

TRI-TROPHIC STUDIES USING CRY1AC- RESISTANT DIAMONDBACK  
MOTH, *Plutella xylostella* (L.) DEMONSTRATE NO ADVERSE EFFECTS OF  
CRY1 AC ON THE ENTOMOPATHOGENIC NEMATODE,  
*Heterorhabditis bacteriophora* POINAR

A Thesis

Presented to the Faculty of the Graduate School  
of Cornell University

In Partial Fulfillment of the Requirements for the Degree of  
Master of Science

by

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January 2014

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## ABSTRACT

*Plutella xylostella* L. (Lepidoptera: Plutellidae), is an important insect pest of brassica crops. This pest is globally distributed and reported to cause annual losses of US \$4-5 billion to the world economy. Continuous and intensive use of many classes of insecticides has led to resistance development in some populations of *P. xylostella*, particularly in the tropics and subtropics where brassica crops are grown year-round and insect pressure is intense. Long distance migration, its ability to establish in new habitats and elimination of local natural enemies from the agroecosystem due to the intense use of broad-spectrum insecticides are some of the reasons that have contributed to outbreaks of this devastating pest.

The development and commercialization of insect-resistant genetically modified (IRGM) crops expressing insecticidal crystal (Cry) proteins from the bacterium *Bacillus thuringiensis* (Bt) have revolutionized insect pest management in two field crops, cotton and corn. In 2012, these crops were grown on more than 69 million hectares worldwide. Several studies have reported that *Bt* genes coding for insecticidal Cry toxins, when introduced in brassica crops, confer resistance to *P. xylostella*. Introduction of Bt brassica crops along with other pest management tactics including cultural, chemical and natural enemies could be a feasible solution to keep *P. xylostella* under control in the near and long term. However, assessing the environmental safety and compatibility of Bt crops with other control measures, particularly with biological control agents, is imperative.

Entomopathogenic nematodes (EPN) in the family Steinernematidae and Heterorhabditidae are important biological control agents for many insect pests.

Laboratory and field studies have reported the potential use of EPN for the control of *P. xylostella*. In the future, if Bt brassica crops are commercialized, EPN applied against *P. xylostella* may become exposed to Bt proteins directly through root exudates or indirectly by feeding on *P. xylostella* that developed on Bt crops.

The main purpose of this research project was to assess the potential effects of Cry1Ac on *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) under tri-trophic conditions. Using Cry1Ac-resistant *P. xylostella* larvae as hosts, we evaluated the potential impact of Cry1Ac-expressing broccoli on several fitness parameters of *H. bacteriophora*. Virulence, reproductive potential, time of emergence and preference of *H. bacteriophora* for the host (*P. xylostella*) were not significantly affected when Cry1Ac-resistant *P. xylostella* larvae were reared on leaves of Cry1Ac or non-Bt broccoli. Also the above-mentioned parameters of the subsequent generation of *H. bacteriophora* did not differ between nematodes obtained from *P. xylostella* reared on Cry1Ac broccoli compared to those obtained from *P. xylostella* reared on non-Bt broccoli.

## **BIOGRAPHICAL SKETCH**

Saurabh Gautam was born July 21, 1988 village Lalpur, Haryana, India. He was raised in a small town, Kosi Kalan. He graduated from the Mahatma Phule Agricultural University, India in 2011 with a Bachelor of Science in Horticulture. As a part of under-graduation requirements, he worked for a year in the floriculture industry in Pune. His interest in insects led him to pursue masters in Entomology under a dual degree program offered jointly by Cornell University and Tamil Nadu Agricultural University. Upon completion of this master's, he will continue his studies at the Tamil Nadu Agricultural University.

## DEDICATION

This manuscript is dedicated to Lord Venkateswara, Tirupati Balaji.

## **ACKNOWLEDGMENTS**

I would like to express my gratitude to my advisor, Anthony Shelton, whose expertise, knowledge, experience and patience made my graduate experience a memorable one. I am very thankful for his constant guidance and assistance in research and thesis writing. I would also like to thank other members of my committee, Dr. Brian Nault for his interest in my work. I would also like to thank Jun-Ce Tian, and Daniel Olmstead, for their help and support at various levels of the project. In addition, I would like to thank Hilda Collins, without her editing assistance, I would not have been able to complete the thesis.

All this would not have been possible, if not for the generous funding by Cornell-Sathguru Foundation to take up my master's degree at Cornell and I express my sincere gratitude to the Cornell-Sathguru Foundation for Development.

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# CHAPTER 1

## INTRODUCTION

### I. *Plutella xylostella* L. (Lepidoptera: Plutellidae)

#### **A. Pest Status, Current Management Strategies and Economic Importance**

*Plutella xylostella* L. (Lepidoptera: Plutellidae) is the most widely distributed pest of all Lepidoptera (Talekar and Shelton 1993). It is a specialist insect pest of the Cruciferae. Members of this family are cultivated in temperate and tropical regions as vegetable and field (e.g. canola) crops and *P. xylostella* occurs wherever these crops are grown. It sustains itself on cruciferous weeds in the absence of favored hosts (Talekar and Shelton 1993). Sulfur-containing compounds such as glucosinolates present in crucifers help *P. xylostella* to identify a potential host and act as oviposition stimulants (Renwick 2002). *Plutella xylostella* is believed to have originated along with crucifers in the Mediterranean region (Harcourt 1954). However, On the basis of presence of host plant and natural enemy diversity, Kfir (1988) assumed that *P. xylostella* could have originated in South Africa and liu et al (2000) on the basis of similar argument linked *P. xylostella* origin to China.

The inherent ability for long distance migrations and better adaptability to new environments compared to its natural enemies is one of the principal reasons for its present pest status. The continuous and indiscriminate use of insecticides in some areas has led to resistance development, control failure and elimination of natural enemies from the agro-ecosystem. *Plutella xylostella* has the distinct ability to develop resistance to insecticides when applied continuously. It was the first insect pest to

develop resistance to field application of the bacterial insecticide *Bacillus thuringiensis* (Kirsch and Schmutterer 1988, Tabashnik et al. 1990, Hama 1992, Shelton and Wyman 1992). In 2012, the Arthropod Pesticide Resistance Database (APRD 2012) reported that some populations of *P. xylostella* have evolved resistance to 82 chemical compounds, including relatively newer and selective insecticides such as indoxacarb, avermectins, spinosad, benzoylureas, and chlorantraniliprole.

Cultivation practices for crucifers vary considerably between developed and developing nations. In developing countries the majority of the crucifer farms are small and located near an urban setting to supply fresh vegetables to cities. Insecticide application is the main crop protection measure employed in these areas, and because of the high cosmetic value of crucifers, farmers spray pesticides frequently and rarely follow protective measures while spraying. These two factors put both producers and consumers at the risk of increased insecticide exposure. In the larger scale farming scenario of industrialized countries, control measures remain more or less the same as in developing countries with strong reliance on chemical insecticides (Talekar and Shelton 1993). In the tropics where crucifers are grown year-round, *P. xylostella* may be able to complete 20 generations per year (Talekar and Shelton 1993). Resistance development and control failure problems are more severe in these areas.

Indiscriminate and frequent use of insecticides has resulted in loss of natural enemies (Furlong et al. 2004b), and caused considerable damage to the environment.

Moreover, chemical control of *P. xylostella* is very costly. According to a recent study by Zalucki et al. (2012), the annual cost of controlling *P. xylostella* in *Brassica* vegetables is US \$1.4 billion worldwide, rising to US \$2.7 billion if yield losses due to

*P. xylostella* are taken into account and to US \$4-5 billion if losses and control costs of *P. xylostella* in the canola industry are included.

## **B. Biology and Ecology**

Female moths start laying eggs immediately after mating and continue for approximately 4 days, laying 11-188 eggs (Harcourt 1954). Immediately after hatching, neonate feed on foliar tissue except for the leaf veins. The larva completes three more molts before pupating in the cocoon on the leaf surface. Depending on the temperature, the pupal period may vary from 4-15 days (Harcourt 1957, Abraham and Padmanaban 1968, Lu and Lee 1984, Chelliah and Srinivasan 1986, Hoy 1988), and adults after emergence feed on water droplets. Long distance migration is one of the major reasons for its global presence. The annual migration to the Baltic and southern Finland is one of the most studied migration patterns of *P. xylostella*, where adult moths migrate more than 3000 km (Talekar and Shelton 1993). Unlike tropical and subtropical regions where *P. xylostella* hosts are available all through the year, perennial occurrence of *P. xylostella* in temperate regions of the world has led to speculation that *P. xylostella* pupae might hibernate in the debris of host plants in temperate regions (Talekar and Shelton 1993). However, whether *P. xylostella* hibernate or diapause remains debated.

