Compatibility of biological control with Bt maize expressing Cry3Bb1 in controlling corn rootworms

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Compatibility of biological control with \textit{Bt} maize expressing Cry3Bb1 in controlling corn rootworms

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Abstract

The western corn rootworm, *Diabrotica virgifera virgifera*, is a serious maize pest in the USA and Europe. Genetically engineered (GE) maize producing insecticidal Cry3Bb1 protein derived from *Bacillus thuringiensis* (*Bt*) to control this pest has been commercialized in the USA. One crucial part of the environmental safety assessment of GE plants is the evaluation of potential risks for non-target species, including biological control agents.

Toxicity of the Cry3Bb1 protein to non-target species can only occur when the *Bt* protein is ingested in a biologically active form. The insecticidal activity of Cry3Bb1 expressed in different tissues of *Bt* maize, contained in maize herbivores, and in spiked soil was confirmed in sensitive insect bioassays using larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*). Bioassays with the same concentration of Cry3Bb1 per ml diet suggested that nutritional quality of food and degradation processes may influence Cry protein toxicity.

A risk assessment for a generalist predator, the spider *Theridion impressum* was conducted. The spider was found to be exposed to the *Bt* protein after analyzing its prey spectrum and the Cry3Bb1 concentration in potential prey species collected in a *Bt* maize field. As feeding studies with *Bt* maize fed prey and *Bt* maize pollen did not indicate adverse effects on adult and juvenile spiders, it can be concluded that *Bt* maize most likely poses a negligible risk for *T. impressum*.

The interaction of an entomopathogenic fungus, *Metarhizium anisopliae*, with *Bt* maize and *D. v. virgifera* was studied in the laboratory. While feeding on *Bt* maize delayed the development of *D. v. virgifera* larvae, there was no difference in fungal infection rates between *Bt* and control maize treatments for larvae and adults. This implies that Cry3Bb1-expressing *Bt* maize is compatible with biological control by this entomopathogenic fungus.

The presented results together with previously published laboratory and field studies on non-target species demonstrate that rootworm-resistant *Bt* maize contributes to sustainable agriculture by maintaining ecological services including biological control provided by spiders and entomopathogenic fungi.
Zusammenfassung

Der westliche Maiswurzelbohrer, *Diabrotica virgifera virgifera*, ist ein bedeutender Maisschädling in den USA und Europa. Gentechnisch veränderter (GV) Mais, der das insektenwirksame Cry3Bb1-Protein aus *Bacillus thuringiensis* (*Bt*) produziert, wird bereits kommerziell angebaut, um den Schädling zu kontrollieren. Ein wichtiger Teil der Umweltverträglichkeitsprüfung von insektenresistenten GV Pflanzen ist die Beurteilung möglicher Risiken für Nicht-Zielorganismen, wie solche, die zur biologischen Schädlingskontrolle beitragen.

Das Cry3Bb1-Protein kann nur dann giftig für Nicht-Zielorganismen sein, wenn es in biologisch aktiver Form aufgenommen wird. Die insektizide Wirksamkeit von Cry3Bb1 in *Bt*-Mais Gewebe, in Herbivoren, die an *Bt*-Mais gefressen haben, sowie in angereichertem Boden, wurde in Fütterversuchen mit Larven des Kartoffelkäfers (*Leptinotarsa decemlineata*) nachgewiesen. Versuche mit der gleichen Cry3Bb1-Konzentration pro ml Kunstdiät deuteten an, dass die Nahrungsqualität sowie Abbauprozesse die Toxizität beeinflussen können.

Für die räuberisch lebende Spinne *Theridion impressum* wurde eine Risikoanalyse durchgeführt. Nachdem das Beutespektrum analysiert wurde und die Cry3Bb1-Konzentrationen in möglichen Beutetieren in einem *Bt*-Maisfeld gemessen wurden, stellte sich heraus, dass die Spinne das *Bt*-Protein aufgenommen hatte. Fütterversuche im Labor mit Beute, die zuvor *Bt*-Mais gefressen hatte, und mit *Bt*-Maispollen ergaben jedoch keine Hinweise auf negative Effekte. Demnach stellt *Bt*-Mais für *T. impressum* vermutlich ein vernachlässigbares Risiko dar.


Chapter 1
Introduction

Corn rootworms – major pests of maize

Leaf beetles of the genus Diabrotica (Coleoptera: Chrysomelidae) are known as corn rootworms and have become major pests of maize in North America and Europe. Central and South America is presumed to be the centre of origin of corn rootworm species harbouring the highest biodiversity (Tallamy et al. 2005). When the spreading cultivation of maize monocultures in North America in the early 20th century met with naturally occurring corn rootworm species, populations started to build up quickly until the beetles became the most devastating maize pest complex in the USA (Tallamy et al. 2005; Ward et al. 2005; Hellmich et al. 2008). Presumably in the late 1980s, the Western corn rootworm (WCR) (Diabrotica virgifera virgifera) was accidentally introduced into Europe where it was first recorded in Serbia in 1992. Since then, populations have been spreading rapidly (Berger 2001; Kiss et al. 2005; Miller et al. 2005).

The univoltine WCR develops best on maize even though other cereals and grasses may serve as alternative hosts (Moeser et al. 2005; Clark & Hibbard 2004; Oyediran et al. 2004). After diapausing as eggs in the soil, larvae hatch early in the summer, seek maize roots to feed on and develop through three larval stages before pupation (Chiang 1973). Infested plants suffer from reduced nutrient and water supply and decreased stability results in lodging and subsequently yield losses. Emerging adult D. v. virgifera mate and oviposit for several weeks mainly in maize fields. They feed on pollen, maize silk and young ears which may reduce plant fertility and affect grain quality (Chiang 1973; Krysan 1986; Levine & Oluomi-Sadeghi 1991; Ward et al. 2005).

Options to control corn rootworms

Crop rotation is most effective to control corn rootworms. Because eggs are laid almost exclusively in the soil of maize fields, hatching larvae encounter another crop in the next season. However, beetles managed to adapt to biannual rotations of maize with soybean, which are often found in the USA. Females increasingly show preference to oviposit in soybean (where maize is cultivated in the next year) or produce eggs that remain in diapause for more than one winter (Levine et al., 2002).

In regions where crop rotation is not an option due to resistant beetles or economical or technical constraints, rootworm damage is usually reduced with soil insecticides against larvae and occasionally also with foliar insecticide applications targeting adults. In the USA, 60% of total insecticides used on
maize are applied against *D. v. virgifera*, which is also named “billion dollar bug” because of high insecticide costs and crop losses (Ward et al. 2005; Hellmich et al. 2008). However, broad spectrum insecticides are known to have adverse effects on soil arthropods and success in reducing rootworm damage is depending on many abiotic factors (e.g., organic matter, soil characteristics, temperature), biotic factors (e.g., microbial degradation) and operational aspects (e.g., planting date, tillage practice, application rate and method). Reduced effectiveness of insecticides has been frequently observed within a few years due to increased microbial degradation and the evolution of resistance in beetle populations (Levine & Oluomi-Sadeghi 1991).

In agricultural fields, naturally occurring enemies like predators, parasitoids and pathogens attack most herbivores and provide natural pest control that keeps populations of most species below the economic level of damage (Sunderland et al. 1997; Symondson et al. 2002). Corn rootworm eggs, larvae and adults are likely to be consumed by a range of rather opportunistic generalist predators (Levine & Oluomi-Sadeghi 1991; Kuhlmann & van der Burgt 1998; Tóth et al. 2002). A number of pathogens, nematodes, predators and parasitoids are known from Central and South America (Kuhlmann & van der Burgt 1998). In contrast, no effective specialist natural enemies were found to attack *D. v. virgifera* in a survey conducted in Central Europe, even though entomopathogenic nematodes and fungi were reported from field collected larvae and adults in low frequencies (Toepfer & Kuhlmann 2004; Pilz et al. 2008). This lack of effective specialist enemies and pathogens is typical for invasive species and one important factor for population densities building up (Toepfer & Kuhlmann 2004).

One strategy to respond to this lack of specialists is to mass rear and release natural enemies to suppress the pest. Field trials with entomopathogenic nematodes have demonstrated promising reductions in root damage and effort has been made to develop suitable application techniques (Toepfer et al. 2008). Similarly, entomopathogenic fungi show potential for biological control as laboratory and field studies revealed that certain strains were able to kill *D. v. virgifera* larvae and beetles effectively and found to persist in the soil (Krueger & Roberts 1997; Mulock & Chandler 2000; Pilz et al. 2007; Pilz 2008). A tachinid fly which parasitizes *D. v. virgifera* adults has been identified in Central America and reared in the laboratory (Zhang et al. 2003; Kuhlmann et al. 2005), even though the potential for biological control is low due to difficulties in mass rearing and handling (Stefan Toepfer, personal communication).

**Corn rootworm-resistant Bt maize**

In 2003, genetically engineered (GE) maize expressing insecticidal Cry3Bb1 protein from the bacterium *Bacillus thuringiensis* (*Bt* maize) entered commercial production in the USA (Vaughn et al. 2005). In the meantime, constructs expressing Cry34/35 or modified Cry3A were also commercialized.
(Hellmich et al. 2008). In all available *Bt* maize varieties, the insecticidal protein is expressed constitutively in the plant and prevents root damage by WCR larvae due to feeding deterrence and/or direct toxicity. Similar to insecticidal GE crops against lepidopteran pests, acreages planted to rootworm-resistant maize are continuously increasing in the USA (James 2007). Growing *Bt* maize has the potential to increase yields and reduce insecticide applications (Demont & Tollens 2005; Hellmich et al. 2008). However, maize plants expressing Cry3Bb1 are not completely protected against corn rootworm larvae and it is still unclear if adults are affected by the Cry3Bb1 protein or not.

The continuous exposure to a single *Bt* protein may lead to the development of resistance, as it happened frequently with chemical insecticides (Levine & Oloumi-Sadeghi 1991). A recently published study on WCR larvae demonstrated that the LC$_{50}$ (lethal concentration causing 50% mortality) in a colony that was reared for only 3 generations on *Bt* maize increased 22-fold compared to a colony not exposed to Cry3Bb1 (Meihls et al., 2008). To delay development of resistance, a certain percentage of conventional maize is usually grown as a “refuge” adjacent to the *Bt* crop. Thus, beetles developing successfully on *Bt* plants and carrying commonly recessive resistance alleles are likely to mate with adults from the non-*Bt* refuge, which results in heterozygous offspring lacking resistance to the *Bt* crop (Tabashnik et al. 2003; Ferré et al. 2008; Meihls et al., 2008). To reduce the likelihood of resistance development, plants expressing different insecticidal proteins with different modes of action targeting the same pest (pyramided genes) are developed (Hellmich et al. 2008).

**Non-target risk assessment of GE insect-resistant crops**

Before new GE crops can be cultivated commercially, risks to human health and the environment have to be assessed and evaluated by regulatory agencies (pre-market risk assessment). For potential risks that cannot be excluded in the pre-market risk assessment, case-specific monitoring plans are foreseen to accompany commercial production. Furthermore, general surveillance is often required by legislation to detect unexpected consequences for the environment at an early stage (Sanvido et al. 2005). In addition to regulatory studies that are needed for product registration and conducted by the applicant, scientific publications are readily incorporated in the risk assessment as they provide basic data on the properties of the insecticidal compounds and on the ecology of plants, pests, beneficial species and their interaction in agroecosystems.

One crucial part of the environmental safety assessment of transgenic insect-resistant crops is the evaluation of potential risks to non-target species. The focus is on organisms providing ecosystem services, like decomposition, pollination and biological control. The characterization of risk includes the determination of exposure to the active compound and the hazard of being
exposed (Conner et al. 2003; Raybould 2007; Romeis et al. 2008). Many beneficial species are exposed to the insecticidal protein by feeding directly on plant material (including pollen). When natural enemies feed on prey which has previously consumed Bt plants, the GE product is transferred along the food chain (Romeis et al. 2009). Additionally, species living below ground encounter Bt protein remaining in plant residues, residue-leachates or root exudates (Saxena et al. 2002; Icoz & Stotzky 2008). However, a certain species is only at risk if the transgenic protein shows toxicity at a realistic level of exposure (Raybould 2007). While estimating exposure requires knowledge on the concentration of biologically active insecticidal protein in the food (Romeis et al. 2009), toxicity is usually tested on a range of selected surrogate species using laboratory assays (Garcia-Alonso et al. 2006; Romeis et al. 2008).

**Bt maize and biological control**

In integrated pest management (IPM), sustainable cropping systems are established by combining environmentally friendly control strategies to minimize the need for harmful, broad-spectrum chemical insecticides. In Bt maize, naturally occurring biological control agents are needed to control herbivores outside the order of Chrysomelidae, which are not killed by Cry3 proteins. To avoid insecticide applications against secondary pests, it is thus important that natural enemies remain unharmed in Bt maize fields (Romeis et al. 2009). Because Bt maize does not control corn rootworm larvae completely (Al-Deeb & Wilde 2005; Oyediran et al. 2007), the release of biological control agents in the field might increase efficiency of rootworm control and reduce the likelihood of resistance development. In addition, biological control agents could be released to control beetle populations in the refuge strips adjacent to Bt maize. A prerequisite for the successful combination of biological control agents with Bt maize is the lack of negative interactions in controlling the pests.

**Scope of this thesis**

The scope of this thesis is to assess the impact of Cry3Bb1 expressing maize on biological control agents. Both the evaluation of exposure to the biologically active Bt protein and the hazard of being exposed are part of the risk assessment procedure. The objectives of the PhD project are:

1) Establishment of new methods to evaluate effects of insecticidal Bt maize on biological control agents and to measure the biological activity of Cry3Bb1.

2) Assessment of the risk that corn rootworm-resistant Bt maize poses for selected biological control agents.

**Thesis outline**

In order to estimate how non-target arthropods are exposed to insecticidal proteins along the food chain, one has to know the concentration of
biologically active protein in the food of the organisms of interest. To detect and quantify Cry proteins expressed by Bt crops, immunological methods are widely used. Enzyme-linked immunosorbent assays (ELISA) allow the very sensitive detection of low Bt protein concentrations but may result in positive signals also for fragments or denatured molecules that have lost biological activity. To confirm the biological activity of Cry proteins, bioassays using sensitive insects can be used. In Chapter 2 of this thesis a procedure to test biological activity of fluid and pulverized test substances using larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*; Coleoptera: Chrysomelidae) is presented. Data on the biological activity of Cry3Bb1 contained in different maize tissues, herbivorous arthropods and soil are compared with ELISA measurements.

Previous research on biological control agents conducted with Cry3Bb1-expressing maize clearly focused on coleopteran predators in the families of Coccinellidae and Carabidae. Other predator groups, like spiders, have been generally neglected in risk assessment research of Bt crops. Spiders are abundant in maize agroecosystems and considered as beneficial for pest suppression (Sunderland 1999). However, only one laboratory hazard study (Ludy & Lang 2006) with *Araneus diadematus* (Araneae: Araneidae) and Cry1Ab-expressing Bt maize has been published. In Chapter 3, a risk assessment of corn rootworm-resistant maize for *Theridion impressum* (Araneae: Theridiidae), a common European spider, is presented. The prey spectrum of this spider and the Cry3Bb1 concentration in a range of potential prey species are analyzed. Furthermore, feeding studies in the laboratory were conducted. Juvenile and adult *T. impressum* were fed for almost 2 months with Bt maize pollen or prey that was reared on Bt maize.

While most non-target studies have been conducted with arthropods, little is known about the interactions between Bt crops, herbivores and entomopathogens. This is surprising because entomopathogenic fungi, bacteria or viruses may contribute considerably to biological control and may become important for resistance management. The only studies available so far are from Lawo et al. (2008) and Johnson et al. (1997a,b), who worked on the interaction of genetically engineered chickpea or tobacco plants expressing Cry2Aa or Cry1Ac, respectively, a lepidopteran pest, and entomopathogenic fungi. Chapter 4 reports on a laboratory study examining the interaction of Cry3Bb1-expressing maize, larvae and adults of *D. v. virgifera* and the entomopathogenic fungus *Metarhizium anisopliae*.

Recently, a number of publications on the effects of rootworm-resistant maize on non-target invertebrates became available. In Chapter 5 data from peer-reviewed publications and registration documents of Cry3Bb1-expressing maize are compiled and discussed. Laboratory and glasshouse experiments as well as field trials published until November 2008 are covered.

Finally, the experimental findings of this thesis and the results of the literature review are discussed and general conclusions on the compatibility of
corn rootworm resistant maize with biological control agents are drawn in Chapter 6.

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Chapter 1 – Introduction


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Chapter 1 – Introduction


Chapter 2

Insecticidal activity of Cry3Bb1 expressed in *Bt* maize on larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

Meissle, M. & Romeis, J.

(acceptable after minor revision at Entomologia Experimentalis et Applicata)

Abstract

The environmental risk assessment for genetically modified crops producing insecticidal Cry proteins derived from *Bacillus thuringiensis* (*Bt*) includes the evaluation of adverse effects on non-target organisms. While ELISA concentration measurements indicate the presence of Cry proteins, sensitive insect bioassays determine whether there is biological activity. The insecticidal activity of the coleopteran-active Cry3Bb1 expressed in different tissues of *Bt* maize, contained in maize-fed herbivores, and in spiked soil was measured in sensitive insect bioassays using larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* (Say); Coleoptera: Chrysomelidae). Biological activity of Cry3Bb1 contained in pulverized *Bt* maize pollen, roots, leaves, silk, and *Bt* maize-fed spider mites and western corn rootworm adults was confirmed. When test substances were incorporated into artificial diet in order to obtain the same concentration of Cry3Bb1/ml diet (measured by ELISA), maize pollen and leaf litter exhibited lower toxicity than fresh plant material and maize-fed arthropods. This suggests that nutritional quality of food and degradation of Cry proteins may influence toxicity to insects. When soil was spiked with Cry3Bb1, the *Bt* protein was highly adsorbed and retained its full biological activity. Because toxicity of Cry proteins contained in different matrices cannot always be determined from ELISA values alone, sensitive insect bioassays can improve hazard and exposure assessments in environmental risk assessment of *Bt* crops.

Keywords

arthropods, *Bacillus thuringiensis*, bioassay, biological activity, Cry toxin, *Diabrotica virgifera virgifera*, environmental risk assessment, soil, transgenic crops, western corn rootworm
Chapter 2 – Biological activity of Cry3Bb1

Introduction

Most insect-resistant genetically modified (GM) crops grown today are transformed with cry genes derived from the bacterium Bacillus thuringiensis (Bt). Cotton and maize varieties expressing Cry1 or Cry2 proteins targeting lepidopteran pests lead the worldwide cultivation of insecticidal GM crops (James, 2007). In addition, adoption rates of maize expressing the coleopteran-specific Cry3Bb1 protein targeting the western corn rootworm (WCR) (Diabrotica virgifera virgifera LeConte, Coleoptera: Chrysomelidae), have increased rapidly since commercialization in 2003 (Hellmich et al., 2008). Before new GM crops are commercialized, the potential risk to the environment needs to be evaluated (Nap et al., 2003; Conner et al., 2003). This includes the potential impact on natural enemies which contribute to the regulation of herbivore populations (Romeis et al., 2006; 2009). Because GM crops expressing Bt Cry proteins target insect pests, an important part of the environmental risk assessment is their potential impact on non-target arthropods. Arthropods may be exposed to the insecticidal protein by feeding directly on plant material above or below ground, such as the beneficial arthropods that feed on pollen or other plant tissue. Decomposers ingest Bt protein remaining in plant residues. Species living below ground may furthermore be exposed to Bt protein from root exudates or leachates from plant residues, which are present in soil (Saxena et al., 2002; Icoz & Stotzky, 2008). In addition, when natural enemies feed on prey containing the plant-expressed insecticidal protein, the GM product may be transferred along the food chain (Romeis et al., 2009). However, regardless of the route of exposure, a certain arthropod species is only at risk if the transgenic protein causes toxic effects at a realistic level of exposure (Raybould, 2007).

While in regulatory risk assessment toxicity is initially tested on a range of surrogate species in the laboratory (Garcia-Alonso et al., 2006; Romeis et al., 2008), estimating exposure requires knowledge of consumed food (plant material, residues, prey) and the concentration of the insecticidal protein in the food (Romeis et al., 2009). To detect and quantify Cry proteins expressed by Bt crops, immunological methods are widely used. Several studies using enzyme-linked immunosorbent assays (ELISA) to measure Bt protein concentrations in fresh plant material, litter and arthropods have been published (e.g., Head et al., 2001; Zwahlen et al., 2003; Obrist et al., 2006). ELISA techniques allow sensitive detection of low Bt protein concentrations in the range of ng/g sample. One potential weakness of this technique is the fact that it is based on antibodies recognizing certain regions of the Cry protein, which may result in positive signals not only for intact, biologically active protein, but also for fragments or denatured Cry molecules that have lost biological activity. When testing toxicity, however, it has to be ensured that the provided insecticidal protein is intact and active. Also for evaluating how non-target arthropods are exposed to insecticidal proteins along the food chain, knowledge of both the concentration in the food sources and the biological
activity of the protein is important. For GM crops expressing Cry1 and Cry2 protein (Bt maize and cotton), biological activity has been tested in bioassays using larvae of sensitive lepidopteran species, like Ostrinia nubilalis (Hbn.) (Lepidoptera: Crambidae), Heliothis virescens (Fabricius) (Lepidoptera: Noctuidae) or Manduca sexta L. (Lepidoptera: Sphingidae). To test for biological activity of coleopteran-active Bt proteins, like Cry3 as expressed in corn rootworm-resistant maize, larvae of the Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say) (Coleoptera: Chrysomelidae), have proved to be suitable for testing. Handling is relatively easy, larvae can be mass-reared, artificial diet is available (Gelman et al., 2001), and sensitivity to Cry3 proteins is relatively high in contrast to, e.g., Diabrotica spp. (Donovan et al., 1992; Keller & Langenbruch, 1993; Monsanto, 2004).

