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Biological Control Methods for Damping-off of Tomato Seedlings Caused by *Pythium myriotylum*

Miranda Marshall Clark
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Miranda Marshall Clark entitled "Biological Control Methods for Damping-off of Tomato Seedlings Caused by *Pythium myriotylum*." 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, Craig H. Canaday

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Accepted for the Council:

Anne Mayhew

Vice Chancellor and Dean of
Graduate Studies

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BIOLOGICAL CONTROL METHODS FOR DAMPING-OFF OF TOMATO SEEDLINGS CAUSED BY *PYTHIUM MYRIOTYLUM*

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Miranda Marshall Clark

May 2006

DEDICATION

This thesis is dedicated to my best friend, my husband, Roger Clark, who supported me, loved me, and made me laugh when I needed it most. I am so grateful to have such an amazing person to share these moments with. I love you, always. I also dedicate this work to my parents, Doug and Marsha Marshall, for their unending financial and emotional support. You are the most giving, honest, and selfless people I know, and I am so lucky to have had you in my life. Your encouragement, confidence, and love have given me the determination to succeed. Without my family, nothing I have accomplished would have been possible.

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time, support, and advice; I could never thank you enough for helping me achieve this goal. I appreciate you, always.

ABSTRACT

Pythium damping-off has the potential to cause severe loss in greenhouse and field grown tomatoes. Species of *Pythium* are found in soils from all climates, and capable of surviving for long periods without a host. Infectious structures of *Pythium* species are motile, and therefore able to travel through irrigation water and runoff. *Pythium myriotylum* thrives in warm, humid environments such as that of the Southeastern United States, and was thus chosen for this study. Currently, no tomato varieties with resistance to damping-off are available. In addition, the agriculture industry is striving for sustainable and biological methods of control of plant pests and pathogens. Therefore, biological controls that are capable of simultaneously protecting plants from pathogens and pests are needed. To that end, the first part of this investigation for biological control of tomato damping-off involves the seed application of an entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, along with a commercial plant growth-promoting rhizobacteria formulation (BioYield) that is known to induce systemic resistance in plants to herbivores and pathogens, and a soil amendment with *Monarda* sp. containing essential oils that are fungicidal to many soilborne pathogens. The objectives of the first study were to determine the following: (i) if herbage of *Monarda didyma* used as a soil amendment is capable of suppressing damping-off of tomato seedlings; (ii) if conidia of *Beauveria bassiana* isolates used as seed coatings are capable of suppressing damping-off of tomato seedlings; (iii) if a commercial form of plant growth promoting rhizobacteria used as a seed drench is

capable of suppressing damping-off of tomato seedlings; and (iv) if combinations of the herbage and the seed treatments are synergistic or antagonistic.

Results from the first study indicated cultivar specificity with *Beauveria bassiana* and herbage treatments. Survival was increased in 'Mountain Spring' tomato seedlings treated with either *B. bassiana* 11-98 or BotaniGard when challenged with the pathogen, but no similar effects were observed in 'Celebrity' seedlings. There was also an increase in stem diameter in *Beauveria*-treated 'Mountain Spring' that was not seen in 'Celebrity.' When 'Celebrity' seedlings were grown in media amended with 'Puerto Purification,' there was a significant decrease in disease index when challenged with the pathogen. This effect was not observed in 'Mountain Spring.' 'Violet Queen' had negative effects on 'Celebrity' seedling growth, seen as a decrease of survival and increase in disease index. Treatment with PGPR had no significant effects in either cultivar.

The second part of this research investigated dried, ground leaves (herbage) from 16 *Monarda* varieties as amendments for biological control against Pythium damping-off in tomato. The objectives of this study were to determine the following: (i) if *Monarda* essential oil constituents could inhibit growth of *P. myriotylum in vitro*; (ii) if herbage amendments could suppress Pythium damping-off; (iii) if herbage amendments had any adverse or beneficial effects on tomato seedling growth.

When essential oil constituents of *Monarda* were tested for toxicity against *P. myriotylum*, thymol and carvacrol inhibited mycelial growth at low and high concentrations (5 and 50 µl, respectively). GC-MS analysis of the herbage used in this study showed concentration of thymol and carvacrol to be variable among varieties.

‘Croftway Pink’ was high in thymol; ‘Sioux’ was approximately equal in thymol, carvacrol, and the sesquiterpene thymoquinone. ‘Mohawk’ had a high concentration of thymoquinone and Rose Geranium had no detectible amounts of thymol, carvacrol, or thymoquinone. Treatments with four of sixteen *Monarda* varieties were successful in decreasing disease index and increasing survival of ‘Mountain Spring’ seedlings when challenged with the pathogen. ‘Croftway Pink’ dominated the varieties with significantly increased shoot height, stem diameter, and survival, as well as decreased disease index in tomato seedlings. Three other amendments, ‘Sioux’, ‘Mohawk’, and Rose Geranium, had no negative effects on seedling growth and increased seedling survival.

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PART I

LITERATURE REVIEW

Introduction

The tomato, (*Lycopersicon esculentum* Mill.), a member of the nightshade family (Solanaceae), that includes potato, pepper, and eggplant, was first thought to be poisonous to humans (Swiader and Ware, 2002). Over the centuries, however, humans realized the great versatility of the fruit in the culinary realm, as well as the potential health benefits from the fruit's antioxidants. Tomatoes are now grown all over the world throughout the year. In the United States production of tomato is second only to potato. The National Agriculture Statistics Service reported the value of fresh market tomatoes in 2005 to be over $1.6 billion (Anonymous, 2006).

Although tomato is commercially grown across the globe, there is no place where the plant is free of disease. One of the major causes of seedling loss is damping-off, a disease that is caused by a variety of fungi, including the fungal-like organism, *Pythium myriotylum* Drechsler. *Pythium* spp. threaten tomato production because of their ability to travel through water and to survive long, harsh periods as resting spores. In the past, farmers have relied strictly on fungicides to reduce disease incidence. Now, however, the public's increasing concern for environmental health makes alternative management strategies more desirable.

Secondary Metabolites

Description

In the writings of all early civilizations, as far back as 4000 B.C., there have been documentations on the preparation and use of plants for medicinal uses, and the

antimicrobial properties of some of these plants have been documented for efficacy in combating disease (Bishop and MacDonald, 1951). In agriculture, essential oils have traditionally been used as protectants for stored grains or legumes and for flying insects in and around the home. Essential oils are extracted form foliage by steam distillation, but even the foliage itself of some aromatic plants has been used for crop protection and insect deterrence (Isman, 2000).

Secondary metabolites, or plant natural products, influence ecological interactions between the plant and its environment (Croteau et al., 2000). These compounds play a pivotal role in the protection of plants, attraction of pollinators and seed-dispersing animals, and influence competition among plant species (Rodríguez-Concepción and Boronat, 2002). Plant natural products generally belong to one of four chemical groups: alkaloids, terpenoids, and the phenylpropanoids, including phenolic compounds. Phenolic compounds are formed through the shikimate or malonate/acetate pathways, and the group consists of about 8,000 compounds (Croteau et al., 2000). There are over 12,000 known alkaloids, which are primarily biosynthesized from amino acids (Croteau et al., 2000). Terpenoids are derived from the five-carbon units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), an isomer of IPP (Croteau et al., 2000; Rodríguez-Concepción and Boronat, 2002). Isoprene is one of the simplest of these compounds. Monoterpenes, composed of two isoprene units, make up as much as 5% of the essences of spices, herbs, and flowers (Croteau et al., 2000). There are many secondary metabolites other than monoterpenes that are built from the isoprene unit, such as sesquiterpenes (Rodríguez-Concepción and Boronat, 2002). These compounds contain

three isoprene units, and many of these are antibiotic compounds produced in response to microbial attack. Compounds produced in response to microbial attack are termed phytoalexins (Croteau et al., 2000). Plant essential oils may be obtained through distillation or extraction of flowers, leaves, and/or stems. Although the individual compounds of a plant's natural products are called essential oils, the tradition of naming the entire mixture after the source plant (i.e. lavender oil, thyme oil, etc.) will be followed in this text. Compounds such as thymol, carvacrol, or citrol, for example, are the constituents of an essential oil.

Importance

Many secondary metabolites found in plants have been studied for efficacy against plant pathogens and pests. For example, in a study investigating fumigant abilities of 22 essential oils, those from *Thymus serpyllum* (rich in thymol and carvacrol) were the most toxic to the bean weevil (*Acanthoscelides obtecus*) (Regnault-Roger et al., 1993). The monoterpenic phenols, carvacrol, eugenol, and thymol, were strongly associated with the antifungal activity of essential oils screened against *Botrytis cinerea* (Wilson et al., 1997). In an analysis of essential oils from numerous Indian herbs for inhibition of twenty phytopathogenic fungi, significant inhibition of all fungi was observed with cymbopogan, ajowan, and dill seed oils (Sridhar et al. 2003). The primary bioactive ingredients were identified as geraniol, thymol, and carvone, respectively (Sridhar et al. 2003). When applied as a seed soak, the essential oils of *Chenopodium ambrosioides* (Chenopodiaceae) reduced damping-off of tomato in soil infested with *Pythium aphanidermatum* or *P. debaryanum* by 67 and 100%, respectively (Kishore and

Dubey, 2002). Oils from *Lippia alba* (Verbenaceae) reduced damping-off of tomato in soil infested with *P. aphanidermatum* or *P. debaryanum* by 89 and 71%, respectively (Kishore and Dubey, 2002). The oils from both *C. ambrosioides* and *L. alba* did not inhibit seed germination or seedling growth, and the oils were more effective in pathogen inhibition than synthetic pesticides (Kishore and Dubey, 2002).

In a vapor contact study in sealed vessels, 6.3 µg ml^{-1} and 63 µg ml^{-1} air of seven essential oils were tested for their inhibitory effects on hyphal growth of *Aspergillus fumigatus* (Inouye et al., 1998). The results indicated that citron, lavender, and tea tree oils were fungistatic at the higher dose; they stopped apical growth and then allowed re-growth after the vapor was removed. Lemongrass and perilla oils were fungicidal at the higher dose, and cinnamon bark and thyme oils retarded or stopped hyphal growth at the low and high dose, respectively. The main constituents of the oils as determined using gas chromatography were limonene (83%) in citron, linalool (38%) in lavender, and carvacrol (80%) in wild thyme. Oil deposition suppressed apical growth of the fungus (Inouye et al., 1998).

Populations of *Phytophthora nicotianae* were reduced in soil treated with 1, 5, or 10% aqueous emulsions of formulations containing clove oil, pepper extract and mustard oil, cassia extract, or synthetic cinnamon oil after 21 days, compared to the untreated control (Bowers and Locke, 2004). Metalaxyl and neem oil also were tested, but did not reduce populations of the pathogen compared to the control. When compared to the un-treated infested soil, 10% aqueous emulsions of pepper extract-mustard oil formulation, a cassia extract, and the synthetic cinnamon oil formulation each suppressed Phytophthora

blight in the greenhouse after 35 days, and plant health was significantly greater (Bowers and Locke, 2004).

Monarda

Characteristics

The genus *Monarda* is named for Nicholas de Monardes, a Spanish physician who first described the plant while visiting the New World in the 16th century. His interest in medicinal herbs prompted his inquiry about this soothing tea (Oswego tea is a common name for the plant) that the Native Americans used to treat fevers and chills. The plant gained the name balm from the use of its leaves as a topical compress to ease the pain of insect bites (Bakalar and Morrison, 1991).

A member of the Lamiaceae (mint) family, *Monarda* consists of 16 species, distributed throughout North America, from the Atlantic coast to the Rocky Mountains and from central Mexico to Canada (Prather et al., 2002). *Monarda* is distinguished from other Lamiaceae by combinations of visual characteristics. Species of *Monarda* are known to cross freely with one another. Such hybridization is speculated to be the evolutionary key in *Monarda* morphology. Based on morphological characteristics, *Monarda media* may be a hybrid of *M. clinopodia* L. and *M. didyma* L. (Prather et al., 2002); however, others have speculated that it is a hybrid of *M. clinopodia* or *M. didyma* with *M. fistulosa* L. (Whitten, 1981; Scora, 1967), and that the complex patterns of diversity of *Monarda* in the Appalachian region are resultant of this triangle of hybridization (Duncan, 1959; Scora, 1967). Hybridizations have been hypothesized

among other species (e.g., *Monarda fistulosa* and *M. lindheimeri* Engelm. and A. Gray) (Scora, 1967). Information generated from DNA technology has further challenged the concept of species relationships in the genus. Analysis of sequences of internal transcribed spacer regions of nuclear ribosomal DNA revealed little molecular diversity among *Monarda* spp. (Prather et al., 2002). Hybridization between *Monarda fistulosa* and *M. lindheimeri* was also confirmed with this technique (Prather et al., 2002).

Most commercial *Monarda* varieties are classified as *Monarda didyma* or *M. fistulosa*; both *M. didyma* and *M. fistulosa* are in the subgenus Monarda. *Monarda didyma* (bee balm) is a perennial that grows to an average height of 1 meter, depending on the cultivar, and has very aromatic opposite leaves. The leaves of this species are used for making the herbal tea that gave the genus its common name (Prather et al., 2002). The flowers are lovely tubular appendages in clusters atop bracts. The plant prefers shady moist soil, but performs well in sun if adequately watered (Bakalar and Morrison, 1991). Wild bergamot (*M. fistulosa*) prefers drier soil than *M. didyma*, and has a more lavender-pink flower instead of scarlet. Many commercial varieties of *Monarda* did not arise from documented breeding programs but are simply selected by nurseries from attractive plants. Given the tendency of interspecies hybridization within the genus, it is likely that many varieties called *M. didyma* by the grower are actually *M. didyma* x *M. fistulosa* hybrids. Few varieties have parental lineage documented in the literature, but those that are documented have both *M. didyma* and *M. fistulosa* in their parentage (Collicutt, 1989; Collicutt and Davidson, 1999; Davidson, 2002). In contrast to most other *Monarda* species that belong to the subgenus *Monarda*, spotted bee balm or horse

mint (*M. punctata* L.) is a member of subgenus *Cheilyctis* (Prather et al., 2002). *Monarda punctata* has slender, less aromatic leaves and pale-pink bracts with yellow flowers.

Essential oil composition of *Monarda*

Monarda varieties are known for the aromatic nature of their leaves and flowers. The essential oils of *Monarda* are well documented, and the composition of the essential oil obtained from *Monarda* is diverse. *Monarda* essential oil contains terpenes, phenols, and alcohols. Each species of *Monarda* contains different amounts of each compound, and the varying proportions give the plants slightly different aromas and herbal properties.

Preliminary research

Greenhouse and laboratory studies have shown that adding herbage (dried and ground leaves and flowers) of *Monarda* sp. to planting medium reduced disease-loss to *Rhizoctonia* and kills the sclerotia of *Sclerotinia* (Gwinn et al., 2003; Gwinn, unpublished data). Herbage of two *Monarda* varieties ('Elsie's Lavender' and 'Marshall's Delight') increased percent germination and plant height of tomato in *Rhizoctonia*-infested medium (Gwinn et al., 2003). There was also a decrease in disease index with the addition of 'Elsie's Lavender' in tomato (Gwinn et al., 2003).

Studies have also shown an effect on tomato fruit when plants are grown in media amended with herbage. 'Elsie's Lavender' and the control with no herbage produced greater numbers and weight in Grade 1 (diameter 8.2 cm or larger) fresh market tomatoes than 'Marshall's Delight' in greenhouse studies (Greene, 2005). An equal number and

weight of Grade 4 (diameter 5.4 -5.8 cm) processing tomatoes was observed in treatments with 'Marshall's Delight' without the pathogen (*Pythium myriotylum*), and in treatments with 'Elsie's Lavender' challenged with the pathogen, when compared to the untreated, uninfested control (Greene, 2005).

Beauveria bassiana (Balsamo) Vuillemin

Beauveria bassiana (Balsamo) Vuillemin (Deuteromycota) is a soilborne necrotrophic parasite that has been documented as an entomopathogenic fungus for centuries. The fungus was first discovered around 900 AD in silkworms found in Japan (Boucais and Pendland, 1998). A fungus similar to the present-day description of *B. bassiana* was also found in a worker ant buried in amber and estimated to be 25 million years old (Poinar and Thomas, 1984). Antiseptic properties have also been noted for *B. bassiana* and it has been used for the treatment of sore throats and wounds (Boucais and Pendland, 1998). In 1834, Italian scientist Antonio Bassi de Lodi demonstrated that the white muscardine disease of silkworms was caused by a fungus. (Boucais and Pendland, 1998). The fungus was originally named *Botrytis paradoxa* by Balsamo, but it was later changed to *Botrytis bassiana* in honor of Bassi. The genus was changed to *Beauveria* in 1912 by Vuillemin, which led to the current binomial, *Beauveria bassiana* (Balsamo) Vuillemin (Steinhaus, 1949; 1975; Alexopoulos et al., 1996; Boucias and Pendland, 1998). A relatively new finding is that *Beauveria bassiana* can grow endophytically in plants. At this time, it is unknown whether the responses of tomatoes to *Beauveria bassiana* resemble systemic acquired resistance or induced systemic resistance or if it

more closely resembles true plant endophytes like grass-*Neotyphodium* interactions. Currently, an isolate of the fungus is used as a biocontrol agent of insect pests and is marketed under the names BotaniGard, Mycotrol, and Naturalis. Although these formulations are routinely sprayed on plants, the degree of host infection and subsequent endophytism is unknown.

