Compatibility of *Bt*-transgenic crops with biological control

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Compatibility of Bt-transgenic crops with biological control

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Contents

Summary

One of the most widely discussed ecological effects of genetically engineered (GE) crops is their impact on non-target organisms including natural enemies that are important for pest regulation and thus of economic value. Prior to commercialization of a GE crop a detailed risk assessment for potential adverse effects on non-target organisms has to be conducted. Finding surrogate species is therefore of major importance. A simplified arthropod food web including the most important herbivores and their natural enemies for Indian pigeonpea was constructed. Using *Bt* pigeonpea, expressing Cry1Ac/Cry2Ab proteins derived from *Bacillus thuringiensis* (*Bt*) as an example, an indication of which organisms would need to be addressed in a regulatory risk assessment are given.

Since *Bt* proteins are active only after ingestion, a dietary bioassay was developed to test direct toxic effects on the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). Larvae were exposed to different insecticidal proteins dissolved in a sucrose solution. Besides other insecticidal proteins, Cry1Ab and Cry1Ac proteins were tested. Neither did caused a negative effect on *C. carnea*, which is consistent with earlier studies.

Further, the direct effect of *Bt* cotton was examined for the non-target herbivore *Aphis gossypii* (Hemiptera: Aphididae). Besides the evaluation of aphid performance on three Indian *Bt* cotton varieties (expressing Cry1Ac) and their non-transformed near-isolines, we investigated weather aphids pick up any *Bt* protein. Further, the sugar composition of aphid honeydew was analyzed to evaluate its suitability for honeydew-feeders. The studies revealed no influence on cotton aphid performance due to *Bt*-expression and only slight variation among varieties which could be explained by the different trichome densities. None of the aphid samples contained *Bt* protein. However, a variation in the nutritional balance of aphid honeydew was detected due to the factors "transformation" and "variety" but it remains unknown whether this shift is of ecological relevance.

As previous studies suggest that *Bt*-fed lepidopteran pests can lower predator performance due to a reduced prey quality, studies were performed to investigate whether prey nutritional composition causes indirect (prey-quality mediated) effects on *C. carnea.* Conducting feeding studies with *Bt* and non-*Bt* fed Cry1Ac-resistant and susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae, confirmed the observation of prey-quality mediated effects when the predator was fed with susceptible *Bt*-fed prey larvae but

not when feeding on *Bt*-fed Cry1Ac-resistant lepidopterans. Biochemical analyses of the glycogen and lipid content as well as the amino acid and sugar content and composition were conducted aiming to detect differences in the nutrients between Cry1Ac-resistant and susceplible food sources. Except for a significant difference in the sugar composition of *Bt*-fed susceptible larvae, no shift could be detected. This was the first attempt to evaluate prey-quality mediated effects. However, other nutrients are important in insect nutrition, and therefore more studies are necessary.

As the *Bt* technology does not provide 100% control against caterpillars, evaluation of the compatibility between *Bt* crops and biological control agents will be important. Therefore, bioassays were conducted to understand the interactions between a Cry2Aa-expressing chickpea line, either susceptible or Cry2A-resistant *H. armigera* larvae, and the entomopathogenic fungus *Metarhizium anisopliae*. Different laboratory studies allowed the conclusion that *M. anisopliae* was compatible in controlling Cry2A-resistant and susceptible *H. armigera* larvae. Further, it appeared that the *Bt* resistance did not cause a fitness cost and that *M. anisopliae* had an enhanced effectiveness on sublethally *Bt*-affected caterpillars.

Summarizing this thesis, it appeared that *Bt* crops are compatible with biological control for the organisms tested.

Résumé

L'un des risques les plus largement débattus en matière de transgenèse réside dans l'impact potentiel des plantes génétiquement modifiées (GM) sur les organismes non ciblés. Les ennemis naturels des organismes nuisibles en font partie, car ils jouent un rôle majeur dans la régulation des ravageurs et ont donc une grande importance sur le plan économique. Avant de commercialiser une plante GM, il est donc nécessaire d'analyser sérieusement les risques d'effets secondaires pouvant toucher les organismes non ciblés et de choisir des espèces représentatives pour cette étude. Dans ce but, un réseau trophique simplifié des principaux ravageurs et de leurs ennemis naturels a été créé pour un champ de pois pigeons indiens (*Cajanus cajan*). En examinant par exemple un pois pigeon *Bt* qui exprime les protéines Cry1Ac et Cry2Ab produites par la bactérie du sol *Bacillus thuringiensis,* nous obtenons des indications sur les organismes qui devraient être considérés dans une analyse des risques.

Etant donné que les protéines *Bt* ne sont actives qu'après leur ingestion, une méthode a été développée pour tester les effets toxiques directs sur la chrysope verte *Chrysoperla carnea* (Neuroptera: Chrysopidae), un insecte utile. Les larves ont été exposées à diverses protéines, dont la Cry1Ab et la Cry1Ac, dissoutes dans une solution de saccharose. Aucune des deux protéines *Bt* n'a eu d'effet négatif sur *C. carnea*, ce qui confirme les résultats d'autres études.

En outre, l'effet direct du coton *Bt* sur l'herbivore non ciblé *Aphis gossypii* (Hemiptera: Aphididae) a été étudié. L'évolution des pucerons a été suivie sur trois variétés de cotons indiens exprimant la Cry1Ac et sur les variétés non transgéniques correspondantes. Nous avons également examiné si les aphidés absorbaient la protéine *Bt* présente dans la plante. Par ailleurs, la composition du sucre contenu dans le miellat des aphidés a été analysée pour savoir s'il convient à l'alimentation des consommateurs de miellat. Notre étude révèle que la *Bt* n'influe pas sur l'évolution des pucerons; seules de légères variations s'observent entre les variétés de coton, ce qui pourrait être dû aux différentes densités des poils glandulaires. Aucun des échantillons de pucerons ne contenait de la *Bt*. Toutefois, une variation a été constatée dans la composition nutritionnelle du miellat; elle est due à la transformation de la *Bt* et dépend de la variété.

Des études antérieures avaient montré que les larves *Bt* perdaient de leur qualité nutritive, ce qui se répercute indirectement sur l'aptitude prédatrice des insectes utiles,

comme les larves de la chrysope verte. Cette hypothèse a été vérifiée dans une étude sur le régime alimentaire de *C. Carnea*. L'insecte utile a été nourri de larves de *Helicoverpa armigera* (Lepidoptera: Noctuidae) sensibles ou résistantes à la Cry1Ac et qui s'étaient alimentées auparavant sur un cotonnier *Bt* ou non transgénique. Un effet indirect a été constaté sur les larves de *C. Carnea* ayant consommé des chenilles sensibles à la *Bt* mais pas sur celles qui s'étaient nourries de chenilles résistantes à la Cry1Ac et qui avaient absorbé de la *Bt* auparavant. Des analyses biochimiques des compositions et des teneurs en glycogène, en lipide, en saccharose et en acide aminé ont été réalisées afin de déceler des différences dans les nutriments entre les sources de nourriture sensibles à la Cry1Ac et les autres résistantes à cette protéine. Hormis une différence significative dans la composition du saccharose chez des larves sensibles à la *Bt,* aucune modification n'a été constatée. Cette première évaluation des effets indirects nécessite des études complémentaires, car beaucoup d'autres substances nutritives ont leur importance dans l'alimentation des insectes.

Etant donné que la technologie *Bt* n'offre pas une protection à 100% contre les lépidoptères nuisibles, il importe d'évaluer l'interaction entre les plantes *Bt* et la protection phytosanitaire biologique. Des études ont donc été réalisées à propos de la compatibilité entre des pois chiches (*Cicer arietinum*) exprimant la Cry2Aa, des larves de *H. armigera* résistantes ou sensibles à la Cry2A et le champignon entomopathogène *Metarhizium anisopliae.* Différents essais en laboratoire nous permettent de conclure que *M. anisopliae,* associé à des pois chiches *Bt,* obtiennent un taux de mortalité élevé parmi les ravageurs sensibles ou résistants à la Cry2A. En outre, il apparaît que la résistance à la *Bt* n'exerce aucun effet sur l'évolution des larves et que *M. anisopliae* a une meilleure efficacité sur des chenilles intoxiquées par la protéine *Bt* sublétale.

En résumé, cette étude montre que les plantes *Bt* sont compatibles avec la protection biologique des organismes testés.

Zusammenfassung

Einer der am häufigsten diskutierten Risiken von gentechnisch veränderten (GV) Kulturpflanzen, ist ihr potentieller Einfluss auf Nichtzielorganismen, einschließlich natürlicher Gegenspieler von Schadorganismen. Letztere spielen eine große Rolle bei der natürlichen Regulierung von Schädlingen und sind daher von großer ökonomischer Bedeutung. Vor der Freisetzung einer GV Kulturpflanze ist es deshalb notwendig eine genaue Risikoanalyse bezüglich möglicher Nebeneffekten auf Nichtzielorganismen durchzuführen. In diesem Zusammenhang ist die Wahl von repräsentativen Arten von Bedeutung. Zu diesem Zwecke wurde ein vereinfachtes Nahrungsnetz der wichtigsten Schädlinge und deren natürlichen Gegenspielern für ein indisches Straucherbsenfeld (*Cajanus cajan*) erstellt und am Beispiel einer *Bt* Straucherbse, welche die Proteine Cry1Ac and Cry2Ab des Bodenbakteriums *Bacillus thuringiensis* exprimiert, wird diskutiert, welche Organismen in einer Risikobewertung berücksichtigt werden sollten.

Da *Bt* Proteine erst durch Verdauung aktiv werden, wurde ein Testverfahren für den Nützling *Chrysoperla carnea* (Neuroptera: Chrysopidae) (Grüne Florfliege) entwickelt in dem direkte toxische Effekte von durch die Nahrung aufgenommenen Proteinen getestet werden können. Den Insektenlarven wurden dazu verschiedene Proteine, einschließlich Cry1Ab and Cry1Ac in einer Zuckerlösung angeboten. Keines der beiden *Bt* Proteine schädigte die Florfliegenlarven. Dieses Ergebnis ist konsistent mit anderen Studien.

In einer weiteren Studie wurde der direkte Effekt von *Bt* Baumwolle auf den Nichtziel-Schädling *Aphis gossypii* (Hemiptera: Aphididae) untersucht. Auf drei verschiedenen indischen Cry1Ac-exprimierenden Baumwollarten und den entsprechenden nicht transgenen Sorten wurde die Entwicklung der Blattläuse untersucht. Ausserdem wurde erfasst, ob Blattläuse das in der Pflanze vorkommende *Bt* Protein aufnehmen. Zusätzlich wurde die Zuckerzusammensetzung des Blattlaushonigtaus auf seine Nahrungseignung für Honigtaukonsumenten evaluiert. Unsere Studien lassen keinerlei Beeinflussung auf die Entwicklung der Blattläuse durch die *Bt* Expression vermuten. Allerdings wurden kleine Unterschiede zwischen den verschiedenen Baumwollarten gefunden, was vermutlich auf einen Unterschied in der Blatthaardichte zwischen den Arten zurückzuführen ist. Keine der untersuchten Blattlausproben enthielt *Bt* Protein. Allerdings konnte eine Variation in der Honigtaunährstoffzusammensetzung aufgrund der *Bt*-Transformation und der Sorte festgestellt werden.

Frühere Studien hatten ergeben, dass Raupen, die zuvor mit *Bt* Protein gefüttert wurden eine reduzierte Nahrungsqualität aufweisen, was negative (indirekte) Auswirkungen auf räuberische Insekten wie Florfliegenlarven hat. Diese Hypothese wurde in Fütterungsstudien mit *C. carnea* Larven überprüft. Dazu wurde in unseren Untersuchungen der Nützling mit anfälligen oder Cry1Ac-resistenten *Helicoverpa armigera* (Lepidoptera: Noctuidae) Raupen gefüttert, die zuvor entweder auf einer konventionellen oder einer *Bt* Baumwollpflanze gefressen hatten. Ein indirekter Effekt konnte bestätigt werden, nachdem die Florfliegenlarven *Bt* gefütterte anfällige Raupen konsumiert hatten. Eine Schädigung blieb jedoch aus, wenn die Larven von *C. carnea* Cry1Ac-resistente Raupen frassen die vorher mit *Bt* gefüttert wurden. Durch biochemische Analysen, die den Lipid- und Glykogengehalt sowie den Zucker- und Aminosäuregehalt und deren Kompositionen untersuchten, sollte eine durch die unterschiedlichen Nahrungsquellen bedingte Variation in den Raupen detektiert werden die für die Effekte auf den Räuber verantwortlich sein könnten. Ausser einem statistisch signifikanten Unterschied in der Zuckerzusammensetzung bei *Bt* gefütterten Raupen konnte allerdings keinerlei Veränderung beobachtet werden. Da dies die ersten Versuche waren um einen indirekten Effekt nachzuweisen und viele weitere Nährstoffe eine wichtige Rolle in der Insektenernährung spielen, sind für eine abschließende Bewertung noch weitere Studien notwendig.

Da die *Bt* Technologie keinen 100%igen Schutz vor Schad-Lepidopteren bietet sind Studien wichtig, die das Zusammenspiel von *Bt* Kulturpflanzen und biologischem Pflanzenschutz untersuchen. Aus diesem Grund wurden Untersuchungen mit dem Ziel durchgeführt die Verträglichkeit zwischen Cry2Aa-exprimierender Kichererbsen (*Cicer arietinum*), Cry2A-resistenten und anfälligen *H. armigera* Raupen sowie dem entomopathogenen Pilz *Metarhizium anisopliae* zu untersuchen. In verschiedenen Laborversuchen erzielte *M. anisopliae* gemeinsam mit *Bt* Kichererbsen eine hohe Mortalitätsrate bei anfälligen und Cry2A-resistenten Schädlingen. Weiterhin zeigten sich keine Anzeichen, dass die *Bt* Resistenz Auswirkungen auf die Entwicklung der Raupen hatte. Allerdings erzielte *M. anisopliae* eine erhöhte Mortalität bei durch das *Bt* Protein sublethal geschädigter *H. armigera* Raupen.

Zusammenfassend ergibt sich, dass eine gute Verträglichkeit von *Bt* Pflanzen mit den untersuchten Organismen des biologischen Pflanzenschutzes gezeigt werden konnte.

Chapter one

General introduction

The PhD thesis was conducted within the "Pulse Network" of the "Indo-Swiss Collaboration in Biotechnology" (ISCB). One objective of the Pulse Network is the development of insect-resistant genetically engineered (GE) pulses to control the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) in India (http://iscb.epfl.ch).

Before describing the different study systems and questions evaluated in this thesis, an introduction to insect-resistant GE plants and their ecological risk assessment is provided, followed by a description of the field situation in India.

GE crops

As a result of consistent and substantial benefits during the first dozen years of the commercialization of GE crops (from 1996 to 2007), farmers have continued to plant more every year. In 2007, 114.3 million hectares of GE crops were grown in twelve developing and eleven industrial countries and this number is constantly increasing. The countries with the largest GE crop area are the USA, Argentina, Brazil, Canada and India (James 2007). Todays GE crops are altered for agronomic traits, such as herbicide-tolerance, insect- or virus-resistance. Thus far, herbicide-tolerance has consistently been the dominant trait. In 2007, it was deployed in soybean, maize, canola, cotton and alfalfa and occupied 63% of the global GE crop area. In addition, plants are under development altered for a higher resistance to abiotic stresses such as salt, drought, and water (Wang et al. 2003; Yamaguchi and Blumwald 2005). Further traits of interest are the increase of the level of essential nutrients in food and feed [such as golden rice, (Al-Babili and Beyer 2005)] to reduce levels of naturally occurring antinutrients and allergens. Furthermore, there are attempts to use biotechnology to generate plants that produce specific plant-derived pharmaceuticals, products that are traditionally synthesized using recombinant microbes or transformed mammalian cells (Gomord et al. 2005; Ma et al. 2005).

Insect-resistant GE plants and *Bacillus thuringiensis*

Currently, the only insect-resistant GE crops that are grown commercially are so-called *Bt* crops. *Bacillus thuringiensis* Berliner is a gram positive naturally occurring soil bacterium, which produces crystallized proteins (Cry) that are toxic to different insect orders. A large number of *Bt*-subspecies are known each producing specific *Bt* proteins. Depending on their insecticidal properties and molecular relationships, the Cry proteins were classified (Höfte and Whiteley 1989; Crickmore et al. 1998). In general Cry1, Cry2,

and Cry9 proteins affect Lepidoptera, Cry3, Cry7, and Cry8 proteins are active against Coleoptera, and Cry4, Cry10, and Cry11 proteins affect Diptera. The Cry proteins are known to have a narrow spectrum of insecticidal activity, e.g. only a specific insect order is sensitive. In Lepidoptera, *Bt* crystals containing the protoxins dissolve in the alkaline midgut of the larvae. Once dissolved, digestive proteinases cleave the protoxin to generate the actual toxic fragment of 60-65kDa (Choma et al. 1990). Activated toxins in the gut lumen bind to specific receptor sites on the membranes of midgut columnar cells, creating pores which interfere with ion transport systems across the midgut wall (Glare and O'Callaghan 2000). The resulting desequilibrium in ion concentrations causes the lysis of cells, which can lead to insect death. The carcasses of the dead insects form a substrate for the growth of *Bt* spores (Manthavan et al. 1989).

Up to now, *Bt* genes have been transferred to a large number of plant species, such as cotton, potato, rice, eggplant, oilseed rape (Ely 1993) as well as chickpea (Sanyal et al. 2005; McPhee et al. 2007; Acharjee et al. unpublished data) and pigeonpea (Sharma and Oritz 2000; Sharma et al. 2001). However, in 2007, only *Bt*-transgenic cotton and maize varieties expressing either lepidopteran or coleopteran specific Cry proteins were grown on 42.1 million hectares worldwide (James 2007).

Impact of insect-resistant GE crops on biological control agents

The impact of insect-resistant GE crops on non-target organisms including biological control agents is one of the most widely discussed ecological effects. Natural enemies are of major concern as they often play an important role in natural pest regulation, and are therefore of economic value. They can be affected by the insecticidal protein either directly (toxic effect) or indirectly (change in the prey/host-quality or abundance) (Romeis et al. 2006; 2008b). Therefore, prior to commercialization, a detailed risk assessment for potential adverse effects on non-target organisms has to be conducted (Conner et al. 2003; Garcia-Alonso et al. 2006; Romeis et al. 2008a). For practical reasons, only a small portion of the non-target species can be considered for regulatory studies. It is therefore necessary that appropriate surrogate species are selected that are representatives of ecologically and economically important taxa in the crop, represent different ecological functions, are most likely to be sensitive to the expressed insecticidal protein, and are amenable to testing (Raybould et al. 2007; Romeis et al. 2008a). A non-target risk assessment is commonly conducted within a stepwise (tiered) framework that proceeds

from relatively simple, controlled lower tier tests to increasingly complex higher tier assessments (Dutton et al. 2003; Garcia-Alonso et al. 2006; Raybould et al. 2007; Romeis et al. 2008a). Toxicity studies are generally used at the initial stage to identify potential hazards of insecticidal proteins at elevated dose exposure conditions (often referred to as tier-1 studies). These studies are typically conducted under controlled laboratory conditions with high levels of replication that increase the likelihood that a hazard will be detected if one is present.

Exposure of biological control agents to *Bt*

The risk that a *Bt* crop poses for a biological control agent depends on the toxicity of the insecticidal protein (hazard) as well as on the likelihood at which an agent is exposed to the compound (exposure) (Dutton et al. 2003; Poppy and Sutherland 2004; Garcia-Alonso et al. 2006; Romeis et al. 2008a). As the *Bt* protein targets the insect midgut, it needs to be ingested to be effective.

Non-target organisms can be exposed to insecticidal proteins in different ways: (i) direct exposure as a result of herbivory, e.g. when a natural enemy feeds on pollen or plant sap from a transgenic plant, or (ii) exposure through honeydew, e.g. in contrast to current *Bt* crops, certain experimental lectin or protease inhibitor expressing plants are known to transport insecticidal proteins in the phloem. When sap sucking insects, such as aphids, feed on such plants, the insecticidal proteins are likely to appear in their honeydew (Shi et al. 1994; Kanrar et al. 2002; Rahbe et al. 2003), or (iii) indirect exposure when a natural enemy feeds or parasitizes a target herbivore containing the transgenic product, e.g. when a natural enemy feeds or parasitizes a *Bt*-fed caterpillar, or (iv) indirect exposure when a natural enemy feeds or parasitizes a non-target herbivore containing the transgenic product, e.g. when a natural enemy feeds or parasitizes a *Bt*-fed thrips (Schuler et al. 1999).

India dealing with *Helicoverpa armigera* to protect its most important crops

One of the most important insect pests in the Old World is the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) due to its mobility, high polyphagy, short generation duration and high reproductive rate (Fitt 1989; Sharma 2005). Its larvae, particularly the later instars, preferentially feed on the reproductive organs which lead to serious yield losses. Minimizing the extent of damage, most farmers rely on broad-spectrum chemical pesticides to control this pest on crops including cotton (King

1994; Durairay et al. 2005) chickpea, and pigeonpea (Shanower et al. 1999; Sharma et al. 2007). There is a steady increase in the amount of pesticide applied to control *H. armigera*, which has resulted in the development of resistance to almost all the insecticides used for its control (Forrester et al. 1993; Kranthi et al. 2002). Furthermore, these chemical sprays are also of environmental concern and responsible for human health problems (Pray et al. 2002; Qaim et al. 2008). Thus alternative control methods are increasingly being employed. The use of GE crops that express *Bt* proteins provide a powerful option to control pest Lepidoptera (Shelton et al. 2002). A successful application of this technology is applied in cotton plants to protect them from damage by the budworm/bollworm complex (*Helicoverpa/Heliothis* spp., *Pectinophora gossypiella*). These *Bt* cotton plants, expressing either the gen *cry1Ac* alone or in combination with *cry2Ab*, are highly resistant to damage by lepidopteran pests and consequently, the application of chemical insecticides has been greatly reduced. Australian data from four seasons during the deployment of Bollgard II™ (Monsanto Company, St Louis, USA) cotton plants, expressing the Cry1Ac and the Cry2Ab proteins, showed an average reduction of 80-90% in the number of sprays and a reduction of 65-75% in the active ingredients applied when compared to conventional cotton crops (Fitt 2008). This makes *Bt* cotton a valuable component of integrated pest management programs with many environmental, economical and health benefits (Qaim et al. 2008).

Since 2002, *Bt* cotton hybrids expressing one Cry protein are cultivated on an increasing area in India. Almost tripling the area to 3.8 million hectares in 2006, India became the largest *Bt* cotton growing country in the world. 131 *Bt* cotton hybrids are commercially available in India (James 2007). By 2007 the *Bt* cotton area had reached a total of 6.2 million hectares grown by 3.8 million small and resource-poor farmers (James 2007). In a recent analysis, Qaim et al. (2006) used data for 2002/03 across four Indian states to examine the economic and insecticide impacts of *Bt* cotton. A yield increase of 34% on average was reported with an average reduction of 50% in insecticide applications. However, the benefits differ from year to year as pod borer infestation varies.

As with cotton, the expression of *Bt cry* genes is an option to protect the important food legumes pigeonpea and chickpea from *H. armigera* (Romeis et al. 2004). Pigeonpea and chickpea play a major role in the diet of the Indian society and are also used as animal fodder. For pigeonpea, India is the largest producer, contributing to around 75% of the world total production (3.65 million tonnes) (FAO 2008). Besides its main use as dhal

(dry, dehulled, split seed used for cooking), pigeonpea's tender green seeds are eaten as a vegetable, while crushed dry seeds and green leaves serve as fodder (Nene and Sheila 1990). Chickpea is the premier pulse crop of India, producing 5.77 million tonnes (Ali and Kumar 2005). Its seeds are eaten as snack food or are ground and the flour is used as soup, dhal, or to make bread. Chickpea plants that express either Cry1Ac or Cry2Aa or both proteins are currently under development and could become commercially available in the next decade (Sanyal et al. 2005; McPhee et al. 2007; Acharjee et al. unpublished data)

Scope of the thesis

Unfortunately larvae of *H. armigera* are not very sensitive to Cry1A proteins when compared to other Lepidoptera species (Akhurst et al. 2003), and surviving larvae have been observed on *cry1Ac*-expressing cotton, especially late in the season (Fitt et al. 1994). In addition, the potential of *H. armigera* to develop resistance to *Bt* proteins has already been demonstrated under laboratory conditions (Kranthi et al. 2000; Akhurst et al. 2003). Furthermore, recently a relatively high baseline frequency of resistance alleles for Cry2Ab has been reported from an Australian *H. armigera* population (Mahon et al. 2007a; b). Studies in Australia have demonstrated that Cry1Ac resistance in *H. armigera* resulted in some fitness costs to the insect including a delayed larval development and reduced size (Akhurst et al. 2003). In contrast, Cry2A-resistant larvae did not show any fitness cost in different life-table parameters measured (Rod Mahon unpublished data).

Thus, studies on the compatibility of *Bt* crops with biological control agents is of utmost importance for their sustainable deployment, and to address concerns regarding the ecological consequences of a large-scale deployment of these plants.

Thesis outline

The subject of this thesis was to evaluate the compatibility of *Bt* crops with biological control agents. In a first step, a simplified ecological food web for an Indian pigeonpea crop was constructed, representing the most abundant herbivores and their predators and parasitoids (**chapter two**). Based on this food web, it is discussed which species would need to be addressed in the non-target risk assessment of insect-resistant GE pigeonpeas.

Chapter three deals with the development of a standardized test method to evaluate any direct effects of orally active insecticidal proteins to the predatory larvae of

Chrysoperla carnea (Neuroptera: Chrysopidae). As it is known that the predator also consumes carbohydrate sources, larvae were provided with a sucrose solution in which different insecticidal toxins were mixed. Providing *C. carnea* with this sucrose solution and an additional protein source allowed the observation of the whole larval development period. By calculating the weight change of the larvae the toxin dose *C. carnea* received was calculated.

In a next step, the direct effect of *Bt* cotton was examined for the non-target herbivore *Aphis gossypii* (Hemiptera: Aphididae) which serve as hosts or prey for a variety of parasitoids and predators. Therefore, effects on this herbivore would have potential consequences on a range of beneficial organisms. Besides the evaluation of aphid performance on three Indian *Bt* cotton varieties (expressing Cry1Ac) and their non-transformed near isolines, we investigated whether aphids pick up any *Bt* protein. Further, the sugar composition of aphid honeydew was analyzed to evaluate its suitability for honeydew-feeders.

As previous studies (e.g. Dutton et al. 2002) suggested that herbivores that are sublethally damaged by the *Bt* protein can lower predator performance due to a reduced prey quality, studies were performed to investigate whether prey nutritional composition causes indirect (prey-quality mediated) effects on *C. carnea* (**chapter five**). Lacewing larvae were fed with either *Bt* or non-*Bt* fed Cry1Ac-resistant or susceptible *H. armigera* prey larvae to allow the separation of indirect and direct *Bt* effects. Further biochemical analyses were conducted to evaluate any shift in the lipid and glycogen content as well as the sugar and amino acid content and composition in Cry1Ac-resistant and susceptible *H. armigera* larvae.

Since not only parasitoids and predators are important for the biological control of herbivores, the compatibility of *Bt* crops and an entomopathogenic fungus (*Metarhizium anisopliae* var. *anisopliae*) to control pest Lepidoptera (*H. armigera*) was studied in **chapter six**. Bioassays were conducted to understand the interactions between a Cry2Aa-expressing chickpea line, either susceptible or Cry2A-resistant *H. armigera* larvae, and the entomopathogenic fungus *Metarhizium anisopliae* with a focus on a potential fitness cost in Cry2A-resistant larvae.

Chapter seven summarizes the most important findings in this thesis and discusses the future of *Bt* crops with a focus on non-target risk assessment.

Fig. 1.1 provides an overview of the relevant organisms in the single chapters.

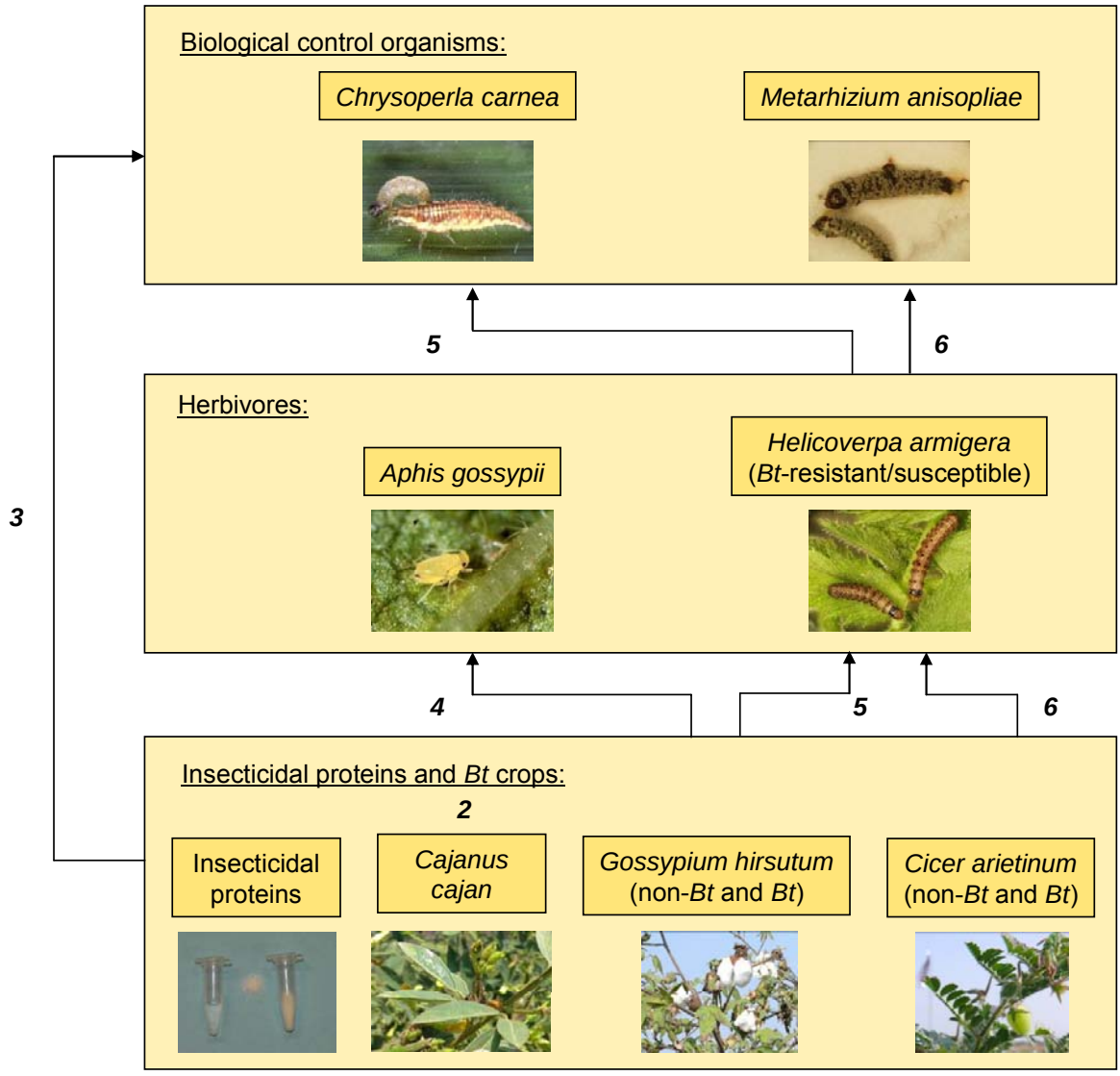

Figure 1.1 Overview of the relevant organisms examined in chapters two to six. Organisms are ordered according their tropic level and italic numbers refer to the relevant chapters.

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Chapter two

Making effective use of existing data for case-by-case risk assessments of GE crops: non-target organism risk assessment of insect-resistant pigeonpeas[1]

[1] Based on: Lawo NC, Raybould A, Romeis J. Making effective use of existing data for case-by-case risk assessments of GE crops: non-target organism risk assessment of insect-resistant pigeonpeas. In preparation.

Abstract

Increasing areas are planted worldwide with insect-resistant genetically engineered (GE) crops. Potential adverse effects of these crops on natural enemies are of major concern as they play an important role in natural pest regulation and are therefore of economic value. Consequently, effects of the GE crop on these non-target organisms are assessed as part of the environmental risk assessment that precedes the commercial release of any GE crop. For practical reasons, only a small portion of the non-target species can be considered for regulatory studies. It is therefore necessary that appropriate surrogate species are selected that are representatives of ecologically and economically important taxa in the crop, represent different ecological functions, are most likely to be sensitive to the expressed insecticidal protein, and are amenable to testing. Reassembling a simplified arthropod food web including the most important herbivores and their parasitoids and predators in Indian pigeonpea, provides the information that helps to identify those non-target organisms that should be considered in a regulatory risk assessment.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is the sixth most important grain legume crop grown in the semi-arid tropics of Asia, Africa and the Caribbean (Nene and Sheila 1990). In 2006, 3.65 million tones were produced worldwide with India being the largest producer, contributing to around 75% of the world total production, followed by Myanmar and Kenya (FAO 2008). Pigeonpea is cultivated as an annual or semi-perennial crop, usually in mixed cropping systems. It is well suited for intercropping, as pigeonpea grows slowly and thus does not compete with shorter-season crops. In addition to cereals, pigeonpea is also intercropped with other legumes and with fiber and root crops (Ali 1990). The nitrogen-fixing ability of pigeonpea makes it an important component in sustainable cropping systems (Kumar Rao 1990).

Besides its main use as dhal (dry, dehulled, split seed used for cooking), pigeonpea's tender green seeds are eaten as a vegetable, while crushed dry seeds and green leaves serve as fodder. The stems are used for fuel wood and to provide a basis for a variety of daily goods (Nene and Sheila 1990). In the vegetarian societies of South Asia, pigeonpea is an important source of dietary protein. Studies in India have shown that in some villages, pigeonpea provides half of all protein consumed (Ryan et al. 1984).

However, pigeonpea is not only a nutritious food for humans, but is also an attractive food for many insects in the field and during storage (Reed and Lateef 1990; Shanower et al.

1999). More then 200 species of insects have been reported to live and feed on pigeonpea. Some of these insects cause large yield losses and are considered major pests, however, the majority are too rare to cause serious damage. Most insects that feed on pigeonpea during the vegetative stage have little or no effect on grain yield as the plants are able to tolerate or recover from the loss of leaf area and even flowers and young pods (Shanower et al. 1999). So only pests that are present continuously or that attack the plant at the middle or end of the reproductive crop cycle cause considerable yield losses (Reed and Lateef 1990). One of the most devastating pests in pigeonpea is the legume pod borer *Helicoverpa armigera* (Lepidoptera: Noctuidae) causing up to 85% yield losses (Durairay et al. 2005), accounting for US$ 317 million economic loss to farmers in the semi-arid tropics (ICRISAT 1992). Its key pest status is due to larval feeding on reproductive parts such as flowers, growing tips and pods which are rich in nitrogen (Fitt 1989). Its pest status is exacerbated by its mobility, polyphagy, short generation time and high reproductive rate (Fitt 1989; Sharma 2005). Currently, the application of chemical insecticides is the most common method of controlling *H. armigera* (Sharma 2005), however, there is a need to find alternative methods to control this pest as *H. armigera* has developed resistance to almost all the insecticides used for its control (Forrester et al. 1993; Kranthi et al. 2002) and the chemical sprays are of concern due to their detrimental effects on the environmental and human health (Qaim et al. 2008).

The use of genetically engineered (GE) crops that express insecticidal genes such as those derived from the soil bacterium, *Bacillus thuringiensis* (*Bt*) provide a powerful option to control lepidopteran pests (Shelton et al. 2002). This technology is successfully applied to protect cotton plants by the expression of *Bt cry* genes, i.e., *cry1Ac* and *cry2Ab* either alone or in combination, from damage by the budworm/bollworm complex (*Helicoverpa/Heliothis* spp., *Pectinophora gossypiella*). Adoption rates of *Bt* cotton are high, reaching 95% of the total area under cotton production in Australia; 72% in the USA; 69% in China; and 66% in India in 2007 (James, 2007).

Overall, the use of *Bt* cotton has greatly reduced the application of chemical insecticides (Fitt 2008; Naranjo et al. 2008). Brookes and Barfoot (2008) estimated that during the first eleven years of *Bt* cotton production (1996-2006) the volume of insecticide active ingredient applied to the global cotton crop was reduced by 22.9%. Insecticide reductions were most significant in China and India where the increased pest control also leads to significant increases in yield (Fitt 2008). In addition, due to less insecticide exposure during spraying operations, farmers and farm laborers gain health benefits, and consumers profit through lower pesticide residues in food and water (Qaim et al. 2008).

As with cotton and other crops, the expression of *cry* genes would be an option to protect pigeonpeas from feeding damage of *H. armigera*. Attempts have already been made to develop *Bt* pigeonpeas (Surekha et al. 2005; Sharma et al. 2006). As *H. armigera* is known for its potential to develop resistance to insecticidal compounds and as laboratory colonies that are resistant to certain Cry proteins have already been selected (Ferré et al. 2008), it is advisable from a resistance management point of view that *Bt* pigeonpea plants should express a minimum of two *cry* genes that are sufficiently different and do not show signs of cross-resistance (Bates et al. 2005; Ferré et al. 2008). Currently, the most likely genes to be deployed in *Bt* pigeonpeas are *cry1Ac* and *cry2Ab* that are successfully expressed in the available double gene *Bt* cotton (Bollgard II^{TM}). The fact that *H. armigera* is a highly polyphagous pest that moves among a number of crops throughout the year and also thrives on non-crop plants (Manjunath et al. 1989; Durairaj et al. 2005; Trivedi et al. 2005), will also mitigate the development of resistance by acting as an unstructured refuge (Bates et al. 2005; Ravi et al. 2005). We thus focus on *Bt* pigeonpeas expressing these two *cry* genes in the following. We furthermore anticipate that the *cry* genes will be driven by a constitutive promoter and consequently the insecticidal proteins are expected to be present in all plant tissues.

Assessing non-target effects of GE crops

Potential adverse effects of GE crops on the environment need to be assessed prior to commercialization. Protection of biodiversity, or non-target species in particular, is one of the management goals defined by Indian (DBT 2008) and international (SCBD 2000) regulations. Because insecticidal GE crops target insect pests, an important part of the environmental risk assessment is their potential impact on non-target arthropods (Conner et al. 2003; Garcia-Alonso et al. 2006; Romeis et al. 2008a, b). Those include organisms providing important ecological services such as the control of herbivores. This function should be maintained in a GE crop field to prevent other herbivores from reaching pest status and thus to ensure a sustainable use of the GE variety as part of an integrated pest management system (Kennedy 2008; Romeis et al. 2008b; c).

