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Potential of *Beauveria bassiana* 11-98 as a biological control agent against tomato pests; and detection of the mycotoxic metabolite beauvericin in tomato plants using HPLC.

A Thesis
Presented for the
Master of Science
Degree
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Wesley Aaron Powell
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Dedication

This thesis is dedicated to my major professor, Dr. Bill Klingeman, my sisters, Christi McNeely and Sarah Thompson, my parents, Marvin and Cathy Powell, my grandparents, Ailene Powell and the late Aaron Powell and Sally Beddingfield and the late C. E. Beddingfield, my fiance and her parents, Sabrina , Kieth and Silvia Roberts, Patrick Kimbrough, Tim Ewart and the rest of my family and friends. Thank you for your prayers, love, guidance, support and theropy during these past two years and throughout my life. I could not have made it without you.

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Abstract

Beauveria bassiana (Balsamo) Vuillemin is an entomopathogenic fungus with an extensive insect host range. The isolate *Beauveria bassiana* 11-98 also endophytically colonizes tomato and cotton plants. In our research, *B. bassiana* 11-98 was evaluated for its potential to control insect pests of tomato in laboratory assays. Potato aphids, *Macrosiphum euphorbiae* (Thomas) exposed to different formulations of *B. bassiana* 11-98 conidia in direct contact and contact-while-feeding assays using target concentrations of 1 x 10^4, 10^5, 10^6 and 10^7 conidia/ml. After exposure for 10 days, aphid mortality did not exceed 40% and rate of reproduction was not affected in any treatment. *B. bassiana* 11-98 provided insufficient control of aphid populations in our assays.

Foliage feeding tomato fruitworms, *Helicoverpa zea* (Boddie) were exposed to initial concentrations of 1 x 10^6 and 10^7 *Beauveria bassiana* 11-98 conidia/ml in direct contact and consumption of viable conidia assays. Larvae in all assays were observed for mycosis 5, 10 and 30 days after treatment (DAT). Larvae that consumed viable conidia had greater and more rapid mortality when compared to larvae in the topical application assay. The highest mortalities in topical application and consumption assays were 21 and 77%, respectively, observed 30 DAT.

A separate bioassay examined the effect of *H. zea* feeding on endophytic tomato foliage established by coating seeds with *B. bassiana* 11-98 and growing tomato seedlings under gnotobiotic conditions. Starved (24 h) larvae were fed foliage from 10-wk-old tomato plants in both choice and no-choice assays. In choice assays, larvae were offered leaf disks from treated and untreated plants and allowed to feed for 6 and 24 h. Larvae in no-choice assays were fed within bags for 48 and 120 h. While 3rd instar *H.*

zea larvae showed no clear preference for treated or untreated foliage, they exhibited a slight trend for less consumption of foliage from *B. bassiana* 11-98-treated plants. Larvae that fed on *B. bassiana* 11-98 -treated foliage did have mortality (17%) after feeding for 120 h.

Finally, foliage from 8-wk-old, endophytically-colonized tomato plants grown from *B. bassiana* conidia-coated seeds was assayed for presence of the fungus and beauvericin using mycological media cultures and high pressure liquid chromatography, respectively. In the fungal isolation assay, 3 leaves were collected from the top, middle and bottom sections of 40 plants treated with *B. bassiana* and 10% of those plants demonstrated endophytic growth on media cultures after 4 wk. The mycotoxic metabolite beauvericin was recovered from lateral foliage of 22 out of 26 eight-wk-old, endophytically-colonized tomato plants.

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Chapter 1

Literature Review

Beauveria bassiana

Beauveria bassiana (Balsamo) Vuillemin is a cosmopolitan, soil-borne entomopathogenic fungus in the phylum Deuteromycota. Known as white muscardine fungus, its characteristics include white mycelia with dry, hyaline conidia. Conidia are globose to oval in shape and can be produced either on the main hyphal branches or on a lateral, right-angled extension of a conidiophore. Flask shaped conidiophores can occur singly or be grouped in whorled clusters (Boucias and Pendland 1998; Steinhaus 1949). Conidia are the infectious stage of the life cycle on susceptible insect hosts and serve as fungal propagules.

A white muscardine fungus was observed in Japan, around 900 A.D. on silkworms, *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) (Boucias and Pendland 1998). A 25 million-year-old worker ant was found embedded in amber covered with a fungus similar to the present-day *B. bassiana* (Poinar and Thomas 1985). In silkworms, muscardine disease was attributed to meteorological conditions or rearing techniques. Not until the 1830's was the disease considered to be parasitic in nature and to be transmittable to other insects (Steinhaus 1949). Discovery of these facts is generally credited to Italian scientist Antonio Bassi de Lodi. In 1935, Bassi showed that the disease was caused by a fungus that multiplied on and in the body of the silkworm. These new findings established the germ theory of disease. The fungus was described by Balsamo, who named it *Botrytis paradoxa,* which was later changed to *Botrytis bassiana*

to honor Bassi. In 1912, Vuillemin changed the genus to *Beauveria* (Steinhaus 1949; 1975; Alexopolous 1996; Boucias and Pendland 1998).

Beauveria bassiana conidia infect susceptible insect hosts in one of two ways, through the cuticle or through the trachea. Most commonly, conidia contact and attach to the host's outer integument. Provided that temperature and humidity are optimal, conidia attach to the cuticle, germinate and produce a series of proteases, chitinases and lipases that aid in degradation of the cuticle. The germ tube penetrates the integument and grows toward the hemocoel. Once inside the hemocoel, the fungus produces mycotoxins, aiding in the formation of blastospores and after 60-70 h, tissue damage becomes visible in the gut and malphigian tubules (Boucias and Pendland 1998). *Beauveria bassiana* conidia have also been shown to infect susceptible hosts through tracheal openings or the alimentary tract (Steinhaus 1949). The fungus continues, with the help of mycotoxins, to grow inside the insect where death occurs within 6-7 days. Within 24-48 h of death, the cadaver of the insect is covered with white mycelia that have grown through the intersegmental membranes (Pekrul and Grula 1979). Outside the body of the insect, mycelia will produce conidiophores and conidia that will continue to infect other hosts.

Beauveria bassiana has an extremely wide range of susceptible insect hosts, more than any other deuteromycete, infecting individuals from most of the orders, at all stages of development (Boucias and Pendland 1998). Since 1925, when approximately 11 million pounds of cocoons of silkworms were lost in northern Italy due to this fungus (Steinhaus 1949), special attention has been given to *B. bassiana*. As a result of its extensive host range and its potential for mass devastation, *B. bassiana* has been the focus of many microbial control tests. Some successfully controlled insects include:

citrus root weevil, *Artipus floridanus* (Horn) (Coleoptera: Curculionidae) (Eyal et al. 1994), sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Homoptera, Aleryrodidae) (Eyal et al. 1994), lesser stalk borer, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) (McDowell et al. 1990), European corn borer, *Ostrinia nubilalis* (Hubner) (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). Also, due to increased popularity of microbial control, several large research projects have taken place in the past few decades, throughout Europe and Asia using *B. bassiana,* that have aided in the control of codling moth, *Cydia pomonella* (L.), European corn borer and Colorado potato beetle (McCoy et al. 1985).

Recent research has also shown that *B. bassiana* is a plant endophyte (Wagner and Lewis 2000). It has been isolated from corn, *Zea mays* (L.) (Bing and Lewis 1991; 1992a; 1992b; 1993) and potato, *Solanum tuberosum* (L.) (Jones 1994). A preliminary investigation has also confirmed, using polymerase chain reaction, that *B. bassiana* is an endophyte of tomato, *Lycopersicon esculentum* (Mill.) (Leckie 2002; Ownley et al. 2004).

Endophytic *B. bassiana* also has the potential to control arthropod populations and feeding damage. Tunneling by larval European corn borer was reduced by 50.6% in *B. bassiana* infected plants and the fungus acted as a systemic bioinsecticide for six to eight weeks (Bing and Lewis 1991). Subsequently, *B. bassiana* moved upward in the

plant through the vascular system suggesting potential for season-long protection from European corn borer (Bing and Lewis 1992). Wagner and Lewis (2000), demonstrated that the fungus enters the plant through natural stomatal openings or through direct penetration into epidermal cell walls when foliarly applied. No adverse affects to the plant due to the introduction of *B. bassiana* have been reported.

Bioinsecticides containing *B. bassiana* strain GHA are commercially available in the United States under trade names BotaniGard, OrganiGard and Mycotrol O (Emerald BioAgriculture Corp.) (Organic Materials Review Institute 2005). These products are labeled for control of ants, borers, whiteflies, thrips, aphids, mealybugs and various lepidopteran and coleopteran pests. Commercial products are formulated as conidial suspensions and applied as a foliar spray.

Toxins Produced by *Beauveria bassiana*

Throughout the insect infection process, *B. bassiana* produces toxins and metabolites to aid normal functions of the fungus. Mycotoxins produced by *B. bassiana* include beauvericin, a cyclic depsipeptide, has the ability to increase permeability of cell membranes and disrupt the function of intact cells (Boucias and Pendland 1998). Bassianolide, another cyclic depsipeptide, is toxic to lepidopteran larvae and oosporein is a red pigmented dibenzoquinone that acts as an antimicrobial agent allowing the fungus to proliferate inside an insect (Boucias and Pendland 1998). *Beauveria bassiana* also produces cyclosporin, a cyclic nonribosomal peptide that is an immunosuppressant and blocks the T-cell signal transduction pathway (Boucias and Pendland 1998). Mycotoxins may also potentially accumulate inside the plant and deter insect herbivory.

Recent research has examined the insect toxicity of the fungal metabolites of *B.*

bassiana. Quesada-Moraga and Vey (2003) injected seven strains of *B. bassiana* fermentation broth into locust, *Locusta migratoria* (L.). All but one were pathogenic, causing 57.5-94.4% percent mortality within 10 days. Ingestion of beauvericin by Colorado potato beetle larvae resulted in an LC_{50} of 633 ppm and LC_{90} of 1,196 ppm (Gupta et al. 1991). An injection of beauvericin into adults of blowfly, *Calliphora erythrocephala* (Meig.) (5μg/ml) and larvae of the mosquito, *Aedes aegypti* (L.) (10 μg/ml) resulted in 15% and 39% mortality after 48 h, respectively (Grove and Pople 1980). When a suspension (0.1 mg/ml) of beauvericin was added to water, 44% of larvae of the northern house mosquito, *Culex pipiens autogenicus* (Roubaud), were killed after 48 h. When examined with optical microscopy, tissues of all organs showed different levels of destruction, vacuolization and disorganization of muscle, while the greatest damage was observed in the midgut (Zizka and Weiser 1993). Topical applications of oosporein fermentation broth to hibiscus leaves infested with mealy bugs resulted in 49.8% mortality within 3 days (Eyal et al. 1994).

Entomopathogenic properties of pure *B. bassiana* metabolites have directed our attention to naturally-produced beauvericin. If endophytic *B. bassiana* does produce metabolites within plants, insect populations and/or feeding damage could be suppressed with only one application.

Fungivory by Insects

Feeding tests involving insects ingesting fungi have yielded promising results for the possibility of endophytic *B. bassiana* to control insect population. Worker termites, *Reticulitermes* sp., were placed to feed on whole cultures of *Metarhizium anisopliae*, *Gliocladium virens* and *B. bassiana.* All termites were dead within 1, 10 and 5 days,

respectively (Kramm and West 1982). *Serangium parcesetosum* (Sicard) (Coleoptera: Coccinellidae) larvae, a predator of whiteflies, were fed prey every day for 10 days that had been exposed, 24 h previous, to leaves sprayed with *B. bassiana* (approximately 1,000 conidia/mm). The number of emerging adult predators was reduced by 84.2%. In a separate assay, a single prey inoculated 24, 48, 72 and 96 h previous, reduced the number of emerging adult predators by 88.9, 71.4, 75.0 and 55.5%, respectively (Poprawski et al. 1998). The migratory grasshopper, *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) suffered 100% mortality when fed a mixture of wheat bran and 5.4×10^6 and 1.1×10^7 spore suspensions of *B. bassiana* (Jeffs et al. 1997). When a leaf disk (1.7 cm diameter) was treated with 40 µl of a conidial suspension of *B. bassiana*, LC_{50} and LC_{90} for third instar lesser cornstalk borers, *Elasmopalpus lignosellus* (Zeller) were 5.07×10^1 and 1.87×10^4, respectively (McDowell et al. 1990). Also, 80% mortality was observed in diamondback moth larvae, *Plutella zylostella* (L.) (Lepidoptera: Plutellidae) after 7 days, when a conidial suspension of *B. bassiana* was sprayed on broccoli plants at 625 spores/cm^2 (Vandenberg et al. 1998a). These studies demonstrate the potential for foliarly applied and endophytic *B. bassiana* to control insects that consume plant foliage.

Detection of *Beauveria bassiana* and Its Metabolites in Plants

Many fungi have the ability to outcompete *B. bassiana* in culture, which may make the fungus difficult to isolate. Doberski and Tribe (1980) developed a selective medium, which allows *B. bassiana* to grow at its optimum capacity. The most frequently reported method of detecting *B. bassiana* in plants has been to place samples of the plant on selective media (Doberski and Tribe 1980; Bing and Lewis 1991, 1992a, 1992b,

1993). Endophytes have also been detected by using electron and light microscopy (Clay 1987; Wagner and Lewis 2000). Recent studies have detected *B. bassiana* in tomato using polymerase chain reaction (PCR) (Leckie 2002; Ownley et al. 2004).

Fungal production of toxic metabolites or mycotoxins can be monitored using different procedures. In Europe, beauvericin and oosporein which are also produced by *Fusarium* species were detected using high-performance liquid chromatography with diode array detection (HPLC-DAD), gas chromatography mass spectrometry (GC-MS) and GC electron capture detection (ECD) (Thrane et al. 2003). Oosporein was detected in submerged cultures of *Beauveria brongniartii* and barley kernels using HPLC (Strasser et al. 2000b). When *B. bassiana* was cultured on corn and wheat kernels, beauvericin was isolated using HPLC (Josephs et al. 1999; Logrieco et al. 2002).

***Helicoverpa (=Heliothis) zea* (Boddie) (Lepidoptera: Noctuidae)**

Helicoverpa zea, also known as corn earworm, tomato fruitworm, bollworm, sorghum headworm and vetchworm, is considered by some to be the most damaging pest in agricultural crops due to its extensive host range and the fact that it typically feeds on the harvested portion of host plants. It is found throughout North America below 40°N latitude, but is highly dispersive and can be found farther north during the growing season (Capinera 2004). In Tennessee, *H. zea* overwinters as pupae two to four inches below the soil surface. Adult moths begin to emerge in early May. After mating females lay eggs singly on suitable host plants. Each female may lay anywhere from 450 to 3000 eggs during adulthood. Eggs hatch in two to five days and neonate larvae crawl to the foliage of the plant and begin to feed. Larvae develop through five, sometimes six, instars while feeding for two to three weeks (Hale 2005). Butler (1976) reported total

larval development times at 31.8, 28.9, 22.4, 15.3, 13.6 and 12.6 days when reared at 20.0, 22.5, 25.0, 30.0, 32.0 and 34.0°C, respectively. *Helicoverpa zea* is a major pest of tomato and many other economically-important agricultural crops. Understanding the biology of this insect is crucial to any control practice used against it.

***Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae)**

The potato aphid attacks over 200 vegetable and ornamental crops as well as a number of weeds (Kessing and Mau 1992). It is widespread across North America affecting both field and greenhouse crops. Adult potato aphids may be green, pink, green and pink or light green with a dark green stripe. Aphids overwinter as eggs on a perennial host, emerge in spring and migrate to a suitable host. Females are parthenogenic and have the ability to bear live young, thus shortening the life cycle and increasing reproductive capabilities to one generation every two to three weeks. Aphids feed by sucking sap from the xylem of the host plant usually in clumps on the more tender growth of the plant. Aphids vector several viral diseases, including mosaic viruses. Production of honeydew results in the growth of a black sooty mold on leaves, which is capable of clogging the plant's natural openings and also blocking sun light, decreasing respiration and photosynthesis. When a plant is fully infested with aphids, apterous females are produced which migrate and re-infest new host plants (Delahaut 2001). The rapid rate of reproduction in potato aphids could limit the effects of microbial control measures. Little is known about the effects of microbial control on aphid fecundity, but reduced rates of nymph production could greatly affect the development of aphid populations (Wang and Knudsen 1993).

Objectives

The objectives of this research were:

1. To determine effects of *B. bassiana* 11-98 conidia administered directly to potato aphids, *M. euphorbiae,* and to tomato foliage, *L. esculentum,* fed to aphids.

2. To determine effects of *B. bassiana* 11-98 conidia administered directly to the mid-dorsal epidermal surface of tomato fruitworms, *H. zea,* and to tomato foliage, *L. esculentum,* fed to fruitworms.

3. To determine effects of tomato foliage, *L. esculentum* 'Mountain Spring' grown from seed treated with *B. bassiana* 11-98 conidia on tomato fruitworms, *H. zea*.

4. To develop plant tissue extraction and HPLC protocols to confirm presence of beauvericin in tomato foliage (*L. esculentum* 'Mountain Spring').

Chapter 2

Direct and indirect effects of *Beauveria bassiana* 11-98 on *Macrosiphum euphorbiae* (Thomas) feeding on tomato.

Introduction

Beauveria bassiana (Balsamo) Vuillemin is a soil-borne entomopathogenic fungus with worldwide distribution. Isolates of this fungus have an extensive host range of insects across several economically important orders, including Homoptera (Dorschner et al. 1991; Eyal et al. 1994; Poprawski et al. 2000). Conidia typically infect insect hosts through the outer integument by attachment, germination and penetration (Boucias and Pendland 1998). Aided by mycotoxins produced *in-vivo*, the fungus continues to grow inside the body cavity of the insect host eventually breaking through the outer membrane of the epidermis (Pekrul and Grula 1979). In several recent studies, pathogen efficacy to aphids was examined using direct application of conidia to the insect epidermis (Feng et al. 1990; Feng and Johnson 1991; Vandenberg 1996; Hatting et al. 2004).

Entomopathogenic control on aphids has proved successful. Early efforts suggested that pest inoculation be accomplished by exposing aphids to spores discharged from infected insects or *in-vitro* cultures (Wilding 1976). Four entomophthoralean fungi, including *Zoophthora radicans* (Brefeld) Batko, *Conidiobolus thromboides* (Drechsler), *C. coronatus* (Costantin) and *Pandora neoaphidis* (Remaudiere and Hennebert), were applied as a spore shower to control Russian wheat aphid, *Diuraphis noxia* (Kurdj.), and rose-grain aphid, *Metopolophium dirhodum* (Wlk.). Inoculum rates of approximately 65 spores/mm^2 yielded 25-98% and 40-100% mortality, respectively (Feng and Johnson

1991). A spray application of 2,456 *B. bassiana* spores/mm^2, resulted in 86% mortality to brown citrus aphids, *Toxoptera citricida* (Kirkaldy) (Homoptera: Aphididae), 10 days after treatment (Poprawski et al. 1999).

Because females of potato aphid, *Macrosiphum euphorbiae*, are parthenogenic and can attain reproductive maturity quickly, populations are able to increase very rapidly (Delahaut 2001), potentially limiting the effects of microbial control measures. Although fecundity is a critical aspect of aphid population dynamics, very little is known about the effects of pathogens on aphid reproduction rates. Reduced rates of nymph production caused by infection of a pathogen would greatly affect the development of aphid populations (Wang and Knudsen 1993).

Entomopathogenic successes have directed an assessment of the virulence of *B. bassiana* 11-98, found in Scott County Tennessee by Roberto Pereira, against the potato aphid, a common pest of tomato. Initial experiments involving the application of *B. bassiana* 11-98 to tomato seed, enabling endophytic growth (Leckie 2002; Ownley et al. 2004), were not effective against *M. euphorbiae* (Canaday et al. Unpub. data). The specific objectives of this research were to optimize the treatment formulation and determine effects of four rates of *B. bassiana* 11-98 on mortality and fecundity of *M. euphorbiae* when applied directly to the epidermis of the insect and when applied to tomato foliage on which aphids were placed.

