

Insect-resistant transgenic plants in a multi-trophic context

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Summary

So far, genetic engineering of plants in the context of insect pest control has involved insertion of genes that code for toxins, and may be characterized as the incorporation of biopesticides into classical plant breeding. In the context of pesticide usage in pest control, natural enemies of herbivores have received increasing attention, because carnivorous arthropods are an important component of insect pest control. However, in plant breeding programmes, natural enemies of herbivores have largely been ignored, although there are many examples that show that plant breeding affects the effectiveness of biological control. Negative influences of modified plant characteristics on carnivorous arthropods may induce population growth of new, even more harmful pest species that had no pest status prior to the pesticide treatment. Sustainable pest management will only be possible when negative effects on non-target, beneficial arthropods are minimized. In this review, we summarize the effects of insect-resistant crops and insect-resistant transgenic crops, especially Bt crops, from a food web perspective. As food web components, we distinguish target herbivores, non-target herbivores, pollinators, parasitoids and predators. Below-ground organisms such as Collembola, nematodes and earthworms should also be included in risk assessment studies, but have received little attention. The toxins produced in Bt plants retain their toxicity when bound to the soil, so accumulation of these toxins is likely to occur. Earthworms ingest the bound toxins but are not affected by them. However, earthworms may function as intermediaries through which the toxins are passed on to other trophic levels. In studies where effects of insect-resistant (Bt) plants on natural enemies were considered, positive, negative and no effects have been found. So far, most studies have concentrated on natural enemies of target herbivores. However, Bt toxins are structurally rearranged when they bind to midgut receptors, so that they are likely to lose their toxicity inside target herbivores. What happens to the toxins in non-target herbivores, and whether these herbivores may act as intermediaries through which the toxins may be passed on to the natural enemies, remains to be studied.

Keywords: Bt, host plant resistance, biological control, food web, ecology, predators, parasitoids

Introduction

Agro-ecosystems consist of organisms that interact in so-called food webs (Dicke and Vet, 1999; Janssen *et al.*, 1998; Mayse and Price, 1978; Poppy, 1997; Price, 1981; Price, 1997; Price *et al.*, 1980; Weires and Chiang, 1973) (Figure 1). Herbivores and carnivores may compete for the same food sources or may partition resources through niche segregation. While lepidopterans are usually economically important pests, insects from other orders such as aphids and thrips or mites may also be important pests.

Potential pest arthropods may be naturally suppressed by their natural enemies in agro-ecosystems, or biological control agents may be specifically introduced to control pest arthropods. Biological control is an important component of durable crop protection programmes (DeBach, 1974; Lewis *et al.*, 1998; Van Lenteren, 1993). Hampering the effects of carnivorous arthropods as a side-effect of pest control methods may induce new pests (DeBach and Rosen, 1991; Van Lenteren, 1993) and should

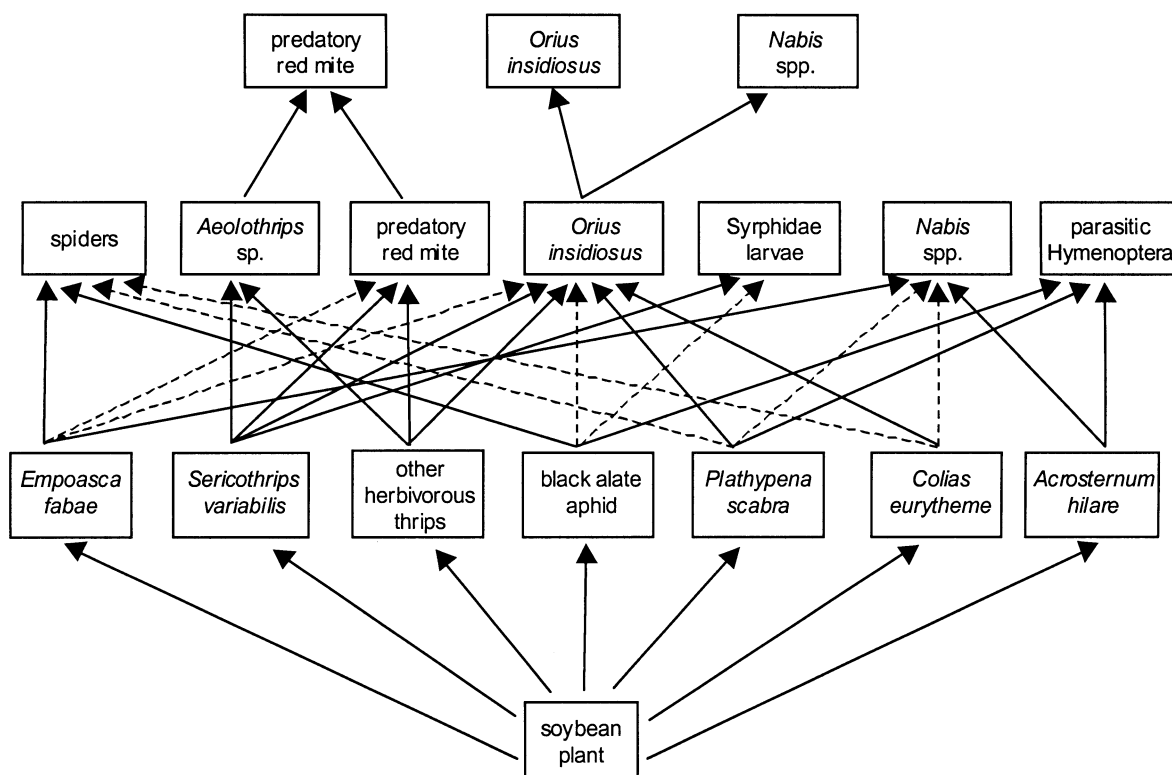


Figure 1 Food web in a soybean agro-ecosystem in Illinois, adapted from Mayse and Price (1978).

therefore be avoided. Natural enemies of herbivores commonly live and/or search for herbivores on plants. Consequently, plant characteristics can affect the behaviour, physiology and ecology of carnivorous arthropods.

Crop protection may be achieved by host plant resistance and biological control. Conventional plant breeding and biological control have been developed independently, but they may interact in various ways (Hare, 1992). When aiming for durable crop protection, one should address these interactions (Dicke, 1999; Hare, 1992; Schuler *et al.*, 1999c). With the development of genetic engineering technology, a whole new array of possibilities has been made available to develop pest-resistant plants, i.e. the introduction of genetic material from any species into crop plants (Duck and Evola, 1997). In the context of insect pest control, this technology has been applied by inserting genes that code for toxins. The δ -endotoxins produced by *Bacillus thuringiensis* (Bt) are the best known example, and genes encoding these endotoxins have been transferred to major crops (Jenkins, 1999; Merritt, 1998). Although genes from other groups of organisms and plants are currently being explored for their potential use in developing pest-resistant plants, so far commercialized insect-resistant transgenic plants only contain genes coding for (an activated form of) a Bt toxin. Consequently, this review is focused on transgenic Bt plants.

When insect-resistant transgenic plants are evaluated, possible effects of host plant resistance on biological control should be considered in detail. The toxins that insect-resistant transgenic plants produce may have similar effects on carnivores as toxins that are naturally produced in a plant. Therefore, this review includes the effects of plant allelochemicals on biological control agents. This review does not include (possible) interactions with microbial organisms.

Conventional host plant resistance

Secondary plant metabolites (allelochemicals) that are part of natural plant defence have been used in breeding programmes to develop cultivars that produce larger quantities to protect the plants from herbivorous arthropods (Panda and Khush, 1995; Smith *et al.*, 1994). Generalist herbivores usually cannot detoxify or sequester specific secondary plant compounds (Barbosa, 1988; Dicke, 1999). Specialist herbivores may overcome the natural plant defence by sequestration of the secondary plant metabolites and use them for their own defence against natural enemies (Barbosa, 1988; Duffey and Scudder, 1972; Ferguson and Metcalf, 1985; Krieger *et al.*, 1971; Pasteels and Daloz, 1977; Pasteels *et al.*, 1986). When very large amounts of allelochemicals are produced

by a new cultivar, specialist herbivores may be negatively affected by these compounds as well. Also, these allelochemicals may be passed on to biological control agents either directly or indirectly, in the same way as Bt toxins in transgenic plants, and (positively or negatively) affect the natural enemies as well.