Environmental risk assessment begins with problem formulation, in which biologically significant differences between the GE plant and its non-GE counterparts are identified in order to focus the risk assessment on the areas of greatest concern or uncertainty (Raybould 2006; 2007; Romeis et al. 2008a). In cases where no other meaningful differences have been identified, such as the risk assessment can focus on the insecticidal protein as the stressor of

concern. Pigeonpea contain compounds with known toxicity to insects and other organisms such as pheonlic acids (Nahar et al. 1988), amylase inhibitors (Giri and Kachole 1998) and protease inhibitors (Mulimani and Paramjyothi, 1992; Pichare and Kachole, 1996); therefore, the concentration of these compounds should be measured in GE pigeonpea, and if the concentration of particular compounds is outside the natural variation, the likelihood of that difference being harmful should also be assessed.

The risk that an insect-resistant GE crop poses for a non-target organisms depends on the toxicity of an insecticidal protein (hazard) as well as on the likelihood at which an organism is exposed to the compound (exposure) (Dutton et al. 2003; Garcia-Alonso et al. 2006; Romeis et al. 2008a). Non-target arthropods can be exposed to the plant-produced insecticidal proteins through various routes, but mainly by directly feeding on the plant or herbivores that have consumed GE plant material (Raybould et al. 2007; Romeis et al. 2008b).

An assessment of the environmental fate of the transgenic protein is, in effect, a test of a hypothesis of no exposure of non-target organisms to the protein. Environmental fate studies include measurements of the concentration of the transgenic protein in various plant tissues at several developmental stages of the crop, including anthesis and seed set. Other considerations are the potential for the protein to persist and accumulate in soil, be present in a following crop due to GE volunteer weeds, and to spread and persist outside agricultural fields though feral populations of the GE crop or via gene flow from the GE crop to wild relatives.

For releases of crops for which the likelihood of persistent volunteer and feral populations, and of gene flow to wild relatives, is minimal, and for which the transgenic protein is readily degraded in soil, the only non-target organisms likely to be exposed are those that occur in fields where the GE crop is cultivated (Raybould et al. 2007). In these cases, the hypothesis of no exposure of non-target organisms outside the field where the GE crop is cultivated is corroborated and minimal risk can be concluded because of minimal exposure. Where gene flow or feral populations are likely, non-target organisms outside agricultural fields may need to be considered; however, it is likely that feral populations or wild relatives will be associated with very similar non-target organisms to the crop, particularly if feral populations and wild relatives occur close to areas where the crop is cultivated. Pigeonpea is not very competitive and likely to disappear without human intervention (van der Maesen 1990). Also, while pigeonpea has sexually compatible wild relatives in the genus *Cajanus*, gene flow from cultivated pigeonpea and wild relatives

appears rare based on differentiation in seed storage proteins (Jha and Ohri 1996), random amplified polymorphic DNA (Ratnaparkhe et al. 1995) and diversity array technology markers (Yang et al. 2006); this may be because hybridization with wild relatives is rare when pigeonpea is the male parent, even though hybrids are readily formed with pigeonpea as the female parent (Reddy et al. 2003). In a study funded by the Department for International Development (DFID), UK, no gene flow from cultivated pigeonpea varieties to wild relatives was detected; however some genes from wild relatives were detected in cultivated varieties based on morphological parameters and some DNA markers. Scientists suggested growing three to five rows of sorghum or millets around a pigeonpea field to ensure no gene flow (KK Sharma personal communication). Consequently the risk assessment can focus on non-target organisms present in the field since exposure to the insecticidal trait outside the actual crops will be negligible.

If negligible risk to non-target organisms cannot be concluded from minimal exposure, it is necessary to evaluate the likelihood of adverse effects of the transgenic protein at concentrations in the field. Such an evaluation may be made from knowledge of the mode of action of the transgenic protein (e.g., if the protein is not intended to be insecticidal), or from laboratory hazard studies (Romeis et al. 2008a). For practical reasons only a small portion of the non-target species can be considered for regulatory studies. It is therefore necessary that appropriate surrogate species are selected that are representatives of ecologically and economically important taxa that may be exposed, represent different ecological functions, are most likely to be sensitive to the expressed insecticidal protein, and are available and amenable to testing (Raybould et al. 2007; Romeis et al. 2008a).

It is also in the problem formulation phase that relevant risk hypotheses are formulated on how the stressor of concern, i.e. the expressed insecticidal proteins, will affect the non-target species. Information on the stressor of concern that is already available can be taken into account to narrow down the risk hypotheses as much as possible to focus the assessment on the remaining uncertainties. Especially for the *Bt* crops that are grown commercially today the knowledge on non-target effects is substantial (Romeis et al. 2006; Sanvido et al. 2007; Wolfenbarger et al. 2008). In addition to peer-reviewed publications, information will be retrieved for example from earlier regulatory dossiers and expert knowledge.

The non-target risk assessment is commonly conducted within a stepwise (tiered) framework that proceeds from relatively simple, controlled lower tier tests to increasingly complex higher tier assessments (Dutton et al. 2003; Garcia-Alonso et al. 2006; Romeis et al. 2008a). Toxicity studies are generally used at the initial stage to identify potential hazards of

insecticidal proteins at elevated dose exposure conditions. The concentrations tested are usually a factor of ten or more higher than the highest plant expression levels (Raybould et al. 2007; Romeis et al. 2008a). Since the insecticidal proteins get diluted within the arthropod food web (Romeis et al. 2008b), i.e. decline with higher trophic levels, this concentration should provide a conservative assessment of potential effects on arthropod predators and parasitoids. This, together with the fact that these studies are typically conducted under controlled laboratory conditions, i.e. investigate the impact of the stressor of concern in isolation, and high levels of replication increases the likelihood that a hazard will be detected if one is present (Garcia-Alonso et al. 2006; Raybould et al. 2007; Romeis et al. 2008a).

Arthropod food web

To evaluate which natural enemies might be exposed to the insecticidal protein in GE pigeonpea fields in India and potentially would be at risk, a simplified arthropod food web was compiled to identify and prioritize non-target arthropods for risk assessment (Fig. 2.1). Basis for the food web is the peer reviewed literature. Since it is a simplification, not every insect occurring in the field has been mentioned; furthermore, the taxonomic status of many species might be incorrect in the literature (see Romeis and Shanower 1996 for examples). Members of the listed taxonomic groups and guilds can, at various levels of likelihood, become exposed to the insecticidal protein expressed in GE pigeonpea.

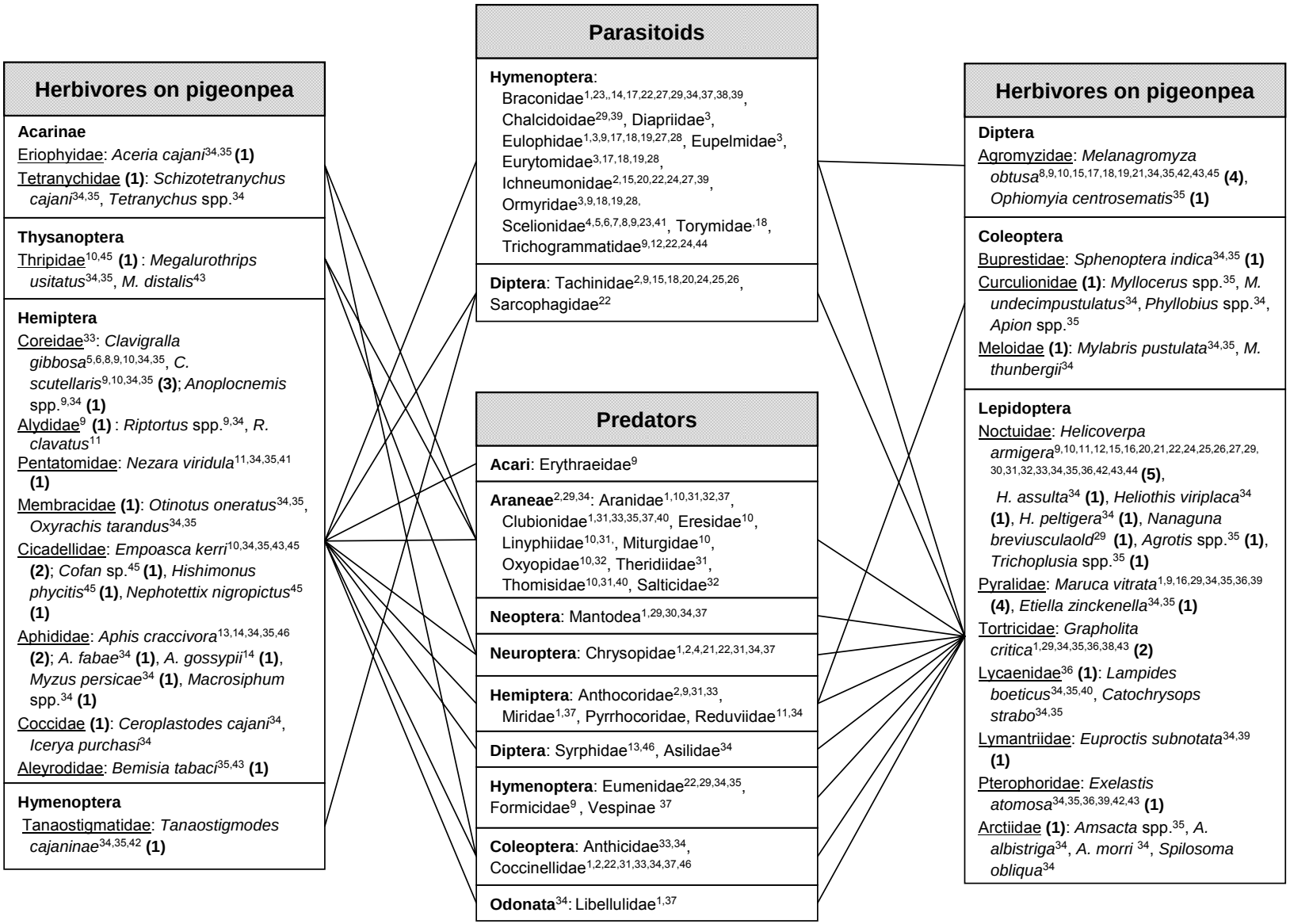
Herbivores on pigeonpea
Acarinae
Eriophyidae: Aceria cajani[34,35] (1)
Tetranychidae (1): Schizotetranychus cajani[34,35], Tetranychus spp.[34]
Thysanoptera
Thripidae[10,45] (1) : Megalurothrips usitatus[34,35], M. distalis[43]
Hemiptera
Coreidae[33]: Clavigralla gibbosa[5,6,8,9,10,34,35], C. scutellaris[9,10,34,35] (3); Anoplocnemis spp.[9,34] (1)
Alydidae[9] (1) : Riptortus spp.[9,34], R. clavatus[11]
Pentatomidae: Nezara viridula[11,34,35,41] (1)
Membracidae (1): Otinotus oneratus[34,35], Oxyrachis tarandus[34,35]
Cicadellidae: Empoasca kerri[10,34,35,43,45] (2); Cofan sp.[45] (1), Hishimonus phycitis[45] (1), Nephotettix nigropictus[45] (1)
Aphididae: Aphis craccivora[13,14,34,35,46] (2); A. fabae[34] (1), A. gossypii[14] (1), Myzus persicae[34] (1), Macrosiphum spp.[34] (1)
Coccidae (1): Ceroplastodes cajani[34], Icerya purchasi[34]
Aleyrodidae: Bemisia tabaci[35,43] (1)
Hymenoptera
Tanaostigmatidae: Tanaostigmodes cajaninae[34,35,42] (1)
Parasitoids
Hymenoptera:
Braconidae[1,23,,14,17,22,27,29,34,37,38,39], Chalcidoidae[29,39], Diapriidae[3], Eulophidae[1,3,9,17,18,19,27,28], Eupelmidae[3], Eurytomidae[3,17,18,19,28], Ichneumonidae[2,15,20,22,24,27,39], Ormyridae[3,9,18,19,28], Scelionidae[4,5,6,7,8,9,23,41], Torymidae[18], Trichogrammatidae[9,12,22,24,44]
Diptera: Tachinidae[2,9,15,18,20,24,25,26], Sarcophagidae[22]
Predators
Acari: Erythraeidae[9]
Araneae[2,29,34]: Aranidae[1,10,31,32,37], Clubionidae[1,31,33,35,37,40], Eresidae[10], Linyphiidae[10,31], Miturgidae[10], Oxyopidae[10,32], Theridiidae[31], Thomisidae[10,31,40], Salticidae[32]
Neoptera: Mantodea[1,29,30,34,37]
Neuroptera: Chrysopidae[1,2,4,21,22,31,34,37]
Hemiptera: Anthocoridae[2,9,31,33], Miridae[1,37], Pyrrhocoridae, Reduviidae[11,34]
Diptera: Syrphidae[13,46], Asilidae[34]
Hymenoptera: Eumenidae[22,29,34,35], Formicidae[9] , Vespinae [37]
Coleoptera: Anthicidae[33,34], Coccinellidae[1,2,22,31,33,34,37,46]
Odonata[34]: Libellulidae[1,37]
Herbivores on pigeonpea
Diptera
Agromyzidae: Melanagromyza obtusa[8,9,10,15,17,18,19,21,34,35,42,43,45] (4), Ophiomyia centrosematis[35] (1)
Coleoptera
Buprestidae: Sphenoptera indica[34,35] (1)
Curculionidae (1): Mylloceru spp.[35], M. undecimpustulatus[34], Phyllobius spp.[34], Apion spp.[35]
Meloidae (1): Mylabris pustulata[34,35], M. thunbergii[34]
Lepidoptera
Noctuidae: Helicoverpa armigera[9,10,11,12,15,16,20,21,22,24,25,26,27,29,30,31,32,33,34,35,36,42,43,44] (5), H. assulta[34] (1), Heliothis viriplaca[34] (1), H. peltigera[34] (1), Nanaguna breviusculaold[29] (1), Agrotis spp.[35] (1), Trichoplusia spp.[35] (1)
Pyralidae: Maruca vitrata[1,9,16,29,34,35,36,39] (4), Etiella zinckenella[34,35] (1)
Tortricidae: Grapholita critica[1,29,34,35,36,38,43] (2)
Lycaenidae[36] (1): Lampides boeticus[34,35,40], Catochrysops strabo[34,35]
Lymantriidae: Euproctis subnotata[34,39] (1)
Pterophoridae: Exelastis atomosa[34,35,36,39,42,43] (1)
Arctiidae (1): Amsacta spp.[35], A. albistriga[34], A. morri [34], Spilosoma obliqua[34]

Figure 2.1 Simplified arthropod food web of pigeonpea (*Cajanus cajan*) in India including the most important herbivore species and their parasitoids and predators. To simplify the food web, arthropods were grouped into orders and families. For herbivores, their ecological and economic relevance or abundance is given in parenthesis on a scale from 1 to 5 (1 = low level of relevance; 5 = high level). Lines indicate an interaction between different orders of herbivores and predators or parasitoids reported in the literature. Superscript numbers refer to references in which the corresponding species were mentioned. References: (1) Kumar and Nath (2003a); (2) Romeis et al. (2000); (3) Shanower et al. (1997); (4) Kalariya et al. (1999); (5) Durairay et al. (2003); (6) Ombir et al. (1996); (7) Mitchell et al. (2004); (8) Dhudashia et al. (1985); (9) Shanower et al. (1999); (10) Arora and Monga (1993), (11) Claver and Ambrose (2003); (12) Romeis and Shanower (1996); (13) Joshi et al. (1999); (14) Singh and Sinha (1983); (15) Bhatnagar et al. (1984); (16) Rao et al. (2004); (17) Sah and Mehra (1986); (18) Sithanantham et al. (1987); (19) Sebastian (1993); (20) Singh et al. (1991); (21) Hegde and Lingappa (1998); (22) Srinivas and Jayaraj (1989); (23) Romeis and Shanower (1996); (24) Dayakar and Ray (1999); (25) Chaudhari (2000); (26) Davies et al. (1979); (27) Singh and Balan (1986); (28) Singh (1991); (29) Sahoo and Senapati (2000); (30) Pawar et al. (1989); (31) Duffield and Reddy (1997); (32) Borah and Dutta (2003); (33) Sigsgaard and Ersbøll (1999); (34) Ranga Rao and Shanower (1999); (35) Reed and Lateef (1990); (36) Lal et al. (1997); (37) Kumar and Nath (2003b); (38) Mohapatra and Sahu (2003), (39) Lateef and Reddy (1984); (40) Singh and Mavi (1984); (41) Nema et al. (2002); (42) Bhuvaneswari and Balagurunathan (2002); (43) Sanap et al. (1995); (44) Duffield (1994); (45) Rangaiah and Sehgal (1984); (46) Joshi et al. (1997).

Most abundant herbivores in pigeonpea

Besides *H. armigera*, the polyphagous bean pod borer, *Maruca vitrata* (Lepidoptera: Pyralidae), is regarded as a serious lepidopteran pest in pigeonpea, causing an estimated $US 30 million economic loss in the semi-arid tropics annually (ICRISAT 1992). Larvae feed from inside a webbed mass of leaves, flowers, and pods (Shanower et al. 1999). Chemical control is thus complicated as larvae are well protected (Ranga Rao and Shanower 1999). Especially varieties flowering early in the season suffer heavy damage (Sharma 2005). Laboratory studies revealed that the species is particularly susceptible to Cry1Ab (LC_{50} of 0.207ppm) while the LC_{50} of Cry1Ac and Cry2Aa was 1.666 and 1.058ppm, respectively (Srinivasan 2008). Consequently *M. vitrata* would probably be little affected by Cry1Ac/Cry2Ab-expressing pigeonpeas when leaf-expression levels where in the order of magnitude as those reported from *Bt* cotton [Cry1Ac 1.56µg/g fresh weight (f.w.) ± 0.15 (Monsanto 2002); Cry2Ab 23.9µg/g f.w. ± 6.3 (Monsanto 2003)]. The damage of the leaf webber, *Grapholita critica* (Lepidoptera: Tortricidae) is very obvious, causing farmers to worry about possible yield losses. However, even if the damage is highly visible, it seldom causes any serious loss in grain yield (Ranga Rao and

Shanower 1999). The larvae of *Lampides boeticus* (Lepidoptera: Lycaenidae) and *Catochrysops strabo* (Lepidoptera: Lycaeninae), and *Exelastis atomosa* (Lepidoptera: Pterophoridae) are common in several parts of Asia and can cause substantial losses in some years due to pod and flower feeding (Reed and Lateef 1990). There is presently no information available on the extend to which the species would be affected by Cry1Ac/Cry2Ab.

There are a number of other insect herbivores that can cause significant damage to pigeonpea which are unlikely to be affected by Lepidoptera-active *Bt* proteins. The pigeonpea pod fly, *Melanagromyza obtuse* (Diptera: Agromyzidae) exclusively feeds on pigeonpea. The maggots feed inside the seeds which will not germinate and are unsuitable for human consumption. Due to the concealed mode of action, pod fly infestation often remains unnoticed by the farmers and thus is difficult to control (Moudgal et al. 2008). So far, no specific control strategy has been universally implemented for pod fly management (Ranga Rao and Shanower 1999). In North India several landrace varieties are grown which have some level of resistance to this pest. Thus, damage and yield losses vary across seasons, regions and cultivars. Damage levels range from 10 to 50% (Shanower et al. 1999) and it is a more serious pest in the northern and central areas than in other parts of India. The bean bug, *Clavigralla* sp. (Hemiptera: Coreidae), causes economical loses which occasionally exceed 50%. Adults and nymphs pierce the pod wall to suck on the developing seeds (Shanower et al. 1999). The most commonly recorded insect attacking pigeonpea during the vegetative growth state are jassids, particularly *Empoasca kerri* (Reed and Lateef 1990). However, as mentioned earlier pests attacking the crop during the vegetative stage are of minor importance. Rare pests in pigeonpea fields are aphids as normally natural enemies and abiotic factors keep them at low levels (Ranga Rao and Shanower 1999). However, occasionally aphids, particularly *Aphis craccivora*, build up in large numbers and thus slow down plant growth, particularly in dry periods (Reed and Lateef 1990). Other pests feeding on pigeonpea in the field cause seldom any serious losses and hardly need any control.

A group of arthropods causing a considerable loss in pigeonpea under storage conditions are bruchids from the family *Callosobruchus* spp. (Coleoptera: Bruchidae). Following bruchid feeding, the seeds lose their viability and are unacceptable for human consumption (Ranga Rao and Shanower 1999).

Important insect parasitoids and predators in pigeonpea

Most information about a pigeonpea insect pest and its natural enemies is available for the pod borer *H. armigera*. *Campoletis chloridae* (Hymenoptera: Ichneumonidae) is reported to be an important mortality factor of *H. armigera* in India, parasitizing preferentially second instar larvae (Dhillon and Sharma 2007; Sharma et al. 2008). One other genus of hymenopteran larval parasitoids, *Eriborus* spp. (Hymenoptera: Braconidae) can cause significant mortality in the first- to third instars of *H. armigera*; up to 23% parasitism has been reported from pigeonpea (Romeis and Shanower 1996). It is striking that egg parasitoids belonging to the genus *Trichogramma* (Hymenoptera: Trichogrammidae) only parasitized very few eggs of *H. armigera* in pigeonpea fields as compared to other crops (Bhatnagar et al. 1984; Reed and Lateef 1990; Romeis and Shanower 1996; Romeis et al. 1999). This low parasitization efficacy is due to the fact that *H. armigera* deposits the majority of eggs on plant structures, flower buds and pods that are covered by trichomes and hinder movements by the tiny parasitoids (Romeis et al. 1998) and by plant volatiles that deter the wasps (Romeis et al. 1997). In general, dipteran parasitoids of *H. armigera* appear to be more active in pigeonpea when compared to Hymenoptera (Davies et al. 1979; Bhatnagar et al. 1984; Reed and Lateef 1990; Romeis and Shanower 1996; Shanower et al. 1997; 1999). Tachinids are the most important group of dipteran parasitoids. They parasitize older instars and emerge from sixth instar larvae or pupae (Romeis and Shanower 1996). Therefore, it is argued that dipteran parasitoids are of less importance to protect the crop since they kill the larvae in the prepupal or pupal phase after the caterpillars have already caused pod damage (Davies et al. 1979; Bhatnagar et al. 1984; Srinivas and Jayaraj 1989). However, since tachinids are one of the few antagonists attacking large *H. armigera* larvae, they might help to prevent the spread of resistant genes if tolerant or resistant *H. armigera* larvae survive on GE plants.

For the lepidopteran pest *M. vitrata*, several parasitoids of the orders Diptera and Hymenoptera have been recorded on different legumes including pigeonpea in Africa and Asia (Sharma 1998).

The only reported natural enemies of the pigeonpea pod fly, *M. obtuse* are parasitic Hymenoptera, whereas the most studied parasitoids belong to the families of *Euderus* spp. (Eulophidae), *Eurytoma* sp. (Eurytomidae) and *Ormyrus* spp. (Ormyridae) (Shanower et al. 1998). For *Euderus* spp. parasitism rates of 25% have been reported throughout India and 12.5% for *Ormyrus orientalis* in central India (Thakur and Odak 1982). *Eurytoma* sp. is of

minor importance, only parasitizing less than 2% of *M. obtusa* in India and Sri Lanka (Sithanantham et al. 1983).

In India, the Braconidae *Trioxys indicus* is the most important aphid parasitoid in pigeonpea (Waterhouse 1998). A single *T. indicus* female can parasitize 100 to 150 aphids in three to five days. Up to 87% of *Aphis craccivora* is parasitized by *T. indicus* in Indian pigeonpea fields (Waterhouse 1998).

Even if there are a number of predators in pigeonpea fields, relative little attention has been devoted to them. The most abundant predatory groups are Neuroptera, mainly chrysopids (*Chrysoperla* spp.), Anthocoridae, mainly *Orius* spp., Coleoptera (Coccinellidae and Anthicidae) and a variety of spiders (Romeis and Shanower 1996; Sigsgaard and Ersbøll 1999). As most predators are polyphagous they feed on a variety of insect pests. In the literature it has been reported that predators feed on other small-bodied herbivores as well as on young *H. armigera* larvae (Romeis and Shanower 1996; Shanower et al. 1999). However, birds, such as the Indian Myna, egrets, drongos, or the king crow also prey on large pod borer larvae (Kumar and Nath 2003b; Ranga Rao and Shanower 1999), grasshoppers (Kumar and Nath 2003b) or blister beetles *Mylabris* spp. (Ranga Rao and Shanower 1999). Studies from India indicate that ants might be important predators of *H. armigera* pupae (Romeis and Shanower 1996). Studies from Africa and Asia reported that predators of the orders Dermaptera, Dictyoptera, Coleoptera, Hemiptera, Hymenopteran, and Araneida prey on *M. vitrata* in different legumes including pigeonpea (Sharma 1998).

As pigeonpea is partially cross-pollinated, pollinators such as honeybees (Hymenoptera: Apidae), bumble bees (Hymenoptera: Apidae), and wasps (Hymenoptera: Apidae) play an important role (Arora et al. 2004; Singh et al. 2003). Different species of *Apis* spp., *Megachile lanata*, *Xylocopa* sp. and *Ceratina* sp. were reported on pigeonpea, where the honeybee *A. dorsata* and *M. lanata* were the most important pollinators (22.67% and 27.94%) (Singh et al. 2003).

Implication for non-target arthropod risk assessment of insecticidal GE pigeonpea

Above, a simplified arthropod food web for Indian pigeonpea fields was presented. Based on this, a proposition can be given which non-target arthropods would be exposed either directly or indirectly to a transgenic trait and thus should be considered in a non-target risk assessment of, for example, a Cry1Ac/Cry2Ab-expressing pigeonpea. It is

unlikely that sap-feeding arthropods such as aphids and planthoppers are exposed to the *Bt* proteins as they are not transported in the phloem sap of *Bt* crops (Romeis et al. 2008b). Although this hypothesis will be tested with a protein expression study of the actual pigeonpea events. If confirmed, predators preferentially feeding on aphids, such as larvae from Syrphidae, most Chrysopidae and Coccinellidae adults and larvae are unlikely to be at risk and would thus not need to be tested for potential toxic effects of the *Bt* Cry proteins. A second hypothesis predicts that egg parasitoids belonging to the family of *Trichogrammatidae* will not be exposed to the *Bt* protein as insect eggs have so far not been found to contain any Cry proteins. Consequently, only predators or parasitoids that directly consume plant material or that attack herbivores that have fed plant tissue would potentially be exposed to the insecticidal proteins.

Prior to the commercialization of *Bt* cotton expressing *cry1Ac/cry2Ab* (Bollgard II^{TM}), feeding studies with purified *Bt* proteins were conducted by the applicant to assess toxic effects of Cry1Ac and Cry2Ab on different orders of non-target organisms (Monsanto 2002, 2003). These studies revealed no toxic effects of the two Cry proteins at concentrations exceeding the expected environmental concentration to the pollinator *Apis mellifera* (Hymenoptera: Apidae), the polyphagous predators *Chrysoperla carnea* (Neuroptera: Chrysopidae) and *Hippodamia convergens* (Coleoptera: Coccinelidae), the parasitic wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), and soil organisms of the order Collembola and Lumbricidae. Based on these regulatory risk assessment studies and a large number of scientific peer-reviewed studies (Romeis et al. 2006; Wolfenbarger et al. 2008), it appears that the two Cry proteins have a high degree of specificity and no effect on organisms outside the order of Lepidoptera. Once it has been confirmed that the Cry proteins expressed in pigeonpea are structurally and functionally equivalent to the proteins expressed in *Bt* cotton and that expression levels are comparable, non-target risk assessment data collected for *Bt* cotton can directly be implemented in the risk assessment for *Bt* pigeonpea. Since pigeonpea and cotton harbor similar groups/guilds of natural enemies one may conclude that pigeonpea plants expressing *cry1Ac/cry2Ab* pose negligible risk to non-target organisms without requiring additional non-target risk assessment studies. One also needs to take into account that, following commercialization, potential disturbances or failures in biological control caused by undetected non-target effects of *Bt* pigeonpea will be noticed by farmers when it leads to outbreaks of non-target-pests herbivores that are not controlled by the insecticidal trait (Sanvido et al. 2009).

In the case of the deployment of GE pigeonpeas expressing other insecticidal traits, additional non-target studies would need to be conducted. The simplified food-web presented in Fig. 2.1 together with information on the level and localization of expression of the insecticidal protein will help to identify species that are potentially at risk. This together with information on the mode of action and spectrum of activity of the expressed *Bt* protein will help to identify species to serve as surrogate test species.

For example, in case of GE pigeonpeas expressing a Diptera- or Coleoptera-specific Cry protein, non-target organisms of those orders might be at risk and would have to be considered in a non-target risk-assessment. However, in the case of plants expressing insecticidal proteins that have a broader spectrum of activity, e.g. protease inhibitors (PI), lectins, predictions regarding the magnitude of risk to non-target arthropod orders are more difficult to make. However, some information on the activity spectrum of these proteins is available in the scientific literature or can be derived from herbivore-screening studies conducted by the developer of the GE plant. For example, the inhibitory spectrum of PIs is usually limited to proteases in one mechanistic class (serine-, cysteine-, aspartate-, and metallo-PIs) (Michaud 2000). Due to the fact that different insect orders have mainly one type of proteases in their gut, a prediction can be given which order would be most likely to be affected by a certain PI (Cowgill and Atkinson 2003). So far, experimental transgenic plants express either serine (Gatehouse et al. 2000) or cysteine PIs (Arai and Abe 2000). For serine PIs it is known that they are most likely to affect insects of the order Lepidoptera and Coleoptera while cysteine PIs appear to be particularly effective against insects of the order Coleoptera (Malone et al. 2008). Mannose-binding lectins such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) and the garlic leaf lectin (*Allium sativus*, ASAL) have received some attention since they have been shown to provide partial protection against phloem-feeding pests such as aphids in a variety of crops including legumes (Romeis et al. 2004; Malone et al. 2008). However, this class of lectins was found to affect important life-table parameters in many different orders including Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, and Neuroptera (Malone et al. 2008).

Thus, the arthropod food web (Fig. 2.1) can help to identify appropriate surrogate species that can be deployed to assess the potential risks of insecticidal proteins with a broader range of activity. Hymenoptera are of particular interest as they have an important function as parasitoids (e.g. Ichneumonidae), predators (e.g. Eumenidae), and pollinators (Apidae) and could therefore be exposed to an insecticidal protein via several routes. In

addition to honeybees, for which standardized protocols to conduct toxicity studies are available, *Campoletis chlorideae* (Hymenoptera: Ichneumonidae) might be a good surrogate since this species is relatively well studied and can be reared and tested in the laboratory (Murugan et al. 2000; Dhillon and Sharma 2007; Sharma et al. 2008). Another group of interest is the Hemiptera, e.g. the omnivorous bug *Orius* spp. (Hemiptera: Anthocoridae) as it is known to feed both on herbivores and on pollen and plant tissue (Alomar and Wiedenmann 1996) and is consequently exposed to higher toxin doses when compared to other predators (Rose 2007).

We here show that using information on the receiving environment (the arthropods present in the GE crop) together with knowledge about the insecticidal trait helps to guide the non-target risk assessment of insecticidal GE crops. In cases where the non-target risks of specific insecticidal traits have already been assessed in a different context (different plant, different region) no additional non-target data may be required to come to a conclusion of negligible risk.

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Chapter three

Assessing the utilization of a carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*[2]

[2] Based on: Lawo NC, Romeis J (2008) Assessing the utilization of a carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*. Biological Control 44:389-398.

Abstract

A concern with the widespread use of insecticidal transgenic crops is their potential to adversely affect non-target organisms, including biological control agents such as larvae of the green lacewing, *Chrysoperla carnea* (Neuroptera: Chrysopidae). Since the insecticidal proteins expressed by the current transgenic plants are active only after ingestion, dietary bioassays are required to test direct effects on nontarget organisms. After showing that *C. carnea* larvae utilize carbohydrate foods, we exposed them to insecticidal proteins dissolved in a sucrose solution. Feeding on snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) as a model compound, the larvae were negatively affected in a number of life-table parameters. Interestingly, GNA caused a prolongation in first instar development, but had no effect on subsequent utilization of prey resulting in an increased weight of second instars. Comparable studies with avidin, a biotin-binding protein, revealed strong effects on *C. carnea* survival at the concentration tested. Despite the fact that the proteolytic digestion of *C. carnea* larvae is reported to be dominated by serine proteases, ingestion of soybean trypsin inhibitor (SBTI) did not cause any detrimental effects. Similarly, two Cry proteins derived from *Bacillus thuringiensis* (Cry1Ac and Cry1Ab) did not cause negative effects on *C. carnea*, what is consistent with earlier studies. The here presented bioassay provides a valuable tool to assess direct impacts of insecticidal proteins to *C. carnea* larvae and other predators that are known to feed on carbohydrate solutions.

Introduction

In recent years, various proteins that cause insect resistance have successfully been engineered into plants (Jouanin et al. 1998; Hilder and Boulter 1999; Ferry et al. 2004; O'Callaghan et al. 2005). These include Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*), protease and a-amylase inhibitors, lectins, and avidin. Currently the only insecticidal transgenic crops that are grown commercially express *Bt* Cry proteins. In 2006, *Bt*-transgenic maize and cotton varieties were grown on 32.1 million hectares worldwide (James 2006).

One of the risks associated with growing insect-resistant transgenic crops is their potential to adversely affect non-target organisms. These include a range of arthropod species that fulfill important ecological functions such as biological control. The potential for adverse non-target effects is evaluated prior to the commercial cultivation of an insecticidal transgenic crop as part of the environmental risk assessment (Conner et al.

2003; Garcia-Alonso et al. 2006). For practical reasons, only a small portion of the non-target species can be considered for regulatory studies. It is therefore necessary that appropriate surrogate species are selected that are representatives of ecologically and economically important taxa in the crop, represent different ecological functions, are most likely to be sensitive to the expressed insecticidal protein and available and amenable to testing (Raybould et al. 2007; Romeis et al. 2008). The non-target risk assessment is commonly conducted within a stepwise (tiered) framework that proceeds from relatively simple, controlled lower tier tests to increasingly complex higher tier assessments (Dutton et al. 2003; Garcia-Alonso et al. 2006; Romeis et al. 2008). Toxicity studies are generally used at the initial stage to identify potential hazards of insecticidal proteins at elevated dose exposure conditions (often referred to as tier-1 studies). These studies are typically conducted under controlled laboratory conditions, provide high levels of replication and increase the likelihood that a hazard will be detected if one is present (Garcia-Alonso et al. 2006; Raybould 2007).

One of the species that has extensively been studied for potential *Bt* maize effects is the green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), a common predator in many crops grown in western and central Europe (Romeis et al. 2006). The larvae preferably prey on aphids but may consume a wide range of soft-bodied arthropods. They are potentially exposed to insecticidal proteins expressed in insect-resistant genetically modified plants through their prey. *C. carnea* is available and amenable to testing. Validated and standardized protocols exist to assess the impact of chemical (contact) insecticides (Vogt et al. 2000). There is, however, a need to develop bioassays that account for the oral exposure pathway of plant-expressed insecticidal proteins to assess their potential hazard to non-target organism such as *C. carnea*.

Chrysopid larvae are known to utilize carbohydrate sources such as extra-floral nectar or honeydew to gain energy (Limburg and Rosenheim 2001; Romeis et al. 2004; Hogervorst et al. 2008). One aim of the present study was to assess to what extent the provision of a carbohydrate food source allows the larvae to compensate for a limitation in prey availability. Following two different approaches, we developed a method to ensure the development of *C. carnea* and the intake of a sucrose solution with dissolved insecticidal protein. In the first approach, we fed first instars with a restricted diet consisting of a defined number of moth eggs (prey) and a sucrose solution at the same time. In the second approach, we provided the predator larvae with a sucrose solution and

subsequently moth eggs *ad libitum*. To verify the quality of the test method, a sucrose solution containing 1% (weight:volume, w:v) snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) was used as earlier studies by Hogervorst et al. (2006) showed direct effects on *C. carnea* larvae at this concentration. To validate our proposed method, a range of insecticidal proteins were tested for direct effects on *C. carnea*.

Materials and methods

Insect material

C. carnea were collected in Bolligen near Bern (Switzerland) in 1993 and have since been maintained in the laboratory with no additions of field-collected insects. Larvae of *C. carnea* were reared on pea aphids, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae); and adults were fed an artificial diet containing honey, brewers yeast and water (7:4:4). Rearing conditions were 22 ± 3°C, 70 ± 5% r.h. with a 16-h photoperiod. Eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) were supplied by Biotop (Valbonne, France) and stored for 2 to 4 weeks at 4°C.

Insecticidal proteins

Insecticidal proteins used in the different bioassays include: (i) lyophilized GNA isolated from snowdrop bulbs, obtained from Els van Damme (Ghent University, Belgium) (van Damme et al. 1987); (ii) recombinant avidin, purified from transgenic corn, ≥12 U/mg protein (Sigma–Aldrich, Switzerland); (iii) soybean trypsin inhibitor (SBTI) isolated from Glycine max, ~10,000 BAEE U/mg protein (Sigma–Aldrich, Switzerland); and (iv) Cry1Ab and Cry1Ac proteins. Lyophilized Cry proteins were obtained from Marianne Pusztai-Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, OH, USA). The protoxins from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 were expressed as single gene products in *Escherichia coli*. Inclusion bodies containing protoxin were dissolved and trypsinized. Afterwards the toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al. 1994).

Insecticidal proteins were provided to the *C. carnea* larvae dissolved in a 2M sucrose (Merck; Darmstadt, Germany) solution. That the dissolved proteins retain their biological activity has been shown in previous studies, i.e., GNA (Romeis et al. 2003; Hogervorst et al. 2006), avidin (Dhillon et al. 2008), SBTI (Babendreier et al. 2005; 2008) and Cry1Ab (Romeis et al. 2004).

Experimental conditions

All bioassays were conducted in a climate chamber at 23 ± 1 °C, 85% r.h. with a 16-h photoperiod. If not stated otherwise, bioassays were conducted with 14 to 16 h-old *C. carnea* larvae that were kept individually in a Petri dish (5cm diameter; 1cm high) the lid of which contained a vent hole covered with a fine-mesh netting.

Prey consumption of *C. carnea* larvae

The aim of these bioassays was to examine the exact number of *E. kuehniella* eggs that are consumed by an individual *C. carnea* larva during the three different larval stages (L1: n=11; L2: n=14; L3: n=14). For this purpose freshly hatched or molted larvae (≤15 min-old) were used and individually fed with *E. kuehniella* eggs *ad libitum* following a designated time scheme. Within the first 8h, Petri dishes with eggs were changed every 2h. Subsequently, they were changed at least twice a day. Before eggs were provided to the predator they were screened under a binocular microscope (WILD, Heerbrugg, Switzerland) and any damaged eggs were removed. During the bioassay, the number of *E. kuehniella* eggs consumed (i.e., depleted egg-shells) by the predator was counted with a binocular microscope. After the first larva had molted or pupated, observations were conducted every hour during day-time.