Using these sensitive insect species, biological activity of Cry proteins contained in Bt plant material, i.e., pollen, leaf, root or seed, has been documented (Sims & Berberich, 1996; Sims & Holden, 1996; Sims & Ream 1997; Huang et al., 2006; Li et al., 2008; Shan et al., 2008). In maize residues collected from the field, biological activity of Cry1Ab and Cry3Bb1 could be confirmed in sensitive insect bioassays up to several months after harvest (Zwahlen et al., 2003; Zurbrügg, 2008). Herbivorous arthropods feeding on lepidopteran-active Bt maize contained biologically active Cry1Ab and the response of O. nubilalis corresponded well to concentrations measured by ELISA (Head et al., 2001; Obrist et al., 2006). Sensitive insect bioassays were also used to confirm the biological activity of Cry proteins purified from B. thuringiensis or GM microbes when mixed into artificial insect diets (MacIntosh et al., 1990; Romeis et al., 2004; Duan et al., 2006; Stacey et al., 2006; Duan et al., 2008).

A large number of studies addressed the biological activity of lepidopteran-active Cry proteins in soil (Icoz & Stotzky, 2008). Soil is known to adsorb large amounts of Bt protein which bind mainly to clay particles and humic substances (Icoz & Stotzky, 2008). This leads to major difficulties for the quantification of Bt protein using ELISA procedures, as the extraction efficacy with commonly used buffers is low (Palm et al., 1994; Shan et al., 2005). However, biological activity was retained when purified Bt protein was mixed into soil (Tapp & Stotzky, 1998; Dubelman et al., 2005; Shan et al., 2008) or adsorbed to humic acids (Crecchio & Stotzky, 1998) or clay (Tapp & Stotzky, 1995). Thus sensitive insect bioassays help to refine exposure assessments, which may underestimate potential exposure for soil organisms to Bt proteins when based on ELISA data alone.

Most research has focused on the biological activity of lepidopteran-active Bt proteins while data on coleopteran-active Bt proteins are scarce and no detailed testing protocol has been published. Furthermore, toxicity of Bt protein contained in plant material and arthropods has only been compared with ELISA measurements for Cry1Ab (Head et al., 2001; Obrist et al., 2006). In the paper we present a procedure to test biological activity of Bt protein in fluid
and pulverized test substances using CPB larvae. We provide data on the biological activity of Cry3Bb1 contained in different maize tissues, herbivorous arthropods and soil. Those data are important to assess exposure of above and below ground non-target organisms. The specific objectives of the study are:

1. To develop a method to incorporate Cry3Bb1-containing plant material, arthropod material or soil into artificial diet for CPB larvae.
2. To establish concentration-response relationships when artificial diet is replaced by plant material, arthropod material or soil with or without Cry3Bb1.
3. To test how biological activity of Cry3Bb1 in different maize tissues and arthropod samples relates to concentrations measured by ELISA.
4. To test whether Cry3Bb1 adsorbed to soil particles remains biologically active.

**Materials & Methods**

*Preparation of test material*

Transgenic maize DKC5143Bt (Event MON 88017, Monsanto Company, St. Louis, USA) and the corresponding non-transformed near isoline DKC5143, were grown in the glasshouse. DKC5143Bt expresses the cry3Bb1 gene driven by the constitutive enhanced 35s cauliflower mosaic virus promoter (Monsanto, 2004). Plants were grown individually in 12 l plastic pots and fertilized with 40 g slow release fertilizer (Osmocote Exact, 16 % N: 11 % P₂O₅: 11 % K₂O, Scotts UK Professional, Bramford, UK) before sowing and weekly with Vegesan standard (80 g N, 70 g P₂O₅ and 80 g K₂O per liter, Hauert HBG Dünger AG, Grossaffoltern, Switzerland) thereafter. Shortly before plants started to shed pollen, tassels were confined in air-permeable cellophane bags (19.5 × 37.5 cm, Cellolclair AG, Liestal, Switzerland). Pollen was collected from those bags, dried at room temperature for 1 day, subsequently sifted (0.2 mm) to remove anthers and contaminants and stored at -20°C. Silk, leaves and roots were cut from plants after anthesis. Roots were washed to remove soil. Silk, leaves and roots were lyophilized (Beta 1-8, Christ, Osterode am Harz, Germany), pulverized (Cyclotec 1093 Sample Mill, Foss, Hillerod, Denmark) and frozen at -20°C. For collecting litter (senescent leaves), plants were grown in a climatic chamber until the leaves were dry and brown. After cutting, senescent leaves were pulverized and stored at -20°C. Spider mites (*Tetranychus urticae* Koch, Acari: Tetranychidae) were reared in the glasshouse on maize plants after anthesis. Infested *Bt* and control plants were kept separate to reduce cross-contamination. Spider mites were collected over a period of several weeks by beating the plants over a plastic tray. Debris (small leaf pieces, anthers, aphids, etc.) falling into the tray was removed using forceps. After transfer of mites to 2 ml microreaction tubes, samples were frozen at -20°C shortly after each collection. Laboratory reared
western corn rootworm (WCR) adults were provided by CABI Europe (Delémont, Switzerland). The beetles were kept in groups (15 to 200 beetles) and fed with a mixture of silk and leaves from Bt or control maize. Food was changed once after 2 or 3 days. After 4 or 5 days of feeding, beetles were transferred to 2 ml microreaction tubes and frozen. Spider mites and WCR adults were lyophilized and subsequently pulverized in a mixer mill MM300 (Retsch, Haan, Germany) fitted with 24 tube-adapter s for micro reaction tubes (Quiagen, Hombrechtikon, Switzerland) using one 5 mm tungsten carbide ball per tube. All pulverized samples were stored at -20°C until further use.

Quantification of Cry3Bb1

The concentration of Cry3Bb1 in plant and arthropod samples was measured in double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA), commercially available from Agdia (Elkhart, Indiana, USA). The test, originally designed for qualitative use, was modified by creating a standard curve with purified Cry3Bb1 solution of known purity provided by Monsanto. Three or five replicates of 3-5 mg were taken from each control or Bt test substance, respectively (silk, pollen, roots, leaves, litter, spider mites, WCR adults). One tungsten carbide ball was added to each sample together with phosphate buffered saline with 0.05% Tween-20 (PBST) at a ratio of 1 mg dry weight (DW) per 60-100 µl PBST. The samples were mixed for 30 sec at 30 Hz in the MM300 mixer mill. After centrifugation at 13000 × g for 5 min, the supernatant of samples from Bt treatments was diluted 20-500 × with PBST while samples from control maize were used undiluted. Antibody coated 96-well microtiter plates were loaded with 100 µl enzyme conjugate (both provided with the kit) and 100 µl sample extract per test well. According to the manufacturer’s instructions, the plates were incubated for 2 h and washed 7 times with PBST. Ten minutes after adding the (provided) substrate solution, the optical density (OD) was measured at 620 nm light wave length using a SpectrafluorPlus plate reader (Tecan, Männedorf, Switzerland). Cry3Bb1 concentrations in µg/g DW were calculated from the standard curve using regression analysis. In order to clearly separate positive from negative results, the limit of detection (LOD) was based on the standard deviation of 12 buffer-only ODs multiplied by 3 (ICH, 2005). Considering the slope of the regression line, sample weight and amount of buffer added, the LOD was at least 0.02 µg Cry3Bb1/g sample DW.

Colorado potato beetle larvae

Eggs of CPB were shipped from the Alampi Beneficial Insect Laboratory (State of New Jersey, Department of Agriculture, Trenton, USA). Upon arrival, egg batches were divided in several groups and incubated for 3-7 days at 12°C to ensure that newly hatched larvae were available for 3-4 consecutive days. After removing from the cold, eggs were disinfected for 30 sec with diluted sodium hypochlorite solution (2.6 ml of 14 % NaOCl in 150 ml water and 3-4 drops liquid soap), washed thoroughly, and placed in 9 cm Petri
dishes lined with filter paper. Eggs were incubated in a climatic chamber at 25 ± 1°C, 70 ± 10 % relative humidity and a light: dark cycle of 16:8 h until hatching. Neonate larvae were used within 24 h, after they had finished feeding on their egg shells and started to move.

Bioassay procedure

The test substances were incorporated into artificial CPB diet commercially available from Bio-Serv (Frenchtown, USA). The ready to use diet mix contains casein, cellulose (fiber), Lepidoptera Vitamin Mix, ascorbic acid, inositol, locust bean gum, cholesterol, choline dihydrogen citrate, casein hydrolysate, sucrose, fructose, Wesson salt mix and corn oil. Disposable 20 ml plastic syringes were used to mix the diet for each treatment. After removing the plungers and blocking the orifice of the syringes, test substance and diet mix was weighed into the syringe tube. For the diet-only treatment, 1.4 g of diet mix was added. For all other treatments, appropriate amounts of diet mix were replaced by the test substance (e.g., 0.7 g diet mix and 0.7 g test substance resulted in a 50 % treatment). Thereafter, ca. 1.5 mg nystatin and 5 mg methyl hydroxybenzoate (both Sigma-Aldrich, Buchs, Switzerland) were added to each syringe to prevent fungal growth. Agar solution was prepared as necessary for the number of treatments tested. For 200 ml solution (enough for 16 treatments), 2.64 g agar-agar (provided by Bio-Serv with the diet mix) was added to 98 ml deionised water and heated until boiling. After removing from the heat, the agar solution was cooled with 71 ml deionised water. As preservatives 0.9 ml potassium hydroxide solution (18 % w:w) and 0.5 ml formaldehyde solution (37 %) were added. To delay solidification, the agar solution was kept in a water bath at 55°C. To each syringe containing diet mix, test substance and fungicides, 8.5 ml of the agar solution was added (total volume ca. 10 ml) and all ingredients were mixed thoroughly for several seconds using an overhead drill (2000 rpm, RW20, Janke & Kunkel, Staufen, Germany) with a fixed plastic paddle fitting into the syringe tubes. While the agar was still liquid, the syringe orifice was unblocked and the content was pressed into two transparent plastic containers (4.3 × 2.2 × 1.0 cm) using the plunger. The solidified diet was either used immediately or covered and left at 4°C until the next morning. The diet was taken out of the containers and the water film on the surface was removed with paper tissue. Using a scalpel, the diet from each container was cut into 21 cubes, which were transferred to 128 well PETG bioassay trays (Bio-Serv). With a fine brush, one CPB larva was added to the diet cube in each well and the wells were sealed with 16 cell tray covers (Bio-Serv). After incubation for 7 days at 25 ± 1°C, 70 ± 10 % RH and 16:8 h light: dark, mortality was recorded and weight of surviving larvae was determined on a microbalance (Mettler Toledo MX5).

Assays were considered valid when mortality in the diet only treatment was below 20 % and less than 10 % of the wells were dried out or overgrown by mould. Two out of the 30 performed assays were invalid and had to be repeated.
Chapter 2 – Biological activity of Cry3Bb1

Concentration-response assays

To characterize the relationship between test substance concentration and CPB larval response (mortality and growth) after 7 days, a series of bioassays was conducted with Bt and non-Bt (control) test substances. Concentration-response curves with control material provide information on how much of a certain substance is tolerated by CPB larvae. The relationships between Cry3Bb1 concentrations in the diet and the larval responses reveal the sensitivity of the larvae to the Bt protein.

For concentration-response assays with control material, 1.25-50 % silk, roots and leaves, 1.25-35 % spider mites and 5-100 % pollen were incorporated into artificial CPB diet. Cry3Bb1 containing silk and leaves were tested at concentrations of 0.31-10 %, roots at 0.62-20 %, spider mites at 0.31-20 % and pollen at 2.5-100 %. Half of the diet was used for bioassays on the day of preparation and the other half on the next day. In addition, each assay was repeated with a different shipment of larvae. For roots, all assays were conducted with the same shipment of larvae. For pollen, one shipment of larvae was used and all diet was used on the same day. For silk, an additional assay with a third shipment of larvae was performed. This procedure ensured that variation in the fitness of larvae between days and shipments was leveled out to some degree as data were pooled for each treatment. The number of replicates (N) per treatment was 38-121. A diet-only treatment (negative control, no Cry3Bb1) and a 1.25 % Bt silk treatment (positive control with Cry3Bb1 causing ca. 50 % mortality) were included in all assays. The concentrations causing 20 % and 50 % mortality (LC 20 and LC 50) and corresponding 95 % confidence intervals were calculated for each test substance by probit analysis using Polo Plus (Version 1.0, LeOra Software, Petaluma, USA).

Same concentration assays

To compare bioactivity of different plant materials, arthropods and litter more accurately, the amount of test substance incorporated into artificial diet was adjusted so that each diet contained 0.5 µg Cry3Bb1/ml (based on ELISA). For each Bt test substance, a corresponding control without Bt protein was included. The bioassay was conducted simultaneously with silk, pollen, roots, leaves, litter, spider mites and WCR adults and repeated 3 times on consecutive days, resulting in a total of 104-126 replicates per treatment. Larval mortality was compared with Logit regression analysis (Wald statistics) within the control and Bt treatments. If this analysis was significant, 21 pairwise comparisons were performed. Furthermore, Bt and control treatments were compared for each of the 7 tested substances. Significance levels were adjusted to multiple testing (Bonferroni-Holm adjustments). Larval weight was analyzed using two-way ANOVA (factors Bt/control and test substance) and means were separated by Tukey HSD tests.
Biological activity of Cry3Bb1 adsorbed to soil

Artificial diet was replaced stepwise with soil to establish how much soil is tolerated by the larvae. Soil (25 % clay, 35 % sand, 40 % silt, pH 7.5) typical for agricultural fields in the Swiss plateau was obtained from Ricoter (Aarberg, Switzerland). This concentration-response bioassay was conducted as described above including *Bt* silk and diet only treatments as positive and negative controls, respectively. Based on the results (Fig. 3A), artificial diet containing 10 % soil had been selected for a spiking bioassay.

Purified Cry3Bb1 (provided by Monsanto) was produced by fermentation of *E. coli* containing the Pmon72735 expression plasmid and purified using SDS-PAGE/ densitometry. From CPB bioassays conducted with the same batch of Cry3Bb1, Monsanto reported a LC50 of 0.4 µg/ml diet. To minimize Cry3Bb1 losses due to binding to the tubes, 2 ml protein low-bind tubes (Vaudaux-Eppendorf, Schönenbuch, Switzerland) were used for dilutions and incubations. After the stock solution (7.6 mg total protein/ml, 83 % purity) was diluted to 5 µg Cry3Bb1/ml, aliquots were frozen until further use. For the preparation of the treatments, 1 ml deionised water or Cry3Bb1 solution (5 µg/ml) was added to 140 mg soil. In parallel, 1 ml water and 1 ml Cry3Bb1 solution without soil were prepared. Cry3Bb1 was allowed to adsorb to the soil particles for 1 h on a platform shaker. Thereafter, the content of each tube was transferred to 1.4 g diet mix (with fungicides) prepared in a syringe as described above. To obtain 10 ml diet, 7.5 ml liquid agar (with preservatives) was added. This resulted in the treatments a) diet only; b) diet containing 0.5 µg Cry3Bb1/ml; c) diet containing 10 % soil; and d) diet containing 0.5 µg Cry3Bb1/ml and 10 % soil. Subsequently, the CPB bioassay procedure was followed as described above. The bioassay was conducted 3 times on consecutive days with 42 larvae per treatment per day. Data were pooled for analysis leading to a total N = 126 per treatment. Mortality and larval weight was compared between and among *Bt* and control treatments using Logit regression analysis (Wald statistics) and t-tests, respectively. Significance levels were adjusted for 4 pair-wise comparisons.

Adsorption of Cry3Bb1 to the soil was verified using ELISA. Soil and purified Cry3Bb1 solution (5 µg/ml) from the same aliquots as used for the bioassay were mixed in the ratio described above. After 1 h incubation, the samples were centrifuged for 5 min at 13000 × g and 60 µl of the supernatant were mixed with 240 µl PBST. In parallel, 60 µl of purified Cry3Bb1 solution from the aliquots (5 µg/ml) were diluted with 240 µl PBST. After adding one 5 mm tungsten carbide ball to the diluted supernatant and Cry3Bb1 solution, the samples were mixed for 1 min at 30 Hz in the MM300 mixer mill and centrifuged for 5 min at 13000 × g. While the Cry3Bb1 solution was further diluted 200 times, the supernatant was analyzed directly. ELISA was conducted as described above. All samples were prepared and analyzed in 5 replications.
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Results

**Cry3Bb1 concentration in test substances**

ELISA measurements revealed that *Bt* maize silk had the highest Cry3Bb1 concentration (mean ± SE; 252 ± 7.7 µg/g DW), followed by roots (194 ± 7.1 µg/g DW), leaves (126 ± 4.7 µg/g DW), spider mites (112 ± 16.7 µg/g DW) and litter (68.8 ± 0.97 µg/g DW). Lowest values were obtained for WCR adults (20.7 ± 0.69 µg/g DW) and pollen (10.3 ± 1.39 µg/g DW). Trace amounts of *Bt* protein were detected in control spider mites (0.09 ± 0.021 µg/g DW) and silk (0.04 ± 0.011 µg/g DW) most likely due to contamination of the samples. All other measurements of control test substances were under the LOD (0.02 µg/g DW).

**Concentration-response assays**

In all diet-only treatments, CPB larval survival was high at 94-97.5 % and the mean weight ranged between 5.1 mg and 6.7 mg. Larval weights in the other treatments are presented as percentages of the weight in the diet-only treatment to obtain a response value that is comparable with mortality (Fig. 1).

When replacing artificial diet with increasing concentrations of test substance from the non-*Bt* treatments, survival decreased. LC 20 values (and 95 % confidence intervals) show that silk was least tolerated by CPB larvae, followed by roots and leaves (Table 1). Values could not be calculated for pollen and spider mites due to a lack of concentrations causing high mortality (Table 1). The concentration-response curves indicate that at least 35 % spider mites could be mixed into the diet with larval mortality remaining below 20 % (Fig. 1E). Highest tolerance was observed for pollen, as survival dropped below 90 % only when the artificial diet mix was substituted completely (Fig. 1B).

Similar to survival, larval weight decreased with increasing concentrations of control test substances incorporated into the diet. An exception was pollen, which had a positive effect on CPB larval weight up to a concentration of 50 % (Fig. 1B).

In the *Bt* treatments, concentrations in the diet necessary to increase mortality to 50 % (LC 50) ranged from 1.5 % for silk to 60 % for pollen (Table 1), corresponding to Cry3Bb1 concentrations between 0.52 and 0.86 µg/ml diet. Similarly the LC 20 values were lowest for *Bt* silk and highest for pollen (Table 1). No significant differences in the LC values based on Cry3Bb1 concentrations were found among *Bt* test substances (overlapping 95 % confidence intervals). LC values based on percentages of test substance in the diet were lower for *Bt* than for control maize silk, roots and leaves (Table 1). In the case of pollen and spider mites, where LC values were not available for comparison, larval survival between *Bt* and control treatments was compared for 80 % pollen and 20 % spider mites in the artificial diet using
Chapter 2 – Biological activity of Cry3Bb1

Figure 1. Survival and weight response (in % of diet only weight) of Leptinotarsa decemlineata larvae fed artificial diet containing increasing concentrations of non-Bt and Cry3Bb1-containing maize A) silk, B) pollen, C) roots, D) leaves; or E) maize-fed spider mites. Concentrations are given in % (w/w) of dry artificial diet mix replaced by test substance and in μg Cry3Bb1/ml diet based on ELISA measurements of pulverized test substances. Neonate larvae were allowed to feed for 7 days. Diet only and 1.25 % Bt maize silk served as negative and positive controls in each assay, respectively. N = 38-121.
Logit regression (Fig. 1B, E). This allowed to demonstrate biological activity of Cry3Bb1 in pollen (Wald statistic = 45.8, p < 0.0001) and spider mites (Wald statistic = 65.4, p < 0.0001).

The positive control (1.25 % Bt maize silk) resulted in 29-48 % survival and a mean larval weight of 2.6-4.0 mg (Fig. 1).

Table 1. Concentrations resulting in 20 % (LC 20) and 50 % mortality (LC 50) of Leptinotarsa decemlineata larvae after feeding for 7 days on artificial diet with Cry3Bb1-containing maize tissues or maize-fed spider mites. Confidence intervals (95 %) are provided in parenthesis.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>µg Cry3Bb1/ml diet</th>
<th>% Bt substance in diet</th>
<th>% control substance in diet</th>
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<tr>
<td></td>
<td>LC 20</td>
<td>LC 50</td>
<td>LC 20</td>
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<td>Silk</td>
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<td>(0.62-1.06)</td>
<td>(19-54)</td>
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<td>(0.39-0.71)</td>
<td>(0.51-1.4)</td>
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<td>(0.53-0.73)</td>
<td>(1.6-2.7)</td>
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<tr>
<td>Spider mites</td>
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<td>(0.33-0.52)</td>
<td>(0.66-0.92)</td>
<td>(2.1-3.3)</td>
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</table>

**Same concentration assays**

Test substances were incorporated into artificial diet in a way that the Cry3Bb1 concentration (based on ELISA results) was 0.5 µg/ml diet in all treatments. For example, only 1.4 % diet mix had to be replaced with silk, while 35 % pollen were necessary to obtain the same level of Cry3Bb1. Survival of CPB larvae within the non-Bt treatments did not differ statistically (Logit regression, p = 0.4; Fig. 2). In contrast, survival differed within the Bt test substances (Fig. 2; Wald statistic = 118, p < 0.0001) and subsequent pair-wise comparisons singled out the pollen and litter treatments resulting in significantly higher survival (adjusted p < 0.002). No differences were found between pollen and litter, or between the other Bt treatments (p > 0.2). Survival in control treatments differed from Bt treatments for most test substances (Wald statistic silk = 54.3, roots = 50.1, leaves = 55.9, spider mites = 47.0, WCR = 44.0, p < 0.0001) but not for pollen and litter (p > 0.1).

