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Effects of *Beauveria bassiana* mycelia and metabolites incorporated into synthetic diet and fed to larval *Helicoverpa zea*; and detection of endophytic *Beauveria bassiana* in tomato plants using PCR and ITS primers

Brian Michael Leckie
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Brian Michael Leckie entitled "Effects of *Beauveria bassiana* mycelia and metabolites incorporated into synthetic diet and fed to larval *Helicoverpa zea*; and detection of endophytic *Beauveria bassiana* in tomato plants using PCR and ITS primers." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Bonnie Ownley, Major Professor

We have read this thesis and recommend its acceptance:

Carl Jones, William Klingeman

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis
Presented for the
Masters of Science
Degree
The University of Tennessee, Knoxville

Brian Michael Leckie
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Dedication

This thesis is dedicated to my parents, David and Sara Leckie, who have done everything to provide me with a loving family, and to give me every possible opportunity in life; and to my dog, Jackson, who stands by me night and day.

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I would like to thank all the people that helped me complete this thesis and my degree in Entomology and Plant Pathology. I thank Dr. Bonnie Ownley for helping me through the many obstacles in completing my thesis. I thank Dr. Carl Jones for allowing me to stay around and for providing me with guidance throughout the completion of my degree. I thank Dr. Bill Klingeman for serving on my committee. I thank Ledare Habera, Malissa Ament, and Tina Richey for teaching me the molecular techniques needed to complete my research project.

Abstract

Beauveria bassiana (Balsamo) Vuillemin is an entomopathogenic deuteromycete that has been used in biological control of insect pests. Recent studies have revealed that *B. bassiana* is an endophyte of corn plants, and that plants colonized by the fungus had a reduction in tunneling from the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). In addition to corn, *B. bassiana* has been observed to grow endophytically in potato plants. *Beauveria bassiana* may also have the ability to colonize closely related plants and potentially reduce insect feeding on them.

This study had three objectives. The first was to evaluate the effect of mycelia from the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*, when incorporated at different rates into a synthetic diet and fed to neonate corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae. Larvae fed the highest rates (1 and 5%) of fungal diet experienced delayed development and suffered high mortality. These insects also had lower larval and pupal weights than larvae fed the lower concentrations of mycelia. Insects fed low rates (0.1 and 0.5%) of fungus suffered low mortality and developed at an accelerated rate, compared to fungus free controls, indicating increased nutrition in low rate fungal diets. Insects fed diets containing *B. bassiana* isolate 11-98 suffered the highest mortality indicating that 11-98 may be more toxic than the other isolates.

In the second study, the effect of *Beauveria bassiana* was evaluated when metabolites were incorporated at different rates into a synthetic diet and fed to neonate corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae. All larvae fed

diets containing metabolites of *B. bassiana* experienced low mortality, but had delayed development. Those insects fed the highest rate (0.5%) of metabolite-amended diet had significantly lower percent pupation and developed at a slower rate than those insects fed the 0.1% rate.

A third study was designed to establish a technique to detect the presence of endophytic *Beauveria bassiana* in tomato plants. After seed-treating tomato plants with *B. bassiana* conidia and allowing them to grow for two weeks in test tubes under gnotobiotic conditions, PCR techniques were used to amplify ITS regions of the plant and fungus from the plant shoot. The presence of *B. bassiana* in treated plants was confirmed by the PCR amplification of a 550-bp ribosomal RNA gene segment. The amplified product was sequenced using ITS1 and ITS4 primers. The resulting sequence data had 100% homology with a previously published sequence for *B. bassiana*.

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Literature Review

Beauveria bassiana

Beauveria bassiana (Balsamo) Vuillemin is a ubiquitous soil-inhabiting entomopathogenic fungus in the phylum Deuteromycota, and is characterized by white mycelia and hyaline conidia that are globose to oval in shape. Conidia are formed from zigzag-shaped extensions on conidiophores, which are located singly or in whorls (Boucias and Pendland 1998). In addition to being an entomopathogen, *B. bassiana* commonly occurs as a saprophyte in soil (McCoy 1985). In its typical form, *B. bassiana* will grow initially as a white mycelial mat, which later produces conidiophores and then conidia. Conidia serve to spread the fungus and are the infective unit on susceptible insect hosts.

In Japan, around 900 A.D., a white muscardine fungus was first recognized as a disease of the silkworm, *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) (Boucias and Pendland 1998). Silkworm cadavers infected with *B. bassiana* were used as antiseptics and antitoxins in oriental medicine. In 1834, Antonio Bassi and Giuseppe Balsamo Crivelli determined that silkworms were infected by *B. bassiana*, giving rise to the germ theory of disease. The causative organism was first named *Botrytis paradoxa* in 1835 by Balsamo and later renamed *Botrytis bassiana*. In 1912, Vuillemin renamed the genus *Beauveria* (Steinhaus 1975).

The infection cycle of *B. bassiana* in an insect begins with the contact of a conidium with the cuticle of a susceptible host. The conidium germinates and the fungus

produces an array of enzymes that help degrade the outer integument. The fungus produces a germ tube that grows through the integument and toward the hemocoel. Once the hemocoel is entered, blastospore formation and toxin production begin (Boucias and Pendland 1988). As the fungus proliferates, the host dies and becomes mummified by hyphal growth that will later extrude from the cadaver through intersegmental membranes (Pekrul and Grula 1979). Death usually occurs in three to seven days and is attributed to nutrient deficiency, water loss, or the action of toxins (Boucias and Pendland 1988). Production of conidiophores and conidia occurs outside the host, and infective propagules disseminate to new hosts.

A variety of insects, at all stages of development are susceptible hosts of *B. bassiana* (McCoy et al. 1985). Due to its wide host range, *B. bassiana* has been tested as a microbial control agent against most of the important insect pests. Pests that have been effectively controlled by *B. bassiana* include: the lesser stalk borer, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) (McDowell et al. 1990), European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Bing and Lewis 1991; Feng et al. 1988), hop aphid, *Phorodon humuli* (Schrank) (Homoptera: Aphididae) (Dorschner et al. 1991), greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae) (Poprawski et al. 2000), and Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Jaros-Su et al. 1999). Interest in the use of *B. bassiana* as a biological control agent has spurred several large-scale research programs. In France, it has been used in attempts to control the codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae). In the People's Republic of China, *B. bassiana* was used to control *O. nubilalis*; and, in 1977, the former U.S.S.R. produced 22

metric tons of a *B. bassiana* product for control of Colorado potato beetle (McCoy et al. 1985).

Beauveria bassiana is commercially available in the United States under the trade names Mycotrol and Naturalis. Suggested target pests for these products include beetles, whiteflies, aphids, leaf and planthoppers, and stem-boring lepidopterans. These bioinsecticides are produced as conidial suspensions and applied as a foliar spray. The conidial suspensions serve as a source of inoculum for the natural disease cycle. However, foliar applications of *B. bassiana* may be challenged by reduced viability of the conidia due to effects of the environment. These conditions include unfavorable temperatures, low rainfall (Bing and Lewis 1991), and high levels of ultraviolet light (Copping and Menn 2000).

Endophyte Plant Mutualism

Endophytes are non-pathogenic fungi that live inside healthy plants (Alexopoulos et al. 1996). The mutualistic association provides nutrients and moisture for the fungus, and imparts stress tolerances to the plant (Bacon 1993). The majority of work with fungal endophytes involves tall fescue (*Festuca arundinacea*) (Schreber) (Cyperales: Poaceae), and *Neotyphodium coenophialum* [(Morgan-Jones and W. Gams) Glenn, Bacon and Hanlin comb. nov.] (Clavicipitales), an endophyte responsible for fescue toxicosis, which affects grazing horses and cattle. Work involving *B. bassiana* as an endophyte of corn (*Zea mays*) has focused on colonization and suppression of *O. nubilalis* (Lewis and Bing 1991, Bing and Lewis 1992a, 1992b). The aforementioned authors have not studied the physiological interactions between plants and *B. bassiana*, but work with other

endophytes (Arachevaleta et al. 1989) has revealed that a variety of factors may influence the development of plants and their endophytes.

Nitrogen availability has multiple effects on endophyte-infected plants. In a study that examined soil nitrogen levels, endophyte-infected (*N. coenophialum*) tall fescue plants, with low nitrogen availability, had 25% thicker leaf blades at 160 days than endophyte-free fescue. At high levels of soil nitrogen, endophyte-infected fescue had 67% greater herbage yields. In the same study, drought stress was evaluated at different nitrogen levels. At the highest levels of nitrogen and a soil matric potential of -0.50 MPa, endophyte-free plants had 75% mortality and infected plants had no mortality (Arachevaleta et al. 1989).

Endophytic infection with *N. coenophialum* has promoted growth and germination in some plants. Infected tall fescue had twice the number of filled seeds, and seeds of infected fescue and perennial rye grass had 10% greater germination rate (Clay 1987). When compared against endophyte-free plants, fescue regrowth height 14 days after harvest increased by 38% in endophyte-infected plants grown at a soil matric potential of –0.03 MPa and 111% at –0.50 MPa (Arachevaleta et al. 1989). In a study exploring the effects of the removal of endophytes by fungicidal treatment from tall fescue, dry root matter decreased 25% in plants where the endophyte was not present. Herbage growth and tiller number also decreased, 18% and 12% respectively, when the endophyte was removed (De Battista et al. 1990).

Drought stress tolerance also is increased in plants colonized by *N. coenophialum*. In addition to the study by Arachevaleta and coworkers (1989), a study involving pastures with differing percentages of infected fescue (Read and Camp 1986) had two of

three stands with low levels of endophyte infection lost due to drought, while no stands were lost in fields with high levels of endophytic infection. This observed drought stress tolerance may be attributed to the increased production of free polyols, common fungal metabolites, which are osmotic regulators in vascular plants (Bacon 1993).

Endophytic infection may also provide increased protection from insect herbivory in many plant species. In a feeding test involving endophyte-infected tall fescue and the oat bird cherry aphid, *Rhopalosiphum padi* (Linnaeus) (Homoptera: Aphididae), aphid mortality after 66 hours was 20% among aphids feeding on non-infected segments and 100% in those feeding on infected plants (Johnson et al. 1985). House crickets, *Acheta domesticus* (Linnaeus) (Orthoptera: Gryllidae) introduced to perennial ryegrass, *Lolium perenne* (L.) (Cyperales: Poaceae), infected with the endophyte *Neotyphodium loliae* [(Latch, Christensen and Samuels) Glenn, Bacon & Hanlin comb. nov.], formerly *Acremonium loliae*, were shown to have 100% mortality in 84 hours. Insect death was attributed to failure of the alimentary tract (Ahmad et al. 1985). Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), survival, when reared on *Cyperus pseudovegetus* (Steud.) (Cyperaceae) infected with the endophyte *Balansia cyperi* (Clavicipitales), exhibited a significantly higher mortality (89%) when compared with those fed endophyte-free *C. pseudovegetus* (76%) (Clay et al. 1985). Among fall armyworms feeding on leaves of tall fescue, 70.8% of the insects preferred feeding on non-infected leaves (Hardy et al. 1986).

Reductions of insect herbivory may be due to accumulation of fungal metabolites, which serve as insect toxins. Peramine hydrobromide, a toxic metabolite of *N. lolii*, has been implicated as an insect toxin (Rowan et al. 1990). Feeding by the Argentine stem

weevil, *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae), was significantly deterred by as little as 0.1 μg/g of peramine hydrobromide in an agar diet.

Beauveria bassiana as an Endophyte

Research on *B. bassiana* has demonstrated that this fungus may grow endophytically in corn (Poaceae) (Lewis and Bing 1991), which is in the same plant taxonomic family as fescue. *Beauveria bassiana* also has been reported as an endophyte of potatoes (Solanaceae) (Jones 1994) and therefore may be expected to grow endophytically in other plants in the family Solanaceae, such as tomatoes. After foliar application to corn plants at the V8 stage, *B. bassiana* was recovered from the pith of plants. Percentage of plants with recovered *B. bassiana* was negatively correlated (r = -0.376) with insect damage per plant. Over the two-year study, plants treated with *B. bassiana* exhibited suppression of tunneling by larval European corn borer (*O. nubilalis*) ranging from 37.0% to 50.6% (Lewis and Bing 1991). In a later study, granular formulations of *B. bassiana* conidia, applied to the foliage of corn at the whorl-stage, grew into and colonized up to 98.3% of plants (Bing and Lewis 1991). Once established in the plant, the fungus again decreased tunneling of *O. nubilalis*. Foliar applications of *B. bassiana* were also effective in establishing this fungus as an endophyte in potato plants. Endophytic *B. bassiana* was recovered from 10% of potato plants receiving foliar applications of conidial suspensions (Jones 1994).

Injection of conidial suspensions was also an effective means for inoculation of corn plants. After injecting plants with a conidial suspension in mid-June, all plant segments tested at harvest contained *B. bassiana*. *Beauveria bassiana* was recovered

from internal areas of 65% of the plants receiving foliar applications, but not from the outer surfaces, indicating that the fungus persisted inside the plant. The fungus was recovered from the outside of 1.9% of injected plants, but this may be due to naturally occurring inoculum from the soil (Bing and Lewis 1991). *Beauveria bassiana* has been recovered from the bark of elm (Doberski and Tribe 1980) and ironwood trees, *Carpinus caroliniana* (Walter) (Betulaceae) (Bills and Polishook 1990), but these isolations were attributed to either conidia found in the bark or to the presence of *B. bassiana* as a facultative saprophyte.

Timing of *B. bassiana* application may be important in establishment of the fungus as an endophyte and its effect on insect control. Applications of *B. bassiana* to the pretassel stage of corn resulted in significantly less tunneling by *O. nubilalis* (0.93 cm tunneling/plant), than did applications to the whorl (1.42 cm tunneling/plant) or late-whorl stages (1.23 cm tunneling/plant). However, the same study revealed no significant difference in percent colonization by *B. bassiana* (Bing and Lewis, 1992a). *Beauveria bassiana* recovered from the pith of colonized plants maintained virulence to larvae of *O. nubilalis* (Bing and Lewis, 1993).