Materials and Methods

Propagation, Culture and Collection of *Beauveria bassiana*

Beauveria bassiana 11-98 was collected and isolated from an infected click beetle (Coleoptera: Elateridae) found in Scott County, TN by Roberto Pereira. Due to decreased virulence of entomopathogenic fungi when cultured on mycological media (e.g. Quesa-Moraga and Vey 2003), *B. bassiana* 11-98 was not used in any experiment until the fungus was enhanced by inoculating insects with a conidial suspension. Mycelia that were observed growing from the insect epidermis were isolated and cultured. Host-enhanced *B. bassiana* 11-98 was cultured on Sabouraud's dextrose agar (SDA) (Difco™, Becton, Dickenson & Co., Sparks, MD) and incubated at 20°C (68°F) for approximately three weeks. Conidia were harvested by brushing the surface of sporulating cultures with a round, camel's hair brush (Fisher Scientific, Pittsburgh, PA) and funneling spores into a glass vial. Vials of conidia were stored in the dark in a second airtight jar on top of a layer of Drierite™ (W. A. Hammond Drierite Comapany, Xenia, OH). Jars were stored at 4°C in a laboratory refrigerator until spores were needed.

Maintenance of *Macrosiphum euphorbiae* Experimental Colony

Macrosiphum euphorbiae were collected as gravid adults from wild populations on tomato plants grown in the University of Tennessee Gardens in Knoxville, TN (35° 58' 22" N, 83° 56' 32" W). Aphids were transferred onto greenhouse grown tomatoes, *Lycopersicon esculentum* 'Mountain Spring', cultured in an aphid rearing chamber (Forbes et al. 1985). Aphids were confirmed to be *M. euphorbiae* by Dr. S. E. Halbert with the Florida Department of Agriculture Division of Plant Industry, to whom voucher specimens were submitted. To restrict parasitoid contamination, adult females were

removed after bearing live young for 48 h. Parasitic wasps were isolated from aphid colonies and destroyed and colonies were further monitored for evidence of parasitism (aphid mummies). Rearing chambers were kept under a fluorescent light bank for a photoperiod of 16:8 (light:dark) h and at a temperature of 24°C (75.2°F) with 50% RH. Aphids used for experiments were transferred onto new plants with a camel's hair brush (Adams and Van Emden 1972).

Preparation of Conidial Suspensions

Preliminary trials investigated several surfactants at different percentages (v/v) including Suffusion, Triton X-100, pure vegetable oil, Tween 20, Tween 80, and Tween 85 to determine the best method for suspension of hydrophobic conidia. These trials established optimal suspensions of viable conidia using Tween 85 at 0.05% (v/v) (data not shown). For each bioassay, concentrations of *B. bassiana* 11-98 at 1×10^4, 1×10^5, 1×10^6 and 1×10^7 spores/ml sterile, de-ionized water (w/v) were tested against *M. euphorbiae*. Within the 4 conidial concentrations, 4 formulations were established using Tween 85 at 0 or 0.05% (v/v) (Sigma Chemical Co., St. Louis, MO) as a surfactant and kaolin clay (Suprex™, Kentucky-Tennessee Clay Co., Langly, SC) as a carrier at 0:1 or 0.05:1 to *B. bassiana* 11-98 spores (w/w). Tween 85 surfactant was filter-sterilized (0.45-µm pore size, Whatman International Ltd., Maidstone, England) before use. Kaolin clay was sterilized by spreading it evenly in a large petri dish under ultraviolet light in the biosafety hood three times for 10 min with 30-min resting periods between exposures. Kaolin was stirred and re-spread during each resting period. To prepare experimental stock suspensions of *B. bassiana* 11-98, 1 liter of sterile, de-ionized water was used. A spatula was used to mechanically disperse the clumps of conidia before adding to the

stock solution. Once cool, 300 ml sterile, de-ionized water was placed in a 400-ml beaker, 2.4 g of *B. bassiana* 11-98 spores were added and the solution was mixed on a stir plate for 20 min. Initial conidial concentrations were 1 x 10^4, 1 x 10^5, 1 x 10^6 and 1 x 10^7 conidia/ml achieved using step-wise dilutions from the stock suspension. Concentrations were evaluated for viability, and active dosages were calculated. Mean colony forming units (cfu) was the average of the number of colonies growing on two cultures after 4 days incubation; mean cfu values were calculated separately for each treatment. Experimental controls were 300 ml of sterile de-ionized water with 1.5 ml Tween 85 surfactant.

Aphid Mortality and Reproduction Following Contact-While-Feeding Exposure

Beauveria bassiana 11-98 was evaluated at four initial concentrations (1 x 10^4, 1 x 10^5, 1 x 10^6 and 1 x 10^7 conidia/ml for efficacy against the potato aphid. A 15-cm (6-in) long terminal tomato stem with intact leaflets was cut from 10-wk-old plants and placed in a water pick to maintain tissue turgor. Water picks were glass vials (60 x 17 mm) filled with de-ionized water, a piece of dental cotton roll (Patterson Dental Supply Inc., Saint Paul, MN) sliced to wrap around the tomato stem as a plug. A 5-cm (2-in) section of a coffee stir straw provided a channel to replenish water as needed. Parafilm-M (American National Can., Greenwich, CT) was wrapped around the top of vials and stems to prevent evaporative water loss. Tomato foliage was submerged into one of 20 treatment formulations and air-dried for approximately 2 h. Dry, treated tomato foliage was enclosed in a 15 cm-long (6-in), 42-mm (1.65-in) diam clear mylar tube. Approximately 60% of the tube surface was excised and covered with organdy fabric for ventilation. Foam plugs were cut from a 2.5-cm (1-in) thick sheet and used to plug both

tube ends. Before plugging the tube top, 15 similarly-sized and aged adult aphids were starved for 12 h, placed on the foliage and allowed to begin feeding.

Treatments were replicated 6 times in a randomized complete block design. Blocks were placed in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) and held at 24°C (75.2°F) with a 16:8 (light:dark) h photoperiod. Aphids were observed for direct mortality 7 and 10 days after treatment (DAT). Dead aphids were surface sterilized by submersion in 10% sodium hypochlorite (NaOCl) solution for 30 seconds and incubated on a plate of *B. bassiana* selective media (Doberski and Tribe 1980), to observe mycelial growth.

Selective media was prepared by adding 1 liter of de-ionized water to 40 g glucose (Mallinckrodt Inc., Paris, KY), 10 g neopeptone (Difco™, Becton, Dickenson & Co., Sparks, MD), 15 g agar (Sigma Chemical Co., St. Louis, MO) and 0.01 g crystal violet (Sigma Chemical Co., St. Louis, MO) and autoclaving for 45 min (Doberski and Tribe 1980). Cyclohexamide (0.25 g) was autoclaved separately and added along with 0.5 g chloramphenicol to the sterilized medium once cool. Live aphids and nymphs were euthanized and sterilized for 30 sec in 70% ethanol and a 10% NaOCl solution, then counted and placed on selective media. Cultures were incubated in the dark at ambient laboratory temperature (20°C) for 7 days.

Aphid Mortality and Reproduction Following Direct Topical Exposure

To assay aphid mortality following direct contact with *B. bassiana* 11-98 conidia, the same four initial concentrations described above were used. Only the formulations with 0% kaolin clay (Formulation A and B) were used in this bioassay. Experimental units of 10 aphids were placed in a 29.6-ml (1-oz) plastic cup and submerged in 1 ml of

one of the 10 treatments for 10 sec. Exposed aphids were placed on an excised terminal tomato stem inserted in a water pick and maintained in a petri dish (110 x 25 mm) with a friction-fit lid.

Each treatment was replicated 6 times and arranged in a randomized complete block design. Blocks were placed in a growth chamber for 10 days held at 24°C (75.2°F) with a 16:8 (light:dark) h photoperiod. Aphid mortality was observed at 10 DAT and insects were sterilized and incubated as described above. For all bioassays, aphid mortality in the controls was corrected using Abbott's formula (Abbott 1925) (graphed values).

$$\text{Abbott's corrected percentage control} = \frac{X - Y}{X \times 100}$$

Where X = % survival in untreated control.
Y = % survival in treated sample.

All aphid mortality and reproduction data were tested for distribution normality using Shapiro-Wilk's test. If Shapiro-Wilk's test resulted with $W<0.9$, data were transformed using arcsin square root before analysis. Results were compared among treatments using mixed model ANOVA in SAS (SAS Institute Inc., Cary NC). Significant effects were further analyzed using Fisher's least significant difference test at $\alpha=0.05$.

Results

Aphid Mortality and Reproduction Following Contact-While-Feeding Exposure

All mortality data for 7 and 10 DAT were tested and did not violate assumption of normality by Shapiro-Wilk's test (W=0.969 to 0.988). While aphid mortality in this bioassay was observed as early as day 7, formulated rates did not differ by 7 DAT (F =

0.75, df = 12,95, $P = 0.70$). All treatments within all formulation groups yielded low aphid mortality and were not different by 7 DAT (Table 2-1). The highest mortality (29%) was achieved in the 8.0 x 10^3 cfu/ml concentration of formulation A (Table 2-1). There were no differences in aphid mortality among formulation groups by 10 DAT ($P = 0.38$). Aphid mortality was also not different for any treatment within formulation groups 10 DAT (Table 2-1). At 10 DAT the highest mean aphid mortality was observed in formulation D at the highest concentration (Table 2-1). Regardless of formulation *B. bassiana* 11-98 did not affect the rate of nymph production by adult females (Figure 2-1). Untreated aphids produced an average of 17.5 and 51 neonates across all formulation groups by 7 and 10 DAT, respectively. But, newborn production in aphids that were exposed to *B. bassiana* 11-98 was typically higher than that of the untreated aphids. The greatest mean number of neonates (81) was observed 10 DAT for formulation C at 5.5 x 10^4 cfu/ ml concentration (Figure 2-1).

Aphid Mortality and Reproduction Following Direct Topical Exposure

Although treatment effects differed ($F = 4.52$, df = 4,45, $P = 0.004$), aphid mortality following direct exposure to *B. bassiana* 11-98 conidia was almost as low as mortality observed in the previous bioassay. Formulation had no effect 10 DAT ($F = 0.48$, df = 3,45, $P = 0.49$). By 10 DAT, the higher concentration of 3.5 x 10^6 cfu/ml yielded 34% mean mortality among treated aphids in formulation A (Table 2-1). Formulation C had 33% mortality by 10 DAT (Table 2-1). As concentration increased in formulation A, mortality also increased by 10 DAT. This was not the case in Formulation C, but mortality via treatment effect still differed (Table 2-1).

As in the first bioassay, aphid fecundity was not affected by applications of *B.*

Table 2-1. Aphid mortality following topical and contact-while-feeding exposure to experimental concentrations and formulations of *Beauveria bassiana* 11-98 conidia and Tween 85 or kaolin clay.

Formulation	Concentration (w/v)	Active Dose[a] (cfu/ml)	Tween 85	Kaolin Clay: *Bb* 11-98 conidia (w/w)	Contact while Feeding (% Mortality [b, c]) 7DAT	Contact while Feeding (% Mortality [b, c]) 10DAT	Topical Exposure (% Mortality [b, c, d]) 10DAT
A	1×10^4	2.25×10^3	0%	0:1	10.0 ± 4.1a	15.3 ± 12.3 a	9.7 ± 3.5 c
	1×10^5	8.0×10^3			28.6 ± 10.7 a	33.3 ± 12.2 a	15.5 ± 6.9 abc
	1×10^6	5.85×10^4			9.7 ± 5.0 a	5.6 ± 5.6 a	33.2 ± 10.2 a
	1×10^7	3.5×10^5			8.3 ± 5.3 a	19.5 ± 16.3 a	34.2 ± 8.9 a
B	1×10^4	5.0×10^3	0%	0.05:1	23.0 ± 14.7 a	15.9 ± 11.2 a	Not Tested
	1×10^5	1.65×10^3			6.9 ± 4.4 a	10.7 ± 8.2 a	
	1×10^6	7.0×10^4			12.6 ± 4.5 a	3.8 ± 2.5 a	
	1×10^7	1.65×10^5			14.1 ± 3.5 a	5.0 ± 5.0 a	
C	1×10^4	1.05×10^3	0.05%	0:1	17.8 ± 7.6 a	36.1 ± 20.4 a	11.4 ± 6.8 bc
	1×10^5	1.5×10^3			7.6 ± 4.3 a	19.4 ± 9.0 a	31.2 ± 12.2 abc
	1×10^6	5.5×10^4			17.8 ± 13.2 a	33.3 ± 16.7 a	22.0 ± 6.7 abc
	1×10^7	7.5×10^5			14.4 ± 6.1 a	24.6 ± 11.1 a	32.8 ± 8.0 ab
D	1×10^4	5.0×10^3	0.05%	0.05:1	11.5 ± 4.5 a	21.4 ± 16.0 a	Not Tested
	1×10^5	7.0×10^3			9.8 ± 5.0 a	14.5 ± 8.0 a	
	1×10^6	3.0×10^4			19.8 ± 11.3 a	31.7 ± 14.7 a	
	1×10^7	4.0×10^5			8.2 ± 7.0 a	40.0 ± 18.2 a	

[a] Active Dose values represent the mean viable conidia values from counts (2 petri dishes per treatment rate) of *B. bassiana* 11-98 colony forming units (cfu) that grew on selective agar within 4 days of inoculation.

[b] Abbott's corrected mortality of aphids ± SE in treatments containing viable *B. bassiana* 11-98 conidia (Abbott 1925).

[c] Conidia-free control percentage mortalities that were used for Abbott's correction were: 21.1 ± 7.2, 34.4 ± 9.8, 33.3 ± 12.3 and 30.0 ± 13.2, 7 DAT (in Formulations A, B, C and D, respectively); 55.6 ± 9.7, 65.6 ± 14.2, 65.6 ± 11.7 and 43.3 ± 15.1, 10 DAT (in Formulations A, B, C and D, respectively); and 21.7 ± 4.8 and 26.7 ± 9.5, 10 DAT (in Formulations A and B, respectively).

[d] Across formulations, *M. euphorbiae* mortality for treatment rates tested by topical exposure were separated using Fisher's least significant difference test at α=0.05. Means followed by similar letters are not significantly different.

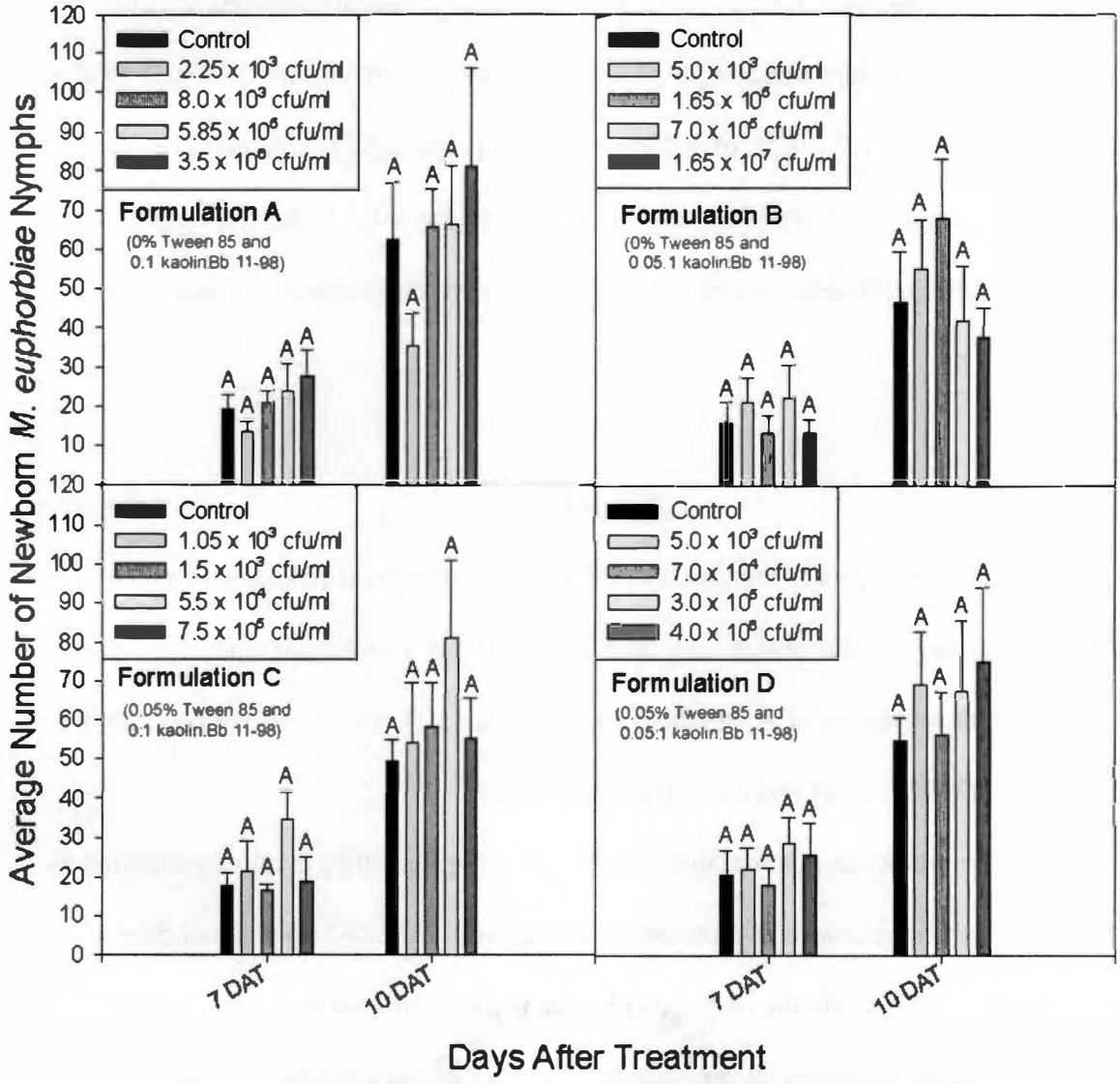


Figure 2-1. Average newborn *M. euphorbiae* nymphs following contact-while-feeding exposure to *B. bassiana* 11-98 conidia in formulations of Tween 85 at 0 or 0.05% (v/v) and 0:1 or 0.5:1 kaolin clay to *Bb* 11-98 conidia (w/w). Error bars represent the standard error. Data were determined to violate assumptions of normality (Shapiro-Wilk's W= 0.88 to 0.89), thus statistical analysis were conducted on arcsin squareroot transformed data. Data in graphs are presented using unadjusted mean values. Means by day were separated using Fisher's least significant difference test at $\alpha = 0.05$. By day, means reported with similar letters are not significantly different.

bassiana 11-98 conidia. Reproduction by *M. euphorbiae* did not differ as a result of formulation ($F = 3.88$, df = 1,45, $P = 0.055$). Treatment effects in both formulation groups were not different when compared to experimental controls that contained no *B. bassiana* 11-98 spores ($F = 0.37$, df = 4,45, $P = 0.83$) (Figure 2-2). Untreated aphids produced an average of 42 nymphs in formulation A by day 10, a higher reproductive yield than all other treatments except 8.0×10^3 cfu/ml, which averaged 45 newborns (Figure 2-2).

Discussion

While formulations of kaolin clay or Tween 85 offered no entomopathogenic benefit in this research, our preliminary tests indicated that a surfactant was necessary to counter the hydrophobicity of *B. bassiana* 11-98 conidia. Therefore, only a formulation of Tween 85 at 0.05% (v/v) was used in further assays.

Aphid mortality in this research was never higher than 40% in any formulation by 10 DAT. Aphids treated with *B. bassiana* 11-98 generally showed no signs of disease immediately after death and the pathogen was recovered from less than 5% of surface sterilized and plated cadavers. It is unknown if surface sterilization had an effect on *B. bassiana* 11-98 inside the insect.