Restricted diet bioassay with L1 *C. carnea*

Utilization of sucrose in the presence of prey

Predator larvae were exposed to a minimum diet that allowed larval development but ensured a sucrose solution was ingested. Therefore *E. kuehniella* eggs and sucrose solution were provided at the same time, to determine at what number of eggs the larvae also utilized the sucrose solution. Egg numbers were chosen based on the results from the previous bioassay. Neonate *C. carnea* received 2, 4, 6, 10 or ad libitum undamaged *E. kuehniella* eggs per day until they molted to the L2. Within each of the treatments, half of the larvae received two 0.5µl droplets of a 2M sucrose solution in addition to the eggs; the other half received only eggs. Larval survival and development to the L2 were recorded in the morning and the evening. A larva was considered dead when it did not respond to mechanical stimulation with a fine sable brush. After the L2 molt was completed, larvae were frozen at -20°C and subsequently dried at 50°C for at least four days. Finally, insect dry weight (d.w.) was determined on a microbalance (Mettler Toledo MX5, division

d=1µg; tolerance ±2µg). The bioassay was repeated twice resulting in a total of 20 to 23 larvae per treatment.

***Impact of GNA on L1* C. carnea**

The aim of these studies was to evaluate the effect of GNA on L1 *C. carnea* while feeding on a restricted diet. Neonate *C. carnea* larvae received 2, 4, or 6 undamaged *E. kuehniella* eggs per day until development to L2. In addition to the eggs, half of the larvae received two 0.5µl droplets of a sucrose solution (2M) and the other half received a sucrose solution containing 1% GNA (w:v; 1mg GNA dissolved in 100µl sucrose solution). The previous experiment had revealed that *C. carnea* larvae ingest the sucrose solution in the presence of this low prey number. Studies of Hogervorst et al. (2006) reveal that GNA does not affect food intake by *C. carnea* larvae when provided in a sucrose solution. As in the previous bioassay, larval development, survival and L2 d.w. were recorded. The bioassay was repeated three times resulting in a total of 30 to 40 larvae per treatment.

Assessing total *C. carnea* imaginal development

Impact of GNA on total* C. carnea *imaginal development

To examine the effect of GNA on the imaginal development of *C. carnea*, a method was established that allowed the insect to complete its development. *C. carnea* larvae received two droplets of a 2M sucrose solution (L1: 0.5µl; L2: 2µl, L3: 3µl) or a sucrose solution containing 1% GNA (w:v) on the first day of each larval stage. After 24h, larvae were transferred to clean Petri dishes and subsequently fed with *E. kuehniella* eggs *ad libitum*.

Larvae were observed twice a day in the morning and in the evening. Parameters recorded included survival and development time (i.e., days required to reach the pupal and adult stages). Larval development was considered completed when cocoon formation was entired. Insects that developed without completing cocoon formation were excluded from further analyses. After emergence adults were sexed, frozen at -20°C and dried in the oven at 50°C for at least four days. Subsequently, adult d.w. was determined on a microbalance. The bioassay was repeated twice, resulting in a total of 69 to 71 insects per treatment.

***Impact of different insecticidal proteins on imaginal* C. carnea**

To test the effects of five different orally active insecticidal proteins on different *C. carnea* imaginal life-table parameters, a bioassay was conducted as described previously. *C. carnea* larvae were either fed a pure sucrose solution (2M), a 2M sucrose solution containing GNA (1%, w:v), avidin (1%), SBTI (1%), Cry1Ab (0.1%) or Cry1Ac (0.1%) during the first 24h of each larval stage and subsequently fed *E. kuehniella* eggs. Aiming to perform a worst-case hazard study, protein concentrations were chosen which had been found to cause effects on other insects in previous studies. In the case of avidin, a range of insects belonging to different orders have been found to be affected at a 1% concentration (Morgan et al. 1993; Kramer et al. 2000; Markwick et al. 2001, Zhu et al. 2005). A number of studies have revealed lethal or sublethal effects of SBTI to herbivores and pollinators at a concentration of 0.1% or 1% (Johnston et al. 1993; Pham-Delègue et al. 2000; Babendreier et al. 2005; 2008). In the case of the two *Bt* Cry proteins, a 0.1% solution should expose the predators to protein amounts that are significantly higher than the expected environmental concentration (Romeis et al. 2004).
The bioassay was repeated twice resulting in a total of 51 to 58 insects per treatment.

Intake of sucrose solution by *C. carnea* larvae

The goal of this experiment was to assess how much sucrose solution, and therefore insecticidal protein, an individual *C. carnea* larva ingests. Freshly hatched or molted larvae (≤15h) were weighed on a microbalance and subsequently either were provided with two droplets of a 2M sucrose solution (L1: 0.5µl; L2: 2µl, L3: 3µl) or were starved. After 24h larvae were reweighed to assess weight change. The discrepancy between insects that fed on sucrose and those that received nothing was equated to the quantity of food they consumed. Excluded from the analysis were insects which excreted an alarm secretion during handling. Additionally, the weight of 1µl sucrose solution (2M) was determined in order to estimate the amounts of sucrose solution consumed by each larva. In total 22 to 32 insects were tested for each of the three larval stages per treatment.

Data analysis

Data on the development time of L1 *C. carnea* were analyzed pair-wise (by number of eggs) using the Mann-Whitney-*U* test, adjusted for ties. Data covering the whole larval or pupal development were calculated using the Cox proportional hazard model (event:

developed to pupal or adult stage). Insects lost during the observation period were evaluated as censored data. Significance levels were corrected for multiple pair-wise comparisons, using the Bonferroni-Holm procedure. Survival was analyzed by performing a two-sided Fisher`s exact test. The Bonferroni-Holm correction was applied for multiple pair-wise comparisons. Dry weight data were checked for normal distribution and homogeneity of variances prior to the analysis. When all assumptions were met, they were analyzed using analysis of variances (ANOVA). Otherwise, data were examined using the Mann-Whitney-*U* test, adjusted for ties. *P*-values were adjusted by the Bonferroni-Holm correction for multiple pair-wise comparisons. When bioassays were repeated and an ANOVA or the Cox proportional hazard model was calculated, data were analyzed for experimental effects. They will be mentioned whenever they were found to be significant. For all tests the α-level was set at 5%. Exact *P*-values are given for $0.001 \geq P \leq 0.05$. Statistical power analyses were conducted for selected results using the PASS software program (NCSS, Kaysville, UT). All statistical analyses were conducted using the software package Statistica (Version 6, StatSoft Inc., Tulsa, OK, USA).

Results

Prey consumption of *C. carnea* larvae

During the first instar, a single *C. carnea* larva consumed on average (± SE) 38.9 ± 2.12 *E. kuehniella* eggs (Fig. 3.1 A). Most of these eggs (47.6%) were consumed within 24-48h following larval emergence. The first larvae developed to L2 within 76-78h. After 96h all larvae had completed the L1 stage. The first 4h after hatching larvae consumed an average of just 1.7 ± 0.45 eggs (4.4% of the total number of eggs consumed). Before molting (72-96h), only 2 ± 0.50 eggs were consumed (1.8%).

The average egg consumption of L2 was 118.4 ± 5.15 (Fig. 3.1 B). Within the first 4h, L2 larvae consumed an average of just 5.5 ± 0.89 eggs (4.6%). During the first 24h, the larvae consumed 70% of the total eggs consumed during the second instar. After 72h the first larvae developed to L3. The following morning (96h), all larvae had developed to L3. Second instar larvae stopped egg consumption after 56h.

During L3, an average of 1173.0 ± 35.15 eggs were consumed per larva (Fig. 3.1 C), most of them during the first 48h (59.6%). Within the first 4h, L3 larvae consumed an average of 20.0 ± 3.63 eggs (1.7%). After 78h, all the larvae had started spinning a cocoon,

by the following morning (96h) all had pupated successfully. In contrast to the previous stages, L3 did not reduce feeding prior to pupation.

Restricted diet bioassay with L1 of *C. carnea*

Utilization of sucrose in the presence of prey

The experiment revealed that L1 *C. carnea* benefited from the additional provision of a sucrose solution when they received 6 or less *E. kuehniella* eggs per day (Table 3.1). For larvae receiving 2 or 4 eggs, development time was significantly ($P<0.001$) shortened when sucrose solution was provided. While larvae that received 2 eggs per day suffered high mortality, survival was significantly increased by the sucrose feeding ($P<0.001$). For all the other treatments, survival was not affected by sucrose feeding ($P>0.05$). Dry weight of the L2 was significantly increased by additional sucrose feeding when the L1 larvae received 6 ($P=0.002$) or 4 ($P=0.043$) eggs per day. Interestingly, no sucrose effect on d.w. was observed when larvae consumed 2 eggs per day ($P>0.05$).

***Impact of GNA on L1* C. carnea**

Independent of the number of eggs provided per day, the additional provision of GNA dissolved in a 2M sucrose solution caused a significant prolongation in *C. carnea* larval development (6 eggs: $P=0.016$; 4 and 2 eggs: $P<0.0001$) (Table 3.2). Larval survival was significantly affected by GNA when larvae ingested 4 eggs ($P=0.042$). Dry weight of freshly emerged L2 was significantly increased in GNA-fed larvae that had received 6 ($P=0.003$) or 4 ($P=0.012$) eggs when compared to larvae that were provided with eggs and a pure sucrose solution. Interestingly, GNA ingestion did not affect the d.w. of L2 that had received 2 eggs ($P>0.05$).

Table 3.1 Mean L1 development time (days) of *Chrysoperla carnea*, survival (%) and mean dry weight (µg) of freshly emerged L2.

Number of eggs	Sucrose solution	n	L1 development (days ± SE)	L1 survival (%)	L2 dry weight (µg ± SE)
ad libitum	Yes	21	3.67 ± 0.053 a	100 a	180.6 ± 12.58 a
ad libitum	No	23	3.76 ± 0.062 a	100 a	206.8 ± 16.12 a
10	Yes	22	3.71 ± 0.054 a	95.5 a	158.2 ± 11.42 a
10	No	22	3.74 ± 0.064 a	95.5 a	152.1 ± 9.23 a
6	Yes	20	3.90 ± 0.045 a	100 a	135.1 ± 5.57 a
6	No	20	4.00 ± 0.000 a	95.0 a	107.2 ± 3.66 b
4	Yes	21	3.92 ± 0.558 a	100 a	103.7 ± 5.72 a
4	No	21	5.43 ± 0.163 b	100 a	88.1 ± 4.34 b
2	Yes	21	6.50 ± 0.218 a	85.7 a	89.5 ± 5.26 a
2	No	20	17.10 ± 0.838 b	25.0 b	92.6 ± 6.65 a

Larvae were provided daily with different numbers of *Ephestia kuehniella* eggs. Half of the larvae were fed additionally with a 2M sucrose solution. Values within groups ("number of eggs") followed by different letters differ significantly ($P<0.05$).

Table 3.2 Impact of snowdrop lectin (GNA) on development time (days) and survival (%) of L1 *Chrysoperla carnea*, and dry weight (µg) of L2.

Number of eggs	Food solution	*n*	L1 development (days ± SE)	L1 survival (%)	L2 dry weight (µg ± SE)
6	Sucrose	40	3.96 ± 0.038 a	97.50 a	114.3 ± 2.73 a
6	Sucrose + GNA	40	4.15 ± 0.083 b	90.24 a	130.6 ± 3.92 b
4	Sucrose	37	4.35 ± 0.082 a	100 a	92.8 ± 3.05 a
4	Sucrose + GNA	32	5.75 ± 0.198 b	87.50 b	111.3 ± 5.17 b
2	Sucrose	30	6.71 ± 0.161 a	96.67 a	90.7 ± 2.36 a
2	Sucrose + GNA	30	9.72 ± 0.778 b	90.00 a	101.6 ± 9.80 a

Larvae were provided daily with different numbers of *Ephestia kuehniella* eggs and additionally with a 2M sucrose solution containing GNA (1%, w:v) or a pure sucrose solution. Values within groups ("number of eggs") followed by different letters differ significantly ($P<0.05$).

Assessing total *C. carnea* imaginal development

Impact of GNA on total C. carnea *imaginal development*

GNA was found to cause a significant detrimental effect on *C. carnea* imaginal larval development and survival (Table 3.3). Mean larval development time was significantly prolonged by 21% ($P<0.0001$), while larval survival was reduced by 14%

(*P*=0.047) in GNA-fed insects. There were no significant differences (*P*>0.05) in pupal development time, pupal survival, and adult d.w. Statistical power analysis was conducted using the observed control means and variances for adult lacewing d.w. to calculate the power to detect a 20% difference between treatment groups. The power was found to be >99% at α=0.05 both for female (effect size *d*=1.17) and male (*d*=1.43) weights. A significant difference between the bioassays was observed for larval (*P*<0.0001) and pupal development times (*P*=0.012); however, in both bioassays, GNA caused a prolongation in larval development time.

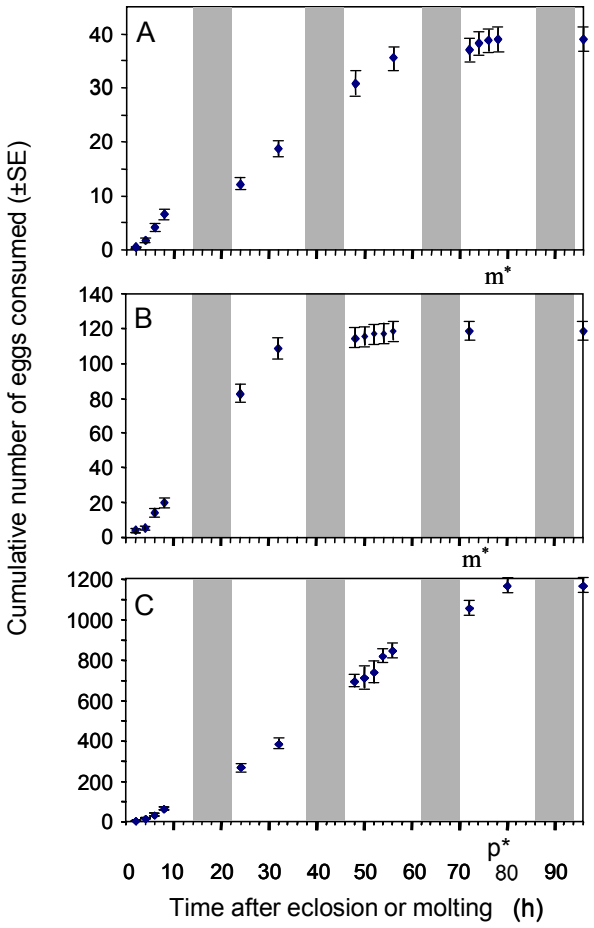

Figure 3.1 Average (±SE) cumulative consumption of *Ephestia kuehniella* eggs by (A) L1 (*n*=11), (B) L2 (*n*=14), and (C) L3 (*n*=14) *Chrysoperla carnea*. Grey areas refer to the night, white areas to the day. m*, first insects molt to next larval stage, p*, first insects pupated. After 96h all insects were either molted or pupated.

Table 3.3 Impact of snowdrop lectin (GNA) on larval development time (days), survival (%), and adult dry weight (µg) of *Chrysoperla carnea*.

Food solution	*n*	Larval development[*1] (days ± SE)	Larval survival (%)	Pupal development[*2] (days ± SE)	Pupal survival (%)	Adult dry weight (µg ± SE) female	male
Sucrose	69	11.2 ± 0.16 a	97.10 a	11.1 ± 0.08 a	82.09 a	3059 ± 97.1 a	2518 ± 69.0 a
Sucrose + GNA	71	13.6 ± 0.18 b	83.10 b	11.1 ± 0.09 a	81.36 a	3113 ± 99.9 a	2712 ± 110.6 a

Larvae were fed a 2M sucrose solution containing 1% GNA (w:v) or a pure sucrose solution on the first day of each larval stage. Subsequently, larvae were fed exclusively with *Ephestia kuehniella* eggs *ad libitum* to continue development. Values followed by different letters differ significantly ($P<0.05$).

Impact of different insecticidal proteins on imaginal C. carnea

Ingestion of avidin had a strong toxic effect on *C. carnea* larvae (Table 3.4). Out of 58 larvae, only two reached the pupal stage. These two larvae also suffered a longer development time (prolongation of 39%) when compared to sucrose-fed control larvae. 24% of the larvae died during the L1 stage and mortality during the L2 was 75%. Most larvae died during the molting process. When comparing the sucrose treatment (negative control) and GNA (our standard), a significant prolongation in larval development time of 13% was observed ($P<0.0001$), while larval survival was not affected ($P>0.05$). Ingestion of SBTI or the two Cry proteins did not affect larval development ($P>0.05$) or survival ($P>0.05$) when compared with the negative control. Pupal development time, pupal survival, and adult d.w. were not affected by any of the insecticidal proteins tested ($P>0.05$).

There was a significant difference between the two bioassays conducted for larval development time ($P=0.002$).

Intake of sucrose solution by *C. carnea* larvae

After 24h of sucrose feeding the weight gained by the different larval stages was 17.0µg, 207.5µg and 715.0µg for L1, L2 and L3, respectively. Assuming that 1µl sucrose solution (2M) weighs 1120.65µg, L1 consumed 0.015µl, L2: 0.185µl, and L3: 0.638µl of the provided food solution. The amount of toxin insects consumed on the first day of each

instar can be estimated from these weights. As the 2M sucrose solution contained 1% insecticidal protein, an individual larva consumed a total of 8.383µg protein during its development (L1: 0.152µg, L2: 1.852µg, L3: 6.380µg). In the case of the two Cry proteins that were provided at a concentration of 0.1%, larvae consumed 0.839µg toxin in total.

Table 3.4 Impact of different insecticidal proteins on development time (days), survival (%), and adult dry weight (µg) of *Chrysoperla carnea*.

Food solution	***n***	**Larval development (days ± SE)**	**Larval survival (%)**	**Pupa development (days ± SE)**	**Pupal survival (%)**	**Adult dry weight (µg ± SE)**	
						female	**male**
Sucrose	54	12.8 ± 0.12	92.59	11.0 ± 0.09	72.00	3107 ± 87.0	2612 ± 94.2
Sucrose + GNA	57	14.5 ± 0.13 *	87.72	11.1 ± 0.12	78.00	3169 ± 43.4	2445 ± 40.0
Sucrose + SBTI	51	13.0 ± 0.12	88.24	11.2 ± 0.08	75.56	3257 ± 113.5	2807 ± 92.4
Sucrose + Cry1Ab	57	12.8 ± 0.14	87.72	11.1 ± 0.07	60.00	3300 ± 121.1	2594 ± 99.2
Sucrose + Cry1Ac	57	12.7 ± 0.12	78.95	11.0 ± 0.07	66.67	3522 ± 114.5	2668 ± 100.0
Sucrose + avidin	58	17.8 ± 0.05 [1]	3.45% *	11.5 ± 0.00 [1]	100 [1]	-	1734 ± 45.6 [1]

[1] Excluded from statistical analysis due to low number of replications.

Larvae were fed a 2M sucrose solution containing either one of five different proteins or a pure sucrose solution on the first day of each larval stage. Subsequently, larvae were fed exclusively with *Ephestia kuehniella* eggs *ad libitum* to continue development. The following insecticidal proteins were dissolved in the sucrose solution: GNA (1%, w:v), SBTI (1%), Cry1Ab (0.1%), Cry1Ac (0.1%) and avidin (1%). Means that differed significantly from the control (sucrose) (corresponding Bonferroni-Holm adjusted *P*-values <0.05) are marked with an asterisk.

Discussion

The aim of this study was to evaluate the combined impact of prey and insecticidal protein on *C. carnea* development. Larvae were fed a combination of prey and sucrose solution allowing development. The intake of the sucrose solution, in which the test proteins could be dissolved, was ensured using two different approaches. In the first approach, *C. carnea* larvae were provided with a restricted number of prey items in

addition to the sucrose solution while in the second approach, larvae were alternately fed the sucrose solution and unrestricted prey.

To understand the feeding behavior of the different instars of *C. carnea*, their prey consumption was examined. A single *C. carnea* larva was found to consume an average of 1330 *E. kuehniella* eggs throughout its larval development when food was provided *ad libitum*. Freshly hatched or molted larvae were observed to consume very few eggs. During the last larval stage (L3), 82% of the whole intake was consumed. This finding is similar to earlier reports by Canard (2001) and Zheng et al. (1993a; b), who studied the influence of aphid or moth egg consumption on several life-table parameters of *C. carnea*. First instars (L1) required about four days to develop to the next larval stage. During this period they consumed an average of 39 eggs, which relates to about ten eggs per day. Based on this information, a restricted diet could be defined for this larval stage to ensure consumption of additionally provided sucrose solution. A beneficial sucrose effect became evident when L1 were provided with six or less *E. kuehniella* eggs per day. When larvae received ten or *ad libitum* eggs per day, no need for additional sucrose feeding became evident. A benefit of sucrose feeding was demonstrated in a shortened development time (when fed two, four eggs) and/or an increased dry weight of the freshly emerged L2 (four, six eggs). Interestingly, L2 dry weight was not increased when larvae were fed with two eggs per day and additionally provided with sucrose solution. This might be due to the fact that larvae that did not receive sugar as a supplemental food developed very slowly and were therefore potentially able to consume more eggs. Sucrose feeding did not have a beneficial effect on larval survival in any of the treatments, except when larvae received two eggs per day. While larvae receiving only two eggs per day suffered about 75% mortality, this was reduced to 14% when sucrose was added. Overall our data indicate that *C. carnea* larvae can utilize a carbohydrate source as an alternative food. It has earlier been reported that larvae feed on extra-floral nectaries in the field (Limburg and Rosenheim 2001), which might benefit them when prey are scarce. Recent studies have revealed that honeydew is also accepted as a food source in the presence of aphid prey (Hogervorst et al. 2008).

Since L1 *C. carnea* provided with two, four or six *E. kuehniella* eggs per day clearly fed on the additionally provided sucrose solution, a bioassay with GNA added to the sucrose solution was conducted. Measurement endpoints were L1 development and survival and dry weight of freshly emerged L2. GNA consumption was found to affect at least one of the recorded life-table parameters independent from the number of eggs provided. While

larval development time was significantly prolonged in the GNA treatment independent from the number of eggs that were provided, survival was only decreased by GNA ingestion when *C. carnea* larvae were fed four eggs per day. Interestingly, dry weight of the L2 was significantly increased by GNA feeding in the four and six egg treatments. This is rather unexpected since the insects that consumed GNA appeared to be sublethally affected resulting in a prolonged developing time. Since *C. carnea* larvae are not able to digest GNA (Hogervorst et al. 2006) they cannot use it as an additional protein source. Our results therefore suggest that the GNA-fed larvae may have gained a nutritional advantage as a result of being able to prey on more moth eggs when compared to larvae that had not fed on the lectin. Consequently, it appears that GNA does not interfere with the insect's utilization of food and that 'sublethally' affected *C. carnea* larvae still would be able to fulfill their role as predators in a GNA-expressing transgenic crop.

Although the described bioassay is useful to assess the impact of insecticidal proteins on *C. carnea* larvae, it is restricted to measurement endpoints of the L1 stage. Since the insects are under constant stress due to limited food supply, results obtained with this bioassay are highly variable and depend for example on the quality of the provided *E. kuehniella* eggs. The latter was found to vary with the age of the moth eggs (unpublished observations). It will thus be difficult to establish a bioassay that ensures defined intake of sucrose solution (and thus insecticidal protein) by all three larval stages.

For this reason, a second approach was followed allowing the complete development of *C. carnea* while ensuring the intake of an additional sucrose solution. The larvae were fed with sucrose solution during the first day of each larval stage and subsequently provided with *ad libitum* prey to continue their development. This approach allowed additional measurement endpoints to be analyzed including the duration of the total larval development and survival as well as pupal development time, adult emergence and adult weight. The experiment revealed a 21% extended larval period in GNA fed larvae, and also a significant increase in larval mortality. Pupal development time and survival and dry weight of the emerging adult lacewings remained unaffected by GNA consumption during the larval stages. After establishing this bioassay, it was used to assess the impact of other insecticidal proteins on *C. carnea*.

When larvae were provided with avidin, most of them were not able to successfully molt and died. This was observed particularly with second instars where in total 75% of the larvae died, more than 60% during molting into the L3. Avidin binds to and prevents the

absorption of vitamins, such as biotin and biotin-dependent carboxylases that are required for the deposition of fat reserves and their subsequent utilization. Larval death may be the consequence of insufficient fat reserves and/or the inability to use them, which becomes most apparent during molting, a phase were no feeding takes place (Markwick et al. 2001; Malone et al. 2002). This bioassay is the first demonstration of a direct effect of avidin on a predatory insect belonging to the order Neuroptera. So far negative effects of avidin have been reported for herbivorous arthropods belonging to the orders of Coleoptera, Lepidoptera, Acaridae, and Orthoptera at concentrations comparable to the one used in our study (Levinson et al. 1992; Morgan et al. 1993; Kramer et al. 2000; Markwick et al. 2001; Malone et al. 2002, Zhu et al. 2005). The ingestion of the protease inhibitor SBTI did not cause any effects on the assessed *C. carnea* life-table parameters. This is surprising since it has been shown that the proteolytic digestion of *C. carnea* larvae is dominated by serine proteases that should be inhibited by SBTI (Ferran et al. 1976; Yazlovetsky 1992; Mulligan 2006). One explanation for the lack of effects could be that *C. carnea* larvae are able to adapt their digestive metabolism to counteract the presence of protease inhibitors in food of their herbivorous prey. This has been reported for other insect predators including the predatory stinkbug *Perillus bioculatus* (Fabricius) (Hemiptera: Pentatomidae) (Bouchard et al. 2003a; b), the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (Ferry et al. 2003), and different carabid beetles (Burgess et al. 2002; Ferry et al. 2005; Mulligan et al. 2006).

In addition to these rather unspecific insecticidal proteins, two Lepidoptera-specific *Bt* toxins, Cry1Ab and Cry1Ac, were investigated. The *Bt* toxins did not cause any effect on the *C. carnea* larvae confirming earlier results (Romeis et al. 2004; Rodrigo-Simón et al. 2006). The lack of effects for the two Cry1A proteins is not surprising due to their mode of action (Schnepf et al. 1998) and the fact that the midgut of *C. carnea* lacks the specific receptors that are a pre-requisite for toxicity (Rodrigo-Simón et al. 2006). So far no evidence has been provided that *Bt*-transgenic crops expressing Cry1Ab or Cry1Ac cause direct toxic effects on insect predators or parasitoids (Romeis et al. 2006). Based on the amount of sucrose solution consumed by a *C. carnea* larvae throughout its larval development we calculated that a single larvae had consumed approximately 0.84µg Cry protein. In the field, maximum exposure is likely through feeding on spider mites [e.g., *Tetranychus urticae* Koch (Acarina: Tetranychidae)], which have been reported to contain high amounts of Cry1Ab (between 2.5 and 6µg protein / g fresh weight) compared to other

herbivores when feeding on *Bt* maize (Dutton et al. 2002; Obrist et al. 2006a; b). It has been reported by Sengonca and Coeppicus (1985) that *C. carnea* require close to 8,000 *T. urticae* (corresponding to about 100mg fresh weight) for their development in the absence of alternative prey. Thus *C. carnea* larvae would consume a maximum of 0.6µg Cry1Ab toxin when feeding exclusively on spider mites that had fed on *Bt* maize. However, the actual exposure in the field is likely to be much lower since *C. carnea* larvae are known to predominately feed on aphids that contain no or only trace amounts of *Bt* protein (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002). Based on this we can conclude that a larva in our bioassay consumed significantly more Cry protein than it could possibly ingest in a *Bt* crop.

Implications

Provision of insecticidal proteins dissolved in a sucrose solution allows to assess the hazards that these proteins pose to *C. carnea* larvae. This method might similarly be applied for other entomophagous arthropods for which no suitable artificial diets are available to conduct toxicity tests. To provide *C. carnea* larvae with a sugar solution and moth eggs as prey allows the larvae to develop until the adult stage, and to assess a range of important life-table parameters. This is an advantage over the method used earlier by Romeis et al. (2004) where *C. carnea* larvae were exclusively fed with a sucrose solution containing an insecticidal protein but no additional protein source. Thus they were confined to a certain instar. Because *C. carnea* larvae are able to compensate for poor food quality (Zheng et al. 1993 a; b), effects of the insecticidal proteins that are ingested only during the first day of each larval stage might be partly compensated by the subsequent *ad libitum* feeding of a high quality prey (moth eggs), potentially masking small effects. Nevertheless, we believe that the low larval mortality (≤8%) makes our approach superior to the use of sub-optimal artificial diets that have been found to cause control mortalities up to 21% (Hilbeck et al. 1998). Ideally, a good quality artificial diet would be developed that allows constant exposure of the *C. carnea* larvae to high concentrations of insecticidal protein. Since in our bioassays pupal mortality was consistently found to be relatively high, pupal weight might be selected as the final measurement endpoint. At the concentrations tested, both GNA and avidin have resulted in consistent effects on two major measurement endpoints, i.e., larval development and survival. These two proteins could thus serve as positive controls for orally active toxins when novel insecticidal proteins are assessed.

GNA was found to prolong the larval development by around 20% (Table 3.3). Statistical power analysis revealed that a total of 40 larvae per treatment would be sufficient to detect this difference in larval development at α=0.05 with a power of 80%.

Even though our study has revealed significant effects of both GNA and avidin on different life-table parameters of *C. carnea* larvae this does not imply that transgenic plants expressing these proteins would pose a risk to this predator under field conditions. Hazard assessment studies that are conducted as part of a regulatory risk assessment are generally initiated with toxicity tests at elevated exposure doses, for example at 10 times the expected environmental exposure (EEC) (Garcia-Alonso et al. 2006; Raybould et al. 2007; Romeis et al. 2008). The EEC is defined as the worst-case concentration of the insecticidal protein to which a particular species may be exposed in the field. If effects are observed, additional studies are conducted to confirm whether an effect is still present under more realistic rates and routes of exposure to the protein (Garcia-Alonso et al. 2006; Romeis et al. 2008). The protein expression levels in the transgenic plant are the basis to define the EEC. This adds an additional safety factor to the assessment since insecticidal proteins expressed in transgenic plants do not appear to accumulate in herbivores but get more diluted as has been shown for *Bt* Cry proteins (Harwood et al. 2005; Vojtech et al. 2005; Obrist et al. 2006a; Torres et al. 2006) but also for protease inhibitors and avidin (Bell et al. 2003; Christeller et al. 2005). Since GNA- and avidin-expressing plants for insect pest control have not been commercialized, expression levels in the plant and thus the EEC for plant-dwelling predators is difficult to predict. In the case of GNA, different studies have shown that a concentration of 0.1% is required to achieve a detectable impact on aphids, one of the main targets of this protein (Down et al. 1996; Sauvion et al. 1996; Couty et al. 2001). Consequently, GNA-expressing plants that target aphid pests may need to contain higher GNA levels in the phloem sap, making our 1% GNA concentration a realistic dose for testing. The situation is different for avidin. Studies with avidin-expressing GE maize plants have revealed that expression levels of about 300 ppm would be required to provide good control of major stored product insect pests while individual transformed plants contained up to 2500 ppm of avidin (Kramer et al. 2000). In this context our 1% concentration (10,000 ppm) appears to be rather high. Thus once plants expressing GNA or avidin become available additional studies would be required to assess if they actually pose a risk to *C. carnea* under more realistic exposure conditions.

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Chapter four

Performance of cotton aphids on Indian *Bt* cotton varieties[3]

[3] Lawo NC, Wäckers FL, Romeis J. Performance of cotton aphids on Indian *Bt* cotton varieties. Submitted.

Abstract

Bt cotton varieties expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) are grown worldwide for the management of pest Lepidoptera. To prevent non-target pest outbreaks and to retain the biological control function provided by predators and parasitoids, the potential risk that *Bt* crops may pose to natural enemies is addressed prior to their commercialization. Aphids play an important role in agricultural systems since they serve as prey or host to a number of predators and parasitoids and their honeydew is an important energy source for several arthropods. To explore possible indirect effects of *Bt* crops we here examined the impact of *Bt* cotton on aphids and their honeydew.

In climate chambers we accessed the performance of cotton aphids, *Aphis gossypii* (Hemiptera: Aphididae) when grown on three Indian *Bt* (Cry1Ac) cotton varieties (MECH 12, MECH 162, MECH 184) and their non-transformed near isolines. Furthermore, we examined whether aphids pick up the *Bt* protein and analyzed the sugar composition of aphid honeydew to evaluate its suitability for honeydew-feeders.

Plant transformation did not have any influence on aphid performance. However, some variation was observed among the three cotton varieties which might partly be explained by the variation in trichome density. None of the aphid samples contained *Bt* protein. As a consequence, natural enemies that feed on aphids are not exposed to the Cry protein. A significant difference in the sugar composition of aphid honeydew was detected for the factors cotton variety as well as transformation. However, it is questionable if this variation is of ecological relevance, especially as honeydew is not the only sugar source parasitoids feed on in cotton fields.

Our study allows the conclusion that *Bt* cotton poses a negligible risk for aphid antagonists and that aphids should remain under natural control in *Bt* cotton fields.

Introduction

Heliothine caterpillars, such as *Helicoverpa* spp. (Lepidoptera: Noctuidae) or *Heliothis virescens* (Lepidoptera: Noctuidae) and the pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) are key pests of cotton world wide. To control these polyphagous herbivores, farmers routinely use large amounts of broad-spectrum chemical insecticides, killing many non-target arthropods in the process. However, since Heliothine caterpillars have a track-record of developing resistance to almost all the insecticides used

for their control (Forrester et al. 1993; Kranthi et al. 2002; Terán-Vargas et al. 2005), alternatives have to be developed. One option is the use of insect-resistant genetically engineered (GE) varieties expressing lepidopteran-active Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*). So-called *Bt* cotton plants are grown commercially since 1996. Most of today's varieties express the *Bt* protein Cry1Ac either alone or in combination with Cry2Ab, protecting plants from damage by the main pest Lepidoptera (Naranjo et al. 2008).

In 2007, *Bt* cotton was grown in nine countries (USA, Argentina, Brazil, India, China, South Africa, Australia, Mexico, and Colombia) (James 2007). As cotton is among the most intensively sprayed of all field crops, the introduction of *Bt* cotton has had a tremendous impact in terms of reducing insecticide use resulting in economic, environmental and human health benefits (Fitt 2008; Qaim et al. 2008). Brookes and Barfoot (2008) estimated that the first eleven years of *Bt* cotton production (1996-2006) have resulted in a 22.9% reduction in insecticide active ingredient application in cotton world wide. Insecticide reductions were most significant in India (Qaim et al. 2006) and China where the improved pest control also related to significant increases in yield (Fitt 2008). In India, *Bt* cotton hybrids expressing the *Bt* gene *cry1Ac* are cultivated on an increasing area since their introduction in 2002. Almost tripling the area to 3.8 million hectares in 2006, India became the largest *Bt* cotton growing country in the world and in 2007, 131 *Bt* cotton hybrids were grown on a total of 6.2 million hectares (James 2007).

Due to the reduction of broad spectrum insecticides in *Bt* cotton, secondary pests which are not targeted by the *Bt* protein survive and occasionally reach pest status (Naranjo et al. 2008). To retain the biological control function provided by naturally occurring antagonists of herbivores, i.e. predators and parasitoids, and to prevent non-target pest outbreaks, the potential risk that GE crops may pose to natural enemies is addressed as part of the environmental risk assessment prior to the commercial release of any novel GE crop (Garcia-Alonso et al. 2006; Romeis et al. 2008a). Several studies examined the effect of *Bt* crops on herbivores and arthropod natural enemies in recent years confirming the high selective mode of action of the deployed *Bt* Cry proteins (Romeis et al. 2006; Wolfenbarger et al. 2008).

Aphids generally play an important role in agricultural food webs since they serve as hosts or prey for a variety of parasitoids and predators. Consequently, the question whether aphids are affected by the *Bt* crop and whether they expose their natural enemies

to the plant-expressed *Bt* protein is of high relevance. Studies available to date provide no evidence that *Bt* crops, expressing Cry1 proteins for the control of pest Lepidoptera, cause direct adverse effects on aphids (Raps et al. 2001; Dutton et al. 2002; Schuler et al. 2005). This is not surprising, since the *Bt* protein does not appear to be present in the phloem on which the aphids feed (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002). However, occasionally studies have reported *Bt* Cry proteins in aphid samples (Zhang et al. 2006; Burgio et al. 2007) and thus a route of exposure to natural enemies exists.

Further, aphids and other phloem feeders produce honeydew which is an important source of carbohydrates for sugar feeding arthropods, including hymenopteran parasitoids and aphid predators (Wäckers 2005; Hogervorst et al., 2007). Sugars can enhance parasitoid reproductive fitness by increasing their longevity, fecundity and/or parasitism rate (Fadamiro and Heimpel 2001; Siekmann et al. 2001; Wäckers 2001; Winkler et al. 2006). However, honeydew can be a relatively unsuitable sugar source (Wäckers et al. 2008) as a result of unfavorable sugar composition (Wäckers 2001; Hogervorst et al. 2007). Honeydew nutrient composition could also be altered as a result of plant transformation.

Therefore, we investigated in standardized laboratory bioassays if cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) performance was affected on three Indian *Bt* cotton varieties (MECH 12, MECH 162, MECH 184), expressing the Cry1Ac protein and their corresponding non-transformed near isolines. In addition to examining several aphid life-table parameters, we clarified whether aphids pick up the *Bt* protein. To gain any insight on the impact of *Bt* on the nutrient composition of aphid honeydew the sugar composition of honeydew was examined.

Material and Methods

Aphis gossypii

A. gossypii were provided by Syngenta (Stein, Switzerland) and were subsequently reared continuously on cotton (*Gossypium hirsutum*) in a climate chamber at 25±1°C, 70±10% r.h., and a 16-h photoperiod.

Cotton plants

Three *Bt*-transgenic *Gossypium hirsutum* varieties (MECH 12, MECH 162, and MECH 184; event BG-I, Mahyco Seeds Ltd.), expressing the Cry1Ac protein (*Bt* plants)

and their corresponding non-transformed near isolines (non-*Bt* plants) were used in the bioassays. All three *Bt* varieties have been commercially grown in India since 2002.
Plants were grown individually in humus-rich sterilized soil in plastic pots (3l) and fertilized weekly with 10N:10P:8K at a concentration of 20ml/l. Plants were grown in a climate chamber, illuminated by metal halide lamps (EYE Clean-Ace lamps, MT400/DL/BH; Iwasaki Electric Co., Ltd.) at 25°C±1°C day/20°C±1°C night, 70%±10% r.h. and a 16-h photoperiod (20,000 lux during daytime). Changes between day and night conditions occurred gradually to stimulate natural dusk and dawn. Metal halide lamps featured a light color close to daylight. To guarantee stable humidity, plants were placed individually in plant dishes (16cm in diameter; 3cm high). Until the eighth leaf stadium, plants were watered once a day, thereafter twice a day. Prior to bioassays cotton plants were controlled for any insect damage to avoid unintended induction of the cotton innate resistance mechanisms. If pests were found, such plants were excluded from the bioassays.