## **C. Host Plant Interaction**

The non-volatile compounds, glucosinolates, dominate the interaction between *Brassica* crops and *P. xylostella* (Hopkins et al. 2009, Textor and Gershenzon 2009). Glucosinolates remain in inactive form in the plant tissues and in the event of mechanical damage they react with the enzyme myrosinase resulting in the formation

of toxic compounds like isothiocyanates. However, *P. xylostella* is endowed with glucosinolate sulfatase in the gut lumen that prevents myrosinase hydrolysis, thus inhibiting the formation of toxic compounds (Ratzka et al. 2002). Thus, *P. xylostella* can feed on *Brassica* hosts without any harm, provided there is no increase in myrosinase activity (Li et al. 2000).

Sulfur containing glucosinolates, allylisothiocyanates act as powerful oviposition stimulants for *P. xylostella* (Renwick et al. 2006). The spontaneous degradation of glucosinolate into isothiocyanate, which is absorbed by phylloplane waxes, is considered the likely cause for this effect (Renwick et al. 2006). *Plutella xylostella* can develop an ability to oviposit on non-host plants with experience. In 1990 in Kenya, *P. xylostella* were reported to be feeding on *Pisum sativum* L. (Fabaceae). The exact reason for this event is not yet clear but close planting of *P. sativum* with cruciferous crops may have led to this situation (Lohr 2001). In subsequent laboratory studies, Lohr and Gathu (2002) showed that *P. xylostella* larvae feeding on pea grows well on both plants, i.e. cabbage and pea. Since this population can complete its life cycle on pea, it is known as pea host-strain (P-strain).

#### **D. Biological Control**

All stages of *P. xylostella* are attacked by a wide range of natural enemies, including parasitoids, predators, and entomopathogenic microbes. The majority of the work on biological control of *P. xylostella* has been centered on hymenopteran parasitoids. Approximately 90 species of parasitoids are known to attack *P. xylostella* (Goodwin 1979); however only 60 species are frequently recovered from the field (Talekar and Shelton 1993). From the known parasitoid complex, larval parasitoids of

the genera *Diadegma* and *Cotesia* are the most common. Several *Diadromus* spp., the majority of which are pupal parasitoids, help to regulate populations of *P. xylostella* (Talekar and Shelton 1993).

Introduction of exotic parasitoids (classical biological control) for *P. xylostella* was started with the introduction of the larval-pupal parasitoid *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) and the pupal parasitoid *Diadromus collaris* (Hymenoptera: Ichneumonidae) in New Zealand from the United Kingdom (Talekar and Shelton 1993). Subsequent introductions of both species from New Zealand to Australia (Wilson 1960), Malaysia (Ooi 1992) and Indonesia have been made. From Indonesia, *D. semiclausum* was introduced into Taiwan (Talekar et al. 1992), leading to the successful introduction of the parasitoid from Taiwan to the Philippines (Ventura 1997), followed by India (Chandramohan 1994), Laos, Vietnam, China, (Talekar 2004), and Kenya (Lohr et al. 2008). Hence, *D. semiclausum* that originated in the United Kingdom has been successfully introduced into Asia and Australia. Similarly, *Cotesia (Plutellae) vestalis* (Hymenoptera: Braconidae), a larval parasitoid, has been introduced in different parts of the world for classical biological control programs (Delvare 2004, Talekar 2004).

Harcourt (1986) constructed a comprehensive life table study on *P. xylostella* and reported that precipitation, parasitism by *Diadegma insulare* (Cresson), and variable female fecundity were the major factors regulating *P. xylostella* population, but made no mention of predator-induced mortality. These results are in contrast to several studies from the United States (Muckenfuss et al. 1992), Australia (Furlong et al. 2004a), China, and North Korea (Furlong et al. 2008) that have reported that

predators can decrease the *P. xylostella* population in *Brassica* crop agro-ecosystems, but the exact impact of the predator complex on *P. xylostella* population is not known. Despite the scarcity of data on predators compared to parasitoids, few studies have reported that predators have the potential to exert sufficient pressure to check the *P. xylostella* population buildup (Furlong et al. 2004a, Furlong et al. 2008). However, further research is required to fully exploit this natural resource. Control of *P. xylostella* using natural enemies holds considerable potential but the widespread use of broad-spectrum insecticides remains a major challenge for biological control programs in the field.

The heavy reliance on broad-spectrum insecticides and the growing concern about them in the environment has stimulated research on alternative control measures. Development of sustainable integrated pest management (IPM) strategies for the control of *P. xylostella* has been discussed for decades (Talekar and Griggs 1986, Talekar 1992, Grzywacz et al. 2010). The integrated pest management program requires conservation of natural enemies, regular monitoring of populations of pests and natural enemies, and threshold level-based application of selective insecticides. Since parasitoids are the main natural enemies that regulate the population buildup of *P. xylostella* (Sarfraz et al. 2005), conservation of parasitoids is crucial for the development of an effective IPM program.

#### **E. Genetic Control Technique**

The Sterile Insect Technique (SIT), which involves the mass release of radiation-sterilized insects to reduce the target pest population, has proved to be futile against *P. xylostella* (Furlong et al. 2013). Application of SIT for controlling moths

was primarily limited by three factors: lack of a mass rearing system, lack of reliable sex separation methods (Bloem and Carpenter 2001), and the high dose of radiation required to sterilize the moths (Bloem and Carpenter 2001, Blomefield et al. 2010). However, recent developments in recombinant DNA technology can aid in SIT. A technique called Release of Insects carrying a Dominant Lethal (RIDL) relies on the mass release of the insects that are homozygous for one or more lethal heritable gene constructs (Thomas et al. 2000, Alphey 2007, Alphey et al. 2007), rather than radiation-induced lethal mutations. Male insects carrying female-specific homozygous lethal genes have been developed for the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Fu et al. 2007), pink bollworm, *Pectinophora gossypiella* and *P. xylostella* (Jin et al. 2013). The production of the male-only cohort will be an essential prerequisite for successful use of RIDL. Transgenesis has been used to address this issue by inducing death at a particular life stage (female only) by using certain genetic systems. Among them, the tetracycline-regulated system is the most promising (Gossen and Bujard 1992, Gossen et al. 1994, Gossen and Bujard 2002). The presence of tetracycline in the larval diet inactivates the original trans-activator, thus preventing the expression of target genes (Urlinger et al. 2000). RIDL males would mate with wild females whose resulting progeny would not be viable.

Theoretical models and recent work in the Shelton lab by members of Oxitec indicate that such ‘species-specific strategies’ can contribute significantly to overcoming insecticide resistance and could be a part of an IPM program, particularly in Bt crops where the high-dose and refuge strategies are the accepted cornerstones of

resistance management (Bates et al. 2005). In the case of *P. xylostella*, such strategies need to be field-evaluated.

## II. Insect-Resistant Transgenic Bt Crops

### **A. Commercialization and Economics of Bt Crops.**

With genetic engineering it has become possible to introduce a desired trait in an organism, in order to enhance its performance for the benefit of humans.

Genetically engineered plants that produce insecticidal crystal (Cry) proteins from the soil bacterium *Bacillus thuringiensis* (Bt) have revolutionized the management of coleopteran and lepidopteran pests in two field crops, cotton and corn (Shelton 2012). The area planted under Bt crops increased at an unprecedented rate from 1.6 million hectares in 1996 to 69 million hectares in 2012 (James 2012). Other Bt crops that are in the pipeline for commercialization are eggplant, cauliflower, cabbage and rice (Shelton et al. 2008, Chen et al. 2011, Shelton 2012)

The economic benefits of genetically modified crops (GM) are well documented but may vary between countries and even within a country. Barfoot and Brookes (2008) reported a 13.15% increase in a producer's income with the cultivation of insect-resistant cotton. This gain in income proved to be substantial, despite the additional cost of GM seeds. Developing countries have benefited more from these crops, as insect pest outbreaks are generally more severe and there are fewer options for pest management in these countries compared to industrialized nations.

