12-2012

COMPATIBILITY OF ESSENTIAL OILS WITH THE BIOCONTROL FUNGUS, BEAUVERIA BASSIANA

Wanjing Liu
wliu17@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Wanjing Liu entitled "COMPATIBILITY OF ESSENTIAL OILS WITH THE BIOCONTROL FUNGUS, BEAUVERIA BASSIANA." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Kimberly D. Gwinn, Major Professor

We have read this thesis and recommend its acceptance:

Bonnie H. Ownley, Ernest C. Bernard, Arnold M. Saxton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

COMPATIBILITY OF ESSENTIAL OILS WITH THE BIOCONTROL FUNGUS, *BEAUVERIA BASSIANA*

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Wanjing Liu

December 2012

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major professor, Dr. Kimberly D. Gwinn, for her support, patience, and encouragement, and for giving me the opportunity to pursue this degree. I also thank my committee members, Dr. Ernest Bernard, Dr. Arnold Saxton and Dr. Bonnie Ownley for their advice, support and guidance. I thank Mary Dee and David Trently for helping me whenever I needed them; and I thank the entire Entomology and Plant Pathology department for all of their help and much of what I learned from this experience.

I must thank all of the graduate students, for their friendships and support, which I will remember forever. Special appreciation is expressed to Marei Abdelkarim, Oluseyi Fajolu, Andrea Vu, Jonathon Mixon, Sandesh Shrestha, Haley Smith, Qunkang Chen and Jonathan Black for their tireless assistance and the friendships, which I will remember forever.

I would also like to express gratitude to my family for their support, encouragement, and love. I feel extremely blessed to have you in my life.

ABSTRACT

The tomato seedling damping-off pathogens *Rhizoctonia* and *Pythium* have the potential to cause severe loss in the greenhouse and field. Both seed application of *Beauveria bassiana* and soil amendment with bioactive monarda herbages are sustainable approaches that can play a role in suppressing damping-off of tomato seedlings. The objectives of this research were to determine the compatibility of essential oils and *B. bassiana*, and to determine the impact of the two when used together as a seed treatment in greenhouse experiments.

Different concentrations of five essential oils that were active against damping-off pathogens **(**cymene, carvacrol, thymol, borneol, and geraniol) were tested. Germ tube emergence was monitored every four hours after germination began. Germ tube lengths of *B. bassiana* conidia treated with each oil concentration were determined. Cymene had a stimulatory effect on germ tube development of *B. bassiana* at low concentrations but inhibited germ tube development at high concentrations. Thymol was the least inhibitory of all tested oils. All oils reduced germ tube length at the highest concentration at 24 h. In a separate study spore germination of *B. bassiana* treated with oils for 24 h was also determined. Germination percentage was decreased across concentration in all five oils treatment. Germ tube development with fresher spores (used in the second germination study) was similar to that for aged conidia (used in the first germination study) but at 24 h, fresher spores had a shorter average tube length.

In the second study, five bioactive monarda cultivars (‘Trinity Purple’,

‘Rose-scented’, ‘Violet Queen’, ‘Cerise’, and *Monarda clinopodia*) representing five chemotypes (cymene, geraniol, carvacrol, borneol, and thymol, respectively) were selected based on the GC-MS analyses. Colonization by *B. bassiana* of seedlings grown in media amended with the five cultivars was determined. All five cultivars had a slight negative impact on the ability of *B. bassiana* to colonize tomato. Treatment with *Monarda clinopodia* (thymol chemotype) resulted in very low germination of tomato seeds. Four other monarda cultivars had no negative impact on tomato seed germination and so have the potential to be used with *B. bassiana* as biological control agents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

LITERATURE REVIEW

Introduction:

Pathogenic microorganisms and pests of plants can reduce or eliminate profits in crop production systems. Effective environmentally-friendly tools for the management of diseases and noxious insect pests are needed for improving economic and environmental performance in sustainable agriculture. Both microbial pesticides such as *Beauveria bassiana* and bioactive herbage including monarda, can provide pest and disease control and have less potential for causing damage to the environment or to non-target organisms than chemical interventions.

Essential oils are well known natural pesticides, and it has been shown that bioactive herbage (dried, ground leaves of *Monarda* spp.) can protect against damping-off diseases in tomato seedling production. Technology for seed treatment with *Beauveria bassiana* has also been developed, and it has been shown that plants derived from treated seed are protected from seedling diseases (Ownley *et al.* 2004, 2008) and insects because they are colonized with the fungus. However, there are few data on the compatibility of natural products and entomopathogenic fungi, and no data on the impact of the two when used as a seed treatment in greenhouse experiments. Demonstration that the two technologies are compatible is needed in a pest management system.

This study will be used to produce models that will predict the effect of bioactive natural products on *B. bassiana*; this approach will allow selection of bioactive natural products that are compatible with *B. bassiana*. This is the first step in the development of systems that increase sustainability, productivity, and farm profits.

1.1 Essential oils

Essential oils are mixtures of different terpenoid compounds and their oxygenated derivatives. They are highly volatile substances synthesized and stored in glandular trichomes of odiferous plants. These oils are known for their broad-spectrum antimicrobial activity against both human and plant pathogens. The antimicrobial action of essential oils generally depends upon their hydrophilic or lipophilic character. Also, some terpenoids affect the activities of membrane enzymes and interfere with respiratory pathways. Certain components of essential oils can even interrupt ADP phosphorylation (primary energy metabolism) and inhibit the synthesis of DNA, RNA, proteins and polysaccharides in fungal and bacterial cells (Knobloch *et al*.1989). The type of microorganism, its cell wall structure and outer membrane arrangement may determine the mode of action of antimicrobial agents. For instance, *Pseudomonas aeruginosa*, an opportunistic bacterium associated with hospital-acquired infections of humans, is resistant to a wide variety of essential oils, because the hydrophobic macromolecules of essential oils constituents are unable to penetrate the hydrophilic surface of the bacterial outer membrane (Hiroshi 1994).

Antifungal essential oils reduce hyphal growth and also induce lysis and cytoplasmic evacuation in fungi. Growth inhibition by essential oils often involves induction of changes in cell wall composition, plasma membrane disruption, mitochondrial structure disorganization, and interference with enzymatic reactions of the mitochondrial membrane, such as respiratory electron transport, proton transport,

and coupled phosphorylation steps (Kishore *et al.* 2007). The essential oils of some plants and their constituents are effective as antifungal agents against plant pathogenic fungi.

Many plants with oils that inhibit the growth of phytopathogenic fungi belong to the Laminaceae family. Those species containing phenolic components, thymol or carvacrol, as the main constituent, have high antifungal activity. The antifungal activity and chemical composition of the essential oil isolated from *Origanum acutidens* (87.0% carvacrol as main constituent) completely inhibited mycelia growth of seventeen species of *Botrytis, Fusarium* and *Alternaria* spp. (Kordali *et al.* 2008). Also, oils collected by steam distillation from *Thymbra spicata, Satureja thymbra* and *Origanum minutiflorum* that contained more than 234.8 mg/mL thymol and/or carvacrol inhibited *in vitro* mycelia growth of several soilborne plant pathogenic fungi (*Fusarium moniliforme, Rhizoctonia solani, Sclerotinia sclerotiorum,* and *Phytophthora capsici)* (Müller-Riebau *et al*. 1995). Furthermore, essential oils of *Thymus vulgaris* and *Mentha piperita* (50.06% thymol as the main component) reduced *in vitro* growth of the pathogenic fungi, *R. solani, Pythium ultimum* var. *ultimum, Fusarium solani* and *Colletotrichum lindemuthianum.* These oils also caused degeneration of fungal hyphae, and hyphae appeared empty of cytoplasmic content materials. When *C. lindemuthianum* and *F. solani* were treated with thyme oil, several changes in fungal growth and reproduction occurred (e.g., alterations in the morphology of the hyphae, reductions of conidial number, and reductions in hyphal diameter) (Zambonelli *et al*., 1996).

In addition to thymol and carvacrol, essential oils from mints that have high concentrations of other constituents reduce growth and reproduction of phytopathogenic fungi. The essential oil compounds of the aerial parts of *Rosmarinus officinalis* (44.02% ρ-cymene, 20.5% linalool as main components) inhibited *in vitro* growth of *Alternaria alternata, Botrytis cinerea* and *Fusarium oxysporum* (Ozcan and Chalchat 2008). In addition, other essential oil constituents that potentially can be used as fungicides have been studied. Oils from the aerial parts of *Salvia rosifolia* [α-pinene (15.7-34.8%) and 1,8-cineole (16.6-25.1%) identified as major constituents] strongly inhibited growth of the strawberry anthracnose-causing fungal plant pathogens, *Colletotrichum acutatum, C. fragariae,* and *C. gloeosporioides* (Ozek *et al.* 2010). The essential oil of *S. fruticosa* (main components - 1,8-cineole and camphor) effectively inhibited mycelia growth and reduced the radial growth of *R. solani, Sclerotinia sclerotiorum* and *F. solani, in vitro* (Pitarokili *et al*.,2003). The essential oil of *Ocimum sanctum* (56.07% eugenol) completely inhibited growth of *Aspergillus niger* and *A. flavus* (Pandey *et al*., 2010). Essential oil extracted from the leaves of *Mentha arvensis* (43.45% menthol) was evaluated *in vitro,* and found to completely inhibit growth of two pathogenic filamentous fungi *A. niger* and *A. flavus* (Pandey *et al.* 2010).

Fungicidal properties of plants belonging to other families have also been tested. Piperitone and trans-ethyl cinnamate, which are two essential oil compounds extracted from *Artemisia judaica* (Asteraceae), inhibited the mycelia growth of *Botrytis fabae, F. oxysporum, Pythium debaryanum,* and *R. solani.* The antifungal

activity of the two isolated compounds against *F. oxysporum* and *R. solani* was greater than borneol and cineole, but weaker than thymol, carvacrol, and geraniol (Abdelgaleil *et al*.,2008). The leaf essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* (Cupressaceae) [main componets - α-pinene (44.2%) and limonene (21.6%)] strongly inhibited growth of the damping-off pathogens, *R. solani* and *F. oxysporum, in vitro*, and also efficiently inhibited the mycelial growth of leaf pathogens *(C. gloeosporioides*, *Pestalotiopsis funerea*) and root-rot pathogens (*Ganoderma australe and F. solani*) (Chang *et al.* 2008). The essential oils of *Syzygium aromaticum* (Myrtaceae) and *Foeniculum vulgare* (Apiaceae) were active against *Botrytis cinerea* (Peighami-Ashnaei *et al*., 2009). Eugenol [the primary constituent of essential oil isolated from *Syzygium aromaticum* (Myrtaceae)] completely inhibited mycelial growth of four apple pathogens (*B. cinerea*, *Monilinia fructigena*, *Penicillium expansum* and *Phlyctema vagabunda*) at a concentration of 150 µl/l but was ineffective in reducing disease incidence after being mixed with soy lecithin (Amiri *et al*., 2008). Essential oils extracted from star anise (Schisandraceae), which contained 89.5% trans-anethole, strongly inhibited mycelial growth of plant pathogenic fungi (*Alternaria solani, Bipolaris maydis, F. graminearum, Pythium aphanidermatum* and *R. solani)* in the *in vitro* direct contact assay (also called the mycelial radial growth inhibition assay) (Huang *et al.* 2010). The efficacy of the essential oil and extracts derived from the flower and leaves of *Magnolia liliflora* against plant pathogenic fungi was evaluated *in vitro* and *in vivo*. The oil inhibited radial growth of *B. cinerea, C. capsici, F. oxysporum, F. solani, Phytophthora capsici,*

R. solani and *Sclerotinia sclerotiorum* by 38 to 65.6%, and had strong detrimental effects on spore germination of all tested plant pathogens (Bajpai and Kang 2009). Essential oil compounds of asafoetida (*Ferula assafoetida*) (Apiaceae) and black cumin seed (*Nigella sativa*) (Ranunculaceae) were effective at controlling colony growth of eight seed-borne fungi including *A. niger, A. flavus, F. oxysporum, F. moniliforme, F. nivale, F. semitectum, Drechslera hawaiinesis* and *Alternaria alternata* (Sitara *et al.* 2008). The essential oil extracted from eucalyptus (*Eucalyptus camaldulensis*) (Myrtaceae) completely inhibited mycelial growth of *Pythium ultimum* and *R. solani* (Katooli *et al.* 2011).

Essential oils also reduced growth of other fungi (e.g., dermatophytes, various molds, and yeasts). The main components β-bisabolene (17.6-51.0%) and 11-α-(H)-himachal-4-en-1-β-ol (9.0-21.6%) of extracts from both flowers and mature umbels with seeds of wild *Daucus carota* subsp. *carota* (Apiaceae) reduced growth of five dermatophytes (*Epidermophyton floccosum*, *Microsporum canis*, *M. gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*) (Maxia *et al.* 2009). The essential oil of *Achillea millefolium* (Asteraceae) containing 42.15% chamazulene suppressed the development of *Aspergillus nidulans* spores and increased number of yellow and white mitotic recombinants of the diploid strain (De Sant'anna *et al.* 2009). Many essential oils from genera of Laminaceae, including several species of *Thymus* (*T. numidicus, T. vulgaris, Thymus* x *viciosoi, T. algeriensis*) and *Origanum* (*O. vulgare, O. majorana*) that had thymol, carvacrol, cymene, linalool or α-pinene as the main constituent, effectively inhibited growth of yeast (e.g., *Saccharomyces cerevisiae,*

Cryptococcus neoformans or *Candida albicans*) (Giordani *et al*., 2008; Van Vuuren *et al*., 2009; Pozzatti *et al*., 2010).