For larval weights, ANOVA revealed differences for the factor Bt/control ($F_{1,1228} = 357$, p < 0.0001), test substance ($F_{6,1228} = 26.6$, p < 0.0001), and a significant interaction between the two factors ($F_{6,1228} = 10.8$, p < 0.0001;
Fig. 2). Within the control treatments, pollen showed the highest larval weights with significant differences to roots and WCR adults, while CPB larvae in the WCR adult treatment also weighed significantly less than in the leaves and litter treatments (Tukey HSD, p < 0.05). Within the Bt treatments, larvae feeding on diet containing Bt pollen and litter weighed more than in the other treatments (Tukey HSD, p < 0.05). Differences between pollen and litter or between the other Bt treatments were not significant (p > 0.05). Larval weight in control treatments differed from Bt treatments (p < 0.0003) for all test substances except litter (p > 0.05; Fig. 2).

![Graph showing survival and weight response of Leptinotarsa decemlineata larvae fed artificial diet containing different concentrations of test substances to obtain 0.5 µg Cry3Bb1/ml (based on ELISA) or test substances without Bt protein. Neonate larvae were allowed to feed for 7 days. In the Bt maize pollen and litter treatments, larvae showed higher survival (Logit regression) and weight (ANOVA) than in the other Bt treatments. For control diet-fed larvae, pollen resulted in higher larval weight than roots and western corn rootworm (WCR) adults and in addition, WCR adults lead to lower weight than leaves and litter. Bt treatments differed from control treatments for all test substances for larval survival (except pollen and litter) and weight (except litter). N = 104-126.]

**Biological activity of Cry3Bb1 adsorbed to soil**

With increasing amounts of artificial diet replaced by soil, CPB larval survival and weight decreased (Fig. 3A). Survival remained above 80 % when 35 % diet were replaced by soil and weight was relatively constant up to 20 % soil in the diet but started to decrease at 35 %.

In the spiking bioassay, CPB larvae showed lower survival when fed with diet containing Cry3Bb1 compared to the controls, both when Bt protein solution was incorporated directly into the diet and when the protein was absorbed to soil (Fig. 3B; Logit regression, Wald statistic = 18 or 28,
respectively, adjusted $p < 0.01$). There was no difference in survival between diet only and soil treatments, both when Cry3Bb1 was present (Logit regression, $p = 0.7$) and when no Bt protein was used (Logit regression, $p = 0.08$). CPB larval weight was lower in the presence of Bt protein (t-test, $t = 7.9$ for diet only, $t = 9.0$ for soil in diet, adjusted $p < 0.01$). No difference was observed, however, when comparing diet only and soil in diet treatments with Cry3Bb1 (t-test, $p = 0.75$) or without Bt protein (t-test, $p = 0.99$).

After Bt protein solution was incubated for 1 h with soil, only trace amounts of Cry3Bb1 were detected in the supernatant after centrifugation ($< 0.1$ % of the Cry3Bb1 concentration in the solution without soil) confirming almost complete adsorption to soil particles.

**Figure 3.** Survival and weight response (in % of diet only weight) of *Leptinotarsa decemlineata* larvae fed A) increasing concentrations of soil mixed into artificial diet and B) purified Cry3Bb1 solution mixed into artificial diet directly or after adsorption to soil, resulting in the treatments: diet without Cry3Bb1; diet containing 0.5 µg Cry3Bb1/ml; diet containing 10 % soil without Cry3Bb1; and diet containing 0.5 µg Cry3Bb1/ml and 10 % soil. Neonate larvae were allowed to feed for 7 days. In the presence of Cry3Bb1, survival (Logit regression) and weight were decreased (t-test). There was no difference in survival and weight between free and adsorbed Cry3Bb1. N = 126.

**Discussion**

When increasing amounts of test substances containing Bt protein were mixed into artificial diet, CPB larval survival and weight decreased and biological activity of Cry3Bb1 contained in Bt maize material (silk, pollen, roots, and leaves) as well as in arthropods that had fed on Bt maize (spider mites and WCR adults) was demonstrated.
For concentration-response curves, non-linear regression analysis is commonly used to calculate characteristic values like LC50 (lethal concentration causing 50% mortality) or EC50 (effect concentration resulting in 50% growth inhibition). In our study, several concentrations around the 50% survival were tested for Cry3Bb1-containing substances, thus the concentration-response function could be estimated and the LC50 values calculated. For growth inhibition, however, concentrations causing more than 50% effect resulted in high larval mortality and a low number of replications. Thus the EC50 for larval weight could not be estimated. Consequently, for bioassays using CPB larvae, survival should be selected as measurement endpoint. Researchers working with the lepidopteran species \textit{M. sexta} also used mortality as a parameter for testing the biological activity of Cry proteins (e.g., Saxena & Sotzky, 2002; Icoz et al., 2008), because it was as sensitive as growth inhibition (MacIntosh et al., 1990). However, more common in short duration bioassays with lepidopteran larvae is the finding that toxicity is already evident in growth inhibition at concentrations not leading to mortality (Huang et al., 2006; Marçon et al., 1999). For example, EC50 values for \textit{H. virescens} were reported to be 2-3 orders of magnitude lower than LC50 values (MacIntosh et al., 1990; Head et al., 2002).

Lepidopteran species commonly used in sensitive insect bioassays, are highly sensitive to Cry1 protein. For example, the concentration of purified Cry1Ab causing 50% weight reduction (EC50) when mixed into artificial diet was reported to be as low as 5 ng/ml diet for \textit{O. nubilalis} (Romeis et al., 2004; Li et al., 2008) and 2.3 ng/ml diet for \textit{H. virescens} (Sims & Berberich, 1996). In contrast, the sensitivity of CPB is 2 orders of magnitude lower, as shown by LC50 values between 0.2 and 0.9 \(\mu\)g/ml diet reported in the present study and in the literature (Monsanto, 2004, Duan et al., 2008). WCR, the target of Cry3Bb1-expressing maize, is even less sensitive with a LC50 of 100 \(\mu\)g/ml diet (Monsanto, 2004).

To ensure CPB larval mortalities not exceeding 20%, a maximum of 13-33% could be incorporated into the artificial diet for most test substances. In contrast, pollen was tolerated up to 80%, which might be because maize pollen is highly nutritional and a preferred food source for many arthropods (e.g., Lundgren & Wiedenmann, 2004; Li et al., 2008). Another factor might be the fresh weight to dry weight ratio of the test substances. For example, fresh silk and roots contain 80-95% water, while the water content in pollen is usually between 20 and 50% only (Meissle & Romeis, unpublished). In our study, test substances were added to artificial diet based on dry weight. Consequently, silk and root powder took up a relatively large amount of water from the added agar solution, and the diet was observed to be thicker than in other treatments. This might explain the low LC20 for silk and the rapid decrease in larval weight in the root treatment.

When designing a sensitive insect bioassay, the amount of test substance mixed into the artificial diet needs to contain enough \textit{Bt} protein to reach the
sensitivity level of the test species, otherwise no effects can be expected. Compared to Lepidoptera, relatively high concentrations of \( Bt \) protein need to be incorporated into the artificial diet for CPB larvae to cause measurable effects. For a given \( Bt \) test substance with a LC 50 of 0.5 \( \mu g \) Cry3Bb1/ml diet that is tolerated at maximal concentrations of 20 % in the diet, the Cry3Bb1 concentration in the substance needs to be at least 17.9 \( \mu g/g \) to reach a response of 50 % mortality. A response to concentrations lower than the LC 50 may be detectable to a certain point, but statistical testing might require a high number of replicates.

As the concentration-response curves in our study had to be conducted with different shipments of CPB larvae, the variability in the data was relatively high. This resulted in large confidence intervals of the LC values and no statistical differences between the different \( Bt \) test substances. Therefore, a bioassay was conducted where each test substance was incorporated into the diet in a way that the Cry3Bb1 concentration was the same (based on ELISA) and approximately at the LC 50 value of 0.5 \( \mu g/ml \). This allowed comparing ELISA measurements with bioactivity of Cry3Bb1 in different plant materials, arthropods and litter more accurately. Diet-incorporated pollen resulted in higher CPB larval survival and weight than other fresh plant material and arthropods. This is fitting to the high LC 50 value from the concentration-response. Probably, sensitivity of the CPB larvae to the \( Bt \) protein was decreased due to the high nutritional quality of pollen (as discussed above). Another explanation would be reduced exposure to the \( Bt \) protein because less diet (and thus \( Bt \) protein) needed to be ingested due to higher nutritional value compared with other substances, or because pollen passed the gut of the larvae partly undigested. Furthermore, biological activity of Cry3Bb1 in litter could not be confirmed even though litter contained the same concentration of Cry3Bb1 (based on ELISA) as the other treatments. Either nutritional quality of litter was also higher than of fresh maize leaves or the \( Bt \) protein in litter might have lost bioactivity due to degradation. When 4 times higher concentrations of litter were incorporated into artificial diet, biological activity of Cry3Bb1 had been shown by Zurbrügg (2008) using the same setup and materials. However, for pollen and litter it cannot be excluded that differences in \( Bt \) protein extraction efficacies contributed to the observed results. Cry3Bb1 contained in arthropods (spider mites and WCR adults) revealed similar toxicity as in plant material. The digestion processes in the herbivore guts apparently did not influence the relation between \( Bt \) protein detectable by ELISA and in the sensitive insect bioassays. Similar results were reported by Obrist et al. (2006) for Cry1Ab in \( O. nubilalis \) bioassays using extracts of spider mites, \textit{Spodoptera littoralis} Boisduval (Lepidoptera: Noctuidae) and leaf material with the same Cry1Ab concentration based on ELISA. For the exposure assessment of non-target arthropods, this implies that ELISA measurements of arthropods seem to report biologically active \( Bt \) protein.
Chapter 2 – Biological activity of Cry3Bb1

Cry3Bb1 added to soil was almost completely adsorbed within 1 hour as only traces of the protein were detected by ELISA in the supernatant after centrifugation. However, the biological activity was fully conserved and similar to pure Cry3Bb1 solution without soil. This indicates that the CPB larvae were able to resorb intact and biologically active Bt protein from the soil particles. Biological activity of soil-bound Bt proteins was also shown in spiking experiments with lepidopteran-active Bt proteins (Tapp & Stotzky, 1998; Dubelman et al., 2005; Icoz & Stotzky, 2008). In the field, however, the concentration of Bt proteins entering the soil from root exudates or plant residue leachates is low and further reduced by degradation due to physical (e.g., temperature) and biochemical (e.g., enzymes of microbes) factors. Laboratory microcosm studies indicated that Bt protein from plant biomass is rapidly degrading in soil (e.g., Sims & Holden, 1996; Sims & Ream, 1997) and several studies using soil from Bt crop fields failed to show biological activity in sensitive insect bioassays (e.g., Head et al., 2002; Dubelman et al., 2005; Shan et al., 2008). On the other hand, adsorption and binding to clay particles as well as low temperatures in the field may protect Bt proteins from degradation under certain conditions (Zwahlen et al., 2003; Icoz & Stotzky, 2008; Zurbrügg, 2008). Consequently, some researchers were able to show Bt proteins persisting and retaining insecticidal properties even after several months in soil under field conditions (e.g., Tapp & Stotzky, 1998; Icoz et al., 2008; Zwahlen et al., 2003).

Conclusions

Bioassays using CPB larvae are suitable for measuring biological activity of Cry3Bb1 in plant, litter, arthropod and soil samples assuming that they contain enough Bt protein to reach the sensitivity range of the larvae. Nutritional properties of the food and degradation of Cry proteins are likely to influence toxicity to sensitive species, when compared with ELISA measurements, as suggested for pollen and litter in the present study. Cry proteins are particularly difficult to extract for ELISA measurements after adsorption to soil, but toxicity to sensitive species is fully retained. Thus toxicity of Cry proteins contained in different matrices cannot always be concluded from ELISA values alone. For environmental risk assessment of Bt crops, sensitive insect bioassays can add certainty to toxicity (hazard) studies with non-target species since they confirm exposure to biologically active protein. Furthermore, sensitive insect bioassays can improve the exposure assessment of non-target species when conducted in addition to ELISA measurements, especially when Bt protein is contained in degrading material or soil.

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Chapter 3

The web-building spider *Theridion impressum* (Araneae: Theridiidae) is not adversely affected by *Bt* maize resistant to corn rootworms

Meissle, M. & Romeis, J.

Abstract

Growing genetically engineered maize that produces the insecticidal protein Cry3Bb1 from *Bacillus thuringiensis* (*Bt*) is an effective method to control corn rootworms (*Diabrotica* spp.), which are threatening maize production in North America and Europe. In this study, the risk of Cry3Bb1-expressing maize for the predatory spider *Theridion impressum*, a common species in European maize fields, was assessed. Quantification of Cry3Bb1 in potential prey species collected in *Bt* maize plots and prey spectrum analysis revealed that *T. impressum* ingests Cry3Bb1 in the field. Exposure to the *Bt* protein, however, was highly variable because some potential prey species, like phloem-feeding herbivores and predators, contained little or no Cry3Bb1, while leaf-feeding herbivores contained high concentrations. Adult and juvenile *T. impressum* spiders were fed with Cry3Bb1-containing food (prey or maize pollen) for 8 weeks in the laboratory to examine the toxicity of the *Bt* protein. No differences in mortality, weight development, or offspring production were observed between spiders provided food containing or not containing Cry3Bb1. Retrospective power analysis indicated that our bioassays were sufficiently sensitive to detect meaningful differences. Even though Cry3Bb1 was ingested in the field, our data provide no evidence for toxicity. Consequently, growing corn rootworm-resistant *Bt* maize appears to pose no risk for *T. impressum*.

Keywords

*Bacillus thuringiensis*, corn rootworm, Cry3Bb1, feeding bioassay, non-target risk assessment
Introduction

Maize is one of the most important crops grown for human food and livestock feed worldwide. For protection of the crop against major pests, resistant plants producing insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (*Bt*) were engineered using gene technology. *Bt* maize varieties expressing Cry1 proteins to control stem boring Lepidoptera have been grown commercially in many countries all over the world for more than a decade (Hellmich *et al.* 2008). Since 2003, varieties expressing Cry3 proteins have provided effective protection against corn rootworms (*Diabrotica* spp.), a coleopteran pest complex causing severe economic damage in the USA and Europe mainly due to root feeding (Vaughn *et al.* 2005; Hellmich *et al.* 2008).

Like other pest control technologies, genetically engineered crops expressing insecticidal proteins could negatively affect beneficial species that provide important ecosystem services, and such potential negative effects should be assessed (Raybould 2007; Romeis *et al.* 2008, 2009). Beneficial species in crop production systems include the naturally occurring biological control agents that prey on or parasitize herbivores and thus provide natural pest regulation (Sunderland *et al.* 1997; Nyffeler & Sunderland 2003). One such group of predators in maize and other crops are spiders because they consume various herbivores and occur in relatively large numbers (Sunderland 1999).

A non-target species is only affected by an insecticidal compound expressed in a GE crop if it ingests a toxic concentration (Raybould 2007; Romeis *et al.* 2008). The major route of exposure for many predators including spiders in *Bt* crops is via the prey that they consume (Romeis *et al.* 2009). During anthesis, however, many predatory species consume maize pollen in addition to prey, because the pollen is readily available and highly nutritious (Romeis *et al.* 2009). Previous studies demonstrated that spiders consume pollen from maize (Ludy 2004) and other plants (Smith & Mommsen 1984; Vogelei & Greissl 1989). Spiders may ingest pollen actively or passively when they recycle their web or feed on pollen-covered prey.

Even though exposure to Cry1Ab was confirmed for the garden spider *Araneus diadematus* (Araneae: Araneidae) when fed on *Bt* maize pollen sticking to their webs in the laboratory, no detrimental effects were observed (Ludy & Lang 2006a). Despite this study, data on the toxicity (hazard) of Cry proteins to spiders are scarce. One reason for this lack of studies may be that suitable test systems are currently not available (Candolfi *et al.* 2000a; Romeis *et al.*, 2008). Consequently, the risk assessment of *Bt* maize for spiders has so far focused on field evidence, i.e., spider abundance in genetically engineered vs. conventional crops. A number of European studies reported no difference in the spider community in lepidopteran-active, Cry1Ab-expressing *Bt* maize vs. conventional maize fields (Volkmar & Freier 2003; Meissle & Lang 2005; Ludy & Lang 2006b; Řezáč *et al.* 2006; Toschki *et al.* 2007). For corn

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rootworm-resistant, Cry3Bb1-expressing maize, however, few field data on spider communities are available and no details for different spider families have been published (Meissle & Romeis 2009). Because data from the field are highly variable and influenced by many environmental factors, the statistical power to detect toxic effects is low and a high number of replicated field sites is necessary. Toxicity can be investigated more efficiently in laboratory studies under defined conditions (Romeis et al. 2008).

In the present study, a detailed risk assessment including exposure and hazard analysis was conducted for a common species of European spider in corn rootworm-resistant Bt maize. Theridion impressum (Araneae: Theridiidae) was selected as a surrogate species because it is one of the most frequent spiders found in the canopy of maize in Europe and has a well-known biology (Pekár 2000; Árpás et al. 2005; Meissle & Lang 2005). In addition, it feeds on corn rootworm beetles and contributes therefore to the biological control of this pest (Árpás et al. 2005). Immature T. impressum immigrate to maize fields early in the growing season, mature, mate, and reproduce (Pekár 1999). Early juvenile stages are fed and protected by their mothers before they disperse. Juvenile spiders can be found in maize fields until the end of the growing season (Meissle & Lang 2005).

To estimate exposure of adult T. impressum to Cry3Bb1 via prey, we analyzed the prey spectrum of the spider and measured the Cry3Bb1 content of potential prey species collected in Bt maize plots. The hazard of feeding on Cry3Bb1-containing prey was assessed in laboratory long-term feeding studies with adults and juveniles. Because juvenile T. impressum are present in the field during anthesis, we determined whether juveniles are able to use maize pollen as food and whether there is a hazard when the pollen contains Cry3Bb1.

**Materials & Methods**

**Experimental Bt maize field**

From 2005 to 2007, genetically engineered maize (event MON88017) expressing a modified cry3Bb1 gene derived from Bacillus thuringiensis subsp. kumamotoensis under the constitutive enhanced 3SS cauliflower mosaic virus promoter (Monsanto 2004, ‘Bt maize’), and the corresponding non-transformed near-isogenic variety DKC 5143 (‘control maize’) were grown in an experimental field near Würzburg, Germany. The 4-ha field was divided into 32 plots (40.5 by 31.5 m each), surrounded by a 10-m-wide buffer of conventional maize. In a modified randomized complete block design, eight plots each of Bt maize, control maize, and two commercial varieties were planted. For more details on the design and management of the field experiment, see Rauschen et al. (2008).
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Collection of *T. impressum* spiders and webs

To test for potential hazards of Cry3Bb1-containing prey to adults of *T. impressum*, we collected females that had not yet produced an egg sac by hand in Bt, control and conventional maize in 2006 and 2007 before anthesis. Provided with a piece of maize leaf as a shelter, spiders were starved for 2 weeks at 12°C before being used for adult feeding experiments. In addition, adult *T. impressum* females were collected from Bt maize plots before anthesis in 2005 and 2007 and frozen individually at -20°C to measure the concentration of Cry3Bb1.

For determination of the prey spectrum of *T. impressum*, the so called ‘pocket-part’ of the webs of adult spiders was analyzed in the laboratory. This pocket-part, which the spider builds from consumed prey items and uses as a shelter, represents the prey spectrum of *T. impressum* (Árpás et al. 2005). In addition to the spider webs collected in Germany, webs from maize fields near Zürich, Switzerland, were analyzed. A binocular loupe was used to count prey items of each insect order or highest recognizable taxon. The mean numbers of prey specimens per web were transformed to percentages.

To obtain juvenile spiders for feeding experiments, we collected female *T. impressum* with egg sacs in conventional maize fields near Zürich in July 2006 and 2007. Spiders and egg sacs were kept individually in ventilated 1.3-l plastic cylinders at 24°C, 60% humidity, and a 16:8 h light: dark cycle until juveniles hatched and started to disperse after the second moult. Fruit flies (*Drosophila melanogaster*, Diptera: Drosophilidae) as food and water were provided regularly.

Cry3Bb1 content in potential prey species

For assessment of the Cry3Bb1 concentration in potential spider prey, the most frequent plant-dwelling arthropod species were collected in four Bt and two control maize plots in 2005 before (July 13/14), during (August 8/9), and after anthesis (September 19-21). Additional samples were collected before anthesis on June 28/29 2006 and 2007. Arthropods were collected by hand or by using a beat sheet or sweep net. Leaf tissue was sampled in 2005 from four plants in each of four Bt plots before, during, and after anthesis. Pollen was sampled in 2005. Shortly after collection, all samples were frozen at -20°C. After taxonomic identification, several individuals (two to ca. 830 depending on size and availability) of most arthropod taxa collected on the same date in the same plot were pooled for Cry3Bb1 measurements (Supplementary Table 1). Adult *T. impressum*, *Cantharis lateralis* (Coleoptera: Cantharidae), and in 2006 collected *Oulema melanopus* (Coleoptera: Chrysomelidae) were analyzed individually.

Plants and insects for feeding assays

Bt and control maize was grown in the glasshouse to obtain food for the prey species used in feeding assays with *T. impressum*. Plants were cultivated
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individually in 12-l pots. The soil in the pots was mixed before sowing with 40 g of slow-release fertilizer (Osmocote Exact, 16% N: 11% P₂O₅: 11% K₂O, Scotts UK Professional, Bramford, UK) and plants were fertilized weekly after sowing with Vegesan standard (80 g N, 70 g P₂O₅ and 80 g K₂O per l, Hauert HBG Dünger AG, Grossaffoltern, Switzerland). Shortly before pollen shedding, the tassel of each plant was confined in an air-permeable cellophane bag (19.5 × 37.5 cm, Celloclair AG, Liestal, Switzerland). Pollen was collected every other day, air dried at room temperature for 2 days, sifted to remove anthers and contaminants, and frozen until used.