Brookes and Barfoot (2013) reported the positive impact of Bt crops on farm income in the United States. The major gains in income in the corn sector are largely

due to increases in yield, whereas in the cotton sector it is due to the cumulative effect of both yield gain and reduced cost of cultivation. Since cultivation of cotton warrants frequent sprays of insecticides when compared to other crops, decrease in the cost of plant protection measures in cotton is more evident compared to corn. The net farm income gain due to the adoption of Bt corn increased from US \$13.54 million in 1996 to US \$3.19 billion in 2011, and the cumulative gain over the same period was US \$14.4 billion. In the case of cotton, the average increase in profitability was US \$53 to US \$153 per ha with Bollgard I, between 1996 and 2002. With the introduction of Bollgard II (containing two Bt genes in its genome to impart broader spectrum control against insect pests) in 2003, per hectare profitability was boosted from US \$87 to US \$175 from 2003- 2011 that resulted in a net gain of US \$502 million in 2011. The cumulative gains with the cultivation of Bt cotton between 1996 and 2011 were reported to be US \$3.77 billion. As a whole, Bt crops increased farm income of US farmers by US \$18.17 billion during 1996-2011.

Bt cotton is the only commercially available GM crop in India and its adoption by farmers has made India the largest producer of Bt cotton globally. Although cotton is being planted only on 5% of the total cultivated area of the country, it requires almost 50% of the total insecticides used (Manjunath 2004). Bt cotton was first adopted in India in 2002 when 44,000 hectares were grown, but that increased to 10.8 million hectares in 2012 (James 2012). Major economic benefits have resulted in an increase in yields of 30-50% (Bennett et al. 2004, Gruere et al. 2008, Qaim 2009, Herring and Rao 2012). Since 2002, the net economic benefits at the national level have been estimated to be US \$9.4 billion (Brookes and Barfoot 2013).

## **B. Bt Crops and Ecosystem**

An ecosystem is a collection of biological organisms that live, feed, reproduce and interact with each other, as well as with the abiotic factors present in the area.

Ecosystems supply all the basic requirements of humans (Daily et al. 1997). A change in the biodiversity of an ecosystem can affect species richness, the number of individuals of each species, the function of the species in the ecosystem, and species interaction with biotic and abiotic factors (Symstad et al. 2003). A relationship between the stability of the ecosystem and biodiversity does exist (Chapin et al. 1997, Loreau et al. 2001). However, determining the role of individual species within the complex ecosystem is challenging.

In the 21<sup>st</sup> century, depletion of biodiversity is projected to continue due to drastic changes in land use patterns and introduction of exotic organisms (Sala et al. 2000). The potential impact of GM crops on biodiversity *vis-a-vis* non-target organisms has been a concern even before their commercialization. The introduction of transgenic crops can affect biodiversity or the ecosystem either by changing farming practices or by their interaction with biotic and abiotic factors. Alterations in the agro-ecosystem may arise if Bt toxins produce lethal or sub-lethal effects on non-target organisms. Potential effects of Bt crops on the agro-ecosystem should be assessed in tandem with the baseline agricultural system replaced by Bt crops. Furthermore, risk of Bt crops to beneficial arthropods depends on the transgenic event, level of exposure, and the role of that particular species in the ecosystem (pollinators, decomposers or natural enemies) (Wolfenbarger and Gonzalez-Espinosa 2004).

In order to assess the non-target risk of Bt crops on beneficial arthropods, three models are illustrated by Andow and Hilbeck (2004): 1. the ecotoxicology model (tiered approach to measure acute toxicity), 2. the non-indigenous species model (validates the risk posed by the introduction of Bt crops to the local species), 3. the ecological model employed to measure the effects of long term exposure of Bt toxins to the representative species having ecological and anthropocentric relevance through tiered approach. Tiered methodology (the ecotoxicology model) provides scientific rationale for non-target risk assessment and there is general consensus among regulatory authorities and the scientific community that this approach is the most suitable and relevant (Naranjo 2009).

Romeis et al (2008) discussed an international tiered-based framework for more rigorous non-target risk analysis of Bt crops and the need to harmonize the international risk assessment guidelines. They stressed the development of a ‘clearly stated’ and relevant risk hypothesis linked to a well-defined endpoint that could adequately characterize the risk but not hamper the implementation of novel pest management strategies. In these tiered approaches, the first step is to identify the area of concern, followed by formulation of a relevant risk hypothesis, and then provide a scientifically valid protocol using representative surrogate species from important functional groups of arthropods. After 16 years of commercialization, except for a very few laboratory studies that have erroneously reported the host-mediated effects as the direct consequence of Bt toxins (e.g., Lovei et al. 2009), the plethora of literature published has not shown any negative effects of Bt proteins to non-target organisms (Romeis et al. 2006, Marvier et al. 2007, Wolfenbarger et al. 2008, Naranjo 2009). Lu

et al. (2012) carried out a long-term and comprehensive assessment of ecological effects of Bt cotton at 36 sites in six provinces of China during 1990-2010 and reported an increase in the abundance of predators and decrease in aphid population with widespread planting of Bt cotton. Many other studies agree with these results, demonstrating that Bt crops can promote the activity of biological control agents in any agro-ecosystem due to the reduced application of insecticides and the selective toxicity of Bt proteins (Shelton et al. 2002, Carriere et al. 2003, Cattaneo et al. 2006, Wolfenbarger et al. 2008, Wu et al. 2008, Hutchinson et al. 2010).

### **C. Bt Crops and Natural Enemies**

Detailed analysis of any potential negative effects of Bt crops on non-target organisms, including natural enemies, is commonly carried out with the tiered approach discussed above. Design of an appropriate approach depends on the test product, local cultivation practices, ecological community, crop, and the test organism's biology. Exposure of insecticidal proteins to natural enemies can be direct: with Bt toxins present in the transgenic plant, with pollen containing Bt toxins, and with Bt toxins present in the soil, or can be indirect through tri-trophic interactions. Mode of entry and the level of exposure also depend on the biology of the test organism. The level of exposure through indirect means is far lower than direct exposure, presumably due to dilution effects (Head et al. 2001, Dutton et al. 2002). Furthermore, phloem-feeding herbivores ingest only a negligible amount of Bt protein (Head et al. 2001, Raps et al. 2001). Thus natural enemies feeding on these prey species will be exposed to a minimal amount of Bt protein. Generalist natural enemies usually prey on several different species that may not feed solely on Bt crops. This

further decreases the chance of exposure of predators to Bt proteins. On the other hand, predators that also feed directly on Bt plant parts (e.g. pollen) may be more exposed.

Parasitoid and host interaction is intimate as parasitoids complete their life cycle in a single host, eventually killing their host. Parasitoids developing inside a host that are fed Bt crops may be exposed to Bt toxins for a greater part of their life cycle compared to generalist predators. A parasitoid developing in a Bt-susceptible host will not complete its life cycle owing to premature death of the host, and this has been wrongly interpreted by some (e.g., Lovei et al. 2009) to indicate a direct hazard to the natural enemy by the Bt protein. Using Bt-resistant hosts overcomes this methodological problem (Romeis et al. 2011, Tian et al. 2012, Tian et al. 2013).

#### **D. Mode of Action of Bt**

Over 150 insecticidal Cry proteins have been reported from *Bacillus thuringiensis* and *Bacillus cereus* (Schnepf et al. 1998). Bt genes encode specific proteins that are toxic to specific insect orders: Cry I (Lepidoptera), Cry II (Lepidoptera and Diptera), Cry III (Coleoptera) and Cry IV (Diptera) (Gill et al. 1992). In Lepidoptera, the midgut epithelium is lined with the protective peritrophic membrane and the pH in the midgut is in the range of 10 to 11 (Chapman 1998). On the other hand, phytophagous insects require an extremely alkaline midgut in order to prevent the inactivation of digestive enzymes by tannins present in leaf tissues. The goblet cells in the midgut epithelium secrete potassium carbonate that helps to maintain a high pH. These cells also help to maintain a high  $K^+$  ion concentration in the gut lumen by active transportation of  $K^+$  from the hemolymph. These two

individual gradients, pH and K<sup>+</sup> concentration, help in nutrient absorption by columnar cells of epithelium (Gringorten 2001).

*Bacillus thuringiensis* species contain endotoxins and other helper factors (Estruch et al. 1996, Kumar and Venkateswerlu 1998, Agaisse et al. 1999) that play a major role in Bt-mediated toxicity. The Cry endotoxin obtained from crystal inclusion of *B. thuringiensis* spores is present in the inactive pro-toxin form (Choma et al. 1990, Choma et al. 1991). Therefore, for toxicity to occur, the pro-toxin must be processed by a proteolytic enzyme, which happens only in the presence of high pH.