Experimental set-up

For one bioassay, 15 metal trays (45x90cm) were placed in two rows on the floor of the climate chamber, each containing six, three-week old plants (two to five true leaves). The plants, one of each *Bt* and non-*Bt* varieties, were ordered randomly per tray (complete block design). The bioassay was repeated twice resulting in a total of 30 plants per treatment.

Aphid performance

A group of approximately 50 reproductive aphids from the permanent culture were allowed to settle on a plant and to give birth to nymphs. After 6h, two to three newborn nymphs (F_1) were brushed carefully onto the last fully expanded leaf of the respective variety and covered with a clip cage (2cm in diameter; 1cm high). Clip cages featured a hole sealed with fine-mesh netting to provide air-circulation. After three days, surplus nymphs were removed at random, to ensure that the performance of a single nymph was monitored on each plant. Every morning and evening, aphid mortality was recorded. After reaching adulthood the F_1 nymphs were counted and removed daily. This procedure was conducted until the aphid died.

The following aphid life-table parameters were obtained this way: nymphal development time (time from birth to first nymphal production; D), number of nymphs

produced during a time span equal to D starting at nymph production (FD), mean reproductive rate during the reproductive period observed (daily fecundity; DF), total fecundity per female during the period observed (TF), adult longevity (AL), total longevity (TL), and intrinsic rate of population increase (r_m). The r_m was estimated based on the daily age-specific fecundity (m_x) and the age-specific survival rate (l_x) and using the equation of Birch (1948) (1) thereafter.

$$\sum_x l_x \cdot m_x \cdot e^{-r_m x} = 1 \quad (1)$$

Aphids which died before producing any nymphs were excluded from the analysis. Aphids which were lost during the observation period were censored.

Trichome density

After the death of the aphid, the leaf below the one exposed to the aphid was collected and stored at -20°C until further examination. For each leaf, three trichome counts were taken from a central section, excluding the primary and secondary leaf veins. Depending on trichome density, measurements were done on an area of 16 or 64mm² under a binocular microscope (WILD, Heerbrugg) with the help of an ocular measuring grid (Leica). Six to eight samples were counted per treatment and subsequently data were calculated as trichome density per cm².

Quantification of Cry1Ac protein in leaves and aphids

To confirm Cry1Ac expression of the transgenic cotton plants used in both bioassays, a total of 12 to 13 leaves per variety of the *Bt* plants and three leaves per variety of the non-*Bt* plants were collected after the end of the bioassays. Approximately 100mg fresh weight (f.w.) of the leaves on which *A. gossypii* had fed were sampled, flash frozen, weighed, lyophilized and weighed again (approximately 20mg dry weight).

To quantify the level of Cry1Ac in *A. gossypii*, 60 to 70 reproductive aphids were brushed on the last fully expanded leaf of three-week old *Bt* or non-*Bt* cotton plants and allowed to reproduce for five weeks. Subsequently, leaves infested with aphids were transferred to two to three-week old cotton plants of the same variety and transformation status and reared under the same climatic conditions. Aphids were allowed to settle and reproduce for one to two additional weeks. Thereafter, 30 to 80mg of aphids was collected using a flexible tube on which gauze and a tip were attached. Before weighing and

lyophilizing, each sample was checked under a binocular microscope to confirm that there was no contamination with other pests e.g. spider mites and/or thrips or leaf pieces. Three to five samples per *Bt* variety and one per non-*Bt* variety were analyzed. From the same plants on which aphids were kept, samples from two of the most heavily infested leaves were also taken for *Bt* protein measurements.

The amount of Cry1Ac protein in leaf and aphid material was measured using an enzyme linked immuno-sorbent assay (ELISA) from Agdia (Elkhard Indiana, USA). After adding phosphate buffered saline with Tween buffer (PBST, provided in the kit) at a ratio of 1:10 (sample material f.w.:buffer) and a 5mm tungsten carbide bead, leaf samples were macerated for 100sec at 15Hz and aphid samples for 40sec at 30Hz, using a mixer mill MM300 (Retsch, Haan, Germany) fitted with 24 tube-adapters (Qiagen, Hombrechtikon, Switzerland). Samples were centrifuged for 5min at 13,000 × g and leaf samples were diluted 1:15 with PBST, while aphid samples were not diluted. Subsequently, instructions from the kit were followed. After stopping the color development with 3M sulfuric acid, spectrophotometric measurements were conducted with a microtiter plate reader (SpectrafluorPlus, Tecan, Männedorf, Switzerland) at 450nm. A standard curve with purified high quality Cry1Ac provided by M. Pusztai-Carey (Dept. Biochemistry, Case Western Reserve University, Cleveland, OH, USA) was established. Concentrations were calculated using linear regression analysis. The limit of detection for aphid extracts was calculated by multiplying three times the standard deviation of eleven buffer-only and control ODs with the slope of the standard curve. Based on µg/g f.w., the limit of detection (LOD) was 0.002.

Sugar analysis of aphid honeydew

For sugar analysis, approximately 50mg of aphids was collected from the rearing colony in a clip cage (5.2cm in diameter; 1cm high) in the afternoon and allowed to settle overnight on the youngest fully expanded cotton leaf. The cage was removed the following morning and the aphids were allowed to settle one additional day.

For honeydew collection, Petri dishes were placed under the aphid infested cotton leaves for 5 to 6h. Thereafter, honeydew-sprinkled Petri dishes were placed upside down at 23±1°C and 85±5% r.h., with a water-saturated piece of cotton wool on the bottom. After 2h, when the viscosity of the honeydew was reduced through hygroscopy, approximately 0.5µl honeydew was collected with 5µl micropipettes (Blaubrand, intra Mark).

Subsequently, the honeydew was dissolved in 20µl ethanol (70%) and stored at -80°C until further analysis.

For sugar analysis of aphid honeydew, five to seven samples per treatment were tested for 16 sugars: sucrose, fructose, glucose, erlose, trehalose, mannitol, sorbitol, melibiose, raffinose, stachyose, lactose, melezitose, mannose, rhamnose, maltose and galactose. Before analysis, samples were diluted 400-fold with 18MΩ water and homogenized using a pestle. Subsequently, samples were filtered through a chromacol syringe filter. Samples were analyzed using a Dionex ICS 3000 Ion Chromatography system (Dionex Corp., Sunnyvale, CA, USA) and concentrations of the individual sugars were calculated using the programm PEAKNET Software Release 5.1 as described by Steppuhn and Wäckers (2004). The limit of quantification for any honeydew sample was set at 0.001µg. Measurements below 0.001µg were set to 0.

Data analysis

Since all assumptions were met, FD, DF, and TF were tested with a multivariate ANOVA including the factors *Bt*/non-*Bt*, variety, and experiment. Means were subsequently compared using the Tukey-Kramer Test. D, AL, and TL were analyzed by means of a Cox regression model including the factors *Bt*/non-*Bt*, variety, and experiment. Insects which were lost over the observation period were censored. To estimate the confidence intervals for the r_m, the bootstrap percentile method with 10,000 resamples was performed (Davison and Hinkley, 1997).

Trichome density was compared among all treatments with a Kruskal-Wallis ANOVA followed by pair-wise comparisons using the Mann-Whitney-*U* test, adjusted for ties. The two-sided exact *P*-value was subsequently corrected with the Bonferroni-Holm procedure. Statistical analyses were conducted using the software package Statistica (version 6, StatSoft Inc., Tulsa, OK, USA) or R (bootstrap). The α-level was 5% in all statistical analyses.

To calculate the sugar composition of the different *Bt* and non-*Bt* varieties, firstly, the lengths of gradient was calculated. Since the value was <3, the use of linear models was justified. Therefore, an unconstrained linear ordination (indirect gradient analysis) using Principal Components Analysis (PCA) on sugar percentages (species data) was conducted to visualize the important patterns in the data. Additionally, the distribution of the sugar concentrations was investigated by constrained linear ordination (direct gradient

analysis), using Redundancy Analysis (RDA) to analyze the variability between *Bt* and non-*Bt* plants and/or among varieties based on the composition of different sugars. The transformation (*Bt*/non-*Bt*) and variety (MECH 12, MECH 162, MECH 184) were used as explanatory variables. The significance of each axis of the RDA was tested using a Monte Carlo permutation test with unrestricted permutations (n=999), followed by forward selection to determine the relative importance and significance of each environmental variable.

The software package CANOCO 4.5 was used to conduct the multivariate analysis (ter Braak and Šmilauer 2002).

Results

Aphid performance

With one exception, statistical analyses showed neither a *Bt*-transformation nor a cotton variety effect for any of the aphid life-table parameters assessed (P>0.05; Table 4.1). The exception was a significant variety effect for FD (P=0.033) which appears to be due to a discrepancy between the varieties MECH 12 and MECH 184. A significant experimental effect was calculated for the parameters FD, DF, and TL; however no interaction among the different factors occurred.

Trichome density

There was a significant difference in the trichome density among the six different cotton plants (P=0.033) (Fig. 4.1). Conducting pair-wise comparison, a significantly greater trichome density was found for *Bt* MECH 184 compared to the other two *Bt* cotton varieties (*Bt* MECH 12; P=0.015 and *Bt* MECH 162; P=0.010). For the control varieties a higher trichome density was observed for MECH 184 compared to MECH 12 (P=0.010). No difference in the trichome density was observed between *Bt* and non-*Bt* leaves for any of the three pairs (P>0.05).

Table 4.1 Performance of *Aphis gossypii* on *Bt* and non-*Bt* cotton varieties (*n*=24 to 30).

Parmeter [a]	Non-*Bt*			*Bt*			Variety effect	Trans-formation effect
	MECH 12	MECH 162	MECH 184	MECH 12	MECH 162	MECH 184		
rm [b]	0.333 (0.300 to 0.343)	0.366 (0.327 to 0.377)	0.371 (0.310 to 0.392)	0.335 (0.320 to 0.348)	0.355 (0.306 to 0.364)	0.368 (0.320 to 0.383)		
FD	14.6 (13.1 to 16.1)	13.1 (11.5 to 14.6)	11.6 (9.9 to 13.4)	14.0 (12.0 to 16.0)	14.1 (12.2 to 16.0)	12.6 (10.9 to 14.4)	*P*=0.033	*P*=0.499 [c]
DF	2.03 (1.82 to 2.23)	1.99 (1.77 to 2.20)	1.72 (1.54 to 1.89)	1.91 (1.67 to 2.14)	1.92 (1.69 to 2.15)	1.88 (1.66 to 2.10)	*P*=0.181	*P*=0.968 [c]
TF	31.5 (26.9 to 36.1)	28.2 (23.6 to 32.8)	27.7 (22.8 to 32.7)	32.5 (26.5 to 38.5)	26.9 (22.1 to 31.6)	31.4 (26.2 to 36.6)	*P*=0.164	*P*=0.527 [c]
D	6.3; 6.0 to 6.5	6.0; 5.0 to 6.5	6.5; 5.5 to 7.0	6.0; 5.5 to 7.0	6.0; 5.5 to, 7.0	6.0; 5.0 to 6.5	*P*=0.889	*P*=0.815 [d]
AL	18.3; 14.5 to 21.0	15.0 13.5 to 18.5	18.0 15.0 to 21.5	17.5 14.5 to 23.0	15.8 13.0 to 18.0	19.8 15.3 to 23.3	*P*=0.385	*P*=0.759 [d]
TL	24.0 21.0 to 27.0	21.0 18.5 to 24.5	24.0 17.5 to 27.0	22.8 19.0 to 29.0	21.5 19.0 to 24.5	25.3 19.5 to 28.5	*P*=0.255	*P*=0.571 [d]

Printed estimate refers to median and variability to first to third quartile in case of Cox-proportional hazard analysis, and to 95% confidence interval of the mean otherwise.

[a] (r_m) intrinsic rate of increase (days); (FD) number of nymphs produced during D; (DF) daily fecundity; (TF) total fecundity; (D) nymphal developing time (days); (AL) adult longevity (days); (TL) total longevity (days).

[b] bootstrap percentile method.

[c] 3-way ANOVA with experiment, cotton variety and *Bt*-transformation as factors.

[d] Cox-proportional hazard analysis with experiment, cotton variety and *Bt*-transformation as factors.

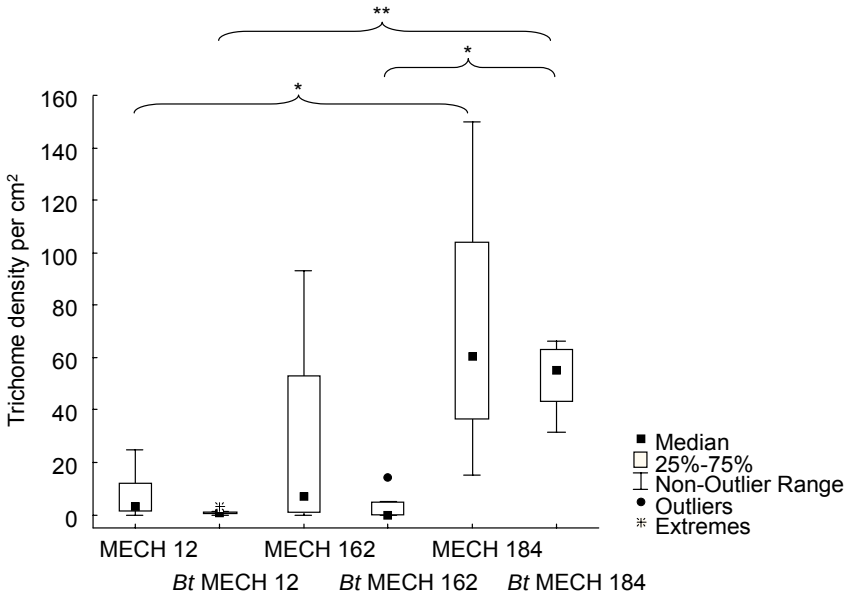

Figure 4.1 Boxplot figures showing the median trichome density per cm^2 (n=6 to 8). Brackets indicate a significant difference between two treatments; *, $P<0.05$; **, $P<0.01$. The outlier range is the range of values that fall above the upper outlier limit (+1.5 x the height of the box) and below the lower outlier limit (-1.5 x the height of the box).

Quantification of Cry1Ac protein in leaves and aphids

Leaves of cotton plants on which aphids had fed during the bioassay were expressing the Cry1Ac protein at the following levels (mean ±SE); *Bt* MECH 12: 0.58 ± 0.060µg Cry1Ac/g f.w., *Bt* MECH 162: 0.73 ± 0.089µg Cry1Ac/g f.w. and *Bt* MECH 184: 0.82 ± 0.065µg Cry1Ac/g f.w.

All ELISA readings revealed that aphids that had been kept on *Bt* cotton did not contain detectable Cry1Ac protein; i.e. readings were below the LOD of 0.002µg/g f.w. Leaves from which aphids were collected for ELISA analysis expressed the following amounts of Cry1Ac: *Bt* MECH 12: 0.34 ± 0.100µg Cry1Ac/g f.w., *Bt* MECH 162: 0.62 ± 0.191µg Cry1Ac/g f.w. and *Bt* MECH 184: 0.38 ± 0.156µg Cry1Ac/g f.w. As expected, none of the non-*Bt* cotton leaves or aphid samples contained any *Bt* protein.

Sugar analyses in honeydew

As it was not possible to determine the exact amount of sugar in honeydew samples in µg/ml, the sugar composition was presented as percentage of total sugar content.

A total of eleven sugars were found in the aphid honeydew. Dominant were the phloem sugars, sucrose and fructose, as well as the aphid-synthesized sugar erlose, that

together made up 73% (*Bt* MECH 184) to 94% (MECH 162) of the total sugar content. Smaller amounts of glucose, trehalose, maltose, mannitol, melibiose, and stachyose were detected in all samples. Sugar composition appeared to differ between *Bt* and non-*Bt* plants as well as among varieties (Fig. 4.2). Whereas, fructose levels were higher in the honeydew from non-*Bt* cotton, glucose was amplified in honeydew from *Bt* cotton (especially for *Bt* MECH 184). Interestingly, erlose levels differed noticeably among the cotton varieties. While it was the dominant sugar in the honeydew collected from MECH 162 and *Bt* MECH 162, a much lower proportion of erlose was found in the honeydew of MECH 184 and *Bt* MECH 184. The greatest proportion of maltose was detected in MECH 12 and sorbitol was measured in all non-*Bt* samples (even though at very low levels) but in none of the *Bt* varieties. Raffinose occurred just in two out of the six treatments (MECH 12 and *Bt* MECH 162).

Performing a Principle Components Analysis (PCA) to visualize the data, a negative correlation between the sugars with the greatest influence on data variability (those with the longest vector, namely erlose, glucose, and fructose) could be observed. Glucose, sucrose, and trehalose were positively correlated with each other and negatively with fructose, maltose, and raffinose (Fig. 4.3 A). A positive correlation among the amount of erlose and sorbitol and a negative correlation with melebiose, stachyose, and mannitol were observed in the data set. Looking at clusters in the biplot graphic, it was assumed that the first axis was best explained by the factor cotton variety (56%) and the second axis by the factor *Bt*-transformation (19%; Fig. 4.3 A). Performing a Redundancy Analysis (RDA) to analyze the influence of the evaluated explanatory variables [transformation (*Bt*/non-*Bt*) and variety (MECH 12, MECH 162, MECH 184)] showed a strong positive correlation between erlose, trehalose, raffinose, and stachyose with the variety MECH 162, and a negative correlation with the variety MECH 184 (Fig. 4.3 B). Glucose, trehalose, raffinose, and stachyose were positively correlated with *Bt* cotton plants, and negatively with the variety MECH 12. The sugars mannitol, melebiose, and sucrose were positively correlated with the variety MECH 184 and negatively with the variety MECH 162. Sorbitol, maltose and fructose were positively correlated with the variety MECH 12 and non-*Bt* cotton plants and negatively with *Bt* plants. The Monte Carlo permutation test revealed a significant difference in sugar composition of honeydew due to the variety MECH 184 (P=0.0010; F=8.60) as well as the *Bt* transformation (P=0.0040; F=3.97; Fig. 4.3 B). The fact that the first two axes of the RDA only explained 32% of the variance (first axis=0.249; second

axis=0.066), indicated that there were variables other then transformation and variety that could explain the differences in honeydew sugar composition.

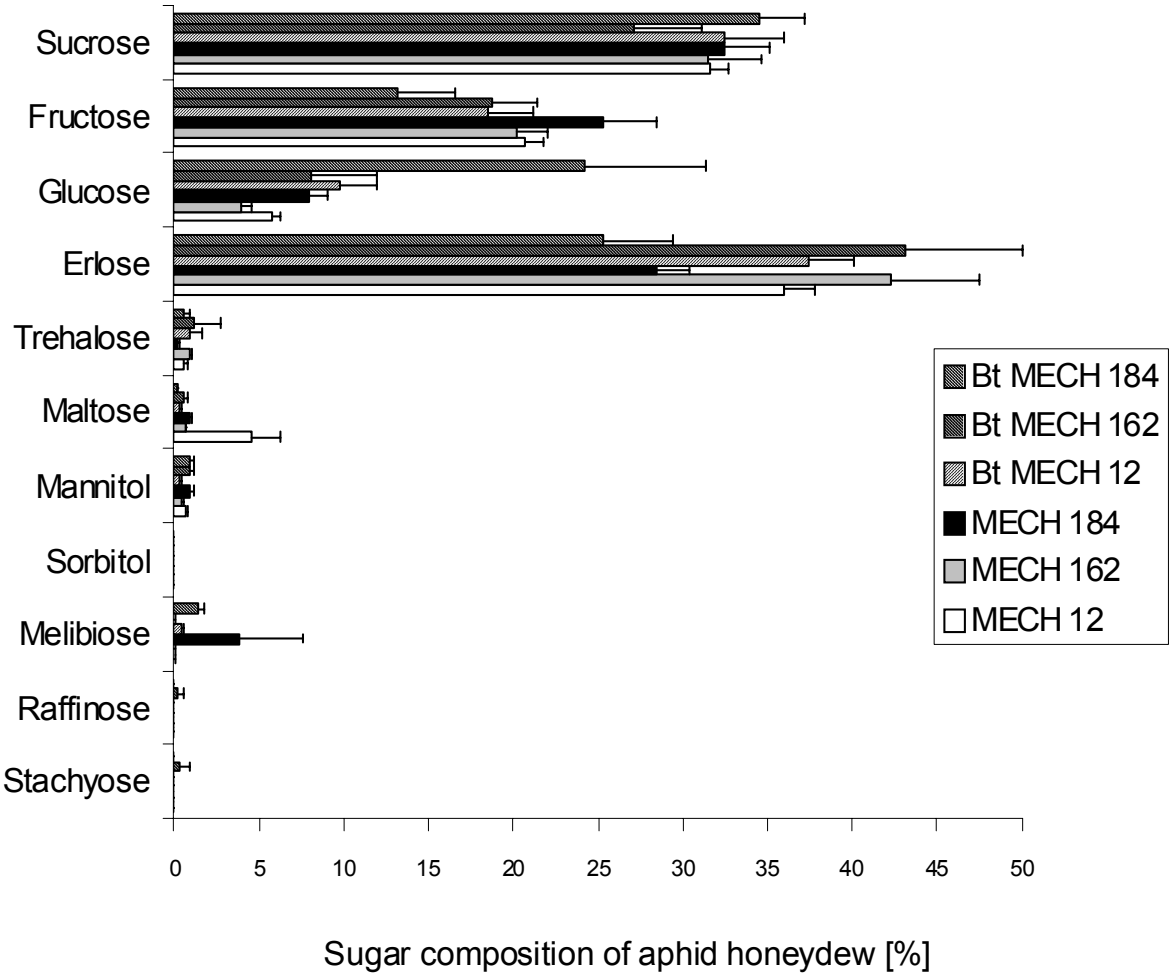

Figure 4.2 Relative sugar composition (mean percentage ± SE) of *Aphis gossypii* honeydew collected over a 5 to 6h interval from either *Bt* (*Bt* MECH 12, *Bt* MECH 162, *Bt* MECH 184) or the corresponding non-*Bt* cotton plants (MECH 12, MECH 162, MECH 184) (*n*= 5 to 7). Lactose, melezitose, mannose, rhamnose, and galactose were not found in any of the samples.

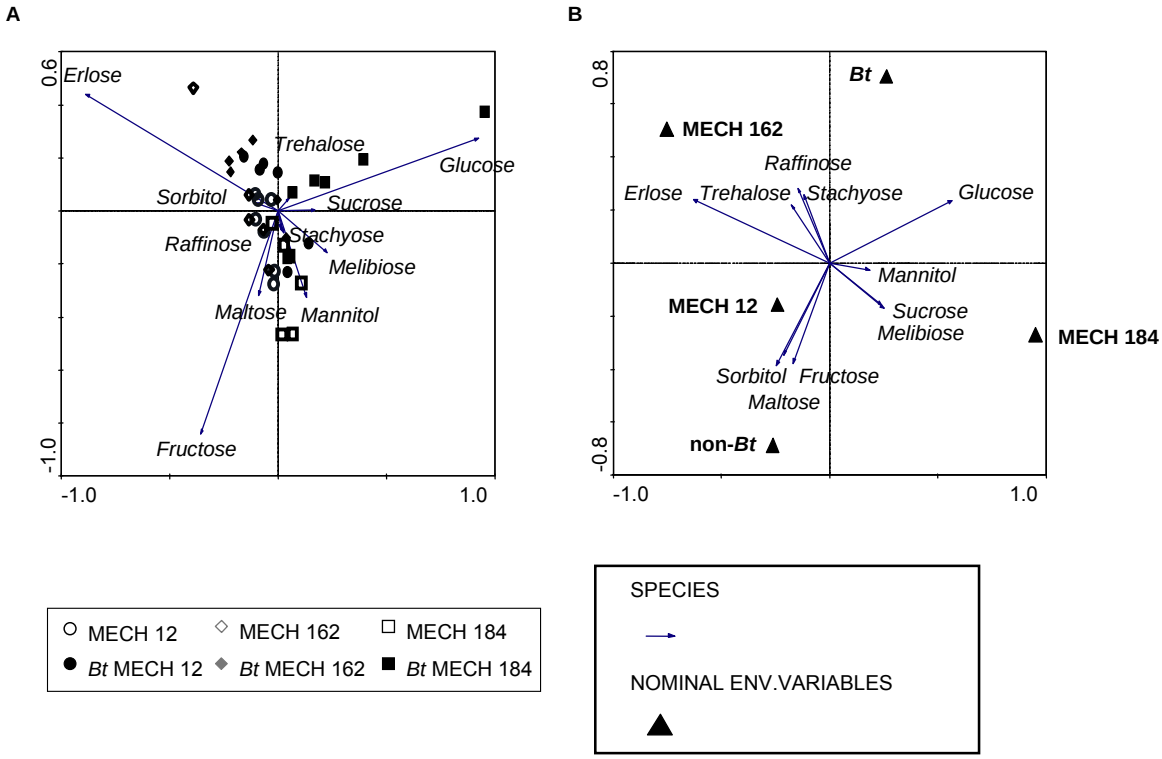

Figure 4.3 Distribution of sugar composition of honeydew samples from *Aphis gossypii* feeding on *Bt* (*Bt* MECH 12, *Bt* MECH 162, *Bt* MECH 184) or the corresponding non-*Bt* cotton plants (MECH 12, MECH 162, MECH 184), in the ordination biplot of a PCA (Fig. 4.3 A; eigenvalues: axis 1: 0.560, axis 2: 0.193) and a RDA (Fig. 4. 3 B; eigenvalues: axis 1: 0.249, axis 2: 0.066). The straight lines of the vectors represent the influence of the different sugars (species; Fig. 4.3 A; B), the triangles the centroids of the environmental variables (variety and *Bt*; Fig. 4.4 B). Sugars were expressed as percentage of total sugar.

Discussion

Aphid performance did not differ between *Bt* and non-*Bt* cotton plants and none of the aphid life-table parameters assessed was influenced by the expression of the *Bt* protein. However, there was a significant difference among cotton varieties for the number of nymphs produced during a time span equaling the nymphal developing time (FD). Furthermore, a slight variation in the r_m-values suggested a small difference among cotton varieties. As the r_m is difficult to interpret, the formula $e^{x\ days * r_m}$ was used to calculate aphid population growth for one week. According to this formula, a population would have increased by a factor of ten on *Bt* and non-*Bt* cotton plants of the variety MECH 12 while population increase would have been more pronounced on the varieties MECH 162 (factor 12 or 13) and MECH 184 (factor 13). To detect differences among cotton varieties is not surprising since disparities are known to be caused by different plant characteristics, e.g. gossypol level (Du et al. 2004) and trichome density (Weathersbee and Hardee 1994). The

latter effect was also found in our study. Xue et al. (2008) reported differences in the trichome density between *Bt* and non-*Bt* cotton plants which could not be confirmed for the three cotton varieties used in our study.

Previous glasshouse studies addressing the performance of *A. gossypii* on *Bt* and non-*Bt* cotton plants have revealed mixed results. While Zhang et al. (2008) reported no difference in aphid performance, Liu et al. (2005) detected for some life-table parameters a variation among three consecutive generations. Studies comparing aphid populations in *Bt* and non-*Bt* cotton fields are also inconsistent. While some studies recorded no difference in aphid populations (Bambawale et al. 2004; Mellet and Schoeman 2007), others found either increased (Cui and Xia 2000; Deng et al. 2003) or decreased aphid densities (Wu and Guo 2005) in the *Bt* crop. Given the results from glasshouse/climate chanber studies, changes in aphid populations in cotton fields are unlikely to be caused directly by the expression of the *Bt* Cry protein. Rather they may be due to other confounding factors, such as an increased overall health of the *Bt* crop or changes in the use of insecticides (Naranjo et al. 2008; Romeis et al. 2008b). In *Bt* maize, a study by Faria et al. (2007) has shown aphids to perform significantly better (expressed as increase in aphid numbers) on different *Bt* maize varieties with different transformation events than on their respective non-transformed control varieties. While slight *Bt* maize effects had earlier been reported by Lumbierres et al. (2004) other glasshouse studies did not show evidence of such effects (Lozzia et al. 1998; Dutton et al. 2002; Ramirez-Romero et al. 2008). In the field, no or only minimal differences in aphid densities on *Bt* and non-*Bt* maize have been recorded (Bourguet et al. 2002; Pons et al. 2005).

Our ELISA analyses revealed that none of the aphid samples contained detectable *Bt* protein. This finding is in accordance with many other studies which reported either no *Bt* protein or only trace amounts of protein in sap-sucking insects of the order Hemiptera after feeding on different *Bt* plants, including maize (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002; Harwood et al. 2005; Obrist et al. 2006), oilseed rape (Schuler et al. 2005), cotton (Torres and Ruberson 2006; Torres et al. 2006), and rice (Bai et al. 2006). In contrast to the studies listed above, Burgio et al. (2007) detected 3% of the Cry1Ac protein content expressed by *Bt* oilseed rape (plant expression level: 64ng Cry1Ac/g f.w.) in all of four aphid samples that had been collected in the greenhouse, but only in one out of eight samples collected from plants kept in a climate chamber (2% of the Cry1Ac protein content expressed by *Bt* oilseed rape). Even higher levels were reported from greenhouse

studies by Zhang et al. (2006) conducting ELISA analyses of aphids which previously had fed on a medium (plant expression level: 49ng Cry1A/g f.w.) or a high (plant expression level: 94ng Cry1A/g f.w.) expressing *Bt*-cotton line. Surprising in this study was the fact that all ten aphid samples contained *Bt*-protein after feeding on the medium *Bt*-expressing line, whereas only four out of ten samples after feeding on the high *Bt*-expressing line. Furthermore the positive aphid samples from the medium expressing *Bt* cotton plants contained 12% of the *Bt* content present in the plant while the aphid samples collected from the high-expressing *Bt* cotton contained only 4% of the *Bt* amount found in the plants. It is notable that (with one exception) all positive samples in the two studies listed above were collected in the greenhouse. We argue that one likely reason for the *Bt* proteins detected in these studies is contamination of their samples by other herbivores such as spider mites or thrips or their feces which contain large amounts of *Bt* protein (Obrist et al. 2005; 2006; Torres and Ruberson 2008). In a preliminary study, aphids were collected from *Bt* cotton that was contaminated with thrips. Great care was taken to check all aphid samples under the binocular microscope, both before releasing the aphids on the cotton plants and again on recollection from the plants to ensure that all herbivores other then aphids were removed. Nevertheless subsequent ELISA analyses still detected some *Bt* protein in eleven out of twelve samples (0.02 to 0.06µg Cry1Ac/g f.w. aphids, corresponding to 13-25% of the amount detected in cotton leaves; Lawo unpublished data). These protein levels were 10 to 30 times higher than the limit of detection of the ELISA used here. These findings underline that very low levels of contamination are sufficient to produce false positives, especially in samples that contain only traces, or no *Bt* protein, like aphids.

To control for contamination, our experiment was conducted in a climate chamber and not in the greenhouse to prevent any thrips or spider mite infestation. To guarantee similar expression levels between the climate chamber and the greenhouse a light spectrum simulating outdoor conditions was used. Based on the fact that no *Bt* protein could be detected in any of the aphid samples collected in the climate chambers, even though the *Bt* cotton plants were expressing well, it can be concluded that aphids feeding on *Bt* cotton do not ingest the *Bt* protein and consequently cannot pass it on to their natural enemies.

In the case of aphids, the performance of natural enemies can also be influenced indirect through the nutritional value of aphid honeydew (Wäckers 2000). The suitability of honeydew as a sugar source can be affected by honeydew nutrient composition

(Wäckers, 2000), as well as by the presence of secondary metabolites (Wäckers 2005) or insecticidal proteins (Romeis et al. 2003; Hogervorst et al. 2008). Honeydew sugar composition has been found to vary not only with aphid and plant species (Byrne and Miller 1990; Hendrix et al. 1992; Völkl et al. 1999; Hogervorst et al. 2007), but also with aphid development stage (Arakaki and Hattori 1998; Costa et al. 1999) and age (Fischer et al. 2002), ant attendance (Fischer and Shingleton 2001; Yao and Akimoto 2001), the presence of bacterial symbionts in the digestive tract (Wilkinson et al. 1997) as well as rate and duration of aphid infestation on a plant (Faria et al. 2008).

Using multivariate statistics to analyze the sugar composition of honeydew collected from *A. gossypii* feeding on the different varieties of *Bt* and non-*Bt* cotton, both variety and transformation were found to have a significant influence. This bioassay is the first demonstration of such a variation in honeydew sugar composition due to these factors. The fact that honeydew sugar composition differs among cotton varieties is not surprising given the fact that the plants differ in a range of parameters including trichome density or the gossypol level as mentioned above. The variation in sugar composition due to the factor transformation might be explained by the fact that *Bt* cotton plants and their non-transformed counterparts generally differ by more than the few intentionally introduced novel genes due to the variety selection and development phase that follows the transformation process. The changes are unlikely due to the expression of the *Bt* protein since we could clearly show that the aphids are not even exposed to the insecticidal compound. The fact that more glucose was detected in honeydew collected from *Bt* plants (especially *Bt* MECH 184) compared to that from the controls may be based on variation in phloem sap composition. Alternatively, it may indicate that the carbohydrate assimilation by the aphids was somewhat affected (Ashford et al 2000). Either way, it is interesting that this difference did not have any influence on aphid performance. However, for a proper evaluation of the nutritional quality of the phloem sap the amino acid composition should directly be examined. In contrast to our results, Faria et al. (2007) did not find any difference in sugar composition in aphid honeydew collected from different *Bt* and non-*Bt* maize varieties. However, the authors reported marginal differences in the amino acid composition.

Since the plant-derived sugars, mainly sucrose, glucose and fructose, are the most suitable for honeydew-feeding natural enemies (Wäckers 2001), honeydew from the cotton variety MECH 184 might have increased nutritional properties compared to the other two

varieties tested. However, we believe that this effect should not be overestimated since cotton features multiple nectar and extra-floral nectar sources (Wäckers and Bonifay 2004), which are frequently exploited by parasitoids (Stapel et al. 1997). When given a choice, parasitoids may ignore honeydew, and select extrafloral nectar. Among *Microplitis croceipes* (Cresson) collected from fields with and without nectar sources, only individuals from fields lacking nectar contained honeydew-specific sugars (Williams and Wäckers unpublished data).

Our studies allow the conclusion that aphid performance is not affected by Cry1Ac expressing *Bt* cotton plants. This, together with the fact that aphids do not ingest the insecticidal protein when feeding on *Bt* cotton, indicates that aphid antagonists are unlikely to be affected either directly or indirectly when attacking aphids in a *Bt* cotton field and that the biological control function they provide should not be compromised. In accordance to our laboratory studies, Bambawale et al. (2004) observed no difference in cotton aphid abundance between Indian fields with *Bt* cotton (MECH 162) and its corresponding near isoline under Integrated Pest Management. Consequently, aphids are likely to remain under biological control in *Bt* cotton which is important for the sustainable deployment of this technology.

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Chapter five

Effects of *Bt* cotton on *Helicoverpa armigera* larvae and the determination of prey-quality mediated effects on *Chrysoperla carnea*[4]

[4] Lawo NC, Wäckers FL, Romeis J. Effects of *Bt* cotton on *Helicoverpa armigera* larvae and the determination of prey-quality mediated effects on *Chrysoperla carnea*. In preparation.

Abstract

One of the most widely discussed ecological effects of *Bt* crops, expressing Cry proteins derived from *Bacillus thuringiensis* (*Bt*) is their potential impact on non-target organisms including biological control agents that are important for natural pest regulation. Possible effects of *Bt* crops could either be direct, due to ingestion of the insecticidal protein during direct feeding on plant tissue (e.g. pollen), or indirect due to the impact of the *Bt* protein on host/prey population or host/prey quality.

As previous studies suggested that lepidopteran pests feeding on *Bt* crops can lower predator performance due to a reduced prey quality, studies were performed to investigate whether prey nutritional composition causes indirect (prey-quality mediated) effects on *Chrysoperla carnea* (Neuroptera: Chrysopidae).

Conducting feeding studies with non-*Bt* and *Bt*-fed susceptible and Cry1Ac-resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae confirmed the observation of prey-quality mediated effects when the predator was fed with susceptible *Bt*-fed lepidopterans but not when feeding on *Bt*-fed Cry1Ac-resistant caterpillars. Conducting biochemical analyses of the glycogen and lipid content as well as the sugar and amino acid content and composition aimed to detect differences in the nutrients of susceptible and Cry1Ac-resistant non-*Bt* and *Bt* fed *H. armigera* larvae. Except for a significant difference in the sugar composition of susceptible larvae correlated to *Bt* feeding, no differences in nutrients could be detected.

Introduction

Bt crops, expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis*, are the only insecticidal genetically engineered (GE) plants that are currently grown commercially. In 2007, more than 42 million ha of *Bt*-transgenic cotton and maize varieties, expressing either lepidopteran- or coleopteran-specific Cry proteins, were grown worldwide (James 2007).

Insect-resistant GE varieties can be an important component of integrated pest management (IPM) systems (Romeis et al. 2008b). Of particular interest in this respect is the impact of GE crops on non-target organisms that fulfill important ecological functions, such as the natural control of pest herbivores by arthropod predators and parasitoids. The potential risk that insect resistant GE plants may pose to those non-target organisms is typically part of the environmental risk assessment prior to the commercial release of any

novel GE crop (Garcia-Alonso et al. 2006; Romeis et al. 2008a). To date, considerable information has been collected on the impact of *Bt* crops on non-target organisms, with particular emphasis on biological control agents (US-EPA 2001; Romeis et al. 2006; 2008b; Wolfenbarger et al. 2008). Possible effects of *Bt* crops on non-target organisms could either be direct, due to ingestion of the insecticidal protein during feeding on plant tissue (e.g. pollen), or indirect due to the impact of the *Bt* protein on host/prey population or host/prey quality.

There is no evidence that the Cry proteins expressed in today's *Bt* crops cause direct toxic effects on natural enemies (US-EPA 2001; Romeis et al. 2006). However, adverse effects in terms of reduced predator or parasitoid survival have been reported in studies where susceptible herbivores, such as caterpillars, were used as host or prey (Romeis et al. 2006). It can thus be assumed that a reduction in the food quality caused a reduced survival, rather than the *Bt* protein itself.

Clear evidence of such prey-quality mediated effects was reported by Dutton et al. (2002) who observed reduced lacewing larvae (*Chrysoperla carnea*, Neuroptera: Chrysopidae) survival when the predator was reared with *Bt* (Cry1Ab) maize-fed caterpillars (*Spodoptera littoralis*, Lepidoptera: Noctuidae) but not when provided with *Bt* maize-fed spider mites (*Tetranychus urticae*, Acari: Tetranychidae), both containing biologically active *Bt* protein (Obrist et al. 2006). As caterpillars are affected by the *Bt* protein while spider mites are not, the authors concluded that *C. carnea* survival was compromised as a result of eating sublethally affected ("sick") prey, rather than by the *Bt* protein. Subsequent studies confirmed that Cry1A does not cause a direct effect on *C. carnea* larvae (Romeis et al. 2004; Rodrigo-Simon et al. 2006; Lawo and Romeis 2008) and adults (Li et al. 2008), contradicting earlier reports of direct toxicity (Hilbeck et al. 1998). Since the above studies only provided an indirect proof for prey-quality mediated effects, we here set out to investigate whether prey nutritional composition causes these indirect effects on a predator.