In a study by Wagner and Lewis (2000), light and electron microscopy were used to observe the mode of *B. bassiana* colonization into and throughout corn plants. The conidia, once applied by foliar application to the surface of the corn plant, germinated and the germ tube penetrated the plant. Entry was achieved by growth through natural openings such as stomates, or by direct penetration through small holes. Direct penetration was facilitated by enzymatic activity and mechanical pressure produced by the fungus. Once inside the plant, the mycelium branched and grew throughout the

epidermal regions and into the palisade parenchyma. Mycelial growth was observed also in the xylem of the plant, which may indicate a means of passive movement throughout the plant. Injection of corn plants with conidial suspensions, below the node of the primary ear, revealed that *B. bassiana* moves into the pith and then upward throughout the plant. *Beauveria bassiana* was most often isolated at the internode below the primary ear (Bing and Lewis 1992b). *Beauveria bassiana* appeared to have no adverse effects on the corn plants.

Toxins Produced by *Beauveria bassiana*

As in other endophyte-infected plants that show a reduction of insect herbivory, *B. bassiana* toxins produced as metabolites may build up in the plant and deter feeding. *Beauveria bassiana* produces beauvericin, bassianolide, and the red pigmented toxin oosporein. Cyclosporin is also produced as a secondary metabolite and is a known immunosuppressant produced by other fungi (Boucias and Penland 1998). Beauvericin (5 μg/fly) injected into adult blowflies, *Calliphora erythrocephala* (Meig.), resulted in 15% mortality by day 2. When injected into larval yellow fever mosquitoes, *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), at a rate of 10 μg/ml, mortality reached 39% at 48 hours (Grove and Pople 1980). Suspensions of beauvericin (0.1 mg/ml) added to water containing larval northern house mosquitoes, *Culex pipiens autogenicus* (Linnaeus) (Diptera: Culicidae), killed 44% of the larvae by 48 hours (Zizka and Weiser 1993). Fermentation broth obtained from the production of *B. bassiana* and containing the red pigment oosporein caused 49.8% mortality of insects in three days, when applied topically to leaves infested with mealy bugs (Eyal et al. 1994).

Oral toxicities of some *B. bassiana* metabolites have been evaluated. Beauvericin, when applied to leaf disks and fed to Colorado potato beetles, had an LC_{50} of 633 ppm and an LC_{90} of 1196 ppm (Gupta et al. 1991). Conversely, beauvericin was shown to have no oral toxicity to silkworms at levels as high as 1000 ppm (Kanaoka et al. 1978). In this same study, bassianolide was also administered orally to silkworms and was lethal at 8 ppm.

Fungivory by Insects

Feeding tests involving insects consuming fungi have produced various results. *Orehesella cincta* (Linnaeus) (Collembola: Entomobryidae) fed *Cladosporium cladosporioides* (Fres.) de Vries mycelia that was produced on different levels of nitrogen, exhibited a significantly greater rate of growth when fed on high nitrogen mycelia (Lavy and Verhoef. 1996). Insects fed high nitrogen (4%) mycelia had a mean dry weight of 0.448 mg, while those fed low nitrogen (2.2%) mycelia resulted in a mean dry weight of 0.384 mg. *Proisotoma minuta* (Tullburg) (Collembola: Isotomidae) and *Onychiurus encarpatus* (Denis) (Collembola: Onychiuridae) both suffered 100% mortality when fed on *Gliocladium virens* (Miller et al.) and *Trichoderma harzianum* (Rifai) (Lartey et al. 1989). The mortality of these collembolans was attributed to direct infection by the fungi or to ingestion of toxic metabolites. *Folsomia candida* (Willem) (Collembola: Isotomidae) were fed conidia and mycelium of *B. bassiana* (Broza et al. 2001). Feeding on the entomopathogen resulted in no mortality, however, the conidia-fed collembolans were smaller ($38 \pm 3\mu g$) than those fed mycelium ($149 \pm 12\mu g$), and conidia fed insects laid fewer eggs. In a study involving the entomopathogenic fungus,

Metarhizium anisopliae (Metsch.) Sorokin, orally administered conidia were shown to cause up to 20% mortality in 1st instar larvae of the root weevil, *Diaprepes abbreviatus* (Linnaeus) (Quintela and McCoy 1997).

Detection of *Beauveria bassiana*

The traditional detection method used to determine the presence of *B. bassiana* in plant tissue is plating samples on a selective medium (Doberski and Tribe 1980; Bing and Lewis 1991; 1992a; 1992b; 1993). Plating of samples relies on the absence of other organisms that may outcompete the fungus targeted for isolation. Detection of fungal endophytes in grasses has been done using tissue print-immunoblot (Gwinn et al. 1991), a technique that could be used to detect endophytes associated with other plants. Immunoblot detections may be subject to interference due to variable specificity in antibodies. Techniques utilizing polymerase chain reaction (PCR) and gel electrophoresis have been used to detect *B. bassiana* in the cadavers of the migratory grasshopper (*Melanoplus sanguinipes*) (Hegedus and Khachatourians 1996). More recent PCR work, using primers specific to the internally transcribed spacer regions (ITS) of ribosomal RNA genes, allow for the detection of fungal DNA. This technique also produces a single-band product enabling species identification. Species confirmation is possible due to the highly variable regions of genetic code located between the conserved ribosomal RNA genes (White et al. 1990).

Development of *Helicoverpa zea*

The normal developmental cycle of *Helicoverpa zea* begins with a gravid adult female laying eggs singly on a suitable host plant. The egg hatches in a few days and the larval corn earworm starts feeding on the plant. Larvae develop for two to four weeks and then pupate in the soil (Hunt and Baker 1982). In a study of larval *H. zea* fed synthetic diet, larvae had a mean days to pupation of 14.6 days and experienced 7% larval mortality (Guerra et al. 1990). In a similar study where larval *H. zea* were fed cotton flower buds, larval growth rate was in the range of 8.2 to 33.5 mg/day (Halcomb et al. 1996). The pupating insects then emerge into adults and mate, starting the life-cycle again.

Objectives

The objectives of this research were:

1. To determine the effects of *Beauveria bassiana* mycelia incorporated into an artificial diet on corn earworms, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae).

2. To determine the effects of *B. bassiana* metabolites incorporated into an artificial diet on corn earworms (*H. zea*).

3. To develop a PCR-based technique to confirm colonization of tomato plants by the entomopathogenic fungus *B. bassiana.*

Chapter 2

Effects of *Beauveria bassiana* mycelia incorporated into synthetic diet and fed to larval *Helicoverpa zea*.

Introduction

The ubiquitous fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin has been used as a biological control agent against many major insect pests (Jaros-Su et al. 1999, Mc Dowell et al. 1990, Poprawski et al. 2000). The focus of those studies was insect infection by direct application of conidia. However, it has been reported that *B. bassiana* is an endophyte of corn plants (Bing and Lewis 1991) and this observation opened new possibilities for use of this fungus. Bing and Lewis (1991) reported that *B. bassiana* persisted in the plant following successful endophytic colonization and provided protection against tunneling of the European corn borer *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). Suppression of tunneling was attributed to direct infection by the fungus or to fungal metabolites produced in plant tissues.

Besides direct infection of susceptible insect hosts, the presence of *B. bassiana* in plant tissue may affect insects that ingest the fungus. Endophytic fungal growth provides protection from insect herbivory in some plant species (Ahmad et al. 1985, Clay et al. 1985, Hardy et al. 1986, Johnson et al. 1985). In these studies, reductions in insect herbivory have been credited to the build-up of fungal metabolites, but based on research involving feeding fungi to insects, a fungal diet may affect insect development and survivorship (Broza et al. 2001, Lartey et al. 1989, Lavy and Verhoef 1996). Tests involving another entomopathogenic fungus, *Metarhizium anisopliae* (Metsch.) Sorokin,

have shown that orally-administered conidia can cause up to 20% mortality in the root weevil *Diaprepes abbreviatus* (Linnaeus) (Quintela and McCoy 1997).

The aim of the present study was to investigate the effects of ingested *B. bassiana* and *M. anisopliae* on corn earworms (also commonly known as tomato fruitworms), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) by feeding the insects a synthetic diet into which mycelia of these fungi had been incorporated. The specific objective was: 1) to determine the effects of four rates of diet-incorporated mycelia from two *B. bassiana* isolates and one isolate of *M. anisopliae* on mortality, growth, and pupation of corn earworms.

Materials and Methods

Propagation and Collection of Entomopathogenic Fungi

The entomopathogenic fungi included in this study were *Beauveria bassiana* (Bb) isolate 11-98, which was obtained in Scott County, TN, from an infected click beetle (Coleoptera: Elateridae), *B. bassiana* isolate 3-00, and *Metarhizium anisopliae* (Ma) isolate 2-00, the latter isolates were cultured from Japanese beetle cadavers [*Popillia japonica* (Newman)(Coleoptera: Scarabiidae)] collected in Warren County, TN. Fungi were grown on Sabouraud dextrose agar (Difco, Sparks, MD) + 0.5% yeast extract (SDAY), incubated at 25°C for approximately 3 weeks. Conidia were harvested by brushing the surface of the plates with a stenciling brush and passing the conidia through a #100-mesh sieve (150-µm opening).

Mycelial Production

Inocula were prepared by adding 2.5 g yeast extract and 2.5 g dextrose to 250 ml de-ionized water in a 500-ml Erlenmeyer flask. Flasks with medium were autoclaved and cooled before inoculation with conidia. Inoculated flasks were incubated in a shaker (150-200 RPM) for 4-5 days at room temperature.

Mycelia were produced in a BIOFLO 2000 fermenter (New Bruswick Scientific, Edison, NJ). The fermenter vessel contained 7-8 L of de-ionized water with 1% dextrose and 1% yeast extract. After autoclaving the vessel with medium, the fermenter was incubated at 24°C, with 200-RPM agitation, and 4 L compressed air/min aeration. The medium was agitated and aerated for one day to confirm sterility. After this period, approximately 100 ml of fungal inoculum was injected into the fermenter vessel and the fungus was incubated for seven days. Total dry weight of Bb 11-98 mycelia produced was 66 g in 7 L of media. Dry weight of Bb 3-00 was 74 g from 8 L of media and isolate Ma 2-00 produced 68 g in 8 L.

The mycelia were harvested by vacuum filtration through a #18-mesh sieve covered with #4 Whatman filter paper. After removal of liquid, the filter paper with the mycelial mat was removed from the sieve and placed on a rack in a vertical flow hood to air-dry. The filter paper was carefully removed and the mycelia were allowed to dry overnight. Dried mycelia were blended to a fine powder and passed through a #60-mesh sieve (250-µm opening).

Insects

Eggs of the Corn earworm, *Helicoverpa zea* (Boddie), were obtained from Agripest (Zebulon, NC). Approximately 75-100 eggs were placed in each of several Petri dishes on filter paper moistened with de-ionized water. Petri dishes were placed in an incubator at 25°C for two days or until larvae hatched. Neonate larvae were collected for use in experiments.

Preparation of Diet

Artificial beet armyworm diet mix (Product #F9219) was obtained from Bio-Serv Inc. (Frenchtown, NJ). To prepare the diet, a 250-ml beaker with 82 ml of de-ionized water was placed on a hot plate and the water was boiled. Agar (2 g) was added to the boiling water and the solution was heated until it cleared. The agar solution was then placed into a blender with 16.12 g of dry diet mix. The mixture was blended thoroughly and cooled for about 1 min before addition of mycelial powder. Dry mycelial powder was added to the diet at 0.1, 0.5, 1, and 5% weight by volume. Diets with mycelia were mixed vigorously, poured into Petri dishes, and stored in a refrigerator (4.5°C) until used. Control diet containing only synthetic diet was prepared also.

Diet Feeding Tests

The three entomopathogenic isolates were evaluated separately in repeated tests. Five rates (0, 0.1, 0.5, 1.0, and 5.0) of mycelia incorporated into diet were tested for each isolate. Treatments were arranged in a randomized complete block design. Neonate corn earworm larvae were exposed to the entomopathogenic mycelia incorporated into the

artificial diet. Each neonate corn earworm was placed into a 29.6 cm^3 plastic cup. Plugs of the diet were prepared with a cork borer (1-cm inside diameter). One plug of diet was added to each cup. Each treatment had 20 replicates; each replicate consisted of a single insect in a cup. Cups were placed in an incubator and held at 24°C. Additional diet was added to the cups when needed, to provide an excess of diet and maintain freshness. Larvae were observed daily and mortality, pupation and pupal weight were recorded. Larval weights were recorded for all treatments in all tests on day 15. The experiment was terminated after the surviving insects in the control treatment reached 100% pupation. Feeding test 1 ended on day 19 and feeding test 2 was terminated on day 20.

Larval mortality, pupal weight, and percent pupation of surviving larvae were compared among treatments using the PC-SAS GLM procedure (SAS Institute Inc., Cary, NC). The response to the quantitative factor "rate of mycelia" was evaluated with single-degree-of freedom orthogonal polynomials.

Results

Larval Mortality

With one exception, as the percentage of *B. bassiana* and *M. anisopliae* mycelia incorporated into synthetic diets increased, mortality of *Helicoverpa zea* increased significantly (Figures 2-1, 2-2, 2-3) The exception came for those insects fed Ma 2-00 in the second test, where higher rates of mycelia had significantly less mortality (Figure 2-3).