Insect-resistant Bt crops contain the gene coding for Cry endotoxins in their genome, resulting in the expression of Cry pro-toxins in their tissues. When a susceptible larva feeds on a Bt crop, Cry pro-toxins present in the plant tissue are solubilized and activated by gut proteases, generating an approximately 60kDa fragment of toxin. The activated toxin undergoes a complex binding sequence with gut proteins such as cadherin (CAD), aminopeptidase N (APN) and alkaline phosphatase (ALP) (Pigott and Ellar 2007, Soberon et al. 2009). Activated Cry protein consists of three domains: Domain I (the N-terminal domain), Domain II, and Domain III (C-terminal domain). First, activated protein binds with ALP and APN present on the membrane in a low-affinity interaction. APN binds with the 3<sup>rd</sup> loop of Domain II and ALP interacts with beta-16 loop of Domain III (Masson et al. 1995, Pacheco et al. 2009, Arenas et al. 2010). Binding with ALP and APN leads to the aggregation of toxins on the microvilli membrane, where toxins bind with CAD (Pacheco et al. 2009, Arenas et al. 2010). This interaction with CAD leads to the proteolytic cleavage of N-terminal and helix alpha-1 of Domain I. This sequential

cleavage of toxins promotes oligomerization of Cry proteins to form pre-pore oligomers (Gomez et al. 2002, Atsumi et al. 2008). The oligomerization increases the affinity of Cry proteins to ALP and APN by 200-fold. Enhanced binding leads to insertion of the pre-pore oligomer into the membrane resulting in pore formation and eventually cell lysis (Pardo-Lopez et al. 2006, Arenas et al. 2010). Insects are killed not merely due to direct action of Bt toxins but due to rapid gut paralysis and feeding inhibition (Gringorten 2001). The selective mode of action of Bt has three prerequisites for toxicity to occur: high pH, protease enzymes, and the presence of specific receptor proteins on the midgut epithelium. To complement the mode of action, some studies have reported the role of midgut bacteria and their significance in expressing the toxicity of Cry proteins (Broderick et al. 2006, Broderick et al. 2009). However, the author reported that the synergism between gut microbes and Bt toxins did not have any implication on risk assessment to non-target organisms.

### **E. Integrated Pest Management and Bt Crops**

Integrated pest management (IPM) is a concept of crop protection that involves need-based use of appropriate pest control strategies, either individually or in combination, to keep the local pest population below the economic injury level. The IPM approach generally involves various combinations of biological control agents, host plant resistance, cultural practices, chemical control and manipulation of pest behavior. These methods are used in conjunction with reliable pest monitoring methodologies. Host plant resistance developed against insect pests through conventional breeding methods have proven to be effective in many crops like rice, wheat, sorghum, and alfalfa (Dhaliwal et al. 2005, Smith 2005). Host plant resistance

has several added advantages: safe to non-target organisms, upon planting resistant seeds they do not require any further intervention for the target pest and compatibility with other crop protection measures. Despite these advantages, host plant resistance through conventional breeding has not been widely realized due to limited availability of elite cultivars having high levels of insect resistance. The advent of genetic engineering to develop insect-resistant crops has overcome these constraints to a great extent (Kennedy 2008).

With recombinant DNA technology, transfer of genes encoding insect resistance traits into the desired crop plants can take place virtually from any organism. Bt crops are the first genetically engineered trait for insect resistance that express the gene encoding insecticidal Cry proteins obtained from the soil bacterium *Bacillus thuringiensis* (Bt) (Shelton 2012). Cry toxins are biodegradable, can be expressed in specific tissues of the plant and are generally species- specific and pose minimum risk to non-target organisms in different orders than the target insect (Gatehouse et al. 1991, Malone et al. 2008). Wide adoption and increasing area of cultivation of Bt crops over the past sixteen years demonstrates its potential and success in controlling pest Lepidoptera and Coleoptera. However, this host plant resistance can only be used as a preventive measure for pest control rather than a holistic tool to control all insect pests. Further, Bt crops are widely planted and exhibit high levels of resistance and thereby exert high selection pressure on the local pest population. Therefore, in order to control non-target pests and to minimize chances of resistance development, it is recommended to use Bt crops as one of the tools in IPM programs.

IPM mandates the implementation of need-based control measures which in turn require adequate pest monitoring. Economic injury level (EIL) and economic threshold level (ETL) have been used in conventional IPM to decide the timing of control measures based on the pest population in the field at any given point of time (Stern et al. 1959). The key difference with host plant resistance measures is that they are implemented before the pest outbreak. Evaluating the risk/cost ratio might be one way to make a well-informed decision on whether host plant resistance should be used prophylactically. One such model developed in the USA for Bt maize estimates the net benefits likely to be generated from planting Bt maize based on assessment of the projected population of *Ostrinia nubilalis* (Lepidoptera: Crambidae), cost of Bt seeds, projected yield, price for produce and expected level of pest population suppression (Kennedy 2008).

Implementation of high dose/refuge strategy is recommended to regulate resistance development in the target pest. This strategy involves the use of Bt plants expressing a high level of Bt toxins that can kill homozygous susceptible (ss) and heterozygous (rs) individuals. Roush (1997, 1988) states that the r allele is most likely present in the rs genotype prior to the evolution of resistance and that the emphasis should be on removing it by using a dose that is sufficient to control rs individuals from the population. In addition to the high dose strategy, planting of non-Bt plants (refuge) in the same field area is essential for avoiding resistance (Tabashnik 1994, Roush 1997). Theoretically, mating of insects that survive on Bt crops (that hold potential to develop resistance) with susceptible insects surviving on refuge crops will result in heterozygous offspring that cannot survive on Bt crops. The rate of resistance

development is directly proportional to the frequency and survival of heterozygous strains (Roush 1997). However, the high dose/refuge strategy has several challenges. First, due to possible disparity in the developmental time between resistant and susceptible strains on Bt and refuge plants, there may be assortative mating (Liu et al. 2001). Second, contamination of non-Bt plants by Bt pollen may decrease the refuge size and increase resistance development (Chilcutt and Tabashnik 2004). Third, high-dose refuge strategy is not practically possible in developing countries like India and China where the land holdings are mostly marginal/small scale, and growers are reluctant or unable to set aside land for refuges.

### III. *Heterorhabditis bacteriophora*

#### **A. Biology and Ecology**

The insect parasitic (entomopathogenic) nematode *Heterorhabditis bacteriophora* (Heterorhabditidae) was first reported in 1975 (Poinar 1975) as an obligate host for the symbiotic bacteria *Photorhabdus luminescens*, and it is used as a biological control agent (Forst et al. 1997). The interaction between *H. bacteriophora* and *P. luminescens* is obligate since the transmission of bacteria by infective juveniles (IJs) is a prerequisite for successful parasitization of the host (Han and Ehlers 2000). Bacteria facilitates a favorable environment for growth and reproduction of *H. bacteriophora* by producing antibiotics that suppress the growth of other secondary organisms, and by digesting host body parts that serve as a nutrient source for IJs. In return, the nematode facilitates the bacterium's entry into the host and provides protection from the external environment (Kaya and Gaugler 1993). Third stage infective juveniles (IJs) are the only stage of the *H. bacteriophora* life cycle that occurs outside the host

cadaver and can tolerate environmental stress. Infective juveniles, once inside the host, release the symbiotic bacteria into the insect hemocoel (Ciche et al. 2003) and reproduce inside the host, which leads to the death of the infested host within 48 hrs by septicemia (Kaya and Gaugler 1993). Infective juveniles develop into hermaphrodite females and complete 2-3 generations within the host cadaver, feeding on digested host tissues and bacteria, eventually producing environmental-resistant IJs within a time span of 10 days (Adams and Nguyen 2002). The life cycle of *Heterorhabditis* is complex, with hermaphroditic and amphimictic generations occurring in the same host (Burnell and Stock 2000).

EPNs are soil-inhabiting worms that provide excellent control of insects found in moist and cryptic soil habitats (Bedding and Miller 1981, Lindegren et al. 1981). Considerable efforts have been made to use EPN against foliage-feeding insects with a certain level of success. Since 90% of all insects spend at least a part of their life cycle in the soil, priority has been given to soil application of EPNs. The ecology of EPNs remains poorly understood owing to constraints in ascertaining the nematodes *in situ* in the soil.