Essential oils also inhibit growth of phytopathogenic bacteria, drug-resistant bacteria, food-related bacteria and diverse bacterial human pathogens. Essential oils are effective as plant or seed disinfectants for the prevention of infections caused by phytopathogenic bacteria. *Thymus fallax* and *Satureja spicigera* (Lamiaceae), both which have a high content of thymol, carvacrol, ρ-cymene and γ-terpinene, inhibited growth of a wide range of agricultural bacterial pathogens such as *Erwinia, Pseudomonas* and *Xanthomonas* (Kotan *et al.* 2010). Essential oils also inhibit drug-resistant bacteria. Species of Laminaceae including *Lavandula* sp., *Salvia rosifolia, Thymus vulgaris, Zataria multiflor, L. angustifolia, L. latifolia Medik, L. luisieri* (Müller-Riebau *et al*. 1995; Roller *et al*. 2009; Ozek *et al*. 2010; Mahboubi & Bidgoli, 2010; Tohidpour et al. 2010) and Myrtaceae including *Cleistocalyx operculatus* and *Eucalyptus globules* (Dung *et al*., 2008; Tohidpour *et al*., 2010) effectively inhibited the growth of drug-resistant bacterial strains. Essential oils from *Helichrysum italicum* (Asteraceae) that contained high concentrations of geraniol, had inhibitory activity against *Acinetobacter baumannii, Enterobacter aerogenes, Escherichia coli,* and *Pseudomonas aeruginosa* (Lorenzi *et al.* 2009). Oil of *Melaleuca alternifolia* (Myrtaceae) that primarily contained terpinen-4-ol was active against coagulase-negative staphylococci (Loughlin *et al*., 2008).

Essential oils added to food products may extend shelf life due to their activity against food-borne bacteria. The essential oils of *Artemisia incana,*

Chaerophyllum operculatus, Laurus nobilis, *Mentha pulegium* and *Nandina domestica* were active against a wide range of the most important food-borne microorganisms including *Listeria monocytogenes, Enterobacter aerogenes, Enterobacter cloacea*, *Escherichia coli*, *Enterococcus faecalis*, *Salmonella typhimurium* and *S. enteritidis*. (Bajpai *et al.* 2008; Dung *et al.* 2008; Erkmen and Ozcan 2008; Mahboubi and Haghi 2008; Cetin *et al.* 2009)

Essential oils have also been used for the treatment of diverse human pathogens. Limonene, the primary component in *Abies koreana*, various citrus species, and *Fortunella japonica* was active against *Propionibacterium acnes* and *Staphylococcus epidermidis,* which are linked to the occurrence of skin infections (Kim *et al*., 2008; Yoon *et al*., 2009; Yang *et al*., 2010). Oils from *Apium nodifloru, Plinia cerrocampanensis,* and *Thymus caramanicus* inhibit growth of the stomach bacterium, *Helicobacter pylori* (Eftekhar *et al*., 2009; Menghini *et al*., 2010; Vila *et al*., 2010). Essential oils of *Achillea ligustica, Mentha longifolia* and *Hyptis pectinata* inhibit growth of two dental bacteria, *Streptococcus mutans* and *S. pyogenes* (Nascimento *et al.* 2008; Al-Bayati 2009; Maggi *et al.* 2009). High antibacterial activity of essential oil of *Cuminum cyminum* (Apiaceae) was observed against several human pathogens, including *Staphylococcus aureus, Streptococcus faecalis, Brochothrix thermosphacta, Pseudomonas fragi* and *Escherichia coli* (Derakhshan *et al*., 2008).

1.2 Bioactive monarda herbage

The genus *Monarda* is limited to the North American continent in its present natural distribution. The numerous species occupy a large geographical area, from the Canadian prairies in the north to Michoacán in the south and throughout the breadth of the North American continent. In most parts of their range, species of this genus have been utilized by man as garden plants, food and flavoring additives, and for medicinal purposes (Scora 1967).

Damping-off of seedlings caused by species of *Pythium* and *Rhizoctonia* can reduce number and quality of tomato seedlings. Plants in the genus *Monarda* produce complex essential oils that contain antifungal compounds. Selections of bioactive monarda herbage (ground dried leaves harvested from selected *Monarda* cultivars) were grouped into five chemotypes based on essential oil composition and antifungal activity; seedling survival in *Rhizoctonia solani*-infested media was increased from 10% (no herbage) up to 80% (‘Croftway Pink’) when bioactive monarda herbage was added to the medium (Gwinn *et al.* 2010). Losses due to *Pythium myriotylum* were reduced when selected bioactive monarda herbages were added to greenhouse growing medium (Clark *et al*., 2006). Cymene was the major essential oil component of ‘Trinity Purple’, geraniol was the major essential oil component of ‘Rose-scented’, carvacrol was the major essential oil component of ‘Violet Queen’, borneol was the major essential oil component of ‘Cerise’, and thymol was the major essential oil component of *M. clinopodia* (Gwinn *et al*., 2010).

1.3 *Beauveria bassiana*

The fungus, *Beauveria bassiana*, was discovered and named by the research scientist Agostino Bassi de Lodi in 1835 while he was studying the white muscadine disease in silkworms. It was formerly known as *Tritirachium shiotae*.

Beauveria bassiana is the anamorph of *Cordyceps bassiana* (Ascomycota). It lives in soils throughout the world, where it grows as multicellular mycelia by absorbing nutrients from decaying matter (Germain and Summerbell 1996). In culture, mycelia grew best on Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) (Senthamizhlselvan *et al*., 2010).

Although sexual reproduction occurs at a low frequency (Meyling *et al*., 2009), *B. bassiana* produces copious asexual non-pigmented spores (conidia) that are wind dispersed. The conidia are single-celled, haploid, hydrophobic, and measure 2 μm to 4 μm. On most common culture media, conidia appear in dry, powdery white spore balls. Conidia are generally considered relatively more resistant to unfavorable environmental conditions than other cells. When released into the environment, they remain dormant or in a non-vegetative state until appropriate environmental conditions activate germination. When favorable environmental conditions exist, the spore absorbs water and starts to swell; its size increases until it reaches a critical volume when a small protrusion (germ tube) starts to form on one side of the spore. This protrusion will develop into a branching germination tube that will then develop into the fungal hyphae. There are three different germination types for conidia of *B. bassiana* on SDA: unidirectional, bidirectional and multidirectional

(Talaei-Hassanloui *et al*., 2007). Spore germination is the most vulnerable part of the *B. bassiana* lifecycle and is therefore a good stage for monitoring the effect of essential oils on fungal growth. There is a general consensus that spore germination and mycelial growth *in vitro* are useful criteria for testing the side effects of fungicides on beneficial entomopathogenic fungi (Gatarayiha *et al*., 2010)

1.4 Spore germination of *B. bassiana*

Spore germination of *B. bassiana* occurs when a spore is exposed to a conducive environment (free water, optimal temperature range, and nutrients). Temperature is a major factor in activation of conidial germination independent of a host (Boucias *et al*., 1988). The temperature range for germ tube development of most entomogenous fungi is 20 to 30℃. At 25 to 30℃, 95 % of conidia of most *B. bassiana* strains germinated in 14 to 25 h (Hywel-Jones and Gillespie 1990; Tefera and Pringle 2003).

Availability of water (high humidity) is another major factor in the germination of conidia. Freely available water (relative humidity of 100%) is most favorable for spore germination and mycelial growth, but in laboratory tests, germination of spores of certain strains of *B. bassiana* occurred at a relative humidity as low as 56.8% (Teng 1962).

Although temperature and water availability have major impacts upon the conidial germination in *B. bassiana*, other environmental factors also influence germination and mycelial growth. Radial mycelial growth of *B. bassiana* is increased

in the light, but density of the mycelium is reduced (Teng 1962). Germination capacity of spores of *B. bassiana* was not lost after accumulative exposure to direct sunlight for about 150 h (Teng 1962). Spore germination was positively correlated with oxygen concentration (Teng 1962). Mycelial growth under adequate oxygen supply was increased, but spore formation was decreased (Teng 1962). Optimal mycelial growth occurred from pH 4.0 to 5.0. The pH range for spore germination is pH 3.0 – 9.4; the range for growth is pH 2.4 to 10.0. The optimum pH for spore germination is about 4.4. There is no specific nitrogen requirement for the germination of *B. bassiana*. Among the inorganic sources, nitrate nitrogen is more utilizable than ammonium nitrogen. Organic nitrogen increases spore production in the light but not in the dark (Teng 1962; Roberts and Campbell 1977).

1.5 Compatibility of *B. bassiana* with fungicides and herbicides

Besides environmental and nutritional factors, conidial survival and germination can also be affected by interactions with agrochemicals or biopesticides, and therefore, these agents may disrupt natural epizootics of *B. bassiana*. In *in vitro* studies, conidial germination of *B. bassiana* was inhibited by nine fungicides and 14 insecticides (Olmert and Kenneth 1974). Conidial germination is an effective criterion to evaluate compatibility of *B. bassiana* with insecticides since conidia are responsible for the occurrence of the first disease foci in the field (Neves *et al*., 2001). Many conventional pesticides are compatible with *B. bassiana.* Activity of fungicides on *B. bassiana* has been reported to vary from strong to non-inhibitory depending on

the chemistry of the fungicide (Gatarayiha *et al.* 2010).

Unlike traditional pesticides, there are few studies on the impact of pest management strategies with natural products and *B. bassiana*. The plant growth regulators Silaid, paclobutrazol, and flurprimidol significantly inhibited germination and growth of *B. bassiana in vitro* (Storey and Gardner 1986). Neem oil was compatible with *B. bassiana* (Islam *et al.* 2010). High concentrations of azoxystrobin (a natural product fungicide) inhibited spore germination and growth of *B. bassiana* but did not reduce *B. bassiana* activity against two-spotted spider mites (Gatarayiha *et al.* 2010). When *B. bassiana*-coated seed were planted into a water-soaked greenhouse growing medium used to stimulate disease losses due to *P. myriotylum*, there were no significant interactions between *B. bassiana* and monarda herbage treatments (Clark *et al.* 2006).

1.6 Biological control activity of endophytic *B. bassiana*

Beauveria bassiana has been reported as an endophytic colonist of many plant species including maize, potato, cotton, common cocklebur, jimsonweed, tomato, ironwood, western white pine, opium poppy, date palm, banana and cacao (Vega 2008). Presence of *B. bassiana* in plant tissues causes feeding deterrence or antibiosis and may produce metabolites that protect plants against some soilborne plant pathogens (Ownley *et al*., 2008, 2010). Application of conidia mixed in methylcellulose solution to tomato seed results in endophytic and epiphytic colonization of seedlings and suppressed plant losses, caused by the damping-off

pathogen, *R. solani* (Ownley *et al*., 2004). The initial population density of *Beauveria* conidia established on seed influenced the subsequent extent of endophytic colonization of tomato. Rates that were most effective in disease control also resulted in the greatest degree of plant colonization. In tomato, all plants grown from seed treated with 1×10^7 CFU conidia per seed were colonized by *B. bassiana* (Ownley *et al.* 2008). Endophytic colonization can be detected with standard plating techniques onto selective medium, PCR of colonized plant tissues, and scanning electron microscope (Ownley *et al.* 2008). Viable *B. bassiana* was detected in root, stem, and leaf sections of surface-sterilized 18-week old tomato seedlings produced from treated seeds (Ownley *et al.* 2008).

Competition and induced systemic resistance are likely mechanisms of *B. bassiana* in suppression of plant disease. Competition for space and resources is a possible mechanism for its biological control activity against *R. solani* and other plant pathogens, since the fungus is a plant colonist, and will actively compete against plant pathogens for niche/infection sites, carbon, nitrogen, and various microelements within the plant. Induced systemic resistance is another possibility. Plants colonized by *B. bassiana* reduce or alleviate disease by triggering a rapid expression of defense-related genes. Numerous secondary metabolites produced by isolates of *B. bassiana* may also have activity in suppression of plant disease (Ownley *et al.* 2004, 2010).

Objectives

The overall objective of this research was to evaluate compatibility of *Beauveria bassiana* both with monarda herbages that reduce plant disease incidence and severity, and with the essential oils that represent the major chemotypes of these monarda herbages. The first objective of this research was to determine the impact of five essential oils (thymol, carvacrol, geraniol, borneol, and cymene) on spore germination and germ tube development of *Beauveria bassiana* at various concentrations and times. The second objective was to determine the effects of *B. bassiana* (used as a seed treatment) and bioactive monarda herbage (used as a greenhouse growing medium amendment) on *B. bassiana* colonization, seed germination, and growth of tomato.

Hypotheses

The null hypothesis of the first objective was that essential oils active against damping-off pathogens have no impact on the spore germination and germ tube development of *Beauveria bassiana*. The alternative hypothesis was that essential oils active against damping-off pathogens have an impact on the spore germination and germ tube development of *Beauveria bassiana.* The null hypotheses of the second objective were that *B. bassiana* does not have the ability to colonize tomato in the presence of bioactive monarda herbage from each of the five chemotypes (thymol, carvacrol, geraniol, borneol, and cymene) and that these two biological control methods cannot be used together as a disease management

treatment. Alternative hypotheses were that the fungus *B. bassiana* has the ability to colonize tomato in the presence of bioactive monarda herbage from each of the five chemotypes (thymol, carvacrol, geraniol, borneol, and cymene) and that these two biological control methods can be used together as a disease management treatment.

PART II

IMPACT OF FIVE ESSENTIAL OILS (THYMOL, CARVACROL, GERANIOL, BORNEOL, AND CYMENE) ON SPORE GERMINATION AND GERM TUBE DEVELOPMENT OF *BEAUVERIA BASSIANA*

Abstract

Losses due to seedling damping-off caused by *Rhizoctonia* and *Pythium* can be reduced both by seed treatment with *Beauveria bassiana* isolate TN11-98 (Bb) and by use of essential oils within bioactive monarda herbages. Five concentrations of cymene, carvacrol, or thymol, ranging from 0 to 500 mM, and six concentrations of borneol or geraniol, ranging from 0 to 50 mM were tested. Ethanol was used as the control. Spore suspensions of *B. bassiana* were placed on water agar in the presence or absence of an essential oil. Spore germination and germ tube development were observed up to 24 h. Germ tube lengths (including the diameter of the spore) were determined. Each oil was tested as a separate experiment designed as a factorial experiment; with varied numbers of concentrations (5 to 6) × four time periods × 10 to 20 microscope views. Each experiment was repeated twice. In all experiments, germ tube length was greatest in control treatment at 24 h. The ρ-cymene had a stimulatory effect on germ tube development of *B. bassiana* at low concentrations but inhibited germ tube development at high concentrations. Germ tube length was reduced by approximately 50% at 24 h for spores exposed to a 50 mM cymene. No spores germinated in treatments with borneol (50 mM), carvacrol (50 mM and 500 μM) or cymene (500 mM). Thymol was the least inhibitory of all tested oils. A second study with all oils at all concentrations, but observed at only one time period (24 h), was also performed with spores collected from 6 to 8-week-old cultures. Germination percentage was decreased across concentration in all five oil treatments. Germ tube development trends were similar to the first time course study, with the average tube

length less than those of the aged conidia used in the first study. The five essential oils active against damping-off pathogens had a negative impact on spore germination and germ tube development of *Beauveria bassiana*.

