Cry proteins in soil needs to be determined in greater detail, especially to explain the reported differences in the persistence of the proteins in soil.

Repeated and large-scale use of *Bt* crop plants could also lead to accumulation and persistence of plant-produced Cry proteins in aquatic environments. The *cry1Ab* gene derived from fields of *Bt* maize was found in nearby streams and rivers and sometimes several kilometers downstream, indicating contamination by agricultural transgenic DNA (Douville et al., 2007). However, the Cry1Ab protein was degraded more rapidly in water than in soil. The *cry1Ab* gene from *Bt* maize persisted for more than 21 and 40 d in surface water and sediment, respectively. In general, the Cry1Ab protein was not commonly found in aquatic environments and was present in only trace concentrations when it was detected (Douville et al., 2005, 2007).

5. Conclusions and future directions

Concern about *Bt* plants is that increased levels of insecticidal Cry proteins in soil could damage beneficial organisms, such as earthworms, collembolans, nematodes, and microorganisms, necessary for plant and soil health, litter decomposition, and nutrient cycling. In general, the Cry proteins released in root exudates and from plant residues of *Bt* crops appear to have no consistent, significant, and long-term effects on the microbiota and their activities in soil. Some differences in total numbers and community structure of microorganisms in soil between *Bt* and non-*Bt* crops have been indicated. However, many of these observations were not statistically significant, were transient, were not related to the inserted transgene, or were the result of altered plant characteristics (e.g., lignin content). Although Cry proteins bind rapidly on clays and humic substances, there is little evidence for the accumulation of the proteins in soils in the field, even after years of continuous cultivation of *Bt* crops. However,

a few studies have reported significant differences in microbial community structure in soils with *Bt* and non-*Bt* crops using various classical and molecular techniques. These contradictory results of the effects of *Bt* plants on microbes may be the result of differences in the type of Cry protein, plant variety, and experimental methods used, as well as in soil type and environmental factors. The species and functional diversity of microbial communities in soil is influenced by numerous direct and indirect environmental factors. Direct effects depend on both the spectrum of activity of the proteins encoded by the transgenes (Oger et al., 1997) and the quantities of the proteins that accumulate in the environment. In contrast, indirect effects are mediated by changes in the chemical composition of plant biomass and root exudates that result from modifications in the normal metabolic pathways in plant tissues. The direct effects are probably of primary concern, as the introduction of transgenes for resistance to pests involves the production of chemical substances that are potentially toxic to nontarget and beneficial soil organisms. Indirect effects caused by changes in the chemical composition are also possible consequences of transgenic plants, but they are more difficult to evaluate, as many factors affect the composition of plant tissue, root exudates, and microbial community structure in soil (Liu et al., 2005).

The results of the studies discussed in this review can be summarized as follows:

- Although only a few studies (e.g., Ahl Goy et al., 1995; Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Clark and Coats, 2006; Lang et al., 2006; Vercesi et al., 2006) have investigated the effects of Cry proteins on earthworms (*L. terrestris*, *E. fetida*, and *A. caliginosa*), all showed that the Cry1Ab protein had no significant effects on their survival, growth, and reproduction, even though the protein was detected in the gut and feces of the earthworms, indicating that the protein was ingested by the worms.
- No toxic effects of Cry proteins on woodlice, collembolans, and mites have been reported.
- In general, few negative effects of Cry proteins from *Bt* crops on populations of soil nematodes have been reported. However, only a few studies have investigated the population dynamics of individual nematode species, and some have indicated that *C. elegans* showed some sensitivity to the Cry1Ab protein from *Bt* maize, in that growth and reproduction were significantly affected by the presence of the protein in soil (e.g., Manachini and Lozzia, 2003; Manachini et al., 2004; Griffiths et al., 2005, 2006; Lang et al., 2006).
- No toxic effects of the Cry proteins on protozoa have been observed (e.g., Donegan et al., 1995; Saxena and Stotzky, 2001b; Griffiths et al., 2006; Icoz et al., 2007), except in a study by Griffiths et al. (2005), who reported a significant, but transient, decrease in the numbers of protozoa in soil with *Bt* maize under field conditions. However, studies in the greenhouse showed significantly higher numbers of protozoa in soils with *Bt* than with non-*Bt* maize (Griffiths et al., 2006).
- Different effects, ranging from no effects to minor and significant effects, of *Bt* plants on microbial communities in soil have been reported, but they were mostly the result of differences in geography, temperature, plant variety, and soil type. In general, differences in microbial community structure were transient and not related to the presence of the Cry proteins. Only one study found consistent significant differences between soils with *Bt* and non-*Bt* maize (Castaldini et al., 2005).
- The rhizosphere bacterial community of *Bt* and non-*Bt* plants were characterized using several techniques, including viable counts, DGGE, CLCP, CLPP, PLFA, ARISA, and T-RFLP. The culturing techniques did not detect any differences in the soil microbiota between soils with *Bt* and non-*Bt* plants, but some molecular techniques indicated that the community structure differed in soils with *Bt* and non-*Bt* plants. Root exudates of *Bt* plants resulted in the development of bacterial communities in soil that differed from those associated with exudates of near-isogenic non-*Bt* plants. However, it was suggested that the exudates of *Bt* plants differ from those of non-*Bt* plants in several ways, not only in the content of the Cry protein (Brusetti et al., 2004).
- Fungi appear to be the organisms most affected by Cry proteins in soil. The roots of *Bt* maize (event 176) were less colonized with mycorrhizae than their non-*Bt* near-isogenic counterpart (Turrini et al., 2004; Castaldini et al., 2005), and, therefore, *Bt* maize may not only lose an important symbiont that contributes to plant nutrition, but the plants might be even more susceptible to insect pests because without mycorrhizae, maize attracts fewer natural enemies of the pests. These studies found lower mycorrhizal infection for event 176 but not for event Bt11 compared with their respective near-isogenic non-*Bt* counterparts.
- No significant inhibitory effects of Cry proteins from *Bt* plants on the activity of some enzymes (e.g., arginine deaminase, arylsulfatases, dehydrogenases, dimethylsulphoxide reductase, catalase, β-glucosidase, phosphatases, proteases, urease) have been reported.
- The evolution of CO_2 from soils amended with biomass of *Bt* maize or cultivated with *Bt* maize was generally lower than that from soils amended with biomass of or cultivated with the respective non-*Bt* isolines (e.g., Dinel et al., 2003; Devare et al., 2004; Flores et al., 2005; Castaldini et al., 2005).
- The chemical composition (e.g., the content of starch, soluble N, proteins, carbohydrates, lignin) of *Bt* maize hybrids was sometimes different from that of their isogenic non-*Bt* counterparts. For example, higher lignin content (Masoero et al., 1999; Saxena and Stotzky, 2001a; Flores et al., 2005; Poerschmann et al., 2005), lower lignin content (Escher et al., 2000), and no differences in lignin content (Folmer et al., 2002; Jung

and Sheaffer, 2004; Mungai et al., 2005; Lang et al., 2006) have been reported. However, one study reported a slower degradation of *Bt* plants (canola, cotton, maize, potato, rice, and tobacco) in soil (Flores et al., 2005), presumably because of a higher lignin content in *Bt* plants, whereas other studies showed no differences in decomposition or N-mineralization between *Bt* and non-*Bt* plants.

- Laboratory and field studies have shown differences in the persistence of Cry proteins in soil. The degradation and persistence of Cry proteins in soil depend largely on microbial activity, which is affected by soil type, pH, temperature, and other physicochemical and biological characteristics of soil. For example, the proteins appear to persist longer in acidic soil, as a result of decreased microbial activity at the lower pH (Tapp and Stotzky, 1998; Wang et al., 2006). The degradation and persistence of Cry proteins in soil also depend on the type and amount of Cry proteins released, the crop species (e.g., chemical composition and differences in C:N ratio), crop management practices (e.g., till vs. no-till with roots remaining in the soil), and other factors that vary with location and climate zones. Moreover, the production of Cry proteins in *Bt* plants varies with season and with parts of the plant and can be influenced by numerous environmental factors (e.g., Dutton et al., 2004; Jehle, 2007), which emphasizes the importance of doing studies on the fate and effects of Cry proteins in *Bt* plants under local climatic conditions and with local varieties (Jehle, 2007).