Mycelium of the fungus is white, yielding the common name white muscardine fungus. Conidia are hyaline, dry, and globose to oval shaped; they are found on the main hyphal branches or as an extension of the conidiophore. The infectious conidia may be found on the flask-shaped conidiophore in clusters, whirls, or singly, in an apical zigzag formation known as a rachis. The sexual stage (teleomorph) of *B. bassiana* is *Cordyceps* sp. Filiform, multiseptate ascospores are found in the asci of the perithecium, or reproductive structure, of the teleomorph (Boucais and Pendland, 1998).

Beauveria bassiana can infect insects of most orders and has world-wide distribution, unlike most other deuteromycetes. The fungus colonizes insects with the aid of mycotoxins, such as beauvericin and oosporein, then continues to grow out of the cadaver, forms conidiophores and subsequently releases conidia for dispersal (Steinhaus, 1949; Boucias and Pendland, 1998). Endophyte colonization of plants was first noted when corn (*Zea mays* L.) plants were treated with *Beauveria* to control European corn borer (*Ostrinia nubilalis* (Hübner)) (Bing and Lewis, 1991). The fungus colonized the plant when applied to foliage as either a granular formulation or injected as a conidial suspension. *Beauveria bassiana* colonized the xylem vessels (Bing and Lewis, 1992). When movement of the fungus through the corn plant is monitored using light and

electron microscopy, germinating hyphae from a foliar application grew and penetrated the corn leaf surface at random (Wagner and Lewis, 2000). Invasion was achieved primarily through direct penetration of the epidermal cell wall rather than through stomata. Once inside, the cuticular ultrastructure of the plant cell wall was noticeably distorted, and growth was observed in the air spaces between the parenchyma cells (Wagner and Lewis, 2000). Hyphal structures were observed in the xylem elements, but primarily following the leaf apoplast away from the point of penetration (Wagner and Lewis, 2000). The observed structures in the xylem vessels indicate the possibility of movement throughout the plant and subsequent protection from insects.

More recent investigations have shown that *B. bassiana* isolate 11-98 (Bb 11-98) endophytically colonizes cotton (Griffin et al., 2005), tomato (Leckie, 2002; Ownley et al., 2004), and snap bean (Ownley et al., unpublished data) when seeds were treated with conidial suspensions of the fungus. *Beauveria* has been isolated from all three plants using selective media (Griffin et al., 2005; Ownley et al., 2004; Doberski and Tribe 1980). Using polymerase chain reaction (PCR) endophytic growth was confirmed in tomato seedlings grown from Bb 11-98 coated seeds (Leckie, 2002).

Seed were coated with Bb 11-98 and grown in medium infested with mycelium of the fungus; subsequent seedlings did not differ from non-coated seed grown in un-infested media in terms of survival, shoot weight, and root weight when not challenged with *Rhizoctonia solani*. However, damping-off was reduced in the Bb 11-98 treated compared to the pathogen control (Seth, 2001). In addition, Bishop (1999) found treatment of 'Mountain Spring' seeds with *Beauveria bassiana* suppressed pre-

emergence damping-off caused by *Rhizoctonia solani*. More recently, when leaf aphid, *Macrosiphum euphorbiae,* fed on tomato foliage treated with conidial suspensions of Bb 11-98, mortality was 29 and 40% at 7 and 10 days after treatment, respectively (Powell, 2005).

When *Beauveria bassiana* 11-98 is applied as a tomato seed treatment, plant stand in *Rhizoctonia solani*-infested media does not differ from plant stand in media that is not infested with the pathogen. (Ownley et al., 2000; Ownley et al., 2004; Ownley et al., unpublished data). *Beauveria bassiana* has been proven time and again to be an effective entomopathogen. Additionally, the endophytic ability of the fungus also gives it outstanding promise as a biological control against other fungi, bacteria, and viruses, by decreasing competition from plant pathogenic organisms.

Systemic Responses of Plants

Systemic acquired resistance

Because plants are sessile, they must employ sophisticated metabolic pathways to survive. These pathways are involved in plant growth, senescence, reproduction and defense, all of which are affected by the others. The complexity of plant metabolism has prompted an intensive area of research on metabolic pathways - function, control, compounds utilized or produced, and interpathway interactions.

Lesion formation, usually from a necrotizing pathogen or hypersensitive response (HR), causes a local accumulation of salicylic acid (SA) that stimulates a signal to the rest of the plant, and the plant becomes resistant to pathogens in areas distant from the

original infection. When a plant responds in this way, it is called systemic acquired resistance (SAR). A phloem-mobile signal is induced after SA concentrations increase, causing the release of methyl-salicylic acid (a volatile compound). The release of methyl salicylate may serve as a signal to nearby plants. In response to the phloem-mobile signal, various pathogen-related (PR) proteins are produced *de novo* throughout the plant, inducing an incompatible plant-pathogen interaction. These PR proteins include the antifungal chitinases, β-1, 3-glucanases, and cysteine-rich proteins, as well as PR-1 and PR-5 proteins that have anti-oomycete activity (Verberne, et al., 2000).

Systemic acquired resistance can be induced by several means. Inoculation of tomato plants with the biocontrol agent *Pythium oligandrum* caused an accumulation of phenolic compounds after 3 h (Le Floch et al., 2005). When pea roots treated with *Bacillus pumilus* strain SE34 were inoculated with *Fusarium oxysporum* f. sp. *pisi*, the pathogen was restricted to the outermost root tissues and unusual structures were deposited at the sites of attempted fungal entry (Benhamou et al., 1996). In addition, large amounts of phenolic compounds were found in the intercellular spaces, but were not found in control tissues. An even coating of chitin was observed on the walls of invading hyphae (Benhamou et al., 1996), indicating that the bacteria aid the plant to "turn on" defenses when under pathogen attack (Benhamou et al., 1996). Abiotic stresses and chemical applications may also activate SAR. Fungicides such as metalaxyl, fosetyl-Al, probenzole, and triazoles have been documented as SAR inducers.

Induced systemic resistance

Induced systemic resistance (ISR), another method of resistance, is one which requires ethylene and jasmonic acid accumulations in the plant, rather than SA. The compounds released due to ISR are usually found in specific cellular compartments and do not accumulate pathogenesis-related proteins or salicylic acid. Research has focused on ISR produced by root-colonizing bacteria that naturally occur in soil. These bacteria, referred to as plant growth-promoting rhizobacteria (PGPR), are known to increase plant growth and induce resistance to pathogens in plants. Generally, plants treated with PGPR have increased shoot and root growth, increased stem diameter, rapid development of new roots, and less transplant shock (Kloepper et al., 2004). Rhizobacteria inhabit the area immediately surrounding plant roots and the roots themselves. Reports of pathogen inhibition have been related to PGPR production of siderophores, antibiotics, hydrogen cyanide, and cell wall degrading enzymes along with the activation of ISR (Kloepper et al., 2004). Several species of bacteria have been noted for their beneficial root-colonizing properties, including *Bacillus*, *Pseudomonas*, *Azospirillum*, and *Azotobacter* (Gupta et al., 1995; Kloepper et al., 2004; Ramamoorthy et al., 2002). Initially, rhizobacteria were studied individually in different systems, in order to find the organism with the most diverse host range. However, since each organism may induce different responses, combining them may prove to be the answer for broad host range capabilities.

Many studies have been published based on the ISR phenomenon. In greenhouse assays, incidence and severity of damping-off of tomato seedlings caused by *Rhizoctonia solani* were significantly reduced when seeds were inoculated with rhizobacteria isolated

previously from the rhizosphere of tomato plants (Gupta et al., 1995). Plants that have activated ISR in turn produce different signaling compounds that are responsible for resistance to other plant pests and pathogens. When challenged with *Pythium aphanidermatum*, and pre-treated with *Pseudomonas florescens* isolate Pf1, tomato and hot pepper (*Capsicum annuum* L.) plants showed increased and earlier activities of the defense-related enzymes phenylalanine ammonia lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) (Ramamoorthy et al., 2002). Also, plants that were pre-treated with Pf1 and challenged with *P. aphanidermatum* had a higher accumulation of phenolic compounds (Ramamoorthy et al., 2002).

Research has shown that combinations of PGPR are more effective in disease suppression via induced resistance than individual strains of rhizobacteria. This research has led to the availability of some PGPR as commercial biocontrol products for controlling plant disease; these are used as seed treatments or soil drench. An investigation of two *Bacillus* strains (*B. subtilis* GB03 and *B. amyloliquefaciens* IN937a) and chitosan added as a soil amendment in transplants of tomato, bell pepper, cucumber and tobacco, resulted in the commercialization of the mixture, called BioYield (Kloepper et al., 2004; Kokalis-Burelle et al., 2003; Kokalis-Burelle et al., 2002). *Bacillus subtilis* GB03 produces antibiotics and *B. amyloliquefaciens* IN937a elicits induced systemic resistance. When treated with BioYield, resistance was induced, and the plants exhibited increased shoot and root growth, enhanced stem diameter, rapid development of new roots, and less transplant shock (Kloepper et. al, 2004). Other early-season improvements were seen in seedling germination, plant vigor, shoot weight, plant height,

stand health, early bloom, and increased nodulation in legumes (Kloepper et. al, 2004). *Bacillus subtilis* GB03 has biological control activity against numerous pathogens, including *Rhizoctonia solani* and *Fusarium* spp. (Kloepper et. al, 2004). *Bacillus amyloliquefaciens* IN937a also has activity against numerous pathogens, including, *Erwinia tracheiphilia*, *Colletotrichum obiculare*, *Pseudomonas syringae* pv. *tomato*, cucumber mosaic virus, and tomato mottle virus (Kloepper et. al, 2004).

Research has shown other PGPR combinations to be effective inducers of resistance as well. A combination of PGPR strains (*Bacillus pumilus* strain INR7, *B. subtilis* strain GB03, *Curtobacterium flaccumfaciens* strain ME1) suppressed three pathogens of cucumber (*C. obiculare*, *P. syringae pv. lachrymans*, *E. tracheiphila*) and provided more consistent disease suppression than individual applications (Raupach and Kloepper, 1998). The same treatments were compared to treatment with acibenzolar-s-methyl (Actigard, a synthetic chemical that triggers SAR). Actigard-treated plants had less growth promotion than the bacterial treatments (Raupach and Kloepper, 1998). In a study investigating biological controls for cucumber diseases in fields fumigated with or without methyl bromide, there was marked disease suppression of two naturally occurring pathogens, *C. obiculare* and *P. syringae* pv. *lachrymans*, as well as growth promotion in those plants treated with a PGPR combination consisting of *B. pumilus* strain INR7, *B. subtilis* strain GB03, and *C. flaccumfaciens* strain ME1(Raupach and Kloepper, 2000).

Several studies have investigated complimentary or antagonistic roles of SAR and ISR in plants. For example, tomato ('Solar Set') transplants treated with Actigard, PGPR

(*B. pumilus* Se 34, *P. putida* 89B61, BioYield, and Equity), or soil amendment with a sulfur mixture were evaluated in greenhouse experiments; bacterial wilt caused by *Ralstonia solanacearum* (race 1 biovar 1), resulted in reduced bacterial wilt by all treatments except Equity (Anith et al., 2004). *Arabidopsis thaliana* was more resistant to the foliar pathogen *P. syringae* pv. *tomato* (Pst), when SAR and ISR were activated simultaneously (van Wees et al., 2000). When ISR or SAR was blocked in *Arabidopsis*, there was no induced resistance. Constitutive expression of NPR1, a regulatory protein that is key for both SAR and ISR, was adequate for concurrent expression of both forms of resistance (van Wees et al., 2000). The expression of SAR marker gene *PR-1*was not affected in plants with induction of ISR, therefore demonstrating that the two pathways are compatible without significant cross-talk (van Wees et al., 2000). Pathogenesis-related proteins and salicylic acid are probably not required during ISR in contrast to SAR. Also, SAR is effective in many plant species, but ISR appears to have a higher degree of host specificity (Vallad and Goodman, 2004).

Some have hypothesized that the biochemical responses of plants could be predicted based on the type of pathogen attacking it (necrotrophic or biotrophic). Evidence supporting that idea was shown when susceptibility to *Phytophthora infestans*, *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pseudomonas syringae*, and *Xanthamonas campestris* was reduced by the jasmonate response in a wild type tomato with no known resistance genes to the pathogens (Thaler et al., 2004). The same response was seen in jasmonate-deficient mutant tomatoes when rescue treatments were used (Thaler et al., 2004).

Plant growth-promoting rhizobacteria induce resistance and compete for space and nutrients. Induced resistance occurs throughout the plant even in distal foliage and may possibly last for several weeks. PGPR serve as biological controls for soil-borne pathogens because they inhabit the roots where many pathogens attack. PGPR provide a means of controlling plant disease without causing selective pressure on pathogen populations, due to their lack of direct antimicrobial activity. Because of this, PGPR show great promise for disease suppression in conventional agriculture by a sustainable mechanism.

Pythium

Description

Pythium species are "fungal-like" hemibiotrophic organisms and members of the family Pythiaceae. This family (phylum Oomycota, kingdom Stramenopila) includes many other economically important phytopathogens, including *Phytophthora infestans*, the causal agent of tomato blight and culprit of the infamous Irish potato famine of the 1800's. Both *Pythium* and *Phytophthora* are capable of moving through water as zoospores via flagella, and are commonly referred to as water molds. The genus *Pythium* contains species that range from non-pathogenic saprophytes, to highly pathogenic species with limited host ranges.

Survival and reproduction

Ordinarily, *Pythium* species survive saprophytically in soil because of their inability to compete with other plant pathogens (Hendrix and Campbell, 1973).

However, if oversaturated soil containing potential hosts is available (i.e. seeds or seedlings), infection can occur. The fungus is able to spread from the diseased seedling due to the separation of the host cells from the breakdown of the middle lamella. Breakdown of these cell components is likely the result of pectinolytic enzymes secreted from the hyphal tips of *Pythium* (Webster, 1970). Within the host, asexual reproduction can occur when the coenocytic mycelia develops sporangia containing numerous nuclei. Cytoplasm is transferred to the vesicle formed on top of the sporangium, and within minutes numerous zoospores are formed from cytoplasmic cleavage (Webster, 1970). The zoospores eventually jostle their way out of the vesicle, and swim away with the aid of two flagella. One tinsel and one whiplash flagella are cast off and the aquatic spore encysts. At this point, a germ tube will develop and the cycle starts again (Webster, 1970).

All *Pythium* species are homothallic. Sexual reproduction occurs when the female oogonium and male antheridium of the same hypha (homothallism) fuse together allowing their nuclei to unite and form a single zygote. The zygote, or oospore, will germinate and develop into mycelium at high temperatures (28°C). Lower temperatures (10 to 17°C) result in the formation of a vesicle, and the subsequent development of infectious zoospores (Alexopoulos et al., 1992).

Symptoms and signs

Two types of damping-off disease occur in plants susceptible to *Pythium* – pre-emergence and post-emergence. Pre-emergence damping-off results in death of the germinating seed and therefore no emergence. When the seed is able to germinate and

the seedling emerges from the soil, the plant may still be susceptible to infection. Successful infection at this stage is referred to as post-emergence damping-off and is characterized by cotyledon and leaf chlorosis, along with a watery rot in the taproot and hypocotyl at or near the soil line. Plants with damaged root systems may continue to grow, and possibly remain green for a few weeks, or appear stunted to varying degrees. Eventually the seedling will collapse.

In addition to damping-off disease, *Pythium* species can cause disease in older seedling and mature plants. In these plants, the fungus is usually limited to the cortex, and the plant may not die. Nonetheless, the root system has been compromised by the pathogen, and therefore the plant will exhibit slower growth and a reduced yield. Root rot of mature plants results in death of feeder roots followed by development of lesions up to 2 cm long on the lateral roots (Agrios, 1997). The size of the lesions increases and the plant shows aboveground symptoms of wilt, chlorosis and necrosis. In cucurbit root rots, fruit becomes exposed to sunburn due to leaf wilt and fruit quality is reduced (Agrios, 1997). A cottony growth can appear on fleshy fruits that come in contact with infested soil and the interior will become a rotted mass called leak (Agrios, 1997).