For the feeding experiments with adult Theridion impressum, prey consisted of adult green lacewings (Chrysoperla carnea, Neuroptera: Chrysopidae) and adult western corn rootworms (D. v. virgifera, Coleoptera: Chrysomelidae). Adult lacewings from a laboratory culture (Romeis et al. 2004) were kept individually and fed with pollen collected from Bt or control maize from the glasshouse. Adults of a non-diapausing strain of D. v. virgifera were obtained from a laboratory culture at the CABI Bioscience Centre in Delémont, Switzerland. Beetles were kept in ventilated plastic cylinders in groups of less then 30 beetles and fed with fresh leaf strips and silk from Bt and control maize collected in the glasshouse from plants at the silking stage. Lacewings and beetles were allowed to feed on the Bt or control maize material for at least 5 days before they were fed to the adult spiders.

For the feeding assay with juvenile spiders, two-spotted spider mites (Tetranychus urticae, Acarina: Tetranychidae) were cultured on fully grown Bt and control maize plants in separated glasshouse compartments. Spider mites were harvested as needed by beating the mite-infested maize leaves over a plastic tray.

Feeding assays with adult T. impressum

The Theridion impressum females that were collected from Bt and control maize plots and starved for 2 weeks at 12°C were weighed, placed in ventilated transparent plastic cylinders (6.5 cm × 8 cm), and kept in a climate chamber at 24 ± 1°C, 60 ± 10% RH, and a 16:8 h light: dark cycle. Within 1 day, spiders started to build a web in the cages. Water was sprayed into all cages every 2-3 days. Spiders collected from the Bt plots received prey fed with Bt maize, and spiders collected from the control plots received prey fed with control maize. Every 4-5 days, one adult lacewing or one corn rootworm beetle was placed into the spider webs in alternation. Prey species were alternated because the prey spectrum of spiders in the field is broad and spiders usually benefit from mixed feeding (Toft 1999). Spiders collected in conventional maize were not fed (starved) to estimate energy reserves of the spiders (see Table 1 for the number of replicates). Every 2-3 days, spiders were checked for mortality and the presence of egg sacs. When offspring emerged from the egg sacs, the adult spider was moved to a new cage and living offspring were counted. On day 58 (2006) and 50 (2007) after the start of the experiment, all spiders in the
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Bt and control treatment obtained one lacewing as prey. After 4-5 more days, half of the spiders were fed with another lacewing while the other half received a corn rootworm beetle. One day later (day 64 or 56 in 2006 or 2007, respectively), all spiders were weighed and frozen at -80°C to measure Cry3Bb1 concentrations. For quantification of Bt protein contained in the prey, lacewings (N = 27 and 32 in 2006 and 2007, respectively) and corn rootworm beetles (N = 40 and 17), as used in the experiment, were frozen individually and processed for Bt concentration measurement.

Feeding assays with juvenile T. impressum

From each of six (in 2006) or seven (in 2007) female T. impressum, 30 juveniles were selected after they started to disperse. Each juvenile was weighed and transferred to a ventilated 3.0 × 3.5 cm transparent plastic cylinder. In 2006, the juveniles were a) fed with spider mites reared on Bt maize; b) fed with spider mites reared on control maize; or c) not fed. Feeding portions (ca. 200 spider mites per juvenile) were transferred to the web of the spiders using a fine brush. After 4 and 8 weeks, spiders were weighed 1 day after feeding. In 2007, juveniles were: a) fed with Bt maize pollen; b) fed with control maize pollen; and c) not fed. Approximately 10 mg of pollen was added to each cylinder. Spiders were weighed every 2 weeks. In both experiments, prey or pollen was provided every 2-3 days, water was sprayed carefully into the cages, and mortality was checked. After 8 weeks, spiders of each treatment were pooled to two samples per mother and frozen for Cry3Bb1 measurements. During the experiment, spider mites and pollen were sampled for Cry3Bb1 quantification.

Quantification of Cry3Bb1

The concentration of Cry3Bb1 was measured in double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA), commercially available from Agdia (Elkhard, Indiana, USA). The test, originally designed for qualitative use, was modified by creating a standard curve, which was calibrated with a purified Cry3Bb1 solution of certified purity and quality (provided by Monsanto). However, unquantified sources of variation (e.g., protein extraction efficacy, protein degradation during the process, inaccuracy of loading and washing the plates, and quality of coated plates and chemicals) limit the absolute validity of reported Cry3Bb1 concentrations.

Arthropods collected in the field experiment (except aphids and thrips), pollen-fed juvenile T. impressum, and pollen-fed lacewings were washed with deionised water to minimize contamination by debris and pollen. After the fresh weight was recorded, samples were lyophilized and weighed again for dry weights (DW). Phosphate-buffered saline Tween (PBST) at a ratio of 1:30 - 1:100 mg DW/ml buffer was added to the samples. If samples were below 3 mg DW, 300 µl buffer was used. For maceration, a 5-mm tungsten carbide ball was added to each sample and the samples were shaken for 2-3 min at 30 Hz.
in a mixer mill MM300 (Retsch, Haan, Germany) fitted with 24-tube adapters for micro reaction tubes (Quiagen, Hombrechtikon, Switzerland). After centrifugation at 13000 × g, the supernatants were diluted with PBST according to the expected Cry3Bb1 concentration. Remaining supernatants were stored at -80°C for repeated analysis when necessary. Antibody coated plates were loaded with enzyme conjugate (both provided with the kit), diluted sample extracts, and Cry3Bb1 standards. Subsequently, the plates were incubated for 2 h under ambient conditions and washed seven times with PBST. Provided substrate solution was added and optical density (OD) was measured after 10-14 min at 620 nm light wavelength and after adding 50 µl of 3 M sulphuric acid per well at 450 nm with a SpectrafluorPlus plate reader (Tecan, Mannedorf, Switzerland). Cry3Bb1 concentrations in µg/g DW were calculated using regression analysis. For clear separation of positive readings from controls, the limit of detection of the test was determined based on the standard deviation of the OD values of buffer-only controls multiplied by 3 (ICH 2005). Subsequently, the detection limit of each sample was calculated with the dilution, sample weight, and amount of added buffer.

Because arthropod and plant samples from the control maize treatment in the field and laboratory did not show OD values systematically different from those of buffer-only controls, no cross reaction of arthropod proteins with the ELISA was apparent.

Statistics

Differences in initial weight between adult spiders collected in the experimental Bt plots, control plots, and conventional maize were analyzed by 2-way ANOVA (factors treatment and year) after log transformation of the data. Subsequent analysis was conducted with spiders from the Bt and control treatment of the feeding assay only, excluding the no-food treatment. Survival of adult spiders was compared with Gehan’s Wilcoxon procedure. The number of spiders producing viable offspring was tested using Fisher’s exact test ($\chi^2$-test) and the number of living offspring produced per spider was tested with 2-way ANOVA (factors treatment and year) including only spiders that produced viable offspring.

Juvenile spiders that died within 3 days after placement in individual cages (18 died in the spider mite assay, 0 in the pollen assay) were excluded from analysis because they were most likely damaged by handling. Five individuals weighing more than 450 µg were also excluded in the spider mite assay to ensure comparable starting conditions of the spiders in all treatments. The effect of the three treatments (Bt prey or pollen, control prey or pollen, no food) and of the mother spider (six in 2006 and seven in 2007) on initial juvenile weight was determined by 2-way ANOVA on log-transformed data. Weight development was compared between Bt and control treatments using repeated measures ANOVA with three time points in the spider mite assay and five in the pollen. In the pollen assay, mortality was compared between Bt and
control treatments with Gehan’s Wilcoxon test. In the spider mite assay, $X^2$-statistics were used because no spider died in the Bt treatment and, therefore, Gehan’s Wilcoxon test could not be used. All mean values are presented with standard errors (mean ± SE).

To avoid committing type II errors, i.e., failing to reject a false null hypothesis, we conducted retrospective power analyses on non-significant results ($p > 0.05$) using PASS (Version 2005, NCCS, Kaysville, USA). The observed control means and standard deviations and the true sample sizes were used to calculate the detectable differences (percentage difference of detectable treatment means relative to control means) for $\alpha = 0.05$ and a power of 80%. Detectable differences for initial weight of adult T. impressum, weight of juveniles at the end of the experiment, and number of offspring per fertile female were calculated based on t-tests. Spider mortality data and data on proportions of adult females producing viable offspring were analyzed with $X^2$-tests.

Results

Prey spectrum

The prey spectra of T. impressum females collected in 2005-2007 in Germany and 2007 in Switzerland were combined with data from 3 years from a maize field in Sóskút, Hungary (Árpás et al. 2005) (Fig. 1). Sternorrhyncha (aphids) were the most numerous prey items in T. impressum webs. Heteroptera (bugs), represented mainly by Miridae (Trigonotylus spp., Lygus spp.) and to a lesser extent Nabidae and Anthocoridae (Orius spp.), were found frequently in the webs in all years and locations, while Thysanoptera (thrips) were only frequent in 2 years in Germany. The same observation was made for Diptera (flies and midges), which were frequent in 2007 in Switzerland but constituted less than 5% of the prey in all other years and locations. Auchenorrhyncha (leafhoppers) and Coleoptera (beetles) constituted up to 12% of the prey items in the webs. Beetles were represented mainly by Alticinae and Chrysomelidae (Oulema spp.), but also by Coccinellidae, Cantharidae, Staphylinidae, Elateridae, and Dermestidae. Hymenoptera, Neuroptera (lacewings), Araneae, and Lepidoptera (small butterflies) were also found in the spider webs, but proportions remained below 5%. Acari (mites) were reported from only three spider webs in 1 year in Hungary (personal communication F. Tóth, Szent István University, Gödöllő, Hungary), which indicates that they are not a common prey for T. impressum adults.

Cry3Bb1 concentration in field-collected arthropods

Sternorrhyncha, representing the most frequent prey in T. impressum spider webs, did not contain measurable amounts of Cry3Bb1, except Rhopalosiphum padi (Sternorrhyncha: Aphididae) collected during anthesis (Fig. 2). In contrast, Thysanoptera and Heteroptera (except Nabidae)
contained high concentrations of Cry3Bb1. One sample of *Trigonotylus caelestialium* (Heteroptera: Miridae) contained more than 100 µg/g DW, which was in the range of the Cry3Bb1 concentrations in maize leaves. While adult frit flies (*Oscinella frit*, Diptera: Chloropidae) contained 17 µg/g DW, other Diptera contained 0.4 µg/g DW or less. Concentrations in Auchenorrhyncha ranged from approximately 1 µg/g DW to values below the detection limit. In the collected beetles Coleoptera, chrysomelids contained 7-33 µg Cry3Bb1/g DW, while concentrations in cantharids and coccinellids remained below 0.08 µg/g DW, except for adult *Propylea quatuordecimpunctata* (Coleoptera: Coccinellidae), which contained 0.2 µg/g during anthesis. For lacewings (Neuroptera: Chrysopidae), concentrations of Cry3Bb1 also increased during anthesis (1.0-1.5 µg/g DW), while Cry3Bb1 was hardly detectable before and after anthesis.

Spiders of the families Araneidae, Linyphiidae, and Tetragnathidae contained low concentrations of Cry3Bb1 (0.1-0.4 µg/g DW) and Cry3Bb1 was not detected in juvenile Theridiidae collected after anthesis. Adult *T. impressum*, analyzed before anthesis in 2005 and 2007, had mean concentrations of ca. 1 µg of Cry3Bb1/g DW, with concentrations ranging from 0 to 6.6 µg/g DW among individual spiders.

Maize leaf tissue contained 160-220 µg Cry3Bb1/g DW, and maize pollen contained 27 µg/g DW. Cry3Bb1 was not detected in any arthropod sample (except one *O. melanopus* individual in 2006) from control maize plots.

![Figure 1](image_url)

**Figure 1.** Prey spectrum of *Theridion impressum* in maize fields based on analysis of the pocket part of webs as determined during 3 years in Würzburg, Germany (*N* = 34-232 webs) and 1 year in Zürich, Switzerland (*N* = 119 webs). Published data from 3 years in Sóskút, Hungary (*N* = 145-558 webs) are included (Árpás et al., 2005).
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Figure 2. Cry3Bb1 concentrations measured in potential prey species of Theridion impressum, in juvenile Theridiidae and adult T. impressum, and in maize plants. Samples were collected in experimental Bt maize (event MON88017) plots in Würzburg, Germany between 2005 and 2007 before, during, and after anthesis. For details on replicates and individuals per sample see Supplementary Table 1. Data plotted left of the y axis denote Cry3Bb1 measurements below the limit of detection (LOD).

Adult feeding assays

At the beginning of the experiment, there was no difference in weight among female spiders collected in the control plots, Bt plots, or conventional maize (Table 1) or between spiders collected in both years (2-way ANOVA, p = 0.27). Starved spiders (collected in conventional maize) died within 64 days in 2006 and 56 days in 2007, except one spider in 2006 and three spiders in 2007 that
survived until the end of the experiment. Mortality of females in the Bt and control treatment was not significantly different (Table 1). More spiders died during the experiment in 2007 than in 2006 (Gehan’s Wilcoxon test, \( Z = p < 0.0001 \)). Kaplan-Meier plots showing survival in the different treatments and years are presented in Supplementary Fig. 1A, B. While in 2006 none of the starved females produced offspring, three spiders produced an egg sac in 2007 and 16-27 juveniles hatched. Most females in the Bt and control treatment had one or two egg sacs, and three spiders were able to produce three. There was no significant difference between the Bt and control treatment in the proportion of spiders producing viable offspring (Table 1), but fewer spiders were fertile in 2007 than in 2006 \( (X^2 = 15.1, p = 0.0001) \). The number of juveniles produced per fertile female was not influenced by food treatment (Table 1) or year (2-way ANOVA, \( p = 0.8 \)). Pooled across all years and treatments, a mean of 31.7 ± 1.24 juveniles emerged per egg sac.

With the given means, proportions, and standard deviations of the control data, the difference detectable with a statistical power of 80% at \( \alpha = 0.05 \) ranged from 13% to 28% for the different parameters (Table 1).

Lacewings feeding on Bt maize pollen contained 22 times less Cry3Bb1 than corn rootworms feeding on Bt maize silk and leaves (Table 2). The Cry3Bb1 concentration in female T. impressum receiving a lacewing as last prey was 18\% of the concentration in lacewings (Table 2). In contrast, the Cry3Bb1 concentration in spiders receiving a corn rootworm as last prey was 32\% of the Bt protein contained in corn rootworms (Table 2). Cry3Bb1 was not detected in lacewings, corn rootworm beetles, and adult spiders from the control treatment.

**Juvenile feeding assay**

There were no significant differences in juvenile spider weights among the three treatments at the beginning of the spider mite and pollen feeding assay (Table 1). The weights of juveniles taken from different mothers were significantly different (2-way ANOVA, spider mite assay: \( F_{6,173} = 21.8; \) pollen assay: \( F_{5,162} = 54.3; \) \( p < 0.001 \)). When fed spider mites or pollen, juveniles gained weight with time (RM-ANOVA, spider mite assay: \( F_{2,218} = 1937.4; \) pollen assay: \( F_{4,344} = 683.9; \) \( p < 0.0001 \)), but there was no difference in weight gain between the Bt and control treatments \( (p > 0.15) \). In both experiments, weight depended on the mother (spider mite assay: \( F_{6,109} = 8.8; \) pollen assay \( F_{5,86} = 13.1, p < 0.0001 \)) but this influence decreased with time, as indicated by the significant time × mother interaction (spider mite assay: \( F_{12,218} = 11.0; \) pollen assay: \( F_{20,344} = 11.9, p < 0.0001 \)). Starved juveniles died within 37 and 29 days in the spider mite and pollen experiment, respectively. By the end of the spider mite experiment, only one juvenile in the control treatment died and all individuals survived in the Bt treatment. In both treatments of the pollen assay, eight juveniles died by the end of the experiment (Table 1, Kaplan-Meier survival plots are presented in Supplementary Fig. 1C, D).
Table 1. Spider weight, mortality, and offspring production in *Theridion impressum* that were starved or provided with *Bt* (event MON889017) or control (DKC5143) maize-fed prey or *Bt* maize or control maize pollen for 8-9 weeks. Adult spiders were provided with adult lacewings (*Chrysoperla carnea*) and corn rootworms (*Diabrotica virgifera virgifera*) in alternation, juveniles received either maize pollen or spider mites (*Tetranychus urticae*). Statistical analysis was performed only with *Bt* and control maize treatments except initial weight, where all three treatments were compared. Means are presented with SE.

<table>
<thead>
<tr>
<th></th>
<th>Starved</th>
<th>Control</th>
<th>Bt</th>
<th>Analysis</th>
<th>p value</th>
<th>Statistic</th>
<th>Det. diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adult <em>T. impressum</em> fed lacewings and corn rootworms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>50</td>
<td>76</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial weight [mg]</td>
<td>10.6 ± 0.48</td>
<td>10.0 ± 0.36</td>
<td>10.2 ± 0.39</td>
<td>2-way ANOVA(b)</td>
<td>0.6 F(2,197) = 0.54</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>Mortality until end [%]</td>
<td>92.0</td>
<td>42.1</td>
<td>37.7</td>
<td>Wilcoxon-test(c)</td>
<td>0.3 Z = 1.0</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>Spiders with offspring [%]</td>
<td>6.0</td>
<td>61.8</td>
<td>64.9</td>
<td>(X^2)-test</td>
<td>0.7 (X^2) = 0.16</td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td>Offspring per fertile female</td>
<td>22.3 ± 3.28</td>
<td>54.1 ± 3.83</td>
<td>51.7 ± 4.27</td>
<td>2-way ANOVA</td>
<td>0.7 (F_{1,93}) = 0.15</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td><strong>Juvenile <em>T. impressum</em> fed spider mites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>70</td>
<td>57</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial weight [µg]</td>
<td>235 ± 8.2</td>
<td>246 ± 8.2</td>
<td>236 ± 7.7</td>
<td>2-way ANOVA(b)</td>
<td>0.1 (F_{2,173}) = 2.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Weight at end [µg]</td>
<td>-</td>
<td>748 ± 21.0</td>
<td>728 ± 16.9</td>
<td>RM-ANOVA(b,d)</td>
<td>0.2 (F_{1,109}) = 2.1</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Mortality until end [%]</td>
<td>100</td>
<td>1.8</td>
<td>0</td>
<td>(X^2)-test</td>
<td>0.5 (X^2) = 1.2</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td><strong>Juvenile <em>T. impressum</em> fed pollen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial weight [µg]</td>
<td>189 ± 7.2</td>
<td>186 ± 6.4</td>
<td>188 ± 6.7</td>
<td>2-way ANOVA(b)</td>
<td>1.0 (F_{2,162}) = 0.03</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Weight at end [µg]</td>
<td>-</td>
<td>440 ± 19.2</td>
<td>451 ± 17.5</td>
<td>RM-ANOVA(b,d)</td>
<td>0.4 (F_{1,94}) = 0.70</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>Mortality until end [%]</td>
<td>100</td>
<td>14.0</td>
<td>13.8</td>
<td>Wilcoxon-test(c)</td>
<td>1.0 Z = -0.002</td>
<td>23%</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) detectable difference for means based on t-tests, for percentages based on \(X^2\)-tests.

\(b\) analysis conducted on log-transformed data.

\(c\) survival analysis on mortality data recorded every 2-3 days.

\(d\) repeated measures ANOVA conducted with initial weight and weight after 4 and 8 weeks for spider mites, and after 2, 4, 6, and 8 weeks for pollen.
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The detectable differences in weight and mortality with statistical power of 80% at \( \alpha = 0.05 \) feeding experiments with juvenile *T. impressum* were between 5% and 23% (Table 1).

Concentrations of Cry3Bb1 measured in spider mites were ca. ten times higher than in pollen (Table 2) and juvenile spiders contained also ten times more Cry3Bb1 when fed with spider mites than when fed with pollen. Cry3Bb1 concentrations in juvenile spiders were approximately 3% of the concentrations in spider mites or pollen. In the control treatments, Cry3Bb1 was not detected in juvenile spiders and maize pollen. Spider mites reared on control maize contained Cry3Bb1 (most likely due to contamination), but concentrations were more than 1500 times lower than for spider mites reared on *Bt* maize.

Table 2. Concentration of Cry3Bb1 (based on ELISA) in spiders and food in 8 to 9-week-long feeding assays with adult and juvenile *Theridion impressum*. Lacewing (*Chrysoperla carnea*) adults feeding on *Bt* maize (event MON88017) pollen and corn rootworm (*Diabrotica virgifera virgifera*) adults feeding on *Bt* maize silk and leaves were provided to adult spiders in alternation while *Bt* maize pollen or spider mites (*Tetranychus urticae*) feeding on *Bt* maize served as food for juvenile *T. impressum*. Means are presented with SE.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Food</th>
<th>Cry3Bb1 concentration [( \mu g/g \text{ dry weight} )] and number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in food</td>
</tr>
<tr>
<td>Adult <em>T. impressum</em></td>
<td>Last prey lacewing</td>
<td>0.74 ± 0.117</td>
</tr>
<tr>
<td></td>
<td>Last prey corn rootworm</td>
<td>16.4 ± 1.78</td>
</tr>
<tr>
<td>Juvenile <em>T. impressum</em></td>
<td>Spider mites</td>
<td>96.1 ± 10.31</td>
</tr>
<tr>
<td></td>
<td><em>Bt</em> maize pollen</td>
<td>8.33 ± 0.342</td>
</tr>
</tbody>
</table>

**Discussion**

*Exposure of T. impressum to Cry3Bb1*

**Prey spectrum of T. impressum** - Mainly herbivores like aphids, thrips, mirid bugs, leaffoppers, and chrysomelid beetles are found in the webs of adult *T. impressum* in maize fields and other crops (Nyffeler & Benz 1979; Pekár 2000). For this reason, the species is generally considered as beneficial, even though predatory thrips (e.g., *Aeolothrips intermedius*), predatory bugs (Anthocoridae and Nabidae), coccinellid beetles, lacewings, as well as hymenopteran parasitoids and bees were occasionally caught by the spiders. The relative abundance of the different arthropod groups in the spider webs was highly variable among years and field sites and most likely influenced by (a) the crop and its dominating arthropod fauna; (b) the location...
Chapter 3 – Risk of Bt maize for Theridion impressum

of the field, e.g., proximity to open water bodies (Nyffeler & Benz 1979); and (c) the population dynamics of the different arthropods in the specific year.

