EFFICACY OF BIOPESTICIDES FOR ORGANIC MANAGEMENT OF CUCUMBER BEETLES

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Mary A Rogers
December 2012

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ACKNOWLEDGEMENTS

Deep gratitude is due to my loving husband, Matthew Charles Amundsen, for all his patience, understanding and undying support during the entirety of our marriage and my educational endeavors.

I owe much thanks and appreciation to my major professor, supervisor and mentor, Annette L. Wszelaki for her countless gifts to me. I thank her for edits and technical advice. For generously sharing her knowledge, for guidance, optimism and for believing in me, cheering me on and letting me pursue my own path. I have flourished under her mentorship.

I am grateful for the conversations and wise advice from Dr. Pasco B. Avery whose work inspired me to take on this research project, and whom I hope to work with more in the future.

Great thanks and appreciation to the members of my committee, Dr. Juan Luis Jurat-Fuentes and Dr. David M. Butler for advice, guidance and critical review of my research, and to Dr. Bonnie H. Ownley for the same, in addition to generously allowing me to work in her lab.

Special thanks to Mary Dee and Andrea Vu in Dr. Ownley's lab for technical assistance, and to Bill Lively, Bobby Terry, Lee Ellis and Bobby Simpson at the Organic Crops Unit for assistance with field work.

I appreciate the help of all the organic interns and undergraduate students. It was a pleasure to spend the summers with you. In no particular order: Daniel Priddy, Alex Minderman, Kelly Corum, Jessica Gates, Elias Attea, Mechelle Kneidinger, Jordan Norton, Becca Smith, Philip Moore, Nora Hutchison, Alexandra Spaulding, DJ Mitchell, Tiffany Kingston Morrison, Mary Clare and Dara Smith.

Thanks to the members of the organic crops team for assistance, encouragement and good times: Jeff Martin, Grant McCarty, Sarah Eichler Inwood, Heather Toler, Susannah Amundson and Fiona McAnally.

This dissertation was funded in part through awards from the IR-4 Project (Rutgers, NJ) and a SARE Graduate Student Grant.

Last but not least thanks to my parents, Tom and Gail Rogers, for all their love and encouragement.

ABSTRACT

Organic growers are limited in crop protection techniques for cucumber beetle management. Spotted (*Diabrotica undecimpunctata howardi*) and striped (*Acalymma vitatta*) cucumber beetles are significant pests of cucurbits in the U.S. Feeding results in aesthetic damage and reduction in marketable yields as well as transmission of bacterial wilt that can result in plant mortality. Biopesticides are products formulated from naturally occurring organisms such as fungi and bacteria that are pathogenic or toxic to insect pests. Advantages to these products are that they have low environmental risk, low risk to non-target organisms including mammals and beneficial insects, and can help reduce resistance to pesticides when used in an integrated pest management program. The overall goal of this dissertation was to examine the potential of microbial products to reduce mortality and feeding by cucumber beetles for the benefit of organic producers. Chapter one is a review of the biopesticide industry, biology of microbial agents for insect pest management, the role of biopesticides in sustainable agriculture, and constraints to their use. Chapter two covers the field experiment conducted on Galia melons in 2010 and 2011 using *Chromobacterium subtsugae* and *Beauveria bassiana*. Chapter three covers the laboratory assays using *Beauveria bassiana* and the laboratory and field experiments using *Isaria fumosorosea*. Chapter four is the final experiment on the effects of these microbial agents on cucumber beetles and squash bugs in organic pumpkin production. The results indicated anti-feedant effects by *Chromobacterium subtsugae* and *Beaveria bassiana* in the laboratory assays, but field trial results were inconclusive and did not show a reduction in beetle populations or a yield increase resulting from spray applications of these microbial agents. Complications in the field studies arose from plant pathogens and physiological factors

independent from cucumber beetle population and damage. Recommendations are to improve biopesticide efficacy through improving formulation and delivery, by additional screening and testing to determine efficacy on multiple life stages of the pest, and research to increase the understanding of ecological roles and interactions of microbial biopesticides in the environment.

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CHAPTER I

The role of microbial biopesticides for insect pest management in sustainable agriculture: applications and limitations

1.1 Introduction

Biopesticides are naturally derived substances or microbes used to manage pests including insects, weeds and diseases. These plant protectants are viewed as more environmentally benign than their synthetically- produced chemical counterparts as they often do not persist in the environment, do not affect vertebrates, and usually have high host selectivity (Gupta and Dikshit 2010). Use of biopesticides is expected to grow in importance in the future as consumers demand more sustainably produced foods; pest resistance to synthetic chemical pesticides increases, and we face new threats by exotic pest species (Chandler et al. 2008). 'Biopesticides' can be broadly defined and include all or some of the following: living organisms (insect predators, parasitoids, nematodes and microorganisms) and the products they produce (secondary metabolites produced by microorganisms), viruses, genes (transgenics), insect pheromones and mating disrupters, and plant extracts/botanicals (Chandler et al. 2008; Copping and Menn 2000). These products are not meant to be used in the same way as synthetic chemical pesticides, but are best used when incorporated into a well-designed integrated pest management (IPM) program. Section 1.1 gives an overview of the biopesticide industry in the U.S., and describes successes and failures that the industry currently faces. Section 1.2 defines the terminology used by the Environmental Protection Agency (EPA) in the U.S. Section 2 discusses microbial biopesticides with particular emphasis on products containing

fungal, bacterial, viral and microsporidial organisms including their secondary metabolites/toxins that are currently registered by the EPA and are commercially available for use in the U.S. for insect pest management in horticultural and agronomic crops. Plant-incorporated-protectants are genetic materials incorporated into transgenic plants and are regulated as biopesticides by the EPA. Plant-incorporated-protectants, in addition to biological control by "macrobials", predation or parasitism by arthropods or nematodes or plant-based crop protectants, are beyond the scope of this review and will not be discussed. In section 3, the role of biopesticides within sustainable agriculture will be addressed, with a major focus on examples of successful usage as well as the issue of compatibility with natural enemies. Limitations and constraints of field efficacy using biopesticides are discussed in section 4, with an emphasis on the effect of both abiotic and biotic factors.

1.1.1 The biopesticide industry

Worldwide, the biopesticide industry comprises just 1 to 2% of the world market for crop protection products (Ravensberg 2011c), and projections for growth in 2010 were at 4.2%, with sales reaching $1 billion (Thakore 2006). The majority of the market is driven by sales of *Bt*-based products (Chandler et al. 2008). Currently, the orchard industry is the largest sector using biopesticides, accounting for 55% of all biopesticides applied (Thakore 2006). Organic farming is a critical market for this industry, and is in itself a growing market, so sales of biopesticides are expected to continue to increase. However, biopesticde use is not restricted to organic markets, and sales of synthetic pesticides are decreasing as the industry responds to consumer concern over pesticide residues and increasing environmental awareness (Thakore 2006). At

the same time, insect resistance to synthetic chemicals, secondary pest outbreaks, environmental pollution and contamination, safety risks for humans and animals, withdrawals of synthetic pesticides, and the threat of exotic insect pests that warrant novel management methods are driving research and development in the biopesticide industry (Chandler et al. 2008; Lacey et al. 2001). There are currently 200 registered biopesticide products for use in the U.S. (Chandler et al. 2008), and new products are being developed to meet the growing demand, but the industry is new and faces many challenges in the development, implementation and commercialization of these products. Some of the roadblocks include: the need to identify effective strains of pathogens and their host range, problems with production and formulation of effective products, lack of understanding on how these organisms can fit into an IPM program and their interactions with the environment, a too-simple pesticide paradigm that compares biopesticides with synthetic chemical pesticides without appreciation for their attributes, and acceptance by growers and the general public (Lacey et al. 2001). It is unlikely that the biopesticide industry will thrive until these roadblocks are addressed, and these challenges have contributed to the unsteady history of the biopesticide industry. In this section a brief history of the successes and failures of the biopesticide industry will be outlined; the major companies, agencies and organizations involved with biopesticide research and development and promotion will be identified; and factors affecting successful product commercialization will be addressed.

Interest in biopesticide development first peaked in the U.S. in the 1980s and 1990s, but due to lack of market insight, projected sales were never realized and many of the larger agrochemical companies abandoned the effort and changed their focus to transgenic crops (Ravensburg 2011c). In a review of the last 50 years of biopesticide production, three main eras have been identified: (1) the pioneering era, (2) the era in which large agrochemicals entered the venture and failed, and (3) the era (since 1995) in which small, diverse companies control the market (Gelernter 2005). It was concluded in this review that failure in this enterprise is largely due to incorrectly perceiving the market size. Ravensberg (2011c) lists other reasons why companies failed to be successful in this sector, and these are: (1) incorrectly assuming that biopesticides would be easy to develop, (2) overestimating their performance over other crop protection products, and (3) underestimating the amount of money and time to develop new products. Some examples of companies in the U.S. that have abandoned or their biopesticide efforts include: Abbot, Biosys, CropGenetics, Ecogen, Ecoscience, Eastman Kodak, Mycogen, Mycotech, Taensa, Thermo Trilogy, Troy Biosciences and WR Grace (Ravensberg 2011c). However, many large companies in the U.S. have actively registered commercial biopesticide products, including: Bio Works, Certis, Dow AgroSciences, Laverlam, Marrone Bio Innovations, Syngenta and Valent BioSciences, and there are currently 200 biopesticide products registered in the U.S. (Chandler et al. 2008). In the U.S., the EPA and individual states register biopesticides for use. The Biopesticide and Pollution Prevention Division (BPPD) is a separate division of the EPA that promotes the use of biopesticides in IPM programs and coordinates with the Pesticide Environmental Stewardship Program (PESP), a group that is concerned with reducing negative environmental effects associated with pesticide use

(Chandler et al. 2008). The Interregional Research Project (IR-4 Project) provides funds for biopesticide research to public researchers and private companies to foster development of new products. The Biopesticide Industry Alliance (BPIA) in the U.S. promotes adoption of biopesticides as well through increased awareness of their efficacy when used correctly in an IPM program.

Factors that companies should consider for successful development of biopesticides are outlined in *A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products for Control of Arthropods*, and include: an accurate business plan that focuses on integrity of data and best-and worst-case scenarios; starting small with modest investments; a strong focus on a single product; developing deep market and customer knowledge; allocating sufficient budgeting for both product development and marketing; careful estimation of time and costs of registration; early involvement of distributors; developing knowledge on compatibility and IPM systems; and balancing risks, progress and debts (Ravensberg 2011a). It is likely that companies that follow these factors will be successful; however, from the consumer side, more cost-benefit analysis and education on the proper role of biopesticides within an IPM program will be needed in order for growers to adopt these products, currently this information is lacking. Efficacy for many of these microbial products remains an issue, and this will be discussed in further detail in section 4.

1.1.2 Regulation and Terminology

Regulation and definitions of biopesticides vary by institution and to avoid confusion, the appropriate terminology needs to be identified prior to discussion. In the United States,

biopesticides are regulated by the EPA, and three classes are recognized. These are: (1) microbial pesticides, (2) plant-incorporated-protectants (PIPs), and (3) biochemical pesticides. Microbial pesticides are defined as "a microbial agent intended for preventing, destroying, repelling, or mitigating any pest, or intended for use as a plant regulator, defoliant, or desiccant, that: (1) is a eukaryotic microorganism, including, but not limited to, protozoa, algae, and fungi; (2) is a prokaryotic microorganism, including, but not limited to, Eubacteria and Archaebacteria, or (3) is a parasitically replicating microscopic element, including, but not limited to, viruses. Plant-Incorporated-Protectants (PIPs) are "pesticidal substances that plants produce from genetic material that has been added to the plant". In the case of transgenic plants expressing the Bt toxin, the protein and its genetic material are regulated, but not the plant itself (www.epa.gov). Biochemical pesticides are "naturally occurring substances that control pests by non-toxic mechanisms". These include sex pheromones and plant extracts used for attraction (www.epa.gov).

Entomopathogenic nematodes are often grouped along with microorganisms, as they share similarities in mass production, product development, application and research discipline (insect pathology), but are not regulated the same way as microbials (Environmental Protection Agency 2007; Ravensberg 2011a). Discussion in this review will be limited to microbial biological control as defined by the EPA on insect pests specifically, and will not include nematodes or other "macrobials" such as parasitic and predacious arthropods. The terms *biological control* and *biocontrol* will be used interchangeably following the definition proposed by Eilenberg et al. (2001), where biocontrol is: "the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less

damaging than it would otherwise be". Additionally, biopesticides in this review will be discussed with the understanding that they primarily fall within the category of *inundation biocontrol*, where "living organisms [are used to] control pests when control is achieved exclusively by the released organisms themselves." In other words, where pest management is the result of direct application of the biopesticide and not on the expectation that control will be sufficiently maintained by natural reproduction in the environment, the latter is defined as *inoculation biocontrol* (Eilenberg et al. 2001). In reality, microbial biopesticides may exhibit a continuum between *inundation* and *inoculation* biocontrol, based on the formulation, the persistence of the organism in the environment, the availability of the host, and other biotic and abiotic factors (Chandler et al. 2008). The term *biopesticide* in this review will be synonymous with *microbial biopesticide*, or *microbial* as defined above, following Ravensberg (2011a).

1.2 Microbial Biopesticides

1.2.1 Entomopathogenic fungi

Most entomopathogenic fungi belong to two orders, Entomophthorales and Hypocreales (formerly Hypomycetes) (Hajek and St. Leger 1994). Entomophthoralean fungi may produce sexual zygospores and asexual azygospores, and most are obligate parasites with narrow host ranges. Many of these species suppress insects through epizootics, and the most common genera include *Entomophaga*, *Entomophthora* and *Zoophthora* (Goettel et al. 2000). These fungi can be used in inoculation biological control, but their use is limited because they are costly and difficult to mass produce, as they need to be collected and reared on insect hosts; in

addition, epizootics are density dependent, requiring a critical threshold level of hosts in order to develop (Hajek and St. Leger 1994). As a result, more attention has recently been paid to developing mycoinsecticides based on Hypocrealean fungi. Significant fungi in this order that will be discussed include *Beauveria*, *Metarhizium* and *Isaria* (formerly *Paecilomyces*) as products from these organisms are currently labeled for managing a variety of insect pests. Although specific strains of these fungi are thought to exhibit a narrow host range, it has been demonstrated that single species are actually a polymorphic species complex exhibiting varying specificity, resulting in an overall broader host range (Fegan et al. 1993; Wang et al. 2005; Zimmermann 2008). Commercial products based on *Beauveria*, *Metarhizium* and *Isaria* are discussed below.

Beauveria bassiana is considered a broad spectrum biopesticide that can infect a diverse group of insects, and is currently the most ubiquitous mycopesticide used in the U.S. (Faria and Wraight 2007). Labels that are currently approved for greenhouse/nursery and vegetable, ornamental and turf production include Mycotrol O™ (Strain GHA, 10.9% AI, Laverlam International Corporation, Butte, MT), BotaniGard ES™ (Strain GHA, 11.3% AI, Mycotech/ Laverlam International Corporation, Butte, MT), BotaniGard 22WP™ (Strain GHA, 22% AI, Laverlam International Corporation, Butte, MT) and Naturalis L™ (Strain ATCC 74040, 7.16% AI, Troy Biosciences Inc., Pine Level, NC). Labels for these products state that they can be used to manage a wide variety of insect orders including members of: Orthoptera (except for BotaniGard), Thysanoptera, Hemiptera, Lepidoptera (except for BotaniGard) and Coleoptera; and the label for Naturalis L™ also include Diptera and Acari (mites). Both BotaniGard labels are not registered for agronomic or field crop use.

Products made from *Metarhizium anisopliae* are not as widely available in the U.S. as those for *B. bassiana*, but these could be forthcoming. Roots Met-52™ (Strain F52, 2% AI), a granular biopesticide label, was registered by Novozymes Biologicals Inc. (Salem, VA) in 2009 and is available to control black vine weevil grubs (*Otiorhynchus sulcatus*), thrip pupae (order Thysanoptera) and ticks (order Ixodida) in the soil and on turf and ornamentals. Recently, a product called 'Green Muscle' was developed from *M. anisopliae* var. *acridum* by the International Institute of Tropical Agriculture's LUBILOSA (Lutte Biologique contre les Locustes et auteriaux) project to control locusts and grasshoppers, major crop pests in Africa (Douthwaite2001).

Two current labels exist at this time for one strain of *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*). The first, PFR-97™ 20% WDG (Apopka strain 97, 20% AI, Certis, Columbia, MD), is labeled for the organic management of a variety of insects and mites for vegetables, fruits and other food crops. Preferal™ (Apopka strain 97, 20% AI, SePRO, Carmel, IN) is labeled for the management of insect and mite pests on vegetables, fruits and ornamental plants grown in greenhouses or nurseries.

1.2.2 Bacterial based biopesticides

The primary entomopathogenic bacteria are in the families Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococcaceae; however, the most widely used and commercially available biopesticides have been formulated from the genus *Bacillus* (Garczynski and Siegel 2007), with the species *Bacillus thuringiensis* (Bt) dominating the commercial market for these products (Ravensberg 2011a). *Bacillus thuringiensis* is a Gram

positive, rod-shaped, aerobic, endospore- producing bacterium, and different isolates (or subspecies) exhibit toxicity to specific insect orders, depending on the insecticidal crystal proteins (ICPs) they produce (Garczynski and Siegel 2007). In 1998, there were 200 registered Bt products (including viable and nonviable biopesticides, and plant-incorporated-protectants), effective against a number of lepidopterans and a selective list of coleopterans (27 products were for dipteran management) (Schnepf et al. 1998). Biopesticide products based on naturally occurring strains of Bt that are currently available for pests of agricultural crops are listed here. Subspecies *kurstaki* is specific to lepidopteran pests, and currently labeled products include Condor® (Strain EG2438, 24.5% AI, Certis, Columbia, MD); Deliver®(Strain SA-12, 85% AI, Certis, Columbia, MD), Javelin WG® (Strain not specified, 7.5% AI, Certis, Columbia, MD); DiPel Pro DF® (Strain ABTS-351, 54% AI, Valent BioScience Corp., Libertyville, IL); Foray® 48B (Strain ABTS-351, 12.65% AI, Valent BioScience Corp., Libertyville, IL); Biobit® HP (Strain ABTS-351, 58.2% AI, Valent BioScience Corp, Libertyville, IL); and Thuricide (Strain not specified, 0.8% AI, Southern Agricultural Insecticides Inc., Hendersonville, NC). These labels manage agriculturally significant lepidopteran pests including but not limited to: diamondback moth (*Plutella xylostella*), imported cabbageworm (*Pieris rapae*), armyworms (many species in the family Noctuidae), European corn borer (*Ostrinia nubilalis*) and corn earworm (*Helicoverpa zea*). Another subspecies of Bt that expresses toxicity to lepidopteran pests is *aizawai*, and XenTari ® (Strain ABTS-1857, 54% AI, Valent BioScience Corp., Libertyville, IL), and Agree® WG (Strain GC-91, 3.8% AI, Certis, Columbia, MD) are commercial formulations that target a broad number of species including those listed above. *Bacillus thuringiensis* subsp. *tenebrionis* is toxic to some leaf beetles (family Chyrsomelidae) including Colorado potato beetle (*Leptinotarsa*

decimlineata). Novodor® (Strain not specified, 10% AI, Valent BioScience Corp, Libertyville, IL) is a commercial product for Colorado potato beetle in solonaceous crops and elm leaf beetle (*Pyrrhalta luteola*) on shade trees and ornamentals.

Insect pest resistance is increasing due to the wide use of Bt products. Laboratory resistance to Bt *kurstaki* has been reported for diamondback moth larvae, tobacco budworm (*Heliothis virescens*) and beet armyworm (Schnepf et al. 1998). More recently, field resistance to Bt has been shown in western corn rootworm (*Diabrotica virgifera virgifera*) (Gassmann et al. 2011).

Actinomycetes are filamentous, aerobic, Gram positive, rod-shaped bacteria that form mycelia, similar to fungi, and are naturally occurring in soils, where they often perform beneficial functions (Higa and Parr 1994). Some species within this order have insecticidal properties, and two species in particular, *Saccharopolyspora spinosa* and *Streptomyces avermitilis*, have been used to formulate biopesticides. Spinosad is produced from aerobic fermentation of *S. spinosa*, and the insecticidal properties are due to spinosyns, secondary metabolites that are fractionated into spinosyn A (about 85%) and spinosyn D (about 15%) (Thompson et al. 2000). Spinosad (Conserve® SC, 11.6% AI; and SpinTor® 2SC, 22.8% AI, Dow AgroSciences, Indianapolis, IN) is less broad range than many conventional pesticides, but is still toxic to a wide variety of insect orders, and is labeled to manage lepidopteran pests including European corn borer, armyworms, certain leaf beetle larvae, including asparagus leaf beetle (*Crioceris asparagi*), flea beetles (many species within subfamilies Acticinae and Galerucinae), Colorado potato beetle, as well as suppression of thrips. The products Entrust® (80% AI, Dow AgroSciences, Indianapolis, IN), and Monterey Garden Insect Spray™ (0.5% AI, Lawn and Garden

Products, Inc., Fresno, CA) are labeled for certified organic production, as well as a fruit fly bait (GF -120® NF Naturalyte®, 0.02% AI, Dow AgroSciences, Indianapolis, IN).

