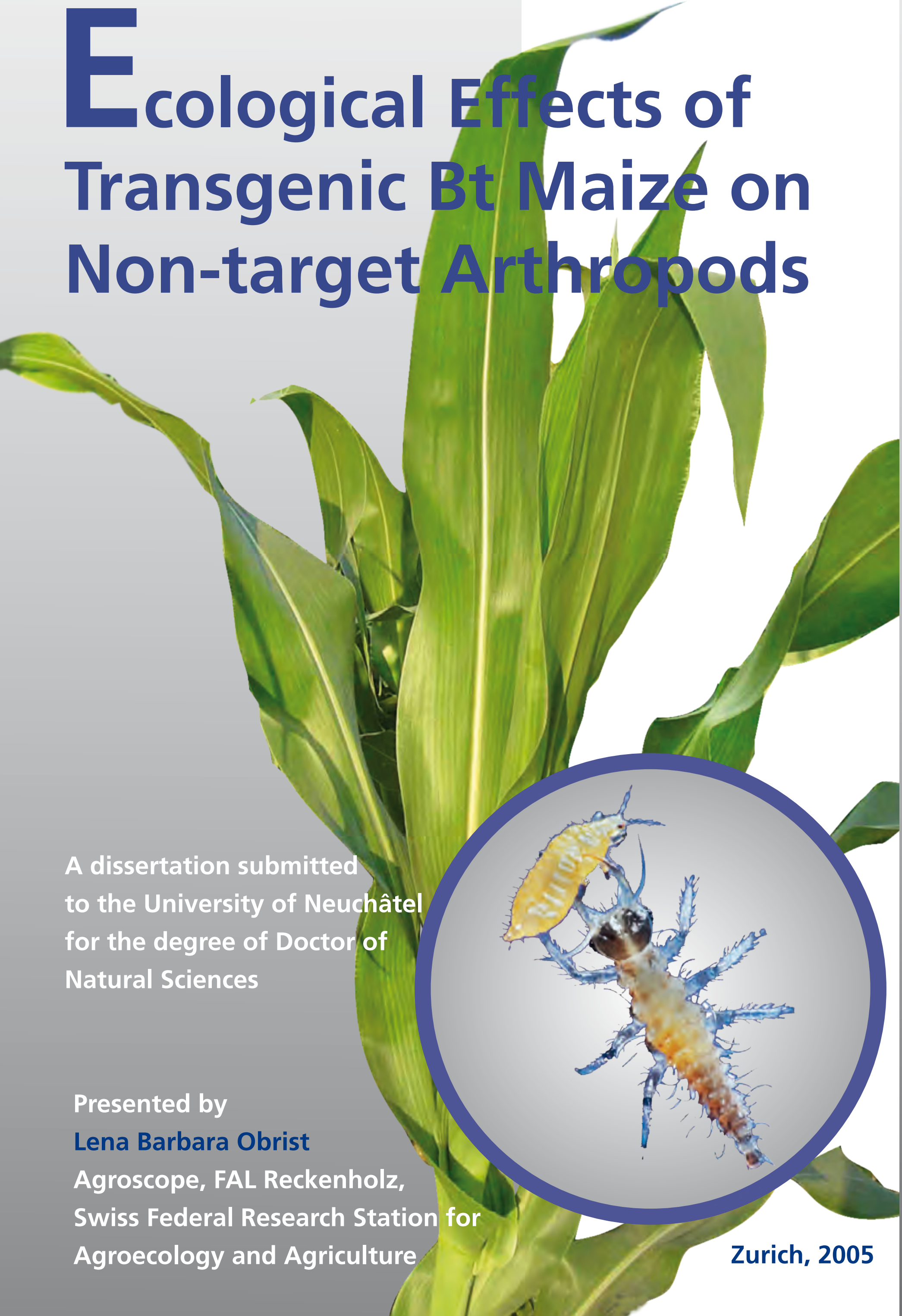
Ecological Effects of Transgenic Bt Maize on Non-target Arthropods



A dissertation submitted
to the University of Neuchâtel
for the degree of Doctor of
Natural Sciences

Presented by
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Agroecology and Agriculture

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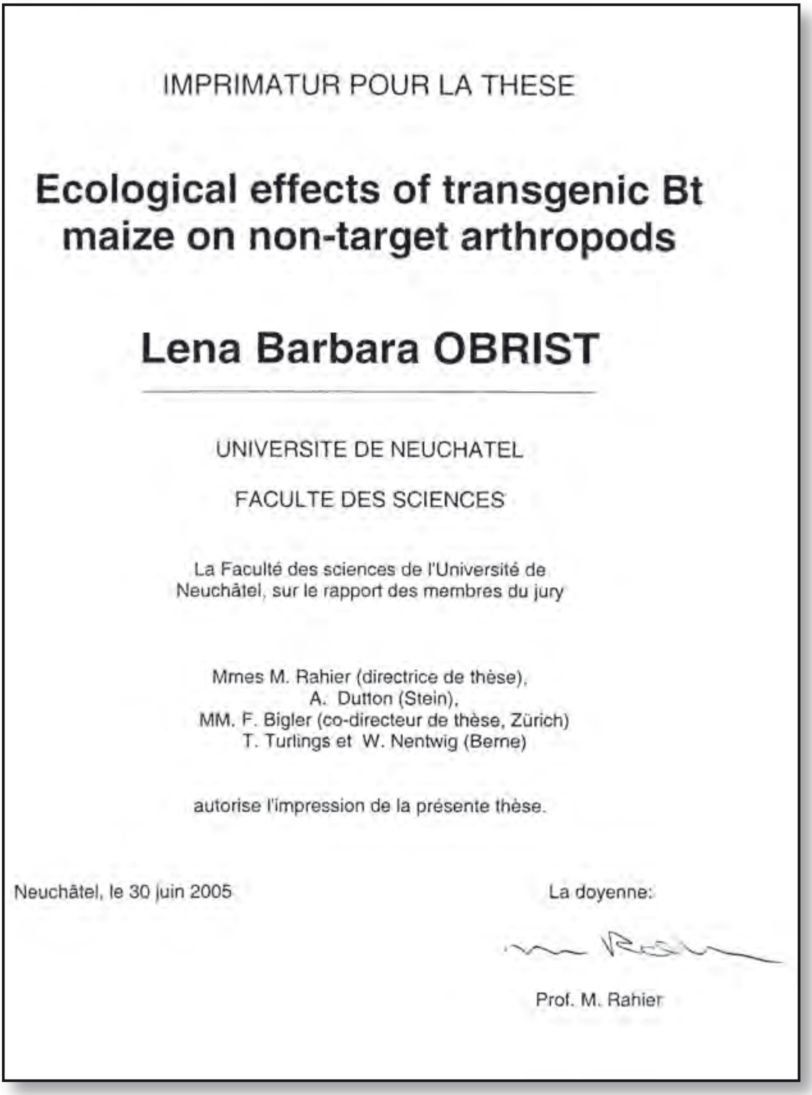

IMPRIMATUR POUR LA THESE

Ecological effects of transgenic Bt maize on non-target arthropods

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UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

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May, 2005

Geheimnisvoll am lichten Tag,
Lässt sich Natur des Schleiers nicht berauben,
Und was sie deinem Geist nicht offenbaren mag,
Das zwingst du ihr nicht ab mit Hebeln und mit Schrauben.

J. W. Goethe, Faust Teil 1, Z. 673

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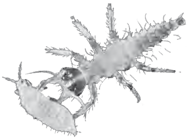
SUMMARY



The use of transgenic Bt maize entails economic benefits as these plants are resistant against corn borers, which are severe lepidopteran pests in maize. The insect resistance is based on the expression of proteins (Bt toxin) originating from the soil bacterium *Bacillus thuringiensi*s. Bt toxins are considered safe for non-target organisms, due to their specific mode of action. However, the insertion of a modified *B. thuringiensis* gene into a plant genome, and the subsequent high and consistent toxin expression, arouse concern as such plants could hold potential risks for non-target organisms including biological control agents. Investigations to determine ecological risks of insect resistant transgenic plants should include the assessment of potential hazards for non-target organisms, taking into consideration their exposure to the insecticidal protein. The aim of this study was to investigate direct and indirect effects of the Cry1Ab toxin expressing Bt maize on non-target arthropods (thrips and predatory mites) and to assess the degree of exposure of herbivores and natural enemies to the toxin.

Thrips are common inhabitants of maize fields and represent a prey for various arthropod predators. An important and widespread thrips species in maize is *Frankliniella tenuicornis.* The studies showed that performance of the thrips was not affected when feeding on Bt maize. This leads to the conclusion that *F. tenuicornis* is not susceptible to the Cry1Ab toxin and that indirect prey-quality mediated effects on natural enemies are unlikely to occur when feeding on thrips. In addition, this herbivore did not contain high toxin levels when reared on Bt maize and the persistence of the toxin in them was very short. Based on these studies, the risks for predators feeding on thrips in or next to a Bt maize field, can be estimated as minimal.

*Amblyseius cucumeris i*s an omnivorous predatory mite species, which is also used as a biological control agent. The impact of Bt maize on this predator was tested by offering either spider mites *(Tetranychus urticae)* reared on Bt or non-Bt maize and Bt or non-Bt maize pollen as food sources. Spider mites were selected as a prey for *A. cucumeris*, since these contain high levels of Cry1Ab toxin when kept on Bt maize. In contrast, toxin levels in pollen of this transgenic cultivar (Bt11) are negligible. No effects on *A. cucumeris* were found when offered Bt-containing spider mites as a prey, indicating that this predator is not susceptible to Cry1Ab toxin. Minor differences in the performance of female *A. cucumeris* were observed when fed with Bt and non-Bt maize pollen. These may be attributed to possible alterations in the nutritional quality of Bt maize pollen.

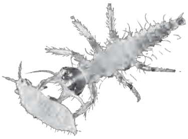
Direct toxic effects of Cry1Ab toxin on predators can be assessed by offering them Bt-containing prey as a food source. So far, it has not been verified whether the tested predators ingest the bioactive Cry1Ab toxin. Using ELISA, the uptake of toxin by predators (*Chrysoperla carnea* and *Orius majusculus*) when feeding on Bt-containing herbivores was confirmed. Feeding bioassays with *Ostrinia nubilalis* revealed that the Cry1Ab toxin ingested by herbivores after feeding on Bt maize remained as active as it was in the plant.

Many risk assessment studies have been performed in the laboratory as well as in the field, independent of the affirmation that the selected species would effectively get in contact with the toxin under natural conditions. By measuring the Bt content in arthropods collected from Bt maize fields in Spain, it could be shown that the toxin can pass through the food chain to predators (e.g., *Orius* spp., *Chrysoperla* spp., *Stethorus* sp.). However, their level of exposure strongly depend on the presence of pollen or spider mites in the field as these food sources contained significant toxin levels. In some predators (e.g., *Nabis* sp., *Demetrias* sp.) the toxin was not detectable, independent of the availability of pollen or spider mites.

The studies conducted in this project represent a further step in the risk assessment of Bt maize. The knowledge will help to elucidate previous results on effects of Bt maize on non-target arthropods and provides relevant information for future studies with Bt maize or transgenic plants expressing other proteins than the Cry1Ab toxin.

Chrysoperla carnea larva feeding on *Frankliniella tenuiconris larva*

RÉSUMÉ



Le maïs transgénique (Bt) permet d’énorme bénéfice économique au système agricole du fait de sa résistance contre des insectes nuisible des cultures tel que la pyrale de maïs. La résistance du maïs Bt face à ces ravageurs est du à l’incorporation d’un gène provenant de la bactérie du sol *Bacillus thurigiensis* qui code pour la protéine Cry1Ab. Les toxines Bt sont considérées inoffensives contres les organismes non-ciblés du fait de leur mode d’action très spécifique. Cependant de telles plantes pourraient représenter des risques potentiels pour les organismes non-ciblés tel que les insectes utiles à cause de leur haut niveau d’expression continue. De plus des effets inattendus, provoqués par l’insertion d’un gène dans le génome de la plante, pourraient avoir lieu. L’objectif de cette recherche était d’étudier les effets directs et indirects du maïs Bt exprimant la toxine Cry1Ab sur les arthropodes non-ciblés et d’évaluer le degré d’exposition des herbivores et des ennemis naturels à cette toxine.

Les thrips sont des insectes se retrouvant fréquemment sur les cultures de maïs et s’avèrent être des proies potentielles pour de nombreux arthropodes prédateurs. Une espèce de thrips commune et bien répandue est celle de *Frankliniella tenuicornis.* Les études ont démontré que les thrips qui se nourrissaient du maïs Bt n’étaient nullement affectés par la toxine produite par la plante. Ceci nous a amené à la conclusion que *F. tenuicornis* n’est pas sensible à la protéine Cry1Ab. Par conséquent la probabilité que les prédateurs naturels des thrips soient affectés par une réduction de la qualité nutritive de sa proie, est minimale. De plus ces phytophages ne contenaient pas de grandes quantités de toxines Bt lorsqu’ils se nourrissaient du maïs transgéniques et cette toxine a montré une persistence limitée dans les thrips. Cette étude nous a permis de mettre en évidence que les risques liés aux prédateurs naturels des thrips localisés sur le maïs Bt ou à proximité étaient minimaux.

Amblyseius cucumeris est une espèce de mite prédatrice omnivore qui est aussi employée dans la lutte biologique. L’impact du maïs Bt sur ce prédateur a été évalué au travers de deux types de traitements. Le premier consistait à mesurer les effets sur *A. cucumeris* alimentés d’acariens jaunes (*Tetranychus urticae*) ayant consommé du maïs Bt ou non-Bt. Le second partait du même principe mais le prédateur était cette fois ci nourri à partir de pollen de maïs Bt ou non-Bt. Alors que de grandes concentrations de Cry1Ab étaient retrouvées dans les acariens jaunes élevées sur du maïs Bt, celles du pollen étaient négligeables. Aucun effet ne fut trouvé chez *A. cucumeris* lorsque des acariens jaunes contenant Cry1Ab leurs étaient offerts en tant que proies, indiquant par la même qu’ils n'étaient pas susceptibles à la toxine Cry1Ab. Des différences mineures dans le développement des femelles nourries avec du pollen de maïs Bt et non-Bt ont été observées. Ces différences pourraient s'expliquer par une variation de la qualité nutritionnelle du pollen du maïs Bt.

Les effets toxiques directs de la protéine Cry1Ab sur les prédateurs peuvent être vérifiés en les nourrissant à partir de proies contenant des toxines Bt. Toutefois il n'a jamais été démontré si les prédateurs testés ingéraient ou non la toxine Cry1Ab sous sa forme active. En utilisant le test ELISA, la prise de toxine par des prédateurs tel que *Crysoperla carnea* et *Orius majusculus* qui se nourrissaient d'herbivores contenant la toxine a été confirmée. Des tests alimentaires avec *Ostrinia nubilalis* ont révélé que la toxine Cry1Ab restait active après son ingestion par les herbivores, au même titre que si elle se trouvait dans le maïs Bt.

De nombreux essais ont été soumis sur les risques que comportait le maïs Bt aussi bien en laboratoire que dans les champs, mais en ignorant le fait que les espèces sélectionnées auraient pu se trouver en contact avec la toxine en conditions naturelles. En prenant en compte les quantités de toxine trouvées dans les arthropodes des champs de maïs Bt en Espagne, la protéine Cry1Ab s'est avérée passer à travers la chaîne alimentaire pour se retrouver dans les prédateurs (par expemple *Orius* spp., *Chrysoperla* spp., *Stethorus* sp.). Cependant le taux d'exposition des predateurs dépendait fortement de la présence du pollen ou des acariens jaunes dans les champs. Ces ressources alimentaires, contenaient des concentrations considérables de toxine. Dans certains prédateurs (*Nabis* sp., *Demetrias* sp.) la toxine n'était pas détectable, en dépit de la présence du pollen ou des acariens jaunes.

Les études entreprises pour ce projet représentent une étape essentielle dans l'estimation des risques potentiels du maïs Bt pour les arthropodes non-ciblés. Les connaissances obtenues lors de cette recherche permettent d'élucider les résultats fournis par les études antérieures traitant de ce sujet. De plus elles apporteront des informations pour les études à venir sur le maïs Bt ou sur d'autres plantes transgéniques exprimant des toxines différentes de la protéine Cry1Ab.

GENERAL INTRODUCTION

BACKGROUND

Bacillus thuringiensis (Bt)

Bt toxins are insecticidal proteins produced by the soil bacterium *Bacillus thuringiensis* Berliner in the form of crystalline inclusions. *Bacillus thuringiensis* is a gram positive, spore forming bacterium which was first isolated by Ishawata (1901) and described by Berliner (1911).

The crystalline inclusions, which can amount up to 20 – 30% of the dry weight of the sporulated cells, mainly consist of δ-endotoxins. Individual *B. thuringiensis* strains vary in the number and type of toxins they produce. More than 170 different genes encoding δ-endotoxins have been identified from *B. thuringiensis*. In addition to the δ-endotoxins, Bt strains can produce a range of other toxins such as Cyt endotoxins (cytolytic endoproteins), β-exotoxins, haemolysins, enterotoxins, exoenzymes and vegetative insecticidal proteins (Glare and O'Callaghan, 2000).

Δ-Endotoxins are classified according to a system of nomenclature originally proposed by Höfte and Whiteley (1989) and later modified by Crickmore et al. (1998). Toxins were classified into types designated Cry1, Cry2, etc., and further subclassified into toxin types designated Cry1A, Cry1B, etc., depending on their insecticidal properties and molecular relationships. In general, Cry1, Cry2, and Cry9 toxins affect Lepidoptera, Cry3, Cry7, and Cry8 toxins affect Coleoptera, and Cry4, Cry10, and Cry11 toxins are active against Diptera. But there are also Cry toxins affecting more than one insect family. In most cases, the inclusions produced by a particular Bt strain contain a mixture of different, however related, δ-endotoxins. For example, *Bacillus thuringiensis kurstaki* HD-1 contains three Cry1 (130 kDa) and two Cry2 (70 kDa) endotoxins in the same crystal (Schnepf et al., 1998; Glare and O'Callaghan, 2000).

Each type of δ-endotoxin is active against a limited number of insects (Lereclus et al., 1993) and affects them in a highly specific way. In Lepidoptera, Bt crystals containing the protoxins of the classes Cry1, Cry2 or Cry9 dissolve in the alkaline midgut of the larvae (Aronson, 1991). Upon dissolution, specific gut proteinases cleave the protoxin in several steps to generate the actual toxic fragment of 60 – 65 kDa (Choma et al., 1990). The activated toxin binds to specific glycoprotein receptors present in the membranes of epithelial midgut cells, and then inserts itself into the gut cell membrane (Gill et al., 1992). Finally the bound toxin induces the formation of pores in the cell membrane, allowing free passage of ions (van Rie et al., 1990; Lee et al., 1992; Knowles and Dow, 1993). The resulting desequilibrium in ion concentrations causes the lysis of cells, which can lead to death of the insect.

As a consequence of the complex mode of action, susceptibility of an insect to a particular δ-endotoxin is depending on at least four parameters: 1. Solubility of the crystalline toxin in the insect gut, determined by midgut environment and molecular properties of the toxin, 2. Presence of proteolytic gut enzymes capable to break down the protoxins, 3. Presence of specific receptors in the cell membrane of the midgut epithelium, and 4. Binding characteristics of the toxin to the midgut (Hofmann et al., 1988; De Maagd et al., 2001; Federici et al., 2002).

Due to its high specificity, implicating minimal effects on non-target organisms and human health, Bt sprays are the most widely used biopesticides in the world (Swaneder, 1994). In the late 1920s already, first attempts to use Bt for insect control were made, and during the 1950s extensive commercial production developed. The first commercial *B. thuringiensis* strain was marketed as "Thuricide". By discovering new and more potent Bt strains, such as HD1 isolated by Dulmage (1970), the insecticidal efficiency was improved and in 1980s world-wide use of Bt sprays was well established. The subspecies *B. thuringiensis kurstaki* has been used most frequently in commercial products for agricultural applications. However, several factors have limited the use of Bt in crop protection: The specificity of Bt entails that many important pest species are not susceptible to Bt, or not affected by Bt spray application due to their cryptic feeding habits. The narrow spectrum of insecticidal activity is desirable from the environmental point of view, but also limits its market potential. Another disadvantage of Bt, compared to chemical insecticides, is that Bt has to be ingested by the pest, which makes efficacy more variable, especially when considering the lack of persistence of Bt in the field (Behle et al., 1997). This implicates the need of frequent application, particularly for controlling polyphagous species or species with long oviposition periods (van Frankenhuyzen, 1993).

Transgenic Bt Plants

In the early 1980s attempts to engineer plants expressing Bt toxins were realised in order to avoid problems associated with the Bt spray application mentioned above. One of the great advantages of this procedure was to target pests which feed internally i.e., on plant tissues inaccessible to conventional Bt sprays. For example in maize, larvae of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), or the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidptera: Noctuidae), migrate to the whorls and bore into the stalk (Ely, 1993; Conzàlez-Núñez et al., 2000).

The first Bt transgenic plants to come up were tobacco (Barton et al., 1987; Vaeck et al, 1987) and tomato (Fischhoff et al., 1987). For these plants an *Agrobacterium tumefaciens*-mediated DNA transfer system was used to insert genes encoding δ-endotoxins into plant genomes.

Insertion of genes coding for the complete protoxin resulted in very low or even undetectable expression levels. The levels of protoxin expressed by the transformed plants, were below 0.0001 ng mg^{-1} of total protein content, and thus not sufficient to induce any insecticidal effects (Barton et al., 1987; Vaeck et al., 1987). In order to obtain higher expression levels, it was necessary to modify the *cry1Ab* gene, and thus to express the truncated gene that only coded for the active part of the protein. In addition, the original bacterial promoter and terminator sequences were replaced by a constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell et al., 1985), and by a suitable terminator. The content of Bt toxins in these plants was high enough to enhance their resistance to certain insect pests but was still low (0.01% of the total protein content). To increase expression levels, the truncated δ-endotoxin genes were reconstructed. The typical bacterial genome characterised by high content of A-T codons was modified into plant specific genomes, rich in G-C codons. Additionally, aberrant processing signals were removed. Reconstruction of the toxin genes was successful, leading to 0.3% Bt toxin of total protein expression in transgenic plants (Perlak et al., 1991), and thus resulting in significantly enhanced resistance to susceptible insects (Wilson et al., 1992). In 1993, Koziel et al. reported the insertion of a modified and truncated version of the *cry1Ab* gene in maize using microprojectile bombardment, which allowed for high toxin expression resulting in effective control of the target pest. As a consequence, various gene constructs expressed by different promoters were inserted in different maize varieties. The transformed varieties are all designated with different “event numbers”, which represent a unique genetic transformation event. For example, Bt11 and MON810 express the toxin under the CaMV 35S promoter during the whole season in leaf, pollen, tassel, silk and kernels (Armstrong et al., 1995), whereas Event176 expresses the toxin mainly in green tissue and pollen (Fearing et al., 1997) driven by two different tissue specific promoters (Koziel et al., 1993). The protein expression in a plant can vary due to various factors including environmental conditions, transgene instability or silencing (Al-Kaff et al., 2000; Coviella et al., 2000; Schubert et al., 2004).

Bt genes have been transferred into a large number of plant species, such as cotton, maize, potato, rice, eggplant and oilseed rape (for a review see Ely, 1993). However, at present only Bt transgenic cotton and maize are commercially available. In transgenic cotton, Bt genes are expressed in order to affect their major pests, the larvae of the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), tobacco budworm, *Heliothis virescens*, (Fabricius) (Lepidoptera: Noctuidae) and the budworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). The target pest of transgenic maize,expressing Cry1Ab toxin, is the lepidopteran European corn borer (*O. nubilalis*) (Koziel et al., 1993). In addition, the Mediterranean corn borer (*S. nonagrioides*) a severe pest in Mediterranean countries can successfully be regulated with Bt maize (Conzàlez-Núñez et al., 2000).

Transgenic Bt plants and ecological risks

Transgenic Bt plants and microbial Bt spray application differ in several points and therefore, ecological risks of these plants should be investigated, in spite of the profound knowledge about the risks of Bt sprays for the environment. In Bt transgenic plants the Cry1Ab toxin is expressed in a modified, truncated and thus more activated form than in *B. thuringiensis*. This implicates that certain steps in the activation process located in the insect midgut become redundant for the plant derived toxin. This may in turn influence its toxicity for non target-herbivores. In addition, the transgene is available during most of a plant's life span whereas the microbial Bt spray degrades within two to seven days (Ignoffo and Garcia, 1978; Behle et al., 1997). Furthermore, the insertion of a gene into the plant genome may inactivate or alter the expression of endogenous genes, leading to unintended effects which are not related to the altered trait (Alonso et al., 2003).

For such reasons transgenic plants hold the potential to cause ecological effects, which are roughly classified into the following groups.

(1) Resistance of target pest species: The continuous expression of the toxin can allow pest insects to evolve resistance (Gould, 1998; Johnson and Gould, 1992). If resistance occurs the efficacy of Bt transgenic crops will decrease. In addition, efficacy of microbial Bt sprays, which are particularly important for organic growers, might decrease. Different strategies for resistance management were developed to delay occurrence of resistant insect populations, e.g., high dose/refuge strategy, toxin stacking (pyramiding) or low dose toxin combined with other control agents (Gould, 1998). Such strategies are effective with regard to economical aspects, but may entail additional risks from an ecological point of view.

