

UTILIZATION AND ENHANCEMENT OF ENTOMOGENOUS FUNGI IN THE  
MANAGEMENT OF THE PECAN WEEVIL (COLEOPTERA: CURCULIONIDAE)

by

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(Under the Direction of Wayne A. Gardner)

ABSTRACT

The pecan weevil, *Curculio caryae* (Horn), is a major pest of pecans, *Carya illinoensis* (Koch) Wangenh. Current control tactics are foliar sprays of chemical insecticides that killed natural enemies resulting in increased populations of secondary pests. The entomogenous fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin, are being developed as alternatives to these insecticides; however, the residual activity of the fungi must be extended to be economically competitive. Laboratory studies demonstrated that both fungi persisted significantly longer in composted cow manure, ground pine bark and composted biosolids than in a Faceville series soil and killed *Tenebrio molitor* L. larvae through 28 d postinoculation. Field trials in 2006 failed to demonstrate persistence and significant suppression of adult weevils. Microscopy studies of fungal activity in a Faceville series soil showed conidial germination up to 28 d with mycelial growth beginning as early as 3 d after inoculation.

INDEX WORDS: Pecan weevil, *Curculio caryae*, Microbial control, *Beauveria bassiana*, *Metarhizium anisopliae*, Pecans

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

## INTRODUCTION

The entomogenous fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff), have been proposed as potential microbial control agents in managing the pecan weevil, *Curculio caryae* (Horn). These naturally-occurring fungi cause weevil mortality in pecan orchards. However, levels of both fungi in pecan production systems must be augmented to realize efficacious weevil control. Furthermore, several limiting factors, including placement and persistence of infective conidia in the cropping environment, must be addressed for optimal and economic use of these fungi as viable alternatives to current insecticidal control of weevils. The research proposed herein is directed to improve the persistence of infective conidia in orchard soils while adult weevils are emerging from pupation sites in the soil. Understanding the fate of the fungi in soils and extending their residual activity will improve the performance of these microbial agents against this key pest.

## LITERATURE REVIEW

The pecan weevil, *Curculio caryae* (Horn) (Coleoptera: Curculionidae), is a major pest of pecans, *Carya illinoensis* (Koch) Wangenh. In 2004, this pest caused annual crop losses estimated at \$260 million in the U.S. (USDA 2004), \$9.2 million of that in Georgia (Guillebeau et al. 2005). Crop loss is caused by adult weevils eating and ovipositing within developing nuts and larval consumption of the kernel after oviposition and eclosion.

Adults emerge from the soil from mid-August to mid-September usually after a rainfall of at least 1.27-3.81 cm (Harp and Van Cleave 1976a). Presumably, the rainfall event softens the soil facilitating emergence. Emergence dates also are related to soil profile, soil type and geography. The majority of the emerging weevils fly to the tree trunk and crawl up the trunk to the canopy. Others either fly to the crown of the tree, crawl to and up the trunk, or never reach

the tree (Rainey and Eikenbary 1968). Those weevils reaching the tree feed on developing nuts in the canopy and mate. Inseminated females oviposit 3-4 eggs into each nut after chewing through the shuck and consuming part of the kernel. One female weevil can oviposit 35–55 eggs in 8–10 nuts on average and visit up to 25 nuts post emergence for approximately 24 days (Harp and Van Cleave 1976b, Harris and Ring 1979). Larvae feed and develop within the shell for approximately 6 wks. Fourth-instar larvae exit the shell, usually after the nut falls to the ground. The impact of the fall presumably triggers the larvae to exit the nut. One larva creates the exit hole by chewing through the shell and exits the nut; the remaining 2-3 larvae use the same exit hole. Larvae burrow 8 to 25 cm into the soil profile. The depth to which they burrow is dependent upon soil type, soil moisture, and other factors. Using radiographic techniques, Harrison et al. (1993b) demonstrated that larvae burrowed vertically (67-92 mm) in orchard soils (Cecil, Tifton, and Lakeland series), even through hardpans of compressed soil. More dense structures (i.e., rocks) limited vertical movement. Each larva eventually creates an earthen cell in which it remains through pupation and until adult emergence. About 90% of the larvae will pupate over a 3-wk period in the late summer or early fall of the following year after which they emerge as adults. The remaining 10% experience a delayed development in which pupation occurs in the second year after adult emergence resulting in a 2-year life cycle for that group (Harris 1985). Control of the pecan weevil has traditionally been directed to the adult stage using conventional chemical insecticides applied to tree foliage when adults are present (Table 1) (Shapiro-Ilan et al. 2002, Tedders et al. 1973). Carbaryl has a longer residual than cypermethrin and requires 3 to 4 applications per season in comparison to 6 to 7 applications required with cypermethrin to provide protection while adults are present. Cypermethrin, therefore, is often used in years with sparse or low rainfall when adult emergence is usually low. All have proven

deleterious to beneficial insects, especially aphid predators, yielding increased aphid populations (Dutcher and Payne 1985). As a result three aphid species - *Monelliopsis pecanis* (Bissel), *Monellia caryella* (Fitch), and *Melanocallis caryaefoliae* (Davis) - have been elevated to the status of key pests (Dutcher and Payne 1985).

Given these problems associated with foliar applications of approved chemical insecticides, alternative control tactics are being considered and evaluated for the pecan weevil. Microbial control is one such alternative. In general, Third World and developing countries have been more successful than developed countries in implementing large-scale and efficacious microbial control programs. The first attempts of large-scale microbial control in the U.S. were with *Beauveria bassiana* (Balsamo) Vuillemin, an entomogenous fungus. It reportedly occurred naturally in the Midwest grain crops during the late 19<sup>th</sup> Century primarily in chinch bug, *Blissus leucopterus* (Say), populations (Lord 2005). An epizootic was reportedly initiated by spreading sporulating cadavers across a field in Minnesota, although many believed the fungal epizootic was a natural phenomenon (Lord 2005). This reported success led to a statewide program to distribute *B. bassiana*. At first, the program offered encouraging results, but later it was discontinued because the naturally-occurring fungus was so prevalent that the introduced fungus had no added impact on reducing chinch bug populations (Lord 2005). Yet, much was learned from these early attempts at microbial control, including the importance of microhabitat conditions in initiating and sustaining disease epizootics.

Introduction and augmentation of the soil-inhabiting bacteria - *Bacillus popilliae* (Dutky) and *Bacillus lentimorbis* (Dutky) known as “milky disease” - proved successful against the grubs of the invasive Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Lord 2005, Weeden et al. 2005). This demonstrated the feasibility of augmenting natural inoculum of

insect pathogens in soil systems and led to the creation of a USDA-ARS laboratory focusing on microbial control where the potent strain of *Bacillus thuringiensis* var. *kurstaki* Berliner was discovered. Several strains of *B. thuringiensis* are now commercialized and marketed worldwide. All are microbial insecticides and are, in general, applied and used like their conventional chemical insecticide counterparts (Lord 2005). In fact, the development and implementation of microbial control in this country have been hampered by the availability of efficacious and inexpensive chemical insecticides.

Entomopathogenic nematodes, unlike other microbial agents, are exempt from the EPA's exclusion clause. Several have been evaluated against curculionids including the pecan weevil (Shapiro-Ilan et al. 2004) and the citrus root weevil, *Diaprepes abbreviatus* (L.) (McCoy et al. 2000). Shapiro-Ilan et al. (2004) concluded that *Steinernema carpocapsae* (Weiser) had a greater infection potential against pecan weevil adults compared to that of *Heterorhabditis indica* Poinar, Karunakar & David. McCoy et al. (2000) found that *Steinernema riobrave* (Cabanillas, Poinar & Raulston), *Heterorhabditis bacteriophora* (Poinar), and *H. indica* effectively controlled citrus root weevils. However, parasitism of weevils by naturally-occurring nematodes was common as evidenced in the control plots. There is potential for using entomopathogenic nematodes in managing the pecan weevil; however, applications of infective dauers must be directed to soil-inhabiting stages of the pest.

Sri-Arunotai et al. (1975) reported *B. bassiana* and *Metarhizium anisopliae* (Metchnikoff) Sorokin naturally infect pecan weevil larvae. Harrison and Gardner (1991) isolated naturally-occurring *B. bassiana* from Georgia pecan orchard soils and suggested that the observed 10-20% natural infection of adults each season was due to exposure to the fungus as they emerged. Shapiro-Ilan et al. (2002) isolated entomogenous fungi from the soils of 16 of 21

orchards surveyed in several southeastern states. All were either strains of *B. bassiana* or *M. anisopliae*, with some orchards yielding more than one isolate of each fungus. Strains of both fungi are and have been commercially formulated for microbial control of insect pests including several curculionids (Table 2).

Historically, *B. bassiana* was first observed infecting insects around 900 A.D. in silkworm, *Bombyx mori* (L.), colonies in Japan (Pendland and Boucias 2004). Agostino Bassi eventually postulated the modern germ theory based upon his studies of this fungus in silkworm production. The conidia, which occur singly or clumped, are the infective units which appear globose, colorless, and tend to be dry (Pendland and Boucias 2004).

*Metarhizium anisopliae* was first identified in 1884 and was used to control the wheat cockchafer, *Anisoplia austriaca* (Herbst), in the Ukraine (Bidochka 2004). *M. anisopliae* has been successfully used against scarab beetle, *Aphodius tasmaniae* (Hope), larvae in Tasmania and against spittlebugs (Hemiptera: Cercopidae) in Brazil (Bidochka 2004). Conidia have an ellipsoidal shape in a columnar arrangement (Bidochka 2004). Colonies growing on semi-solid artificial media tend to be white at first and then turn green as the conidia mature.

Infection usually occurs through the host integument. When conditions are conducive, the conidia attach to the cuticle and germinate to form a germ tube (Deacon 1983, Bidochka 2004). The germ tube releases proteases, chitinases, and lipases that digest the cuticle allowing the germ tube to penetrate into the hemocoel. The germ tube of *M. anisopliae* further differentiates into an appressorium, a hold-fast structure that secures the hyphae to the cuticle (Bidochka 2004, Deacon 1983). A similar structure may occur with *B. bassiana*, but is not necessary for the hyphae to penetrate the cuticle. This infection process takes approximately 48 h to complete. Once in the hemocoel, fungal mycelia grow throughout the insect often forming

blastospores, or yeast-like hyphae (Goettel and Inglis 1997). Death occurs as a result of nutrient depletion, invasion of fungi into organs, release of fungal toxins, obstruction of blood flow, or any combination of these factors (Goettel and Inglis 1997).

Both fungi produce toxins that can expedite host death (Bidochka 2004). Many toxic compounds released by the fungi have been isolated and identified. Their role is unclear, but they appear to be antimicrobial and, thus, inhibit bacterial growth in the insect cadaver thereby ensuring the success of the fungus (Deacon 1983).

Both occur naturally in soils in a variety of geographic locations and climatic conditions. Naturally-occurring *M. anisopliae* and *B. bassiana* can be extracted from the soil using different methods. Selective agar-based media containing dodine, chloramphenicol, and crystal violet have proven to be effective and reliable *in vitro* methods for isolating these deuteromycetes from soils (Bidochka 2004, Deacon 1983, Schaerffenberg 1963). An *in vivo* method of isolating *M. anisopliae* and *B. bassiana* from soil is the “Galleria bait method” that involves placing waxworm larvae, *Galleria mellonella* (L.), in soil samples. Larvae become infected, and the fungi may be isolated from the cadaver (Zimmerman 1986, Bidochka 2004, Klingen et al. 2002). A limiting factor with this method is the vulnerability of the waxworm larvae to infection by many other soil-borne microorganisms.

*Beauveria bassiana* and *M. anisopliae* are promising biological control agents for managing the pecan weevil (Gottwald and Tedders 1983, Shapiro-Ilan et al. 2002, Tedders et al. 1973). Sri-Arunotai et al. (1975) observed larvae infected with *M. anisopliae* in lab conditions exhibited similar symptoms as larvae infected with naturally-occurring fungi in the field. Gottwald and Tedders (1983) reported that *M. anisopliae* appeared to have no effect on larval mortality, but they observed an adult mortality of 49.8% caused by the fungus. Tedders et al.



(1973) recorded larval mortality levels of 82.6% caused by *B. bassiana* and 73.9% caused by *M. anisopliae* 28 days after exposure to conidia in laboratory assays. Mortality levels in field trials using either fungus were slightly lower than those levels observed in the laboratory (Tedders et al. 1973). Gottwald and Tedders (1983) further demonstrated control of pecan weevil adults and larvae with *B. bassiana* and *M. anisopliae* in field and greenhouse studies. They also achieved significant levels of infection when adults were caged with a single adult contaminated with either fungus. Harrison et al. (1993a) killed all adults and larvae following exposure to  $\geq 10^5$  *B. bassiana* conidia/mL in the laboratory. Uniform mixtures of soil and conidia ( $10^7$  conidia/g) caused 67-73% mortality, while applying a 5-cm barrier of fungal conidia directly in the soil profile did not significantly affect mortality of exposed weevils (Harrison et al. 1993a).

Larval stages may be targeted for control by applications of the fungi to soil when larvae are emerging from the nut and burrowing into the soil prior to formation of the earthen cell (Harrison and Gardner 1997). Gottwald and Tedders (1984) inoculated earthen soil cells with either *B. bassiana* or *M. anisopliae*. Both fungi successfully infected larvae within the treated cells, although *B. bassiana* caused higher larval mortality than *M. anisopliae*. Furthermore, *B. bassiana* hyphae growing from larval cadavers in the cells penetrated the earthen wall and infected neighboring larvae. *M. anisopliae*, on the other hand, did not spread from the cells. Growth of hyphae in the soil is postulated to increase the fungal inoculum in orchard soils.