Epidemiology

Pythium species have been isolated from soil of arable land, pastures, forests, nurseries, marshes, swamps, and water (Agrios, 1997; Trigiano et al., 2004). They occur most abundantly in cultivated soils near the root region in superficial soil layers, and less commonly in uncultivated or acid soils where fungistasis keeps them suppressed (Agrios, 1997; Trigiano et al., 2004). Soil from a given field may contain several pathogenic

Pythium species. Excessive soil moisture of 70% or greater, low light, poor nutrient availability, and temperature all play a role in the survival of Pythium. Moist soil is particularly important for *Pythium* species. Water is required for the movement of spores. Moist soil is conducive to disease development because the low oxygen level encourages the release of exudates from seeds. *Pythium ultimum* Trow, *P. irregulare* Buisman, and *P. debaryanum* Hesse inhabit cool, moist soil as saprophytes on crop residues (Ben-Yephet and Nelson, 1999; Van der Plaats-Niterink, 1981). *Pythium aphanidermatum* (Edson) Fitzp. and *P. myriotylum* occur in warm, moist soil (Ben-Yephet and Nelson, 1999; Van der Plaats-Niterink, 1981). These exudates stimulate the growth of *Pythium* towards the emerging plant tissue. As seedlings continue to grow, the risk of plant death from post-emergence damping-off decreases.

Control

Sanitation is important for controlling *Pythium* spp. because the resting spores can survive in dust, planting medium, water, or in soil particles on greenhouse floors and in flats and pots (Abawi and Widmer, 2000). Removal and disposal of diseased plants is essential for control. Growing medium also must be sterilized in order to ensure that all possibilities for dispersal are eliminated. Steaming the growing medium (60°C; 30 minutes) is a safe cultural control method (Abawi and Widmer, 2000). Alternatively, adding organic matter can help decrease disease incidence by increasing competitive microbial populations (Abawi and Widmer, 2000). Other environmentally acceptable means of control include crop rotation, better soil drainage and air circulation, and reduced nitrogen fertilization (Agrios, 1997). Raised beds often are used to aid draining

of the soil immediately surrounding field plants (Anonymous, 2005). There are no *Pythium*-resistant cultivars available at present.

Chemical treatments are available for control of plant diseases caused by *Pythium*. Plants and growing media can be chemically treated to aid in disease control (Abawi and Widmer, 2000). Metalaxyl, chloranil, and captan are commercially available for seed, bulb, and seedling treatments. Other fungicides for seed and bulbs only include chloroneb, mancozeb, and thiram (Agrios, 1997; Trigiano et al., 2004; Holmes et al., 2005). Chemicals commonly used for field tomatoes in the southeast are fosetyl-Al and mefenoxam (Holmes et al., 2005).

***Pythium myriotylum* Drechsler**

Pythium blight of tomato caused by *P. aphanidermatum* is recognized as one of the major diseases in the production of transplants (Jaworski, et al., 1967). At 35°C, both *P. myriotylum* and *P. aphanidermatum* cause severe damage to tomato plants, but *P. myriotylum* causes more severe rot at 23°C (Littrell and McCarter, 1969). Since environmental conditions in the Piedmont and mountain regions of the southeastern United States tend to be cooler than the coastal plain, *P. myriotylum* may be much more destructive in these areas of the southern U.S. (Littrell and McCarter, 1969).

Pythium myriotylum is one of the most common species found in greenhouse production systems due to its broad host range (Ben-Yephet and Nelson, 1999). *Pythium myriotylum* isolated from root systems of bell peppers was also pathogenic to tomato, causing severe weight loss (Chellemi et al., 2000). Four *Pythium* species were isolated

from diseased hydroponic tomato roots, one of which was *P. myriotylum* (Jenkins and Averre, 1983).

Research Goals

The first goal of this research was to determine the impact of *Monarda* herbage, *Beauveria bassiana*, and PGPR on damping-off of tomato seedlings caused by *Pythium myriotylum*. The specific objectives were to determine: (i) if herbage of *Monarda* sp. used as a soil amendment is capable of suppressing damping-off of tomato seedlings; (ii) if conidia of *B. bassiana* isolates used as seed coatings could suppress Pythium damping-off of tomato seedlings; (iii) if a commercial form of plant growth promoting rhizobacteria used as a soil drench could suppress Pythium damping-off of tomato seedlings; (iv) if combinations of treatments are synergistic or antagonistic. A second goal of this research was to compare 16 *Monarda* herbage treatments for suppression of damping-off of tomato seedlings caused by *Pythium myriotylum*. The specific objectives were to determine: (i) if herbage of any of 16 *Monarda* varieties could suppress Pythium damping-off of tomato seedlings; (ii) if the herbage caused any significant effects in tomato growth.

PART II

EVALUATION OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN, BIOYIELD (A COMMERCIAL PGPR), AND *MONARDA* SP. AS BIOLOGICAL CONTROLS FOR DAMPING-OFF OF TOMATO SEEDLINGS CAUSED BY *PYTHIUM MYRIOTYLUM*

Abstract

Diseases of tomato transplants cause significant economic losses each year. Pythium damping-off is difficult to control in transplant production because it is dispersed by irrigation water and is well adapted to greenhouse temperatures. In the first part of this study, *Beauveria bassiana* (TN isolate 11-98 and BotaniGard), a mixture of plant growth-promoting rhizobacteria (PGPR; BioYield), and three varieties of *Monarda* ('Cerise,' 'Puerto Purification,' and 'Violet Queen') were investigated for efficacy as biological controls against *Pythium myriotylum*, a causal agent of damping-off in tomato (*Lycopersicon esculentum* Mill. 'Celebrity' and 'Mountain Spring'). Tomato seeds were coated with or without one of the *Beauveria* treatments, drenched with or without the PGPR treatment, placed in medium amended with or without one of the three *Monarda* herbage (dried flowers and leaves) treatments, and challenged with or without *Pythium myriotylum*. A sub-sample of each *Monarda* herbage sample was collected at the beginning of each repetition of the experiment; sub-samples were extracted in hexane and analyzed by gas chromatography-mass spectrometry. When challenged with the pathogen, *Beauveria* treatments increased survival (%) of 'Mountain Spring' seedlings. Herbage from 'Puerto Purification' increased growth (stem diameter and shoot height) and decreased disease in 'Celebrity' seedlings. Treatments with PGPR had no effect on either cultivar. Results with 'Puerto Purification' herbage indicate that *Monarda* herbage has potential as a biological control against Pythium damping-off in tomato seedlings. Further studies are necessary to identify the capabilities of *Monarda* herbage in response to other pathogens.

Introduction

Pythium sp. threaten tomato transplant production because of the ability of the zoospores to travel through water and survive harsh periods as resting spores. *Pythium myriotylum* can cause economic loss of tomato transplant production, particularly in the Piedmont and mountainous regions of the southern United States.

Beauveria bassiana has the potential to act as a dual-purpose biological control organism. Isolates of *B. bassiana* are available commercially as BotaniGard, Mycotrol, and Naturalis, and function effectively as entomopathogens. *Beauveria bassiana* isolate 11-98 (Bb 11-98) endophytically colonized cotton (Griffin et al., 2005), tomato (Leckie, 2002; Ownley et al., 2004), and snap bean (Ownley et al., unpublished data) when seeds were treated with conidial suspensions of the fungus. Seed treatment with BotaniGard was not known to be endophytic, although it has been observed (Ownley et al., unpublished data). *Beauveria* was isolated from all three plants (Griffin et al., 2005; Ownley et al., 2004) using selective medium. (Doberski and Tribe, 1980). Tomato seed were coated with *B. bassiana* conidia and grown in medium inoculated with mycelium of the fungus; those seedlings had the greatest survival compared to other treatments when challenged with *Rhizoctonia solani* (Seth, 2001).

Monarda varieties are known for the aromatic nature of their leaves and flowers. The essential oils of *Monarda* are well documented, and the composition of the essential oil obtained from *Monarda* is diverse. Many essential oils and their specific constituents are known for having antifungal properties (Sridhar et al., 2003; Kishore and Dubey, 2002). Adding herbage (dried and ground leaves and flowers) of *Monarda* sp. to planting

medium reduces disease-loss to *Rhizoctonia* and kills sclerotia of *Sclerotinia* (Gwinn et al., 2003; Gwinn et al., unpublished). Herbage of two *Monarda* varieties ('Elsie's Lavender' and 'Marshall's Delight') increased percent germination and plant height of tomato in *Rhizoctonia*-infested medium (Gwinn et al., 2003).

Plant growth-promoting rhizobacteria (PGPR) are known to increase plant growth and induce resistance to pathogens in plants. Rhizobacteria inhabit the area immediately surrounding plant roots and the roots themselves. Generally, plants will have increased shoot and root growth, an increase in stem diameter, rapid development of new roots, and less transplant shock (Kloepper et al., 2004). In greenhouse assays, incidence and severity of damping-off of tomato seedlings caused by *Rhizoctonia solani* was significantly reduced when seeds were inoculated with rhizobacteria isolated previously from the rhizosphere of tomato plants (Gupta et al., 1995). When challenged with *Pythium aphanidermatum*, and pre-treated with *Pseudomonas florescens* isolate Pf1, tomato and hot pepper (*Capsicum annuum* L.) plants showed increased and earlier activities of the defense-related enzymes, phenylalanine ammonia lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) (Ramamoorthy et al., 2002). An investigation of two *Bacillus* species (*B. subtilis* GB03 and *B. amyloliquefaciens* IN937a) and chitosan added as a soil amendment in transplants of tomato, bell pepper, cucumber and tobacco, resulted in the commercialization of the mixture, called BioYield, which is used as a seed or soil drench (Kloepper et al., 2004; Kokalis-Burelle et al., 2003; Kokalis-Burelle et al., 2002).

In order to investigate possibilities for using *Monarda*, *Beauveria*, and PGPR as biological controls for damping-off of tomato seedlings, experiments were conducted to test each type of control alone and in combination. The specific objectives were to determine: (i) if herbage of *Monarda* sp. used as a soil amendment could suppress Pythium damping-off of tomato seedlings; (ii) if conidia of *Beauveria bassiana* isolates used as seed coatings could suppress Pythium damping-off of tomato seedlings; (iii) if a commercial form of plant growth promoting rhizobacteria used as a seed drench could suppress Pythium damping-off of tomato seedlings; and (iv) if combinations of the herbage and the seed treatments are synergistic or antagonistic.

Materials and Methods

GC-MS analysis of herbage

Three varieties of *Monarda didyma* ('Cerise', 'Violet Queen', and 'Puerto Purification') were grown in the Monarda Evaluation Gardens – UT Gardens, Knoxville, TN. Herbage consisted of leaves and flowers, dried and ground to pass a 5-mm mesh sieve, and then stored in sealed Mason jars (Ball Corporation, Broomfield, CO). Prior to experiments, herbage samples of each variety from 2003 and 2004 were mixed, and mixtures were used in all experiments except replicate 1, in which single collections from 2003 were used. Each time a jar of herbage was opened for soil amendment, a sub-sample was collected and stored at -80°C. A 5-mg aliquot was shaken in 5 ml hexane for 24 h, and 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. Essential oil content was

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 a 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.

Growth chamber study

***Beauveria bassiana* preparation.** *Beauveria bassiana* isolate 11-98 (Bb 11-98) (B. H. Ownley, University of Tennessee) was originally isolated by Roberto Pereira from an infected click beetle (Coleoptera: Elateridae) in Scott County, TN. Conidia from Bb 11-98 were re-isolated from seedlings grown from treated tomato seed and used to establish stock cultures (see Fungal isolation). The fungus was grown on Sabouraud's dextrose agar (SDA) (Difco, Becton, Dickenson & Co., Sparks, MD) at room temperature for approximately 4 weeks. Conidia were harvested by brushing the plate surface with a camel hair brush (Fisher Scientific, Fair Lawn, NJ) and collecting conidia in sterile glass vials. Vials were stored at 4°C. BotaniGard 22 WP, a registered, commercially available *Beauveria bassiana* conidial preparation (strain GHA, BioAgriculture Corporation, Butte, MT) was stored at 4°C.

Seed treatments. Tomato seed (250) 'Mountain Spring' or 'Celebrity' (Syngenta Seeds, Inc., Downers Grove, IL) were coated with *Beauveria bassiana* using a 2% methylcellulose solution containing conidia. Seeds were sterilized in a Petri dish for 15 min in 10% Clorox, rinsed with deionized water, and then rinsed again for 2 min in deionized water. Seeds were allowed to air dry. Methylcellulose (2g) (Sigma Chemical Co., St. Louis, MO) was stirred into 100 ml of deionized water that had been autoclaved for 30 min. The mixture was stirred until a cloudy solution formed, then transferred to an ice bath and stirred until clear. Methylcellulose solution was mixed with the *Beauveria* conidia (0.067 g Bb 11-98 or 0.0228 g BotaniGard) and 10 µL of Tween 20 (Fisher Scientific, Fair Lawn, NJ), along with the tomato seeds (250) to obtain 1.4 x 10^5 conidia per seed. Seeds were stirred until coating appeared uniform and left to dry in a biological safety cabinet (Sterilgard hood, the Baker Co., Inc, Sanford, ME) overnight. All seeds were stored at 4°C until use.

Fungal isolation. In order to confirm the endophytic capability of Bb 11-98 in tomato, 'Mountain Spring' seeds were coated as previously described and grown under gnotobiotic conditions. Test tubes [24-mm (1-in) O. D. and 15-cm (6-in) long] were filled with 20 cm^3 medium grain vermiculite and 20 ml deionized water, capped, and sterilized for 30 min by autoclave. One coated seed was placed in each tube and placed in continuous light for 72 h. The tubes were then placed in a growth chamber (Environmental Growth Chambers, Model # Q113a2, Chagrin Falls, OH) at 25°C with a 12/12 light/dark regimen. Seedlings were grown for 2 weeks, then surface sterilized in a 95% ethanol soak for 1 min, followed by a 20% bleach soak for 3 min, and a final 95%

ethanol soak for 1 min. Plants were then rinsed with deionized water and placed in a sterile Petri dish to dry in a laminar flow hood. Pieces (approx. 5 mm length) of the seedlings were excised with a sterile scalpel and placed on Sabouraud's dextrose agar or *Beauveria*-selective media (Doberski and Tribe, 1980). Selective medium was 40 g glucose (Mallinckrodt Inc., Paris, KY), 10 g neopeptone (Difco, Becton, Dickenson & Co., Sparks, MD), 15 g agar (Sigma Chemical Co., St. Louis, MO) and 0.01 g crystal violet (Sigma Chemical Co.) in 1 liter deionized water. Formulated medium was autoclaved for 45 min. Cyclohexamide (0.25 g) was autoclaved separately, mixed with 0.5 g chloramphenicol, and added to cooled, sterilized medium. Leaves on both selective and nonselective media were monitored weekly for endophytic growth.

Soil amendment with *Monarda didyma* herbage. Germination mix (BM2, Berger Peat Moss, Inc. Saint Modeste, Quebec) was autoclaved for 90 min on 2 consecutive days. Herbage (10% v/v) of the appropriate variety was added to the sterile mix.

Preparation of plant growth-promoting rhizobacteria. BioYield Flowable (Gustafson LLC, Plano, TX), a PGPR solution, (0.0003% in deionized H_2O) was used as a seed drench.

Pathogen preparation. A culture of *Pythium myriotylum* acquired from B. H. Ownley (University of Tennessee, Knoxville) was maintained on potato dextrose agar (Fisher Scientific, Fair Lawn, NJ) at room temperature until use. Zoospore production was induced with a protocol modified from Mitchell and Rayside (1986). A 5-mm plug of *P. myriotylum* was placed on V-8 juice agar (200 ml clarified V-8 juice; 3 g CaCO3;

15 g agar; 10 g maltose; 800 ml deionized water) and allowed to grow for 2 days at room temperature. Three 5-mm plugs acquired from the edges of the 2-day-old culture were placed in 15 ml V-8 juice broth (200 ml clarified V-8 juice; 3 g $CaCO_3$; 10 g maltose; 800 ml deionized water) in Petri plates. Broth cultures were incubated at 25°C in the dark. After 24 hours, plates were drained and washed 3 times with 25 ml aliquots of sterile MES (2 – [N – Morpholino] ethanesulfonic acid, ACROS Organics USA, Morris Plains, NJ) buffer (0.1 g MES/5 L deionized water, pH 6.2). After washing, plates were flooded with 15 ml MES buffer and incubated at 25°C under light for 18 to 24 hours. Plates were washed 3 times with 25 ml-aliquots of MES buffer and then incubated in 15 ml MES buffer at 25°C in light. After 5 hours, zoospores were released, and the liquid was added to the Cone-tainers (Cone-tainers, Stuewe and Sons, Corvallis, OR). Cone-tainers were plugged with absorbent cotton balls (Fisher Healthcare, Houston, TX) to avoid soil loss without eliminating drainage.

Experimental design. The experiment was designed as a 2 x 3 x 4 x 2 (pathogen/no pathogen x 11-98/ BotaniGard/none x 3 *Monarda*/none x PGPR/none) factorial in a randomized complete block (RCB). The RCB design was generated using Statistical Analysis System (SAS Institute, Inc., Cary, NC). One block consisted of 48 randomized treatments (Table 2.1; all tables and figures are located in Appendix I and II) of one cultivar of tomato seeds (Mountain Spring or Celebrity). There were a total of 6 blocks per cultivar and two observations (seeds) per treatment. Cone-tainers were filled with 200 ml of sterilized germination mix or germination mix amended with herbage (10%) as described above. To achieve flooding conditions conducive to pathogen

growth, latex sleeves (Durex Consumer Products, Norcross, GA) were placed on the Cone-tainers, and each was watered with 75 ml deionized H_2O. Two seeds were added to each cone-tainer. For PGPR treatments, seeds were treated with 1 ml of the 0.0003% solution. Pathogen (15ml) prepared as described above was added to 24 of the 48 Cone-tainers. Germination mix or amended germination mix was placed over the seeds. Cone-tainers were covered with clear wrap and incubated at 25°C with 12/12 light/dark condition. After 48 hours, the latex sleeves were removed. The clear wrap was removed when seedlings were visible, and Cone-tainers were watered thereafter as needed. Plants were grown in the growth chamber for 6 weeks, and were fertilized once at 4 weeks with Peters Professional 20-20-20 (Spectrum Group, St. Louis, MO).