(2) Gene transfer: Transfer of genetic material from GM plants to other organisms is

considered as another potential risk (Conner et al., 2003). Out-crossing or vertical gene transfer is based on a sexual process (e.g., by pollinisation) and takes place between transgenic plants and their cultivated or wild relatives (Bergelson, 1998; Ellstrand, 2001). In case of Bt maize, the potential for out-crossing with wild indigenous relatives is limited to Mexico and Central America, where it has already been reported to occur (Quist and Chapela, 2001). The gene transfer from plants to other organisms such as bacteria is based on asexual mechanisms (e.g. conjugation) and is called horizontal gene transfer. It is assumed that it mainly depends on the availability of genetic material (DNA) in the soil where it may be transferred, for example, to soil bacteria (Shelton et al., 2002).

(3) Impact on non-target organisms: Organisms, which are not supposed to be affected by an insecticide, are called non-target organisms. Non-target arthropods are subdivided into four classes comprising herbivores, natural enemies, pollinators and soil organisms (Groot and Dicke, 2002). As this thesis deals with the impact of Bt maize on herbivores and predators, mainly the first and second group of arthropods are discussed more in detail.

How to assess risks of insect resistant transgenic plants for non-target arthropods?

To assess the risks that an insect resistant transgenic crop may pose to non-target arthropods, several factors have to be taken into account (Groot and Dicke, 2002; Dutton et al., 2003; O'Callaghan et al., 2005). As a fist step, it should be determined which arthropods are ecologically and economically important in a given crop system. Emphasise is generally laid on the investigation of natural enemies, as these are necessary for natural pest regulation in the crop (e.g., DeBach and Rosen, 1991). Furthermore, their role in Bt maize crops is considered important as secondary pest may evolve due to the reduction in insecticide application in the transgenic crop (Romeis et al., in preparation). As next step, it should be verified which arthropod species and stages may get in contact with the insecticidal protein expressed by the plant (exposure). Herbivores can be exposed to the toxin when feeding on transgenic plants. The degree of exposure is influenced by the spatial and temporal expression of the toxin but also depends on their feeding behaviour. Natural enemies (predators and parasitoids) can be exposed to the toxin when feeding on prey items containing the toxin. Omnivorous predators or adult parasitoids may additionally get in contact with the toxin when feeding directly on plant tissues (e.g., pollen or leaves) (Head et al., 2001; Raps et al, 2001; Dutton et al., 2002).

In order to study effects of a transgenic plant on a selected non-target organism, a tiered testing scheme has been recommended, which was derived from that applied for conventional pesticides (Cowgill and Atkinson, 2003; Dutton et al., 2003; Poppy and Sutherland, 2004). In the first tier, potential toxic effects should be studied in laboratory bioassays. In these studies the organism should be exposed to excessive concentrations of artificially produced toxin or to maximal amounts of plant-derived toxin that an organism can be expected to encounter in the field. Such "worst case" studies can be followed by standardised behavioural or physiological studies. If effects are observed in the laboratory studies, second tier studies should be performed. These should be conducted under semi-field conditions, which allow undisturbed behaviour, choice among food sources, species interaction and realistic levels of exposure. Finally, field studies can be performed in a third tier. Such studies are difficult to perform and require expensive and time-consuming procedures. In addition, the abundance of organisms is generally assessed without taking into account their level of exposure to the toxin in the field. As a consequence, differences between abundance of arthropods in transgenic and non-trangenic crops, are difficult to interpret and their ecological significance cannot or hardly be estimated.

Potential effects of transgenic plants on non-target arthropods

Different types of adverse effects of transgenic plants on non-target arthropods may be distinguished as those having a direct and those having an indirect effect. Direct toxic effects on herbivores can only occur if they get in contact with the toxin when feeding plant parts containing the toxin. Such effects depend on the susceptibility of the species and their degree of exposure. Arthropods of higher trophic levels (predators and parasitoids) can also be directly affected when feeding on food sources containing the toxin (e.g. herbivores or pollen). In addition, they may be indirectly affected, when feeding on prey or hosts, which are susceptible to the toxin. Such arthropods can represent a food source of reduced abundance, size or quality for natural enemies. This may affect their antagonists, even if they are not susceptible to the toxin themselves. Moreover, behaviour of herbivores can be influenced when sublethally affected upon feeding on insect resistant transgenic plants. The herbivores may reveal reduced escape or defence behaviour, which makes them an easy prey for antagonists. This partial plant resistance was shown to be advantageous for synergistic interactions between transgenic plants and natural enemies (Johnson, 1997; Bell et al., 2001) on condition that negative effects due to the consumption or parasitism of the sublethally affected prey are not deleterious. In addition, the behaviour of the beneficial insects may be influenced when facing an intoxicated prey/host. This was shown by Schuler et al., (1999) who reported that host searching behaviour of the parasitoid, *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae), was reduced when confronted with Bt susceptible *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) larvae feeding on Cry1Ac expressing oilseed rape leaves, compared to larvae of a Bt-resistant *P. xylostella* strain feeding on the same leaves. The lack of response to Bt susceptible larvae was attributed to the reduced consumption rate of Bt leaves by these larvae, involving reduced volatile delivery by damaged leaves.

Finally, unintended changes in the transgenic plant may occur and affect non-target arthropods. Gene insertions can lead to interferences with the host genome and cause phenotypic changes in the plant, which are not directly related to the toxin (Bhat and Chopra, 1999; Alonso et al., 2003; Cellini et al., 2004). In different events of transgenic Bt maize the lignin content was shown to be higher than in the respective non-Bt isolines (Saxena and Stotzky, 2001; Flores et al., 2005). Such phenotypic alterations may affect performance of some herbivore species feeding on maize. Even the presence of marker genes which are inserted along with the gene of interest to distinguish successful transformation, can cause unintended non-target effects. This was reported by Lecardonnel et al. (1999) showing that the insertion of a mere marker gene in potato plants enhanced foliage consumption by Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae).

Selected studies on effects of transgenic Bt maize on non-target herbivores

A herbivore species, which was thoroughly investigated regarding non-target effects of Bt maize, is the bird cherry-oat aphid, *Rhopalosiphum padi*, (L.) (Homoptera: Aphididae). Aphids ingest phloem sap when feeding on plants and therefore, the Cry1Ab toxin content in phloem sap of Bt maize, in *R. padi* reared on this maize, and subsequently in the honeydew they produce, was analysed (Raps et al., 2001). Only traces of toxin were found in the phloem sap of Bt maize and in aphids when reared on Bt maize. These findings agree with the results of other studies (Head et al., 2001; Dutton et al., 2002), showing that *R. padi* reared on transgenic Bt maize did scarcely contain Cry1Ab toxin after feeding on Bt maize. The investigation of effects of Bt maize on performance of *R. padi* revealed inconsistent results. Whereas Lozzia et al. (1998) and Dutton et al. (2002) did not detect effects on performance of *R. padi* when reared on Bt maize, a study by Lumbierres et al., (2004) reported enhanced performance of offsprings of alates, and inhibited performance of offsprings of apterous *R. padi* when reared on Bt maize. In addition, the authors detected some differences in abundance of *R. padi* in the field, mostly showing more alates in Bt maize than in non-Bt maize in the early season. Given that it is unlikely that the toxin could be responsible for such effects, the authors conclude that some unintended changes in the plants may have altered attractiveness of the transgenic plants to the aphids. In a field study, the cicadellids, *Zyginidia scutellaris* (H.-S.) (Homoptera: Cicadellidae) were also observed to be more abundant in Bt maize fields than in control fields (Pons et al., 2005).

Another herbivore on which effects of Bt maize were investigated is the two-spotted spider mite, *Tetranychus urticae* (Koch) (Acari: Tetranychidae). Dutton et al. (2002) showed that *T. urticae* ingested the toxin when feeding on Bt maize but that their performance was not affected.

A publication by (Losey et al., 1999) of the potential risk of Bt maize pollen for larvae of the monarch butterfly *Danaus plexippus* (L.) (Lepidoptera: Danaidae) raised the attention of the public. The authors report that "larvae of the monarch butterfly reared on milkweed leaves dusted with pollen from Bt corn (Bt11), ate less, grew more slowly and suffered higher mortality than larvae reared on leaves dusted with untransformed corn pollen or on leaves without pollen". In a more accurate study (Jesse and Obrycki, 2000), also found negative effects of Bt maize pollen on survival of monarch larvae. As a consequence, several studies were conducted in a collaborative work under the instruction of the EPA (U.S. Environmental Protection Agency) in order to develop a formal risk assessment which should verify the ecological significance of these findings (Hellmich et al., 2001; Oberhauser et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Zangerl et al., 2001). Factors such as acute toxic effects of Bt maize pollen, the degree to which monarch larvae would effectively be exposed to pollen when feeding on milkweed in proximity of a transgenic maize field, and expression levels of Bt in the pollen of different events, were taken into account. Hellmich et al. (2001) reported that particularly first instar larvae were susceptible to purified Cry1A proteins and to the transgenic maize pollen of Event176, but not to the transgenic pollen from Bt11 and MON810, characterised by low expression levels in pollen (Koziel et al., 1993; Fearing et al., 1997). In fact, these findings were not unexpected since the susceptibility of several lepidopteran larvae to Bt toxins is already known (Miller 1990; Johnson et al., 1995; Peacock et al., 1998). The extended studies involving the full range of risk factors (exposure and toxicity) in the field, suggest that the ecological risks of Cry1Ab expressing Bt maize with regard to the monarch butterfly are negligible. Similar results were found in a study performed by (Wraight et al, 2000), who investigated the ecological effects of Bt maize pollen (MON810) on the black swallowtail butterfly, *Papilio polyxenes* (Fabricius) (Lepidoptera: Papilionidae).

Selected studies on effects of transgenic Bt maize on natural enemies

Emphasis is placed on the investigation of the potential impact of transgenic plants on natural enemies as these are important for natural pest regulation in crops (e.g., DeBach and Rosen, 1991). Accordingly, various studies investigating the impact of transgenic Bt maize on natural enemies have been conducted in the laboratory and in the field (for review see Romeis et al., submitted). A few of them are discussed in more detail.

A study conducted by Hilbeck et al., (1998a) arouse particular interest as it reported that survival of larvae of the green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), an important predator species, was reduced when fed on an artificial diet containing purified and activated Cry1Ab toxin at high concentrations. Another study showed that the predacious larvae were also affected when feeding on *O. nubilalis* and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), when these were previously fed with Bt maize (Hilbeck et al., 1998b).

More recent investigations showed that the predator is not affected when feeding on *T. urticae*, although the toxin levels measured in this herbivore were significantly higher than those in *S. littoralis* (Dutton et al., 2002). The study also showed that *S. littoralis* larvae were affected by the toxin themselves and that they represented a bad quality food source for *C. carnea* even when reared on non-Bt maize. For this reason, it is assumed that effects observed on *C. carnea* are caused by reduced prey quality rather than by the toxin itself. The assumption was also supported by Romeis et al., (2004) who could not detect any detrimental effects on this predator when the toxin was offered in a sugar solution. Some adverse effects were also reported on other predators or parasitoids, when these were offered Bt-intoxicated prey or host species (Bernal et al., 2002; Prütz and Dettner, 2005; Meissle et al., 2005; Vojtech et al., 2005). Given that all prey species used in these studies were lepidopteran, being affected by the toxin themselves, it is assumed that a reduction of prey quality was responsible for effects.

An important predator group in maize is that of *Orius* spp. (Heteroptera: Anthocoridae). Laboratory studies with *Orius majusculus* (Reuter) reported that this predator was not affected when feeding on the thrips, *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae), reared on Bt maize (Zwahlen et al., 2000). The question arouse, whether *O. majusculus* was effectively exposed to Bt toxin in the experiment when feeding on thrips, as the toxin uptake by this herbivore was not verified.

In addition to the laboratory studies, a large number of field studies had been performed with Bt maize, most of which measuring the abundance of various beneficial arthropod species. So far, no consistent negative effects on abundance of predators were reported (e.g., Orr and Landis, 1997; Pilcher et al., 1997; Bourguet et al., 2002; Jasinski et al., 2003; Musser and Shelton, 2003; Candolfi et al., 2004; de la Poza et al., 2005).

OUTLINE

Although Cry1Ab expressing Bt maize is the subject of various investigations dealing with non-target effects, many questions remain open. This may be partly due to the fact, that transgenic plants are relatively novel crops, and there is no regulatory procedure in place yet to assess their risks with a systematic and standardised protocol. On the other hand, previous studies have raised new questions, which require further investigations. The aim of this study is to address both issues that is, to make a further step in the risk assessment of Bt maize by investigating direct and indirect effects on selected arthropods taking into account their exposure to Cry1Ab toxin.

Effects of Bt maize on *Frankliniella tenuicornis* and exposure of thrips predators to prey-mediated Bt toxin

Thrips are common inhabitants of maize fields. An important and widespread thrips species is *Frankliniella tenuicornis*. This herbivore is preyed upon by various natural enemies such as anthocorids, chrysopids, coccinellids or phytoseiids. This study was performed to investigate the effects of Bt maize on thrips and to assess if thrips fed with Bt maize could mediate risks to their antagonists. The specific objectives of this study were to

- determine if thrips are affected when feeding on Bt maize
- verify if thrips ingest the Bt toxin when feeding on Bt maize and if differences exist among different thrips stages regarding their Bt content
- measure the persistence of Bt toxin in adult thrips and the toxin content in faeces
- investigate if exposure of a generalist predator (*Chrysoperla carnea*) can be influenced when faced with different thrips stages

Assessing the effects of Bt maize on the predatory mite *Amblyseius cucumeris*

Predatory mites are efficient predators of small arthropods such as spider mites or young thrips larvae, and some species are used as biological control agents. *Amblyseius cucumeris* is an omnivorous predator which is also known to feed on pollen. So far, effects of Bt maize on predatory mites have not been investigated. The objectives in this study were to

- evaluate an optimal testing system for *A. cucumeris* for tritrophic feeding experiments with *T. urticae* as a prey, which takes into account the state of the prey (alive or dead) and the toxin content in the food source
- assess sensitivity of *A. cucumeris* to Bt toxin by offering *Tetranychus urticae* reared on Bt maize
- assess the suitability of Bt maize (Bt11) pollen for *A. cucumeris*

Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*

The toxicity of Cry1Ab protein to predators can be investigated by exposing them either to purified and activated toxin produced by transformed *Escherichia coli* or to plant-derived toxin. Plant derived toxin cannot be obtained as a pure product and therefore needs to be offered to the predator either via herbivores or plant tissues (e.g., pollen) containing the toxin. In any case, it is important to ensure that the transgene product is present in a biologically active form in the offered food source and that it is ingested by the predator. The objectives of this study were to

- verify if *Chrysoperla carnea* larvae ingest Bt toxin when feeding on herbivores (*Tetranychus urticae* and *Spodoptera littoralis*) containing the toxin after feeding on Bt maize
- establish a feeding bioassay with *Ostrinia nubilalis* to determine whether Bt toxin remains biologically active when ingested by herbivores
- determine whether differences exist between plant-derived Cry1Ab toxin and purified toxin produced by *E. coli* bacterium

Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields

To date, many studies have been conducted to assess effects of Bt maize on various herbivores and predators. Although the potential exposure of arthropods was assessed in some of the laboratory studies, the selection of the species to be tested was rarely based on the likelihood of exposure in the field. The objectives of this study were to

- verify the passage of Cry1Ab toxin through the food chain and thus assess exposure of predators to the toxin in Bt maize fields in Spain
- investigate if toxin is transferred to neighbouring alfalfa fields by predator dispersal
- confirm the Bt toxin ingestion by the omnivorous predator *Orius majusculus* when feeding on different Bt-containing food sources (*Tetranychus urticae*, pollen, leaf) and determine persistence of the toxin in the predator in complementary laboratory studies

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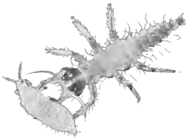
CHAPTER 1



EFFECTS OF BT MAIZE ON *FRANKLINIELLA TENUICORNIS* AND EXPOSURE OF THRIPS PREDATORS TO PREY-MEDIATED BT TOXIN[1]

ABSTRACT

As a part of a risk assessment procedure, the impact of Bt maize expressing Cry1Ab toxin on the thrips *Frankliniella tenuicornis* was investigated, and the potential risks for predators feeding on thrips on Bt maize were evaluated. The effects of Bt maize on *F. tenuicornis* were assessed by measuring life-table parameters when reared on Bt and non-Bt maize. The content of Cry1Ab toxin in different stages of *F. tenuicornis* reared on Bt maize, and the persistence of the toxin in adults was determined in order to evaluate possible exposure of predators when feeding on thrips. In addition, *Chrysoperla carnea* was used as a model predator to assess how the behaviour of prey and predator may influence the exposure of a natural enemy to the Bt toxin. Life-table parameter results showed that *F. tenuicornis* was not affected when it was reared on Bt maize. This indicates that the potential for prey quality-mediated effects on predators is low. Bt content was highest in thrips larvae and adults, and negligible in the non-feeding prepupal and pupal stages. The persistence of the Cry1Ab toxin in adult *F. tenuicornis* was short, resulting in a decrease of 97% within the first 24 h. Predation success by young *C. carnea* larvae varied among the thrips stages, indicating that exposure of predators to Bt toxin can additionally depend on the prey stage. When combining the current knowledge of the susceptibility of major thrips predators with our findings showing no potential for prey quality-mediated effects, relatively low toxin content in thrips as well as short persistence, it can be concluded that the risks for predators when feeding on thrips in or next to Bt maize fields are negligible.

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[1] Based on: Obrist, L.B., H. Klein, A. Dutton and F. Bigler. 2005. Effects of Bt maize on *Frankliniella tenuicornis* and exposure of thrips predators to prey-mediated Bt toxin. Entomologia Experimentalis et Applicata 115: 409-416.

INTRODUCTION

Risk assessment studies of an insect-resistant transgenic crop should include investigations of non-target effects on ecolocially and economically important species living in the crop. The effects of Bt maize expressing Cry1Ab have been tested on various non-target herbivore arthropods and their natural enemies (e.g., Hilbeck et al., 1998; Lozzia et al., 2000; Dutton et al., 2002). Thrips, although common inhabitants of maize fields (Schmitz and Bartsch, 2001), are often neglected since they normally do not inflict severe damage on maize and are therefore not considered as major pests in this crop (Steffey et al., 1999).

Frankliniella tenuicornis (Uzel) (Thysanoptera: Thripidae) is an important thrips species in central Europe. Its presence in maize crops was first described in 1926 by Hood and Herrick (taken from Köppä, 1970). This widespread species is known to recurrently cause considerable yield loss in small grain cereals in Northern Europe (Köppä, 1970; Kobro et al., 2000) and it also occurs in the USA (Beshear, 1979).

Thrips are reported to have many natural enemies in the field (Sabelis and van Rijn, 1997). The most well known of these are predatory bugs, including *Orius* spp. (Heteroptera: Anthocoridae), and predatory mites including *Amblyseius* spp. (Acari: Phytoseiidae). These predators are used as biological control agents against thrips and are therefore commercially available (Gillespie, 1989; van Houten et al., 1995). Chrysopids (Neuroptera: Chrysopidae) and coccinellids (Coleoptera: Coccinellidae) are important generalist predators which are also known to feed on thrips (Sabelis and van Rijn, 1997; Chang, 1998; Triltsch, 1999). All these predators occur naturally in maize fields (Hadam et al., 1986; Jarvis and Guthrie, 1987; Coll and Bottrell, 1991; Ayyappath et al., 1996; Iraola et al., 1997; Orr and Landis, 1997; Schmitz and Bartsch, 2001; Wold et al., 2001).

Herbivores can be negatively affected by a toxin when feeding directly on an insect resistant transgenic plant. In natural enemies, the insecticidal protein can also cause direct toxic effects both, when feeding on plant parts (e.g., pollen), or when ingesting herbivores containing the toxin. In addition, natural enemies can be "indirectly" affected by transgenic plants due to a reduction in prey quality or quantity (Ashouri et al., 2001; Couty et al., 2001; Bernal et al., 2002). These effects apply when the prey species is affected by the toxin itself, as it was shown for several non-target lepidopteran species, when feeding on Bt maize or Bt maize pollen (Hellmich et al., 2001; Dutton et al. 2002; 2005). For example, the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) was reported to be negatively affected when feeding on Bt-intoxicated prey (Hilbeck et al., 1998; Dutton et al., 2002). Recent studies have suggested, that these effects are more likely due to a reduction in prey quality than to the direct toxicity of the Cry1Ab protein to this predator (Romeis et al., 2004).

An important factor to consider when determining the risks of insect-resistant transgenic plants to natural enemies is the issue of whether the latter group is exposed to the insecticidal protein. The exposure of natural enemies depends on their feeding habits, as well as on the availability of food sources (e.g., prey) containing the toxin. Studies with herbivores feeding on Bt maize have revealed that large differences in the quantity of ingested Cry1Ab toxin exist among species (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002). Moreover, the exposure of natural enemies to a transgene product depends on the persistence of the toxin in the prey. Assuming that natural enemies will not only be present in the transgenic crop itself, but also in bordering vegetation, in adjacent fields, or in refuge areas used for resistance management (e.g., Johnson and Gould, 1992; Andow and Hutchinson, 1998; Gould, 1998), the persistence of the toxin in herbivores moving out of the transgenic crop should also be considered.

A study conducted with *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) fed with thrips [*Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae)] reared on Bt maize showed no negative effects on development and mortality of the predator (Zwahlen et al., 2000). However, it is not clear from this study whether the absence of effects was due to the unsusceptibility of *O. majusculus* to the Cry1Ab toxin or due to the absence of the toxin in the prey, as the toxin ingestion by thrips was not verified. In addition, the effects of Bt maize on thrips themselves have not yet been evaluated. The verification of the impact of Bt maize on the performance of thrips has also gained in importance, since Bourguet et al. (2002) have found inconsistent results when assessing the abundance of thrips in Bt and non-Bt maize fields.

The objectives of this study were to evaluate (a) the effects of Bt maize on life-table parameters of *F. tenuicornis* in order to assess the potential for prey-mediated effects on thrips predators; (b) the Bt toxin content and persistence in different stages of *F. tenuicornis* in order to assess the exposure of predators to Bt toxin when feeding on thrips; (c) the predation of different thrips stages by larvae of *C. carnea* in order to assess how the behaviour of prey and predator may influence the exposure of a natural enemy to the Bt toxin.

MATERIAL AND METHODS

Plants. Transgenic Bt maize (Bt11, N4640Bt; Syngenta, formerly Northrup King) expressing a truncated Cry1Ab toxin was used (from now on referred to as Bt^+) and the corresponding non-transformed near isogenic line (N4640) as a control ($Bt^–$). The Cry1Ab expression is driven by the constitutive CaMV 35S promotor. Plants were grown in individual plastic pots (3 l) and fertilized with 16%N: 6%P_2O_5: 26%K_2O at a concentration of 1g l^{-1} of water. Plants were kept in the greenhouse at environmental conditions of 24 ± 4 °C, 70 ± 10% RH. Four to five-week-old plants (5 – 7th leaf stage, 60 – 80 cm high) were used for the thrips colonies, and seven to nine-week-old plants (10 – 12th leaf stage, 95 – 140 cm high) were used for the experiments.

Insects. Insects were kept in climatic chambers at environmental conditions of 25 ± 1 °C, 70 ± 10% RH, and L16:D8 photoperiod. Experiments were conducted under the same conditions. *Frankliniella tenuicornis* was collected from a maize field at the Swiss Federal Research Station near Zurich in August 2001. Colonies were reared in cages on either Bt^+ or $Bt^–$ maize plants. *Chrysoperla carnea* eggs were collected from our permanent laboratory colony (Romeis et al., 2004) and kept individually until they hatched. Experiments were conducted with newly hatched (1 – 12 h-old) larvae.

Performance of *Frankliniella tenuicornis*

Life-table parameters, including egg development and hatching rate, preimaginal mortality and development, and female preoviposition time, longevity, number of eggs, and weight were assessed on thrips reared on $Bt^–$ or Bt^+ plants.

Egg development and hatching rate. Adult females of *F. tenuicornis* (100 – 200) were collected from $Bt^–$ and Bt^+ colonies and transferred into cellophane bags that were fixed on the 6th leaf of a respective plant ($Bt^–$ / Bt^+) as described above. Females were left 8 h to oviposit in the leaf. Subsequently, the females and bags were removed and the number of eggs was counted using a light-transmitting binocular. Eggs were marked and checked twice daily to monitor hatching. A total of 126 eggs on $Bt^–$ and 226 eggs on Bt^+ plants were used for calculating hatching rate and time.

Preimaginal development and mortality. To obtain thrips larvae of a similar age, 60 adult females per treatment were kept individually in clip-cages (6 x 8 x 19 mm) fixed on the 7th or 8th leaf of either Bt$^{–}$ or Bt^{+} maize plants, and allowed to lay eggs for 24 h. Subsequently the cages were removed, and all but one egg per cage-site were destroyed using a pin. After three days the leaf cages were reattached on the sites containing the egg, and the hatching time was recorded. Individuals were checked daily for survival and development until adult emergence. After verifying that there was no significant difference between the development time of males and females in either treatment (Mann-Whitney U-test, $U = 25.5$, $P = 0.24$ for Bt^{-}, $U = 45$, $P = 0.37$ for Bt^{+}), as had already been shown by Köppä (1970) and Malchau (1990), the data for male and female development time and preimaginal mortality was pooled. The experiment was repeated twice to get enough data to analyse the preimaginal development time. Total sample size was therefore 19 for Bt$^{–}$ and 22 for Bt^{+} treatment.