Avermectins are pesticidal compounds resulting from fermentation of the soil Actinomycete *Streptomyces avermitilis*. The two types that have been commercialized to manage insects, mites and plant pathogenic nematodes in crops are abamectin and emamectin benzoate (Pitterna et al. 2009). Abamectin is a mixture of avermectin B_1a (>80%) and avermectin B_1b (<20%), and Emamectin benzoate is produced through catalytic conversion of avermectins (Molnár et al. 2005). Current labels for abamectin include Agri-mek® 0.15 EC (2% AI, Syngenta Crop Protection, Greensboro, NC) for management of mites and Colorado potato beetle on a number of fruit and vegetable crops; and Zephyr® 0.15 EC (2% AI, Syngenta Crop Protection, Greensboro, NC), for management of mites on cotton. Commercial products containing Emamectin benzoate include Proclaim® (5% AI, Novartis Crop Protection, Greensboro, NC) for the management of lepidopteran larvae on *Brassica* vegetables, celery and head lettuce; and Affirm® (17 g/L AI in 20 L, Syngenta Crop Protection, Greensboro, NC), for the management of boll worms (*Helicoverpa armigera* and *Helicoverpa punctigera*), and suppression of green mirids (*Creontiades dilutus*) and mites in cotton.

A new fermentation product was recently developed from *Chromobacterium subtsugae* strain PRAA4-1 and is currently labeled (Grandevo™) for management of lepidopteran larvae, aphids, mites, thrips, and whiteflies on vegetable and fruit crops (30% AI, Marrone Bio Innovations, Davis, CA).

1.2.3 Virus based biopesticides

The most studied group of viruses that are pathogenic to invertebrates are members of Baculoviridae. These are rod-shaped viruses with ds DNA that form occlusion bodies that are dissolved by the insect gut, wherein the virus multiplies (Cory and Evans 2007). The two genera that have been studied for biological control are *Polyhedrovirus* and *Granulovirus*, known together as the nucleopolyhedroviruses (NPV and GV) (Cory and Evans 2007). A few commercial products based on these organisms have been developed for the management of key agricultural pests, including Cyd-Xe®, (*Cydia pomonella* granulovirus, 0.06%, Certis, Columbia, MD) for organic management of codling moth in apples, pears, plums, prunes and walnuts; Spod-X® LC, (OBs of NPV of *Spodoptera exigua*, 0.64% AI, Certis, Columbia, MD) for organic management of beet armyworm in ornamental, vegetable and agronomic crops; and Gemstar® LC (OBs of NPV of *Helicoverpa zea*, Certis, Columbia, MD) for organic management of corn earworm, cotton bollworm, tomato fruitworm and tobacco budworm (all *Heliocoverpa* spp.) on a variety of vegetable crops.

1.2.4 Biological control by Microsporidia

Microsporidia (formerly aligned with Protozoa) are eukaryotic, unicellular, spore-producing obligate parasites that are closely related to fungi (Solter and Becnel 2007). These organisms are important naturally-occurring insect pathogens but are not well suited for inundation biological control, as they have complicated life cycles and may require alternate hosts and, as obligate parasites, are difficult to mass produce; furthermore, their effects on insects tend to be chronic rather than acute (Solter and Becnel 2007). *Nosema locustae*, which is specific to

grasshoppers, is the only microbial in this class that is registered as a biopesticide, formulated as a bait product. Semaspore Bait™ (0.05% AI, Planet Natural, Bozeman, MT) and Nolo Bait™ (0.05% AI, M&R Durango, Bayfield, CA) are currently available for commercial use.

1.3 The potential of biopesticides in sustainable agriculture

Pest management in sustainable agriculture should incorporate a variety of control measures to manage pests below economic injury levels, and the inundative use of entomopathogenic fungi should not be thought of as a therapeutic control like a typical chemical pesticide, but rather, as a form of biological control that should be used in tandem with other management practices, such as using insect predators and parasitoids and other cultural techniques as part of a comprehensive integrated pest management program (Jaronski 2009; Ravensberg 2011b). For sustainable pest management, the goal should be to maximize preventive strategies based on ecological principles to avoid pest buildup, with the occasional use of focused, biorational controls applied at the correct time for therapeutic management of pest outbreaks (Lewis et al. 1997). Microbial based biopesticides have received increased research attention due to the advantages they have over synthetically produced, broad-spectrum traditional insecticides. These advantages include: (1) relative safety to humans, (2) decrease in toxic pesticide residues in the environment, (3) host specificity and limited effects on non-target organisms, (4) limited pest resistance by the target pest species, (5) no secondary pest outbreaks, (6) compatibility with other biological control agents, (7) potential for long-term control, (8) ease of application, and (9) no pre-harvest interval (Kaya and Lacey 2007, Lacey et al. 2001; Siegel 2001; Tanada and Kaya 1993;). The disadvantages of biopesticides include: (1) specificity to one target organism

and the potential need for additional products for other pests, (2) narrow timing windows for application, (3) little “knock-down” effect, (4) short field persistence due to environmental factors(discussed in further detail in section 4), (5) difficulty in formulation and mass production of obligate parasites and pathogens, (6) short shelf life, (7) development of resistance to Bt products, and (8) economic constraints to use (Kaya and Lacey 2007). Examples of successful use of microbial biopesticides for inundation biological control will be discussed in section 3.1, compatibility of biopesticides with insect natural enemies are discussed in Section 3.2, and the factors that limit the use of biopesticides in agriculture will be discussed in section 4.

1.3.1 Biopesticide efficacy studies

Biopesticides based on microorganisms can be effective when used at the proper time and development stage of the target pest. It may take many years to develop effective formulations and application techniques of these products, and it is important to realize the potential of these organisms is in the suppression of pest populations and indirect effects on pest growth and reproduction, rather than a quick knockdown. One of the benefits of using microbials is the compatibility and potential synergistic effect of these pest protectants with other natural enemies and other microbials. Examples of successful applications of biopesticides are discussed in this section.

Effective use of biopesticides is often based on proper timing of application at the optimal population density and life stage of the target pest. In a study by Poprawski et al. (1997), applying unformulated conidia of *Beauveria bassiana* (mixed in water, with a 0.01% Silwet surfactant suspension, at the rate of 5×10^{13} viable conidia/ha) with four applications at

3-4 day intervals, resulted in a significant reduction of Colorado potato beetle larvae early in the growing season. Mycosis was observed to be >90% after the last application, and defoliation was significantly reduced and provided equally acceptable levels of control as the conventional treatments. The authors attributed this to the proper timing of the applications and good coverage obtained by the spray equipment used (Poprawski et al. 1997). Susceptibility of the target pest to microbial pesticides can vary depending on pest life stage and by isolate, dose and temperature. Vandenberg et al. (1998) found mortality of diamondback moth larvae was highest when sprayed with *B. bassiana* strain GHA during the 4th instar, and during the 3rd instar when sprayed with isolate ARSEF 4543, and that survival times were reduced when infection occurred during the 2nd and 3rd instars. Furthermore, increasing the dose of both isolates increased mycoses and mortality, decreased survival time, and temperatures of 25°C were most effective for mycosis to occur (Vandenberg et al. 1998).

It may take many years to develop a successful biopesticide, as exhibited by the LUBILOSA project (Douthwaite et al. 2001). The use of *Metarhizium anisopliae* spores to manage migratory locusts in Africa is an example of the successful use of a biopesticide. After resolving initial technical issues regarding mass production, formulation and field studies, researchers were able to achieve infection and mortality in 70 to 90% of treated locusts within 14 to 20 days of application, without affecting non-target organisms (Lomer et al. 2001; Shah and Pell 2003). This work resulted in the development of “Green Muscle” for commercial use, after 12 years of research and $17 million in project funding (Shah and Pell 2003).

In a greenhouse experiment, spray applications of *Isaria fumosorosea* blastospores were effective in managing greenhouse whitefly (*Trialeurodes vaporariorum*) on beans (*Phaseolus vulgaris*); and was compatible with the use of whitefly parasitoid *Encarsia formosa* and in some cases, the combination treatment was more effective than either treatment used alone (Avery et al. 2008). Another laboratory bioassay showed that blastospores of *I. fumosorosea* were more effective than conidia at infecting Mexican bean beetle larvae (*Epilachna varivestis*), indicating that efficacy can depend on the type of propagule (Behle et al. 2006). Field studies are currently lacking for this organism on food crops. One study showed that *I. fumosorosea* has the potential to reduce feeding and increase mortality of the Asian citrus psyllid (*Diaphorina citri*), on grapefruit leaves in the laboratory (Avery et al. 2011). *Isaria fumosorosea* (PFR 97™) killed 95% of first and third instar nymphs of the potato psyllid (*Bactericera cockerelli*), a pest of solanaceous crops that is responsible for vectoring 'zebra chip' disease (Lacey et al. 2009). Manipulating the substrate media could be a way to increase efficacy of entomopathogenic fungi. *Isaria fumosorosea* reduced larval growth, feeding rates, and adult emergence mortality of diamondback moth larvae; and was enhanced when isolates were cultured with 1% (w/v) chitin (Ali et al. 2010). The authors concluded that using chitin as a carbon source when culturing fungi can increase chitin degrading enzymes and increase efficacy (Ali et al. 2010).

Combinations of biopesticides can have a synergistic effect on suppressing pest populations. Commercial formulations of *Beauveria bassiana* (Mycotrol) and *Bacillus thuringiensis tenebrionis* (Novodor) were more effective when used together to manage Colorado potato beetle larvae (Wraight and Ramos 2005). Combinations of a commercial formulation of Bt (XenTari) and nucleopolyhedrosis virus (Spod-X) were more effective against

beet armyworm than when used alone (Kolodny-Hirsch et al. 1997). *Isaria fumosorosea* (PFR 97™) and abamectin B (Agri-Mek) were effective in reducing potato psyllids (*Bactericera cockerelli*) that vector a bacteria responsible for zebra chip disease of potato, and combinations of PFR 97™ and Trilogy® (70% clarified hydrophobic extract of neem oil, 70% AI, Certis, Columbia, MD) resulted in higher yields of potatoes than when PFR 97™ was used alone (Lacey et al. 2011).

Spinosad is a relatively newer biopesticide, and has shown good efficacy in field and greenhouse studies. A commercial formulation of spinosad (Conserve) was highly toxic to immature and adult western flower thrips (*Frankliniella occidentalis*) on greenhouse-grown cucumbers (Jones et al. 2005). A field study showed that spinosad (SpinTor 2 SC) was effective in killing eggplant flea beetle (*Epitrix fuscula*) on eggplant foliage, but did not persist on the foliage compared to conventional treatments of thiamethoxam and chlorfenapyr (McLeod et al. 2002). This illustrates that comparable control can be achieved with biopesticides than with broader spectrum pesticides, while reducing the impact on some beneficials. Biocompatibility of these products will be discussed further in the next section.

1.3.2 Biocompatibility

The compatibility of microbial biopesticides with beneficial arthropods and pollinators varies by the selectivity, mode of action, formulation and application of the active ingredient, biology and exposure of the non-target organisms, timing and dose of application, in addition to abiotic and biotic conditions and environmental persistence. Efforts should be made to reduce the impact on beneficial insects whenever possible. Assessing impacts on non-target organisms can be

difficult due to a number of factors. Laboratory bioassays focus on high doses and direct, acute effects in a controlled setting, far different from field conditions, and fail to address sublethal and indirect effects (Glare and O'Callaghan 2003). Microbial biopesticides based on Bt (excluding transgenically modified organisms for this discussion) and baculoviruses are considered highly selective and safe to natural enemies, consequently there are very few reports of Bt directly infecting non-target organisms in the field (Glare and O'Callaghan 2003; Ravensberg 2011b). *Bacillus thuringiensis* is compatible with *Trichogramma* wasps (Takada et al. 2001), and using these egg parasitoids can increase control of lepidopteran pests. Furthermore, indirect effects due to host competition can be avoided by sequential rather than simultaneous application of these organisms when they are used for augmentation biocontrol (Navon 2000). Similarly, baculoviruses have been used for many years without direct negative effects on natural enemies (Cory and Myers 2003). However, indirect effects on larval parasitoids due to competition for hosts could also occur, and could especially limit parasitoid populations if and when viral epizootics occur. Compared to conventional pesticides, bacterial biopesticides are considered "soft" or "biorational" on beneficial insects and pollinators, due to the lack of direct effects.

Entomopathogenic fungi have a broader host range and greater potential impact on natural enemies and pollinators. Most of these studies have been done in the laboratory on *Beauveria bassiana* and *Metarhizium anisopliae*, and fewer field studies exist (Vestergaard et al. 2003). *Beauveria bassiana* has been isolated from beneficial insects in the field, including ground beetles (Carabidae), spiders (Araneae, Lycosidae and Salticidae), and lady beetles (Coccinellidae); but it was concluded that the risk was low, especially when careful isolate

selection and spacio-temporal factors to reduce exposure to natural enemies were considered (Vestergaard et al. 2003). There is some concern that both *B. bassiana* and *M. anisopliae* can be harmful to pollinators, including bumblebees (*Bombus* spp.) and honey bees (*Apis mellifera*), and care should be taken to avoid exposing these species to entomopathogens (Hokkanen et al. 2003). The pesticide label for BotaniGard ES (*B. bassiana*) has a honey bee warning, stating that there is potential for infection on honey bees, and spraying near hives and when bees are foraging should be avoided. In a greenhouse study, both BotaniGard WP and Naturalis-L (*B.bassiana*) were compatible with beneficial mites (*Amblyseius cucumeris*) when used to manage western flower thrips (*Frankliniella occidentalis*) on cucumbers (Jacobsen et al. 2001). BotaniGard was also found compatible with both *E. formosa* parasitoids and *Dicyphus hesperus* predators when used in combination to manage greenhouse whiteflies (*Trialeurodes vaporariorum*) on tomato plants without significant interference, and these can be applied together when whitefly populations are higher than typically recommended for effective biological control (Labbé et al. 2009).

However, significant mortality has also been observed in greenhouse studies using lady beetles (Coccinellidae) for biological control. For example, the mortality rate of predatory beetle *Serangium parcesetosum* that fed on whiteflies infected with *B. bassiana* (strain GHA) was 86%, compared to 13% for control beetles (Poprawski et al. 1998); and BotaniGard reduced survival of the mealybug predator *Cryptolaemus montrouzieri* in interiorscapes (Smith and Krischik 2000). Field studies show that *Metarhizium anisopliae* is capable of infecting beneficial ground (Carabidae) and rove (Stapylinidae) beetles, however, incidence of infection was low, and in laboratory tests at ideal conditions, only high doses of *M. anisopliae* infected non-target

species (Vestergaard et al. 2003). Furthermore, the granular formulation of Roots Met-52 applied directly for managing vine weevils should limit the exposure to natural enemies that occur in the phyllosphere.

A few studies on the compatibility of *Isaria fumosorosea* with natural enemies have been published, and results look promising for the organisms studied. Sterk (1995a, b and 2002) examined the effects of PreFeRal (*I. fumosorosea*, Apopka 97 strain, not labeled for use in the U.S.) on four natural enemies, predatory mites *Phytoseiulus persimilis*; predatory bugs *Orius laevigatus* and *Macrolophus caliginosus*; parasitoid *E. formosa;* and bumblebee pollinator *Bombus terrestris* and determined that this biopesticide is harmless (<25% mortality) to these species in laboratory and semi-field trials. Whitefly insect predators are important for inundation biological control. PFR 97^{TM} (*I. fumosorosea* Apopka 97 strain) did not interfere with predation of greenhouse whiteflies by *D. hesperus* (Alma et al. 2007), and *Isaria fumosorosea* (strain 612) did not have significant detrimental effects on the whitefly coccinellid predator *S. parcesetosum* (Poprawski et al. 1998) or *Hippodamia convergens* (Pell and Vandenberg 2002). Similarly, strain PF01-N4 did not affect survival of coccinellid beetle *Axinoscymnus cardilobus* in the laboratory (Zhou et al. 2010). Parasitism of whiteflies (*T. vaporariorum*) by *E. formosa* was not inhibited by PreFeRal in control chambers (Hamdi et al. 2011). Furthermore, parasitism of the brown citrus aphid (*Toxoptera citricidus*) by the native parasitoid wasp *Lysiphlebus testaceipes* was not affected by applications of PFR 97 ^{TM} in cage studies (Pick et al. 2012). The majority of the studies on *I. fumosorosea* at this point do not show negative non-target effects on natural enemies.

The research indicates that there is potential for negative direct effects with entomopathogenic fungi, although this can be mitigated by proper timing and avoidance of natural enemies. There are many different isolates of these organisms, some yet to be identified, and testing should be performed on insects at the species level to confirm compatibility (Vestergaard et al. 2003). Up to this point, fungal-based biopesticides are considered safe for non-target insects in the field, although caution is warranted due to the host range potential.

Spinosad is known to be toxic to beneficial insects. In a field study on effects of low-doses of granular spinosad (Tracer Naturalyte, Dow AgroSciences, Indianapolis, IN) for management of armyworm (*Spodoptera frugiperda*) in corn, Staphylinid beetles (*Aleochara bilineata*) and earwig (*Doru taeniatum*) predators were killed by consuming either dead armyworms or via exposure to the granules in the soil, with mortality levels ranging from 48 to 98% depending on dosage (Cisneros et al. 2002). Lacewings (*Chrysoperla carnea*), however, were conserved because they were not exposed to the soil-applied granules. There was differential mortality on beneficials used to manage western flower thrips on cucumbers in the greenhouse, with low toxicity to predatory mites (*Amblyseius cucumeris*), moderate toxicity to minute pirate bug predators (*Orius insidiosus*), and high toxicity to *E. formosa* one day after spraying Conserve (120 SC, Dow AgroSciences, Calgary, AB, Canada). In toxicology studies, spinosad is considered harmful (>75% mortality) to bumblebees when they are sprayed directly and via ingestion from contaminated sugar water (Sterk et al. 2002). Sublethal effects on non-target insects have also been reported, such as reduced oviposition by parasitoid wasps (*Colpoclypeus florus*) (Brunner et al. 2001), and decreased survival, weight gain, fecundity and

fertility in a laboratory bioassay on Asian lady beetles (*Harmonia axyridis*) (Galvan et al. 2005). Due to the number of reports of non-target effects of spinosad on natural enemies and pollinators, caution should be used in applying this biopesticide and attempts should be made to avoid exposing beneficials to this product.

Conservation of natural enemies and pollinators is imperative in a sustainable pest management program, thus it is important to understand the range of compatibility of biopesticides with these non-target organisms. It is generally accepted that these formulae pose less risk than traditional broad-spectrum synthetic pesticides to both beneficial insects and vertebrates; however some biopesticides are "softer" than others and this can depend on the selectivity of the active ingredient, the formulation, application technique and dose, the biology and exposure of the non-target organism and the environmental fate of these products. Biopesticides are best used in an IPM system, and in some cases can act synergistically with natural enemies to increase pest management. Limitation and constraints of biopesticides are discussed in the next section.

1.4 Limitations and constraints of microbial biopesticides

Inundative biocontrol can only be achieved by "winning the numbers game," where infective propagules are introduced in sufficient numbers to reduce pest populations, a feat that is sometimes easier accomplished in a controlled greenhouse setting rather than in the field (Jaronski 2009). The widespread use of fungi as biocontrol agents to manage insect pests is currently constrained by environmental and biological factors. Environmental factors such as sunlight, rainfall, temperature and humidity can impact the viability of fungal propagules, and

ideal conditions may differ on the leaf surface microhabitat versus the macrohabitat (Jaronski 2009). Soil factors such as humidity, temperature, pore size and organic matter content can influence fungal populations and diversity, and this is complicated by interactions with other soil microbes as well as plant root exudates and secondary plant metabolites (Bruck 2009; Hesketh et al. 2009; Meyling and Eilenberg 2006). The effects of abiotic and biotic factors on efficacy and adoption of biopesticides are discussed in this section.

1.4.1 Abiotic factors

Sunlight, rain, temperature and humidity all determine the rate of decline of microbial products once they are applied to foliage in the field. The UV-A and UV-B components of sunlight are major contributors to degradation of fungal propagules and largely responsible for short field persistence (Jaronski 2009). The half-life of fungal conidia exposed to outdoor sunlight can be as low as 3 to 4 hours (Braga et al. 2001), although other studies have shown viable conidia of *M. acridum* 8 to 14 days after application in subtropical and semi-arid environments (Jaronski 2009; Van der Valk 2007). The susceptibility of fungal entomopathogens to sunlight varies by organism and strain. For example, conidia of *I. fumosorosea* are more susceptible to degradation by UV light than *M. anisopliae* and *B. bassiana* (Fargues et al. 1996). Factors that can increase field persistence include the following: reducing exposure of propagules to light by focusing applications on the undersides of leaves and using sunscreens and protectants, and increasing photo stability of these products by using natural plant extracts (Eyheraguibel et al. 2010), soy-based sunscreens, and water-soluble lignins as adjuvants for UV protection (Behle et al. 2011).

Rainfall can wash fungal propagules from the leaf surface and reduce field persistence, decreasing efficacy. In one study, 25 to 47% of *B. bassiana* conidia were removed from alfalfa and 51 to 56% from wheat leaves under 30 minutes of simulated rain fall (Inglis et al. 1995). Oil-based surfactants and emulsifiable spray formulations can help preserve propagules from being washed out by rainfall (Jaronski 2009; Wraight and Carruthers 1999). In addition, fungal propagules require warm temperatures and high humidity conditions to germinate and infect insects, and oil and emulsion formulations can increase efficacy at low field humidities (Smith 1997); although optimal thresholds may vary by organism, generally infection occurs at warm temperatures and high humidity. Humidity and temperature levels for optimal infection for *B. bassiana* is 25°C and 65% RH (Athanassiou and Steenburg 2007); for *M. anisopliae*, is 25°C and >96% RH (Arthurs and Thomas 2001); and for *I. fumosorosea*, is 25 to 28°C and 75% RH (Behle et al. 2006; Hallsworth and Magan 1999; Zimmermann 2008). Sunlight affects viability of entomopathogens and is considered the most important factor in field persistence (Behle 2011; Ignoffo 1992; Jaronski 2009). When exposed to simulated and natural sunlight, the half-lives of various propagules from bacteria, fungi, protozoans and viruses ranged from one hour to 96 hours (Ignoffo 1992). Many of these natural pathogens are derived from soil environments, and may not have evolved mechanisms to tolerate exposure to UV light. In general, abiotic factors will influence the persistence and efficacy of biopesticides in the field, and these factors may be mitigated by improvements in adjuvants and surfactants to increase longevity of propagules and infective agents, such as selecting strains that are more resilient to degradation. Besides abiotic factors, the interaction of entomopathogenic microorganisms with their insect hosts,

plants and other microorganisms can influence the success or failure of these organisms for biological control. These factors are discussed in the next section.