Data collection and analysis. GC-MS analysis of herbage was repeated at the beginning of each experiment. Constituent concentrations were calculated from a standard curve. Arithmetic mean and standard error was calculated for each component. Germination data were recorded every 2-3 days for 2 weeks once the first seedlings emerged. Any decline of seedlings was recorded thereafter. At the end of six weeks, plants were removed, shoot height and diameter were measured, and a disease rating was assigned. Ratings were assigned on a scale of 1 to 7 (Table 2.2). All data were analyzed using PROC MIXED. Significant effects were further analyzed with an F-protected LSD ($P = 0.05$) (SAS Institute, Inc., Cary, NC).

Results

GC – MS analysis of herbage

All retention times and concentrations of oils are found in Appendix I (Table 2.3, 2.4, 2.5). Borneol, bornyl acetate, cineole, linalool, and myrcene were found in 'Cerise,' but in very small (less than 2 μM) amounts in the other two varieties. Terpineol was present in small concentrations in all varieties. Carvacrol concentration in 'Violet Queen' was at least 30-fold greater than the other two varieties. Concentration of cymene in 'Puerto Purification' was approximately half of the concentration found in 'Cerise' and 'Violet Queen.' Concentration of limonene was similar in all varieties. Concentration of 1-octen-3-ol was lowest in 'Puerto Purification' (34 μM) and highest in 'Cerise' (122 μM). Concentrations of α-pinene and γ-terpinene in 'Cerise' were 4 and 6 times greater, respectively, than in 'Violet Queen,' and present only in small amounts in 'Puerto Purification.' Thymol (105 μM) and thymoquinone (402 μM) concentrations were highest in 'Puerto Purification.' Relative concentrations of thymol and thymoquinone were reversed in 'Violet Queen' and 'Cerise.' 'Cerise' had ca. 10 fold more thymol than thymoquinone, whereas 'Violet Queen' had ca. 80 fold more thymoquinone than thymol (Table 2.3, 2.4, 2.5)

Growth chamber study

Achieved P-values for all tomato seedling experiments are in Table 2.6.

Stem diameter. *Beauveria* had no significant effect on stem diameter of 'Celebrity' or 'Mountain Spring' tomato seedlings (Table 2.6). With the addition of 'Cerise' or 'Puerto Purification' herbage, stem diameter was not different from the

'Celebrity' control, whereas addition of 'Violet Queen' herbage decreased stem diameter in 'Celebrity' seedlings (Table 2.7). In 'Mountain Spring' seedlings, addition of 'Cerise' herbage significantly decreased stem diameter compared to all other treatments, which were not different (Table 2.8). Pathogen treatment was significant in both tomato cultivars, with a decrease in stem diameter in the presence of *Pythium* (Table 2.7 and 2.8). A significant interaction was present with *Beauveria* and pathogen treatment in 'Mountain Spring' seedlings (Table 2.6). Stem diameters of treatments with either Bb 11-98 or BotaniGard without the pathogen were not different from the untreated, uninfested control (Table 2.8). When compared to the untreated, pathogen-infested control, both *Beauveria* treatments had significantly higher stem diameters (Table 2.9). PGPR had no effect on 'Celebrity' or 'Mountain Spring' stem diameters, either alone or in combination with the other treatments. Also, there was no significant interaction observed with herbage and pathogen treatment in either tomato cultivar (Table 2.6).

Shoot height. Treatment with *Beauveria* had no significant effect on shoot height of 'Celebrity' or 'Mountain Spring' tomato seedlings (Table 2.6). 'Celebrity' seedlings had decreased shoot height, compared to the untreated control, with the addition of 'Cerise' or 'Violet Queen' herbage, while addition of 'Puerto Purification' was not different from the control (Table 2.10). Shoot height of 'Mountain Spring' seedlings was decreased with the addition of 'Cerise', 'Puerto Purification', and 'Violet Queen' compared to the untreated control (Table 2.11). Both 'Celebrity' and 'Mountain Spring' tomato seedlings had significantly decreased shoot height when challenged with *P. myriotylum* (Table 2.10 and 2.11). PGPR had no significant effect on the shoot height of

either cultivar, whether alone or in combination with other treatments. No significant interactions were observed with any treatments in either cultivar.

Disease index. Disease index of 'Celebrity' tomato seedlings was significantly affected by *Beauveria*, herbage, and *Pythium* treatments (Table 2.12). Both Bb 11-98 and BotaniGard slightly increased disease index. All herbage treatments were not different from the control, except for 'Violet Queen,' which increased disease. There was a significant increase in disease index in both 'Celebrity' and 'Mountain Spring' when the pathogen was present (Table 2.12 and 2.13). No other treatments were significant in 'Mountain Spring,' including PGPR, which was also not significant in 'Celebrity.' There was a significant interaction between herbage and pathogen in 'Celebrity' disease index, with 'Violet Queen' increasing disease index in the uninfested plants compared to all other uninfested treatments (Table 2.14). In pathogen-treated seedlings, disease index was not different with the addition of 'Cerise' or 'Violet Queen' herbage compared to the control. 'Puerto Purification' herbage significantly decreased disease index when compared to the control. No other interaction resulted from the analysis.

Survival. Treatment with BotaniGard decreased survival in 'Celebrity' and increased survival in 'Mountain Spring' (Table 2.15 and 2.16). Survival was not different than control or BotaniGard with Bb 11-98 treatment in either cultivar. There was a significant effect of herbage (Table 2.6) in 'Celebrity' although none of the treatments were different from the control (Table 2.15). Pathogen presence significantly decreased survival in 'Celebrity' and 'Mountain Spring' seedlings (Table 2.15 and 2.16). PGPR, alone or in combination with other treatments, had no significant effect in either

cultivar. A significant interaction between *Beauveria* and *Pythium* was observed in 'Mountain Spring' (Table 2.17). In the uninfested treatments, both Bb 11-98 and BotaniGard had the same survival as the untreated control. Survival was increased with *Beauveria* treatment in the presence of the pathogen when compared to the infested control (Table 2.17).

Discussion

Composition of essential oils of *Monarda* is diverse. In our laboratory, hexane extracts are used to evaluate chemical constituents of large numbers of *Monarda* samples, because this method is inexpensive and rapid. Essential oil profiles generated by this method should be used only for comparison among varieties because only compounds soluble in hexane are extracted. Our data are consistent with chemical compositions reported by Duke (2006).

Many essential oils and their specific constituents are active against *Pythium* spp. (Sridhar et al., 2003; Kishore and Dubey, 2002). *Pythium myriotylum* was sensitive to most compounds listed in Tables 2.3, 2.4, and 2.5 (Clark, unpublished). When applied as a seed soak, the essential oils of *Chenopodium ambrosioides* (Chenopodiaceae) reduced damping-off of tomato in soil infested with *P. aphanidermatum* or *P. debaryanum* by 66.7 and 100%, respectively (Kishore and Dubey, 2002). Oils from *Lippia alba* (Verbenaceae) reduced damping-off of tomato in soil infested with *P. aphanidermatum* or *P. debaryanum* by 88.9 and 71.3%, respectively (Kishore and Dubey, 2002). Essential oil of thyme (*Thymus vulgaris* L.) that contained 50% thymol inhibited growth of

Pythium ultimum (Zambonelli et al., 1996). Similarly, the best control of *P. myriotylum* in this study was achieved by 'Puerto Purification,' the variety with the highest thymol content. Results from the present study are strong indicators of the capabilities of plant natural products as biological controls for soilborne pathogens.

Although there was a significant pathogen effect in both 'Celebrity' and 'Mountain Spring' tomato seedlings, based on the survival (%) rates in pathogen treatments (50.7 and 63.9%, respectively), it is evident that 'Celebrity' is slightly more susceptible to *P. myriotylum*. Disease index was also higher in 'Celebrity.' 'Celebrity' had a disease index of 4.6, and 'Mountain Spring' had 4.1. Both 'Mountain Spring' and 'Celebrity' have been cultivated because of their resistance to Fusarium and Verticillium wilts, but there are no known cultivars resistant to Pythium damping-off (Sanders, 2001).

Treatment with BotaniGard had a negative impact on 'Celebrity,' with an increase in disease index and a decrease in survival. Although Bb 11-98 also increased disease index, there was no difference in survival with treatment of Bb 11-98 compared to the control. Stem diameter (mm) of 'Mountain Spring' seedlings treated with Bb 11-98 or BotaniGard was not different when compared with the control. Survival (%) was increased with BotaniGard and not different with Bb 11-98 when compared to the control. However, when challenged with the pathogen, the treatments with Bb 11-98 or BotaniGard increased stem diameter and survival compared to the infested control. Seth (2001) found that 'Mountain Spring' seeds treated with *B. bassiana* 11-98 conidia had a seedling survival rate of 70% when challenged with *Rhizoctonia solani*, although germination was delayed. However, Bishop (1999) found treatment of 'Mountain

Spring' seeds with *B. bassiana* significantly suppressed pre-emergence damping-off caused by *R. solani*.

The present results indicate that treatment with *Beauveria* may be cultivar specific, since plant stand of 'Mountain Spring' was increased in response to treatment, whereas 'Celebrity' was not. However, it is difficult to conclude that *Beauveria* treatment in 'Celebrity' was completely ineffective, due to the flooded environment and amended soil to which the seeds were subjected. It is possible that a higher rate of conidia would prove more protective in 'Celebrity.' In an experiment investigating the effectiveness of seed treatments on tomato stand when challenged with *Rhizoctonia solani*, *Beauveria* treatment significantly increased plant stand in 'Mountain Spring' when compared to the infested control (Bishop, 1999). In light of the results with 'Mountain Spring,' it would be beneficial to conduct more studies with *Beauveria* treatments in this cultivar when challenged with *P. myriotylum* and other pathogens.

Treatment with herbage had significant effects in both 'Celebrity' and 'Mountain Spring.' Greenhouse and laboratory studies have shown that adding herbage (dried and ground leaves and flowers) of *Monarda* sp. to planting medium inhibits growth of *Rhizoctonia* and *Fusarium*, and kills sclerotia of *Sclerotinia* (Gwinn et al., 2003; unpublished data). Herbage from 'Puerto Purification' increased plant growth and survival in 'Celebrity', while 'Violet Queen' had the opposite effect; there was a decrease in plant growth and survival. When challenged with the pathogen, 'Puerto Purification' suppressed disease, whereas 'Violet Queen' enhanced disease compared to the infested control. Disease index in 'Celebrity' uninfested treatments was the same for control

'Cerise' and 'Puerto Purification.' The disease index was increased in the uninfested 'Violet Queen' treatments. Although 'Puerto Purification' and 'Violet Queen' did not differ in stem diameter compared to the control, all three herbage treatments slightly decreased shoot height in 'Mountain Spring' seedlings. This result is not unusual; tests have shown that amendment with *Monarda* herbage often delays germination (Gwinn et al., unpublished data). It is possible that the release of essential oil constituents into the medium caused initial stunting. Over time, this effect may be lost by flushing the growing medium as plants are watered. Plants were fertilized once at 4 weeks and measured at 6 weeks, which may have had a bearing on the small plant size as well. These results differ from those found by Gwinn et al., (2003), where herbage of two *Monarda* varieties ('Elsie's Lavender' and 'Marshall's Delight') increased percent germination and plant height of tomato in *Rhizoctonia*-infested medium.

The difference in herbage treatments is likely due to the chemical composition of the plants. The main constituents found in 'Violet Queen' are carvacrol, cymene, and thymoquinone. Carvacrol has inhibitory effects against several pathogens, including *Pythium myriotylum* (this study). 'Puerto Purification' contains mostly thymol, thymoquinone, and cymene. Thymol has also shown inhibitory effects against *Pythium myriotylum* and several other pathogens (Sridhar et al., 2003; this study). Thymol and carvacrol are isomers, and therefore it is not unusual that herbage high in one of the compounds would have much less of the other. In addition, the fact that 'Violet Queen' (high in carvacrol) appears to be toxic to both plant and fungus, 'Puerto Purification' (high in thymol) was not toxic to the plant, and 'Cerise' (without a dominant constituent)

had little effect either way, demonstrates that the diversity of constituent concentrations among *Monarda* varieties is important. Therefore, combining varieties that differ chemically should be investigated as a means of biological control of soilborne pathogens.

PART III

EVALUATION OF PATHOGEN SUPPRESSION OF TOMATO SEEDLINGS CHALLENGED WITH *PYTHIUM MYRIOTYLUM* USING DRIED HERBAGE OF *MONARDA* SP. AS A SOIL AMENDMENT

Abstract

Damping-off in tomato seedlings causes significant losses in crop production. Herbage (dried ground leaves) from 16 different *Monarda* varieties was tested for efficacy as a soil amendment for biological control of damping-off of tomato (*Lycopersicon esculentum* cv. Mountain Spring) seedlings caused by *Pythium myriotylum*. A sub-sample of each *Monarda* herbage sample was collected at the beginning of each repetition of the experiment; sub-samples were extracted in hexane and analyzed by gas chromatography-mass spectrometry. Thirteen compounds identified in hexane extracts were tested for activity against *P. myriotylum*. All constituents inhibited mycelial growth of *P. myriotylum* at the highest dose (50µl/plate). Beta-pinene, cineole, 1-octen-3-ol, borneol acetate, terpineol, linalool, thymol, and carvacrol also inhibited growth at the lowest dose (5µl/plate). One variety, *M. didyma* 'Croftway Pink,' significantly enhanced tomato seedling growth and reduced damping-off. Three other amendments, 'Sioux', 'Mohawk', and Rose Geranium, had no negative effects on seedling growth and increased seedling survival.

Introduction

Pythium blight of tomato caused by *P. aphanidermatum* is recognized as one of the major diseases in the production of transplants (Jaworski et al., 1967). *Pythium myriotylum* is one of the most common species found in greenhouse production systems due to its broad host range (Ben-Yephet and Nelson, 1999), and the disease may spread to other plants. Isolates of *P. myriotylum* from root systems of bell peppers (*Capsicum*

annuum L.) was also pathogenic to tomato *(Lycopersicon esculentum* Mill.), causing severe weight loss (Chellemi et al., 2000). Four *Pythium* species were isolated from diseased hydroponic tomato roots, one of which was *P. myriotylum* (Jenkins and Averre, 1983).

Monarda is known for the aromatic nature of leaves and flowers. Essential oils of *Monarda* are well documented, and the composition of the essential oil obtained from *Monarda* is dependant upon species, variety and environment (Gwinn et al., 2003; Gwinn et al., unpublished data). Typically, *Monarda* spp. as well as other mints contain high concentrations of monoterpenes and alcohols (e.g. thymol, carvacrol, or cymene).

Many essential oils and their specific constituents are antifungal. In an analysis of essential oils from numerous Indian herbs for inhibition of 20 phytopathogenic fungi, significant inhibition of all fungi was observed with cymbopogan, ajowan, and dill seed oils (Sridhar et al., 2003). The bioactive ingredients of the oils were identified as the simple monoterpenes geraniol, thymol, and carvone, respectively (Sridhar et al., 2003). The oils from both *Chenopodium ambrosioides* (Chenopodiaceae) and *Lippia alba* (Verbenaceae) had no phytotoxic effects when assayed using seed germination and seedling growth as parameters, and they also proved more efficient in pathogen inhibition than synthetic pesticides (Kishore and Dubey, 2002).

Greenhouse and laboratory studies have shown that adding herbage (dried and ground leaves and flowers) of *Monarda* sp. to planting medium inhibits growth of *Rhizoctonia*, and kills sclerotia of *Sclerotinia* (Gwinn et al., 2003; Gwinn et al., unpublished data). Herbage of two *Monarda* varieties ('Elsie's Lavender' and

'Marshall's Delight') increased percent germination and plant height of tomato in *Rhizoctonia*-infested medium (Gwinn et al., 2003). There was also a decrease in disease index with the addition of 'Elsie's Lavender' in tomato (Gwinn et al., 2003). Fruit quality and quantity were affected when plants were grown in media amended with herbage (Greene, 2005). In order to test the potential of *Monarda* sp. as a biological control for damping-off caused by *Pythium myriotylum*, 16 varieties were analyzed for chemical composition and utilized as soil amendments in growth chamber assays with tomato seedlings.