In both the studies involving isolate Bb 11-98, insects fed the 5% rate had significantly higher mortalities than all other rates on every day tested (Table 2-1). Bb

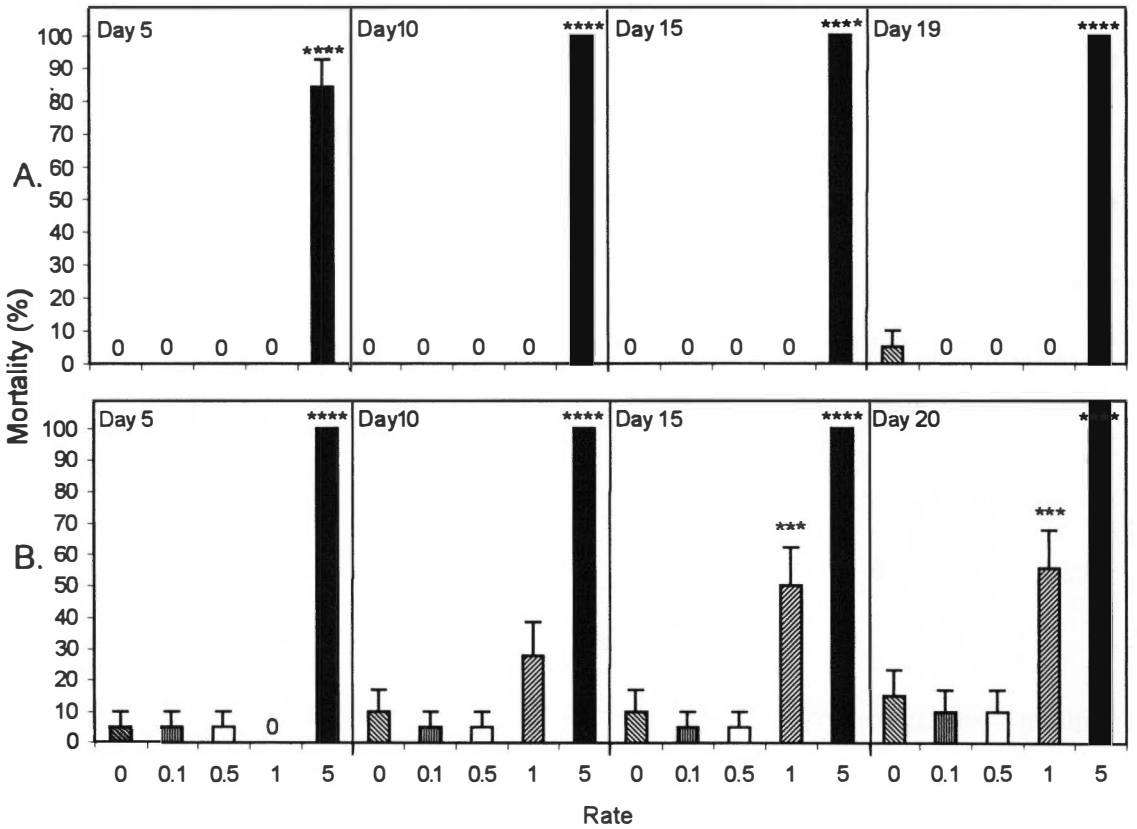

Figure 2-1. Percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 11-98 (A=1st test; B=2nd test). Error bars = standard error of the mean. In both tests, on each evaluation day, the effect of rate was significant. Using single-degree-of-freedom orthogonal contrasts, rates of mycelia were compared with the "0" rate control for the effects on larval mortality; *, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

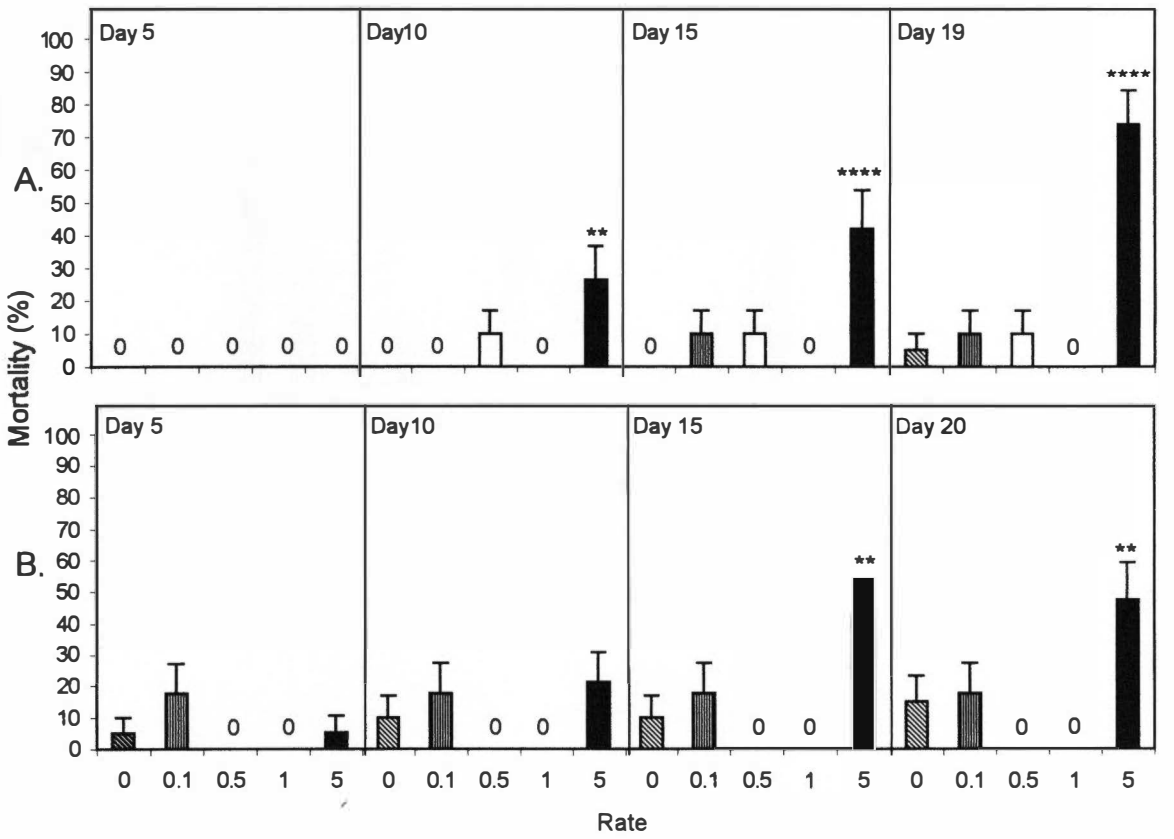

Figure 2-2. Percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 3-00 (A=1st test; B=2nd test). Error bars = standard error of the mean. The effect of rate was significant on Day 10, 15, and 19 in test 1, and on day 15 and 20 in test 2. Using single-degree-of-freedom orthogonal contrasts, rates of mycelia were compared with the "0" rate control for the effects on larval mortality; *, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

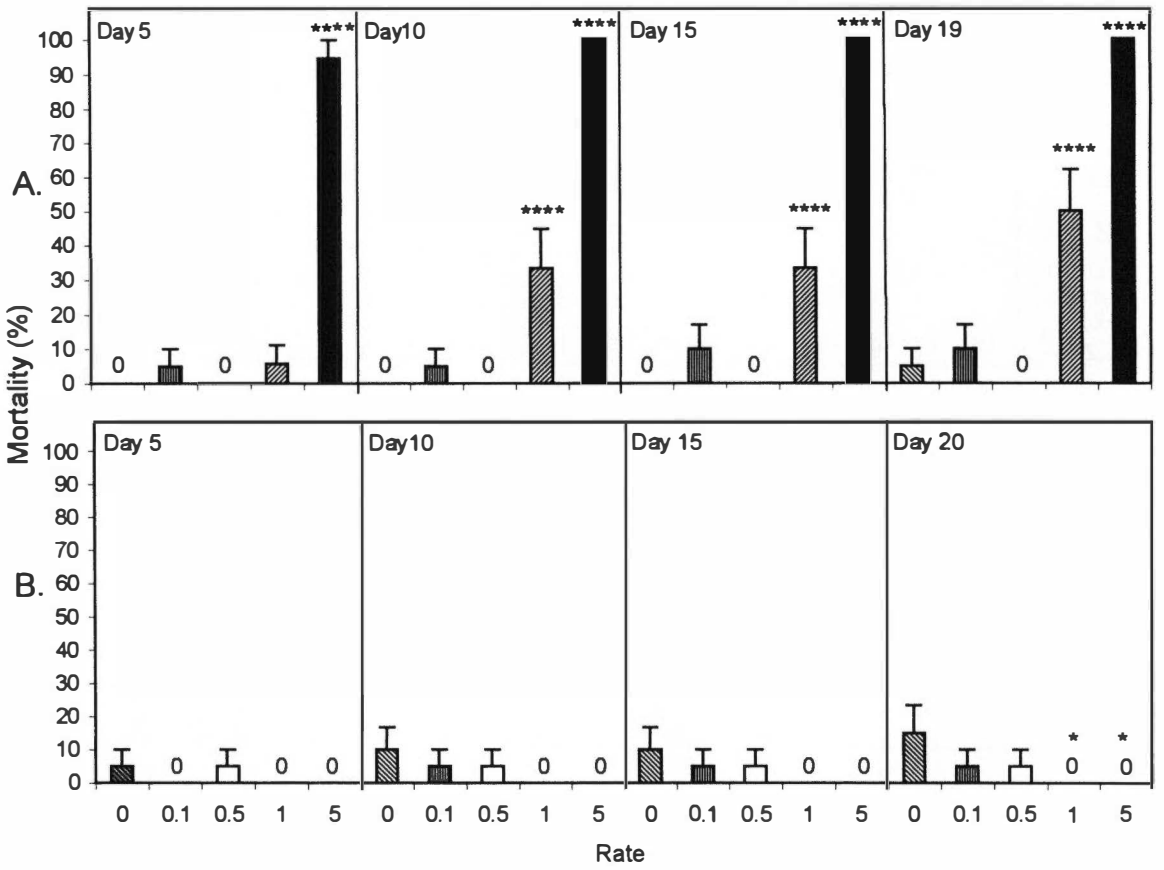

Figure 2-3. Percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Metarhizium anisopliae* isolate 2-00 (A=1st test; B=2nd test). Error bars = standard error of the mean. The effect of rate was significant on all days of test 1 but not in test 2. Using single-degree-of-freedom orthogonal contrasts, rates of mycelia were compared with the "0" rate control for the effects on larval mortality; *, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

Table 2-1. Rate contrast of percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 11-98.

Rate Contrast	Study 1				Study 2			
	Day 5	Day 10	Day 15	Day 19	Day 5	Day 10	Day 15	Day 20
0 vs. 0.1	NS	-	-	NS	NS	NS	NS	NS
0 vs. 0.5	NS	-	-	NS	NS	NS	NS	NS
0 vs. 1	NS	-	-	NS	NS	NS	***	***
0 vs. 5	****	****	****	****	****	****	****	****
0.1 vs. 0.5	NS	-	-	NS	NS	NS	NS	NS
0.1 vs. 1	NS	-	-	NS	NS	*	****	****
0.1 vs. 5	****	****	****	****	****	****	****	****
0.5 vs. 1	NS	-	-	NS	NS	*	****	****
0.5 vs. 5	****	****	****	****	****	****	****	****
1 vs. 5	****	****	****	****	****	****	****	****

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrasts.
- = values were 0 and contrasts could not be calculated.

11-98 at the 5% rate resulted in insects reaching 100% mortality by days 5 and 10, in the second and first tests, respectively (Figure 2-1). Insects fed lower rates of Bb 11-98 suffered no mortality in the first test and had low mortality in the second study, except for those insects fed the 1% rate, which reached 50% mortality by day 20 (Figure 2-1).

Insects fed diets containing Bb 3-00 at the 5% rate had significantly higher mortalities than Bb 3-00 diets at lower rates by day 10 and day 15, in the first and second tests, respectively (Table 2-2). Although insects fed the 5% rate of Bb 3-00 had high mortality, these insects suffered lower mortality than insects fed Bb 11-98 at 5%. Insects fed Bb 3-00 diets with low rates of mycelia exhibited low mortality (Figure 2-2).

Insects fed the *M. anisopliae* diets had low mortality, except in the first test where, by day 19, the 1% and 5% treatments had mortalities of 47% and 100%, respectively (Figure 2-3). Both the 1% and 5% rates had significantly higher mortality than all other rates of diet, in the first study (Table 2-3). The second study resulted in the 1% and 5% rates with mortalities significantly lower by day 20 than those insects fed the control diet (Table 2-3, Figure 2-3).

Larval Weights

In general, adjusted larval weight ratios of *H. zea* fed mycelia of *B. bassiana* and *M. anisopliae* were greatly reduced as concentrations of mycelia in diets increased (Tables 2-4, 2-5, 2-6). Larvae fed mycelia of Bb 11-98 followed this trend, but due to the loss of all larvae with the highest concentration of mycelia, growth rates could not be calculated (Table 2-4). Larvae fed the 1% rate were significantly smaller than insects fed

Table 2-2. Rate contrast of percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 3-00.

Rate Contrast	Study 1				Study 2			
	Day 5	Day 10	Day 15	Day 19	Day 5	Day 10	Day 15	Day 20
0 vs. 0.1	-	NS	NS	NS	NS	NS	NS	NS
0 vs. 0.5	-	NS	NS	NS	NS	NS	NS	NS
0 vs. 1	-	NS	NS	NS	NS	NS	NS	NS
0 vs. 5	-	**	****	****	NS	NS	**	**
0.1 vs. 0.5	-	NS	NS	NS	*	NS	NS	NS
0.1 vs. 1	-	NS	NS	NS	*	NS	NS	NS
0.1 vs. 5	-	**	**	****	NS	NS	*	**
0.5 vs. 1	-	NS	NS	NS	NS	NS	NS	NS
0.5 vs. 5	-	*	**	****	NS	*	****	****
1 vs. 5	-	**	****	****	NS	*	****	****

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrasts.
- = values were 0 and contrasts could not be calculated.

Table 2-3. Rate contrast of percent larval mortality by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Metarhizium anisopliae* isolate 2-00.

Rate Contrast	Study 1				Study 2			
	Day 5	Day 10	Day 15	Day 19	Day 5	Day 10	Day 15	Day 20
0 vs. 0.1	NS	NS	NS	NS	NS	NS	NS	NS
0 vs. 0.5	NS	NS	NS	NS	NS	NS	NS	NS
0 vs. 1	NS	****	***	****	NS	NS	NS	*
0 vs. 5	****	****	****	****	NS	NS	NS	*
0.1 vs. 0.5	NS	NS	NS	NS	NS	NS	NS	NS
0.1 vs. 1	NS	***	**	****	NS	NS	NS	NS
0.1 vs. 5	****	****	****	****	NS	NS	NS	NS
0.5 vs. 1	NS	****	****	****	NS	NS	NS	NS
0.5 vs. 5	****	****	****	****	NS	NS	NS	NS
1 vs. 5	****	****	****	****	NS	NS	NS	NS

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrasts.
- = values were 0 and contrasts could not be calculated.