1.4.2 Biotic factors

The interaction between the entomopathogen and the insect host can be complex. In order to be effective in pest management, the pathogen must overcome the insect immune system causing an infection that results in feeding inhibition and reduced function, ideally causing death. The insect host can respond behaviorally to infection by pathogens and these responses include induced fever, elevation seeking, reduced or increased activity, reduced response to semiochemicals and changes in reproductive behavior (Roy et al. 2006). In laboratory assays, the generalist predator *Anthocoris nemorum* actively detected and avoided pea aphids (*Acyrthosiphon pisum*) that were infected with *B. bassiana*, preferring instead to feed on healthy aphids (Meyling and Pell 2006). Behavioral alterations may allow the insect to escape or avoid infection either physically or through the immune response and can reduce the efficacy of biopesticides for pest management. The behavioral response by insects to entomopathogens reflects the coevolutionary relationship between the agonist (insect) and antagonist (pathogen) and the complexity at the base of biological control, and can explain in part why biopesticides may fail to work.

Entomopathogenicity in fungi is thought to have evolved more than once, and is particularly pronounced in hemipteran insect hosts such as scales (Coccidae), aphids (Aphididae) and whiteflies (Aleyrodidae) that are closely associated with their plant hosts and mainly immobile (Humber 2008; Vega et al. 2009). Little is known on the life history of these

microorganisms (Roy et al. 2010) but evidence suggests that some of them may obtain nutrients from plant sources in addition to their insect hosts, and it is known that *Beauveria, Metarhizium* and *Isaria* can act as plant endophytes and interact with plant roots in the rhizosphere (Vega 2008; Vega et al. 2009), and can be antagonistic to plant disease-causing pathogens (Ownley et al. 2008). Most research on entomopathogens has been focused on the pathogen-insect interaction, excluding the role of the plant. Understanding the role the plant plays in these interactions and how plant chemicals interact with these organisms will help in designing more effective pest management strategies (Jackson et al. 2009).

Secondary plant metabolites can have an allelopathic effect on entomopathogenic organisms, resulting in reduced efficacy of biopesticides. Catechol and salicylic acid reduced germination of *P. fumosoroseus* (= *I. fumosorosea*) blastospores in *in vitro* studies, at concentrations of 100 ppm; levels that are much lower than what would be found in plant tissue (Vega et al. 1997). Lacey and Mercadier (1998) tested the chemicals tomatine, solanine and camptothecin (alkaloids), xanthotoxin (furanocoumarin), and tannic acid (phenolic) for their effects on germination and colony growth rates of *P. fumosoroseus*. All of the allelochemicals tested in this study resulted in germination inhibition relative to the controls and camptothecin, tomatine and xanthotoxin reduced colony size significantly. Isothiocyanates are synthesized by crops in the family Brassicaceae, and were shown to have toxic effects on *M. anisopliae* based on germination and growth (Inyang et al. 1999). In addition, phenylethyl-isothiocyanate volatiles reduced pathogenicity of the fungi on mustard beetles (*Phaedon cochleariae*) (Inyang et al. 1999). *In vivo* studies also show that certain host plants can influence mycosis based on plant chemistry. For example, two different populations of whitefly nymphs

(*Bemisia argentifolii*) were reared on cotton and melon host plants and introduced to *B. bassiana* and *P. fumosoroseus* in a laboratory assay (Poprawski and Jones 2000). The authors found that germination of fungal conidia of both organisms was inhibited on nymphs reared on cotton only, and this was attributed to the influence of the terpenoid gossypol in cotton and the possibility of sequestration of this compound by *Bemisia* for insect defense. Plant chemistry may play an important role in the efficacy of biological control and more research in this area is needed to understand the underlying mechanisms at work in these tritrophic interactions.

1.5 Conclusion

Successful management with biopesticides is currently more commonly realized in controlled settings such as interiorscapes and greenhouses. The ecological interactions with entomopathogenic fungi in managed cropping systems and the ability to infect insect pests is a complicated process that needs further study. Applied research to investigate the practicality of using these pathogens in agricultural pest management programs focuses on the effective formulation and dispersal of infective propagules in the field and whether this is economically feasible. Even if efficacy is satisfactory, economic factors may constrain adoption of biopesticides. These products can be expensive to produce and have a short shelf life, which may limit their practical use. More research is needed to understand the compatibility of biopesticides with natural enemies in the field and understand the complex biotic factors that influence efficacy. More work needs to be done on the consumer end to educate growers on the proper uses of these products within an integrated pest management program and cost-

benefit analyses of adopting biopesticides need to be developed in order for this industry to thrive in both conventional and organic farming systems.

Chapter 2

Field efficacy of strains of *Chromobacterium subtsugae* and *Beauveria bassiana* for management of *Diabrotica undecimpunctata howardi* (Barber) and *Acalymma vittata* (Fabricius) (Coleoptera: Chrysomelidae) in organically grown F1 Galia muskmelon (*Cucumis melo* L. cv. *reticulatus* Ser.)

2.1 Introduction

Spotted and striped cucumber beetles (*Diabrotica undecimpunctata howardi* and *Acalymma vittata*) are economically significant pests of cucurbits and other crops in the southeast region of the U.S. where they overwinter and have multiple generations per year. In contrast to adult preference for cucurbits, larvae of *D. undecimpunctata howardi* are generically known as southern corn rootworm and cause devastating damage when feeding on roots of corn. However, *A. vittata* is considered a cucurbit specialist (Ellers-Kirk and Fleischer 2006), and larvae only develop on cucurbit roots (Bach 1980).

Adults of *D. undecimpuntata howardi* and *A. vittata* are long-lived; surviving up to 125 days, and females may lay up to 4 eggs per day (Ellers-Kirk and Fleischer 2006). Beetles damage cucurbit crops such as pumpkin, melon, summer squash and cucumber by feeding directly on the fruit, leaves and stems, while larvae can damage roots and fruit that are in contact with the soil. Apart from feeding damage, adults of both species also vector bacterial wilt caused by *Erwinia tracheiphila*, which can cause significant mortality of both cucumber and muskmelon. Squash, pumpkin and watermelon are less commonly affected by this disease (Cline et al. 2008; Yao et al. 1996). The pathogen is present in beetle feces and enters the plant via feeding

wounds (Leach 1964). Incidence of bacterial wilt is directly proportional to the number of cucumber beetles in the field (Yao et al. 1996), highlighting the importance of effective management of the cucumber beetle vectors to prevent disease.

Currently, cucumber beetles are managed conventionally with the use of pyrethroids, neonicotinoids and carbamates (Kemball 2011). These compounds are effective but may have negative impacts on aquatic organisms, beneficial insects and pollinators (Kovach et al. 2012). Options for controlling cucumber beetles in organic agriculture is far more limited, and alternatives are urgently needed as a result of increasing consumer demand for vegetables that are grown without synthetic chemicals (Organic Trade Association 2012). While pyrethrum is effective in killing cucumbers beetles in organic systems, it is considered a broad spectrum pesticide that may affect pollinators and beneficial insects (Casida 1980). Techniques to manage cucumber beetles organically that may be combined in integrated pest management programs include the use of row covers prior to planting, crop rotation, companion planting, trap cropping, using reflective and colored mulches and spraying pyrethrum (Andino et al. 2004; Cline et al. 2008; Caldwell et al. 1999; Platt et al. 1999; Santos et al. 1995). The limited availability of pesticides to control cucumber beetle species in organic farming highlights the importance of developing effective biological pesticides for the control of these pests without negatively affecting pollinators or beneficial insects.

Biopesticides are made or extracted from naturally occurring microorganisms that kill pestiferous arthropods while minimizing the negative impacts on other animals, including beneficial insects. A new biopesticide developed by Marrone Bio Innovations Incorporated (Grandevo™, 30% AI, Marrone Bio Innovations; Davis, CA) is now labeled for management of

lepidopteran larvae, aphids, mites, thrips, and whiteflies on vegetable and fruit crops. This biopesticide is formulated using the secondary metabolites produced from the *Chromobacterium subtsugae* bacterial strain PRAA4-1, and preliminary laboratory tests demonstrated toxicity on chewing insect pests, such as Colorado potato beetle larvae (*Leptinotarsa decemlineata*), yellow margined leaf beetles (*Microtheca ochroloma*), and striped cucumber beetles (*A. vittata*). *Chromobacterium* sp. isolated from soil were found to be toxic to larvae of *L. decemlineata*, adult southern green stink bug (*Nezara viridula*) as well as two diabroticite beetles *D. virgifera*, and *D. undecimpuntata* in laboratory bioassays (Martin et al. 2004; 2007).

Beauveria bassiana is a soil dwelling broad spectrum entomopathogenic fungus with worldwide distribution, wide insect host range, and efficacy against cucumber beetles (Bruck and Lewis 2001). Conidia of the fungus germinate on the insect cuticle, penetrate the exoskeleton and produce toxins that eventually lead to insect death. After death, under high humidity conditions, the fungus will proliferate on the insect body, and mycelia may cover the insect entirely (Pekrul and Grula 1979). Due to its activity and safety, *B. bassiana* is commonly used in greenhouse environments (BotaniGard™ ES, Mycotech, Butte, MT), where it is used against foliar insect pests such as whiteflies, aphids, thrips and mealybugs. In addition, some *B. bassiana* formulations (Mycotrol™O, Laverlam International Corporation, Butte, MT) may also be applied for biocontrol of ornamental and vegetable lepidopteran and leaf-chewing beetle pests including cucumber beetles, although few scientific reports are currently available to support field efficacy of this product.

The objective of this study was to determine the efficacy of two biopesticides, *C. subtsugae* strain PRAA4-1 (MBI 203=Grandevo™) and *Beauveria bassiana* (Mycotrol™O) to reduce damage caused by *D. undecimpunctata howardi* and *A. vittata* on organically grown F_1 Galia muskmelons (*Cucumis melo* L.), in comparison with carbaryl (Sevin 80 S) as a conventional standard. In addition, varying application rates and spray rotations were used to determine optimal application practices and efficacy.

2.2 Materials and Methods

Field trials were performed in 2010 and 2011 at the Organic Crops Unit (OCU) of the University of Tennessee in Knoxville, TN.

2.2.1 Field Experiment 2010

On 19 May 2010, untreated melon (Galia 'Diplomat' F1) seeds (Johnny's Selected Seeds, Winslow, ME) were sown in SunGro Sunshine Organic Blend Professional Growing Mix (Sun Gro Horticultural, Bellevue, WA) in plastic pots (11.4 cm in diameter) in the greenhouse. Seedlings were hand fertilized weekly with 200 mL Rain Grow (4N-0.87P-2.5K) liquid fertilizer (Oliver, BC, Canada) in 1 L water delivering 75 mL per pot. The greenhouse temperature settings were 18°C/ 21° F night/day with a photoperiod of 16:8. In the field, 0.57 hectares of a cowpea cover crop was flail mowed (Alamo SH74, Alamo Industrial, Seguin, TX) and tilled on 28 May with a rotary tiller (Bush Hog, Selma, AL), and again on 14 Jun to manage regrowth. The cowpea cover crop was estimated to provide 113 kg N/ha, based on 3.5% N in aboveground biomass. On 14 Jun, plastic mulch (0.9 m wide and 1-mil thick; Pliant Corp., Chippewa Falls, WI) and drip irrigation (10 mil thick, with emitters set every 30.5 cm to provide 59 L/h of water at 562.4

g/cm^2 (Netafim, Tel Aviv, Israel) was laid in rows. Plants were set out in the field by hand on 16 Jun. Plots were 6 m long with 3 m between rows, set at 0.6 m in-row spacing. There were ten plants per plot, and four replicates. A 1.2 m wide strip of buckwheat was seeded on either side of the plots with an Almaco light duty grain drill (Almaco, Nevada, IA) at the rate of 45 kg/ha to encourage pollination and to serve as a buffer strip. Nature Safe course ground fertilizer (10N-1.7P-3.3K, Griffin Industries Inc., Cold Spring, KY) was side dressed to each transplant by hand at planting to deliver 25 kg of additional N per hectare.

The spray treatments began on 2 July and were repeated weekly for six weeks. The spray treatments and schedule are listed in Table 2.1. A Bellspray™ backpack sprayer (Bellspray Inc., Opelousas, LA) with 2 kg CO_2 cylinder, 4.2 kg/cm^2 regulator, and a 4-nozzle boom (48 cm spacing between nozzles) was used for all treatments. The biopesticide formulations were mixed in designated 2-L plastic bottles. The sprayer was calibrated to deliver 218 L/ha at walking speed, and the boom was held at 46 cm above the plant canopy. Spray applications began with the lowest concentration to the highest, and the sprayer was fully rinsed with water between each application.

Insect scouting began on 29 June, and was conducted weekly on the day before spraying, until 10 Aug., for a total of seven scouting dates. Both *D. undecimpunctata howardi* and *A. vittata* were counted on two random plants (20% of plot). Harvesting began on 23 July, and was repeated twice weekly until 17 Aug., comprising a total of eight harvest dates. Total fruit per plot was sorted into marketable and unmarketable categories, counted and weighed. Plant mortality was recorded for each plot on the same days insects were scouted. The complete schedule of field activities is listed in Table 2.2.

Table 2.1 Spray rotation schedule for experiments conducted in 2010 and 2011 at the University of Tennessee Organic Crops Unit in Knoxville, TN.

Treatment-Rate (trade name, AI and manufacturer)	**Treatment notation**	**Year**	
		2010	**2011**
Beauveria bassiana-2.4 L/ha (strain GHA, Mycotrol O, 10.9% AI, Laverlam International, Butte, MT)	**B1X**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.
Beauveria bassiana-4.8 L/ha (strain GHA, Mycotrol O, 10.9% AI, Laverlam International, Butte, MT)	**B2X**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.
Chromobacterium substsugae-22.25 L/ha (strain PRAA4-I, MBI 203, 94.5% AI, Marrone Bio Innovations, Davis, CA)	**M1X**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.
Chromobacterium substsugae-66.75 L/ha (strain PRAA4-I, MBI 203, 94.5% AI, Marrone Bio Innovations, Davis, CA)	**M3X**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.
Chromobacterium substsugae-22.25 L/ha (alternated with *Beauveria bassiana*- 2.4 L/ha)	**MB**	2, 14, 28 July (7, 22 July; 4 Aug.)	28 June; 12, 26 July; 9 Aug. (5, 19 July; 2, 16 Aug.)
Chromobacterium substsugae-22.25 L/ha (alternated with carbaryl-2.4 L/ha)	**MSV**	2, 14, 28 July (7, 22 July; 4 Aug.)	28 June; 12, 26 July; 9 Aug. (5, 19 July; 2, 16 Aug.)
Carbaryl-4.8 L/ha (Sevin concentrate, 22.5% AI, TechPac LLC, Lexington, KY)	**SV**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.
Unsprayed Control	**UC**	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.

Table 2.2 Schedule of activities for experiments conducted in 2010 and 2011 at the University of Tennessee Organic Crops Unit in Knoxville, TN.

	Year	
Activity	**2010**	**2011**
Melon seeds sown in the greenhouse	18 May	13 May
Cover crops flail mowed and spaded	28 May	23 May
Dripline and plastic mulch laid	14 June	17 June
Melons transplanted	16 June	17 June
Insect scout dates	29 June	27 June
	6, 13, 20, 27 July	5, 11, 18, 25 July
	3, 10 Aug.	1, 8, 15, 22 Aug.
Harvests	23, 27, 30 July	5, 8, 10, 15, 20, 22, 25 Aug.
	3, 5, 9, 12, 17 Aug.	

2.2.2. Field experiment 2011

On 13 May 2011, untreated melon (Galia 'Diplomat' F1) seeds were sown in McEnroe's Premium Lite Growing Mix (McEnroe Organic Farm, Millerton, NY) in 50-cell plug trays in the greenhouse. The media contained compost and nutrients sufficient for germination and no additional fertilizers were used. Greenhouse conditions were the same as described in 2010. On 23 May, a field planted in winter rye and crimson clover at the OCU was flailed mowed and spaded (Imants, Reusel, The Netherlands) to prepare for planting. Plot size was reduced to five

plants per plot due to an increase in treatments. Plants were at the same spacing as described in 2010. Black plastic mulch and drip irrigation was established as described in 2010, and buckwheat was again sown in alleys. Field transplanting was done on 17 June, and plants were side-dressed with 0.9 kg of soybean meal (7N-0.87P-0.83K, TN Farmers Co-op, LaVergne, TN) to deliver 90 kg of N/ha.

The spray treatments began on 28 June and were repeated weekly for eight weeks, as described in 2010. Insect scouting was performed weekly for nine weeks, starting on 5 July Harvesting in 2011 began on 5 Aug., and was repeated twice weekly until 25 Aug., comprising a total of seven harvest dates (Table 2.2).

2.2.3 Statistical analysis

The experiment was analyzed as a randomized block design with eight treatments replicated four times. Beetle counts per plant (from two plants per plot) in both years were fitted to a Poisson distribution or square root transformed and analysis was done using PROC GLIMMIX (Generalized Linear Mixed Models) ANOVA in SAS (9.1, Cary, NC). A Fisher's Least Significant Difference (LSD) test was used to determine differences between means. Yield was factored on a total per plot basis in 2010 (ten plants per plot) and a total per plant basis in 2011 (five plants per plot), to compensate for additional treatments added in 2011. Fruit were graded into marketable and unmarketable categories and weighed. Yield data were analyzed using PROC GLIMMIX and log transformed or analyzed non-parametrically if the assumptions of equal variance could not be met. Survival data was also non-parametric and ranks were analyzed as a repeated measures design using PROC GLIMMIX, with an LSD test signifying differences

between means. Polynomial regression analysis was done to compare the response of yield to beetle populations.

2.3 Results

2.3.1. Field experiment 2010

Overall, populations *of A. vitatta*, *D. undeimpunctata* and both beetle species combined did not differ per treatment in 2010, with beetle numbers ranging from <1 to 2 beetles per plant on average (Table 2.3). However, *D. undecimpunctata* were found in much lower numbers than *A. vittata* (Table 2.3). There were differences in total and marketable melon fruit per hectare in 2010 by treatment (Table 2.4). Total and marketable yield were highly variable, with yields ranging from 19,123 to 36, 528 and 5,835 to 13,613 fruit per hectare, respectively. The B2X and MB treatments had significantly fewer marketable fruit harvested than the unsprayed control plots. The M3X treatment had significantly more total fruit harvested than the B2X, M1X label rate, MB and C treatments. There was no clear trend between beetle populations and marketable fruit yield or plant survival. There was a significant difference in melon plant survival by treatment (Fig. 2.1). By the last harvest date, fewer than 50% of the melons in the MB and UC treatment plot were alive, compared to around 80% for B1X, SV and M1X treatments (Fig. 2.1). There was a trend between plant mortality and yield that was not explained by beetle populations, based on a quadratic model ($p<.0001$), with only 31% of the variation in yield explained by beetle populations.