Soils are home to a diverse range of life and are complex and dynamic biological systems (e.g., Stotzky, 1997). Therefore, it is often difficult to determine the composition of microbial communities in soil and their response to perturbations of this ecosystem. However, any changes, even small, in the composition of the microbial community should be considered as an early warning indicator for risk assessment. Although recent methodological advances, especially molecular techniques, are helping to understand soil communities, many aspects of these communities are still not sufficiently understood (Kowalchuk et al., 2003). Moreover, the use of a wide variety of techniques has, paradoxically, often made comparisons among and between studies difficult (Bruinsma et al., 2003). In spite of gaps in the understanding of plant–soil systems, current knowledge suggests that some microbial groups can be indicators for effects induced by GM plants, and several have now become accessible with the advent of culture-independent, molecular ecological methods. Because it is not possible to monitor all components of a soil ecosystem for their response to a GM crop, the identification of some important microbial groups and their activities as indicators is crucial (Kowalchuk et al., 2003), as trends in such indicators measured regularly can provide clues about the response of the soil ecosystem to GM plants. These indicators should be chosen for their agronomic relevance, ecological significance, and responsiveness to perturbations, as well as on the basis of the availability of practical assays. Some keystone indicators have been suggested (e.g., Lottmann et al., 1999; Nannipieri et al., 2003; Bruinsma et al., 2003; Kowalchuk et al., 2003; O'Callaghan et al., 2005). For example, Kowalchuk et al. (2003) and Bruinsma et al. (2003) proposed a case-by-case approach within a framework that targets both indicators potentially vulnerable to Cry proteins and general community parameters for assessing the impact of GM plants on soil microorganisms. The vulnerable groups suggested were arbuscular mycorrhizal fungi, symbiotic N_2-fixing bacteria, antagonists of plant pathogens (e.g., pseudomonads and *Trichoderma*), wood-decaying fungi, and nitrifying bacteria. The general community parameters included evaluation of microbial groups, such as bacterial counts or total microbial biomass, and activity-based assays, such as respiration, N-mineralization, general soil suppressiveness, substrate-induced respiration, enzymatic potential, community-level physiological profiles, molecular fingerprinting (e.g., DGGE), and phospholipid fatty acid analysis. Although the general parameters provide additional information about changes in community structure, they do not identify affected groups, and, therefore, effects detected using general community parameters should be studied in more detail to assess their significance (Kowalchuk et al., 2003).

In addition to indicators, it is important to compare protective GM crop systems to other crop protection systems, such as current insecticides, to estimate whether GM crops pose a direct threat to soil ecosystems. A comprehensive review by O'Callaghan et al. (2005) on the effects of plants genetically modified for insect resistance on nontarget organisms suggested that studies should also include unrelated plant cultivars as an additional control for comparison, as many studies have found that even when differences between GM and unmodified near-isogenic plant lines were observed, these were generally not greater than those observed between different cultivars. The extent of exposure of nontarget organisms to insecticidal proteins depends on the persistence of the proteins in soil, and methods, such as immunological detection of the proteins, should be coupled with bioassays that provide information on the ecological impact of any protein remaining in soil (O'Callaghan et al., 2005; Icoz et al., 2007). In addition, key ecological processes in soil, such as respiration (e.g., CO_2 evolution) and the activity of some specific groups of decomposers (e.g., lignin- or cellulose-degrading organisms), should be evaluated. Moreover, because of the ecological significance of population shifts (e.g., significant differences in community structure resulting from seasonal changes), studies are necessary at the field level, on a large scale, and over a sufficiently long period to account for environmental variability.