There is increasing evidence that a change of plant characteristics in a breeding programme affects natural enemies of herbivores. These effects may be positive, negative or neutral (see Table 1 for a review of the literature). Such specific breeding programmes have aimed for (a) physical plant barriers, such as an altered wax layer or glandular trichomes, (b) plant allelochemicals that negatively affect herbivore performance, and (c) specifically in cotton: nectariless cultivars, as nectar may attract herbivores (Table 1).

An example of positive effects of host plant characteristics on carnivores are plant digestibility reducers that slow down herbivore development. Because the herbivores remain in a stage that is susceptible to their enemies for a longer period of time, this may increase carnivore effectiveness (Dicke, 1999; Haggstrom and Larsson, 1995; Loader and Damman, 1991; but see Benrey and Denno, 1997). Plant structures such as domatia that are used by ants or mites to provide shelter, may also enhance the effectiveness of carnivores (O'Dowd and Willson, 1989).

Negative effects of host plant resistance on carnivores may be found when herbivores sequester plant toxins and subsequently employ them in their defence against their carnivorous enemies (Barbosa, 1988; Björkman and Larsson, 1991; Mendel *et al.*, 1992; Pasteels *et al.*, 1988; Peterson *et al.*, 1987). This may especially occur in specialist herbivores (Dicke, 1999). Specialist carnivores in turn are generally better able to cope with herbivore-sequestered plant toxins than generalist carnivores (Barbosa, 1988; Price, 1991). Natural enemies may also come into contact directly with plant toxins, because they may use various plant tissues and plant products, such as floral and extra-floral nectar, plant sap and pollen, as sources of nutrition (Dicke, 1999).

Transgenic host plant resistance

Bt crops may be considered as having a specific form of host plant resistance, as a toxin is produced in the plant for defence against herbivores. However, there are a few important differences from conventional plant breeding, apart from the fact that in Bt crops a gene from another organism (the bacterium) is incorporated into the genome of the plant. In conventional plant breeding, closely related plant species or cultivars are crossed, and it is largely unknown which or how many genes are involved, or even which plant characteristics are responsible for increased insect resistance. Consequently, it is difficult to specifically

determine their environmental impact. In Bt crops, host plant resistance is determined by one major gene (i.e. vertical resistance), and this gene and its product are understood in great detail. Another major difference is the expression level of the insect-resistant plant characteristics in the newly developed lines. In conventional plant breeding, the expression level of plant allelochemicals, for example, is lower than the maximum occurring in nature. In Bt crops, the expression level of the gene encoding the Bt toxin is determined by the (modified) promoter (Koziel *et al.*, 1993; Perlak *et al.*, 1990; Perlak *et al.*, 1991) and the number of gene copies inserted in the plant genome (Kota *et al.*, 1999). In this way, the level of Bt toxins in transgenic plants may become as high as 2–5% of the total protein level in the plants.

The main crops into which Bt genes have been incorporated to control certain pest insects are maize, rice, cotton, potato, tomato, tobacco, soybean and *Brassica* species (cabbage, oilseed). Bt toxins are expressed constitutively throughout the Bt plant. Bt toxins are only active in insects when ingested orally, as their mode of action is in the midgut. What causes the specificity of the Bt toxins has not yet been completely elucidated. When insects come into contact with and ingest the crystalline inclusions, these inclusions as well as the toxins present in these inclusions (i.e. cry(stal) protoxins; Cry) first need to be proteolytically processed before binding to receptors is possible (Boucias and Pendland, 1998; Pietrantonio and Gill, 1996; Watson *et al.*, 1992). Hence, the specificity of Bt strains may be caused by the presence or absence of specific proteases in an insect midgut. This is an important difference from the mode of action of Bt toxins produced in a Bt plant. Transgenic Bt plants produce toxins instead of protoxins, as only the part of the gene that codes for the active toxin is inserted in the plant genome. As a result, after ingestion, the δ -endotoxins may bind directly to specific receptors on the midgut membrane, without the involvement of insect proteolytic enzymes. In the case of Bt plants, specificity may be based on the presence of specific receptors. So far, the identified receptors to which δ -endotoxins may bind are general receptors, i.e. aminopeptidase-N proteins, cadherin-like proteins or glycolipids (Denolf, 1996; Ferré *et al.*, 1991; Gahan *et al.*, 2001; Van Rie *et al.*, 1990). Binding of the toxins to the receptors is a prerequisite for their insecticidal activity, although binding alone is not sufficient for toxicity (De Maagd *et al.*, 1999, 2001; Denolf, 1996; Schnepf *et al.*, 1998). The rate at which the ligand is inserted into the target membrane decisively influences its relative toxicity (Boucias and Pendland, 1998). However, only when binding is followed by pore formation in the midgut membrane, are midgut cells disrupted and the insect dies (De Maagd *et al.*, 1999; De Maagd *et al.*, 2001; Jenkins *et al.*, 2000; Liang *et al.*, 1995; Schnepf *et al.*, 1998).

Table 1 Effects of host plant resistance on biological control agents

Natural enemy	HPR type ^a	+/-/0 ^d	Reference
Hymenoptera			
Aphelinidae			
<i>Encarsia formosa</i>	P ^b : hairs (cucumber)	-	1, 2
Braconidae			
<i>Aphidius</i> spp.	P: glandular pubescence (potato)	-	3
<i>A. rhopalosiphi</i>	C: hydroxamic acids (Poaceae)	(+)	4, 5
<i>Cardiochiles nigriceps</i>	C: lack of extra-floral nectar (cotton)	-	6
	P/C: trichomes + methyl ketones (tomato)	0	7
<i>Cotesia congregata</i>	C: nicotine	-	8, 9, 10
	P/C: trichomes + methyl ketones (tomato)	-	7
<i>C. marginiventris</i>	C: lack of extra-floral nectar (cotton)	-	6
	P/C: trichomes + methyl ketones (tomato)	0	7
<i>Diaeretiella rapae</i>	C: allyl isothiocyanate (Brussels sprout)	+	11
<i>Microplitis croceipes</i>	? ^c : breeding (soybean)	0	12
	C: lack of extra-floral nectar (cotton)	-	6, 13, 14
<i>Lysiphlebus testaceipes</i>	?: breeding (barley and sorghum)	+	15
<i>Praon</i> sp.	P: glandular pubescence (potato)	-	3
Encyrtidae			
<i>Neodusmetia sangwani</i>	?: breeding (rhodesgrass)	+	16
Eulophidae			
<i>Pediobius foveolatus</i>	?: breeding (soybean)	0	12
Ichneumonidae			
<i>Campoletis sonorensis</i>	C: lack of extra-floral nectar (cotton)	(-)	13
	C: nectariless + high terpenoid (cotton)	0	17
<i>Hyposoter annulipes</i>	C: nicotine	-	8, 18
<i>Hyposoter exiguae</i>	C: α -tomatine	-	19
Scelionidae			
<i>Telenomus sphingis</i>	P: glandular trichomes (tobacco)	(-)	20
	P/C: trichomes + methyl ketones (tomato)	-	7
<i>T. podisi</i>	?: breeding (soybean) (via host eggs)	(-)	21
Trichogrammatidae			
<i>Trichogramma</i> spp.	P/C: trichomes + methyl ketones (tomato)	-	7
<i>T. minutum</i>	P: glandular trichomes (tobacco)	-	20, 22
<i>T. pretiosum</i>	C: nectariless + high terpenoid (cotton)	0	17
	C: lack of extra-floral nectar (cotton)	-	23
Diptera			
Tachinidae			
<i>Archytas marmoratus</i>	P/C: trichomes + methyl ketones (tomato)	-	24, 25
<i>Eucelatoria bryani</i>	P/C: trichomes + methyl ketones (tomato)	(-)	24
	P/C: trichomes + methyl ketones (tomato)	0	25
<i>Lydella thompsoni</i>	C: plant volatiles (corn)	+	26
Coleoptera			
Coccinellidae			
<i>Coleomegilla maculata</i>	P: glandular pubescence (potato)	-	3
	P/C: trichomes + methyl ketones (tomato)	-	27
	C: lack of extra-floral nectar (cotton)	0	13
<i>Cryptolaemus</i> sp.	C: lack of extra-floral nectar (cotton)	+	28
<i>Hippodamia convergens</i>	C: lack of extra-floral nectar (cotton)	-	13
	?: breeding (resistant aphids, sorghum)	-	29
Hemiptera			
Anthocoridae			
<i>Orius insidiosus</i>	C: lack of extra-floral nectar (cotton)	-	13
	C: nectariless + high terpenoid (cotton)	(-)	17
Lygaeidae			
<i>Geocoris punctipes</i>	C: lack of extra-floral nectar (cotton)	0	13
<i>Geocoris pallens</i>	C: lack of extra-floral nectar (cotton)	0	13
Miridae			
<i>Cyrtorhinus lividipennis</i>	?: breeding (partially resistant rice)	+	30
Nabisidae			
<i>Nabis</i> spp.	C: lack of extra-floral nectar (cotton)	-	13
Pentatomidae			