Materials and Methods

GC-MS analysis of herbage

Sixteen varieties of *Monarda* sp. (Table 3.1) were grown in the Monarda Evaluation Gardens – UT Gardens, Knoxville, TN. Leaves were collected in summer 2005, dried and ground to pass a 5-mm mesh sieve (herbage), and then stored in sealed Mason jars (Ball Corporation, Broomfield, CO). Each time a jar was opened for soil amendment, a sub-sample of herbage was collected and stored at -80°C. A 5-mg aliquot was shaken in 5 ml hexane for 24 h, and 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. Essential oil content was analyzed 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 a 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.

Essential oil assay

Toxicity against *P. myriotylum* of 13 essential oils found in *Monarda* spp. was assayed prior to investigations with dried herbage as a soil amendment for the biological control of Pythium damping-off in tomato seedlings. The inverted Petri dish technique was used for evaluation (Maruzzella et al., 1959; 1960). One 5-mm plug of *P. myriotylum* was placed on a plate of potato dextrose agar (Fisher Scientific, Fair Lawn, NJ), with a layer of Whatman No. 1 filter paper containing 0, 5, or 50 µL of one essential oil in the lid above the plug. Plates were incubated at 25°C. Colony diameter (cm) was measured after 3 days.

Growth chamber study

Soil amendment with *Monarda didyma* herbage. Germination mix (BM2, Berger Peat Moss, Inc. Saint Modeste, Quebec, Canada) was autoclaved for 90 min on 2 consecutive days. Herbage (10% v/v) was added to the sterile mix.

Pathogen preparation. A culture of *Pythium myriotylum* acquired from B. H. Ownley (University of Tennessee, Knoxville) was maintained on potato dextrose agar (Fisher Scientific, Fair Lawn, NJ) at room temperature until use. Zoospore production

was induced with a protocol modified from Mitchell and Rayside (1986). A 5-mm plug of *P. myriotylum* was placed on V-8 juice agar (200 ml clarified V-8 juice; 3 g $CaCO_3$; 15 g agar; 10 g maltose; 800 ml deionized water) and allowed to grow for 2 days at room temperature. Three 5-mm plugs acquired from the edges of the 2-day-old culture were placed in 15 ml V-8 juice broth (200 ml clarified V-8 juice; 3 g CaCO3; 10 g maltose; 800 ml deionized water) in Petri plates. Broth cultures were incubated at 25°C in the dark. After 24 hours, plates were drained and washed 3 times with 25 ml aliquots of sterile MES (2 – [N – Morpholino] ethanesulfonic acid, ACROS Organics USA, Morris Plains, NJ) buffer (0.1 g MES/5 L deionized water, pH 6.2). After washing, plates were flooded with 15 ml MES buffer and incubated at 25°C under light for 18 to 24 hours. Plates were washed 3 times with 25 ml-aliquots of MES buffer and then incubated in 15 ml MES buffer at 25°C in light. After 5 hours, zoospores were released, and the liquid was added to the Cone-tainers (Cone-tainers, Stuewe and Sons, Corvallis, OR). Cone-tainers were plugged with absorbent cotton balls (Fisher Healthcare, Houston, TX) to avoid soil loss without eliminating drainage.

Experimental design. The experiment was designed as a completely random split-split block. The main plot was divided by pathogen/no pathogen treatment, and the sub-plot was divided based on *Monarda* treatment (with or without). Each sub-plot contained 16 completely randomized treatments, giving the entire block a total of 64 treatments. There were a total of 6 reps with two observations (seeds) per treatment. Cone-tainers were filled with 200 ml of sterilized germination mix or germination mix amended with herbage (10%) as described above. To achieve flooding conditions

conducive to pathogen growth, latex sleeves (Durex Consumer Products, Norcross, GA) were placed on the Cone-tainers, and each was watered with 75 ml deionized H_2O. Two tomato seeds (*Lycopersicon esculentum* Mill. cv. Mountain Spring, Park Seed Wholesale, Inc., Greenwood, SC) were added to each Cone-tainer. Pathogen (15 ml) prepared as described above was added to 32 of the 64 Cone-tainers. Germination mix or amended germination mix was placed over the seeds. Cone-tainers were covered with clear plastic wrap and incubated at 25°C with 12/12 light/dark condition. After 48 hours, the latex sleeves were removed. The clear wrap was removed once seedlings were visible, and Cone-tainers were watered thereafter as needed. Plants were maintained in the growth chamber for 6 weeks and fertilized once at 4 weeks with Peters Professional 20-20-20 (Spectrum Group, St. Louis, MO).

Data collection and analysis. Data from the essential oil assay were obtained by measuring colony growth (diameter in cm). For analysis, percentage growth was then calculated in reference to an untreated control [(treated/control) x 100]. GC-MS analysis of herbage was repeated at the beginning of each experiment, and constituent concentrations were calculated from standard curves. Arithmetic mean and standard error was calculated for each component. Germination data were recorded every 2-3 days for 2 weeks once the first seedlings emerged. Any decline of seedlings was recorded thereafter. At the end of six weeks, plants were removed, shoot height and diameter were measured, and a disease rating was assigned. Ratings were assigned on a scale of 1 to 7 (Table 2.2). All data were analyzed using PROC MIXED. Significant

effects were further analyzed with an F-protected LSD of $P = 0.05$ (SAS Institute, Inc., Cary, NC).

Results

GC – MS analysis

Hexane extracts contained hydrocarbons, straight-chain alcohols, phenolics, and a sesquiterpene. Myrcene, cymene, γ-terpinene, and limonene were present in various percentages in Monarda varieties (Fig. 3.1). For most varieties, cymene was the primary hydrocarbon, however, for Rose Geranium and Lavender, myrcene was the primary hydrocarbon. Percentage of straight-chain alcohols varied among varieties (Fig. 3.2). Concentration of 1-octen-3-ol was the highest in all varieties except 'Marshall's Delight,' Mixed Purple, Red, and 'Prairie Night'. Linalool was the primary straight-chain alcohol in Mixed Purple and 'Prairie Night'. Percentage of bornyl acetate and linalool was approximately equal in Red. Borneol and α-terpineol were approximately equal in 'Marshall's Delight.' 'Croftway Pink' and Mahogany had the highest concentrations of thymol among the varieties (2700 and 1000 μM, respectively). Mahogany, *M. clinopodia*, *M. menthifolia*, and *M. fistulosa* had high amounts of thymoquinone ca. 3000 to 4000 μM (Fig. 3.3). Rose Geranium and Lavender did not contain carvacrol, thymol, or thymoquinone (Fig. 3.3). 'Fishes,' 'Trinity Purple,' *M. menthifolia*, Red, Lavender, and *M. fistulosa* did not contain thymol. 'Marshall's Delight,' *M. didyma*, Rose Geranium, Mixed Purple, 'Prairie Night', Lavender, and Mahogany did not contain carvacrol.

Essential oil assay

Mycelial growth decreased at one or both concentrations (5 or 50 µl/plate) of constituents commonly found in essential oils of *Monarda* (Fig. 3.4). Carvacrol eliminated fungal growth at both concentrations. Growth was eliminated when a 50 µl treatment of cineole, linalool, 1-octen-3-ol, terpineol, or thymol was used. At the highest dose (50 µl/plate), there was greater than 50% inhibition of mycelial growth with borneol, β-myrcene, γ-terpinene, thymoquinone, or α-pinene. Treatment with 5 µl of linalool, terpineol, or thymol inhibited growth by more than 90%. Cineole, β-pinene, p-cymene, or 1-octen-3-ol decreased fungal growth less than 35% in 5 µl treatments. All other treatments inhibited mycelial growth when treated with 50 µl, although not to the same extent.

Growth chamber study

Achieved P-values for all tomato seedling experiments are in Table 3.2.

Stem diameter. Stem diameter of tomato seedlings grown in medium amended with *Monarda didyma* 'Croftway Pink' was significantly increased compared to all other treatments (Fig. 3.5). No other herbage treatments were significantly different from control. When challenged with the pathogen, 'Croftway Pink' again had the most significant effect, although other treatments were significant as well. Herbage from 'Croftway Pink,' 'Mohawk,' 'Sioux,' and 'Marshall's Delight' each increased stem diameter when compared to the infested control (Fig. 3.6). Stem diameter of seedlings grown in pathogen-infested media with amendments of 'Croftway Pink,' 'Sioux,' 'Mohawk,' Rose Geranium, Mixed Purple, 'Marshall's Delight,' 'Fishes,' *M. clinopodia*,

M. menthifolia, 'Prairie Night', Lavender, and Mahogany were not different from seedlings grown in uninfested media amended with the same varieties (Fig. 3.6).

Shoot height. Shoot height of tomato seedlings grown in medium amended with 'Croftway Pink' was significantly increased compared to all other treatments. Again, there was no difference in the other herbage treatments compared to the control (Fig. 3.7). Treatment with herbage from 'Croftway Pink,' 'Mohawk,' or 'Sioux' increased shoot height when challenged with the pathogen, compared to the infested control (Fig. 3.8). Shoot height of seedlings grown in pathogen-infested media with amendments of 'Croftway Pink,' 'Sioux,' 'Mohawk,' Rose Geranium, Mixed Purple, *M. clinopodia*, and *M. menthifolia* were not different from seedlings grown in uninfested media amended with the same varieties (Fig. 3.8).

Disease index. Disease index was significantly reduced in seedlings grown in media amended with herbage from 'Croftway Pink' or Rose Geranium compared to the other treatments, which were not different from the control (Fig. 3.9). When challenged with the pathogen, herbage from 'Croftway Pink,' 'Mohawk,' 'Sioux,' 'Fishes,' Rose Geranium, or Mixed Purple significantly reduced disease index compared to the infested control (Fig. 3.10). Disease index of seedlings grown in pathogen-infested media with amendments of 'Croftway Pink,' 'Sioux,' 'Mohawk,' *M. didyma*, 'Fishes,' Rose Geranium, Mixed Purple, *M. menthifolia*, and Lavender were not different from seedlings grown in uninfested media amended with the same varieties (Fig. 3.10).

Survival. There was a significant increase in survival of seedlings grown in the presence of herbage from 'Croftway Pink,' 'Mohawk,' or Rose Geranium, compared to

the untreated control (Fig. 3.11). Survival of seedlings grown in pathogen-infested media with amendments of 'Croftway Pink,' 'Sioux,' 'Mohawk,' 'Marshall's Delight Night,' *M. didyma*, 'Fishes,' Rose Geranium, Mixed Purple, *M. clinopodia*, *M. menthifolia*, and Lavender were not different from seedlings grown in uninfested media amended with the same varieties (Fig. 3.12).

Discussion

Several plant essential oils inhibit growth of *Pythium* species. When applied as a seed soak, the essential oils of *Chenopodium ambrosioides* (Chenopodiaceae) reduced damping-off of tomato in soil infested with *P. aphanidermatum* or *P. debaryanum* by 67 and 100%, respectively (Kishore and Dubey, 2002). Oils from *Lippia alba* (Verbenaceae) reduced damping-off of tomato in soil infested with *P. aphanidermatum* or *P. debaryanum* by 89 and 71%, respectively (Kishore and Dubey, 2002).

In assay of *Monarda* essential oil constituents, every compound tested decreased mycelial growth of *Pythium myriotylum* by some degree, and most compounds were substantially inhibitory. The straight-chain alcohols, except borneol, and the monoterpenic phenols inhibited growth at both the high and low doses, whereas the hydrocarbons tended to inhibit only at the high dose. In other studies, monoterpenic phenols, carvacrol, eugenol, and thymol, were strongly associated with antifungal activity of essential oils screened against the fruit pathogen *Botrytis cinerea* (Wilson et al., 1997). Essential oil of thyme (*Thymus vulgaris* L.) that contained 50% thymol inhibited growth of *Pythium ultimum* (Zambonelli et al., 1996).

In this study, one herbage treatment, 'Croftway Pink,' was superior for plant growth and disease protection from *P. myriotylum* in tomato seedlings. In previous research, herbage of two other *Monarda* varieties ('Elsie's Lavender' and 'Marshall's Delight') increased percent germination and plant height of tomato in *Rhizoctonia*-infested medium (Gwinn et al., 2003; Greene, 2005). There was also a decrease in disease index with the addition of 'Elsie's Lavender' in tomato (Gwinn et al., 2003; Greene, 2005). Hexane extracts of 'Elsie's Lavender' flowers and leaves used in that study contained high concentrations of carvacrol (2.6 mM) and thymoquinone (490 µM) (Greene, 2005). In contrast to the 'Marshall's Delight' used in this study, the 'Marshall's Delight' flowers and leaves used by Gwinn et al. (2003) contained essentially no thymol and high concentrations of carvacrol (255 µM) (Greene, 2005). Compared to other varieties in both studies, 'Croftway Pink' had the highest concentration of thymol in hexane extracts (1.3 mM) (Fig. 3.3). 'Croftway Pink' also had a high concentration of cymene and 1-octen-3-ol (data not shown). These compounds are all volatile and relatively insoluble in water. Concentration released by herbage was not measured; however, release was sufficient to reduce disease loss, but not to inhibit germination or stunt plant growth, both of which are reduced by thymol (Vaughn and Spencer, 1993) and probably by carvacrol (Angelini et al., 2003). The major constituents found in 'Croftway Pink', inhibited *P. myriotylum* at the high concentration tested in this study, but only thymol (a monoterpenic phenol) and 1-octen-3-ol (a straight-chain alcohol) were inhibitory at the low concentration.

Three additional *Monarda* varieties that have potential to control *P. myriotylum* damping-off in tomato seedlings and that do not reduce growth as compare to controls were identified: 'Sioux', 'Mohawk', and Rose Geranium (Figs. 3.6, 3.8. 3.10). Chemical components of these varieties and 'Croftway Pink,' are noticeably similar in many ways. In hexane extracts, cymene constituted 80% of the total hydrocarbons in all 4 varieties, except Rose Geranium (Fig. 3.1), and 1-octen-3-ol constituted 80% of the straight-chain alcohols, in all varieties except Rose Geranium (Fig. 3.2). 'Sioux' extract had about equal amounts of thymol and carvacrol (ca. 400 µM), and thymoquinone concentration of ca. 250 µM. 'Mohawk' had negligible concentrations of carvacrol. Concentration of thymol was ca. 250 µM and thymoquinone was ca. 1500 µM. Hexane extracts of Rose Geranium contained almost exclusively myrcene, a compound that inhibited *P. myriotylum* only at the highest concentration.

Results from this research are strong indicators of the capabilities of *Monarda*-based products as biological controls for soilborne pathogenic fungi. Development of these products requires a balance between the antifungal activity and phytotoxicity effects of essential oil constituents. A mixture high in cymene, 1-octen-3-ol, thymol, and thymoquinone appeared superior in the suppression of damping-off in tomato seedlings. High concentrations of thymol in the mixture did not appear to limit germination as in other studies (Vaughn and Spencer, 1993; Angelini et al., 2003). All experiments in this study used 10 %v/v Monarda as an amendment. Other varieties might be suitable if the rate is reduced or if varieties are mixed to maximize pathogen control and minimize phytotoxicity.

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APPENDIX I

Table 2.1. Treatment assignments for experiment testing efficacy of soil amendment with one of three *Monarda* sp. varieties, seed treatment with *Beauveria*, and/or soil drench with commercial plant growth-promoting rhizobacteria against *Pythium myriotylum* damping-off of tomato. Experiment was arranged as a randomized complete block.

Treatment[a]	Herb[b]	*Beauveria*[c]	BioYield	Pathogen[d]
1	None	No Bb	No BioYield	No Pathogen
2	None	Bb11-98	No BioYield	No Pathogen
3	None	BOT	No BioYield	No Pathogen
4	None	No Bb	BioYield	No Pathogen
5	None	Bb11-98	BioYield	No Pathogen
6	None	BOT	BioYield	No Pathogen
7	Mon10	Bb11-98	No BioYield	No Pathogen
8	Mon29	Bb11-98	No BioYield	No Pathogen
9	Mon44	Bb11-98	No BioYield	No Pathogen
10	Mon10	BOT	No BioYield	No Pathogen
11	Mon29	BOT	No BioYield	No Pathogen
12	Mon44	BOT	No BioYield	No Pathogen
13	Mon10	No Bb	BioYield	No Pathogen
14	Mon29	No Bb	BioYield	No Pathogen
15	Mon44	No Bb	BioYield	No Pathogen
16	Mon10	Bb11-98	BioYield	No Pathogen
17	Mon29	Bb11-98	BioYield	No Pathogen
18	Mon44	Bb11-98	BioYield	No Pathogen
19	Mon10	BOT	BioYield	No Pathogen
20	Mon29	BOT	BioYield	No Pathogen
21	Mon44	BOT	BioYield	No Pathogen
22	Mon10	No Bb	No BioYield	No Pathogen
23	Mon29	No Bb	No BioYield	No Pathogen
24	Mon44	No Bb	No BioYield	No Pathogen

[a] Treatment #: Numbers were used for statistical randomization of the 48 treatments for each tomato cultivar (Celebrity or Mountain Spring).
[b] Herb: Mon10 = 'Cerise'; Mon29 = 'Puerto Purification'; Mon44 = 'Violet Queen'; None = No herbage.
[c] Beauveria: Bb 11-98 = *Beauveria bassiana* 11-98; BOT = BotaniGard; No Bb = No *Beauveria.*
[d] Pathogen: *Pythium myriotylum*.