Boethel and Eikenbary (1979) suggested that these deuteromycetes might be applied to orchard soils to target either adults emerging from the soil or larvae exiting nuts and burrowing into the soil. Storey and Gardner (1987) demonstrated >90% of viable colony forming units (CFUs) were recovered in the upper 15 cm of the soil profile following applications of an aqueous suspension of the commercially-formulated *B. bassiana* ABG6112 strain. Field and

greenhouse studies corroborated these laboratory findings and further demonstrated that >90% of conidia applied to soil surfaces actually remain within the upper 2-5 cm of the profile (Storey and Gardner 1988, Storey et al. 1989). Based upon these results, it might be postulated that deuteromycete conidia applied to soil surfaces could establish an infective inoculum in the upper few cm of the soil profile (Storey et al. 1987).

Persistence of the infective conidia in soils is limited. Several studies show that the number of viable CFUs recovered from treated soils increases exponentially within 72 h of application. However, these numbers generally decrease to 85-95% of the original concentration within 10-12 d of application (Lingg and Donaldson 1981, McCoy 1986, Storey et al. 1989, Krueger and Roberts 1997). Presumably, these increases and subsequent decreases in fungal propagules in treated soils are due to the natural germination of the conidia soon after application (Richards 1991).

Various abiotic and biotic factors regulate growth, sporulation and field stability of entomogenous fungi (Hallsworth and Magan 1999, Walstad et al. 1970). Relative humidity, soil moisture, temperature, light, and microbial interactions affect the persistence and germination of *B. bassiana* and *M. anisopliae* in the soil (Lingg and Donaldson 1981, Walstad et al. 1970).

Most entomogenous fungi require  $\geq 95\%$  relative humidity to germinate and infect susceptible hosts (Hallsworth and Magan 1999). Gottwald and Tedders (1982) observed that *B. bassiana* produced 10-fold greater conidia than *M. anisopliae* as a result of a rapid increase in relative humidity. Lingg and Donaldson (1981) noted that relative humidity in soil systems is never below 98.9%. They further observed that at the soil-air interface the relative humidity is >95% and concluded that the atmospheric relative humidity would not be a critical limiting factor in the germination of *B. bassiana* conidia in soils.

The optimal temperature range for both *M. anisopliae* and *B. bassiana* to germinate is between 15<sup>0</sup>C and 35<sup>0</sup>C (Walstad et al. 1970, Ekesi et al. 2003). Walstad et al. (1970) further observed that sporulation of both fungi was inhibited below 10<sup>0</sup>C and above 35<sup>0</sup>C. At temperatures of 49<sup>0</sup>C and 50<sup>0</sup>C, spores of both fungi did not survive (Walstad et al. 1970). Lingg and Donaldson (1981) reported that the half-life of *B. bassiana* decreased as temperature and soil moisture increased yielding the highest mortality of susceptible hosts at 25<sup>0</sup>C and a moisture saturation of 75%. They also noted a positive correlation of soil moisture with conidial viability. Increasing soil moisture content decreases available oxygen, thereby increasing carbon dioxide, which aids in establishing a prime habitat for microorganisms (Keller and Zimmerman 1989).

The hyaline conidia of *B. bassiana* are killed or damaged by sunlight, specifically by UV-B radiation (Daoust and Pereira 1986). Exposure to UV-B rays can result in either damage to DNA, which causes deletions or mutations, or production of byproducts such as peroxides (Pearlman et al. 1985, Ignoffo and Garcia 1994). This sensitivity negates the application of conidia to foliage or other exposed surfaces. Inglis et al. (1995) observed that a coating of conidia with clay increased the persistence of the conidia by protecting them from harmful UV. In a similar study, Edgington et al. (2000) reported that a mixture of milk powder and egg albumen showed potential in protecting *B. bassiana* conidia against UV-B radiation. Leland and Behle (2005) demonstrated lignin-coated *B. bassiana* (GHA strain) conidia in an oil formulation protected them from UV; however, the lignin-coated conidia exhibited a 10X lower rate of germination and a lower level of virulence to the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois).

Organic soil amendments may have negative or positive effects on biotic conditions and, thus, microbial viability and persistence. Soil amendments can increase populations of

microorganisms that are antagonistic to *B. bassiana* and *M. anisopliae*. The antagonistic microorganisms are capable of inhibiting the growth of other microorganisms by releasing toxic metabolites (Hornby 1978). This possibly indicates that antagonistic microorganisms did not affect *B. bassiana* virulence and growth, but that temperature ranges exert a larger role on the fungal activity in soil. Walstad et al. (1970) observed that germination of *B. bassiana* and *M. anisopliae* was inhibited by other microorganisms when grown on unsterilized soil, duff, and leaf litter. Lingg and Donaldson (1981) observed a fungistatic effect when using non-sterile soil and adding carbon and nitrogen amendments to the soil. Autoclaving soil, organic amendments, and filtering soil extracts were cited as enhancing germination of *B. bassiana* and *M. anisopliae* (Walstad et al. 1970), further implicating soil microbes as inhibitory agents. However, Studdert et al. (1990) reported that, at a rate of  $1 \times 10^8$  conidia/cm<sup>3</sup>, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) at 16°C larval mortality due to *B. bassiana* was higher at a 24°C rather than a 16°C soil temperature.

The survival of conidia in the soil profile is related to high moisture and organic matter (Studdert et al. 1990, Li and Holdom 1993). One of the first studies to incorporate an amendment to enhance germination of *B. bassiana* was performed by Clerk (1969) with the addition of either a 1% dextrose solution, silk extract, or insect material. Insect cuticles, which are composed of chitin and other compounds that are fungal nutrients, stimulated conidial germination of *B. bassiana* and *M. anisopliae* (Walstad et al. 1970). Groden and Lockwood (1991) compared conidial germination of *B. bassiana* on colloidal chitin and Colorado potato beetle, *Leptinotarsa decemlineata* (Say), elytra. They concluded there was no significant increase in germination with these substances. Schippers and Palm (1973) postulated that the breakdown of chitin can release secondary substances, such as ammonia, which has an inhibitory

effect on the germination of *B. bassiana* conidia, while Groden and Lockwood (1991) speculated that the availability of other nutrients (i.e., protein) derived from insects can stimulate germination of conidia. Sharapov and Kalvish (1984) showed a relationship between increasing fungistasis and increasing organic matter which can be achieved in nutrient-poor soil by adding amendments.

In a study conducted by Rosin et al. (1996), fertilizers rich in nitrogen were examined to see if they affected *B. bassiana*. They concluded that fresh cow manure had an inhibitory effect, composted manure had a stimulatory effect, and urea had no significant effect. Furthermore, Shapiro-Ilan et al. (2002) reported that manganese levels in soil was directly related with the presence of *B. bassiana*, while calcium and magnesium levels were positively correlated with *M. anisopliae* presence in orchard soils. Bruck (2005) observed a gradual decline in numbers of fungal propagules in peat-based and bark-based potting media amended with chitin over 342 days, while noting significant levels of infection of last-instar black vine weevil, *Otiorhynchus sulcatus* (F.), larvae. Various chemical pesticides (herbicides, fungicides, and insecticides) are deleterious to these fungi (Storey and Gardner 1985, Storey and Gardner 1986, Harrison and Gardner 1992, Osborne and Boucias 1985). Osborne and Boucias (1985) reported that xylene-based insecticides and pyrethroid insecticides were inhibitory to *B. bassiana*. Herbicides such as paraquat, simazine, diuron, norflurazon, and terbacil inhibited conidial germination, mycelial growth or both of *B. bassiana* (Storey and Gardner 1985, Harrison and Gardner 1992).

In summary, some generalizations can be made from the literature: (1) *B. bassiana* and *M. anisopliae* are effective microbial control agents that infect pecan weevil larvae and adults; (2) both fungi occur naturally in soils in Georgia and other pecan-producing areas; (3) weevil mortality due to these fungi occurs naturally in orchard systems; (4) both fungi can be mass-

produced and applied to orchard soils to target the pecan weevil; and (5) when applied to soils, fungal conidia appear to accumulate within the upper few cm in the soil profile. Yet, their use as microbial control agents in pecan weevil management is limited by their sensitivity to abiotic and biotic factors including UV radiation, humidity, microbial competition, and chemicals, as well as, their relatively short persistence. Persistence and application must be examined to ensure the use of either *M. anisopliae* or *B. bassiana* as efficacious, sustainable, and economically feasible microbial control agents.

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Table 1. Insecticides currently used in Georgia pecan orchard systems for control of the pecan weevil (Guillebeau 2006)

<b>Insecticide</b>	<b>Class of Insecticide</b>
Sevin 80S (carbaryl)	carbamate
Sevin XLR (carbaryl)	carbamate
Mustang Max (zeta-cypermethrin)	pyrethroid

Table 2. Entomogenous fungi associated with curculionids as cited in the literature.

<b>Curculionid Host</b>	<b>Fungi</b>
rice weevil <i>Sitophilus oryzae</i> (L.)	<i>M. anisopliae</i>
alfalfa weevil <i>Hypera postica</i> (Gyllenhal)	<i>B. bassiana</i>
sweet potato weevil <i>Cylas formicarius elegantulus</i> (Summers)	<i>B. bassiana</i>
chestnut weevil <i>Curculio proboscideus</i> (F.)	<i>M. anisopliae</i>
pales weevil <i>Hylobius pales</i> (Herbst)	<i>M. anisopliae</i> <i>B. bassiana</i>
citrus root weevil <i>Diaprepes abbreviatus</i> (L.)	<i>M. anisopliae</i> <i>B. bassiana</i>
black vine weevil <i>Otiorhynchus sulcatus</i> (F.)	<i>M. anisopliae</i> <i>B. bassiana</i>
pecan weevil <i>Curculio caryae</i> (Horn)	<i>M. anisopliae</i> <i>B. bassiana</i>

## CHAPTER 2

UTILIZATION OF *TENEBRIO MOLITOR* L. FOURTH INSTARS TO DETECT INFECTIVE  
PROPAGULES OF THE ENTOMOGENOUS FUNGI *BEAUVERIA BASSIANA* (BALSAMO)  
VUILLEMIN AND *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN IN SOIL

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**Abstract** Laboratory bioassays were conducted to determine the feasibility of using fourth-instar *Tenebrio molitor* L. (Coleoptera:Tenebrionidae) to detect infective propagules of entomogenous fungi in soil. Eight concentrations each of *Beauveria bassiana* (Balsamo) Vuillemin (GHA strain) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (F52 strain) were included in a filter paper bioassay to establish median lethal concentrations (LC50's) of  $2.3 \times 10^4$  conidia/mm<sup>2</sup> for *B. bassiana* and  $1.9 \times 10^2$  conidia/mm<sup>2</sup> for *M. anisopliae* against *T. molitor* larvae. Mortality of fourth instars placed in a Faceville series sandy loam soil treated with either *B. bassiana* or *M. anisopliae* ( $10^1$  to  $10^7$  conidia/g of soil) was lower and more variable than that obtained in the filter paper bioassays. Based upon these results, *T. molitor* fourth-instar larvae can be placed in soil to detect the presence of infective propagules of *B. bassiana* GHA and *M. anisopliae* F52. The method, however, lacks the ability to predict or estimate quantities of infective propagules in soil.

**Key Words** bioassay, entomogenous fungi, *Tenebrio molitor*, *Beauveria bassiana*, *Metarhizium anisopliae*, concentration/mortality response



## Introduction

*Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) are entomopathogenic hypomycete fungi that invade the insect host through the cuticle, develop within the hemocoel, and eventually sporulate externally to disperse infective conidia (Tanada and Kaya 1993). Both are commercially available for use as microbial insecticides against a variety of insect pests including members of the orders Homoptera, Lepidoptera, Thysanoptera, Coleoptera, Isoptera, and Orthoptera (Flexner and Belnavis 2000). Both fungi are promising alternatives to chemical insecticides for controlling soil-inhabiting and other cryptic insects including the pecan weevil, *Curculio caryae* (Horn) (Coleoptera: Curculionidae).

In southeastern pecan culture, *C. caryae* is commonly controlled with foliar applications of chemical insecticides (e.g., carbaryl) directed to adults (Hudson et al. 2002). These late-season applications often interfere with natural enemies causing aphid population resurgence, resulting in the elevation of these aphid species to key pest status (Dutcher and Payne 1985). Therefore, several laboratories have investigated the potential of *B. bassiana* and *M. anisopliae* as microbial control agents for *C. caryae*.

Studies have shown that *B. bassiana* and *M. anisopliae* occur naturally in pecan orchard soils (Harrison and Gardner 1991, Shapiro-Ilan et al. 2002), *B. bassiana* is easily transmitted from infected to healthy weevil adults and larvae in orchard soils (Gottwald and Tedders 1983, 1984), and field efficacy of soil-applied *B. bassiana* conidial preparations is higher against adult weevils than larvae (Tedders 1985, Harrison et al. 1993). Furthermore, enumeration of viable propagules of *B. bassiana* recovered from soil following application of conidial suspensions demonstrated a logarithmic increase in fungal inoculum within 3 days of application followed by a depletion of inoculum within 12 to 21 days (Lingg and Donaldson 1981, McCoy 1986, Storey

et al. 1989). Clearly, the short-term persistence of fungal conidia in soils is a limiting factor to the success of these fungi as microbial control agents against *C. caryae* and other soil-inhabiting pests (Storey et al. 1987, 1989, Krueger et al. 1991). Shapiro-Ilan et al. (2004) suggest that fungal persistence in soil must be increased to effectively manage *C. caryae* with these entomogenous fungi.

Studies aimed at extending the persistence of infective propagules of *B. bassiana* and *M. anisopliae* in soil are limited by the lack of an effective method of monitoring the propagules in soil over time. While selective media (e.g., oatmeal-dodine agar) are used to isolate and recover *B. bassiana* and *M. anisopliae* from soil (Liu et al. 1993, Beilharz et al. 1982, Goettel and Inglis 1997), these methods provide only counts of viable colony-forming units (CFUs) with no indication of infectivity of the propagules. Infectivity is best identified by exposure of target insect hosts to the soils or extracts of the soils. However, due to its extended life cycle, *C. caryae* is not reared in laboratory cultures; therefore, larval and adult weevils are not readily available for such assays, and a substitute host must be used.