Introduction

The various risk factors associated with the use of conventional chemical fungicides such as development of resistance, adverse effects on non-target organisms, accumulation of pesticide residues in the food chain, environmental pollution, health risks and high costs have driven scientists to develop alternative eco-friendly techniques for disease management. The entomogenous fungus, *Beauveria bassiana*, and monarda bioactive herbage are potential biological control agents that could be used in crop protection and in production of agricultural commodities free from pesticide residues. Disease management would be enhanced if these two biological control strategies were compatible and could be combined.

The literature provides many examples of essential oils that have effects on the development of fungi, inhibitory or stimulatory. The essential oil isolated from *Origanum acutidens* that contains the phenolic component carvacrol as the main constituent, completely inhibited mycelia growth of seventeen species of *Botrytis*, *Fusarium* and *Alternaria* (Kordali *et al.* 2008). Essential oils of *Thymus vulgaris* and *Mentha piperita*, which contains 50% thymol as the main component, reduced *in vitro* growth of the pathogenic fungi, *R. solani* and *F. solani*, and caused degeneration of

fungal hyphae (Zambonelli *et al.* 1996). The essential oil compounds extracted from the aerial parts of *Rosmarinus officinalis* (44% ρ-cymene) inhibited *in vitro* growth of *Alternaria alternata, Botrytis cinerea,* and *Fusarium oxysporum* (Ozcan and Chalchat 2008). The purpose of this study was to evaluate the impact of essential oils (ρ-cymene, borneol, geraniol, carvacrol, and thymol) on the spore germination and germ tube development of *B. bassiana.*

Materials and Methods

Time Course Germination Study

Beauveria bassiana. *Beauveria bassiana* 11-98 was isolated from a click beetle (Coleoptera: Elateridae), characterized at University of Tennessee, Knoxville (strain TN11-98) by Roberto Pereira and maintained by Bonnie H. Ownley. For production of sporulating cultures, spores of *B. bassiana* were placed on Difco Sabouraud Dextrose Agar, spread evenly with an autoclaved spreading rod, and grown for 60 days at room temperature.

Spore Suspension Preparation. Spores of *B. bassiana* from cultures up to 6-months-old were transferred to a 15mL test tube with water (ca. 3mL) and vortexed. Tween-20 was purchased from Fisher Scientific (Fair Lawn, NJ). Since spores tend to cling together in water, two drops of diluted Tween-20 (0.006 %) were added to the suspension. The suspension was sonicated for 10 min.

Essential oils Preparation. Five essential oils that define the monarda herbage chemotypes, including cymene, carvacrol, borneol, geraniol and thymol, were

purchased from Sigma-Aldrich Co. (St. Louis. Mo). The 95% ethanol was purchased from Fisher Scientific (Fair Lawn, NJ). Five essential oils were each combined with ethanol to produce different concentrations. A 500 mM solution of each essential oil was prepared (Table 2.1), and the final amount of essential oil in each dish for the 500 mM treatment was 5.0 μmoles/dish. A dilution series of the 500 mM solution was used for additional treatments. Five concentrations (0, 0.5, 5.0, 50, and 500 mM) of cymene, carvacrol, and thymol, and six concentrations of borneol and geraniol (0, 0.005, 0.05, 0.5, 5.0, and 50 mM) were tested. Tubes were shaken until the mixtures were visually homogeneous. Pipette tips were changed between dilutions.

Table 2.1. Preparation of the 500 mM essential oil treatments.

Essential Oil	Molecular Weight**	Specific Density**	Oil (μL)	Ethanol (μL)	Solid (g)	Total solution (μL)
Borneol	154.24	1.011	10	121	NA	131
Carvacrol	150.21	0.976	10	120	NA	130
ρ-cymene	134.21	0.860	10	118	NA	128
Geraniol	154.24	0.889	10	105	NA	115
Thymol*** (stock)	150.21	-	NA	1000	751	1000
Control (ethanol)	-	-	NA	100	NA	100

* In experiments, antibiotic discs (1/plate) were treated with 10μL essential oils, which resulted in a final treatment concentration of 5.0 μmoles per dish. Other treatments were prepared from a dilution series of the original solution. The amounts showed here resulted in 500 mM concentrations.

**Data from Sigma-Aldrich.

***Solution shown is a 5.0 M stock solution. Treatments were performed with a 1:10 dilution of this stock.

Experimental Design. Agar blocks (1.5% water-agar) were used to provide a water source that is essential for fungal spore germination and growth. Molten agar was

poured into a plastic form (12.5 cm × 8.4 cm), and after solidification, the agar was cut into blocks; blocks were placed on a clean microscope slide in a 100 × 15mm Petri dish. A suspension of *B. bassiana* spores (10 µL) was placed on the agar blocks. A 10µL drop of essential oil solution (0 – 500 mM) (Table 2.1) was added to an antibiotic testing disc located beside the glass slide in the dish. Petri dishes were closed and incubated on wet sponges at 25℃ to maintain high relative humidity. Separate dishes were used for each concentration at each time period since sampling destroyed the micro-atmosphere inside the Petri dish.

Based on previous experiments, germ tube initiation was expected to first be detectable at 12 h or 16 h, and the length of germ tube in controls was expected to exceed the microscope field after 24 h. Under 1000× magnification of a brightfield microscope (Nikon Alphaphot YS, Nikon Instruments, Melville, NY), the spore germination and germ tube length were observed at 12, 16, 20, and 24 h (cymene, carvacrol, thymol) or 16, 20, 22, and 24 h, (borneol or geraniol). Microscope views were photographed with a Nikon Coolpix P5100 (Nikon Instruments, Melville, NY). Photographs of a stage micrometer were taken prior to viewing of slides of each concentration and at each period of time to calibrate spore dimensions. At least ten microscope fields containing 10 or more spores were photographed for each field.

Data Collection and Analysis. Photographs were printed, and spore diameter and germ tube length were determined by use of a Scale Master Pro XE (Calculated Industries, Carson City, NV). Measurements included both the spore and the germination tube. Actual size was determined by calibration with the micrometer.

Only germinated spores were measured in each photograph (Fig. 2.1). While in some high concentration treatments, where no germination was observed, spore diameters were measured to ensure positive measurements. There were one to ten observations (spore length) per treatment due to the low germination rate of spores in some treatments. Each experiment was performed three times. All data were recorded in Microsoft Excel and analyzed with PROC MIXED (SAS Institute, Inc., Cary, NC). Log_{10} of essential oil concentrations were used in analyses.

Figure 2.1. Spores of *Beauveria bassiana* 20 h after treatment (control)
All germinated spores in a microscope view were measured from photographs. Dimensions were calibrated based on measurements from a stage micrometer.

Twenty-Four Hour Germination Study

Experimental Design. In order to determine the impact of five essential oils (thymol,carvacrol, geraniol, borneol, and cymene) on percent germination of fresh *Beauveria bassiana* spores, a second study was performed in the same manner as the first, except that only one time period was tested, and spores from 6 to 8 week old cultures were used. After incubation for 24 h, ten microscope fields were photographed for each field, twenty spores were selected randomly for each photograph (Fig. 2.2), and the spore germination observed and germ tube lengths were measured, recorded and analyzed for each concentration, as described above. The experiment was a 5 × 5 factorial design with five concentrations (control, 0.5 mM, 5 mM, 50 mM, 500 mM) of five essential oils (cymene, carvacrol, borneol, thymol, and geraniol) at one time period (24 h). The experiment was performed three times.

Figure 2.2. Spores of *Beauveria bassiana* 24 h after treatment (control)
In each microscope view, twenty spores were selected at random and measured from photographs. Spore dimensions were calibrated based on measurements from a stage micrometer.

Data Collection and Analysis. All data were recorded in Microsoft Excel and analyzed using PROC MIXED (SAS Institute, Inc., Cary, NC). Simple linear regression was used to model the relationship between the length of germinated tubes (including the diameter of spores) of *Beauveria bassiana* (dependent variable y) and concentrations of essential oils (explanatory variable x) at each time period. Values for concentrations were logarithm transformed. Unknown model parameters, including intercepts (a) and slopes (b) were estimated from the data with SAS (Appendix 1).

Results

Time of exposure and concentration of the five essential oils had significant effects on germ tube development of *Beauveria bassiana* ($P < 0.0001$). For all oils, germ tube length increased with time and decreased with concentration (Appendix 1-Tables A.1 – A.15). Time course studies were performed over a 6-month time period and during this time, spores of *B. bassiana* aged, and percentage of germination decreased (data not shown).

Time Course Germination Study.

Borneol. Treatment with borneol reduced spore germ tube elongation (Fig. 2.3); no germination was observed in the 50 mM treatment. Length of germ tubes in controls was greater than treatments for all borneol treatments. At 16 h, mean germ tube length was not different among treatments with low concentrations (0.005 mM to 5 mM), but by 24 h, all treatments were significantly different from each other.

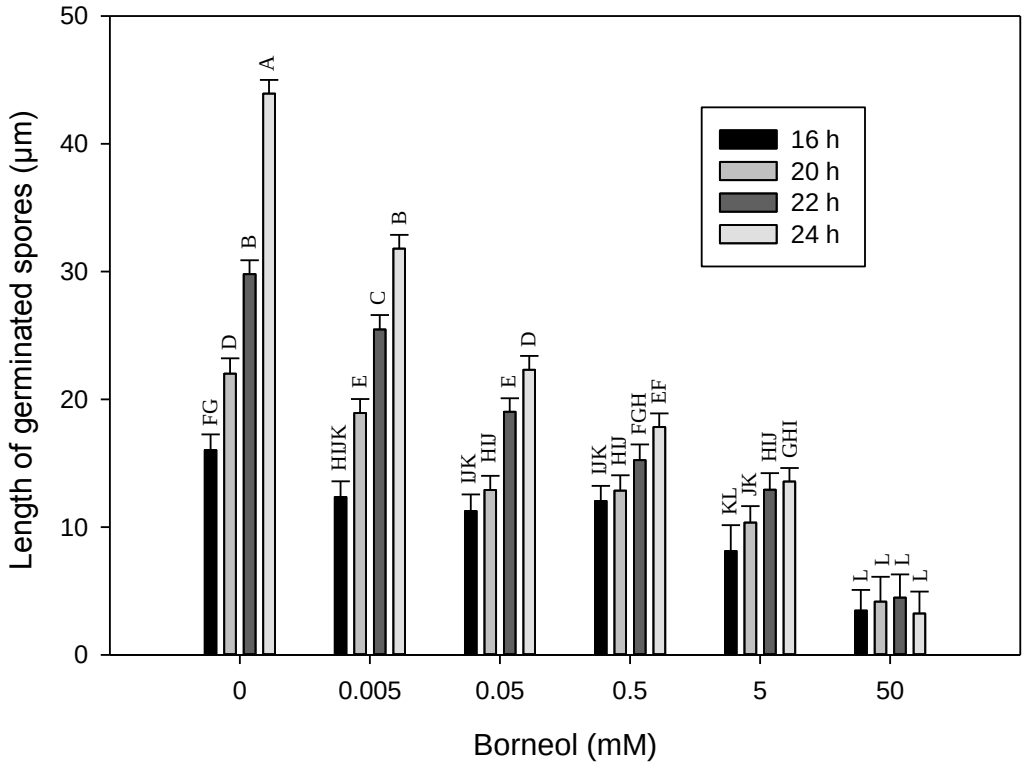

Figure 2.3. Effects of borneol on germ tube elongation of *Beauveria bassiana*
Conidia were exposed to a micro-atmosphere of varying concentrations of borneol, and germ tube lengths were measured at 4 h intervals. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Geraniol. There were no significant differences in mean germ tube length among five different concentrations and the control at 16 h, and few differences observed at 20 h (Fig. 2.4). At 22 h and 24 h, germ tubes were longest in the control and shortest in the 50 mM treatments. Geraniol did not inhibit germ tube development at 16 h and 20 h significantly, but had an inhibitory effect at 22 h and 24 h.

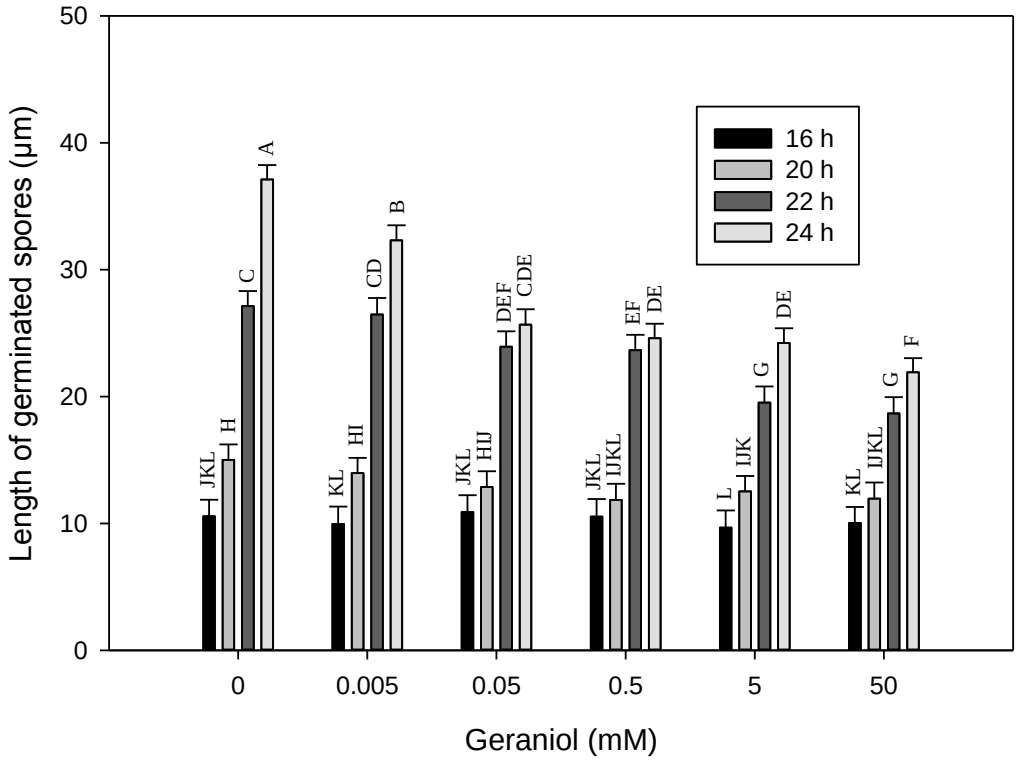

Figure 2.4. Effects of geraniol on germ tube elongation of *B. bassiana*
Conidia were exposed to a micro-atmosphere of varying concentrations of borneol, and germ tube lengths were measured at 4 h intervals. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Carvacrol. Germ tube length was less than the control in the 0.5 mM treatment at 24 h (Fig. 2.5). In the 5 mM treatment, germ tube length was reduced at 16, 20 and 24 h. There was no germination at either 50 mM or 500 mM (measurements include spore diameters, and are always positive even without germination).