Table 1 (continued)

Natural enemy	HPR type ^a	+/-/0 ^d	Reference
<i>Podisus maculiventris</i>	C: α -tomatine	-	31, 32
	C: chlorogenic acid	-	32
	C: rutin (tomato)	(-)	32
	C: combination of the three above	(-)	33
	C: iridoid glycosides (via prey)	-	34
	? : breeding (soybean) (via prey)	-	21
Neuroptera			
Chrysopidae			
<i>Chrysoperla carnea</i>	P: glandular pubescence (potato)	-	3
	C: lack of extra-floral nectar (cotton)	-	13
Dictyoptera			
Mantidae			
<i>Tenodera sinensis</i>	C: cardenolides (via prey)	-	35
Arachnida			
Phytoseiidae			
<i>Phytoseiulus persimilis</i>	P: glandular trichomes (tomato)	-	36
	P: trichomes (gerbera)	-	37
	P: trichomes (apple)	+	38
<i>Typhlodromus pyri</i>	P: trichomes (apple)	+	38
Salticidae			
<i>Phidippus audax</i>	C: iridoid glycosides (via prey)	-	34

^aHost plant resistance type; ^bP, physical plant defence; C, chemical plant defence; ^cPlants bred for higher insect resistance, but resistance type is not specified; ^d+, positive effect of HPR on biological control agent(s); -, negative effect of HPR on biological control agent(s); 0, no effect of HPR on biological control agent(s). References: ¹Van Lenteren (1991); ²Van Lenteren *et al.* (1995); ³Obyrcki *et al.* (1983); ⁴Fuentes-Contreras and Niemeyer (1998); ⁵Campos *et al.* (1990); ⁶Lewis and Takasu (1990); ⁷Farrar *et al.* (1994); ⁸Barbosa *et al.* (1986); ⁹Thorpe and Barbosa (1986); ¹⁰Thurston and Fox (1972); ¹¹Van Emden (1986); ¹²Herzog and Funderburk (1985); ¹³Schuster and Calderon (1986); ¹⁴Stapel *et al.* (1997); ¹⁵Schuster and Starks (1975); ¹⁶Schuster and Dean (1973); ¹⁷Lingren *et al.* (1978); ¹⁸El-Heneidy *et al.* (1988); ¹⁹Campbell and Duffey (1979); ²⁰Rabb and Bradley (1968); ²¹Orr and Boethel (1986); ²²Elsy and Chaplin (1978); ²³Treacy *et al.* (1987); ²⁴Farrar *et al.* (1992); ²⁵Farrar and Kennedy (1993); ²⁶Franklin and Holdaway (1966); ²⁷Barbour *et al.* (1993); ²⁸Adjei-Maafa (1980); ²⁹Rice and Wilde (1989); ³⁰Kaneda (1986); ³¹Traugott and Stamp (1996); ³²Stamp *et al.* (1997); ³³Bozer *et al.* (1996); ³⁴Strohmeier *et al.* (1998); ³⁵Paradise and Stamp (1990); ³⁶Van Haren *et al.* (1987); ³⁷Krips *et al.* (1999); ³⁸Roda *et al.* (2000).

How may non-target arthropods come into contact with Bt toxins?

Beneficial, non-target arthropods may come into contact with toxins produced in transgenic plants in several ways; by feeding on plant parts themselves, through feeding on target or non-target herbivorous insects, or via the environment, i.e. the soil when toxins persist and do not lose their toxicity after plant parts or insects have died.

Through feeding on the plants themselves

Carnivorous arthropods such as predators and parasitoids may feed on plant parts or pollen, and thus may directly ingest the toxin from these plant parts (Godfray, 1994; Hagen, 1986; Weiser and Stamp, 1998). Generally, floral nectar and pollen are fed upon by many different groups of carnivorous insects and mites (see Van Rijn *et al.*, 2002 and references therein) that rely on these food sources for energy and in some cases also for egg production (Hagen, 1986). Dipteran and hymenopteran parasitoids are ento-

mophagous in their larval stage, but the adults usually feed on (extra)floral nectar or honeydew (Godfray, 1994; Hagen, 1986). Bt toxins are not present in (extra)floral nectar and honeydew, and thus adult parasitoids are not likely to come into contact with Bt toxins. A number of predators (e.g. coccinellid beetles, tachinid flies, pentatomid bugs) may feed on pollen or green plant parts when prey is not available or present in low densities (e.g. Andow, 1990; Coll and Bottrell, 1991; Coll and Bottrell, 1992). Thus, these predators will consume Bt toxins as adults.

In conclusion, natural enemies may feed directly on plant parts in which Bt toxins are present; however, nectar does not contain Bt toxins.

Through feeding on insects that have ingested the toxin

Carnivorous arthropods may also ingest the Bt toxins in a more indirect way when they feed on herbivorous insects that have ingested the toxins from the plant. In target insect herbivores, the toxins are bound to receptors in the

Table 2 Effects of Bt on biological control agents

Natural enemy	Bt effects studied		L/F ⁱ	+/-/0 ^j	Reference
	Form of Bt toxin offered	Parameter quantified			
Hymenoptera					
Braconidae					
<i>Cardiochiles nigriceps</i>	Cry1A(b)-fed(p) ^a hosts	Parasitism rate	F	+	1
	Cry1A(b)-fed(p) hosts	Parasitism rate	F	0	2
<i>Cotesia marginiventris</i>	Btk-fed ^b hosts	Emergence rate	L	-	3
	Btk spray ^{c,*}	Parasitism rate	F	0	4
	Btk spray*	Emergence rate	F	0	4
<i>C. melanoscela</i>	Btk-fed hosts	Parasitism rate	L	+	5
	Btk-fed hosts	Emergence rate	L	-	5
<i>C. plutellae</i>	Cry1A(c) rape	Attraction	L	0	6
	Cry1A(c)-fed(p) hosts	Emergence rate	L	-	7
<i>Diaeretiella rapae</i>	Cry1A(c)-fed(p) aphids	Parasitism rate	L	0	6, 8
<i>Macrocentrus grandii</i>	Cry1A(b) corn*	Parasitism rate	F	0	9
<i>Microplitis croceipes</i>	Btk-fed hosts	Development	L	-	10
Eulophidae					
<i>Neochrysocharis punctiventris</i>	Btk-treated leaves ^d	Mortality	L	0	11
<i>Diglyphus intermedius</i>	Btk-treated leaves	Mortality	L	-	11
Ichneumonidae					
<i>Camponotus sonorensis</i>	Cry1A(b)-fed(p) hosts	Parasitism rate	F	+	1,2
	Cry1A(b)-fed(p) hosts	Parasitism rate	L	+	12
<i>Diadegma insulare</i>	Bta-treated plants	Parasitism rate	F	0	13
<i>Eriborus terebrans</i>	Cry1A(b) corn*	Parasitism rate	F	0	9
Pteromalidae					
<i>Nasonia vitripennis</i>	Cry1A(c) diet	Mortality	L	0	14
	Cry2A diet	Mortality	L	0	15
Trichogrammatidae					
<i>Trichogramma</i> spp.	Btk sprays	Parasitism rate	F	-	16
Diptera					
Tachinidae					
<i>Myiopharus doryphorae</i>	Btt-fed hosts	Larviposition	L	0	17
Coleoptera					
Carabidae					
	Btk sprays	Density	F	0	18
	Cry1A(b) corn	Density	F	0	19
<i>Lebia grandis</i>	Cry3A potato	Density	F	-	20
Coccinellidae					
<i>Coleomegilla maculata</i>	Btsd ^e -treated pollen	Predation rate	L	-	21
	Cry1A(b) pollen (corn)	Development	L	0	22
	Cry3A-fed(p) prey	Predation rate	L	0	23
	Cry3A-fed(p) prey ^f	Development	L	0	23
	Cry3A potato	Density	F	0	20
<i>Coccinella septempunctata</i>	Btt-treated leaves	Mortality	L	-	24
<i>Hippodamia convergens</i>	Cry1A(c) diet	Mortality	L	0	14
	Cry2A diet	Mortality	L	0	15
	Cry3A-fed(p) aphids	Fitness ^h	L	0	25
Hemiptera					
Anthocoridae					
<i>Orius majusculus</i>	Cry1A(b)-fed(p) prey	Development and mortality	L	0	22
<i>Orius insidiosus</i>	Cry1A(b) pollen(corn)	Development	L	0	23
Berytidae					
<i>Jalysus wickhami</i>	Cry1A(b)-fed(p) prey	Predation rate	F	+	1
Pentatomidae					
<i>Perillus bioculatus</i>	Btt-fed prey	Predation rate		+	27