The impact on predator fitness could be explained by its nutritional requirements, as arthropods need a range of compounds including protein and/or the ten essential amino acids, B-vitamin complexes and other water-soluble growth factors, certain fat soluble vitamins, cholesterol, minerals, and an energy source such as carbohydrates and/or lipids (Thompson and Hagen 1999). As lipids, such as cholesterol, play an important role in insect growth, they are considered to be one of the most important nutrient groups for

insects (House 1961). Insects accumulate lipids in high concentrations, especially at molting or pupation (Dadd 1973) or, in the case of parasitoids, during the pupal stage (Giron and Casas 2003). Furthermore, fatty acids are necessary components of phospholipids on which the structural integrity and functioning of probably all external and internal cellular membranes depend (Dadd 1973). Together with lipids, carbohydrates play an important role in energy provision (Dadd 1985; Grenier et al. 1986). They are essential for growth, longevity and fecundity (House 1961). Sugars, as an energy source, are especially important for parasitoids (Olsen et al. 2000). Carbohydrates can be assembled into large macromolecules of glycogen, mostly by predators, and stored within fat bodies (Dadd 1985), the crop, or as easily accessible haemolymph sugars. With respect to protein as a nutritional source, the ten essential amino acids (arginine, histine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are more or less indispensable for the growth and reproduction of insects (House 1961; Dadd 1985).

In our studies, susceptible and Cry1Ac-resistant *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae were allowed to feed on non-*Bt* or *Bt* (Cry1Ac)-transgenic cotton plants. Subsequently, caterpillars were fed to *C. carnea* larvae to verify prey-mediated effects. To clarify the mechanism underlying the reduced prey quality, the lipid and glycogen content and sugar and amino acid content and composition in non-*Bt* and *Bt* cotton-fed caterpillars were examined. To determine whether observed effects were due to the presence of the *Bt* protein, supplementary studies were conducted with *H. armigera* larvae fed artificial diet containing pure Cry1Ac at two concentrations, one below (approximately by a factor of ten) and one exceeding the plant expression level (approximately by a factor of ten; Supplementary Fig. 5.1).

Materials and methods

Insect material

Pupae of the Cry1Ac-resistant *H. armigera* strain were provided by CSIRO Entomology (Canberra, Australia) and pupae of the susceptible strain by Bayer CropScience (Monheim, Germany). Twenty pairs of adults were reared in one 2.5l plastic container. For egg deposition, tissue papers were placed in the container. Tissue papers and plastic containers were changed daily and moths were offered a solution containing 20g honey, 20g sugar and 6g ascorbic acid per liter through a cotton plug at the bottom of the container. The solution was replaced every two to three days. Rearing conditions were 25 ±

1°C, 60 ± 5% r.h. with a 14-h photoperiod. Eggs were stored at the same climatic conditions until hatch or kept at 12°C for up to eight days to delay development.

C. carnea were collected in Bolligen near Bern (Switzerland) in 1993 and have since been maintained in the laboratory without any further introductions of field-collected insects. Larvae of *C. carnea* were reared on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs provided by Biotop (Valbonne, France), and adults were kept on an artificial diet containing honey, brewers yeast and water (7:4:4). Rearing conditions were 22 ± 3°C, 70 ± 5% r.h. with a 16-h photoperiod.

Plants

Bt cotton (*Gossypium hirsutum*) plants (MECH 12; event BG-I, Mahyco Seeds Ltd.), producing Cry1Ac protein (*Bt* plants) and their corresponding non-transformed near isoline (non-*Bt* plants) were used in the bioassays. Plants were grown individually in humus-rich sterilized soil in 3l plastic pots. Plants were fertilized weekly with 10N:10P:8K at a concentration of 20ml/l and kept in a climate chamber at 25 ± 1°C, 70 ± 5% r.h. with a 16-h photoperiod. Three to four week old plants were transferred to the greenhouse (25 ± 3°C; 16-h photoperiod) for one week, before being used in the bioassays. At the time of the experiments, plants had four to five fully developed leaves.

Purified Cry1Ac protein

Lyophilized Cry1Ac protein was obtained from Marianne Pusztai-Carey (Dept. Biochemistry, Case Western Reserve University, Cleveland, OH, USA). The Cry1Ac protoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 was expressed as a single gene product in *Escherichia coli*. Inclusion bodies containing protoxin were dissolved and trypsinized. Subsequently, the toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al. 1994).

Feeding studies with *H. armigera* and *C. carnea* larvae

Bioassay with* C. carnea *larvae

The aim of this study was to assess prey-quality mediated indirect effects on *C. carnea* when kept on *Bt* cotton-fed *H. armigera* larvae. For this purpose freshly hatched lacewing larvae (≤14-h old) were provided with susceptible or Cry1Ac-resistant one-day old *H. armigera* larvae until they molted to the third instar (L3). *C. carnea* larvae were

kept individually in a Petri dish (5cm diameter; 1cm high), containing a hole in the lid covered with a fine-mesh netting for ventilation. Caterpillars were kept on the third, fourth, or fifth leaf of non-*Bt* or *Bt* cotton for one day (24h). The leaves were covered with air-permeable bags (20x24cm). Subsequently, prey larvae were offered to the predator together with a piece of cotton leaf of the corresponding treatment in the morning and in the evening. For the first 8h of the bioassay freshly-hatched *C. carnea* larvae were provided with *H. armigera* larvae that had fed for only 12 to 14h on non-*Bt* or *Bt* cotton plants. This was necessary since freshly-hatched *C. carnea* larvae have difficulties dealing with one-day old *H. armigera* larvae (personal observations). Development and survival of the *C. carnea* larvae were recorded twice a day, in the morning and in the evening, and freshly-molted L3 were weighed on a microbalance (Mettler Toledo MX5). Bioassays with larvae of the susceptible and the Cry1Ac-resistant *H. armigera* strain were performed separately and were conducted with 31 to 46 *C. carnea* larvae per treatment.

***Consumption rate of* C. carnea**

Petri dishes, leaves, and lepidopteran larvae were changed every 24h. To determine the amount of prey consumed by individual *C. carnea* larvae, twice a day all living *H. armigera* larvae were counted and subtracted from the total number provided previously. To ensure that missing lepidopterans had been eaten by the predator and not by fellow *H. armigera* larvae, extra Petri dishes with *H. armigera* larvae only were examined. Ten, 20 or 30 susceptible larvae and 10, 20 or 25 Cry1Ac-resistant larvae were placed together with a non-*Bt* or *Bt* cotton leaf piece in a Petri dish. After 24h survivors were counted. Because susceptible *Bt* cotton-fed *H. armigera* larvae showed an increased tendency for cannibalism (Chilcutt 2006), 12 to 14 Petri dishes were examined. Five to six repetitions were conducted for the other three treatments. On average, one susceptible non-*Bt* cotton-fed larva was eaten by its fellows, three susceptible *Bt* cotton-fed larvae, half a Cry1Ac-resistant non-*Bt* cotton-fed *H. armigera* larva and one Cry1Ac-resistant *Bt* cotton-fed lepidopteran larva were eaten. The median number of larvae lost due to cannibalism was multiplied by the total development time of individual *C. carnea* larvae. Subsequently, this number was subtracted from the hypothetical total consumption rate of individual predator larvae. To calculate the biomass *C. carnea* consumed during L1 and L2, the median fresh weight (f.w.) of non-*Bt* and *Bt* cotton-fed one-day old prey larvae was determined (n=42 to

63). Subsequently, *H. armigera* larvae weight was multiplied with the corrected consumption rate of the predator.

Statistics of feeding studies

C. carnea larval survival (event=dead) as well as development time (event=developed to the third instar) were analyzed by a log-rank test including the factor non-*Bt* vs. *Bt* cotton. For the larval survival analysis, insects that were lost or developed to the third instar during the observation period were included as censored data and for the analysis of larval development time, insects that were lost during the observation period were included as censored data. Weight data of surviving *C. carnea* larvae was checked for normality and homogeneity of variances prior to analysis. As all assumptions were met, data were analyzed with a student *t*-test (factor: non-*Bt* vs. *Bt* cotton). Since data on the prey number consumed by predatory larva were not normally distributed, a Mann-Whitney-*U* test, adjusted for ties, was performed. Statistical analyses were conducted using the software package Statistica (version 6, StatSoft Inc., Tulsa, OK, USA). For all tests the α-level was set at 5%.

Biochemical analysis

Assessment of Cry1Ac protein content

To confirm that predatory larvae were exposed to Cry1Ac protein when preying on *Bt* cotton-fed lepidopterans, 6 to 8mg of prey larvae were analyzed for their *Bt* content (fifteen larvae per strain of the *Bt*-fed lepidopterans and three to four larvae per strain of the non-*Bt* fed lepidopterans as a control). Surviving chrysopidae larvae were pooled (12 to 16mg) to assess their *Bt* content ("*C. carnea* fed susceptible *H. armigera*" (*n*=1), "*C. carnea* fed Cry1Ac-resistant *H. armigera*" (*n*=4) and *n*=2 to 3 "susceptible or Cry1Ac-resistant non-*Bt* cotton-fed" *C. carnea*). For comparison, additional samples of five-week old *Bt* cotton leaves were analyzed (*n*=16).

To quantify Cry1Ac protein content, samples of cotton leaves, chrysopidae larvae and caterpillars were analyzed with an enzyme linked immuno-sorbent assay (ELISA) from Agdia (Elkhard Indiana, USA). After measuring the fresh weight and lyophilizing cotton leaf samples, phosphate buffered saline with Tween buffer (PBST, provided in the kit) was added at a ratio of 1:10 (sample material f.w.:buffer). Thereafter, a 5mm tungsten carbide bead was added and leaf samples were macerated for 100sec at 30Hz, using a

mixer mill MM300 (Retsch, Haan, Germany) fitted with 24 tube-adapters (Quiagen, Hombrechtikon, Switzerland). Lacewing and caterpillar samples were crushed with a plastic pestle after adding PBST at a ratio of 1:40 for lacewings and 1:20 for caterpillars. Insect samples were incubated at room temperature for 2 to 3h. All samples were centrifuged for 5min at 13,000 x g and leaf samples were diluted 1:15 with PBST, while insect samples were not diluted. Subsequently, instructions from the kit were followed. After stopping the color development with 3M sulfuric acid, spectrophotometric measurements were conducted with a microtiter plate reader (SpectraflourPlus, Tecan, Männedorf, Switzerland) at 450nm. Together with the samples, purified *Bt* protein provided with the kit was diluted to obtain a range of standards. A standard curve with purified high quality Cry1Ac provided by M. Pusztai-Carey (Dept. Biochemistry, Case Western Reserve University, Cleveland, OH, USA) was established. Concentrations were calculated using linear regression analysis. The limit of detection for insect extracts was calculated by multiplying three times the standard deviation of thirteen buffer-only and control ODs with the slope of the standard curve. The limit of detection for lacewing samples was 0.006µg/g fresh weight (f.w.); that for caterpillars was 0.012µg/g f.w.

Nutrient content and composition of one-day old* H. armigera *larvae

To examine the nutritional quality of prey larvae after feeding on non-*Bt* or *Bt* cotton plants for one day, the lipid and glycogen content as well as the sugar and amino acid content and composition were examined (for details see below). Five to 56 neonate susceptible or Cry1Ac-resistant *H. armigera* larvae were brushed on the two youngest fully enlarged cotton leaves of four to five-week old non-*Bt* and *Bt* cotton plants and subsequently five to 14 *H. armigera* larvae were covered with one clip-cage (2cm in diameter; 1cm high), resulting in one or two clip-cages per leaf. The numbers of lepidopteran larvae depended on the strain (susceptible or Cry1Ac-resistant) and plant (non-*Bt* or *Bt)*. A total of 15 to 20 plants were tested per strain and plant. After one day, four to ten larvae were collected from one plant, pooled to reach a weight around 1000µg (for lipid and glycogen determination; n=14 to 22) or 2000µg (for sugar and amino acid determination; n=14 to 19) and frozen at -80°C until further analysis. To examine the *Bt* expression level of the plants, samples were taken from two to three additional plants which had been planted at the same time as the plants used in the bioassay, from the two youngest leaves at the beginning of the bioassay.

Lipid and glycogen content of H. armigera *larvae*

Frozen *H. armigera* larvae were transferred to 2ml glass vials and 30µl of a 2% sodium sulphate solution as well as 270µl chloroform-methanol (1:2) was added. Subsequently, larvae were crushed with a plastic pestle, vortexed, and centrifuged at 8,000 x g for 8min. Thereafter, the supernatant was transferred to a glass tube (12mm x 75mm). Lipid and glycogen analyses were conducted as described by Olsen et al. (2000). Standard solutions and reagents were made following the recipe described by van Handel (1985a; b). Soybean oil was used to generate the lipid standard curve and rabbit liver glycogen (Sigma-Aldrich; Switzerland) was used for the glycogen standard curve. Lipid solutions were prepared in amounts of 0, 5, 10, 25, and 50µg/ml and brought to a total volume of 100µl with chloroform (Merck; Darmstadt, Germany). Before 50µl sulphuric acid (Merck) and 1ml vanillin reagent were added, the glass tubes were heated at 90°C until all chloroform had evaporated. Glycogen solutions were prepared in amounts of 0, 5, 10, 25, and 30µg/ml and brought to a total volume of 100µl with MilliQ water. Subsequently, 0.9ml anthrone reagent was added. After preparing the solutions the whole sample was transferred to disposable cuvettes (1.5ml; 12.5 x 12.5 x 45mm; Plastibrand, Switzerland). The OD from every sample was recorded from both sides of the cuvettes. The absorbance at 525nm was recorded for the lipid analysis and at 625nm for the glycogen analysis using a spectrophotometer (Spekol 1100; Zeiss, Switzerland).

Sugar and amino acid content and composition of H. armigera *larvae*

H. armigera larvae samples were analyzed for twelve sugars: sucrose, fructose, glucose, trehalose, mannitol, sorbitol, melibiose, raffinose, stachyose, melezitose, maltose, and galactose. Before analysis, samples were diluted 400-fold with 18MΩ water and homogenized using a pestle. Subsequently, samples were filtered through a chromacol syringe filter. Samples were analyzed using a Dionex ICS 3000 Ion Chromatography system (Dionex Corp., Sunnyvale, CA, USA) and concentrations of the individual sugars were calculated using the program PEAKNET Software Release 5.1 as described by Steppuhn and Wäckers (2004). Fifteen to 19 samples per treatment were analyzed for lepidopteran larvae that were exposed to either non-*Bt* or *Bt* cotton plants. The limit of quantification for any caterpillar sample was set at 0.001µg. Measurements below 0.001µg were set to 0.

To analyze the amino acid composition, samples were diluted by adding 580µl 18MΩ water to a total volume of 600µl and homogenized using a pestle. Subsequently, samples were filtered through a chromacol syringe filter [17-SF-02(T)]. Sample analysis was via pre-derivatisation with the OPA reagent. The OPA reagent was prepared by dissolving 5mg of o-phthaldehyde in 100µl of methanol, followed by dilution with 900µl of a borate buffer. Before injection, 15µl of this reagent was added to 15µl of the sample. For each sample, 20µl was injected into a Dionex ICS 3000 Ion Chromatography system (Dionex Corp., Sunnyvale, CA, USA). The system was equipped with a ICS 3000 dual pump, an Acclaim 120 analytical column (2.1 x 150mm), as well as a Dionex RF 2000 fluorescence detector set at an excitation wavelength of 300nm and emission detection at 450nm (Dionex, Leeds, UK). Two eluents were used for the mobile phase. Line A contained an aqueous phosphate buffer consisting of 10mM NaH_2PO_4: 10mM $Na_2B_4O_7.10H_2O$: 0.5mM NaN_3. Line B contained by volume 45% acetonitrile, 45% methanol, and 10% water. Run time was set at 45 minutes at a flow of 0.4ml/min. The column was kept at 45°C during analysis. Daily reference curves were obtained for 15 amino acids: lysine, leucine, isoleucine, phenylalanine, methionine, valine, tyrosine, alanine, threonine, glycine, arginine, histidine, serine, glutamate, aspartate at 0.4ppm, 0.2ppm, 0.1ppm, 0.05ppm, 0.025ppm and 0.01ppm. Fourteen to 17 samples of lepidopteran larvae were examined. The limit of quantification for any caterpillar sample was set at 0.001µg. Measurements below that were set to 0.

The amino acids were analyzed by capillary electrophoresis with a Beckman P/ACE MDQ system equipped with a 488nm argon-ion laser module (Picometrics, France, 25mW). The data were collected and analyzed by Beckman P/ACE MDQ 1.5 or 1.2 software (Beckman-Coulter, Fullerton, CA, USA). Half an hour before analysis, the caterpillar samples were put at room temperature. For the analysis, 15 to 45µl of the Dissolving Matrix (Sodium phosphate monobasis, Sodium phosphate dibasis, Glycine-Glycine) were added to the sample. Thereafter, the sample was mixed with 2.5 to 7.5µl 50mM NBD-F and heated at 60ºC for 3min, after which it was mixed with 15 to 45µl DOPAC to quench the reaction and cooled to room temperature before analysis. During capillary electrophoresis, the sample was injected by pressure at 0.5psi for 5sec. The applied voltage for CE separation was 20.6kV (0 to 16min) and 30kV (17 to 25min). CE experiments were conducted at 20ºC.

To clarify which nutrient changes were due to the *Bt* protein rather than secondary plant compounds, biochemical analysis were conducted using *H. armigera* larvae that had fed artificial diet with or without Cry1Ac (Supplemental material).

C/N analysis

To examine the carbon (C) and nitrogen (N) ratio of non-*Bt* and *Bt* cotton plants, the third, fourth, and fifth leaf of five week old cotton plants were rolled in Eppendorf tubes, flash frozen in liquid nitrogen and lyophilized (n=10). For analysis, 3 to 6mg of macerated leaf material (as described above for the ELISA analysis) was burned in an elemental analyzer (HEKAtech GmbH, Wegberg, Germany). The analysis is based on the principle of dynamic flash combustion followed by gas chromatographic separation of the resultant gases. Calculations were done with the Callidus^{TM} 2E3 Software (HEKAtech GmbH, Wegberg, Germany).

Statistics for biochemical analysis

As data on the *Bt* content, larval weight, total nutrition content and nutrient composition were not normally distributed, data were analyzed separately for each strain by pair-wise comparisons (factor non-*Bt* vs. *Bt* cotton) using the Mann-Whitney-*U* test, adjusted for ties. Significance levels were corrected for multiple pair-wise comparisons, using the Bonferroni-Holm procedure. Statistical analyses of *Bt* content, total lipid and glycogen content and the C/N ratio were conducted using the software package Statistica (version 6, StatSoft Inc., Tulsa, OK, USA). Statistical analyses of the sugar and amino acid content and composition was performed using the software package SPSS (version 11.5.1, SPSS Incorporation). For all tests the α-level was set at 5%.

Results

Feeding studies with *H. armigera* and *C. carnea* larvae

Bioassay with C. carnea *larvae*

Feeding on *Bt* cotton-fed susceptible prey caused a significant decrease in *C. carnea* larvae survival compared to the control (18.9% vs. 87.1%; P<0.0001, Fig. 5.1). Furthermore, the consumption of *Bt* cotton-fed susceptible *H. armigera* larvae prolonged *C. carnea* median development time (L1 to L3) significantly from 9.5 to 14 days compared to non-*Bt* fed susceptible lepidopterans (P<0.0001; Fig. 5.2). No difference in predator

survival (72.2% vs. 71.0%; *P*=0.9120; Fig. 5.1) or development time (8.0 vs. 8.75 days; *P*=0.9249; Fig. 5.2) was observed when feeding non-*Bt*- or *Bt* cotton-fed Cry1Ac-resistant *H. armigera* larvae. In none of the treatments was the fresh weight of newly eclosed L3 *C. carnea* influenced by *Bt* cotton-fed prey (susceptible strain: 2240 vs. 2184µg; *P*=0.7396; Cry1Ac-resistant strain: 2210 vs. 2257µg; *P*=0.6530).

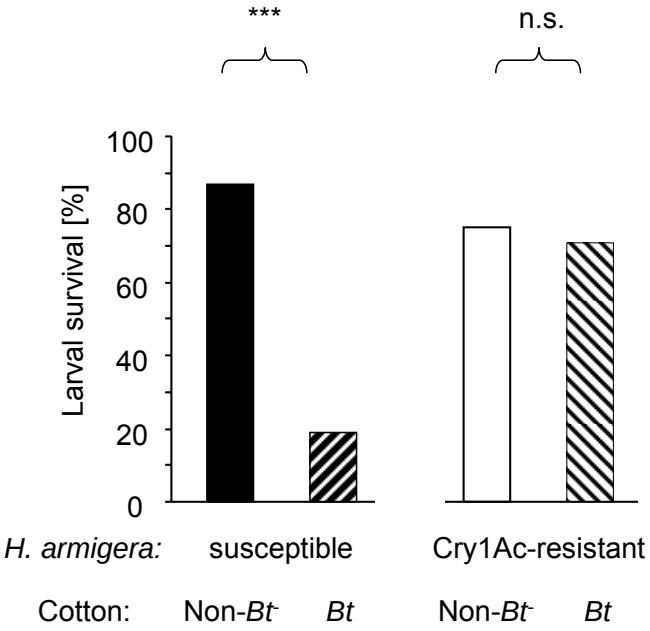

Figure 5.1 Survival of *Chrysoperla carnea* larvae (percentage reaching the third instar). Predator larvae were fed either with susceptible or Cry1Ac-resistant non-*Bt* or *Bt* cotton-fed *Helicoverpa armigera* larvae. *** *P*<0.0001; n.s. – not significant (*P*>0.05); *n*= 31 to 46.

***Consumption rate of* C. carnea**

The number of *H. armiger*a larvae and the total biomass consumed by *C. carnea* larvae differed in both strains due to the food source (number of lepidopterans consumed: susceptible larvae *P*<0.0001, Cry1Ac-resistant larvae *P*<0.0001; consumed larval biomass: susceptible larvae *P*<0.0001, Cry1Ac-resistant larvae *P*=0.0010) (Table 5.1). The median *H. armigera* larval weight was significantly reduced in the susceptible strain after *Bt* cotton feeding (*P*<0.0001), but no difference was observed in the Cry1Ac-resistant strain (*P*= 0.2848).

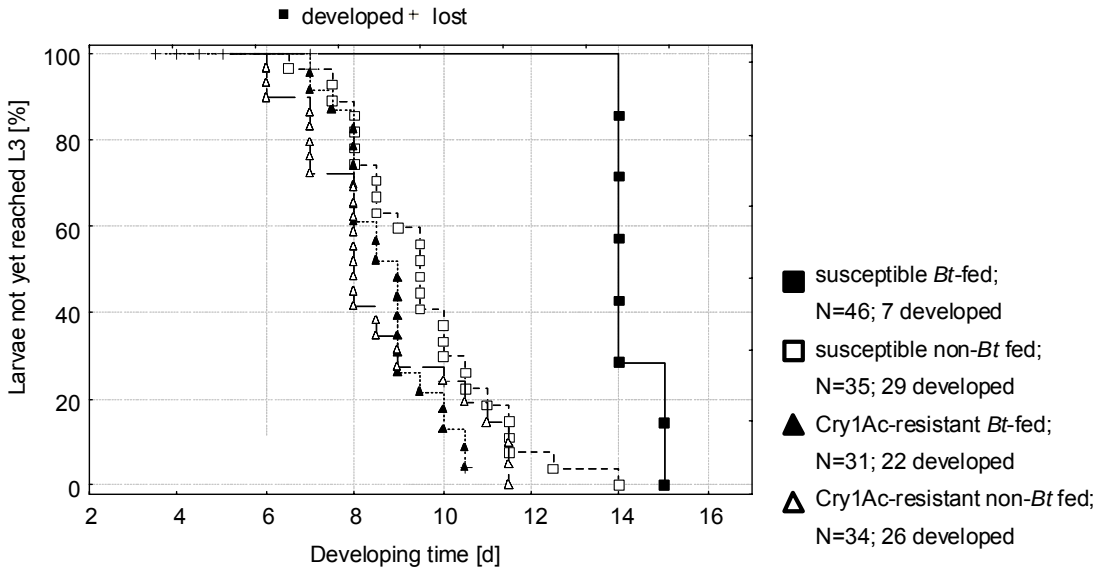

Figure 5.2 Development time of *Chrysoperla carnea* larvae (days to reach the third instar). Predator larvae were fed with susceptible or Cry1Ac-resistant non-*Bt* or *Bt* cotton-fed *Helicoverpa armigera* larvae. *** $P<0.0001$; n.s. – not significant ($P>0.05$); n= 31 to 46.

Table 5.1 Median (first to third quartile) number of *Helicoverpa armigera* larvae and biomass consumed by individual *Chrysoperla carnea* larva during the first two larval stages and weight of individual one day old non-*Bt* or *Bt* cotton-fed *H. armigera* larvae.

***H. armigera* strain**	**Plant**	**Number of lepidopterans consumed**	**Biomass consumed [g]**	**Weight of *H. armigera* larvae**
Susceptible	non-*Bt*	53 (44 to 64) b	9.4 (7.9 to 11.4) b	179.88 (132 to 222) a
Susceptible	*Bt*	224 (209 to 239) a	17.5 (16.4 to 18.7) a	78.31 (66 to 101) b
Cry1Ac-resistant	non-*Bt*	66 (47 to 73) b	12.4 (8.8 to 13.8) b	189.00 (161 to 232) a
Cry1Ac-resistant	*Bt*	85 (70 to 89) a	15.4 (12.7 to 16.1) a	182.22 (155 to 221) a

Values within groups ("susceptible" or "Cry1Ac-resistant") followed by different letters differ significantly ($P<0.05$).

Biochemical analysis

Assessment of Cry1Ac protein content

Bt cotton plants contained significantly more Cry1Ac protein compared to susceptible *H. armigera* larvae (17 times more; $P<0.0001$) and compared to Cry1Ac-resistant *H. armigera* larvae (four times more; $P<0.0001$) (Table 5.2). In *Bt* cotton-fed Cry1Ac-resistant *H. armigera* larvae a four times higher Cry1Ac protein concentration was detected compared to susceptible *Bt* cotton-fed larvae ($P<0.0001$). *C. carnea* larvae which fed on Cry1Ac-resistant prey contained five times less Cry1Ac protein than Cry1Ac-resistant *H. armigera* larvae themselves. No *Bt* protein was detected in chrysopidae larvae feeding on susceptible *Bt* cotton-fed lepidopterans (Table 5.2).

Table 5.2 Mean (±SE) Cry1Ac protein content in *Bt* cotton leaves, susceptible and Cry1Ac-resistant neonate *Helicoverpa armigera* larvae that had fed on *Bt* cotton for one day, and *Chrysoperla carnea* larvae after feeding on susceptible or Cry1Ac-resistant *H. armigera* larvae.

Treatment (*n*)	**Cry1Ac content [µg Cry1Ac/g f.w.]**
Bt cotton leaves (*n*=16)	1.170 (±0.085)
Susceptible *H. armigera* (*n*=15)	0.071 (±0.008)
Cry1Ac-resistant *H. armigera* (*n*=15)	0.285 (±0.039)
C. carnea fed susceptible *H. armigera* (*n*=1)	<0.004[*1]
C. carnea fed Cry1Ac-resistant *H. armigera* (*n*=4)	0.060 (±0.024)

[*1] below limit of detection

Nutrient content and composition of one-day old H. armigera *larvae*

Lipid and glycogen content of H. armigera *larvae*

In the susceptible *H. armigera* strain, a significant weight differential was observed between non-*Bt* and *Bt* cotton-fed larvae ($P<0.0001$; Fig. 5.3 A and B). However, this had no consequences on total lipid ($P=0.5411$; Fig. 5.3 A) and glycogen ($P=0.7178$; Fig. 5.3 B) content. In the case of the Cry1Ac-resistant *H. armigera* strain, weight did not differ significantly between non-*Bt* and *Bt* cotton-fed larvae ($P=0.0769$; Fig. 5.3 A and B). No changes in the total lipid level due to *Bt* feeding were detected ($P=0.9486$; Fig. 5.3 A).

However, a significant glycogen differential was observed between non-*Bt* and *Bt* cotton-fed larvae (*P*=0.0079; Fig. 5.3 B).

Sugar content and larval weight of H. armigera *larvae*

In susceptible *H. armigera* larvae, *Bt* cotton feeding significantly reduced larval weight (*P*<0.0001) as well as the total sugar content (*P*=0.0058; Fig. 5.3 C). Even though the weight of Cry1Ac-resistant larvae was also reduced (*P*=0.0338) due to *Bt* feeding, it had no effect on the total sugar composition (*P*= 0.4610; Fig. 5.3 C).

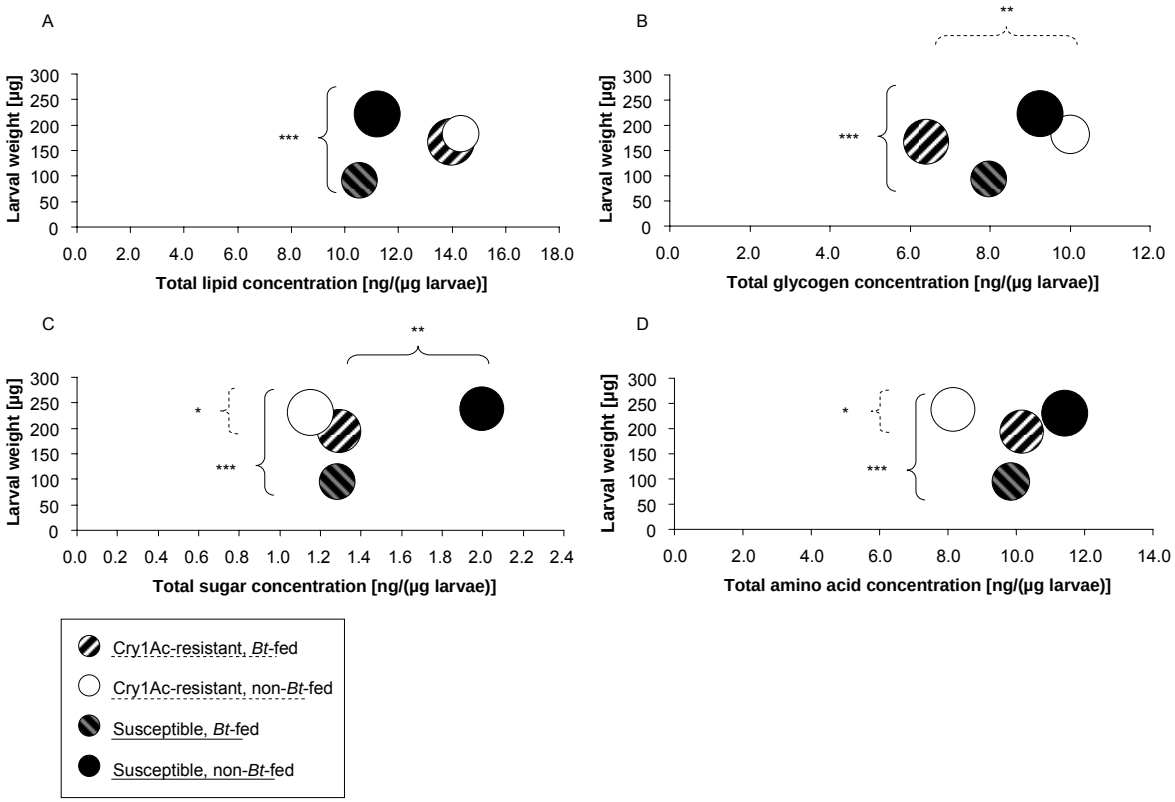

Figure 5.3 Total lipid (A, *n*=14 to 28), glycogen (B, *n*=14 to 28), sugar (C, *n*=15 to 16) and amino acid (D, *n*=14 to 17) concentration/besser content in susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding on non-*Bt* or *Bt* cotton plants for one day in dependence to the larval weight (f.w.). Diagrams are presented as bubbles where the centre is determined by the mean larval weight and mean nutrition concentration of the respective group. The diameter of the bubble shows the pooled standard error of nutrition concentration and weight. This was calculated as √ [(variance of nutrient concentration / number) + (variance of weight / number)]. Statistical differences of *H. armigera* larval weight and nutrient content between non-*Bt* and *Bt* cotton-fed *H. armigera* larvae: **P*<0.05; ***P*<0.01; ****P*<0.001; Mann-Whitney-*U* test; susceptible and Cry1Ac-resistant larvae were analyzed separately. Full lined bracket indicate significance for the susceptible *H. armigera* strain, dashed brackets for the Cry1Ac-resistant strain. All other comparisons were not significant (*P*>0.05).

Sugar composition of H. armigera *larvae*

A total of eleven sugars (glucose, sucrose, fructose, stachyose, maltose, galactose, melibiose, raffinose, trehalose, sorbitol, mannitol) were found in susceptible *H. armigera* larvae (Fig. 5.4). Dominant on average were stachyose (non-*Bt* cotton-fed 61%; *Bt* cotton-fed 52%) and glucose (non-*Bt* cotton-fed 27%; *Bt* cotton-fed 40%). Only trace amounts of sorbitol and mannitol were detected in non-*Bt* cotton-fed susceptible larvae and traces of sorbitol and trehalose in *Bt* cotton-fed lepidopterans. Melezitose was absent from all samples while trehalose was not detected in non-*Bt* cotton-fed susceptible larvae and melebiose and raffinose in *Bt* cotton-fed larvae. In susceptible *H. armigera* larvae the amount of galactose ($P<0.0001$), sucrose ($P=0.0017$) and stachyose ($P=0.0292$) was significantly decreased after *Bt* cotton feeding when compared to non-*Bt* cotton-fed larvae (Fig. 5.4).

In Cry1Ac-resistant larvae a total of 11 sugars were found (glucose, sucrose, fructose, stachyose, maltose, galactose, melibiose, raffinose, sorbitol, mannitol, melizitose). As for the susceptible strain, stachyose (non-*Bt* cotton-fed 69%; *Bt* cotton-fed 82%) and glucose (non-*Bt* cotton-fed 21%; *Bt* cotton-fed 9%) were dominant in all samples. Only trace amounts of sorbitol and mannitol were detected. Trehalose was absent from all samples while melibiose was not detected in non-*Bt* cotton-fed lepidopterans. *Bt* cotton-fed larvae did not contain any raffinose. A significant reduction in the amount of glucose ($P=0.0067$) was found in *Bt* cotton-fed larvae when compared to the control (Fig. 5.4).

Amino acid content and larval weight of H. armigera *larvae*

A significant weight reduction was observed in susceptible *H. armigera* larvae due to *Bt* cotton feeding ($P<0.0001$; Fig. 5.3 D) as well as in Cry1Ac-resistant larvae after *Bt* cotton feeding ($P=0.0142$; Fig. 5.3 D). For both strains no difference in the total amino acid content was observed due to *Bt* cotton feeding ($P>0.05$; Fig. 5.3 D).

Amino acid composition of H. armigera *larvae*

A total of 15 amino acids were analyzed in *H. armigera* larvae. Valine was only detected in two out of 16 samples in susceptible non-*Bt* cotton-fed larvae, and no valine was found in susceptible *Bt* cotton-fed lepidopterans. The dominant amino acids were glutamate, histidine, arginine (non-*Bt* cotton-fed 13 to 16%; *Bt* cotton-fed 13 to 19%). For

non-*Bt* cotton-fed Cry1Ac-resistant larvae no aspartate, tyrosine, valine, or phenylalanine was detected, while phenylalanine was not detected in *Bt* cotton-fed lepidopterans and aspartate, tyrosine, and valine were only detected in one out of 15 samples. As for susceptible *H. armigera* larvae glutamate, histidine, arginine were the dominant amino acids (non-*Bt* cotton-fed 14 to 20%; *Bt* cotton-fed 14 to 16%). No significant difference was detected for any amino acid in both strains due to *Bt* cotton feeding (P>0.05; Fig. 5.5).

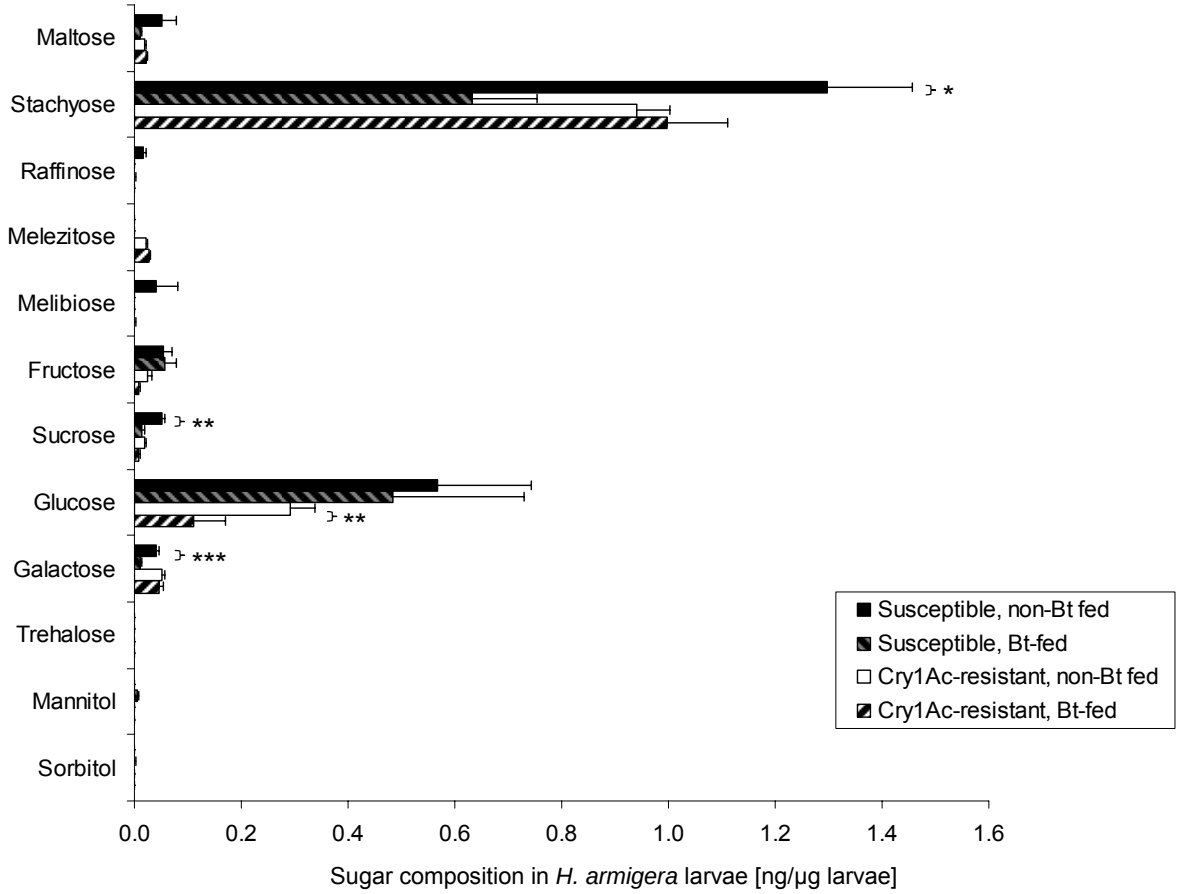

Figure 5.4 Mean sugar composition (ng/µg larvae f.w.; +SE) in susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding on non-*Bt* or *Bt* cotton plants for one day.