Adult females: preoviposition time, number of eggs, longevity and weight. Forty female pupae originating from each colony (Bt$^{–}$ / Bt^{+}) were introduced in clip cages placed on the distal third of the 7th leaf each with two adult males from the respective colony. Three days after female emergence, males were removed and the leaf-cage was placed on the distal third of the 8th leaf of the same plant. After four days the cages were transferred to the 7th leaf of a new plant of the respective age. Throughout the experiment, cages were transferred every three or four days to new plants, always using the 7th and the 8th leaf. Preoviposition time, survival and number of eggs laid by each female (29 Bt$^{–}$ and 30 Bt^{+}) were recorded daily for the first seven days. Thereafter, the females were checked for survival and deposited number of eggs every three or four days until death. Dead females were dried (60 °C, for 24 h) and weighed on a microbalance (Mettler Toledo, MX5, d = 1 µg; ± 2 µg).

Quantification of Cry1Ab toxin

Cry1Ab content in different stages of *Frankliniella tenuicornis*. Thrips were collected from Bt^{+} plants and frozen (–80 °C) to be subsequently separated by stage (larvae, prepupae, pupae and adults) and stored at –80 °C. An additional sample consisted of newly emerged adults, which had never been in contact with food from their pupation. Therefore, pupae originating from the Bt^{+} colony were collected, provided with water and kept in the climatic chamber until adult emergence. Newly emerged adults were frozen at

–80 °C. As a negative control, samples consisting of either adult thrips or a mixture of all preimaginal stages were collected from Bt⁻ plants and stored at –80 °C.

To determine the levels of Cry1Ab an Enzyme-Linked Immuno-Sorbent Assay (ELISA) was performed using a kit from EnviroLogix Inc. (Portland, Maine). Cry1Ab standards at concentrations of 0, 0.5, 2.5, and 5 ppb were used as calibrators. Spectrophotometric measurements were conducted with a microtiter plate reader (Dynatech MR 5000) at 450 nm and the data were analysed using the software package Biolinx 2.0 (Dynatech Laboratories Inc.) and Dynex Revelation G 3.2 (Dynex Technologies).

The samples under analyses were homogenised in extraction buffer at a concentration of 4 mg ml^{-1} extraction buffer. In addition, the extraction solutions containing adults and larvae were diluted by a factor two. Mean toxin content in the Bt^{+} samples was calculated from three to five replicates collected at different dates.

Persistence of Cry1Ab toxin in adult *Frankliniella tenuicornis* and toxin content in faeces. Adult thrips reared on Bt^{+} plants were collected and separated into five groups each containing approximately 100 individuals. One group (designated day 0) was immediately frozen at –80 °C. The remaining four groups were transferred into four cellophane bags (20.5 x 40 cm), which were fixed on the distal part of the 6th leaf of a Bt^{-} plant using two foam-coated wooden sticks sealed with clips. The bags were left on the plant in the greenhouse (24 ± 4 °C, 70 ± 10% RH) for either one, two, four or eight days (day 1, day 2, day 4, day 8). On each of these days one bag was removed from the plant, the living adult thrips were sorted out and stored at –80 °C. This experiment was repeated three times and the samples were analysed with an ELISA as explained above. The concentrations (mg material per ml extraction buffer) of the different samples under analyses were: 4 and 2 mg ml^{-1} day 0 and 8 mg ml^{-1} day 1, 2, 4 and 8. Mean toxin content in the samples (day 0, day 1, day 2, day 4, day 8) was calculated from the three replicates.

Dry thrips faeces were abraded from dry leaves originating from either Bt^{-} or Bt^{+} thrips colonies. Three samples from the Bt^{+} treatment were analysed with ELISA at a concentration of 0.125 mg ml^{-1} extraction buffer, whereas one sample was analysed from the Bt^{-} treatment at a concentration of 0.25 mg ml^{-1}. Because thrips faeces dry immediately after deposition it was not possible to determine the dry / fresh weight ratio.

Stage-dependant predation by *Chrysoperla carnea*

Neonate *C. carnea* larvae were placed individually in Petri dishes (25 mm in diameter, and 7 mm high) and offered either two 2nd instar thrips, two prepupae, two pupae or two adult thrips. Upon introducing individual *C. carnea* larvae into the Petri dishes, these were checked after 0.5, 1, 2, 3, 4, 5, and 6 h to record the time at which the first successful attack (killing the prey and feeding) occurred. Between 83 and 85 *C. carnea* were tested for each thrips stage.

Statistical analyses. All statistical analyses were computed in STATISTICA (version 5 and 6). For a comparison of the means, data sets were tested for normal distribution using the Shapiro-Wilk test. Given that all parameters contained at least one data set which was not normally distributed, only Mann-Whitney U-tests were performed. For comparisons of mortality and hatching rate,Chi-square tests were done. The stage-dependant predation by *C. carnea* was analysed using a logistic regression model (logit). The model assessed whether predation by *C. carnea* larvae as a function of time depended on the thrips stage offered and was used because it accounts for the binomial distribution of predation success (success = 1 / failure = 0). Therefore, stage was defined as a categorical and time as a continuous predictor. Verification of the interaction 'stage x time' was included by testing the homogeneity of slopes. The α - level was 0.05 for all tests.

RESULTS

Performance of *Frankliniella tenuicornis*

All life-table parameters are presented in Table 1.1. Egg hatching rate, as well as egg development time, were similar in both treatments. Thrips preimaginal mortality was high but not statistically different between treatments. Mortality was greatest during the first two days after hatching in both treatments. Mean development time and preoviposition time of the females were also not statistically different between the two treatments. Four females from each treatment did not lay eggs. A Chi-square test (χ^2 = 0.00, d.f. = 1, P = 0.99) confirmed the consistency between the two treatments. The average number of eggs per female was approximately 11 for both treatments and was not significantly different. A high variation of the longevity of females was observed, ranging from 2 – 42 days on Bt^- and 2 – 36 days on Bt^+ plants. However, no statistically significant difference between means was detected. No difference between treatments was found regarding the dry weight of females.

Table 1.1 Life-table parameters and adult weight of *Frankliniella tenuicornis* reared on Bt$^+$ and Bt$^-$ maize

Parameter	**Bt$^-$**	**Bt$^+$**	**Statistics**
Hatching rate (%)	91.3 (n = 126)	91.6 (n = 226)	χ^2 = 0.00, P = 0.92$^{1)}$
Mean egg development time (days ± SE)	4.4 ± 0.02 (n = 115)	4.5 ± 0.02 (n = 217)	U = 10630, P = 0.11$^{2)}$
Preimaginal mortality (%)	61.5 (n = 26)	62.5 (n = 22)	χ^2 = 0.00, P = 0.94$^{1)}$
Preimaginal development time (days ± SE)	8.4 ± 0.15 (n = 19)	8.0 ± 0.24 (n = 22)	U = 156, P = 0.16$^{2)}$
Mean preoviposition time (days ± SE)	2.6 ± 0.4 (n = 25)	2.4 ± 0.2 (n = 26)	U = 316, P = 0.87$^{2)}$
Mean number of eggs per female	11.9 ± 2.1 (n = 25)	11.1 ± 1.4 (n = 26)	U = 416, P = 0.77$^{2)}$
Mean female longevity (days ± SE)	21.9 ± 1.9 (n = 29)	24.1 ± 1.5 n = 30)	U = 375, P = 0.36$^{2)}$
Mean female dry weight (µg ± SE)	15.1 ± 0.6 (n = 25)	14.0 ± 0.8 (n = 29)	U = 294, P = 0.23$^{2)}$

1) Chi-square test, d.f. = 1 **2) Mann-Whitney U-test**

Quantification of Cry1Ab toxin

Cry1Ab content in different stages of *Frankliniella tenuicornis*. The highest Cry1Ab toxin content was measured in larvae, followed by adult *F. tenuicornis* (Table 1.2). Very low toxin levels were observed in the non-feeding prepupal and pupal stages, and Cry1Ab toxin was not detectable in newly emerged adult thrips, which were not provided with food after their pupation. The trace amounts of Cry1Ab detected in adults kept on Bt$^-$ maize could be due to cross reactions with other proteins or contamination.

Persistence of Cry1Ab toxin in adult *Frankliniella tenuicornis* and toxin content in faeces. A rapid decrease of toxin levels in adult thrips was observed over time after transfer from Bt$^+$ plants to Bt$^-$ plants (Figure 1.1). Within the first day (day 1), the toxin content decreased to only a fraction (3%) of the original Cry1Ab toxin content (day 0). After eight days of thrips feeding on Bt$^-$ plants no more toxin was detected.

The concentrations of Cry1Ab toxin measured in the faeces of *F. tenuicornis* were high but variable (Table 1.2). No toxin was detected in the faeces collected from the Bt$^-$ colony used as a negative control.

Table 1.2 Average Cry1Ab toxin concentrations (µg g^{-1} fresh weight ± SE) measured in different development stages of the thrips *Frankliniella tenuicornis* collected from Bt$^+$ and Bt$^-$ maize plants (n.d. = not detectable)

Developmental stage	Host maize plant	Replicates (n)	Toxin concentration
Larvae	Bt$^+$	3	1.38 ± 0.03
Prepupae	Bt$^+$	3	0.09 ± 0.02
Pupae	Bt$^+$	3	0.033 ± 0.006
Newly emerged adults	Bt$^+$	3	n.d.
Adults	Bt$^+$	5	0.98 ± 0.18
Preimaginal stages	Bt$^-$	4	n.d.
Adults	Bt$^-$	3	0.001 ± 0.000
Faeces$^{1)}$	Bt$^+$	3	32.01 ± 14.49
Faeces$^{1)}$	Bt$^-$	1	n.d.
Leaf$^{2)}$	Bt$^+$	5	3.77 ± 0.05
Leaf$^{2)}$	Bt$^-$	5	n.d.

1) Dry weight **2) Taken from Dutton et al. (2002)**

Stage-dependant predation by *Chrysoperla carnea*

Data of stage-dependant predation is represented in Figure 1.2. Logistic regression analyses revealed that differences existed in the ability of *C. carnea* to prey on different thrips stages as a function of time (Wald-statistic = 68.96, d.f. = 3, $P < 0.001$). Prepupae and pupae which are the least mobile thrips stages, were mostly attacked and killed by *C. carnea* within the first 30 min (79.5% and 84.4%, respectively). Second instar *F. tenuicornis*, the more mobile stage, were caught less successfully (55.4% after 30 min), and only 15.8% of the *C. carnea* larvae were able to prey on adults within the first 30 min.

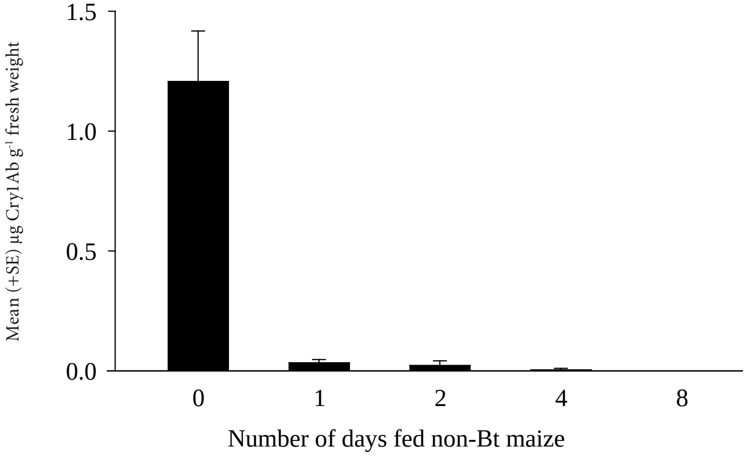

Figure 1.1 Average Cry1Ab toxin concentrations (µg^{-1} g fresh weight ± SE) measured in adult *Frankliniella tenuicornis* reared on Bt^{+} maize plants and subsequently fed on Bt^{-} maize plants for different periods of time (n = 3).

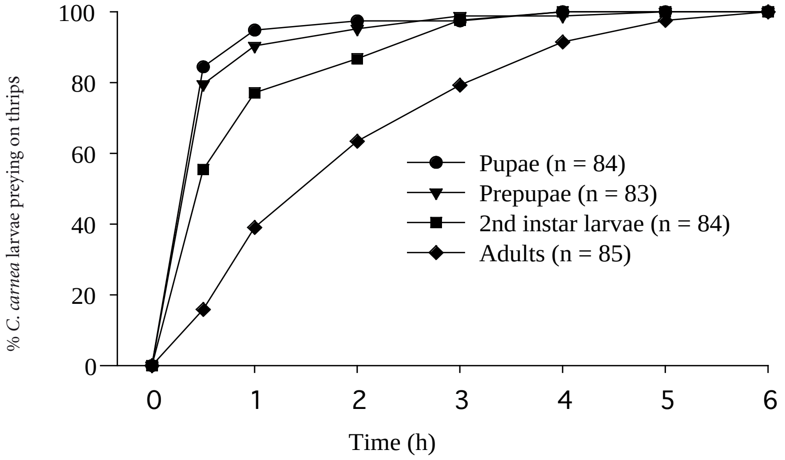

Figure 1.2 Predation rate of 1st instar *Chrysoperla carnea* on different *Frankliniella tenuicornis* stages as a function of time. (n = number of 1st instar *C. carnea*).

DISCUSSION

Our results demonstrate that potential prey-mediated effects of Bt maize on thrips predators are unlikely to occur, as thrips are not adversely affected when kept on transgenic plants. Although none of the life-table parameters measured for thrips kept on Bt^+ and Bt^- maize were shown to differ, some of the parameters (e.g., number of eggs per female, and longevity) were clearly lower than those reported in the literature where experiments were conducted on cereals (wheat and oat) (Köppä, 1970; Malchau, 1990). This could be due to either maize being a less suitable host plant for *F. tenuicornis* or to the experimental design used in our study. As our experiment differed from previous studies in both aspects, it was not possible to determine the conclusive reason for this difference. However, the fact that no differences in the life-table parameters were found between the treatments (Bt^+ and Bt^-), even under suboptimal conditions, indicates that the Cry1Ab protein expressed in Bt maize is not toxic to *F. tenuicornis*. Moreover, unintended changes in the plant, e.g., the higher lignin content in Bt maize as detected by Saxena and Stotzky (2001), were not shown to influence the performance of thrips on Bt maize. This finding suggests that the probability of prey quality-mediated effects occurring on predators when feeding on *F. tenuicornis* in Bt maize fields is minimal.

Our results show that Cry1Ab toxin concentrations vary substantially among the different thrips stages. This variation can be explained by the biology of the different stages of *F. tenuicornis*. Larvae and adults are the only feeding stages, and were shown to contain the highest toxin concentration. Second instar thrips stop feeding and undergo the two non-feeding prepupal and pupal stages. Until adult emergence, therefore, this herbivore does not feed, which accounts for the decreasing toxin concentrations in these stages. By the time adults emerged, the toxin was no longer detectable. Similar observations were made by Howald et al. (2003) with the herbivore *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) on Bt oilseed rape. Different herbivore species were also found to contain variable amounts of Bt toxin, depending on their feeding habits and metabolism (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002). For example, in spider mites the toxin concentrations were of the same order of magnitude as in Bt^+ leaf material (Dutton et al., 2002). In contrast, the toxin concentrations measured in larvae and adults of *F. tenuicornis* are relatively low, implying a low exposure and thus a reduced risk to natural enemies.

Cry1Ab toxin content in thrips decreases rapidly after cessation of feeding on Bt^+ plants. This was shown on the one hand by the decreasing toxin content in the non-feeding thrips stages (prepupae and pupae) and on the other by the short persistence of the toxin in the adult thrips. When the latter were transferred from Bt^+ to Bt^- plants, a reduction of

97% within the first 24 hours was observed. The short persistence of the Bt toxin in thrips reduces the risk for thrips predators being exposed to the toxin. This applies in particular to thrips in bordering vegetation or adjacent fields. The decrease of Bt toxin concentrations in thrips could be due to excretion or degradation of the toxin. The fact that very high concentrations of toxin were found in dry thrips faeces indicates that at least part, if not most, of the toxin is excreted. Bt toxin excretion has already been demonstrated for other non-target herbivores (Raps et al., 2001; Howald et al., 2003). The finding that high toxin levels are contained in faeces, which can, at heavy infestation, cover large leaf areas, reveals another route of exposure of the Bt toxin to non-target organisms (e.g., when licking dew or honeydew on leaves). This possible route of exposure should be taken into account in future risk assessment procedures.

The variability of toxin concentrations in different thrips stages suggests that the exposure of natural enemies to Bt toxin depends not only on the prey species (Dutton et al., 2002), but also on the prey stage they feed upon. Among other things, the behaviour of both predator and prey is a crucial factor influencing predation. Our studies have shown that pupal and prepupal thrips, which are constraint in their mobility, were killed most successfully whereas older larvae and winged adults were more likely to escape attacks of *C. carnea* larvae. The latter stages are more mobile and able to defend themselves by jerking their abdomen and secreting a droplet of rectal fluid (Bakker and Sabelis, 1989; Lewis, 1997). However, it has to be considered that in a natural system there are additional factors influencing predator-prey relationships (e.g., plant factors) and therefore the significance of these results in a field situation needs to be determined. In a natural system, prepupae and pupae are most likely to hide in protected plant parts such as in leaf sheaths (Köppä, 1970; personal observations). To what extent *C. carnea* larvae are able to enter such sites is not known and may likewise depend on their developmental stage. Our experiments showed that *C. carnea* larvae require more time to kill adult thrips than the other stages, and it was observed that the latter were hardly ever caught at the first attempt. However, in our experimental set-up, thrips could not escape and were thus killed after a while. In nature, the probability that an adult thrips escapes such attacks is high, except if it is “trapped” e.g., when about to lay an egg into leaf tissue.

Based on our studies and the current knowledge of the susceptibility of major predators including anthocorids, chrysopids, coccinellids and phytoseiids to Cry1Ab protein (Pilcher et al. 1997; Zwahlen et al., 2000; Al-Deeb et al., 2001; Dutton et al., 2002; Obrist et al., submitted), the risks for most predators when feeding on thrips in or next to a Bt maize field can be estimated as minimal due to the low toxin contents in thrips, the short persistence of the toxin in thrips and the absence of the potential for prey quality-mediated effects. In contrast, the high toxin levels found in thrips faeces, imply that arthropods can be exposed to Bt toxin when ingesting dew or honeydew on affected leaves. We suggest that this possible route of exposure should be taken into account for future risk assessment studies.

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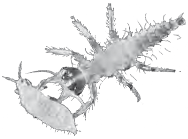

CHAPTER 2

ASSESSING THE EFFECTS OF BT MAIZE ON THE PREDATORY MITE *AMBLYSEIUS CUCUMERIS*[2]

ABSTRACT

The investigation of *Amblyseius cucumeris* in the context of the ecological risk assessment of insect resistant transgenic plants is of particular interest as this omnivorous predatory mite species is commercially available and considered important for biological control. In a multitrophic feeding experiment we assessed the impact of Bt maize on *A. cucumeris* by offering spider mites (*Tetranychus urticae*) reared on Bt (Bt11, Syngenta) or non-Bt maize (near isogenic line) and Bt or non-Bt maize pollen as a food source and measuring various life-table parameters (mortality, development time, oviposition rate). Spider mites were used as a prey for *A. cucumeris*, since these herbivores are known to contain levels of Cry1Ab toxin similar to those found in the transgenic leaf material when reared on Bt maize. In contrast, toxin levels in pollen of this transgenic cultivar are negligible. No differences in any of the parameters were found when *A. cucumeris* was fed with spider mites reared on Bt and non-Bt maize. Pollen was shown to be a less suitable food source for this predator as compared to spider mites. Moreover, subtle effects on female *A. cucumeris* (9% longer development time and 17% reduced fecundity) were detected when fed with pollen originating from Bt maize as compared to non-Bt maize pollen. Our findings indicate that the predatory mite *A. cucumeris* is not sensitive to the Cry1Ab toxin as no effects could be detected when offered Bt-containing spider mites. Thus, effects that were found when the predator was kept on Bt maize pollen were probably due to altered food quality rather than to the toxin. The significance of these findings is discussed with regard to the ecological relevance for risk assessment of transgenic plants.

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[2] Based on: Obrist, L.B., H. Klein, A. Dutton and F. Bigler. submitted. Assessing the effects of Bt maize on the predatory mite *Amblyseius cucumeris*.

INTRODUCTION

The adoption of insect resistant transgenic plants has caused many concerns and with those, the demand for detailed investigations of their potential risks for non-target organisms arouse. Investigations to determine the ecological risks of such plants for non-target organisms should include the assessment of potential hazards taking into consideration the exposure to the insect toxin (Groot and Dicke, 2002; Dutton et al., 2003a). Different types of adverse effects can be distinguished. Firstly, insecticidal proteins expressed by plants may cause direct toxic effects on a non-target organism provided that this organism is susceptible to the toxin. Secondly, organisms of higher trophic levels (e.g. predators) may be indirectly affected by reduced prey quality. Such prey-mediated effects can occur if the prey is affected by the toxin, which may lead to a reduction in prey abundance, size, or nutritional value (Ashouri et al., 2001; Couty et al., 2001; Bernal et al., 2002; Bell et al., 2003). Finally, non-target organisms may be indirectly affected by unintended changes in the plant caused by interferences of the inserted gene with the plant genome (Lecardonnel et al., 1999; Lumbierres et al., 2004). Such alterations are not directly related to the transgene product and can affect any phenotypic trait in the plant (Bhat and Chopra, 1999; Gutierrez-Campos et al., 2001; Saxena and Stotzky, 2001; Cellini et al., 2004).

When direct toxic effects of an insecticidal protein on non-target organisms are assessed, it is of importance to consider the degree of exposure to the transgene product. Species of higher trophic levels such as predators may be exposed to toxin expressed by plants in different ways. Arthropod predators can get in contact with the insecticidal protein when feeding on prey (e.g. herbivores) containing this toxin. Some predators are omnivorous and may be exposed to the toxin when feeding directly on plant parts (e.g., leaf, pollen) provided that the toxin is present in these tissues (Dutton et al., 2003a).

Bt maize expressing Cry1Ab toxin is cultivated in many maize growing areas since the commercialisation of transgenic plants in 1996 (James, 2004). The economic benefits of its cultivation can be attributed to its resistance against larvae of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae), two severe lepidopteran pests in maize (Conzàlez-Núñez et al., 2000; Brookes, 2002). Different Bt maize varieties exist in which toxin expression is driven by diverse promoters. Bt11 or MON810 express the toxin under the Cauliflower Mosaic Virus promoter (CaMV 35S) leading to significant expression levels in most of the plant tissues but not in pollen. In contrast, in Event176 the toxin is expressed in green leaves and pollen due to the presence of two maize specific promoters, but toxin expression is low in roots, pith and kernels (Koziel et al., 1993). The toxin expression pattern in plants influences the toxin ingestion by herbivores. This was demonstrated by measuring the toxin content in various herbivores using Enzyme-Linked Immuno-Sorbent Assays (ELISA) (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002; Obrist et al., 2005). Spider mites, *Tetranychus urticae* (Koch) (Acari: Tetranychidae) kept on transgenic maize contained highest Cry1Ab toxin levels as compared to all other chewing and sucking herbivores tested so far. In laboratory studies, the toxin levels measured in spider mites were of the same order of magnitude as those measured in maize leaves. In samples collected from the field, the levels of Bt toxin exceeded those of the plant by a factor of three (Obrist et al., in press a). Moreover, the toxin in spider mites was shown to remain biologically active after ingestion (Obrist et al., in press b).

In a risk assessment scheme, the arthropod predators to be tested for adverse effects should be selected according to their likelihood of exposure to the toxin, and to their economic and ecological importance in the agro-ecosystem (Dutton et al., 2003a). To date, the impact of Bt maize on many important predators has been investigated (e.g., Pilcher et al., 1997; Hilbeck et al., 1998; Zwahlen et al., 2000; Dutton et al., 2002), but predatory mites have not yet been taken into account although their presence in many crops including maize is recognised as well as their potential to regulate pests (McMurtry et al., 1970; Hadam et al., 1986; Pickett and Gilstrap, 1986; Messenger et al., 2000; Croft et al., 2004). Previous studies reported some negative effects on predatory mites when sprayed with a microbial Bt formulation containing Cry1Ab toxin (Dipel, *Bacillus thuringiensis* var. *kurstaki* HD-1 strain) (Hassan et al., 1987; Chapman and Hoy, 1991). However, such Bt formulations contain a mixture of Cry-toxins (δ-endotoxins) and other types of toxins (exotoxins) (Glare and O'Callaghan, 2000). Furthermore, it cannot be ruled out that other components of commercial preparations such as carriers, UV screens or inert ingredients were responsible for such effects (Croft and Flexner, 1990).

The generalist predatory mite *Amblyseius cucumeris* (Oudemans) (Acari: Phytoseiidae) is a cosmopolitan species which is also reported to occur in maize (Iraola et al., 1997). This predator is commercially available mainly for thrips control in greenhouses (Gillespie, 1989; Hoy and Glenister, 1991; van Houten et al., 1995). Being a generalist predator, *A. cucumeris* also feed on small arthropods including all stages of phytophagous mites (McMurtry and Croft, 1997; Blackwood et al., 2001). In addition, this species is known to feed on pollen and is able to develop and reproduce when kept exclusively on this food source (van Rijn and van Houten, 1991; McMurtry and Croft, 1997).