In preliminary investigations *M. euphorbiae* were fed an artificial diet (Dadd and Mittler 1965) containing *B. bassiana* 11-98 conidia at initial concentrations of 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/ml diet solution (Klingeman, unpub. data). Results of preliminary trials indicated that conidia growing within artificial diet do not directly affect aphid mortality after 120 h of feeding. However, difficulties with

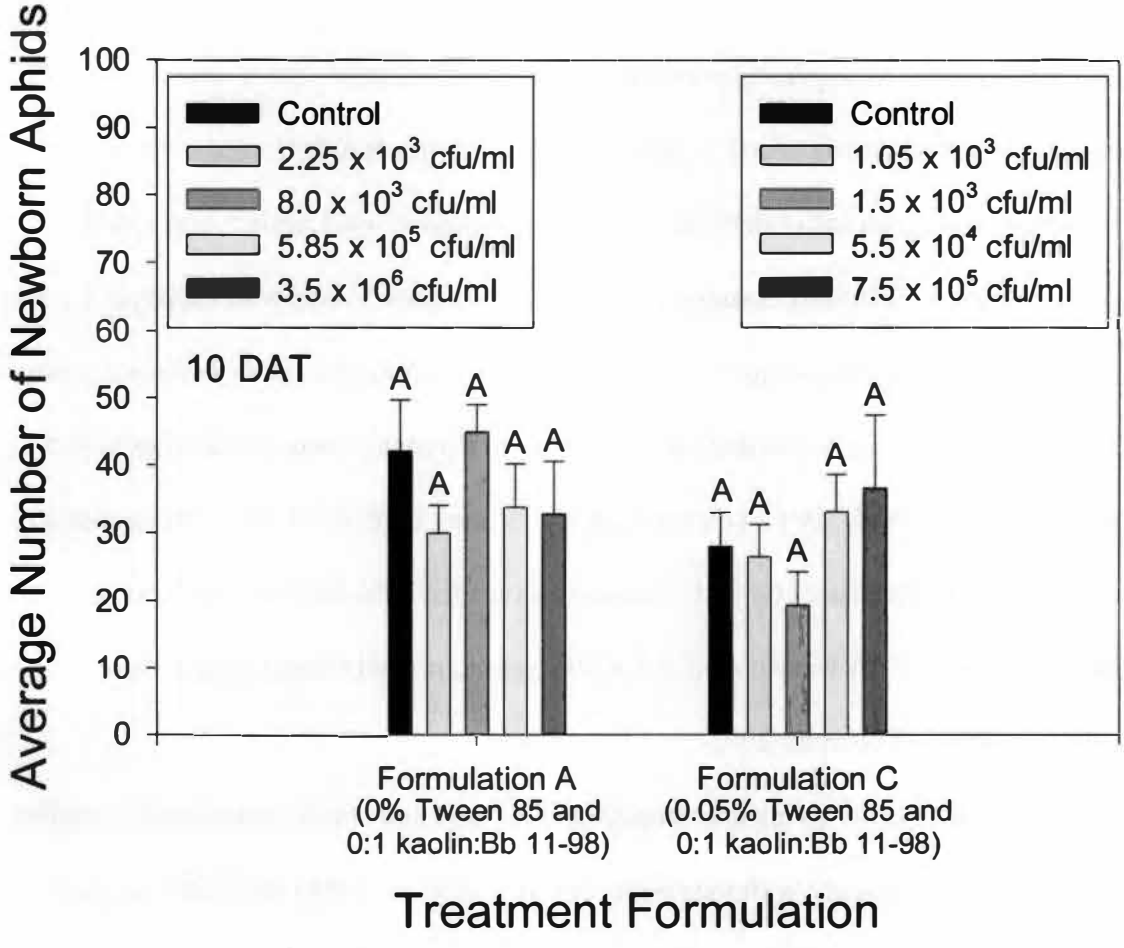


Figure 2-2. Average newborn *M. euphorbiae* nymphs following direct topical exposure to *B. bassiana* 11-98 conidia in formulations of Tween 85 at 0.05% (v/v) and 0:1 or 0.5:1 kaolin clay to *Bb* 11-98 conidia (w/w). Error bars represent the standard error. Data were determined to violate assumptions of normality (Shapiro-Wilk's W= 0.89), thus statistical analysis were conducted on arcsin squareroot transformed data.. Data in graphs are presented using unadjusted mean values. Means by formulation group were separated using Fisher's least significant difference test at $\alpha = 0.05$. By formulation, means reported with similar letters are not significantly different.

maintaining conidia suspended solutions were experienced at the higher rates of 1 x 10^8 and 1 x 10^9 conidia/ml. Conidia of *B. bassiana* 11-98 are hydrophobic and tend to clump together at high densities.

Mortality to *M. euphorbiae* resulting from direct contact with *B. bassiana* 11-98 was much lower when compared to other studies involving aphids treated with similar rates. For example, an aphid-derived isolate of *B. bassiana* used against hop aphids, *Phorodon humuli* (Schrank), resulted in 98% mortality after 5 days when applied at a rate of 1 x 10^8 conidia/ml (Dorschner et al. 1991). A spray application of *B. bassiana* strain GHA at 5 x 10^{13} spores/ha yielded 78% mortality to Russian wheat aphids after only 5 days (Vandenberg et al. 2001), however colony forming units (cfu), which are a measure of spore viability, were not counted. Mortality exceeded 80% for two species of aphids when *B. bassiana* GHA was applied at 1 x 10^8 conidia/ml and treated aphids were incubated at 23°C (Yeo et al. 2003).

No bioassays involving entomopathogenic fungi and aphid contact-while-feeding could be found to compare with the results of this research. Aphid mortality was high in control groups and was variable in all conidial treatments on both days of observation. Contact exposure may be influenced by where aphids settled to feed on the treated plant tissue. Aphids may have avoided areas where conidia were present and after the stylet is inserted into the plant, aphids typically do not move to other feeding sites, especially in only 10 days (Forbes 1977). Low aphid mortality in our trials may also indicate that *B. bassiana* conidia (approximately 3 μm in diam) (Viaud et al. 1997) were not ingested due to the diameter of aphid stylets (approximately 1 μm) (Forbes 1977). Also, hyphal

penetration of the insect integument may be superficial if aphids escape infection by casting the infectious inoculum during molting (Ferron 1981).

Female aphids are parthenogenic and have the ability to bear live young, thereby shortening their life cycle and increasing reproductive capabilities (Delahaut 2001). Although reproduction of *M. euphorbiae* was not affected in either assay by *B. bassiana* 11-98 conidia due to variability, there was a trend for reduced fecundity. Average reproduction observed in these experiments were similar to that of Wang and Knudsen (1993) during which adult female aphids produced an average of 3 nymphs per day. Such results suggest that *B. bassiana* 11-98 and its fungal metabolites affect aphid fecundity differently than other insects and protozoan pathogens. For example, fecundity of European corn borer, *Ostrinia nubilalis* (Hubner), was significantly reduced after infection of protozoan *Perezia pyraustae* (Paillot) (Kramer 1959). Also, *H. zea* adults experienced reproductive abnormalities where eggs were laid in masses instead of singly after consuming *B. bassiana* 11-98 mycelia incorporated into artificial diet (Leckie 2002).

After evaluation of *B. bassiana* 11-98 conidia on *M. euphorbiae*, it is evident that the host-enhanced isolate alone would not be an effective means of controlling aphid populations in the field. Although mortality was moderate and fecundity was not reduced, *B. bassiana* 11-98 still shows potential to be successful when applied along with another control measure as part of an IPM system for controlling aphid populations.

Chapter 3

Effects of *Beauveria bassiana* 11-98 conidia administered directly to the epidermis of larval *Helicoverpa zea* and to tomato foliage fed to tomato fruitworms.

Introduction

Many studies have evaluated the potential for *Beauveria bassiana* (Balsamo) Vuillemin to function as a biological control agent against lepidopteran pests (McDowell et al. 1990; Poprawski et al. 2000). *Beauveria bassiana* is a soil-dwelling entomopathogenic fungus in the phylum Deuteromycota (Alexopolous et al. 1996). *Beauveria bassiana* demonstrates pathogenic properties towards many economically-important pests in most insect orders and at all stages of development (Boucias and Pendland 1998). Infection by *B. bassiana* typically begins with contact and attachment of conidia to the insect cuticle where, under favorable conditions, conidia germinate and penetrate the epidermis. *Beauveria bassiana* has also demonstrated infection through the tracheal openings and alimentary tract (Steinhaus 1949). However, intestinal flora within the gut of an insect generally inhibits fungal growth (Boucias and Pendland 1998.) Inside the host, *B. bassiana* produces several different mycotoxins (Boucias and Pendland 1998). The most notable toxin is beauvericin, a cyclic depsipeptide that has the ability to dissolve lipid bilayers, increase permeability of cell membranes and disrupt the normal function of intact cells or organelles such as mitochondria. *Beauveria bassiana* also produces bassianolide, another cyclic depsipeptide and cyclosporein, a cyclic nonribosomal peptide, which are both pathogenic to many lepidopteran species. Oosporein, a red pigmented dibenzoquinone, is typically produced by *B. bassiana in-vivo*

and acts as an anitmicrobial agent allowing the fungus to proliferate inside an insect host without competition from bacteria within the gut (Boucias and Pendland 1998).

Aided by the mycotoxins, *B. bassiana* continues to grow, eventually killing the insect and filling the body cavity (Steinhaus 1949; Boucias and Pendland 1998). Within 24-48 h of death, the cadaver of the insect is covered with white mycelia that have grown through the intersegmental membranes (Pekrul and Grula 1979). Outside the insect body, mycelia continue to grow and produce conidiophores and conidia that are disseminated to other hosts.

Mortality also occurs when insect pests consume *B. bassiana* (Kramm and West 1981; Jeffs et al. 1997; Poprawski et al. 1998; Leckie 2002). Conidia and mycelia of *B. bassiana* have been formulated with artificial diet (Kramm and West 1982; Jeffs et al. 1997; Leckie 2002) and on infected prey (Poprawski et al. 1998). Infection of insects may occur through viable conidia trapped in and around the mouthparts and anus (Jeffs et al. 1997) and conidia attachment in the foregut (Kramm and West 1982).

An isolate of *B. bassiana* was collected from an infected click beetle (Coleoptera: Elateridae) found in Scott County, TN by Roberto Pereira in 1998 and designated isolate 11-98. In early trials, *B. bassiana* 11-98 was tested against corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Larvae were maintained on an artificial beet armyworm diet mix (Bio-Serv, Inc., Frenchtown, NJ), supplemented with 0, 0.1, 0.5, 1.0 and 5.0% dried mycelia of *B. bassiana* 11-98 (w/v) and several other isolates. After 10 days the highest rate of *B. bassiana* 11-98 yielded 100% mortality among treated 3rd instar *H. zea* larvae (Leckie 2002). Yet, *H. zea* also experienced increased larval and pupal weights due to nutritional gain from feeding on diet containing dried *B. bassiana*

mycelia from less toxic isolates (Leckie 2002). In order to sustain epizootic levels of control in nature (e. g. Miller et al. 1983), further research with conidia of *B. bassiana* was needed. A bioassay was conducted involving a direct application of conidia and blastospores of *B. bassiana* to larval *Diatraea saccharalis* (Lepidoptera: Crambidae) (Alves et al. 2002). Larval mortality increased in time and with dose rates regardless of the structure of the cells used for inoculum. Higher mortality was observed in the highest treatments at 1×10^7 and 1×10^8 cells/ml, and virtually no fungal infection was observed at the lower concentrations of 1×10^5 and 1×10^6 cells/ml.

To address effects of *B. bassiana* 11-98 conidia on *H. zea*, the specific objectives of this research were to: 1) determine effects of *B. bassiana* 11-98 conidial solutions administered directly to the epidermis of tomato fruitworm. and 2) determine effects of conidia ingested by feeding *H. zea* tomato foliage treated with suspensions of *B. bassiana* 11-98.

Materials and Methods

Propagation, Culture and Collection of *Beauveria bassiana* 11-98

Due to decreased virulence of entomopathogenic fungi when cultured on mycological media (e.g. Quesa-Moraga and Vey 2003), *B. bassiana* 11-98 was not used in any experiment until the fungus was enhanced by inoculating insects with a conidial suspension. Mycelia that were observed growing from the insect epidermis were isolated and cultured. Host-enhanced *B. bassiana* 11-98 was cultured on Sabouraud's dextrose agar (SDA) (Difco™, Becton, Dickenson & Co., Sparks, MD) and incubated at 20°C (68°F) for approximately 3 weeks. Conidia were harvested by brushing the surface of

sporulating cultures with a round, camel's hair brush (Fisher Scientific, Pittsburgh, PA) and funneling spores into a glass vial. Vials of conidia were stored in the dark in a second airtight jar on top of a layer of Drierite™ (W. A. Hammond Drierite Comapany, Xenia, OH). Jars were stored at 4°C in a laboratory refrigerator until spores were needed.

Maintenance of *Helicoverpa zea* Experimental Colony

A colony of tomato fruitworm, *H. zea*, was established from eggs obtained from Agripest (Zebulon, NC). Approximately 100 eggs were placed on filter paper moistened with de-ionized water in each of several petri dishes. Petri dishes were incubated (Percival Mfg. Co., Boone, IA) at 24°C (75.2°F) until larvae hatched. Artificial beet armyworm diet (Bio-Serv Inc., Product #F9219B, Frenchtown, NJ), was prepared by adding 19.8 g agar to 820 ml de-ionized water in a 1liter beaker, placing the solution on a hot plate and heating until clear. Heated agar was placed into a blender with 161.6 g dry beet armyworm diet, blended thoroughly and 5-ml aliquots were poured into each cell of a 32 cell rearing tray (Bio-Serv Inc., Frenchtown, NJ). Diet was allowed to cool and harden for 30 min before neonate larvae were introduced with a small camel's hair brush. Rearing trays were placed in an incubator at 24°C (75.2°F) until larvae reached the 3rd instar experimental studia in approximately 8 days. Additional larvae were retained on diet to maintain the laboratory colony.

Preparation of Conidial Suspensions

Preliminary trials investigated the use of several concentrations (v/v) of surfactants including Suffusion, Triton X-100, pure vegetable oil, Tween 20, Tween 80, and Tween 85 to determine the best method for suspending hydrophobic conidia in solution. These trials established optimal spore suspensions using Tween 85 at 0.05%

(v/v) (data not shown). For each experiment, harvested spores were taken from cold storage at 4°C and clumps were mechanically dispersed with a sterile spatula before adding to stock solution. For stock solutions of 1 x 10^7 conidia/ml, 50 ml de-ionized water was sterilized in an autoclave for 30 min and allowed to cool for 1 h. In a 25-ml test tube, 10 ml sterile, de-ionized water was mixed with 0.05 ml Tween 85 (Sigma Chemical Co., St. Louis, MO) that was filter sterilized (0.45-μm pore size, Whatman International Ltd., Maidstome, England) and *B. bassiana* 11-98 spores. Stock solutions were vortexed for 2 min to suspend conidia.

Larval Mortality Following Direct Topical Application

To assay larval mortality following direct contact with *B. bassiana* 11-98 conidia, two conidial concentrations of the host-enhanced isolate and an experimental control of de-ionized water and Tween 85 were tested against *H. zea* in two laboratory bioassays replicated in time. Initial conidial concentrations (w/v) were 1 x 10^6 (1 ml stock in 9 ml sterile de-ionized water and 0.045 ml Tween 85) and 1 x 10^7 conidia/ml (stock).

Bioassay 1 was replicated in two trials and 1 ml of each concentration was set aside for dilution and colony forming unit (cfu) counts. Actual cfu concentrations used in both trials of Bioassay 1 were 2.35 x 10^6 and 2.3 x 10^7 cfu/ml in 0.05% (v/v) Tween 85. Each treatment was replicated 4 times and arranged in a completely randomized design. To test larvae, ten 3rd instar *H. zea* were placed individually in a 59.2-ml (2-oz) plastic cup (Solo Cup Co., Urbana, IL) and 10 μl of each conidial suspension was topically applied to the mid-dorsal epidermal surface using a micropipetter.

For Bioassay 2, greater larval numbers enabled 5 replicates arranged in a completely randomized design. *Beauveria bassiana* 11-98 treatments were 6.05 x 10^6

and 5.2 x 10^7 cfu/ml in 0.05% (v/v) Tween 85 with a de-ionized water and Tween 85 experimental control. Each replicate consisted of 30 individual, 3rd instar *H. zea* larvae placed in 59.2-ml (2-oz) plastic cups and starved for 24 h to induce immediate feeding on experimental foliage.

Responding to low larval mortality observed during the first bioassay, the inocula was increased 4-fold (i.e, a 40 µl suspension of each treatment was topically applied to the mid-dorsal epidermal surface of each larva). Larvae were allowed to air-dry in the cup for 1 h at ambient laboratory temperature (20°C) after which they were returned to their original diet cell. Trays were maintained in an incubator (Percival Mfg. Co., Boone, IA) at 24°C (75.2°F) with 16:8 (light:dark) h. Larvae were observed 5, 10 and 30 days after treatment (DAT) for mortality, pupation or eclosion. Observations were terminated after 30 days or when 100% of surviving larvae in the control treatment emerged as moths.

Larval Mortality Following Consumption of Treated Tomato Foliage

To assay larval mortality following consumption of tomato foliage treated with *B. bassiana* 11-98 conidia, two conidial suspensions of the host-enhanced isolate were assayed in two laboratory experiments using the same initial concentrations described above with an identical sterile de-ionized water and 0.05% Tween 85 control.

Bioassay 1 was replicated in two trials. Stock conidial solution was prepared with 300 ml sterile de-ionized water, dry *B. bassiana* 11-98 conidia and Tween 85. Serial dilutions yielded experimental treatment rates of 2.96 x 10^6 and 4.99 x 10^7 cfu/ml for both trials of Bioassay 1 and 3.14 x 10^6 and 5.19 x 10^7 cfu/ml for Bioassay 2. Four replicates of 30 individual larvae were tested in both trials of Bioassay 1 and 75

individual larvae in 5 replicates were tested in Bioassay 2. In both bioassays, individual 3rd instar *H. zea* larvae were placed in 59.2-ml (2-oz) plastic cups and starved for 24 h to induce immediate feeding on experimental foliage. A 15-cm (6-in) long petiole with intact tomato leaflets was cut from the 10-wk-old parent plant and dipped in one of three treatments. Treated foliage was air-dried for approximately 2 h, after which 12-cm-diameter leaf disks were taken. One leaf disk was placed in each cup and larvae were allowed to feed for 6 h after which they were returned to their original diet cells. Trays were incubated (Percival Mfg. Co., Boone, IA) at 24°C (75.2°F). Larvae were observed 5, 10 and 30 DAT for mortality, pupation or eclosion and observations were terminated after 30 days or when 100% of surviving larvae in the control treatment emerged as moths.

All larval mortality data were tested for distribution normality using Shapiro-Wilk's test. If Shapiro-Wilk's test resulted with $W<0.9$, data were transformed using arcsin square root before analysis. When larval mortality in the control treatments exceeded 5%, treatment mortality was corrected using Abbott's formula (Abbott 1925). Results were compared among treatments using mixed model ANOVA in SAS (SAS Institute Inc., Cary NC). Significant effects were further analyzed using Fisher's least significant difference test at $\alpha=0.05$.