Table 2 (continued)

Natural enemy	Bt effects studied		L/F ⁱ	+/-/0 ^j	Reference
	Form of Bt toxin offered	Parameter quantified			
Neuroptera					
Chrysopidae					
<i>Chrysoperla carnea</i>	Cry1A(c) diet	Mortality	L	0	14
	Cry2A diet	Mortality	L	0	15
	Cry1A(b) pollen (corn)	Development	L	0	23
	Cry1A(b) diet	Mortality	L	-	28
	Cry1A(b) diet	Development	L	0	28
	Cry1A(b)-fed(p) prey	Mortality	L	-	29
	Cry1A(b)-fed(p) prey	Development	L	(-)	29
	Cry#-fed prey ^g	Mortality	L	-	30
	Cry#-fed prey	Development	L	-	30
	Cry1A(b)-fed <i>S. littoralis</i>	Mortality	L	-	31
	Cry1A(b)-fed <i>S. littoralis</i>	Development	L	-	31
	Cry1A(b)-fed <i>T. urticae</i>	Mortality	L	0	31
	Cry1A(b)-fed <i>T. urticae</i>	Development	L	0	31
	Cry1A(b)-fed(p) prey	Prey preference	L	(-)	32
	Cry1A(b)-fed(p) aphids	Prey preference	L	0	32
	Odonata				
Libellulidae					
<i>Erythemis simplicicollis</i>	Bti-fed hosts	Development	L	(-)	33
	Bti-fed hosts	Predation rate	L	0	33
Plecoptera					
Perlidae					
<i>Acroneuria lycorias</i>	Bti-killed hosts	Predation rate	L	0	34
Megaloptera					
Corydalidae					
<i>Nigronia serriocornis</i>	Bti-killed hosts	Predation rate	L	0	34
Acari					
Phytoseiidae					
<i>Metaseiulus occidentalis</i>	Btt spray	Mortality	L	-	35

^aHost fed Bt toxin present in plant; ^bhost fed Btk in artificial diet; ^cfield plots treated with Btk sprays; ^dLeaves dipped in Btk solution;

^eBt var. *san diego*; ^fplus pollen offered; ^gthree Cry toxins tested (separately): Cry1A(b) toxin, Cry1A(b) protoxin, Cry2A protoxin: effects for the three toxins were similar; ^hFitness: survival, aphid consumption, development, reproduction; ⁱL, Laboratory experiments; F, field experiments; ^j+, Bt had a positive (indirect) effect on effectiveness of biological control agent(s); -, Bt had a negative (indirect) effect on effectiveness of biological control agent(s); 0, Bt had no effect on biological control agent(s). *No difference in hosts between treated and untreated plots. ¹Johnson & Gould 1992; ²Johnson 1997; ³Atwood *et al.*, 1997; ⁴Young *et al.*, 1997; ⁵Chenot & Raffa 1998; ⁶Schuler *et al.*, 1999a; ⁷Schuler *et al.*, 2001; ⁸Schuler *et al.*, 1999b; ⁹Orr & Landis 1997; ¹⁰Blumberg *et al.*, 1997; ¹¹Schuster 1994; ¹²Johnson *et al.*, 1997b; ¹³Riggin-Bucci & Gould 1997; ¹⁴Sims 1995; ¹⁵Sims 1997; ¹⁶Campbell *et al.*, 1991; ¹⁷Lopez & Ferro 1995; ¹⁸Riddick & Mills 1995; ¹⁹Lozzia & Riggamonti 1998; ²⁰Riddick *et al.*, 1998; ²¹Giroux *et al.*, 1994; ²²Zwahlen *et al.*, 2000; ²³Pilcher *et al.*, 1997; ²⁴Riddick & Barbosa 1998; ²⁵Keller & Langenbruch 1993; ²⁶Dogan *et al.*, 1996; ²⁷Cloutier & Jean 1998; ²⁸Hilbeck *et al.*, 1998a; ²⁹Hilbeck *et al.*, 1998b; ³⁰Hilbeck *et al.*, 1999; ³¹Dutton *et al.*, 2002; ³²Meier & Hilbeck 2001; ³³Painter *et al.*, 1996; ³⁴Merritt *et al.*, 1991; ³⁵Chapman & Hoy 1991.

midgut epithelium, after which they are structurally rearranged (De Maagd *et al.*, 1999; De Maagd *et al.*, 2001; Gazit *et al.*, 1998; Masson *et al.*, 1999) and thus most likely lose their toxicity to natural enemies. In non-target herbivores, however, the toxins do not bind to midgut receptor cells or do not cause pore formation. They may or may not be digested by proteolytic enzymes in the digestive tract, so that the toxins may remain active and subsequently affect entomophagous natural enemies.

In conclusion, natural enemies may not come into contact with Bt toxins via target herbivores, but via non-target herbivores.

Through the environment

Which species come into contact with Bt toxins of transgenic plants depends to a large extent on the persistence of δ -endotoxins in the plant, in insects and in the soil. Several studies have shown that Bt toxins are released from transgenic plants or microbial biomass bind to the soil (Crecchio and Stotzky, 1997; Koskella and Stotzky, 1997; Palm *et al.*, 1996; Saxena *et al.*, 1999; Sims and Holden, 1996; Tapp and Stotzky, 1995a; Tapp and Stotzky, 1995b; Tapp and Stotzky, 1998; Tapp *et al.*, 1994; West *et al.*, 1984). The amount of extractable toxins decreased

rapidly in all studies. Free toxins purified from Dipel (66 kDa) (Dipel being a commercial formulation of Bt based on Bt var. *kurstaki*) were readily utilized as a carbon source by a mixed microbial culture of *Proteus vulgaris* and *Enterobacter aerogenes* (both Proteobacteria, Enterobacteriales), while bound toxins remained toxic after exposure to the microbes (Koskella and Stotzky, 1997).

The toxicity of bound toxins has been established in bioassays, where insects were exposed to free, adsorbed or bound toxins, which were diluted and distributed over the surface of a food medium (Crecchio and Stotzky, 1998; Koskella and Stotzky, 1997; Sims and Holden, 1996; Tapp and Stotzky, 1995a). In this way, the researchers (Tapp and Stotzky, 1995a; Tapp and Stotzky, 1998; Koskella and Stotzky, 1997) found that bound toxins from Btk (66 kDa) (Bt var. *kurstaki*) purified from Dipel remained toxic to *Manduca sexta* even after 234 days, while bound toxins from Btt (68 kDa) (Bt var. *tenebrionis*) purified from M-One (a commercial formulation of Bt based on Bt var. *san diego*) remained toxic to *Leptinotarsa decemlineata* after 3 days (the longest periods tested). Toxins from Bt plants also bind to the soil (Palm *et al.*, 1996; Sims and Holden, 1996; Sims and Ream, 1997). For example, Palm *et al.* (1996) found that 25–30% of the Cry1A(c) proteins produced by Bt cotton leaves remained bound in the soil (pH 6.2) even after 140 days.