The aim of this study was to assess the impact of Bt maize on the performance of *A. cucumeris* using food sources from different trophic levels. The experiment consisted in measuring different life-table parameters of *A. cucumeris* when offered spider mites (*T. urticae*) reared on Bt or non-Bt maize and Bt and non-Bt maize pollen. Prior to the experiment, an optimal testing system for the tri-trophic experiment was evaluated taking into account the state of the prey (alive or dead) and toxin concentration in the food source.

MATERIAL AND METHODS

Plants. Transgenic Bt maize (NT4640Bt; Syngenta, formerly Northrup King) expressing a gene encoding a truncated version of the Cry1Ab toxin (from now on referred to as Bt$^+$) was used as well as the corresponding non-transformed near isogenic variety (NT4640) as a control (Bt$^-$). Plants were grown individually in plastic pots (3 l volume) in the greenhouse at environmental conditions of 24 ± 4 °C, 70 ± 10% RH and fertilized with 16%N: 6%P_2O_5: 26%K_2O at a concentration of 1 g l^{-1} of water. Pollen was collected by placing individual inflorescences of 30 plants into air-permeable cellophane bags (20.5 x 40 cm) sealed with paper clips. After 24 h, the cellophane bag containing the inflorescence was cut and the pollen collected in the bag was sieved and dried for 24 h at ambient conditions. The dried pollen was pooled and stored at –80 °C until use.

Mites. Spider mites (*T. urticae*) were kept in separate colonies on either Bt$^-$ or Bt$^+$ maize plants (older than 10 weeks) in the greenhouse at environmental conditions of 24 ± 4 °C, 70 ± 10% RH. Spider mites were collected in a tray kept underneath infested leaves by shaking them with a stick. The collected spider mites were transferred into plastic boxes (13 x 10.5 x 5 cm), which were put on a frigistor in order to restrict their mobility during handling.

Predatory mites (*A. cucumeris*) were obtained from Andermatt Biocontrol (Switzerland). Colonies were kept in a climatic chamber at 25 ± 1 °C, 85 ± 10% RH, 16:8 L:D and provided with *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs. For experiments, protonymphs of the same age were used. To synchronize the colony, 80 – 100 gravid females were transferred into 8 – 10 cages (12.5 x 10 x 5 cm) and provided with food, water and a few fibres of cotton wool as a substrate for oviposition. After 24 h the eggs were removed and transferred individually into cages (2.7 x 1 cm, diameter x height) containing 5 µl of water. *Amblyseius cucumeris* pass through a non-feeding larval stage and only start foraging after the 1st molt i.e., as protonymphs. Therefore, the vials were checked twice a day for hatching of protonymphs, and if necessary supplied with water.

Cry1Ab toxin content in food sources

Four samples of each of the food source were taken for measuring the Bt toxin content using ELISA. In addition, the variation of Cry1Ab toxin concentration in *T. urticae* over a period of 24 h was measured, in order to verify the exposure of *A. cucumeris* when kept in different testing systems. Therefore, spider mites were collected from Bt^+ maize and subsequently divided into three groups. The first group was kept alive at 5 °C for 30 minutes, whereas the other two groups were killed either by freezing at –80 °C or by exposing the mites to acetic acid ethyl ester (from now on referred to as AAEE) for 30 minutes. One initial sample of living spider mites was taken and stored at –80 °C. Then, the spider mites from each treatment were distributed into five boxes containing a water soaked filter paper and kept in the climatic chamber (25 ± 1 °C, 85 ± 10% RH, 16:8 L: D). A sample (individuals originating from one box) of each treatment was taken after 1, 2, 4, 8, and 24 h.

To determine the levels of Cry1Ab toxin, ELISA was performed using kits from EnviroLogix Inc. (Portland, Maine USA). Cry1Ab standards at concentrations 0, 0.5, 2.5, and 5 ppb were used as calibrators. Spectrophotometric measurements were conducted with a microtiter plate reader (Dynatech MR 5000) at 450 nm and data was analysed using the software package Biolinx 2.0 (Dynatech Laboratories Inc.) and Dynex Revelation G 3.2 (Dynex Technologies). Pollen was analysed at a concentration of 30 mg ml^{-1} extraction buffer. Spider mites were extracted at a concentration of 2 mg ml^{-1} extraction buffer and four times diluted for the spectrophotometric measurements. Control samples (Bt^- pollen and spider mites) were extracted at the respective concentrations but analysed undiluted, to verify possible cross-reactions with other proteins.

Influence of prey state on performance of *Amblyseius cucumeris*

In order to determine an optimal testing system for *A. cucumeris*, we assessed the influence of the prey state on development time of *A. cucumeris* in a preliminary experiment. Spider mites reared on Bt^- maize were used as a prey which was offered alive or killed either by freezing at –80 °C or using AAEE as described above.

Cages (2.7 x 1 cm, diameter x height) were prepared containing 2 µl of water and sufficient *T. urticae* (8 – 15 according to their size) which were either alive, killed by freezing or AAEE. Only males or non-ovipositing stages of spider mites were used to prevent that eggs were laid by adult females, as the toxin content in eggs is not known. Molting was checked daily until adult eclosion. For each treatment 23 *A. cucumeris* were tested.

All statistical analyses were computed in STATISTICA (Version 5.5, StatSoft Inc., Tulsa, USA). Comparisons between means of development time were made using Kruskal-Wallis ANOVA. Pairwise comparisons were made using Mann-Whitney U-test adjusted for ties and an adjusted α–level obtained from sequential Bonferroni tests (Holmes procedure).

Performance of *Amblyseius cucumeris* on Bt$^-$/Bt$^+$ spider mites

Life-table parameters including preimaginal mortality, development time, preoviposition time, and fecundity of *A. cucumeris* were assessed when fed with *T. urticae* reared on Bt$^-$ or Bt$^+$ maize plants. We used living *T. urticae* as a food source in this experiment, since the preliminary experiment indicated that this state of prey is the most suitable (see results).

A total of 90 newly emerged protonymphs were transferred into individual cages containing 2 µl of water and sufficient (8 – 15 according to their size) non-ovipositing stages of *T. urticae* originating from either Bt$^-$ or Bt$^+$ plants. The cages were exchanged daily and checked twice a day for exuvie, until adult eclosion. The development time of the feeding preimaginal stages (protonymph and deutonymph) was taken as one parameter. Adults were sexed, and males were removed from the experiment. Each female was provided with two males and food as described above. To determine the preoviposition time, the cages were checked twice a day. Thereafter, oviposition was recorded until eight days after reaching adulthood. A minimum of forty eggs of known deposition time (± ½ day) were collected and transferred individually into cages and provided with sufficient water. The eggs were checked twice a day for hatching of larvae and protonymph. The development time of egg and larvae of the progeny was taken as one parameter as the latter stage was extremely short (< 1 d).

Performance of *Amblyseius cucumeris* on Bt$^-$/Bt$^+$ maize pollen

The same experiment as described above was repeated with pollen originating from either Bt$^-$ or Bt$^+$ maize. The cages containing pollen were prepared at least one hour before the transfer of the arthropods. Using a small spatula, pollen (approximately 50 – 100 grains) was added to cages containing 2 µl of water. The cages were incubated in the climatic chamber in order to swell the dried pollen before use. The experiment was conducted in an analogous way as that with spider mites and repeated three times. The first experiment started with a total of 44, the second with 56 and the third with 80 protonymphs per treatment. In the first experiment, an additional Bt^- spider mite treatment (initial n = 44) was performed as a reference. This was done to compare spider mites to pollen as a food source for *A. cucumeris* in the same experiment as experimental effects were expected.

All arthropods that escaped or were killed due to handling were discarded for statistical analyses. The data for development time, preoviposition time and egg/larval development time was transformed by subtracting a random number between 0.0 and 0.5 (accounting for the time span between two observation events). This was done, as the development time in our experiment was short and synchronistic which led to an unfavourable distribution for analyses. Comparisons of means were made using one-way or factorial ANOVA depending on the number of categorical predictors that needed to be tested [e.g., treatment (Bt^-/Bt^+), sex, experiment effect]. Chi-square tests were applied to compare ratios.

Amblyseius cucumeris and *Tetranychus urticae* (left)

RESULTS

Cry1Ab toxin content in food sources

The mean toxin concentrations measured in *T. urticae* reared on Bt^+ maize was 5.13 ± 0.68 µg g^{-1} fresh weight (± SE). Trace amounts of toxin were detected (0.15 ± 0.02 µg g^{-1}; ± SE) in spider mites reared on Bt^- maize. This was also found in previous studies and could be attributed to cross-reactions with other proteins (Dutton et al., 2002; Obrist et al., in press b). In Bt^+ pollen trace amounts of toxin were measured (below quantification limit) and no toxin was detected in Bt^- pollen. Toxin content in Bt^+-fed spider mites, which were kept for 24 h in cages, when either kept alive, or killed by freezing or using AAEE is presented in Figure 2.1. After 24 h, toxin was still present in spider mites from all treatments even though some reduction in toxin concentration (approximately 35%) occurred when they were kept alive

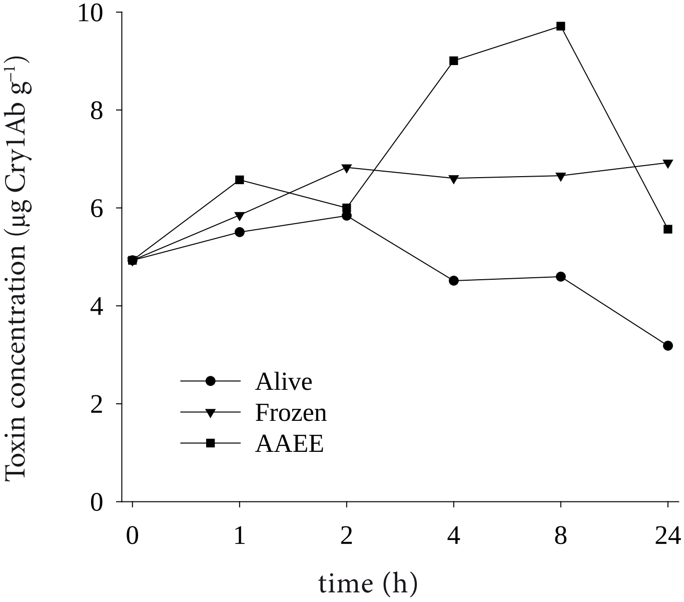


Figure 2.1 Cry1Ab toxin concentration (µg g^{-1} fresh weight) in *Tetranychus urticae* at 1, 2, 4, 8, and 24 h after being kept in cages either alive or dead. Dead individuals were killed by freezing at −80 °C or by exposure to acetic acid ethyl ester (AAEE).

Influence of prey state on performance of *Amblyseius cucumeris*

Mean development times (± SE) of *A. cucumeris* were 4.0 ± 0.13, 4.52 ± 0.12, and 4.57 ± 0.18 days when fed with spider mites kept alive, killed by freezing or AAEE respectively. Development times depended on the state of the offered spider mites (Kruskal-Wallis test; H = 10.7, d.f. = 2, n = 69, P = 0.005). Predatory mites kept on living spider mites developed significantly faster than those kept on frozen spider mites (Mann-Whitney U-test adjusted for ties and sequential Bonferroni tests; U = 147.5, P = 0.002) and those killed using AAEE (U = 164, P = 0.007). No differences were found between development time of *A. cucumeris* kept on dead spider mites, killed by freezing or AAEE (U = 254, P = 0.79).

Performance of *Amblyseius cucumeris* on Bt^-/Bt^+ spider mites and pollen

When fed with spider mites, none of the life-table parameters measured for *A. cucumeris* was shown to depend on treatment (Bt^-/Bt^+) (Table 2.1). In addition, no differences

Table 2.1 Life-table parameters of *Amblyseius cucumeris* when kept on Bt^+ and Bt^- maize-fed spider mites. (PN = protonymph; DN = deutonymph; E = egg; L = larvae)

Parameter	Bt^-	Bt^+	Treatment effect
Mortality (PN+DN; %)	0 (n = 45)	0 (n = 45)	– *
Mean female development time (PN+DN; d ± SE)	3.96 ± 0.06 (n = 23)	3.86 ± 0.05 (n = 26)	$F_{1,47}$ = 0.003; P = 0.96 2)
Mean male development time (PN+PN; d ± SE)	3.84 ± 0.06 (n = 22)	3.87 ± 0.05 (n = 18)	$F_{1,38}$ = 0.61; P = 0.44 2)
Mean preoviposition time (d ± SE)	3.38 ± 0.12 (n = 21)	3.63 ± 0.29 (n = 24)	$F_{1,43}$ = 0.62; P = 0.43 2)
Mean number of eggs per female	7.0 ± 0.86 (n = 21)	8.75 ± 0.77 (n = 24)	$F_{1,43}$ = 2.3; P = 0.14 2)
Non-ovipositing females (%)	8.7 (n = 23)	0 (n = 24)	χ^2 = 2, P = 0.16[1]
Mean progeny development time (E+L; d ± SE)	2.89 ± 0.04 (n = 41)	2.96 ± 0.03 (n = 46)	$F_{1,85}$= 0.66, P = 0.42[2]

1) Chi-square test, d.f. = 1 **2) One-Way ANOVA** ***no difference**

were detected between male and female development time (Factorial ANOVA for sex; $F_{1,85}$ = 1.01, P = 0.32). When fed with pollen, treatment effects were observed for female *A. cucumeris* but not for males (Table 2.2). Moreover, factorial ANOVA revealed that differences between male and female development time existed (sex; $F_{1,85}$ = 31.0, P < 0.0001) as well as an interaction between treatment and sex (treatment * sex; $F_{1,314}$ = 8.7, P = 0.003). This interaction was caused by the fact, that significant differences in male and female development time could only be detected for the Bt^+ pollen treatment [Factorial ANOVA sex (Bt^+ pollen); $F_{1,154}$ = 37.2, P < 0.0001].

A strong experimental effect (all P < 0.0001) was found for all parameters except for egg and larval development time of the progeny ($F_{2,125}$ = 2.6, P = 0.08). A comparison between pollen and spider mites (both Bt^-) as a food source for *A. cucumeris* was therefore only done with the data from the additional Bt^- spider mite treatment that was done simultaneously with the first pollen experiment (Table 2.3). Spider mite feeding significantly shortened male and female development time of *A. cucumeris* as compared to pollen feeding. In addition, females had a shorter preoviposition time and laid more eggs when kept on spider mites. Development time of males and females was similar when fed with spider mites (Factorial ANOVA for sex; $F_{1,81}$ = 1.4, P = 0.24).

Table 2.2 Life-table parameters of *Amblyseius cucumeris* when kept on Bt^+ and Bt^- maize pollen (PN = protonymph; DN = deutonymph; E = egg; L = larvae)

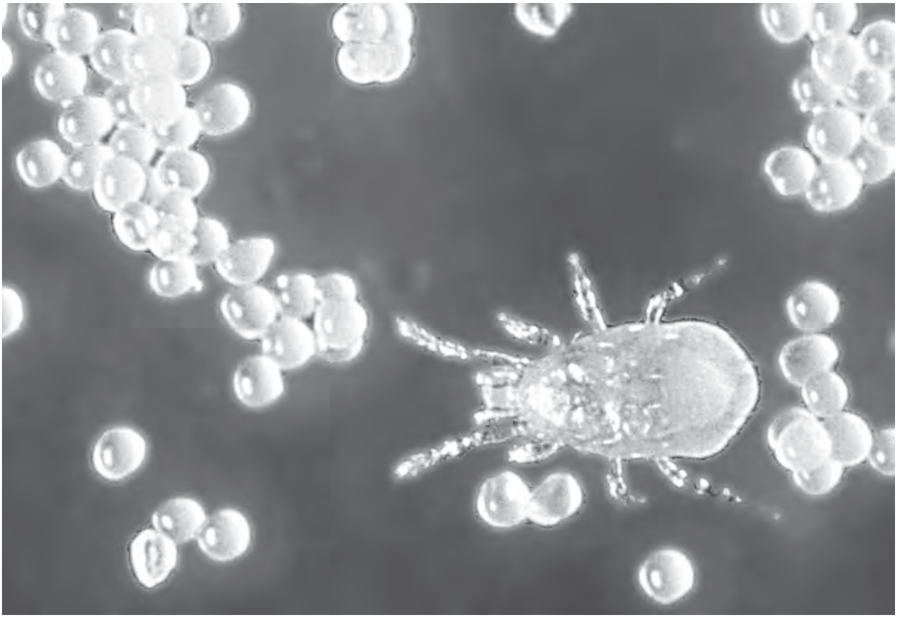
Parameter	**Bt^-**	**Bt^+**	**Treatment effect**
Mortality (PN+DN; %)	1.1 (n = 170)	5.3 (n = 170)	χ^2 = 2.68, P = 0.10¹⁾
Mean female development time (PN+DN; d ± SE)	4.1 ± 0.06 (n = 78)	4.48 ± 0.11 (n = 70)	$F_{1,142}$ = 13.3;P < 0.0004²⁾
Mean male development time (PN+PN; d ± SE)	3.93 ± 0.09 (n = 88)	3.87 ± 0.07 (n = 90)	$F_{1,172}$ = 0.41; P = 0.52²⁾
Mean preoviposition time (d ± SE)	2.55 ± 0.06 (n = 73)	2.76 ± 0.09 (n = 60)	$F_{1,127}$ = 3.6; P = 0.06²⁾
Mean number of eggs per female	6.17 ± 0.26 (n = 66)	5.08 ± 0.34 (n = 63)	$F_{1,123}$ = 8.0 = 0.006²⁾
Non-ovipositing females (%)	1.4 (n = 74)	9.1 (n = 66)	χ^2 = 4.4, P = 0.036¹⁾
Mean progeny development time (E+L; d ± SE)	3.05 ± 0.05 (n = 73)	3.07 ± 0.08 (n = 58)	$F_{1,125}$ = 0.05, P = 0.82²⁾

1) Chi-square test, d.f. = 1 **2) Factorial ANOVA**

Table 2.3 Life-table parameters of *Amblyseius cucumeris* when kept on spider mites (*Tetranychus urticae*) and maize pollen (both Bt$^-$). (PN = protonymph; DN = deutonymph; E = egg; L = larvae)

Parameter	Bt$^-$	Bt$^+$	Treatment effect
Mortality (PN+DN; %)	0 (n = 44)	2.3 (n = 44)	χ^2 = 0.99, P = 0.32[1]
Mean female development time (PN+DN; d ± SE)	3.7 ± 0.10 (n = 23)	4.5 ± 0.12 (n = 18)	$F_{1,39}$ = 29.7; P < 0.000[2]
Mean male development time (PN+PN; d ± SE)	3.40 ± 0.10 (n = 21)	4.36 ± 0.25 (n = 23)	$F_{1,42}$ = 10.9; P = 0.002[2]
Mean preoviposition time (d ± SE)	2.32 ± 0.14 (n = 22)	2.82 ± 0.18 (n = 17)	$F_{1,37}$ = 4.55; P = 0.040[2]
Mean number of eggs per female	6.17 ± 0.26 (n = 21)	5.08 ± 0.34 (n = 16)	$F_{1,35}$ = 5.58 = 0.023[2]
Non-ovipositing females (%)	0 (n = 22)	0 (n = 17)	– *
Mean progeny development time (E+L; d ± SE)	2.86 ± 0.04 (n = 49)	2.91 ± 0.05 (n = 48)	$F_{1,95}$ = 0.063, P = 0.80[2]

1) Chi-square test, d.f. = 1 **2) One-Way ANOVA** *** No difference**



Amblyseius cucumeris on maize pollen

DISCUSSION

When using multitrophic testing systems to assess effects of a transgene product on non-target organisms, it is important to consider the toxin concentrations in the food source. Our ELISA measurements confirmed that spider mites contained Cry1Ab toxin when reared on Bt maize. In addition, we have shown that 65% of the toxin is still present in spider mites after 24 h of food deprivation. A constant exposure of *A. cucumeris* to Bt toxin in the subsequent experiments was therefore ensured when providing predatory mites with living spider mites. The reduction in toxin concentration in living spider mites during 24 h was unexpectedly low. Previous studies with the herbivorous thrips *Frankliniella tenuicornis* (Uzel) (Thysanoptera: Thripidae) revealed a much shorter persistence with a reduction of 97% within 24 h (Obrist et al., 2005). This discrepancy could partly be due to the fact that the thrips were kept on Bt^- maize during this period. Food ingestion may have influenced their metabolism and stimulated excretion of the toxin. In contrast to spider mites, Bt^+ maize pollen contained negligible toxin levels. This finding was expected given that the Cry1Ab protein is only expressed at minimal levels in pollen of Bt11 (Koziel et al., 1993).

The state of the food is an important factor influencing the development of *A. cucumeris*. This was shown in the preliminary experiment in which the development time of the predator was clearly faster when offered living spider mites as compared to dead mites. For this reason, spider mites were kept alive in the following experiments. It appears interesting that living spider mites represent a better food source for *A. cucumeris*, as difficulties with predation due to intensive webbing by spider mites could have been expected (Abdallah et al., 2001; Vantornhout et al., 2005). The prolonged development time of *A. cucumeris* when fed with dead spider mites could possibly be due to a variation of the water balance in the prey. Although kept under equal conditions, it was more difficult to maintain the water balance constant in cages containing dead prey.

In spite of the high toxin levels that were measured in Bt^+ maize-fed spider mites, no detrimental effects were detected on *A. cucumeris* when kept on this herbivore. This indicates that *A. cucumeris* is not sensitive to the Cry1Ab toxin at the concentrations they were exposed to in this study. Additionally, it suggests that previously reported effects of some microbial Bt formulations on predatory mites (Chapman and Hoy, 1991) are probably rather due to other compounds contained in the Bt formulation than to the Cry1Ab protein. This assumption is fortified by analogous findings for *T. urticae,* which were only affected by Bt spray (Dipel) but not by Cry1Ab toxin in plants (Dutton et al., 2002; 2003b).

Maize pollen was a less suitable food source for *A. cucumeris* as compared to spider mites. Moreover, subtle differences in the suitability of Bt^- and Bt^+ maize pollen were observed. The effects were only noted for females, which had prolonged development time (+9%) and reduced fecundity (-17%) when kept on Bt^+ maize pollen. Interestingly, we also detected a difference between male and female development time but only when kept on Bt^+ maize pollen. Based on literature, equal development time of male and female *A. cucumeris* was expected (Dosse, 1955; Castagnoli, 1989). As the toxin content in Bt^+ maize pollen is negligible, in particular, when compared to that in spider mites, the effects we measured cannot be attributed to the Cry1Ab protein. A more plausible explanation is that Bt^+ maize pollen represented a food source of altered quality for *A. cucumeris* and therefore the findings should rather be classified into the category of unintended effects. Indeed, we cannot rule out that they may be due to a genetic divergence of the Bt^+ strain from the parent line as a consequence of breeding procedures after the transformation process (Conner, 2003; Kok and Kuiper, 2003).

The suitability of pollen as a food source for *Amblyseius* spp. is highly depending on the plant species (Schausberger, 1992; van Rijn and Tanigoshi, 1999; Pratt et al., 1999). This might be attributed to the variable nutritional value and digestibility of pollen coming from different plant species (Roulston and Cane, 2000). Moreover, the nutritional profile of pollen was shown to be variable among different maize hybrids (one transgenic and several non-transgenic) (Lundgren and Wiedenmann, 2004). When *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) was fed with pollen originating from these hybrids, the authors observed different mortality rates which strongly correlated with the nutritional profile of the pollen. Dutton et al. (A. Dutton, M. D'Alessandro, J. Romeis, D. Babendreier and F. Bigler, in preparation) also observed differences in nitrogen and total amino acid content in different maize varieties. In the same study they showed that the amino acid composition tended to be slightly lower in Bt^+ maize pollen (MON810) when compared to the near isogenic hybrid. Although these differences were not statistically confirmed, and their significance can hardly be estimated, it is conceivable that differences in the nutritional profile exist between pollen of the transgenic variety we tested and the near-isogenic line. These could have been responsible for the effects that measured on the performance of female *A. cucumeris*.

The effects that we measured when *A. cucumeris* was kept on Bt^+ pollen, should be interpreted with care. Firstly, our testing system allowed for detecting very subtle effects and it can be assumed that pollen originating from different maize varieties would cause similar differences. The relevance of our findings should therefore be determined by comparing the suitability of pollen of several maize varieties (Kok and Kuiper, 2003; O'Callaghan et al., 2005). Secondly, in a natural system, many additional factors may influence the quality of pollen. Age and state of degradation may considerably affect the nutritional quality of pollen as even storing pollen for a few days results in altered amino acid composition (Linskens and Pfahler, 1973). Such factors would probably have stronger impacts on performance of these predators in the field than differences between Bt^- and Bt^+. Moreover, one has to keep in mind that *A. cucumeris* feeds on a mixture of plant and arthropod sources of varying quality. This may compensate or mask the impact on its performance caused by variable quality of pollen originating from different maize varieties.

The present study shows that Cry1Ab toxin is not toxic for the predatory mite *A. cucumeris* as individuals performed equally on Bt^+ and Bt^--fed spider mites. In addition, it was shown that maize pollen as a food source represents a lower quality food source for this predator when compared to spider mites. Some differences in the performance of female predators when fed with Bt^- or Bt^+ maize pollen were observed which may be attributed to possible alterations in the nutritional quality of Bt^+ pollen. The biological relevance of this finding appears minor, as nutritional profile of pollen originating from different maize varieties is known to be variable. Moreover, in a field situation, the predator feeds on a range of food sources and pollen can only be used during a restricted time period.