Results

Larval Mortality Following Direct Topical Application

In all tests, direct application of *B. bassiana* 11-98 conidia to the insect epidermis yielded low larval mortality. Treatments with *B. bassiana* 11-98 in both trials of

Bioassay 1, did not differ ($P = 0.08$) nor was the trial by treatment interaction different ($P = 0.39$), thus data were pooled. Mortality in Bioassay 1 was observed as early as day 5 ($F = 2.33$, df = 2,18, $P = 0.13$) but treatments did not differ from controls until 30 DAT ($F = 4.25$, df = 2,18, $P = 0.03$) (Figure 3-1). On each day, higher larval mortality was observed at the highest conidial rate. But by 30 DAT, *H. zea* mortality was only 15% and 21% for 2.35×10^6 and 2.3×10^7 cfu/ml treatments, respectively (Figure 3-1). In Bioassay 2, mortality was not observed until 10 DAT and treatment mortality differed from controls ($F = 10.77$, df = 2,8, $P = 0.0054$). Mortality was similar, regardless of conidial rate, on 10 and 30 DAT, but average mortality still did not exceed 13% following direct topical exposure to *B. bassiana* 11-98 conidia (Figure 3-2).

Larval Mortality Following Consumption of Treated Tomato Foliage

In contrast with topical applications of 1×10^6 and 1×10^7 (w/v) *B. bassiana* 11-98 conidia, *H. zea* larvae were much more likely to develop an infection and die if they consumed tomato foliage treated with similar initial rates of the host-enhanced isolate. Consumption of viable conidia would have allowed for multiple modes of *H. zea* infection. Mortality was greater at both rates in the consumption bioassay than in any previous experiment. Differences in mortality were observed 5 DAT, much earlier than in the direct exposure bioassays. Increasing the rate of conidia applied to tomato foliage increased mortality in all bioassays on each day (Figures 3-3, 3-4). While trial by treatment interactions did not differ ($P = 0.35$), larval mortality differed between the two initial tests for mortality following consumption of treated tomato foliage and treatments ($P = 0.05$), thus data were not pooled. By 5 DAT, *H. zea* larvae fed the highest rate of *B. bassiana* 11-98 had 41% mortality in trial A and 40% in trial B (Figure 3-3). Effect of

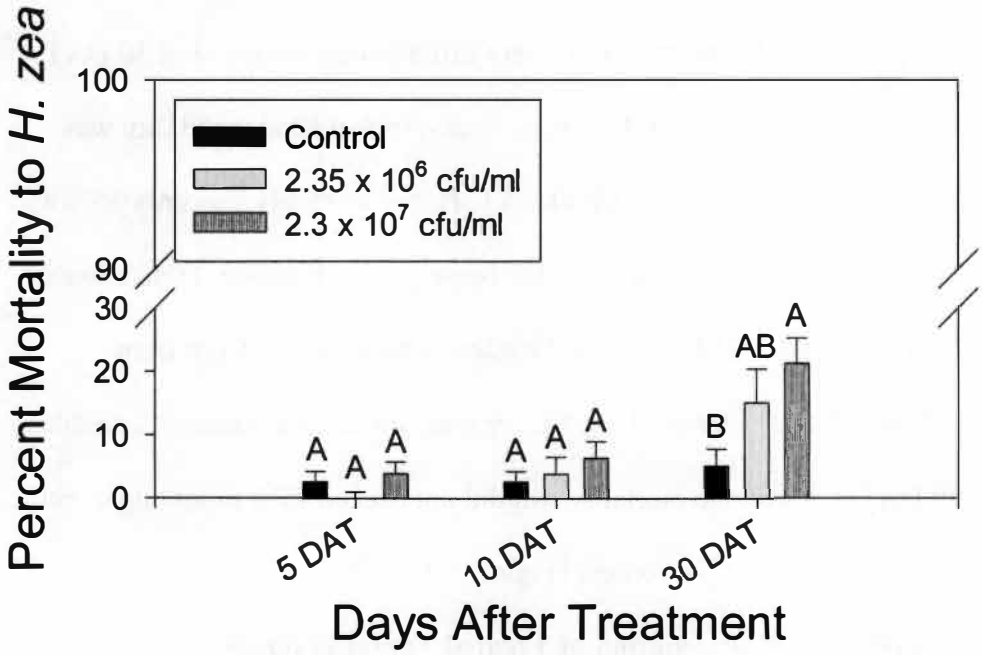


Figure 3-1: Percent mortality of *H. zea* following topical application of *B. bassiana* 11-98 in Bioassay 1. Error bars represent the standard error. Means within day were separated using LSD mean separation (α=0.05). By day, means without similar letters reported are significantly different.

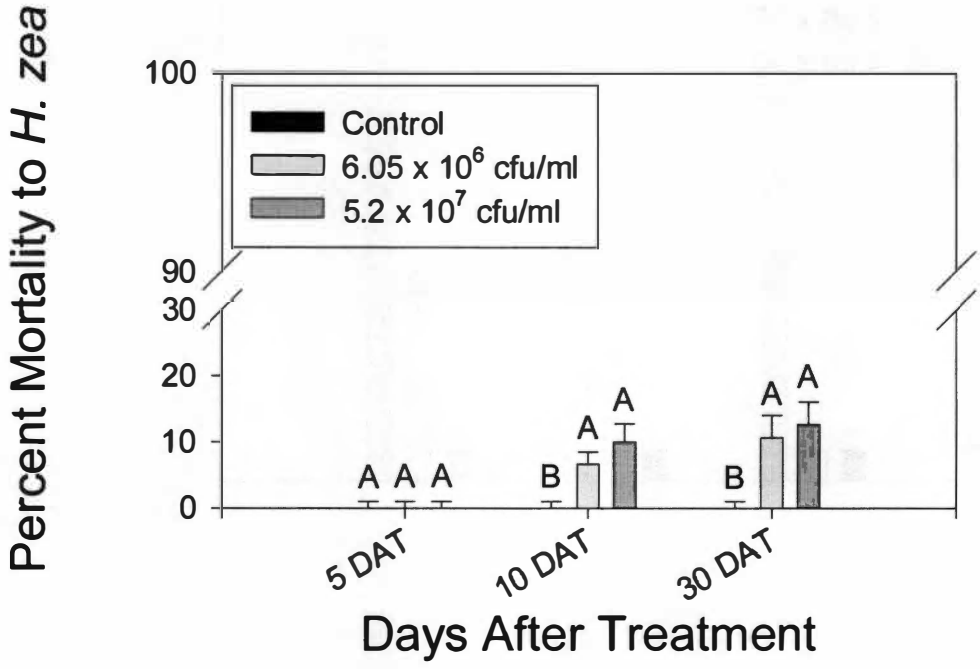


Figure 3-2: Percent mortality of *H. zea* following topical application of *B. bassiana* 11-98 in Bioassay 2. Error bars represent the standard error. Means within day were separated using LSD mean separation (α=0.05). By day, means without similar letters reported are significantly different.

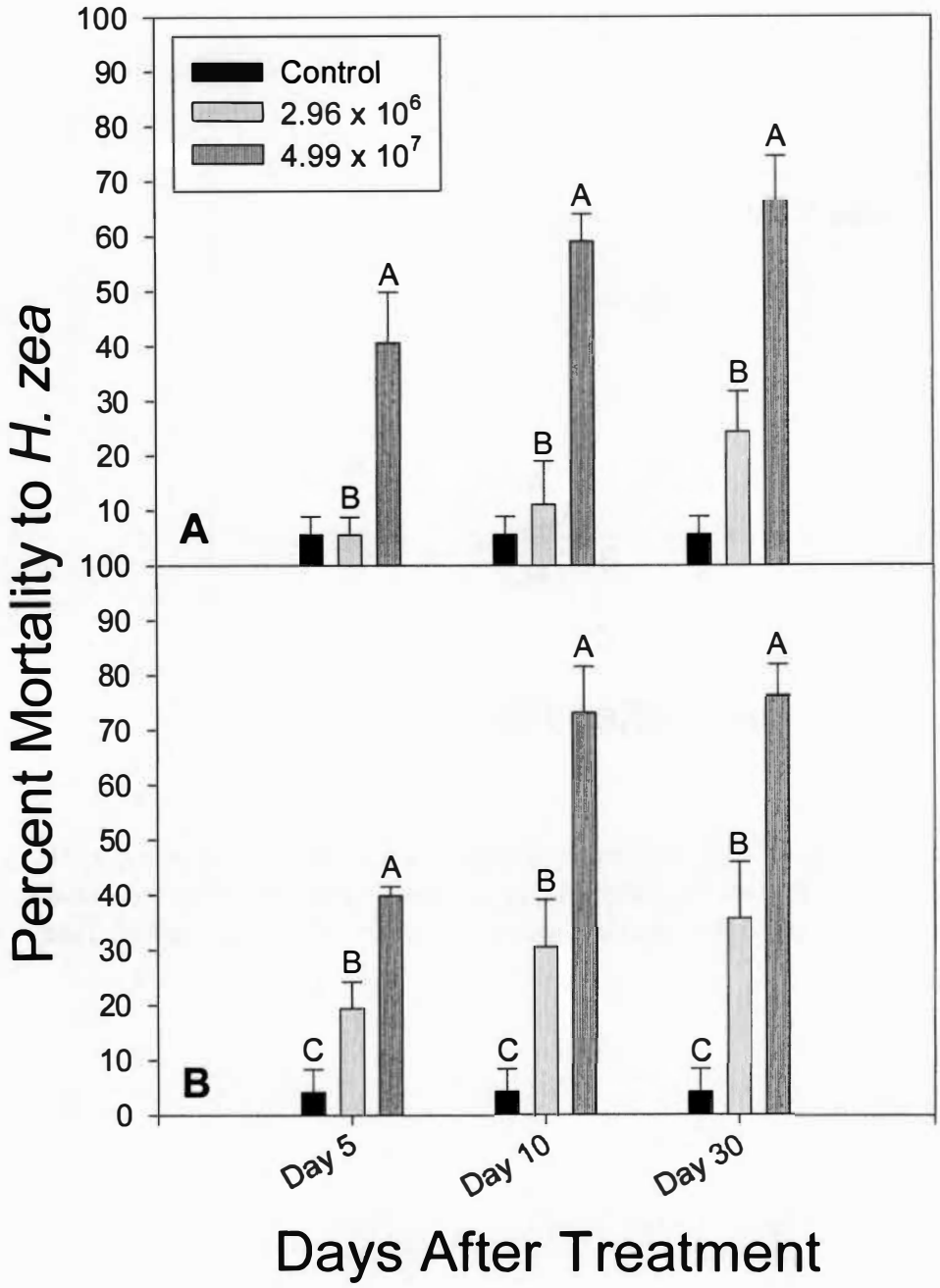


Figure 3-3: Percent mortality of *H. zea* following consumption of *B. bassiana* 11-98-treated tomato foliage in repeated trials for Bioassay 1. Data were not pooled due to differences among treatments in trials A and B. Error bars represent the standard error. For trial A, control mortalities exceeded 5% and were subjected to Abbott's correction thus, were not included in mean separation. Means within day were separated using LSD mean separation (α=0.05). By day, means reported without similar letters are significantly different.

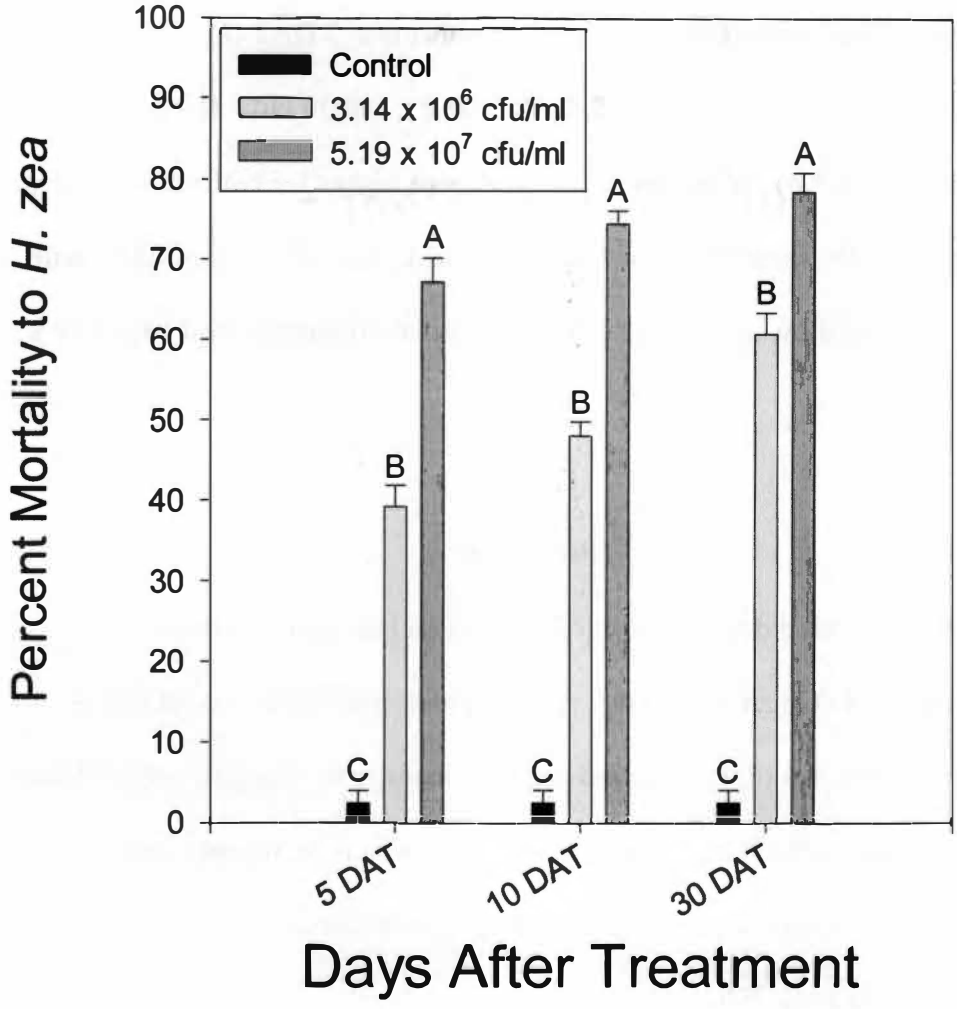


Figure 3-4: Percent mortality of *H. zea* following consumption of *B. bassiana* 11-98-treated tomato foliage in Bioassay 2. Error bars represent the standard error. Means within day were separated using LSD mean separation (α=0.05). LSD values are 5 DAT=6.20, 10 DAT=6.53 and 30 DAT=8.73. By day, means reported without similar letters are significantly different.

rate on mortality differed in both trials on each day (Figure 3-3). The highest larval mortality was achieved at the highest rate at 30 DAT with 67 and 76%, in trials A and B respectively (Figure 3-3).

In Bioassay 2, there were differences in all treatments at 5 DAT ($F = 309.44$, df = 2,8, $P < 0.0001$), 10 DAT ($F = 326.56$, df = 2,8, $P < 0.0001$), and 30 DAT ($F = 309.13$, df = 2,8, $P < 0.0001$) (Figure 3-4). Mortality in this study was higher for both conidial rates than any other study on the same day. The highest mortality was observed 30 DAT with 78% of 125 larvae dead following the consumption of tomato foliage treated with 5.19 x 10^7 cfu/ml.

Discussion

Our present research did not use artificial diet as an inoculation method. In preparation of artificial diet, agar is heated to approximately 100°C before diet mix is added. Extreme temperatures of the prepared diet may damage the integrity and viability of conidia and after diet is cool it hardens not allowing conidia to be mixed evenly. Also, lower concentrations of conidia were not used in our research due to lack of infection mentioned earlier (Alves et al. 2002).

Consumption of *B. bassiana* 11-98 conidia resulted in greater and more rapid mortalities on all days when compared with topical applications of similar rates. Larvae fed treated tomato foliage had consistent mortalities and times to mortality with those of similar studies (McDowell et al. 1990; Vandenberg et al. 1998a). Third instars of lesser cornstalk borer larvae, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae), had LC_{50} and LC_{90} values of 5.07 x 10^1 and 1.87 x 10^4 cfu/cm^2, respectively when fed a 1.7

cm diameter (0.67 in) soybean leaf disk treated with 40 μl of conidial suspensions (McDowell et al. 1990). Second instar diamondback moth larvae fed broccoli foliage that was sprayed with 5.0 x 10^{13} *B. bassiana* spores/hectare, using a backpack sprayer delivering 280.5 liters/hectare, had 80% mortality 7 days after treatment (Vandenberg et al. 1998a).

Mortality of *H. zea* in these experiments may not be the result of conidial penetration of the gut wall. The environment in the gut is typically not suitable for fungal growth (Boucias and Pendland 1998). Low oxygen availability, high pH levels and harsh digestive enzymes in the gut may have inhibited normal growth of *B. bassiana* 11-98.

Infection of an insect by *B. bassiana* depends upon numerous factors. For example, younger insects are typically more susceptible to infection than older larvae (Boucias and Pendland 1998), and optimal germination of conidia requires temperature to be at least 25°C and humidity 75% (Luz and Fargues 1997; Fargues and Luz 1998, 2000). Most larvae in these experiments first demonstrated fungal growth on or around the mouth or anus, indicating that infection was not through the gut wall. These observations correlate with those seen in larval cockchafer, *Melolontha melolontha* (L.), where the most sensitive sites to infection of *Beauveria* sp. were the mouth and anus (Delmas 1973). Microenvironments on or adjacent to the mouthparts are favorable sites for host infection due to increased moisture levels, which allows for germination of conidia (Jeffs et al. 1997). This case may have been similar to that of Jeffs et al. (1997), where grasshopper larvae were infected through the integument by viable conidiospores in the insect's fecal matter. Death in *H. zea* is likely due to nutrient depletion, dehydration, or

toxin production by the fungus (Boucias and Pendland 1998).

Larvae of *H. zea* had much smaller mortality percentages than previous experiments using similar methods when other isolates of *B. bassiana* conidia were topically applied (Vandenberg et al. 1998b; Zurek and Keddie 2000, Hicks et al. 2001). For example, late instar pine beauty moth larvae, *Panolis flammea* (Dennis & Schiffermuller) (Lepidoptera: Noctuidae) demonstrated 75-100% mortality within 11 days after 4 µl of conidial suspensions of *B. bassiana* at 4.8×10^3, 4.8×10^4 and 4.8×10^5 were applied directly to the mid-dorsal integument surface (Hicks et al. 2001). Early and late instar satin moth larvae, *Leucoma salicis* (L.) (Lepidoptera: Lymantriidae), treated with topical applications of 10 µl of a 2.0×10^4 conidial suspension of *B. bassiana,* yielded 76 and 90% mortality within 4 days, respectively (Zurek and Keddie 2000). Second, 3rd and 4th instar diamondback moth larvae, *Plutella xylostella* (Lepidoptera: Plutellidae), sprayed with 625 *B. bassiana* spores/cm had 53, 86 and 72% mortality, respectively after 7 days (Vandenberg et al. 1998b). Also, larval obliquebanded leafrollers, *Choristoneura rosaceana* (Lepidoptera: Tortricidae), were subjected to a direct conidial applications of several *B. bassiana* isolates at 1×10^7 conidia/ml and demonstrated a combined larval and pupal mortality of 60% after 60 days (Todorova et al. 2002).

Low mortality in the present direct topical exposure experiments may be the result of the lack of *in-vivo* blastospore formation (Pendland and Boucias 1997). *Beauveria bassiana* yeast cells and conidia were compared in a mortality bioassay against *Diatraea saccharalis* (Lepidoptera: Crambidae) (Alves et al. 2002). At concentrations of 1×10^7 and 1×10^8 cells/ ml, suspensions containing yeast-like cells caused significantly higher

mortality than the suspensions containing conidia. Also during molting, penetration of the old integument may be superficial and the insect can escape infection by casting the infectious inoculum (Ferron 1981). Insects may further cause mechanical damage to the inoculum by natural movements, for example continuously scraping off infectious propagules (Ferron 1978).

These studies were conducted to simulate a field spray with possible dual means of infection. This isolate was not evaluated at higher concentrations due to difficulties in keeping the conidia suspended in solutions during preliminary trials. Mortality was low for *H. zea* larvae in the direct contact study, but feeding insects were susceptible to *B. bassiana* 11-98. Although the infection process is not fully understood, *B. bassiana* 11-98 was effective in controlling feeding *H. zea* larvae. Further research with direct applications may also be necessary to evaluate application site, temperature, humidity and epidermal germination of *B. bassiana* 11-98.