Table 2.3 *Acalymma vitatta* and *Diabrotica undecimpunctata howardi* adults and both species combined per plant on Galia melons by treatment across eight sampling dates in 2010

Treatment	*A. vitatta**	*D. undecimpunctata**	Both species combined*
Beauveria bassiana label rate, B1X	1.58 ± 0.18	0.38 ± 0.12	1.96 ± 0.24
Beauveria bassiana double label rate, B2X	1.48 ± 0.22	0.43 ± 0.08	1.91 ± 0.23
Chromobacterium subtsugae label rate, M1X	1.25 ± 0.18	0.34 ± 0.10	1.59 ± 0.21
Chromobacterium subtsugae triple label rate, M3X	1.92 ± 0.25	0.25 ± 0.08	2.17 ± 0.26
Chromobacterium subtsugae alternated with *Beauveria bassiana,* MB	1.52 ± 0.23	0.39 ± 0.09	1.91 ± 0.27
Chromobacterium subtsugae alternated with carbaryl, MSV	1.46 ± 0.22	0.30 ± 0.06	1.76 ± 0.23
Carbaryl label rate, SV	1.26 ± 0.20	0.32 ± 0.07	1.58 ± 0.22
Unsprayed control, UC	1.41 ± 0.20	0.38 ± 0.09	1.79 ± 0.22
F; d,f; p	*0.94; 7,56; 0.4811;*	*0.51; 7,56; 0.8197*	*1.10; 7,56; 0.3786;*

*Values are untransformed means ± standard error

Table 2.4 Yield and average fruit weight (kg) of Galia melons by treatment in 2010

Treatment	Total fruit number per hectare*+		Marketable fruit number per hectare*+		Mean marketable melon wt (kg) per fruit
Beauveria bassiana label rate, B1X	28813 ± 2740	**abc**	8750 ± 1705	**abc**	2 ± 0.2
Beauveria bassiana double label rate, B2X	19123 ± 1338	**d**	5835 ± 838	**bc**	1 ± 0.2
Chromobacterium subtsugae label rate, M1X	26448 ± 4278	**bcd**	6483 ± 918	**abc**	1 ± 0.2
Chromobacterium subtsugae triple label rate, M3X	36528 ± 1923	**a**	6808 ± 1438	**abc**	2 ± 0.1
Chromobacterium subtsugae alternated with *Beauveria bassiana,* MB	21910 ± 1918	**cd**	5835 ± 2215	**c**	1 ± 0.2
Chromobacterium subtsugae alternated with carbaryl, MSV	33318 ± 5290	**ab**	13613 ± 2875	**a**	2 ± 0.1
Carbaryl label rate, SV	30530 ± 5475	**abc**	11020 ± 1543	**ab**	2 ± 0.3
Unsprayed control, UC	26740 ± 3973	**bcd**	12965 ± 1908	**a**	1 ± 0.1
F; d,f; p;	*3.09; 7,21; 0.0272*		*2.59; 7,21; 0.0429;*		*0.58; 7,21; 0.7642;*

* Based on a population of 9075 plants per hectare

+Values are untransformed means ± standard error; mean separation by LSD test at α = 0.05

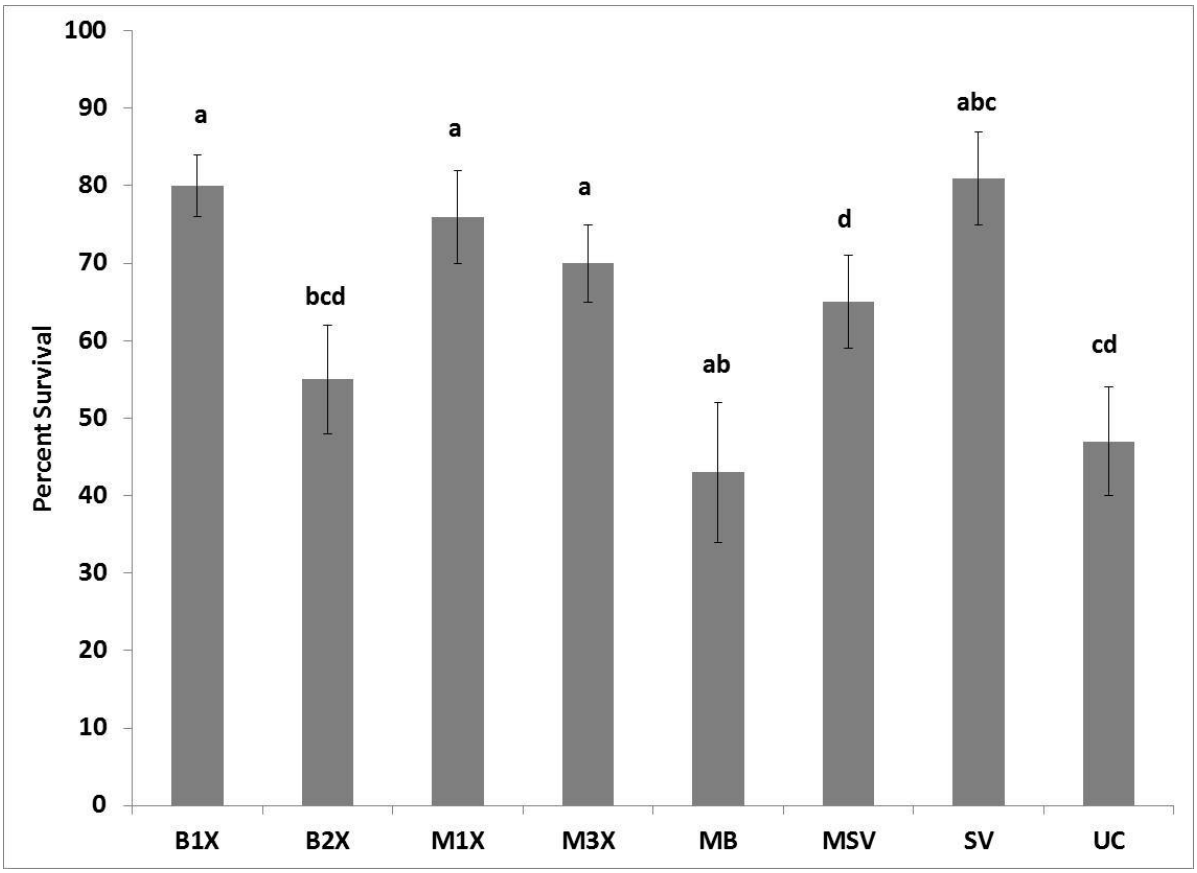

Fig. 2.1 Percent survival of F_1 Galia melon when sprayed weekly with biopesticides and a standard insecticide comparison at the University of Tennessee Organic Crops Unit in Knoxville, TN in 2010. Mean ± standard error is reported. Values followed by different letters were significantly different ($P \leq 0.05$). B1X = *Beauveria bassiana* label rate; B2X = *Beauveria bassiana* twice label rate; M1X = *Chromobacterium subtsugae* label rate; M3X = *Chromobacterium subtsugae* triple label rate; MB = *Chromobacterium subtsugae* alternated with *Beauveria bassiana*; MSV = *Chromobacterium subtsugae* alternated with carbaryl; SV = Carbaryl label rate; UC = Unsprayed control

2.3.2 Field experiment 2011

In contrast to 2010, populations of *D. undecimpunctata howardi*, *A. vittata* and both beetle species combined differed by treatment, with the SV label rate treatment having fewer beetles than the unsprayed control plots in all cases (Table 2.5). As was the case in the previous year, the species predominantly found was *A. vittata* (Table 2.5). The SV control and the B1X treatments had fewer beetles than the unsprayed control, however the higher rate (B2X) had higher numbers of beetles (Table 2.5). However, in contrast to 2010, there were no differences in marketable or total fruit yield per acre in 2011 (Table 2.6). This indicates that lower beetle infestations did not result in increased yield. As found during the 2010 experiment, plant mortality was not significant and plants had >90% survival (Fig. 2.2).

Table 2.5 *Acalymma vitatta* and *Diabrotica undecimpunctata* adults and both species combined per plant on Galia melons by treatment across eight sampling dates in 2011

Treatment	*A. vitatta**	*D. undecimpunctata**	Both species combined*
Beauveria bassiana label rate, B1X	0.80 ± 0.14 **bc**	0.25 ± 0.06 **bc**	1.03 ± 0.15 **cd**
Beauveria bassiana double label rate, B2X	1.24 ± 0.17 **a**	0.45 ± 0.08 **a**	1.69 ± 0.19 **a**
Chromobacterium subtsugae label rate, M1X	1.09 ± 0.14 **abc**	0.34 ± 0.08 **ab**	1.43 ± 0.17 **abc**
Chromobacterium subtsugae triple label rate, M3X	0.85 ± 0.13 **bc**	0.24 ± 0.06 **bc**	1.08 ± 0.15 **bcd**
Chromobacterium subtsugae alternated with *Beauveria bassiana,* MB	0.95 ± 0.13 **abc**	0.23 ± 0.06 **bc**	1.18 ± 0.14 **bcd**
Chromobacterium subtsugae alternated with carbaryl, MSV	1.14 ± 0.14 **a**	0.11 ± 0.04 **c**	1.25 ± 0.14 **b**
Carbaryl label rate, SV	0.70 ± 0.12 **c**	0.14 ± 0.04 **c**	0.84 ± 0.13 **d**
Unsprayed control, UC	1.14 ± 0.15 **a**	0.33 ± 0.08 **ab**	1.47 ± 0.16 **ab**
F; d,f; p	*3.19; 7,56; 0.0066;*	*3.14; 7,56; 0.0072;*	*2.48; 7,56; 0.0272;*

*Values are untransformed means ± standard error; mean separation by LSD test at $\alpha = 0.05$

Table 2.6 Yields and average fruit weight (kg) of Galia melons by treatment in 2011

Treatment	Total fruit number per hectare*	Marketable fruit number per hectare*	Avg. marketable melon wt (kg) per fruit
Beauveria bassiana label rate, B1X	25575 ± 4380	11550 ± 3258	1 ± 0.1
Beauveria bassiana double label rate, B2X	24805 ± 3805	12100 ± 3025	2 ± 0.9
Chromobacterium subtsugae label rate, M1X	31495 ± 4053	10143 ± 3100	1 ± 0.2
Chromobacterium subtsugae triple label rate, M3X	26090 ± 2855	11345 ± 2113	1 ± 0.1
Chromobacterium subtsugae alternated with *Beauveria bassiana*, MB	27225 ± 3705	83778 ± 2403	1 ± 0.1
Chromobacterium subtsugae alternated with carbaryl, MSV	22040 ± 4005	12965 ± 3258	1 ± 0.1
Carbaryl label rate, SV	25575 ± 2685	15675 ± 3888	1 ± 0.2
Unsprayed control, UC	20943 ± 3148	9773 ± 2403	1 ± 0.2
F; d,f; p	*1.20; 7,20; 0.3499;*	*0.77; 7,20; 0.6167;*	*0.88; 7,20; 0.5383;*

* Based on a population of 9075 plants per hectare

+Values are untransformed means ± standard error; mean separation by LSD test at $\alpha = 0.05$

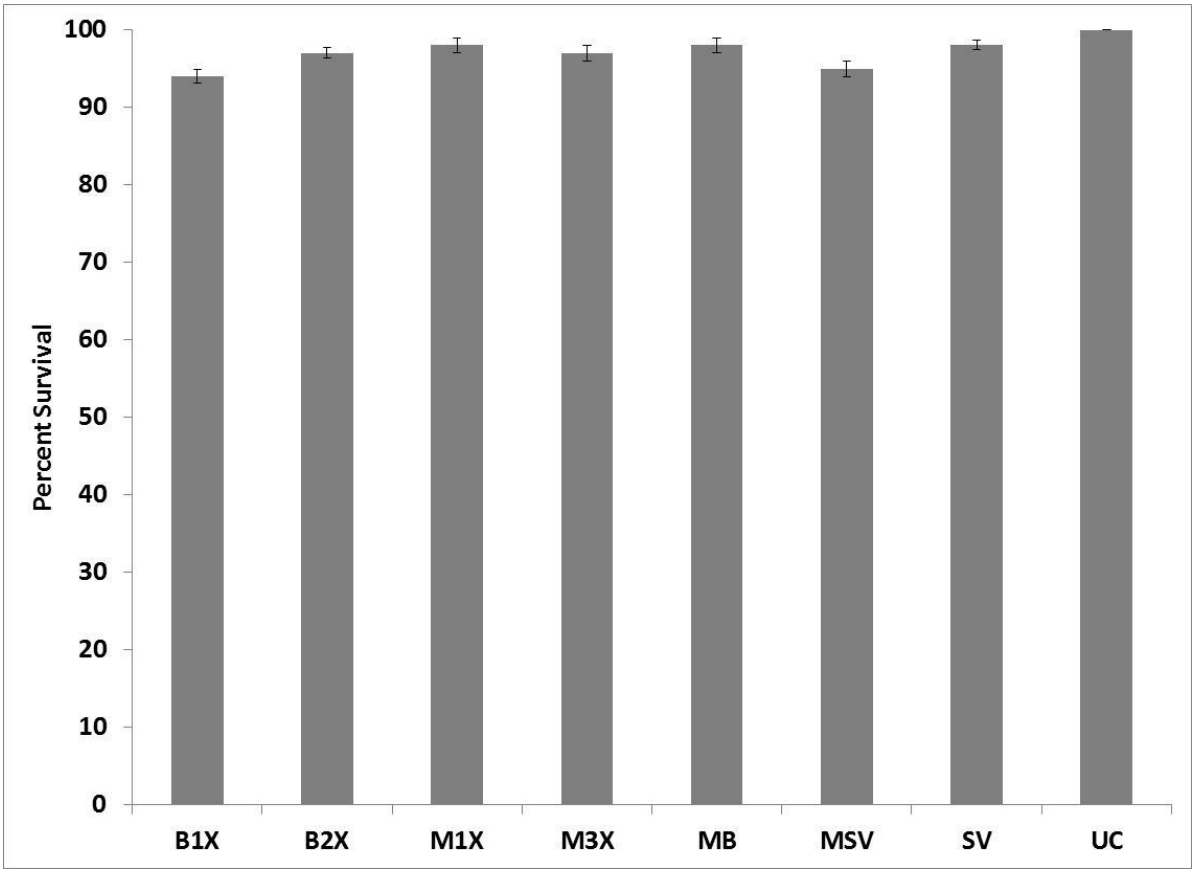

Fig. 2.2 Percent survival of F1 Galia melon when sprayed weekly with biopesticides and a standard insecticide comparison at the University of Tennessee Organic Crops Unit in Knoxville, TN in 2011. Mean ± standard error is reported. B1X = *Beauveria bassiana* label rate; B2X = *Beauveria bassiana* twice label rate; M1X = *Chromobacterium subtsugae* label rate; M3X = *Chromobacterium subtsugae* triple label rate; MB = *Chromobacterium subtsugae* alternated with *Beauveria bassiana*; MSV = *Chromobacterium subtsugae* alternated with carbaryl; SV = Carbaryl label rate; UC = Unsprayed control

2.4 Discussion and Conclusion

During both of our field experiments we found higher numbers of *A. vittata* compared to *D. undecimpunctata howardi*, which is in agreement with results from Platt et al. (1999) and Cline et al. (2008). We found that none of the insecticidal spray applications were able to consistently manage *A. vittata* below the economic threshold of one beetle per plant (Brust and Foster 1999) for the duration of the spray-scout interval. However, according to field trials that led to threshold recommendations given by Brust and Foster (1999), no yield loss was associated with beetle densities lower than four *A. vittata* beetles per plant on cantaloupe. In this experiment, *A. vittata* populations would fluctuate from week to week (data not shown), but were never higher than four beetles per plant and on average stayed within 1 to 1.5 beetles per plant. These relatively low numbers of beetles may make it more difficult to discern treatment differences and may have little impact on yield. In addition, cucumber beetles are extremely mobile, and move between host plants and field edges throughout the day (Luna and Xue 2009). Insect scouting was done on the day before spraying, and this may have made it more difficult to discern temporal treatment differences due to emigration or immigration from field plots. The application of Sevin concentrate resulted in the lowest numbers of both cucumber beetle species in 2011.

The *A. vittata* population in our experiment may have been reduced by natural enemies attracted to the buckwheat alleys. Buckwheat flowers are known to attract beneficial insects, and were reported to reduce populations of cucumber beetles on muskmelon when used as a companion plant due to increases in predation and parasitism (Cline et al. 2008). Natural

enemies of cucumber beetles include the Pennsylvania leatherwing (*Chauliognathrus pennsylvanicus*) and the tachinid flies *Celatoriae diabrocitae* and *C. setosa*, all of which are attracted to buckwheat (Cline et al. 2008; Platt et al. 1999). Average parasitism rates of *A. vittata* beetles by *C. setosa* were reported at 42% in the field (Elsey 1988). However, correlations cannot be made as natural enemy populations and species were not monitored in this study.

Marketable and total fruit numbers per acre were not correlated with cucumber beetle populations. Therefore, in these field studies, we can infer that yields were not significantly reduced by cucumber beetle feeding or transmission of bacterial wilt disease. This is likely due to beetle populations being lower than economic injury levels. Yields were lower than ideal in this study. On average, Galia melons should produce from 3-5 marketable fruit per plant under ideal conditions (Shaw et al. 2012) and yields would range from 20,400 to 34,000 fruit per acre at 2.4 m between- row spacing and 0.6 m in-row spacing (Kemball 2011). Yields in 2010 ranged from 9,773 to 15,675 marketable fruit per hectare and 5,835 to 13,613 marketable fruit per hectare in 2011. The main reasons for unmarketable fruit were sunscald, poor pollination/deformed fruit, soft rots, animal feeding, excessive scarring due to cucumber beetle feeding, or lesions and water soaked spots indicating disease. Yield may also have been affected by slow uptake of organic nutrients and nitrogen deficiency. Yields were lower on muskmelon when grown organically with composted cotton trash for fertility versus a commercial fertilizer (Brosius et al. 1998). The variety 'Diplomat' was chosen for powdery mildew resistance. Muskmelons are very susceptible to diseases including gummy stem blight, anthracnose, downy mildew and fusarium wilt. Fungicides were not used in this study due to

potential interactions that may have occurred with the biopesticides, and disease lowered yields.

Due to their higher sensitivity to environmental conditions, biopesticides may be less field-stable than synthetic conventional pesticides, and efforts to increase field stability will improve these products (Eyheraguibe et al. 2010). Environmental factors such as sunlight, rainfall, temperature and humidity can impact the viability of fungal propagules, and ideal conditions may differ on the leaf surface microhabitat versus the macrohabitat (Jaronski 2009). Besides abiotic factors, the ecological interactions of entomopathogenic fungi in managed cropping systems and the ability to infect insect pests is a complicated process that is not well understood (Hesketh et al. 2009; Roy et al. 2010; Vega et al. 2009). Applied research to investigate the practicality of using these pathogens in agricultural pest management strategies focuses on the effective formulation and dispersal of infective propagules in the field and whether this is economically feasible (Jackson et al. 2009; Wraight and Carruthers 1999). Economic constraints were listed as a major obstacle in using dry mycelia particles of *B. bassiana* to manage corn rootworm larvae (*D. undecimpunctata howardi*) in corn, as large quantities of propagules were required to infect larvae in corn fields (Krueger and Roberts 1997).

This study failed to provide evidence that *B. bassiana* strain GHA and *C. substugae* strain PRAA4-I are effective in reducing cucumber beetle populations in the field under the specific conditions used. This low effectivity may be due to low field persistence and/or low efficacy on diabroticite beetles. In addition, it is possible that low populations of beetles may have limited

detection of effective control. Additional controlled laboratory and greenhouse studies on these substances will help determine efficacy on cucumber beetles.

Chapter 3

Efficacy of entomopathogenic bacteria *Chromobacterium subtsugae* and fungi *Beauveria bassiana* and *Isaria fumosorosea* as biological control agents of cucumber beetles (Coleoptera: Chrysomelidae)

3.1 Introduction

The development and production of botanical and microbial-based biopesticides is a growing industry (Thakore 2006). New pest management products are important as novel modes of action allow for a more complex pesticide rotation regime that will discourage pest resistance (Tabashnik 1989). Additionally, biopesticides are appealing to both organic and conventional producers as they have a shorter pre-harvest interval and may have a lower environmental impact and be safer than synthetic conventional pesticides (Chandler et al. 2008; Lacey et al. 2001; Rimando and Duke 2006; Thakore 2006). Biopesticides may be made from living organisms such as fungi, bacteria or the substances that they produce (Chandler et al. 2008; Copping and Menn 2000). Plants may also produce toxic compounds. Common examples of effective biopesticides include pyrethrums, extracted from *Chrysanthemum cinerariifolium,* oils from the neem tree (*Azadirachta indica)*, spinosad from *Saccharopolyspora spinosa*, a soil bacterium; and the Cry toxins from the bacterium *Bacillus thuringiensis* (Bt). These organisms and insecticidal molecules have been developed into different products that are used widely on organic farms and in residential landscapes. The continued discovery of novel biopesticides can help improve pest management programs for difficult-to-manage pests in a variety of settings. In order to be effective in pest management, biopesticides should reduce survival of the target

insect pest or prevent herbivory. Some disadvantages of biopesticides include low field persistence, little “knock-down” effect, host specificity (too broad or narrow) and economic constraints (Lacey et al. 2001).

Diabroticite beetles are considered one of the most damaging agricultural pests in the U.S. in both the larval and adult stages. They are destructive pests of cucurbit and other crops as adults, when they chew leaves, stems and fruit and can vector bacterial wilt disease caused by *Erwinia tracheiphila* (Ellers-Kirk and Fleischer 2006; Leach 1964). Larvae of the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*, are known as the southern corn rootworm, and can cause significant damage to roots of corn, peanuts and other vegetable crops (Campbell and Emery 1967). The striped cucumber beetle (*Acalymma vittata*) is considered a cucurbit specialist (Bach 1980; Ellers-Kirk and Fleischer 2006), and larvae only develop on cucurbit roots (Bach 1980; Smyth and Hoffman 2003). Cucumber beetles are difficult to manage in both organic and conventional systems. In conventional systems, cucumber beetles are primarily managed using pyrethroid, neonicotinoid and carbamate pesticides (Kemball 2011), while *Diabrotica* spp. larvae were managed by soil-applied organophosphate and pyrethroid pesticides and more recently by the use of transgenic corn expressing Bt toxins (Moellenbeck et al. 2001). Organic growers cannot use traditional pesticides to manage cucumber beetles. Techniques in organic systems are focused on prevention and cultural controls, such as the use of reflective mulches, row covers, companion planting, crop rotation and trap cropping (Andino et al. 2004; Cline et al. 2008; Caldwell et al. 1999; Platt et al. 1999; Santos et al. 1995). Row covers show good promise as they provide a physical barrier to prevent cucumber beetle feeding; but covers need to be removed at

flowering for pollination. This system may create a microclimate that can be beneficial to microbial-based biopesticides by increasing humidity and filtering sunlight. The economic relevance, need for organic management tools, and risk of evolved resistance in conventional systems highlight the importance of finding effective biopesticides against *Diabrotica* spp.