Bt toxins may not only get into the soil via decaying plant material or insects, but also via root exudates, as shown by Saxena *et al.* (1999). They tested the toxicity of root exudates of Bt corn producing Cry1A(b), using *M. sexta*, and found a 90–95% mortality after 5 days. Soil samples remained toxic even after 25 days. The concentration of Cry1A(b) protein in soil samples from the rhizosphere of seedlings was about $95 \mu\text{g g}^{-1}$ soil (Saxena *et al.*, 1999), which is much more than Sims and Ream (1997) calculated for Bt toxins that enter the soil via decaying Bt plants. Sims and Ream (1997) calculated a maximum expected field load of $1.6 \mu\text{g g}^{-1}$ soil when Bt cotton with 60 000 plants per acre is uniformly incorporated into the top 7.6 cm of soil, because they found a Cry2A concentration of about $34 \mu\text{g g}^{-1}$ fresh weight in Bt cotton tissue, which amounted to 8 mg of toxin per plant. When root exudates are taken into account as well, the maximum field load may be 60 times higher.

As Bt toxins may persist in the soil for at least as long as 234 days (the longest period analysed; Tapp and Stotzky, 1998), soil organisms such as Collembola and Carabidae are exposed to the toxins for prolonged periods of time. Due to accumulation of toxins over time during degradation of plant biomass, the doses of Bt toxin to which these soil organisms are exposed may increase with time as well. Soil bacteria, fungi, protozoa, nematodes and earthworms do not seem to be

affected by Bt toxins, as Saxena and Stotzky (2001) did not find mortality or weight differences between these organisms in Bt soil and non-Bt soil. However, earthworms do ingest Bt toxin bound to the soil: Saxena and Stotzky (2001) detected Cry1A(b) in the guts and casts of earthworms that were kept in jars with soil containing biomass of Bt corn for 45 days. Hence, similar to non-target herbivores, these earthworms may serve as intermediates through which Bt toxins may be passed on to organisms feeding on these earthworms.

A soil mite and collembolan were not negatively affected when fed on Bt cotton or potato or when exposed to Cry1A, Cry2A or Cry3A toxins (Sims and Martin, 1997; Yu *et al.*, 1997).

In conclusion, Bt toxins are only extractable from soils for a very short period, after which they bind to the soil. These bound toxins retain their toxicity, as has been shown by all relevant studies published since 1996, even after frost. Whether soil organisms are affected by these bound toxins remains to be studied. The bound Bt toxins may be passed on to other organisms feeding on earthworms.

Effects of Bt crops on different food web components

The Bt genes in crops are generally used to control specific target pest insects belonging to one of the three insect orders Lepidoptera, Coleoptera or Diptera. As mentioned before, many other (secondary) pests may occur in these crops apart from the target pests for which Bt crops are developed. Non-target pests will feed on the same plants and may be influenced by the Bt toxins. If they are not influenced, biological control agents may be important to reduce the possibility of a new pest developing. Also, many species belonging to the Coleoptera and Diptera are not phytophagous but carnivorous or saprophagous, and a number of them are important biological control agents. Specifically, about 35% of coleopteran species are phytophagous while 65% are carnivorous or saprophagous, and only about 30% of the dipteran species are phytophagous while 70% are carnivorous or saprophagous (Strong *et al.*, 1984). Of the Lepidoptera, more than 99% are phytophagous. However, this order of insects includes not only pest species but also species that are important in terms of conservation, such as the monarch butterfly (*Danaus plexippus*) in the United States and swallowtails (*Papilio* spp.) in the United States and Europe. In determining possible effects of Bt plants on non-target insects, it is important to recognize the food webs and their components that may be present. Non-target arthropods comprise non-target Lepidoptera, other non-target herbivorous pests, pollinators, and parasitoids and predators.

Non-target Lepidoptera

The study that drew a lot of attention to (possible) effects of Bt plants on butterflies that are important for conservation was that by Losey *et al.* (1999). They reasoned that, as Bt corn also expresses the toxin in the pollen, and pollen can be dispersed to and deposited on other plants near corn fields, non-target organisms that feed on these other plants may ingest Bt toxins and be affected as well. When milkweed leaves were dusted with pollen from N4640 Bt corn (Bt11 event), *D. plexippus* larvae consumed significantly less from these leaves compared to leaves dusted with untransformed pollen, and in 4 days almost half of the tested larvae died, which was significantly more than on the leaves with untransformed pollen where none of the tested larvae died.

The study of Losey *et al.* (1999) received a lot of criticism, as most likely it did not address the dynamics of endotoxin encounters under field conditions. However, recently, a number of large field studies have been conducted to determine (a) the amount of pollen from corn that is deposited on milkweed leaves inside and outside corn fields (Pleasants *et al.*, 2001; Stanley-Horn *et al.*, 2001), (b) what amounts of pollen from which Bt corn events were toxic to monarchs as well as swallowtails (Hellmich *et al.*, 2001; Zangerl *et al.*, 2001), (c) where the monarchs occur during their breeding season (Oberhauser *et al.*, 2001), and (d) what percentage of the population of monarchs is possibly affected by the Bt toxins in Bt corn in areas where Bt corn is presently grown (Sears *et al.*, 2001). Three different Bt corn events are currently grown in the USA, i.e. Bt11, Mon810, and, at a decreasing rate, event 176. The three events differ in the amount of Bt toxin that is expressed. Pollen from Bt11 and Mon810 express less than 0.09 µg Cry1A(b) per g pollen (<http://www.epa.gov/scipoly/sap/2000/october/>), while pollen from event 176 express up to 7.1 µg Cry1A(b) per g pollen (<http://www.epa.gov/pesticides/biopesticides/factsheets/fs006458t.htm>). The amount of corn pollen naturally deposited on milkweed leaves ranged from 150–400 grains cm⁻² inside corn fields, and 95% of the leaf samples had pollen densities below 600 grains cm⁻² (Pleasants *et al.*, 2001). Outside corn fields, i.e. 0, 1, 2 and 4–5 m from the field edge, 95% of the samples had pollen densities below 300, 200, 75 and 25 grains cm⁻², respectively (Pleasants *et al.*, 2001). Pollen densities between the three corn events did not differ (Stanley-Horn *et al.*, 2001). Pollen densities declined dramatically after rain (Pleasants *et al.*, 2001; Zangerl *et al.*, 2001).

As the amounts of toxin differ in the three Bt corn events, the effect of pollen from these corn plants on monarchs differed as well. Pollen from Bt11 and Mon810 did not have larvicidal effects on monarchs, while pollen from event 176 did (Hellmich *et al.*, 2001; Stanley-Horn

et al., 2001). Monarch larvae were significantly smaller when feeding on leaf discs treated with pollen densities as low as 5–10 grains cm⁻² from event 176 (Hellmich *et al.*, 2001). In event 176 corn fields, fewer than 7% of monarch larvae survived, although this rate of decline slowed after systematically removing predatory arthropods (Zangerl *et al.*, 2001). For swallowtails, pollen from this event resulted in significant mortality when present at densities of 100 grains cm⁻² and higher (Zangerl *et al.*, 2001).

Monarch butterflies naturally occur in agricultural habitats throughout their breeding season, where their densities were as high as or higher than in non-agricultural habitats (Oberhauser *et al.*, 2001). Thus, agricultural practices in general could have a large impact on monarch populations. In the case of Bt corn event 176, the amount of grains deposited on milkweed leaves in and around corn fields is sufficient to detrimentally affect monarch larvae. Seed companies producing event 176 chose not to seek US EPA re-registration in 2001 (<http://www.epa.gov/pesticides/biopesticides/factsheets/fs006458t.htm>), which means that this type of corn will be commercially available only until 2003.

In conclusion, monarch larvae are affected by the Bt toxin Cry1A(b). Swallowtails seem to be affected by Bt toxins in pollen only when offered at higher doses. One other study determined the effect of Bt on non-target Lepidoptera, i.e. the tree-feeding *Papilio glaucus*, *P. canadensis* and *Callosamia promethea* (Johnson *et al.*, 1995). This study relates to sprays of Bt var. *kurstaki*. In forests in the US and Canada, Btk sprays are used against the forest defoliator, the gypsy moth *Lymantria dispar*, and therefore non-target lepidopterans in these areas may be affected as well. When first- and early-second-instar larvae of the three non-target Lepidoptera were placed on host trees that were or were not sprayed with Bt, significantly fewer caterpillars were alive on the Bt-treated trees after 5 days. With *P. glaucus*, an extra experiment was conducted, in which first- and second-instar larvae were placed on Bt-treated trees 0, 10, 20 and 30 days after spraying. This species showed significantly higher mortality after 8 days for all treatments, i.e. after 8, 18, 28 and 38 days, respectively, compared to controls (Johnson *et al.*, 1995), which indicates that the Bt sprays used in this study were more persistent than usually assumed.