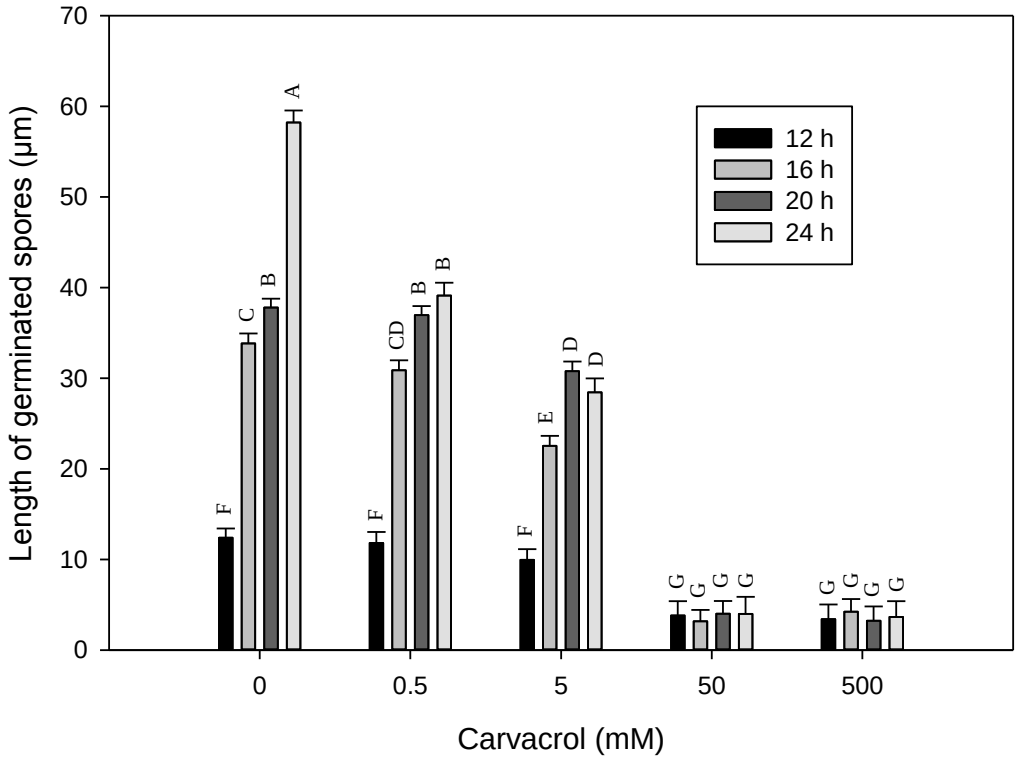

Figure 2.5. Effects of carvacrol on germ tube elongation of *B. bassiana*
Conidia were exposed to a micro-atmosphere of varying concentrations of borneol, and germ tube lengths were measured at 4 h intervals. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Thymol. Exposure to thymol slowed germ tube development but did not kill spores at any of the six concentrations from 0 to 500 mM (Fig.2.6). Germ tube length in the highest concentration (500 mM) was not different from the length in the 10-fold dilution (50 mM) at any sampling time.

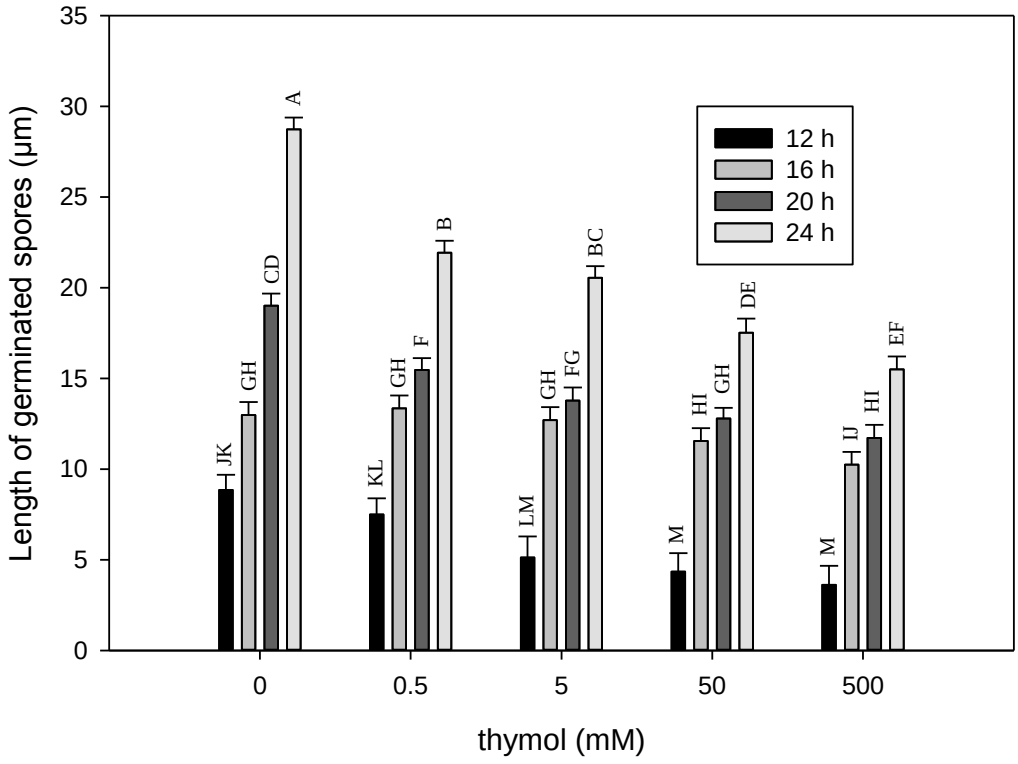

Figure 2.6. Effects of thymol on germ tube elongation of *B. bassiana*

Conidia were exposed to a micro-atmosphere of varying concentrations of borneol, and germ tube lengths were measured at 4 h intervals. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Cymene. Cymene had a stimulatory effect on germ tube development of *B. bassiana* at the lowest concentration tested (0.5mM) but inhibited germ tube development at high concentrations (Fig. 2.7). At 16 and 24 h, germ tube length was greater in the 0.5 mM treatment than in the control. Length of germ tubes at all other concentrations was lower than the control. In the 50 mM treatment, germ tube lengths were reduced, and almost no germination was observed at 500 mM.

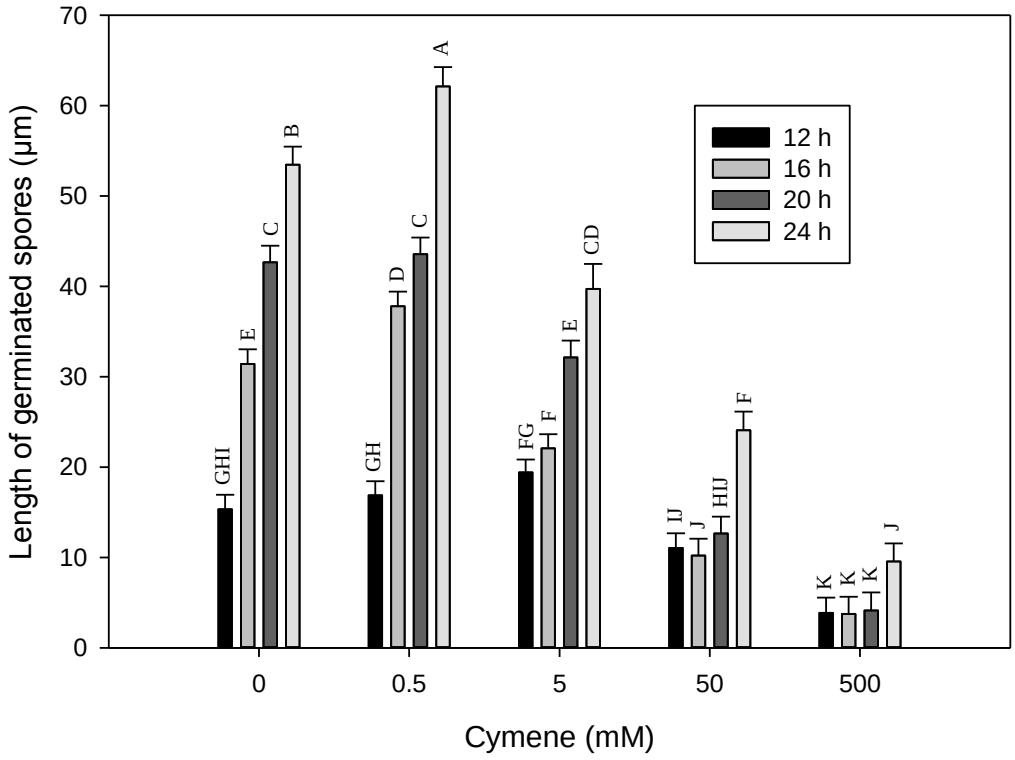

Figure 2.7. Effects of cymene on germ tube elongation of *B. bassiana*
Conidia were exposed to a micro-atmosphere of varying concentrations of borneol, and germ tube lengths were measured at 4 h intervals. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Comparison of oils. At 24 h, spore germ tube elongation was inhibited by carvacrol and borneol at all sample times (fungicidal), but the highest concentration of thymol, geraniol, and cymene were fungistatic (Fig. 2.8). Regression models that best fit each oil at the 24 h sample time were used to calculate half maximal effective concentration (EC-50) values (Table 2.2). The y-intercepts were mean length of spore germ tubes in control, and line slopes were negative (i.e., length would decrease with increases in concentration).

Table 2.2. Calculated EC_{50} values at the 24 hour observation time.

Essential Oils	EC-50
Borneol	0.64 mM
Carvacrol	2.88 mM
Cymene	374.30 mM
Geraniol	>500 mM
Thymol	>500 mM

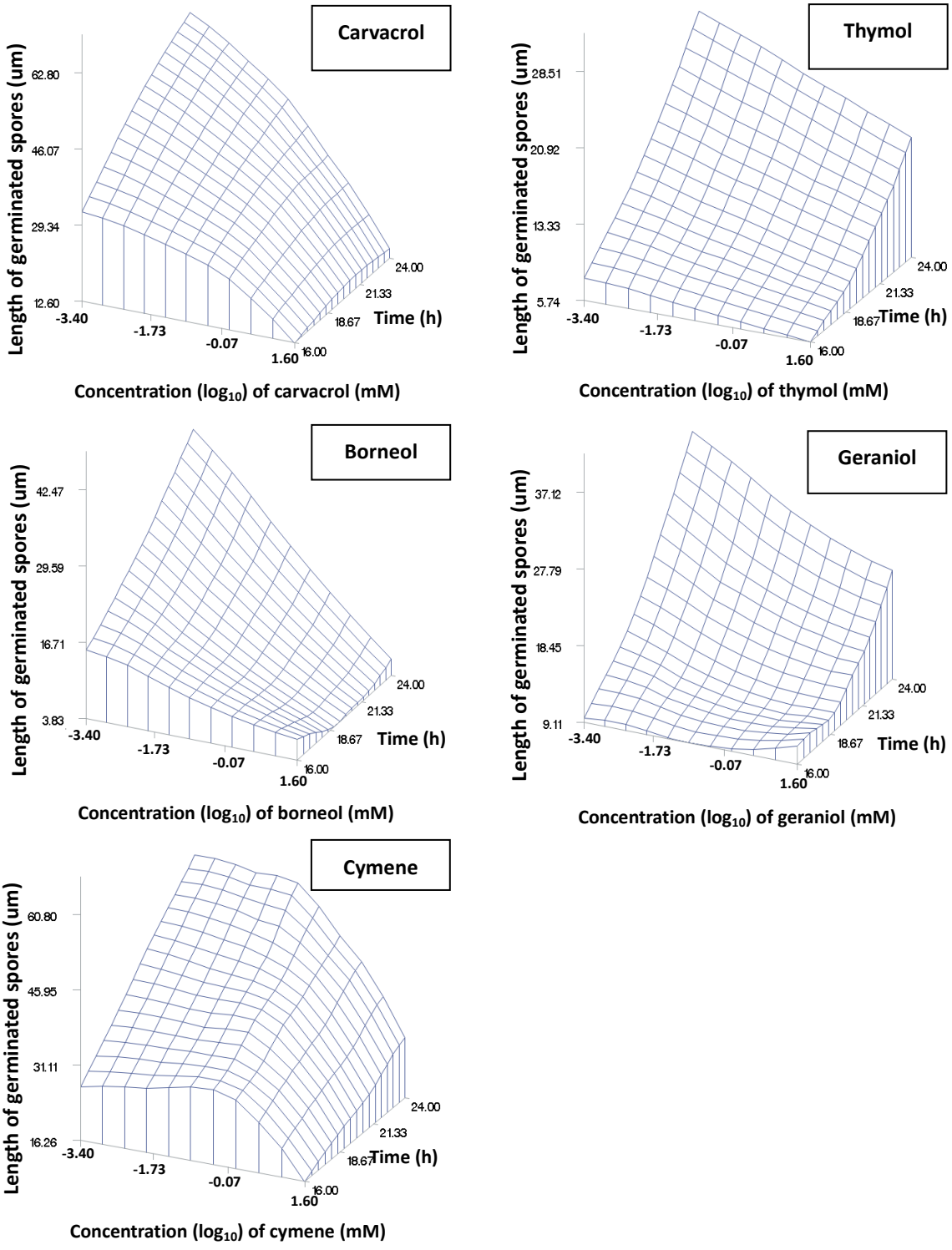

Figure 2.8. Response surface plot of effect of time and concentration of five essential oils (monoterpenes) found in monarda herbage on germ tube lengths of *B. bassiana*; oils are arranged by oxidation state - carvacrol and thymol (phenolic alcohols); borneol (secondary alcohol); geraniol (allylic primary alcohol); and cymene (hydrocarbon). Because there were no differences between the 50 and 500 mM concentration in the carvacrol and thymol treatments, and few in the cymene, the values for 500 mM are not shown.