Research is needed to identify organisms that have the potential to be developed into biopesticides against *Diabrotica* spp., to determine efficacy via laboratory and field tests, and to increase shelf life and activity. Currently, there is a lack of information substantiating efficacy of three entomopathogenic microbes with potential for activity against *Diabrotica* spp.: *B. bassiana*, *C. substsugae* and *I. fumosorosea.*

Chromobacterium subtsugae is a motile, gram-negative violet-pigmented bacterium associated with soil and water. The strain PRAA4-1 of *C. subtsugae* displays oral toxicity to adult and larvae of the spotted cucumber beetle (*Diabrotica undecimpunctata howardi*), Colorado potato beetle (*Leptinotarsa decemlineata*), larvae of the small hive beetle (*Aethina tumida*), adults and nymphs of the sweet potato whitefly (*Bemisia tabaci*), and southern green stink bug (*Nezara viridula*) (Martin et al. 2004, 2007). This strain has been developed into a broad spectrum biopesticide (Grandevo™ WP, 30% AI, Marrone Bio Innovations; Davis, CA), labeled for psyllids, thrips, mealybugs, leaf miners, stinkbugs, lygus bugs, leaf beetles, white grubs, armyworms and other pests in vegetable crops, fruits and ornamentals.

Both *B. bassiana* and *I. fumosorosea* (formerly *Paecilomyces fumosorosea*) are entomopathogenic fungi that have been developed into biopesticides. While *B. bassiana* is a well-known fungus with a broad host range, less information exists on *I. fumosorosea.* Labels of *B. bassiana* that are approved include Mycotrol O (Laverlam International Corporation, Butte,

MT) and Naturalis L (Troy Biosciences Inc., Pine Level, NC), which are labeled for control of aphids, thrips and whiteflies; leaf feeding beetles such as cucumber beetles, Japanese beetles, Colorado potato beetles, and flea beetles; plant bugs and borers in field, agronomic, vegetable and orchard crops as well as greenhouses. In comparison, *I. fumosorosea* has been isolated from a variety of insect hosts including Colorado potato beetle, elm leaf beetle, aphids, thrips and whiteflies among others (Zimmerman 2008). Two current labels for products containing *I. fumosorosea* presently exist: PFR-97 20% WDG (Apopka strain 97, 20% AI, Certis, Columbia, MD), for the management of a variety of insects and mites in vegetables, fruits and other food crops; and Preferal (Apopka strain 97, 20% AI, SePRO, Carmel, IN) labeled for insect and mite pests on vegetables, fruits and ornamental plants grown in greenhouses or nurseries.

The objective of our project was to test the efficacy of *C. subtsugae*, *B. bassiana*, and *I. fumosorosea* on spotted cucumber beetles in the laboratory by measuring mortality, feeding activity and mycosis. Field trials of *I. fumosorosea* were included to determine effects on spotted and striped cucumber beetles in the field based on yield data of melon host plants and beetle populations.

3.2 Materials and Methods: laboratory bioassays

Newly eclosed adult spotted cucumber beetles (*D. undecimpuncata*) were purchased from a laboratory reared colony (French Agricultural Research, Inc., Lamberton, MN) In all assays, beetles were used within one week of delivery.

Melons are a host plant of spotted cucumber beetles, and adults readily fed on Galia melon leaf disks in preliminary trials. Untreated melon seeds (‘Diplomat’ Galia F1, Johnny’s

Selected Seeds, Winslow, ME) were sown in McEnroe's Premium Lite Growing Mix (McEnroe Organic Farm, Millerton, NY) in 4.5-inch pots in the greenhouse. The greenhouse temperature settings were 18°C/21°C night/day with a photoperiod of 16:8. For bioassays, fresh leaves were harvested from pre-flowering plants (approximately 3-4 weeks old) and cut into 5.5 cm diameter leaf disks. The leaf disks were surface sterilized in 10% Clorox/sterile water solution for 2 minutes. In all assays, melon leaf disks were added to sterile, moistened filter paper in Petri dishes (15 mm x 100 mm, Fisher Scientific, Pittsburgh, PA). Two beetles were added to each Petri dish prior to spraying. Each leaf disk was then sprayed three times (to runoff) with an aerosol sprayer (Nalgene Nunc International, Rochester, NY). All dishes were sealed with Parafilm and incubated at 23°C, photoperiod 16:8 L:D. Beetle mortality and percent leaf area consumed were assessed at 24 hour intervals. Percent leaf area consumed was rated using a modified Horsfall-Barratt scale, a quantitative grading system for measuring plant disease symptoms (Horsfall and Barratt 1945), and can be modified to measure herbivory by insects (Elle et al. 1999)

Beetle cadavers from the *B. bassiana* and *I. fumosorosea* assays were collected and saved to determine infection. Cadavers were surface sterilized in a 10% sodium hypochlorite (Clorox, 5.2%) solution for two minutes and plated onto Sabouraud dextrose agar (SDA) (Fisher Scientific, Pittsburgh, PA). The agar was prepared by adding 52 g of SDA per 800 mL sterile water and adding 4 mL chloramphenicol antibiotic and 8 µL Danitol 2.4 EC to manage mites (Valent BioSciences, Walnut Creek, CA). After two weeks, fungal colonies were observed and identified. Pure colonies were saved for later testing.

Bioassays were analyzed as a completely randomized design with repeated measures and sampling. Data were non-parametric and analyzed using PROC GLIMMIX (Generalized Linear Mixed Models) ANOVA with a Wilcoxon Rank Sum Test. Arithmetic means with standard error are reported. A Fisher's protected LSD (Least Significant Difference) test was used to determine differences between means (SAS 9.1, Cary, NC).

The strains and formulated product used in this study were chosen based on reports of efficacy (Avery 2008; Martin 2007), cultures collected from diabroticite hosts (USDA ARSEF catalogue), and the pesticide label (Mycotrol O). The procedure for each assay is described by organism in the sections below.

3.2.1 Lab bioassays: Chromobacterium subtsugae

The *C. subtsugae* product was obtained as a liquid formulation (strain PRAA4-I, MBI 203, 94.5% AI, Marrone Bio Innovations, Davis, CA). Assays consisted of equal numbers of sterile water control treatments, 1X treatment (5 mL of product to 100 mL sterile water) and 2X treatments of MBI 203 (10 mL of product to 100 mL sterile water). Bioassays ran for 96 hours to measure toxicity. Four replicate experiments were performed with the liquid formulation. Each experiment contained equal numbers of control and treatment dishes (experiment one: n=100; experiment two: n=64; experiment three: n=34; experiment four: n=80).

An assay (37 Petri dishes per treatment) was performed using the newly labeled wettable powder formulation of *C. subtsugae* (Grandevo WP, 30% AI, Marrone Bio Innovations; Davis, CA) to compare to the liquid formulation. Rates used were 100 mg in 100 mL sterile

water (1X) and 300 mg in 100 mL sterile water (3X). All else was the same as described for the liquid formulation assays. This assay was not repeated.

3.2.2 Lab bioassays: Beauveria bassiana

Strain 11-98 was obtained from colonies from the laboratory of Dr. Bonnie H. Ownley at the University of Tennessee. To prepare the spray, 0.8 g of conidia from 8-10-week old colonies were added to 100 mL sterile water with 25 µL of Tween 20 as surfactant (Sigma Aldrich, St. Louis, MO), and mixed with a vortex for approximately 1 min to suspend. A ten-fold dilution series was performed at the time of assays to determine the rate as 7.6 x 10^6 colony forming units (CFUs)/mL. Two replicates of the experiment were performed with this strain, containing 50 plates per treatment per replicate and equal numbers of sterile water control plates.

The commercial formulation Mycotrol O (Strain GHA, 10.9% AI, Laverlam International, Butte, MT) was purchased from Arbico Organics (Oro Valley, AZ) and an assay was completed using 35 Petri dishes per treatment. The Mycotrol O assays consisted of sterile water control treatments and 1X treatment (0.5 mL in 50 mL sterile water) on melon leaf disks. All assays ended at 144 hours to allow time for mycosis, with newly sprayed leaves given at 96 hours to account for leaf degradation and consumption using the same population of beetles introduced at the start of the assay. This assay was not repeated.

3.2.3 Lab bioassays: Isaria fumosorosea

Two different strains of *I. fumosorosea* were used in laboratory assays: strain 3581 and strain 1506. Dry blastospores of strain 3581 packed in diatomaceous earth were obtained from Dr. Mark Jackson, from the USDA-ARS (Peoria, IL). For the *I. fumosorosea* strain 3581 assays, 0.5 g

of blastospore/diatomaceous earth were weighed and added to 50 mL of sterile water. The solution was left for 15 minutes to settle at room temperature. The top layer containing the suspended blastospores was pipetted into an aerosol sprayer and applied to the melon leaf disks as described. Tween surfactant was not used, as blastospores are hydrophilic and suspend readily in water. Two replicates were prepared with this strain, and included 50 Petri dishes of sterile water control leaves and 50 dishes of blastopore sprayed leaves for each replicate. A ten-fold dilution series was performed at the time of assays and the rate was determined as 3.7 x 10^5 CFUs/mL. The assays ran for 144 hours to allow time for mycosis, with newly sprayed leaves given at 96 hours to account for leaf degradation and consumption using the same population of beetles introduced at the start of the assay.

A colony of strain 1506 was obtained from Dr. Richard Humber at the USDA ARS collection of entomopathogenic fungal cultures (Ithaca, NY) and transfers were made using SDA agar prepared as per the procedure described. For the *I. fumosorosea* strain 1506 assays, 0.5 g of conidia from 8-10-week old colonies were added to 50 mL sterile water with a drop of Tween 20 surfactant and mixed with a vortex to suspend. Three replicates were done with this strain, with 55, 59 and 51 Petri dishes per replicate for both the sterile water control and 1506 treatments. A ten-fold dilution series determined the rate as 6.75 x 10^7 CFUs/mL. The assays ran for 144 hours with newly sprayed leaves given at 96 hours to account for leaf degradation and consumption using the same population of beetles introduced at the start of the assay.

3.2.4 Field experiments

Isaria fumosorosea strain 3581 was tested in field trials on F_1 Galia muskmelons (*Cucumis melo*), in 2011 and 2012 at the Organic Crops Unit at the University of Tennessee in Knoxville, TN. The 2011 field trial experimental design is described in detail in Chapter 2. In 2011, four treatments were applied to melon plants: *I. fumosorosea* strain 3581 (6.67 g blastospores in 667 mL water with 0.5 mL sticker (Nu-Film-P, 96% AI, Miller Chemical & Fertilizer Corporation, Hanover, PA); *I. fumosorosea* strain 3581 with AG-19 row cover (Agribon, Polymer Group Inc., Charlotte, NC); row cover alone, and a water control. The same four treatments were repeated in 2012, with the addition of a 2X rate of *I. fumosorosea* strain 3581 (13.34 g blastospores in 667 mL water), and a rotation between *I. fumosorosea* strain 3581 and *C. subtsugae* (Grandevo 1X rate, 4.16 g in 667 mL water). No surfactant was used in 2012 as it was unnecessary for blastospore suspension and caused minor phytotoxic symptoms on melon plants. Field activities for 2011 and 2012 are listed in Table 3.1. The field trials were designed as a randomized block replicated four times. Fruit yield was factored on a per plant basis and cucumber beetles were counted on two whole-plant samples per plot in both years. Yield data were analyzed using PROC GLIMMIX and log transformed or analyzed non-parametrically if the assumptions of equal variance could not be met. Beetle counts were fitted to a Poisson distribution and analysis was done using PROC GLIMMIX.

Table 3.1 Schedule of activities for field trials conducted in 2011 and 2012 at the University of Tennessee Organic Crops Unit in Knoxville, TN.

	Year	
Activity	**2011**	**2012**
Melon seeds sown in the greenhouse	13 May	14 May
Cover crops flail mowed	23 May	NA
Field spaded	23 May	7 June
Dripline and plastic mulch laid	17 June	7 June
Melons transplanted and row covers added	17 June	7 June
Row covers removed	11 July	9 July
Insect scout dates	27 June; 5, 11, 18, 25 July;	18, 25 June; 2, 9, 16, 24, 30 July;
	1, 8, 15, 22 Aug.	6, 13 Aug.
Spray dates	28 June; 5, 12, 19, 26 July;	22, 27 June; 3, 13, 25 July;
	2, 9, 16 Aug.	1, 8 Aug.
Harvests	5, 8, 10, 15, 20, 22, 25 Aug.	16, 20, 24 July; 2, 6 Aug.

[z]cover crops not used in 201

3.4 Results

The results of this study show that *C. subtsugae*, *B. bassiana* and *I. fumosorosea* were not effective at killing cucumber beetles in the laboratory. Percent mortality did not differ compared to the control regardless of treatment (Tables 3.2 to 3.6). MBI 203 resulted in reduced leaf area consumed at the 2X rate, yet the 3X rate of Grandevo did not result in reduced feeding in 96 hour assays (Table 3.2). The Mycotrol O product and *Beauveria bassiana* strain 11-98 showed reduced leaf area consumed (Tables 3.3 and 3.4). There were no treatment effects on leaf area consumed in the *I. fumosorosea* strain 3581 and 1506 assays (Tables 3.5 and 3.6).

A high percentage of mycosis was confirmed (53 to 86%) from the beetle cadavers collected from the fungal treatments showing that these strains of *B. bassiana* and *I. fumosorosea* are able to infect *D. undecimpunctata howardi* adults under controlled laboratory conditions (Table 3.7). However, infection did not result in reduced feeding or mortality within the 144 hour period. Mycosis was determined from beetle cadavers from the sterile water control treatments, indicating contamination of the control beetles (Table 3.7). Applications of *C. subtsugae* are not expected to result in mycosis due to the formulation of the product, so were not included in Table 3.7.

The 2011 field study on *I. fumosorosea* showed no difference in either *A. vittata* or *D. undecimpunctata howardi* as the water or row cover control plots (Table 3.8), and yield was unaffected (Table 3.9). Results for the field trials on *C. subtsugae* and *B. bassiana* were similar and are included in Chapter 2 Table 6. In the 2012 field study on *I. fumosorosea* (1X and 2X

rates) and the *C. subtsugae*/*I. fumosorosea* rotation, there was no difference in their control of cucumber beetle populations (Table 3.10) or melon yield (Table 3.11) versus the controls.

Table 3.2 Mortality of adult *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Chromobacterium subtsugae* (MBI 203 and Grandevo) during a 96-hour assay

		Mortality							
		Evaluation Period (hour)							
		24		48		72		96	
Treatment	**Total # of beetles**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**
Control (sterile water)	358	15	4	29	8	46	13	46	13
MBI 203 1X	362	29	8	34	9	41	12	61	17
MBI 203 2X	262	22	8	37	14	39	14	49	18
Grandevo 3X	74	4	5	3	4	6	8	8	11
Chi-Square *Pr <= P*		*NS*[z]		*0.0407*		*NS*		*NS*	

[z]Not significant at α =0.05

Table 3.3 Mortality of adult *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Beauveria bassiana* (Mycotrol O strain GHA) during a 144-hour assay

		Mortality											
		Evaluation Period (hour)[z]											
		24		48		72		96		120		144	
Treatment	**Total # of beetles**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**
Control (sterile water)	70	0	0	4	6	4	6	8	11	8	11	10	14
B. bassiana (Mycotrol O)	70	1	1	5	7	5	7	8	11	13	19	17	24
Chi-Square *Pr <= P*		*NS*[y]		*NS*		*NS*		*NS*		*NS*		0.0106	

[z]Newly sprayed leaves given at 96 h

[y]Not significant at α =0.05

Table 3.4 Mortality of adult *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Beauveria bassiana* (strain 11-98) during a 144-hour assay

		Mortality											
		Evaluation Period (hour)[z]											
		24		48		72		96		120		144	
Treatment	**Total # of beetles**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**
Control (sterile water)	96	2	2	3	3	7	7	10	10	9	9	9	9
B. bassiana (strain 11-98)	100	3	3	3	3	2	2	9	9	9	9	12	12
Chi-Square *Pr <= P*		*NS*[y]		*NS*		*NS*		*NS*		*NS*		*NS*	

[z]Newly sprayed leaves given at 96 h

[y]Not significant at α =0.05

Table 3.5 Mortality of adult *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Isaria fumosorosea* (strain 3581) in a 144-hour assay to measure infection and mycosis

		Mortality											
		Evaluation Period (hour)[z]											
		24		48		72		96		120		144	
Treatment	**Total # of beetles**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**
Control (sterile water)	145	5	3	15	10	35	24	49	34	82	57	93	64
Isaria fumosorosea (strain 3581)	142	6	4	27	19	27	19	44	31	64	45	90	63
Chi-Square *Pr <= P*		*NS*[y]		*NS*		*NS*		*NS*		*NS*		*NS*	

[z]Newly sprayed leaves given at 96 h

[y]Not significant at α =0.05

Table 3.6 Mortality of adult *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Isaria fumosorosea* (strain 1506) during a 144-hour assay

		Mortality											
		Evaluation Period (hour)[z]											
		24		48		72		96		120		144	
Treatment	**Total # of beetles**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**	**#**	**%**
Control (sterile water)	330	8	2	25	8	73	22	79	22	100	30	109	33
Isaria fumosorosea (strain 1506)	338	7	2	15	4	44	13	88	26	96	28	109	32
Chi-Square *Pr <= P*		*NS*[y]		*NS*		*0.0056*		*NS*		*NS*		*NS*	

[z]Newly sprayed leaves given at 96 h

[y]Not significant at α =0.05

Table 3.7 Percent of beetle cadavers plated onto SDA agar and percent of cadavers exhibiting fungal infection by *Beauveria bassiana* and *Isaria fumosorosea* at the end of assays

Treatment	Cadavers plated/total cadavers (%)	Mycosis of plated cadavers (%)
B. bassiana (Mycotrol O)	16/17 (46%)	81
B. bassiana (Mycotrol O) CONTROL PLATES	NA	NA
B. bassiana (strain 11-98)	12/12 (100%)	86
B. bassiana (strain 11-98) CONTROL PLATES	9/9 (100%)	17
I. fumosorosea (strain 3581)	37/90 (41%)	81
I. fumosorosea (strain 3581) CONTROL PLATES	34/93 (37%)	79
I. fumosorosea (strain 1506)	61/109 (56%)	53
I. fumosorosea (strain 1506) CONTROL PLATES	57/109 (52%)	54

Table 3.8 *Acalymma vittata* and *Diabrotica undecimpunctata howardi* per plant on Galia melons treated with *Isaria fumosorosea* strain 3581 across eight sampling dates in 2011

Treatment	Both species combined*	*A. vittata**	*D. undecimpunctata howardi**
Control (water)	1.0 ± 1.0	0.8 ± 1.0	0.2 ± 0.4
Control (row cover)	1.1 ± 1.7	0.8 ± 1.4	0.3 ± 0.6
I. fumosorosea (strain 3581)	1.1 ± 1.2	0.8 ± 1.1	0.2 ± 0.6
I. fumosorosea (strain 3581) + row cover	0.9 ± 1.0	0.6 ± 1.1	0.2 ± 0.5
F; d,f; p	*0.46; 3,13; 0.7128*	*0.62; 3,14; 0.6141*	*0.32; 3,11; 0.8110*

*Values are untransformed means ± standard error

Table 3.9 Yields and average fruit weight (lb) of Galia melons treated with treated with *Isaria fumosorosea* strain 3581 in 2011

Treatment	Marketable fruit number per acre*+	Total fruit number per acre*+	Mean marketable fruit wt (lb)
Control (water)	5082 ± 3177	12385 ± 6028	3.9 ± 1.3
Control (row cover)	5349 ± 3704	12801 ± 10293	4.1 ± 0.8
I. fumosorosea (strain 3581)	3086 ± 3177	9075 ± 3994	3.5 ± 0.5
I. fumosorosea (strain 3581) + row cover	3449 ± 4509	9438 ± 37998	3.3 ± 0.6
F; d,f; p	*2.86; 3,9; 0.0969*	*0.50; 3,9; 0.6944*	*2.14; 3,9; 0.1655*

*Based on a population of 3630 plants per acre; +Values are untransformed means ± standard deviation

Table 3.10 *Acalymma vittata* and *Diabrotica undecimpunctata howardi* per plant on Galia melons treated with *Isaria fumosorosea* strain 3581 and *Chromobacterium subtsugae* across eight sampling dates in 2012

Treatment	Both species combined*	*A. vittata**	*D. undecimpunctata howardi**
Control (water)	2.1 ± 02.3	1.9 ± 2.0	0.2 ± 0.7
Control (row cover)	1.7 ± 2.0	1.5 ± 2.0	0.1 ± 0.4
I. fumosorosea (strain 3581) 1X	2.5 ± 2.2	2.3 ± 2.1	0.2 ± 0.4
I. fumosorosea (strain 3581) 2X	2.1 ± 1.8	1.9 ± 1.7	0.2 ± 0.5
Chromobacterium substsugae alternated with *I. fumosorosea* (strain 3581)	1.9 ± 2.3	1.6 ± 2.2	0.2 ± 0.5
I. fumosorosea (strain 3581) + row cover	1.4 ± 1.8	1.3 ± 1.8	0.1 ± 0.2
F; d,f; p	*2.42; 5,16; 0.0813*	*2.20; 5,17; 0.1027*	*1.86; 5,26; 0.1365*

*Values are untransformed means ± standard deviation

Table 3.11 Yields and average fruit weight (lb) of Galia melons treated with *Isaria fumosorosea* strain 3581 and *Chromobacterium subtsugae* in 2012