Table 2-4. Development of larval *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* (Bb) isolate 11-98.

Isolate	Rate[a]	Adjusted larval weight ratio[b]	Larval growth rate (mg/day)	Number of larvae remaining on Day 15[c]	Adjusted pupal weight ratio[d]	Mean days to pupation	At conclusion of study		
							Number of larvae pupated	Number of larvae dead	Number of larvae remaining
Study 1									
Control	0	1.00 ± 0.0540	23 ± 1.2	7	1.00 ± 0.0359	14.8 ± 0.308	19	1	0
Bb 11-98	0.1	-[e]	-	0	1.08 ± 0.0267	13.5 ± 0.177	19	0	0
Bb 11-98	0.5	0.898 ± 0.0937	20 ± 2.1	12	0.896 ± 0.0387	15.7 ± 0.323	16	0	4
Bb 11-98	1	0.280 ± 0.0517	6.3 ± 1.2	20	-	-	0	0	20
Bb 11-98	5	-	-	0	-	-	0	19	0
Study 2									
Control	0	1.00 ± 0.0655	18 ± 1.2	7	1.00 ± 0.0318	15.5 ± 0.322	17	3	0
Bb 11-98	0.1	0.950 ± 0.0687	18 ± 1.3	10	1.09 ± 0.0405	15.5 ± 0.355	17	2	1
Bb 11-98	0.5	1.02 ± 0.0807	19 ± 1.5	19	1.12 ± 0.0882	18.6 ± 0.245	5	2	13
Bb 11-98	1	0.0484 ± 0.0184	0.90 ± 0.34	9	-	-	0	10	8
Bb 11-98	5	-	-	0	-	-	0	20	0

[a] Rate = Percent weight by volume of *Beauveria bassiana* isolate 3-00 mycelia incorporated into a synthetic diet.
[b] Adjusted larval weight ratio = mean weight of treatment larvae on day 15/ mean weight of larvae in the control.
[c] Number of larvae on Day 15 = n for calculations of adjusted larval weight and growth rate.
[d] Adjusted pupal weight ratio = mean weight of treatment pupae / mean weight of pupae in the control.
[e] Not determined.

Table 2-5. Development of larval *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* (Bb) isolate 3-00.

Isolate	Rate[a]	Adjusted larval weight ratio[b]	Larval growth rate (mg/day)	Number of larvae remaining on day 15[c]	Adjusted pupal weight ratio[d]	Mean days to pupation	At conclusion of study		
							Number of larvae pupated	Number of larvae dead	Number of larvae remaining
Study 1									
Control	0	1.00 ± 0.0540	23 ± 1.2	7	1.00 ± 0.0359	14.8 ± 0.308	19	1	0
Bb 3-00	0.1	1.19 ± 0.368	27 ± 8.3	2	1.00 ± 0.0325	13.4 ± 0.380	18	2	0
Bb 3-00	0.5	0.843 ± 0.0614	19 ± 1.4	12	0.758 ± 0.0363	14.9 ± 0.379	10	2	8
Bb 3-00	1	0.932 ± 0.133	21 ± 3.0	10	0.880 ± 0.0416	15.0 ± 0.469	14	0	6
Bb 3-00	5	0.00763 ± 0.00251	0.17 ± 0.057	11	-[e]	-	0	14	5
Study 2									
Control	0	1.00 ± 0.0655	18 ± 1.2	7	1.00 ± 0.0318	15.6 ± 0.322	17	3	0
Bb 3-00	0.1	1.00	19	1	1.35 ± 0.0698	14.4 ± 0.199	14	3	0
Bb 3-00	0.5	1.08 ± 0.110	20 ± 2.0	8	1.20 ± 0.0807	15.1 ± 0.373	20	0	0
Bb 3-00	1	1.18 ± 0.108	22 ± 2.0	5	1.48 ± 0.0682	15.0 ± 0.254	19	0	1
Bb 3-00	5	0.0419 ± 0.00612	0.78 ± 0.11	11	-	-	0	9	11

[a] Rate = Percent weight by volume of *Beauveria bassiana* isolate 3-00 mycelia incorporated into a synthetic diet.
[b] Adjusted larval weight ratio = mean weight of treatment larvae on day 15/ mean weight of larvae in the control.
[c] Number of larvae on Day 15 = n for calculations of adjusted larval weight and growth rate.
[d] Adjusted pupal weight ratio = mean weight of treatment pupae / mean weight of pupae in the control.
[e] Not determined.

Table 2-6. Development of larval *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Metarhizium anisopliae* (Ma) isolate 2-00.

Isolate	Rate[a]	Adjusted larval weight ratio[b]	Larval growth rate (mg/day)	Number of larvae remaining on day 15[c]	Adjusted pupal weight ratio[d]	Mean days to pupation	At conclusion of study		
							Number of larvae pupated	Number of larvae dead	Number of larvae remaining
Study 1									
Control	0	1.00 ± 0.0540	23 ± 1.2	7	1.00 ± 0.0359	14.8 ± 0.308	19	1	0
Ma 2-00	0.1	0.833 ± 0.0523	19 ± 1.2	7	0.797 ± 0.0403	15.1 ± 0.347	18	2	0
Ma 2-00	0.5	0.802 ± 0.107	18 ± 2.4	20	0.713	19.0	1	0	19
Ma 2-00	1	0.0716 ± 0.0309	1.6 ± 0.70	12	-	-	0	9	9
Ma 2-00	5	-[e]	-	0	-	-	0	19	0
Study 2									
Control	0	1.00 ± 0.0655	18 ± 1.2	7	1.00 ± 0.0318	15.6 ± 0.322	17	3	0
Ma 2-00	0.1	0.608	11	1	1.66 ± 0.0413	12.9 ± 0.235	18	1	1
Ma 2-00	0.5	1.06	20	1	1.61 ± 0.0539	12.7 ± 0.297	19	1	0
Ma 2-00	1	1.58 ± 0.000539	29 ± 0.010	2	1.64 ± 0.0391	13.3 ± 0.315	20	0	0
Ma 2-00	5	1.25 ± 0.152	23 ± 2.8	7	1.62 ± 0.0656	14.9 ± 0.228	19	0	1

[a] Rate = Percent weight by volume of *Metarhizium anisopliae* isolate 2-00 mycelia incorporated into a synthetic diet.

[b] Adjusted larval weight ratio = mean weight of treatment larvae on day 15/ mean weight of larvae in the control.

[c] Number of larvae on Day 15 = n for calculations of adjusted larval weight and growth rate.

[d] Adjusted pupal weight ratio = mean weight of treatment pupae / mean weight of pupae in the control.

[e] Not determined.

lower rates. In the first test, the lowest rate of Bb 11-98 had no larvae on day 15, due to pupation, and a larval growth rate could not be calculated (Table 2-4).

For insects fed Bb 3-00 there was a significant decrease in the adjusted larval weight ratios in the 5% treatments (Table 2-5). In the first test, larvae fed the 5% rate had the lowest recorded adjusted larval weight ratio (0.00763 ± 0.00251) and larval growth rate (0.17 ± 0.057 mg/day). The second study had a similar result; insects fed the 5% rate had an adjusted larval weight ratio of 0.0419 ± 0.00612. In the first study, larvae fed the 0.1% rate had the highest adjusted larval weight ratio (1.19 ± 0.368) of all treatments containing Bb 3-00.

In the first study, treatments containing *M. anisopliae* had the same general trend, with the 1% rate having a significantly lower adjusted larval weight ratio (0.0716 ± 0.0309) than insects fed the lower rates (Table 2-6). The 5% treatment, in the first study, had no larvae remaining by day 15 and a growth rate could not be calculated. The trend was reversed in the second study, where insects fed increased percent mycelia had the larger adjusted larval weight ratios. The 1% rate produced the largest larvae, which had an adjusted larval weight ratio of 1.58 ± 0.000539. The lowest adjusted larval weight ratio (0.608) in the second study came from the 0.1% group, which had only one remaining larva on day 15.

Pupation of Surviving Larvae

In the first study, insects fed Bb 11-98 at the three highest rates (0.5, 1, and 5%) had significantly lower pupation rates than the control diet-fed insects by day 15 (Figure 2-4). The lowest rate of diet (0.1%) had a significantly higher percent pupation than

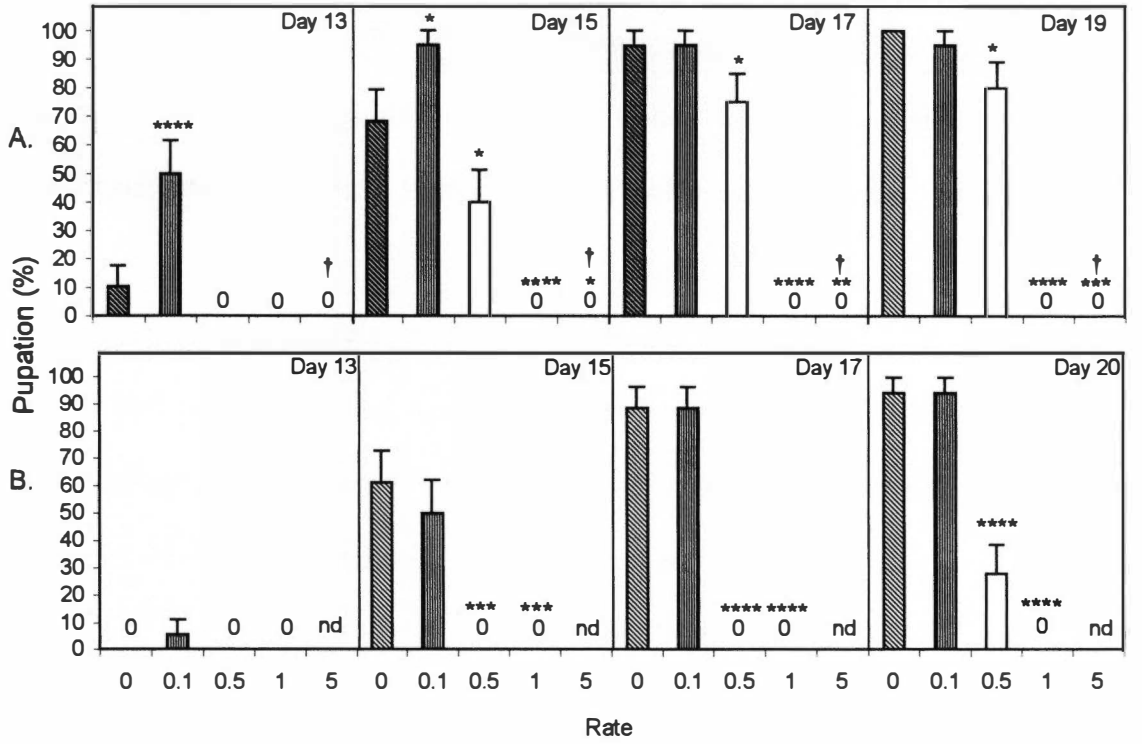

Figure 2-4. Percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 11-98 (A=1st test; B=2nd test). Error bars = standard error of the mean. In test 1, the effect of rate was significant ($P < 0.0001$) on all days. In test 2, rate was significant on day 15, 17, and 20 at $P < 0.0001$. Using single-degree-of-freedom contrasts, treatments were compared with the "0" rate control. *, **, ***, **** = significant at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively. nd = not determined due to death of all larvae. † = only one surviving larvae.

insects fed control diet by day 13 (50%), but by day 17 the control group had reached 95% and the 0.1% rate was no longer significantly higher (Figure 2-4, Table 2-7). The second test had insects in the 0.5% and 1% treatments with significantly lower percent pupation by day 15 and the 5% rate had no larvae pupate, therefore no comparisons could be made.

Insects fed Bb 3-00, in the first study, had significantly less larvae pupating than the control fed insects in all treatments by day 17, except the lowest rate (0.1%), which had 100% pupation by day 17 (Figure 2-5) (Table 2-8). The second study had only one treatment (5%) with significantly less larvae pupating than the control. All other rates had high percent pupation ranging from 82 to 100%.

In the first test involving Ma 2-00, all rates except the 0.1% treatment had significantly lower percent pupation than the control by day 15 (Figure 2-6) (Table 2-9). The 0.1% rate resulted in 100% of the larvae pupating. All Ma 2-00 fed insects in the second study had the highest combined percent pupation of all treatments in all studies. All treatments had at least 95% pupation by day 17, resulting in no significant differences at the end of the study.

Pupal Development

Pupal weights of insects fed Bb 11-98 were lowest in the first test, where the 0.5% treatment had an adjusted pupal weight ratio of 0.896 ± 0.0387 (Table 2-4). In the second study, the 0.5% rate had the largest adjusted pupal weight ratio (1.12 ± 0.0882). In both tests, the two highest rates had no pupae to weigh and the 0.5% rate had the longest days to pupation (Table 2-4).

Table 2-7. Rate contrast of percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 11-98.

Rate Contrast	Study 1				Study 2			
	Day 13	Day 15	Day 17	Day 19	Day 13	Day 15	Day 17	Day 20
0 vs. 0.1	****	*	NS	NS	NS	NS	NS	NS
0 vs. 0.5	NS	*	*	*	NS	****	****	****
0 vs. 1	NS	****	****	****	NS	***	****	****
0 vs. 5	NS	*	**	***	-	-	-	-
0.1 vs. 0.5	****	****	*	NS	NS	***	****	****
0.1 vs. 1	****	****	****	****	NS	**	****	****
0.1 vs. 5	NS	**	**	***	-	-	-	-
0.5 vs. 1	NS	**	****	****	NS	NS	NS	*
0.5 vs. 5	NS	NS	*	**	-	-	-	-
1 vs. 5	NS	NS	NS	NS	-	-	-	-

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrasts.
- = values were 0 and contrasts could not be calculated.