There is no published information on the prey spectrum of juvenile T. impressum or closely related species. Most probably, they feed on small arthropods like aphids, leafhoppers, thrips, and spider mites. Because juveniles disperse at the time of pollen shedding (Pekár 1999) and can grow on maize pollen alone (as shown in the present study), it is likely that they also consume pollen in the field.

**Cry3Bb1 concentrations in potential prey species** - While no Bt protein was detected in aphids (the most numerous prey items in T. impressum webs) before anthesis, low concentrations were detected in R. padi collected after pollen shedding. Probably, R. padi was contaminated with pollen, plant remains, or arthropod faeces because no Cry3Bb1 was detected in R. padi in a climate chamber experiment under clean conditions with the same maize variety (six replicates, Cry3Bb1 concentration below the limit of detection of 0.005 µg/g DW). Aphids feed on the phloem sap (Douglas 2003), which contains no or very low amounts of Bt protein, as shown for Cry1Ab-expressing maize (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002; Dutton et al. 2004). Similarly, leafhoppers (e.g., Psammotettix alienus, Hemiptera: Auchenorrhyncha) feeding on phloem sap ingested little or no Cry3Bb1. In contrast, the leafhoppers Empoasca pteridis, Eupteryx atropunctata, and Zyginidia scutellaris (Hemiptera: Auchenorrhyncha) feed also on mesophyll cells (Nickel 2003) and thus do ingest the Bt protein. This was shown previously for Z. scutellaris feeding on Cry1Ab-expressing maize (Dutton et al. 2004; Obrist et al. 2006a). Mesophyll-feeding thrips, mirid bugs, and tissue-feeding chrysomelid beetles contained considerably higher Cry3Bb1 concentrations, almost reaching the level detected in maize tissue. That Bt protein levels in herbivores can reach the concentrations in maize leaves has been reported also for Cry1Ab-expressing maize in Spain (Obrist et al. 2006a). In a study of Bt maize in the USA, mesophyll-feeding herbivores were scarce but the chrysomelid beetles present (including D. v. virgifera) contained high amounts of Cry1Ab (Harwood et al. 2005).

Predatory thrips (Aeolothrips spp.), bugs (Orius spp. and Nabis spp.), beetles (Cantharis lateralis and Coccinellidae), fly larvae (Syrphidae), and lacewing larvae (Chrysoperla spp.) generally contained less Cry3Bb1 than the herbivores feeding directly on maize. Bt protein concentrations are often one order of magnitude lower in predators than in their prey because the prey’s gut (where the Bt protein is located) represents only part of the consumable prey and because some Bt protein is digested and excreted (Romeis et al. 2009). In addition to prey, many predators also feed on maize pollen (Romeis et al. 2009). This can explain why Orius spp., Chrysoperla spp., and coccinellid beetles contained relatively high Cry3Bb1 concentrations during anthesis compared to before or after pollen shed. Similar observations were made in Cry1Ab-expressing maize in Spain (Obrist et al. 2006a). Harwood et al. (2005)
also reported considerable amounts of Cry1Ab in *Orius insidiosus* and in some coccinellid beetles collected in a *Bt* maize field in the USA, but it remains unclear whether pollen feeding played a role because the collection date was not presented. In the current study, spiders of different families contained detectable concentrations of Cry3Bb1 (except juvenile Theridiidae before anthesis) and adult *T. impressum* contained a mean concentration of ca. 1 µg/g DW. Measurable concentrations of Cry1Ab were also reported from spiders (mainly Linyphiidae) in *Bt* maize in the USA (Harwood *et al.* 2005).

In arthropods collected in non-*Bt* maize plots of the present study, generally no Cry3Bb1 was detected. This indicates that arthropods (including flying species with high Cry3Bb1 concentrations) did not change frequently from *Bt* to control maize plots.

The data presented here demonstrate that the prey spectrum of adult *T. impressum* includes species with very low amounts of Cry3Bb1, like phloem feeders, predators, and species that are unlikely to feed directly on *Bt* maize (e.g., most Diptera and Hymenoptera). In addition, the spiders frequently consume herbivores with high Cry3Bb1 concentrations, like mirid bugs, thrips, and chrysomelid beetles. Consequently, environmental exposure concentrations may occasionally approach the expression level in *Bt* maize tissue, but the average exposure will be considerably lower. For this reason, Raybould *et al.* (2007) suggested to use the *Bt* protein concentration in plant tissue divided by five as an estimated realistic environmental exposure concentration for generalist predators in risk assessment.

*Hazard for *T. impressum* when feeding on food containing Cry3Bb1*

Before the feeding experiment started, the weight of adult spiders collected from *Bt* and control maize plots was similar. Furthermore, the number of spiders collected in a certain period of searching time was similar for four *Bt* plots (10-16 spider/h) and four control maize plots (9-18 spiders/h) in 1 year (M. Meissle & J. Romeis, unpublished data). This suggests that previous exposure to Cry3Bb1 in the field did not influence the spiders. Some adults kept in the laboratory without food were able to survive for more than 10 weeks until the end of the feeding experiment and in some rare cases even managed to produce viable offspring. Apparently *T. impressum* had substantial energy reserves at the beginning of the laboratory experiment.

When female *T. impressum* were fed prey reared on *Bt* or control maize, no effect on mortality and offspring production was detectable. However, 38% of the *T. impressum* females that were fed with lacewings and corn rootworms were unable to produce viable offspring. They either died before producing an egg sac, were not mated, or eggs were infertile. The number of juveniles that emerged per egg sac in the laboratory study was less than half of the number emerging from egg sacs collected in Swiss maize fields (72.9 ± 3.13, N = 34; unpublished data). This indicates that the conditions were less favourable in the laboratory than in the field. One possible reason may be that the prey
provided in our experiment did not fully meet the nutritional requirements of the spiders. Because feeding a mix of different prey species can improve the nutrient balance for spiders (Riechert & Harp 1987; Toft 1999), we provided two prey species in alternation. Furthermore, the 2-week starvation period before the start of the experiment and suboptimal humidity, temperature or light conditions might have reduces spider fecundity.

Cry3Bb1 measurements at the end of the feeding experiment confirmed that most spiders had ingested the *Bt* protein. To simulate the field situation, we fed *T. impressum* adults in alternation with corn rootworms containing relatively high Cry3Bb1 concentrations and with lacewings containing considerably lower Cry3Bb1 concentrations. At the end of the experiment, spiders contained less than one third of the Cry3Bb1 concentrations in the last prey. This indicates that the *Bt* protein was excreted or digested and not accumulated over time. Given that the laboratory feeding experiment lasted 8-9 weeks from the time of collection (ca. 4 weeks before anthesis), spiders consumed Cry3Bb1-containing prey longer in the laboratory experiment than they would in the field. Because corn rootworms contained 22 times more Cry3Bb1 than lacewings, they represent the major source of *Bt* protein ingestion for the spiders in the feeding experiment. The biological activity of Cry3Bb1 contained in adult corn rootworms fed with *Bt* maize silk and leaves was confirmed in sensitive insect bioassays (Meissle & Romeis, submitted). The lack of negative effects on spiders in the *Bt* treatment therefore suggests that Cry3Bb1 exposure in the field represents no hazard for adult *T. impressum*.

Juvenile *T. impressum* survived and gained weight when fed exclusively with spider mites or maize pollen and no negative impact of Cry3Bb1 was evident. Earlier studies have shown that the spiders *Thomisus onustus* (Araneae: Thomisidae) and *A. diadematus* survived 1.5-2 times longer when feeding on pollen from Asteraceae (*Bellis perennis*, *Erigeron annuus*) or birch trees (*Betula papyrifera*) than when receiving no food (Smith & Mommsen 1984; Vogelei & Greissl 1989). In our study with maize pollen, starved *T. impressum* juveniles died within 4 weeks, while more than 85% of pollen-fed spiders were alive after 8 weeks. In addition, spider weight at the end of the experiment more than doubled. This indicates a high nutritional quality of maize pollen. With pollen shedding lasting for at most 2-3 weeks in a maize field, the 8-week duration of the pollen feeding assay is extended compared to the field situation.

Spider mites contained one order of magnitude more Cry3Bb1 than pollen and concentrations were the range of those in the leaves. This has previously been shown for Cry1Ab-expressing maize (Dutton *et al.* 2002; Obrist *et al.* 2006a,b). Because concentrations are higher in spider mites than in other small arthropods, like thrips, leafhoppers, or aphids, exposure of the juvenile spiders to Cry3Bb1 contained in spider mites can be considered as a realistic worst-case exposure scenario. Juvenile spiders hatching in mid-July will be in...
the maize crop at latest until the end of September, when maize is usually harvested. Thus, the 8 weeks of the feeding experiment represents a realistic time frame. The biological activity of Cry3Bb1 contained in Bt maize pollen and in spider mites feeding on Bt maize was confirmed in sensitive insect bioassays (Li et al. 2008; Meissle & Romeis, submitted). The lack of detrimental effects in the Bt spider mite and pollen treatments indicates that Cry3Bb1 does not pose a hazard to juvenile *T. impressum*.

Because of the absence of statistical differences between Bt and control treatments for any parameter in the feeding experiments with *T. impressum*, retrospective power analyses were conducted to calculate the difference between control and treatment means that could have been detected with a power of 80% at a 5% type I error rate. The detectable differences were smallest for juvenile and adult spider weights (5-14%), followed by mortality (14-23%) and the proportion of adult spiders with offspring (24%). The number of offspring per female was the least sensitive parameter, with a detectable difference of 28%.

A difference of 20% in important life table parameters between treatments is considered ecologically meaningful for generalist predators in agroecosystems and is therefore commonly used as a trigger value for risk assessment (Candolfi et al. 2000b). Consequently, the statistical power in the experimental setup we used was sufficient to detect meaningful differences if they had been present. For future toxicity studies, the use of juveniles can be recommended for the following reasons: 1) Experiments with adults required more replicates than those with juveniles to obtain similar statistical power because field-collected adults showed higher variability in most parameters than juveniles hatched in the laboratory; 2) More effort was needed to search for the high number of individuals in the field for the adult feeding experiment than was required for the breeding of juveniles from a few field-collected mothers; 3) Tests with juveniles can last for several months with low control mortality; 4) Juveniles can be exposed to relatively high concentrations of toxin using pollen or spider mites as food.

**Implications for risk assessment**

The information on Cry3Bb1 concentrations in arthropods collected in Bt maize can be used for future Bt exposure assessments of other predators with known prey spectrum. The most frequent arthropods and the measured Bt protein concentrations in arthropods relative to the expression levels in the plants were comparable in a Cry3Bb1-expressing maize field in Germany (this study) and a Cry1Ab-expressing maize field in Spain (Obrist et al. 2006a). This suggests that arthropod food webs and Bt protein transfer show similar patterns in European maize fields. Although the food web in American maize fields contained different species and apparently fewer herbivores with considerable Bt protein concentrations, the concentration measured in the different guilds (mesophyll feeders, predators, etc.) were comparable to the
data from Europe (Harwood et al. 2005). Furthermore, that predators contained generally lower amounts of Cry3Bb1 than mesophyll-feeding herbivores confirms that Bt and other insecticidal proteins are diluted in the food chain (Romeis et al. 2009).

Even though *T. impressum* is exposed to the Bt protein in the field, long-term feeding studies with juvenile and adult spiders revealed no detrimental effects of prey or pollen containing Cry3Bb1. Thus, the cultivation of corn rootworm-resistant Bt maize appears to represent no risk for *T. impressum*. In comparison, susceptibility of the species to the broad-spectrum insecticides used for pest control in conventional crop production has been demonstrated (Pekár 2002). Like the present study, other laboratory and field studies have found no evidence for adverse effects of different Bt crops expressing Cry1 or Cry3 proteins on a range of natural enemies, including spiders (Romeis et al. 2006; Wolfenbarger et al. 2008). Our data for Cry3Bb1-expressing maize therefore confirm the specificity of currently deployed Bt crops. The present study also shows that the impact of plant-expressed insecticidal proteins can be tested under controlled laboratory conditions using *T. impressum* as a surrogate for spiders. Such tests may be part of a regulatory risk assessment if a potential risk to this group of predators can be hypothesized.

**Acknowledgements**

We thank the German Project “Biosafety research on transgenic *Diabrotica*-resistant Bt maize” funded by the Federal Ministry of Education and Research for access to their field experiment and the staff in Würzburg, who helped whenever needed. We acknowledge Ferenc Tóth for discussing and sharing field data on *T. impressum* prey spectrum, Mario Waldburger for analyzing spider webs, and Andrea Czollner, Simon Knecht, and Marco Peter for technical assistance. Further thanks go to Eva Sprecher, Denise Wyninger, Bernhard Merz, Stefan Rauschen, and Gerald Moritz for taxonomic expertise and for fruitful discussions on arthropod ecology. We thank Sabine Eber, Wolfgang Nentwig, and Joe Huising for valuable comments on an earlier version of the manuscript. We are grateful to the Swiss Innovation Promotion Agency (CTI) for funding this research (project 7487.1 LSPP-LS).

**References**


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Meissle M, Romeis J (submitted) Insecticidal activity of Cry3Bb1 expressed in Bt maize on larvae of the Colorado potato beetle, Leptinotarsa decemlineata (Coleoptera: Chrysomelidae). Entomologia Experimentalis et Applicata, in review.


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### Supplementary Table 1. Cry3Bb1 concentrations in maize and arthropods collected in Würzburg (Germany) before anthesis (in 2005, 2006, and 2007) and during and after anthesis (in 2005); ELISA results below the limit of detection ( LOD) are indicated as ‘<’ and the corresponding LOD value.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Taxon / Species</th>
<th>Stage</th>
<th>Dry weight per individual [mg]</th>
<th>Ratio dry weight/fresh weight [%]</th>
<th>Before anthesis</th>
<th>During anthesis</th>
<th>After anthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.07.2005</td>
<td>29.06.2006</td>
<td>29.06.2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>177.048 ± 51.060</td>
<td>27.329 ± 6.461</td>
<td>163.083 ± 16.057</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>(4)</td>
<td>(4)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

**Order**
- Araneae
  - Araniidae
    - Araniella sp.
      - juvenile: 0.19 ± 0.11 (4; 60)
    - adult: 3.43 ± 4.4 (4; 0-9)
  - Linyphiidae
    - juvenile & adult: 0.71 ± 0.71 (4; 0-9)
  - Tetragnathidae
    - juvenile: 0.24 ± 0.24 (4; 0-9)
    - adult: 3.25 ± 3.25 (4; 0-9)
  - Theridiidae
    - juvenile: 0.19 ± 0.19 (4; 0-9)
    - adult: 3.43 ± 4.4 (4; 0-9)

**Order**
- Coleoptera
  - Cantharidae
    - Cantharis lateralis L.
      - adult: 3.25 ± 3.25 (4; 0-9)
  - Chrysomelidae
    - Oulema lichenis (Voet.)
      - adult: 2.38 ± 2.38 (4; 0-9)
    - Oulema melanopus (L.)
      - adult: 2.38 ± 2.38 (4; 0-9)
  - Phyllotreta sp.
    - adult: 0.30 ± 0.30 (4; 0-9)
  - Coccinellidae
    - Coccinella septempunctata L.
      - larva: 6.72 ± 6.72 (4; 0-9)
      - adult: 13.81 ± 13.81 (4; 0-9)
    - Propylea quatuordecimpunctata (L.)
      - larva: 2.12 ± 2.12 (4; 0-9)
      - adult: 3.41 ± 3.41 (4; 0-9)

**Order**
- Diptera
  - Chloropidae
    - Oulema lichenis (Voet.)
      - adult: 3.25 ± 3.25 (4; 0-9)
  - Chrysopidae
    - larva: 4.16 ± 4.16 (4; 0-9)
    - adult: 0.15 ± 0.15 (4; 0-9)
    - larva: 0.07 ± 0.07 (4; 0-9)
    - adult: 0.07 ± 0.07 (4; 0-9)

**Order**
- Heteroptera
  - Anthocoridae
    - Orius sp.
      - larva: 0.08 ± 0.08 (4; 0-9)
      - adult: 0.20 ± 0.20 (4; 0-9)
  - Miridae
    - Lygus rugulipennis Popp.
      - juvenile: 0.94 ± 0.94 (4; 0-9)
      - adult: 2.38 ± 2.38 (4; 0-9)

**Order**
- Hemiptera
  - Sternorrhyncha
    - Metaplistothripus dehodum (Walk.)
      - exalate: 0.09 ± 0.09 (4; 0-9)
      - exalate: 0.07 ± 0.07 (4; 0-9)
    - Rhopalothripus padi (L.)
      - alate exalate: 0.08 ± 0.08 (4; 0-9)
      - alate exalate: 0.08 ± 0.08 (4; 0-9)

**Order**
- Neuroptera
  - Chrysopidae
    - Chrysoperla sp.
      - larva: 1.48 ± 1.48 (4; 0-9)
      - adult: 0.02 ± 0.02 (4; 0-9)
    - Chrysoperla sp.
      - larva: 1.48 ± 1.48 (4; 0-9)
      - adult: 0.02 ± 0.02 (4; 0-9)
    - Chrysoperla sp.
      - larva: 1.48 ± 1.48 (4; 0-9)
      - adult: 0.02 ± 0.02 (4; 0-9)

**Order**
- Thysanoptera
  - Aeolothripidae
    - Aulothrips intermedius
      - larva: 0.03 ± 0.03 (4; 0-9)
      - adult: 0.04 ± 0.04 (4; 0-9)
    - Aulothrips intermedius
      - larva: 0.03 ± 0.03 (4; 0-9)
      - adult: 0.04 ± 0.04 (4; 0-9)
    - Aulothrips intermedius
      - larva: 0.03 ± 0.03 (4; 0-9)
      - adult: 0.04 ± 0.04 (4; 0-9)

**Order**
- Nematoda
  - Nematidae
    - Nabis sp.
      - adult: 0.19 ± 0.19 (4; 0-9)
      - larva: 0.20 ± 0.20 (4; 0-9)
    - Nabis sp.
      - adult: 0.19 ± 0.19 (4; 0-9)
      - larva: 0.20 ± 0.20 (4; 0-9)
Supplementary Figure 1. Survival of *Theridion impressum* when provided with prey fed on *Bt* maize (event MON88017 expressing Cry3Bb1) or control maize (non-transformed near-isoline, DKC5143), *Bt* maize pollen or control pollen, or when receiving no food. Adult spiders were fed lacewings and corn rootworms A) in the year 2006, B) in the year 2007. Juvenile spiders were fed C) spider mites or D) maize pollen.
Chapter 4

Susceptibility of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to the entomopathogenic fungus *Metarhizium anisopliae* when feeding on Cry3Bb1-expressing *Bt* maize

Meissle, M., Pilz C. & Romeis, J.

Abstract

Genetically engineered maize producing the insecticidal protein Cry3Bb1 from *Bacillus thuringiensis* (*Bt*) is protected against corn rootworms (*Diabrotica* spp.), which are serious maize pests in North America and Europe. The aim of the present study was to investigate the interaction of *Bt* maize (Event MON88017) and the entomopathogenic fungus *Metarhizium anisopliae* for controlling *D. v. virgifera*. Exposure to Cry3Bb1 expressed in *Bt* maize seedlings resulted in decreased weight gain in *D. v. virgifera* larvae but did not influence susceptibility to *M. anisopliae*. Adult beetles were not affected by Cry3Bb1 in their food, but mortality when feeding on maize leaves was higher than when feeding on silk. Adults were more susceptible to the fungus than larvae. These results indicate that *Bt* maize will not interfere with the biological control provided by entomopathogenic fungi.

Keywords

*Bacillus thuringiensis*; biological control; ELISA; invasive species; species interactions; western corn rootworm, resistance management
Introduction

One of the most devastating maize pests in North America is the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). Since the early 1980s, the beetle has been introduced several times to Eastern and Central Europe, where it is spreading rapidly (Berger, 2001; Kiss et al., 2005; Miller et al., 2005). After overwintering as eggs in the soil, larvae of this univoltine species hatch early in the maize growing season, seek maize roots to feed on, and develop in three larval stages before pupation (Chiang, 1973). Infested plants suffer from reduced nutrient and water supply, and the decreased plant stability often results in lodging. Further yield reductions can be caused by adults feeding on silk and kernels (Krysan, 1986; Levine & Oloumi-Sadeghi, 1991).

Options for controlling the western corn rootworm include crop rotation and the application of insecticides (Levine & Oloumi-Sadeghi, 1991). However, *Diabrotica* spp. have developed resistance against both measures (Levine & Oloumi-Sadeghi, 1991; Spencer et al., 2005). Since 2003, *Diabrotica*-resistant transgenic maize expressing the *cry3Bb1* gene from the bacterium *Bacillus thuringiensis* (*Bt*) has been grown commercially in the USA (Vaughn et al., 2005; James, 2007; Hellmich et al., 2008). The deployment of *Bt* maize has huge potential for reducing insecticide applications and yield losses in the USA as well as in Europe (Rice, 2004; Demont & Tollens, 2005). The continuous exposure to the *Bt* protein, however, poses high selection pressure on the pest population and may lead to the development of resistance, as has frequently occurred with chemical insecticides (Levine & Oloumi-Sadeghi, 1991; Gould 1998; Shelton et al., 2002; Tabashnik et al., 2003). To delay development of resistance to *Bt* crops in the field, a certain percentage of conventional maize is usually grown as a “refuge” adjacent to the *Bt* crop. It is expected that resistant individuals surviving on *Bt* maize will mate with a large number of susceptible beetles emerging from the refuge. For this strategy to be effective, however, resistance has to be recessive and the toxin concentration in plants has to be high enough to kill resistance heterozygous insects (Tabashnik et al., 2003; Ferré et al., 2008). The concentration of Cry3Bb1 expressed in *Bt* maize, however, is not considered a high dose for *D. v. virgifera* and larval survival was equivalent to near-isogenic control maize after only three generations of selection on *Bt* maize in the greenhouse, (Meihls et al., 2008).