Previous studies have drop-plated soil washes on oatmeal-dodine agar (ODA) to estimate numbers of CFUs per unit of soil (Storey and Gardner 1987, Storey et al. 1987, 1989, Harrison and Gardner 1991), and last-instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae were placed in soil samples to determine the presence of infective propagules in two of these studies (Harrison and Gardner 1991, Storey et al. 1989). Indeed, *G. mellonella* larvae are routinely used as host insect “baits” or “traps” to isolate entomopathogenic microorganisms and nematodes from soil (Zimmerman 1986). Shapiro-Ilan et al. (2002) used *G. mellonella* to isolate *B. bassiana*, *M. anisopliae* and entomopathogenic nematodes from pecan orchard soils in the southeastern United States. However, the high sensitivity of *G. mellonella* larvae to these

pathogens (Zimmerman 1986) restricts their use to either indicating simply the presence or absence of infective propagules in soils or as pathogen “traps”.

We report here the evaluation of mealworm, *Tenebrio molitor* L. (Coleoptera:Tenebrionidae), larvae for such usage primarily because of the ease and cost-effectiveness of maintaining laboratory cultures of the insect. The objectives were to determine the concentration-mortality response of fourth-instar *T. molitor* to *B. bassiana* and *M. anisopliae* and to assess the suitability of these larvae as indicators of infective fungal propagules in soil.

### **Materials and Methods**

All *T. molitor* larvae used in these bioassays were originally obtained as fourth instars from Southeastern Insectaries (Perry, GA). Larvae were maintained at ambient room temperature and conditions in rectangular (32L x 24W x 10D cm) plastic containers with dried baby oatmeal as a food source and dampened paper towels as a moisture source.

The *M. anisopliae* and *B. bassiana* used in the bioassays were isolated from commercial preparations of each fungus. The *M. anisopliae* was the F52 strain obtained from Earth BioSciences (Fairfield, CT). This strain was originally isolated from a codling moth, *Cydia pomonella* (L.) (formerly *Laspeyresia pomonella* (L.) and *Carpocapsa pomonella* (L.)) (Lepidoptera: Tortricidae), larva in Germany and designated as Ma43. It was later designated as F52 by Bayer Corporation as they initiated its development as a microbial insecticide. The *B. bassiana* was the GHA strain obtained from Emerald BioAgriculture (Butte, MT). This strain was originally isolated from *Diabrotica undecimpunctata howardi* (Barber) (Coleoptera: Chrysomelidae) on green beans grown in a greenhouse in Corvallis, OR.

Cultures of each fungus were routinely maintained on Sabouraud’s dextrose agar plus 1% yeast extract (SDAY) at 24±1°C with periodic passage through *T. molitor* larvae to prevent

attenuation of virulence. Conidia used in the bioassays were harvested from 14-day-old cultures on SDAY by scraping the agar surface with a sterile glass microscope slide. The fungal material obtained was then suspended in 40 mL of sterile distilled water (SDW) containing a wetting agent (0.01% v/v, Tween 80™), mixed for 20 min with a magnetic stirrer, and filtered through a double layer of cheese cloth. The number of conidia per mL of the resulting solution was determined using an Improved Neubauer™ hemacytometer. Conidial viability approached 95% for each stock solution.

A filter paper technique was used to first determine the concentration-mortality response of *T. molitor* fourth instars to each fungus. Eight concentrations ranging from  $10^1$  to  $10^8$  conidia/mL were established by serially diluting the stock solution of each fungus. SDW was used as the control in each bioassay.

Individual Whatman® filter paper disks (55.0 mm diam) in Petri dishes (60x15 mm) were treated with 0.5 mL of the appropriate conidial concentration. Fourth-instar *T. molitor* larvae were placed individually in the Petri dishes (30 larvae/concn/fungus). Larvae were monitored and deaths were recorded daily for 7 days. Cause of death was confirmed by characteristic fungal growth or typical symptoms of fungal infection of insect hosts (i.e., subcuticular tanning, mummification, conidiophore formation, sporulation).

These bioassays were repeated three times for each fungus, each time with fresh conidial suspensions. A test for homogeneity of variance (Snedecor and Cochran 1989) revealed no significant variability among the tests for each fungus, and data were combined for analysis. All percentage data were transformed by arcsine of the square root prior to analysis. Cumulative mortality data after 7 days of exposure were subjected to probit analysis (PROC PROBIT, SAS 2006) using  $\log^{10}$  of the concentrations.

Bioassays also were conducted to determine the mortality response of *T. molitor* fourth instars exposed to soil containing each fungus. The soil used in these assays was a sandy loam Faceville series soil (sand:silt:clay, 84:10:6; pH = 6.1; organic matter 2.8%, w/w) from Byron (Peach Co.), GA. The soil was crushed using a spatula to insure uniform coverage of soil particles with each fungal suspension and was oven-dried prior to addition of the conidia.

Four conidial suspensions of  $10^2$ /mL,  $10^4$ /mL,  $10^6$ /mL, and  $10^8$ /mL were established as previously described. Ten mLs of each suspension were added individually to 100 g of soil in individual 1-L Nalgene<sup>®</sup> containers and mixed by rotating the container by hand for 2 min or until the soil was evenly moistened with the suspension. This established conidial concentrations of  $10^1$ ,  $10^3$ ,  $10^5$ , and  $10^7$  conidia per g of soil. SDW was used as a control. Gravimetric analysis determined that the resultant soil moisture was 10% (w/w).

One gram of treated soil was placed individually in 30-mL clear plastic creamer cups, and a fourth-instar *T. molitor* larva was added to each cup. The cups were capped and placed in covered plastic containers (36L x 30W x 25D cm) lined with moistened paper towels to insure a moisture-saturated environment. These were maintained at ambient room temperature. Larvae were monitored and deaths recorded daily for 7 days. Cause of death was confirmed by characteristic fungal growth or typical symptoms of fungal infection of insect hosts (i.e., subcuticular tanning, mummification, conidiophore formation, sporulation).

Each concentration for each fungus was replicated three times with 30 larvae per replicate. Cumulative mortality data were subjected to analysis of variance using the General Linear Models Procedure (PROC GLM, SAS 2006) and when statistical differences were demonstrated, the treatment means were separated by the least significant differences procedure.

## Results and Discussion

The median lethal concentration (LC50) of *B. bassiana* GHA against *T. molitor* fourth instars as determined with the filter paper bioassay was  $2.3 \times 10^4$  conidia per  $\text{mm}^2$  (SE =  $\pm 21.7$ ; 95% FL =  $1.3 \times 10^4 - 4.1 \times 10^4$ ). The LC50 of *M. anisopliae* F52 against fourth-instar *T. molitor* was  $1.9 \times 10^2$  conidia per  $\text{mm}^2$  (SE =  $\pm 10.1$ ; 95% FL =  $1.1 \times 10^2 - 3.2 \times 10^2$ ). Based upon these values, the F52 strain of *M. anisopliae* is >100x more virulent than the GHA strain of *B. bassiana* against *T. molitor* fourth instars. A similar pattern of differential virulence between these two strains also was observed by Bruck et al. (2005) with the soil-inhabiting cabbage maggot, *Delia radicum* (L.). In that study, mortality of second instars following exposure to  $10^6$  conidia per g of soil was almost 2x higher with *M. anisopliae* F52 than with *B. bassiana* GHA, while mortality at  $10^5$  conidia/g was 10x higher with F52 than with GHA.

Mean mortality of *T. molitor* fourth instars placed in soil treated with *M. anisopliae* F52 ranged from 9 to 71% depending upon the concentration of conidia in the soil (Table 3). Mortality response to conidial concentration was variable, and the relationship was not linear. Mean mortality of *T. molitor* larvae following exposure to soil treated with *B. bassiana* GHA ranged from 3 to 29%, and variability in mortality response was minimal with standard errors associated with the means ranging from 0.3 to 1.5.

Although a proportion of fourth-instar mealworms were infected and killed by *M. anisopliae* following exposure to treated soil, the lack of a linear or proportional response limits the use of *T. molitor* in providing a quantitative estimate of infective propagules in the soil. The method of exposing the fourth instars to the treated soil, however, appeared sufficient for detecting the presence or absence of infective propagules of *M. anisopliae* F52 in soil samples.

The utility of *T. molitor* larvae in detecting *B. bassiana* in soil also appears limited. Exposure of mealworms to *B. bassiana* GHA at concentrations of  $10^1$  to  $10^5$  conidia/g of soil yielded mortality levels ranging from only 3 to 9%. Mortality increased to 29% when the concentration was increased to  $10^7$  conidia/g. Richards (1991) reported that *T. molitor* larvae did not effectively detect *B. bassiana* AF4 in a sandy loam soil and concluded that mealworm larvae are not suitable indicators of infective propagules of this pathotype in soil.

The concentration-mortality bioassays on filter paper yielded LC50's that appeared to fall within a range that should be sufficient for using *T. molitor* fourth instars in detecting infective propagules of *M. anisopliae* F52 and *B. bassiana* GHA in soil. However, when larvae were exposed to soil containing conidia at concentrations encompassing and including the LC50 of each fungus, mortality was lower and more variable than that observed in the filter paper assays. The soil matrix apparently alters the probability of exposure of the larvae to the fungal propagules, alters host susceptibility to the fungus, or alters the ability of the fungus to infect the host. Lingg and Donaldson (1981) observed a fungistatic effect when using non-sterile soil. These interactions prevent the usage of *T. molitor* fourth instars as quantitative measures of infective propagules of *B. bassiana* GHA and *M. anisopliae* F52 in soil. However, *T. molitor* can be used to detect the presence or absence of infective propagules of these two fungi.

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Table 3. Mean ( $\pm$ SE) percent mortality of *Tenebrio molitor* fourth instars following exposure to soil containing various concentrations of *Metarhizium anisopliae* and *Beauveria bassiana*.

Fungus concn (conidia/g)	% mortality <i>Metarhizium anisopliae</i>	% mortality <i>Beauveria bassiana</i>
0	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b
10	23.3 $\pm$ 10.7 b	3.3 $\pm$ 2.0 b
10 <sup>3</sup>	71.0 $\pm$ 22.7 a	9.0 $\pm$ 5.0 b
10 <sup>5</sup>	9.0 $\pm$ 1.0 b	7.7 $\pm$ 1.0 b
10 <sup>7</sup>	22.3 $\pm$ 1.0 b	29.0 $\pm$ 3.0 a

Means within columns followed by the same letter are not significantly different (LSD,  $P=0.05$ ).

## CHAPTER 3

### PERSISTENCE OF THE ENTOMOGENOUS FUNGI *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN AND *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN IN SELECTED SOIL AMENDMENTS

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Entomology

**Abstract** The persistence of *Beauveria bassiana* (Balsamo) Vuillemin (GHA strain) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (F52 strain) were compared in composted cow manure, ground pine bark mulch, composted biosolids, and a Faceville series sandy loam soil. Drop-plating of serially diluted washes of the soil or soil amendments onto the selective oatmeal dodine agar medium at 0, 1, 3, 4, 7, 14, 21 and 28 days post inoculation (dpi) indicated that the number of viable colony-forming units (CFUs) of *B. bassiana* was greatest at 1 to 4 dpi in the soil, ground pine bark, and composted biosolids and greatest at 14 and 21 dpi in the composted cow manure. Exposure of fourth-instar *Tenebrio molitor* L. (Coleoptera:Tenebrionidae) larvae to these soil samples further demonstrated that a large proportion of the *B. bassiana* CFUs recovered in the soil throughout the sampling period were not infective and that the three amendments extended the persistence of *B. bassiana* infective propagules through 14 and 21 dpi. Numbers of *M. anisopliae* CFUs recovered from the soil and the amendments was low in the initial phases (0 through 7 dpi) of the sampling period. However, larval mortality demonstrated the presence of infective propagules during this time. All three soil amendments extended the persistence of infective *M. anisopliae* propagules.

**KEY WORDS** entomogenous fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, persistence

## Introduction

*Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin are entomogenous fungi that are commercially available for use as biological control agents against a variety of insect pests (Flexner and Belnavis 2000). Both are being investigated as potential alternatives to chemical insecticides for controlling the pecan weevil, *Curculio caryae* (Horn) (Coleoptera: Curculionidae). Investigations of pecan weevil biology (Boethel and Eikenbary 1979) and efficacy of the fungi against the weevil (Harrison et al. 1993) indicate that *B. bassiana* and *M. anisopliae* might best be used when adult weevils are emerging from soil in pecan orchards.

Currently, *C. caryae* is controlled with multiple foliar sprays of insecticides (e.g., carbaryl) directed at the adult stage as it feeds and mates in tree canopies. Hudson et al. (2002) note that these applications provide suppression for at least 7 to 10 days and are relatively economical (approximately \$US40/ha). The efficacy, cost and residual activity of any alternative tactic must be comparable. Yet, the short-term persistence of *B. bassiana* in soil limits its potential use for the microbial control of *C. caryae* (Storey et al. 1989, Krueger et al. 1991). Extending the persistence of these entomogenous fungi can help optimize application frequency and costs.

In general, the number of viable propagules of *B. bassiana* recovered from treated soils increases exponentially within 72 h of application (McCoy 1986, Storey et al. 1989). Numbers generally decrease to 85 to 95% of the original concentration within 10 to 12 d of application (Lingg and Donaldson 1981, McCoy 1986, Storey et al. 1989, Krueger and Roberts 1997). Survival of the propagules in soil is related to moisture and organic matter (Studdert et al. 1990, Li and Holdom 1993) and high levels of nutrients (i.e., nitrogen, carbon) in soil amendments

(Clerk 1969, Lingg and Donaldson 1981, Rosin et al. 1996). Thus, the study reported herein was undertaken to define the persistence of *B. bassiana* and *M. anisopliae* in selected soil amendments versus soil. An underlying assumption was that, if fungal persistence could be increased with the addition of soil amendments or mulches, then the fungi might be combined with the soil amendments for ease of application to orchard soils while gaining some benefit for the pecan crop by increasing soil organic matter and other properties (Foshee et al. 1999).