Twenty-Four Hour Germination Study

Percentage germination and germ tube length of fresh spores *Beauveria bassiana* spores did not differ among five oils at 24 h (P=0.6375).

Percentage Spore Germination. Percent germination of fresh untreated control spores ranged from 35-45% (Table 2.3). In general, percent germination of spores decreased across concentration. There was no germination in treatments with 50 mM geraniol or 500 mM cymene, geraniol, or carvacrol. The high concentration of cymene, geraniol and carvacrol inhibited spore germination.

Table 2.3. Percent germination of *Beauveria bassiana* treated with six different concentrations of five essential oils (Chi-Square, p<0.0001).

Concentration (mM)	Borneol	Cymene	Thymol	Geraniol	Carvacrol
	Percent germination				
0	44.0	39.1	35.8	35.2	35.3
0.5	32.5	28.8	33.2	36.1	35.3
5	35.0	25.5	35.7	27.8	25.6
50	27.5	25.5	32.2	0	30.5
500	28.8	0	24.5	0	0
	Percentage of control				
0	100	100	100	100	100
0.5	74	74	93	100	100
5	80	65	100	79	73
50	63	65	90	0	86
500	65	0	68	0	0

Percent weight of germinated spores. An estimated weight for accumulated lengths of germinated spores in each treatment was examined using SAS. The percent weight of the lengths for germinated spores decreased across concentration (Table 2.4).

Table 2.4. Weighted* germination of *B. bassiana* spores in six different concentrations of five essential oils (Method=Chi-Square, p<0.0001).

Concentration (mM)	Borneol	Cymene	Thymol	Geraniol	Carvacrol
	Percent germination				
0	84.6	82.5	80.1	79.5	79.4
0.5	75.3	75.9	77.7	78.3	78.1
5	73.7	68.3	75.3	69.4	69.5
50	65.0	57.9	67.9	0	63.2
500	63.9	0	57.2	0	0
	Percentage of control				
0	100	100	100	100	100
0.5	89	92	97	98	98
5	87	83	94	87	88
50	77	70	85	0	80
500	75	0	71	0	0

*Each numeric value provides an estimated weight for each observation in the data. PROC FREQ statement in SAS assumes that an observation represents n observations, where n is the value of variable. The value is not required to be an integer.

Germ tube length. Germ tube length of the spores in the five oil treatments was similar; the lengths were decreased across all concentrations (Fig. 2.9). In the control, the greatest length was ca. 11μm. In the 0.5 mM treatments, germ tube length was not significantly decreased by borneol, geraniol, carvacrol or cymene; whereas, in the 5 mM treatment, only spores in the geraniol treatment did not have decreased germ tube length compared to other treatments. Almost no germ tube emergence was observed in treatments with 500 mM geraniol, 50 mM and 500 mM thymol, and 500 mM carvacrol.

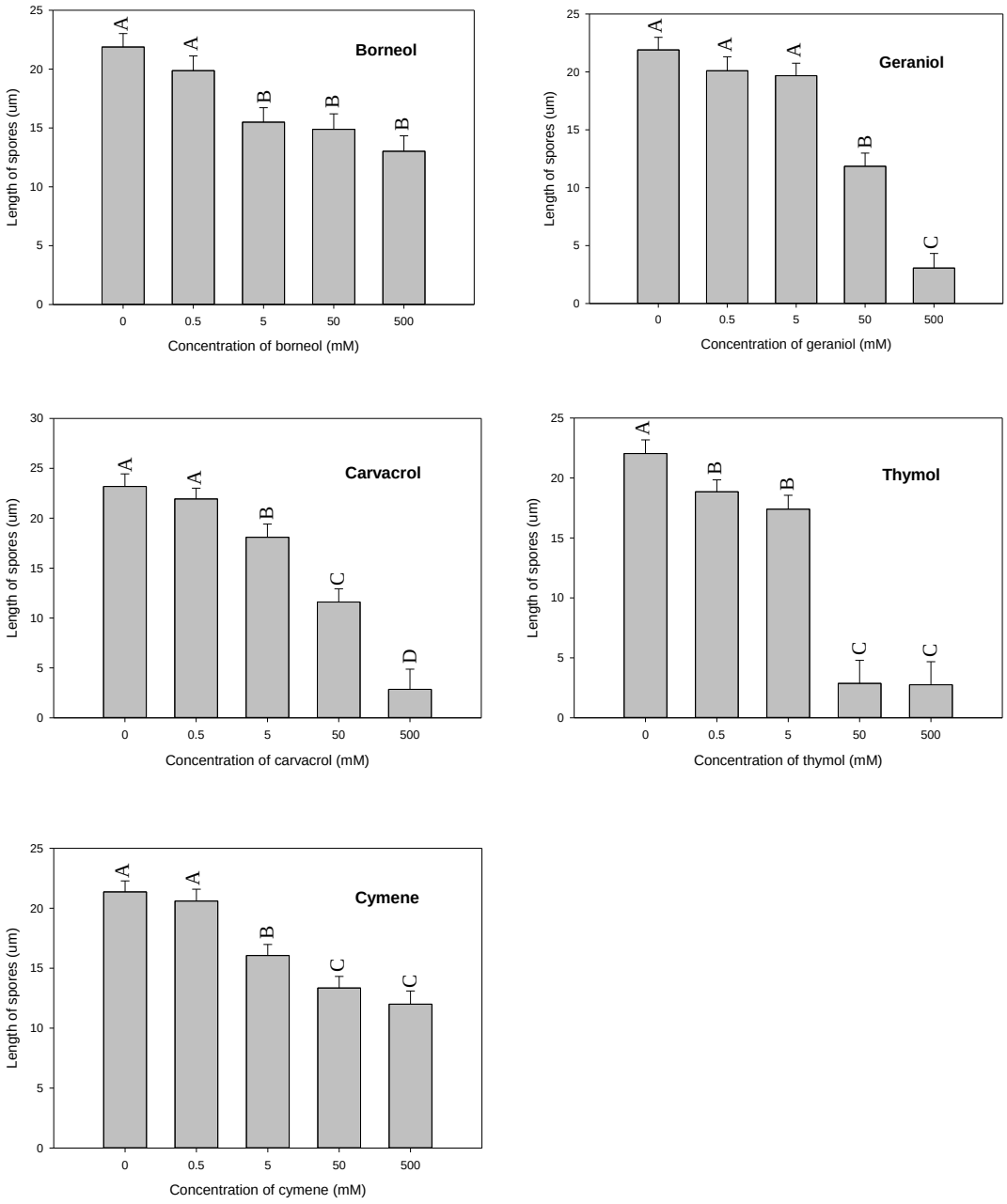

Figure 2.9. Effect of five concentrations of five essential oils on germ tube length of *B. bassiana* at 24 h after exposure. Conidia were exposed to a micro-atmosphere of varying concentrations of five essential oils including borneol, geraniol, carvacrol, thymol and cymene, and germ tube lengths were measured after 24 h exposure. Bars with the same letter are not significantly different according to an F-protected least significant difference at $\alpha = 0.05$.

Discussion

Sensitivity of the conidia of the biocontrol fungus *Beauveria bassiana* to several chemical pesticides has been reported (Olmert and Kenneth 1974; Neves *et al.* 2001; Gatarayiha *et al.* 2010). Essential oils extracted from natural product commonly used in disease management have various effects on spore germination and replication of *B. bassiana* (Storey and Gardner 1986; Islam *et al.* 2010; Gatarayiha *et al.* 2010). Compatibility of biocontrol pathogens with natural products additives is necessary for optimal use of the agent in disease management programs.

Spore germination and mycelial growth were used as criteria for testing the effects of essential oils on *B. bassiana* (Gatarayiha *et al*., 2010). Spores were considered germinated when the germ tube length reached at least double the size of the spore (5µm). All essential oils tested in this research except for cymene had an inhibitory effect on germ tube development of *B. bassiana* across time. High concentrations of carvacrol and cymene were considered harmful for the spores of *B. bassiana*. Although some of the spores observed had bidirectional germination at 24 h, only one tube was measured for those spores. Germination was higher in control treatment than in essential oil treatments, but germination was still very low even under control conditions. These spores may have aged past ideal germination percentages. In the first experiment, each individual spore was an observation, so only germ tube length was tested. To test the effect of different concentration of five essential oils on the percent germination of spores, another experiment was done. Fresher spores were used to achieve higher percentage germination. The longest

testing time, 24 h, was determined to be the best time to examine the effect of oils on fungal growth. Both the percent spore germination and the percent weight germination for all oils decreased across concentration. The germ tube development trends of fresh spores were similar to the aged ones used in the time course study. Of the five oils tested, only geraniol and thymol did not completely inhibit conidial germination at both low and high concentrations; cymene oil even stimulated germination at low concentration.

Application of essential oils possessing fungicidal properties should be carefully monitored to avoid disruption of the field efficacy of *B. bassiana* conidia. Low concentrations of the essential oils have the potential to be used together with *B. bassiana* as biological control agents. Further studies are necessary to evaluate this combination on plants in the greenhouse and field studies.

PART III

EFFECTS OF *BEAUVERIA BASSIANA* (USED AS A SEED TREATMENT) AND BIOACTIVE MONARDA HERBAGES (USED AS A GREENHOUSE GROWING MEDIA AMENDMENT) ON *B. BASSIANA* COLONIZATION, SEED GERMINATION, AND GROWTH OF TOMATO

Abstract

Plant-based natural products and fungi such as *Beauveria bassiana* are more sustainable than insecticides due to their lower risk of resistance, reduced environmental impact, and longer control time. The objective of this research was to determine the compatibility of essential oils and *B. bassiana* and the impact of the two when *B. bassiana* is applied as a seed treatment in greenhouse planting mix containing monarda herbage. Bioactive monarda herbage (ground dried leaves harvested from selected cultivars of *Monarda* spp.) representing five chemotypes (thymol, carvacrol, geraniol, borneol, and cymene), were used in this study. Tomato seeds coated with methylcellulose or with *B. bassiana* spores embedded in methylcellulose were planted into greenhouse growing medium that contained either no herbage or 10% bioactive herbage. Germination was delayed in all monarda herbages. At four weeks, seedlings were harvested, surface-sterilized, and plated on selective medium. Since seed planted in medium amended with *Monarda clinopodia* (thymol chemotype) had very low germination (5.56%), plants from this treatment were not tested on the selective medium. Levels of colonization by *B. bassiana* of seedlings grown in media amended with 'Trinity Purple' (cymene chemotype), 'Rose-scented' (geraniol chemotype), and 'Violet Queen' (carvacrol chemotype) were not different (ca. 40%) from the no-herbage control; colonization levels of seedlings grown in medium amended with 'Cerise' (borneol chemotype) was less than all other treatments (22.22%).

Introduction

Plant diseases need to be controlled in order to maintain the quality of crops produced by growers around the world. Growers often rely heavily on chemical pesticides and fungicides; however, problems with agrochemicals (e.g., environmental pollution) have led researchers to focus efforts on developing alternatives for controlling plant diseases. Both plant-based natural products including *Monarda* spp. and microbial organisms such as *B. bassiana* can play a significant role in sustainable crop production due to their lower risk of resistance.

Monarda spp. have been utilized by man as garden plants, food and flavoring additives, and for medicinal purposes (Scora 1967). Damping-off of seedlings caused by species of *Pythium* and *Rhizoctonia* can reduce the number and quality of tomato seedlings. Plants in the genus *Monarda* produce complex essential oils that contain antifungal compounds. Significant inhibition of seedling disease caused by *Rhizoctonia solani* was observed when adding herbage (dried and ground leaves and flowers) of *Monarda* sp. to greenhouse growing medium in greenhouse and laboratory studies (Gwinn *et al.* 2010). This research was undertaken to determine the ability of *B. bassiana* to colonize tomato in the presence of bioactive monarda herbages from each of the five chemotypes: cymene, borneol, geraniol, carvacrol, and thymol.

Materials and Methods

GC-MS Analysis of Herbage

Thirteen varieties of *Monarda sp.* (‘Puerto Purification’, *Monarda clinopodia*, ‘Mohawk’, ‘Mahogany’, ‘Croftway Pink’, ‘Lavender’, ‘Violet Queen’, ‘Rose Geranium’, ‘Rose-scented’, ‘Prairie Night’, ‘Mixed Purple’, ‘Cerise’, and ‘Trinity Purple’) were grown in the UT Gardens, Knoxville, TN, harvested, dried and grounded to pass a 5-mm mesh sieve, and then stored in sealed Mason jars (Ball Corporation, Broomfield, CO). Prior to experiments, 5 mg of each herbage variety was shaken in 5 ml hexane for 24 h. The liquid was filtered through a 0.45-µm nylon membrane, 4-mm syringe filter (Fisher Scientific, Fair Lawn, NJ) into a glass vial for analysis. Concentrations of essential oil components were determined by gas chromatography-mass spectrometry (GC-MS) analysis. One microliter of hexane eluent was introduced with an automatic sample injector (Model 7683, Agilent Technologies, Palo Alto, CA) into an Agilent 6850 series GC system with quadrupole MS Detector (Model 5973) coupled through a HP-5MS column (J & W Scientific, Agilent Technologies Palo Alto, CA) 30-m long, 0.25-mm internal diameter, and 0.25-µm film thickness. The starting temperature of 60°C was held for 1 min and then increased by 4°C every min until reaching 90°C. After 3 min at 90°C, the temperature was increased by 2°C per min up to 121°C. The temperature was held for 2 min at 121°C, followed by a third increase of 6°C per min until it reached 182°C. The final temperature was held for 1 min to complete the program (Gwinn *et al*., 2010).