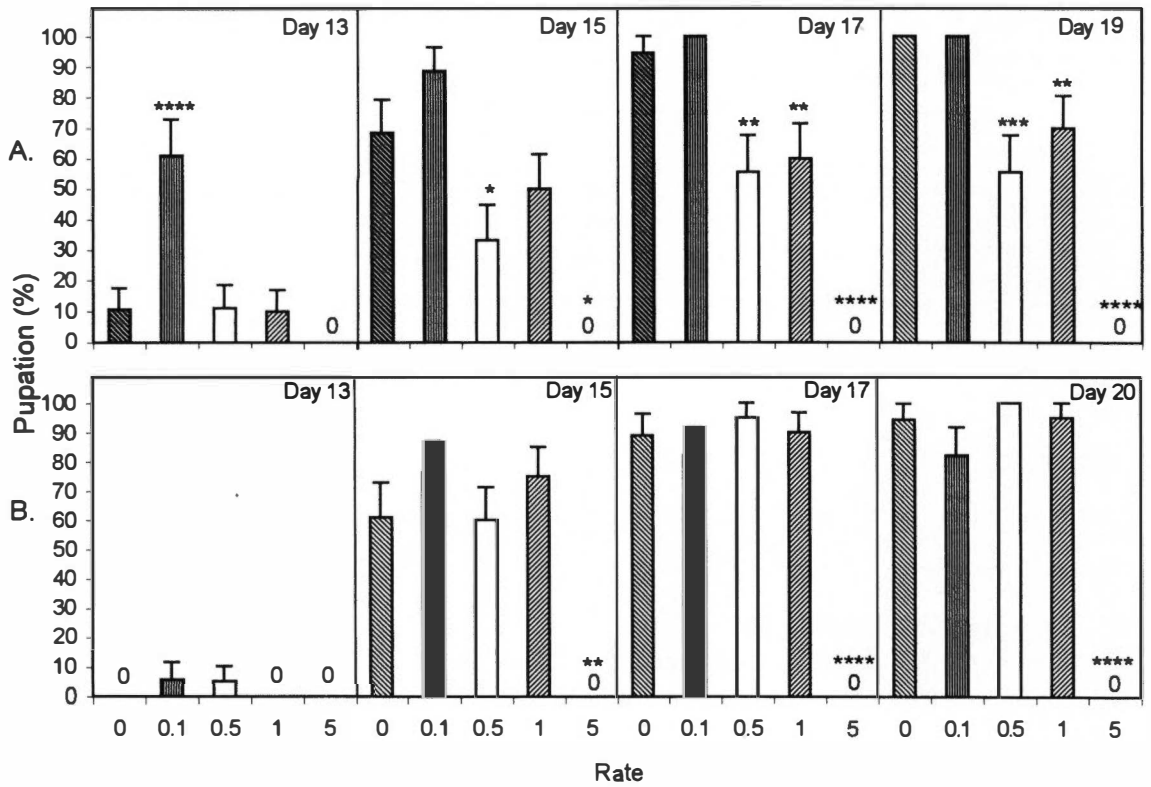

Figure 2-5. Percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 3-00 (A=1st test; B=2nd test). Error bars = standard error of the mean. In test 1, the effect of rate was significant on day 13, 17, and 19 at $P \leq 0.0001$ and on day 15 at $P = 0.0006$. In test 2, rate was significant on day 15 ($P = 0.015$), 17 and 20 ($P \leq 0.0001$). Using single-degree-of-freedom contrasts, the effect of treatment on percent pupation was compared with the "0" rate control. *, **, ***, **** = significant at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

Table 2-8. Rate contrast of percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Beauveria bassiana* isolate 3-00.

Rate Contrast	Study 1				Study 2			
	Day 13	Day 15	Day 17	Day 19	Day 13	Day 15	Day 17	Day 20
0 vs. 0.1	****	NS	NS	NS	NS	NS	NS	NS
0 vs. 0.5	NS	*	**	***	NS	NS	NS	NS
0 vs. 1	NS	NS	**	**	NS	NS	NS	NS
0 vs. 5	NS	*	****	****	NS	**	****	****
0.1 vs. 0.5	****	***	***	***	NS	NS	NS	*
0.1 vs. 1	****	**	**	**	NS	NS	NS	NS
0.1 vs. 5	**	***	****	****	NS	****	****	****
0.5 vs. 1	NS	NS	NS	NS	NS	NS	NS	NS
0.5 vs. 5	NS	NS	**	***	NS	**	****	****
1 vs. 5	NS	NS	**	****	NS	****	****	****

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrasts.
- = values were 0 and contrasts could not be calculated.

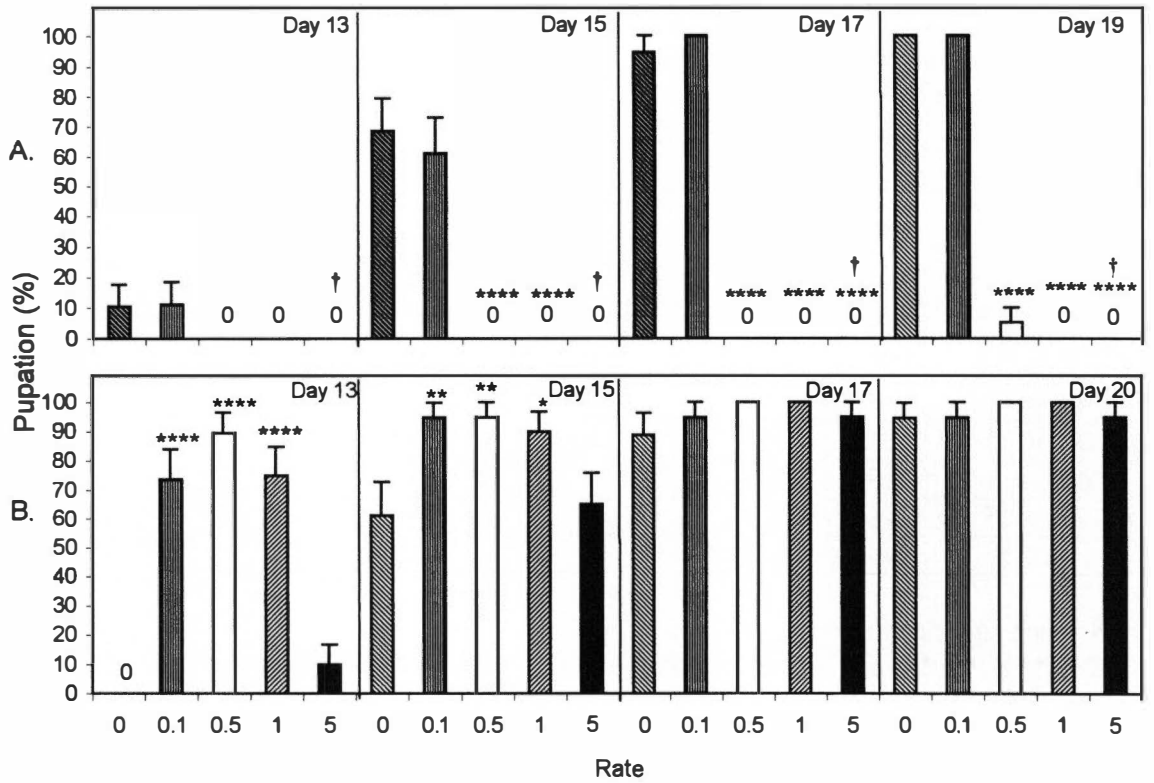

Figure 2-6. Percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Metarhizium anisopliae* isolate 2-00 (A=1st test; B=2nd test). Error bars = standard error of the mean. In test 1, the effect of rate was significant on day 15, 17, and 19 at $P \leq 0.0001$. In test 2, the effect of rate was significant on Day 13 ($P \leq 0.0001$) and 15 ($P = 0.0095$). Using single-degree-of-freedom contrasts, the effect of treatment on percent pupation was compared with the "0" rate control. *, **, ***, **** = significant at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively. † = only one surviving larvae.

Table 2-9. Rate contrast of percent pupation by day of *Helicoverpa zea* fed synthetic diets containing 0, 0.1, 0.5, 1, or 5% (weight/volume) mycelia of the entomopathogenic fungus *Metarhizium anisopliae* isolate 2-00.

Rate Contrast	Study 1				Study 2			
	Day 13	Day 15	Day 17	Day 19	Day 13	Day 15	Day 17	Day 20
0 vs. 0.1	NS	NS	NS	NS	****	**	NS	NS
0 vs. 0.5	NS	****	****	****	****	**	NS	NS
0 vs. 1	NS	****	****	****	****	*	NS	NS
0 vs. 5	NS	NS	****	****	NS	NS	NS	NS
0.1 vs. 0.5	NS	****	****	****	NS	NS	NS	NS
0.1 vs. 1	NS	***	****	****	NS	NS	NS	NS
0.1 vs. 5	NS	NS	****	****	****	*	NS	NS
0.5 vs. 1	NS	NS	NS	NS	NS	NS	NS	NS
0.5 vs. 5	NS	NS	NS	NS	****	*	NS	NS
1 vs. 5	NS	NS	NS	NS	****	*	NS	NS

NS = not significantly different.
*, **, ***, **** = significant differences at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively, based on single-degree-of-freedom orthogonal contrast.
- = values were 0 and contrasts could not be calculated.

When percent mycelia in the diet was increased, insects fed Bb 3-00 in the first study, had decreased pupal weight and an increase in days to pupation (Table 2-5). In the second study all treatments had larger adjusted pupal weight ratios than the control group. The largest adjusted pupal weight ratio (1.48 ± 0.0682) occurred in the 1% treatment, in which 19 insects pupated (Table 2-5).

Insects fed Ma 2-00 had differing results in the two tests. The first test had lower adjusted pupal weight ratios in all treatments when compared to the control group (Table 2-6). In the second test, all treatments had considerably larger pupae than the control. The largest adjusted pupal weight ratio occurred in the 0.1% treatment (1.66 ± 0.0413). In both tests, mean days to pupation tended to increase with more mycelia in the diets.

Discussion

Although negative effects produced by the ingestion of entomopathogenic fungi are expected, insects feeding on diets with mycelia may actually gain nutritional resources from the fungus. When compared to insects fed fungus-free diets, some insects fed diets containing the entomopathogens *B. bassiana* and *M. anisopliae* experienced increases in weights of pupae and larvae. Insects that had larger pupal and larval weights occurred mainly in those fed diets containing low concentrations of fungal mycelia. Developmental time to pupation was also shorter in insects fed diets that resulted in additional weight gain. Weight gains and shorter developmental times may indicate that the diets were supplemented by the presence of the fungus, or by metabolites remaining inside or on the fungal mycelia. These findings remain consistent with those of Lavy and Verhoef (1996), where collembola feeding on hyphae of *Cladosporium cladosporioides*,

which contained varying levels of nitrogen, grew faster and had increased body weight, when feeding on the higher nitrogen diets.

The anticipated deleterious effects were observed in insects fed diets that contained high concentrations of the isolates of entomopathogenic fungi. Deleterious effects, which included high mortality and delayed development of corn earworms, may be attributed to the toxicity of the fungal mycelia or metabolites produced by the fungus. Similar results were found by Lartey et al. (1989), where the toxic effects of fungal metabolites or direct parasitism by the fungus caused high mortality of collembola feeding on plant pathogens. Delayed development of some larvae may also be attributed to starvation of some insects that avoided diets containing high concentrations of fungal mycelia, such as the high number of insects fed Bb 11-98 at 1% remaining in the larval stage. Some insects that died, were observed to be stuck to the plastic cups by a translucent fluid, which emanated from the anus of the insect. These observations may be similar to those noted by Ahmad et al. (1985), where house crickets, *Acheta domesticus* (Linnaeus), suffered from complete failure of the alimentary process due to feeding on perennial ryegrass infected with *Neotyphodium loliae* [(Latch, Christensen and Samuels) Glenn, Bacon & Hanlin comb. nov.], previously known as *Acremonium loliae*.

When pupae were allowed to develop in a rearing cage, the emerging adults were observed to suffer from reproductive abnormalities. While normal *Helicoverpa zea* would lay eggs singly on a suitable surface, some individuals were observed to lay masses of eggs, surrounded by several adults. These adults were adhered to the mass of eggs, probably from the glue-like substance secreted by the adult to attach eggs to plant

surfaces. Although this behavior may be the result of the inability to regulate egg laying, it may also be due to the lack of a suitable surface of a host plant.

Based on this study, it is probable that insects feeding on entomopathogenic fungi may be subjected to both the toxic effects of metabolites and the increased nutrition provided by the fungus. Insects fed diets with low concentrations of mycelia tend to benefit from the increased nutrition and low toxicity of the diet. As concentrations of entomopathogenic mycelia increased in diets fed to corn earworms, the insects had delayed development. Insects fed diets with the highest concentrations of fungi suffered detrimental effects due to increases in toxicity, which outweighed the added nutrition and usually resulted in the death of the corn earworm larvae.

In the present study, toxicity of fungal diets may be dependent on the isolates that are evaluated. Insects fed diets containing Bb 11-98 suffered higher mortality, and developed more slowly than those fed diets containing isolate Bb 3-00. Differences in the effects of different isolates may be attributed to the relative amounts of toxins produced by each isolate. Variations in production of toxic metabolites have been documented for a variety of entomopathogenic fungi and are not unusual (Strasser et al. 2000).

Variation in mortality and pupation results observed in insects fed diets containing Ma 2-00 indicate that, by the time of the second test, toxicity of these diets were greatly reduced. This reduction in toxicity may be attributed to the breakdown of fungal metabolites during storage and may indicate that the use of older preparations of entomopathogenic fungi for control of insects by oral toxicity may not be effective and may even enhance the development of insects feeding on the fungus. This reduction in toxicity was observed only in the Ma 2-00 diet and may indicate that *M. anisopliae* toxins

are less stable than those of *B. bassiana*. If *M. anisopliae* toxins breakdown quickly, this fungus may not be well suited for this mechanism of control.