### **Materials and Methods**

**Fungi.** The fungi used in these assays were isolated from commercial preparations of *M. anisopliae* and *B. bassiana*. The *M. anisopliae* was the F52 strain obtained from Earth BioSciences (Fairfield, CT) and was originally the Ma43 strain isolated from a codling moth larva, *Cydia pomonella* L. (formerly *Laspeyresia pomonella* L. and *Carpocapsa pomonella* L.) (Lepidoptera: Tortricidae), in Germany. It was renamed F52 by Bayer Corporation as it was initially developed as a commercial microbial insecticide. The *B. bassiana* was the GHA strain obtained from Emerald BioAgriculture (Butte, MT) and was originally isolated from *Diabrotica undecimpunctata howardi* (Barber) (Coleoptera: Chrysomelidae) in Oregon, USA.

Cultures of each fungus were maintained on Sabouraud's dextrose agar plus 1% yeast extract (SDAY) at 24±1°C with periodic passage through *T. molitor* larvae to prevent attenuation of virulence. Conidia were harvested from 14-d-old cultures using a sterile glass microscope slide to scrape the agar surface. The fungal material obtained was suspended in 40 mL of sterile distilled water (SDW) containing a wetting agent (0.01% v/v, Tween 80™), mixed for 20 min on a magnetic stirrer, and filtered through a double layer of cheese cloth. The number of conidia/mL of the solution was determined using an Improved Neubauer™ hemacytometer. Conidial viability approached 95% for each stock solution.

**Soil and amendment treatments.** The amendments were composted cow manure (Black Kow™, Black Gold Compost Co., Oxford, FL), ground pine bark (Nature's Helper™, Garick Smith Garden Products, Cleveland, OH), and composted biosolids (peanut hulls and municipal waste) (Erthfood™, ErthProducts LLC, Peachtree City, GA). All amendments were purchased from garden retail centers. The soil was a Faceville series sandy loam soil (sand:silt:clay, 84:10:6; pH = 6.1, organic matter 2.8%, w/w) obtained from Peach Co., GA.

Three hundred grams of either the soil or amendments were placed individually in Ziploc™ bags (26.8 x 27.9 cm, S.C. Johnson & Son, Racine, WI). Ten mL of a conidial suspension ( $\times 10^5$  conidia/mL) of either *B. bassiana* or *M. anisopliae* were added to each bag and mixed by hand for 2 min or until the soil or amendment was uniformly moistened. Controls were treated with 10 mL of SDW. These mixtures were transferred individually into 6.1-L plastic containers (Rubbermaid™) and maintained at ambient room temperature. Each treatment was replicated 4 times.

**Assays.** Numbers of viable colony-forming units (CFUs) of the fungi were determined at 0, 1, 3, 4, 7, 14, 21 and 28 d after treatment of the soil or amendments. At each sampling interval, 1 g of soil or amendment was removed from each container, placed in 9 mL of SDW, vortexed twice for 60 s, serially diluted, and drop-plated on oatmeal dodine agar (ODA) (Beilharz et al. 1982, Liu et al. 1993). Counts of either *B. bassiana* or *M. anisopliae* colonies were made 7 days later and used to calculate the numbers of CFUs per g of soil or amendment at that sampling interval.

The presence or absence of infective fungal propagules in the treated soil or amendments was determined by exposing fourth-instar *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae to soil or amendments removed from the containers 0, 1, 3, 4, 7, 14, 21 and 28 d after



treatment. Larvae were obtained from Southeastern Insectaries (Perry, GA) and were maintained in colonies in rectangular plastic containers (32L x 24W x 10D cm) at ambient room temperature with baby oatmeal as a food source and dampened paper towels as a moisture source. Thirty larvae were placed in Petri dishes (100 x 15 mm) containing 10 g of soil or amendment each. There were 4 replicates per treatment per sampling interval. Dishes containing larvae and soil or amendments were placed in covered plastic containers (36L x 30W x 25D cm) lined with moistened paper towels to insure a saturated environment maintained at ambient room temperature. Larvae were checked 10 d later and daily thereafter for mortality. Cause of death by mycosis was confirmed by characteristic fungal growth or typical fungal infection symptoms, such as mummification or subcuticular tanning.

**Statistical analysis.** CFU counts were analyzed as a randomized complete block design and subjected to analysis of variance using the General Linear Models procedure (PROC GLM, SAS 2006) within each sampling interval. Larval deaths were recorded as percent mortality. These were arcsine transformed prior to analysis of variance (General Linear Model) but were re-transformed as percent mortality for presentation herein. All significant ( $P < 0.05$ ) differences among means were separated using the least significant differences.

## **Results and Discussion**

The survivorship of *B. bassiana* following its addition to the Faceville series soil was characteristic of, and corroborated by previously reported survivorship studies of the fungus in various soil systems (Lingg and Donaldson 1981, McCoy 1986, Storey and Gardner 1988, Bruck 2005). Mean numbers of CFUs recovered on the selective ODA decreased initially (1 day postinoculation [dpi]) but increased  $>10\times$  by 3 and 4 dpi. Thereafter, the numbers of viable CFUs recovered gradually declined over the 28-day sampling period.

Survivorship of *M. anisopliae* in soils is not well documented. In our study, the numbers of viable CFUs of *M. anisopliae* recovered on the ODA were lower than the number of *B. bassiana* CFUs recovered. *Metarhizium anisopliae* CFUs increased approximately 10 fold by 1 dpi, decreased at 3 and 4 dpi, and rebounded to numbers 10x higher by 7 dpi. Thereafter, the numbers of viable CFUs declined gradually over the remainder of the 28-d sampling period.

Survival of *B. bassiana* in the ground pine bark and in the composted biosolids generally exhibited the same survivorship pattern as that observed in the Faceville series soil (Fig. 1A). Numbers of viable CFUs recovered on the ODA reflected an initial decline in numbers at 1 dpi, followed by an increase by 3 dpi. Thereafter, the numbers recovered from both of these amendments declined through the remainder of the 28-d sampling period.

The numbers of *B. bassiana* CFUs recovered on ODA from the composted cow manure differed from numbers recovered from either the soil or the other two amendments (Fig. 1A). During the initial phase of the sampling period (0 through 3 dpi), the numbers recovered were lower than those recovered from the other treatments. However, the mean number of CFUs recovered from the composted cow manure was >4x higher than the mean numbers recovered from the other treatments at 14 dpi and 10x higher than those recovered from either ground pine bark or composted biosolids at 21 dpi.

The different survivorship pattern exhibited in the composted cow manure might be attributed to several factors. In the initial phase of the sampling period, the low numbers of CFUs recovered on the ODA were at least partially due to the presence of an aggressive bacterium that often obliterated *B. bassiana* colonies on the agar surface. However, a fungistatic effect may occur in the composted cow manure, given the higher numbers recovered at 14 and 21 dpi (Lockwood 1964).

The mean numbers of *M. anisopliae* CFUs recovered from the amendments remained comparatively low from 0 through 7 dpi (Fig. 1B). At 14 and 21 dpi, the numbers recovered from the three amendments were significantly higher than the number recovered from the Faceville series soil. Furthermore, it is apparent that the activity of *M. anisopliae* in the soil and amendments (as measured by recovery of CFUs on ODA) lags behind that of *B. bassiana*.

While enumerating viable CFUs recovered from a wash of soil or amendment provides an indication of fungal survivorship, the method fails to provide an indication of the infectivity of the CFUs present in the soil or amendment when the sample is obtained. Infectivity of fungal propagules in the soil or amendment is best identified by exposure of the target insect pest to a sample of the soil or the amendment. However, due to its extended life cycle, *C. caryae* is not available in laboratory culture, and a suitable alternative insect host must be used. In a previous study, Scocco et al. (unpubl. data) concluded that the relative susceptibility of fourth-instar *T. molitor* to *B. bassiana* and *M. anisopliae* facilitates the use of these larvae to detect the presence of infective propagules of these fungi in the Faceville series soil. Thus, we assessed the presence of infective propagules of the fungi using fourth-instar *T. molitor*.

Mortality of *T. molitor* larvae due to mycosis following exposure to soil or amendments treated with either *B. bassiana* or *M. anisopliae* indicated that infective propagules were present in these amendments and soil at most of the sampling intervals (Fig. 2). Larval mortality caused by exposure to the three amendments treated with *B. bassiana* remained high through 7 dpi and decreased only slightly at 14 and 21 dpi (Fig. 2A). In contrast, the level of mortality following exposure to the Faceville series soil treated with *B. bassiana* was comparatively low for the entire sampling period, with no mortality occurring at 14, 21 and 28 dpi. Clearly, a large

proportion of the relatively high numbers of viable CFUs recovered from the treated soil are either not infective or may be compromised in the soil-fungus-insect interaction.

Larval mortality following exposure to the amendments treated with *M. anisopliae* also remained high through 14 (all amendments) and 21 (composted biosolids) dpi (Fig. 2B). These high levels of mortality throughout most of the sampling period further indicate that fungistasis may be occurring in these amendments during the early phases (0 through 7 dpi) of the sampling period.

Regardless of the fungus employed, the three soil amendments evaluated herein clearly extended the persistence of infective fungal propagules in comparison to simply adding the fungal conidia directly to soil. Of these amendments tested, the composted cow manure may prove problematic in this usage in southeastern U.S. pecan culture. Public and grower concern of potential crop contamination with human pathogens that may be present in manure amendments (Muirhead et al. 2006, Mukherjee et al. 2006) will limit the utility of manure-based amendments in these systems. Composting presumes to kill any pathogenic microorganisms, but there remains at least a perception of the risk of pathogens contaminating the amended soil or the crop and placing human health and food security at risk (Lemunier et al. 2005). Furthermore, ground pine bark poses the risk of decreasing the soil pH to less than optimal or even detrimental levels for nut crop production.

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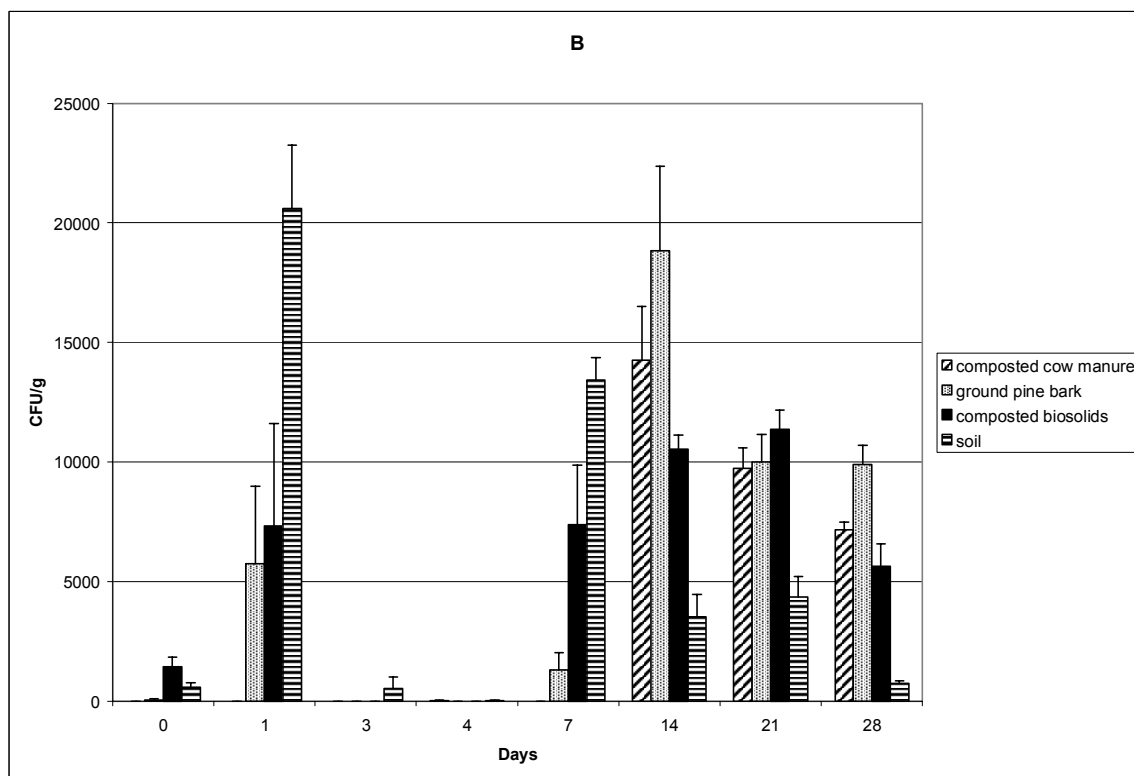
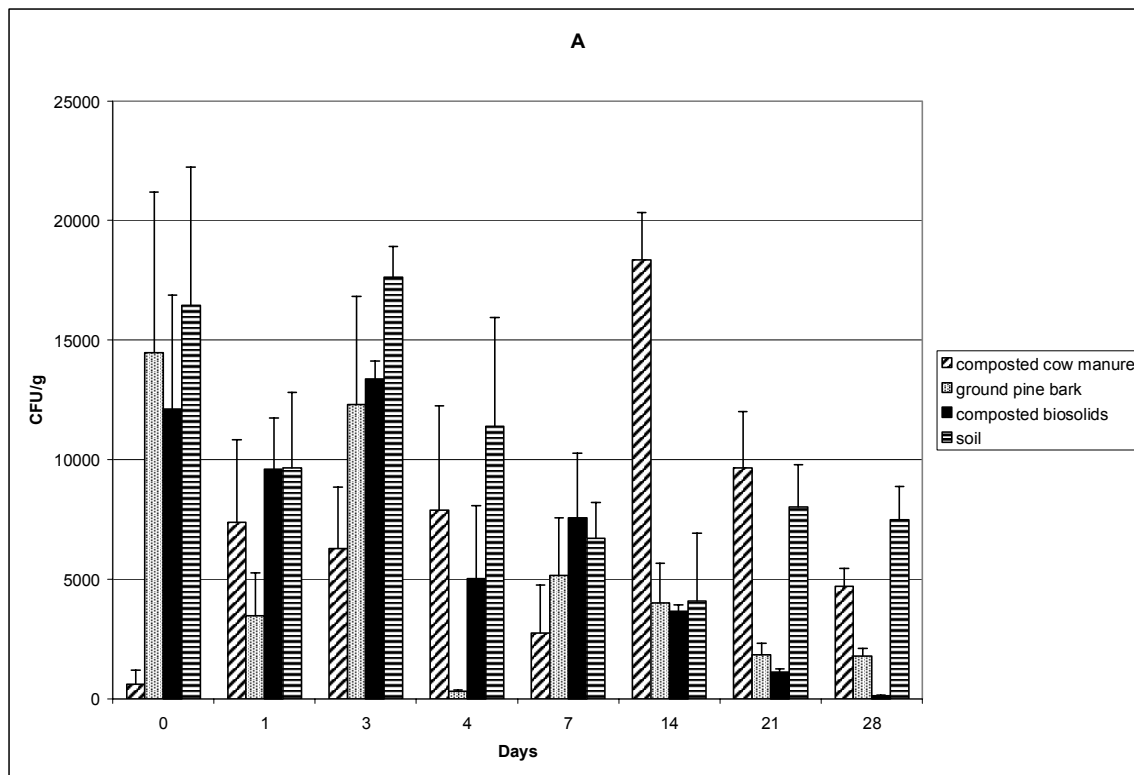
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**Figure 1**