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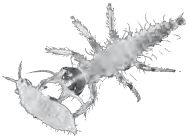
Chapter 3



Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*[3]

Abstract

A major concern regarding insect resistant transgenic plants is their potential impact on non-target organisms, in particular on beneficial arthropods such as predators. To assess the risks that such plants pose to predators, various experimental testing systems can be used. When using tritrophic studies, it is important to verify the actual exposure of the tested predator, that is to say, the passage of biologically active toxin through the herbivorous arthropod (prey). We therefore investigated the uptake of Cry1Ab toxin by larvae of the green lacewing, *Chrysoperla carnea*, when feeding on two Bt maize-fed herbivores (*Tetranychus urticae* and *Spodoptera littoralis*) by means of an immunological test (ELISA), and the activity of the Cry1Ab toxin following ingestion by the herbivores. Moreover, we compared the activity of Cry1Ab toxin produced by Bt maize to that of purified toxin obtained from transformed *Escherichia coli*, which is recommended to be used in toxicity studies. The toxicity was assessed in feeding bioassays with larvae of the European corn borer, *Ostrinia nubilalis*, the target pest of Cry1Ab expressing maize. ELISA confirmed the ingestion of Bt toxin by *C. carnea* larvae when fed with either of the two prey species. The feeding bioassays showed that the biological activity of the Cry1Ab toxin ingested by herbivores is maintained. These findings are discussed in the context of previous risk assessment studies with *C. carnea*. The purified Cry1Ab protein was more toxic to *O. nubilalis* compared to the plant-derived Cry1Ab toxin when applied at equal concentrations according to ELISA measurements. A possible reason for this finding could be the different origin of production of the toxin (plant and bacterium).

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[3] Based on: Obrist, L.B., A. Dutton, J. Romeis and F. Bigler. Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. BioControl (in press).

INTRODUCTION

A major concern regarding the deployment of insect resistant transgenic plants is their potential impact on non-target arthropods, in particular on natural enemies, as they are considered important for natural pest regulation (Groot and Dicke, 2002; Dutton et al., 2003). To assess the risks of insect resistant transgenic plants, it has been suggested to follow a tiered testing system going from laboratory "worst case" to field studies (Cowgill and Atkinson, 2003; Dutton et al., 2003; Poppy and Sutherland, 2004). "Worst case " laboratory studies in which the insecticidal protein is offered directly to the test organisms at excessive concentrations should be followed by extended laboratory studies in multitrophic testing systems which include natural routes of exposure to the toxin as well as species interactions. When assessing non-target effects of insect resistant transgenic plants on natural enemies by performing such multitrophic feeding experiments it is important to verify that representatives of the lower trophic level (prey or host arthropod) contain the insecticidal protein, given that an organism is only at risk if it is actually exposed to the hazard source. For Bt maize, it has been shown that different herbivorous arthropod species ingest variable amounts of Cry1Ab toxin (Head et al., 2001; Raps et al., 2001; Obrist et al., 2005). Using Enzyme-linked Immuno-Sorbent Assays (ELISA), Dutton et al. (2002) measured Cry1Ab concentrations in *Tetranychus urticae* (Koch) (Acarina: Tetranychidae) which were of the same order of magnitude as those found in Bt maize. In contrast, first instar *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) contained only a fourth of these concentrations after feeding on Bt maize.

To assess the risks of Bt maize on natural enemies, various studies have been performed in different tiers (for review see Dutton et al., 2003; O'Callaghan et al., 2005). In particular, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), an important predator in many maize growing areas, has thoroughly been studied, since this predator was found to be negatively affected by Cry1Ab in a first tier ("worst case") study by Hilbeck et al. (1998a). The authors reported reduced survival of immature *C. carnea* when purified and trypsinized Cry1Ab toxin produced by *Escherichia coli* was mixed into an artificial diet at a concentration of 100 µg ml^{-1}. However, another study by Romeis et al. (2004) revealed that larvae of *C. carnea* were not affected when toxin from the same source was incorporated in a sucrose solution. In tritrophic studies, *C. carnea* was found to be negatively affected in both development and survival when fed lepidopteran larvae [*S. littoralis* and *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)] reared on Bt maize (Hilbeck et al. 1998b; Dutton et al., 2002). In contrast, Dutton et al. (2002) found no detrimental effects when the predator was offered Bt maize-fed *T. urticae*, a herbivore unaffected by the Cry1Ab toxin. This finding was surprising since the amount of toxin measured in *T. urticae* was four times higher than that measured in *S. littoralis*. Dutton et al. (2003) and Romeis et al. (2004) suggested that the negative effects observed on *C. carnea* were most likely due to the fact that the lepidopteran larvae were affected by the toxin, resulting in prey quality-mediated effects as have been reported in other studies with insect resistant transgenic plants (Ashouri et al., 2001; Couty et al., 2001; Bernal et al., 2002; Bell et al., 2003).

The discrepancy of the observed effects on *C. carnea* when feeding on *T. urticae* and *S. littoralis* may also be due to other factors. The toxin may, for example, be broken down by salivary enzymes during ingestion by a natural enemy before reaching the gut, as suggested by Armer et al. (2000). In particular, predators using extraoral digestion such as Heteropteran, Neuropteran or Coleopteran species (Cohen, 1995) may break down the toxin during ingestion. The actual exposure of a natural enemy when feeding on Bt-containing prey can be verified by measuring the toxin content in the natural enemy itself. Another possible explanation is that the toxin in certain herbivores may be deactivated or degraded by gut proteases into a non-toxic form, which is still measured with ELISA. In fact, different protein detection methods have already been reported to deliver inconsistent results. For example, Pang et al. (1999) showed that digested *B. thuringiensis* δ-endotoxin (Cry1Aa) appeared to be intact when analyzed by native protein techniques. The protein however, exhibited no insecticidal activity and sequencing data revealed a cleavage site in domain II. Furthermore, effectiveness of Bt toxins can be influenced by secondary plant compounds (Sivamani et al., 1992; Appel and Schultz, 1994; Olsen and Daly, 2000) or ingested particles in the herbivore gut (Ben-Dov et al., 2003). All these factors could influence the actual exposure of natural enemies to biologically active Cry1Ab contained in herbivores.

The objective of this study was to investigate the actual exposure of a predator to Cry1Ab toxin, using the system Bt maize – *T. urticae* / *S. littoralis* – *C. carnea*. The uptake of Cry1Ab toxin by *C. carnea* when feeding on *T. urticae* and *S. littoralis* kept on Bt maize was measured using ELISA, and the biological activity of Cry1Ab toxin following ingestion by each of the two herbivore species was investigated by means of a feeding bioassay with the target lepidopteran pest *O. nubilalis*. Moreover, the activity of Cry1Ab toxin expressed by Bt maize was compared to that of purified Cry1Ab toxin produced by transformed *E. coli* which has been used for first tier toxicological studies (MacIntosh et al., 1990; Hilbeck et al., 1998a; Mendelsohn et al., 2003; Romeis et al., 2004).

MATERIAL AND METHODS

Plants. Transgenic Bt maize (Bt11, NT4640Bt; Syngenta, formerly Northrup King) (referred to as Bt^+) expressing a synthetic gene encoding a truncated version of the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and the corresponding non-transformed near isogenic line (NT4640) (Bt^-) as a control were used for insect rearing and experiments. In this transgenic maize variety the Cry1Ab expression is driven by the constitutive CaMV35S promoter. Plants were grown individually in plastic pots (3 l) in the greenhouse at 24 ± 4 °C, 70 ± 10% RH and fertilised using 6% N: 6% P_2O_5: 26% K_2O at a concentration of one gram per litre with every watering. Seven to nine week old plants were used for all experiments (6th – 9th leaf stage). Plant material used for ELISAs and feeding bioassays was collected from the 5th oldest leaf and frozen at –80 °C.

Insects. Eggs of *O. nubilalis* were obtained from INRA (Institut National de la Recherche Agronomique, Le Magneraud, France). Egg masses were stored at 5 °C for a maximum of two days and subsequently kept in a climatic chamber (25 ± 1 °C, 70 ± 10% RH, 16:8 L: D) until egg hatch and initiation of the experiment.

Separate colonies of *T. urticae* were kept on either Bt^- or Bt^+ maize plants in the greenhouse, at environmental conditions of 24 ± 4 °C, 70 ± 10% RH. Spider mites were collected in a tray kept underneath infested leaves by shaking the leaves using a stick. Thereafter, they were either offered to *C. carnea* or transferred into Eppendorf tubes and stored at –80 °C for ELISA, and for extraction solutions.

Eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland) and kept at 25± 1 °C, 70 ± 10% RH, 16:8 L:D. Upon emergence, larvae from 2 – 3 egg masses were transferred into a cellophane bag (20.5 x 40 cm) which was fixed on the 5th leaf of a whole maize plant (7 – 9 weeks old, 6th – 9th leaf stage) and sealed with clips to prevent escape. After having fed for 24h on Bt^- or Bt^+ plants, larvae were collected and either offered alive to *C. carnea* or stored as described above for *T. urticae.*

Eggs of *C. carnea* were collected from our permanent laboratory colony (Romeis et al., 2004) and kept separately in a climatic chamber (25 ± 1 °C, 70 ± 10% RH, 16:8 L:D) until they hatched. Experiments were conducted with food deprived 1st instars (12 – 24 h old).

ELISA. Quantification of Cry1Ab toxin was conducted with ELISA kits from EnviroLogix Inc. (Portland, Maine, USA). Cry1Ab standards at concentrations 0, 0.5, 2.5 and 5 ppb were used as calibrators. Spectrophotometric measurements were conducted with a microtiter plate reader (Dynatech MR 5000) at 450 nm and data were analysed using the software package Biolinx 2.0 (Dynatech Laboratories Inc.) and Dynex Revelation G 3.2 (Dynex Technologies).

Purified Cry1Ab toxin. Purified Cry1Ab toxin (M. Carey, Dept. Biochemistry, Case Western Reserve University, Cleveland, Ohio) was produced from Cry1Ab protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1 that was expressed as a single gene product in *E. coli*. Inclusion bodies containing Cry1Ab protoxin were dissolved and trypsinized and the Cry1Ab toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al., 1994). All experiments were performed with protein from the same aliquot.

Bt uptake by first instar *Chrysoperla carnea*

In order to verify if Bt content in the predator correlates with the amount of ingested Bt-containing prey, individual weights of first instar *C. carnea* were measured on a microbalance (Mettler Toledo, MX5, division d = 1 µg; tolerance ± 2 µg, Switzerland). Larvae were subsequently placed individually in vials (25 mm Ø, 7 mm high) containing either adult *T. urticae* (sorted out under the binocular) or 1st instar *S. littoralis* which had been kept on Bt^- or Bt^+ (as described above) and allowed to feed *ad libitum* for 3.5 hours. After feeding, larvae were weighed again and then frozen at –80 °C. To obtain enough material for quantifying Bt toxin in predators using ELISA, 70 – 80 whole ground *C. carnea* fed with *S. littoralis* larvae, and 30 – 40 *C. carnea* fed with *T. urticae,* were taken for one sample. For all Bt^+ treatments three samples were collected to obtain three independent replicates of the ELISA. One sample was taken for the Bt^- treatment to verify that there was no cross-reaction in the negative samples. Furthermore, three samples of the respective prey (approximately 8 mg of *S. littoralis* and 1 mg of *T. urticae*) were taken to determine the Bt toxin concentration. Bt^+ samples were extracted at the following concentrations (mg material per ml extraction buffer): 8 and 1 mg ml^{-1} for Bt^+-fed *S. littoralis* larvae and *T. urticae*, respectively; 16 and 2 mg ml^{-1} for *C. carnea* after consuming Bt^+-fed *S. littoralis* larvae or *T. urticae*, respectively. Each extraction solution was diluted (2 x and 4 x) for ELISA measurements. All control samples (Bt^-) were analysed undiluted.

Activity of Cry1Ab toxin in herbivores

In order to verify the activity of Cry1Ab toxin upon ingestion by *T. urticae* and *S. littoralis*, we conducted two bioassays. For both bioassays extraction solutions of Bt^+-fed *T. urticae* and *S. littoralis* larvae were incorporated into an artificial diet, which was subsequently offered to *O. nubilalis* larvae. Weight of *O. nubilalis* larvae was used as a parameter for the activity of the Cry1Ab toxin, as susceptibility of neonate larvae is reflected by a restricted increase of larval weight at sublethal toxin concentrations (Sims and Holden, 1996; Marcon et al., 1999; Head et al., 2001).

Extraction solutions for *Ostrinia nubilalis* feeding bioassays. Prior to the assays, seven extraction solutions were prepared, representing the following treatments: *S. littoralis* larvae (Bt^- and Bt^+), *T. urticae* (Bt^- and Bt^+), maize leaf (Bt^- and Bt^+) and purified Cry1Ab toxin. The solutions containing *T. urticae*, *S. littoralis* and maize leaves were made at equal proportions of 120 mg sample material per ml ELISA extraction buffer. For this purpose 1.2 g *S. littoralis* larvae, 0.8 g *T. urticae* and 0.8 g leaf material (both Bt^- and Bt^+ for each sample type) were macerated in 10, 6.67, and 6.67 ml extraction buffer respectively. After centrifugation (10 min, 1200 g) the supernatant was stored at 5 °C. The solution with purified Cry1Ab toxin was prepared by adding 100 µg to one ml extraction buffer and by diluting it to get a solution of 200 ng toxin ml^{-1}. In addition, a solution consisting of pure extraction buffer was prepared for a control diet to determine whether the added arthropod or plant materials (Bt^-) had any impact on *O. nubilalis*.

An ELISA was performed with all the extraction solutions as described above. Each Bt^+ solution was diluted (3 x, 6 x, 12 x, 24 x and 48 x for *S. littoralis*, 25 x, 50 x, 100 x, 200 x and 400 x for *T. urticae* and leaf, 10 x, 20 x, 40 x, 80 x, 160 x for purified toxin) in order to get a range of measurable concentrations. The control solutions (Bt^- arthropod and leaf material) were measured undiluted.

First bioassay. The purpose of the first bioassay was to determine whether the Cry1Ab toxin contained in *S. littoralis* larvae and *T. urticae* is active when compared to Cry1Ab in Bt^+ maize and to a control (Bt^- samples). Therefore, equal amounts of each sample material (i.e. extraction solutions containing *S. littoralis*, *T. urticae,* maize leaf each Bt^- and Bt^+ respectively) were incorporated in meridic diet and offered to neonate *O. nubilalis* larvae.

A standard quantity of 2.5 ml from each extraction solution (Bt^- and Bt^+ from *S. littoralis,* *T. urticae*, maize leaf respectively) was mixed each with 2.5 ml extraction buffer. This quantity was chosen as preliminary experiments have shown that the expected toxin quantities contained in these solutions caused a measurable impact on weight increase of *O. nubilalis* larvae. Each of the prepared 5 ml solutions was incorporated into 45 g of liquid agar based Ivaldi-Sender diet (Bathon et al., 1991). To avoid degrading the Bt toxin, the temperature of the liquid diet was kept below 60 °C. The diets were distributed in Petri dishes (9 x 1.6 cm; diameter x height) and covered with tissue paper. After cooling down, the solidified diets were cut into pieces of approximately 0.5 g and distributed into cells of bioassay trays (C-D International, Pittman, NJ). Individual 1 – 12 hours old *O. nubilalis* larvae were introduced to each of the cells, which were subsequently sealed with a vented acetate cover (C-D International, Pittman, NJ). Forty *O. nubilalis* larvae were tested per treatment. After seven days of incubation in the climatic chamber (25 ± 1 °C, 70 ± 10% RH, 16:8 L:D) the weight of each *O. nubilalis* was recorded. Since mortality was low (< 5%) and did not differ among treatments (Chi-square-test; $\chi^2_3 = 0.006$, P = 1.0), dead larvae were not included in the analyses. Larvae that escaped during the experiment were also discarded.

All statistical analyses were computed in STATISTICA (version 6, Statsoft Inc., Tulsa, USA). After verifying that there were no statistical differences among *O. nubilalis* weights of all control groups (pure extraction buffer, Bt^--fed *S. littoralis*, Bt^--fed *T. urticae*, and Bt^- leaves) (ANOVA, $F_{3,154} = 1.7$, P = 0.17) these datasets were pooled for further analyses. Since variances of Bt^+ treatments were not homogeneous, comparison of mean weights of *O. nubilalis* feeding on the different Bt^+ diets and the control were analysed using Kruskal-Wallis ANOVA. *Post hoc* comparisons of mean ranks were made using Mann-Whitney U-test with Bonferroni correction. Six pairwise comparisons of all treatments lead to an adjusted $\alpha = 0.008$.

In addition, a dose-response curve was drawn for the decreasing *O. nubilalis* weights as a function of increasing Cry1Ab toxin concentrations in the different Bt^+ diets. The concentrations in the diets were derived from the ELISA measurements of the extraction solutions. The model was determined with purified Cry1Ab toxin prior to the assay using non-linear regression analyses and was described with the function $Y = (a + bX^c)^{-1}$.

Second bioassay. The aim of the second bioassay was to determine whether Cry1Ab toxin in Bt^+ maize-fed *S. littoralis*, *T. urticae* and in Bt^+ maize have equal degrees of biological activity. Therefore, the Cry1Ab toxin concentration was kept constant in all *O. nubilalis* diets by adapting the quantities of extraction solutions to be added to the diets.

These quantities were calculated based on the ELISA measurements of the extraction solutions. In addition, the activity of plant-derived Cry1Ab toxin was compared to that of purified toxin using the same method.

Solutions of identical Cry1Ab concentration were prepared by mixing the appropriate quantity of each extract (4.22 ml of *S. littoralis*, 0.67 ml of *T. urticae* and 1.0 ml of leaf solution each Bt^- and Bt^+, and 4.71 ml of the solution containing purified Cry1Ab) with extraction buffer to yield a total of 5 ml of extraction solution of a toxin concentration of 350 ng ml^{-1}. This solution was added to 45 g of meridic diet as described above. This resulted in a concentration of 7 ng g^{-1} diet. This concentration was chosen because it caused easily detectable effects on weights of *O. nubilalis* larvae in preliminary assays. The following steps in this experiment were performed as described for the first feeding bioassay.

After verifying that there were no statistical differences among *O. nubilalis* weights of all control groups (buffer, Bt^--fed *S. littoralis*, Bt^--fed *T. urticae*, and Bt^- leaves) (ANOVA; $F_{3,139} = 0.83$, $P = 0.48$) these datasets were pooled for further analyses. Larvae that escaped or died during the experiment were discarded from the analyses, as mortality did not differ among treatments (Chi-square-test; $\chi^2_4 = 0.006$, $P = 1.0$). Comparison of means were made as described above, but with one additional treatment (purified Cry1Ab toxin) leading to ten pairwise comparisons with a Bonferroni-adjusted $\alpha = 0.005$.

RESULTS

Bt uptake by 1st instar *Chrysoperla carnea*

Mean weights (± SE) of *C. carnea* larvae increased from 59.7 ± 0.5 to 113.1 ± 2.0 mg (n = 124) after feeding on *T. urticae* and from 60.7 ± 0.4 to 115.5 ± 1.5 mg (n = 250) after feeding on *S. littoralis* larvae for 3.5 h. This corresponds to a weight increase of a factor of approximately 1.9. ELISA measurements revealed that the toxin content in Bt^+-fed *T. urticae* was approximately ten times higher than that in Bt^+-fed *S. littoralis*. Accordingly, the Bt toxin concentration in *C. carnea* was about ten times higher when fed with Bt$^+$-fed *T. urticae* than when fed with Bt$^+$-fed *S. littoralis* (Figure 3.1). No Cry1Ab toxin was detected in *C. carnea* larvae fed control herbivores (Bt$^-$-fed *S. littoralis* and Bt$^-$-fed *T. urticae*).

Activity of Cry1Ab toxin in herbivores

ELISA of extraction solutions. Highest Bt toxin concentrations were measured in the extraction solution containing Bt$^+$-fed *T. urticae* (525.4 ng ml^{-1}), followed by the Bt$^+$ leaf extraction solution (350.1 ng ml^{-1}) and the Bt$^+$-fed *S. littoralis* extraction solution (82.9 ng ml^{-1}). A concentration of 74.4 ng ml^{-1} was measured in the solution containing purified Cry1Ab toxin. This corresponds to approximately 37% of the value that would have been expected from the amount added to the solution (200 ng ml^{-1}). Traces of Cry1Ab toxin (below the limit of quantification ranging from 0.5 to 1 ng ml^{-1}) were measured in the control extraction solutions. Trace amounts in Bt$^-$-fed spider mites have been detected earlier (Dutton et al., 2002), and could be attributed to cross-reaction with other proteins resulting from the high sample / buffer ratio at which the undiluted controls were analysed.

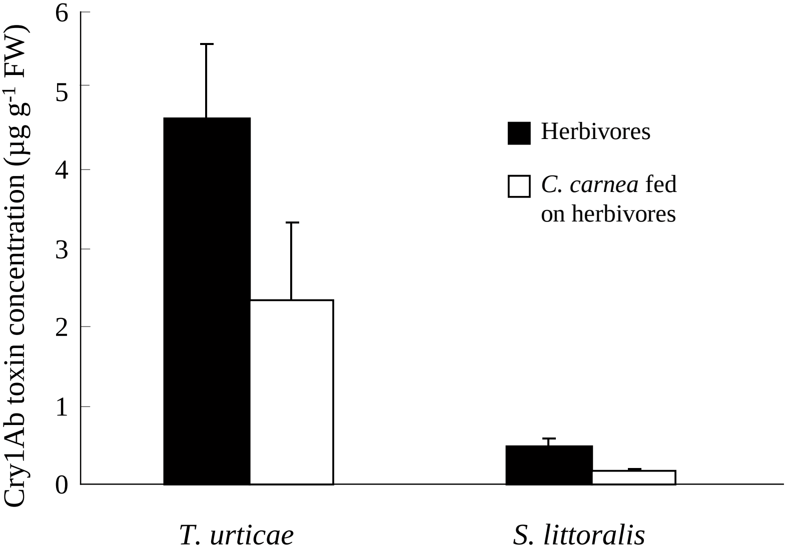

Figure 3.1 Mean (+SE) Cry1Ab toxin concentrations (µg g^{-1} fresh weight) in Bt$^+$ maize-fed herbivores (*Tetranychus urticae and Spodoptera littoralis*) and in 1st instar *Chrysoperla carnea* after having fed on each herbivore (n = 3).

First bioassay. The levels of Cry1Ab toxin in the diets offered to *O. nubilalis* were calculated from the ELISA measurements of the extraction solutions, since the concentrations in the diets were too low for reliable measurements. The resulting concentrations were 4.1, 26.3 and 17.5 ng g^{-1} diet for the Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, and Bt^+ leaf, respectively.

Mean weights of *O. nubilalis* larvae feeding for seven days on diets containing an equal quantity of either Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, Bt^+ leaf material or control extraction solutions differed significantly among treatments (Kruskal-Wallis ANOVA, $H_{3,269} = 139.9$; $P < 0.0001$) (Figure 3.2). Pairwise comparisons of means showed that weights of control insects were significantly higher compared to those of any of the Bt^+ treatments and revealed a clear difference between weights of *O. nubilalis* fed on

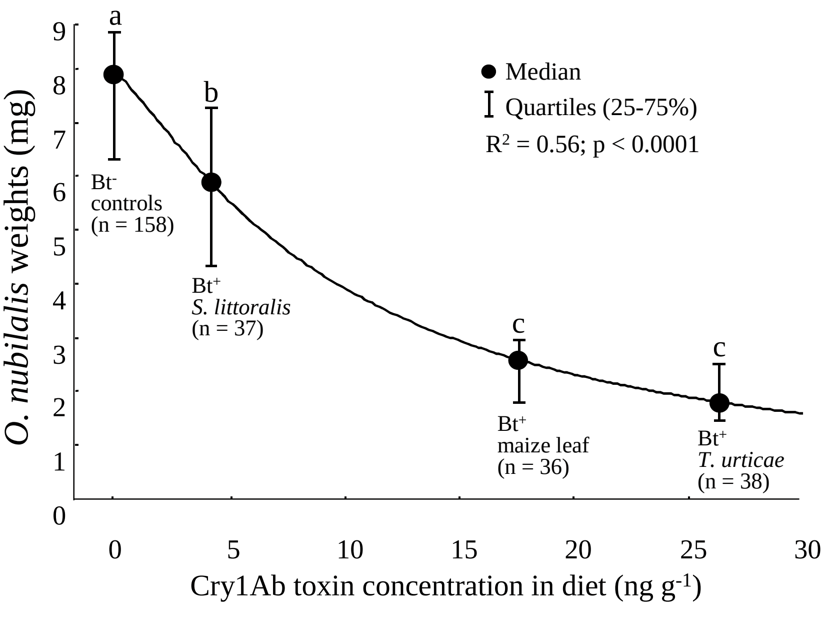

Figure 3.2 Median of weights of *Ostrinia nubilalis* larvae (mg quartiles) after feeding seven days on diets containing different quantities of Cry1Ab due to the addition of equal quantities of Bt^+-fed *Spodoptera littoralis*, Bt^+-fed *Tetranychus urticae*, Bt^+ maize leave, or control (Bt^-) extraction solutions. Different letters above the bars represent differences among treatments (Mann-Whitney U-test with Bonferroni-adjusted $\alpha = 0.008$). In addition, a dose-response curve representing *O. nubilalis* weights as a function of the Cry1Ab toxin concentrations in the diets is shown. The model is described by the function $Y = (0.135+0.006X^{1.255})^{-1}$.