In agricultural fields, most herbivorous arthropods are attacked by a number of naturally occurring antagonists. This contribution to pest control is considered important because it keeps populations of many herbivores below the economic level of damage. A number of different antagonists (microbial pathogens, nematodes, arthropod predators, and parasitoids) from Central and South America, which has the highest diversity of *Diabrotica* species and is presumably the region of origin (Tallamy et al., 2005), are known to attack corn rootworms (Kuhlmann & van der Burgt, 1998). In Europe and North
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America, generalist predators are also likely to feed non-specifically on D. v. virgifera, but no effective indigenous parasitoids or other specialized antagonists were found (Levine & Oloumi-Sadeghi, 1991; Toepfer & Kuhlmann, 2004; Toth et al., 2002). In addition, infections by entomopathogenic nematodes and the fungal species Beauveria bassiana (Bals.) Vuill. and Metarhizium anisopliae (Metsch.) Sorokin were reported in field-collected D. v. virgifera larvae and adults, but frequencies were very low (Toepfer & Kuhlmann, 2004; Pilz et al., 2008).

Entomopathogenic fungi have a long history as biological control agents. They are used to control a variety of pests, and a number of commercial products are registered worldwide (Butt et al., 2001; Lord, 2005). They also show a high potential for biological control of D. v. virgifera because they were able to kill larvae and beetles effectively in the laboratory and the field (Krueger & Roberts, 1997; Mulock & Chandler, 2000; Pilz et al., 2007).

In integrated pest management (IPM), sustainable cropping systems are established by combining environmentally friendly control strategies to reduce the application of harmful, broad-spectrum chemical insecticides. Because Cry3Bb1-expressing Bt maize does not control D. v. virgifera larvae completely (Al-Deeb & Wilde, 2005; Oyediran et al., 2007) and rapid evolution of resistance has been demonstrated in the greenhouse (Meihls et al., 2008), biological control by entomopathogenic fungi in combination with Bt maize might help to delay resistance development by decreasing the number of surviving beetles.

When different pest control methods, like plants with resistance to herbivores, pathogens and biopesticides, are combined, interactions may lead to synergistic, antagonistic, or additive effects. For example, when biopesticides containing Bt derivates were applied together with entomopathogenic fungi, the effects on herbivores were additive (Costa et al., 2001), or even synergistic (Brousseau et al., 1998; Wraight & Ramos, 2005). Similarly, host plants with resistance to herbivores due to antibiosis or antixenosis in combination with B. thuringiensis derivates, entomopathogenic fungi, or viruses resulted in synergistic or additive effects for herbivore control (Hare, 2002). The interactions of Bt proteins produced in genetically modified plants and entomopathogenic fungi, however, have been examined only for lepidopteran pests. Lawo et al. (2008) demonstrated that Bt-susceptible caterpillars were more sensitive to the fungus when feeding on Bt chickpea than when feeding on control plants, while infection rates were similar for a Bt-resistant strain. In addition, Johnson et al. (1997a,b) reported that Bt-susceptible caterpillars showed higher infection rates than Bt-resistant ones when feeding on Bt tobacco. The interactions of any commercialized Bt plant with entomopathogenic fungi for controlling beetle pests and potential consequences for biological control have not been studied to date.

When corn rootworm-resistant Bt maize is applied together with entomopathogenic fungi for controlling Diabrotica spp., interactions could be
synergistic, antagonistic or additive. Larvae that are sublethally damaged by the \textit{Bt} protein might have a weaker immune system that allows easier infection by entomopathogenic fungi, which would be most desirable for pest control. Alternatively, one can hypothesize that only corn rootworm larvae with a high level of fitness can survive on \textit{Bt} maize, making infection by the entomopathogen more difficult, which would limit the efficacy for pest control. Finally, both pest control methods may be compatible in a way that they act independently with additive effects on the pest.

We present laboratory data on the interaction of \textit{Bt} maize as the host plant, \textit{D. v. virgifera} as the herbivore, and \textit{M. anisopliae} as the entomopathogen. The specific aims of the study were (1) to assess exposure of \textit{D. v. virgifera} larvae to root-expressed Cry3Bb1 protein; (2) to compare the susceptibility of \textit{Bt} maize-fed larvae versus control maize-fed larvae to \textit{M. anisopliae}; and (3) to compare the susceptibility of adults fed with \textit{Bt} or control maize silk or leaves to \textit{M. anisopliae}.

### Materials & Methods

#### Plants and insects

Transgenic maize DKC5143Bt (Event MON88017, Monsanto Company, St. Louis, USA) and the corresponding non-transformed near isoline DKC5143 were used for the experiments. DKC5143Bt expresses the cry3Bb1 gene driven by the constitutive enhanced 35s cauliflower mosaic virus promoter (Monsanto, 2004). To quantify \textit{Bt} protein concentrations in roots and to obtain silk and leaves to feed adult \textit{D. v. virgifera}, maize plants were grown individually in 12 l plastic pots and fertilized with 40 g of slow-release fertilizer (Osmocote Exact, 16\% N: 11\% P$_2$O$_5$: 11\% K$_2$O, Scotts UK Professional, Bramford, UK) before sowing and weekly with Vegesan standard (80 \text{g} N, 70 \text{g} P$_2$O$_5$ and 80 \text{g} K$_2$O per l, Hauert HBG Dünger AG, Grossaffoltern, Switzerland) thereafter. Silk and leaves above the ears were harvested from the same plants. To obtain maize seedlings for the quantification of Cry3Bb1 and for infection studies with \textit{D. v. virgifera} larvae, we disinfected seeds for 30 sec with a sodium hypochloride solution (1 – 2 ml Javel water in 1 l of water) and washed them thoroughly thereafter. Seeds were soaked in water for ca. 12 h and transferred to 9 cm Petri dishes lined with moist filter paper. After incubation at 25\degree C and 70\% RH in the dark for 2 days, germinated seedlings were used for the experiments or stored at 4\degree C until further use. Organic maize (variety Gavott, KWS Mais GmbH, Einbeck, Germany) was used to rear \textit{D. v. virgifera} because it showed little fungal infection (T. Haye, personal communication).

A non-diapausing strain of \textit{D. v. virgifera}, derived from the USDA-ARS North Central Agricultural Research Laboratory (Brookings, USA), was reared in the quarantine facilities of CABI Europe (Delémont, Switzerland). The strain had not been selected for Cry3Bb1 resistance. Larvae were obtained in ventilated
transparent plastic containers (24 × 16 cm) lined with filter paper and filled with germinated maize. Pupae were provided in rearing containers filled with soil instead of maize. Emerging beetles were collected and used for the experiments within 48 h.

**Fungus cultivation, spore preparation, and viability testing**

For infection studies, we used *M. anisopliae* strain Bipesco 5, obtained from the Institute of Microbiology, University of Innsbruck, Austria. This strain, originally isolated from *Cydia pomonella* L. (Lepidoptera: Tortricidae) in Austria, is commercially available worldwide and has been cultivated in our institute since 2005. The fungus was passed through *D. v. virgifera* to ensure infectivity to the beetle (F0). Spores were transferred to selective Sabouraud-2-glucose-agar (S2GM) (Sigma Aldrich, Buchs, Switzerland) containing cyclohexamide, dodine, streptomycin, and tetracycline (Strasser et al., 1996) and incubated at 25 ± 1°C in the dark. Plates with sporulating colonies (F1) were stored at 4°C. The strain was propagated on a weekly basis from the F1 colonies on pure S2GM medium (F2). After incubation for ca. 10 days, fresh F2 spores were used for the experiments.

Spores were washed from the medium plates using 15 – 20 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany), homogenized for at least 30 min on a magnet stirrer, and filtered through gauze to eliminate mycelium and agar residues. The spores were counted in a Thoma counting chamber (haemocytometer), and spore suspensions were diluted to a final concentration of $2.5 \times 10^8$ (high dose) and $5 \times 10^7$ (medium dose) spores per ml. Tween 80 (0.1%) served as a control.

To test for spore viability, molten 10% S2GM in agar was pipetted onto clean microscope glass slides and left to solidify. Drops of diluted spore suspension (ca. $10^5$ spores per ml) were dispersed on the medium. Slides were transferred to plastic containers lined with wet filter paper and incubated at 25 ± 1°C for 24 h. Thereafter, 100 spores were checked for germination. Germination rates were 95% or higher in all experiments.

**Effect of Bt maize on infection of *D. v. virgifera* larvae by *M. anisopliae***

Second instar *D. v. virgifera* larvae were used for testing whether feeding on *Bt* maize influences the susceptibility to *M. anisopliae*. Compared to neonates, second instars have the advantage that mortality on *Bt* maize is lower and that they are less susceptible to damage by handling.

Glass vials (1.5 × 7.5 cm) were filled with ca. 1.7 ml of sand (sieved 1 mm, dried, and sterilized for 2 d at >100°C) and 300 µl of deionized water to ensure moist conditions without excess of water. One maize seedling (*Bt* or control) was added to each vial, with the root tip pointing to the sand surface. Second instar *D. v. virgifera* with a body weight of 2 – 6 mg were selected from the rearing container and each larva was transferred to an individual vial. Vials
were closed with a dry cotton wick and placed into plastic racks holding 7 × 7 vials. Bt and control maize treatments were alternated between rows. The racks were stored in boxes lined with moist paper towels to maintain humidity above 90%. Larval mortality was recorded 2 days after the start of the experiment, and living larvae were transferred into new vials with new maize seedlings. On day 4, larvae were weighed and assigned alternately to one of two M. anisopliae spore suspensions (5 × 10^7 or 2.5 × 10^8 spores per ml) or the control (0.1% Tween 80). Larvae were submerged in the spore suspension for 3 – 5 s and transferred into a new vial with a maize seedling. Thereafter, larval mortality was recorded, and vials with maize seedlings were changed every other day. Four days after the fungus treatment, larvae were weighed. When larvae stopped feeding and started to build a pupation chamber, the maize seedling was removed and no transfer to new vials was done. The experiment ended when all larvae died or adults emerged from the pupae. Dead larvae were transferred to Petri dishes (3.5 cm) lined with moist filter paper. Dishes were stored in a humid plastic box, and the presence of fungal mycelium was checked and recorded every other day.

The experiment was repeated 3 times with different batches of larvae. From each batch, 150 – 200 larvae were selected and after 2 days another 100 – 200 larvae reaching the suitable weight were used. Altogether, 476 larvae were fed with Bt maize while 455 larvae were fed with control maize. After 4 days of feeding, 27 larvae from one batch feeding on Bt maize were frozen and lyophilized for quantification of Cry3Bb1. The remaining larvae, which have not died and were not damaged by handling within the first 4 days, were divided into the fungal treatments, which resulted in 6 treatments (2 maize variants × 3 spore concentrations) with 118 – 133 larvae each.

**Effect of Bt maize on infection of D. v. virgifera adults by M. anisopliae**

Susceptibility of adult D. v. virgifera to M. anisopliae was tested with beetles feeding on Bt maize leaves or silk. Previous studies have shown that leaves are of low nutritional quality for adult D. v. virgifera, while silk is of high quality (Ludwig & Hill, 1975; M. Meissle and J. Romeis, unpublished). This allowed the impact of the Bt treatment to be tested in combination with stress due to low nutritional quality. Adults emerging from the rearing containers were sexed and assigned alternately to the following treatments: 1) Bt silk, 2) Bt leaves, 3) control silk, and 4) control leaves. Leaves were harvested in the greenhouse and cut into ca. 5 cm long strips. For the leaf and silk treatments, one leaf strip or 5 – 20 silk threads were provided per beetle. Beetles were kept individually in 25 ml transparent plastic cylinders (6.5 × 8.0 cm). Water was added to the wall of the cylinders using a spray bottle, and cylinders were sealed with a plastic lid. No ventilation was provided to ensure the high humidity necessary for fungal growth. Beetle mortality was recorded daily, and beetles were transferred to a clean container with new food every other day. On day 4, half of the beetles from each food treatment were dipped into a 0.1% Tween 80
solution (control) and the other half into a spore suspension containing $5 \times 10^7$ spores per ml as described earlier. Before beetles were returned to the containers, they were put on a paper towel to remove excess moisture. Dead beetles were transferred to Petri dishes with moist filter paper for development of fungal mycelium as described earlier.

The experiment was conducted with beetles emerging over a period of 16 days and new replicates were set up every second day, resulting in a total of 75 – 76 beetles each for the treatments $Bt$ and control leaves and $Bt$ and control silk. After day 4, when surviving beetles were either treated or not treated with fungal spores, 33-36 beetles were in each of the 8 treatments (4 maize variants $\times$ 2 spore concentrations). Because the experiment was terminated on the same day for all replicates, the experiment lasted 70 days for the first replicates and 54 days for the last ones.

All beetles from the $Bt$ treatments that survived the experiment were lyophilized and stored at -20°C for quantification of Cry3Bb1. This resulted in 23 beetles for silk and no beetle for leaves.

**Cry3Bb1 expression in plant material**

Because *D. v. virgifera* larvae can feed on maize roots for approximately 2 months in the field, Cry3Bb1 concentrations were measured in roots sampled every 2 weeks for a total of 8 weeks from plants grown in the glasshouse. Two weeks after sowing, plants had reached growth stage 12 (2 leaves unfolded, see Meier, 2001), and after 4, 6, and 8 weeks, plants had reached stage 14 – 15 (4 – 5 leaves unfolded), stage 16 – 18 (6 – 8 leaves unfolded), and stage 34 – 35 (4 – 5 nodes detectable), respectively. For each of the 4 sampling dates, soil was gently removed from 5 $Bt$ and 2 control plants. One long root per plant was chosen (ca. 30 – 100 cm long, depending on the plant age), cut from the plant, washed thoroughly under running water, and dried with tissue paper. Each root was divided into 4 sections of equal length, each section representing one sample from one of four positions from root tip to base.

To measure the amount of Cry3Bb1 protein in the maize seedlings fed to *D. v. virgifera* larvae in the present experiment, the seedlings were cut from the 25 $Bt$ and 4 control seeds, which had been germinated in the laboratory as described previously. The concentration of Cry3Bb1 in the leaves and silk as used for the feeding study with adult *D. v. virgifera* was measured in 6 samples each from different $Bt$ maize plants grown in the glasshouse.

After the fresh weights of root sections, seedlings, leaf pieces and silk were recorded, the samples were lyophilized in 2 ml microreaction tubes and the dry weights (DW) were determined. Samples were cut into small pieces and reduced to a maximum of 6 mg DW if necessary. Until analysis of Cry3Bb1 contents, the samples were stored at -20°C.
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**Quantification of Cry3Bb1**

The concentration of Cry3Bb1 (in seedlings; in silk, leaf, and root tissues of older plants; and in *D. v. virgifera* larvae and adults) was measured using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA), commercially available from Agdia (Elkhard Indiana, USA). One tungsten carbide ball was added to each sample together with phosphate buffered saline with Tween (PBST) at a ratio of 60 – 300 µl per mg dry weight. The samples were homogenized for 3 min at 30 Hz in a MM300 mixer mill (Retsch, Haan, Germany). After centrifugation at 13000 × g for 5 min, the supernatants were diluted with PBST 500 – 1000 times for *Bt* maize samples and 50 – 100 times for *D. v. virgifera* samples. Supernatants of control samples were used undiluted. Antibody-coated, 96-well microtiter plates were loaded with 100 µl of enzyme conjugate (both provided with the kit) and 100 µl of sample extract per test well. In addition, purified Cry3Bb1 solutions of attested purity and quality provided by Monsanto were loaded in 5 concentrations between 0.3 and 20 ng/ml to construct a standard curve. According to manufacturer instructions, the plates were incubated for 2 h and subsequently washed 7 times with PBST. Fourteen minutes after adding the (provided) substrate solution, the optical density (OD) was measured at 620 nm wavelength using a SpectrafluorPlus plate reader (Tecan, Männedorf, Switzerland). Cry3Bb1 concentrations in µg per g dry weight (DW) were calculated from the standard curve using regression analysis. The limit of detection of the test system, based on the standard deviation of buffer-only OD values multiplied by 3 (ICH, 2005), was 0.2 µg of Cry3Bb1 per ml of extract added to the test well.

**Data analysis**

All data were analyzed using the Statistica 7.1 software package (StatSoft Inc, Tulsa, USA). In the larval feeding experiment, weight data were log transformed before conducting a 2-factor ANOVA with maize (*Bt*, control) and fungal dose (0, medium, high) for each time point. Larval mortality until fungal treatment (day 4) was compared between *Bt* and control maize using frequency tables (Chi²-test). Survival of larvae after fungal treatment was compared in pairwise Gehan’s Wilcoxon tests including data until day 17 after the treatment, when the first beetle emerged. Missing or damaged larvae were considered as censored for survival analysis and were removed for all other analyses. Each fungal dose was compared within *Bt* and control maize treatments, and the same doses were compared between maize treatments. P values were sequentially adjusted for 9 comparisons using the Bonferroni-Holm method (Holm, 1979). Frequencies of emerging beetles versus dead larvae and pupae were compared by Chi²-tests using the same pairwise comparisons and adjustments as for larval survival. Fungal development in dead larvae was compared in 4 pairwise Chi²-tests with p values sequentially adjusted.
Survival of adults was analyzed separately for leaves and silk using pairwise Gehan’s Wilcoxon tests adjusted for 4 comparisons (fungal treatments within each maize variety and maize variety within each spore treatment).

Cry3Bb1 concentrations in maize roots from the glasshouse were analyzed by 2-way ANOVA with the factors position (4 root sections from tip to base, see Fig. 4) and plant age (2 – 8 weeks). Tukey HSD post-hoc tests were conducted for significant factors.

Results

Effect of Bt maize on infection of D. v. virgifera larvae by M. anisopliae

Larval weight at the beginning of the experiment was similar in all treatments for the factors Bt and fungal treatment (ANOVA, p > 0.3). After 4 days of feeding, weight gain was significantly lower in the Bt treatment than in the control ($F_{1,749} = 54.89, p < 0.0001$). On day 8, i.e., 4 days after treatment with the fungus, larvae weighed more if they had fed on control maize rather than on Bt maize (Fig. 1) and fungus-treated larvae gained less weight than untreated larvae (factor maize $F_{1,575} = 54.17, p < 0.0001$; factor fungus $F_{2, 575} = 4.69, p = 0.01$).

Figure 1. Weight of D. v. virgifera larvae (mean ± SE) at the start of the experiment (N = 118–133), 4 days after feeding on Cry3Bb1-expressing maize (Event MON88017) or corresponding non-transformed maize (N = 118–133), and 4 days after treatment with spore suspensions of M. anisopliae (8 days on Bt and control maize) (N = 77–115).
Survival of *D. v. virgifera* larvae during the first 4 days of the experiment (before fungal treatment) was 89% when fed on *Bt* maize seedlings and slightly lower (82%) on control maize (*Chi^2* = 7.93; *p* = 0.005). Within 17 days after fungal treatment, survival of larvae in both maize treatments was less with the high spore dose than with the medium dose, and less with the medium dose than with no spores (Gehan’s Wilcoxon tests, adjusted *p* < 0.006 - 0.012) (Fig. 2). For any spore concentration, survival was similar for *Bt* and control maize (*p* > 0.3).

![Proportion of living larvae](image)

**Figure 2.** Survival of *D. v. virgifera* larvae feeding on Cry3Bb1-expressing maize (Event MON88017) or corresponding non-transformed maize seedlings after treatment with spore suspensions of *M. anisopliae*. Survival was analyzed until day 17 after infection, when the first adult beetle emerged (*N* = 118–133).

*Metarhizium anisopliae* grew from 67% and 78% of the dead larvae in the high dose treatments for *Bt* and control maize, respectively, and from 40% and 51% in the medium dose treatments (Table 1). While there was no difference between *Bt* and non-*Bt* maize-fed *D. v. virgifera* larvae (*p* > 0.07), fungal growth was observed more frequently in the high dose than in the low dose treatments (pairwise *Chi^2* tests, adjusted *p* < 0.0125 – 0.017).

Exposure to the fungus decreased the number of emerging beetles (Table 1), and all differences between the three spore doses were significant regardless of the maize treatment (pairwise *Chi^2* tests, adjusted *p* < 0.006 –
0.012). In contrast, there was no difference in adult emergence between \( Bt \) and control maize without fungus (\( p = 0.29 \)), in the medium dose (\( p = 0.06 \)), or in the high dose treatment (\( p = 0.12 \)).

After feeding on \( Bt \) maize seedlings for 4 days, \( D. \ v. \ virgifera \) larvae contained a mean (± SE) of 95 ± 13.4 µg of Cry3Bb per g DW.

**Table 1.** Effects of Cry3Bb1-expressing \( Bt \) maize on infection of \( D. \ v. \ virgifera \) larvae by \( M. \ anisopliae \). Total number of larvae exposed to the fungus, number of dead larvae, observed fungal development in dead larvae, and number of adult beetles emerging are presented. Pairwise Chi\(^2\)-tests were conducted for the number of dead larvae with observed fungal development (4 comparisons) and the number of beetles emerged (9 comparisons). Fungal doses within maize treatments and maize treatments for each fungal dose were compared. Different letters denote significant differences within columns.