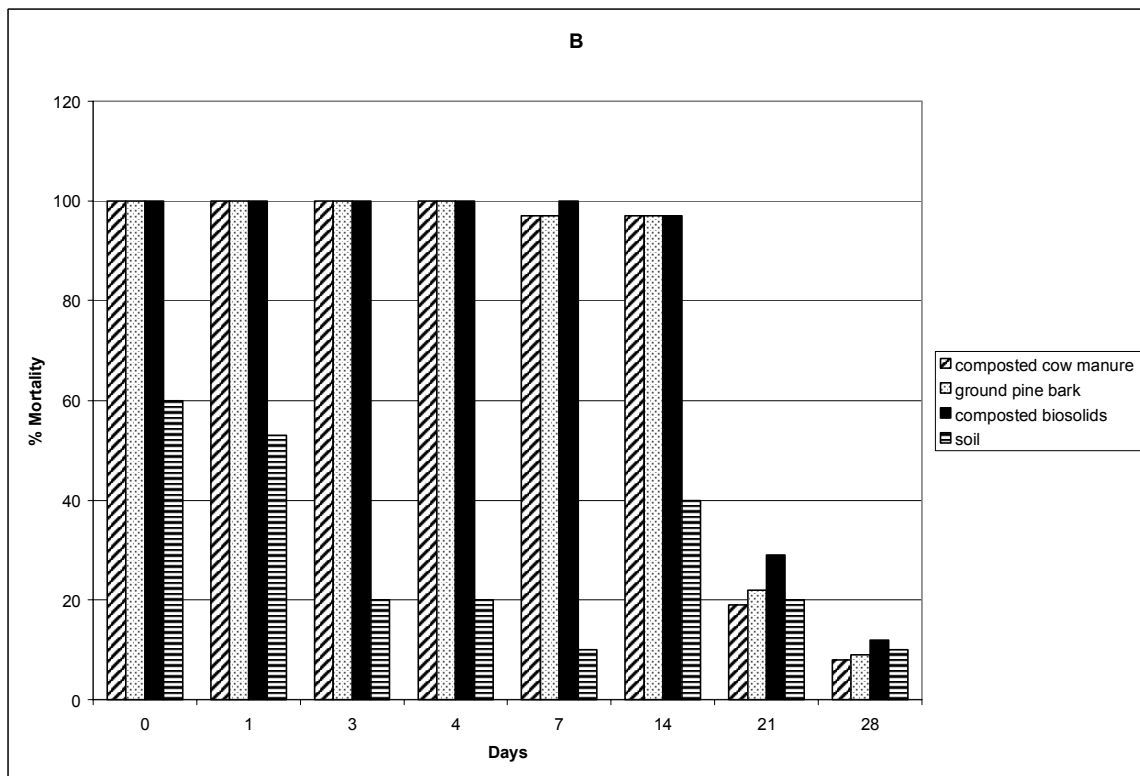
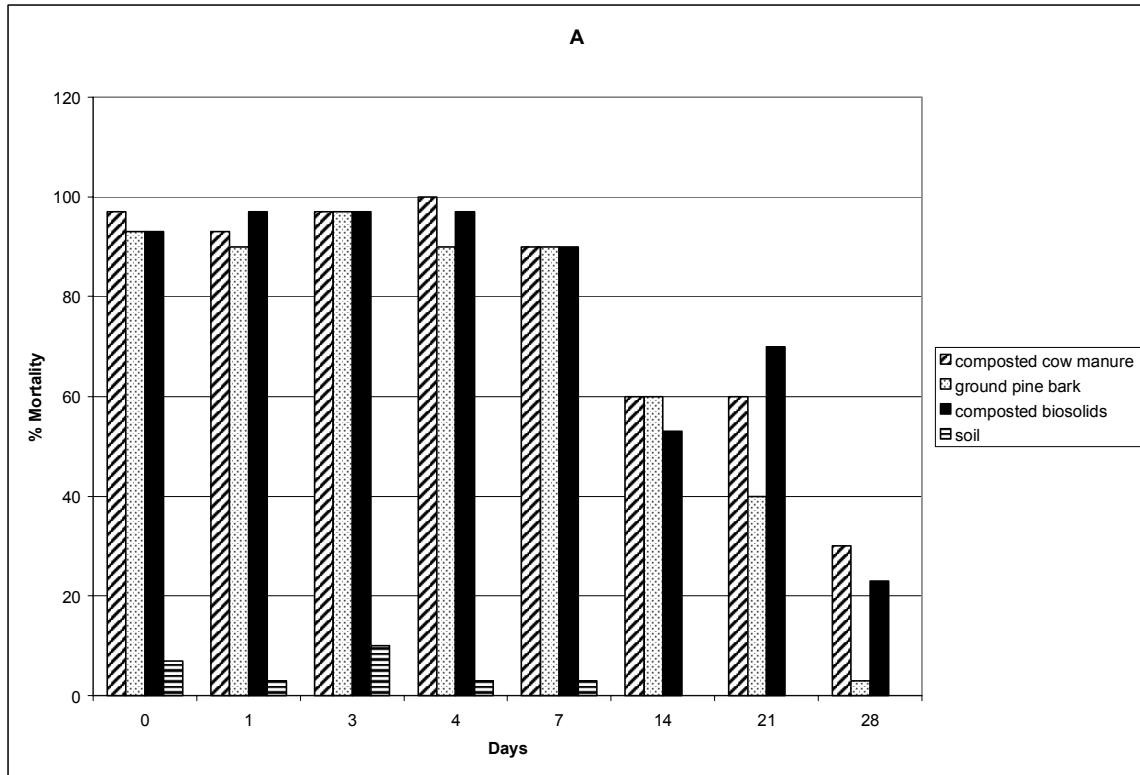
**Mean numbers of colony-forming units (CFUs) of the entomogenous fungi *Beauveria bassiana* (A) and *Metarhizium anisopliae* (B) recovered from a Faceville series soil and three soil amendments (bracket above bars indicates SE).**





**Figure 2**

**Mean mortality of fourth-instar *Tenebrio molitor* larvae exposed to a Faceville series soil or three soil amendments treated with either *Beauveria bassiana* (A) or *Metarhizium anisopliae* (B) (brackets above bars indicate SE)**



CHAPTER 4

SUPPRESSION OF ADULT PECAN WEEVIL (COLEOPTERA: CURCULIONIDAE) WITH  
FIELD APPLICATIONS OF THE ENTOMOGENOUS FUNGI *BEAUVERIA BASSIANA*  
(BALSAMO) VUILLEMIN AND METARHIZIUM ANISOPLIAE (METCHNIKOFF)

SOROKIN

Scocco, E.A., W.A. Gardner, and D.I. Shapiro-Ilan. To be submitted to Journal of Entomological  
Science

**Abstract** Methods of applying commercially-formulated strains of *Beauveria bassiana* (Balsamo) Vuillemin (strain GHA) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (strain F52) were evaluated for control of adult pecan weevils, *Curculio caryae* (Horn), in central Georgia. Methods included application of conidial suspensions to the ground under the trees (*B. bassiana* and *M. anisopliae*), spreading a mixture of composted biosolids and conidia on the ground under trees (*B. bassiana* and *M. anisopliae*), and either spraying the tree trunk with a conidial spray (*B. bassiana*) or encircling tree trunks with a cloth band containing conidia (*M. anisopliae*). Control plots were treated with water applied to the ground under the trees. Adult weevils were captured in Circle cone traps attached to the tree trunks at 1, 3, 8, 10 and 15 d after application and later assessed for mycosis and mortality. Based upon fungus-induced mortality among these weevils, the application of *B. bassiana* conidia in sprays to the tree trunks and the placement of cloth bands containing *M. anisopliae* conidia to tree trunks appear to have potential for causing significant levels of infection in those adult weevils crawling up tree trunks.

**Key Words** *Beauveria bassiana*, *Metarhizium anisopliae*, pecan weevil, field efficacy, fungus-induced mortality, *Curculio caryae*

## Introduction

The pecan weevil, *Curculio caryae* (Horn), is a key pest of pecans in the southeastern U.S., Oklahoma, Kansas and Texas (Harris 1999). The insect exhibits a 1- to 2-year life cycle. Adults emerge from the soil in late July and August and move to pecan tree canopies where they feed upon and oviposit into developing nuts. Larvae develop within the nuts, and, in late fall, fourth instars emerge from the nuts, and, burrow to depths ranging from 8 to 25 cm, and overwinter. Approximately 90% emerge as adults during the following late summer and early fall, while the remaining 10% remain in the soil to emerge the following year (Harris 1985).

Current control recommendations consist of foliar applications of chemical insecticides (e.g., carbaryl) directed at the adults in the tree canopies (Hudson et al. 2002). However, these foliar applications destroy natural enemies, frequently causing secondary pest outbreaks (e.g., mites and aphids) (Dutcher and Payne 1985). Mitigation of these deleterious impacts requires development of alternative management tactics.

Two entomogenous fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff), show great potential for development as alternatives to chemical insecticides for managing the pecan weevil. Both occur naturally in the soils of pecan production systems (Harrison and Gardner 1991, Shapiro-Ilan et al. 2002), and, as a result, natural mortality of the weevil reportedly ranges between 10-20% (Sri-Arunotai et al. 1975). However, these levels are insufficient to maintain populations below economic thresholds (Harris 1999).

Based upon previous research on environmental constraints on these fungi (Fuxa 1987), susceptibility of weevil life stages to the fungi (Gottwald and Tedders 1983, Harrison et al. 1993), and biology of the weevil (Boethel and Eikenbary 1979), the most promising approach for

controlling pecan weevil with these fungi is to target the adults as they emerge from the soil. In fact, Shapiro-Ilan et al. (2004) observed up to 95% *B. bassiana*-induced mortality of adult weevils within 3 d of application of the fungus in a 2-m band around the base of the trees. However, limiting factors continue to be the persistence of viable infective units in the system following application and the management of production and application costs (Shapiro-Ilan et al. 2004). Residual time and economics must be comparable to those of chemical insecticides for the successful adoption of these fungi as alternative management tactics for *C. caryae*.

The objective of the study reported herein was to compare the effectiveness of different delivery methods for suppression of adult *C. caryae* with *B. bassiana* and *M. anisopliae* in pecans. The methods included application of conidial suspensions to the ground encircling the base of the trees, application of a mixture of compost + conidia to the ground encircling the base of the trees, and either the application of a conidial suspension of *B. bassiana* to the tree trunks or the placement of bands containing with *M. anisopliae* conidia around the tree trunks.

### **Materials and Methods**

Field trials to determine the effectiveness of these delivery methods in suppressing *C. caryae* were conducted in two pecan orchards located on the USDA-ARS Southeastern Fruit and Tree Nut Research Unit facility at Byron, GA (Peach Co.) in 2006. Both orchards consisted of mature ‘Stuart’ variety trees that were approximately 60 yrs old. Trees in these orchards had an average circumference of approximately 2 m (at 1 m in height) and were spaced 20 m apart. The soil was a Faceville series sandy loam with a sand:silt:clay composition of 80:16:4 and a pH of 6.1.

***Beauveria bassiana* applications.** One of the two orchards was used to evaluate delivery methods with *B. bassiana*. Treatments in this trial were: (1) ground application of an aqueous

suspension *B. bassiana* conidia; (2) trunk application of a *B. bassiana* conidial spray; (3) ground application of *B. bassiana* conidia incorporated into composted biosolids, and; (4) a control of water applied to the ground. Treatments were applied on 15 August 2006 in a randomized complete block design with four replications per treatment (1 tree per replicate). Blocks (rows) were separated by 1 row of untreated trees, and treated trees within a block (row) were separated by at least 1 untreated tree.