It is important to note that all above-mentioned studies refer to non-target lepidopterans that do not feed on Bt plants themselves. Green plant parts contain much higher levels of Bt toxins (Hellmich *et al.*, 2001). Non-target lepidopterans that do feed on Bt plant parts but are not affected by the toxin ingest these toxins and may subsequently act as intermediates through which these toxins are passed on to the third trophic level, their predators and parasitoids, in a similar way as naturally occurring plant allelochemicals.

Non-target herbivores

Apart from the Lepidoptera, Diptera and Coleoptera, there are pest arthropods from other orders as well, the most important ones being aphids and other sucking insects (Homoptera, Hemiptera), thrips (Thysanoptera), and spider mites (Tetranychidae). Most herbivorous insects and mites will ingest the toxins when feeding on the transgenic plants. The major exceptions are aphids, because they do not feed on plant cells but on phloem sap, in which Bt toxins are not expressed (Raps *et al.*, 2001). After ingestion, herbivorous non-target arthropods may be affected by the Bt toxin synthesized in the plant, depending on the presence of receptor cells in the midgut epithelium and whether binding to receptors subsequently results in pore formation. Although specific receptors for the toxic proteins on the midgut epithelium appear to be necessary, so far it is unclear whether such receptors are present in non-target organisms (De Maagd *et al.*, 2001; Tapp *et al.*, 1994).

One major unanswered question so far is what happens to Bt toxins after ingestion by non-target herbivores. As these toxins may not bind to receptors so that their structure remains unchanged, it is conceivable that the toxins will remain intact and therefore toxic. If so, natural enemies parasitizing or feeding on these herbivores may come into contact with and be affected by the toxins. An example illustrating this relates to spider mite–predatory mite interactions. Spider mites do not seem to be affected by Bt toxins (Chapman and Hoy, 1991), although they do ingest the toxins when feeding from transgenic plants (Dutton *et al.*, 2002). However, Chapman and Hoy (1991) did find a toxic effect on the predatory mite *Metaseiulus occidentalis* when leaf disks with *Tetranychus urticae* were sprayed with suspensions of Bt var. *tenebrionis* (i.e. protoxin). Predatory mites are important biological control agents of spider mites (Helle and Sabelis, 1985). Thus, if toxins from transgenic plants are passed on via the herbivore to the predator, the predatory mites may be negatively affected when feeding on Bt-fed spider mites which may result in outbreaks of spider mites.

In conclusion, so far it is largely unknown what happens to Bt toxins in non-target herbivores and/or whether these herbivores may act as intermediaries through which the toxins may be passed on to predators and parasitoids.

Pollinators

Pollinators are essential for most fruit and vegetable growers. The honey bee *Apis mellifera* is the pollinator most commonly used, although solitary bees such as *Osmia* spp. are also used. In the current transgenic crops, Bt toxins are produced in the pollen as well; therefore it is important to determine whether honey bees are affected

by these toxins. In collecting pollen, bees visit many flowers per flight, so that their body is often covered with pollen grains. Because of this behaviour, gene flow between transgenic crops and non-transgenic plants may occur (e.g. Ramsay *et al.*, 1999). However, it is beyond the scope of this review to cover the possibilities and dangers of gene flow between transgenic and non-transgenic plants. The bees that may be affected by the Bt toxins are the larvae and younger bees (up to 10 days of age) for which the pollen are collected. The bees collecting the pollen feed from floral nectaries (nectar is also collected for the production of honey). As transgene products have not been detected in nectar, bees are not exposed to the toxin when feeding on this plant product (Malone and Pham-Delègue, 2001). Toxicity of Bt toxins should thus be determined for larvae and young bees of up to 10 days old.

Several studies have been conducted to determine the toxicity of Bt toxins to pollinators. Vandenberg (1990) tested a solution of Bt (Bt var. *tenebrionis*) in sucrose that was fed to newly emerged (< 24 h) adult *A. mellifera* in cages, and found no significant differences in mortality after 5 days when compared to adults fed plain sucrose. Sims (1995, 1997) conducted toxicity tests on larval and adult *A. mellifera* that were fed sugar solutions with the activated Cry1A(c) toxin (Sims, 1995) or the activated Cry2A toxin (Sims, 1997). In neither case were significant differences found in mortality between bees fed the Bt sugar solutions or the sugar solution without Bt toxins. Arpaia (1997) mixed Cry3B toxin in supplemental syrup that was offered to *A. mellifera* colonies. Each week, larval survival and pupal dry weight were recorded, and no toxic effects were found for this toxin either. Neither did Malone and Pham-Delègue (2001) find differences in longevity and flight activity when *A. mellifera* bees were fed with purified Cry1B(a) toxins.

In conclusion, there is no evidence that *A. mellifera* larvae or adults are affected by Bt toxins. Other pollinators have not been tested so far.

Parasitoids and predators

When the third trophic level is considered, primary parasitoids and predators may feed on one or several herbivorous insect species, depending on their degree of specialization. Parasitoids are usually specialists and thus will mostly parasitize only a few species belonging to one family (e.g. Hawkins, 1994). When the population of target insects diminishes due to the Bt crop, their parasitoids may move to other sites where more hosts will be encountered (Croft and Messing, 1990). This effect will be similar for all pest control methods that lead to a reduction in a pest population.

When investigating whether effects of herbivore population reductions on natural enemies occur, it is important

to first verify whether treated plots contain a significantly smaller population of the target herbivore. This is not always the case. For example, Orr and Landis (1997) and Pilcher *et al.* (1997) did not find a difference in population density of predators between transgenic corn (containing Cry1A(b) toxin) and isogenic corn. However, no significant differences in egg densities and egg masses of the target herbivore *Ostrinia nubilalis* were found either (Orr and Landis, 1997; Pilcher *et al.*, 1997), but the percentage of individual eggs within egg masses that hatched was significantly lower in transgenic plots (Orr and Landis, 1997).

Only a few field studies have been conducted that aimed to determine not the overall population density differences of parasitoids and predators in general, but of specific predator or parasitoid species between Bt and non-Bt crops. For example, Riddick *et al.* (1998) determined differences in abundance of *Lebia grandis* and *Coleomegilla maculata* adults between isogenic, 100% Cry3A-transgenic potato fields, and mixed fields. The specialist predator *L. grandis* was significantly less abundant in seed-mixed and pure 100% transgenic potato fields than in non-transgenic potato fields, and probably dispersed from transgenic fields due to low densities of *leptinotarsa decemlineata* (Riddick *et al.*, 1998). The generalist predator *C. maculata* was as abundant in seed-mixed and pure 100% transgenic potato fields as in the isogenic potato fields. When *L. decemlineata* is present in low densities, most likely *C. maculata* turns to alternative prey or to plant pollen (Riddick *et al.*, 1998). However, when feeding on pollen, this predator may still encounter Bt toxin.

Effects on carnivorous arthropods may especially be expected when Bt toxins are used in crops against dipteran and coleopteran pests, because many Diptera and Coleoptera are important generalist predators. For example, Bt toxins in potato aimed against the coleopteran *Leptinotarsa decemlineata* may affect an important group of predators that prey upon potential non-target pest species, such as aphids. Spiders are also important generalist predators of a large number of insect species, although their role is not always recognized (Bogya, 1999; Mayse and Price, 1978; Riechert and Lockley, 1984; Schmaedick and Shelton, 2000).

Published effects of Bt toxins on predators and parasitoids are summarized in Table 2, and discussed below.

Effects of toxins in Bt plants on biological control agents

Table 2 summarizes not only the studied effects of Bt toxins in plants, but also effects of Bt sprays, because these studies indicate whether effects of δ -endotoxins on biological control agents may be expected. However, the results of these studies cannot be translated completely to

possible effects of Bt toxins produced in transgenic plants, due to (a) the possible presence of more than one endotoxin in Bt sprays, and (b) the presence of spores, which have been reported to act synergistically with the δ -endotoxins in their toxic activity (Dubois and Dean, 1995; Johnson and McGaughey, 1996; Miyasono *et al.*, 1994; Moar *et al.*, 1989). Also, the doses used in the Bt spray studies are difficult to compare with doses of toxins in Bt plants. In studies with Bt sprays, the doses used may be expressed in kg ha^{-1} (varying from 0.68 kg ha^{-1} (Young *et al.*, 1997) to 2.3 kg ha^{-1} (Riddick and Mills, 1995)), in international toxicity units mg^{-1} water (e.g. Giroux *et al.*, 1994; Painter *et al.*, 1996), or in $\text{mg Bt toxin g}^{-1}$ diet (e.g. Blumberg *et al.*, 1997). In studies with Bt plants, the toxin concentrations have been expressed as a percentage of the total amount of proteins produced by the plant (e.g. Perlak *et al.*, 1990; Perlak *et al.*, 1991) or as $\mu\text{g g}^{-1}$ dry weight of the Bt plant (e.g. Meier and Hilbeck, 2001; Pilcher *et al.*, 1997).