Bt^+-fed *S. littoralis* and Bt^+-fed *T. urticae* diet (Mann-Whitney U-test for all comparisons $P < 0.0001$). No statistically significant difference was found between weights of *O. nubilalis* fed on Bt^+-fed *T. urticae* and Bt^+ leaf diet when using the adjusted $\alpha = 0.008$ ($U = 492$, $P = 0.037$). When *O. nubilalis* weight is plotted against the Cry1Ab concentrations in the diets calculated from ELISA measurements of extraction solutions, a strong dose-response curve is obtained [$Y = (0.135+0.006\ X^{1.255})^{-1}$, $R^2 = 0.56$, $F_{3,266} = 887.7$, $P < 0.0001$] (Figure 3.2).

Second bioassay. Mean weights of *O. nubilalis* larvae feeding for seven days on diets containing equal toxin concentration (7 ng g^{-1}) originating from Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, Bt^+ leaf, purified Bt toxin and control extraction solutions were different among treatments (Kruskal-Wallis ANOVA, $H_{4,290} = 94.0$; $P < 0.0001$) (Figure 3.3). Pairwise comparisons showed that none of the treatments containing either Bt^+ plant or

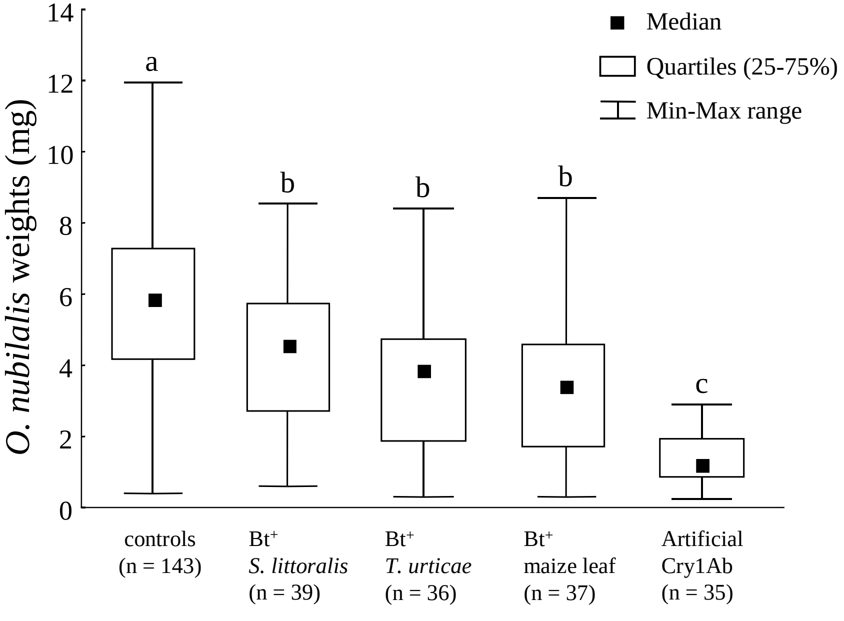

Figure 3.3 Median of weights of *Ostrinia nubilalis* larvae (mg ± quartiles) after feeding seven days on diets containing Cry1Ab at a concentration of 7 ng g^{-1} caused by the addition of different quantities of Bt^+-fed *Spodoptera littoralis*, Bt^+-fed *Tetranychus urticae*, Bt^+ maize leaf, or artificial Cry1Ab toxin extraction solutions compared to a control. Different letters represent differences among treatments (Mann-Whitney U-test with Bonferroni-adjusted $\alpha = 0.005$).

Bt^+-fed arthropod extraction solutions differed from each other when using the adjusted $\alpha = 0.005$ (Mann-Whitney U-test for *S. littoralis* versus *T. urticae*: $U = 498.5$, $P < 0.03$; *S. littoralis* versus leaf: $U = 523$, $P = 0.04$; *T. urticae* versus leaf: $U = 634$, $P < 0.72$). However, all of these treatments were statistically different from the control (*S. littoralis*: $U = 1930$, $P = 0.003$; *T. urticae*: $U = 1252.5$, $P < 0.0001$; leaf: $U = 1370$, $P < 0.0001$). Purified Cry1Ab toxin was shown to cause a significantly stronger effect on weights of *O. nubilalis* larvae compared to the plant-derived Bt^+ treatments, although toxin concentrations in the diets were equal according to ELISA.

DISCUSSION

Risk assessment of insect-resistant transgenic plants with respect to natural enemies should include investigations of their exposure as well as their susceptibility to the transgene product. The exposure of species of higher trophic levels occurs following ingestion of food (e.g. herbivores) containing the active, insecticidal protein. Our results confirm that the predator *C. carnea* ingests the Cry1Ab toxin, when feeding on Bt maize-reared *T. urticae* and *S. littoralis* larvae. In addition, we have shown that the Cry1Ab toxin uptake by *C. carnea* varied according to the amount of toxin measured in the herbivores entailing a ten times higher exposure when feeding exclusively on Bt^+-fed *T. urticae* than when feeding on Bt^+-fed *S. littoralis*. Cry1Ab toxin concentrations measured in *C. carnea* (approximately half of the concentration measured in the respective prey) corresponded well to the concentrations we would expect according to their food uptake (weight gain of a factor of 1.9 in a 3.5 h period). This indicates that *C. carnea* takes up the Cry1Ab toxin equally, not depending on the herbivore species or the toxin concentration in the herbivore. From our results it appears that the toxin is not modified when ingested by the predator and we assume that exposure of *C. carnea* will correlate with Cry1Ab toxin concentration in any other herbivore it may consume.

Our feeding bioassays have shown that the biological activity of Cry1Ab toxin ingested by both *T. urticae* and *S. littoralis* was maintained resulting in measurable effects on weights of *O. nubilalis* larvae which consumed diet incorporating extracts from the respective prey. The growth inhibition of susceptible *O. nubilalis* larvae as a function of the toxin concentrations (measured with ELISA), corresponded well to the model which was established to describe the dose-response curve of Cry1Ab toxin ($Y = (a + bX^c)^{-1}$). Moreover, all diets containing toxin originating from maize plants (Bt^+-fed *T. urticae*, Bt^+-fed *S. littoralis*, Bt^+ leaf) caused similar effects on weights of *O. nubilalis* larvae when applied at the equal concentrations (based on ELISA measurements). These results show that the activity of Cry1Ab toxin in both Bt$^+$-fed *T. urticae* and Bt$^+$-fed *S. littoralis* remains the same as in Bt$^+$ maize, and that the toxin is not deactivated in the herbivore gut. The findings that Bt toxin ingested by herbivores remains active stands in agreement with previous studies by Head et al. (2001), who tested the activity of purified Cry1Ab toxin following ingestion by *O. nubilalis* and aphids.

The fact that *C. carnea* is exposed to higher concentrations of biologically active Cry1Ab toxin when feeding on Bt$^+$-fed *T. urticae* than when feeding on Bt$^+$-fed *S. littoralis*, indicates that other mechanisms are responsible for the previously reported negative effects on *C. carnea* when offered Bt$^+$-fed lepidopteran larvae (Hilbeck et al., 1998b; Dutton et al., 2002). The most probable explanation is that *C. carnea* is affected by feeding on a food source of reduced quality rather than by the toxin itself. This assumption is supported by the studies by Romeis et al. (2004) who could not detect direct toxic effects on *C. carnea* larvae, when these were fed with sucrose solution containing purified Cry1Ab at 10,000 fold higher concentrations than those found in *S. littoralis* larvae. Chrysopids are known to have prolonged development when feeding on low quality food (Prinicpi and Canard, 1984). *Spodoptera littoralis*, as a lepidopteran species, was shown to be adversely affected by the Cry1Ab toxin when feeding on Bt maize (Dutton et al., 2002; 2005). Given that *S. littoralis* is a poor quality prey even without having been exposed to the toxin (Dutton et al., 2002) such prey-quality mediated effects on *C. carnea* are likely to occur when feeding on sublethally affected (sick) *S. littoralis*.

An alternative hypothesis, to explain that effects on *C. carnea* were only detected when fed with Bt-containing *S. littoralis* larvae is that the Cry1Ab toxin in the mid-gut of this herbivore may be present in a more active form than in Bt maize or Bt-fed *T. urticae.* The Cry1Ab protein in maize is expressed in a truncated form compared to the protoxin expressed by *B. thuringiensis* (Koziel et al., 1993). However, this does not necessarily mean that the plant-derived toxin is solubilised and in a biologically active form (Federici, 2002). Solubilisation of the Cry1Ab is mainly caused by the lepidopteran-specific alkaline pH of the gut juices whereas truncation is performed by various mid-gut proteases (Choma et al., 1990; Gill et al., 1992). Since *S. littoralis* is susceptible to the Cry1Ab (Dutton et al., 2002; 2005), it can be assumed that activation processes (solubilisation and/or cleavage) occur in its mid-gut. The fully activated toxin in the *S. littoralis* gut may cause adverse effects on *C. carnea* whereas the original, less processed protein in the mid-gut of *T. urticae* would not elicit such effects. However, to our knowledge, no evidence supporting this hypothesis has been provided to date and recent studies revealing that the toxin does not bind in *C. carnea* mid-gut make this hypothesis even less plausible (Rodrigo et al. 2004;

R. de Maagd, personal communication, 2004).

Our results show that the purified Cry1Ab toxin produced by *E. coli* is more toxic to *O. nubilalis* larvae than the plant-originated Cry1Ab toxin when applied at equal concentrations according to the ELISA measurements. This difference could either be based on aberrant ELISA measurement leading to variable toxin concentrations in the diets (i.e. the activity of the purified toxin was underestimated by the ELISA), or could be caused by differing activities of Cry1Ab at the same concentration (i.e. the biological activity of the purified toxin exceeds that of the plant-derived toxin). The ELISA constantly detected only about one third of the *E. coli*-produced toxin that was actually added to the solution. This was also found in previous analyses at various dilutions (unpublished results) and may lead to the conclusion, that the ELISA underestimates the toxicity of the purified toxin. Although the source of the discrepancy in our ELISA data could not be determined, speculations explaining differences in the properties of plant-derived and *E. coli*-produced toxin can be made, for example by considering the method of production and / or state of activity. Differences may exist in the stability of the Cry1Ab toxins of different origin which could influence their degradation. Indications for this assumption are given by Palm et al. (1994) who reported that plant-derived toxin degraded within a shorter period than purified Cry1Ab toxin obtained from transformed *E. coli* in the soil. Finally, the discrepancy could also be attributed to the presence of plant or arthropod material in the diet which may reduce the susceptibility of the lepidopteran larvae (Ben-Dov et al., 2003) or to toxin-plant compound interactions influencing the toxicity of the Cry1Ab protein (Olsen and Daly, 2000).

In our studies, we confirm that the Cry1Ab toxin remains biologically active when ingested by two herbivores species and that the toxin is more concentrated in *T. urticae* than in *S. littoralis* larvae. *Chrysoperla carnea* was therefore exposed to higher concentrations of Cry1Ab toxin when consuming Bt$^+$ maize-fed *T. urticae* than when consuming Bt$^+$ maize-fed *S. littoralis* larvae. The results of the present study provide further evidence that *C. carnea* is not susceptible to Cry1Ab, because no effects were found on the predator when fed with spider mites containing the toxin (Dutton et al., 2002). Thus, previously reported negative impacts on *C. carnea* following consumption of Bt$^+$ maize-fed *S. littoralis* resulted from a reduction in the prey quality since the lepidopteran larvae were affected by the Bt toxin themselves.

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CHAPTER 4

EXPOSURE OF ARTHROPOD PREDATORS TO CRY1AB TOXIN IN BT MAIZE FIELDS[4]

ABSTRACT

1. To assess the risks of an insect resistant transgenic plant for non-target arthropods, it is important to verify which arthropods can be exposed to the transgene product. Exposure of predators in the field depends on the toxin levels in food sources as well as on their feeding ecology and that of their prey.

2. To verify the transmission of Cry1Ab toxin through the food chain and thus exposure of predators in the field, samples from different plant tissues, herbivores, and predators in Bt maize fields in Spain (Event176) were collected at different periods over the season and the toxin content was measured using ELISA. Complementary laboratory studies were performed with the omnivorous predator *Orius majusculus* to assess the toxin uptake and persistence after feeding on variable Bt-containing food sources.

3. Field results revealed that toxin content in some herbivores was negligible (aphids, thrips, leafhoppers) compared to those in spider mites (*Tetranychus urticae*). The latter herbivore only occurred after pollen shed and contained three times higher toxin levels than Bt maize leaves.

4. Our data confirmed that the Bt toxin can be transferred to predators, that is to say to *Orius* spp. *Chrysoperla* spp., *Stethorus* sp.. But this only applies when Bt maize pollen or spider mites are available. The passage of Bt toxin to *O. majusculus* via these two food sources was also confirmed in the laboratory. Contrastingly, some predators in the field (hemerobiids, *Nabis* spp., *Adonia* sp., *Demetrias* sp.) contained no or negligible toxin levels even when pollen or spider mites were present.

5. In addition to information on potential exposure of various arthropod predators to Bt toxin, this study provides an insight into the feeding ecology of different arthropods in the maize system.

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[4] Based on: Obrist, L.B., H. Klein, A. Dutton and F. Bigler. Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. Ecological Entomology (in press).

Introduction

To assess potential risks that insect resistant transgenic plants pose to non-target arthropods, several factors have to be taken into account. Besides the investigation of the toxicity of an insecticidal protein expressed by a transgenic plant, it is important to assess the extent to which non-target organisms are exposed to the toxin (U.S. EPA, 1998; Cowgill and Atkinson, 2003; Dutton et al., 2003). Exposure of predators to the insecticidal protein may be indirect when preying on herbivores containing the toxin, or direct when feeding on plant parts containing the toxin (Groot and Dicke, 2002). The latter route of exposure applies for omnivorous predators (e.g. many heteropteran species) as they can feed on pollen and/or other plant tissue (Alomar and Wiedenmann, 1996). To assess the exposure of arthropods to an insecticidal protein, the level of toxin contained in their food source has to be determined (Dutton et al., 2002; 2003). Laboratory studies with herbivores feeding on Cry1Ab expressing Bt maize revealed large differences in the quantity of ingested toxin among species (Head et al., 2001; Raps et al., 2001; Obrist et al., 2005). Using Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dutton et al. (2002) measured Cry1Ab toxin concentrations in *Tetranychus urticae* (Koch) (Acarina: Tetranychidae) which were similar to those found in Bt maize leaves. Lower levels were found in *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvae and no or only trace amounts of toxin were detected in aphids, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae), reared on Bt maize. Accordingly, toxin levels measured in first instar *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) were shown to correlate with the amount of toxin in the Bt maize-fed herbivores after feeding on them (Obrist et al., in press).

To date, many field studies have been performed with Bt maize, which primarily assess its impact on the abundance of numerous arthropod species (for review see Romeis et al., submitted). However, little is known about the extent to which beneficial arthropods are effectively exposed to Bt toxin in the field. Exposure under field conditions is influenced by numerous factors such as variable expression rates in different plant parts and over the growing season, feeding ecology, behaviour and mobility of herbivores and predators. Moreover, various environmental factors such as wind or rain may influence exposure in the field and complicate a quantitative estimation based on laboratory studies.

In Europe, transgenic Bt maize expressing Cry1Ab toxin is predominantly grown in Spain where it has been commercialised since 1998. Increasing areas are covered with Bt maize which is resistant against the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae), and the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), two severe pest species, in particular in Mediterranean countries.

Until 2002, Compa (CB® Event176 Syngenta, Switzerland) was the only Bt maize variety that was commercially grown in Spain (Brookes, 2002). This transgenic cultivar is known to express significant levels of Cry1Ab toxin in leaves and pollen, but not in roots, pith and kernels (Koziel et al., 1993; Fearing et al., 1997).

A number of predator species are regularly present in Spanish maize fields (Iraola et al., 1997; Asin and Pons, 1999; Albajes et al., 2003; de la Poza et al., 2005) among which *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) is one of the predominant species. *Orius* spp. are known to be generalist and omnivorous predators, which feed on various arthropods like thrips, spider mites, leafhoppers, aphids and lepidopteran eggs or young larvae (McMurtry et al., 1970; Coll and Bottrell, 1991; Corey et al., 1998). Pollen can be an important alternative food source for them in maize fields (Dicke and Jarvis, 1962; Corey et al., 1998; Musser and Shelton, 2003a) and some *Orius* spp. were shown to be able to develop exclusively on pollen (Pilcher et al., 1997; Vacante et al., 1997). The role of green plant tissue feeding for *Orius* spp. is not entirely manifested but it is assumed that it is at least used for water provisioning (Armer et al., 1998). The impact of Bt maize on *Orius* spp. was investigated in several studies, all of which detecting no detrimental effects on this predator group (Pilcher et al., 1997; Zwahlen et al., 2000; Bourguet et al., 2002; Jasinski et al., 2003; Musser and Shelton, 2003b; Pons et al., 2004; de la Poza et al., 2005). Due to their complex feeding ecology, their exposure to Bt toxin in the field remains unclear. *Orius* spp. and other predatory species usually found in maize are also recorded in other crops, like alfalfa, that is grown in rotation with maize in irrigated Mediterranean areas. Toxin acquired in Bt maize may thus be transferred to alfalfa neighbouring fields.

The present study was conducted to assess the exposure of relevant arthropod predators to Cry1Ab toxin in a transgenic maize ecosystem in Spain by considering the pathways through which the toxin may reach predators. In addition, preliminary studies were conducted to evaluate the possibility of toxin transfer to neighbouring fields by predator dispersal. Samples of three successive trophic levels, that is, maize plants, arthropod herbivores and predators were collected in Bt maize fields at different periods in the growing season and Cry1Ab toxin content was determined using ELISA. The toxin content in predators collected from adjacent alfalfa fields was also determined. Complementary laboratory studies were performed with the omnivorous predator *O. majusculus* to verify the toxin uptake when fed with different Bt-containing food sources and to assess the persistence of the toxin in adults.

MATERIAL AND METHODS

Laboratory experiments

Plants. Commercial cultivars of transgenic Bt maize (Event176, Compa CB®, Syngenta, Switzerland) (designated as Bt$^+$) expressing a gene coding for a truncated version of the Cry1Ab protein derived from *Bacillus thuringiensis* Berliner subsp*. kurstaki* HD-1 and the corresponding non-transformed near isogenic variety (Dracma®, Syngenta) (Bt$^-$) as a control were used for experiments. In this transgenic maize variety the Cry1Ab expression is driven by the constitutive PEPC promotor as well as a pollen-specific promotor. Plants used for experiments were grown individually in plastic pots (3 l volume) in the greenhouse at 24 ± 4 °C, 70 ± 10% RH and used when at vegetative stages V10 – V12 (6 – 9 weeks) according to Ritchie et al. (1993). All plants were fertilized (10% N: 10% P_2O_5: 8% K_2O) every two weeks with ½ l of a 0.2% aqueous solution. Additional fertilizer (15 – 20 g of 27.5% N) was given after 3 – 4 weeks. Plants used for pollen collection were grown in plastic pots of 10 l volume under the same environmental conditions. Pollen was collected by placing individual inflorescences into air-permeable cellophane bags (20.5 x 40 cm) for 3 – 4 days upon emergence. Pollen was subsequently sieved and dried for 24 h at ambient conditions. Then, the pollen was transferred into Eppendorf tubes and stored at –24 °C until use.

Arthropods. Spider mites, (*T. urticae*, originating from a permanent rearing at the Agroscope FAL Reckenholz) were kept in separate colonies on either Bt$^-$ or Bt$^+$ maize plants (older than two months) in the greenhouse at environmental conditions of 24 ± 4 °C, 70 ± 10% RH. Spider mites were collected in a tray which was kept underneath infested leaves while shaking the leaves.

Colonies of *O. majusculus* (provided by Andermatt Biocontrol, Switzerland) were kept in a climatic chamber at 25 ± 1 °C, 75 ± 10% RH, and L: D 16:8 h photoperiod and fed with *Ephestia kuehniella* (Zeller) (Leptidoptera: Pyralidae) eggs and provided with water. Potato tubers (*Solanum tuberosum L.*) and green bean pods (*Phaseolus vulgaris* L.) were supplied as a substrate for oviposition. For experiments, either fifth instar or adult *O. majusculus* were used. For this purpose, old fourth or fifth instars respectively were separated from the colony, and kept individually in Plexiglas cages (2.7 x 10 mm, diameter x height) containing each 10 µl of water. Cages were kept in a climatic chamber (see above) for a total of 48 h. After the first 24 h, the water in the cages was removed using a cotton swab and the cages were kept in the climatic chamber for another 24 h. Only individuals that have moulted during the two days of food deprivation and thus have reached the next developmental stage (fifth nymphal stage or adult respectively) were used for experiments.

ELISA analyses. To determine the levels of Cry1Ab protein, ELISAs were performed using kits from EnviroLogix Inc. (Portland, Maine, USA). Cry1Ab standards at concentrations 0, 0.5, 2.5 and 5 ppb were used as calibrators. Spectrophotometric measurements were conducted with a microtiter plate reader (Tecan, Spectrafluor PLUS for samples from the lab and Titertek Multiskan(R) PLUS MKII for samples from the field) at 450 nm. The toxin was defined as not detectable if the measured optical density value was lower than the intercept of the Y-axis of the standard regression line. Quantification limit was at approximately 0.03 µg g^{-1} sample material (dry weight) when analysed at the maximal concentration of 15 mg ml^{-1} (sample material per ml extraction buffer). Sample extracts were diluted for analyses if necessary when too high toxin levels lead to measurements out of a quantifiable range.

Nymphs and adults of *Orius majusculus* feeding on different food sources containing Bt toxin. These experiments were conducted to assess Bt toxin content in different stages (adults and nymphs) of *O. majusculus* when provided with different food sources containing Cry1Ab toxin (spider mites, pollen and leaf) for different periods of time.

Spider mites. Cages (2.7 x 1 cm, diameter x height) were prepared containing each a minimum of 15 adults or deutonymphs of *T. urticae* collected from the Bt^+ maize colony. Predators were allowed to feed for 3 h, then transferred into Eppendorf tubes and frozen at –24 °C.

Pollen. Approximately 5 – 10 mg dry pollen was given into cages (2.7 x 1 cm, diameter x height) and 6 µl of water were added. The cages were closed and incubated for at least one hour in the climatic chamber in order to swell the pollen with water and make it edible for *O. majusculus*. Before placing the insects individually in the cages, all water was thoroughly removed using a cotton swab. Two additional treatments were made with pollen, both lasting for 24 h. In one of the two treatments individuals were transferred into cages containing pollen as described above but provided with water (6 μl). In the other treatment, individuals were transferred into a cellophane bag (20 x 40 cm) which was fixed on a pollen shedding tassel of a Bt^+ maize plant.

Maize leaf. Nymphs of *O. majusculus* were transferred individually into clip-cages (1 x 1 cm, diameter x height) which were fixed for 3 h on the sixth or seventh leaf cut-off a plant. One third of the clip-cages was additionally supplied with water-soaked cotton balls (0.3 cm diameter) another third with *E. kuehniella* eggs *ad libitum*.

For each Bt^+ treatment at least four samples collected at different times were analysed using ELISA. One sample consisted of a minimum of 30 nymphs or adults respectively. The toxin content in the food source offered to each predator sample was analysed accordingly. In addition, a control was made which consisted of two samples of individuals fed with the corresponding Bt^- food source. In order to verify if food ingestion is related to toxin uptake, the weight gain was determined by measuring the weights of at least 40 individuals (a minimum of ten from each sample) per treatment before and after feeding using a microbalance (Mettler Toledo, MX5 Greifensee, Switzerland, d = 1 µg; ± 2 µg). As the food uptake in some treatments was very restricted or even negative, the weight loss within 3 h of a food and water deprived control group was measured.

All statistical analyses were computed in STATISTICA (Version 6, Statsoft Inc., Tulsa, USA). One-way and/or main effects ANOVA were performed to determine the influence of "treatment" and "stage". The data for weight changes were transformed by Log_e (x + 100), the data for toxin uptake by Log_e (x + 1) prior to analyses.

Persistence of Bt toxin in adult *Orius majusculus*. Food deprived adults (150 – 200 individuals) were allowed to feed on *T. urticae* originating from the Bt^+ colony for 3 h as described above. Thereafter, 30 randomly chosen individuals were frozen at –24 °C whereas the rest was distributed in five boxes (13 x 10.5 x 5 cm) containing water and leaves infested with spider mites originating from the Bt^- colony. After 24 h, one third of the *O. majusculus* was collected and new spider mites were supplied to the remaining individuals. The same was repeated after two days and the remaining individuals were collected after four days. This experiment was repeated three times in order to have three ELISA replicates.

Field experiments

Experimental fields. Sampling of plant material and arthropods was done during the growing season 2004 in commercial fields of Bt^+ and Bt^- maize (same cultivar as used in laboratory experiments) belonging to a farm located 35 km to the West of Lleida (Catalunya, Spain). The crop was managed according to common growing practices in the region (for details see Lumbierres et al., 2004) but without insecticide application. Field size was variable ranging from 0.4 – 0.7 ha. Samplings were made in three Bt^+ and one Bt^- maize (control) fields. The distance between the experimental Bt^+ fields was at least 200 m. The Bt^- field was next to one of the Bt^+ fields, but since fields were arranged in terraces, this field was at least 1.5 m higher than the bordering Bt^+ field.