The *B. bassiana* used in this trial was the GHA strain commercially-formulated as an emulsifiable oil containing  $2 \times 10^{13}$  conidia per 946-mL container (Emerald BioAgriculture, Butte, MT). Viability of the preparation exceeded 90% as determined by methods described by Goettel and Inglis (1997). Watering cans were used to apply an aqueous suspension of  $5 \times 10^{12}$  viable conidia in 15 L of water to the ground in a 5-m radius encircling each tree ( $\sim 6.4 \times 10^{11}$  conidia/m<sup>2</sup>). The control plots were treated in the same manner (15 L) with water only. Conidial sprays containing  $1.6 \times 10^{13}$  conidia/mL were applied in 3 L of water to a height of 1.25 m on the trunks of individual trees using a CO<sub>2</sub>-powered backpack sprayer. In addition, commercially-formulated *B. bassiana* was mixed thoroughly with composted biosolids (composted peanut hulls and municipal waste, Erthfood™, ErthProducts, Peachtree City, GA) and applied at a rate of  $5 \times 10^{12}$  conidia per tree in 27 kg of compost equally distributed to a depth of ~3 cm within the 5-m radius encircling each treated tree.

***Metarhizium anisopliae* applications.** The trial evaluating *M. anisopliae* application methods was initiated on 22 August 2006 in the second orchard. The F52 strain of *M. anisopliae* was originally obtained from Earth BioSciences (Fairfield, CT) and was grown on rice by Jared Leland (USDA-ARS, Stoneville, MS) for use in this trial. The treatments were arranged in a randomized complete block design and included: (1) a ground application of an aqueous



suspension *M. anisopliae* conidia; (2) a ground application of *M. anisopliae* conidia incorporated into composted biosolids; (3) a water control, and; (4) the attachment of cloth bands impregnated with *M. anisopliae* conidia to tree trunks. There were four replications per treatment (1 tree per replicate). Blocks (rows) were separated by 1 row of untreated trees, and treated trees within a block (row) were separated by at least 1 untreated tree.

The ground applications of conidia, conidia + compost, and water were made in the same manner and at the same rates as previously described for both fungi. In the band treatment, two cloth bands (~ 2 in width) impregnated with *M. anisopliae* conidia were stapled around individual tree trunks at 0.6 and 1.2 m heights. The production and composition of these bands are proprietary (J. Leland, USDA-ARS, Stoneville, MS). However, methods by Goettel and Inglis (1997) determined that all preparations of *M. anisopliae* used in this trial exceeded 90% viability.

**Mycosis and mortality assessments.** In each trial, adult weevils were collected in Circle trunk traps attached to the tree trunks as described by Mulder et al. (1997) and Cottrell and Mulder (2001). These traps were constructed of wire mesh (1.5-mm mesh) with an opening (~45 cm diam) facing toward the ground surface and tapering to a removable top. Traps were attached to the tree trunks in such a manner that the entire trunk was encircled and the large opening was ~30 cm from the soil surface. This positioning decreased the probability of capturing weevils that had flown to the trunk while maximizing the passive capture of weevils crawling up the trunk from the soil below (Shapiro-Ilan et al. 2004). The removable tops of the traps were positioned atop the traps 24 h prior each collection. Adult weevils were, thus, collected 1, 3, 8, 10 and 15 d following initiation of each trial. At each collection, 10 weevils per tree were removed from the cone traps and placed individually in labeled 30-mL clear plastic

creamer cups along with a moistened cotton wick and a small slice of apple. Collected weevils were maintained in an incubator at 25°C and checked at 7 and 14 d after collection. Mycosis was confirmed by external fungal growth or sporulation.

**Statistical analysis.** Differences among treatments compounded throughout the duration of each trial were analyzed using repeated measures analysis and LSMEANS (Proc Mixed, SAS 2006). Treatment effects also were analyzed by collection date using analysis of variance (PROC ANOVA) and the Student-Newman-Keuls tests (SAS 2006). All percentage data were transformed by arcsine of the square root prior to analysis.

## Results and Discussion

***Beauveria bassiana.*** When the treatment effects were averaged over the entire experimental period, statistically significant differences ( $F = 6.92$ ;  $df = 3,9$ ;  $P = 0.0061$ ) in *B. bassiana*-induced weevil mortality were noted among the application methods with trunk application > ground application > compost application > control (Table 4). Analysis of mortality data by days postinoculation (dpi) showed that, at 3 dpi, fungus-induced mortality resulting from the trunk application was significantly higher than all other treatments ( $F = 12.36$ ;  $df = 3,9$ ;  $P = 0.0015$ ). No other significant differences ( $P > 0.05$ ) among the treatments for any of the individual collection dates were detected (Table 4).

***Metarhizium anisopliae.*** When treatment effects were averaged over the experimental period, *M. anisopliae*-induced mortality occurring in the control plots was significantly lower than levels observed in response to the other three treatments ( $F = 6.16$ ;  $df = 3,9$ ;  $P = 0.0145$ ) (Table 5). At 10 dpi, *M. anisopliae*-induced mortality in response to the attachment of conidia-bands on tree trunks was significantly higher than in all other treatments ( $F = \infty$ ;  $df = 3,9$ ;  $P =$

0.0001). No other significant differences ( $P > 0.05$ ) among the treatments for any individual collection dates were detected (Table 5).

Shapiro-Ilan et al. (2004) observed up to 95% *B. bassiana*-induced mortality of adult *C. caryae* within 3 d after application of the fungus to a 2-m diam area of the ground encircling the base of pecan trees. They recommended that methods needed to be developed to extend the persistence of the fungus in the soil in order for this tactic to be a viable alternative to chemical insecticides for control of *C. caryae*. Our trials were conducted as an extension of that study using *B. bassiana* and *M. anisopliae*; however, the results of our trials with these fungi demonstrated only limited success in improving the suppression of adult pecan weevils with these fungi by extending their persistence after application.

Application of commercially-formulated *B. bassiana* to the tree trunks yielded significantly ( $F = 6.92$ ;  $df = 4, 10$ ;  $P > 0.0061$ ) higher fungus-induced mortality of adult weevils than those levels resulting from application of the fungus to the ground either as an aqueous suspension or in a mixture with composted biosolids. Following trunk applications, adult weevil mortality due to mycosis was 92.9% ( $\pm 7.1$ , SE) at 1 dpi and 83.9% ( $\pm 7.0$ , SE) at 3 dpi. However, fungus-induced mortality gradually declined in subsequent collections probably in response to inactivation of the fungal conidia by ultraviolet radiation and other environmental factors.

The levels of fungus-induced mortality following application of *M. anisopliae* were low. Over the entire experimental period, 49.5% ( $\pm 12.9$ , SE) of the weevils collected after crawling through the cloth bands containing *M. anisopliae* conidia attached to the tree trunks died of infection. This apparatus may prove to be an excellent tool for exposing emerging adult pecan weevils to entomogenous fungi. Placement of the bands around the trunks of the tree forces

weevils crawling up the tree trunk to pass through infective conidia. Mortality observed in the collections made at 10 dpi also indicates that the method may extend the residual activity of conidia in the system.

In laboratory assays, Scocco et al. (unpubl. data) effectively extended the residual activity of *B. bassiana* and *M. anisopliae* in composted cow manure, ground pine bark mulch, and composted biosolids. Composted biosolids were chosen based upon health concerns relating to microbe population in manure (ie. *E. coli*) and the ground pine bark could lower the pH in the system, which is not beneficial for fungal growth. The trials reported herein included one of those amendments – composted biosolids. In addition to providing a matrix for application of the fungal conidia, the amendment could provide an added benefit to the grower in terms of improving soil structure and fertility (McLaurin 2006). Yet, our results show that the composted biosolids were ineffective in extending the residual activity of either *B. bassiana* or *M. anisopliae*. Further research is needed to further evaluate these and additional methods for efficacious and economic use of entomogenous fungi as alternatives to currently used chemical insecticides in pecan insect pest management.

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Table 4. Mean ( $\pm$  SE) percentage mortality of adult pecan weevil adults induced by *Beauveria bassiana* mycosis in response to methods of applying commercially-formulated conidia\*.

Trtmnt	Day 1	Day 3	Day 8	Day 10	Day 15	Avg**
Compost	34.7 $\pm$ 12.0a	32.5 $\pm$ 8.5b	16.3 $\pm$ 10.3a	11.1 $\pm$ 7.9a	20.0 $\pm$ 20.0a	22.4 $\pm$ 5.0a
Control	33.3 $\pm$ 16.7a	5.0 $\pm$ 5.0b	17.8 $\pm$ 9.7a	12.5 $\pm$ 8.0a	5.6 $\pm$ 5.6a	14.1 $\pm$ 4.4a
Ground	61.7 $\pm$ 10.9a	27.5 $\pm$ 9.5b	35.1 $\pm$ 3.1a	30.8 $\pm$ 10.8a	20.8 $\pm$ 12.5a	35.2 $\pm$ 5.1b
Trunk	92.9 $\pm$ 7.1a	83.9 $\pm$ 7.0a	51.7 $\pm$ 18.1a	38.5 $\pm$ 10.3a	6.7 $\pm$ 6.7a	54.0 $\pm$ 9.0c

\*Means within columns followed by the same lower case letter are not significantly different (lsd,  $P=0.05$ ).

\*\*Percentage of weevils exhibiting signs of mycosis averaged over the entire experiment.

Table 5. Mean ( $\pm$  SE) percentage mortality of adult pecan weevil adults induced by *Metarhizium anisopliae* mycosis in response to methods of applying commercially-formulated conidia\*.

Ttmt	Day 1	Day 3	Day 8	Day 10	Day 15	Avg**
Compost	33.2 $\pm$ 11.3a	27.1 $\pm$ 10.4a	43.3 $\pm$ 21.1a	0.0 $\pm$ 0.0b	33.3 $\pm$ 16.7a	27.1 $\pm$ 6.4 ab
Control	0.0 $\pm$ 0.0a	6.2 $\pm$ 6.2a	0.0 $\pm$ 0.0a	0.0 $\pm$ 0.0b	16.7 $\pm$ 16.7a	4.4 $\pm$ 3.2 a
Ground	42.9 $\pm$ 21.7a	0.0 $\pm$ 0.0a	50.0 $\pm$ 28.9a	0.0 $\pm$ 0.0b	70.0 $\pm$ 30.0a	32.4 $\pm$ 10.6 b
Band	64.4 $\pm$ 19.4a	25.0 $\pm$ 25.0a	33.3 $\pm$ 33.3a	100.0 $\pm$ 0.0a	50.0 $\pm$ 50.0a	49.5 $\pm$ 12.9 b

\*Means within columns followed by the same lower case letter are not significantly different (lsd,  $P=0.05$ ).

\*\* Percentage of weevils exhibiting signs of mycosis averaged over the entire experiment.



## CHAPTER 5

# A MICROSCOPIC ASSESSMENT OF THE FATE OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN AND *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN CONIDIA IN SOIL

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**Abstract** Conidia of the entomogenous fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin were placed on Millipore™ filters and buried in a Faceville series sandy loam soil in vials. At specified intervals, filters were extracted from randomly selected vials and observed with light microscopy to elucidate the growth phases of these fungi in soils. Large numbers of conidia were observed on the filters from 0-48 h postinoculation (hpi). Conidia were observed germinating as early as 24 hpi with fungal hyphae and mycelia appearing 48-60 hpi for *B. bassiana* and 7 d postinoculation (dpi) for *M. anisopliae*. Conidia of both fungi were observed on the filters throughout the observation period (28 dpi).

**Key Words** *Beauveria bassiana*, *Metarhizium anisopliae*, entomogenous fungi, survivorship, environmental fate

## Introduction

*Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin are entomogenous fungi that occur naturally in a variety of cropping and pest management systems. Both are commercially formulated for use against a variety of insect pests from the orders Homoptera, Lepidoptera, Thysanoptera, Coleoptera, Isoptera and Orthoptera (Flexner and Belnavis 2000). The sensitivity of the infective conidia to ultraviolet radiation and other environmental factors reduces the residual activity of both fungi on exposed surfaces (Fuxa 1987). Thus, their usage as augmentative or inundative control agents is generally restricted to soil-inhabiting or cryptic insects or those otherwise residing in protected environments.

Once added to soils, the numbers of viable colony-forming units (CFUs) of *B. bassiana* as determined by drop-plating on the selective oatmeal-dodine agar increase logarithmically within 3 days postinoculation (dpi) but are depleted within 12 to 21 dpi (Lingg and Donaldson 1981, McCoy 1986, Storey et al. 1989). McCoy (1986) suggested that the observed increase in CFUs immediately after inoculation was due to germination of conidia and subsequent growth of fungal mycelia in the treated soil. Thus, the increased numbers of CFUs recovered on the selective media within 3 dpi were presumed to be from mycelial fragments.

Although selective media are available, such survivorship curves have not been reported for *M. anisopliae* in soil. However, Scocco et al. (unpubl. data) observed a bimodal survivorship curve for *M. anisopliae* in a Faceville series sandy loam soil from central Georgia. Logarithmic increases in the numbers of CFUs recovered on selective media occurred at 1 and 7 dpi. Numbers of recovered CFUs remained substantially lower at the other sampling intervals (3, 4, 14, 21 and 28 dpi). They also found high levels of mycosis of mealworm, *Tenebrio molitor* L., larvae exposed to the soils only at 1 dpi. Mortality due to mycosis decreased substantially

thereafter. Similar results were obtained with soil inoculated with *B. bassiana*, and they concluded that significant proportions of CFUs of both fungi recovered on selective media are noninfective fungal propagules.

The objective of the study reported herein was to further elucidate the survivorship of *B. bassiana* and *M. anisopliae* in soil. Several techniques have been employed in such investigations. Lumsden (1980) buried nylon mesh embedded with fungi in a sandy loam soil to observe the fungal growth response. The mesh allowed movement of gases, water, root, and invertebrates. Lockwood (1968) reported burying glass slides coated with nutrient media in soil and retrieved the slides at specified intervals to evaluate fungal growth using microscopy. Richards (1991) also used a nylon mesh buried in soil to observe *B. bassiana* growth with scanning electron microscopy.