Effects of different Bt sprays on target and non-target arthropods have been reviewed by Krieg and Langenbruch (1981), Flexner *et al.* (1986), and Glare and O'Callaghan (2000). In determining effects of δ -endotoxins on carnivorous arthropods, the results of many of these studies are of limited use, as β -exotoxins were present in many of the Bt products that were used until the late 1980s (Glare and O'Callaghan, 2000). Because β -exotoxins were found to be toxic to many species, including mammals, Bt sprays are now required to be free from β -exotoxins in most European countries, the US and Canada (Glare and O'Callaghan, 2000). The addition of Ca^{2+} ions has been used to precipitate β -exotoxins. Unfortunately, most studies did not specify the exact content of the Bt sprays used in the experiments. Therefore, we chose to review only the studies conducted in Europe, the US and Canada from 1990 onwards (see Table 2).

The toxins that are produced in Bt plants may be passed on to biological control agents via plant material (pollen or in some cases also plant leaves) or via target or non-target herbivores, depending on which life stage comes into contact with the toxin via the plant or via the herbivore in the field. So far, few studies have been conducted to determine the effects of Bt toxins on natural enemies of both target and non-target herbivores. Most studies have concentrated on natural enemies of target herbivores (see Table 2).

The doses that have been tested varied from 25–100 μg activated toxin g^{-1} diet when artificial diet was used (Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1999; Sims, 1995; Sims, 1997), and from 2.57–7 μg toxin g^{-1} fresh dry weight when plant material was used (Meier and Hilbeck, 2001; Pilcher *et al.*, 1997). In a number of studies, no dose was specified, only the transgenic lines that were used (e.g. Johnson and Gould, 1992; Lozzia and Rigamonti, 1998; Orr

and Landis, 1997; Schuler *et al.*, 1999a). In these transgenic lines, the level of toxins is expressed as a percentage of the total amount of proteins in the plant (Perlak *et al.*, 1990, 1991) or in g mg^{-1} plant protein (Koziel *et al.*, 1993; Vaeck *et al.*, 1987). For example, the first transgenic tobacco lines expressed 42 ng mg^{-1} protein, which caused 100% mortality in *Manduca sexta* larvae after 3 days (Vaeck *et al.*, 1987). Improved tobacco and tomato lines produce 0.02–0.03% of total protein content as Bt toxin protein (Perlak *et al.*, 1991). Bt cotton plants express up to 0.1% of their total protein as Cry1A(b) or Cry1A(c) (Perlak *et al.*, 1990). In Bt corn lines, the level of toxin expressed is up to $4 \mu\text{g Cry1A(b) mg}^{-1}$ soluble protein (Koziel *et al.*, 1993). To be able to compare possible non-target effects of (activated) Bt toxins expressed in different transgenic plant lines, a standardized measure is needed of the amount of toxin in different plant parts. Also, to clarify which effects have been found at which dose, dose–response curves should be constructed, which are lacking so far. If a new generation of Bt crops has much higher expression levels of the Bt toxin (McBride *et al.*, 1995; Kota *et al.*, 1999), then this is likely to have more severe effects on non-target organisms than the effects reported to date.

Fitness

Bt toxins may affect natural enemies either directly, or indirectly by feeding from sub-optimal food. Hilbeck *et al.* (1998a) tried to distinguish between these effects by feeding two different kinds of prey to *Chrysoperla carnea*, i.e. one that is highly susceptible to the toxin (*Ostrinia nubilalis*) and one that is less sensitive (*Spodoptera littoralis*). Significant differences in the overall developmental time of *C. carnea* were found between predators reared on Bt-fed and non-Bt-fed *O. nubilalis* larvae, while the overall developmental time was similar between predators reared on Bt-fed and non-Bt-fed *S. littoralis*. This suggests that the nutritional deficiency of Bt-affected prey has a larger effect on *C. carnea* than the Bt toxins themselves (Hilbeck *et al.*, 1998a). However, *C. carnea* reared on Bt-fed prey had a higher mortality than predators reared on non-Bt-fed prey. Also, Schuler *et al.* (1999a) found a negative effect of Bt hosts on the development of *Cotesia plutellae*, but this was due to the fact that all hosts died before *C. plutellae* could eclose from these hosts. Pilcher *et al.* (1997) did not find fitness effects of Cry1A(b) toxins from transgenic corn on the predator *Coleomegilla maculata* when fed either pea aphids (*Acyrtosiphon pisum*), non-Bt pollen, or Bt pollen. As Cry1A(b) should be specifically toxic to Lepidoptera, it is not surprising that this coleopteran predator was unaffected by this toxin. However, as long as the specificity of Bt toxins is not completely clarified, effects of each Bt toxin on insects belonging to other orders cannot be excluded.

Direct toxicity tests with specific Bt toxins were conducted by Schuster (1994) and Sims (1995, 1997). Schuster (1994) examined a number of insecticides, including Dipel (Btk), by dipping tomato leaflets in an aqueous preparation of the insecticide, air-drying them, and then letting the parasitoids survive on them. With Dipel, adult *Diglyphus intermedius* (Hymenoptera, Eulophidae) showed significant mortality compared to the control, but the larval stage, and the larvae and adults of *Neochrysocharis punctiventris* (of the same family) did not show higher mortality. However, direct toxicity tests are of limited value, because it remains unclear whether natural enemies would be exposed to the toxin in this way in the field, and, even when no direct toxicity is found, the overall fitness and parasitism or predation rate may still be affected.

In conclusion, it seems that the fitness of parasitoids and predators is not affected by Bt toxins expressed in Bt plants, but there is an indirect effect by feeding from sub-optimal food or because of host death.

Parasitism/predation rate

Similar to findings with Bt sprays, field-grown transgenic Bt tobacco plants interacted synergistically with natural enemies to reduce populations of *Heliothis virescens*, due to prolonged development of the prey (Johnson and Gould, 1992; Johnson, 1997; Johnson *et al.*, 1997a; Johnson *et al.*, 1997b). Also, *H. virescens* larvae on Bt plants moved around more than on isogenic plants in the laboratory (Johnson, 1997). If larvae have this restless behaviour on toxic plants in the field as well, they may be more vulnerable to predators and parasitoids (Johnson, 1997; Johnson *et al.*, 1997a; Johnson *et al.*, 1997b).

So far, it seems that predators and parasitoids do not distinguish between Bt-intoxicated prey and non-intoxicated prey. Parasitoids and predators of the Colorado potato beetle (*L. decemlineata*) did not discriminate between Bt-intoxicated and non-intoxicated individuals (Lopez and Ferro, 1995; Riddick and Barbosa, 1998). Neither did the parasitic fly *Myiopharus doryphorae* discriminate between Bt-intoxicated hosts and non-intoxicated hosts; larvae were laid in control *L. decemlineata* as well as lethally or sub-lethally infected *L. decemlineata*, although it took about twice as long to larviposit in the control larvae (Lopez and Ferro, 1995). However, third-instar *Chrysoperla carnea* did feed significantly less from Bt-fed *S. littoralis* than from non-Bt-fed *S. littoralis*, at least when probings lasting up to a minute were not taken into account (with the idea that no substantial amount of food can be ingested in such brief time; Meier and Hilbeck, 2001).

Although natural enemies do not seem to distinguish Bt-intoxicated from unintoxicated prey, attraction to Bt plants may be reduced due to less feeding damage by suscep-

tible, intoxicated herbivores (Johnson, 1997; Schuler *et al.*, 1999a). When Bt-susceptible prey feed less due to intoxication, fewer of the volatile cues from damaged plants used by parasitoids and predators to locate their hosts are produced (Chadwick and Goode, 1999; Dicke and Sabelis, 1988; Dicke *et al.*, 1990; Turlings *et al.*, 1990). Hence, in fields consisting exclusively of toxic plants, a reduction in host-finding cues may cause a depression of natural enemy activity (Schuler *et al.*, 1999a) or even an emigration of natural enemies towards more favourable habitats (Johnson, 1997).