C/N analysis

No difference between non-*Bt* and *Bt* cotton plants were measured for the C and N ratio [median C/N (first to third quartile): non-*Bt* cotton 7.83 (7.75 to 8.09); *Bt* cotton 8.36 (8.26 to 8.75); P=0.0524].

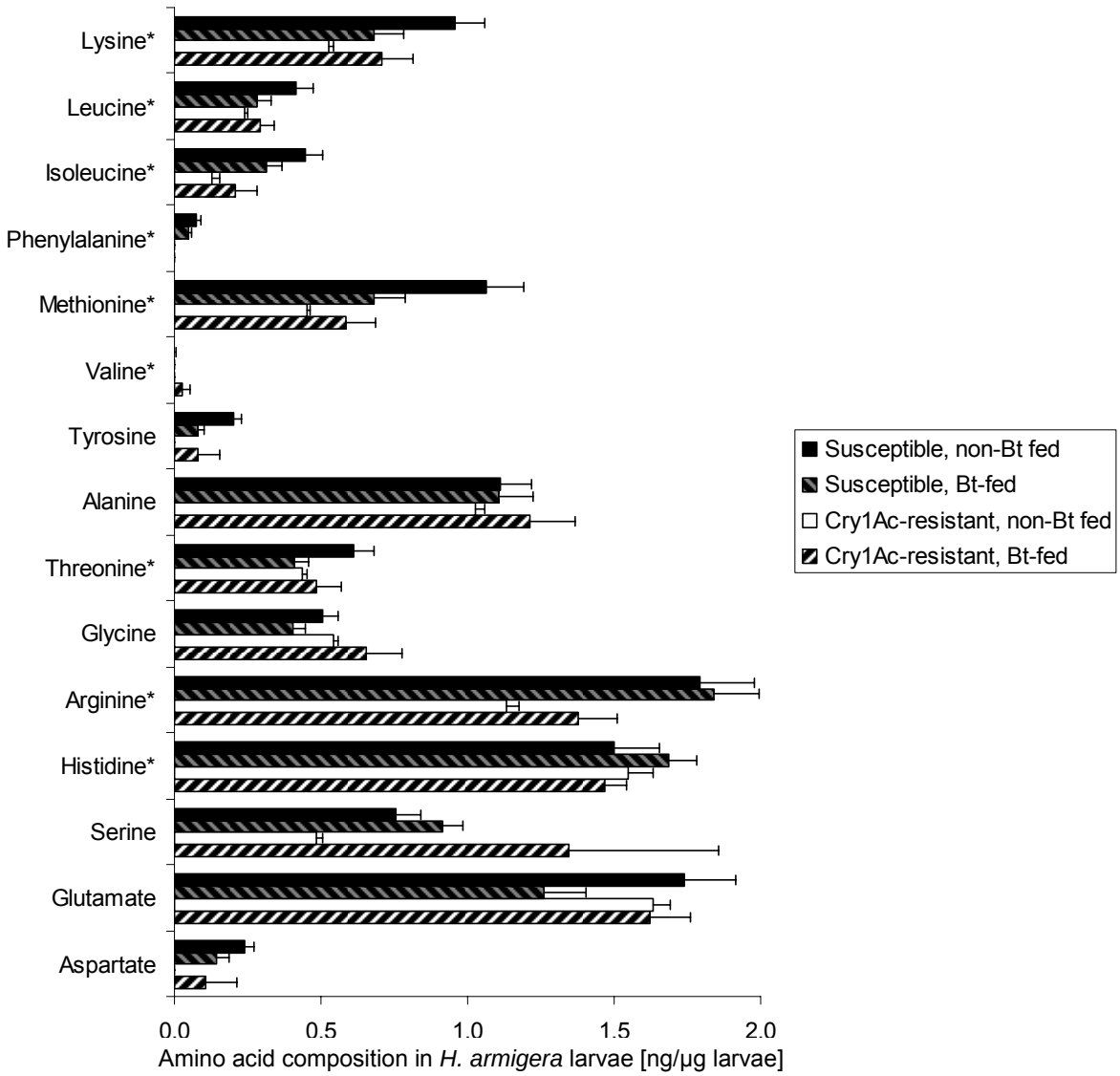

Figure 5.5 Mean amino acid composition (ng/µg larvae f.w.; +SE) in susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding on non-*Bt* or *Bt* cotton plants for one day. The essential amino acids are marked with an asterisk.

Discussion

This study demonstrated a prey-quality mediated effect on *C. carnea* larvae, expressed by an increased mortality and an elongated development time, after feeding on susceptible *Bt* cotton-fed *H. armigera* larvae. In contrast, predator larvae were not affected when feeding on *Bt* cotton-fed Cry1Ac-resistant *H. armigera* larvae even though they contained significantly more Cry1Ac protein then the susceptible lepidopterans. Based on studies of Obrist et al. (2006), where they proved the biological activity of Cry1Ab protein

after ingestion by *T. urticae* we believe that the Cry1Ac protein was still active when present in the gut of Cry1Ac-resistant caterpillars. As *C. carnea* ingested the Cry1Ac protein, direct *Bt* effects can be excluded, confirming earlier direct toxicity studies with Cry1A proteins (Romeis et al. 2004; Rodrigo-Simon et al. 2006; Lawo and Romeis 2008). However, for any assured conclusion feeding studies with a Cry1Ac-sensitive caterpillar, on an artificial diet containing macerated *Bt* cotton-fed Cry1Ac-resistant *H. armigera* larvae would be required.

Evaluating the consumed biomass of *C. carnea* larvae revealed a 1.9 times higher biomass consumption of susceptible *Bt* cotton-fed *H. armigera* larvae compared to non-*Bt* cotton-fed susceptible larvae. This might be due to the fact, that those larvae were smaller and/or more easy to handle for the predator. In case of the Cry1Ac-resistant strain, the predator also consumed more biomass (by a factor of 1.2) of *Bt* cotton-fed larvae when compared to non-*Bt* cotton-fed caterpillars, despite the fact that both *H. armigera* larvae were similar in weight.

One possible explanation for the higher consumption rate of *Bt* cotton-fed prey is that the predators were trying to compensate for any nutritional deficiencies in *Bt* cotton-fed susceptible and less so in Cry1Ac-resistant *H. armigera* larvae. Previous studies by House (1970; 1971a; b) showed that the parasitoid fly, *Agria affinis* (Diptera: Sarcophagidae), was able to select the artificial diet with the best nutrient balance for optimal growth. Specty et al. (2003), who fed coccinellid larvae either with aphids or moth eggs reported that the predator had to consume three times more aphids to achieve a similar development time and final weight. Studies of Mayntz et al. (2005) showed for a ground beetle and two different spiders that carnivores are able to forage selectively for certain nutrients to redress specific nutritional imbalances. Similarly, Zhang et al. (2006) found the parasitoid *Campoletis chlorideae* (Hymenoptera: Ichneumonidae) to be sublethally affected when developing in *Bt* cotton-fed *Bt*-resistant *H. armigera* larvae, indicating a nutritional deficiency of the host larvae. Therefore, we conclude that the Cry1Ac protein also caused some effect on Cry1Ac-resistant *H. armigera* larvae in our study.

Interestingly in our studies, a weight decline in Cry1Ac-resistant caterpillars after one day of *Bt* cotton feeding was observed in some analysis (Fig. 5.3 C and D) but not in others (Fig. 5.3 A and B). This weight reduction in Cry1Ac-resistant *H. armigera* larvae became even more evident when they were exposed to increased amounts of Cry1Ac

protein in a supplemental artificial diet bioassay (Supplementary Fig. 5.1 B; 5.2 A; 5.3 A). Consequently, the larvae have to be regarded as "tolerant" and not "resistant" to Cry1Ac protein.

The strain $ISOC_8$ used in our studies was developed from the strain examined by Bird and Akhurst (2004). The $ISOC_4$ strain was developed by backcrossing a resistant strain (IS) to a susceptible laboratory strain, ANGR, with subsequent re-selection for Cry1Ac-resistance in the F_2 generation and one or two following generations; the backcross and re-selection was repeated four times to produce the $ISOC_4$ strain. Another four backcrosses and re-selections from $ISOC_4$ were used to generate the $ISOC_8$ strain. As there were about 12 to 16 generations between $ISOC_4$ and $ISOC_8$ and four backcrosses, it is reasonable to assume that the genetic backgrounds of $ISOC_4$ and $ISOC_8$ were slightly different to each other and one needs to be careful when drawing conclusions for $ISOC_8$ based on findings reported earlier by Bird and Akhurst (2004) and/or Ma et al. (2005) for $ISOC_4$ (Ray Akhurst personal communication).

Our biochemical analyses revealed no evidence that shifts in lipid, glycogen or amino acid content in the *Bt* cotton-fed *H. armigera* larvae were responsible for the observed effects on *C. carnea*. However, a reduction in the sugar content and a shift in the sugar and amino acid composition were observed due to *Bt* cotton feeding in susceptible lepidopterans. Even if no variation in the lipid content of Cry1Ac-resistant *H. armigera* larvae was observed due to *Bt* cotton feeding, a shift in the lipid content was measured in Cry1Ac-resistant larvae after feeding on an artificial diet containing a high Cry1Ac protein concentration (Supplementary Fig. 5.2 B). The huge variation might partly be explained by the fact that we dealt with a mixed population rather than a homozygous one, varying in their resistance response (O Schmidt personal communication). No shift in glycogen content was noticed after susceptible caterpillars fed on non-*Bt* and *Bt* cotton. Interestingly, a shift in the glycogen content was detected after susceptible *H. armigera* larvae fed on artificial diet containing Cry1Ac at a concentration exceeding the plant level by approximately a factor of ten (Supplementary Fig. 5.2 C). The observation of a significant reduction in the glycogen content in Cry1Ac-resistant *H. armigera* larvae after one day of *Bt* cotton feeding was regarded as inconsiderable as no differences in the glycogen content of Cry1Ac-resistant caterpillars which fed for two, four, or six days on non-*Bt* or *Bt* cotton was evident (data not shown). Further, no shift in the glycogen content was measured in

Cry1Ac-resistant *H. armigera* larvae after feeding on an artificial diet containing Cry1Ac concentrations higher than the plant expression levels (Supplementary Fig. 5.2 C).

The total sugar content was found to be significantly reduced in susceptible *Bt* cotton-fed *H. armigera* larvae, when compared to larvae fed non-*Bt* cotton. Since this difference was not observed for susceptible larvae after feeding on an artificial diet containing different Cry1Ac protein concentration (Supplementary Figure 5.3 B), it was concluded that plant factors other than the *Bt* protein might be the cause. Furthermore, the observed shift in the total sugar amount is unlikely to be a reason for the recorded effects on the *C. carnea* larvae since a rough calculation based on the consumed biomass of single *C. carnea* larvae, the median nutrition content and weight of caterpillar larvae showed that the predator balanced the amount of total adsorbed sugars successfully. Further, analyzing the C/N ratio of non-*Bt* and *Bt* cotton leaves did not provide any evidence of a nutrient shift in the prey larvae feeding on the cotton plants. Our results were in contrast to studies by Flores et al. (2005) who report a significant difference in the C/N ratio between non-*Bt* and *Bt* cotton. One other factor that could have contributed to the observed effects is secondary plant compounds such as gossypol (Faria et al. submitted).

A significant difference in sugar composition was observed for *Bt* cotton-fed caterpillars as well as for caterpillars that had fed on a Cry1Ac-containing artificial diet (Supplementary Fig. 5.4). For susceptible *H. armigera* larvae a reduction in sucrose, stachyose and galactose was observed independently from the fact whether larvae were fed *Bt* cotton or Cry1Ac-containing artificial diet. Interestingly, sucrose (besides fructose) is important for insect growth (Vanderzant 1965; Dadd 1985) and Wäckers (2001) reported a significant increase in parasitoid longevity due to feeding on sucrose, stachyose and galactose. Due to the importance for insect performance, the reduction in these sugars might be one reason for a reduced predator survival. Our artificial diet studies confirmed the reduction in these sugars and revealed further a decrease in susceptible *H. armigera* larvae for maltose, raffinose, and melibiose and an increase in sorbitol. Even if no significant changes in susceptible *H. armigera* larvae could be observed for these sugars after *Bt* cotton feeding, they show a similar trend as that observed for Cry1Ac containing artificial diet. Both maltose and melibiose, for example, increased parasitoid longevity while raffinose did not cause any effect (Wäckers 2001; Winkler et al. 2005). In studies investigating the impact of sorbitol, Ozalp and Emre (2001) reported a negative impact on parasitoid longevity. Analyzing the sugar composition of Cry1Ac-resistant *H. armigera*

larvae, a significant decrease in the glucose level due to *Bt* cotton feeding was assessed. However, this could not be confirmed in the artificial diet studies (Supplementary Fig. 5.4) and was thus probably not due to the Cry1Ac protein. Further, as *C. carnea* larvae were not affected when feeding on *Bt* cotton-fed Cry1Ac-resistant lepidopterans, this observed shift in the glucose concentration appeared not to be of importance. Feeding on a low Cry1Ac-containing diet caused a reduction in the sorbitol and fructose content and after feeding on a high Cry1Ac-containing diet a reduction in melibiose, maltose, and fructose was detected.

No variation was observed in the amino acid composition in any of the *H. armigera* strains correlated to *Bt* cotton feeding. However, for susceptible *H. armigera* larvae a significant reduction in lysine content was observed after the consumption of Cry1Ac protein. An increase in β-alanine, tyrosine, glutamic acid and GABA was observed at Cry1Ac protein concentration that exceeded the plant expression level. Correlating to a high Cry1Ac protein concentration an increase in glutamine-histidine, tyrosine, leucine, glutamic acid, and glycine was observed, and a decrease in β-alanine and GABA. As no shift in the amino acid composition was observed due to *Bt* cotton feeding in either strain, those findings may be of little ecological relevance. In an earlier study, Salama et al. (1983) reported a decrease, increase or constant amount for individual amino acids due to feeding on a *Bt*-containing artificial diet in the haemolymph of third instar *S. littoralis* larvae. It has to be considered that 50 times more *Bt* protein was present in the diet used by Salama et al. (1983) compared to our highest Cry1Ac protein concentration mixed in artificial diet. Further, an increase in some amino acids were reported by Benz (1963) who analyzed *Bt* infected *Melolontha* larvae. A shift in the amino acid pattern was also observed in *S. littoralis* larvae infected with nuclear polyhedrosis virus (Boctor 1980).

Conclusion

C. carnea is lethally affected when feeding on susceptible *Bt* cotton-fed *H. armigera* larvae but not when feeding on *Bt* cotton-fed Cry1Ac-resistant lepidopterans. This clearly gives evidence to a prey-quality mediated effect. A direct toxicity of the Cry1Ac protein could be excluded as *C. carnea* larvae feeding on *Bt* cotton-fed Cry1Ac-resistant *H. armigera* strain were exposed to substantially more Cry1Ac protein compared to feeding on susceptible *Bt* cotton-fed caterpillars.

Since the lipid and glycogen content as well as the amino acid content and composition did not differ between non-*Bt* and *Bt*-fed susceptible *H. armigera* larvae, these parameters do not seem to be responsible for the observed prey-quality mediated effect. However, significant differences in both the total sugar amount and sugar composition could be detected in *H. armigera* larvae fed with *Bt* cotton. And even if *C. carnea* larvae feeding on susceptible *Bt* cotton-fed prey compensated successfully for this discrepancy by increasing the eaten biomass, no assumption can be given regarding the sugar composition. Therefore, these shifts might be one reason for the observed indirect effects on the predatory lacewing larvae. Further an increase in immune and metabolic status of susceptible *Bt* cotton-fed prey larvae might be an explanation for prey-quality mediated effects (Rahman et al. 2004). Beside that many more nutrients than those studied here and their balance play an important role for the development and performance of insect predators (Dadd 1973), further research is necessary. Sterols such as cholesterol are known to be of importance as well as water-soluble vitamins, biotin, the lipogenic growth factor choline, chloride, minute doses of folic acid, inorganic salts, minerals, fat-soluble vitamins A and E and sometimes ribonucleic and nucleic acids (Dadd 1973, 1985).

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Supplemental Material

Effects of *Bt* cotton on lepidopteran larvae and the determination of subsequent prey-quality mediated effects on a lacewing predator

Supplementary Material and Methods

Sensitivity of *H. armigera* to Cry1Ac

To evaluate the sensitivity of both *H. armigera* strains, neonate lepidopteran larvae (≤16-h old) were fed with artificial diet (prepared as described by Akhurst et al. 2003) containing different amounts of Cry1Ac protein: 0, 0.001, 0.01, 0.1, 1, 10 or 100µg Cry1Ac/ml diet. The solidified diet was cut into small pieces (approximately 1ml diet ≈ 1.02g) and placed singly into cells of bioassay trays (C-D International, Pittman, NJ). Individual *H. armigera* larvae were introduced to each of the cells, which were subsequently sealed with a vented acetate cover (C-D International, Pittman, NJ). Larval mortality was determined after seven days. A larva was considered alive when it responded to a mechanical stimulation by a fine hairbrush. Surviving larvae were frozen and subsequently weighed on a microbalance. In total, 63 to 64 susceptible and 114 to 124 Cry1Ac-resistant *H. armigera* larvae were examined. Bioassays were conducted separately in a climatic chamber at 25 ± 1°C, 30 ± 5% r.h. and a 16-h photoperiod. ELISA studies were conducted to verify the Cry1Ac protein amount in the artificial diet provided to the larvae. Three pieces of diet were collected three times from each batch (n=8 to 9 per protein concentration in the range 0.1 to 100µg/ml). Analyses were performed as described in the main text with the exception that samples were diluted at a ratio of 1:10 (sample material f.w.:buffer) and macerated for 50sec at 15Hz. Subsequently, all samples were diluted 1:2, 1:20, 1:200, or 1:2000.

Changes in biochemical composition of Cry1Ac-treated susceptible and Cry1Ac-resistant *H. armigera* larvae with Cry1Ac protein concentration

Separate bioassays aimed to assess how different Cry1Ac protein concentrations caused a change in weight as well as in the nutrition composition in neonate susceptible and Cry1Ac-resistant *H. armigera* larvae after feeding for 24h on artificial diet containing no, a low or a high Cry1Ac protein concentration (0, 0.1 or 10µg Cry1Ac/ml diet).

Concentrations were chosen based on the outcome of the previous laboratory experiment and comparable bioassay conditions were used. After 24h of feeding, surviving larvae were pooled and weighed on a microbalance to reach a total of 800 to 2000µg. Larvae were then stored at -80°C for nutritional analysis. In total, 26 to 43 samples per treatment were collected for lipid and glycogen analysis and 10 to 15 samples for sugar and amino acid analysis. The Cry1Ac protein content in the artificial diet was verified by analyzing three different parts of each batch of diet fed to the larvae (n=5 to 8 per Cry1Ac protein concentration). Samples were prepared as described above. *H. armigera* larvae samples were analyzed for twelve sugars: sucrose, fructose, glucose, trehalose, mannitol, sorbitol, melibiose, raffinose, stachyose, melezitose, maltose, and galactose and 20 amino acids was analyzed in total: lysine, leucine, isoleucine, phenylalanine, tryptophan, valine, methionine, tyrosine, GABA, alanine, β-alanine, threonine, glycine, arginine, glutamine, histidine, serine, aspargine, glutamic acid, and aspartic acid.

Statistics

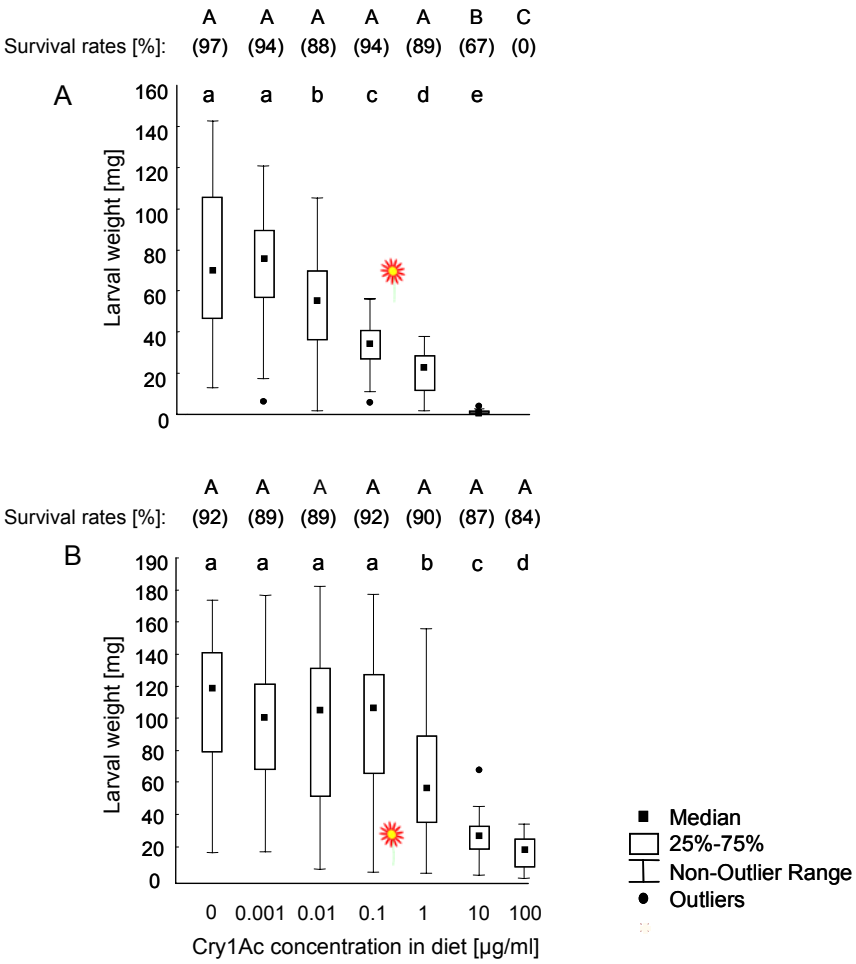
Statistical analyses were conducted for each strain separately. Larval survival was analyzed by performing a two-sided Fisher`s exact test. Since larval weight, and total lipid, glycogen, sugar, and amino acid content and composition were not normally distributed, a Mann-Whitney-*U* test, adjusted for ties, was performed. The Bonferroni-Holm correction was applied for multiple pair-wise comparisons. Nutrient content and composition compared the Cry1Ac protein treatments (0.1 or 10µg Cry1Ac/ml diet) with the control. For all tests the α-level was set at 5%. Statistical analyses were conducted to evaluate the sensitivity of *H. armigera* to Cry1Ac using the software package SAS (version 9.13 by SAS Institute Inc., Cary, NC, USA). Analysis regarding the sugar and amino acid composition were performed using the software package SPSS (version 11.5.1, SPSS Incorporation). For all other statistical analyses the software package Statistica (version 6, StatSoft Inc., Tulsa, OK, USA) was used.

Supplementary Results

Sensitivity of *H. armigera* to Cry1Ac

Mortality of susceptible *H. armigera* larvae significantly increased when diet contained a concentration of 10µg Cry1Ac/ml diet (all P<0.05; Supplementary Fig. 5.1 A). None of the larvae survived feeding on diet containing 100µg Cry1Ac/ml. A significant

weight decline compared to the control was measured from a concentration of 0.01µg Cry1Ac/ml diet onwards (all $P<0.05$). The survival of Cry1Ac-resistant larvae was not influenced by any of the Cry1Ac protein concentrations tested ($P>0.05$; Supplementary Fig. 5.1 B). However, a weight decline was observed at and above a concentration of 1µg Cry1Ac/ml diet (all $P<0.0001$). Nevertheless their weight was a factor 100 times that of the susceptible strain. ELISA studies revealed that 41 to 64% of the initial Cry1Ac content could be extracted from the artificial diet.



Supplementary Figure 5.1 Weight of susceptible (A; n=63 to 64) and Cry1Ac-resistant (B; n=114 to 124) *Helicoverpa armigera* larvae after feeding seven days on artificial diet containing different Cry1Ac protein concentrations. Percentage survival is given in parenthesis. Different lower case letters indicate significant differences between larval weights, capital letters indicate differences in survival ($P<0.05$). Squares represent the median; boxes the first to third quartile; whiskers the non-outlier range; and circles represent outliers and asterisk the extremes. The outlier range is the range of values that fall above the upper outlier limit (+1.5 x the height of the box) and below the lower outlier limit (-1.5 x the height of the box). The flower indicates the approximate Cry1Ac expression in *Bt* cotton.

Changes in biochemical composition of Cry1Ac-treated susceptible and Cry1Ac-resistant *H. armigera* larvae with Cry1Ac protein concentration

Lipid and glycogen content of* H. armigera *larvae

The weight of larvae from both *H. armigera* strains was significantly reduced when feeding on a diet containing 0.1µg Cry1Ac/ml (susceptible strain: $P<0.0001$; Cry1Ac-resistant strain: $P=0.0375$) or 10µg Cry1Ac/ml (susceptible and Cry1Ac resistant prey larvae: $P<0.0001$) compared to the control diet (Supplementary Fig. 5.2 A). For susceptible lepidopterans no significant difference in the total lipid content was observed among the control and Cry1Ac protein feeding treatments (0 vs. 0.1µg/ Cry1Ac/ml: $P=0.1802$; 0 vs. 10µg/ Cry1Ac/ml: $P=0.0702$; Supplementary Fig. 5.2 B). However, a reduction in total glycogen content occurred after feeding on a diet containing a high Cry1Ac protein concentration (0 vs. 0.1µg/ Cry1Ac/ml: $P=0.1324$; 0 vs. 10µg/ Cry1Ac/ml: $P=0.0123$; Supplementary Fig. 5.2 C). In Cry1Ac-resistant larvae the total lipid content was reduced after feeding 24h on a diet containing a high Cry1Ac protein concentration (0 vs. 0.1µg/ Cry1Ac/ml: $P=0.3799$; 0 vs. 10µg/ Cry1Ac/ml $P=0.0009$; Supplementary Fig. 5.2 B), however no difference among treatments was measured in the glycogen content (0 vs. 0.1µg/ Cry1Ac/ml: $P=0.3342$; 0 vs. 10µg/ Cry1Ac/ml $P=0.2326$; Supplementary Fig. 5.2 C).

Sugar and amino acid content of* H. armigera *larvae

In this experiment also, larval weight was significantly reduced due to feeding on a diet containing a high or a low Cry1Ac protein concentration (susceptible *H. armigera* larvae: 0 vs. 0.1µg Cry1Ac/ml $P=0.0017$; 0 vs. 10µg Cry1Ac/ml $P<0.0001$; Cry1Ac-resistant *H. armigera* larvae: 0 vs. 0.1µg Cry1Ac/ml $P=0.0453$; 0 vs. 10µg/ Cry1Ac/ml $P<0.0001$; Supplementary Fig. 5.3 A). However, Cry1Ac protein did not influence the total sugar content in any of the samples tested (susceptible *H. armigera* larvae: 0 vs. 0.1µg Cry1Ac/ml $P=1.0$; 0 vs. 10µg Cry1Ac/ml $P=0.4363$; Cry1Ac-resistant *H. armigera* larvae: 0 vs. 0.1µg Cry1Ac/ml $P=0.6529$; 0 vs. 10µg/ Cry1Ac/ml $P=0.8063$; Supplementary Fig. 5.3 B).

Sugar composition

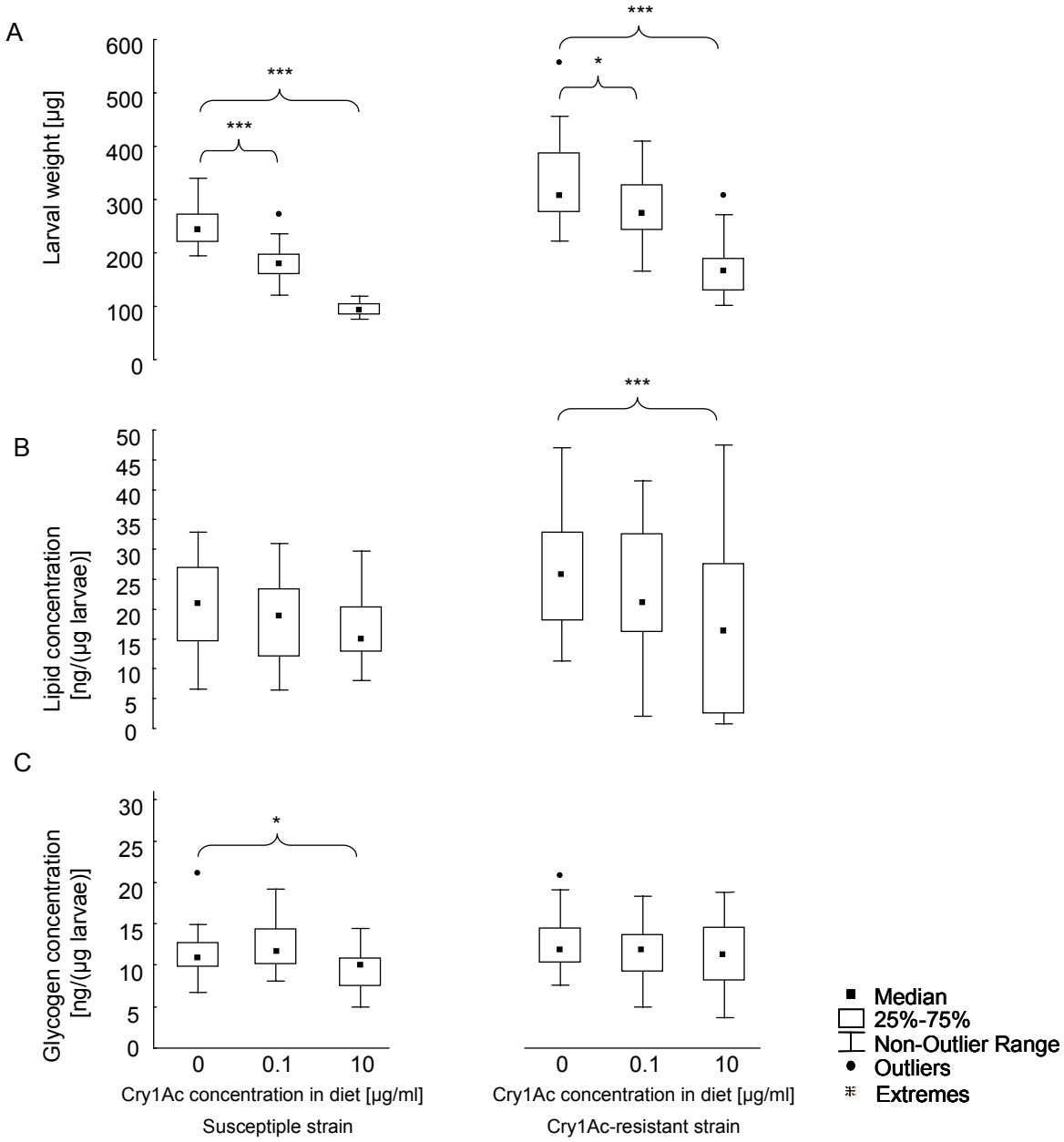
The dominant sugars in both strains and treatments of *H. armigera* larvae were glucose (susceptible larvae: 39 to 60%; Cry1Ac-resistant lepidopterans: 34 to 49%) and

stachyose (susceptible larvae: 16 to 25%; Cry1Ac-resistant lepidopterans: 24 to 30%). Only traces of sorbitol and mannitol were detected in all samples. In the case of susceptible *H. armigera* larvae, no melezitose was found in any of the samples and in Cry1Ac-resistant lepidopterans no melezitose was detected in larvae that fed on a control diet. Otherwise only some samples contained small amounts of melezitose. Significant differences in the sugar composition for susceptible and Cry1Ac-resistant *H. armigera* larvae due to Cry1Ac protein feeding are shown in Supplementary Fig. 5.4.

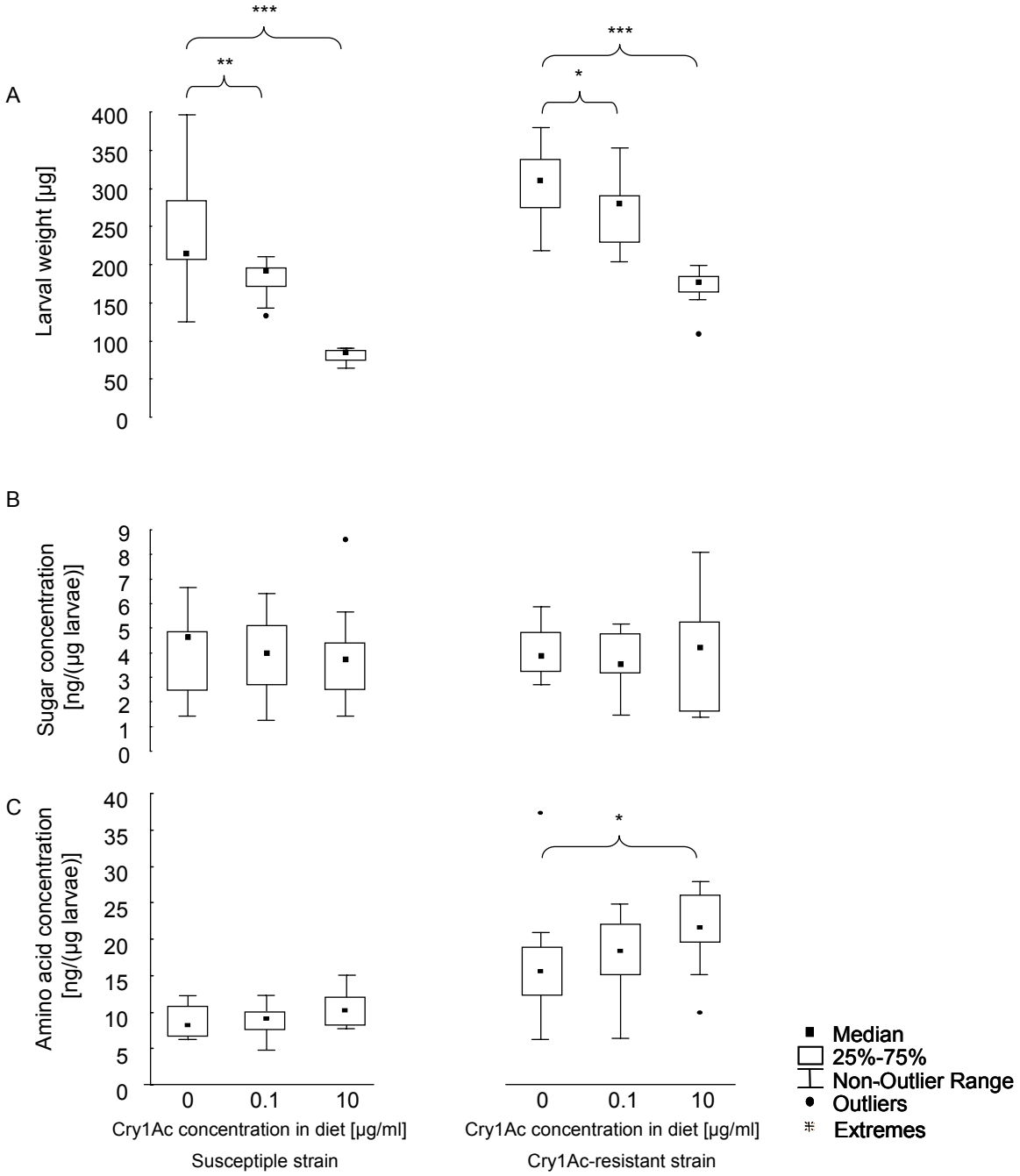
Amino acid composition

The most dominant amino acids in susceptible caterpillars were lysine (10 to 17%), glutamine-histidine (17 to 19%), and glutamic acid (14 to 17%). In all samples of Cry1Ac-resistant larvae the most abundant amino acids were glutamic acid (14 to 15%), alanine (11 to 13%) and leucine (12 to 19%): No lysine was detected in any of the Cry1Ac-resistant samples, no tryptophan and β-alanine in Cry1Ac-resistant caterpillars that had fed on a diet containing a high Cry1Ac protein amount; arginine was detected only in caterpillars that had fed on the control diet. Significant differences in the sugar composition for susceptible and Cry1Ac-resistant *H. armigera* larvae due to Cry1Ac protein feeding are shown in Supplementary Fig. 5.5.

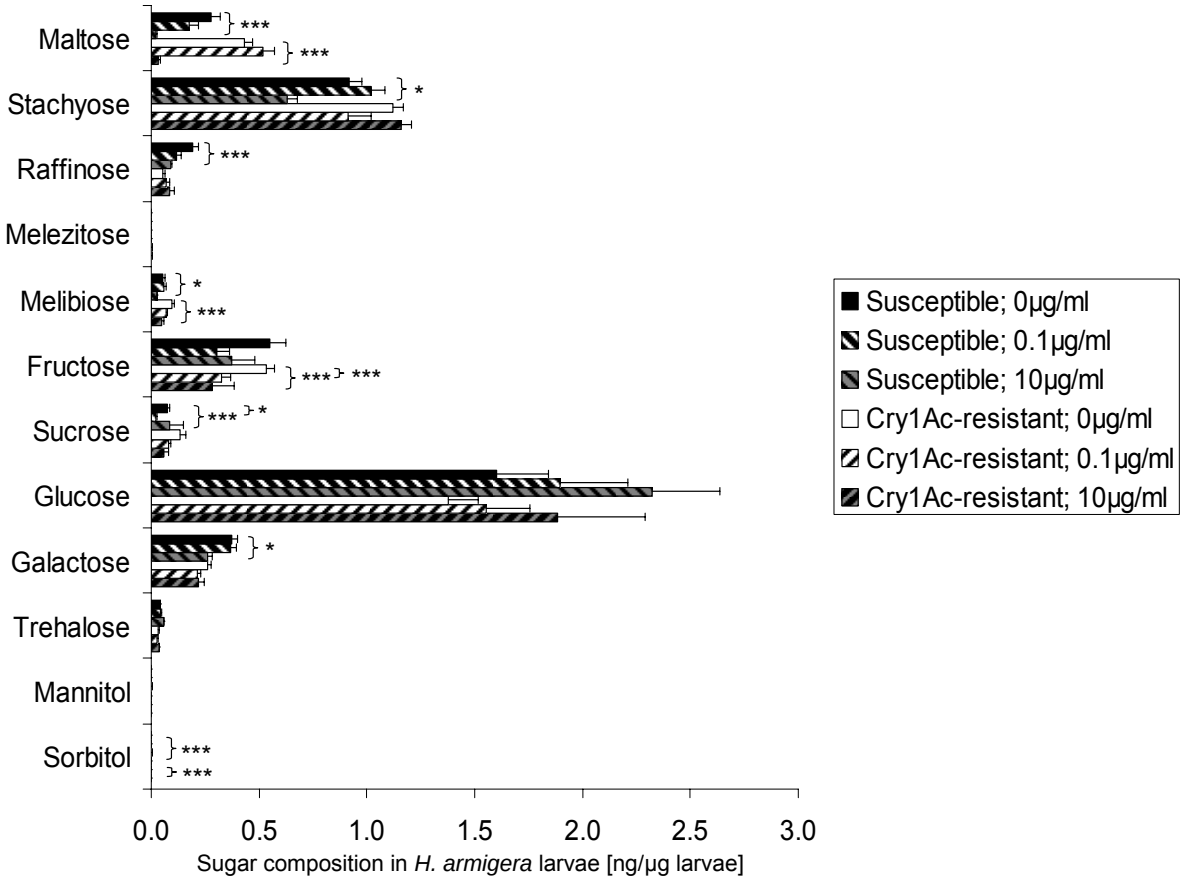
From 80 to 100% of the initial Cry1Ac content was detected in the artificial diet per treatment.