Based on this study, it is likely that in order for entomopathogenic fungi to be used for insect control where the pests are killed by feeding or deterrence from feeding on diets containing entomopathogenic fungi, the amounts of fungus needed would be too large to be practical. Other isolates of entomopathogenic fungi may be selected for this type of application, if they produce higher concentrations of toxins or other deleterious metabolites. On the other hand, although not directly addressed in this study, control of insect herbivory on plants may be attained by endophytic colonization of entomopathogenic fungi, as observed by Lewis and Bing (1991).

In summary, the major findings of this experiment were that *Helicoverpa zea* feeding on mycelia of entomopathogenic fungi may receive extra nutrition or be subjected to deleterious effects from the fungal diet. The effects of the mycelial diet are isolate dependent, and different concentrations of mycelia in diet influence the growth and development of larvae. Further testing with isolates may be necessary to select more toxic ones which are better suited for this type of insect control.

Chapter 3

Effects of *Beauveria bassiana* metabolites incorporated into synthetic diets on larval *Helicoverpa zea.*

Introduction

Beauveria bassiana has been the focus of many studies to evaluate its potential as a biological control agent (Jaros-Su et al. 1999, Mc Dowell et al. 1990, Poprawski et al. 2000). A new method of applying *B. bassiana* provides protection from herbivory through endophytic colonization. In studies involving endophytic *B. bassiana*, protection against insect pests has been attributed to direct infection of the fungus or to insect ingestion of metabolites produced in the plant tissues by *B. bassiana* (Bing and Lewis 1991).

Feeding of insects on fungal mycelia has been shown to alter development and lower survivorship (Broza et al. 2001, Lartey et al. 1989, Lavy and Verhoef 1996). In addition, toxins produced by *B. bassiana*, such as beauvericin and bassianolide, have been shown to cause deleterious effects when ingested by insects (Gupta et al. 1991, Kanaoka et al. 1978,). The objective of this study was to evaluate the effects of ingested *B. bassiana* metabolites produced during liquid culture fermentation, on corn earworms, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), fed a synthetic diet into which fungus-free culture broths of *B. bassiana* isolates had been incorporated.

Materials and Methods

Propagation and Collection of Entomopathogenic Fungi

The entomopathogenic fungi included in this study were *Beauveria bassiana* (Bb) isolate 11-98 obtained from an infected click beetle (Coleoptera: Elateridae) collected in Scott County, TN. *Beauveria bassiana* isolate 8-99 obtained from a cadaver of *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) collected from an unknown locality, *B. bassiana* isolate 1-00, and *B. bassiana* isolate 3-00. The latter isolates were cultured from Japanese beetle cadavers [*Popillia japonica* (Coleoptera: Scarabiidae)] collected in Warren County, TN. Fungi were grown on Sabouraud dextrose agar + 0.5% yeast extract (SDAY), incubated at 25°C for approximately 3 weeks. Conidia were harvested by brushing the surface of the plates with a stenciling brush and passing the conidia through a #100-mesh sieve (150-µm opening).

Fermentation Culture of Entomopathogens

Fermentation broth was prepared by adding 2.5 g yeast extract and 2.5 g dextrose to 250 ml de-ionized water in a 500-ml Erlenmeyer flask. Flasks with medium were autoclaved and cooled before inoculation with conidia. Inoculated flasks were incubated in a shaker (150-200 RPM) for 4-5 days at room temperature.

The fungal isolates were cultured in a BIOFLO 2000 fermenter. The fermenter vessel contained 7-10 L of de-ionized water with 1% dextrose and 1% yeast extract. After autoclaving the vessel with liquid medium, the fermenter was incubated at 24°C, with 200-RPM agitation, and 4 L compressed air/min aeration. The medium was agitated and aerated for 1 day to confirm sterility. After this period, approximately 100 ml of

fungal inoculum was injected into the fermenter vessel and the fungus was incubated for 7 days.

The mycelia were removed by vacuum filtration through a #18-mesh sieve (100-μm opening) covered with #4 Whatman filter paper. The filtered broth was collected in 1-L bottles and refrigerated (4.5°C) until used in preparation of diet.

Preparation of Diet

Artificial beet armyworm diet mix (Product #F9219) was obtained from Bio-Serv Inc. (Frenchtown, NJ). Agar (2 g) was added to boiling water (15 mL) and the solution was heated until clear. The 82 mL volume of de-ionized water that was required to formulate the diet were substituted, in part, with experimentally pre-determined volumes of fermentation broth to achieve the total volume. Volumes of water not substituted were added to the diets, which were cooled before addition of fermentation broth. The agar solution was placed in a blender with 16.12 g of dry beet armyworm diet mix. The mixture was blended thoroughly and cooled. Diets were prepared for isolates Bb 11-98, Bb 8-99, Bb 3-00, and Bb 1-00 at the fermentation broth concentrations corresponding to the volumes of broth that the 0.1 and 0.5% mycelial concentrations, used in experiments described in Chapter 2, would have produced. In addition a broth control diet (DYE) was prepared using uninoculated broth (1% dextrose and 1% yeast extract) at 0.1% and 0.5%.

Quantities of broth to be incorporated into synthetic diet were calculated by taking the total volume of filtered broth and dividing by the total dry weight of mycelia harvested from the fermenter, when the respective isolate was produced. The resulting volume of broth per gram of mycelia was used to calculate the volume of broth that

would contain metabolites equivalent to the addition of 0.1% and 0.5% (w/v) mycelia as used in diets described in Chapter 2. Diets corresponding to 1% and 5% would require too much fermentation broth incorporated into diet, and could not be prepared without significantly changing the diet texture and firmness. For example, isolate Bb 11-98 produced 66 g of mycelia in 7 L of fermentation broth (7000 ml / 66 g = 106 ml broth/g mycelium). Because 0.5 g of mycelia were needed to prepare 100 ml of the 0.5% diet described in Chapter 2, 53 ml of fermentation broth (106 x 0.5 = 53 ml) were used to prepare 100 ml of a fermentation-broth diet corresponding to the 0.5% mycelia diet. The fermentation broth diet corresponding to the 0.1% mycelia for this same isolate received 10.6 ml of the broth. For isolates 8-99, 1-00, and 3-00; 100, 108, and 108 ml, respectively, of fermentation broth were needed to produce 1 gram of mycelia. DYE control diet contained 10 ml of uninoculated broth per 100 ml of diet in the 0.1% treatment and 50 ml per 100 ml of diet for the 0.5% treatment. Diets with cultured broth were mixed vigorously, poured into Petri dishes, and stored in a refrigerator (4.5°C) until used.

Insects

Corn earworm, *Helicoverpa zea* (Boddie), eggs were obtained from Agripest (Zebulon, NC). Approximately 75-100 eggs were placed in each of several Petri dishes on filter paper moistened with de-ionized water. Petri dishes were placed in an incubator at 25°C for 2 days or until larvae hatched. Neonate larvae were collected for use in experiments.

Diet Feeding Tests

Two diet feeding tests were performed. The experiments were designed as 2 x 5 factorials in a randomized complete block design with two rates of fermentation metabolites (0.1 and 0.5%) and five metabolite treatments: four culture broths from fungi (Bb 8-99, Bb 11-98, Bb 3-00, and Bb 1-00) and a fungal metabolite-free control broth (DYE) treatment. Each neonate corn earworm was placed into a 29.6 cm^3 plastic cup. Plugs of the diet were prepared with a cork borer (1-cm inside diameter). One plug of diet was added to each cup. Each treatment combination had 20 replicates; each replicate consisted of a single insect in a cup. Cups were placed in an incubator at 24°C. Additional diet was added to the cups when needed, to provide an excess of diet and maintain freshness. Larvae were observed daily and mortality, pupation and pupal weight were recorded. When one of the treatments first reached 100% pupation among the surviving insects, larval weights were recorded across all treatments. This occurred on Day 18 in both feeding tests. The experiment was terminated after the surviving insects in the control treatment reached 100% pupation. The first test was terminated on Day 22 and the second test on Day 20.

Larval mortality, weight and growth rate of larvae, days to pupation, pupal weight, and percent pupation of surviving larvae were compared among treatments using the PC-SAS MIXED procedure (SAS Institute Inc., Cary, NC). Significant effects were further analyzed with a Fisher's-protected least significant difference test at $P = 0.05$.

Results

Larval Mortality

Insects fed diets containing *B. bassiana* metabolites had low mortality in both tests. Across all isolates, the main effect of rate was significant ($F = 8.30$, df = 1,171, $P = 0.0045$) for larval mortality in the first test with greater mortality in the 0.5% than in the 0.1% treatments (14% versus 3%) (Table 3-1). The broth control (DYE) diets caused no mortality (Table 3-1). The highest mortalities in treatments with *B. bassiana* fermentation broths occurred in the 0.5% treatments of Bb 11-98 and Bb 8-99, both with 25% mortality (Table 3-1).

There were no significant differences in mortality in the second test (Table 3-2). The highest mortality (20%) occurred in insects fed the Bb 3-00 treatment at 0.5%. Insects fed Bb 1-00 broth at 0.5% had no mortality. All other treatments had between 5% and 15% mortality.

Larval Weights and Growth Rates

There were no significant differences among treatments for larval weights (data not shown) or larval growth rates for either test (Tables 3-1 and 3-2).

Days to Pupation

In the first test the effects of rate ($F = 13.58$; df = 1,139; $P = 0.0003$), isolate ($F = 10.00$, df = 4,139, $P < 0.0001$), and the interaction (F = 3.58, df = 4,139, $P = 0.0082$) were significant for mean days to pupation (Table 3-1). In the

Table 3-1. Development of larval *Helicoverpa zea*, in the first test, fed synthetic diets containing 0.1 or 0.5% dextrose yeast extract (DYE) or fermentation broth from the production of *Beauveria bassiana* (Bb) isolates 8-99, 11-98, 3-00, and 1-00.

Isolate	Rate[a]	Percent mortality on Day 22[b,c]	Mean days to pupation[b,d]	Percent pupation of surviving larvae on Day 22[b,e]	Larval growth rate (mg/day)[b]	Weight of pupae (mg)[b]
DYE	0.1	0.0 ± 6.0 b	15.1 ± 0.379 f	100 ± 6.06 a	-[f]	340 ± 12.3
	0.5	0.0 ± 6.0 b	15.7 ± 0.400 ef	90.0 ± 6.06 abc	19 ± 3.5	323 ± 13.0
Bb 8-99	0.1	5.0 ± 6.0 b	17.1 ± 0.400 bcd	94.7 ± 6.21 ab	25 ± 4.2	307 ± 13.4
	0.5	25 ± 6.0 a	17.4 ± 0.453 bc	94.1 ± 6.97 abc	18 ± 2.4	313 ± 14.7
Bb 11-98	0.1	0.0 ± 6.0 b	16.3 ± 0.379 cde	100 ± 6.06 a	20 ± 4.2	313 ± 12.3
	0.5	25 ± 6.0 a	18.7 ± 0.453 a	93.8 ± 6.97 abc	20 ± 4.2	305 ± 14.7
Bb 3-00	0.1	0.0 ± 6.0 b	16.1 ± 0.389 def	95.0 ± 6.06 a	25 ± 4.2	344 ± 12.7
	0.5	10 ± 6.0 ab	17.9 ± 0.453 ab	77.8 ± 6.37 abc	20 ± 2.3	309 ± 14.7
Bb 1-00	0.1	10 ± 6.0 ab	16.0 ± 0.411 def	94.6 ± 6.37 abc	22 ± 4.2	334 ± 13.4
	0.5	10 ± 6.0 ab	15.7 ± 0.453 ef	77.6 ± 6.38 c	23 ± 3.0	333 ± 14.7
	0.1	3.0 ± 2.7 b	16.1 ± 0.175 b	96.9 ± 3.12 ab		
	0.5	14 ± 2.7 a	17.1 ± 0.198 a	86.7 ± 3.28 b		
DYE			15.4 ± 0.275 b			
Bb 8-99			17.2 ± 0.302 a			
Bb 11-98			17.5 ± 0.295 a			
Bb 3-00			17.0 ± 0.299 a			
Bb 1-00			15.9 ± 0.306 b			

[a] Rate = Percent volume of *Beauveria bassiana* fermentation broth or DYE incorporated into a synthetic diet.

[b] Values = mean ± standard error.

[c] Significant differences based on the effect of rate ($F = 8.30$, df = 1,171, $P = 0.0045$). Analysis was performed using the Mixed procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

[d] Significant differences based on rate ($F = 13.58$, df = 1,139, $P = 0.0003$), isolate ($F = 10.0$, df = 4,139, $P < 0.0001$), and the rate + isolate interaction ($F = 3.58$, df = 4,139, $P = 0.0082$). Analysis was performed using the Mixed

Table 3-1. Continued.

procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

[e] Significant differences based on the effect of rate ($F = 6.86$, df = 1,154, $P = 0.0097$). Analysis was performed using the Mixed procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

[f] No remaining larvae.

Table 3-2. Development of larval *Helicoverpa zea*, in the second test, fed synthetic diets containing 0.1 or 0.5% dextrose yeast extract (DYE) or fermentation broth from the production of *Beauveria bassiana* (Bb) isolates 8-99, 11-98, 3-00, and 1-00.