Table 2.1, continued.

Treatment[a]	Herb[b]	*Beauveria*[c]	BioYield	Pathogen[d]
25	None	No Bb	No BioYield	Pathogen
26	None	Bb11-98	No BioYield	Pathogen
27	None	BOT	No BioYield	Pathogen
28	None	No Bb	BioYield	Pathogen
29	None	Bb11-98	BioYield	Pathogen
30	None	BOT	BioYield	Pathogen
31	Mon10	Bb11-98	No BioYield	Pathogen
32	Mon29	Bb11-98	No BioYield	Pathogen
33	Mon44	Bb11-98	No BioYield	Pathogen
34	Mon10	BOT	No BioYield	Pathogen
35	Mon29	BOT	No BioYield	Pathogen
36	Mon44	BOT	No BioYield	Pathogen
37	Mon10	No Bb	BioYield	Pathogen
38	Mon29	No Bb	BioYield	Pathogen
39	Mon44	No Bb	BioYield	Pathogen
40	Mon10	Bb11-98	BioYield	Pathogen
41	Mon29	Bb11-98	BioYield	Pathogen
42	Mon44	Bb11-98	BioYield	Pathogen
43	Mon10	BOT	BioYield	Pathogen
44	Mon29	BOT	BioYield	Pathogen
45	Mon44	BOT	BioYield	Pathogen
46	Mon10	No Bb	No BioYield	Pathogen
47	Mon29	No Bb	No BioYield	Pathogen
48	Mon44	No Bb	No BioYield	Pathogen

[a] Treatment #: Numbers were used for statistical randomization of the 48 treatments for each tomato cultivar (Celebrity or Mountain Spring).
[b] Herb: Mon10 = 'Cerise'; Mon29 = 'Puerto Purification'; Mon44 = 'Violet Queen'; None = No herbage.
[c] Beauveria: Bb 11-98 = *Beauveria bassiana* 11-98; BOT = BotaniGard; No Bb = No *Beauveria.*
[d] Pathogen: *Pythium myriotylum*.

Table 2.2. Disease index used for evaluation of symptoms of damping-off on tomato seedlings caused by *Pythium myriotylum*.

Rating	Description of symptoms
1	no discoloration of the root system
2	1 – 10% of the root system discolored
3	11 – 25% of the root system discolored
4	26 – 50% of the root system discolored
5	>50% of the root system discolored
6	seedling death (post-emergence)
7	no germination (pre-emergence)

Table 2.3. Concentration of essential oil constituents in *Monarda didyma* variety 'Cerise.' Herbage was extracted in hexane and analyzed with gas chromatography-mass spectroscopy. Concentrations were determined by comparison to standard curves.

Essential oil constituent	Retention time	Concentration[a] (µM)
Borneol	11.76	66.24 ± 62.90
Bornyl acetate	17.82	18.57 ± 11.17
Carvacrol	19.16	13.75 ± 24.16
Cineole	7.02	7.37 ± 2.90
Cymene	6.83	380.16 ± 88.71
Limonene	7.11	8.06 ± 11.39
Linalool	9.03	8.49 ± 3.49
β-Myrcene	5.95	24.20 ± 6.91
1-Octen-3-ol	5.66	122.44 ± 47.12
α-Pinene	4.67	36.03 ± 8.52
γ-Terpinene	7.79	66.49 ± 21.43
α-Terpineol	13.45	0.11 ± 0.07
Thymol	18.47	123.33 ± 119.49
Thymoquinone	15.6	14.45 ± 19.33

[a]Average of four separate GC-MS analyses.

Table 2.4. Concentration of essential oil constituents in *Monarda didyma* variety 'Puerto Purification.' Herbage was extracted in hexane and analyzed with gas chromatography-mass spectroscopy. Concentrations were determined by comparison to standard curves.

Essential oil constituent	Retention time	Concentration[a] (µM)
Borneol	11.65	0.15 ± 0.04
Bornyl acetate	17.85	0.25 ± 0.19
Carvacrol	18.95	31.62 ± 42.62
Cineole	7.04	0.12 ±0.10
Cymene	6.82	171.55 ±80.60
Limonene	6.93	5.28 ± 3.87
Linalool	9.01	0.33 ± 0.32
β-Myrcene	5.96	0.14 ± 0.04
1-Octen-3-ol	5.81	34.30 ± 10.51
α-Pinene	4.66	5.12 ± 3.13
γ-Terpinene	7.80	0.26 ± 0.19
α-Terpineol	13.45	0.10 ± 0.05
Thymol	18.46	1047.48 ± 676.25
Thymoquinone	15.86	402.38 ± 139.62

[a]Average of four separate GC-MS analyses.

Table 2.5. Concentration of essential oil constituents in *Monarda didyma* variety 'Violet Queen.' Herbage was extracted in hexane and analyzed with gas chromatography-mass spectroscopy. Concentrations were determined by comparison to standard curves.

Essential oil constituent	Retention time	Concentration[a] (µM)
Borneol	11.65	0.22 ± 0.15
Bornyl acetate	17.83	0.49 ± 0.46
Carvacrol	18.97	993.89 ± 565.68
Cineole	7.04	0.16 ± 0.06
Cymene	6.82	338.29 ± 114.22
Limonene	6.97	5.17 ± 3.72
Linalool	9.02	0.43 ± 0.39
β-Myrcene	5.95	1.43 ± 1.25
1-Octen-3-ol	5.66	54.22 ± 33.33
α-Pinene	4.66	8.66 ± 2.57
γ-Terpinene	7.80	9.44 ± 5.90
α-Terpineol	13.45	0.08 ±0.03
Thymol	18.46	2.05 ± 1.53
Thymoquinone	15.86	165.33 ± 68.14

[a]Average of four separate GC-MS analyses.

Table 2.6. Achieved P–values for treatment means of measurements taken from tomato (*Lycopersicon esculentum*) seedlings grown in soil amended with one of three *Monarda* sp. varieties from seed treated with *Beauveria*, and/or seed drenched with commercial plant growth-promoting rhizobacteria (PGPR) against *Pythium myriotylum* damping-off of tomato.

		'Mountain Spring'	**'Celebrity'**
Measurement	**Treatment**	**P – value[a]**	
Stem diameter (mm)	*Beauveria*	0.0912	0.0996
	Herbage	0.0499	0.0042
	Pathogen	<0.0001	<0.0001
	Beauveria*Pathogen	0.0365	0.8288
	Herbage*Pathogen	0.5500	0.1605
Shoot height (cm)	*Beauveria*	0.2239	0.1127
	Herbage	0.0012	0.0010
	Pathogen	<0.0001	<0.0001
	Beauveria*Pathogen	0.1133	0.5538
	Herbage*Pathogen	0.2771	0.0630
Disease index	*Beauveria*	0.1902	0.0276
	Herbage	0.2190	0.0020
	Pathogen	<0.0001	<0.0001
	Beauveria*Pathogen	0.0645	0.1571
	Herbage*Pathogen	0.8181	0.0413
Survival (%)	*Beauveria*	0.0505	0.0353
	Herbage	0.8771	0.0059
	Pathogen	<0.0001	<0.0001
	Beauveria*Pathogen	0.0335	0.3033
	Herbage*Pathogen	0.6493	0.1031

[a] Values <0.05 are significant.

Table 2.7. Stem diameter of 'Celebrity' tomato seedlings treated with herbage (ground leaves and flowers, $P = 0.0042$) or pathogen ($P < 0.0001$).

Treatment		Stem diameter[a,b] (mm)
Herbage	None	1.443 ± 0.1327 ab
	'Cerise'	1.339 ± 0.1327 bc
	'Puerto Purification'	1.606 ± 0.1327 a
	'Violet Queen'	1.205 ± 0.1327 c
Pythium myriotylum	No pathogen	1.789 ± 0.1201 a
	Pathogen	1.008 ± 0.1201 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.8. Stem diameter of 'Mountain Spring' tomato seedlings treated with herbage (ground leaves and flowers, $P = 0.0499$) or pathogen ($P < 0.0001$).

Treatment		Stem diameter (mm)
Herbage	None	1.457 ± 0.1436 a
	'Cerise'	1.239 ± 0.1436 b
	'Puerto Purification'	1.442 ± 0.1436 a
	'Violet Queen'	1.435 ± 0.1436 a
Pythium myriotylum	No pathogen	1.677 ± 0.1400 a
	Pathogen	1.109 ± 0.1400 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.9. Stem diameter differences ($P = 0.0365$) of 'Mountain Spring' tomato seedlings treated with *Beauveria* and pathogen.

Beauveria	***Pythium myriotylum***	**Stem diameter (mm)**
None	No pathogen	1.718 ± 0.1537 a
Bb 11-98	No pathogen	1.572 ± 0.1537 a
BotaniGard	No pathogen	1.742 ± 0.1537 a
None	Pathogen	0.925 ± 0.1537 c
Bb 11-98	Pathogen	1.167 ± 0.1537 b
BotaniGard	Pathogen	1.234 ± 0.1537 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.10. Shoot height of 'Celebrity' tomato seedlings treated with herbage (ground leaves and flowers, $P = 0.0010$) or pathogen ($P < 0.0001$).

Treatment		Shoot height (cm)
Herbage	None	8.225 ± 0.5951 a
	'Cerise'	7.015 ± 0.5951 b
	'Puerto Purification'	8.269 ± 0.5951 a
	'Violet Queen'	6.188 ± 0.5951 b
Pythium myriotylum	No pathogen	9.923 ± 0.5132 a
	Pathogen	4.926 ± 0.5132 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.11. Shoot height of 'Mountain Spring' tomato seedlings treated with herbage (ground leaves and flowers, $P = 0.0012$) or pathogen ($P < 0.0001$).

Treatment		Shoot height (cm)
Herbage	None	9.726 ± 0.7015 a
	'Cerise'	7.430 ± 0.7015 b
	'Puerto Purification'	8.496 ± 0.7015 b
	'Violet Queen'	8.530 ± 0.7015 b
Pythium myriotylum	No pathogen	10.588 ± 0.6413 a
	Pathogen	6.503 ± 0.6413 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.12. Disease index of 'Celebrity' tomato seedlings treated with *Beauveria* (P = 0.0276), herbage (ground leaves and flowers, P = 0.0020), or pathogen (P < 0.0001).

Treatment		Disease index
Beauveria	None	3.2 ± 0.2029 b
	Bb 11-98	3.6 ± 0.2029 a
	BotaniGard	3.8 ± 0.2029 a
Herbage	None	3.4 ± 0.2253 b
	'Cerise'	3.3 ± 0.2253 b
	'Puerto Purification'	3.2 ± 0.2253 b
	'Violet Queen'	4.2 ± 0.2253 a
Pythium myriotylum	No pathogen	2.4 ± 0.1777 b
	Pathogen	4.6 ± 0.1777 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at P = 0.05.

Table 2.13. Disease index of 'Mountain Spring tomato seedlings treated with the pathogen (P < 0.0001).

Treatment		Disease index
Pythium myriotylum	No pathogen	2.3 ± 0.1900 b
	Pathogen	4.1 ± 0.1900 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at P = 0.05.

Table 2.14. Disease index ($P = 0.0413$) of 'Celebrity' tomato seedlings treated with herbage (ground leaves and flowers) and with or without the pathogen.

Herbage	***Pythium myriotylum***	**Disease index**
None	No pathogen	1.9 ± 0.0413 d
'Cerise'	No pathogen	2.2 ± 0.0413 d
'Puerto Purification'	No pathogen	2.4 ± 0.0413 d
'Violet Queen'	No pathogen	3.3 ± 0.0413 c
None	Pathogen	5.0 ± 0.0413 a
'Cerise'	Pathogen	4.4 ± 0.0413 ab
'Puerto Purification'	Pathogen	4.0 ± 0.0413 bc
'Violet Queen'	Pathogen	5.1 ± 0.0413 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 2.15. Survival (%) of 'Celebrity' tomato seedlings treated with *Beauveria* (P = 0.0353), herbage (ground leaves and flowers, P = 0.0059), or pathogen ($P < 0.0001$).

Treatment		Survival (%)
Beauveria	None	74.5 ± 3.5822 a
	Bb 11-98	65.6 ± 3.5822 ab
	BotaniGard	62.5± 3.5822 b
Herbage	None	67.4 ± 4.0778 ab
	'Cerise'	69.4 ± 4.0778 a
	'Puerto Purification'	76.4 ± 4.0778 a
	'Violet Queen'	56.9 ± 4.0778 b
Pythium myriotylum	No pathogen	84.4 ± 0.4400 a
	Pathogen	50.7 ± 0.4400 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at P = 0.05.

Table 2.16. Survival (%) differences of 'Mountain Spring' tomato seedlings treated with *Beauveria* (P = 0.0505) or with or without the pathogen ($P < 0.0001$).

Treatment		Survival (%)
Beauveria	None	71.4 ± 4.2326 b
	Bb 11-98	77.1 ± 4.2326 ab
	BotaniGard	81.8 ± 4.2326 a
Pythium myriotylum	No pathogen	89.6 ± 3.8618 a
	Pathogen	63.9 ± 3.8618 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at P = 0.05.

Table 2.17. Survival (%) differences ($P = 0.0335$) of 'Mountain Spring' tomato seedlings treated with *Beauveria* and with or without the pathogen.

Beauveria	***Pythium myriotylum***	**Survival (%)**
None	No pathogen	90.6 ± 5.1884 a
Bb 11-98	No pathogen	86.5 ± 5.1884 a
BotaniGard	No pathogen	91.7 ± 5.1884 a
None	Pathogen	52.1 ± 5.1884 c
Bb 11-98	Pathogen	67.7 ± 5.1884 b
BotaniGard	Pathogen	71.9 ± 5.1884 b

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table 3.1. Treatment number assigned to each of 16 *Monarda* species and varieties. Dried leaves from the plants were used as a soil amendment for tomato seedlings challenged with *Pythium myriotylum*.

Treatment number	UT number	Species[a]	Variety
1	35	*Monarda didyma*	'Croftway Pink'
2	17	*Monarda didyma*	'Mohawk'
3	20	*Monarda didyma*	'Sioux'
4	39	*Monarda didyma*	'Marshall's Delight'
5	52	*Monarda didyma*	-
6	28	*Monarda didyma*	'Fishes'
7	27	*Monarda didyma*	Rose Geranium
8	24	*Monarda didyma*	Mixed Purple
9	54	*Monarda clinopodia*	-
10	9	*Monarda menthifolia*	-
11	6	*Monarda didyma*	Red
12	51	*Monarda didyma*	'Trinity Purple'
13	46	*Monarda didyma*	'Prairie Night'
14	5	*Monarda fistulosa*	-
15	50	*Monarda didyma*	Lavender
16	38	*Monarda didyma*	Mahogany

[a]Species are not conclusive due to out-crossing.

Table 3.2. Achieved P–values for treatment effects on stem diameter, shoot height, disease index, and survival of tomato seedlings grown in soil amended with one of sixteen *Monarda* sp. varieties as biological control against damping-off caused by *Pythium myriotylum*.

Measurement	Treatment effect	P - value[a]
Stem diameter (mm)	Herb	<0.0001
	Pathogen	0.0018
	Herb*Pathogen	0.0093
Shoot height (cm)	Herb	0.0008
	Pathogen	0.0002
	Herb*Pathogen	0.0037
Disease index	Herb	0.0550
	Pathogen	0.0004
	Herb*Pathogen	0.0169
Survival (%)	Herb	0.0745
	Pathogen	0.0052
	Herb*Pathogen	0.0509

[a]Values <0.05 are significant.

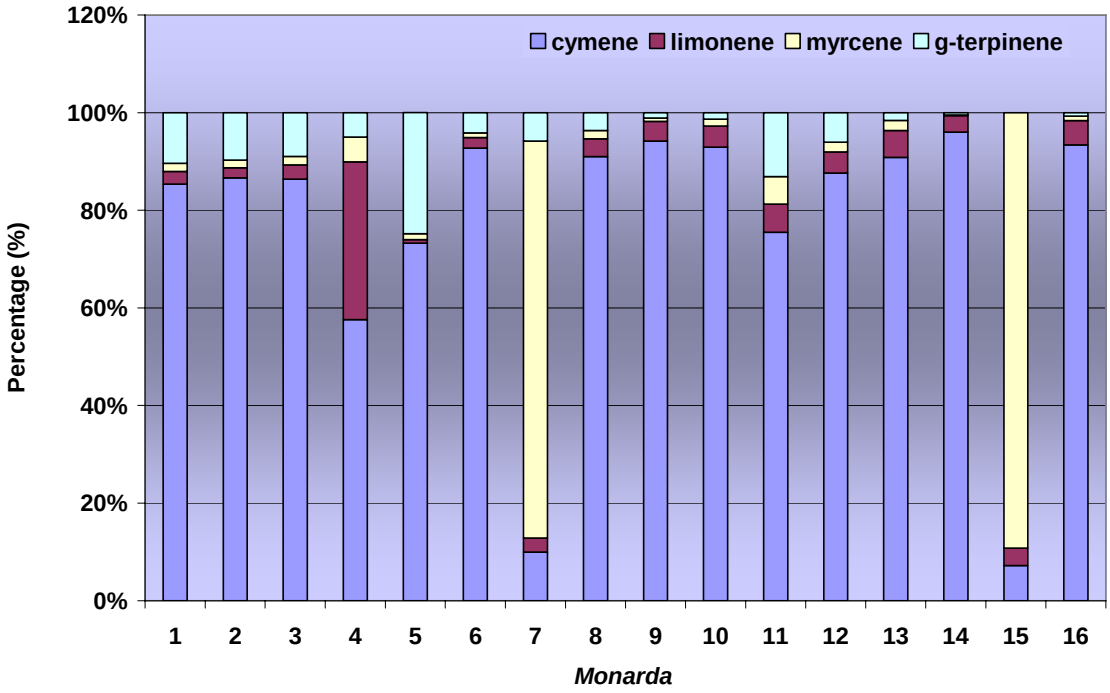

Figure 3.1. Percentage (%) of hydrocarbons in dried leaves from each of 16 *Monarda* sp. determined by GC-MS. X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight Night'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany.