### **Materials and Methods**

For this study, Millipore™ filters were used as the substrate for the two fungi and were buried in vials of soil for observation of fungal growth responses at specified intervals. The fungi were originally isolated from commercially-formulated preparations. The *M. anisopliae* used was the F52 strain obtained from Earth BioSciences (Fairfield, CT), and the *B. bassiana* was the GHA strain obtained from Emerald BioAgriculture (Butte, MT). Cultures were maintained on Sabouraud's dextrose agar plus 1% yeast extract (SDAY) in incubators operated at 24±1°C.

Conidia of each fungus were harvested from SDAY using a sterile glass microscope slide to scrape fungal growth from the agar surface. The material obtained was suspended in 20 mL of sterile distilled water (SDW) with a wetting agent (Tween™, 0.01% v/v), mixed on a magnetic stirrer for 20 min, and filtered through a double layer of cheesecloth. The number of conidia per

mL in the resulting suspension was determined using an improved Neubauer™ hemacytometer and then diluted to 10 conidia per mL using SDW. Conidial viability in these preparations approached 95%.

A Faceville series sandy loam soil (sand:silt:clay, 84:10:6, pH = 6.1, 2.8% [w/w] organic matter) was collected from Peach Co., GA, sterilized by repeated autoclaving, and oven dried. The soil was brought to 10% (v/v) moisture content with SDW, and 5 g were placed individually into the sterile glass vials (10mL). Millipore filters (55mm) were placed individually on top of the layer of soil in each vial, and 1000  $\mu$ L of the fungal suspension was pipetted onto each filter. These treated filters were then covered with another 5 g of soil, and each vial was capped and maintained at a temperature of  $24\pm 1^{\circ}\text{C}$  in a moisture-saturated environment in covered plastic containers.

Four glass vials from each fungus treatment were randomly selected at each observation interval. The filter in each was extracted and observed with microscopy (100x, Olympus® BX60). Digital images were obtained with an attached Olympus® DP12 camera (Olympus®, Center Valley, PA). Observations were made every 12 h during the first 96 h and at 7, 14, 21 and 28 dpi.

## Results and Discussion

Microscopic examination of the filters extracted from the soil at 0, 12, 36 and 24 h postinoculation (hpi) showed that conidia of both fungi remained intact in the soil, but that some were beginning to enlarge and even germinate (Fig. 3A-C, Fig 4A-C). Mycelial growth on the *B. bassiana*-treated filters was observed as early as 36 and 48 hpi. Richards (1991) first observed *B. bassiana* conidial germination between 24 and 36 hpi. In her study, a massive mycelial mat prevented subsequent observation by 48 hpi. However, using light microscopy, we were able to

observe germinating *B. bassiana* conidia throughout the observation period and as late as 28 dpi (Fig. 3D). Mycelia of *M. anisopliae* were observed on the filters from 2 through 28 dpi (Fig. 4C-D), yet we continued to observe conidia germinating through the observation period.

These observations further elucidate the survivorship of these fungi in the Faceville series soil recently reported by Scocco et al. (unpubl. data). In that study, a logarithmic increase in *B. bassiana* CFUs was recovered from the soils 3 and 4 dpi; yet, % mortality of *T. molitor* larvae exposed to these soils decreased from levels observed at 0 and 1 dpi. Clearly, the increase in CFUs at 3 and 4 dpi resulted from mycelial growth, and the low level of *T. molitor* mortality following exposure to the soils at 14, 21 and 28 dpi resulted from the few conidia that persisted during the latter phases of the sampling period. This also substantiates assumptions by McCoy (1986) and others regarding the growth response of *B. bassiana* in soil.

Scocco et al. (unpubl. data) also observed a logarithmic increase in *M. anisopliae* CFUs at 7 dpi. This increase did not correspond with high mortality levels in *T. molitor* larvae exposed to the soils after inoculation when large numbers of conidia were observed on the filters.

In conclusion, care must be taken in defining survivorship of entomogenous fungi in soils based only upon recovery of CFUs using selective media. Results reported herein conclusively show that the increases in *B. bassiana* and *M. anisopliae* CFUs following inoculation of soils are due to recovery of hyphal fragments resulting from germination of conidia.

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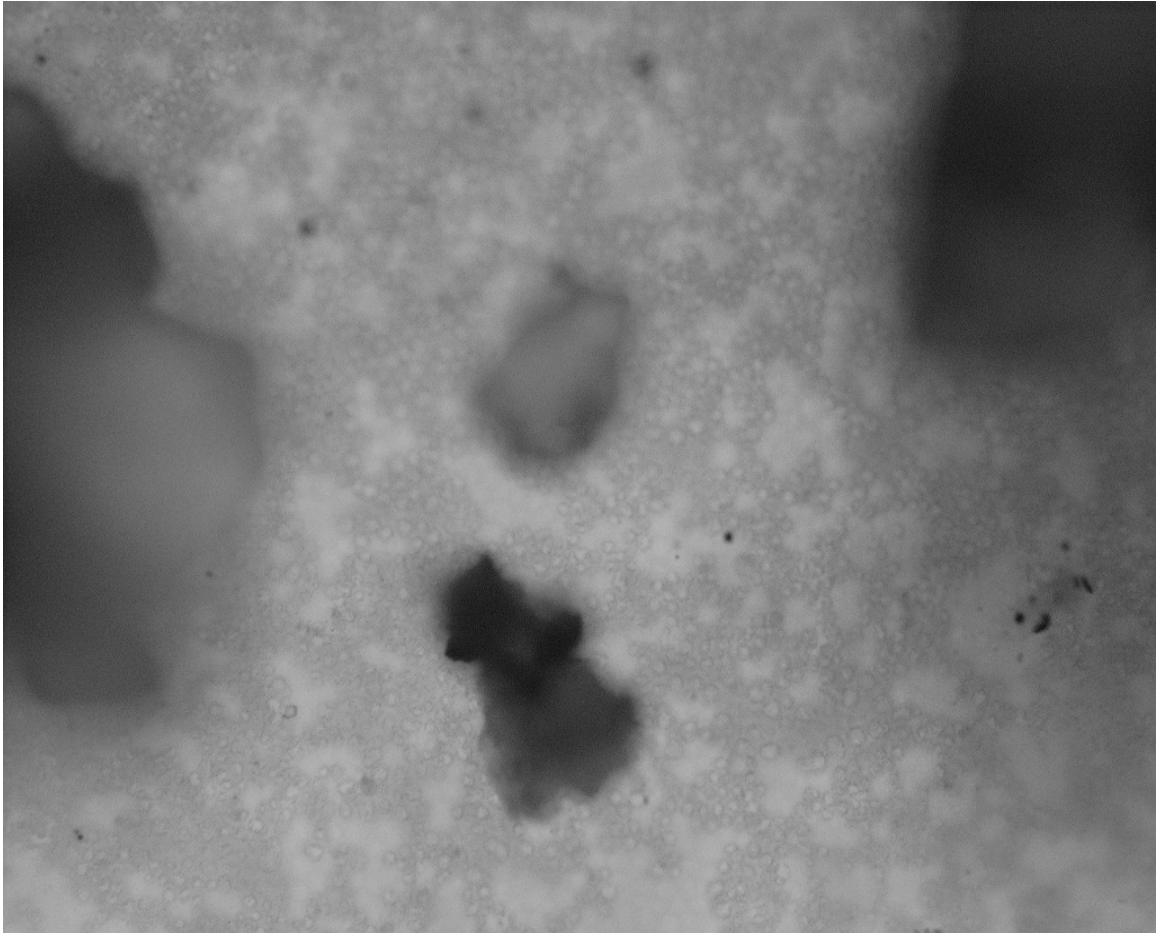
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**Figure 3**

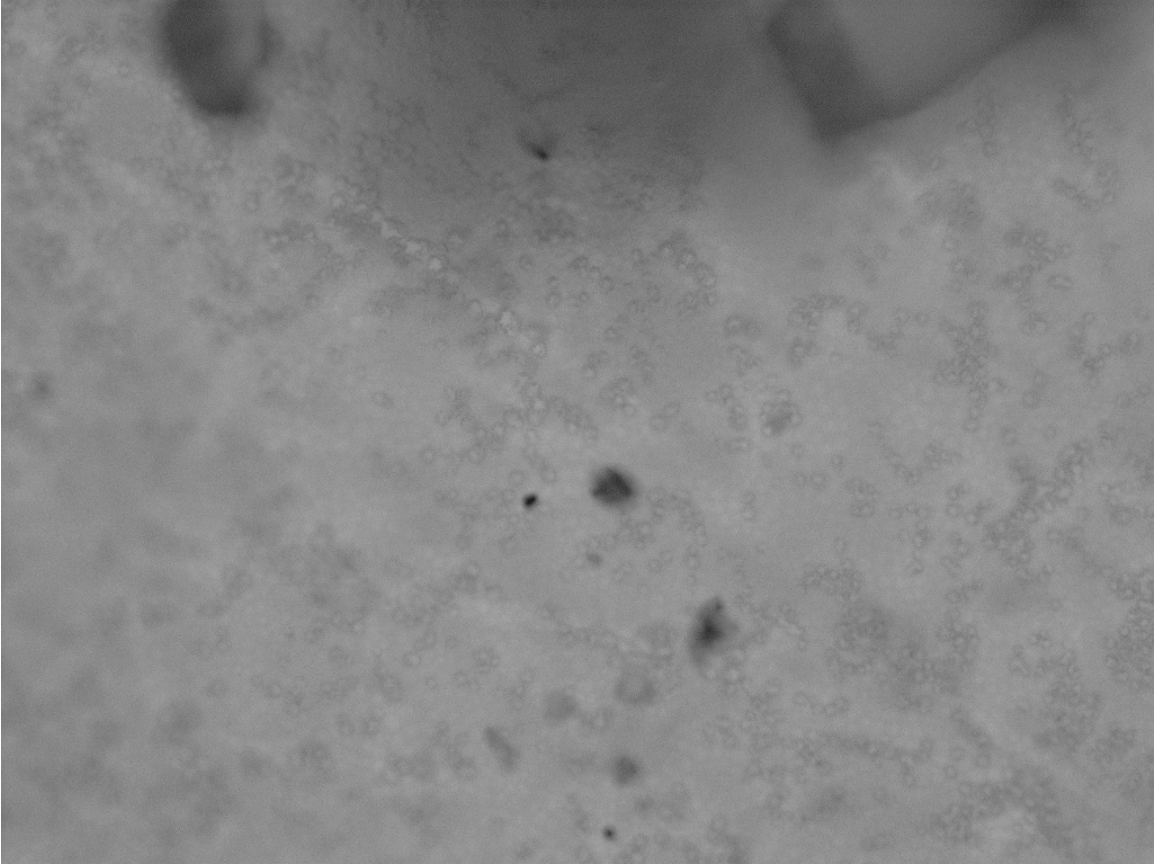
**Digital images of *Beauveria bassiana* on Millipore™ filters buried in a Faceville series sandy loam soil at 0 (A), 24 (B) and 28 (C) days after inoculation.**