Isolate	Rate[a]	Percent mortality on Day 20[b]	Mean days to pupation[b,c]	Percent pupation of surviving larvae on Day 20[b,d]	Larval growth rate (mg/day)[b]	Weight of pupae (mg)[b,f]
DYE	0.1	15 ± 6.3	14.1 ± 0.328 d	100 ± 7.32 a	-[e]	345 ± 12.4 abc
	0.5	10 ± 6.3	14.1 ± 0.329 d	100 ± 7.11 a	-[e]	350 ± 12.0 abc
Bb 8-99	0.1	10 ± 6.3	15.3 ± 0.338 bc	94.4 ± 7.11 a	20 ± 4.1	352 ± 12.4 abc
	0.5	5.0 ± 6.3	15.2 ± 0.338 bc	89.5 ± 6.92 a	27 ± 3.3	350 ± 12.4 abc
Bb 11-98	0.1	10 ± 6.3	15.4 ± 0.338 bc	94.4 ± 7.11 a	22 ± 3.9	347 ± 12.4 abc
	0.5	5.0 ± 6.3	16.9 ± 0.421 a	57.9 ± 6.92 b	21 ± 1.9	353 ± 15.4 abc
Bb 3-00	0.1	5.0 ± 6.3	15.1 ± 0.329 bc	94.7 ± 6.92 a	24 ± 6.0	378 ± 12.0 a
	0.5	20 ± 6.3	16.0 ± 0.441 ab	62.5 ± 7.54 b	16 ± 2.4	324 ± 16.1 bc
Bb 1-00	0.1	5.0 ± 6.3	14.6 ± 0.329 cd	94.7 ± 6.92 a	23 ± 6.0	360 ± 12.0 ab
	0.5	0.0 ± 6.3	15.6 ± 0.329 b	90.0 ± 6.75 a	19 ± 2.4	319 ± 12.0 c
	0.1		14.9 ± 0.150 b	95.7 ± 3.17 a		356 ± 5.47 a
	0.5		15.6 ± 0.168 a	80.0 ± 3.16 b		339 ± 6.13 b
DYE			14.1 ± 0.236 c	100 ± 5.10 a		
Bb 8-99			15.3 ± 0.239 b	92.4 ± 4.83 ab		
Bb 11-98			16.2 ± 0.270 a	92.0 ± 4.96 ab		
Bb 3-00			15.6 ± 0.275 ab	78.6 ± 5.12 bc		
Bb 1-00			15.1 ± 0.232 b	76.2 ± 4.96 c		

[a] Rate = Percent volume of *Beauveria bassiana* fermentation broth or DYE incorporated into a synthetic diet.

[b] Values = mean ± standard error.

[c] Significant differences based on the effects of rate ($F = 9.04$, df = 1,132, $P = 0.0032$) and isolate ($F = 8.94$, df = 4,132, $P < 0.0001$). Analysis was performed using the Mixed procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

[d] Significant differences based on the effects of rate ($F = 12.33$, df = 1,154, $P = 0.0006$), isolate ($F = 4.00$, df = 4,154, $P = 0.00041$), and the rate + isolate interaction ($F = 2.96$, df = 4,154, $P = 0.0217$). Analysis was performed using the

Table 3-2. Continued.

Mixed procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

[e] No remaining larvae.

[f] Significant differences based on the effect of rate ($F = 4.48$, df = 1,132, $P = 0.0361$). Analysis was performed using the Mixed procedure of PC-SAS (SAS Institute Inc., Cary, NC). Values followed by the same letter are not significantly different based on a Fisher's-protected least significant difference test at $P = 0.05$.

second test, both isolate ($F = 8.94$, df = 4,132, $P < 0.0001$) and rate ($F = 9.04$, df = 1,132, $P = 0.0032$) were significant, but the interaction was not (Table 3-2). In both tests, insects fed 0.5% diets had longer developmental times (Tables 3-1 and 3-2). Across both rates, broth from 11-98 resulted in the longest developmental time in both tests (Tables 3-1 and 3-2). For diets with broth from isolate 11-98 except 3-00, there was a significant difference between the 0.1 and 0.5% rates in both tests (Tables 3-1 and 3-2).

In the first test, the shortest time to pupation was 15.1 days observed in the treatment with diet containing DYE at 0.1% (Table 3-1). The longest time to pupation (18.7 days) was observed for insects fed Bb 11-98 broth at 0.5%; this value was significantly longer than all treatments except the diet containing isolate 3-00 at 0.5% (Table 3-1). At the 0.1% rate, diets with Bb 8-99 or Bb 11-98 broth had longer days to pupation than the DYE diet. At 0.5%, diets containing broth from all fungal isolates except Bb 1-00, had significantly longer days to pupation than those insects fed the DYE diet.

In the second test, developmental times were shorter for all treatments in relation to those observed in the first test. The shortest developmental times occurred in the treatments with DYE at 0.5% and 0.1% with 14.1 days to pupation (Table 3-2). The insects fed broth from Bb 11-98 at 0.5% had the longest time to pupation (16.9 days) (Table 3-2). At 0.1% broth from isolates 8-99, 11-98, and 3-00 had longer developmental times that insects fed the DYE control diet (Table 3-2). At 0.5%, insects on diets with broth from all of the fungal isolates had a greater number of days to pupation that insects fed the DYE diet (Table 3-2). Across both rates, the DYE diets had significantly fewer days to pupation than diets containing broth from Bb isolates.

Pupation of Surviving Larvae

In the first test, the effect of rate was significant ($F = 6.86$, df = 1,154, $P = 0.0097$) for percent pupation of surviving larvae on Day 22. Insects fed the 0.5% diets had significantly lower percent pupations than those fed diets at 0.1% (87 versus 97%). All surviving insects pupated that were fed 0.1% DYE, and the diets containing isolates 11-98 at 0.1% (Table 1). The lowest percent pupation occurred in insects fed Bb 1-00 at 0.5% (78%). For Bb 3-00, percent pupation was significantly lower for those fed 0.5% diet than for 0.1% (Table 1).

In the second test the effects of rate ($F = 12.33$, df = 1, 154, $P = 0.0006$), isolate (, $F = 4.00$, df = 4,154, $P = 0.0041$), and the interaction of rate and isolate ($F = 2.96$, df = 4,154, $P = 0.0217$) were significant. All insects fed the DYE diets pupated (Table 2-2). Two treatments, Bb 11-98 at 0.5% and Bb 3-00 at 0.5%, had significantly lower percent pupation than all other treatments with 58% and 63%, respectively. All diets, except for DYE diets, exhibited the same trend as in the first test, where 0.5% diets resulted in lower percent pupations, however the difference was significant for Bb 11-98 and Bb 3-00 only.

Pupal Weights

Statistical analysis of pupal weights revealed no significant differences among all treatments in the first test. All weights for both tests were between 305 and 378 mg per pupae. In the second test, the effect of rate was significant ($F = 4.48$, df =1,132, $P = 0.0361$); the diets at the 0.1% rate resulted in significantly larger pupal weights than the

the 0.5% diets (356 versus 339 mg). Isolate Bb 3-00 and Bb 1-00 had significantly smaller pupae for the 0.5% diets than the 0.1% diets (Table 2).

Discussion

Development of most insects fed diets containing *B. bassiana* metabolites in fermentation broths was delayed. Insects fed diets with the highest concentrations of *B. bassiana* metabolites had significantly longer developmental times to pupation, possibly due to fungal toxins or waste products in the diet. Percent pupation was also lower for insects fed diets with higher rates of fungal broth. Similar studies have demonstrated that *B. bassiana* metabolites may cause mortality at high doses (Gupta et al. 1991, Kanaoka et al. 1978); although mortality in the present study was low, the amounts of deleterious metabolites in the fermentation broths was unknown and may not have been high enough to be effective in killing insects through ingestion.

In this study, positive effects were observed for insects feeding on diets containing uninoculated culture broths. Insects fed the control DYE diets experienced shorter times to pupation, which may be attributed to the increased nutrition of the diet. Insects may also prefer the DYE diets and consume more food.

The low mortality among insects fed diets containing *B. bassiana* metabolites indicates that insect control using orally administered toxins may not be practical with these isolates. Insects experiencing increased developmental times could compound pest problems if larvae continue to feed over a longer time period. Isolates with increased toxicity may prove to be effective in this type of insect control, if the metabolites produced by the isolate caused mortality or deterred feeding.

Chapter 4

Detection of endophytic *Beauveria bassiana* in tomato plants using PCR and ITS primers.

Introduction

Traditionally, biological control with the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin was thought to involve direct infection of the insect by conidia as the sole mode of action (Jaros-Su et al. 1999, McDowell et al. 1990, Poprawski et al. 2000). However, *B. bassiana* has been reported to suppress tunneling of the European corn borer *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) by growing into and throughout the plant as an endophyte (Bing and Lewis 1991). Suppression of tunneling was attributed to direct infection by the fungus or to metabolites produced in the plant tissues by the fungus. A later investigation confirmed fungal colonization of plants by light and electron microscopy (Wagner and Lewis 2000). In general, *B. bassiana* has been detected by plating samples on selective media (Bing and Lewis 1991, 1992a, 1992b, 1993; Doberski and Tribe 1980). This technique may be unreliable due to contamination of plates and competition from other microorganisms in the samples. More recent techniques utilizing polymerase chain reactions, (PCR) and gel electrophoresis have been used to detect *B. bassiana* in the cadavers of the migratory grasshopper, *Melanoplus sanguinipes* (Fabricus) (Hegedus and Khachatourians 1996). Species detection and identification in more complex systems containing a variety of organisms may be performed using PCR, internally transcribed spacer (ITS) region primers, and gel electrophoresis. ITS regions are segments of genetic code that lie between highly

conserved ribosomal RNA genes. These internal regions evolve quickly and are therefore suitable for taxonomic determination at the species level (White et al. 1990). Using ITS primers, a 650-bp PCR product was identified for tomato (Marshall 1999), and a 550-bp product was identified for *B. bassiana* (Shih et al. 1995). Sequence analysis of the PCR products allows species level identification.

The objectives of this study were to determine if *B. bassiana* could colonize tomato endophytically following application to seed, and to determine if the fungus could be detected from tomato shoot tissues with gel electrophoresis, and sequence analysis of PCR products using ITS primers.

Materials and Methods

Preparation of *B. bassiana*

Beauveria bassiana (Bb) isolate 11-98 was isolated from an infected click beetle (Coleoptera: Elateridae), collected in Scott County, TN. Isolate 11-98 was plated on Sabouraud Dextrose Agar + 0.5% yeast extract (SDAY) and incubated at 25°C for approximately 3 weeks. Conidia were harvested by brushing the surface of the plates with a stenciling brush and passing the conidia through a #100-mesh sieve (150-µm opening).

Seed Treatment

Tomato seeds (*Lycopersicon esculentum* Mill.) 'Mountain Spring' (seed lot F8895A) were coated with a mixture of methylcellulose and *B. bassiana* conidia. Methylcellulose solution was prepared by autoclaving 1 L of de-ionized water for 30 min. The hot water was placed on a stirring plate and 20 g of methylcellulose (Sigma, St.

Louis, MO) was added with stirring until a suspension was formed. The 2% methylcellulose suspension was placed in an ice bath until the solution cleared.

Tomato seed was obtained from Novartis Seeds Inc. (Downers Grove, IL). Approximately 40 seeds were placed in a 50-ml beaker. Based on the seed weight (0.096 g), a 2:1 w/v aliquot of 2% methylcellulose solution (0.048 ml) was added to the beaker and mixed with the seeds. Conidia of *B. bassiana* (0.005 g) were added to the beaker and stirred until the coating of seeds was uniform. Seeds were spread on aluminum foil and air-dried in a vertical flow hood for approximately 3 hours.

Growth of Seedlings

Thirty test tubes (19-mm outside diameter and 150-mm in length) were filled with 10 cm^3 of vermiculite and 7 ml of de-ionized water. The test tubes were sealed with plastic Kim-Kaps (Fisher Scientific., Pittsburgh, PA), and autoclaved for 30 min on 2 successive days. Test tubes were allowed to cool and transferred to a vertical flow hood. Tubes were uncapped and seeds were placed approximately 0.5 cm under the surface of the vermiculite. Twenty replicate treated seeds and ten untreated seeds were planted. Tubes were recapped and placed in an incubator with a 12 hour light/ dark cycle at 24°C, and maintained for 14 days under gnotobiotic conditions. Tubes were transferred to a vertical flow hood, where tubes were broken at the base, and the root section was cut at the soil line and discarded. The shoots were washed with water and immediately subjected to the DNA isolation procedure.

Isolation of Plant and Fungal DNA

Individual tomato shoots were placed in a mortar and pestle and covered with liquid nitrogen. The shoot tissue was ground, and liquid nitrogen was added to ensure that the tissue did not thaw. DNA was isolated using a PureGene kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, the ground shoot material was subjected to a cell lysis solution (600 µL) and incubated for 1 h at 65°C. After centrifugation, a protein precipitation solution (200 µL) was added. The DNA was elutriated with 100% isopropanol and then with 70% ethanol. The alcohol was then allowed to evaporate and the DNA was rehydrated with de-ionized water. DNA concentrations were determined on a TD 360 Mini-Flourometer (Turner Designs Instruments, Sunnyvale, CA) at 360 nm according to manufacturer's instructions. DNA samples were stored in a -80°C ultra-low freezer until needed.

Polymerase Chain Reaction

The polymerase chain reaction was performed in a Peltier thermalcycler PTC-200 (MJ Research, Inc., Watertown, MA). The appropriate PCR primers; ITS1, 5' TCCGTAGGTGAACCTGCGG 3', and ITS4, 5' TCCTCCGCTTATTGATATGC 3' (White et al. 1990), were obtained from Dr. Karen Hughes (The University of Tennessee, Knoxville). The PCR reaction mixture contained 5 µl of AmpliTaq buffer (PE Applied Biosystems, Foster City, CA), 5 µl of a 2 mM mixture of dCTP, dGTP, dTTP, and dATP, 5 µl of 3 µM ITS1 primer, 5 µl of 3 µM ITS4 primer, 3 µl of 25 mM $MgCl_2$, 25.5 µl of de-ionized H_2O, 0.5 µl AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) and 1 µl of sample (10 ng/µl). This mixture was placed in a 500-µl

eppendorf tube and a drop of mineral oil was added to reduce condensation. The tube was placed into the thermalcycler and the amplification procedure was conducted. Reaction parameters were as follows; 95°C for 9 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a final 3-min period at 72°C. The reaction mixture was held at 4°C until the tube was removed. Products were removed from the tubes and electrophoretically separated on a 1.5% (w/v) agarose-TBE (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA) gel.