<table>
<thead>
<tr>
<th>Maize treatment</th>
<th>Fungal dose (spores/ml)</th>
<th>No. of larvae exposed to fungus</th>
<th>No. of dead larvae</th>
<th>No. of dead larvae showing infection</th>
<th>No. of beetles emerged</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Bt )</td>
<td>0</td>
<td>131</td>
<td>77</td>
<td>-</td>
<td>48 A</td>
</tr>
<tr>
<td></td>
<td>( 5 \times 10^7 )</td>
<td>133</td>
<td>109</td>
<td>44 a</td>
<td>16 B</td>
</tr>
<tr>
<td></td>
<td>( 2.5 \times 10^6 )</td>
<td>127</td>
<td>119</td>
<td>80 b</td>
<td>4 C</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>123</td>
<td>59</td>
<td>-</td>
<td>49 A</td>
</tr>
<tr>
<td></td>
<td>( 5 \times 10^7 )</td>
<td>118</td>
<td>87</td>
<td>44 a</td>
<td>26 B</td>
</tr>
<tr>
<td></td>
<td>( 2.5 \times 10^6 )</td>
<td>123</td>
<td>115</td>
<td>90 b</td>
<td>0 C</td>
</tr>
</tbody>
</table>

**Effect of \( Bt \) maize on infection of \( D. \ v. \ virgifera \) adults by \( M. \ anisopliae \)**

Mortality of the beetles before the fungal treatment was low and ranged from 5.6 to 8.7%. After being dipped into a spore suspension or the Tween 80 control, beetles fed with leaves died relatively quickly in all treatments (Fig. 3A). When adults were fed with silk, mortality without fungal treatment remained low in contrast to beetles treated with the pathogen (Fig. 3B). At the end of the experiment, 46 silk-fed beetles without fungal treatment and 1 beetle treated with the medium spore concentration survived. No beetle survived when fed leaves (Fig. 3A). Differences in the survival curves between fungal-treated and untreated beetles were significant for silk both for control and \( Bt \) maize (Gehan’s Wilcoxon tests, adjusted \( p < 0.0125 \) - 0.0167) and for leaves from control maize (Gehan’s Wilcoxon test adjusted \( p < 0.0125 \)) with a trend also for leaves from \( Bt \) maize (\( p = 0.057 \)). There was no difference between \( Bt \) and control maize treatments (\( p > 0.16 \)). The fungus was able to
develop in 43 and 58% of the dead beetles feeding on Bt and control leaves, respectively. Fungal development was higher in dead adults fed Bt and control silk, at 77 and 82% respectively.

Beetles in the silk treatment surviving until the last day of the feeding assay contained at maximum 27 µg of Cry3Bb1 per g DW with a mean (± SE) of 12 ± 1.9 µg per g DW.

**Figure 3.** Survival of *D. v. virgifera* adults feeding on Cry3Bb1-expressing maize (Event MON88017) or corresponding non-transformed maize (A) leaves or (B) silk after treatment with spore suspensions of *M. anisopliae*. The experiment was terminated 50–66 days after treatment with fungal spores (N = 33–36).
Cry3Bb1 expression in plant material

Cry3Bb1 concentrations in roots from the glasshouse ranged from 141 ± 3.2 to 300 ± 33.0 µg per g DW (Fig. 4) and differed among the root segments (ANOVA, F_{3,64} = 6.821; p = 0.0005). The root tips contained more of the Bt protein than the other segments (Tukey HSD test, p < 0.012), except for roots sampled 2 weeks after sowing as indicated by a significant position × plant age interaction (F_{9,64} = 3.007; p = 0.0047; Fig. 4). There was no significant difference among roots sampled on different dates (p = 0.45). With younger roots containing more water than older roots, averaged Cry3Bb1 concentrations across root segments based on the fresh weight (FW) increased from 11 µg per g (week 2) to 12 (week 4), 17 (week 6), and 19 µg per g (week 8).

Seedlings of Bt maize germinated in the laboratory had a mean (± SE) Cry3Bb1 concentration of 431 ± 26.7 µg per g DW (Fig. 4). ELISA measurements of silk and leaves revealed 92 ± 6.6 µg per g DW and 65 ± 4.6 µg per g DW, respectively.

**Figure 4.** Cry3Bb1 concentration (mean + SE) in Bt maize (Event MON88017) seedlings (N = 25) and roots (N = 5) sampled every 2 weeks in the glasshouse. Roots were divided into 4 sections from tip to base.
Discussion

Effects of Cry3Bb1 ingestion

Second instar *D. v. virgifera* grew slower when feeding on *Bt* maize seedlings than on non-*Bt* maize but showed no difference in mortality. Interestingly, first instars exposed to purified Cry3Bb1 applied to the surface of an artificial diet had increased mortality without significant sublethal effects on growth (Siegfried et al., 2005). In the field, *Bt* maize expressing Cry3Bb1 effectively protects maize plants from feeding damage by *D. v. virgifera* (Vaughn et al., 2005). Even though *Bt* maize reduces the number of emerging adults by ca. 90%, some beetles manage to develop (Al-Deeb & Wilde, 2005; Oyediran et al., 2007; Meihls et al., 2008). Our data suggest that larvae are likely to survive on *Bt* maize roots once they reach the second instar, although they need a longer time for development.

Adult *D. v. virgifera* died quickly when fed with control maize leaves but not when fed with control maize silk. In the *Bt* maize treatments, mortality of the beetles was similar to the control treatments, but beetles died faster when fed *Bt* maize leaves than when fed *Bt* maize silk, even though Cry3Bb1 concentrations in silk were 30% higher than in leaves. This demonstrates that the nutritional quality of the food had a strong impact on the beetles, while the presence of Cry3Bb1 did not affect them. Overall, our results confirm the previous finding that maize leaves are of low nutritional quality for *Diabrotica* spp. (Lance & Fisher, 1987).

Exposure to Cry3Bb1

A field survey revealed that first instars of *D. v. virgifera* feed on maize roots from mid-May and that third instars are present until mid-July (Toepfer & Kuhlmann, 2006). To estimate the concentration of Cry3Bb1 to which *D. v. virgifera* larvae are exposed during their development, ELISA measurements of roots from maize grown in the glasshouse were conducted over a period of 8 weeks. Working in the glasshouse allowed variation due to environmental conditions to be minimized and thus increased the probability of detecting differences in Cry3Bb1 concentrations with time and among the root sections. ELISA measurements indicated relatively constant Cry3Bb1 concentrations in roots (based on dry weights) over time and higher values at the root tip. Concentrations in maize seedlings, which were fed to the *D. v. virgifera* larvae in our experiment, were almost twice as high as in the roots harvested from the glasshouse. Apparently, young tissue (root tip and seedling) contained higher concentrations of the *Bt* protein than older root tissue.

Adult *D. v. virgifera* feed mainly on silk and pollen but can also consume leaves and immature kernels in the field (Ludwig & Hill, 1975; Moeser & Hibbard, 2005). We selected silk and leaves for the present study because they contain ca. 15 times higher concentrations of Cry3Bb1 than, e.g., pollen (Monsanto, 2004; Li et al., 2008).
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The Cry3Bb1 concentrations measured in plant material from the glasshouse were similar to the concentrations measured in experimental field plots with the same maize variety in Germany (H. Nguyen Thu & J. Jehle, personal communication). Thus in the present study, *D. v. virgifera* larvae and adults were most likely exposed to realistic or even elevated doses of Cry3Bb1 compared to the field.

Ingestion of Cry3Bb1 by *D. v. virgifera* larvae feeding for 4 days on *Bt* maize seedlings and of adults surviving the experiment in the *Bt* maize silk treatment was confirmed by ELISA. Larvae and adults were found to contain lower amounts of Cry3Bb1 when compared to their respective food, i.e., 22% and 13%, respectively. Lower concentrations of Cry3Bb1 in the herbivore than in the food source are typical for most arthropods because the *Bt* protein-containing food is located in the gut and represents only a part of the insect’s body weight, and excretion and digestion by gut enzymes further decrease *Bt* protein concentrations.

**Effects of fungal treatment**

In contrast to the *Bt* protein, the fungus *M. anisopliae* was able to kill second instar larvae and adults of *D. v. virgifera*. Although 18 – 60% of dead larval and adult bodies lacked signs of fungal development, those larvae and adults might still have died because of the fungus, but the fungus may have failed to develop further under the given conditions (e.g., because the body cavity was damaged or because the fungus was unable to compete with other microorganisms in the dead insect). Similar findings were reported by Pilz et al. (2007) and Lawo et al. (2008). Death may also have been caused, however, by other pathogens or nutritional deficiencies, which is evident from the mortality in the control treatments without fungal exposure. A lower percentage of dead adults showed fungal development in the maize leaf treatments than in the silk treatments. This suggests that on maize leaves many beetles died before the fungus was able to develop because of the poor nutritional quality of the food.

Using laboratory bioassays similar to those in the present study, Pilz et al. (2007) demonstrated that several strains of *M. anisopliae* isolated from a range of host species or from soil samples differed in virulence to *D. v. virgifera*. Bipesco 5, the strain used in the present study, was among the most virulent strains, being able to infect more than 40% of *D. v. virgifera* larvae and more than 60% of adults within a period of 14 days. Interestingly, in our study beetles that died up to 49 days after fungal treatment showed clear symptoms of growing fungal mycelium and sporulation on the surface of their bodies. Apparently, the fungus was able to persist in the bodies of these beetles for a relatively long time. Pilz et al. (2007) reported a LT$_{50}$ (time needed for 50% mortality) of 12 days when *D. v. virgifera* adults collected in the field in Hungary were treated with $1 \times 10^7$ Bipesco 5 spores. In contrast, the newly emerged beetles from the laboratory colony used in the present study seemed
to be more resistant to the fungus because mortality of beetles that were fed with silk remained below 50% until day 20 after treatment with a five times higher spore concentration.

While natural infections of *D. v. virgifera* by *Beauveria* spp. and *M. anisopliae* in Europe and the USA seem to be rare (Bruck & Lewis, 2001; Pilz et al., 2008), the application of spores can reduce the number of *Diabrotica* spp. larvae and adults not only in the laboratory, as shown in the present study and by Mulock & Chandler (2001) and Pilz et al. (2008), but also in the field (Krueger & Roberts, 1997; Mulock & Chandler, 2000, Bruck & Lewis, 2002; Pilz, 2008). This demonstrates the potential of entomopathogenic fungi for biological control of *Diabrotica* spp.

**Interaction of Bt maize and *M. anisopliae***

*Diabrotica v. virgifera* larvae and adults responded similarly to the fungus, whether fed on *Bt* maize or non-*Bt* maize. This indicates that the effects of *Bt* maize and *M. anisopliae* on *D. v. virgifera* were additive rather than synergistic or antagonistic in our laboratory assays. The interaction between plant-expressed, genetically engineered Cry2Aa protein, herbivorous *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and *M. anisopliae* was studied by Lawo et al. (2008). Larvae of a Cry2Aa-susceptible *H. armigera* strain fed on chickpea leaves containing the *Bt* protein were more sensitive to the fungus than those fed on control leaves, while sensitivity of larvae from a strain resistant to Cry2Aa was similar on both kinds of leaves. Unlike in our study, this indicates that sublethal damage to *H. armigera* caused by the *Bt* protein enhanced the effectiveness of *M. anisopliae*. Multitrophic relationships may also be influenced by insect movement and behavior. Lawo et al. (2008) found no differences in the feeding behavior of the lepidopteran larvae on *Bt* or control plants, suggesting that the larvae had similar exposure to fungal spores, which were applied to the leaf surface before being provided to the larvae. However, depending on the insect species and the *Bt* protein concentrations, feeding on *Bt* plants may result in reduced movement due to sublethal damage or, in contrast, in higher activity if the larvae search for better food (Romeis et al., 2009). Both might influence exposure to entomopathogens in the field and result in interactions not observed in our simplified laboratory assays. For example, Johnson et al. (1997a) reported that Cry1Ab-resistant *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) larvae were less susceptible to the entomopathogenic fungus *Nomuraea rileyi* (Farl.) Samson than susceptible larvae when feeding on Cry1Ab-expressing tobacco. The increased movement that was observed for susceptible larvae may have increased the probability of encountering lethal doses of fungal conidia. These effects were most likely responsible for the faster adaptation of *H. virescens* larvae to *Bt* tobacco when conidia of *N. rileyi* were present (Johnsson et al., 1997b). Understanding these interactions is important because pests developing resistance to *Bt* crops are a major threat for the continuous use of *Bt* technology.
Conclusions

The present study demonstrated that feeding on Cry3Bb1-expressing maize delayed larval development in *D. v. virgifera* but did not affect mortality of second instars or adults. When larvae and beetles were exposed to *M. anisopliae* in addition to being fed on *Bt* maize, no interaction effects were evident. Our study indicates that *Bt* maize can be combined with biological control by entomopathogenic fungi.

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Chapter 5

Literature review: Effects of Cry3Bb1-expressing maize on non-target invertebrates


Abstract

In this chapter, published literature on non-target effects of corn rootworm-resistant, Cry3Bb1-expressing Bt maize is reviewed. Most laboratory or glasshouse studies demonstrated no adverse effects of Bt maize or purified Cry3Bb1 on non-target invertebrates. Field studies confirmed that Bt maize has no detrimental impact on non-target species. In contrast, chemical insecticides were found to affect non-target invertebrates when applied to plant foliage and occasionally also when used as soil insecticides. Seed coating did not influence invertebrate species. The published literature indicates that Cry3Bb1-expressing Bt maize is compatible with beneficial soil species, pollinators and biological control agents.
Introduction

Genetically engineered (GE) maize expressing the cry3Bb1-gene derived from Bacillus thuringiensis (Bt) has been commercialized in 2003 (Vaughn et al. 2005). The Bt protein is produced constitutively in the maize plant and prevents root damage by corn rootworm larvae (Diabrotica spp., Coleoptera: Chrysomelidae), which cause severe problems in North America and Europe (Hellmich et al. 2008). While Cry3Bb1-producing maize has been the first GE crop targeting corn rootworms, other Bt crops, like cotton and maize varieties producing Lepidoptera-active Cry1 or Cry2 proteins, entered commercial production already in 1996 (James, 2007). One focus of the research on the environmental impact of Bt crops has been on potential effects of the produced insecticidal protein on non-target species. Species that provide important ecosystem services, like biological control, pollination and degradation have received most attention. More than one decade after the first Bt crops have been released to the environment, a large number of studies on non-target species is available.

Romeis et al. (2006) have reviewed published studies investigating effects of Bt crops on above-ground arthropod natural enemies. In laboratory and glasshouse studies, no direct toxic effects were present, but indirect effects occurred when natural enemies fed on susceptible, sublethally damaged herbivores. This was confirmed in field studies showing that natural enemy abundance and biological control function was comparable in Bt and non-Bt crops. In contrast, fields treated with conventional insecticides often showed reduced activity and abundance of biological control agents. For below-ground species, Icoz & Stotzky (2008) reported that in general no or little effects of Bt crops were evident on soil invertebrates including arthropods, earthworms, nematodes and protozoa. Also microbial communities did not seem to be affected by the presence of Cry proteins. Duan et al. (2008a) conducted a meta-analysis on effects of Bt Cry proteins on honey bees and Malone & Burgess (2009) reviewed the literature on GE crops and pollinators. The results indicated no negative effects of Bt crops in laboratory settings.

In most studies compiled in the above listed review articles, lepidopteran-active Cry1 or Cry2 proteins were used. In the recent years, however, a number of publications on effects of corn rootworm-resistant, Cry3Bb1-expressing maize on non-target invertebrates became available. In this chapter, data from peer-reviewed publications and registration documents of Cry3Bb1-expressing maize are presented. Non-target studies published until November 2008 were included in this review.

Laboratory and Glasshouse Studies

Data from the laboratory or glasshouse are available for 7 herbivore and 15 predator species, but only for 1 parasitoid, 1 pollinator, 3 decomposer and 2 aquatic species (Table 1).
Herbivores like larvae of the Monarch butterfly, *Danaus plexippus* (L.) (Lepidoptera: Nymphalidaeae), the ladybird beetle, *Epalachna vigintioctopunctata* (F.) (Coleoptera: Coccinellidae) and the leaf beetle *Galerucella vittaticollis* Baly (Coleoptera: Chrysomelidae) did not suffer when feeding on *Bt* maize pollen provided on leaf discs (EPA 2003; Mattila et al. 2005; Shirai 2006). This is interesting, as the two latter species belong to same insect order and one of them even to the same family as the targets of Cry3Bb1 expressing maize, i.e. corn rootworms. *Bt* maize roots were more attractive to the bulb mite *Rhizoglypus robini* Claparede (Astigmata: Acaridae) than control maize roots in 1-day choice experiments (Carter et al. 2004). However, *Bt* maize seeds were coated with different fungicides than control seeds, which might have influenced the mites. In addition, no-choice experiments indicated similar acceptance by *R. robini* (Carter et al. 2004). Weight of two slug species feeding on *Bt* maize leaves remained similar compared to feeding on leaves of the corresponding near-isoline, even though the experiment lasted only 3 days (Zurbrügg & Nentwig in press). Experiments with the aphid *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) revealed a reduction in body weight when developing on *Bt* maize (Lundgren & Wiedenmann 2005). However, phloem sap, the food aphids feed on, has been shown to transport no or only traces of *Bt* protein, thus aphids were not exposed and effects most likely due to different plant properties of the maize lines compared.

Work on predators focused clearly on coccinellid and carabid beetles, as they are among the most important predators in maize fields and beetles are the target group of rootworm-resistant *Bt* maize. The ladybirds *Coleomegilla maculata* (DeGeer) and *Hippodamia convergens* Gue.-Men. (Coleoptera: Coccinellidae) did not show susceptibility to Cry3Bb1 in a range of feeding assays with purified Cry3Bb1 and maize pollen (Ahmad et al. 2006a; Duan et al. 2002; EPA 2003; Lundgren & Wiedenmann 2002). The only tritrophic study published used *C. maculata* and aphids as prey (Lundgren & Wiedenmann 2005). No effects were observed, but the beetles did not ingest Cry3Bb1 as it was not detectable in the aphids. On the other hand, first instar *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) showed 33% mortality when provided with lepidopteran eggs that were treated with Cry3Bb solution at a concentration of 25 µg/ml (Schmidt et al. in press). However, the study likely suffered from methodological problems indicated by the following points: (1) the control mortality in the first larval stage was high (21%); (2) a higher concentration of 50 µg Cry3Bb/ml did not result in significant differences to the control group; (3) developmental time of surviving larvae was not affected; (4) later larval stages had a low control mortality and did not show effects at any Cry3Bb concentration; and (5) when Cry1Ab (known to be specific for Lepidoptera) was fed to the beetles, reported effects on first instar mortality were even stronger than for the Coleoptera-specific Cry3Bb. Studies with carabid beetles provided with pollen or purified Cry3Bb1 revealed no
detrimental effects (Ahmad et al. 2006a; Duan et al. 2006; Mullin et al. 2005). Similarly, no impact of Cry3Bb1 on the non-beetle predators Orius insidiosus (Say) (Hemiptera: Anthocoridae) and Chrysoperla carnea (Stephens) (Neuroptera: Chrysopidae) was observed (Duan et al. 2008b; EPA 2003; Li et al. 2008).

Data on one parasitoid, Nasonia vitripennis (Walker) (Hymenoptera: Braconidae) and one pollinator, the honey bee Apis mellifera L. (Hymenoptera: Apidae) are available from regulatory risk assessment and did not reveal any effect of purified toxin at high concentrations. The community of decomposers was addressed in studies with earthworms and the collembolan Folsomia candida Willem (Collembola: Isotomidae). No effects were evident at Cry3Bb1 levels exceeding maximal environmental concentrations (Ahmad et al. 2006b; EPA 2003). The aquatic midge Chironomus dilutus Shobanov, Kiknadze, & Butler (Diptera: Chironomidae) showed increasing mortality when provided food amended with increasing amounts of Bt maize root extracts (Prihoda & Coats 2008). However, it remains unclear if effects were due to the presence of Cry3Bb1 or other compounds in the root extracts, as no control treatments with increasing concentrations of non-Bt maize root extracts were included. Another aquatic species, Daphnia magna Straus (Diplostraca: Daphniidae), did not show effects when exposed to high amounts of Bt maize pollen mixed into water (EPA 2003).

Field Studies

The invertebrate fauna in the plant layer, on the ground and in the soil has been investigated in field trials with Cry3Bb1-expressing Bt maize (Table 2). Besides the untreated, non-transformed near isolate, most studies included alternative pest control methods such as seed coating, soil insecticides or foliar insecticides.

The abundance of plant dwelling arthropods including herbivores, predators and parasitoids, measured by visual counts, sticky traps and sweep netting, was generally reported to be similar in plots of the Bt and untreated control maize line (Ahmad et al. 2006a; Al-Deeb & Wilde 2003; Bhatti et al. 2005a; McManus et al. 2005; Rauschen et al. in press). Bhatti et al. (2005a) included D. v. virgifera beetles and another chrysomelid species in their analysis and showed, as expected, reduced numbers in Bt maize. Only one study has directly assessed the biological control function and reported no differences in predation of corn borer egg masses between Bt and control maize (Ahmad et al. 2006a). Seed treatments and soil insecticides had generally no influence on plant dwelling arthropods, in contrast to foliar application of permethrin, which reduced coccinellids, lacewings and nabids and resulted in increased aphid populations (Ahmad et al. 2006a; Al-Deeb & Wilde 2003; Bhatti et al. 2005a; McManus et al. 2005).
Species living on the ground were captured using pitfall traps. Predators, herbivores and detritivores in Bt maize showed similar activity densities when compared to the corresponding non-transformed maize plants (Ahmad et al. 2005; Al-Deeb & Wilde 2003; Bhatti et al. 2005b; Bitzer et al. 2005). While seed treatment did not influence ground dwelling invertebrates, the use of tefluthrin as a soil insecticide resulted in reduced numbers of carabids and spiders in one of 3 studies (Bhatti et al. 2005b) and increased abundance of surface active Collembola (Bitzer et al. 2005). Spiders were furthermore affected by foliar application of permethrin (Bhatti et al. 2005b).