An alfalfa field located next to one of the Bt^+ fields was used for additional predator samplings. The two fields were separated by a weedy strip of approximately 1 m width. Alfalfa had not been treated with pesticides for at least one year.

Toxin content in Bt maize. Samplings were made during three development stages: I) before pollen shed (end of June to beginning of July), II) during pollen shed (mid to end of July) and III) after pollen shed (mid to end of August).

Plant samples consisting of a pool of material from four randomly chosen plants were taken in each of the three Bt^+ fields. In period I, samples were taken from the 6th (during vegetative stages V7 – V10 according to Ritchie et al., 1993), 11th (during V14 – V15), 19th leaf (during tasseling before pollen was shed). In addition, samples of developing, unfolded leaves from the whorl, leaf sheaths and stem were taken during vegetative stages V14 – V15. During pollen shed (period II), samples of pollen and the stem within the tassels (designated as tassel axis) were taken. After pollen shed (period III), additional leaf samples were collected from the 15th and 19th leaf and from silk. Pollen of mixed age was also collected from leaves and axils after the period of pollen shed.

Toxin content in arthropods from Bt maize. Arthropod groups that were collected during the three periods (I – III) are presented in Table 4.1. From each of the three Bt^+ fields, two independent samples were collected from two distinct spots in the centre of the fields. The size of these spots, respectively the number of plants that needed to be inspected to collect sufficient arthropods, depended on their density.

Thrips were sampled during the first and second period. In the first period (I), maize plants were cut and dissected on site and all stages of *Frankliniella* spp., were collected together in a vial (4.7 x 4 cm, diameter x height). A rough estimation, which was done by dissecting 20 plants and counting all visible thrips, showed that preimaginal stages were approximately twice as abundant as adults. The predominant thrips species was *Frankliniella tenuicornis* (Uzel) (Thysanoptera: Thripidae). Samplings during the second period were made by shaking tassels (inflorescences) into a white plastic tray, from where thrips were immediately collected. The predominant species in inflorescences was identified as *Frankliniella occidentalis* (Pergande).

Spider mites (exclusively *T. urticae*) were only found after pollen shed. For collections, infested leaves were cut and transferred into a plastic bag, which was kept in a cooling box for the transport to the laboratory. There, all stages of spider mites were collected together with a camel hair brush directly from the underside of the leaves under the binocular.

All other arthropod groups were collected using a sweep net and / or an exhaustor. The collected arthropods were immediately put on ice in order to reduce the metabolism and the excretion of the toxin. Once in the laboratory, arthropods were frozen at –24 °C. Later, samples were sorted out, and, when needed, pollen and other impurities were removed under the binocular. Subsequently, samples were weighed on a semi-microbalance (A&D Company, Limited. HA-202M D = 10 µg ± 20 µg, Abingdon, Oxford OX14 3YS, UK), lyophilised, and re-weighed to determine their fresh / dry weight ratios. The dried samples were stored at –24 °C until ELISA was performed. The initial amount of sample material that was collected in the field ranged from 10 – 60 mg fresh weight. If individuals were big (e.g., adult chrysopids, chrysomelids, nabids), a minimum of four individuals per sample was collected. In Table 4.1, concentrations at which samples were extracted and dilutions made for the ELISA are provided.

The average toxin concentration from two samples per plot was used to calculate the mean concentration in samples of the three Bt^+ plots. One-way ANOVA was performed to determine the influence of a single categorical factor ("sampling period") and main effects ANOVA of two categorical factors ("sampling period" and "stage"). Data sets were transformed by Log_e (x + 1) prior to analyses.

Estimation of prey abundance. During each sampling period (I – III), the abundance of herbivores was estimated by checking a minimum of 25 randomly chosen plants per plot. The abundance of aphids, leafhoppers and spider mites was estimated according to the following abundance class system: absent = 0; present = 1, few = 2; medium = 3; high = 4; very high = 5. The assessment of leafhoppers was based on the amount of damage (decolored strips) on the leaves, as this insect is very mobile and moves away when approaching a plant. Thrips were individually counted during the first sampling period by dissecting the whole plants. Thrips were also observed to be present during the following sampling periods (II + III) but their abundance was not estimated.

Toxin content in predators collected in alfalfa field adjacent to Bt maize. During pollen shed, samples of selected adult predators were taken from the alfalfa field to verify if the toxin is carried out of the Bt maize field by predators. Therefore, three samples of adult *Orius* spp., *N. provencalis, Chrysoperla* spp., and *A. variegata* were taken at two distances (1 – 3 m and 4 – 6 m from the Bt$^+$ field) from different spots by means of a sweep net.

Table 4.1 Arthropods collected before (I) during (II) and after (III) pollen shed in Bt maize fields. Concentrations at which samples were extracted (mg of lyophilised sample material per ml extraction buffer) are provided together with the dilutions (in brackets) made for ELISA measurements.

Arthropods	Genus or species	Stage[1)]	I	II	III
Heteroptera					
Anthocoridae	*Orius* spp.	N	15	15 (4x)	15 (4x)
		A	15	15 (4x)	15 (4x)
Nabidae	*Nabis provencalis* (Remane)	N	-	-	15
		A	15	15	15
Miridae	*Trigonotylus* spp.	Mix	15[2)] (16x)	15 (16x)	-
Homoptera					
Cicadellidae	*Zyginidia* spp.	Mix	15	-	-
Aphididae	*Metopolophium dirhodum* (Walker)	Mix	15	-	-
Thysanoptera					
Thripidae	*Frankliniella* spp.	Mix	15	15 (4x)	-
Neuroptera					
Chrysopidae	*Chrysoperla* spp.	L	15	15	15 (4x)
		A	-	15 (4x)	15
Hemerobiidae	Not identified	A	15	15	-
Coleoptera					
Carabidae	*Demetrias atricapillus* (L.)	A	15	15	15
Staphylinidae	*Tachyporus* sp.	A	15	-	-
Coccinellidae	*Adonia variegata* (Goeze)	A	-	15	-
	Scymnus spp.	A	-	15	15
	Stethorus punctillum (Weise)	L	-	-	4 (4x)
		A	-	-	8 (16x)
Chrysomelidae	*Oulema melanopus* (L.)	A	15 (25x)	15	15
Acarina					
Tetranychidae	*Tetranychus urticae* (Koch)	Mix	-	-	2 (16x)

1) A = adults, L = larvae, N = nymphs, Mix = mixture of all available stages
2) Samples were only taken from two fields

RESULTS

Laboratory experiments

Toxin content and persistence in *Orius majusculus*. Weight change of *O. majusculus* was depending on the food sources they were offered (One-way ANOVA, nymphs: $F_{5,269} = 38.0$, $P < 0.0001$; adults: $F_{2,176} = 64.035$, $P < 0.0001$) (Figure 4.1). When offered pollen or spider mites, nymphs of *O. majusculus* gained significantly more weight than adults (mean ± SE, nymphs: 57.4 ± 3.8 mg, adults 25.4 ± 3.5 mg; Main-effects ANOVA, $F_{1,330} = 56.1$; $P < 0.0001$). The toxin concentrations measured in nymphs and adults were also significantly different depending on the food sources (One-way ANOVA, nymphs: $F_{6,24} = 39.3$, $P < 0.0001$; adults: $F_{3,13} = 4.37$, $P = 0.025$) (Figure 4.2). Toxin concentrations in nymphs were significantly higher than those in adults after feeding on Bt-containing spider mites and Bt^+ pollen (mean ± SE, nymphs: 0.75 ± 0.11 µg g^{-1}, adults: 0.51 ± 0.10 µg g^{-1}; Main-effects ANOVA, $F_{1,31} = 7.56$, $P = 0.01$). Levels measured in nymphs that were exposed to Bt^+ maize leaves for 3 h were mostly below detection

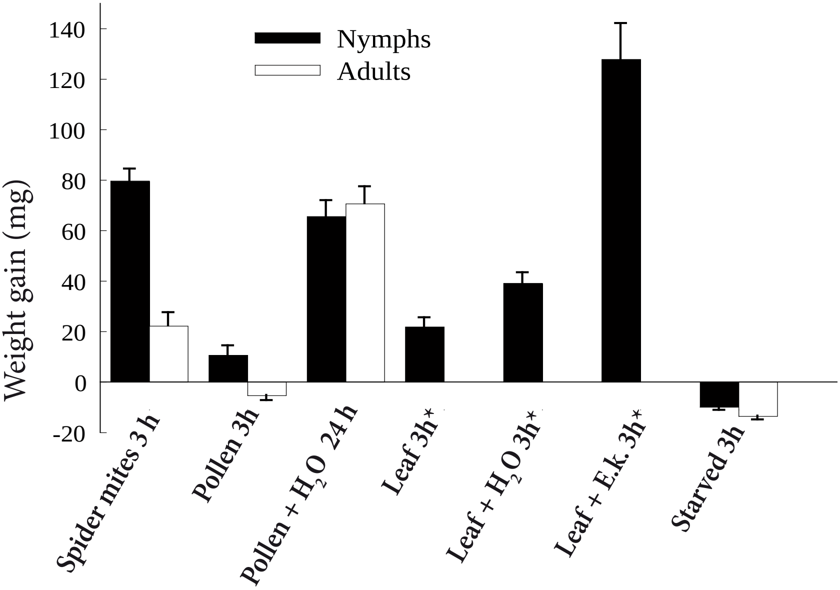

Figure 4.1 Mean weight change (mg + SE) of *Orius majusculus* nymphs and adults after feeding on various food sources (or starved) 3 or 24 h (n = 40 – 80). H_2O = water, E.k. = *Ephestia kuehniella* eggs. * Only nymphs were tested.

level independent of the presence of food (*E. kuehniella* eggs) or water. In none of the *O. majusculus* samples fed on control food sources (Bt^-) the toxin was detected.

Field experiments

Toxin content in Bt maize. Different plant tissues were shown to contain different toxin levels (One-way ANOVA, $F_{10,22} = 90.3$, $P < 0.0001$) (Figure 4.3). Relatively high levels of toxin were measured in leaves (> 4 µg g^{-1} DW) and in fresh pollen (approximately 3 µg g^{-1} DW). Concentrations in old pollen collected from leaves and axils after pollen shed remained comparatively high. Low levels were measured in tissues originating from inner parts of the plant (e.g., whorl, stem, sheath). Toxin concentration in silk was below the quantification limit. The toxin concentrations in leaves collected during the first period (6th, 11th or 19th leaf) was shown to be variable when taking the dry weight as a reference (One-way ANOVA, $F_{2,6} = 16.3$, $P < 0.004$). However, this difference was not detected when toxin concentrations relating to the fresh weight were taken for statistical analyses (One-way ANOVA, $F_{2,6} = 0.46$, $P = 0.65$).

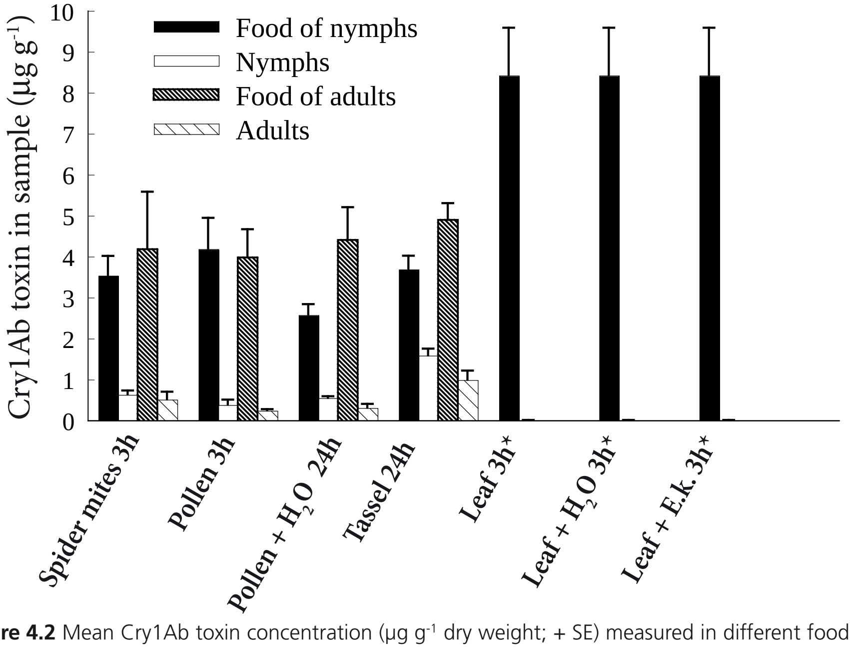

Figure 4.2 Mean Cry1Ab toxin concentration (µg g^{-1} dry weight; + SE) measured in different food sources and in nymphs and adults of *Orius majusculus* fed with the respective food. (n = 4 – 6). H_2O = water, E.k. = *Ephestia kuehniella* eggs. * Only nymphs were tested.

Toxin content in arthropods from Bt maize. Toxin levels in different arthropod groups collected during three different periods in the season (I – III) are presented in Figure 4.4. During the first sampling period, concentrations of Cry1Ab toxin in most of the arthropod groups (including some herbivore groups) were low or not detectable. Only mirids (*Trigonotylus* spp.) and adult chrysomelids (*O. melanopus*) contained substantial levels of toxin. In the second sampling period, when pollen was available, the toxin was additionally detected in several other arthropod groups including *Orius* spp., adults of *Chrysoperla* spp., and *Frankliniella* spp.. In contrast, at that time, the toxin levels were below the quantification limit in adult *O. melanopus*. During the third sampling period, very high toxin levels were found in spider mites (*T. urticae*). These concentrations were almost three times higher than those measured in leaves. Nymphs of the spider mite predator *S. punctillum* contained highest toxin levels compared to those detected in all

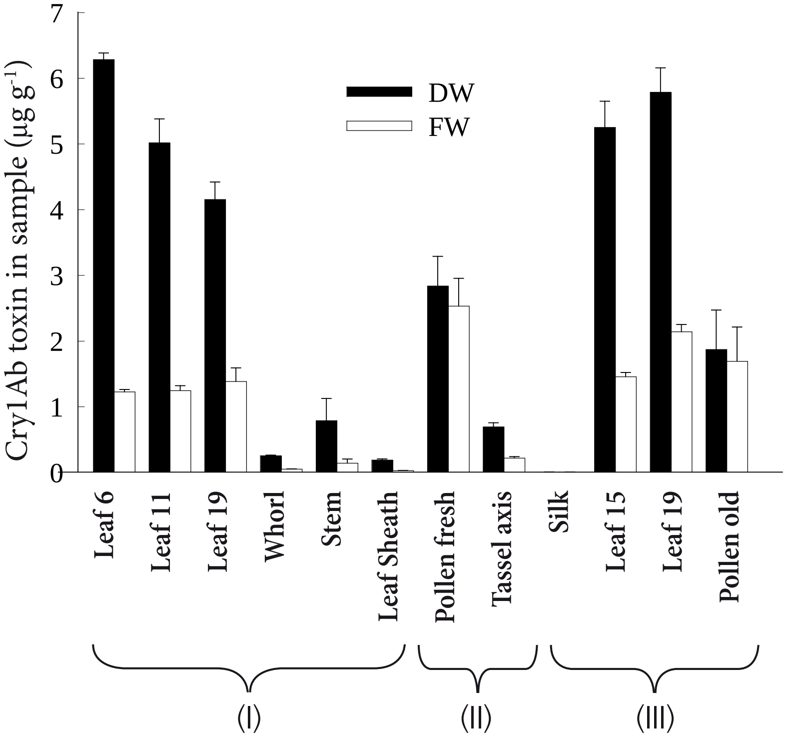

Figure 4.3 Mean Cry1Ab toxin concentrations [µg g^{-1} dry weight (DW) or fresh weight (FW); + SE] measured in different plant parts of Bt maize from the field. Samples were taken before (I) during (II) or after pollen shed (III). (n = 3).

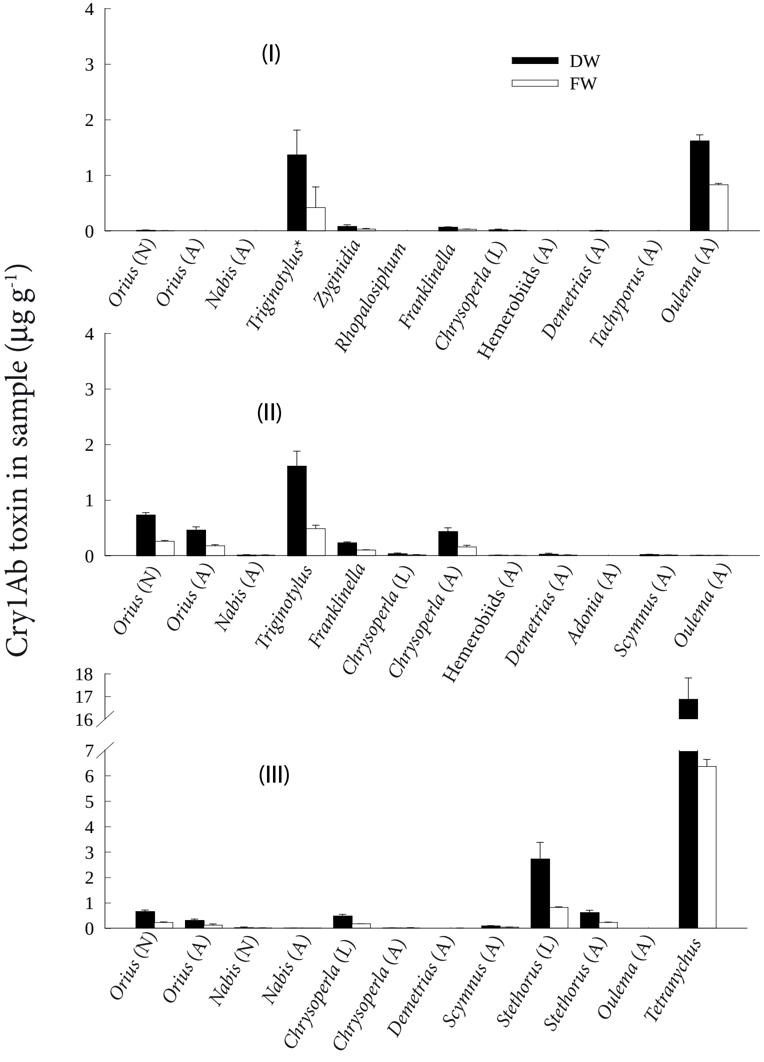

Figure 4.4 Mean Cry1Ab toxin concentrations [µg g^{-1} dry weight (DW) or fresh weight (FW); +SE] measured in different arthropod groups which were collected from Bt maize fields before (I) during (II) and after (III) pollen shed. Taxonomic details can be seen in Table 4.1. (A = adults, L = larvae, N = nymphs). (n = 3 except for Mirids n = 2, designated with *).

other predators. Further, the toxin was found in adults of *S. punctillum*, *Orius* spp. and larvae of *Chrysoperla* spp. to a similar level. In all but one control (Bt^-) arthropod groups no toxin was detected. The exception were spider mites, in which traces of toxin (below quantification level) were detected. Traces in the control group of spider mites were already detected in previous studies and are supposed to be caused by cross-reactions with other proteins (Dutton et al., 2002; Obrist et al., in press).

Statistical analyses over the whole sampling season showed that toxin levels in *Orius* spp. depended on sampling period, being higher in the second and third sampling periods than in the first one (Main effects ANOVA, $F_{2,14} = 74.1$, $P < 0.0001$), and stage as higher concentrations where found in nymphs than in adults ($F_{1,14} = 17.4$, $P < 0.001$). A higher toxin concentration in larvae than in adults was also found for *S. punctillum* ($F_{1,4} = 15.6$, $P = 0.017$). In other predators like *N. provencalis* the toxin concentration was very low, independent of the sampling period (One-way ANOVA, $F_{2,6} = 1.68$, $P = 0.26$) and in *D. atricapillus* the toxin was never detected at all.

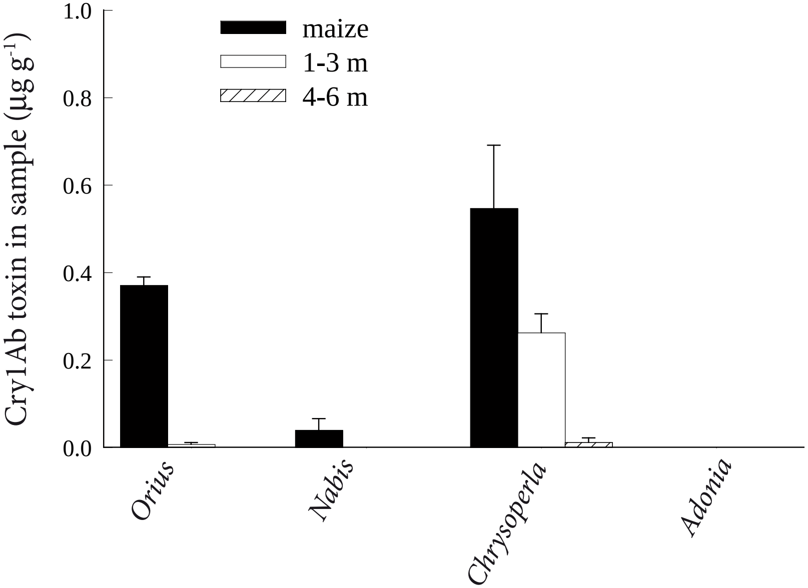

Figure 4.5 Mean Cry1Ab toxin concentrations [µg g^{-1} dry weight (DW); +SE] measured in predatory arthropods collected in Bt^+ maize fields and two distances (1 – 3 m and 4 – 6 m) in the bordering alfalfa fields during maize pollen shed. (n = 3).

Estimation of prey abundance. Mean number of adult thrips per plant during the first sampling period was 16.5 (± 0.75 SE, n = 95). Other prey groups are presented as medians [+; - quartile] of the estimated abundance group during the sampling periods (I-III). Aphids were predominantly found in the beginning of the season (I = 1 [2; 1], II = 1 [0; 1], III = 0 [0; 0]). Leafhoppers were present during all periods (I = 2 [3; 2], II = 2 [3; 2], III = 3 [3; 3]). Spider mites only occurred after pollen shed (I = 0 [0; 0], II = 0 [0; 0], III = 2 [3; 2]).

Toxin content in predators collected in alfalfa field adjacent to Bt maize. Most predators collected in the adjacent alfalfa field contained negligible levels of Cry1Ab toxin (Figure 4.5). Only adults of *Chrysoperla* spp. contained significant Bt toxin levels, in particular, when collected close to the adjacent Bt maize field (1 – 3 m).

Bt maize fields near Lleida (Spain)

DISCUSSION

Risk assessment studies of an insect resistant transgenic crop require previous knowledge of the agroecosystem. Arthropod species to be tested for adverse effects should be selected according to their ecological and economic importance in the crop and the likelihood of exposure to the insecticidal protein (Dutton et al., 2003). Exposure of predators depends on their feeding habits as well as on the feeding ecology of the prey (e.g., herbivores). The toxin ingestion by herbivores depends on the expression levels and pattern in plants. Thus, as a first step, the toxin expression in different plant tissues was verified. As expected from previous studies (Koziel et al., 1993; Fearing et al., 1997) the toxin was mainly present in leaves and pollen.

In order to be able to track the toxin through the trophic levels, the Bt toxin content in the most abundant herbivores found in Bt maize fields was determined. No or negligible amounts of toxin were detected in aphids and leafhoppers. These findings are in agreement with previous laboratory studies (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002; 2004). Thrips collected from Bt maize fields did not contain significant toxin levels either, even not when collected directly from inflorescences during pollen shed. This was not expected, as in a previous laboratory study, the toxin concentrations in larvae of *F. tenuicornis* reared on Bt maize plants (Bt11, Syngenta) were up to 50 times higher than those found in the field before pollen shed (Obrist et al., 2005). The discrepancy with the present study could be due to the different cultivars used in the two studies (Event176 versus Bt11) as they have different expression levels and spatial patterns (see Dutton et al., 2003). This assumption is supported by the fact that before pollen shed, most thrips were found in leaf sheaths and whorl, two parts in which very low toxin levels were measured in Event176. Contrastingly, spider mites (*T. urticae*) collected in the field contained toxin concentrations that were almost three times higher than those measured in plants and four times higher than those in spider mites used in our laboratory study. As fluctuations in the toxin content in spider mites were already observed in previous laboratory studies (Dutton et al., 2002; Obrist et al., in press), it can be assumed that variable proportions of different stages in the population (including diapausing individuals) account for a major part of this variability. Among all herbivores tested to date, spider mites contain by far the highest toxin levels. Moreover, the toxin ingested by them has been shown to remain in an active form (Obrist et al., in press). Thus, these herbivores bear a high likelihood for transferring the toxin to their predators. Another interesting herbivore regarding the toxin content is the chrysomelid beetle *O. melanopus*. In the beginning of the sampling season, considerable toxin concentrations were measured in adults. However, during the following sampling periods, no toxin was detected anymore. This finding can be explained by the biology of *O. melanopus*, of which the F_1 adults were shown to feed on corn from late May to late June and subsequently stop feeding and become less active (Grant and Patrick, 1993).