Growth Chamber Study

Seed Treatments. Spores from 60-day-old cultures of *Beauveria bassiana* isolate 11-98 (Bb 11-98) grown on Sabouraud Dextrose Agar (SDA) were used in these experiments.

Seeds of the tomato hybrid 'Mountain Spring' were purchased from Park Seed Wholesale, Inc. Greenwood, SC. The germination level determined by the producer was 88%. Methylcelluose (Sigma-Aldrich Co., St. Louis, MO) suspensions [2 % (wt/v)] were stirred at room temperature until viscous, then transferred to an ice bath and stirred until clear. Half of the methylcellulose solution was mixed with *Beauveria* conidia and 250 tomato seeds in a Petri dish and stirred until seeds were uniformly coated. Seed were then allowed to air dry for approximately 8 h, and then separated carefully. The other aliquot of the methylcellulose solution was mixed with 250 seeds and treated as the *B. bassiana* treatment above. All seeds were stored at 4°C until use. Prior to use, Mary Dee (University of Tennessee) determined the numbers of viable conidia per seed from seed washings. Conidia from the seed were estimated to be at a concentration of 10^7 CFU/seed based on serial dilutions on SDA.

Soil Amendment Preparation. Greenhouse growing medium was moistened with deionized (DI) water and autoclaved for 90 min each on two consecutive days. *Monarda* plants with high content of each essential oil (thymol, carvacrol, geraniol, borneol, and cymene) were selected to represent each herbage chemotype. Each monarda herbage was mixed into autoclaved greenhouse growing medium [10% (v/v)]. Controls were greenhouse growing medium alone. Cone-tainers

(47-mm-diameter) were filled with 100 ml of growing medium or growing medium amended with herbage, and placed in Cone-tainer trays.

Experimental Design. The experiment was a 6 x 2 (5 Monarda/none × Beauveria/no Beauveria) factorial with a randomized complete block (RCB) design. One block consisted of 12 randomized treatments (Table 3.1). The experiment was repeated twice (n=3).

Table 3.1. Design for experiment testing the effects of *Beauveria bassiana* (used as a seed treatment) and bioactive Monarda herbages (used as a greenhouse growing medium amendment) on *B. bassiana* colonization, seed germination, and growth of tomato.

Treatment number	*Monarda sp.* variety	Herbage code	Essential oil represented	Seed treatment
1	*Monarda clinopodia*	Mon 54	Thymol	*B. bassiana*
2	Violet Queen	Mon 44	Carvacrol	*B. bassiana*
3	Rose-scented	Mon 50	Geraniol	*B. bassiana*
4	Cerise	Mon 10	Borneol	*B. bassiana*
5	Trinity Purple	Mon 52	Cymene	*B. bassiana*
6	No herbage	None	None	*B. bassiana*
7	*Monarda clinopodia*	Mon 54	Thymol	No *Beauveria*
8	Violet Queen	Mon 44	Carvacrol	No *Beauveria*
9	Rose-scented	Mon 50	Geraniol	No *Beauveria*
10	Cerise	Mon 10	Borneol	No *Beauveria*
11	Trinity Purple	Mon 52	Cymene	No *Beauveria*
12	No herbage	None	None	No *Beauveria*

In order to ensure that six seedlings would be available for colonization analyses, eight seeds (1/Cone-tainer) were planted for each treatment. In each Cone-tainer, one tomato seed was planted into growing medium or growing medium amended with herbage at the same depth; DI H_2O (75 ml) was added. A plastic cover was placed on top of each Cone-tainer. Treatments were color coded. All trays were placed in growth

chamber at 24°C with 12h light/12h dark. The plastic cover was removed when seedlings emerged, then plants were watered every day and fertilized with All Purpose Plant Food (Scott Miracle-Gro Products, Inc., Marysville, OH) every two weeks. Some of the seedlings died in a fertilizer overdose accident, but at least six seedlings for each treatment survived. The experiment was performed three times.

To identify *Beauveria* from the seedlings, surface-sterilized seedlings were plated on *Beauveria* selective medium. To a liter of DI water, 32 g glucose, 8 g neopeptone (Difco), 12 g agar, and 800 ml water were added, and after stirring, 0.008 g crystal violet was added. The solution was sterilized in an autoclave for 40 min, then after the solution was cooled to around 50℃, 0.2 g cycloheximide and 0.4 g chloramphenicol were added. The medium was poured into large (150 mm×15 mm) polystyrene Petri dishes (Fisher Scientific Co LLC, Suwanee, GA) and stored at room temperature in the dark.

Data Collection and Analysis. Seedling emergence was recorded every day once the first seedlings emerged. At the end of six weeks, seedlings were harvested; potting mix was washed from roots with DI water. Seedlings were surface-sterilized in 95% ethanol for 1 min, 20% bleach (Clorox, Clorox Company, Oakland, CA) for 3 min, and 95% ethanol for 1 min. Surface-sterilized seedlings were placed on selective medium (Doberski and Tribe 1980); plates were incubated at room temperature in the dark for 2 to 4 weeks. Growth of *B. bassiana* from the plant tissue on the plate was considered positive for colonization. All data were analyzed using PROC MIXED (SAS Institute, Inc., Cary, NC).

Results

GC – MS Analysis of Herbage

Five Monarda herbages were selected from thirteen herbages based on the concentration of essential oils (Tables 3.2 and 3.3) and chemotype (Gwinn *et al*., 2010). *Monarda clinopodia* was selected because it had a higher thymol concentration than other tested thymol chemotypes (Table 3.2). Carvacrol concentration in the carvacrol chemotypes (‘Violet Queen’ and ‘Lavender’) differed slightly; ‘Violet Queen’ was selected. Geraniol concentration in the geraniol chemotype, ‘Rose-scented’ was 17-fold greater than the other variety in this chemotype (‘Rose Geranium’). Concentration of borneol in ‘Cerise’ was 33 and 8 times greater, respectively, than the concentration found in the two other borneol chemotypes (‘Prairie Night’ and ‘Mixed Purple’, respectively). The lone member of the Trinity Purple chemotype had a high concentration of cymene. Complete essential oil profile for the selected monarda herbages is shown in Table 3.3.

Table 3.2. Monarda species selection. Concentration of each essential oil representative of a chemotypes in 13 herbages.

Essential oil represented	Monarda species	Concentration (μM)
Thymol	Puerto Purification	2,646.4
	Monarda clinopodia	19,078.0
	Mohawk	4,916.3
	Mahogany	1,866.1
	Croftway Pink	4,916.8
Carvacrol	Lavender	193.6
	Violet Queen	226.8
Geraniol	Rose Geranium	1.6
	Rose-scented	27.8
Borneol	Prairie Night	33.1
	Mixed Purple	130.5
	Cerise	1,107.0
Cymene	Trinity Purple	10.1

Table 3.3. Concentrations of essential oil constituents in five Monarda species and varieties used in this study (µM).

Essential oils	*Monarda clinopodia*	Violet Queen	Rose-scented	Cerise	Trinity Purple
Borneol	24.23	7.41	0.3	1,107.02	0.09
Bornyl acetate	0.37	2.86	0.36	0.19	0.36
Camphene	0.26	1.71	0.21	4.98	0.19
2-Carene	36.12	0.43	0.27	0.11	0.58
3-Carene	1.27	0.45	6.69	0.77	0.68
Carvacrol	132.29	226.8	0.04	0.08	0.08
Carvone	1.04	1.13	7.02	1.89	0.49
α-Caryophyllene	0.89	1.1	0.4	0.63	0.63
β-Caryophyllene	0.77	0.17	0.18	0.39	0.5
Cineole	0.4	0.04	0.15	0.25	0.16
Cymene	39.24	26.57	0.34	4.49	10.14
Fenchone	0.87	4.13	3.13	0.55	0.35
Geraniol	0.09	0.21	27.82	0.15	0.09
Isoborneol	0.04	0.03	0.04	292.86	0.03
Limonene	3	2.02	1.58	3.07	1.05
Linalool	4.08	0.11	44.63	0.23	0.07
β-Myrcene	49.55	2.29	0.45	0.25	0.99
3-Octanol	0.2	0.51	0.35	0.93	0.51
1-Octen-3-ol	1,021.27	115.6	209.07	36.77	0.92
α-Phellandrene	57.16	5.64	6.23	10.14	3.95
α-Pinene	0.25	0.34	0.28	0.25	0.08
β-Pinene	19.53	0.7	1.96	3.17	0.62
ϒ-Terpinene	0.17	0.31	0.53	0.14	0.1
α-Terpineol	0.16	0.01	0.36	0.66	0.02
Thujone	0.37	0.27	0.54	0.5	0.44
Thymol	19,077.97	68.86	0.08	1.19	0.51
Thymoquinone	0.86	1.19	0.07	0.91	0.47

Growth Chamber Study

Tomato seeds treated with *M. clinopodia* (thymol chemotype) had very low germination (8.33%) so the thymol treatment was removed from the study. Percentage germination was greater than 90% for all other treatments (Table 3.4).

Table 3.4. Final percent germination for tomato seeds in the presence of different Monarda herbages that represent different essential oils (Method=LSD, P<0.05).

Monarda Herbages	Essential Oils	Percent Germination (%)
Violet Queen	Carvacrol	93.75
Cerise	Borneol	97.92
Rose-scented	Geraniol	91.67
Trinity Purple	Cymene	100.00
Monarda clinopodia	Thymol	8.33
No herbage	None	95.83

Seed Germination Time. Germination was delayed in all monarda herbages (Table 3.5, Fig. 3.1). Seeds that were planted in the **'**Violet Queen' (carvacrol chemotype) treatment had a seedling emergence delay of four days. Days to emergence for seeds planted in medium amended with 'Cerise' (borneol chemotype) and Rose-scented (geraniol chemotype) were not different, but less than 'Violet Queen'. Seeds planted in Trinity Purple (cymene chemotype) required the shortest time to germinate. Germination was accelerated by *B. bassiana* except with in the carvacrol treatment (Table 3.6, Fig. 3.1). Seeds that were coated with *B. bassiana* required one sixth less time to germinate than those that were not.

Table 3.5. Days to germination for tomato seeds planted in greenhouse growing medium containing Monarda herbages that represent different essential oils chemotypes (Method=LSD, P<0.05).

Monarda Herbages	Chemotype	Days to Germination	Standard Error	Letter Group
Violet Queen	Carvacrol	5.59	0.17	A
Cerise	Borneol	4.25	0.16	B
Rose-scented	Geraniol	4.11	0.16	B
Trinity Purple	Cymene	2.81	0.16	C
No herbage	None	1.03	0.16	D

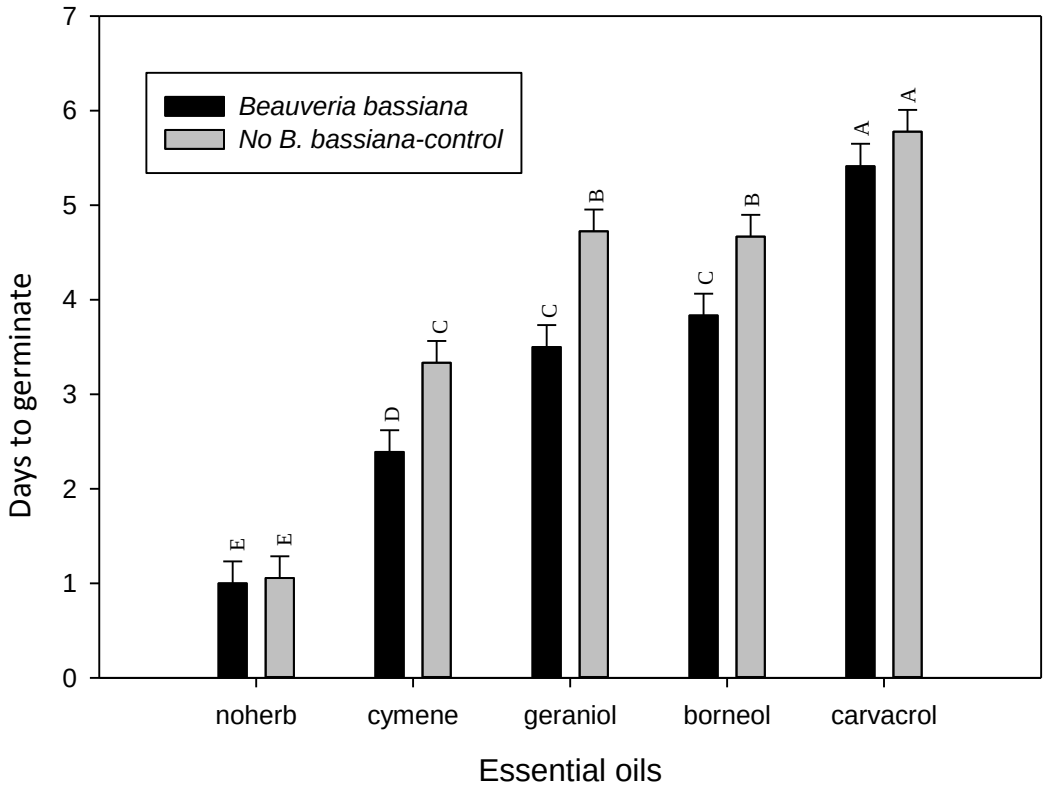

Figure 3.1. Effects of essential oils on tomato seeds germination. Two groups of tomato seeds coated with and without *B. bassiana* were planted in greenhouse growing medium amended with one of four monarda herbages. Each herbage represented a chemotype of monarda bioactive herbage (cymene, geraniol, borneol and carvacrol).Seedling emergence was recorded every day for six weeks. Bars with the same letter are not significantly different according to an F-protected least significant difference at P = 0.05.

Table 3.6. Days to germination for tomato seeds coated with *B. bassiana* suspended in methylcellulose and those seeds that were coated with methylcellulose only (Method=LSD, P<0.05).

Treatment	Days to Germination	Standard Error	Letter Group
Methylcellulose	3.91	0.10	A
Beauveria bassiana	3.23	0.10	B

Colonization of tomato seedlings by *Beauveria bassiana*. Percentage colonization of tomato seedlings by *B. bassiana* in treatments of 'Violet Queen' (carvacrol chemotype), 'Trinity Purple' (cymene chemotype) or 'Rose-scented' (geraniol chemotype) was not different from the 'no herbage' control, but treatment with 'Cerise' (borneol chemotype) reduced the colonization of seeds by *B. bassiana* compared to the 'no herbage' control group (Fig. 3.2). Only one plant in the no-*Beauveria* controls ('Cerise' - borneol chemotype) was positive for *B. bassiana* colonization.