In conclusion, positive effects of Bt toxins on the performance of parasitoids and predators were found when these toxins caused prolonged development in target hosts or prey. Natural enemies are likely to be less attracted to Bt plants infested with Bt-susceptible prey, because reduced feeding by these herbivores causes a reduced production of volatile cues.

Effects of plant allelochemicals on biological control agents

As found with Bt toxins, herbivores, especially generalists, may be influenced by plant allelochemicals in terms of their behaviour or development such that predators or parasitoids can attack them more easily or for prolonged periods (see Table 1). A good example are hydroxamic acids that are present in wheat and other Poaceae. On wheat cultivars with increased levels of hydroxamic acids, aphids showed a longer developmental time and a smaller body size, which negatively influenced their defensive reactions to parasitoids, i.e. kicking behaviour to prevent parasitoid stabbing (Fuentes-Contreras and Niemeyer, 1998).

Nicotine in tobacco may be sequestered by herbivores in their haemolymph, which alters their suitability for natural enemies (Barbosa, 1988; Rowell-Rahier and Pasteels, 1992; Thomas and Waage, 1996). Negative effects of increased levels of nicotine have been found for the parasitoids *Cotesia congregata* and *Hyposoter annulipes*, both parasitizing *Manduca sexta*, a specialist herbivore of Solanaceae (see Table 1). The specialist parasitoid *C. congregata* was less affected by nicotine-fed hosts than *H. annulipes*, a generalist parasitoid of a variety of noctuids (Barbosa *et al.*, 1986; El-Heneidy *et al.*, 1988). However, *C. congregata* was detrimentally affected by the nicotine-fed hosts just prior to and after larval parasitoid emergence, although the amounts of nicotine used caused no significant mortality in the hosts (Barbosa *et al.*, 1986). Also, in the field, a significantly smaller proportion of male and female *C. congregata* adults emerged from *M. sexta* hosts feeding on a high-nicotine tobacco variety compared to a low-nicotine tobacco variety (Thorpe and Barbosa, 1986). These studies indicate

that nicotine is passed on via the herbivore to parasitoids, which are subsequently negatively affected by this plant allelochemical.

In tomato, several plant metabolites have been found to reduce herbivory: α -tomatine (Campbell and Duffey, 1979; Traugott and Stamp, 1996), the methyl ketones 2-tridecanone and 2-undecanone (Barbour *et al.*, 1993; Farrar and Kennedy, 1993; Farrar *et al.*, 1992), and chlorogenic acid and rutin (Stamp *et al.*, 1997). α -Tomatine, a steroidal alkaloid, reduces the growth of various caterpillar species. As for other steroidal alkaloids, tomatine binds to sterols, making them unavailable for structural purposes, steroid hormone synthesis, and reproductive tissues (Bernays, 1989; Campbell and Duffey, 1981). Insect predators rely on their prey as a source of cholesterol or utilizable sterols (Svoboda *et al.*, 1978). Campbell and Duffey, 1979) determined the effect of α -tomatine on the parasitoid *Hyposoter exiguae* when fed *Helicoverpa zea*. When *H. exiguae* was fed on α -tomatine-fed hosts, its larval period was prolonged and percentages of pupal eclosion, adult weight and adult longevity were significantly reduced. Higher concentrations of tomatine resulted in smaller adult parasitoids. α -Tomatine was found in the larval tissues of *H. exiguae*, indicating that it was absorbed from the host.

When herbivores sequester plant toxins, this can affect their susceptibility to pathogens. For instance, tobacco budworm larvae fed diets with low nicotine concentration are highly susceptible to Bt, whereas they are unaffected by the bacterium when feeding on a diet with a high nicotine concentration (Krischik *et al.*, 1988). Similar effects were recorded for the influence of tannins on the susceptibility of gypsy moth or corn earworm larvae to viruses or Bt toxin, respectively (Hunter and Schultz, 1993; Navon *et al.*, 1993).

In conclusion, as with Bt toxins, plant allelochemicals can positively affect the performance of natural enemies due to prolonged developmental time or reduced defensive reactions of the prey. Nicotine and α -tomatine can be passed on via the herbivore to parasitoids, which subsequently may be negatively affected by these plant allelochemicals.

Influence of natural enemies on resistance development in target insects

Natural enemies of herbivorous insects can increase or decrease the rate of herbivore adaptation to host plant resistance (Gould *et al.*, 1991; Gould, 1994). For example, generalist predators may feed on the few early-pupating (i.e. faster-developing and thus resistant) individuals, but become satiated as the masses of (more susceptible) individuals pupate. Such a response would slow the rate of adaptation in the herbivore. By contrast, predators or parasitoids may have a search image for pupae when they

are abundant and not when they are rare. In this case, susceptible individuals are more subject to predation than the faster-developing resistant individuals. Although such synergism between plant resistance and natural enemies may be favourable in terms of the initial efficacy of pest control, it is not favourable for the durability of control. Due to the removal of susceptible individuals, adaptation of the herbivore to insect-resistant (Bt) plants will be accelerated (Johnson and Gould, 1992). Hence, as long as adapted (resistant) herbivorous individuals are less prone to natural enemy mortality than unadapted susceptible individuals, natural enemies will increase the rate of adaptation of the pest population (Gould *et al.*, 1991; Gould, 1994).

Conclusions

Biological control is an important component of durable crop protection strategies, and therefore negative effects of (transgenic) insect resistance on carnivorous arthropods should be avoided. At present, it is difficult to compare different studies of the effects of Bt toxins on non-target, beneficial insects, because in Bt sprays and different transgenic lines the toxin type and toxin dose are reported differently. To be able to compare possible non-target effects of (activated) Bt toxins expressed in different transgenic plant lines, a standardized measure is needed of the amount of toxin in different plant parts. There is a need for dose–response curves. Because of the development of a new generation of Bt crops with much higher expression levels (Kota *et al.*, 1999; McBride *et al.*, 1995), the effects on non-target organisms reported so far are likely to be an under-estimate.

Non-target, beneficial arthropods may come into contact with toxins produced in insect-resistant (transgenic) plants by feeding directly on plant parts in which the toxins are present, with the exception of nectar which does not contain Bt toxins. Bt toxins are persistent in the soil, where they bind especially to clay particles. These bound toxins retain their toxicity, even after frost, as shown by all relevant studies published since 1996. Whether soil insects are affected by these bound toxins remains to be studied. The bound Bt toxins may be passed on to other organisms feeding on earthworms.

When feeding from pollen of Bt corn (event 176), monarch larvae are affected by the Bt toxin Cry1A(b). Swallowtails seem to be affected by Bt toxins in pollen only when offered in high concentrations. There is no evidence that honey bee *A. mellifera* larvae or adults are affected by Bt toxins. Other pollinators have not been tested so far.

Natural enemies may not come into contact with Bt toxins via target herbivores, but via non-target herbivores, because the toxins do not bind to receptors on the midgut

membrane in the non-target herbivores. So far it seems that the fitness of parasitoids and predators is not directly affected by Bt toxins expressed in Bt plants, only indirectly by feeding from sub-optimal food or because of host death. The parasitism or predation rate were positively affected by Bt sprays or toxins expressed in Bt plants when this caused prolonged developmental time or reduced defensive reactions in hosts or prey. However, such an initial positive effect on the efficacy of natural enemies has negative effects on the durability of control. Such removal means that susceptible individuals are selectively removed from the population, which accelerates resistance development in target insects. Negative effects on the parasitism/predation rate have been found in some predators/parasitoids when they came directly into contact with Bt sprays. Other predators were not affected in this way. Differences in overall natural enemy densities between Bt and non-Bt fields are difficult to interpret. Most likely, generalist natural enemies remain in Bt fields as long as alternative food is present, while specialist natural enemies emigrate to sites with higher prey densities.

To make a true contribution to durable crop protection through transgenic plants, it is essential to take food web consequences into account. Therefore, studies on the multi-trophic aspects of transgenic insect-resistant crops are badly needed.

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