### **B. Mode of Infection of Entomopathogenic Nematodes**

The different stages of host infestation by EPN can be classified into three stages: locating a host, gaining entry into the host and successful infestation, followed by defending the host cadaver against opportunistic competitors. The miniature size and lack of appendages are constraints to IJs in locating a potential host. Nematodes are primarily limited to host and environment-related cues to locate a suitable host. Most species crawl on the surface using surface tension associated with the soil or

water (Croll 1970). *Heterorhabditis bacteriophora* exhibit a “cruiser” type of foraging strategy and crawl actively through the soil, scanning the environment for chemical and temperature gradient cues. Cruiser IJs have a relative linear movement in the absence of host cues to expand their searching area (Lewis et al. 1992). In contrast, *Steinernema carpocapsae* IJs exhibit the “ambusher” type of strategy and remain largely stationary. Ambushers also respond to host cues but their response is apparent only when they are in “standing” position. Infective juveniles of *S. carpocapsae* raise the anterior part of their body above the surface, and this standing position facilitates attachment to the host by reducing surface tension, and also aids in scanning the environment for host-related cues (Campbell and Gaugler 1993, Campbell and Kaya 2000). *Heterorhabditis megadis* has been reported to show strong attraction towards cues emanating from plants that are damaged by insects (Rasmann et al. 2005). Lewis et al. (1992) found that IJs of *Steinernema glaseri*, after establishing contact with the potential host, increase their localized search. Lewis et al. (1995) reported that crawling IJs of *S. carpocapsae* respond to host cues only when they make contact with the host cuticle. The response exhibited by IJs after contact with a potential host is considerably higher in comparison to that of less preferred hosts (Lewis et al. 2006). Nematodes are not deterred from infecting a potential host when it is already infected by a con-specific or other species. For example, *S. carpocapsae* and *H. bacteriophora* can co-infect but cannot coexist in a single host (Alatorre-Rosas and Kaya 1990).

Upon locating a suitable host, IJs must change their behavior to gain entry into the host hemocoel. Penetration into an unsuitable host may turn lethal to a nematode, either due to the lethal composition of gut fluid or by the strong response of the host

immune system. Most nematodes react to the chemical stimuli and physical structure of the insect integument (Lewis et al. 2006). However, there are other contributing factors like host physiology, presence of wounds on the insect cuticle, and presence of secondary bacteria inside the host that can stimulate or deter the penetration of nematodes (Hay and Fenlon 1995, Glazer 1997). Since EPNs enter into the host hemocoel through the anus, mouth or through the cuticle, it is speculated that putative cues might be located in these areas. Entry of the nematode through the anus may be restricted either due to width limitation or due to eventual defecation of nematodes along with feces (Eidt and Thurston 1995), whereas nematodes entering through the mouth are exposed to the risk of being crushed by the mandibles (Gaugler and Molloy 1981).

Once the IJs are inside the hemocoel, they may get trapped in cellular or non-cellular capsules, as has been reported in Orthoptera, Coleoptera, Diptera and Lepidoptera (Dowds and Peters 2002). After successful infestation, Steinernematids require at least one member of the opposite sex for reproduction, whereas the Heterorhabditid IJs develop into self-fertile adults. Mass attack may be advantageous for IJs to overcome the host defense system (Peters and Ehlers 1997). After successful parasitism by the nematodes, the dead host remains in the soil for 7-20 days before IJs emergence. Dead hosts may attract other opportunistic saprophages. Baur et al. (1998) and Zhou et al. (2002) reported that foraging ants avoid feeding on nematode-infected cadavers. This deterrence is due to the presence of an ant-deterrent factor (ADF) released by the symbiotic bacteria *Photorhabdus* and *Xenorhabdus* spp. IJs emerging from the host avoid competition by switching to a non-infectious stage, and the

proportion of non-infectious and infectious individuals in the emerging population changes over time ( Hominick and Reid 1990, Bohan and Hominick 1996, 1997).

### **C. Foliar Application of EPN**

Foliar application of nematodes against above-ground pests has been largely unsuccessful, probably due to undesirable conditions like desiccation and harmful UV rays (Grewal and Georgis 1999, Arthurs et al. 2004, Shapiro-Ilan et al. 2006). These abiotic challenges can be minimized by prudent use of nematodes in the evening or in early morning, and through improved formulation such as mixing EPN with protective surfactants and polymers (Schroer and Ehlers 2005). Shapiro-Ilan et al. (2010) reported that follow-up applications of sprayable gel used as a fire protectant improved the efficiency of *S. carpocapsae*. Similarly, better control of codling moth, *Cydia pomonella*, was achieved with a nematode treatment containing fire-gel or wood foam (Lacey et al. 2010). Efficiency of EPN can also be improved by selecting appropriate application approaches such as the use of proper nozzles and pumps to increase the survival and dispersal of nematodes. The level of acceptance of foliar application of EPN has increased with the development of polymeric formulations, better application equipment and low volume application at proper time intervals. In addition to foliar pests, nematodes have also been used against insect pests hiding in cryptic habitats such as inside the plant stem or trunk, which also helps protect them from extreme environmental stress (Begley 1990). New strains of EPN, tailored for foliar placement, can be developed through genetic enhancement via selection, hybridization or genetic engineering (Burnell 2002).

#### **D. EPN and Tri-trophic Interaction**

A tri-trophic interaction refers to an effect on a natural enemy when it feeds on a host that is feeding on a plant or other source of nutrition. Tri-trophic interactions may be caused by compounds in the plant that have some effect on the host (prey). Several studies have reported that the nutritional status of a host and its food source can affect the fitness parameters of EPN under *in vivo* and *in vitro* production systems. For example, under *in vitro* conditions, dispersal and infectivity of *S. carpocapsae* is adversely affected by the protein source (Yang et al. 1997), and heterorhabditid development rate is affected by the lipid source and quantity in the media (Yoo et al. 2000). Similarly, under *in vivo* conditions, the host plant can affect EPN under tri-trophic interactions. Allelochemicals in cucurbitacins negatively affect the growth and development of nematodes developing inside the hosts fed with cucurbits (Barbercheck and Wang 1996). Choice of host species can also affect the efficacy of nematode production (Blinova and Ivanova 1987, Shapiro-Ilan et al. 2005). The size of the host can also affect the number of nematodes produced (Flanders et al. 1996).

#### **IV. Research Objectives**

With the background provided on the importance of *P. xylostella* in crucifer production globally, and the interest in producing Bt crucifers, it is important to evaluate whether biological control agents like EPNs can play a role in an IPM program for Bt crucifers and *P. xylostella*. In the present research project the following objectives were studied: 1) comparing the virulence of *H. bacteriophora* against *P. xylostella* larvae fed with Cry1Ac-expressing broccoli versus those fed with non-Bt broccoli; 2) evaluating the effect of Cry1Ac-fed *P. xylostella* on the

reproductive ability of *H. bacteriophora*; 3) testing whether *H. bacteriophora* can discriminate between *P. xylostella* larvae that developed on Cry1Ac or non-Bt broccoli; and 4) evaluating if Cry1Ac broccoli plants negatively affect the progeny of *H. bacteriophora* that developed in the host fed with Cry1Ac broccoli. This research was undertaken to provide scientific evidence whether Bt crops would affect important fitness parameters of *H. bacteriophora* if they are incorporated into an IPM program with Bt crops.

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## CHAPTER 2

Tri-trophic studies using Cry1Ac-resistant *Plutella xylostella* demonstrate no adverse effects of Cry1Ac on the entomopathogenic nematode, *Heterorhabditis bacteriophora*

### Abstract

**The potential impacts on natural enemies of crops that produce insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) are an important part of an environmental risk assessment. Entomopathogenic nematodes are important natural enemies of lepidopteran pests and the effects of Bt crops on these non-target organisms should be investigated to avoid disruption of their biological control function. The objective of this study was to investigate the effects of Cry1Ac- expressing transgenic Bt broccoli on the entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) under tri-trophic conditions. Using Cry1Ac-resistant *Plutella xylostella* L. (Lepidoptera: Plutellidae) larvae as hosts, we evaluated the potential impact of Cry1Ac-expressing Bt broccoli on several fitness parameters of *H. bacteriophora*. Virulence, reproductive potential, time of emergence and preference of *H. bacteriophora* for the host (*P. xylostella*) were not significantly affected when Cry1Ac-resistant *P. xylostella* larvae were reared on leaves of Cry1Ac or non-Bt broccoli. Also the above-mentioned parameters of the subsequent generation of *H. bacteriophora* did not differ between nematodes obtained from *P. xylostella* reared on Cry1Ac broccoli compared to those obtained from *P. xylostella* reared on non-Bt broccoli. To the best of our knowledge, the present study provides the first clear evidence that Cry1Ac does not affect important fitness parameters of *H. bacteriophora*.**

## INTRODUCTION

The development and commercialization of insect-resistant genetically modified (IRGM) crops producing insecticidal proteins (Cry proteins) from the bacterium, *Bacillus thuringiensis* (Bt), has revolutionized insect management (Shelton et al. 2002) and greatly contributed to integrated pest management (IPM) programs (Romeis et al. 2008a). Currently Bt corn and cotton are the only two commercially available IRGM crops. In 2012, these crops were grown on more than 69 million hectares worldwide (James 2012). Bt eggplant, cauliflower, cabbage and rice are other Bt crops awaiting commercialization (Shelton et al. 2008, Chen et al. 2011, Shelton 2012). However, the safety of Bt crops to non-target beneficial organisms warrants investigation as part of an environmental risk assessment (Romeis et al. 2008b).