Chapter 4

Effects of tomato foliage grown from seed coated with *Beauveria bassiana* 11-98 conidia on mortality and feeding preference of larval *Helicoverpa zea.*

Introduction

Beauveria bassiana (Balsamo) Vuillemin was known to infect insects only through conidial attachment, germination and penetration of the outer integument (Steinhaus 1949; Boucias and Pendland 1998). However, *B. bassiana* has recently been demonstrated as an endophyte of corn using light and electron microscopy (Wagner and Lewis 2000) and by isolating the fungus from plant samples placed on selective media culture (Doberski and Tribe 1980; Bing and Lewis 1991, 1992a, 1992b, 1993; Griffin et al. 2005). Foliar applications of granular formulations or injections of conidial suspensions have been used successfully to inoculate corn plants (Bing and Lewis 1992a). Sprays of 240 ml *B. bassiana* conidial suspensions containing 1.5×10^{11} colony forming units (cfu) to 1-m rows of potato also resulted in endophytic colonization of 78% of the plants (Jones 1994).

As an endophyte, *B. bassiana* was used to suppress tunneling of the European corn borer *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) (Bing and Lewis 1991). Suppression of tunneling in corn stalks may have been attributed to toxic metabolites produced within corn plants. If *B. bassiana* does produce metabolites within the plant, then colonization of tomato plants by *B. bassiana* 11-98 may be a sufficient amount of fungal growth to produce the metabolites necessary to reduce insect herbivory.

Several methods of application of *B. bassiana* fungal metabolites to manage

insects have been investigated. Techniques have included injections of *B. bassiana* inoculated broth (Quesada-Moraga and Vey 1993), direct injections of beauvericin into arthropods (Grove and Pople 1980) and applications of beauvericin to larval aquatic environments (Zizka and Weiser 1993). Beauvericin has also been applied directly to leaf disks in consumption assays using foliage-feeding insects (Gupta et al. 1991). Previous studies have not investigated the use of naturally occurring beauvericin produced by endophytic *B. bassiana.*

Vegetable seeds coated with insecticides or bioinsecticides have yielded promising results when tested against insect pests. A study that investigated the efficacy of systemic insecticide treatments to snap bean seeds yielded significantly less damage by bean leaf beetles, *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae) than did untreated seeds (Koch et al. 2005). Also, a seed dressing of 2% dry conidial powder, containing 1 x 10^{11} *B. bassiana* conidia g^{-1}, to corn induced endophytic colonization of the plants and reduced tunneling by stem boring, *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Cherry et al. 2004).

Recent research has demonstrated that tomato seeds coated with *B. bassiana* 11-98 yielded endophytic growth in tomato (Leckie 2002; Ownley et al. 2004). Leckie (2002) reported that coating the seed with conidia effectively introduced *B. bassiana* 11-98 into tomato seedlings when PCR amplification confirmed endophytic growth in sampled plants.

The objectives of this research were: 1) to assess effects of tomato foliage grown gnotobiotically from seed coated with *B. bassiana* 11-98 conidia when fed to 3rd instar *H zea* larvae and 2) to determine if 3rd instar tomato fruitworm larvae exhibit differential.

feeding activity for treated or untreated tomato foliage in choice and no-choice bioassays.

Materials and Methods

Propagation, Culture and Collection of *Beauveria bassiana* 11-98

Beauveria bassiana (isolate 11-98) was collected from an infected click beetle (Coleoptera: Elateridae) found in Scott County, TN by Roberto Pereira. The fungus was cultured on Sabouraud's dextrose agar (SDA) (Difco™, Becton, Dickenson & Co., Sparks, MD) and incubated at 20°C (68°F) for approximately three weeks. Conidia were harvested by brushing the surface of sporulating cultures with a round, camel's hair brush (Fisher Scientific, Pittsburgh, PA) and funneling spores into a glass vial. Vials of conidia were stored in the dark in a second airtight jar on top of a layer of Drierite™ (W. A. Hammond Drierite Comapany, Xenia, OH). Jars were maintained at 4°C in a laboratory refrigerator until spores were needed.

Maintenance of *Helicoverpa zea* Experimental Colony

A colony of tomato fruitworm, *H. zea*, was established from eggs obtained from Agripest (Zebulon, NC). Approximately 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 24°C (75.2°F) until larvae hatched. Artificial beet armyworm diet (Bio-Serv Inc., Product #F9219B, Frenchtown, NJ) was prepared by adding 19.8 g agar to 820 ml de-ionized water in a 1-liter beaker, placed on a hot plate and heated until clear. Heated agar was placed into a blender with 161.6 g dry diet mix, blended thoroughly and 5 ml aliquots were poured into each cell of a 32 cell rearing tray (Bio-Serv Inc., Frenchtown, NJ). Diet was allowed to cool and harden for 30 min before neonate larvae were

introduced with a small camel's hair brush. Rearing trays were placed in an incubator at 24°C (75.2°F) until larvae reached the 3rd instar experimental studia after about 8 days. Additional larvae were retained on diet to

maintain the laboratory colony.

Treatment of Tomato Seeds with *B. bassiana* Conidia

Tomato seeds (*Lycopersicon esculentum* Mill.) 'Mountain Spring' (Syngenta Seed Inc., seed lot F8895A, Downers Grove, IL) were treated with *B. bassiana* 11-98 by mixing a 2% methylcellulose solution with conidia. Methylcellulose was used so that the hydrophobic conidia could adhere to the seed coat. Seeds were surface-sterilized by soaking in 95% ethanol for 1 min, 66% bleach for 5 min and again in 95% ethanol for 1 min. Seeds were allowed to air-dry on a piece of filter paper placed in a vertical flow hood. Methylcellulose solution was prepared by autoclaving 50 ml of de-ionized water in a 100-ml beaker for 30 min. Sterilized water was placed on a stir plate and 1 g of methylcellulose (Sigma Chemical Co., St. Louis, MO) was added until suspended in solution, after which, the solution was transferred to an ice bath until it cleared. Approximately 80 dry, surface-sterilized seeds were placed in a petri dish. The 2% methylcellulose solution (3 ml) and *B. bassiana* 11-98 conidia (0.067 g) were added to the surface sterilized seeds with 50 μl Tween 85 in the petri dish. The mixture was stirred thoroughly every 30 min for 3 h until there was a uniform coating of the seeds. Seeds were separated from each other in the dish during the last 30 min and allowed to air-dry at ambient laboratory temperature (20°C) in the dark in a vertical flow hood over-night. Dilutions were placed on agar and cfu were determined to be approximately 5.5 x 10^3 per seed.

BotaniGard WP22 (Emerald BioAgriculture Corp., Butte, MT) was also used to coat seeds using the procedure described above. Eighty surface-sterilized seeds were placed in a petri dish along with methylcellulose (3 ml), Tween 85 (50 μl) and BotaniGard (0.0228 g). Dilutions were placed on agar and cfu were determined to be approximately 2.1 x 10^5 per seed. Control seeds were coated with methylcellulose only.

Growth of Gnotobiotic Tomato Seedlings

For each treatment 80 test tubes (24-mm (1-in) O. D. and 15-cm (6-in) in length were filled with 20-ml medium grain vermiculite and 20 ml de-ionized water and capped. Tubes and contents were sterilized by autoclaving for 30 min on 2 consecutive days. Test tubes were placed in a vertical flow hood and allowed to cool. Caps were removed and one seed was placed 5 mm (0.2 in) beneath the surface of the vermiculite in each tube. Tubes were recapped and placed in a growth chamber (Environmental Growth Chambers, Model # Q113a2, Chagrin Falls, OH) at 24°C (75.2°F) with a 12:12 (light:dark) h photoperiod. Seedlings were grown for 2 wk under gnotobiotic conditions, after which seedlings were transplanted into 5-cm (2-in) pots with sterile ProMix (Premier Horticulture, Red Hill, PA) and kept in a sterile growth chamber for 8 wk.

***H. zea* Feeding Preference and No-Choice Bioassays**

To assess the preference among 3rd instar *H. zea* larvae for treated versus untreated foliage, three 12-mm-diameter leaf disks were taken from leaves of 10-wk-old plants in the *B. bassiana* 11-98 and control treatments. Thus, 339.12 mm^2 total treated and untreated leaf area were presented to each larvae. Treated or untreated leaf disks were arranged alternately around the circumference of each of 100 petri dishes with a piece of moistened filter paper and one larvae was introduced to the experimental arena.

Half of the dishes had leaf disks of *B. bassiana* 11-98 treatment placed abaxially and untreated disks placed adaxially. For the remaining dishes, treatment positions in the dish were switched. Alternating treatments and placement of treatments were performed to ensure that larvae had equal opportunities to encounter both treatments and that larval feeding was not influenced by orientation of the leaf disks. This bioassay was performed at ambient laboratory temperature (20°C) with a 12:12 (L:D) h photoperiod. Percent consumption was recorded for each treatment after 6 and 24 h and the experiment was repeated.

To assess 3rd instar *H. zea* larval consumption of treated versus untreated tomato foliage, a no-choice bioassay was conducted. Tomato foliage from 40 eight-week-old plants from each treatment was used in this experiment. On each plant, two 15 x 18 cm (6 x 7 in) polyester cloth bags were placed around separate fully expanded leaf petioles at the 3rd and 4th true leaf position on the tomato plants. One 3rd instar tomato fruitworm was placed in each bag, and the bag was tied off at the base with a 10-cm (4-in) zip-tie. Larvae were allowed to feed in a growth chamber at 24°C (75.2°F) with a 12:12 (L:D) h photoperiod for 48 and 120 h. At the end of the feeding period, the stem was excised, the consumed percentage of available foliage was estimated from the remaining leaf tissue and recorded. Any foliage not eaten by larvae was collected and frozen for future analysis. Larvae were placed back on original diet and observed for mortality on days 5, 10 and 30. This experiment was repeated. To correct for mortality in the control, Abbot's formula was used (Abbott 1925). Data were transformed using arcsin squareroot transformation. Transformed data were analyzed using SAS GLM procedure (SAS Institute Inc., Cary NC) and Fisher's least significant difference test at α=0.05.

Results

Feeding Preference and No Choice Bioassays

Measured by consumption, 3rd instar tomato fruitworms did not demonstrate clear preferences for treated or untreated foliage after 6 h ($F = 0.25$, df = 1, 171, $P = 0.62$) or 24 h ($F = 1.46$, df = 1, 171, $P = 0.23$). Larvae consumed only slightly more untreated than treated foliage in both assays with 18 and 16% of untreated and treated tomato leaf disks ingested, respectively after 6 h, and 78 and 72.5% of available foliage of untreated and treated plants ingested, respectively after 24 h (Figure 4-1).

In no-choice assays, larval mortality and leaf area consumption were compared at 48 and 120 hours after infestation. A proportion of larvae from the no-choice assay emigrated through the polyester cloth bags after consuming all available tomato tissue. Because larvae could subsequently have consumed foliage from adjacent plants, escaped larvae were not counted towards percent mortality. Similarly, larvae that did not feed during trials were not used to calculate mean percent consumption. No larvae died within 48 h, but after feeding for 120 h, *H. zea* larvae that consumed tomato foliage from plants grown from seed treated with *B. bassiana* 11-98 experienced 17% (n=41) mortality compared to 2.5% (n=80) BotaniGard and 0% (n=46) untreated plants within 5 days (Figure 4-2). When incubated, one larva fed foliage from one of the *B. bassiana* 11-98 treated plants had mycelia grow through the integument and cover the epidermis.

Percent of foliage consumed during both trials did not differ between treatments ($P = 0.31$) and in each trial, larvae ate about the same amounts of treated or untreated tomato tissue ($P = 0.57$), thus larvae data for treatments were pooled between trials. No differences in mean percent foliage consumed by *H. zea* larvae were observed after 48 h

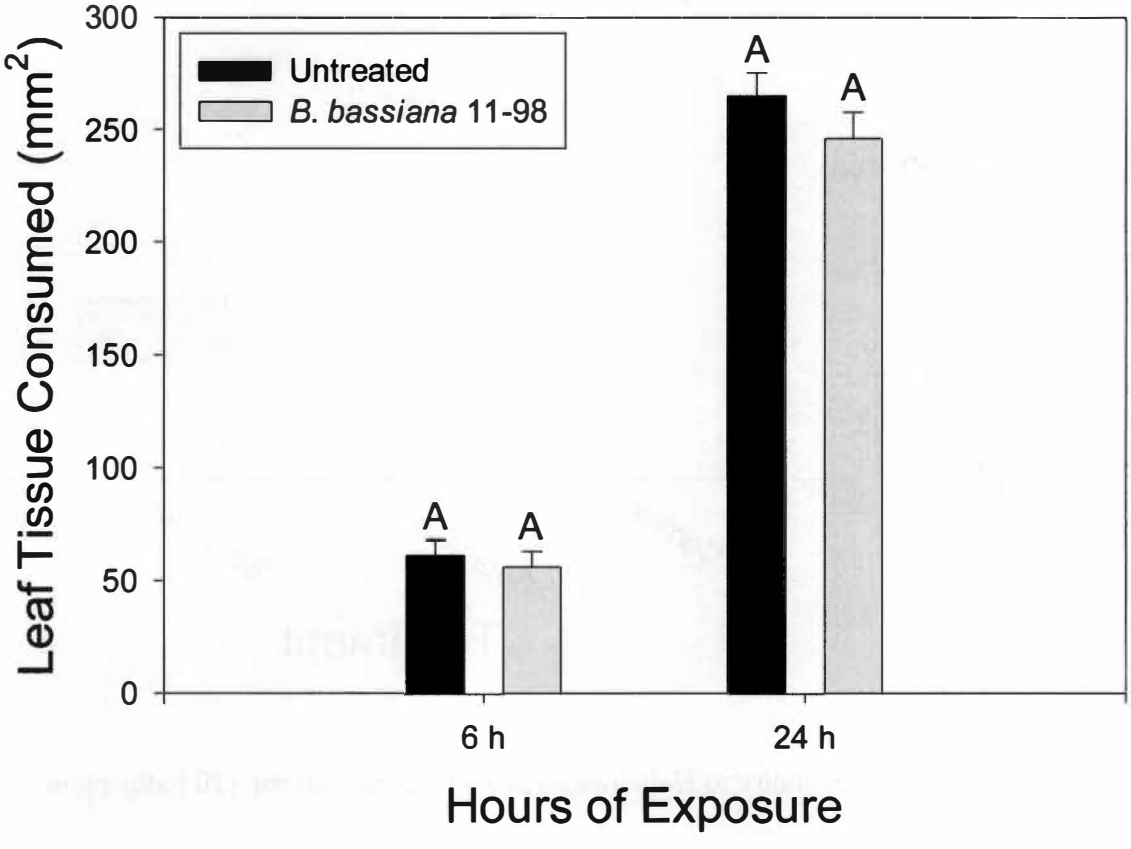


Figure 4-1. Average leaf area consumed by 3rd instar *Helicoverpa zea* larvae during choice preference assays. Error bars represent the standard error. Means within hours of exposure were separated using LSD mean separation (α=0.05). LSD values are 6 h=18.7 mm^2 and 24 h=28.3 mm^2. By hour, means without similar letters reported are significantly different.

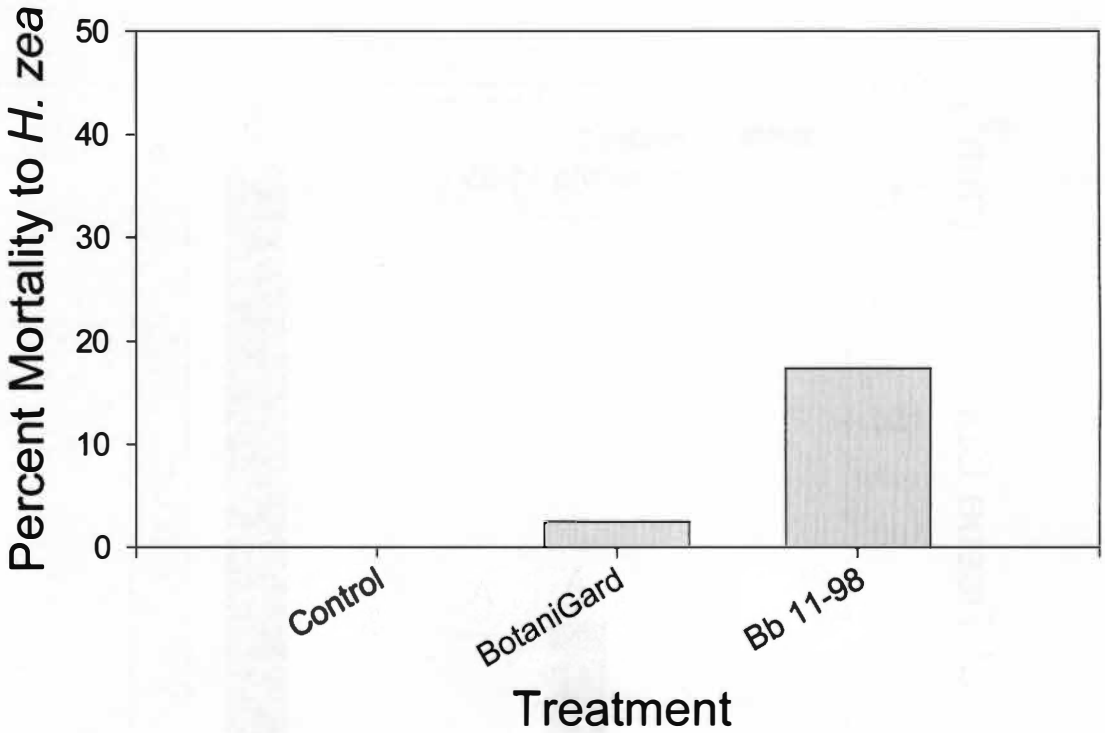


Figure 4-2. Percent mortality to *Helicoverpa zea* larvae that fed for 120 h during no-choice assays.

($F = 0.86$, df = 2,51, $P = 0.43$) or 120 h ($F = 0.89$, df = 2,103, $P = 0.42$). After 48 h, less feeding was evident by larvae provided only untreated foliage (8.5%) and more by larvae provided foliage treated with BotaniGard (17%). However after 120 h, larvae ate 33% of the available tomato leaf tissue treated with *B. bassiana* 11-98, which was the least tissue consumed of all treatments (Figure 4-3).

Discussion

In nature, larvae feed in response to stimuli induced by interactions with the plant and as a result of internal stimuli in the insect (Halcomb et al. 2000). If these stimuli are present and the insect is hungry, then the insect will feed regardless of the presence of a toxin. In our study, *H. zea* larvae consumed heavily and similarly on foliage of both treatments in the free choice assays. *Helicoverpa zea* larvae showed no preference for either treated or untreated tomato leaf tissue, but larvae consumed slightly less foliage from treated plants. Although, these results are similar to those of Jones (1994), in which defoliation of *B. bassiana* colonized potato plants by Colorado potato beetle larvae was 66% and statistically no different from feeding on untreated plants (71%).