Exposure of predators does not only depend on the presence of toxin in herbivores but also on its own feeding habits. *Orius* spp. are important predators in maize, and thus our studies aimed at describing their routes of exposure to Bt toxin in more detail by performing complementary laboratory experiments. These have shown that *O. majusculus* ingests Bt toxin when feeding on pollen or spider mites in a "no-choice" situation, but that toxin persistence in adults is shorter than 24 h. Since a previous study reported that the toxin concentration in *C. carnea* was related to the toxin content in the respective herbivore and also corresponded to the amount of food that was taken up by the predator (Obrist et al., in press), it could have been expected that the toxin uptake by *O. majusculus* was also related to the weight gain. But such a relation was not found when *O. majusculus* was fed with pollen or Bt maize leaves. Tested individuals contained well detectable toxin levels after feeding on pollen for 3 h (0.38 µg g^{-1} for nymphs; 0.24 µg g^{-1} for adults) even though their weight gain was extremely restricted. The present findings therefore suggest that the toxin is rather concentrated in the content of the pollen grain leading to proportionally high toxin uptake by *O. majusculus* when sucking out Bt pollen. In contrast, no toxin was detected in *O. majusculus* nymphs which were fed on Bt maize leaves, although plant feeding was confirmed by the weight gain of insects kept on maize leaves. This finding stands in agreement with a study by Armer et al. (2000) who could not detect Cry3A toxin in *O. insidiosus* after feeding on Bt potato leaves. Armer et al. (1998) have shown in a previous study that the same predator ingests xylem sap. Given that the overall protein concentration in xylem is low and that these proteins are rather specific to the xylem (Biles and Abeles, 1991; Buhtz et al., 2004), it can be assumed that Bt toxin content is negligible in xylem. The fact that the toxin could not be detected in *Orius* spp. after feeding on Bt maize leaves suggests that Cry1Ab toxin ingestion from Bt maize leaves is not relevant as compared to uptake via prey or pollen.

In the field, the toxin concentrations measured in *Orius* spp. are related to those measured in the food sources available during the three sampling periods. Before pollen shed, these predators mainly prey on thrips and occasionally on aphids, leafhoppers, lepidopteran eggs or other predatory arthropods (Dicke and Jarvis, 1962; Coll and Bottrell, 1992; Corey et al., 1998; L.B. Obrist, personal observation). Since none of these herbivores contained significant toxin levels, and the laboratory experiments indicated that toxin uptake by *O. majusculus* through plant feeding is negligible (except from pollen), the exposure of the predator to the toxin before pollen shed can be estimated as being minor.

However, during pollen shed, the toxin in *Orius* spp. was well detectable. These results leave no doubt that pollen was responsible for the toxin found in *Orius* spp., in particular, as our laboratory experiments have shown that *O. majusculus* can take up considerable amounts of toxin from pollen. In the last sampling period, levels of toxin measured in *Orius* spp. were similar to those found during pollen shed. Although *Orius* spp. were often observed feeding on spider mites (L.B. Obrist, personal observation), it cannot be ruled out that they also fed on old pollen. Further investigations would be needed to determine to what extent decomposing pollen could be used as a food source by *Orius* spp. or other arthropods.

The different toxin concentrations found in other heteropteran groups indicate that their feeding habits must differ from those of *Orius* spp.. Mirids (*Trigonotylus* spp.) contained toxin already during the first sampling period, and, when pollen was available, their toxin content exceeded that of all other groups collected during this period. Species of this family are known to be omnivorous, and it even occurred that a species (*Tytthus mundulus* Breddin) considered as a pest in one place, was a highly effective predator in another (DeBach and Rosen, 1991; Naranjo and Gibson, 1996). Apparently, the mirids collected for this study substantially feed on maize leaves and possibly on pollen. In contrast, the toxin content in *N. provencalis* could hardly or not be detected during neither of the sampling periods. For nabids, some plant feeding has been reported (Ridgway and Jones, 1968), but generally they are predacious, presumably preying on small invertebrates including leafhoppers, aphids and various other arthropods (Lattin, 1989). Although we cannot exclude that they ingested the toxin at any time, the exposure of nabids to Bt toxin in maize in Spain can be estimated negligible. A similar situation was found for the carabid beetle, *D. atricapillus*, an efficient predator which is known to climb plants to feed there on soft bodied prey items such as aphids, insect eggs (Sunderland and Vickermann, 1980; Luff, 2002; Sunderland, 2002).

Chrysopids are of particular interest with regard to the risk assessment of Bt maize, since previous laboratory studies revealed inconsistent effects of Cry1Ab on *C. carnea* (Hilbeck et al., 1998; Dutton et al., 2002; Romeis et al., 2004). However, the exposure of this predator to the toxin under field conditions has not been assessed yet. Negligible toxin levels were found in *Chrysoperla* spp. larvae when collected before and during pollen shed. Apparently, larvae did not or only marginally feed on pollen in our fields, even though pollen feeding was reported in no-choice laboratory studies (Pilcher et al., 1997; J. Romeis, personal communication). In contrast, relatively high toxin levels in larvae were measured after pollen shed, possibly as a consequence of consuming spider mites. The toxin content measured in adult *Chrysoperla* spp. differed from that in larvae. Adults of this genus are not predacious and feed primarily on pollen and carbohydrates (McEwen et al., 2001). Accordingly, highest toxin levels in adults were detected during pollen shed whereas negligible levels in them were found after pollen shed. In contrast to the chrysopids, adults of the related family of hemerobiids did not contain any Bt toxin. This family is generally predacious with adults having similar feeding habits as larvae (Stelzl, 1991; McEwen et al., 2001). The total lack of Bt toxin in the hemerobiid samples collected during pollen shed indicates that they did probably not feed on pollen in the maize fields, even though pollen feeding for some species of this family has been reported (Stelzl, 1991). It can also be assumed that pollen feeding for adults of the coccinellid *A. variegata* is of minor importance, given that the Bt toxin could not be detected in them during pollen shed. This is an interesting finding as several coccinellid species are known to feed on pollen (Smith, 1965; Hodek and Honěk, 1996; Lundgren and Wiedenmann, 2002; 2004). Pollen can represent a substantial food source for *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) an important predator in North America and thus, pollen feeding is addressed in many risk assessment studies of transgenic Bt maize (Pilcher et al., 1997; Duan et al., 2002; Lundgren and Wiedenmann, 2002; 2004). *Stethorus punctillum*, another coccinellid that was analysed for the presence of Bt toxin, is a specialist predator feeding exclusively on spider mites (McMurtry et al., 1970; Hodek and Honěk, 1996). As it was expected from the high toxin concentrations in spider mites, this predator contained remarkable toxin concentrations. Exposure of *S. punctillum* to Bt toxin in the field, in particular of the larvae, was therefore highest of all predators that were analysed.

A consistent result of this study is that Bt toxin concentrations in preimaginal stages (nymphs and larvae) of predators are higher than in the respective adults, provided that both stages feed on the same prey items. For *O. majusculus* the data for weight uptake substantiate these results by showing that nymphs ingest significantly more food than adults. The finding that such nymphs or larvae are generally exposed to higher toxin concentrations than adults may be taken into account for risk assessment studies.

In spite of the toxin levels in adult *Orius* spp. when collected in Bt maize fields, the toxin was hardly detectable in individuals collected in the bordering alfalfa field. This could be due to the short persistence of the toxin in adults as indicated by the laboratory experiments, or to lacking movement between the two crops. Bt toxin was still detectable in adult *Chrysoperla* spp. when collected in the alfalfa field at the closest distance (1-3 m) to the Bt maize field. The low concentrations measured in adults collected farther away from the Bt maize field (4-6 m) indicate that the concentrations rapidly decrease with distance. It remains unclear if *Chrysoperla* spp. collected in the margin of the alfalfa field transported the toxin after feeding on pollen in the Bt maize fields or fed on Bt maize pollen which was freighted from the Bt maize field into the margin of the alfalfa field. The second assumption seems less likely when considering the fact, that the toxin was hardly detected in *Orius* spp., another pollen feeding predator that would probably also contain the toxin, if Bt maize pollen was available in this zone.

Our field studies are the first to show the level of exposure of arthropod predators to Cry1Ab in Bt maize in a quantitative analysis. This data, together with the information gained about feeding behaviour of different arthropods, provide the information needed for a science based exposure assessment of relevant predators in Bt maize.

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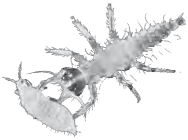
Pollen shedding maize tassels

GENERAL CONCLUSION



Bacillus thuringiensis (Bt) sprays are widely-used biopesticides which are effective against specific insect pests but considered safe for non-target organisms due to their specific mode of action (Glare and O'Callaghan, 2000). Nevertheless, the use of transgenic Bt crops has raised concerns, because the high and consistent toxin expression due to the insertion of a modified and activated *Bacillus thuringiensis* gene into a plant genome, could entail potential risks for non-target organisms (Dale et al., 2002; Federici, 2002). This thesis was conducted to investigate various aspects pertaining to risk assessment of transgenic plants using Bt maize as a model plant. Potential direct and indirect effects of Bt maize on non-target arthropods (thrips and predatory mites) were assessed. The movement of Bt toxin through the trophic levels was investigated under laboratory and field conditions to determine the degree of exposure of herbivores and predators to Bt toxin.

DIRECT AND INDIRECT EFFECTS OF BT PLANTS ON NON-TARGET ARTHROPODS

Transgenic plants expressing an insecticidal protein can elicit direct and indirect effects on non-target organisms. Direct effects are caused by the toxin itself and only occur if the non-target organism is susceptible to the toxin. Given that Cry1Ab toxin expressed by Bt maize is very specific, direct toxic effects on non-target organisms are not likely to occur. The fact that the thrips, *Frankliniella tenuicornis* was not affected when feeding on Bt maize, although toxin ingestion by all feeding stages was confirmed, leads to the conclusion that the Cry1Ab protein is not toxic for thrips. Moreover, no direct toxic effects could be detected on predatory mites (*Amblyseius cucumeris*), as no differences in their performance were found when fed with spider mites containing Bt toxin and a control.

Indirect prey-quality mediated effects of the toxin may occur on organisms of higher trophic levels (e.g., predators) if their prey is affected by the toxin and represents a food source of reduced quality, size, or abundance for the predator. Such effects are strongly related to the sensitivity of the herbivore to the toxin and are assumed to be responsible for previously reported effects on natural enemies when offered Bt-intoxicated lepidopteran prey (Dutton et al., 2002; Meissle et al., 2005; Vojtech et al., 2005). As thrips are not affected by Cry1Ab toxin, it can be ruled out that they could mediate such effects to their antagonists. Spider mites (*Tetranychus urticae*) were already shown to be unaffected when feeding on Bt maize (Dutton et al., 2002). Accordingly, no prey quality-mediated effects on predatory mites were detected when feeding on spider mites reared on Bt maize.

Another category of indirect effects is related to unintended changes in the plant due to the transformation event. Such changes are not due to the toxin expressed by the plant and they could cause favourable or adverse effects on arthropods (Lecardonnel, 1999). For example, Bt maize varieties were reported to have higher lignin contents than their respective control plants (Saxena and Stotzky, 2001; Flores et al., 2005). It cannot be ruled out that such properties could have an influence on performance of herbivores. The present studies showed that feeding on Bt maize caused nor positive nor negative effects on thrips. Unintended changes in the plants can also affect predators, for example those feeding directly on plant parts. In this study, subtle differences were detected in the performance of female *A. cucumeris* when fed with Bt or non-Bt maize pollen. The effects cannot be attributed to direct toxicity of Cry1Ab toxin, as it was not sufficiently present in pollen of this transgenic variety, and caused no effects when offered in spider mites. This leads to the conclusion that the effects were more likely caused by some differences in the nutritional quality of Bt maize pollen, which may be attributed to some unintended changes in the plant or to differences between the transgenic and the near-isogenic line (discussed below). In fact, variation in the nutritional profile of pollen originating from different maize varieties is not unusual and was also found to cause variable mortality rates on *Coleomegilla maculata* (Lundgren and Wiedenman, 2004). In the field, predatory mites are exposed to food sources of different quality and suitability. The ecological relevance of the effects on predatory mites reported in this study therefore appears negligible.

The present findings confirm that direct toxic effects of the Cry1Ab toxin expressed by Bt maize on non-target arthropods belonging to other orders than Lepidoptera are very unlikely. The occurrence of prey quality-mediated effects on predators is not expected, provided that the prey offered in the experiment is not a sensitive lepidopteran species. Indeed, Bt maize appears safer for non-target organisms than microbial Bt spray formulations. The latter were found to harm spider mites as well as predatory mites (Hassan et al., 1987; Chapman and Hoy, 1991; Dutton et al., 2003a). From the present studies, it can be concluded that these effects cannot be attributed to the Cry1Ab toxin. It is more likely that different toxins (δ-endo- or exotoxins), other compounds contained in such spray formulations (e.g., carriers, UV screen) or other unknown factors were responsible for these effects. The findings of this study indicate that indirect effects caused by unintended changes in the plant, not related to the toxin, cannot be ruled out. Such alterations are known from classical plant breeding (Bradford et al., 2005). The visible alterations of transformed plants are generally eliminated by breeding and selection programmes as it is done in classical plant breeding. The assessment of indirect effects on non-target organisms would pose some difficulties. Being not related to the toxin, the effects cannot be detected using a targeted approach. Moreover, they may depend on various environmental conditions and are event-specific, which means that they can differ in each transgenic variety (Alonso et al., 2003; Schubert et al., 2004). Whether and how such indirect effects should be addressed in a risk assessment protocol for non-target effects of transgenic plants remains disputable. Furthermore, the question may arise if such indirect effects on non-target organism should also be taken into account in conventional plant breeding.

EXPOSURE OF ARTHROPODS TO CRY1AB TOXIN

A great part of this thesis is based on the investigation of the exposure of arthropods to Bt toxin, given that the risk of an insect resistant transgenic plant for a non-target arthropod depends on the hazard (potential of a plant to cause adverse effects) and the degree of exposure. The knowledge about the exposure is also needed to confirm and interpret results obtained in studies aimed at determining the actual toxicity of Cry1Ab toxin for non-target organisms. Furthermore, valuable information can be drawn from exposure studies, which can contribute to improve methods for risk assessment experiments.

Uptake of Cry1Ab toxin by predators

Although previous studies have been done to quantify the content of Cry1Ab toxin in herbivores that had been kept on transgenic Bt maize, the transfer of the toxin to the predators has not been verified to date. In a laboratory experiment it was determined to what level predators are exposed to the toxin when feeding on different Bt-containing food sources. The ELISA confirmed that *Chrysoperla carnea* larvae ingest the toxin when feeding on Bt-containing spider mites (*T. urticae*) and lepidopteran larvae (*Spodoptera littoralis*). The toxin ingestion by *C. carnea* was related to the toxin content in the herbivore and also corresponded to the amount of food that was ingested during the experiment. The uptake of Cry1Ab toxin when feeding on spider mites was also confirmed for *Orius majusculus*. This omnivorous predator ingests Bt toxin also to a relatively high extent when feeding on pollen containing the toxin (Event176). In contrast, *O. majusculus* did not contain toxin after feeding on Bt maize leaves, although the weight gain of tested individuals confirmed that they had ingested leaf sap. Green plant tissue feeding by omnivorous predators is a feature of general interest, as it could have implications for their capacity as biological control agents (Naranjo and Gibson, 1996). Our findings agree with those by Armer et al. (1998; 2000) and suggest that *Orius* spp. feed on xylem, most probably for water provisioning. In the context of the risk assessment of Bt maize, the results show that Bt maize leaves as a food source for *Orius* spp. are not recommended when experiments are aimed at assessing direct toxic effects of Cry1Ab toxin. For this purpose, pollen or spider mites are more appropriate.

Exposure of arthropods to Cry1Ab toxin in the field

An important aspect of this work is the assessment of exposure of arthropods under natural conditions. Many field tests have been conducted to assess the impact of transgenic Bt maize on the abundance of various arthropods. Such field studies require high efforts and sometimes provide data which is difficult to interpret. The knowledge of exposure in the field could help to determine if differences in abundance are due to the presence of toxin (direct effects) or to other factors (indirect effects). Moreover, information about exposure may be used as a criterion to select species that should be further tested in the laboratory for direct toxic effects. The field study conducted in Spain revealed that there are great differences in the exposure among herbivores and predators and that not all arthropods are exposed to the toxin in Bt maize fields. Some herbivores (aphids, cicadellids, thrips) did not contain significant toxin levels in spite of feeding on Bt maize plants. The values measured in them were comparable to those determined in the laboratory (Raps et al., 2001; Head et al., 2001; Dutton et al, 2002; Dutton et al., 2004). In contrast, spider mites contained high levels of toxin, which exceeded those in plants by a factor of three and were higher than those found in individuals from the laboratory colony. The presence of this herbivore in the field strongly influenced the exposure of predators. For example, the predacious *Chrysoperla* spp. larvae only contained toxin when spider mites were available. The specialist coccinellid species, *Stethorus punctillum* feeds exclusively on spider mites and therefore contained the highest toxin levels compared to all other predators that were investigated. In addition to spider mites, Bt maize pollen of Event176 represented an important Bt toxin source for omnivorous predators such as for *Orius* spp. or for adult *Chrysoperla* spp.. However, other predators such as adult coccinellids and hemerobiids did not contain toxin when pollen was present. This is an unexpected result because, based on the knowledge of the feeding habits of species belonging to these insect families, it was assumed that they would contain Bt toxin (e.g., Smith, 1965; Stelzl et al., 1991). Other generalist predators such as the heteropteran species *Nabis provencalis* and the carabid *D. atricapillus* did not appear to be substantially exposed to toxin as it was hardly detected in them, independent of the presence of spider mites or pollen. Besides the knowledge required for assessing the risks of Bt maize on non-target arthropods, this study provided interesting information about the feeding ecology of various arthropods in maize fields. However, one has to keep in mind that the study was conducted in Spain, where different arthropod groups may be present, than in other maize growing areas, as for example in the USA. *Coleomegilla maculata,* a species occurring in North America, would probably be exposed to toxin, as it is reported to use pollen as a food source (Smith, 1965; Pilcher et al., 1997; Duan et al., 2002; Lundgren and Wiedenmann, 2004). Furthermore, the exposure of arthropods is strongly influenced by the presence of toxin in pollen. Event176 was grown for many years in Spain now, but is now being replaced by varieties expressing negligible toxin levels in pollen (Brookes, 2002). In the USA, Bt maize varieties expressing Cry1Ab toxin to significant extent in pollen, are not commercially grown anymore. Nevertheless, the information gained in this study can be used as a basis for future studies with transgenic plants, such as Bt maize resistant to *Diabrotica* spp. (Coleoptera: Chrysomelidae), which expresses Cry3Bb1 toxin in pollen.

METHODOLOGICAL ASPECTS

Selection of species and suitability of testing systems: Predatory mites as a case study

When testing organisms of higher trophic levels for non-target effects of transgenic plants in the laboratory, one needs to consider several factors. Besides the selection of a suitable organism for the experiment, an optimal testing system should be established. The selection of an organism should mainly be based on its ecological and economic importance (e.g., conservation needs, potential value for biological control, roles in pollination, or soil function) and on their likelihood of exposure to the toxin (Dutton et al., 2003b; Cowgill and Atkinson, 2003). By taking these prerequisites into account, more practical aspects, such as the availability and amenability of the testing organism, should be considered. Allowing for all these factors, the omnivorous predator *A. cucumeris* appeared a suitable organism for such experiments. The presence of predatory mites including *A. cucumeris* in maize fields is known as well as their potential for biological control (Gillespie, 1989; Hoy and Glenister, 1991; van Houten et al., 1995; Iraola et al., 1997). Bt toxin ingestion by the predator itself could not be verified due to its small size, but it can be assumed that it is exposed to Bt toxin in maize fields as it feeds on young thrips larvae and spider mites, which were shown to contain toxin when feeding on Bt maize. This predator is commercially available and can easily be reared in the laboratory. In addition, the omnivory of this species appeared advantageous, as two different testing systems could be used. One consisted in offering spider mites reared on Bt maize to the predatory mites and the second in exposing them directly to transgenic pollen. The use of spider mites containing Bt toxin as a food source entailed the advantage that a relatively high degree of exposure to the toxin was ensured throughout the experiment, and that prey quality-mediated effects should not occur, given that this herbivore is not sensitive to the toxin (Dutton et al., 2002). In contrast, maize pollen originating from Bt11 hardly contains toxin, and could be used to detect potential indirect effects of transgenic plants on the predator. Preliminary experiments were done to determine if the state of the food source may influence the performance of the predator. Spider mites were therefore offered to *A. cucumeris* either alive or killed. The results showed that *A. cucumeris* is very sensitive to changes in the food quality as living spider mites were a better food source than those which were killed before the experiment. For this reason the spider mites containing the toxin were offered alive. *Amblyseius cucumeris* represents a good indicator for assessing the effects of Bt maize as it complied for all requirements described above and was shown to be sensitive to alterations in the food source.

Control plants

When *A. cucumeris* was tested on pollen, subtle differences between the performance on females were detected when offered pollen originating from Bt and non-Bt maize (discussed above). Independent of the ecological relevance of these effects, the findings point at an important factor that should be considered in risk assessment studies, namely the control plant used in the experimental set up. The control plant can either be an unmodified direct parent line, or a null-segregant which represents a transgenic line that has lost the transgene following out-crossing and chromosome segregation. Using the latter as a control could be advantageous as it only differs from the transgenic line by the presence or absence of the transgene product (Conner et al., 2003). However, the use of null-segregants may also entail disadvantages, as for example, that an appropriate plant may not be available. By using an unmodified control plant for the experiment with predatory mites, it was not possible to distinguish effects, which were directly related to the insertion of the transgene from those caused by genetic divergence from the parental line due to a breeding programme following the transformation event (Kok and Kuiper, 2003; O'Callaghan et al., 2005). Including additional plant cultivars in the experiments could provide an indication about the natural variance among varieties and help to elucidate the significance of the detected differences between the transgenic and non-transgenic line (Kok and Kuiper, 2003; O'Callaghan et al., 2005).

Activity of Cry1Ab toxin ingested by herbivores

A prerequisite for testing direct toxic effects of a transgene product on natural enemies is that the toxin in the food source offered to the predator remains in a biologically active form. This applies particularly for tritrophic feeding experiments, as the bioactivity of the toxin could be altered when ingested by herbivores. It cannot be ruled out that Bt toxin is modified or broken down by gut enzymes even though measurable with ELISA as described by Pang et al. (1999). The bioassay with the target lepidopteran species, *Ostrinia nubilalis* showed that the toxin ingested by *T. urticae* and *S. littoralis* remained as active as it was in the plant. In the context of the debate over the susceptibility of *C. carnea* to *Cry1Ab* toxin expressed by Bt maize, the findings give further evidence that direct toxic effects on the predator are unlikely to occur (see introduction). The fact, that feeding on Bt-containing spider mites caused no effect on this predator, although concentrations of biologically active toxin in spider mites can be up to ten times higher than in *S. littoralis*, strongly suggests that effects observed on *C. carnea* when fed with Bt-intoxicated *S. littoralis* were exclusively prey-quality mediated.

The use of Cry1Ab toxin produced by *Escherichia coli*

Direct toxicity of Cry1Ab protein for non-target organisms can be tested by exposing them to "artificially produced" toxin incorporated in different kinds of diets (MacIntosh et al., 1990; Hilbeck et al., 1998; Mendelsohn et al., 2003; Romeis et al., 2004). Such proteins are obtained from transformed *Escherichia coli* expressing a *cry1Ab* gene identical to that from *B. thuringiensis*. The purified protein is solved and trypsinised to achieve its full state of activity (Pusztai-Carey, 1994). The *cry1Ab* gene in Bt plants was modified before insertion and codes for a truncated version of the Cry1Ab protein (Koziel et al., 1993). Using a bioassay with *O. nubilalis*, the activity of *E. coli* produced and plant derived Cry1Ab protein was compared. The toxin obtained from *E. coli* appeared more toxic for *O. nubilalis* than the plant-derived toxin when applied at equal concentrations based on ELISA. Although it was not possible to elucidate the reason for this inconsistency within the framework of this study, it is an important factor to consider in forthcoming risk assessment studies. The use of toxin produced by *E. coli* can be advantageous as it can be applied in high concentrations, for example, to determine direct toxicity for arthropods in "worst case" experiments, or to investigate processes at the molecular level such as binding or degradation mechanisms. However, the present results lead to the conclusion that findings obtained with toxin produced by the *E. coli* should be interpreted with care and that comparisons with plant derived toxins should be done to determine the significance of results obtained with *E. coli* toxin. Furthermore, it would be interesting to investigate more in detail the origin of the difference in toxicity. Palm et al., (1994) reported that plant-derived Cry1Ab toxin degraded faster in the soil than the purified toxin. It cannot be ruled out that secondary plant compounds influence the toxicity as it was shown to occur in transgenic Bt cotton by Olsen and Daly (2000). A comparison of the degradation rate of the two toxins or the influence of the presence of plant material on the toxicity could be made in additional bioassays analogous to those performed in this study. Differences between the Cry1Ab toxin produced by *E. coli* and by Bt maize may also exist regarding their state of activity (solubilisation and truncation). To date, it is not known to what extent solubilisation and additional truncation of the Cry1Ab toxin expressed by Bt maize is needed for further binding processes in the insect gut (Federici, 2002). Western blots could be performed to determine if differences in size exist between the Bt toxins of different origin, which may account for their state of activity.

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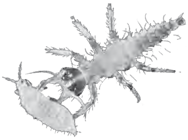

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