*Plutella xylostella* L. (Lepidoptera: Plutellidae), is the major insect pest of brassica crops (Talekar and Shelton 1993) and occurs in every part of the world where brassica crops are grown. *Plutella xylostella* has a long history of becoming resistant to insecticides, beginning with DDT in 1950 (Ankersmit 1953). Since then, no new product has remained effective for more than a few years when applied intensively (Grzywacz et al. 2010). Indiscriminate and intensive use of insecticides has reduced populations of natural enemies important for control of *P. xylostella*, and has contributed to outbreaks of this devastating pest (Talekar and Shelton 1993). As a result, there is an urgent need for development and implementation of cost-effective and environmentally safe alternatives to keep *P. xylostella* populations below economically damaging levels. Introduction of Bt cotton and Bt maize have resulted in considerable reduction in insecticide use, decreases in environmental impact and

increases in profit to growers (Brookes and Barfoot 2012) and less harm to important natural enemies (Wolfenbarger et al. 2008, Naranjo 2009). Our earlier studies (Metz et al. 1995a; Cao et al. 1999, 2002, 2005; Tang et al. 1999, 2001; Shelton et al. 2000; Zhao et al. 2000, 2003, 2005) have shown that *cry1* genes, when introduced in brassica crops, confer resistance to *P. xylostella*, although none have yet been commercialized (Shelton 2012). Furthermore, modeling studies have shown that the introduction of Bt crucifers, in conjunction with biological control agents, can be a long-term solution to managing the pest density of *P. xylostella* and delaying its evolution to Bt plants (Onstad et al. 2013).

Laboratory and field studies have demonstrated the potential use of entomopathogenic nematodes (EPNs) for the control of *P. xylostella* (Shinde and Singh 2000, Somvanshi and Ganguly 2007, Nyasani et al. 2008). Studies have also reported synergism between EPNs and Bt crops (Gassmann et al. 2006, 2008). The potential impacts of Bt proteins in tri-trophic interactions on many arthropod parasitoids and predators have been studied, but little is known about Bt host plant-mediated interactions between herbivores and EPNs, an important source of natural mortality for many insect pests (Kaya and Gaugler 1993).

EPNs have a unique parasitic relationship with their hosts. Third stage infective juveniles (IJs) enter the host hemocoel and release symbiotic bacteria; following this, host mortality occurs within 48 h (Boemare 2002, Dowds and Peters 2002). Symbiotic bacteria produce antibiotics that protect the host cadaver from microorganisms and supply nutrients essential for nematode growth and reproduction (Richardson et al. 1988, Ciche et al. 2001). IJs develop into adults and complete 2-3

generations within the host cadaver, feeding on digested host tissues and bacteria, and eventually produce a new generation of IJs in ca. 10 d, depending upon temperature and initial infestation density (Adams and Nguyen 2002).

If the host of the EPN feeds on Bt plants, it is possible that the EPN, in turn, might also be exposed to the Cry protein and it is important to determine if this will harm the EPN. The virulence and number of the IJs produced might be constrained by the quality and quantity of the host tissues. Using a Bt-resistant insect and a plant producing the same Bt protein is a method that allows investigators to effectively determine any direct and indirect effect (i.e. mediated by poor host quality) of the Bt protein on a natural enemy (Romeis et al. 2011).

The purpose of this study was to investigate the direct effects of the Cry1Ac protein on *H. bacteriophora*. Previous reports have shown that *H. bacteriophora* is very effective against *P. xylostella* on the basis of median lethal dose, LD<sub>50</sub> (9.16 IJs/larva), median lethal time, LT<sub>50</sub> (43.26 h), exposure time and mortality relationship, Lex T<sub>50</sub> (3.24 h), and the propagation potential (271.42 IJs/mg) (Shinde and Singh 2000). In the future, if Bt brassica crops are commercialized for the control of *P. xylostella* (Shelton 2012), *H. bacteriophora* would likely be exposed to *P. xylostella* that have fed on Bt plants. Therefore, the following objectives were addressed in this study: 1) compare the virulence of *H. bacteriophora* against *P. xylostella* larvae fed Cry1Ac-expressing broccoli versus those fed non-Bt broccoli; 2) evaluate the effect of Cry1Ac-fed *P. xylostella* on the reproduction ability of *H. bacteriophora*; 3) test whether *H. bacteriophora* can discriminate between *P. xylostella* larvae that developed on Cry1Ac or non-Bt broccoli; and 4) evaluate if

Cry1Ac broccoli plants negatively affect the ability of the progeny of *H. bacteriophora*, developed in the host fed on Cry1Ac broccoli, to utilize a subsequently provided host.

## MATERIALS AND METHODS

### **A. Insects**

Two strains of *P. xylostella* were used in this study: 1) a laboratory-reared Cry1Ac-resistant strain that can survive on Cry1Ac broccoli plants and 2) a Cry1Ac-susceptible laboratory strain (Geneva 88) that cannot survive on Cry1Ac broccoli plants (Zhao et al. 2005). In order to confirm the expression of Cry1Ac in Bt plants, the susceptible strain was used, whereas in the tri-trophic bioassays the resistant strain was used. Important life table parameters of Cry1Ac-resistant *P. xylostella* have been reported not to be significantly different when its larvae fed on Cry1Ac broccoli or non-Bt broccoli (Liu et al. 2011).

### **B. Nematodes**

Infective juveniles (IJs) of *H. bacteriophora* were obtained from a commercial supplier (The Green Spot Ltd, Nottingham, New Hampshire, USA) and stored at 4 °C until use. Before each bioassay, fresh IJs were harvested by placing the sponge formulation in distilled water in a Petri dish at room temperature.

### **C. Plants**

Two types of broccoli (*Brassica oleracea* L.) were used in this study. The first produced high levels of Cry1Ac (Metz et al. 1995b) and expression was verified by screening 4-5 wk-old plants with susceptible *P. xylostella* neonates (Tang et al. 2001).

The plants on which neonates showed 0% survival were used in the tests. A non-Bt variety, *Brassica oleracea*, var. 'Packman', was used as the control.

#### **D. Virulence bioassays**

Median lethal dose (LD<sub>50</sub>) and median lethal time (LT<sub>50</sub>) were used to compare the virulence of *H. bacteriophora* against Cry1Ac on non-Bt broccoli-fed mid-4<sup>th</sup> instar *P. xylostella*. Tests were conducted in 5 cm Petri dishes lined with filter paper. IJs were suspended in distilled water and the desired concentrations were obtained using serial dilutions. To determine the LD<sub>50</sub>, the following concentrations were used: 1, 4, 8, 16, 32, and 40 IJs per *P. xylostella* larva. Each concentration was added to five Petri dishes (replications) in 0.5 ml of distilled water. Five additional dishes of 0.5 ml distilled water without nematodes served as the untreated control. After 30 min, 10 mid-4<sup>th</sup> instars of *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli were individually placed in each Petri dish. Non-Bt broccoli leaves were washed with distilled water and cut into 5 cm leaf discs and allowed to air-dry for 45 min before being used as the nutrient medium in the Petri dishes. After inoculation, Petri dishes were sealed and placed in a growth chamber maintained at 25 °C, 50% RH and 16:8 h photoperiod. Mortality was recorded 48 h after introduction of the larvae into the Petri dishes.

Median lethal time (LT<sub>50</sub>) values were determined using the Glazer (1992) method where a single nematode concentration of 30 IJs/larva is applied. Ten mid-4<sup>th</sup> instars of *P. xylostella* that developed on Cry1Ac or non-Bt broccoli were kept in contact with IJs for 0.5, 1, 2, 4, 8, 12 and 16 h, as described above. After each exposure period, larvae were transferred to another Petri dish and kept in the growth

chamber under the conditions described above. Mortality was recorded after 48 h. Each treatment had 5 replications (50 larvae per treatment). Insect mortality data were corrected by Abbott's formula (Abbott 1925) and LD<sub>50</sub> and LT<sub>50</sub> values were calculated by probit analysis.