In Jones's study, insects were not monitored to guage mortality after consuming endophytically-colonized plant tissue. However, Cherry et al (2004) did culture dead stem borers to look for mycosis, but when incubated, no signs of fungal infection were observed. In the present study, mortality to *H. zea* larvae was observed in both conidial seed treatments in the 120 h no-choice assays. One larva produced mycelial growth from the cadaver after incubation. This finding reveals that insects consuming endophytically colonized plant tissue can develop fungal mycosis and subsequently die. Larvae that

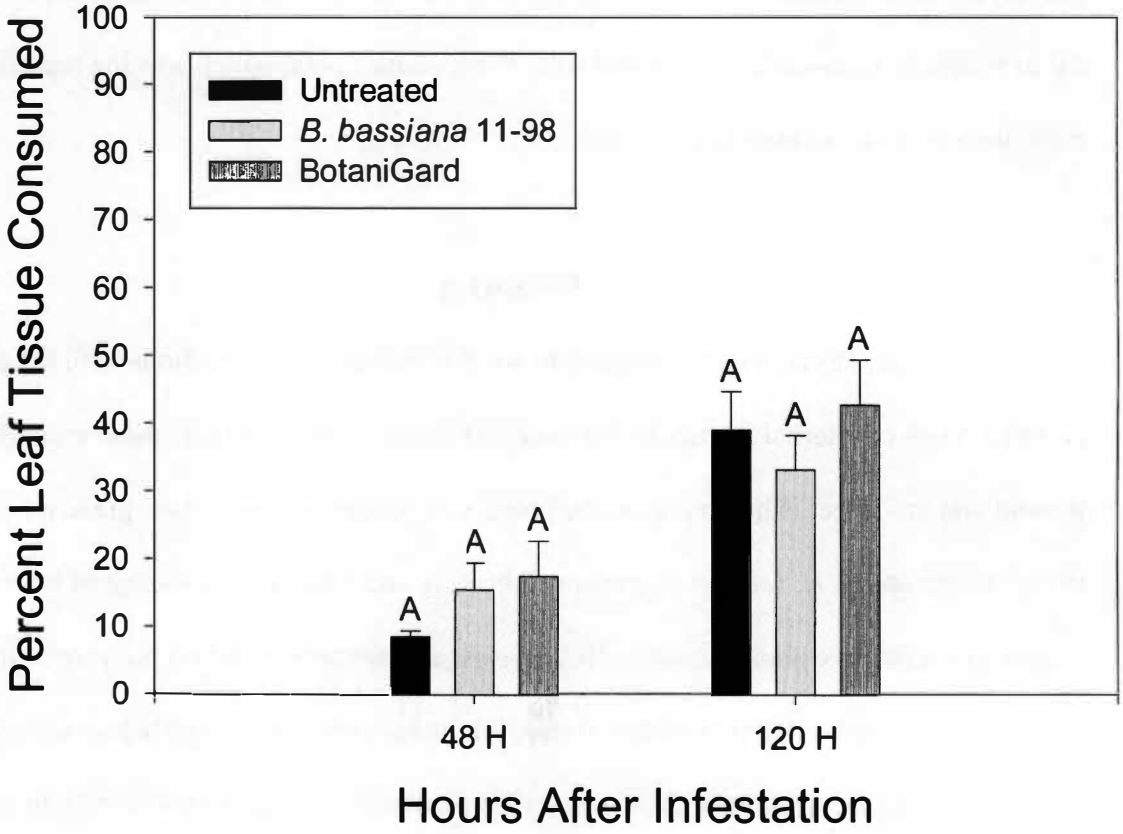


Figure 4-3. Estimated percentage of total tomato leaf tissue consumed by *Helicoverpa zea* larvae after 48 and 120 h during no-choice assays. Error bars represent the standard error. Statistical analysis were conducted using arcsin squareroot transformed data. Data in graphs are presented using unadjusted mean values. Means within hours after infestation were separated using LSD mean separation ($\alpha = 0.05$). LSD values are 48 h = 10.6 and 120 h = 14.9. By hour, means without similar letters reported are significantly different.

died after ingesting treated tissue consumed between 5-100% of available foliage. Mortality at the lower end of this range suggests that larvae may not have died as a result of consuming *B. bassiana* mycelia (Leckie 2002) in the foliage, but rather due to presence of one or more fungal mycotoxins. Presence of beauvericin in treated tomato tissues has been confirmed with high pressure liquid chromatography (Chapter 5).

Toxic fungal metabolites have been examined for lethal effects against several insects. Quesada-Moraga and Vey (1993) injected seven strains of *B. bassiana* inoculated broth into locust, *L. migratoria*. All but one strain was pathogenic, yielding between 57.5 and 94.4% mortality within 10 days. Beauvericin applied to leaf disks at 633 ppm and ingested by Colorado potato beetle larvae, *L. decemlineata* resulted in 50% mortality (Gupta et al. 1991). Beauvericin injected into adult blowfly, *C. erythrocephala* (5μg/ml) and *A. aegypti,* mosquito larvae (10 μg/ml) resulted in 15 and 39% mortality after 48 h, respectively (Grove and Pople 1980). Also, a 0.1-mg/ml suspension of beauvericin was added to water containing larvae of the northern house mosquito, *C. pipiens autogenicus*, resulted in 44% mortality after 48 h (Zizka and Weiser 1993). When examined with optical microscopy, tissues of all mosquito organs showed different levels of destruction, vacuolization and disorganization of muscle though the greatest damage was observed in the midgut. Beauvericin, when tested with the MTT-colorimetric assay against the SF-9 cell lines of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), had a CC_{50} (50% cytotoxicity concentration) of 2.5 μM after 48 h (Fornelli et al. 2004). If enough beauvericin is present in endophytically colonized plants, then coating seeds with *B. bassiana* conidia could be a new method for suppressing insect populations.

Leaf feeding by *H. zea* in no-choice assays did not differ at either 48 or 120 h after infestation. Our results suggest that internal insect and plant stimuli (Halcomb et al. 2000) and the insect state of hunger may have affected feeding behavior, but amounts of foliage in each bagged feeding arena were sufficient to satisfy the state of hunger of the insect before being totally consumed. Therefore, larvae had the opportunity to stop eating toxic tomato foliage. Although not statistically different, larvae consumed slightly less of the *B. bassiana* 11-98 treatment at 120 h after infestation.

Bing and Lewis (1993) found almost 2,000 cfu of *B. bassiana* per gram of soil collected from a corn field under a no-tillage regime, which was believed to have resulted in suppression of natural populations of European corn borer by mycosis. This inoculum concentration is much smaller than the 5.5×10^3 cfu/seed used in our study, which in turn was only half the 1×10^6 cfu/seed we intended to apply to each tomato seed. *Beauveria bassiana* that has been isolated both from plant tissue and *H. zea* (e.g. dual-host-enhanced) may be more effective in colonizing plants through the seed coat and may also be more virulent to insect pests. In turn, this is expected to allow for smaller amounts of conidia to be used in seed applications. Further research is necessary to answer these questions.

Environmental conditions such as light, temperature and relative humidity (Fargues and Luz 1998, 2000), play a major role in the growth and mode of infection of *B. bassiana* (Miller et al. 1983) and also on the growth of tomato plants (Sams 2001). Consequently, effects of field grown tomatoes treated with *B. bassiana* conidia as seeds will also need to be investigated.

Chapter 5

Isolation of *Beauveria bassiana* 11-98 and detection of its metabolite beauvericin in plant tissue.

Introduction

Beauveria bassiana (Balsamo) Vuillemin generally attacks insect hosts by direct penetration through the insect epidermis (Copping and Menn 2000). However, endophytic *B. bassiana* has shown promising results in reducing damage by insect pests. *Beauveria bassiana* has been shown to colonize corn plants endophytically and reduce tunneling of European corn borer and the corn stem borer (Bing and Lewis 1991; Cherry et al. 2004). Tunneling suppression was attributed either to direct fungal infection or to fungal metabolites produced in plant tissues. In 2000, Wagner and Lewis also demonstrated fungal colonization of corn plants through light and electron microscopy. Seed dressing, topical application and stem injections of *B. bassiana* to corn each reduced tunneling by the stem borer *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae), due to endophytic colonization of the plant (Cherry et al. 2004).

Endophytic colonization of *B. bassiana* in plants is typically confirmed by placing tissue samples on selective media cultures and watching for endophytic mycelial growth (Doberski and Tribe 1980; Bing and Lewis 1991, 1992a, 1992b, 1993). This technique may cause difficulties in isolation of *B. bassiana* due to contamination of cultures and competition from other microorganisms in the samples (Leckie 2002; Griffin et al. 2005). However, Bing and Lewis (1991) isolated *B. bassiana* at harvest on selective media from 98.3% of plants that had been treated topically and 95% of injected plants.

Beauvericin, an ionophorous mycotoxin produced by *B. bassiana,* has been shown to cause deleterious effects when tested against insect pests (Grove and Pople 1980; Gupta et al. 1991; Zizka and Weiser 1993). Beauvericin increases permeability of cell membranes to transfer ions into cells disrupting normal functions (Boucias and Pendland 1998). For example, a suspension (0.1 μg/ml) of beauvericin added to water yielded 44% mortality in northern house mosquito larvae, *Culex pipiens autogenicus* (Roubaud), after 48 h. Optical microscopic examination of tissues of all mosquito organs showed different levels of destruction, vacuolization and muscle disorganization. Greatest damage was observed in the midgut (Zizka and Weiser 1993). When *B. bassiana* metabolites were incorporated into artificial diet at 0.5% and fed to corn earworms, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), 25% mortality was observed 20 days after treatment (Leckie 2002).

Fungal production of toxic metabolites or mycotoxins can be monitored using different procedures. In Europe, mycotoxins produced by three *Fusarium* species were detected using high-performance liquid chromatography with diode array detection (HPLC-DAD), gas chromatography mass spectrometry (GC-MS) and GC electron capture detection (ECD) (Thrane et al. 2003). Oosporein, a red pigmented mycotoxin produced by *Beauveria* and *Fusarium* species, has been detected in submerged cultures of *Beauveria brongniartii* and inoculated barley kernels using HPLC (Strasser et al. 2000b). Beauvericin has been isolated from corn and wheat kernels inoculated with *Fusarium* sp. using HPLC (Josephs et al. 1999; Logrieco et al. 2002). It is unknown if *B. bassiana* produces beauvericin when growing endophytically within a host plant. If so, insect pests and their interaction with endophytically-colonized plants could be affected.

The objectives of this research were: 1) to determine if *B. bassiana* 11-98 can be recovered from tomato plants grown gnotobiotically when seeds are coated with conidia of *B. bassiana* 11-98 and 2) to develop plant tissue extraction and HPLC protocols enabling detection of the *B. bassiana* fungal metabolite beauvericin.

Materials and Methods

Propagation, Culture and Collection of *Beauveria bassiana*

Beauveria bassiana (isolate 11-98) was collected from an infected click beetle (Coleoptera: Elateridae) found in Scott County, TN by Roberto Pereira. The fungus was cultured on Sabouraud's dextrose agar (SDA) (Difco, Becton, Dickenson & Co., Sparks, MD) and incubated at 20°C (68°F) for approximately three weeks. Conidia were harvested by brushing the surface of sporulating plates with a round, camel's hair brush (Fisher Scientific, Pittsburgh, PA) and funneling the spores into a glass vial. Vials were stored in an airtight jar on top of a layer of Drierite™ (W. A Hammond Drierite Company, Xenia, OH). Jars were maintained at 4°C (39.2°F) in the dark in a laboratory refrigerator until spores were needed.

Treatment of Tomato Seeds with *B. bassiana* 11-98 conidia and BotaniGard

Tomato seeds (*Lycopersicon esculentum* Mill.) 'Mountain Spring' (Syngenta Seed Inc., seed lot F8895A, Downers Grove, IL) were treated with *B. bassiana* 11-98 and BotaniGard WP22 (Emerald BioAgriculture Corp., Butte, MT) by mixing a 2% methylcellulose solution with conidia. Seed surfaces were sterilized by soaking in 95% ethanol for 1 min, 66% bleach for 5 min and again in 95% ethanol for 1 min. Seeds were allowed to air dry on a piece of sterile filter paper placed in a vertical flow hood.

Methylcellulose solution was prepared by autoclaving 50 ml of de-ionized water in a 100-ml beaker for 30 min. Sterilized water was placed on a stir plate and 1 g methylcellulose (Sigma Chemical Co., St. Louis, MO) was added until suspended and the solution was transferred to an ice bath until clear. Approximately 80 surface-sterilized, dry seeds were placed in a petri dish. The 2% methylcellulose solution (3 ml) and *B. bassiana* conidia (0.067 g) or BotaniGard (0.0228 g) were added to the seeds with 50 µl Tween 85 in the petri dish. The mixtures were stirred thoroughly every 30 min for 3 h until there was a uniform coating of the seeds. Seeds were separated from each other in the dish during the last 30 min and allowed to air-dry at ambient laboratory temperature (20°C) in the dark in a vertical flow hood over-night. Dilutions were placed on agar and cfu were determined to be approximately 5.5×10^3 per seed for *B. bassiana* 11-98 and 2.1×10^5 per seed for BotaniGard. Control seeds were coated with methylcellulose only.

Growth of Gnotobiotic Tomato Seedlings

For each treatment, 80 test tubes (24-mm (1-in) O. D. and 15-cm (6-in) length) were filled with 20-ml medium grain vermiculite and 20 ml de-ionized water then capped. Tubes and contents were sterilized by autoclaving for 30 min on two consecutive days, then placed in a vertical flow hood and allowed to cool. Caps were removed and one seed per tube was placed 5 mm (0.2 in) beneath the vermiculite surface. Tubes were recapped and placed in a growth chamber (Environmental Growth Chambers, Model # Q113a2, Chagrin Falls, OH) at 24°C (75.2°F) with a 12:12 (L:D) h photoperiod. Tomato seedlings were grown for 2 wk under gnotobiotic conditions after which, seedlings were transplanted into 5-cm (2-in) pots with sterile ProMix (Premier Horticulture, Red Hill, PA) and kept in a sterile growth chamber for 8 wk.

Fungal Isolation from 'Mountain Spring' Tomato Leaves

A fully expanded tomato leaf from the terminal stem, a lateral stem in the middle of the plant and a lateral stem on the bottom half of the plant, were excised from 40 eight-wk-old plants and refrigerated for 24 h. Leaves from each plant were surface-sterilized by soaking in 95% ethanol for 1 min, 20% bleach for 3 min and again in 95% ethanol for 1 min (Jones 1994). Finally, leaves were rinsed in de-ionized water for a few seconds before being placed in a sterile petri dish to dry. Two pieces of each leaf from the *B. bassiana* 11-98 treatment and the control were aseptically cut with a sterile scalpel to approximately 5-mm (0.2-in) lengths and placed on selective media and Sabouraud's dextrose agar culture plates. Each dish contained one piece from each section of the plant. Selective media was prepared by adding 1 liter de-ionized water to 40 g glucose (Mallinckrodt Inc., Paris, KY), 10 g neopeptone (Difco™, Becton, Dickenson & Co., Sparks, MD), 15 g agar (Sigma Chemical Co., St. Louis, MO) and 0.01 g crystal violet (Sigma Chemical Co., St. Louis, MO). Formulated media was autoclaved for 45 min (Doberski and Tribe 1980). Cyclohexamide (0.25 g) was autoclaved separately then mixed with 0.5 g chloramphenicol and added to cooled, sterilized medium. Leaves on both selective and no-selective media were monitored weekly for endophytic growth.

Plant Tissue Extraction

Beauvericin was successfully recovered from 'Mountain Spring' tomato leaves using a modified extraction process obtained (after Strasser et al. 2000b). Tomato leaves were collected from the middle third of 10-wk-old plants grown from seeds coated with *B. bassiana* and frozen. Frozen leaves from each plant were lyophilized for 24 h, submerged in liquid nitrogen and ground with mortar and pestle. Ground, dry plant tissue

(1 g) was homogenized in approximately 8 ml ethyl acetate (Mallinckrodt Inc., Paris, KY), vortexed and filtered twice through Whatman No. 1 paper. Filtrate was poured into a 5 ml test tube and dried over Na_2SO_4 (Mallinckrodt Inc., Paris, KY). Supernatant was poured into a round bottom flask and evaporated on a Buchi RE 121Rotovapor (Buchi Laboritories-Technik AG, Flawil, Switzerland) at 60°C (140°F). Dry residue was resuspended by adding 1 ml HPLC grade methanol (Fisher Scientific International Inc., Pittsburgh, PA) in 100-µl aliquots until dissolved. The re-suspended solution was placed in a silanized tube, capped and used for HPLC.

High Pressure Liquid Chromatography

Fungal metabolites were detected by high pressure liquid chromatography (HPLC) (Waters 1525 binary pump and 717 plus autosampler) (Waters Corp., Milford, MA) on a reverse phase column (Xterra RP-18, 4.6 x 150 mm) (Waters Corp. Milford, MA) linked to a UV-VIS detector (Shimadzu SPD-10AV) (Shimadzu Scientific Instruments Inc., Norcross, GA) and quantified using the Waters Breeze 3.1™ HPLC software system (Waters Corp., Milford, MA). Optimum detection wavelength for beauvericin was 205 nm (after Logrieco et al. 2002). Mobile phase was HPLC grade acetonitrile and water (Fisher Scientific International Inc., Pittsburgh, PA) (90:10 v/v), both containing 0.05% trifluoricacetic acid (w/v) at a flow rate of 1.2 ml/min under isocratic conditions (Josephs et al. 1999). Retention time for beauvericin was approximately 2.04 min. Vials of extract were maintained in a sample carousel in the injector at 4°C. Injection volumes of sample extract were 5 µl. Quantification was performed by comparing the retention time of beauvericin with a calibration curve obtained using a beauvericin standard (Sigma Chemical Co., St. Louis, MO) at 1.0, 0.1

and 0.01 mg/ml. All samples were analyzed twice. Independent confirmation of the identity of beauvericin was outside the scope of these experiments but will be performed.

Results

Fungal Isolation

Forty plants treated as seeds with *B. bassiana* 11-98 conidia in the first trial yielded 7 cultures (17.5%) with endophytic *B. bassiana* growth. Only 2 cultures (5%) yielded endophytic *B. bassiana* growth from the BotaniGard WP22-treated seeds (Table 5-1). No *B. bassiana* was recovered from tomato tissues harvested from untreated plants. While a few cultures also yielded unidentified contaminates, none were observed in *B. bassiana* cultures. Also, all sampled zones of tomato plants produced endophytic *B. bassiana* growth. Of 7 *B. bassiana* 11-98 cultures, three grew from tissue taken from the bottom third of the plant, two from the top third, one from the middle third and one from both the middle and top (Table 5-1). BotaniGard WP22 treated plants yielded growth in one culture taken from the top and one from the bottom third of the plant.

In the second trial, only one culture (2.5%) was recovered from plants treated as seeds with *B. bassiana* 11-98 and six (15%) from plants treated as seeds with BotaniGard WP22. Untreated plants did not show endophytic *B. bassiana* growth. Of the plants showing endophytic growth, none was observed from the top third of the plant (Table 5-2). One plant again showed fungal growth from two areas of the plant. Endophytic *B. bassiana* growth was observed after incubation for 4 wk on media. Tomato tissues from both trials did not produce additional *B. bassiana* cultures after 8 wk incubation.

Table 5-1. Isolation of *Beauveria bassiana* from leaves of 'Mountain Spring' tomato plants grown from seeds coated with conidia in the first trial.

Beauveria bassiana Endophytic Growth			
	Number of Original Plants (n = 40)		
Treatment	Top[a]	Middle[b]	Bottom[c]
Control	-	-	-
B. bassiana 11-98	3	2	3
BotaniGard WP22	1	-	1

[a] *Beauveria bassiana* 11-98 plant #s 23, 30 and 12*; BotaniGard WP22 plant # 36.

[b] *Beauveria bassiana* 11-98 plant #s 21 and 12.

[c] *Beauveria bassiana* 11-98 plant #s 3, 19 and 42; BotaniGard WP22 plant # 34.

* Plant # 12 also killed an insect in previous no choice test.

Table 5-2. Isolation of *Beauveria bassiana* from leaves of 'Mountain Spring' tomato plants grown from seeds coated with conidia in the second trial.

Beauveria bassiana Endophytic Growth			
	Number of Original Plants (n = 40)		
Treatment	Top	Middle[a]	Bottom[b]
Control	-	-	-
B. bassiana 11-98	-	-	1
BotaniGard WP22	-	3	3

[a] BotaniGard WP22 plant #s 41, 22 and 61.

[b] *Beauveria bassiana* 11-98 plant # 57; BotaniGard WP22 plant #s 9, 59 and 61.