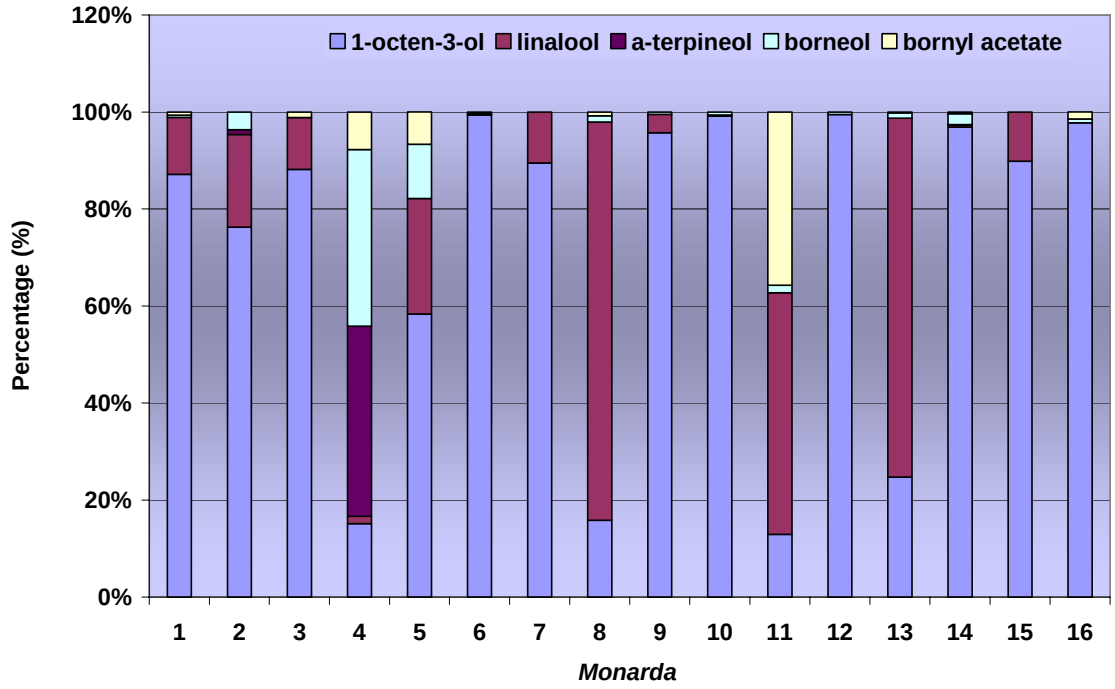

Figure 3.2. Percentage (%) of straight-chain alcohols in dried leaves from each of 16 *Monarda* sp. determined by GC-MS. X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany.

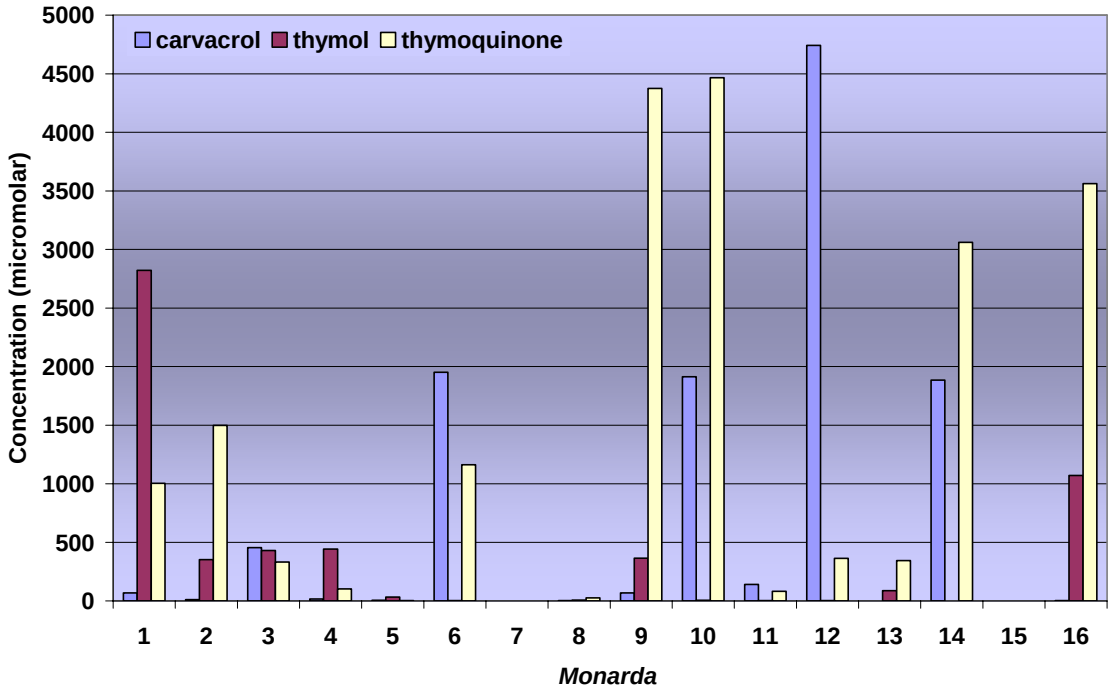

Figure 3.3. Concentration of carvacrol, thymol, and thymoquinone in dried leaves from each of 16 *Monarda* sp. determined by GC-MS. X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany.

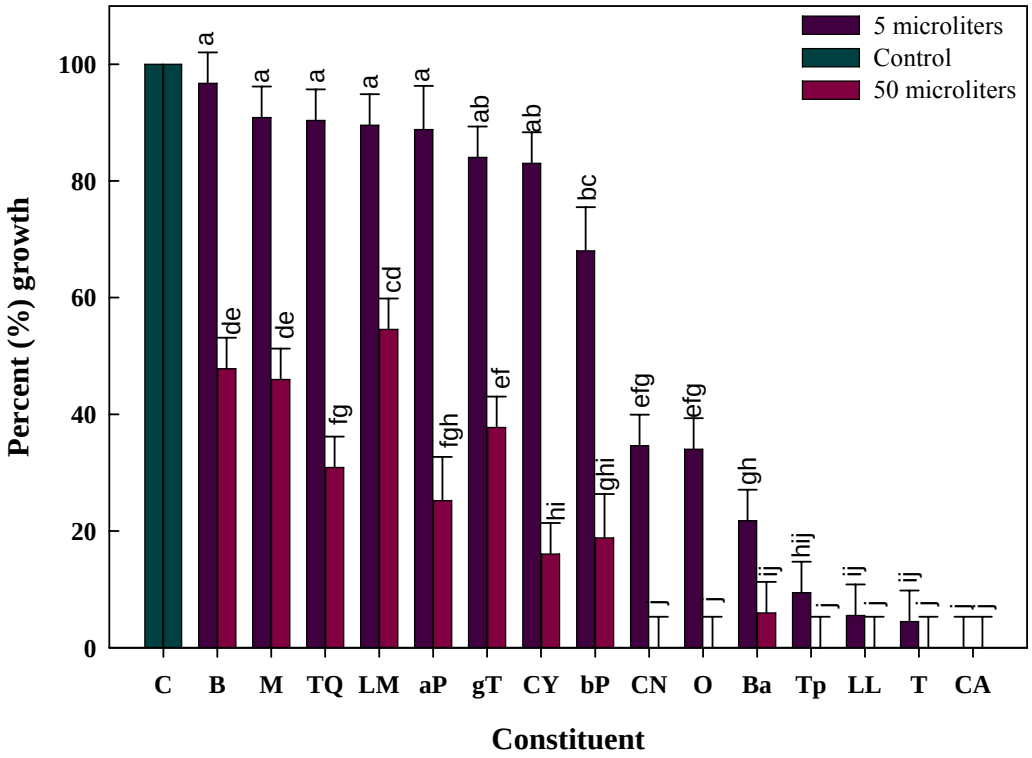

Figure 3.4. Percent growth estimates (mean ± SE) for mycelium of *Pythium myriotylum* in the presence of essential oil constituents of *Monarda* sp., in relation to control (growth = 100%). X-axis abbreviations: C, control; B, borneol; M, beta-myrcene; TQ, thymoquinone; LM, limonene; aP, alpha-pinene; gT, gamma-terpinene; CY, cymene; bP, beta-pinene; CN, cineole; O, 1-octen-3-ol; Ba, bornyl acetate; Tp, terpineol; LL, linalool; T, thymol; CA, carvacrol. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

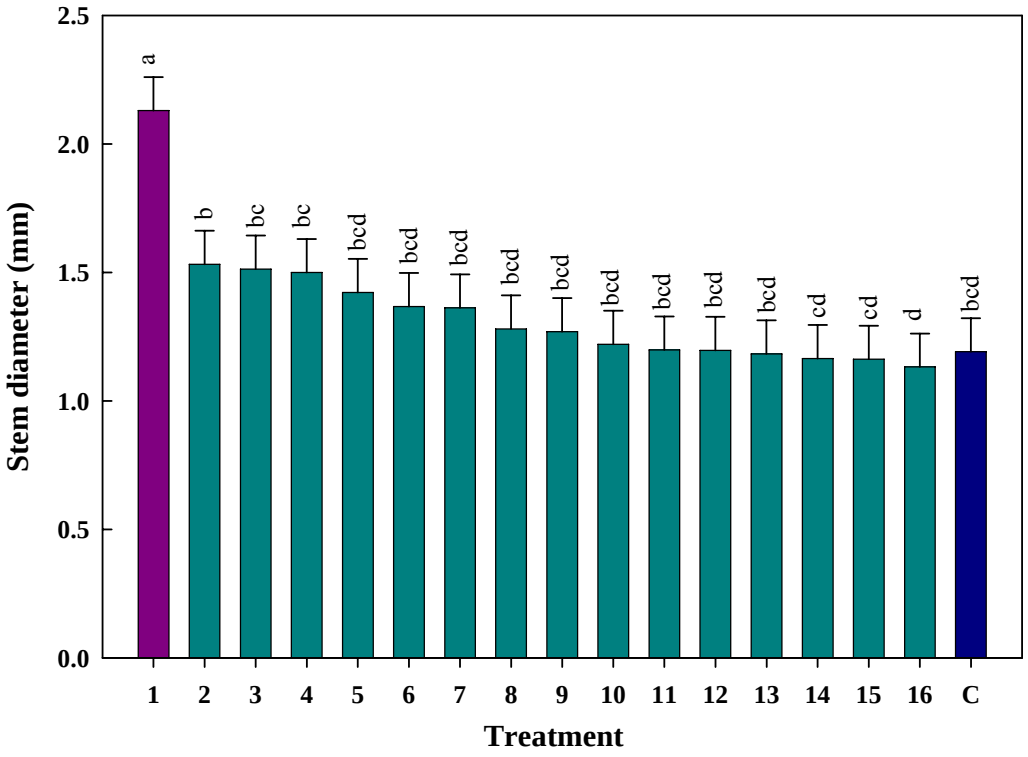

Figure 3.5. Stem diameter (mean ± SE) of 'Mountain Spring' tomato seedlings grown in greenhouse growing medium amended with 10% dried leaves from one of 16 *Monarda* sp. or no herbage (control). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Treatment values are the mean with and without the pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

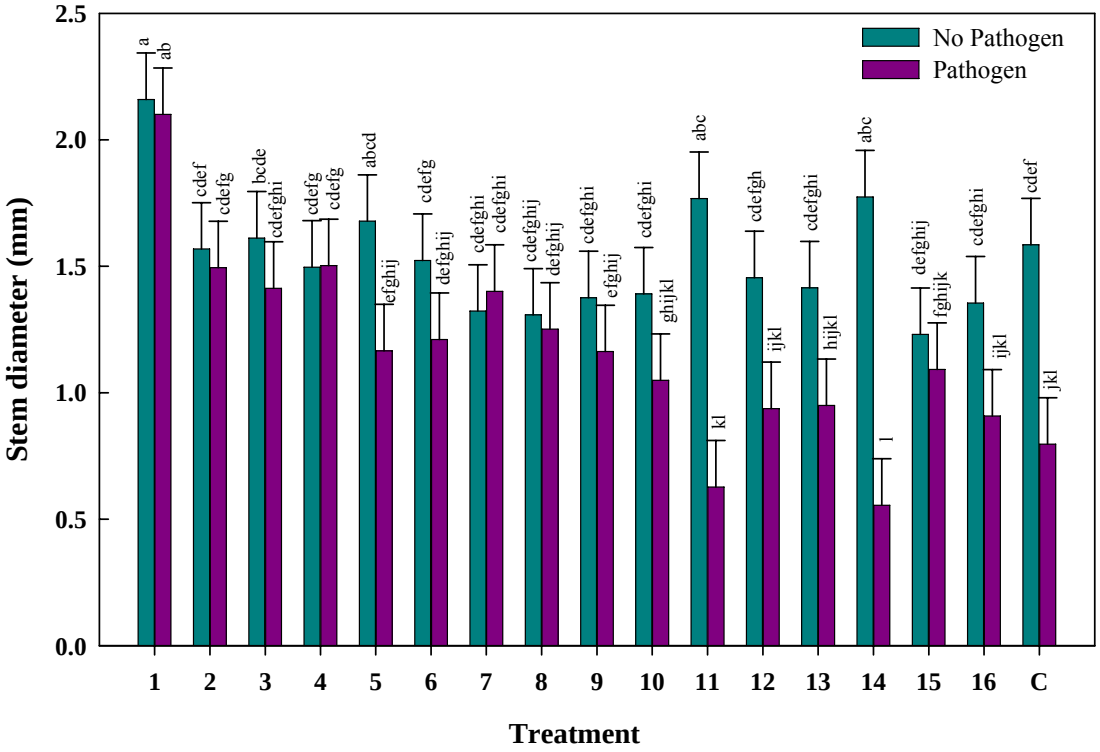

Figure 3.6. Stem diameter (mean ± SE) of 'Mountain Spring' tomato seedlings grown in peat moss amended with or without 10% dried leaves from one *Monarda* sp. and with or without pathogen (*Pythium myriotylum*). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

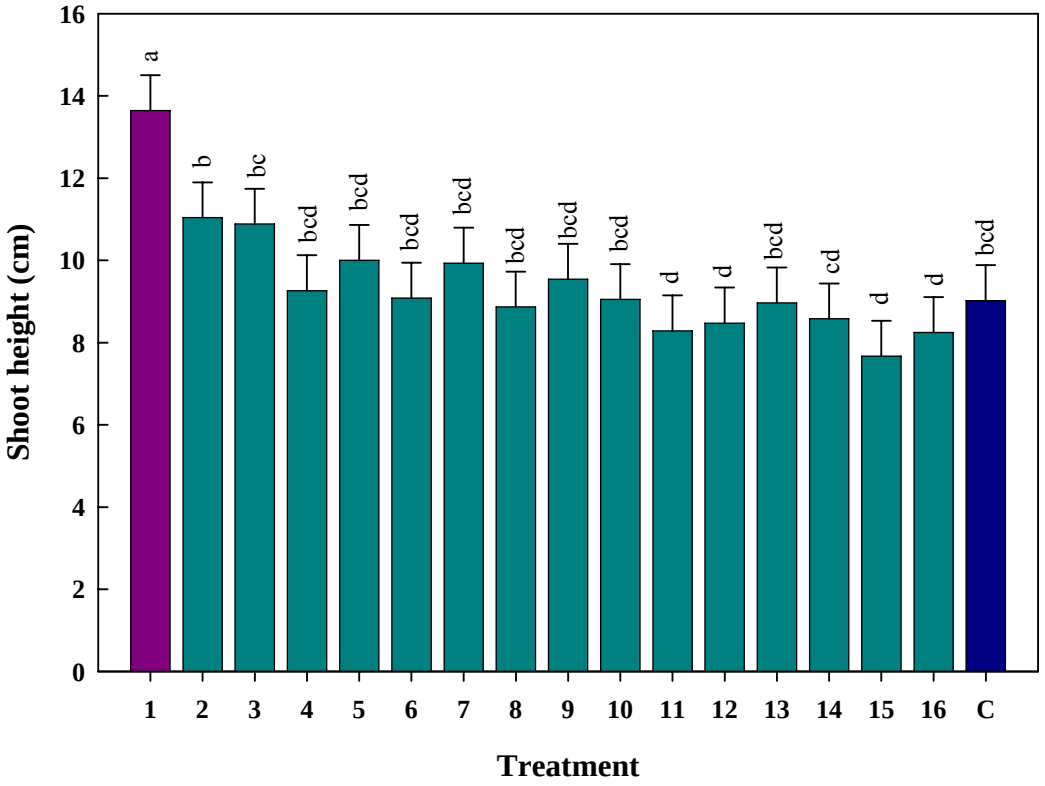

Figure 3.7. Shoot height (mean ± SE) of 'Mountain Spring' tomato seedlings grown in greenhouse growing medium amended with 10% dried leaves from one of 16 *Monarda* sp. or no herbage (control). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Treatment values are the mean with and without the pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