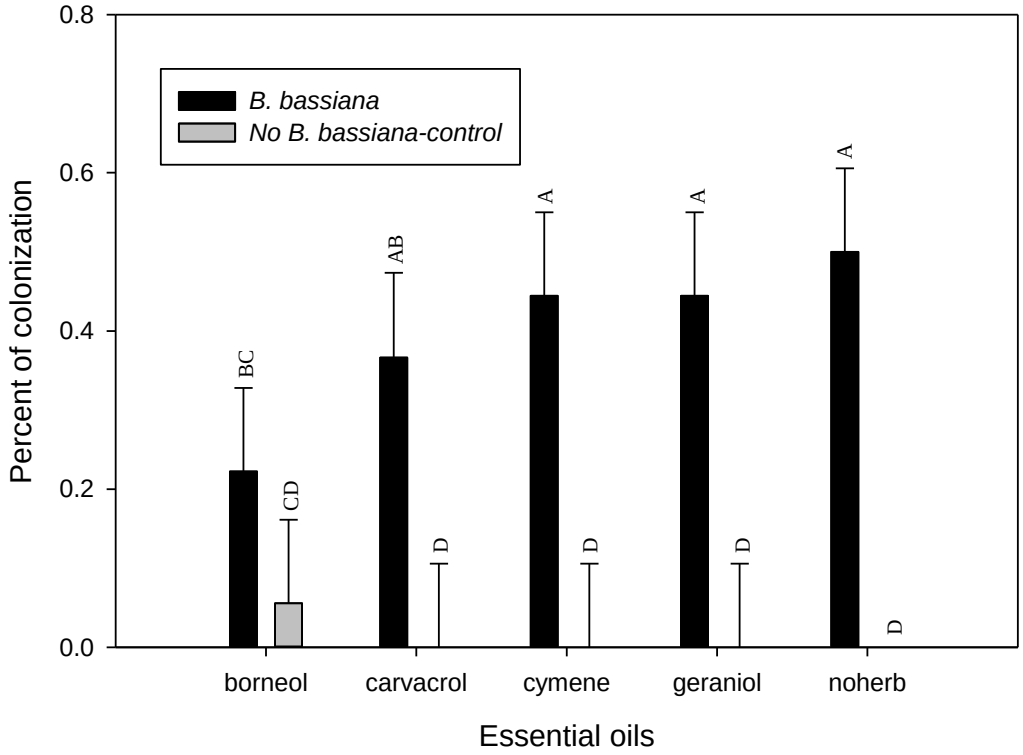

Figure 3.2. Effects of essential oils on ability of *B. bassiana* to colonize tomato seeds. Two groups of tomato seeds (with and without *B. bassiana*) were planted in greenhouse growing medium amended with one of four monarda herbages. Each herbage represented a chemotype of monarda bioactive herbage (cymene, geraniol, borneol and carvacrol). Surface-sterilized seedlings plated onto selective media were considered positive if at least one *B. bassiana* colony grew from the seedling. Percentage colonization of tomato seeds was percentage of positive seedlings. Bars with the same letter are not significantly different according to an F-protected least significant difference at P = 0.05.

Discussion

Chemical composition diversity of *Monarda* resulted in different herbage treatment effects on *Beauveria bassiana*. 'Trinity Purple', the variety with the highest cymene content, is the most favorable greenhouse growing medium amendment for tomato seeds germination compared to the other four monarda cultivars (Table 3.4, Table 3.5, Fig. 3.1). Carvacrol and thymol had inhibitory effects on seed germination and growth of some plants (Kordali *et al.* 2008). In this study, *Monarda clinopodia* (thymol chemotype) is the most unfavorable since it results in very low germination of tomato seeds (Table 3.3, Table 3.4). Of the remaining herbage treatments, seeds planted in 'Violet Queen' (carvacrol chemotype) had the greatest delay to emergence.

Colonization was rarely observed in the no-*Beauveria* controls; plant-to-plant spread rarely occurred in the growth chamber study. 'Trinity Purple' and 'Rose-scented' (geraniol chemotype) showed the best capability with *B. bassiana* based on the percentage of colonization data, but if both the germination time (2.81±0.16 day) and rate of tomato seeds (100 percent) are considered, it is evident that 'Trinity Purple' is slightly more favorable to be used in combination with *B. bassiana* seed treatment.

In previous studies, herbages classified as carvacrol and thymol chemotypes were demonstrated to be highly active against *Rhizoctonia* and *Pythium*, and protected tomato seedings from damping-off disease. These herbages inhibited mycelial growth

of *P. myriotylum* at both low and high concentrations (5 and 50 µl, respectively) (Clark *et al.* 2006), and reduced growth of *R. solani* by more than 65% when tested at 50 to 65µmoles/dish (Gwinn *et al.* 2010). Development of these monarda bioactive herbage products for commercial use requires consideration of their antifungal activity to beneficial fungi and possible toxic effects of essential oil constituents to the plants. Results from this research provide an understanding of the sensitivity of the biocontrol fungus and crop plant to the bioactive monarda herbage, which is needed before this technology can become practical.

In the growth chamber study, the soil construction within each Cone-tainer was not the same, so the essential oils in herbages that evaporated each day were not consistent. As a result, the exact concentration of essential oils to which seeds exposed is not clearly known, which might influence the result.

SUMMARY

Results from this research demonstrated the potential of combining two biological control methods, *Monarda*-based products and the entomopathogenic fungus, *B. bassiana* (as a tomato seed treatment) for disease management. Further development of this plant disease control method will benefit organic or sustainable agriculture systems where alternatives to synthetic fungicides are needed.

The results of this research are based on one fungus isolate and five monarda herbages. Future studies should further investigate other monarda varieties that might be more suitable considering the diversity of constituent concentrations among Monarda varieties. For instance, the monarda cultivar "*Monarda clinopodia*",which has thymol as the main compound, could be replaced by 'Puerto Purification' (also high in thymol), which was not toxic to the tomato plant in previous studies (Clark *et al.* 2006). Another approach would be to adjust the rate (10 %v/v in this study) of monarda in the greenhouse growing medium or to mix different varieties to maximize compatibility with the beneficial fungus.

REFERENCES

Abdelgaleil SA, Abbassy MA, Belal A-SH, Abdel Rasoul M, 2008. Bioactivity of two major constituents isolated from the essential oil of *Artemisia judaica* L. *Bioresource Technology* **99**: 5947–5950.

Al-Bayati FA, 2009. Isolation and identification of antimicrobial compound from *Mentha longifolia* L. leaves grown wild in Iraq. *Annals of Clinical Microbiology and Antimicrobials* **8**: 20.

Amiri A, Dugas R, Pichot AL, Bompeix G, 2008. *In vitro* and *in vivo* [corrected] activity of eugenol oil (*Eugenia caryophylata*) against four important postharvest apple pathogens. *International Journal of Food Microbiology* **126**: 13–19.

Bajpai VK, Kang SC, 2009. Antifungal activity of leaf essential oil and extracts of *Metasequoia glyptostroboides* Miki ex Hu. *Journal of the American Oil Chemists Society* **87**: 327–336.

Bajpai VK, Rahman A, Kang SC, 2008. Chemical composition and inhibitory parameters of essential oil and extracts of *Nandina domestica* Thunb. to control food-borne pathogenic and spoilage bacteria. *International Journal of Food Microbiology* **125**: 117–122.

Boucias DG, Pendland JC, Latge JP, 1988. Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology* **54**: 1795–1805.

Cetin B, Ozer H, Cakir A, Mete E, Tosun M, Oztürk E, Polat T, Kandemir A, 2009. Chemical composition of hydrodistilled essential oil of *Artemisia incana* (L.) Druce and antimicrobial activity against foodborne microorganisms. *Chemistry & Biodiversity* **6**: 2302–2310.

Chang H-T, Cheng Y-H, Wu C-L, Chang S-T, Chang T-T, Su Y-C, 2008. Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin leaf against plant pathogenic fungi. *Bioresource Technology* **99**: 6266–6270.

Clark MM, Gwinn KD, Ownley BH, 2006. Biological control of *Pythium myriotylum*. *Phytopathology* **96**: S25.

Derakhshan S, Sattari M, Bigdeli M, 2008. Effect of subinhibitory concentrations of cumin (*Cuminum cyminum* L.) seed essential oil and alcoholic extract on the morphology, capsule expression and urease activity of *Klebsiella pneumoniae.* *International Journal of Antimicrobial Agents* **32**: 432–436.

Doberski JW, Tribe HT, 1980. Isolation of entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*. *Transaxtions of the Mycological Society* **74**: 95–100.

Dung NT, Kim JM, Kang SC, 2008. Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of *Cleistocalyx operculatus* (Roxb.) Merr and Perry buds. *Food and Chemical Toxicology* **46**: 3632–3639.

Eftekhar F, Nariman F, Yousefzadi M, Hadiand J, Ebrahimi SN, 2009. Anti-*Helicobacter pylori* activity and essential oil composition of *Thymus caramanicus* from Iran. *Natural Product Communications* **4**: 1139–1142.

Erkmen O, Ozcan MM, 2008. Antimicrobial effects of Turkish propolis, pollen, and laurel on spoilage and pathogenic food-related microorganisms. *Journal Of Medicinal Food* **11**: 587–592.

Gatarayiha MC, Laing MD, Miller RM, 2010. In vitro effects of flutriafol and azoxystrobin on *Beauvaria bassiana* and its efficacy against *Tetranychus urticae*. *Pest Management Science* **66**: 773–778.

Germain GS, Summerbell R, 1996. Identifying filamentous fungi - a clinical laboratory handbook. *Star Publishing Company, Belmont, California*: 288 p.

Giordani R, Hadef Y, Kaloustian J, 2008. Compositions and antifungal activities of essential oils of some Algerian aromatic plants. *Fitoterapia* **79**: 199–203.

Gwinn KD, Ownley BH, Greene SE, Clark MM, Taylor CL, Springfield TN, Trently DJ, Green JF, Reed A, Hamilton SL, 2010. Role of essential oils in control of Rhizoctonia damping-off in tomato with bioactive monarda herbage. *Phytopathology* **100**: 493–501.

Hiroshi N, 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**: 382–388.

Huang Y, Zhao J, Zhou L, Wang J, Gong Y, Chen X, Guo Z, Wang Q, Jiang W, 2010. Antifungal activity of the essential oil of *Illicium verum* fruit and its main component trans-anethole. *Molecules* **15**: 7558–7569.

Hywel-Jones NL, Gillespie a. T, 1990. Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. *Mycological Research* **94**: 389–392.

Islam M, Olleka A, Ren S, 2010. Influence of neem on susceptibility of *Beauveria bassiana* and investigation of their combined efficacy against sweet potato

whitefly, *Bemisia tabaci* on eggplant. *Pesticide Biochemistry and Physiology* **98**: 45–49.

Katooli N, Maghsodlo R, Razavi SE, 2011. Evaluation of eucalyptus essential oil against some plant pathogenic fungi. *Journal of Plant Breeding and Crop Science* **3**: 41–43.

Kim S-S, Baik JS, Oh T-H, Yoon W-J, Lee NH, Hyun C-G, 2008. Biological activities of Korean *Citrus obovoides* and *Citrus natsudaidai* essential oils against acne-inducing bacteria. *Bioscience Biotechnology and Biochemistry* **72**: 2507–2513.

Kishore GK, Pande S, Harish S, 2007. Evaluation of essential oils and their components for broad-spectrum antifungal activity and control of late leaf spot and Crown Rot Diseases in peanut. *Plant Disease* **91**: 375–379.

Knobloch K, Pauli A, Iberl N, Weigand N, Weis HM, 1989. Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research* **1**: 119–128.

Kordali S, Cakir A, Ozer H, Cakmakci R, Kesdek M, Mete E, 2008. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and ρ-cymene. *Bioresource Technology* **99**: 8788–8795.

Kotan R, Cakir A, Dadasoglu F, Aydin T, Cakmakci R, Ozer H, Kordali S, Mete E, Dikbas N, 2010. Antibacterial activities of essential oils and extracts of Turkish *Achillea, Satureja* and *Thymus* species against plant pathogenic bacteria. *Journal of the Science of Food and Agriculture* **90**: 145–160.

Lorenzi V, Muselli A, Bernardini AF, Berti L, Pagès J-M, Amaral L, Bolla J-M, 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. *Antimicrobial Agents and Chemotherapy* **53**: 2209–2211.

Loughlin R, Gilmore BF, McCarron PA, Tunney MM, 2008. Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells. *Letters in Applied Microbiology* **46**: 428–433.

Maggi F, Bramucci M, Cecchini C, Coman MM, Cresci A, Cristalli G, Lupidi G, Papa F, Quassinti L, Sagratini G, Vittori S, 2009. Composition and biological activity of essential oil of *Achillea ligustica* All. (Asteraceae) naturalized in central Italy: ideal candidate for anti-cariogenic formulations. *Fitoterapia* **80**: 313–319.

Mahboubi M, Bidgoli FG, 2010. Antistaphylococcal activity of *Zataria multiflora* essential oil and its synergy with vancomycin. *Phytomedicine* **17**: 548–550.

Mahboubi M, Haghi G, 2008. Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil. *Journal of Ethnopharmacology* **119**: 325–327.

Maxia A, Marongiu B, Piras A, Porcedda S, Tuveri E, Gonçalves MJ, Cavaleiro C, Salgueiro L, 2009. Chemical characterization and biological activity of essential oils from *Daucus carota* L. subsp. *carota* growing wild on the Mediterranean coast and on the Atlantic coast. *Fitoterapia* **80**: 57–61.

Menghini L, Leporini L, Tirillini B, Epifano F, Genovese S, 2010. Chemical composition and inhibitory activity against *Helicobacter pylori* of the essential oil of *Apium nodiflorum* (Apiaceae). *Journal of Medicinal Food* **13**: 228–230.

Meyling NV, Lübeck M, Buckley EP, Eilenberg J, Rehner SA, 2009. Community composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and seminatural habitats. *Molecular Ecology* **18**: 1282–1293.