To assess better any ecological risks of GM crops on soil ecosystems, it is also important to know how realistic the experimental and statistical protocols are. Therefore, the pattern, duration, and extent (dosage) of exposure must at

least equal those experienced by the test organisms in nature (Marvier, 2002). Often, species chosen for risk assessment are those that are easily maintained in laboratory culture, and these are not always representative of the environment in which the GM plants will be grown (O'Callaghan et al., 2005). Marvier (2002) suggested that a few simple improvements in experimental design could greatly increase the rigor and the information content of, especially, field studies. For example, many experiments are conducted for short periods, although some of the species tested can live a year or more and could experience much longer periods of exposure. Because small-scale field experiments are generally not sensitive enough to detect anything other than large and obvious effects (NRC, 2002), assessment of risks to biological diversity will need to be conducted on a long-term, large-scale basis. Moreover, the number of replicates used in most studies is generally small (usually 2–6 replicates treatment^{-1}), which results in studies that have a limited chance of detecting real effects, and indicates that sample size and replication should be increased and that nonsignificant results should be accompanied by an analysis of statistical power (Marvier, 2002).

Biotechnology represents a means for enhancing genetic diversity in crop species through the introduction of novel genes. The use of GM crops can positively impact agriculture if the GM crops enable the management of weeds and insect pests more specifically than chemical herbicides and pesticides. In particular, the use of insect-resistant *Bt* crops, expressing highly specific *Bt* proteins, represents an opportunity to replace the use of broad-spectrum insecticides. Concerns related to the risks of GM crops, especially *Bt* crops, to the environment have been extensively assessed worldwide during the past 11 or so years of commercial cultivation of *Bt* crops. Consequently, considerable data on the environmental effects of these *Bt* crops are available. These data have provided no scientific evidence that cultivation of *Bt* crops has caused sustained environmental harm to below-ground microbial and invertebrate communities. Moreover, these data have suggested that the effects resulting from the cultivation of *Bt* crops fall within the normal variation expected in agricultural systems and that they are not as large as those resulting from growing different (conventional) maize cultivars and other crops or from natural differences between sites or times of sampling. However, the lack of evidence of negative effects of *Bt* crops does not mean that other GM plants are without risk. Moreover, the possibility of long-term effects of *Bt* crops cannot be excluded and must be examined on a case-by-case basis, especially as a number of issues related to the interpretation of the scientific data on the effects of *Bt* crops on the environment are still controversially debated.

Among the questions that still need to be addressed is whether the cultivation of *Bt* crops affects the yield of subsequent crops, especially non-*Bt* crops, grown on the same soils on which *Bt* crops have been grown. If effects on subsequent crops are observed, the duration (e.g., number of seasons) of the effects would be of practical interest. Although there appear to be no significant, long-term, detrimental effects of *Bt* plants on below-ground organisms, the potential impact of *Bt* plants on nontarget above-ground organisms (e.g., predators, parasitoids, pollinators, butterflies, herbivores) still remains controversial, as does gene flow from *Bt* crops to compatible local crops and wild relatives.

Because most studies have generally indicated few or no significant detrimental effects on microbes and other organisms in below-ground soil ecosystems, more studies on the risks associated with *Bt* plants, at least those currently available, to these organisms are probably not indicated. The time and money would be better spent on studies of the potential risks associated with the release of transgenic plants genetically engineered to express pharmaceutical and industrial products that, in contrast to Cry proteins, are targeted primarily to human beings and other higher eukaryotic organisms (e.g., Marvier, 2007; Fiorito et al., 2007; Sabharwal et al., 2007; Stotzky and Saxena, 2007).

Acknowledgments

Preparation of this review was supported, in part, by grants from the US Department of Agriculture (USDA) and the New York University Research Challenge Fund (NYURCF). The opinions expressed herein are not necessarily those of the USDA or the NYURCF. We thank two anonymous referees and Chief Editor J.S. Waid for helpful comments.

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