Treatment	Marketable fruit number per acre[*+]	Total fruit number per acre[*+]	Mean marketable fruit wt (lb)
Control (water)	9075 ± 6504	17606 ± 8835	3.3 ± 1.6
Control (row cover)	4175 ± 5682	11253 ± 9633	3.1 ± 1.1
I. fumosorosea (strain 3581) 1X	7079 ± 4160	17061 ± 7270	3.2 ±0.8
I. fumosorosea (strain 3581) 2X	9075 ± 5201	13613 ± 7437	3.4 ± 1.3
Chromobacterium substsugae alternated with *I. fumosorosea* (strain 3581)	8349 ± 6877	15246 ± 9611	3.5 ± 1.0
I. fumosorosea (strain 3581) + row cover	4175 ± 5682	11253 ± 8835	3.1 ± 1.1
F; d,f; p	*1.04; 5,15; 0.4286*	*1.20; 5,15; 0.3548*	*1.37; 5,15; 0.2895*

[*] Based on a population of 3630 plants per acre

[+]Values are untransformed means ± standard deviation

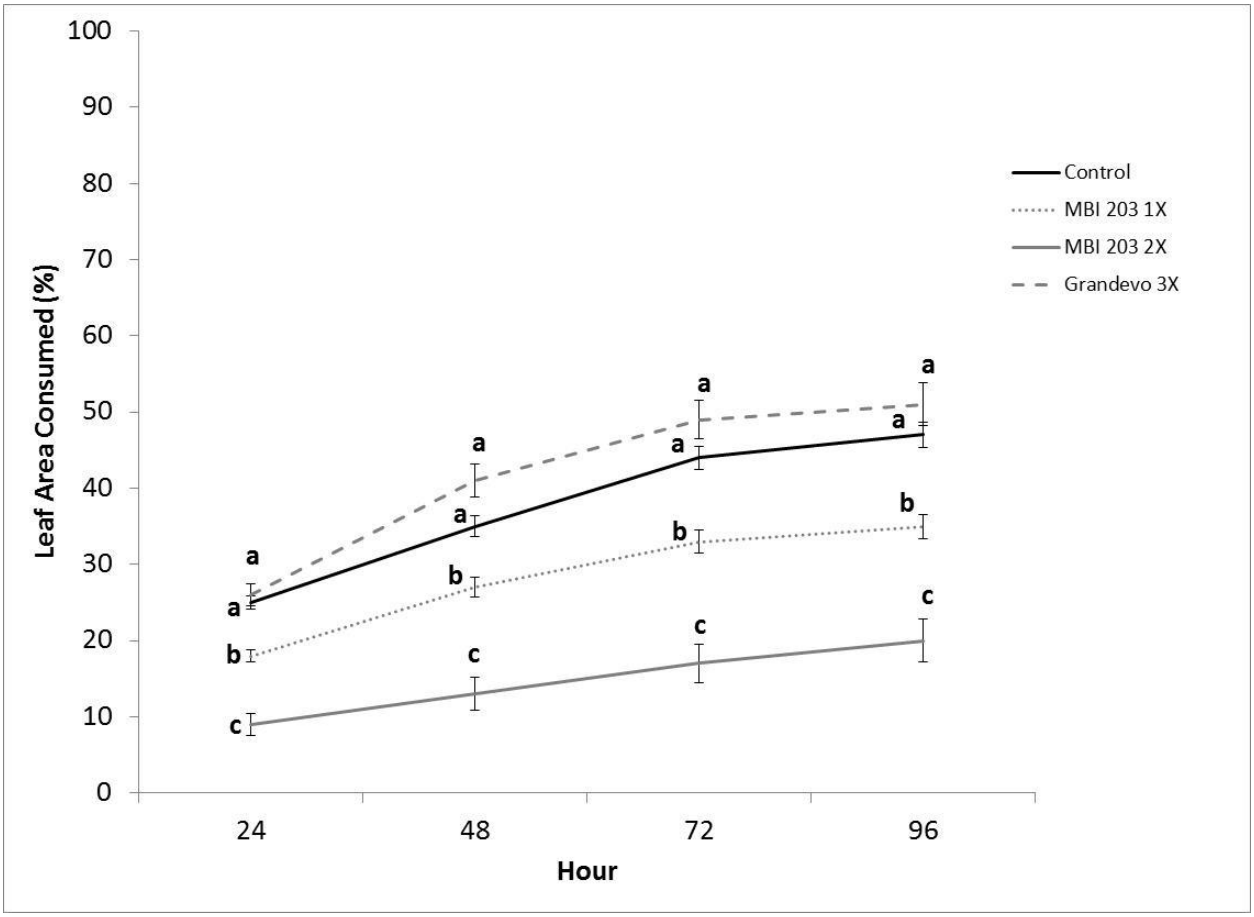

Fig. 3.1 Percent leaf area consumed by *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Chromobacterium subtsugae* (MBI 203 and Grandevo) in 96-hour assay to determine acute toxicity. Different letters indicate differences between means at α = 0.05.

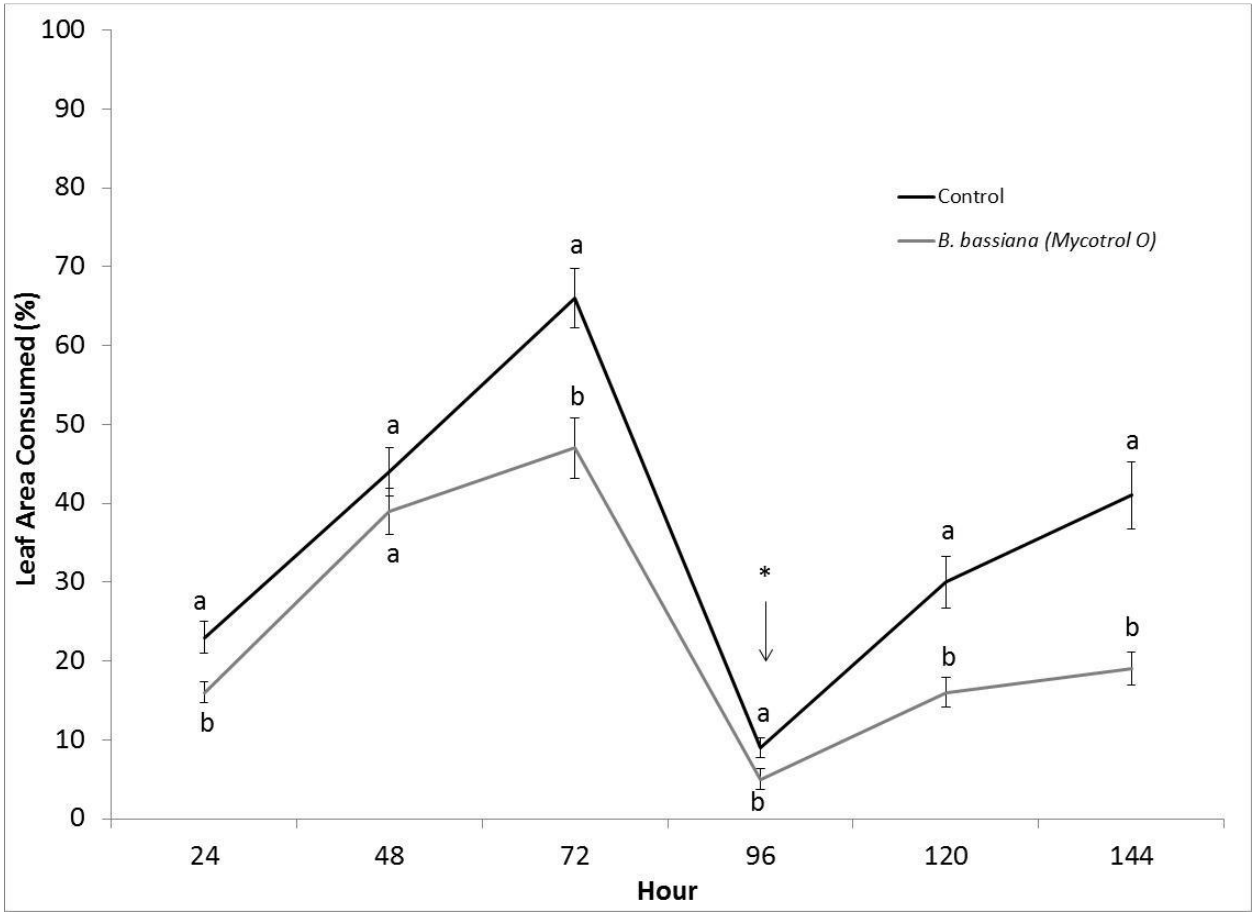

Fig. 3.2 Percent leaf area consumed by *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Beauveria bassiana* (Mycotrol O strain GHA) in a 144-hour assay to measure infection and mycosis. Different letters indicate differences between means at α = 0.05.

*Newly sprayed leaves given at 96 hours to account for leaf degradation and consumption.

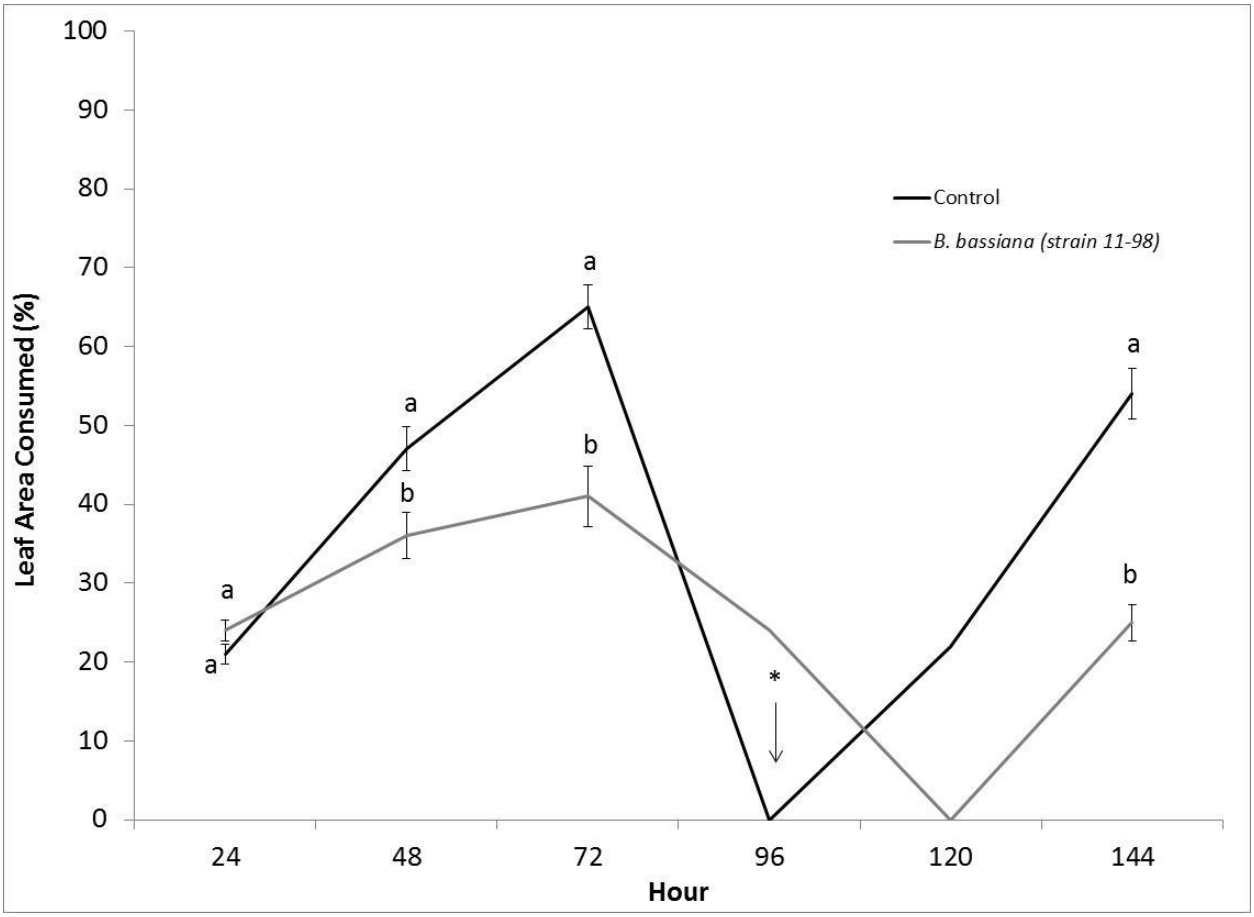

Fig. 3.3 Percent leaf area consumed by *Diabrotica undecimpunctata howardi* on Galia melon leaves treated with *Beauveria bassiana* (strain 11-98) in a 144-hour assay to measure infection and mycosis. Different letters indicate differences between means at α = 0.05. *Newly sprayed leaves given at 96 hours to account for leaf degradation and consumption.

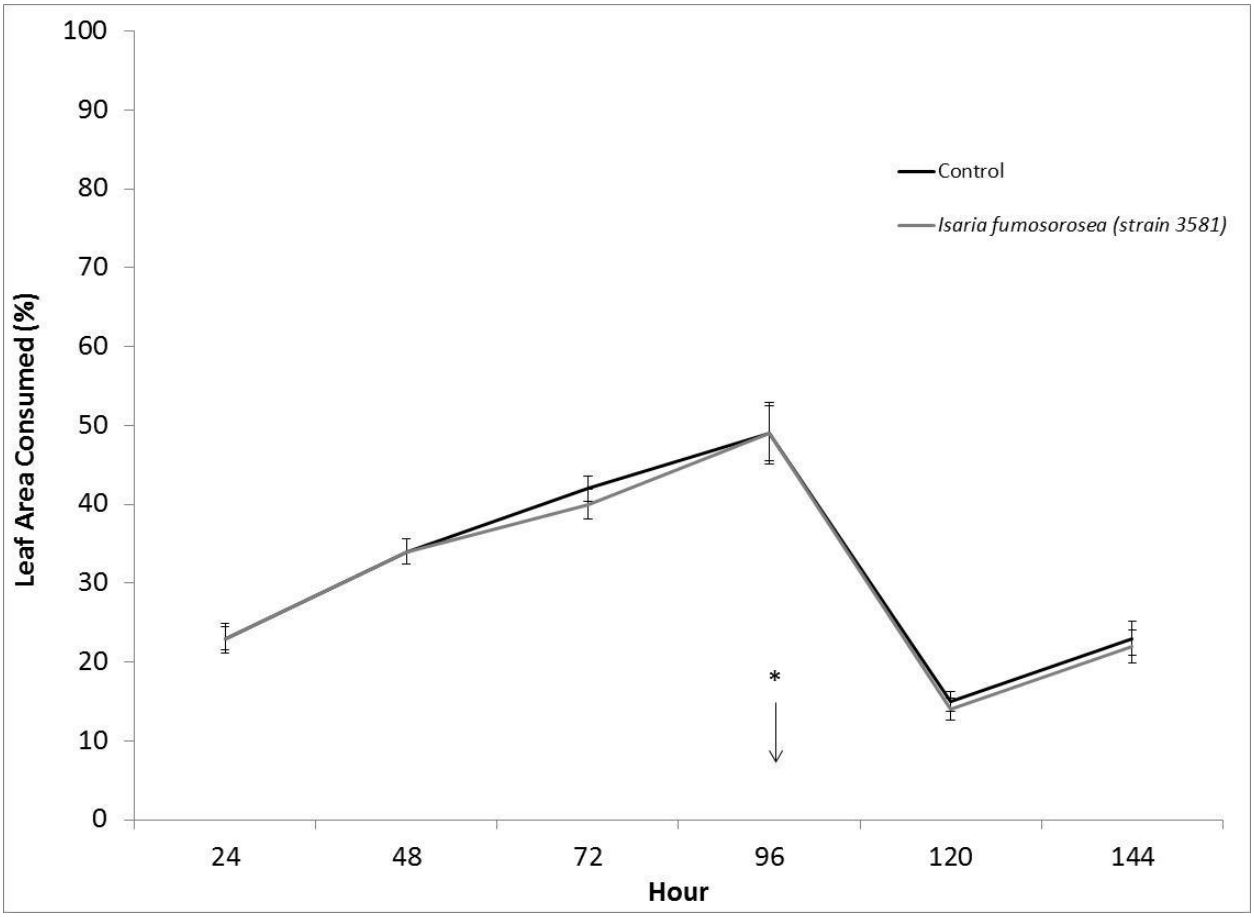

Fig. 3.4 Percent leaf area consumed by spotted cucumber beetles on Galia melon leaves treated with *Isaria fumosorosea* (strain 3581) in a 144-hour assay to measure infection and mycosis. *Newly sprayed leaves given at 96 hours to account for leaf degradation and consumption.

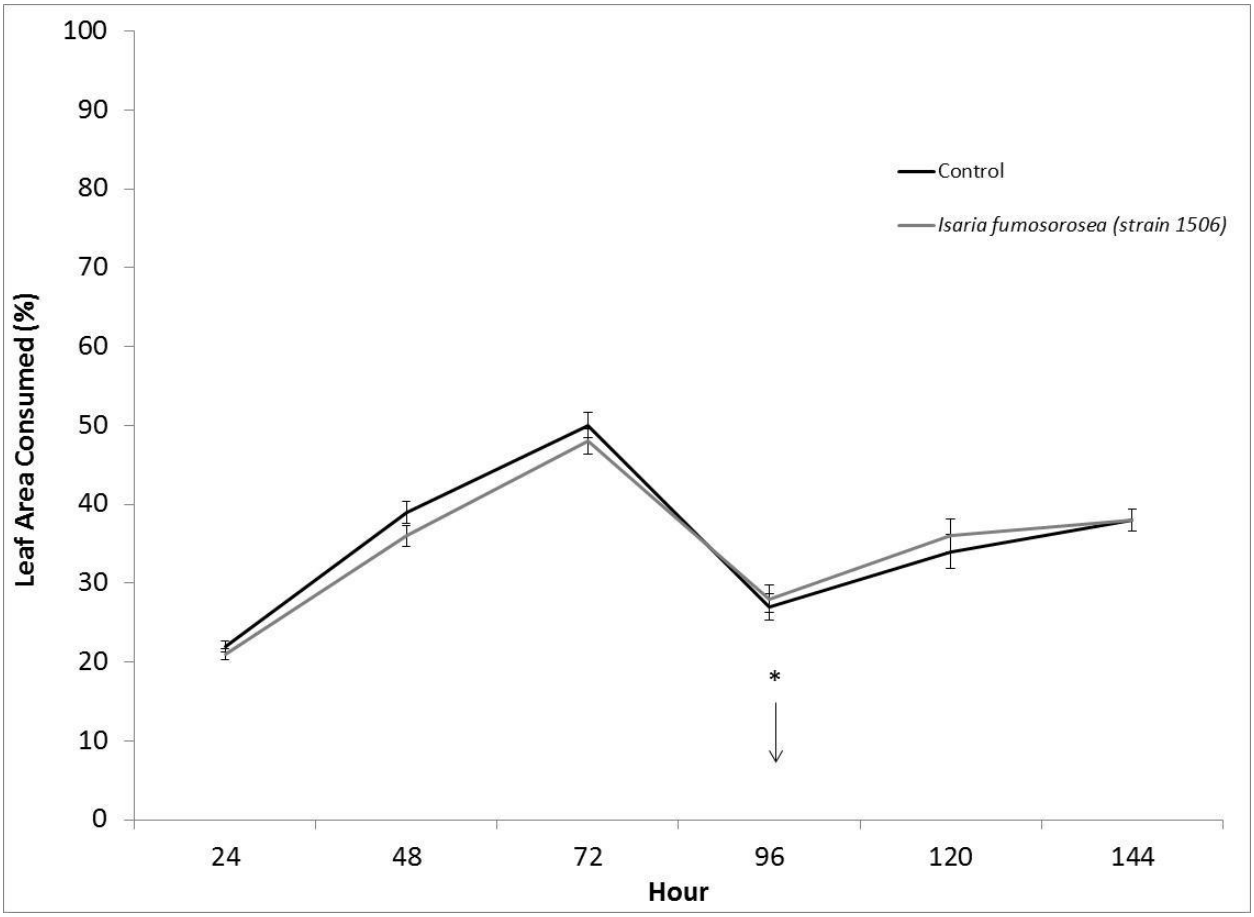

Fig. 3.5 Percent leaf area consumed by spotted cucumber beetles on Galia melon leaves treated with *Isaria fumosorosea (*strain 1506) in a 144-hour assay to measure infection and mycosis. *Newly sprayed leaves given at 96 hours to account for leaf degradation and consumption.

3.5 Discussion and Conclusion

Laboratory bioassays allow testing of new pesticides on target insects in a controlled environment. The assays performed in this experiment showed that under ideal temperatures and humidity, infection of *D. undecimpunctata howardi* is possible when *B. bassiana* and *I. fumosorosea* are sprayed directly on beetles and their food source. Both fungal organisms are naturally occurring in the soil and are found in many climates and habitats (De Faria and Wraight 2007; Zimmerman 2008). Larvae of *D. undecimpunctata howardi* survive in the soil, and *B. bassiana* and *I. fumosorosea* have broad host ranges and are closely associated with hosts that spend some of their life cycle in and around soil (Hesketh et al. 2010; Pell 2010). Therefore, it is not unexpected that these fungi are able to infect *D. undecimpunctata* in the soil, and infection by *B. bassiana* and *Metarhizium anisopliae* has been isolated on larvae (USDA ARSEF) and reported in the scientific literature (Krueger and Roberts 1997; Pereira and Roberts 1991). In fact, the GHA strain of *B. bassiana* from which Mycotrol O was developed, was originally isolated from larval host *D. undecimpunctata howardi*, and designated ARSEF 201 (Bradley et al. 1999). *Isaria fumosorosea* has been identified infecting adult *D. undecimpunctata howardi*, and two strains (CG170 and CG204) from Florida and Mexico were found to be highly infective on eggs of *D. speciosa* (Tigano-Milani et al. 1994).