### **E. Choice bioassays**

For choice bioassays, IJs were presented with a choice of mid-4<sup>th</sup> instars of *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli. Tests were conducted in a cylindrical plastic tube (5 cm high x 2 cm diam). Initially, each tube was filled with 2 cm of moist, autoclaved sand. Then ca. 1,000 IJs of *H. bacteriophora* in 1 ml of distilled water were pipetted over the surface of the sand. After 30 m, additional moist, autoclaved sand was added to the tube so that the final level was 4.5 cm. By placing a plastic sheet (2 cm long x 0.5 cm wide) vertically, the open end of the tube was divided into two equal semicircular chambers. Five mid-4<sup>th</sup> instar *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli were placed on either side of the vertical divider. Subsequently, the tube was sealed with Parafilm and transferred to a growth chamber maintained as described above. After 24 h of exposure to IJs, *P. xylostella* larvae were removed from the tube and placed in Petri dishes provided with a non-Bt broccoli leaf as a source of nutrition until dissection. After 2 d, each *P. xylostella* larva was placed in distilled water and dissected and the number of nematodes was counted using a dissecting microscope at 40x magnification. The experiment was replicated 15 times.

## **F. Reproductive potential**

Ten mid-4<sup>th</sup> instar *P. xylostella* that had developed on Bt or non-Bt broccoli were infested with *H. bacteriophora* in the same way as described in the virulence bioassay, using a single concentration of 30 IJs per larva. After 48 h, five infected cadavers, recognized by their red color, were removed from the Petri dish, rinsed, weighed, transferred to a White trap (White 1927) and incubated in the growth chamber under the conditions described above. After 3 d, observations were made every 6 h and the time was recorded when IJs were observed in the White trap. Emerging IJs were collected from the White traps daily over 10 d and stored in 50 ml plastic tubes at 4°C. The content of each tube (nematode suspension from an individual White trap) was mixed thoroughly with a pipette, 10 samples of 10 µl from each suspension were examined under a dissecting microscope at 40x magnification, and the total numbers of IJs per White trap were calculated. To control for variations in larval weights of *P. xylostella* and its potential to influence production of IJs, the values for IJs produced were calculated per mg of *P. xylostella*. The experiment was replicated 15 times.

## **G. Effect of Bt proteins on 2<sup>nd</sup> generation infective juveniles**

In order to evaluate whether Cry1Ac plants would negatively affect the ability of the progeny of *H. bacteriophora* to utilize a subsequently provided host, the IJs obtained from mid-4<sup>th</sup> instar *P. xylostella* fed on Cry1Ac broccoli or non-Bt broccoli plants were tested against mid-4<sup>th</sup> instar *P. xylostella* that had developed on artificial diet (Shelton et al. 1991). Plants might have subtle difference in their biochemistry that could have an implication on the outcome of the bioassays therefore, in order to

minimize the factors influencing the bioassay we used artificial diet to rear *P. xylostella*. To maintain the genetic uniformity of the first and second generation nematode host (*P. xylostella*), we used Cry 1Ac resistant larvae as second host rather than Bt susceptible *P. xylostella*. In order to mitigate direct or indirect effects on fitness parameters of IJs produced due to initial inoculation density, IJs used for 2<sup>nd</sup> generation bioassays were obtained by infecting mid-4<sup>th</sup> instar *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli with a single concentration of 30 IJs per larva. The experimental design and conditions were the same as described above for the LD<sub>50</sub>, LT<sub>50</sub> and reproductive potential experiments.

### STATISTICAL ANALYSES

Data on LD<sub>50</sub> and LT<sub>50</sub> were analyzed by a generalized linear model (GLM) with the Probit link function. Data on choice bioassays were analyzed with paired *t*-tests. Data on larval weight, time of emergence and reproductive potential of *H. bacteriophora* were analyzed using the Student's *t*-test. All statistical calculations were performed with the R version 2.15.1 package (R Core Team 2012). For all tests,  $\alpha = 0.05$ .

### RESULTS

#### **A. Virulence Bioassay**

The LD<sub>50</sub> and LT<sub>50</sub> values for *H. bacteriophora* against *P. xylostella* larvae that had developed on Cry1Ac broccoli were 9.3 IJs per larva and 3.4 h, respectively, and there were no significant differences compared to the non-Bt broccoli values of 8.8 IJs per larva and 3.6 h (Table 2.1). Similarly, there were no significant differences found for the 2<sup>nd</sup> generation (Table 2.2).

## **B. Choice bioassays**

*Heterorhabditis bacteriophora* exhibited no significant preference for *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli (paired *t*-test,  $t = -0.06$ ,  $df = 14$ ,  $p = 0.95$ ). An average of  $59 \pm 4$  IJs (Mean  $\pm$  SE) were found in each infected larvae from both treatments.

## **C. Reproductive potential**

The number of *H. bacteriophora* produced per mg of *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli were compared and the difference between the numbers of IJs was not significant (Student's *t*-test,  $t = -0.71$ ,  $df = 28$ ,  $p = 0.482$ ) (Table 2.3). The emergence time of IJs from the host cadaver was also not significantly affected by Cry1Ac plants (Student's *t*-test,  $t = 0.315$ ,  $df = 28$ ,  $p = 0.76$ ) (Table 2.3). Similar results were found for the 2nd generation IJs (Table 2.4).

**Table 2. 1** Virulence of *Heterorhabditis bacteriophora* against mid-4<sup>th</sup> instar Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N)

Plant type	LD <sub>50</sub>	95% Fiducial limits		LT <sub>50</sub>	95% Fiducial limits	
		Lower	Upper		Lower	Upper
Bt	9.3 (1500) a	7.5	11.0	3.4 (1750) a	2.6	4.2
N	8.8a (1500) a	7.2	10.4	3.6 (1750) a	2.8	4.4

LD<sub>50</sub> values expressed in number of nematodes per larvae. LT<sub>50</sub>: the time in hours at which 50% of larvae used in the treatment were killed.

LD<sub>50</sub> and LT<sub>50</sub> values followed by the same letter within the same column are not significantly different ( $P < 0.05$ ) (n), sample size

**Table 2. 2** Virulence of 2<sup>nd</sup> generation *Heterorhabditis bacteriophora* emerged from Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N) against mid-4<sup>th</sup> instar Cry1Ac-resistant *P. xylostella* reared on artificial diet

Plant type	LD <sub>50</sub>	95% Fiducial limits		LT <sub>50</sub>	95% Fiducial limits	
		Lower	Upper		Lower	Upper
Bt	7.7(1500) a	6.1	9.3	3.2 (1750) a	2.5	3.9
N	8.3(1500) a	6.7	9.9	3.0 (1750) a	2.4	3.6

LD<sub>50</sub> values expressed in number of nematodes per larvae. LT<sub>50</sub>: the time in hours at which 50% of larvae used in the treatment were killed.

LD<sub>50</sub> and LT<sub>50</sub> values followed by the same letter within the same column are not significantly different ( $P < 0.05$ ) (n), sample size.

**Table 2. 3** Weight, time of emergence and reproductive potential of *Heterorhabditis bacteriophora* from mid-4<sup>th</sup> instar Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N)

Observations	Plant type	(Mean $\pm$ SE)
Larval weight (mg)	Bt	24.4 $\pm$ 3.3 (75) a
	N	25.6 $\pm$ 3.1 (75) a
Mean time to emergence (h)	Bt	154.4 $\pm$ 10.0 (75) a
	N	153.2 $\pm$ 10.8 (75) a
Mean nematodes per White trap	Bt	2918.0 $\pm$ 2014.0 (75) a
	N	3430.0 $\pm$ 1924.0 (75) a
Mean nematodes per mg <i>P. xylostella</i>	Bt	119.0 $\pm$ 78.0 (75) a
	N	136.0 $\pm$ 78.0 (75) a

Within each observation category, means followed by the same letter are not significantly different (Student's *t*-test,  $P < 0.05$ ) (n), sample size.

**Table 2. 4** Weight, time of emergence and reproductive potential of 2<sup>nd</sup> generation *Heterorhabditis bacteriophora* obtained from Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N), from mid- 4<sup>th</sup> instar Cry1Ac-resistant *Plutella xylostella* reared on artificial diet

Observations	Plant type	(mean ± SE)
Larval weight (mg)	Bt	36.0±2.4 (75) a
	N	35.6±2.3 (75) a
Mean time to emergence (h)	Bt	154.0±10.0 (75) a
	N	152.8±11.0 (75) a
Mean nematodes per White trap	Bt	3156.0±1019.0 (75) a
	N	3048.0±1508.0 (75) a
Mean nematodes per mg <i>P. xylostella</i>	Bt	88.0±29.0 (75) a
	N	85.0±39.0 (75) a

Within each observation category, means followed by the same letter are not significantly different (Student's *t*-test,  $P < 0.05$ ) (n), sample size.