Plant Tissue Extraction Efficiency

Plant tissue extraction efficiency was confirmed by extracting beauvericin from several leaves from multiple plants grown from seeds coated with *B. bassiana* 11-98 (1 g dry material). Beauvericin concentration was 0.061 mg/g. Tissue from untreated plants was ground into a fine powder and 1 g dried material was spiked with 0.061 mg beauvericin. Extraction was as described above. Spiked tissue was injected and beauvericin was present at 0.049 mg/g. Thus, our extraction process for recovering beauvericin from tomato leaf tissue was 79.5% efficient.

High Pressure Liquid Chromatography

Leaf tissue from plants that produced endophytic *B. bassiana* growth on selective medium cultures and leaf tissue from plants that killed an insect in the no-choice bioassay (Chapter 4) was extracted for HPLC analysis. Parameters of the HPLC system for beauvericin detection are presented in Table 5-3. Beauvericin or a compound that co-elutes with beauvericin was detected in all samples from *B. bassiana* 11-98 and BotaniGard WP22 treatments (Table 5-4). No beauvericin was detected in tissue from untreated tomato plants (Figure 5-1). Tissue from plants in the *B. bassiana* 11-98 seed treatment where insects died following consumption (Chapter 4) contained 0.086, 0.077, 0.074 and 0.074 mg/g beauvericin for plant numbers 12, 38, 43 and 48, respectively (Table 5-4).

In the second trial, leaf tissue from plants that produced endophytic *B. bassiana* growth on selective medium cultures and leaf tissue from plants that killed an insect in

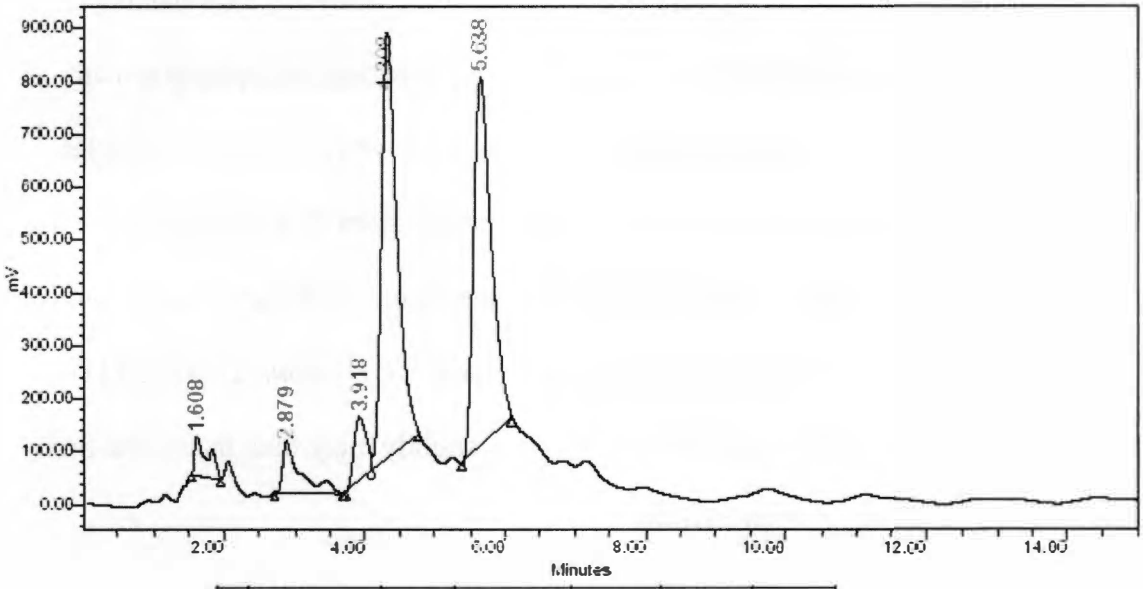
Table 5-3. HPLC component parameters for detecting beauvericin in plant tissue.

Component	Parameter
HPLC Type	Adsorption
HPLC Mode	Reversed-phase
Column	Xterra RP-18 (4.6 x 150 mm)
Mobile Phase Solvents	acetonitrile:water (90:10)
Mobile Phase Buffer	trifloricacetic acid (0.05%)
Mobile Phase Method	Isocratic
Flow Rate	1.2 ml/min
Injection Volume	5 μl
Detector	UV-VIS
Beauvericin Retention Time	2.1 min

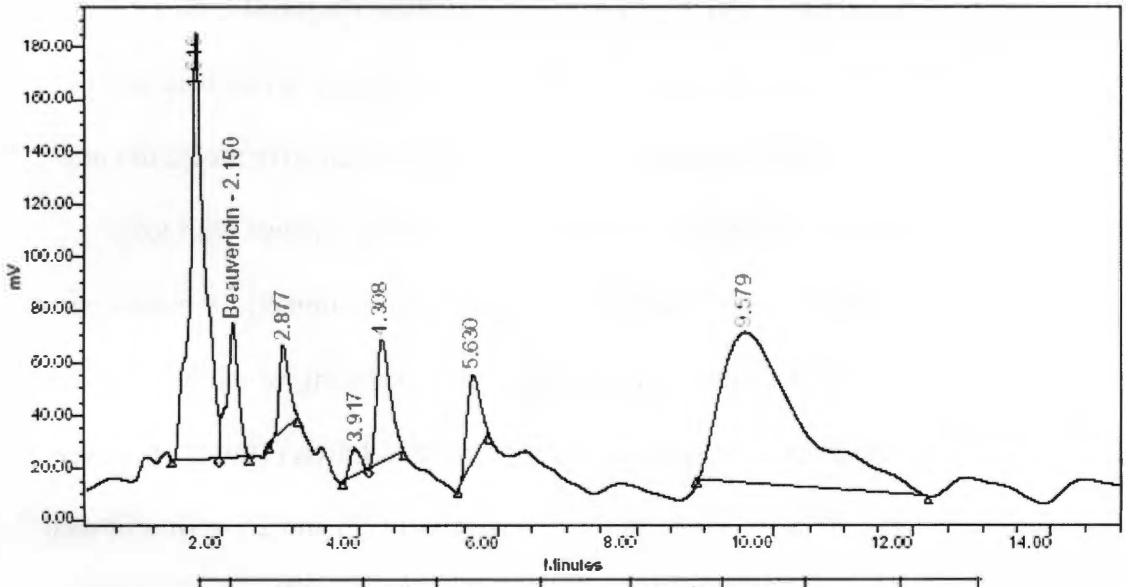
Table 5-4. Detection of the *Beauveria bassiana* metabolite beauvericin in leaves of 'Mountain Spring' tomato plants grown from seeds coated with conidia in the first trial.

HPLC beauvericin detection	
Treatment Plant Code	Beauvericin Concentration (mg/g dried tissue)
Control	-
B. bassiana 11-98	
23 [b]	0.043
42 [b]	0.063
21 [b]	0.064
43 [a]	0.074
48 [a]	0.074
19 [b]	0.075
38 [a]	0.077
4 [b]	0.085
12 [a, b]	0.086
30 [b]	0.142
3 [b]	0.171
BotaniGard WP22	
34 [b]	0.095
36 [b]	0.125

[a] Foliage from these plants killed an insect in no choice bioassay.
[b] Foliage from these plants had endophytic growth on selective media cultures.



	Peak Name	RT (min)	Area (V*sec)	% Area	Height (V)	% Height
1		1.608	992974	3.40	75909	4.19
2	Beauvericin	2.170				



	Peak Name	RT (min)	Area (V*sec)	% Area	Height (V)	% Height	Amount	Units
1		1.616	2417582	26.31	161682	40.96		
2	Beauvericin	2.150	577849	6.29	50692	12.84	0.049	mg/ml

Figure 5-1. HPLC chromatograms (Waters Breeze 3.1 HPLC system, Waters Corp., Milford, MA) of untreated tomato tissue and untreated tomato tissue spiked with beauvericin standard.

the no-choice bioassay (Chapter 4) were extracted for HPLC analysis. Two plants (#22 and #61) from the BotaniGard WP22 seed treatment that produced endophytic growth were damaged during relocation and therefore not analyzed. Only 4 out of 13 samples did not produce beauvericin (two from BotaniGard and two from *B. bassiana* 11-98). In all other samples, including two untreated plants, beauvericin was detected and amounts are listed in Table 5-5. Average beauvericin production by *B. bassiana* 11-98 (0.111 mg/g) and BotaniGard (0.120 mg/g) in this assay was slightly more than that of the first trial (0.061 and 0.097 mg/g, respectively).

Discussion

Beauveria bassiana 11-98 can colonize tomato plants via seed treatment with conidia (Leckie 2002; Ownley et al. 2004). Although the method of inoculation was different, fungal isolation of *B. bassiana* from plant tissue on selective media did not succeed as well as that of Bing and Lewis (1991), where 98% of plants with foliar treatments had endophytic growth at harvest (119 days after treatment). *B. bassiana* 11-98 was isolated from 10% of treated plants 60 days after treatment, which was consistent with the foliarly-treated plants in a seperate trial of Bing and Lewis (1991). However, our inoculum amounts, 5.5×10^3 cfu/seed for *B. bassiana* 11-98 and 2.1×10^5 cfu/seed for BotaniGard WP22, were less than our desired amount of 1×10^6 cfu/seed. With higher inoculum rates, endophytic colonization of tomato plants may have also been higher.

Of plants treated with *B. bassiana* 11-98 that produced endophytic growth on cultures in both trials, fungus was recovered from all sampled positions on the plant.

Table 5-5. Detection of the *Beauveria bassiana* metabolite beauvericin in leaves of 'Mountain Spring' tomato plants grown from seeds coated with conidia in the second trial.

HPLC beauvericin detection	
Treatment Plant Code	Beauvericin Concentration (mg/g dried tissue)
Control	
54 [a]	0.013
5 [a]	0.146
B. bassiana 11-98	
3 [a]	-
7 [a]	-
25 [a]	0.052
57 [b]	0.058
48 [a]	0.065
1 [a]	0.078
BotaniGard WP22	
41 [b]	-
59 [b]	-
9 [b]	0.043
46 [a]	0.009
51 [a]	0.038

[a] Foliage from these plants killed an insect in no choice bioassay.
[b] Foliage from these plants had endophytic growth on selective media cultures.

These results support the hypothesis of Leckie (2002), that plants in the Solanaceae family, including tomato, are ideal for endophytic growth of *B. bassiana.* Roots, shoots and fruit of endophytically-colonized plants were not tested in this assay. *Beauveria bassiana* was isolated from the roots of cotton seedlings grown from seeds coated with conidia of isolate 11-98 (Griffin et al 2005). Host-enhanced *B. bassiana* isolated from plant tissue may be more effective in colonizing plants through the seed coat and may also be more active inside the plant, which could allow for smaller amounts of conidia to be used in seed applications and quicker establishment in the plant.

Beauvericin has demonstrated toxicity to several insects in different insect orders. Beauvericin ingested by Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say) had an LC_{50} of 633 ppm and LC_{90} of 1,196 ppm (Gupta et al. 1991). An injection of beauvericin into blowfly adults, *Calliphora erythrocephala* (Meig.) (5μg/ml) and *Aedes aegypti* (L.) mosquito larvae, (10 μg/ml) resulted in 15 and 39% mortality after 48 h, respectively (Grove and Pople 1980). Plants with endopytic *B. bassiana* 11-98 and BotaniGard WP22 appear to have extractable beauvericin (0.086 and 0.108 mg/g, respectively) in tomato plants within 10 wk; it is possible, but unlikely that the compound isolated is not beauvericin, so independent confirmation is imperative. In chromatograms containing beauvericin, a large peak appears at 9.5 min. This peak may be a polymer of the compounds observed at 4.3 and 5.6 min.

Our in-plant dosages are higher than those administered to *C. pipiens autogenicus, C. erythrocephala* and *A. aegypti* in previous assays (Grove and Pople 1980; Zizka and Weiser 1993). Our results suggest that the amount of beauvericin produced by endophytic *B. bassiana* would be sufficient to influence insect population growth. Plants

were selected based on presence of *B. bassiana* in cultures or insect mortality. Beauvericin recovery was fairly consistent within all selected plants of the same treatment. Beauvericin recovery from untreated plants could be the result of plant colonization due to inoculum traveling in irrigation water through the root ball of treated plants on the upper shelves and splashing onto untreated plants on the lower shelves in the growth chamber. Bing and Lewis (1991) reported colonization of untreated plants in the field due to wind blown inoculum. Alternately, research has suggested that plants systemically infected with fungal pathogens in the class Deuteromycetes can transfer the pathogen from plant to seeds. For example, pepper seeds became infected with *Collectotrichum capsici* following plant infection (Siddiqui et al. 1977). Hyphae of *Fusarium moniliforme* were transferred from corn plants to the basal part of the embryo where the fungus grew throughout the seed (Lawrence et al. 1981). This past research suggests that untreated seeds in present research may have been infected with *B. bassiana* before use in any assay. The fact that beauvericin was also found in plant tissue not showing endophytic growth on selective medium cultures leads to the assumption that beauvericin traveled within the vascular system of the plant. If this is true, then coating tomato seeds with *B. bassiana* 11-98 conidia may provide enough endophytic growth to influence insect activity on the plant.

Beauvericin is just one of many mycotoxins produced by *B. bassiana.* This fungus also produces bassianolide, cyclosporein and oosporein, each of which aid in the infection and subsequent death of infected insects (Boucias and Pendland 1998). Further research is needed to analyze tissue from all other areas of the plant and tissue from older plants, especially fruit, for beauvericin and other metabolites produced by *B. bassiana,* as

well as a positive confirmation of beauvericin.

Only tomato plants were used in this research. More work involving endophytic colonization of *B. bassiana* 11-98 in other plants in the Solanaceae family is needed to evaluate the potential for those plants to host this isolate of *B. bassiana.*

Chapter 6

Summary

Beauveria bassiana is a ubiquitous, soil-inhabiting entomopathogenic fungus in the phylum Deuteromycota that infects a wide range of insects. When not exhibiting entomopathogenic properties, *B. bassiana* is a saprophyte in the soil (McCoy et al. 1985). Conidia typically infect insect hosts through attachment, germination and penetration of the outer integument (Boucias and Pendland 1998). *Beauveria bassiana* conidia have also been shown to infect susceptible hosts through tracheal openings and by penetrating the alimentary tract (Steinhaus 1949). With the aid of fungal metabolites, hyphae proliferate inside the insect body cavity eventually killing the host. Within 24-48 h of death, the insect cadaver is covered with white mycelia that have grown through the intersegmental membranes. Subsequently, conidiophores and conidia are produced and disseminated by wind and rain-splash to other hosts (Pekrul and Grula 1979). Throughout the infection process, *B. bassiana* produces toxic enzymes and metabolites that aid in the normal function of the fungus. Toxins produced by *B. bassiana* include beauvericin, bassianolide, oosporein and cyclosporein.

In this research, assays determined the efficacy of *B. bassiana* 11-98 against two tomato pests, *Macrosiphim euphorbiae* and *Helicoverpa zea.* We detected the fungal metabolite beauvericin in tomato foliage using HPLC and evaluated the effects of endophytic *B. bassiana* 11-98 on larval *H. zea.*

When *M. euphorbiae* were introduced to tomato foliage treated with conidial suspensions of *B. bassiana* 11-98, 29 and 40% mortality was observed at 7 and 10 days after treatment (DAT), respectively, at the higher conidial concentrations. Highest

mortality to *M. euphorbiae* following direct exposure to *B. bassiana* 11-98 conidia 10 DAT was 34%. Results from all aphid assays were much lower when compared to other studies involving similar species and conidial rates (Dorschner et al. 1991; Vandenberg et al. 2001, Yeo et al. 2003). When compared to other insects, reproduction of aphids is intensified due to rapidly maturing, parthenogenic females (Delahaut 2001). Reproduction of treated and untreated adults were not different and at a concentration of 5.5×10^4 conidia/ml *B. bassiana* 11-98, *M. euphorbiae* were able to produce an average of 81 nymphs 10 DAT, which was highest of all treatments. Still, moderate mortality to *M. euphorbiae* suggests that *B. bassiana* 11-98, when integrated into an IPM system could successfully reduce potato aphid populations in the greenhouse and field. Future tests are necessary to evaluate this possibility.

Direct application of *B. bassiana* 11-98 conidia to the epidermis of *H. zea* larvae yielded only 17% mortality 30 DAT for the highest rate of 2.3×10^7 conidia/ml applied. When compared with topical applications of different isolates of *B. bassiana* to lepidopteran pests, isolate 11-98 appears to be a less virulent strain (Vandenberg et al. 1998b; Zurek and Keddie 2000; Hicks et al. 2001). But *H. zea* consumption of tomato foliage treated with *B. bassiana* 11-98 conidia produced higher rates and faster mortality in larvae at levels consistent with similar assays (McDowell et al. 1990; Vandenberg et al. 1998a). Although mosquito larvae experienced penetration and infection in the gut (Zizka and Weiser 1993), Boucias and Pendland (1998) state that the pH within the gut of lepidopterans is too high for growth of *B. bassiana.* Our results can most likely be attributed to trapped, viable conidia in and around the mouth and anus and not penetration of the gut wall. The mouth and anus on larval cockchafer, *Melolontha*

melolontha, were the most sensitive sites to infection of *Beauveria* sp. (Delmas 1973). Further research is necessary to investigate if conidia of *B. bassiana* 11-98 are able to survive, germinate and infect *H. zea* larvae by penetrating the digestive tract.

Beauveria bassiana 11-98 was evaluated also for the ability to colonize gnotobiotically-grown tomato plants after seeds were coated with conidia. Choice and no-choice larval feeding assays, using plants with endophytic *B. bassiana* 11-98 against 3rd instar *H. zea* larvae suggest that larval feeding is not affected. Although more untreated foliage was consumed, feeding tests showed no clear preference for either treated or untreated plants after 6 and 24 h. In no-choice assays, *H. zea* larvae consumed slightly less foliage from plants treated with *B. bassiana* 11-98 after 120 h, but feeding by individual larvae was highly variable and treatments did not differ. Also, larvae that fed for 120 h on tomato foliage from the *B. bassiana* 11-98 treatment experienced 17% mortality.

Foliage from endophytically-colonized tomato plants was extracted and analyzed for presence of the mycotoxin beauvericin using HPLC. Presence of beauvericin was determined by co-elution with an authentic standard; independent confirmation was not done. Beauvericin was present in all but four samples tested from isolate 11-98 and BotaniGard WP22 treatments. Endophytic growth of *B. bassiana* is sufficient for production of metabolites within the plant. Beauvericin also was recovered from plants that neither killed an insect nor showed epiphytic growth on selective media cultures. The recovery of beauvericin from treated plants that yielded no colonization on agar media suggests that beauvericin is produced early in the colonization process and that mycotoxins move within the vascular system of the plant. Beauvericin was recovered

from two untreated plants suggesting that inoculum traveled in irrigation water from treated to untreated plants. Bing and Lewis (1991) experienced a similar phenomenon when untreated plants became endophytically-colonized by wind blown inoculum. Alternately, research has suggested that plants systemically infected with fungal pathogens in the class Deuteromycetes can transfer the pathogen from plant to seeds. For example, pepper seeds became infected with *Collectotrichum capsici* following plant infection (Siddiqui et al. 1977). Hyphae of *Fusarium moniliforme* were transferred from corn plants to the basal part of the embryo where the fungus grew throughout the seed (Lawrence et al. 1981). Deuteromycetes have also been transferred to seedling by fungal structures contained within the viable seed. In soybean, *Colletotrichum truncatum* hyphae were observed in cotyledons and became established inside the plant. Hyphae then penetrated the stem, petioles, leaves and developing seeds and pods (Tiffany 1951). Indeed, embryonic infection generally results in systemic infection of the new plant (Agarwal and Sinclair 1997). If *B. bassiana* can endophytically colonize tomato plants and be transferred from the plant to the embryo, then one conidial seed treatment could be enough inoculum to colonize multiple generations. Histological work is also needed to determine where fungal structures occur within plants and seeds.