Muller-Riebau F, Berger B, Yeged O, Antalya K, 1995. Chemical composition and fungitoxic properties to phytopathogenic fungi of esential oils of selected aromatic plants growing wild in Turkey. *Journal of Agricultural and Food Chemistry* **43**: 2262–2266.

Nascimento PFC, Alviano WS, Nascimento ALC, Santos PO, Arrigoni-Blank MF, Jesus RA De, Azevedo VG, Alviano DS, Bolognese AM, Trindade RC, 2008. *Hyptis pectinata* essential oil: chemical composition and anti-*Streptococcus mutans* activity. *Oral Diseases* **14**: 485–489.

Neves PMO., Hirose E, Tchujo PT, Moino A, 2001. Compatibility of entomopathogenic fungi with neonicotinoid insecticides. *Neotropical Entomology* **30**: 263–268.

Olmert I, Kenneth RG, 1974. Sensitivity of the entomopathogenic fungi, *Beauveria bassiana*, *Verticillium lecanii*, and *Verticillium* sp. to fungicides and insecticides. *Environmental Entomology* **3**: 33–38.

Ownley BH, Griffin MR, Klingeman WE, Gwinn KD, Moulton JK, Pereira RM, 2008. *Beauveria bassiana*: endophytic colonization and plant disease control. *Journal of Invertebrate Pathology* **98**: 267–270.

Ownley BH, Gwinn KD, Vega FE, 2010. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl* **55**: 113–128.

Ownley BH, Pereira RM, Klingeman WE, Quigley NB, Leckie BM, 2004. *Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens. *In: Lartey RT, Caesar A (eds) Emerging Concepts in Plant Health Management. Research Signpost, Kerala*: 255–269.

Ozcan MM, Chalchat J-C, 2008. Chemical composition and antifungal activity of rosemary (*Rosmarinus officinalis* L.) oil from Turkey. *International Journal of Food Sciences and Nutrition* **59**: 691–698.

Ozek G, Demirci F, Ozek T, Tabanca N, Wedge DE, Khan SI, Başer KHC, Duran A, Hamzaoglu E, 2010. Gas chromatographic-mass spectrometric analysis of volatiles obtained by four different techniques from *Salvia rosifolia* Sm., and evaluation for biological activity. *Journal of Chromatography* **1217**: 741–748.

Pandey RR, Dubey RC, Saini S, 2010. Phytochemical and antimicrobial studies on essential oils of some aromatic plants. *African Journal of Biotechnology* **9**: 4364–4368.

Peighami-Ashnaei S, Farzaneh M, Sharifi-Tehrani A, Behboudi K, 2009. Effect of essential oils in control of plant diseases. *Communications in Agricultural and Applied Biological Sciences* **74**: 843–847.

Pitarokili D, Tzakou O, Loukis A, Harvala C, 2003. Volatile metabolites from *Salvia fruticosa* as antifungal agents in soilborne pathogens. *Journal of Agricultural and Food Chemistry* **51**: 3294–3301.

Pozzatti P, Loreto ES, Lopes PGM, Athayde ML, Santurio JM, Alves SH, 2010. Comparison of the susceptibilities of clinical isolates of *Candida albicans* and *Candida dubliniensis* to essential oils. *Mycoses* **53**: 12–15.

Roberts DW, Campbell AS, 1977. Stability of entomopathogenic fungi. *Miscellaneous Publications of the Entomological Society of America* **10**: 19–76.

Roller S, Ernest N, Buckle J, 2009. The antimicrobial activity of high-necrodane and other lavender oils on methicillin-sensitive and -resistant *Staphylococcus aureus* (MSSA and MRSA). *Journal of Alternative and Complementary Medicine* **15**: 275–279.

Sant'anna JR De, Franco CCDS, Miyamoto CT, Cunico MM, Miguel OG, Côcco LC, Yamamoto CI, Junior CC, Castro-Prado MAA De, 2009. Genotoxicity of *Achillea millefolium* essential oil in diploid cells of *Aspergillus nidulans. Phytotherapy Research* **23**: 231–235.

Scora RW, 1967. Study of the essential leaf oils of the genus Monarda (Labiatae). *American Journal of Botany* **54**: 446–452.

Senthamizhlselvan P, Sujeetha JARP, Jeyalakshmi C, 2010. Growth, sporulation and biomass production of native entomopathogenic fungal isolates on a suitable medium. *Journal of Biopesticides* **3**: 466–469.

Sitara U, Niaz I, Naseem J, Sultana N, 2008. Antifungal effect of essential oils on *in vitro* growth of pathogenic fungi. *Pakistan Journal of Botany* **40**: 409–414.

Storey GK, Gardner WA, 1986. Sensitivity of the entomogenous fungus *Beauveria bassiana* to selected plant growth regulators and spray additives. *Applied and Environmental Microbiology* **52**: 1–3.

Talaei-Hassanloui R, Kharazi-pakdel A, Goettel MS, Little S, Mozaffari J, 2007. Germination polarity of *Beauveria bassiana* conidia and its possible correlation with virulence. *Journal of Invertebrate Pathology* **94**: 102–107.

Tefera T, Pringle K, 2003. Germination, radial growth, and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates and their virulence to *Chilo partellus* (Lepidoptera: Pyralidae) at different temperatures. *Biocontrol Science and Technology* **13**: 699–704.

Teng C, 1962. Studies on the biology of *Beauveria bassiana* (Bals.) Vuill. with the reference to microbial control of insects. *Acta Botanica Sinica* **10**: 210–232.

Tohidpour A, Sattari M, Omidbaigi R, Yadegar A, Nazemi J, 2010. Antibacterial effect of essential oils from two medicinal plants against Methicillin-resistant Staphylococcus aureus (MRSA). *Phytomedicine* **17**: 142–145.

Vega FE, 2008. Insect pathology and fungal endophytes. *Journal of Invertebrate Pathology* **98**: 277–279.

Vila R, Santana AI, Pérez-Rosés R, Valderrama A, Castelli MV, Mendonca S, Zacchino S, Gupta MP, Cañigueral S, 2010. Composition and biological activity of the essential oil from leaves of *Plinia cerrocampanensis*, a new source of alpha-bisabolol. *Bioresource Technology* **101**: 2510–2514.

Vuuren SF Van, Suliman S, Viljoen AM, 2009. The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. *Letters in Applied Microbiology* **48**: 440–446.

Yang E-J, Kim SS, Moon J-Y, Oh T-H, Baik JS, Lee NH, Hyun C-G, 2010. Inhibitory effects of *Fortunella japonica* var. *margarita* and *Citrus sunki* essential oils on nitric oxide production and skin pathogens. *Acta Microbiologica et Immunologica Hungarica* **57**: 15–27.

Yoon W-J, Kim S-S, Oh T-H, Lee NH, Hyun C-G, 2009. *Cryptomeria japonica* essential oil inhibits the growth of drug-resistant skin pathogens and LPS-induced nitric oxide and pro-inflammatory cytokine production. *Polish Journal of Microbiology* **58**: 61–68.

Zambonelli A, Aulerio AD, Bianchi A, Albasini A, 1996. Effects of essential oils on phytopathogenic fungi *in vitro**. *Journal of Phytopathology* **94**: 491–495.

APPENDIX

Time Course Studies

Borneol

Table A1.1. Length of germination tubes of *Beauveria bassiana* treated with borneol at different time periods (Method=LSD, P<0.05).

Time (h)	Length Estimate (µm)	Standard Error	Letter Group
16	1.05	0.08	A
20	1.35	0.08	B
22	1.78	0.08	C
24	2.21	0.07	D

Table A1.2. Length of germination tubes of *Beauveria bassiana* treated with different concentrations of borneol (Method=LSD, P<0.05).

Concentration (mM)	Length Estimate (µm)	Standard Error	Letter Group
0	2.79	0.08	A
0.005	2.21	0.08	B
0.05	1.64	0.08	C
0.5	1.45	0.08	D
5	1.12	0.09	E
50	0.38	0.1	F

Table A1.3. Estimate of intercepts and slopes of the linear regression predicting the relationship between the logarithm transformation of germination tube-length of *Beauveria bassiana* (dependent variable y) and logarithm transformation of concentration of borneol (explanatory variable x) at each time period (equation: log10(y)=a+b×log10(x), P =0.05).

Time (h)	Intercept (µm)	Slope	R-Square
16	-0.24±0.07	-0.30±0.04	0.76
20	-0.03±0.06	-0.34±0.03	0.85
22	0.13±0.07	-0.42±0.04	0.85
24	0.24±0.08	-0.50±0.05	0.87

Geraniol

Table A1.4. Length of germination tubes of *Beauveria bassiana* treated with geraniol at different time periods (µm) (Method=LSD, P<0.05).

Time (h)	Length Estimate (µm)	Standard Error	Letter Group
16	1.03	0.09	A
20	1.30	0.09	B
22	2.32	0.09	C
24	2.76	0.09	D

Table A1.5. Length of germination tubes of *Beauveria bassiana* treated with different concentrations of geraniol (µm) (Method=LSD, P<0.05).

Concentration (mM)	Length Estimate (µm)	Standard Error	Letter Group
0	2.25	0.09	A
0.005	2.07	0.10	B
0.05	1.83	0.10	C
0.5	1.77	0.10	CD
5	1.65	0.10	DE
50	1.56	0.10	F

Table A1.6. Estimate of intercepts and slopes of the linear regression predicting the relationship between the logarithm transformation of germination tube-length of *Beauveria bassiana* (dependent variable y) and logarithm transformation of concentration of geraniol (explanatory variable x) at each time period (equation: log10(y)=a+b×log10(x), P =0.05).

Time (h)	Intercept (µm)	Slope	R-Square
16	0.02±0.03	-0.00±0.02	0.01
20	0.24±0.02	-0.04±0.01	0.43
22	0.79±0.02	-0.09±0.01	0.68
24	0.92±0.04	-0.11±0.02	0.55

Carvacrol

Table A1.7. Length of germination tubes of *Beauveria bassiana* treated with carvacrol at different time periods (Method=LSD, P<0.05).

Time (h)	Length Estimate (µm)	Standard Error	Letter Group
12	0.83	0.06	A
16	1.89	0.05	B
20	2.26	0.05	C
24	2.67	0.07	D

Table A1.8. Length of germination tubes of *Beauveria bassiana* treated with different concentrations of carvacrol (µm) (Method=LSD, P<0.05).

Concentration (mM)	Length Estimate (µm)	Standard Error	Letter Group
0	3.56	0.06	A
0.5	2.97	0.06	B
5	2.29	0.06	C
50	0.38	0.08	D
500	0.36	0.08	D
0	3.56	0.06	A

Table A1.9. Estimate of intercepts and slopes of the linear regression predicting the relationship between the logarithm transformation of germination tube-length of *Beauveria bassiana* (dependent variable y) and logarithm transformation of concentration of carvacrol (explanatory variable x) at each time period (equation: log10(y)=a+b×log10(x), P =0.05).

Time (h)	Intercept (µm)	Slope	R-Square
12	-0.19±0.08	-0.29±0.05	0.75
16	0.47±0.16	-0.51±0.09	0.71
20	0.62±0.18	-0.55±0.10	0.71
24	0.77±0.19	-0.62 ±0.11	0.80

Thymol

Table A1.10. Length of germination tubes of *Beauveria bassiana* treated with thymol at different time periods (Method=LSD, P<0.05).

Time (h)	Length Estimate (µm)	Standard Error	Letter Group
12	0.59	0.04	A
16	1.22	0.03	B
20	1.45	0.03	C
24	2.08	0.03	D

Table A1.11. Length of germination tubes of *Beauveria bassiana* treated with different concentrations of thymol (Method=LSD, P<0.05).

Concentration (mM)	Length Estimate (µm)	Standard Error	Letter Group
0	1.74	0.04	A
0.5	1.46	0.04	B
5	1.30	0.04	C
50	1.15	0.04	D
500	1.03	0.04	E
0	1.74	0.04	A

Table A1.12. Estimate of intercepts and slopes of the linear regression predicting the relationship between the logarithm transformation of germination tube-length of *Beauveria bassiana* (dependent variable y) and logarithm transformation of concentration of thymol (explanatory variable x) at each time period (equation: log10(y)=a+b×log10(x), P =0.05).

Time (h)	Intercept (µm)	Slope	R-Square
12	-0.49±0.04	-0.19±0.02	0.87
16	0.21±0.02	-0.05±0.01	0.57
20	0.41±0.01	-0.09±0.01	0.93
24	0.77±0.02	-0.12±0.01	0.94

Cymene

Table A1.13. Length of germination tubes of *Beauveria bassiana* treated with cymene at different time periods (µm) (Method=LSD, P<0.05).

Time (h)	Length Estimate (µm)	Standard Error	Letter Group
12	1.33	0.07	A
16	2.10	0.08	B
20	2.70	0.08	C
24	3.78	0.10	D

Table A1.14. Length of germination tubes of *Beauveria bassiana* treated with different concentrations of cymene (µm) (Method=LSD, P<0.05).

Concentration (mM)	Length Estimate (µm)	Standard Error	Letter Group
0	4.01	0.14	A
0.5	3.57	0.14	B
5	2.83	0.15	C
50	1.45	0.14	D
500	0.53	0.14	E
0	4.01	0.14	A

Table A1.15. Estimate of intercepts and slopes of the linear regression predicting the relationship between the logarithm transformation of germination tube-length of *Beauveria bassiana* (dependent variable y) and logarithm transformation of concentration of cymene (explanatory variable x) at each time period (equation: log10(y)=a+b×log10(x), P =0.05).

Time (h)	Intercept (µm)	Slope	R-Square
12	0.26±0.13	-0.23±0.07	0.46
16	0.67±0.13	-0.43±0.07	0.73
20	0.89±0.14	-0.44±0.08	0.71
24	1.28±0.11	-0.34±0.06	0.72

VITA

Wanjing Liu was born in Nanchang, Jiangxi province, China in 1988. She received her Bachelor of Science in Agriculture degree from Northeast Forestry University in Harbin in 2010. Her major field of study was Forestry Resources Conservation and Recreation. After that, she started her graduate studies majoring in Plant Pathology and worked as a graduate research assistant in the Department of Entomology and Plant Pathology at the University of Tennessee at Knoxville under the direction of Dr. Kimberly D. Gwinn.