## DISCUSSION

The toxicity of Cry1Ab and Cry3Bb to free soil-dwelling nematodes, *Caenorhabditis elegans*, has been reported in the laboratory (Hoss et al. 2008, Hoss et al. 2011). However such effects were only seen as high concentrations and field studies revealed no significant differences in the nematode communities between Bt and non-Bt maize. Liu et al. (2011) confirmed the presence of bioactive Cry1Ac in resistant *P. xylostella* larvae that fed on Bt broccoli and the presence of Cry1Ac in the endoparasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae) that fed on *P. xylostella* larvae. Likewise, in this study it appears that the EPN was also exposed to Cry1Ac since it fed internally on *P. xylostella* that had consumed Cry1Ac. Upon entering the host and reaching its hemocoel, IJs of *H. bacteriophora* release their symbiotic bacteria, ultimately killing the host within 48 h. However, host mortality does not ensure progeny reproduction. In order to become self-fertilized hermaphrodites with a female phenotype (Poinar 1975), IJs must feed within the host hemocoel on the bacteria and host tissues (Kaya and Gaugler 1993) and eventually they give rise to a 2<sup>nd</sup> generation consisting of amphimictic males, females and IJs (Strauch et al. 1994). If nutritive conditions are favorable, IJs will develop into hermaphrodite females; otherwise they emerge from the host. Therefore, it can be concluded that *H. bacteriophora* feeding on mid-4<sup>th</sup> instar *P. xylostella* that had developed on Bt broccoli are also exposed to bioactive Cry1Ac. However, despite being exposed, there were no significant differences in the LD<sub>50</sub> or LT<sub>50</sub> values, time of emergence from the host cadaver, or reproductive potential of *H. bacteriophora* when developing in *P. xylostella* that had fed on Cry1Ac or non-Bt broccoli.

Furthermore, in the present study, our results from choice bioassays also indicate that *H. bacteriophora* could not discriminate between Cry1Ac or non-Bt broccoli-fed hosts.

The components in the insect host diet can also affect the efficacy of IJs of subsequent generations (Shapiro-Ilan et al. 2008). In our study, there were no significant differences in the above-mentioned parameters for the second generation *H. bacteriophora* obtained from Cry1Ac or non-Bt broccoli-fed *P. xylostella* against larvae that developed on artificial diet. Combinations of EPNs and Bt crops have been shown to be synergistic in insect suppression in the field (Gassmann et al. 2006). Our study provides strong evidence there is no effect from Cry1Ac on *H. bacteriophora*, and this has important implications for its role as a natural enemy in IPM.

Arthurs et al. (2004) in an analysis of 136 publications on field and greenhouse trial of *S. carpocapsae*, concluded that efficacy of nematode subjected to foliar application depends on target insect's habitat. Piggott et al in (2003) reported the acceptable control of foliar insect pests, *Spodoptera exigua* (Hübner) and leafminers in the genus *Lyriomyza* at low concentration rate and prudent placement of nematodes using polymeric formulations. Satisfactory control of western flower thrips, *Frankliniella occidentalis* (Pergande) in chrysanthemums was made possible with specially formulated *S. feltiae*. Schroer et al. (2005) reported the enhanced efficiency of EPNs against *P. xylostella*, when applied with additives. Development of polymeric formulations, custom modified spray equipments, low volume and prudent use of EPNs has increased the level of acceptance of EPNs against foliar insect pests.

In conclusion, based on the results from the present study and our previous studies (Cao et al. 1999, 2002; Zhao et al. 2003, Chen et al. 2008, Liu et al. 2011), it has been shown that Cry1Ac brassica crops can effectively control *P. xylostella* but have no effects on important natural enemies due to high specificity of the toxins. Furthermore, the present study provides valuable information to regulatory authorities about the safety of Cry1Ac to entompathogenic nematodes. We expect similar results will be obtained using other lepidopteran-active Bt proteins.

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## CHAPTER 3

### EPILOGUE

The present work was conducted within the broad context of a risk assessment of insect-resistant genetically engineered (IRGM) crops expressing insecticidal crystal (Cry) proteins from the bacterium, *Bacillus thuringiensis* (Bt). The major objective of this research project was to provide science-based information on the direct and indirect effects of Bt crops on soil-dwelling nematodes that are natural enemies of many economically important insect pests. In this study, we used *Heterorhabditis bacteriophora* as an example of a nematode biological control agent.

Entomopathogenic nematodes (EPN) are important biological control agents in soil ecosystems (Yeates and Coleman 1982) and should be protected. Because of their persistence, broad host range and compatibility with some chemical and biological pesticides, EPN are considered good candidates for integrated pest management (Kaya and Gaugler 1993). Traditionally, nematodes have been used primarily against soil- and root-dwelling insect pests. However, with the advancements in sprayer technology and better understanding of nematode biology and ecology, coupled with growing restrictions on chemical insecticides, there is an increased interest in using EPN against aboveground pests.

Use of natural enemies for pest management is complex because it involves three living organisms: the plant, the pest and the natural enemy. Plant biochemistry, morphology, and variety can affect the successful deployment of natural enemies against foliage insect pests (Barbosa et al. 1991). This can occur with arthropods as well as entomopathogens including bacteria (Reichelderfer 1991), fungi (Ramoska and

Todd 1985) and viruses (Richter et al. 1987). Studies suggest that host plants may also affect EPN; this may be the case with IRGM crops which have revolutionized the management of coleopteran and lepidopteran pests in two field crops, cotton and corn (Shelton 2012). The area planted under Bt crops increased at an unprecedented rate from 1.6 million hectares in 1996 to 69 million hectares in 2012 (James 2012). With growing interest in foliar application of EPNs and Bt crops, it is important to assess the potential effects of Bt crops on EPN.

The impact of Bt crops on natural enemies should be subjected to a standard tiered risk assessment approach that involves laboratory and field tests (Sharples 1991, Romeis et al. 2011, Romeis et al. 2013). Lower tier tests are designed to expose the target organisms to a higher dose of toxins than is present under field conditions, thus representing a worst-case scenario. If the effects are significant on non-target organisms in lower tier studies, then higher tier studies involving more elaborate laboratory or greenhouse studies, and possibly field studies, should be undertaken. Risk assessment process can be discontinued at any stage as soon as sufficient information has been gathered to address the risk hypothesis.

We investigated potential effects of Cry1Ac on the development, reproductive potential and other fitness parameters of *H. bacteriophora* under tri-trophic conditions using Cry1Ac-resistant *P. xylostella*. In our bioassays we did not find any negative effects due to Bt toxins on any fitness parameters of *H. bacteriophora*. However, Hoss et al. (2011) documented potential negative effects at high concentrations of Bt crop exudates on *Caenorhabditis elegans*, a common non-infectious, non-pathogenic,

non-parasitic organism that lives in the soil on rotting vegetation and feeds on microbes such as bacteria.

While our studies indicated that *H. bacteriophora* would not be harmed when it fed on Cry1Ac- resistant *P. xylostella* that consumed Cry1Ac broccoli, additional studies on EPN may be warranted. Bt proteins from Bt plants can enter the soil through root exudates, decaying plant residues, and through the feces of animals fed on Bt crops. Once in the soil, Bt proteins can bind with other soil components like clay and humus particles, which will slow their degradation without impairing their biological activity (Koskella and Stotzky 1997). Accumulation of biologically active Bt proteins in the soil may pose a potential hazard for soil-dwelling nematodes, including EPN. Saxena and Stotzky (2001) reported that Bt corn decomposes at a slower rate than non-Bt corn due to its higher lignin content. Thus, it is possible that the habitat preference and foraging strategy of EPN may be negatively influenced by the presence of Bt toxins in the soil. Griffiths et al. (2003) reported that Cry proteins interfere with *C. elegans* physiology by competitive binding with carbohydrate receptors in the intestinal tracts of *C. elegans*. Therefore, a similar study on the gut action of Bt toxins on *H. bacteriophora* may be warranted.

The complex life cycle of *H. bacteriophora* may also be affected in other ways by Bt proteins they may come into contact with in the soil. After successful infestation and establishment in the host, *H. bacteriophora* develops into a female adult inside the host. The adult produces eggs that, upon hatching, develop into infective juveniles (IJs) which in turn feed on the adult as well as the host. During emergence, IJs acquire the symbiotic bacteria *Photorhabdus luminescens* from the maternal body (female

nematode inside the host), apparently not from the host itself (Ciche et al. 2008). Since the symbiotic transmission of bacteria to the IJs is essential for successful infestation of the new host, it may warrant investigation whether the Bt proteins present in the host have any impact on maternal transmission of symbiotic bacteria. Symbiotic bacteria may produce antibiotic and secondary compounds that render protection to the insect cadaver from opportunistic secondary saprophages. The effect of Bt proteins on the ability of bacteria to produce these compounds may warrant further investigation.

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