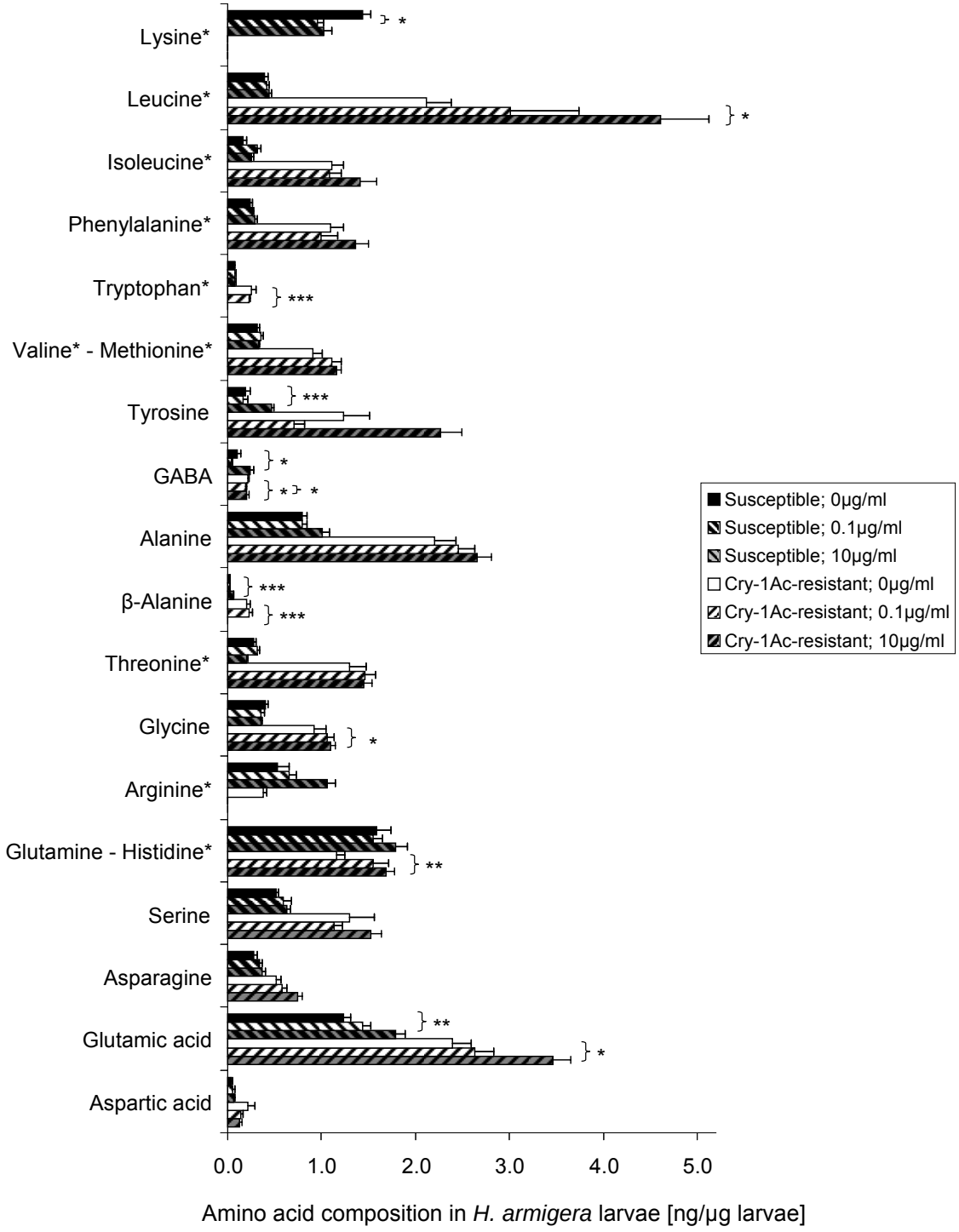
Supplementary Figure 5.2 (A) Fresh weight of susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding for 24h on artificial diet containing 0, 0.1 or 10µg Cry1Ac/ml diet (susceptible *H. armigera* larvae: *n*=26 to 31; Cry1Ac-resistant larvae: *n*=37 to 43). The same larvae were used to examine the lipid (B) and glycogen (C) concentration (ng/µg larvae). Statistical comparisons were made separately for each *H. armigera* strain. Statistical significance is shown between the Cry1Ac protein treatments (0.1 or 10µg Cry1Ac/ml diet) and the control (0µg Cry1Ac/ml diet) ($P<0.05$). Squares represent the median; boxes the first to third quartile; whiskers the non-outlier range; circles represent outliers and asterisks the extremes. The outlier range is the range of values that fall above the upper outlier limit (+1.5 x the height of the box) and below the lower outlier limit (-1.5 x the height of the box); *, $P<0.05$; ***, $P<0.001$.



Supplementary Figure 5.3 (A) Fresh weight and (B) total sugar (C) and amino acid content of susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding for 24h on artificial diet containing 0, 0.1 or 10µg Cry1Ac/ml diet (*n*=15). Statistical comparisons were made separately for each *H. armigera* strain. Statistical significance is shown between the Cry1Ac protein treatments (0.1 or 10µg Cry1Ac/ml diet) and the control (0µg Cry1Ac/ml diet) ($P<0.05$). Squares represent the median; boxes the first to third quartile; whiskers the non-outlier range; circles represent outliers and asterisk the extremes. The outlier range is the range of values that fall above the upper outlier limit (+1.5 x the height of the box) and below the lower outlier limit (-1.5 x the height of the box); *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.



Supplementary Figure 5.4 Mean sugar composition (ng/µg larvae f.w.; +SE) in susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding for 24h on artificial diet containing different Cry1Ac protein concentrations (0, 0.1 or 10µg Cry1Ac/ml diet); *n*=26-43.



Supplementary Figure 5.5 Mean amino acid composition (ng/µg larvae f.w.; +SE) in susceptible and Cry1Ac-resistant *Helicoverpa armigera* larvae after feeding for 24h on artificial diet containing different Cry1Ac protein concentrations (0, 0.1 or 10µg Cry1Ac/ml diet); *n*=10-15.

Chapter six

Effectiveness of *Bacillus thuringiensis*-transgenic chickpeas and the entomopathogenic fungus *Metarhizium anisopliae* in controlling *Helicoverpa armigera* (Lepidoptera: Noctuidae)[5]

[5] Based on: Lawo NC, Mahon RJ, Milner RJ, Sarmah BK, Higgins TJV, Romeis J (2008) Effectiveness of *Bacillus thuringiensis*-transgenic chickpeas and the entomopathogenic fungus *Metarhizium anisopliae* in controlling *Helicoverpa armigera* (Lepidoptera: Noctuidae). Applied and Environmental Microbiology 74:4381-4389.

Abstract

The use of genetically modified (*Bt*) crops expressing lepidopteran-specific Cry proteins derived from the soil bacterium *Bacillus thuringiensis* is an effective method to control the polyphagous pest *Helicoverpa armigera*. As *H. armigera* potentially develops resistance to Cry proteins, *Bt* crops should be regarded as one tool in integrated pest management. Therefore, they should be compatible with biological control. Bioassays were conducted to understand the interactions between a Cry2Aa-expressing chickpea line, either a susceptible or Cry2A-resistant *H. armigera* strain, and the entomopathogenic fungus *Metarhizium anisopliae*.

In a first concentration-response assay, Cry2A-resistant larvae were more tolerant of *M. anisopliae* than susceptible larvae, while in a second bioassay the fungus caused similar mortalities in the two strains fed control chickpea leaves. Thus, resistance to Cry2A did not cause any fitness costs that would become visible in an increased susceptibility to the fungus.

On *Bt* chickpea leaves, susceptible *H. armigera* larvae were more sensitive to *M. anisopliae* than on control leaves. It appeared that sublethal damage induced by the *B. thuringiensis* toxin enhanced the effectiveness of *M. anisopliae*. For Cry2A-resistant larvae the mortalities caused by the fungus was similar when fed either food source.

To examine which strain would be more likely to be exposed to the fungus, their movements on control and *Bt* chickpea plants were compared. Movement did not appear to differ among larvae on *Bt* or conventional chickpea as indicated by the number of leaflets damaged per leaf. The findings suggest that *Bt* chickpeas and *M. anisopliae* are compatible to control *H. armigera*.

Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the most important insect pests in the Old World due to its mobility, high polyphagy, short generation duration and high reproductive rate (Fitt 1989; Sharma 2005). Currently, the application of chemical spray insecticides is the most common method of controlling this pest on crops including cotton (King 1994; Durairay et al. 2005) and chickpea (Shanower et al. 1998; Sharma et al. 2007). *H. armigera* is known to develop resistance to almost all the insecticides used for its control (Forrester et al. 1993; Kranthi et al. 2002). These chemical sprays are also of environmental concern and are responsible for human health

problems (Pray et al. 2002; Qaim et al. 2008). Thus alternative control methods are increasingly being employed. The use of genetically modified (GM) crops that express insecticidal genes such as those derived from the soil bacterium *Bacillus thuringiensis* (*Bt* crops) provide a powerful option to control pest Lepidoptera (Shelton et al. 2002). This technology, for example, is applied to protect cotton plants by the expression of *B. thuringiensis cry* genes, i.e., *cry1Ac* and *cry2Ab* either alone or in combination, from damage by the budworm/bollworm complex [*Helicoverpa/Heliothis* spp., and *Pectinophora gossypiella* (Saunders)]. These *B. thuringiensis*-transgenic cotton plants are highly resistant to damage by lepidopteran pests, and consequently, the application of chemical insecticides has been greatly reduced (Fitt 2008, Naranjo et al. 2008). This makes *Bt* cotton a valuable component of integrated pest management programs with many environmental, economical and health benefits (Pray et al. 2002; Qaim et al. 2008).

As with cotton, the expression of *B. thuringiensis cry* genes is an option to protect chickpeas from damage by *H. armigera* (Romeis et al. 2004). Chickpea plants that express either Cry1Ac or Cry2Aa or both proteins are currently under development and could become commercially available in the future (Sanyal et al. 2005; McPhee et al. 2007).

The deployment of insect-resistant GM plants poses two potential problems. First, the target pest may develop resistance to the expressed insecticidal protein(s) due to the strong and continued selection pressure imposed on the insect populations (Gould 1998; Shelton et al. 2002; Tabashnik et al. 2003). This is particularly the case for *H. armigera* for which populations resistant to single Cry proteins have been selected in the laboratory (Ferré et al. 2008). To manage insect resistance development, the us of high-dose-expressing *Bt* plants, along with an adjacent refuge of non-*Bt* plants, is considered to be the most effective strategy (Tabashnik et al. 2003; Ferré et al. 2008). Most resistance alleles are recessive, and the frequency of such alleles in pest populations is generally very low before resistance becomes evident (Ferré et al. 2008). However, recently, a relatively high baseline frequency of resistance alleles for Cry2Ab (0.0033) has been reported in an Australian *H. armigera* population prior to the widespread adoption of Bollgard II™ (Monsanto Company, St Louis, MO) cotton, which expresses this protein in combination (pyramided) with Cry1Ac (Mahon et al. 2007a; b).

The second area of concern is the possible effect of insect-resistant GM crops on nontarget organisms, especially those that provide important ecological services, such as biological control (Romeis et al. 2008a; b). These organisms are important, since they help

to keep other herbivores that are not affected by the insecticidal GM protein under their economic thresholds, but also because they potentially help to kill target insects that have developed resistance against the GM trait. Biological control of arthropods is thus considered during the environmental-risk assessment of insecticidal GM crops (Romeis et al. 2008b), and a great deal of research has been conducted to assess the impact of *B. thuringiensis*-transgenic crops on arthropod predators and parasitoids (Romeis et al. 2006). Overall, studies have not revealed any direct effects of the *B. thuringiensis* Cry proteins on natural enemies. In contrast to arthropod natural enemies, insect pathogens have received little attention. This needs to be addressed, since it is known that the activity of entomopathogens is affected by host plant resistance factors. Hare (2002) has compiled a comprehensive literature review of the interactions between host plants, herbivores and pathogens. Additive effects of these interactions were reported in over half of the published studies, while approximately one-third reported synergistic effects. However, little attention has been given to the interactions of *Bt* plants with entomopathogenic fungi, despite evidence that *H. armigera* is attacked by a variety of entomopathogens, such as *Nomuraea rileyi*, *Beauveria bassiana*, and *Metarhizium anisopliae* (Grzywacz et al. 2005). Since the application of *M. anisopliae* is a promising method to control *H. armigera* in India (Nahar et al. 2003; 2004a; b), it was selected for our investigations.

To evaluate the complementarity of a pathogen and a *Bt* plant for insect pest control, we studied the interaction of the entomopathogenic fungus *M. anisopliae*, a susceptible and a Cry2A-resistant strain of *H. armigera*, and Cry2Aa-expressing chickpea plants (transformation line BS 5A). Previous bioassays have shown that this line caused approximately 36% mortality among neonate *H. armigera* larvae (Acharjee et al. unpublished data). This low-*cry2Aa*-expressing line was chosen in order to examine the combined effects of *M. anisopliae* and *Bt* chickpea plants on the susceptible *H. armigera* strain. To determine the responses of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae* on control (non-*Bt*) plants, a concentration-response curve was established. Using this knowledge, we examined whether the fungus could complement the mortality induced by the toxin in the *Bt* plant in laboratory and greenhouse studies. Subsequently, we evaluated the feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae when exposed to control and *Bt* chickpeas.

Materials and Methods

Plants

B. thuringiensis-transgenic chickpea (*Cicer arietinum* var. Desi) plants (transformation line BS 5A; families: 5A.6.14.1, 5A.6.17.3, 5A.6.17.4), (Acharjee et al. unpublished data) expressing a full-length Cry2Aa toxin (*Bt* plants) and the corresponding nontransformed near-isoline cv. Semsen (control plants) were used in the bioassays. Seeds were germinated in a climate chamber at 24°C ± 1°C before being planted. The plants were grown individually in sandy soil (70% compost, 15% sand, 15% perlite) in plastic pots (15cm in diameter; 14cm high) at a temperature of 25 to 32°C during the day and 15 to 20°C at night with an ~12-h photoperiod in the greenhouse. Plants 5 to 8 weeks old were used in all bioassays.

Expression of Cry2Aa protein in plants

To ensure that presumptive transgenic chickpea plants were expressing the Cry2Aa protein, Western blot analyses were conducted on a total of 152 plants.

In the laboratory bioassay examining the complementarity of *Bt* chickpeas and *M. anisopliae*, 47 presumptive transgenic plants of the families used in the bioassay were analyzed. In 6% of those plants, no Cry2Aa protein could be detected. Because of the presence of nonexpressors, all transgenic plants were tested before use in subsequent bioassays. A two to three-fold variation in expression of the Cry2Aa toxin among expressors was also observed (Fig. 6.1).

Leaves of untransformed chickpea plants were used as a negative control, while a high-expressing line, BS 6H, which caused 98% mortality in *H. armigera* larvae (Acharjee et al. unpublished data) was used as a positive control. Protein was extracted from 80 to 100mg of a young, fully expanded leaf into 400µl of extraction buffer {0.1M TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid) (Sigma catalog no. T-5691) pH 7.6, 0.2M NaCl, 1mM PMSF, 1mM EDTA}. The suspension was centrifuged at 13,000rpm for 5min, and the resultant supernatant was used for protein determination (Bradford 1976). Forty micrograms of protein (with disulfide bonds reduced in the presence of β-mercaptoethanol) from each sample was separated by size fractionation in a NuPage precast 10% Bis-Tris polyacrylamide gel system (Invitrogen catalog no. NP0315), using a MOPS (morpholinepropanesulfonic acid)-sodium dodecyl sulfate running buffer (50mM MOPS,

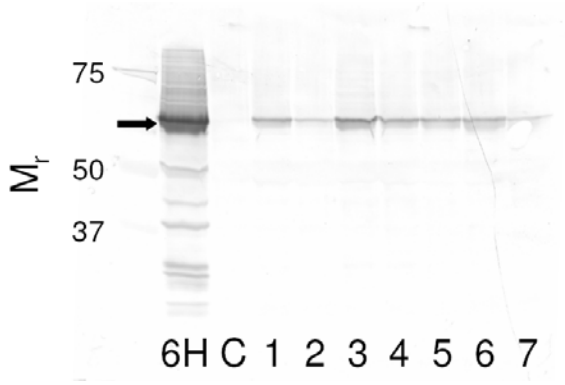


Figure 6.1 Western blot of chickpea leaf proteins (40µg per lane). Lane 6H contained protein from a high-expressing line that caused 98% mortality in *Helicoverpa armigera* larvae, lane C contained protein from a nontransgenic chickpea leaf and lanes 1 to 7 contained proteins from individual *Bt* chickpea plants used in the bioassays. The numbers on the *y* axis (M_r) refer to the relative molecular masses of markers (10^3). The arrow indicates the position of full-length Cry2Aa protein.

50mM Tris-base, 3.5mM sodium dodecyl sulfate, 1mM EDTA). The protein was transferred electrophoretically to a nitrocellulose membrane (200mA for 90min.) using transfer buffer (25mM Bicine, 25mM Bis-Tris, 1mM EDTA, 10% methanol). The nitrocellulose membrane was blocked in Tris-buffered saline solution (20mM Tris, pH 7.5, 0.5M NaCl) and 5% skim milk powder for 1h. The membrane was washed in Tris-buffered saline solution and 0.1% Tween 20 (TTBS). The primary anti-Cry2Aa antibody, raised in rabbit, was diluted in TTBS, and incubated with the membrane for 1h, before being washed briefly with TTBS. The secondary antibody, anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate (Promega catalog no. S3731), was diluted in TTBS and incubated with the membrane for 1h before being washed briefly in TTBS. Cry2Aa protein bands were detected by the addition of BCIP (5-bromo-4-chloro-3-indolylphosphate)/Nitro Blue Tetrazolium substrate (Sigma catalog no. B5655).

Insect material

Strains of susceptible and Cry2A-resistant *H. armigera* were provided by CSIRO Entomology, Canberra, Australia. The Cry2A-resistant strain (SP15) was established from a single *H. armigera* pair collected as eggs on corn near Griffith, NSW (Mahon et al. 2007a). To maintain fitness vigor, the Cry2A-resistant strain was outcrossed with a susceptible strain (GR). After three outcrosses to the susceptible strain, the Cry2A-resistant colony was genetically very similar (87% isogenic) to the susceptible strain (with the exception of the linkage group containing the gene conferring resistance) (Mahon et al. 2007a).

Larvae were reared as described by Teakle and Jensen (1985), except that three or four neonates were kept in each well (3cm x 3cm x 2cm) of a 32-well plastic tray (Oliver Products Company, Grand Rapids, MI) until they reached the third instar. Subsequently, the larvae were separated and kept in individual wells on a fresh diet. Larvae used to evaluate the sensitivity of *H. armigera* to *M. anisopliae* were treated as described above. For bioassays involving *Bt* chickpea plants, ~50 neonates were reared in plastic boxes (12cm in diameter; 6cm high) on control chickpea leaves (10 to 12 weeks old) until they reached the third instar. Two pieces (ca. 5ml) of 2% agar were included in the box to raise the humidity in order to limit leaf desiccation. The larvae were re-fed after 2 to 3 days if necessary. Early-third-instar larvae were used in all bioassays. Adults were housed as described by Mahon et al. (2007b).

Fungus

Metarhizium anisopliae var. *anisopliae* (FI-1248) from the CSIRO Insect Pathogen Culture collection was used in the experiments. The strain was originally isolated from a termite, *Mastotermes darwiniensis*, collected near Darwin, Northern Territory, Australia, in 1997. *M. anisopliae* was grown on Oxoid Sabouraud's dextrose agar plus 1% yeast extract for three weeks at ~24°C under natural daylight conditions. Spores were harvested by scraping them from the agar surface using a loop and stored at 4°C until they were use. Clumps of spores were then dispersed in 0.5% Tween 80 using a magnetic stirrer for 1h. The concentration of conidia was estimated using a Petroff-Hausser counting chamber (Hausser Scientific Partnership, Horsham, PA; 1/400mm^2, 0.02mm deep). The initial suspension was serially diluted with 0.5% Tween 80 to the concentrations used in the experiments.

Prior to each bioassay, a sample of spores was taken to determine viability by germinating conidia on thin plates of Sabouraud's dextrose agar with 0.1% chloramphenicol. A droplet of spore suspension (~10^7 spores/ml) was pipetted onto the plate, covered with a thin coverslip, and incubated at ~28°C in the dark for 24h. The plate was examined using a phase-contrast microscope (Leitz, Wetzlar, Germany) at x400 magnification. One hundred spores were examined at three locations on each plate and scored as either germinated (viable) or not germinated (dead). A spore was considered to have germinated if the germ tube was clearly visible. Germination was >90% in all bioassays.

Sensitivity of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*

First laboratory bioassay

Control chickpea leaves (8 leaflets each) were dipped into six *M. anisopliae* Tween 80-based spore suspensions prepared as five-fold serial dilutions ranging from 9.6 x 10^5 to 3 x 10^9 spores/ml. A 0.5% Tween 80 solution was used as a control. The dipped chickpea leaves were placed on the surface of 4 to 5ml cooled 2% agar in wells of 32-well plastic trays. After 2 to 3h exposure to air (to allow leaves to dry), a single early-third-instar *H. armigera* larva was placed on each leaf. The trays were then heat sealed with a vented acetate cover and maintained at 28°C ± 1°C. To give the fungus optimal conditions for germination, the trays were wrapped in damp tissues and enclosed in a plastic bag to provide a humid environment for the first 24h. On days 2, 4, and 6, dead larvae were removed and fresh untreated leaves (8 to 10 leaflets each) were provided to each living larva. On day 8, survivors from each treatment were pooled, and their fresh weight and larval stage were recorded. Dead larvae were incubated at 28°C and >90% r.h. for up to 10 days and examined regularly for evidence of conidial growth. The bioassay was repeated twice, resulting in the exposure of a total of 55 to 61 larvae to each spore concentration. Slopes and 50% lethal concentration (LC_{50}) estimates for the two *H. armigera* strains were calculated using the software package POLO-PC (LeOra Software, Berkeley, CA).

Bt* chickpeas and *M. anisopliae* in combination to control *H. armigera

Second laboratory bioassays

Control and *Bt* chickpea leaves (8 leaflets each) were either dipped in a Tween 80-based suspension of a 'low' (L) spore suspension (1.2 x 10^8 spores/ml), a 'medium' (M) spore suspension (5.7 x 10^8 spores/ml), or in 0.5% Tween 80 (0) as a control. The L and M spore suspension was chosen to lie approximately midway between the LC_{30}^S (L) or LC_{50}^S (M) of the two *H. armigera* strains, respectively, as determined in the concentration-response assay. The bioassays were set up as described above, except that the larvae used in the experiments were reared on control chickpea leaves until they reached the third instar. The larvae were fed with eight leaflets each, (four leaflets each from two plants to provide a mixture of plants that might have different expression levels). The leaves were changed on days 2, 4, and 6. The parameters assessed were larval survival, weight and instar after 8 days and the proportion of larvae producing *M. anisopliae* spores. The bioassay was repeated four times with 30 to 32 larvae tested at each spore concentration.

Larval survival was analyzed using the Cox proportional-hazard model. Bonferroni-Holm correction was performed when required. Each run was analyzed separately, comparing the fungus treatments (L and M) and the controls (susceptible/Cry2A-resistant strain on control/*Bt* leaves with no fungus application). Where no larvae died in a control group, one additional dead larva was added to each treatment to enable statistical analysis. This procedure was necessary for susceptible larvae, run B on control plants, and for Cry2A-resistant larvae, runs C and D on *Bt* plants. Data on larval weights after 8 days feeding were checked for normality and homogeneity of variances prior to analysis. Since all assumptions were met, data were analyzed for all repeated experiments (runs), together with a three-way analysis of variance (ANOVA) (factors: plant, fungus, and *H. armigera* strain; n=125 to 128). For all tests, the α-level was set at 5%. Statistical analyses were conducted using the software package Statistica (version 6, StatSoft Inc., Tulsa, OK).

Greenhouse bioassay

Half of the available control and *Bt* chickpea plants were sprayed until runoff with a *M. anisopliae* spore suspension containing 5.4 x 10^8 spores/ml. The remainder were sprayed with 0.5% Tween 80 as a control. After plants were allowed to dry for 1h, 10 susceptible early-third-instar larvae were placed on each plant, each on a separate leaf. The plants were then enclosed in a cloth bag which was sealed to the pot to ensure that larvae could not escape. To provide humid conditions, plants were enclosed in a plastic bag for the first 24h. The plants were watered every 2 days by placing the pots into water-filled dishes (17cm in diameter; 2cm deep) for 2 to 3h. The mortality of the larvae was evaluated after 10 days. In total three to five plants were used for each of the four treatments. Plants were placed at randomized positions in the greenhouse. During the experiment, the greenhouse temperature varied between 15°C and 35°C with a ~12-h photoperiod and ~40% r.h.

Feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae on control and *Bt* chickpeas

Control or *Bt* chickpea leaves of similar size and structure (10 leaflets each) were placed in petri dishes (9cm in diameter; 2cm high). Subsequently, one early-third-instar *H. armigera* larvae, either susceptible or Cry2A-resistant, was placed on the lowest leaflet of either control or *Bt* leaves. The petri dishes were stored at 25 ± 1°C, 40 ± 5% r.h., and a

14-h photoperiod. After 24h, the larvae were removed and leaf feeding activity evaluated using a nine-category scale according to the damage inflicted by the feeding larvae (categories: 0, 0% damage; 1, <1%; 2, 2 to 5%; 3, 5 to 10%; 4, 10 to 20%; 5, 20 to 30%; 6, 30 to 50%; 7, 50 to 70%; 8, 70 to 80%; 9, >80%). Feces produced during the exposure period by each larva were collected and stored in a desiccator containing silica gel for at least 24h before storage at -80°C. Samples were dried further at 50°C in an oven for at least 4 days before being weighed on a microbalance (Mettler Toledo MX5; division, 1µg; tolerance ±2µg). The experiment was repeated twice, resulting in a total of 32 to 43 larvae per treatment. The data on feeding damage and feces weight were evaluated by Kruskal-Wallis ANOVA, followed by pairwise comparisons using the Mann-Whitney-*U* test adjusted for ties and Bonferroni-Holm correction. The importance of two factors, plant (control or *Bt*) and strain (susceptible or Cry2A-resistant), was evaluated.

Results

Sensitivity of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*

First laboratory bioassay

The concentration-response of the two *H. armigera* strains to *M. anisopliae* is given in Fig. 6.2 and details of mortality, sporulation, weight, and larval instar of survivors are shown in Table 6.1. The LC_{50} for the susceptible strain was determined to be 1.9×10^8 spores/ml (95% confidence interval [CI], 1.5×10^7, 8.1×10^8) while for the Cry2A-resistant strain, it was 7.8×10^8 spores/ml (95% CI, 4.5×10^8, 1.3×10^9). The slope of the line for susceptible larvae (0.95 ±0.187) differed significantly from that of the Cry2A-resistant strain (1.94 ±0.370; P=0.002).

Bt chickpeas and *M. anisopliae* in combination to control *H. armigera*

Second laboratory bioassays

The performance of larvae on control and *Bt* chickpea leaves at different concentrations of fungal spores is shown in Fig. 6.3. For the control chickpea leaves, mortality rates in the *H. armigera* strains were similar (P>0.05); however, a marked fungus effect (P<0.0001) was observed. For the *Bt* chickpea leaves, both strain (P<0.0001) and fungus (P<0.0001) effects were recorded. The data were thus analyzed separately for each *H. armigera* strain. Since larval survival differed significantly between bioassay runs for

control and *Bt* leaves (P=0.003; P=0.018), each run was evaluated separately (runs A to D in Fig. 6.3).

While the L spore concentration did not increase mortality among susceptible *H. armigera* larvae feeding on control leaves (P>0.05), a significant increase was observed in two of the four runs when the larvae were fed *Bt* leaves (run A, P=0.006; run C, P<0.0001). In contrast, the M spore concentration caused a significant level of mortality in susceptible *H. armigera* larvae on control leaves in three of the four runs (run A, P=0.009; run B, P<0.001; run D, P=0.033) and in all four runs on *Bt* leaves (run A, P=0.035; run B, P=0.022; run C, P<0.0001; run D, P=0.002). Mortality was not increased at the L spore concentration on control plants in the Cry2A-resistant strain in any of the runs (P>0.05). The M spore concentration caused significant mortality among Cry2A-resistant larvae in two runs (run A, P=0.009; run B, P=0.005) when control leaves were fed and in one run (run B, P=0.014) when the larvae were feeding on *Bt* leaves.

A three-way ANOVA evaluating the factors fungus (0, L, or M), *H. armigera* strain (susceptible or Cry2A-resistant), and plant (control or *Bt*) indicated that a significant decrease in larval weight occurred after 8 days due to the factors strain and plant (P<0.0001). However, the factor fungus did not significantly contribute to this decrease (P>0.05; Table 6.2).

Table 6.1 Evaluation of the sensitivities of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*[a].

Spore concn (no. of spores/ml)	*H. armigera* strain	No. of larvae exposed	No. of dead larvae (no. of larvae producing spores)	Mortality (%)	Mean weight (mg)	Distribution of larval instars (%)		
						L3	L4	L5
0	Susceptible	60	6 (0)	10.0	68.0	0	48.2	51.9
	Cry2A-resistant	60	6 (0)	10.0	77.7	0	37.0	63.0
9.6×10^5	Susceptible	59	11 (0)	18.6	68.1	0	51.1	48.9
	Cry2A-resistant	59	5 (0)	8.5	67.2	0	59.3	40.7
4.8×10^6	Susceptible	61	24 (6)	39.3	66.7	0	75.7	24.3
	Cry2A-resistant	61	6 (1)	9.8	75.9	0	65.5	34.6
2.4×10^7	Susceptible	55	19 (6)	34.5	63.3	0	81.1	18.9
	Cry2A-resistant	57	8 (2)	17.0	83.1	0	69.4	30.6
1.2×10^8	Susceptible	59	25 (15)	42.4	59.3	0	88.2	11.8
	Cry2A-resistant	58	16 (8)	27.6	76.9	0	63.4	36.6
6×10^8	Susceptible	60	37 (27)	61.7	63.2	12.0	48.0	40.0
	Cry2A-resistant	57	26 (24)	45.6	72.4	0	56.7	43.3
3×10^9	Susceptible	59	51 (48)	86.4	51.2	0	100	0
	Cry2A-resistant	59	47 (46)	79.7	57.9	9.1	72.7	18.2

[a] Larvae were exposed to chickpea leaves treated with six different spore concentrations for 2 days, differing fivefold in their spore concentration. Subsequently, the larvae were provided with untreated chickpea leaves every other day until day 8. Weight is shown as the mean weight of all survivors per treatment. The larval instar reached by surviving larvae at the completion of the experiment is given as percentage of survivors. *n*=55 to 61.

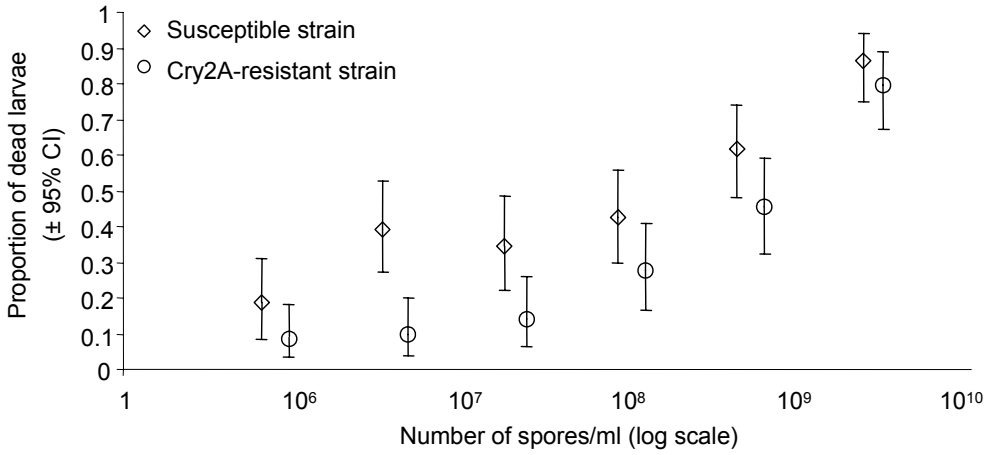


Figure 6.2 Proportions of dead susceptible and Cry2A-resistant *H. armigera* larvae (±95% CI) fed on control chickpea leaves treated with six different spore concentrations of *M. anisopliae* differing fivefold (*n*=55 to 61). Third-instar larvae were treated with the fungus.

Greenhouse bioassay

The greenhouse bioassay was conducted only with susceptible *H. armigera* larvae, since the second laboratory bioassays revealed no interaction of *Bt* plants and fungal efficacy for the Cry2A-resistant *H. armigera* strain. Hardly any larval mortality way observed on the untreated control plants. Approximately 50% of the *H. armigera* larvae died on untreated *Bt* plants and fungus-treated control plants. Combining the *B. thuringiensis* protein and the fungus caused a mortality of 89%. Consequently, the greenhouse bioassay suggested an additive effect of the *B. thuringiensis* toxin and the fungus on the mortality of susceptible *H. armigera* larvae (Fig. 6.4). Of the larvae which had fed on fungus-treated leaves, 25 to 100% of the larvae from the control plants and 20 to 86% of the larvae from the *Bt* plants produced fungal spores. None of the dead larvae on the control treatments produced *M. anisopliae* spores.

Towards the end of the experimental period, an incursion of an unknown insect pathogen, or perhaps insecticide use in neighboring glasshouses, caused mortality to insects in all treatments, including controls. This prevented planned replication of this bioassay. Thus, no statistical analyses were performed.

Table 6.2 Weights and developmental stages of susceptible and Cry2A-resistant *H. armigera* larvae after 8 days of feeding on either control or *Bt* chickpea leaves treated with different spore concentration of *M. anisopliae*.[a]

Plant	Fungus[b]	*H. armigera* strain	Mean wt (mg) ±SE[c]	Distribution of larval instars (%)[d]		
				L3	L4	L5
Control	0	Susceptible	42.0 ± 0.7 a [1]	2.5	41.0	56.6
		Cry2A-resistant	45.1 ± 0.5 a		47.1	52.9
	L	Susceptible	38.6 ± 0.7 a	5.0	57.9	37.2
		Cry2A-resistant	35.8 ± 0.6 a		63.1	36.9
	M	Susceptible	34.3 ± 0.7 a	12.3	53.4	34.2
		Cry2A-resistant	35.6 ± 0.4 a	5.1	73.1	21.8
Bt	0	Susceptible	16.8 ± 0.8 b	41.2	48.2	10.6
		Cry2A-resistant	42.3 ± 0.9 a	1.7	46.6	51.7
	L	Susceptible	28.0 ± 0.2 b	37.8	53.3	8.9
		Cry2A-resistant	41.7 ± 1.1 a	4.5	53.6	41.8
	M	Susceptible	16.0 ± 0.7 b	17.4	60.9	21.7
		Cry2A-resistant	37.8 ± 1.1 a	3.4	50.6	46.0

[a] Data from the four runs (see Fig 6.3) were pooled (n =125 to 128).

[b] 0, control; L, 1.2 x 10^8; M, 5.7 x 10^8 spores/ml.

[c] Weight is shown as the mean weight of all surviving larvae per treatment. Different letters indicate significant differences (P<0.05) between means of a group (i.e., a certain "plant" and "fungus" treatment).

[d] The larval instar reached after 8 days is given as a percentage of the survivors.

Feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae on control and *Bt* chickpeas

There is a high correlation between feeding damage caused by *H. armigera* larvae on the chickpea leaves and the weight of feces they excrete (R^2=0.716). For the susceptible *H. armigera* strain, leaf damage was significantly higher for control leaves than for *Bt* chickpea leaves after 24h of feeding (P<0.001) (Fig. 6.5 A). The difference in feeding activities on the two plant types was also evident in the feces weight measurements (P<0.001) (Fig. 6.5 B). In contrast, the Cry2A-resistant strain inflicted similar levels of feeding damage (P>0.05) and produced similar amounts of feces (P>0.05). On control

plants, the two strains inflicted similar levels of leaf damage; however, significantly more feces were produced by susceptible larvae (P=0.007). Data for the two experimental runs were combined for the analysis, since they revealed a similar pattern.

No significant difference was observed in the number of leaflets damaged per leaf provided to the two strains on either plant type during 24h (means for the susceptible strain 8.1 to 9.3 leaflets damaged/leaf; Cry2A-resistant strain: 8.7 to 9.1 leaflets damaged/leaf).