Invertebrates living in the soil were studied by a range of methods like Tullgren, MacFadyen and pan trap extraction, bulb mite traps and centrifugal flotation. No detrimental effects of Bt maize on predators, herbivores and detritivores were reported (Ahmad et al. 2005; Al-Deeb et al. 2003; Bhatti et al. 2005b; Bitzer et al. 2005; Carter et al. 2004; Hönemann et al. 2008). Only the western corn rootworm was less abundant in soil samples from Bt maize (Bhatti et al. 2005b). Similar to above ground species, seed coating did not seem to influence invertebrate abundance, while one of 5 studies found soil insecticide to reduce herbivorous Nitidulidae (Coleoptera) and predatory Carabidae and Japygidae (Diplura) (Bhatti et al. 2005b). Furthermore, soil-dwelling Collembola were counted more frequently in soil treated with tefluthrin (Bitzer et al. 2005).

Conclusions

In conclusion, most studies conducted in the laboratory or glasshouse demonstrate that Cry3Bb1-expressing Bt maize is specific to Chrysomelidae. No detrimental effects on non-target invertebrates including Coleoptera were reported with the exception of 2 studies on the ladybird beetle A. bipunctata and the midge C. dilutus. However, both studies suffered from serious methodological problems that shed some doubts whether the reported effects were caused by the ingestion of Cry3Bb1. In the field no detrimental impact of Bt maize on non-target species was reported. In contrast, chemical insecticides used to control corn rootworms were found to affect invertebrates when applied to plant foliage and occasionally when used as soil insecticides.

The results of the present review on Cry3Bb1-expressing Bt maize are in accordance to studies including other Bt crops, mainly maize and cotton expressing lepidopteran-active Cry proteins, which showed absence of detrimental effects on above-ground predators and parasitoids (Romeis et al. 2006), bees (Duan et al. 2008a; Malone & Burgess 2008) and below-ground species (Icoz & Stotzky 2008). By preserving non-target arthropods providing ecological services, like biological control, pollination or decomposition, rootworm-resistant Bt maize can contribute to sustainable agriculture.
Table 1. Effects of Cry3Bb1-expressing maize or purified Cry3Bb1 on non-target invertebrates under confined conditions. Unless otherwise stated, *Bt* maize was compared to the corresponding non-transformed near-isoline and diet containing *Bt* protein to pure diet.

<table>
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<tr>
<th>Invertebrate species</th>
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<td>Lepidoptera: Nymphalidae</td>
<td>Larvae</td>
<td>MON863</td>
<td>Milkweed leaves dusted with up to 3200 pollen grains/cm²</td>
<td>No effect on development time, survival, weight and feeding after 4 days exposure</td>
<td>EPA (2003), Mattila <em>et al.</em> (2005)</td>
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<td>Larvae</td>
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<td>Black nightshade leaf discs dusted with up to 2000 pollen grains/cm²</td>
<td>No effect on survival and development after 10 days</td>
<td>Shirai (2006)</td>
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<tr>
<td>Coleoptera: Coccinellidae</td>
<td>Larvae</td>
<td>MON863</td>
<td>Bitter dock leaf discs dusted with up to 2000 pollen grains/cm²</td>
<td>No effect on survival and development after 10 days</td>
<td>Shirai (2006)</td>
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<td><em>Epilachna vigintioctopunctata</em> (F.)</td>
<td>Larvae</td>
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<td>Leaves</td>
<td>Decreased weight on <em>Bt</em> maize</td>
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<td>Leaves</td>
<td>No effect on weight and feeding after 3 days</td>
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<td>Adults</td>
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<td>Leaves</td>
<td>No effect on weight and feeding after 3 days</td>
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<td><strong>Predators</strong></td>
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<td>Coleoptera: Coccinellidae</td>
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<td>Purified</td>
<td>Lepidopteran eggs sprayed with 0, 5, 25 and 50 µg Cry3Bb1/ml solution</td>
<td>Decreased survival of 1st instar at 25µg/ml, no effect at other (including higher) doses and on later instars, development time and weight</td>
<td>Schmidt <em>et al.</em> <em>(in press)</em></td>
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<td><em>Adalia bipunctata</em> (L.)</td>
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<td>MON863</td>
<td>Pollen (50%) mixed with fruit fly eggs and bee pollen</td>
<td>No effect on development time, survival and weight</td>
<td>EPA (2003)</td>
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<td><em>Coleomegilla maculata</em> (DeGeer)</td>
<td>Larvae</td>
<td>MON863</td>
<td>Pollen and 1 aphid in 3rd and 4th instar</td>
<td>No effect on development time, survival and weight, no consequences for adult fecundity and fitness (flip time, walking speed)</td>
<td>Ahmad <em>et al.</em> (2006a)</td>
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<td>Pollen and 3 aphids in 2nd, 3rd and 4th instar</td>
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<td>Duan <em>et al.</em> (2002)</td>
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<td></td>
<td></td>
<td></td>
<td>Pollen (50%) mixed with fruit fly eggs</td>
<td>No effect on development time, survival and weight</td>
<td></td>
</tr>
<tr>
<td>Taxonomy</td>
<td>Species</td>
<td>Life stage</td>
<td>Test Material</td>
<td>Effect</td>
<td>Reference(s)</td>
</tr>
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<tr>
<td><strong>Aphids</strong></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Aphids reared on Bt maize (no exposure as aphids did not contain Cry3Bb1)</td>
<td>No effect on development time, survival, weight, fitness (flip time, walking speed) and fecundity</td>
<td>Lundgren &amp; Wiedenmann (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pollen (50%) mixed with fruit fly eggs</td>
<td>No effect on survival and fecundity during 30 days</td>
<td>Duan et al. (2002), EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400 and 8000 µ Cry3Bb1/ml honey diet</td>
<td>No effect on survival after 10 days</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pollen (50%) in honey</td>
<td>No effect on survival after 15 days</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td><strong>Coleoptera: Carabidae</strong></td>
<td></td>
<td>Adults</td>
<td>MON863 Pollen (50%) mixed with fruit fly eggs</td>
<td>No effect on longevity</td>
<td>Ahmad et al. (2006a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>MON863 Pollen (50%) in honey</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
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<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heteroptera: Anthocoridae</strong></td>
<td></td>
<td>Nymphs</td>
<td>Purified</td>
<td>No effect on development time and survival</td>
<td>Duan et al. (2008b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Larvae</td>
<td>Purified</td>
<td>No effect on survival after 10 days</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival, pre-oviposition period, fecundity, fertility and weight during 28 days</td>
<td>Li et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival, pre-oviposition period, fecundity, fertility and weight during 28 days</td>
<td>Li et al. (2008)</td>
</tr>
<tr>
<td><strong>Hymenoptera: Braconidae</strong></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival at 400 µg/ml, 45% decreased survival at 8000 µg/ml (not significant)</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Larvae</td>
<td>Purified</td>
<td>No effect on development and survival</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td><strong>Hymenoptera: Apidae</strong></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival and behaviour</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Larvae</td>
<td>Purified</td>
<td>No effect on survival and behaviour</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td><strong>Annelida: Clitellata</strong></td>
<td></td>
<td>Adults</td>
<td>MON863</td>
<td>No effect on survival and weight after 12-45 days</td>
<td>Ahmad et al. (2006b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival and weight after 14 days</td>
<td>EPA (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Purified</td>
<td>No effect on survival and weight after 14 days</td>
<td>EPA (2003)</td>
</tr>
</tbody>
</table>

**Notes:**
- **Bt** maize refers to maize plants genetically modified to produce the Cry3Bb1 toxin.
- The effects noted are based on survival, development time, weight, and reproductive success.
- The references cited are studies that have evaluated the effects of Bt maize on various insect species.
### Collembola: Isotomidae

**Folsomia candida** Willem

- Adults MON863
- 0.5, 5 and 50% leaf tissue in granulated brewer’s yeast

| Transformation event of **Bt** maize; “purified” denotes that **Bt** protein produced in microbes was used

### Aquatic species

**Diptera: Chironomidae**

**Chironomus dilutus** Shobanov, Kiknadze & Butler

- Larvae MON863
- **Bt** maize root extracts in artificial diet at 0, 17, 30 and 48 ng Cry3Bb1/ml

<table>
<thead>
<tr>
<th>Reported effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased survival with higher amounts of added <strong>Bt</strong> maize root extract (no comparison with control root extracts), no effect on growth</td>
</tr>
</tbody>
</table>

**Prihoda & Coats (2008)**

**Diplostraca: Daphniidae**

**Daphnia magna** Straus

- Larvae MON858
- 120 mg pollen/l water

<table>
<thead>
<tr>
<th>Reported effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect on mortality and behaviour after 2 days</td>
</tr>
</tbody>
</table>

**EPA (2003)**

---

### Table 2. Effects of Cry3Bb1-expressing maize and insecticide applications on non-target invertebrates under field conditions. Unless otherwise stated **Bt** maize and insecticide treatments are compared with untreated near-isolines.

<table>
<thead>
<tr>
<th>Transform. event</th>
<th>Sampling method</th>
<th>Sampled taxa</th>
<th>Reported effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MON863</td>
<td>Visual counts; predation of moth eggs</td>
<td>Predators (Het: Anthocoridae, <em>Orius insidiosus</em>; Col: Coccinellidae, <em>Coleomegilla maculata</em>, <em>Hippodamia convergens</em>, Scymnus spp.); activity of chewing &amp; sucking predators</td>
<td>No effect of <strong>Bt</strong> maize, seed coat (clothianidin) or soil insecticide (tefluthrin, bifenthrin, pyrethroid) on abundance and egg predation</td>
<td>Ahmad et al. (2006a)</td>
</tr>
<tr>
<td>MON863</td>
<td>Sticky traps</td>
<td>Predator (Col: Coccinellidae, <em>C. maculata</em>)</td>
<td>No effect or slightly increased abundance in <strong>Bt</strong> maize compared to near-isoline (both with seed coat imidacloprid), no effect of soil insecticide (tefluthrin, pyrethroid)</td>
<td>McManus et al. (2005)</td>
</tr>
<tr>
<td>MON862</td>
<td>Visual counts</td>
<td>Herbivore (Hem: Miridae, <em>Trigonotylus caelestialium</em>)</td>
<td>No effect of <strong>Bt</strong> maize, but differences among conventional varieties</td>
<td>Rauschen et al. (in press)</td>
</tr>
<tr>
<td>MON863</td>
<td>Sweep netting</td>
<td>Herbivores (Col: Chrysomelidae; Hem: Aphididae), Predators (Col: Coccinellidae; Hem: Anthocoridae; Nabidae; Neu: Chrysopidae; Dip: Syrphidae; Araneae), Parasitoids (Hym: Braconidae)</td>
<td>Decreased abundance of Chrysomelidae (target group) in <strong>Bt</strong> maize, no effect of <strong>Bt</strong> maize on other taxa; abundance decreased for Coccinellidae, Chrysopidae and Nabidae and increased for aphids after application of foliar insecticide (permethrin), no effect of seed coat (imidacloprid) or soil insecticide (tefluthrin)</td>
<td>Bhatti et al. (2005a)</td>
</tr>
<tr>
<td>Ground-dwelling arthropods</td>
<td>Soil fauna</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>---------------------------</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MON863</strong> Pitfall traps</td>
<td>Detritivores (Acari, Collembola)</td>
<td>No effect of Bt maize, seed coat (clothianidin, neonicotinoid) or soil insecticide (tefluthrin, pyrethroid)</td>
<td>Ahmad et al. (2005)</td>
<td></td>
</tr>
<tr>
<td><strong>MON853</strong> Pitfall traps</td>
<td>Detritivores (Acari, Collembola)</td>
<td>Decreased abundance of Chrysomelidae (taget group) in Bt maize, no effect of Bt maize on other taxa; decreased abundance for Carabidae, Nitidulidae and Japygidae after application of soil insecticide (tefluthrin), no effect of seed coat (imidacloprid)</td>
<td>Bhatti et al. (2005b)</td>
<td></td>
</tr>
<tr>
<td><strong>MON862</strong> Orthoptera: Gryllidae, Predators (Col: Carabidae, Staphylinidae; Araneae), Detritivores (Hym: Formicidae; Collembola)</td>
<td>Nematodes (<em>Helicotylenchus, Pratylenchus, Tylenchorhyncus, Hoplolaimus</em>)</td>
<td>No effect of Bt maize in litter bags, no differences among conventional varieties</td>
<td>Höinemann et al (2008)</td>
<td></td>
</tr>
<tr>
<td><strong>MON863</strong> Pitfall traps</td>
<td>Detritivores (Acari, Collembola, Acaridae, Rhizoglyphus robini)</td>
<td>No effect of seed coat (imidacloprid) or soil insecticide (tefluthrin), no effect of Bt maize with seed coat</td>
<td>Carter et al. (2004)</td>
<td></td>
</tr>
<tr>
<td><strong>MON863</strong> Tullgren extraction</td>
<td>Detritivores (Collembola)</td>
<td>No effect of Bt maize or seed coat (imidacloprid), increased abundance after application of soil insecticide (tefluthrin)</td>
<td>Bitzer et al. (2005)</td>
<td></td>
</tr>
</tbody>
</table>

1 Insect orders are abbreviated as Col (Coleoptera), Hem (Hemiptera), Het (Heteroptera), Hym (Hymenoptera), Neu (Neuroptera)
Chapter 5 – Literature review

References


Chapter 5 – Literature review


Shirai, Y. 2006. Laboratory evaluation of effects of transgenic Bt corn pollen on two non-target herbivorous beetles, Epilachna vigintioctopunctata (Coccinellidae) and Galerucella vittaticollis (Chrysomelidae). Applied Entomology and Zoology 41, 607-611.
Chapter 5 – Literature review


Chapter 6

Synthesis: Compatibility of biological control with *Bt* maize expressing Cry3Bb1

Cry3Bb1 contained in *Bt* maize tissue and herbivores remains biologically active

The insecticidal activity of the coleopteran-active Cry3Bb1 expressed in different tissues of *Bt* maize, contained in maize herbivores, and in spiked soil was measured in sensitive insect bioassays using larvae of the Colorado potato beetle (Chapter 2). Concentration-response assays showed that the *Bt* protein was biologically active in pulverized maize pollen, roots, leaves, and silk. Biological activity was furthermore demonstrated for Cry3Bb1 contained in adult western corn rootworms and spider mites that had consumed *Bt* maize and for Cry3Bb1 adsorbed to soil. Bioassays with the same concentration of Cry3Bb1 per ml diet (as measured by ELISA), suggested that nutritional quality of food and degradation of *Bt* protein may influence Cry protein toxicity to insects.

Negligible risk for the spider *Theridion impressum*

Analysis of the prey spectrum of *Theridion impressum* and the Cry3Bb1 concentration in a range of potential prey species revealed that the spider is exposed to the toxin in *Bt* maize fields, even though exposure is highly variable (Chapter 3). When Cry3Bb1-containing prey or *Bt* maize pollen was fed to juvenile and adult spiders in the laboratory for almost 2 months, no detrimental effects on mortality, offspring production, or weight were observed. Those results suggest that corn rootworm-resistant *Bt* maize poses a negligible risk for the spider.

*Bt* maize is compatible with the entomopathogenic fungus *Metarhizium anisopliae*

Second instar *D. v. virgifera* feeding on Cry3Bb1-expressing *Bt* maize in the laboratory developed slower, but no effects on mortality were evident (Chapter 4). Adults were not influenced by Cry3Bb1 in their food (maize leaves or silk). Infection rates with the entomopathogenic fungus *Metarhizium anisopliae* were depending on the concentration of spore suspensions into which rootworms were dipped. Adults were more susceptible to the fungus than larvae. No differences in susceptibility to the fungus could be observed between *Bt* and control maize-fed insects. This indicates that corn rootworm-resistant *Bt* maize is compatible with biological control by this entomopathogenic fungus.
No evidence of negative effects on non-target invertebrates from the literature

The published literature on the impact of Cry3Bb1-expressing *Bt* maize on non-target invertebrates provides no evidence of negative effects (Chapter 5). Laboratory and glasshouse studies with *Bt* maize or purified Cry3Bb1 protein as well as field studies indicate that Cry3Bb1-expressing *Bt* maize is not harmful to invertebrates providing ecological services including decomposition, pollination and biological control.

Discussion

Natural enemies are commonly fed with purified *Bt* protein, plant material or herbivorous prey for non-target risk assessment. To conclude from those studies that the *Bt* protein does not cause direct toxic effects, one has to know if the protein was biologically active in the food that was provided to the species of concern. Biological activity of Cry3Bb1 in *Bt* maize tissue (including pollen) and arthropods feeding on *Bt* maize (spider mites and western corn rootworm adults) was demonstrated in Chapter 2. Consequently, *T. impressum* spiders received prey which contained biologically active Cry3Bb1 in the laboratory feeding studies presented in Chapter 3. The lack of effects on the spiders therefore shows that they were not susceptible to the provided amount of Cry3Bb1. Furthermore, the lack of effects on adult western corn rootworms when feeding on *Bt* maize compared to control maize in Chapter 4 indicates that they were not susceptible to Cry3Bb1 in silk and leaves, even though Chapter 2 indicates that they ingested and contained biologically active Cry3Bb1 when feeding on *Bt* maize. The ingestion of active *Bt* protein did not result in an interaction with the fungus.

Biological activity of Cry3Bb1 has been shown previously in 2 non-target studies, where Colorado potato beetles were fed with diet containing purified Cry3Bb1 (Duan et al. 2006; 2008a). However, biological activity was not confirmed in studies using *Bt* maize tissue (including pollen) as food for the tested invertebrate species (see Chapter 5).

For non-target risk assessment of Cry3Bb1-expressing *Bt* maize, a number of invertebrates including herbivores, predators, parasitoids, pollinators, decomposers and aquatic species have previously been studied. Predators received most attention as 10 studies were conducted on 14 species (Chapter 5). In most of those studies, *Bt* maize tissue or diet containing purified Cry3Bb1 was provided. This includes predator species which were fed with *Bt* maize pollen and/ or artificial diet. Only one study used a tritrophic system, in which neither prey (aphids) nor predators (*Coleomegilla maculata* ladybird beetles) ingested the *Bt* protein (Lundgren & Wiedenmann 2005). This lack of tritrophic studies is surprising, because most predators are likely to consume *Bt* protein mainly via their prey. Ingestion of plant tissue by predatory arthropods is common, but often limited to certain periods of time, e.g., pollen
shedding, or to certain conditions, e.g., if no prey is available (Romeis et al. 2009). The present study with *T. impressum* is the first tritrophic study, in which biologically active Cry3Bb1 was transferred to a predator via prey.

Most non-target studies on predators focused on carabid and coccinellid beetles. This can be explained by the fact that *Diabrotica* spp., the target of Cry3Bb1-expressing *Bt* maize, belong to the same insect order (Coleoptera) as carabids and coccinellids. Besides studies on beetles, one study on the predatory bug *Orius insidiosus* (Heteroptera: Anthocoridae) (Duan et al. 2008a) and 2 studies on the lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) (EPA 2003; Li et al. 2008) have been published. Even though spiders are important predators in agricultural landscapes (Sunderland 1999), the study presented in Chapter 3 is the first non-target study with Cry3Bb1-expressing maize on this arthropod group. The lack of artificial diets for spiders and the difficulties to rear spiders in the laboratory may be two reasons. In accordance with most laboratory studies on different invertebrate species, the data on *T. impressum* confirm the lack of effects of Cry3Bb1 on species outside the family of Chrysomelidae. This specificity of Cry3Bb1-expressing *Bt* maize was also demonstrated in several field studies (reviewed in Chapter 5). Similar results for other crops, mainly maize and cotton expressing lepidopteran-active Cry proteins, were reported in recent reviews on above-ground predators and parasitoids (Romeis et al. 2006), bees (Duan et al. 2008b; Malone & Burgess 2009) and below-ground species (Icoz & Stotzky 2008).

In contrast to arthropod biological control agents, data on *Bt* plants and entomopathogens are scarce, although they are ubiquitous and contribute to biological control. The interaction of experimental *Bt* tobacco and chickpea plants with entomopathogenic fungi has been examined for lepidopteran pests (Johnson et al., 1997a,b; Lawo et al., 2008) and work on entomopathogenic nematodes has been conducted on *Bt* cotton and the pink bollworm *Pectinophora gossypiella* (Lepidoptera: Gelechiidae; Gassmann et al. 2006; 2008). The present study on *M. anisopliae* and Cry3Bb1-expressing maize is the first on a beetle-active *Bt* protein and the first on *Bt* maize. The lack of antagonistic effects in this study and in the published literature indicates that *Bt* maize is compatible with biological control by those entomopathogens.

**Conclusions**

The work presented in this thesis together with the published literature on laboratory and field studies revealed that *Bt* maize maintains ecological services including biological control provided by spiders and entomopathogenic fungi. This is very important, because selection experiments in the glasshouse demonstrated that *D. v. virgifera* was able to develop resistance against Cry3Bb1-expressing maize within only 3 generations (Meihls et al. 2008). A healthy community of biological control agents can contribute to resistance management by feeding on corn
rootworms that survive on Bt maize. Furthermore, Bt maize often replaces broad-spectrum soil insecticides, which are known to have adverse effects on non-target species. This shows that corn rootworm-resistant Bt maize can improve current agricultural practice and thus contributes to more sustainable agriculture.

References


Chapter 6 – Synthesis


Acknowledgements

During the last 4 years, I had the opportunity to work together with many people, who made a major contribution to the success of my PhD project. My special thanks go to:

- Jörg Romeis for giving me the opportunity to work on this project, for supervising me, and for being always willing to deal with questions and problems immediately, no matter if the pile on his desk was big or very big.

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