Ten shoots from treated seeds and eight from untreated seeds were tested for the presence of *B. bassiana* DNA. For comparisons, PCR products from three untreated plants and three treated plants were placed on an agarose gel. A negative control with all reaction components except the DNA sample was included in each gel to ensure no DNA contamination of the PCR constituents. A positive control of DNA from *B. bassiana* isolate 11-98 was included in each gel. The positive control DNA was sequenced at The University of Tennessee Molecular Biology Resource Facility and was confirmed to be *B. bassiana*. A 1Kb+ DNA ladder (Invitrogen Life Technologies, Carlsbad, CA) was included to determine the size of PCR products from treated and untreated plants. Gels were electrophoresed at 80 volts until bands had separated properly. The gels were stained with ethidium bromide and visualized with a Fisher Biotech 312-nm transilluminator FBTI 816 (Fisher Scientific, Pittsburgh, PA).

DNA Sequence Analysis

Bands were excised from the gels, and processed with a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) using the manufacturer's instructions. Products were

then sent to The University of Tennessee Molecular Biology Resource Facility, and sequenced with ITS1 and ITS4 primers.

Results

All plants shoots, with or without *B. bassiana* seed treatment, produced a single PCR product band of approximately 650-bp (Figure 4-1). In addition, shoots from all treated plants produced a second band of approximately 550-bp (Figure 4-1). Direct sequencing of the 550-bp PCR product from the pure *B. bassiana* 11-98 positive control (Figure 4-2) and the 550-bp PCR product from treated plants produced the same sequence. This sequence is almost identical to the published ITS region (Shih et al. 95) but lacks 4 bps due to 4 single bp deletions and a substitution at 360 bp. The 550-bp PCR product was entered into a GenBank Blast search and had 100% homology to the sequence of *Beauveria bassiana* isolate Bb2515. The sequence of the 650-bp PCR product was entered also into a GenBank Blast search and was confirmed to be the ITS region of tomato (data not shown).

Discussion

Based on PCR amplification, gel electrophoresis, and direct sequencing of DNA from treated and untreated plants in this study, the use of ITS primers will be an important tool in confirming the presence of *Beauveria bassiana* within a system containing a mixture of tomato plant and fungal DNA. Although this procedure can detect the occurrence of the fungal DNA, it cannot determine the specific location of fungus. Real-time PCR could be used to determine the exact location of *B. bassiana* within tomato tissue.

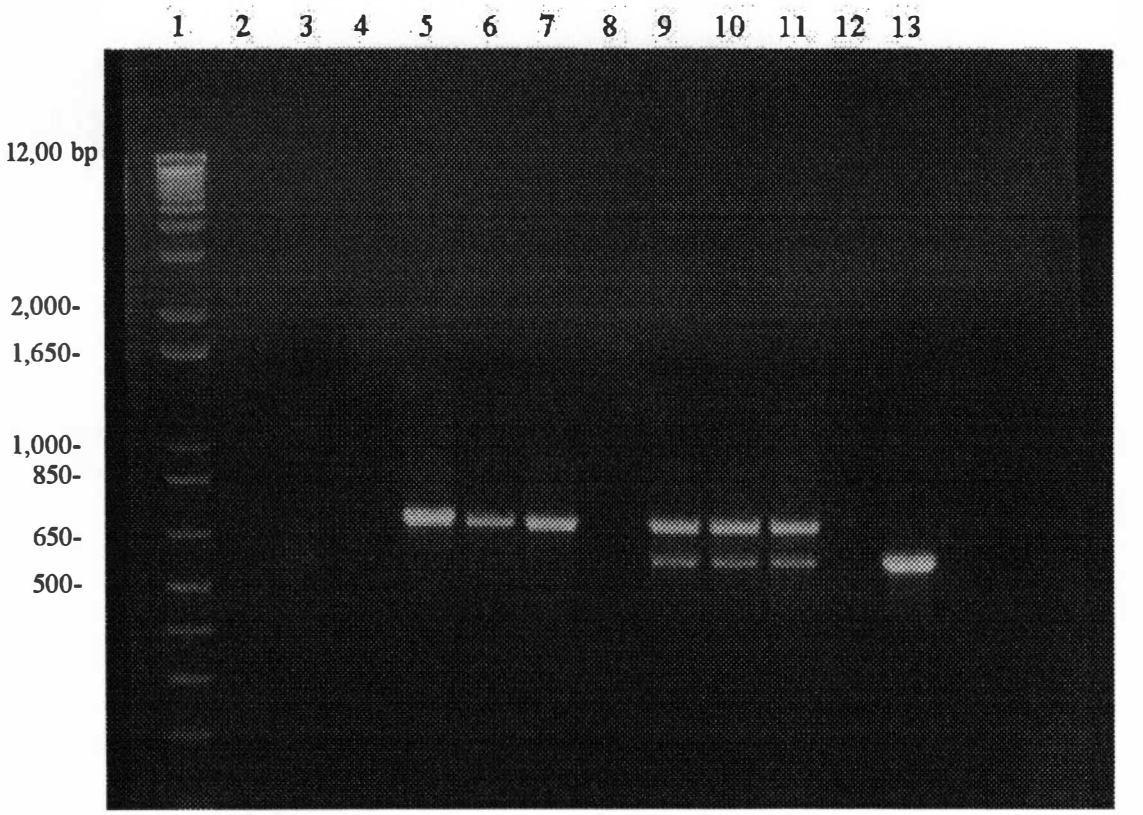

Figure 4-1. Amplification of DNA from treated and untreated plants using ITS1 and ITS4 primers. Samples of products analyzed by electrophoretic separation on a 1.5% agarose gel. The 1Kb + DNA ladder was co-electrophoresed as a size standard (Marker). Lane 1: 1Kb + DNA ladder (Invitrogen Life Technologies, Carlsbad, CA); Lane 2: blank lane; Line 3: blank control; Line 4: blank lane; Lines 5-7: tissue from untreated plants containing one 650-bp product; Lane 8: blank lane; Lanes 9-11: tissue from treated plants containing one 650-bp product and one 550-bp product; Lane 12: blank lane; and Lane 13: *B. bassiana* isolate 11-98 control containing one 550-bp product.

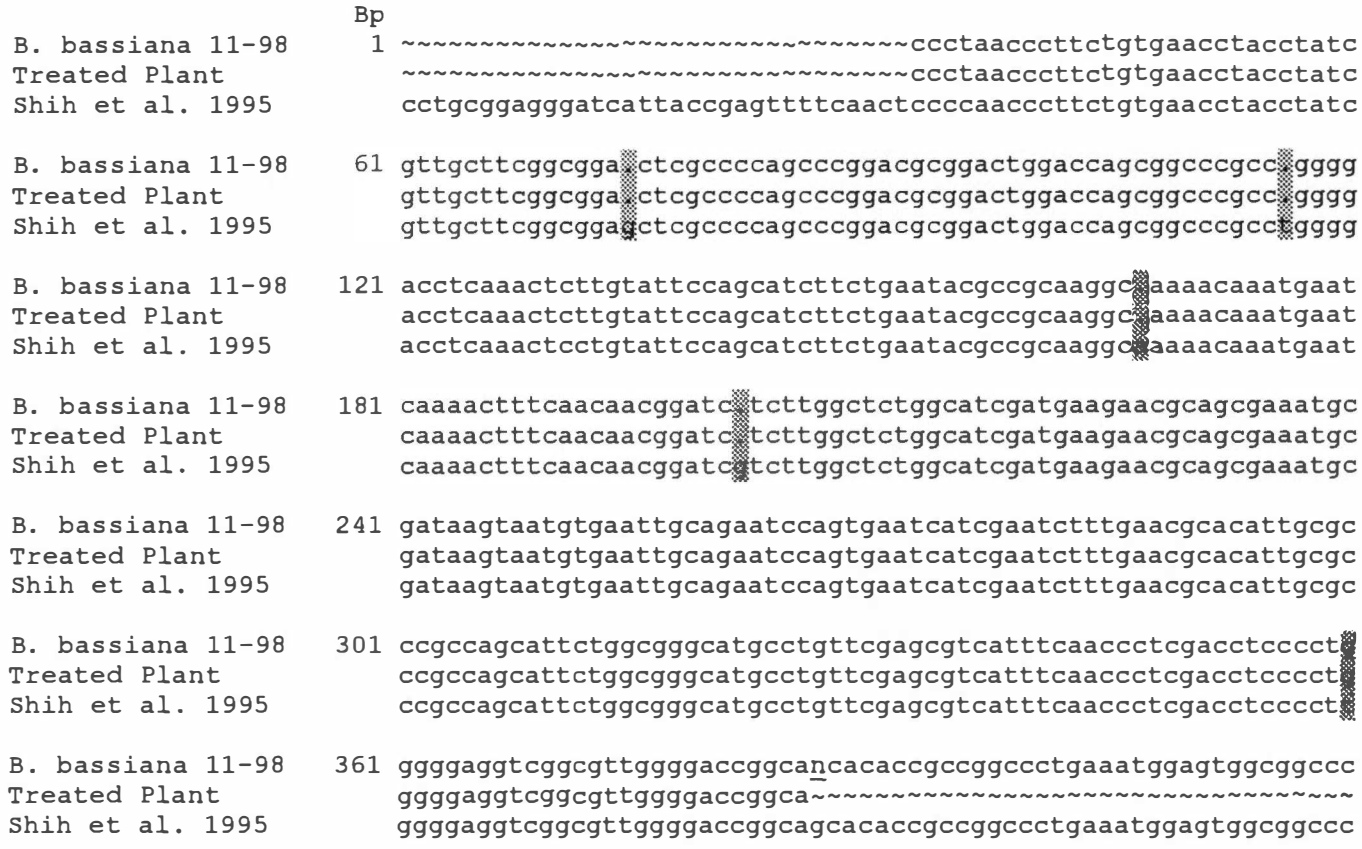

Figure 4-2. Sequence and comparison of internal regions of ITS1 and ITS4 PCR products from *B. bassiana* isolate 11-98, fungal PCR product from tomato treated with *B. bassiana* (Treated Plant), and published ITS region from *B. bassiana* (Shih et al. 1995). Differing nucleotides in sequences are shaded. Undetermined nucleotides are represented by n.

In this study, only the seeds were treated with *B. bassiana* and great care was taken to avoid passive transport of conidia to the upper portions of the shoot. Only seedlings that did not have the seed coat adhering to the cotyledons during development were selected for analysis. Further, shoots were removed for the assay, which eliminated disturbance of the root system.

Wagner and Lewis (2000) observed both endophytic and epiphytic colonization of corn by *B. bassiana*. It is likely that this fungus similarly colonizes tomato. To ensure that only endophytic fungal DNA was collected by this procedure, a control should be added to this protocol that includes an isolation of DNA from the surface of the tomato plant.

This technique does confirm that coating tomato seed with *B. bassiana* conidia is an effective means of introducing this fungal endophyte to the plant. Further work utilizing electron microscopy and histological techniques are needed to substantiate the ability of *B. bassiana* to colonize and grow endophytically throughout tomato plants.

Chapter 5

Summary

In these studies, experiments were conducted to evaluate the ability of *Beauveria bassiana* to endophytically colonize tomato plants and to determine the potential effects of the endophyte when fed to insects. Feeding tests using segments of colonized tomato plants failed. To try and evaluate the possible effects, varying rates of mycelia and spent culture broth were incorporated into synthetic diets and fed to larval corn earworms, *Helicoverpa zea*.

PCR amplification of DNA from the aerial part of seed-treated tomato seedlings produced a 650-bp and a 550-bp product. This confirmed the presence of the fungus in an area of the plant in which to was not introduced. Although it is possible that fungal spores were carried passively up the stem during growth of the plant, the seedlings were washed to remove potential contaminants. Histological work by Wagner and Lewis (2000), demonstrated that the main mode of entry for the fungus in corn to grow through stomata and other natural opening. If viable spores were to be carried up the stem, it is possible that they served as a secondary inoculant. Further work to resolve these questions would include histological verification that the spores will germinate and grow into the plant, and electron microscopy to verify that the fungus grows throughout the plant.

The feeding tests indicated that at low levels, the presence of fungal mycelia might actually benefit the insect by adding nutrition to the diet. At higher rates of fungus, the mycelial diets caused high mortality in larval corn earworms, and disrupted larval development. The quantity of fungal mycelia in a colonized corn plant is unknown and the quantities of mycelia found to negatively impact feeding larvae is relatively high. It is

unlikely that the rates of mycelia needed to lower insect herbivory would be found in the vascular system of tomato plants. This would imply that more than just the presence of mycelia is needed for control. This assumption is based on the fact that living mycelia would affect the larvae in the same way as dead mycelia. Since this is unlikely, testing is needed to evaluate the effects of live mycelia in the diets of insects.

To test the effects of metabolites of *B. bassiana* on corn earworms, spent culture broth was used to make evaluations. In all rates of metabolite diet, insects suffered low mortality, but did experience slower rates of development. The broth contained quantities of metabolites, waste products from the growth of the fungus, and unspent medium from the original fermentation broth. In a plant-endophyte system, the metabolites and wastes of the fungus would be present, but the unspent broth would not. Thus, the addition of nutrients may have outweighed the toxic effects of the metabolites and waste products, resulting in increased larval survivorship. Further testing involving extracted metabolites and waste products is needed to negate the effects of the unspent broth. Tests should also be conducted to determine the concentrations of these toxins in colonized plants in order to correctly design feeding experiments.

Based on these experiments, there is potential for the use of endophytic *B. bassiana* as a control for insects feeding on tomato plants. Although it is not unequivocally confirmed that the fungus will colonize the entire plant, it is likely that the fungus will grow into the shoot of the plant from treated seeds. If the production of metabolites in corn plants is the basis of the reduction of insect damage reported by Lewis and Bing (1991), then colonization of tomato shoots may be a sufficient amount of fungal growth to produce the metabolites needed to reduce herbivory. Further PCR work

involving testing of smaller sections of the aerial parts of colonized tomato plants is needed to determine the extent of colonization. To enhance the validity of these tests, a surface sterilization of the outside of the plant should be done to ensure no contaminants travel up the outside of the plant. Additionally, PCR of swab samples of the plant surface would detect epiphytic *B. bassiana*.

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Vita

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