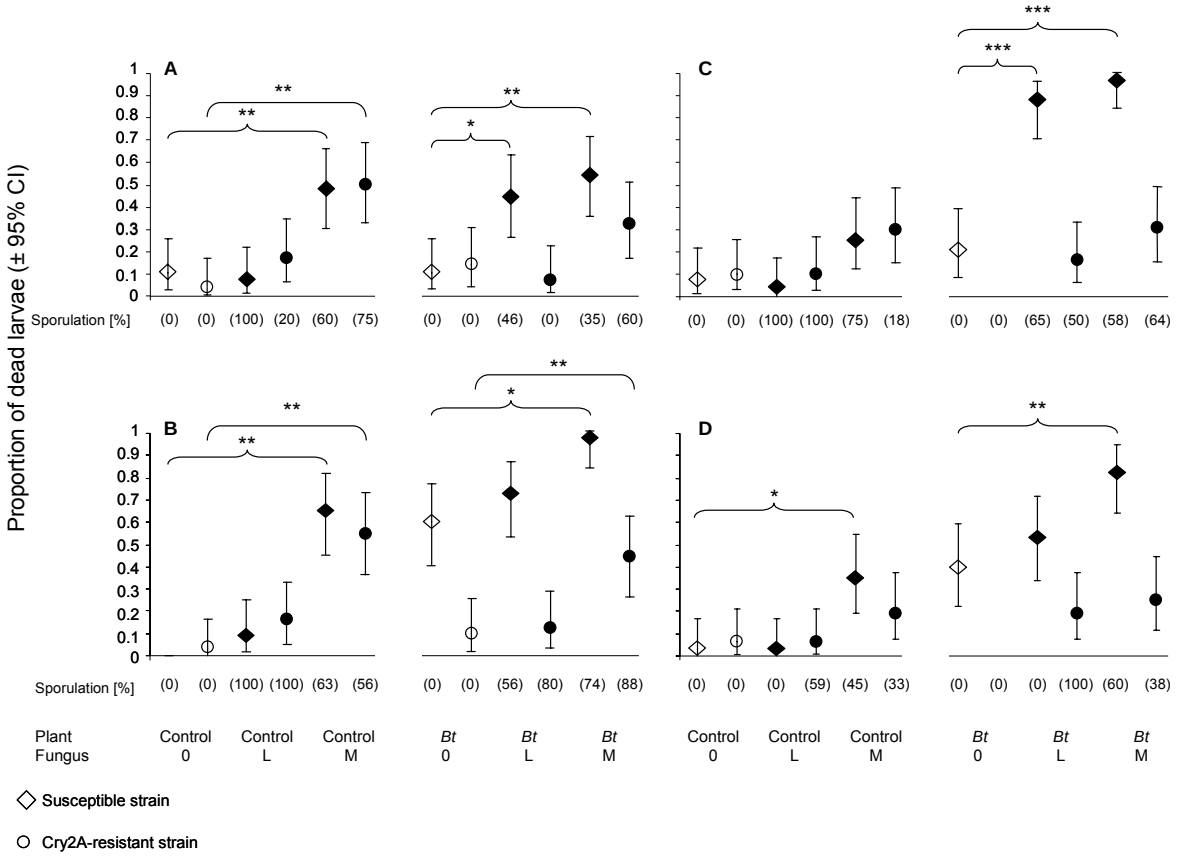


Figure 6.3 Proportions of dead susceptible and Cry2A-resistant *H. armigera* larvae (±95% CI) fed on control or *Bt* chickpea leaves treated with different concentrations of *M. anisopliae* spores [0 (0.5% Tween 80), L (1.2 x 10^8 spores/ml), M (5.7 x 10^8 spores/ml)]. The experiment was repeated four times (runs A to D) with an n of 30 to 32 per run. Statistical comparisons were made separately for each *H. armigera* strain and for control or *Bt* chickpea leaves. Statistical significances are shown between the fungus treatments (L and M) and the controls (susceptible/Cry2a-resistant strain on control/*Bt* leaves with no fungus application). No control mortality occurred for susceptible larvae in run B and on *Bt* leaves for Cry2A-resistant larvae in runs C and D. *, P<0.05; **, P<0.01; ***, P<0.001; Cox proportional-hazard model. Sporulation (percent) in dead larvae is given for each treatment. Open symbols refer to the control treatments, filled symbols refer to the fungus treatments.

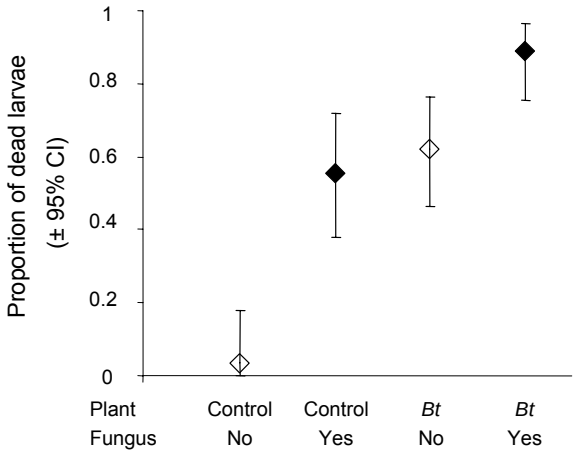


Figure 6.4 Proportions of dead susceptible *H. armigera* larvae (±95% CI) fed on control or *Bt* chickpea leaves treated with a concentration of *M. anisopliae* spores (5.4 x 10^8 spores/ml) or 0.5% Tween 80 in the greenhouse. The mortality of susceptible *H. armigera* larvae was recorded after 10 days. *n*= 3 to 5 plants per treatment.

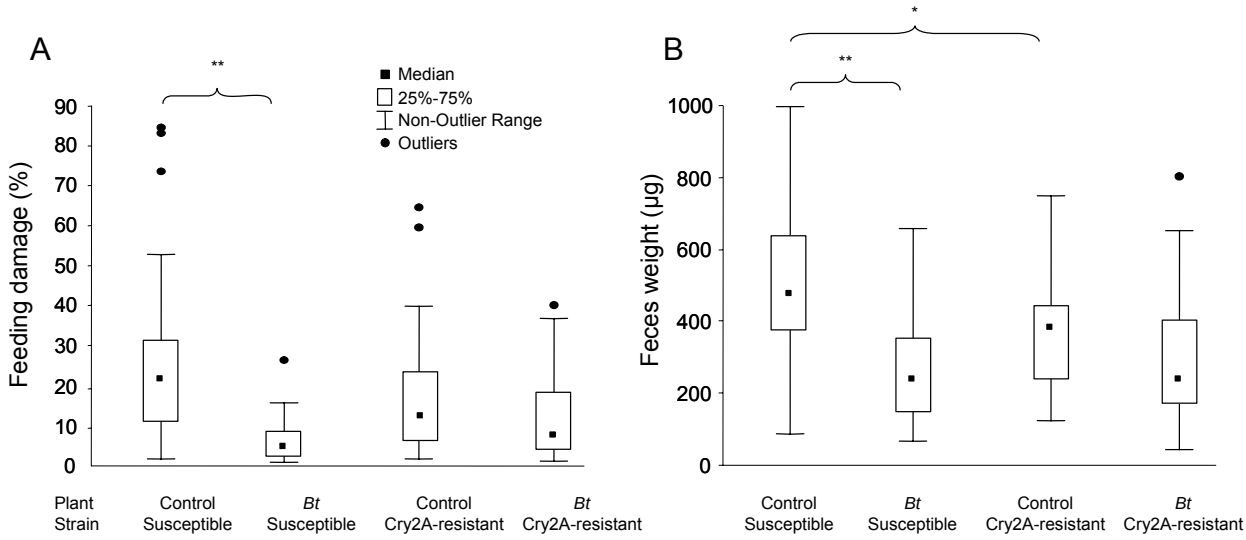


Figure 6.5 Boxplots showing the distribution of feeding damage (percent; *n*=32 to 43) (A) and (B) feces weights (µg; *n*=27 to 40) for susceptible and Cry2A-resistant *H. armigera* larvae fed for 24h on control or *Bt* chickpea leaves. *, *P*<0.01; **, *P*<0.001. The outlier range is the range of values that fall above the upper outlier limit (+1.5 x the height of the box) and above the below-outlier limit (-1.5 x the height of the box).

Discussion

Our studies revealed that *M. anisopliae* is effective in killing both susceptible and Cry2A-resistant *H. armigera* larvae on control and *Bt* chickpeas and that the number of larvae producing *M. anisopliae* spores did not differ between the two *H. armigera* strains. In some treatments, 20 to 100% of larvae that were apparently killed by the fungus did not produce fungal spores. It is known that entomopathogenic fungi can cause high mortality with little sporulation (Shaw et al. 2002). It was suggested in earlier studies that the insects

were killed by toxins, such as destruxins, produced by *M. anisopliae* (Kershaw et al. 1999; Zimmermann 2007).

The Cry2A-resistant strain of *H. armigera* appeared to be more tolerant of *M. anisopliae* than the susceptible strain in the concentration-response bioassay in which larvae were fed control chickpea leaves treated with various spore concentrations of the entomopathogenic fungus. However, this finding was not confirmed in the second laboratory bioassay, in which larvae received control or *Bt* chickpea leaves treated with no fungus or an L or an M spore concentration. In this bioassay, no difference in larval susceptibility to *M. anisopliae* was observed between the two *H. armigera* strains while feeding on fungus-treated control chickpea leaves.

A number of studies have shown significant fitness costs with some laboratory-selected, *B. thuringiensis*-resistant strains of different species of Lepidoptera (Bates et al. 2005). Fitness costs may be expressed in a variety of forms, e.g., reduced survival rates; diminished fertility, fecundity, and mating ability; and increased overwintering mortality and developmental rates. For larvae of a Cry1Ac-resistant strain of *H. armigera*, a reduced survival rate and an increased development time on different host plants were observed (Akhurst et al. 2003; Bird and Akhurst 2007). These fitness costs can also be expressed as an increased susceptibility to natural enemies, such as entomopathogenic nematodes Gassmann et al. (2006) or insect viruses (Raymond et al.; 2007). However, we did not detect a higher susceptibility of Cry2A-resistant *H. armigera* larvae to infection by *M. anisopliae* which is supported by the fact that previous studies had revealed that the two *H. armigera* strains used in our study are indistinguishable in a number of life table parameters (Rod J. Mahon unpublished data). Our findings are consistent with the study by Johnson et al. (1997) who did not find a higher fungal infection with *N. rileyi* in *B. thuringiensis*-resistant *Heliothis virescens* larvae. Likewise, susceptibility to nucleopolyhedrovirus (NPV) infection was not increased in a *B. thuringiensis*-resistant *P. xylostella* strain (Raymond et al. 2006) and *B. thuringiensis*-resistance in larvae of the flour moth *Ephestia kuehniella* had no effect on parasitism by an endoparasitoid (Rahman et al. 2004).

Interestingly, a greater susceptibility to *M. anisopliae* occurred when susceptible *H. armigera* larvae fed on *Bt* chickpea leaves than when they fed on control leaves. On control leaves, the L spore concentration of the fungus did not cause mortality, while a significantly increased mortality (31 to 65%) due to the M spore concentration was

observed in three out of four bioassays (Fig. 6.3 A, B, and D) (the L spore concentration refers to the approximate LC_{30} calculated in the concentration-response curve and the M spore concentration refers to the LC_{50}). When susceptible *H. armigera* larvae fed on *Bt* chickpea leaves treated with *M. anisopliae*, an additive effect occurred at an M spore concentration, with larval mortalities between 53 and 97%; whereas at a L spore concentration, the effect was more than additive in two out of four bioassays, resulting in 72 and 87% mortality (Fig. 6.3 A, and C). Interestingly, this was observed when the untreated *Bt* leaves caused little mortality to susceptible *H. armigera* larvae (9 and 20%). In the two runs (runs B, D) in which larval mortality was already substantial on the *Bt* leaves (39 and 59%), no significant increase in larval mortality due to a L *M. anisopliae* spore concentration was observed (52 and 72%). Varying levels of mortality on *Bt* chickpea leaves in susceptible *H. armigera* larvae probably reflected varying expression levels in the *Bt* chickpea plants to which they were exposed. The variation may be due to segregation of at least two copies of the gene present at different loci (Acharjee et al. unpublished data). As the leaves provided to the *H. armigera* larvae were always taken from two different plants during each feeding regime and a weight reduction in survivors was measured in each experimental run, it can be concluded that larvae always ingested at least low doses of *B. thuringiensis* toxin, which caused sublethal damage that subsequently resulted in the enhanced efficacy of the entomopathogenic fungus. The Cry2A-resistant larvae showed no indication of deleterious effects of very high levels of Cry2Ab toxin and are cross-resistant to Cry2Aa (Mahon et al. 2007a). Therefore, it was not surprising that mortalities of resistant insects induced by *M. anisopliae* did not differ when larvae fed on either control or *Bt* chickpea leaves. Once higher-expressing *Bt* chickpeas are available that are appropriate for *H. armigera* control in the field, additional studies should examine the effect of the entomopathogenic fungus on Cry2A-resistant larvae in more detail. Those data would also be valuable in the context of resistance management.

The application of pathogens as a biopesticide in combination with *B. thuringiensis*-transgenic plants to control pest Lepidoptera has previously been examined in the laboratory. For susceptible *H. virescens* larvae, a synergistic effect was observed between Cry1Ab-expressing tobacco plants and the entomopathogenic fungus *N. rileyi* (Johnson et al. 1997). Unlike bacteria and viruses, fungi can infect insects not only through the gut but also through spiracles and, in particular, through surface of the integument (Ferron 1978). This leads to the possibility of infecting insects independently of their

feeding activity. A previous study reported that susceptible larvae of *H. virescens* moved more than resistant larvae on *B. thuringiensis* (Cry1Ab)-expressing tobacco plants (Johnson et al. 1997). Consequently, when plants were treated with a pathogen, susceptible larvae were more likely to be infected than *B. thuringiensis*-resistant larvae. Similarly, a higher level of activity was reported for *B. thuringiensis*-susceptible *Spodoptera exigua* larvae when feeding on a *B. thuringiensis*-containing diet than on a control diet (Berdegué et al. 1996). Interestingly, in some cases of orally active pathogens such as nucleopolyhedrovirus, antagonistic effects by *B. thuringiensis* were reported (Farrar et al. 2004; Liu et al. 2006; Raymond et al. 2006). This could be due to the feeding-deterrent effect of the *B. thuringiensis* toxin, which reduces the consumption rate of plant material and thus ingestion of the virus.

In our studies, the movements of larvae of both *H. armigera* strains were similar on both non-*Bt* and *Bt* chickpea leaves, as indicated by the number of leaflets damaged per leaf. This finding is supported by behavioral observations made over an 11-h period of the movements of susceptible and Cry2A-resistant larvae on either control or *Bt* chickpea leaves, which did not reveal any obvious differences (data not presented). One possible explanation for the lack of activity differences observed in this study is that the *B. thuringiensis* expression level in the chickpea plants employed may have been simply too low to cause behavioral effects in *H. armigera*, as has been suggested for *H. virescens* on low-expressing *Bt* cotton lines (Benedict et al. 1992). However, the chickpea plants were clearly expressing at some level, as susceptible *H. armigera* larvae caused significantly less damage to *Bt* chickpea leaves than to control leaves. In the case of the Cry2A-resistant strain, no difference in feeding activity between the plant types was observed. In contrast to the leaf damage data, our study revealed a difference in feces production within 24h by both *H. armigera* strains while feeding on control chickpea leaves. A reason for this difference is not obvious, but it could be a discrepancy in the food utilization between strains. If food utilization differences were responsible, one would expect to see differential larval and pupal weights. However, such differences were not seen when the two genotypes were fed an artificial diet, cotton or pigeonpea plants (Rod J Mahon unpublished data).

Our laboratory/glasshouse studies with low-expressing *Bt* chickpea plants and *M. anisopliae* have shown that the two control methods are generally complementary for the control of *H. armigera*. *Bt* chickpea plants that are developed for commercial release will

need to provide much greater control than the plants used in our study. Furthermore, they are likely to express two Cry proteins that are sufficiently different that insects resistant to one would still be susceptible to the other. Such a pyramid of *cry* genes should provide good control, as well as reduce the likelihood of the development of resistance by the target pest (Bates et al. 2005; Ferré et al. 2008). Nevertheless, it is likely that some *H. armigera* larvae will survive in a *Bt* chickpea crop. First, we do not expect that the *Bt* technology will provide 100% control, and therefore, occasional susceptible larvae will survive. This could occur, for example, through selective feeding on lower-expressing tissues, or that the toxin concentration could declines in chickpea plants after flowering, as has been documented in cotton crops (Fitt et al. 1994; Kranthi et al. 2005; Olsen et al. 2005). Secondly, larvae might survive on *Bt* chickpeas through possession of a level of tolerance or resistance to the expressed Cry proteins. In both cases, the impact of natural enemies, such as *M. anisopliae*, will help to kill survivors and potentially decrease the speed of resistance development (Gould et al. 1991; Raymond et al. 2007).

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Chapter seven

General discussion

This thesis was conducted to investigate various aspects concerning the potential non-target effects of insecticidal GE plants with a special focus on:

- The construction of a simplified arthropod food web for the model crop pigeonpea (*Cajanus cajan*) to help with the selection of non-target species that need to be addressed in the non-target risk assessment of an insecticidal GE variety (chapter two).
- Bioassays to investigate the **direct effects** of:
  - Different insecticidal proteins to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) (chapter three);
  - Cry1Ac-expressing cotton plants to the non-target herbivore *Aphis gossypii* (Hemiptera: Aphididae) (chapter four).
- Bioassay to evaluate **indirect effects** on:
  - Natural enemies by analyzing *Bt*-fed aphids on their *Bt* content and the nutritional quality of *Bt*-fed aphid honeydew (chapter four),
  - *C. carnea* larvae feeding on Cry1Ac-cotton fed caterpillars, *Helicoverpa armigera* (Lepidoptera: Noctuidae) (chapter five).
- The **compatibility** of *Bt* chickpeas and the entomopathogenic fungus, *Metarhizium anisopliae* var. *anisopliae* to control *H. armigera* (chapter six).

Simplified arthropod food web to select appropriate non-target species for an environmental risk assessment

In chapter two, a simplified arthropod food web for an Indian pigeonpea crop was constructed. Based on this food web, a proposition was made which non-target arthropods would be exposed to a genetically engineered (GE) trait. Since a number of risk-assessment studies have been conducted so far for a number of insecticidal proteins that could potentially be expressed in GE pigeonpeas, such as Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*), predictions could be made about any potential hazard to certain orders of non-target arthropods. This information together with the information on the receiving environment, i.e. the arthropod food web, will guide the regulatory non-target risk assessment.

Investigating direct toxin effects on a predator

Providing predatory *C. carnea* larvae with different insecticidal proteins [Cry1Ab, Cry1Ac, snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), soybean trypsin inhibitor (SBTI), avidin] dissolved in a sucrose solution allowed the examination of potential hazards under elevated dose exposure conditions (chapter three). The intake of the sucrose solution in which the test proteins could be dissolved, was ensured by using two different approaches. In the first approach, *C. carnea* larvae were provided with a restricted number of prey items in addition to the sucrose solution, while in the second approach, larvae were alternately fed on sucrose solution and unrestricted prey. Feeding *C. carnea* larvae with a sugar solution and a protein source allowed larvae development to the adult stage. Thus, a range of important life-table parameters could be assessed. This represents an advance over the method used earlier by Romeis et al. (2004) where *C. carnea* larvae were exclusively fed with a sucrose solution containing an insecticidal protein but no additional protein source.

Although the first approach is useful to assess the impact of insecticidal proteins on *C. carnea* larvae, it is restricted to measurement endpoints of the first instar. Since the insects may be under constant stress due to limited food supply, results obtained with this bioassay are highly variable and depend for example on the quality of the provided prey items. It will thus be difficult to establish a bioassay that ensures defined intake of sucrose solution (and thus insecticidal protein) by all three larval stages. For this reason, the second approach was followed allowing the complete development of *C. carnea* while ensuring the intake of an additional sucrose solution. This approach allowed additional measurement endpoints as total larval development time/survival, pupae development time/survival, and adult weight and a more secure conclusion regarding any hazard could be given.

Once more it could be proven that Cry1A proteins do not directly effect *C. carnea* larvae. At the concentrations tested, both GNA and avidin caused direct effects on *C. carnea*. However, this does not imply that transgenic plants expressing these proteins would pose a risk to this predator under field conditions. Since GNA- and avidin-expressing plants for insect pest control have not been commercialized, expression levels in the plant are difficult to predict. Thus, once plants expressing GNA or avidin become available, additional studies would be required to assess if they actually pose a risk to *C. carnea* under more realistic exposure conditions.

Investigating indirect food quality related effects

Direct effects on non-target herbivores and possible consequences for natural enemies due to an altered food quality

To evaluate direct effects on a non-target herbivore, cotton aphids, *A. gossypii* were allow to feed on three Indian *Bt* cotton varieties (MECH 12, MECH 162, MECH 184) expressing the Cry1Ac protein, and their non-transformed near isolines (chapter four). Besides the performance of cotton aphids it was investigated whether aphids pick up any *Bt* protein when feeding on the plants. Our studies supported the conclusion that aphids are not affected by *Bt* cotton plants, however some small differences in their performance were observed among the three cotton varieties which might partly be explained by the discrepancy in their trichome density. The fact that aphid performance is not altered in *Bt* cotton and that aphids do not ingest the *Bt* proteins has consequences for a environmental risk assessment. Aphid antagonists are unlikely to be affected in *Bt* cotton and their biological control potential should be retained.

Further the evaluation of the honeydew sugar composition provided evidence that its nutritional quality was influenced due to the factor plant transformation and cotton variety and thus a negative impact on honeydew-feeding natural enemies could exist. However, even if the sugar composition differed significantly, it is questionable if the differentiation is of ecological relevance, especially as honeydew is not the only food source parasitoids feed on.

Indirect effects caused by a reduced food quality and the advantage to work with Bt-*resistant prey*

Susceptible H. armigera *larvae*

By feeding *C. carnea* larvae with *Bt* and non-*Bt* fed susceptible *H. armigera* larvae, an indirect effect on the predator was expressed by an increased mortality and an elongated development time (chapter five). Trying to elucidate the mechanism behind those prey-quality mediated effects, nutrition analysis of the lipid and glycogen content as well as the sugar and amino acid content and composition of the susceptible *H. armigera* larvae were conducted. Interestingly, only investigations of the sugar composition revealed a significant change in the nutrient content in susceptible *Bt*-fed *H. armigera* larvae, expressed in a reduction in sucrose, stachyose and galactose. Since sucrose is besides fructose important for insect growth (Vanderzant 1965; Dadd 1985) and a more or less

pronounced increase in parasitoid longevity was reported after feeding on sucrose, stachyose and galactose (Wäckers 2001), the reduction of these sugars might be one of the causes for a reduced predator survival.

However, as this was only the first attempt of detecting prey-quality mediated effects, further studies are necessary. As several more nutrients also play an important role for insects, further research is necessary. Sterols such as cholesterol are of great importance besides, water-soluble vitamins, biotin, the lipogenic growth factor choline, chloride, minute doses of folic acid, inorganic salts, minerals, fat-soluble vitamins A and E and sometimes ribonucleic acid and nucleic acid (Dadd 1973; 1985).

Cry1Ac-resistant H. armigera *larvae*

Working with Cry1Ac-resistant prey larvae provided several benefits. As Cry1Ac-resistant lepidopterans contained four times more *Bt* protein then susceptible caterpillars, *C. carnea* larvae were exposed to 3.5 times more *Bt* protein when feeding on the Cry1Ac-resistant larvae due to the consumed biomass. As the resistant mechanism in Cry1Ac-resistant larvae is, among other things, due to the loose of a specific binding side (Akhurst et al. 2003), it can be assumed that the Cry1Ac protein was present in the gut of Cry1Ac-resistant prey larvae. Since Obrist et al. (2006) confirmed the biological activity of Cry1Ab protein after ingestion by spider mites or caterpillars, our results allow once more the exclusion of a direct Cry1Ac protein effect on *C. carnea* larvae.

As non-*Bt* and *Bt*-fed Cry1Ac-resistant caterpillars were similar in their size, one can conclude that predatory larvae were aware of a reduced prey quality and tried to compensate this deficiency by higher biomass consumption. However, the conduction of the nutritional analyses did not reveal any significant changes in the Cry1Ac-resistant *H. armigera* larvae caused by the *Bt* feeding.

Compatibility of *Bt* crops and a biological control agent to control a target pest

As larvae of the devastating pest *H. armigera* have a potential to develop resistance against *Bt* crops used for their control (Ferré et al. 2008), a good compatibility of biological control agents and *Bt* crops is warranted to allow a sustainable use of the *Bt* varieties. Several studies report that *Bt*-resistant caterpillars are more susceptible to entomopathogens, such as nematodes, virus diseases, and entomopathogenic fungi

(Gassmann et al. 2006; Raymond et al. 2007; JD Vandenberg personal communication), suggesting that fitness costs are associated with *Bt*-resistance.

To evaluate the complementarity of a pathogen and a *Bt* crop for insect pest control, the interaction of the entomopathogenic fungus *M. anisopliae*, a susceptible and a Cry2A-resistant strain of *H. armigera*, and Cry2Aa-expressing chickpea plants was studied. Conducting a concentration-response bioassay in which larvae were fed control chickpea leaves treated with various spore concentrations of the entomopathogenic fungus, the Cry2A-resistant strain of *H. armigera* appeared to be more tolerant to *M. anisopliae* than the susceptible strain. However, this finding was not confirmed in a second laboratory bioassay, in which larvae received control or *Bt* chickpea leaves treated with no fungus, a low, or a medium spore concentration. In this bioassay, no difference in larval susceptibility to *M. anisopliae* was observed between the two *H. armigera* strains when feeding on fungus-treated control chickpea leaves.

The compatibility of the entomopathogenic fungus and *Bt* chickpeas in controlling *H. armigera* was evaluated in a second laboratory bioassay. Feeding Cry2A-resistant lepidopterans with either *Bt* or non-*Bt* chickpea leaves treated with non, a low or a medium spore concentration, revealed the same susceptibility to *M. anisopliae* on non-*Bt* and *Bt* chickpeas. For susceptible lepidopterans there was an additive or even synergistic effect of *M. anisopliae* on *Bt* chickpeas. In case that the susceptible larvae were only subleathaly affected by the *Bt* protein, more larvae could be killed by the entomopathogenic fungus.

However, as *M. anisopliae* was effective in killing both susceptible and Cry2A-resistant *H. armigera* larvae on control and *Bt* chickpeas the impact of this entomopathogenic fungus will help to kill survivors and potentially decrease the speed of resistance development. Both bioassays revealed that the *Bt* resistance did not cause a fitness cost expressed in a higher susceptibility to the fungus.

Thus, it can be concluded that *Bt* crops do not cause any direct effects on non-target arthropods, such as *C. carnea* larvae and *A. gossypii*. Further, aphids do not pick up any *Bt* protein, and natural enemies are not at risk by this indirect route of exposure. However, in case predators consume *Bt*-fed lepidopterans, which are targeted be the *Bt* protein, indirect effects can be observed. Furthermore, it appears that biological control, such as *M. anisopliae* can be deployed with *Bt* crops to control pest Lepidoptera.

Summarizing a compatibility of Cry1Ac or Cry2A expressing GE crops with biological control can be ensured for the organisms tested.

Future use of *Bt* crops

Even if the first GE crops were already planted in 1996, hardly any field resistance has been observed so far. This is most likely due to a comprehensive and successful resistance management strategy (Bates et al. 2005; Ferré et al. 2008). However, different target pests might develop resistance to *Bt* proteins in the future as it was already demonstrated for several laboratory strains (Ferré et al. 2008) and there are indications that resistance might have already developed in the field (Matten et al. 2008; Tabashnik et al. 2008). Further, a relatively high baseline frequency of resistance alleles for Cry2A has recently been reported for an Australian field population of *H. armigera* (Mahon et al. 2007a; b). Therefore there is an urgent need to develop new control strategies. Fortunately, resistance development against a specific Cry protein can greatly be delayed when another effective toxin is expressed in the plant. As discussed by Ferré et al. (2008), the deployment of two *cry* genes that are sufficiently different in their binding side is advantageous as insects resistant to one would still be susceptible to the other. Great success has been already reported for *Bt*-cotton (Bollgard II^{TM}; Monsanto Company, St Louis, USA), expressing the Cry1Ac/Cry2Ab protein (Naranjo et al. 2008). Currently, new GE crops are under development expressing two or more partly "new" (uncommerzialised) stacked Cry proteins (Moellenbeck et al. 2001; Cao et al. 2008; Narva et al. 2008).

A further option to delay insect resistance development would be the deployment of a new class of insecticidal proteins, such as vegetative insecticidal proteins (Vip). Vip proteins are, in contrast to the Cry proteins, produced during the vegetative growth phase of *Bt* rather then during sporulation. Unlike the Cry proteins, Vip proteins do not need to be solubilize in the insect gut before they can act. Similar to Cry proteins, Vip proteins bind to a specific, but different, receptor in the insect midgut resulting in pore formation (Lee et al. 2003; 2006). Their mode of action make them ideal partners for *cry* genes in GE crops and their use could help to reduce resistance pressure on the Cry proteins currently deployed and thus increase the potential life span of the technology. As the Vip3A protein is effective against a range of Lepidopteran pests (Estruch et al. 1996; ICAC 2003), 'VipCot' cotton (Syngenta Biotech) will express Vip3A probably in combination with a

Cry protein (Kurtz et al. 2007), and is expected to be commercialized in the USA in the near future (Malone et al. 2008).

Another possibility to increase the toxicity of Cry proteins is to manipulate the genes themselves, resulting in novel recombinants. As the three-domain structure is typical for all Cry proteins, having separate roles in the progress of receptor binding and channel formation (Bravo et al. 2007), an increase in toxicity could be achieved by exchanging one of the three domains, a so called "domain-swapping". De Maagd et al. (2000) observed a high mortality in Cry1A-resistant *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae, after exchanging the domain III of a Cry1Ab protein with the one of a mortality causing Cry1Ca protein. By exchanging domains in two lepidopteran specific Cry1 proteins, a toxicity to a different order of insects (Coleoptera: Chrysomelidae) could even be achieved (Naimov et al. 2003).

By generating a mutation of the three-domain Cry proteins an increase in the toxicity towards target pests was observed. This was accomplished by Rajamohan et al. (1996) who mutated the amino acid residues in the loop region of domain II which interacts with the receptors in the insect gut. Soberon et al. (2007) suggested to engineer Cry proteins that lack the requirement of cadherin binding, which is important in the mechanism of toxicity since several lepidopteran pests developed a *Bt* resistance due to a mutation in the midgut cadherin (Gahan et al. 2001; Morin et al. 2003, Tabashnik et al. 2002).

Another chance is to add an accessory substance to a *Bt* protein which does not have a function on its own. Recently, scientists from Georgia, USA discovered that a portion of an insect cadherin enhances the activity of several *Bt* proteins. Expressing a *Bt* protein and a so called *Bt*Booster^{TM} into *Arabidopsis thalia* revealed an enhanced susceptibility of lepidopteran larvae (Taylor et al. 2008).

Further attempts of increasing the toxicity of GE crops can be achieved by deploying fusion genes as it was described for Cry1Ac with the galactose-binding domain of the nontoxic ricin B-chain (RB). This fused protein provides the toxin with additional binding domains and thus increases the toxicity of GE crops to several insects compared to those containing the *Bt* protein alone. Further, the increasing number of binding domains lowers the likelihood of resistance evolving in target insect pest populations (Mehlo et al 2005).

However, regardless which *Bt* crops will be commercialized a special focus has to be given to the potential environmental risks including non-target effects. So far Vip cotton and corn appeared to have well-defined and restricted specifics for Lepidoptera and the crops are likely to cause negligible effects on no-target organisms similar to the currently grown *Bt* maize and cotton varieties (Dively 2005; Fernandes et al. 2007; Whitehouse et al. 2007). Further the *Bt*Booster^{TM} appeared not to cause any effect on arthropods when it was applied alone (Taylor et al. 2008). However, in case of the deployment of new *cry* genes or an increased activity spectrum of the *Bt* protein as it has been described for "domain-swapping" (Naimov et al. 2003), mutagenesis or the fusion gene (Mehlo et al 2005), additional non-target studies will be necessary to avoid unintended adverse effects which could affect the sustainable use of this technology for pest control.

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Curriculum vitae

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2005-2008	NCCR Graduate School "Plants and their Environment", and advanced training courses, e.g. public speaking, poster presentation, scientific writing, "R", multivariate statistics.
2000-2004	Studies in Agriculture at the Christian-Albrechts University Kiel/Germany
2004	Master of Science, Thesis: "Plants cry for help – how carnivores are geared to this" (in German)
2003	Bachelor of Science, Thesis: "Measurements of Morphological Data of the Nematode *Steinernema* sp. (Strain PS4) from Tamil Nadu in India" (in German)
1987-2000	School career in Essen-Ruhr, Karlsruhe, Bremen/Germany and Minnesota/USA

List of publications

- Lawo NC, Mahon RJ, Milner RJ, Sarmah BK, Higgins TJV, Romeis J (2008) Effectiveness of *Bt* chickpeas and the entomopathogenic fungus *Metarhizium anisopliae* in controlling *Helicoverpa armigera* (Lepidoptera: Noctuidae). Applied and Environmental Microbiology 74:4381-4389.
- Lawo NC, Romeis J. (2008) Assessing the utilization of a carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*. Biological Control 44:389-398.
- Lawo NC, Wäckers FL, Romeis J. Performance of cotton aphids on Indian *Bt* cotton varieties. In preparation.
- Lawo NC, Wäckers FL, Romeis J. Effects of *Bt* cotton on lepidopteran larvae and the determination of subsequent prey-quality mediated effects on a lacewing predator. In preparation.
- Lawo NC, Sharma HC, Dhillon MK, Romeis J. Simplified arthropod food web for pigeonpea in India – implications for non-target arthropod risk assessment of genetically engineered varieties. In preparation.
- Dhillon MK, Lawo NC, Sharma HC, Romeis J (2008) Direct effects of *Galanthus nivalis* agglutinin (GNA) and avidin on the ladybird beetle *Coccinella septempunctata*. IOBC/WPRS Bulletin 31:43-49.

List of presentations

Oral presentations:

- Effectiveness of *Bt* chickpeas and an entomopathogenic fungus to control *Helicoverpa armigera* (Lepidoptera: Noctuidae). Talk given at the "41st Annual Meeting of the Society for Invertebrate Pathology", August 3rd – 7th 2008 in Warwick, Great Britain.
- Much ado about nothing: Can biological control agents live with transgenic *Bt* crops? Talk given at the "Kolloquien Forschungsgruppe 33.3, Agroscope ART Reckenholz, April 30th 2008 in Zurich Switzerland.
- Impact of sublethally affected caterpillars on biological control organisms. Talk given at the „NCCR Annual PhD Student Meeting", April 7th 2008 in Neuchâtel, Switzerland.
- *Bt*-transgenic crops: Interaction of sublethally effected caterpillars with biological control organisms. Invited talk given at the "Ökologisches Seminar", Institute of Zoology, March 3rd 2008 in Bern, Switzerland.
- Effectiveness of *Bt* chickpeas and an entomopathogenic fungus to control *Helicoverpa armigera* (Lepidoptera: Noctuidae). Talk given at the "Indo-Swiss Pulse Network Meeting", February 14th & 15th 2008 in Hyderabad, India.
- Assessing *Bt*-related indirect effects on a predator. Talk given at the "Entomologentagung", March 26th – April 1st 2007 in Innsbruck, Austria.
- Compatibility of *Bt*-transgenic pulses with biological control - Assessing *Bt*-related indirect effects on an insect predator. Talk given at the "Indo-Swiss Pulse Network Meeting", Dec 5th – 7th 2006 in Delhi, India.
- Compatibility of *Bt*-transgenic pulses with biological control. Talk given at the "Indo-Swiss Pulse Network Meeting", February 2nd – 4th, 2006 in Hyderabad, India.

Poster presentations:

- Lawo NC, Romeis J. Performance of *Aphis gossypii* on Indian *Bt* cotton varieties. Poster presented at the IOBC/WPRS meeting "Ecological Impact of Genetically Modified Organisms", May 23rd – 25th 2007 in Warschau/Poland.
- Lawo NC, Romeis J. Performance of cotton aphids on *Bt* cotton. Poster presented at the Ninth International Symposium on Biosafety of Genetically Modified Organisms "Biosafety Research and Environmental Risk Assessment", September 24th – 29th 2006 on Jeju Island, Korea.
- Lawo NC, Hogervorst PAM, Romeis J. Development of a test method to assess the effects of insecticidal proteins on *Chrysoperla carnea*. Poster presented at the IOBC/wprs working group meeting "Ecological Impact of Genetically Modified Organisms", June 1st – 3rd 2005 in Lleida, Spain.

Certificate of completion of graduate school

Université de Neuchâtel

PLANT SURVIVAL
National Centre of Competence in Research

Doctoral Programme "Plants and their Environment"

Certificate of Completion

Mrs Nora Lawo

has fulfilled the requirements of the Doctoral Programme.

From February 2005 to September 2008 she obtained 27.5 credit points*
(see attachment).

Ted Turlings
Director of NCCR Plant Survival

Christiane Bobillier
Education officer

** A minimum of 12 credit points is required during the full period of the thesis*

Neuchâtel, 1 October 2008

FNSNF
Schweizerischer Nationalfonds
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Université de Neuchâtel

PLANT SURVIVAL
National Centre of Competence in Research

Doctoral Programme "Plants and their Environment"

Certificate of Completion

From February 2005 to September 2008 Mrs Nora LAWO obtained 12.0 credit points within the Doctoral Programme (DP, minimum 8 required) and 15.5 credit points outside the Doctoral Programme (EX) with the following activities:

Communication activities: 10.0 credit points (6.5 within the Doctoral Programme)

DP	How to make scientific presentations and posters interesting	April 2005	1.5
DP	Preparing for the next step - Improving your CV and practical training to meet your future employer's expectations	January 2006	1.0
DP	Effective public speaking	April 2006	1.0
EX	Scientific writing practice II: writing up research, Bern	April 2006	1.0
DP	Scientific writing clinic	December 2007	2.0
DP	Planning a career strategy - Part 1: Job finding methodology and networking	February 2008	1.0
EX	Einführung in das Projektmanagement, Bern	Jan.-March 2008	1.5
EX	Coaching als Führungsaufgabe, Bern	June 2008	1.0

Research tools activities: 8.5 credit points (5.5 within the Doctoral Programme)

DP	An introduction to the practice of statistics using R	June 2005	3.0
EX	Datenanalyse, Bundesamt für Statistik, Berne	August 2006	2.5
DP	Multivariate statistics in community ecology	February 2007	2.5
EX	Einführung in die Statistik Umgebung R, ETH Zürich	January 2008	0.5

Scientific activities: 8.5 credit points (3.5 within the Doctoral Programme)

EX	Annual pulse network meeting of the Indo-Swiss collaboration in biotechnology at ICRISAT, Patancheru, India (oral presentation)	February 2006	1.0
DP	Annual graduate school meeting (poster)	April 2006	0.5
DP	Interaction of plants with other organisms: friends and foes	February 2006	2.0
EX	Annual pulse network meeting 2006. Indo-Swiss collaboration in biotechnology, Dehli, India (oral presentation)	December 2006	1.0
EX	Entomologentagung 2007, Innsbruck, Austria (oral presentation)	February 2007	1.0
DP	Annual Ph.D. students meeting 2008 (oral presentation)	April 2008	1.0
EX	Pulse network meeting of the Indo-Swiss collaboration in biotechnology, Hyderabad, India (oral presentation)	February 2008	1.0

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EX	41st annual meeting of the society for invertebrate pathology and 9th international conference on Bacillus thuringiensis, Warwick, UK (oral presentation)	August 2008	1.0

Miscellaneous: 0.5 credit points (0 within the Doctoral Programme)

EX	Excel: Pivottabellen, Dübendorf	November 2005	0.5

Professor Ted Turlings
Director of NCCR Plant Survival

Dr Christiane Bobillier
Education officer

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