In this study, infection of *I. fumosorosea* and *B. bassiana* strain 11-98 did not result in increased mortality or reduced leaf feeding when compared to control treatments, which may be due to constraints in the used protocol. For example, when adult *D. undecimpunctata howardi* were given different formulations of dry mycelium of *B. bassiana*, mortality ranged

from 68-100%, compared to only 47% for the control groups in 15-day laboratory assays (Pereira and Roberts 1991). However, the Mycotrol O and *B. bassiana* strain 11-98 used in this study contained infective spores (Mycotrol O contains 2 x 10^{10} viable spores per gram, and strain 11-98 was tested to have 7.6 x 10^{6} CFUs per mL); and it is expected that infection will occur more quickly in assays where conidia are used versus mycelia (Tanada and Kaya 1993). The CFU concentration for *I. fumosorosea* was 3.70 x 10^{5} and 6.75 x 10^{7} for strains 3581 and 1506, respectively, and is within the rates reported for infection to occur on third instar potato psyllid (*Bactericera cokerelli*), which had 83 to 97% mortality when exposed to 10^{5}, 10^{6} and 10^{7} conidia per mL of Pfr 97 (*Isaria fumosorosea* Apopka strain) (Lacey et al. 2009). There is little information on the effects of *I. fumosorosea* on adult beetles. This study is the first to show that *I. fumosorosea* strains 3581 and 1506 are able to infect adult *D. undecimpunctata howardi*. However, it is important to note that 55% of plated cadavers from the sterile water treatments were positive for mycosis. Adult beetles were obtained from laboratory colonies started from field collected beetles and may have been infected before the assays occurred. Molecular analysis would be needed to identify the cause of mycosis.

Chromobacterium subtsugae does not infect, but is toxic when ingested by susceptible insects. Initial laboratory toxicity tests of *C. subtsugae* showed decreased survival of both adult and larvae *D. undecimpunctata howardi* when ingesting freeze-dried corn rootworm diet in a 5-day laboratory assay, and feeding inhibition was listed as a sublethal effect of larvae, at the rate of 100 µL bacterial culture to 10 mL deionized water (Martin et al. 2007). Our study contrasts with Martin et al., showing no difference in adult survival, but does show an anti-feedant effect on adult *D. undecimpunctata* in 96-day assays.

Field studies are necessary to determine efficacy in the natural environment, but are more difficult to control than laboratory studies. The field studies in this work showed no treatment effects. Explanations for this could be environmental conditions such as temperature, sunlight and humidity affecting the activity of the microorganisms used. The organisms used in this study are naturally found in the soil environment, which is very different than the phyllosphere, and may have resulted in decreased persistence (Jaronski 2010). In particular, ultraviolet light is known to degrade fungal propagules (Braga et al. 2001) and adjuvants that act as sunscreens may help increase viability of conidia on the leaf surface (Behle et al. 2011). The insect life stage is also important, and the larval or nymphal stages of insects are often more susceptible to infection from pathogens than the adult stage, likely due to sclerotization and other protective layers of the exoskeleton (Tanada and Kaya 1993). Targeting larvae rather than adults of *D. undecimpunctata howardi* with these organisms may result in better suppression, due to overlapping habitats and physiological factors.

Chapter 4

Field efficacy of biopesticides for *Anasa tristis* (DeGeer) (Hemiptera: Coreidae), *Diabrotica undecimupunctata howadi* (Barber) and *Acalymma vittata* (Fabricius) (Coleoptera: Chrysomelidae) in organically grown pumpkin

4.1 Introduction

Pumpkin (*Curcubita* spp.) is a specialty crop grown primarily for processing and to a lesser extent, for the ornamental and agritourism industries in the U.S. (Geisler 2012). Pumpkin production has rapidly increased over the last 25 years and the farm value of the U.S. pumpkin crop was $170 million from 2004-06 (Lucier and Dettmann 2007). Organic pumpkin production is not widely practiced in the U.S., however markets are increasing for organic baby food, oil seed pumpkins and ornamental uses (Bachmann 2010; Bavec 2007; Delate 2003), and production may increase in response to growing demand for organic produce (Dimitri and Oberholtzer 2009). Pumpkins are difficult to grow organically due to disease and insect pressure and organic growers need novel pest and disease management tactics.

Insect pests may be damaging on pumpkin crops when grown organically on small acreages. Key insect pests on pumpkin include cucumber beetles/corn rootworms (*Diabrotica* spp.), squash vine borer (*Melitta curcurbitae)* and squash bug *(Anasa tristis*). Cucumber beetles vector bacterial wilt caused by *Erwinia tracheiphila*, which is a serious disease in cucumber and melon production, however pumpkins and squash are not as susceptible (Yao et al. 1996). Squash bugs are vectors of cucurbit yellow vine disease caused by bacterium *Serratia marcescens,* which was first observed in southern states of the U.S. in 1988 (Bruton et al. 2003)

and watermelon, cantaloupe, squash and pumpkins are susceptible (Pair et al. 2004). These diseases are prevented by managing the insect vectors, and growers need a variety of management tactics to accomplish this. Conventional growers may use pyrethroids, carbamates, and neonicotinoid pesticides to manage these pests (Kemball 2011), while management in organic systems is based on cultural control strategies including floating row covers, crop rotation, sanitation, intercropping and companion planting, lures and traps; or biological control strategies and chemical control using neem, *Bacillus thuringiensis* (Bt) and pyrethins (Cline et al. 2008; Delate et al. 2005; Jackson et al. 2005; Platt et al. 1999; Santos et al. 1995; Seaman 2012). Preventative techniques may not be effective in keeping pest populations under economic threshold levels once colonization occurs, and curative sprays have low residual activity and must be timed to target immature life stages. The economic threshold for cucumber beetles on pumpkins is five beetles per plant, for squash bugs is one egg mass per plant, and for squash vine borer it is as soon as larval feeding is detected (Brust et al. 1995).

Biopesticides are produced from naturally occurring organisms. They represent a growing industry and have potential for use in both conventional and organic production systems (Thakore 2006). The first step in the development of biopesticides for insect pest management is the screening of potential organisms and strains, followed by laboratory and field studies to verify efficacy (Shapiro-Ilan et al. 2012). *Beauveria bassiana* and *Isaria fumosorosea* are fungal entomopathogens that have been well studied for the development of biopesticides, and insects hosts are known (Vega et al. 2012, Zimmerman 2008), while *Chromobacterium substsugae* was only recently discovered and the host range is not well studied (Martin et al. 2007).

Beauveria bassiana is commercially available for cucumber beetle management in organic vegetable crops (Mycotrol O, Laverlam International Corporation, Butte, MT) and are reported effective in managing adult *Diabrotica* spp. in the field (Bruck and Lewis 2001). Furthermore, a number of *B. bassiana* isolates have been recovered from *Diabrotica* spp. hosts around the world (St Leger et al. 1992). *Beauveria bassiana* has not been reported on squash bugs, but has been recovered from true bugs in other heteropteran families, such as Lygaeidae, Miridae and Pentatomidae (St Leger et al. 1992).

Isaria fumosorosea (formerly *Paecilomyces fumosoroseus*) is less studied than *B. bassiana*, although the two are related and share similar characteristics (Zimmerman 2008). *Isaria fumosorosea* has been recovered from many arthropods including diabroticites *D. speciosa* (Tigano-Milani et al. 1995) and *A. vittata* (Avery 2008), and on heteropteran families Lygaedia, Miridae and Aradidae, but has not been reported on squash bugs (Zimmerman 2008).

Chromobacterium subtsugae is a novel bacterial-based biopesticide that has been recently labeled for a variety of pests in organic vegetable systems (Grandevo WP, 30% AI, Marrone Bio Innovations; Davis, CA), excluding cucumber beetles and squash bugs. However, *C. subtsugae* is reported to result in feeding inhibition and mortality on both larval and adult stages of *D. virgifera* and *D. undecimpunctata howardi* and the mortality of southern green stink bug (*Nezara viridula*) (Martin et al. 2007). Overall, the effects of these three organisms are not well studied on cucumber beetles and squash bugs, significant vegetable pests that vector disease in cucurbits.

The objective of this work was to examine the efficacy of *B. bassiana*, *I. fumosorosea* and *C. subtsugae* in the field for potential management of cucumber beetles and squash bugs for organic pumpkin production.

4.2 Materials and Methods

Field trials were performed in 2010, 2011 and 2012 at the Organic Crops Unit of the University of Tennessee in Knoxville, TN.

4.2.1 Field experiment 2010

Certified organic pumpkin seeds (*Cucurbita pepo* cv. 'Baby Pam') (Johnny's Selected Seeds, Winslow, ME) were sown in SunGro Sunshine Organic Blend Professional Growing Mix (Sun Gro Horticultural, Bellevue, WA) in plastic pots (11.4 cm in diameter)in the greenhouse. Seedlings were hand fertilized with 200 mL Rain Grow 4-2-3 (4N-0.87P-2.5K) liquid fertilizer (Oliver, BC, Canada) in 1 L water delivering 75 mL per pot. The greenhouse temperature settings were 18°C/ 21°C night/day with a photoperiod of 16:8. In the field, 0.57 hectares of a cowpea cover crop was flail mowed (Alamo SH74, Alamo Industrial, Seguin, TX) and tilled with a rotary tiller (Bush Hog, Selma, AL), and tilled again two weeks later to manage regrowth. The cowpea cover crop was estimated to provide 113 kg N/ha per acre, based on 3.5% N in aboveground biomass. Plastic mulch (0.9 m wide and 1-mil thick; Pliant Corp., Chippewa Falls, WI) and drip irrigation (10-mm thick, with emitters set every 30.5 cm to provide 59 L/h of water at 562.4 g/cm2 (Netafim, Tel Aviv, Israel) was laid in rows. Plants were set out in the field by hand on 16 Jun. Plots were 12 m long with 3 m between rows, and 1.2 m in-row spacing. There were ten plants per plot and four replicates. A 1.2 m wide strip of buckwheat was seeded on either side of the

plots with a light duty grain drill (Almaco, Nevada, IA) at the rate of 40 lbs per acre to encourage pollination and to serve as a buffer strip. Nature Safe course ground fertilizer (10N-1.7P-3.3K, Griffin Industries Inc., Cold Spring, KY) was side dressed to each transplant by hand at planting to deliver 25 kg of additional N per hectare.

The treatments were repeated weekly for six weeks. The spray treatments and schedule are listed in Tables 4.1 and 4.2. A backpack sprayer (Bellspray Inc., Opelousas, LA) with 2 kg CO_2 cylinder, 4.2 kg/cm2 regulator, and a 4-nozzle boom (48 cm spacing between nozzles) was used for all treatments. The biopesticide formulations were mixed in designated 2-L plastic bottles. The sprayer was calibrated to deliver 218 L/ha at walking speed, and the boom was held at 46 cm above the plant canopy. Spray applications began with the lowest concentration to the highest, and the sprayer was fully rinsed with water in between each treatment application.

Insect scouting was conducted weekly one day before spraying for a total of seven scouting dates. Squash bugs (*Anasa tristis*) adults, nymphs and egg masses, and cucumber beetle adults were counted on two random plants (20% of plants). Pumpkins were harvested was repeated weekly until 19 Aug, comprising a total of eight harvest dates. Total fruit per plot was sorted into marketable and unmarketable categories based on outward appearance, and counted and weighed on a per-pant basis. Plant mortality from vine borer or disease was recorded for each plot on the same days that insects were scouted.

4.2.2 Field experiments 2011 and 2010

The pumpkin variety was changed in 2011 and 2012 to a powdery mildew tolerant variety (*Cucurbita pepo* cv. 'Cannon Ball' F1 Osborne International Seed Co., Mount Vernon, WA). The

seeds (untreated) were sown in McEnroe's Premium Lite Growing Mix (McEnroe Organic Farm, Millerton, NY) in 50-cell plug trays in the greenhouse. The growing media contained compost and nutrients sufficient for growth and no additional fertilizers were used. Greenhouse conditions were the same as described in 2010. Field preparation was as described in 2010, except plant population was reduced to five plants per plot to compensate for increased treatments. Plants were side-dressed with 0.9 kg of soybean meal (7N-0.87P-0.83K, TN Farmers Co-op, LaVergne, TN) to deliver 90 kg of N/ha in 2011 and 2012. Insect scouting was conducted weekly as described in 2010. The complete schedule of field activities is listed in Table 4.1.

Table 4.1 Schedule of activities for field trials conducted in 2010, 2011 and 2012 at the University of Tennessee Organic Crops Unit in Knoxville, TN.

	Year		
Activity	**2010**	**2011**	**2012**
Pumpkin seeds sown in the greenhouse	18 May	13 May	14 May
Cover crops flail mowed	28 May	23 May	NA
Field spaded	28 May	23 May	7 June
Dripline and plastic mulch laid	14 June	17 June	7 June
Pumpkins transplanted and row covers added	16 June	17 June	7 June
Row covers removed	NA	11 July	9 July
Insect scout dates	29 June; 6, 13, 20, 27 July; 3, 10 Aug.	27 June; 5, 11, 18, 25 July; 1, 8, 15, 22 Aug.	18, 25 June; 2, 9, 16, 24, 30 July; 6, 13 Aug.
Spray dates	2, 7, 14, 22, 28 July; 4 Aug.	28 June; 5, 12, 19, 26 July; 2, 9, 16 Aug.	22, 27 June; 3, 13, 25 July; 1, 8 Aug.
Harvests	3, 5, 9, 12, 17, 23 Aug.	31 Aug.	27, 30 July; 6, 13 Aug.

Table 4.2 Spray treatments for experiments conducted in 2010, 2011 and 2012 at the University of Tennessee Organic Crops Unit in Knoxville, TN.

Treatment-Rate (trade name, AI and manufacturer)	Treatment notation	Year applied
Beauveria bassiana-1 qt/acre (strain GHA, Mycotrol O, 10.9% AI, Laverlam International, Butte, MT)	**B1X**	2010 2011
Beauveria bassiana-2 qt/acre (strain GHA, Mycotrol O, 10.9% AI, Laverlam International, Butte, MT)	**B2X**	2010 2011
Isaria fumosorosea-3 kg/acre (strain 3581)	**IFR1X**	2011
Isaria fumosorosea-6 kg/acre (strain 3581)	**IFR2X**	2012
Isaria fumosorosea-3 kg/acre (strain 3581) + row cover	**IFRC**	2011
Carbaryl-2 qt/acre (Sevin concentrate, 22.5% AI, TechPac LLC, Lexington, KY)	**SV**	2010 2011 2012
Chromobacterium subtsugae-9.5 qt/acre (strain PRAA4-I, MBI 203, 94.5% AI, Marrone Bio Innovations, Davis, CA)	**M1X**	2010 2011 2012
Chromobacterium subtsugae -28.5 qt/acre (strain PRAA4-I, MBI 203, 94.5% AI, Marrone Bio Innovations, Davis, CA)	**M3X**	2010 2011 2012
Chromobacterium subtsugae -9.5 qt/acre alternated with *Beauveria bassiana*-1 qt/acre	**MB**	2010 2011
Chromobacterium subtsugae -9.5 qt/acre alternated with carbaryl-2 qt/acre	**MSV**	2010 2011 2012
Chromobacterium subtsugae-0.5 qt/acre alternated with *Isaria fumosorosea*-3 kg/acre	**MIFR**	2012

Table 4.2 continued

Treatment-Rate (trade name, AI and manufacturer)	Treatment notation	Year applied
Unpsrayed control	**C**	2010
		2011
		2012
Water control	**W**	2011
		2012
Row cover	**RC**	2011
		2012

4.2.3 Statistical analysis

Randomized block experimental designs with four replicates were used in all years. Cucumber beetle and squash bug populations were analyzed using generalized mixed models ANOVA (PROC GLIMMIX) as repeated measures fitted to a Poisson distribution as the assumptions for normality could not be met. The same model was used to analyze yield data. Yield data were square root transformed if the assumptions of equal variance could not be met. Percent survival for each year was analyzed with the same model using a Wilcoxon Rank Sum test because the data did not fit assumptions of equal variance and normality and assumptions were not met with square root or log transformation.

4.3 Results

There was no treatment effective on populations of squash bug adults, nymphs or egg clusters, nor adult cucumber beetles on pumpkin plants in 2010 (Table 4.3). In 2011, there was a treatment effect on squash bug nymphs, with the MB and RC treatments having significantly fewer nymphs per plant than the unsprayed control plot (Table 4.4). There was no treatment effect on the populations of squash bug adults, nymphs or egg clusters, or adult cucumber beetles on pumpkin plants in 2012 (Table 4.5). Squash bug egg clusters were above the action threshold for all treatments, ranging from 1.4 to 2.9 clusters per plant in 2010, 1.4 to 3.7 per plant in 2011, and 5.8 to 9.5 per plant in 2012. Cucumber beetle populations were at the five beetles per plant action threshold in 2010, but were much lower in 2011, with < 1 beetle per plant, and 1 to 2 beetles per plant in 2012.

Table 4.3 *Anasa tristis*, *Diabrotica undecimpuntata howardi* and *Acalymma vittata* per plant on 'Baby Pam' pumpkin by treatment across seven sampling dates in 2010

Treatment	Squash bug adults	Squash bug nymphs	Squash bug egg clusters	Cucumber beetles
Beauveria bassiana label rate, B1X	0.2 ± 0.1	2.1 ± 1.2	1.4 ± 0.3	4.8 ± 0.7
Beauveria bassiana double label rate, B2X	0.3 ± 0.1	1.8 ± 1.1	1.6 ± 0.4	4.7 ± 0.8
carbaryl label rate, SV	0.3 ± 0.1	2.4 ± 1.0	2.8 ± 0.6	3.6 ± 0.6
Chromobacterium subtsugae label rate, M1X	0.1 ± 0.1	2.7 ± 1.2	1.9 ± 0.5	3.7 ± 0.5
Chromobacterium subtsugae triple label rate, M3X	0.2 ± 0.1	2.4 ± 1.0	2.7 ± 0.6	4.6 ± 0.6
Chromobacterium subtsugae alternated with *Beauveria bassiana,* MB	0.6 ± 0.4	1.0 ± 0.6	2.0 ± 0.5	4.0 ± 0.5
Chromobacterium subtsugae alternated with carbaryl, MSV	0.1 ± 0.1	0.7 ± 0.3	1.7 ± 0.3	4.5 ± 0.6
Unsprayed control, UC	0.3 ± 0.1	2.6 ± 0.8	1.6 ± 0.4	4.6 ± 0.6
F;	*0.85;*	*1.02;*	*0.32;*	*0.87;*
d,f;	*7,33;*	*7,19;*	*7,22;*	*7,25;*
p	*0.5523*	*0.4489*	*0.9364*	*0.5439*

*Values are untransformed means ± standard error

Table 4.4 *Anasa tristis, Diabrotica undecimpuntata howardi* and *Acalymma vittata* per plant on 'Cannon Ball' F1 pumpkin by treatment across nine sampling dates in 2011

Treatment	Squash bug adults	Squash bug nymphs	Squash bug egg clusters	Cucumber beetles
Beauveria bassiana label rate, B1X	0.6 ± 0.1	2.5 ± 0.9 **ab**	2.35 ± 0.39	0.65 ± 0.15
Beauveria bassiana double label rate, B2X	0.3 ± 0.1	4.0 ± 1.5 **ab**	2.77 ± 0.48	0.40 ± 0.09
carbaryl label rate, SV	0.6 ± 0.2	2.2 ± 0.8 **ab**	3.65 ± 0.51	0.53 ± 0.12
Chromobacterium subtsugae label rate, M1X	0.4 ± 0.1	2.4 ± 0.9 **ab**	2.40 ± 0.29	0.54 ± 0.10
Chromobacterium subtsugae triple label rate, M3X	0.5 ± 0.1	1.6 ± 0.6 **ab**	3.73 ± 0.56	0.54 ± 0.12
Chromobacterium subtsugae alternated with *B. bassiana*, MB	0.4 ± 0.1	0.7 ± 0.3 **b**	2.88 ± 0.54	0.69 ± 0.15
Chromobacterium subtsugae alternated with carbaryl, MSV	0.5 ± 0.1	1.3 ± 0.5 **ab**	3.13 ± 0.34	0.44 ± 0.10
Isaria fumosorosea, IFR1X	0.4 ± 0.1	2.8 ± 1.0 **ab**	2.67 ± 0.46	0.84 ± 0.24
Isaria fumosorosea with row cover, IFRC	0.1 ± 0.1	1.6 ± 0.6 **ab**	2.63 ± 0.52	0.61 ± 0.24
Unsprayed control, UC	0.4 ± 0.1	5.7 ± 2.1 **a**	2.54 ± 0.33	0.53 ± 0.13
Water control, W	0.4 ± 0.1	3.8 ± 1.4 **ab**	2.24 ± 0.32	0.69 ± 0.16
Row cover, RC	0.2 ± 0.1	0.8 ± 0.3 **b**	1.44 ± 0.34	0.35 ± 0.11
F;	*1.55;*	*2.57;*	*1.49;*	*0.55;*
d,f	*12,39*	*12,38*	*12,32*	*12,34*
p	*0.1483*	*0.0137*	*0.1779*	*0.8666*

*Values are untransformed means ± standard error; mean separation by LSD test at α = 0.05

Table 4.5 *Anasa tristis*, *Diabrotica undecimpuntata howardi* and *Acalymma vittata* per plant on on 'Cannon Ball' F1 pumpkin by treatment across nine sampling dates in 2012

Treatment	Squash bug adults	Squash bug nymphs	Squash bug egg clusters	Cucumber beetles
Carbaryl label rate, SV	1.05 ± 0.20	4.82 ± 1.37	9.50 ± 1.76	0.96 ± 0.25
Chromobacterium subtsugae label rate, M1X	1.65 ± 0.30	6.24 ± 1.88	5.81 ± 0.84	1.56 ± 0.32
Chromobacterium subtsugae triple label rate, M3X	1.44 ± 0.44	5.02 ± 1.74	7.78 ± 1.82	1.52 ± 0.27
Chromobacterium subtsugae alternated with *Isaria fumosorosea,* MIFR	1.75 ± 0.30	6.05 ± 1.15	7.16 ± 0.88	1.79 ± 0.46
Chromobacterium subtsugae alternated with carbaryl, MSV	1.31 ± 0.26	10.58 ± 3.19	6.88 ± 1.05	1.09 ± 0.21
Isaria fumosorosea, IFR1X	1.78 ± 0.35	11.81 ± 3.47	8.17 ± 1.29	1.49 ± 0.35
Isaria fumosorosea double label rate, IFR2X	1.23 ± 0.24	7.25 ± 1.65	9.54 ± 1.38	1.51 ± 0.27
Isaria fumosorosea with row cover, IFRC	0.86 ± 0.21	4.45 ± 1.14	6.76 ± 1.26	1.55 ± 0.32
Unsprayed control, UC	1.40 ± 0.24	9.93 ± 2.59	9.13 ± 1.49	1.35 ± 0.27
Water control, W	1.90 ± 0.45	5.79 ± 1.56	5.96 ± 0.96	1.67 ± 0.32
Row cover, RC	1.56 ± 0.31	12.05 ± 4.71	8.65 ± 1.32	1.52 ± 0.35
F;	*1.10;*	*0.96;*	*0.82;*	*0.18;*
d,f;	*10,33;*	*10,31;*	*10,31;*	*10,32;*
p;	*0.3882*	*0.4991*	*0.6074*	*0.9967*

*Values are untransformed means ± standard error

There was no treatment effect on marketable fruit per acre, total fruit per acre, and marketable pumpkin weight per fruit in 2010, 2011 and 2012 (Tables 4.6, 4.7 and 4.8). In 2010, marketable fruit per acre ranged from 1,207 to 3,046. In 2011 and 2012, marketable yield was lower and much more variable, with ranges of 0 to 1,815 and 0 to 1,210 fruit per acre, respectively. Low marketable and total yields for 2011 and 2012 can be attributed to high plant mortality. In 2010, at 10 weeks after transplanting, survival was at 60 to 88% (Fig. 4.1), whereas survival was 15 to 70% in 2011 (Fig. 4.2), and all plants were dead at 9 weeks after transplanting in 2012.