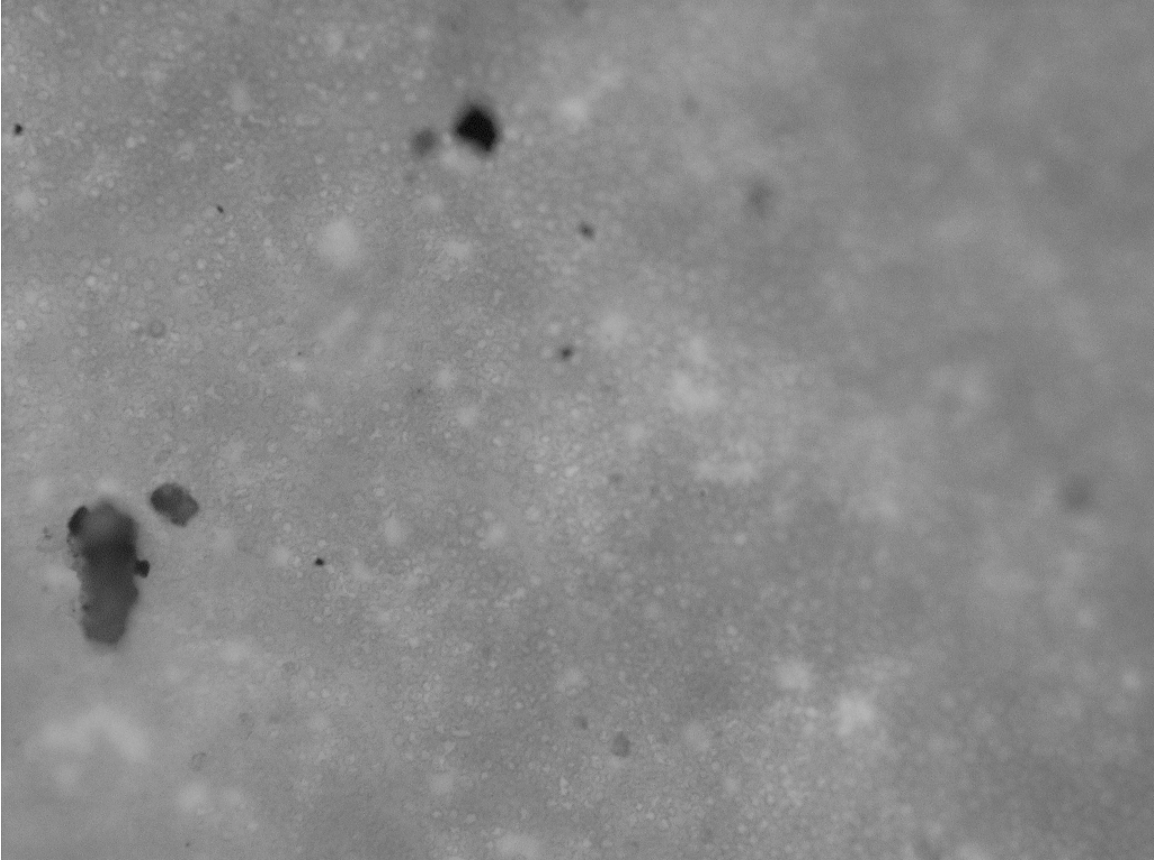
A



**B**



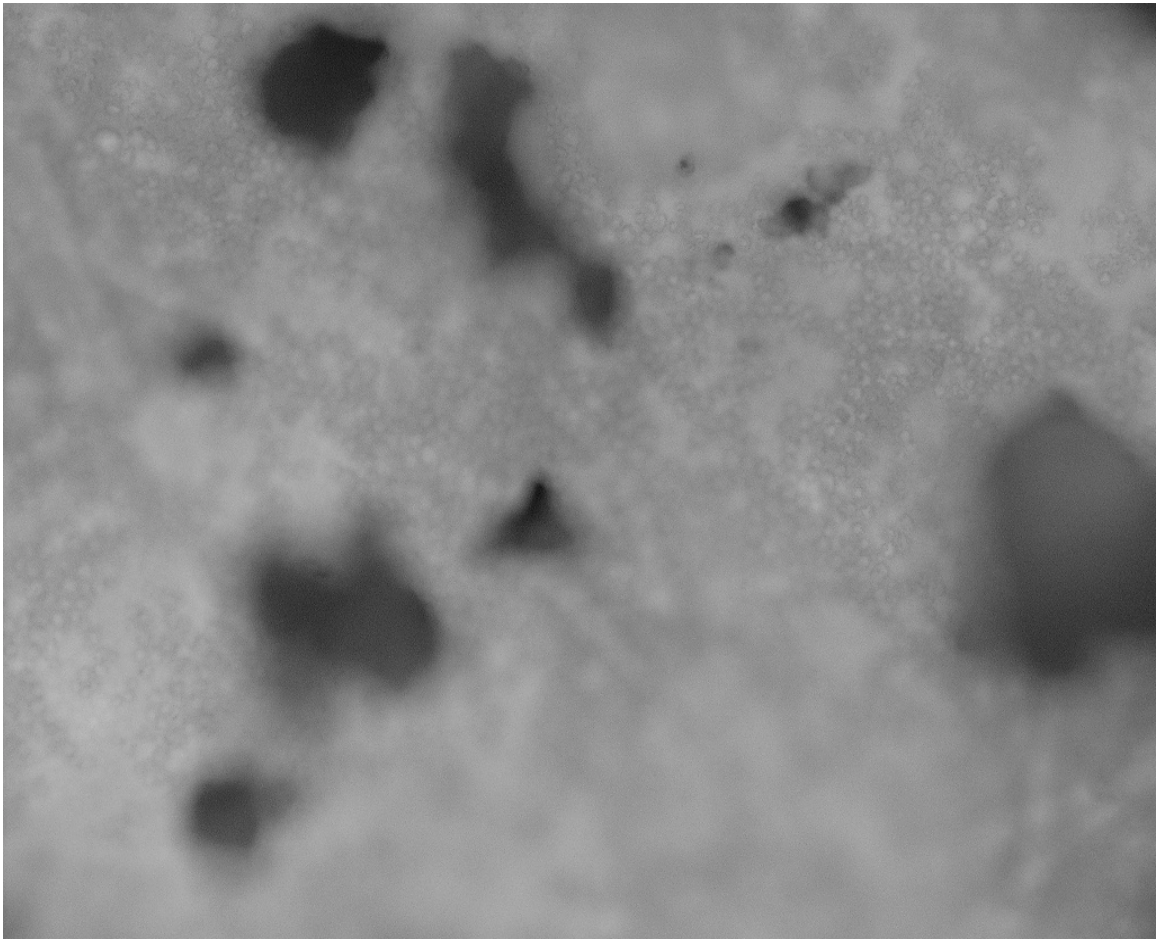
C



**Figure 4**

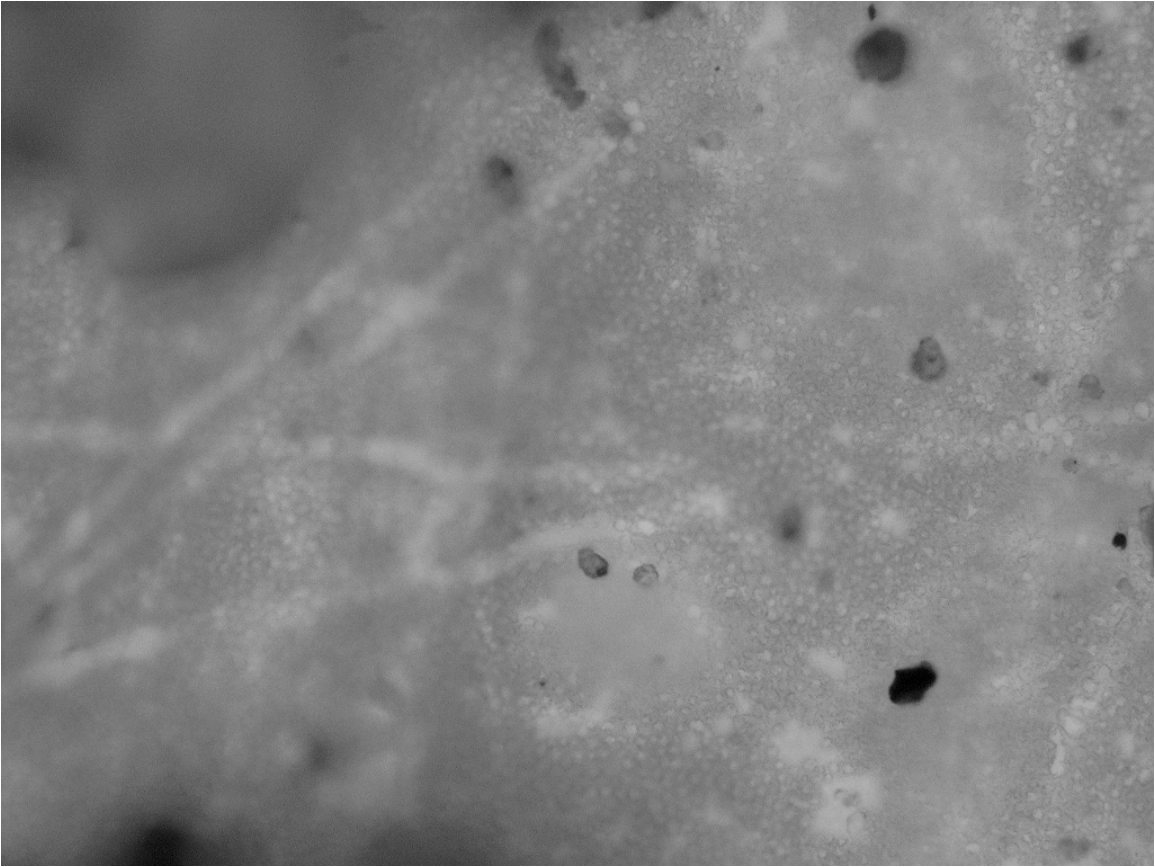
**Digital images of *Metarhizium anisopliae* on Millipore™ filters buried in a Faceville series sandy loam soil at 0 (A), 48 (B), and 28 (C) days after inoculation.**

A

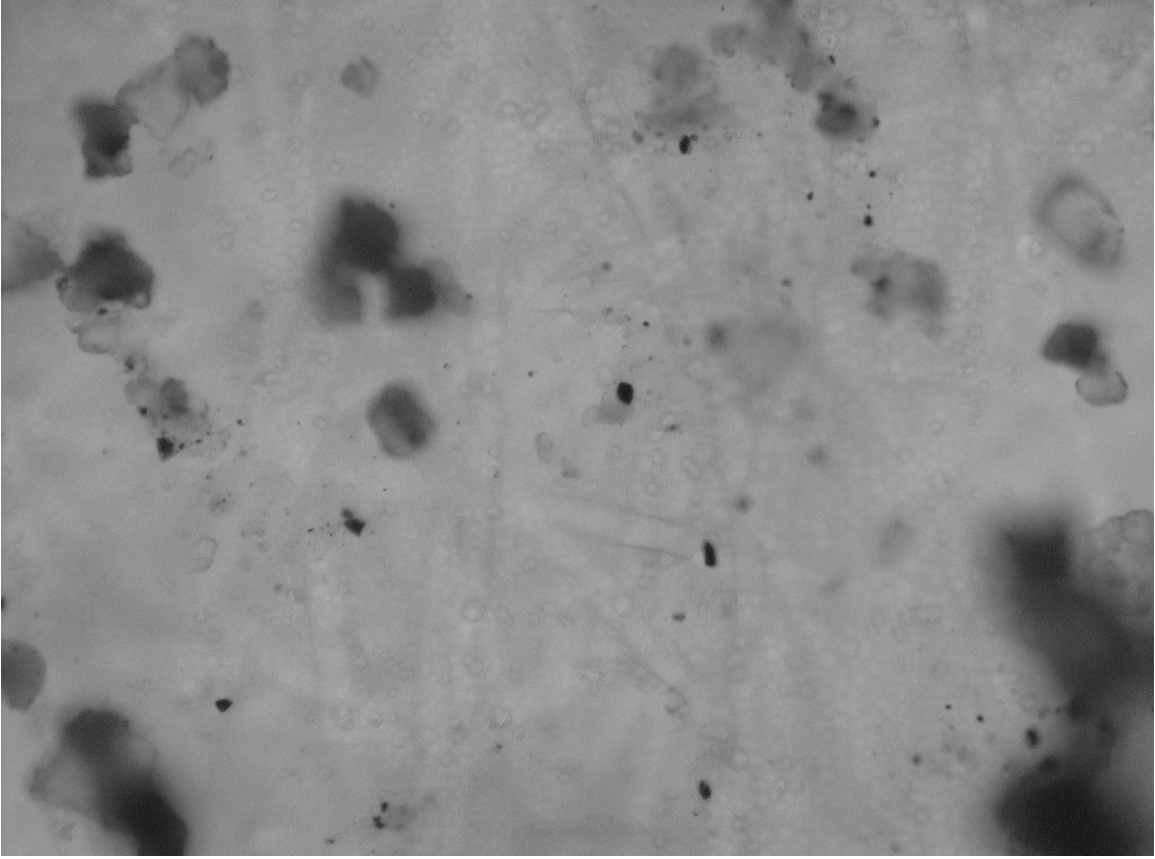




**B**



C



CHAPTER 6  
CONCLUSION



The series of studies reported herein have further elucidated the survivorship and fate of the entomogenous fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin, in soils with an ultimate goal of developing these fungi as viable alternatives to chemical insecticides for managing the pecan weevil, *Curculio caryae* (Horn). Previous research demonstrated that these fungi cause low levels of natural mortality in pecan weevils, and both fungi kill pecan weevil larvae and adults in laboratory assays and field trials. Control using fungi should target emerging adult weevils from soil, thus, applying the fungi directly to soil. Yet, research also shows that the residual activity of the fungi must be extended in order for them to be considered viable economic alternatives to chemical insecticides.

Laboratory assays were conducted to establish the survivorship of the two fungi in soil amendments and soil. The underlying purpose of these assays to determine if the fungi could be mixed with soil amendments or mulches to extend the persistence of the fungi targeted to controlling emerging pecan weevil adults. This mixture could be applied easily by growers and could improve soil fertility. Data from the assays corroborated previously reported survivorship curves of entomogenous fungi in soils developed by using selective media. In general, numbers of viable colony-forming units (CFUs) recovered on the selective media increase within 12 h of application, but steadily decline to background levels within 7 to 14 d after application. In general, numbers of CFUs of both fungi recovered from the compost and mulch amendments exhibited a survivorship pattern that was similar to that observed in the soil.

However, CFUs recovered on selective media are not necessarily from infective fungal propagules. Microscopic examination of fungi on Millipore™ filters buried in the Faceville series soil clearly showed that germinating conidia of both fungi remain in the soil for at least 28 d postinoculation (dpi). Fungal mycelial growth from the germinating conidia was observed as

early as 3 dpi. It was apparent that a large proportion of CFUs recovered on the selective media at 3 dpi result from mycelial fragments rather than infective conidia. Therefore, the presence and quantity of infective fungal propagules in soils or soil amendments must be established using suitable insect hosts that can be easily cultured in the laboratory.

A bioassay established the median lethal concentration (LC50) of *B. bassiana* and *M. anisopliae* against the easily-reared and maintained mealworm, *Tenebrio molitor* L., fourth instars. These insects proved to be suitable for detecting the presence of infective propagules of both fungi in soils.

Based upon these findings, mortality of fourth-instar *T. molitor* larvae exposed to the amendments in the laboratory assays clearly demonstrated that infective *B. bassiana* conidia persisted in the amendments for at least 21 d postinoculation (dpi). Larval mortality exceeded ~90% in these assays through 7 dpi but decreased to between 50 and 70% at 14 and 21 dpi. Some activity remained at 28 dpi with observed mortality  $\leq 30\%$ . Similar responses were observed with *M. anisopliae* with larval mortality exceeding 90% through 7 dpi, decreasing to 30 to 60% on 14, 21 and 28 dpi.

The residual activity of both fungi was significantly ( $P>0.05$ ) extended in composted cow manure, ground pine bark, and composted biosolids relative to soil. Field trials comparing the application of conidia mixed with composted biosolids to the ground encircling the base of the pecan trees with other application methods were conducted in two pecan orchards in 2006. In these trials, the compost + conidia application failed to enhance suppression of emerging pecan weevil adults. Placement of conidia on tree trunks either in aqueous sprays (*B. bassiana*) or in cloth bands encircling the trunks (*M. anisopliae*) resulted in significantly higher fungus-induced mortality of adults in comparison to other treatments.

These studies provide evidence that *B. bassiana* and *M. anisopliae* persist longer in the amendments tested; however, once placed in the soil matrix in a field setting fungal activity was not comparable to results from laboratory assays. Thus, further studies must be conducted to further delineate these responses.