Environmental conditions such as light, temperature, precipitation and relative humidity play a major role in the growth and infection of *B. bassiana* (Bing and Lewis 1991; Fargues and Luz 1998, 2000). Endophytic establishment could eliminate the problems brought on by the environment, but it could also create new problems. For example, beauvericin is toxic to brine shrimp (Strasser et al. 2000a) and several mammalian cell lines including those of humans (Logreico et al. 2002). Further testing

involving the fruit of endophytically colonized plants from coated seeds is crucial to determine if any fungal structures or mycotoxins are present.

Based on these experiments, *B. bassiana* 11-98 has considerable potential to act as a microbial control agent against plant feeding insect pests. Wagner and Lewis (2000) demonstrated that foliar applications of *B. bassiana* conidia to corn are an efficient method for endophytic colonization. Electron microscopy showed that conidia entered the plant through stomata and other natural openings. If a field spray of isolate 11-98 conidia can colonize tomato plants in the same manner, then insects that feed on plant tissue are in double jeopardy due to viable conidia on the surface of leaves and the mycotoxins produced inside the plant.

Literature Cited

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265–267.

Adams, J. B. and H. F. Van Emden. 1972. The biological properties of aphids and their host plant relationship. pp 47-104. *In*: Aphid Technology, H. F. Van Emden [ed]. AP Acad. Press, London, England.

Agarwal, V. K. and J. B. Sinclair. 1997. Principles of Seed Pathology. 2nd ed. CRC Press, Boca Raton, FL.

Alexopolous, C. J., C. W. Mims and M. Blackwell. 1996. Introduction to Mycology. 4th ed. John Wiley and Sons, New York, NY.

Alves, S. B., L. S. Rossi, R. B. Lopes, M. A. Tamai and R. M. Pereira. 2002. *Beauveria bassiana* yeast phase on agar medium and its pathogenicity against *Diatraea saccharalis* (Lepidoptera: Crambidae) and *Tetranychus urticae* (Acari: Tetranychidae). J. Invertibr. Pathol. 81: 70-77.

Bing, L. A. and L. C. Lewis. 1991. Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. Environ. Entomol. 20: 1207-1211.

Bing, L. A. and L. C. Lewis. 1992a. Temporal relationships between *Zea mays*, *Ostrinia nubilalis* (Lepidoptera: Pyralidae) and endophytic *Beauveria bassiana*. Entomophaga 37: 525-536.

Bing, L. A. and L. C. Lewis. 1992b. Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostrinia nubilalis* (Hubner). Biocontr. Sci. Technol. 2: 39-47.

Bing, L. A. and L. C. Lewis. 1993. Occurrence of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimes and in *Zea mays* L. and virulence towards *Ostrinia nubilalis* (Hubner). Agr. Ecosys. Environ. 45: 147-156.

Boucias, D. G. and J. C. Pendland. 1998. Principles of Insect Pathology. Kluwer Academic Publishers, Boston.

Butler, Jr., G. D. 1976. Bollworm: development in relation to temperature and larval food. Environ. Entomol. 5: 520-522.

Canaday, C. H., B. H. Ownley, G. L. Lentz, K. D. Gwinn, D. M. Benson, M. L. Elliot, T. K. Broschat and C. M. Mannion. Unpublished data.

Capinera, J. L. 2004. Corn earworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae). University of Florida IFAS Extension publication EENY-145. Gainesville, FL.

Cherry, A. J., A. Banito, D. Djegui and C. Lomer. 2004. Suppression of the stem-borer *Sesamia calamistis* (Lepidoptera: Noctuidae) in maize following seed dressing, topical application and stem injection with African isolates of *Beauveria bassiana.* Int. J. Pest Manag. 50: 67-73.

Clay, K. 1987. Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea.* Oecologia 73. 358-362.

Copping, L. G. and J. J. Menn. 2000. Biopesticides: a review of their action, applications and efficacy. Pest Manag. Sci. 56: 651-676.

Dadd, R. H. and T. E. Mittler. 1965. Srudies on the artificial feeding of the aphid *Myzus persicae* (Sulzer)-III. Some major nutritional requirements. J. Insect. Physiol. 11: 717-743.

Delahaut, K. A. 2001. Vegetable Aphids. University of Wisconsin Extension publication A3757-E. Madison, WI.

Delmas, J. C. 1973. Influence of the place of tegumentary contamination on the development of *Beauvaria tenella* (Delacr.) Siemaszko (Fungi imperfecti) mycosis in the larvae of the Coleoptera *Melolontha melolontha* L. Comptes Rendus Hebdomudaires des Seances. Academie des Sciences. Serie D. Sciences Naturelles. 277: 433-435.

Doberski, J. W. and H. T. Tribe. 1980. Isolations entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisophliae.* Trans. Br. Mycol. Soc. 74: 95-100.

Dorschner, K. W., M. G. Feng and C. B. Baird. 1991. Virulence of an aphid-derived isolate of *Beauveria bassiana* (Fungi: Hyphomycetes) to the Hop aphid *Phorodon humuli* (Homoptera: Aphididae). Environ. Entomol. 20: 690-693.

Eyal, J., MD A. Mabud, K. L. Fischbein, J. F. Walter, L. S. Osborne and Z. Landa. 1994. Assessment of *Beauveria bassiana* Noc. EO-1 strain, which produces a red pigment of microbial control. Appl. Biochem. Biotechnol. 44: 65-80.

Fargues, J. and C. Luz. 1998. Effects of fluctuating moisture and temperature regimes on sporulation of *Beauveria bassiana* on cadavers of *Rhodnius prolixus.* Biocontr. Sci. Technol. 8: 323–334.

Fargues, J. and C. Luz. 2000. Effects of fluctuating moisture and temperature regimes on the infection potential of *Beauveria bassiana* for *Rhodinus prolixus.* J. Invertibr. Pathol. 75: 202-211.

Feng, M. G. and J. B. Johnson. 1991. Biosaaay of four entomophthoralean fungi (Entomophthorales) against *Diuraphis noxia* and *Metopolophium dirhodum* (Homoptera: Aphididae). Environ. Entomol. 20: 338-345.

Feng, M. G., J. B. Johnson and L. P. Kish. 1990. Virulence of *Verticillium lecanni* and an aphid-derived isolate of *Beauveria bassiana* (Fungi: Hyphomycetes) for six species of cereal-infesting aphids (Homoptera: Aphididae). Environ. Entomol. 19: 815-820.

Feng, Z., R. Carruthers, T. Larkin and D. Roberts. 1988. A phenology model and field evaluation of *Beauveria bassiana* (Bals.) Buillemin (Deuteromycota: Hyphomycetes) mycosis of the European corn borer, *Ostrinia nubilalis* (Hbn.) (Lepidoptera: Pyralidae). Can. Entomol. 120: 133-144.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. Annu. Rev. Entomol. 23: 409-442.

Ferron, P. 1981. Pest control by the fungi *Beauveria.* pp 465-482. *In:* Microbial Control of Pests and Plant Diseases 1970-1980, H. D. Burges [ed.]. Acad. Press, New York, NY.

Forbes, A. R. 1977. The mouthparts and feeding mechanisms of aphids. pp. 83-103. *In*: Aphids as virus vectors, K. F. Harris and K. Maramorosch [eds.]. Acad. Press, New York, NY.

Forbes, A. R., B. D. Frazer and C. K. Chan. 1985. Aphids. pp. 353-359. *In*: Handbook of Insect Rearing Vol. 1, P. Singh and R. F. Moore [eds.]. Elsevier, New York, NY.

Fornelli, F., F. Minervini and A. Logrieco. 2004. Cytotoxicity of fungal metabolites to lepidopteran (*Spodoptera frugiperda*) cell line (SF-9). J. Invertibr. Pathol. 85: 74-79.

Griffin, M., B. Ownley, W. Klingeman and R. Pereira. 2005. Biocontrol of *Rhizoctonia* damping-off of cotton with endophytic *Beauveria bassiana.* Phytopathology 95: S36.

Grove, J. and M. Pople. 1980. The insecticidal activity of beauvericin and the enniatin complex. Mycopathologia 70: 103-105.

Gupta, S., S. Krasnoff, N. Underwood, J. A. Renwick and D. Roberts. 1991. Isolation of beauvericin as an insect toxin from *Fusarium semitectum* and *Fusarium moniliforme* var. *subglutinans*. Mycopathologia 115: 185-189.

Halcomb, J. L., J. H. Benedict, B. Cook, D. R. Ring and J. C. Correa. 2000. Feeding behavior of bollworm and tobacco budworm (Lepidoptera: Noctuidae) larvae in mixed stands of nontransgenic and transgenic cotton expressing an insecticidal protein. J. Econ. Entomol. 93: 1300-1307.

Hale, F. A. 2005. Corn earworm on sweet corn. University of Tennessee Agricultural Extension Service publication SP 341-G. Nashville, TN.

Hatting, J. L., S. P. Wraight and R. M. Miller. 2004. Efficacy of *Beauveria bassiana* (Hyphomycetes) for control of Russian wheat aphid (Homoptera: Aphididae) on resistant wheat under field conditions. Biocontr. Sci. Tech. 14: 459-473.

Hicks, B. J., A. D. Watt and D. Cosens. 2001. The potential of *Beauveria bassiana* (Hyphomycetes: Moniliales) as a biological control agent against the pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae). Forest Ecol. Manag. 149: 275-281.

Jaros-Su, J., E. Groden and J. Zhang. 1999. Effects of selected fungicides and the timing of fungicide application on *Beauveria bassiana*-induced mortality of the Colorado potato beetle (Coleoptera: Chrysomelidae). Biol. Control 15: 259-269.

Jeffs, L. B., M. G. Feng, J. E. Falkowsky and G. G. Khachatourians. 1997. Infection of the migratory grasshopper (Orthoptera: Acrididae) by ingestion of the entomopathogenic fungus *Beauveria bassiana*. J. Econ. Entomol. 90: 383-390.

Jones, K. D. 1994. Aspects of the biology and biological control of the European corn borer in North Carolina. Ph.D. Dissertation. North Carolina State University, Raleigh, NC.

Josephs, R. D., R. Krska, R. Schumacher and M. Grasserbauer. 1999. A rapid method for the determination of the *Fusarium* mycotoxin beauvericin in maize. Fresenius J. Anal. Chem. 360: 130-131.

Kessing, J. L. M. and R. F. L. Mau. 1992. *Macrosiphum euphorbiae* (Thomas). University of Hawaii Extension publication M-EUPHOR. Honolulu, HI.

Klingeman, W. E. Unpublished data.

Koch, R. L., E. C. Burkness, W. D. Huchison and T. L. Rabaey. 2005. Efficacy of systemic insecticide seed treatments for protection of early-growth-stage snap beans from bean leaf beetle (Coleoptera: Chrysomelidae) foliar feeding. Crop Prot. 24:734-742.

Kramer, J. P. 1959. Some relationships between *Perezia pyraustae* Paillot (Sporozoa, Nosematidae) and *Pyrausta nubilalis* (Hubner) (Lepidoptera, Pyralidae). J. Insect Pathol. 1: 25-33.

Kramm, K. R. and D. F. West. 1982. Termite pathogens: Effects of ingested *Metarhizium*, *Beauveria*, and *Gliocladium* conidia on worker termites (*Reticulitermes* sp.). J. Invertibr. Pathol. 40: 7-11.

Lawrence, E. B., P. E. Nelson and J. E. Ayers. 1981. Histopathology of sweet corn seed and plants infected with *Fusarium moniliforme* and *F. oxysporum*. Phytopathology 71: 379pp.

Leckie, B. M. 2002. 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 plant s using PCR and ITS primers. M.S. Thesis. University of Tennesse, Knoxville, TN.

Logrieco, A., A. Rizzo, R. Ferracane and A. Ritieni. 2002. Occurrence of beauvericin and enniatins in wheat affected by *Fusarium avenaceum* head blight. Appl. Environ. Microbiol. 68: 82-85.

Luz, C. and J. Fargues. 1997. Temperature and moisture requirements for conidial germination of an isolate of the hyphomycete *Beauveria bassiana* (Bals.) Vuill., pathogenic to *Rhodnius prolixus* (Ståhl) (Hem., Reduviidae). Mycopathologia 138: 117–125.

McCoy, C. W., R. A. Samson and D. G. Boucias. 1985. Entomogenous fungi, pp. 151-236. *In*: Ignoffo, C. M.and N. B. Mandava. [eds.]. Handbook of Natural Pesticides. Vol. 5. CRC Press, Boca Raton, FL.

McDowell, J. M., J. E. Funderburk, D. G. Boucias, M. E. Gilreath and R. E. Lynch. 1990. Biological activity of *Beauveria bassiana* against *Elasmopalpus lignosellus* on leaf substrates and soil. Environ. Entomol. 19: 137-141.

Miller, L. K., A. J. Lingg and L. A. Bulla, Jr. 1983. Bacterial, viral and fungal insecticides. Science 219:715-721.

Organic Materials Review Institute. 2005. *Beauveria* sp. Eugene, OR.

Ownley, B. H., R. M. Pereira, W. E. Klingeman, N. B. Quigley and B. M. Leckie. 2004. *Beauveria bassiana*, a dual purpose biocontrol organism with activity against insect pests and plant pathogens. pp. 255-269 *In*: Emerging Concepts in Plant Health Management, R. T. Lartey and A. J. Caesar [eds]. Research Signposts, T. C. Kerala, India.

Pekrul, S.and E. A. Grula. 1979. Mode of infection of the corn earworm by *Beauveria bassiana* as revealed by scanning electron microscopy. Invertebr. Pathol. 34: 238-247.

Pendland J. C. and D. G Boucias. 1997. In vitro growth of the entomopathogenic hyphomycete *Nomuraea rileyi.* Mycologia 89:66-71.

Poinar, Jr., G. O. and G. M. Thomas. 1984. A fossil: Entomogenous fungus from Dominican amber. Experientia 40: 578-579.

Poprawski, T. J., J. C. Lefaspi and P. E. Parker. 1998. Influence of entomopathogenic fungi on *Serangium parcensetosum* (Coleoptera: Coccinellidae), an important predator of whiteflies (Homoptera: Aleyrodidae). Environ. Entomol. 27: 785-795.

Poprawski, T. J., P. E. Parker and J. H. Tsai. 1999. Laboratory and field evaluation of Hyphomycete insect pathogenic fungi for control of brown citrus aphid (Homoptera: Aphididae). Environ. Entomol. 28: 315-321.

Poprawski, T. J., S. M. Greenberg and M. A. Ciomperlik. 2000. Effect of host plant on *Beauveria bassiana*- and *Paecilomyces fumosoroseus*-induced mortality of *Trialeurodes vaporariorum.* Environ. Entomol. 29: 1048-1053.

Quesada-Moraga, E. and A. Vey. 2003. Intra-specific variation in virulence and *in vitro* production of macromolecular toxins active against locust among *Beauveria bassiana* strains and effects of *in vivo* and *in vitro* passage on these factors. Biocontr. Sci. Technol. 13: 323-340.

Sams, D. W. 2001. Guide to warm-season garden vegetables. University of Tennessee Agricultural Extension Service publication SP 291-P. Nashville, TN.

SAS Institute Inc. 1999. SAS 9.13 user's guide: statistics. SAS Institued Inc. Cary, NC.

Siddiqui, M. R., D. Singh and A. Gaur. 1977. Prevalence of chili anthracnose fungus on seeds and its effective control. Seed Res. 5: 67 pp.

Steinhaus, E. A. 1949. Principles of Insect Pathology. McGraw-Hill book Company, INC. New York, NY.

Steinhaus, E. A. 1975. Disease in a Minor Chord. Ohio State University Press, Columbus, OH.

Strasser, H., A. Vey and T. M. Butt. 2000a. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? Biocontr. Sci. Tech. 10: 717-735.

Strasser, H., D. Abendstein, H. Stuppner and T. M. Butt. 2000b. Monitoring the distribution of secondary metaboites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. Mycol. Res. 104: 1227-1233.

Thrane, U., A. Adler, P. Clasen, F. Galcano, W. Langseth, H. Lew, A. Logrieco, K. Nielsen and A. Ritieni. 2003. Diversity in metabolite production *by Fusarium langsethiae, Fusarium poae*and *Fusarium sporotrichioides*. Int. J. Food Microbio. 95: 257-266.

Tiffany, L. H. 1951. Delayed sporulation of *Colletotrichum* on soybean. Phytopathology 41: 975 pp.

Todorova, S., D. Coderre, C. Vinvent and J. C. Cote. 2002. Screening of *Beauveria bassiana* (Hyphomycetes) isolates against *Choristoneura rosaceana* (Lepidoptera: Tortricidae). Can. Entomol. 134: 77-84.

Vandenberg, J. D. 1996. Standardized bioassay and screening of *Beauveria bassiana* and *Paecilomyces fumoroseus* against the Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 89: 1418-1423.

Vandenberg, J. D., A. M. Shelton, W. T. Wilsey and M. Ramos. 1998a. Assessment of *Beauveria bassiana* sprays for control of diamondback moth (Lepidoptera: Plutellidae) on crucifers. J. Econ. Entomol. 91: 624-630.

Vandenberg, J. D., L. E. Sandvol, S. T. Jaronski, M. A. Jackson, E. J. Souza and S. E. Halbert. 2001. Efficacy of fungi for control of Russian wheat aphid (Homoptera: Aphididae) in irrigated wheat. Southwestern Entomol. 26: 73-85.

Vandenberg, J. D., M. Ramos and J. A. Altre. 1998b. Dose-response and age- and temperature-related susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae). Environ. Entomol. 27: 1017-1021.

Viaud, M., Y. Couteaudier and G. Riba. 1997. Molecular analysis of hypervirulent somatic hybrids of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria sulfurescens*. Appl. Environ. Microbiol. 64: 88-93.

Wagner, B. L. and L. C. Lewis. 2000. Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. Appl. Environ. Microbiol. 66: 3468-3473.

Wang, Z. G. and G. R. Knudsen. 1993. Effect of *Beauveria bassiana* (fungi: Hyphomycetes) on fecundity of the Russian wheat aphid (Homoptera:Aphididae). Environ. Entomol. 22: 874-878.

Waters Breeze System 3.1. 2000. Waters Corp. Milford, MA.

Wilding, N. 1976. Determination of the infectivity of *Entomophthora* spp., pp. 296-300. *In*: Proceedings of the First International Colloquium on Invertebrate Pathology, T. H. Angus, P. Faulkner and A. Rosenfield [eds.] Kingston, Ontario. Queens University Printing Office.

Yeo, H., J. K. Pell, P. G. Alderson, S. J. Clark and B. J. Pye. 2003. Laboratory evaluation of temperature effects on the germmination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. Pest Manag. Sci. 59: 156-165.

Zizka, J.and J. Wieser. 1993. Effect of beauvericin, a toxic metabolite of *Beauveria bassiana*, on the ultrastructure of *Culex pipiens autogenicus* larvae. Cytobiotics 75: 13-19.

Zurek, L. and B. A. Keddie. 2000. *Beauveria bassiana* (Balsamo) Vuillemin- a promising microbial control agent of the satin moth (Lepidoptera: Lymantriidae). Biocontr. Sci. Technol. 10: 641-644.

Vita

Wesley Aaron Powell was born in Huntsville, Alabama, November 13, 1980. He was raised in Fayetteville, Tennessee and attended Highland Rim Elementary School and Central Junior High School. Throughout High School, Wes ran a small lawn care and landscaping business. Wes graduated from Lincoln County High School in May 1999. From there, he went to Austin Peay State University in Clarksville, Tennessee and graduated with a Bachelor of Science degree in agriculture with a minor in agri-business on May 9, 2003. In January of 2004, Wes began a Master of Science in plant science at the University of Tennessee, which he completed in December of 2005. Currently, Wes plans to move to Clarksville to pursue a career in landscaping and nursery production.

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