Table 4.6. Yields and average fruit weight (lb) of 'Baby Pam' pumpkin by treatment in 2010

Treatment	Marketable fruit number per acre*	Total fruit number per acre*	Avg. marketable melon wt (lb) per fruit
Beauveria bassiana label rate, B1X	1317 ± 416	1848 ± 510	1.6 ± 0.2
Beauveria bassiana double label rate, B2X	2193 ± 692	2501 ± 690	1.9 ± 0.1
Carbaryl label rate, SV	2784 ± 879	2958 ± 816	1.9 ± 0.2
Chromobacterium subtsugae label rate, MSV	2501 ± 789	2937 ± 810	1.9 ± 0.1
Chromobacterium subtsugae triple label rate, M3X	1207 ± 381	1926 ± 531	1.8 ± 0.1
Chromobacterium subtsugae alternated with *B. bassiana,* MB	2447 ± 773	3376 ± 931	1.7 ± 1.0
Chromobacterium subtsugae alternated with carbaryl, MSV	2549 ± 805	3080 ± 849	1.7 ± 0.1
Unsprayed control, UC	3046 ± 962	3251 ± 897	1.9 ± 0.1
F;	*1.31;*	*0.77;*	*0.80;*
d,f;	*7,21;*	*7,21;*	*7,21;*
p;	*0.2939*	*0.6152*	*0.5939*

* Based on a population of 1815 plants per acre

Table 4.7. Yields and average fruit weight (lb) of 'Cannon Ball' F1 pumpkin by treatment in 2011

Treatment	Marketable fruit number per acre*	Total fruit number per acre*	Avg. marketable melon wt (lb) per fruit
Beauveria bassiana label rate, B1X	264 ± 182	676 ± 168	2.8 ± 0.4
Beauveria bassiana double label rate, B2X	0 ± 0	668 ± 236	NA
Carbaryl label rate, SV	0 ± 0	592 ± 180	NA
Chromobacterium subtsugae label rate, M1X	108 ± 104	525 ± 132	2.4 ± 0.3
Chromobacterium subtsugae triple label rate, M3X	213 ± 146	639 ± 146	2.9 ± 0.4
Chromobacterium subtsugae alternated with *B. bassiana*, MB	91 ± 106	509 ± 145	2.8 ± 0.6
Chromobacterium subtsugae alternated with carbaryl, MSV	23 ± 53	528 ± 148	2.4 ± 0.6
Isaria fumosorosea, IFR1X	30 ± 46	491 ± 108	2.7 ± 0.3
Isaria fumosorosea with row cover, IFRC	91 ± 106	530 ± 148	2.9 ± 0.1
Unsprayed control, UC	40 ± 82	801 ± 210	2.8 ± 1.0
Water control, W	91 ± 150	676 ± 236	3.3 ± NA
Row cover, RC	0 ± 0	359 ± 243	2.8 ± NA
F;	*0.65;*	*0.65;*	*1.44;*
d,f	*12,9;*	*12,9;*	*11,4;*
p	*0.7586*	*0.7579*	*0.3884*

* Based on a population of 1815 plants per acre

Table 4.8 Yields and average fruit weight (lb) of 'Cannon Ball' F1 pumpkin by treatment in 2012

Treatment	Marketable fruit number per acre*	Total fruit number per acre*	Avg. marketable melon wt (lb) per fruit
Carbaryl label rate, SV	0 ± 0	363 ± 208	NA
Chromobacterium subtsugae label rate, M1X	91 ± 141	363 ± 303	2.5 ± 0.8
Chromobacterium subtsugae triple label rate, M3X	0 ± 0	363 ± 303	NA
Chromobacterium subtsugae alternated with *Isaria fumosorosea*, MIFR	85 ± 86	267 ± 128	2.7 ± 0.48
Chromobacterium subtsugae alternated with carbaryl, MSV	0 ± 0	726 ± 429	NA
Isaria fumosorosea, IFR1X	0 ± 0	363 ± 214	NA
Isaria fumosorosea with row cover, IFRC	91 ± 81	483 ± 230	2.3 ± 0.32
Unsprayed control, UC	40 ± 77	470 ± 319	1.7 ± 1.0
Water control, W	0 ± 0	363 ± 208	NA
Row cover, RC	0 ± 0	363 ± 303	NA
F;	*0.59;*	*0.19;*	*0.93;*
d,f	*10,3;*	*10,3;*	*10,3;*
p;	*0.7682*	*0.9815*	*0.5968*

* Based on a population of 1815 plants per acre

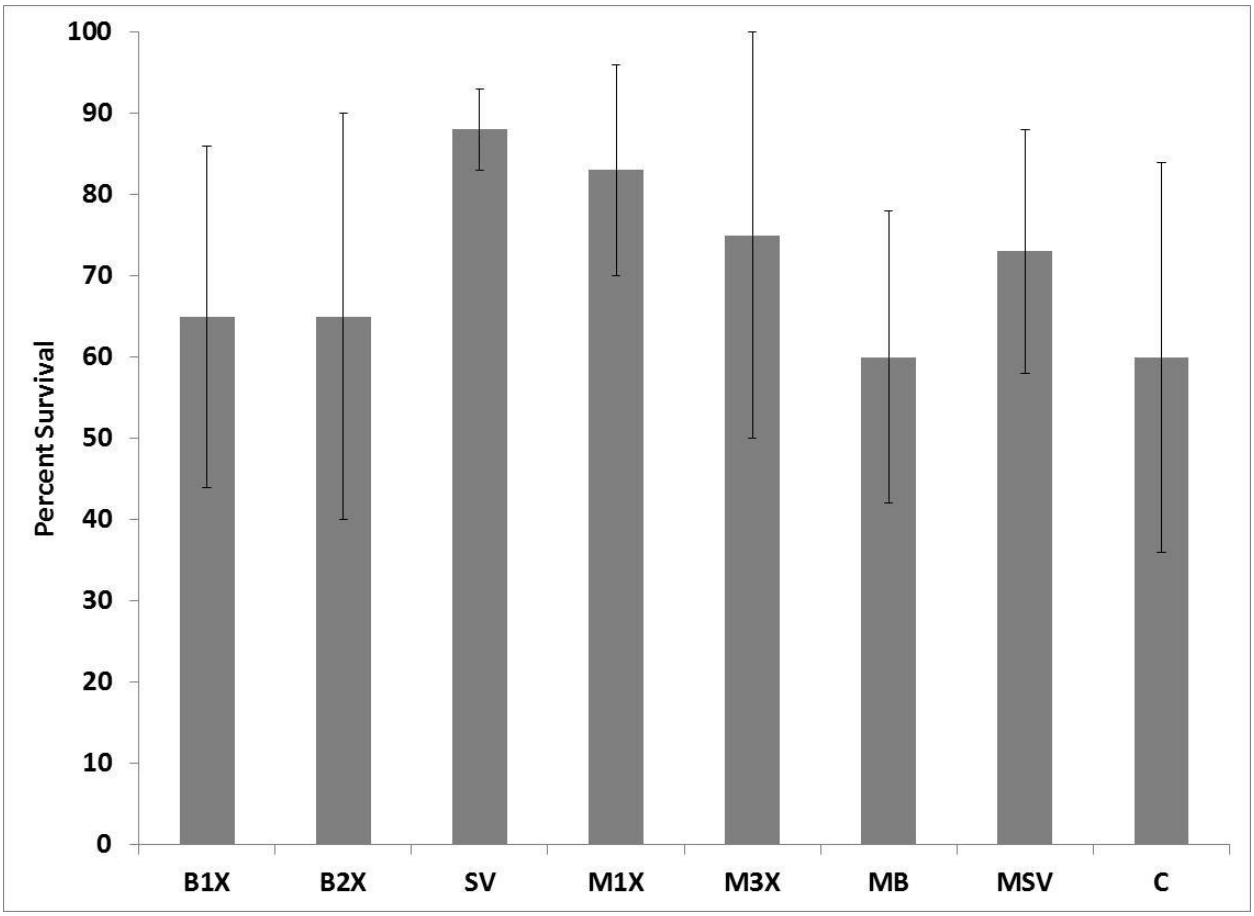

Fig. 4.1 Percent survival of 'Baby Pam' F1 pumpkin when sprayed weekly with biopesticides and a standard insecticide comparison at the University of Tennessee Organic Crops Unit in Knoxville, TN in 2010, at 10 weeks after transplanting. Bars represent mean percent survival ± standard deviation. B1X = *Beauveria bassiana* label rate; B2X = *Beauveria bassiana* twice label rate; M1X = Chromobacterium subtsugae label rate; M3X = *Chromobacterium subtsugae* triple label rate; MB = *Chromobacterium subtsugae* alternated with *Beauveria bassiana*; MSV = *Chromobacterium subtsugae* alternated with carbaryl; SV = Carbaryl label rate; UC = Unsprayed control

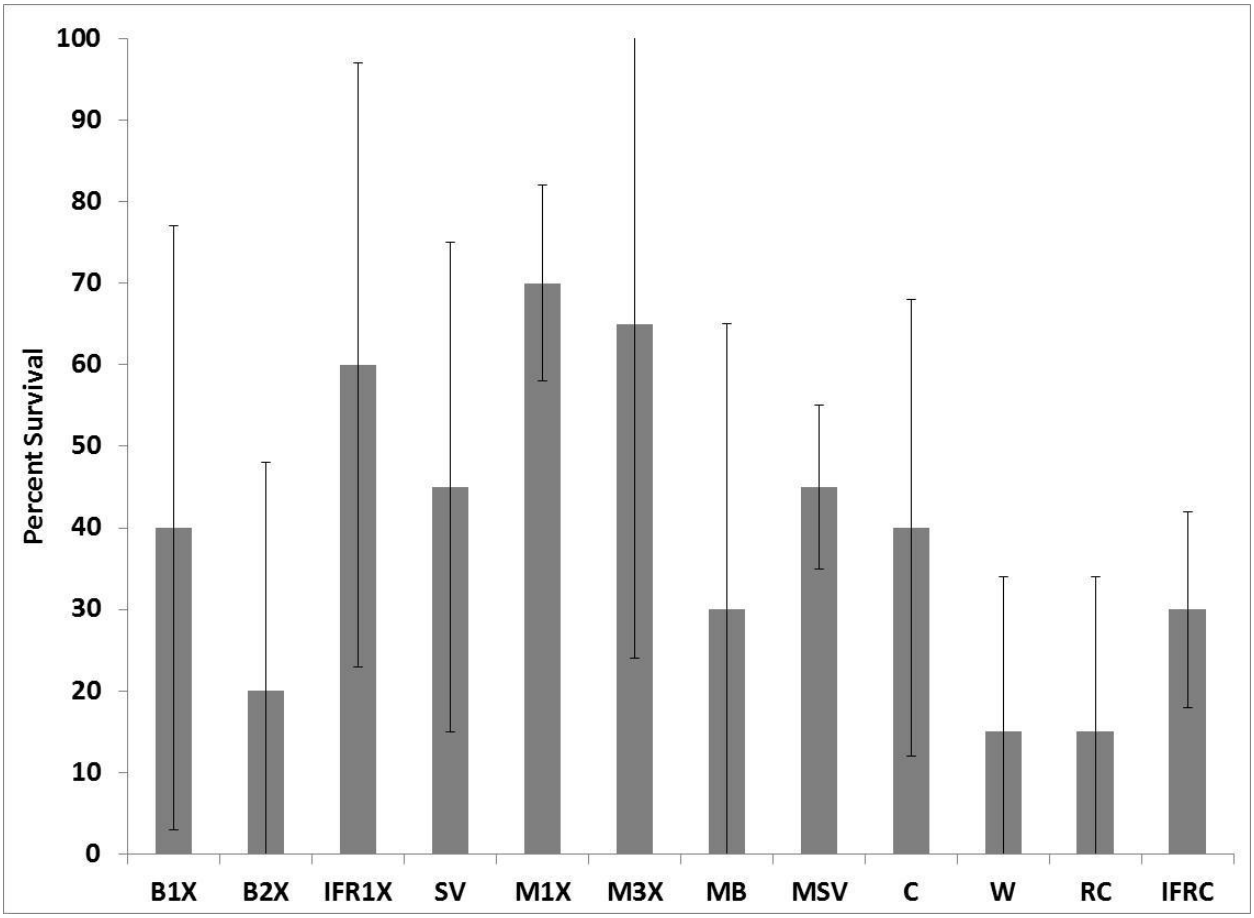

Fig. 4.2 Percent survival of 'Cannon Ball' pumpkin when sprayed weekly with biopesticides and a standard insecticide comparison at the University of Tennessee Organic Crops Unit in Knoxville, TN in 2011, at 10 weeks after transplanting. Bars represent mean percent survival ± standard deviation. B1X = *Beauveria bassiana* label rate; B2X = *Beauveria bassiana* twice label rate; IFR1X = *Isaria fumosorosea* label rate; SV = Carbaryl label rate; M1X = *Chromobacterium subtsugae* label rate; M3X = *Chromobacterium subtsugae* triple label rate; MB = *Chromobacterium subtsugae* alternated with *Beauveria bassiana*; MSV = *Chromobacterium subtsugae* alternated with carbaryl; UC = Unsprayed control; W = Water control; RC = Row cover; IFRC = *Isaria fumosorosea* + row cover

4.4 Discussion and Conclusion

Results from this work show that the biopesticide spray treatments failed to maintain squash bugs under the economic threshold level of one egg cluster per plant on pumpkin in all three years. In 2011, lower populations of nymphs were counted in the C. *subtsugae* rotated with *B. bassiana* plots, but not in either plot alone, indicating that there may be a synergistic effect of these two compounds on nymphs, although this was not seen in 2010. In addition, row covers suppressed the number of squash bug nymphs on pumpkin plants in 2011, which is in agreement with Delate (2002) and Cartwright (1990). Interestingly, the carbaryl plots did not reduce squash bug adults or nymphs compared to the control plots in all three years. Squash bugs are often found on the undersides of leaves and at the base of the plants, so contact with the pesticide spray may have been limited. In general, squash bug populations increased from year to year. In each year, plants were set on black plastic mulch used as a weed barrier. Black plastic mulch has been shown to increase squash bug populations on mulched summer squash versus bare soil, as adults and nymphs will congregate under the mulch and benefit from warmer temperatures, increased soil moisture and protection from natural enemies in this habitat (Cartwright et al. 1990). Overwintering squash bugs harbor cucurbit yellow vine disease and it is recommended that sanitation via clearing crop residue and elimination of overwintering habitat can help mitigate crop damage the following year (Pair et al. 2004). Due to the use of black plastic mulch and the high amount of organic matter and crop residues in the fields, it is likely that squash bug adults were overwintering and increasing in number during the three year period of this study, resulting in high population densities in 2012.

Cucumber beetle populations were on average at threshold levels in 2010, but below threshold levels in 2011 and 2012. This could be due to the change in the pumpkin variety used in 2010 and later years, from 'Baby Pam' to 'Cannon Ball'. Adult beetles are attracted to volatiles from fruit and flowers, which produce cucurbitacins, terpenoid compounds that act as feeding stimulants (Tallamy et al. 1998; Martin and Schroder 2000), and beetles are often found inhabiting pumpkin flowers. It was found that the spotted cucumber beetle, *D. undecimpunctata howardi*, prefers *C. maxima* cultivars over *C. pepo* and *C. moschata*, and *D. v. virgifera* prefers *C. maxima* as well as certain varieties of *C. pepo* better than *C. moschata* (Anderson and Metcalf 1987). While 'Baby Pam' and 'Cannon ball' are both of the species *C. pepo*, they could have differed in the levels of terpenoid compounds produced, which maypossibly explain why more cucumber beetles were found on pumpkin plants in 2010. The buckwheat borders may also have played a role in the suppression of cucumber beetle populations, as natural enemies are attracted to buckwheat strips (Cline et al. 2008; Platt et al. 1999). Row covers may prevent colonization of pumpkin plants by cucumber beetles in the early stages of plant development; however, once they are removed for pollination, insect pests may rapidly colonize the plants (Cartwright et al. 1990; Cline et al. 2008). Cucumber beetles are extremely mobile, and will move between host plants and field edges throughout the day (Luna and Xue 2009).

Pumpkin yield was low in all three years. In this area, average yields for 'Baby Pam' in conventional systems should be around 5,200 fruit per acre, based on a population of 1,815 plants/acre (Mullins 2000). Total fruit per acre in 2010 was 35 to 65% less than what would be expected for conventional yields to differ. Typical yields for 'Cannon Ball' in this region should

be around 4,235 fruit per acre, based on a population of 1,089 plants per acre (Wszelaki and Schulteis, unpublished data). Yield per acre in 2011 and 2012 was very low due to high plant mortality. Plant mortality was high in both years due to incidence of downy mildew and *Plectosporium* blight on pumpkin plants. Disease was the dominate factor on yield and marketability in these two years. Fungal diseases in pumpkin and squash crops are often more serious than insect pests (Brust et al. 1995). Therapeutic disease management in organic systems may include copper fungicides, soaps and oils, or microbial organisms, but these were not used in this study due to the potential of interference with the biopesticides treatments.

Beauveria bassiana, *Isaria fumosorosea* and *Chromobacterium subtsugae* did not decrease squash bugs and cucumber beetles on pumpkins in the field compared to control plots. Fungal disease on pumpkins were a major constraint in this study and influenced yield and marketability more than insect pressure. Future research on testing compatibility of disease suppressing fungicides with biopesticides for organic systems would show whether yields on pumpkins and other susceptible cucurbit crops can be practically achieved.

5 Conclusion and Recommendations

Microbial products for pest management are appealing due to low environmental risks, low risk to non-target organisms, and for resistance management. However, efficacy may be difficult to achieve, and more basic and applied research on these products are needed. Laboratory research is needed, including screening of different species and strains of microbes at the species level and at all lifestages of the pest. Molecular technology will allow increased understanding in distinguishing species and strains and understanding virulence factors.

Research on the immune and behavioral responses of the insect host to entomopathogens, and more work on tri-trophic interactions will be helpful in understanding efficiacy. Improvements in formulation, field delivery methods and environmental modification can increase field efficacy. Recommendations on how biopesticides can be best used in an integrated pest management program are needed. Improving production will help reduce costs of biopesticides. Although there are many environmental benefits to their use, biopesticides will only be sustainable if they can reliably decrease pest pressure and damage on crops in the field in an economically sound manner, and more work is needed to make these improvements.

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Vita

Mary Anne Rogers was born in Sioux Falls, SD, to the parents of Thomas and Gail Rogers. She attended Lincoln High School in Sioux Falls from 1994-1998. After graduation, she studied at the University of Minnesota (UMN) Twin Cities campus, where she received her B.S. in Environmental Horticulture (emphasis in Sustainable Agriculture) in 2003. She joined the Department of Entomology at the UMN in fall of 2005 as a graduate assistant studying integrated pest management for landscape and ornamental plants. Upon receiving her M.S. in 2008, she re-located to Knoxville, TN to work as a Research Associate in the Organic and Sustainable Crops Production Program in the Department of Plant Sciences at the University of Tennessee (UT). Mary graduated with a Ph.D in Plants, Soils and Insects (emphasis in Integrated Pest Management) in December, 2012. Currently, she is continuing her research at UT as a postdoctoral associate.