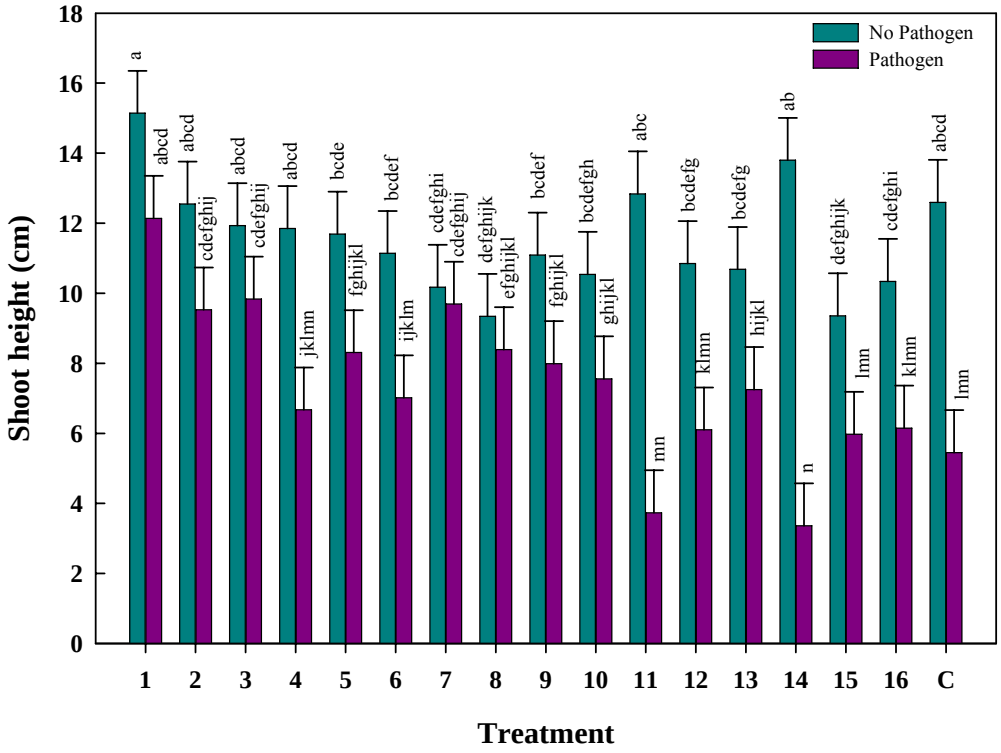

Figure 3.8. Shoot height (mean ± SE) of 'Mountain Spring' tomato seedlings grown in peat moss amended with or without 10% dried leaves from *Monarda* sp. and with or without pathogen (*Pythium myriotylum*). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Treatment values are the mean with and without the pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

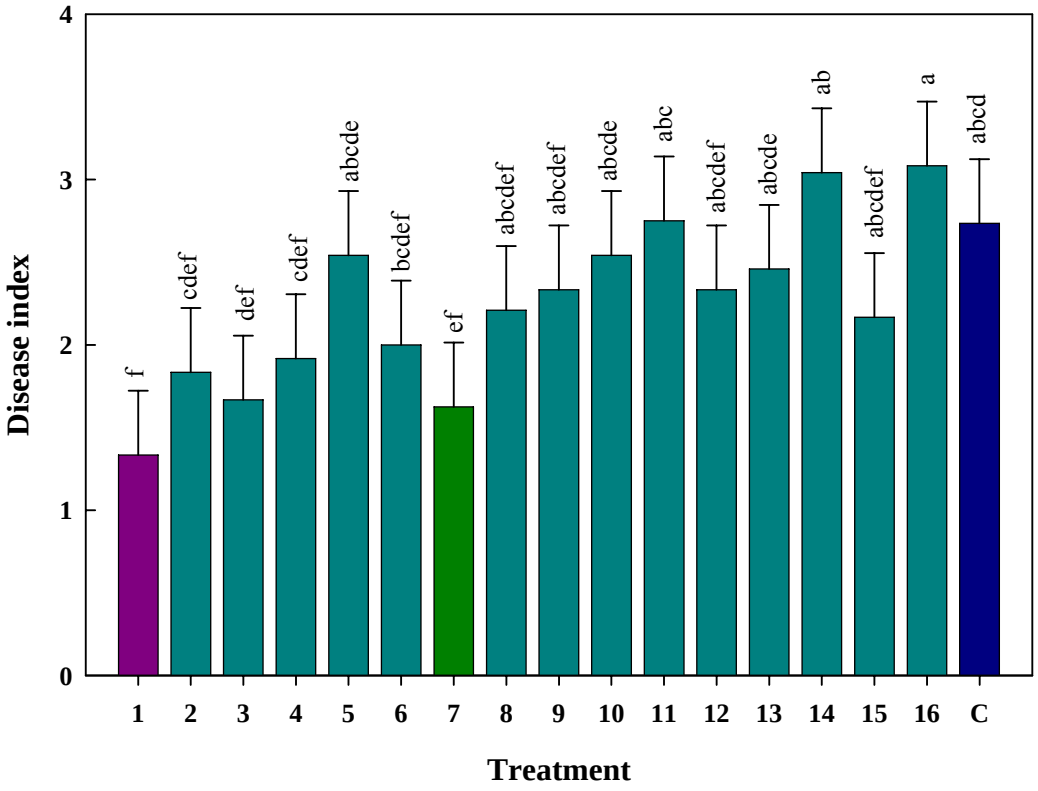

Figure 3.9. Disease index (mean ± SE) of 'Mountain Spring' tomato seedlings grown in greenhouse growing medium amended with 10% dried leaves from one of 16 *Monarda* sp. or no herbage (control). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Treatment values are the mean with and without the pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

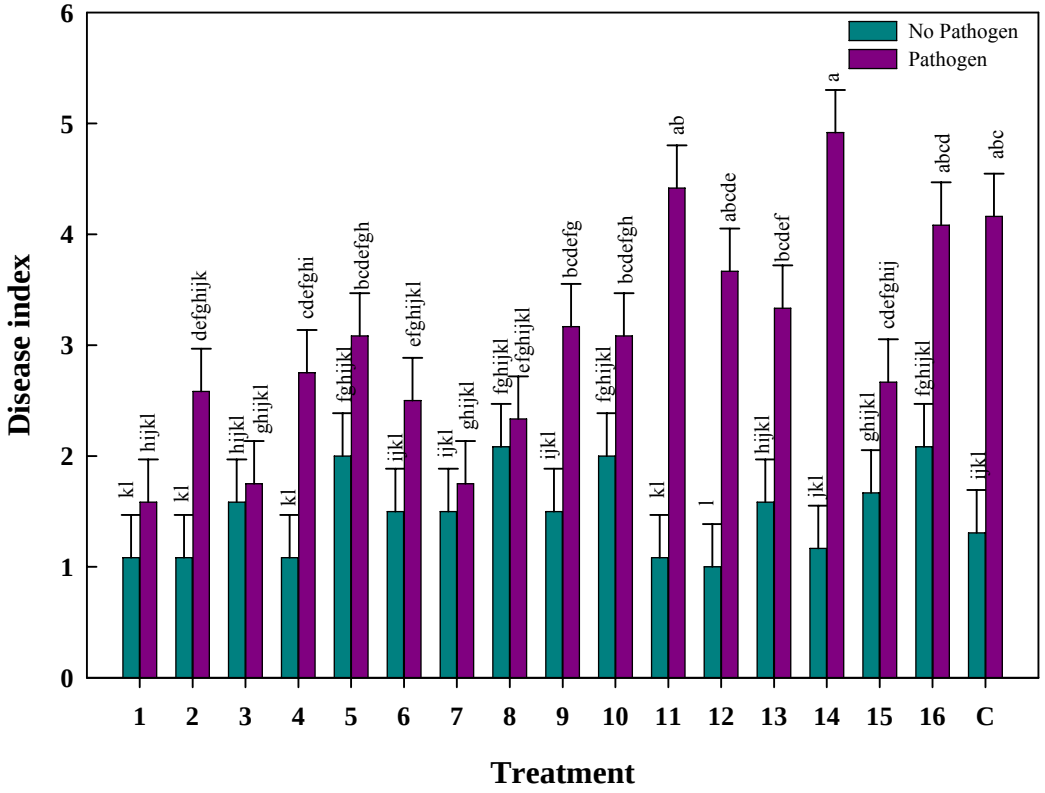

Figure 3.10. Disease index (mean ± SE) of 'Mountain Spring' tomato seedlings grown in peat moss amended with or without 10% dried leaves from *Monarda* sp. and with or without pathogen (*Pythium myriotylum*). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

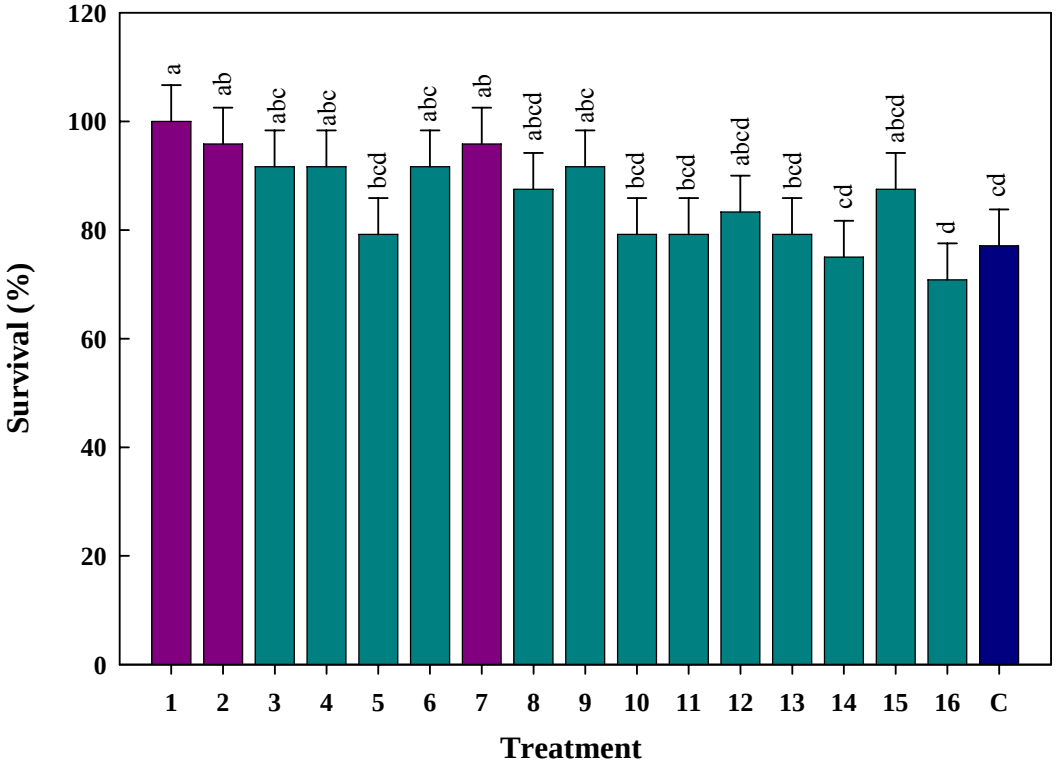

Figure 3.11. Survival (mean ± SE) of 'Mountain Spring' tomato seedlings grown in greenhouse growing medium amended with 10% dried leaves from one of 16 *Monarda* sp. or no herbage (control). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Treatment values are the mean with and without the pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

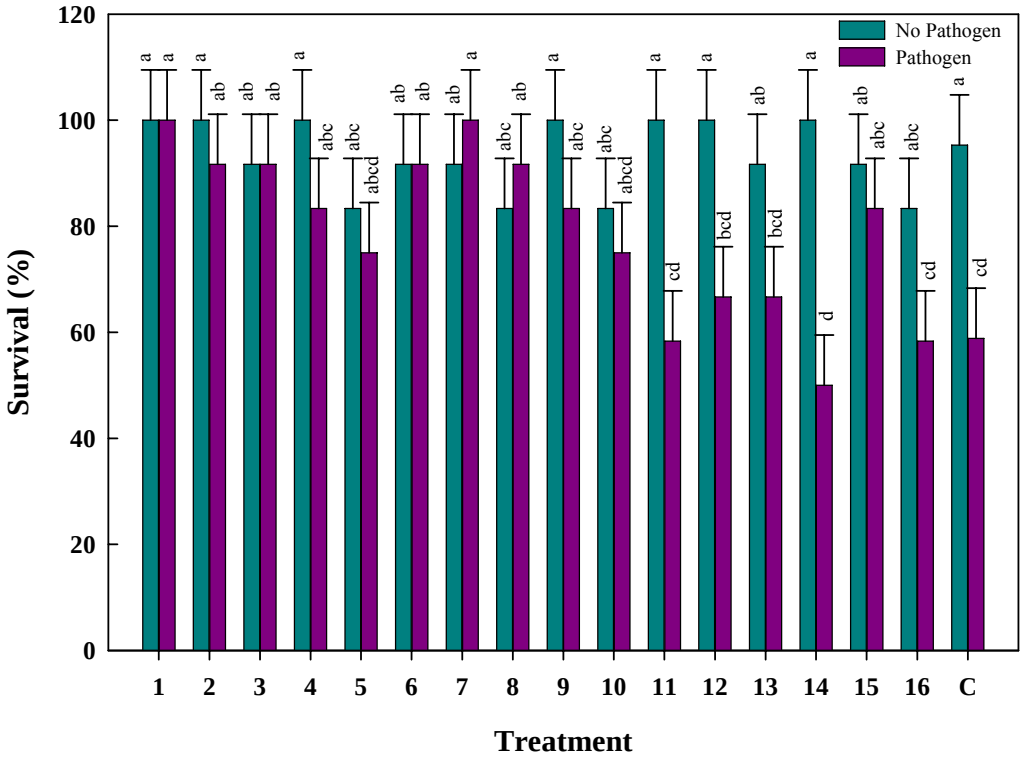

Figure 3.12. Survival (mean ± SE) of 'Mountain Spring' tomato seedlings grown in greenhouse growing medium amended with or without 10% dried leaves from *Monarda* sp. and with or without pathogen (*Pythium myriotylum*). X – axis numerical abbreviations: 1, *M. didyma* 'Croftway Pink'; 2, *M. didyma* 'Mohawk'; 3, *M. didyma* 'Sioux'; 4, *M. didyma* 'Marshall's Delight'; 5, *M. didyma*; 6, *M. didyma* 'Fishes'; 7, *M. didyma* Rose Geranium; 8, *M. didyma* Mixed Purple; 9, *M. clinopodia*; 10, *M. menthifolia*; 11, *M. didyma* Red; 12, *M. didyma* 'Trinity Purple'; 13, *M. didyma* 'Prairie Night'; 14, *M. fistulosa*; 15, *M. didyma* Lavender; 16, *M. didyma* Mahogany; C, Control. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

APPENDIX II

Table II.1. Achieved P–values for treatment effects on stem diameter (mm) measurements taken from tomato seedlings grown in soil amended with one of three *Monarda* sp. varieties from seed treated with *Beauveria* sp., and/or soil drenched with a commercial plant growth-promoting rhizobacteria (PGPR) against *Pythium myriotylum* damping-off of tomato.

	'Mountain Spring'	**'Celebrity'**
Treatment Effect	**P – value[a]**	
Beauveria	0.0912	0.0996
Herbage	0.0499	0.0042
PGPR	0.9799	0.8989
Pathogen	<0.0001	<0.0001
*Beauveria**Herbage	0.8078	0.7696
*Beauveria**PGPR	0.8802	0.8447
*Beauveria**Pathogen	0.0365	0.8288
Herbage*PGPR	0.4610	0.4533
Herbage*Pathogen	0.5500	0.1605
PGPR*Pathogen	0.5320	0.5090
*Beauveria**Herbage*PGPR	0.5241	0.9512
*Beauveria**Herbage*Pathogen	0.8545	0.4704
*Beauveria**PGPR*Pathogen	0.8513	0.5738

[a] Values <0.05 are significant.

Table II.2. Achieved P–values for treatment effects on shoot height (cm) measurements taken from tomato seedlings grown in soil amended with one of three *Monarda* sp. varieties from seed treated with *Beauveria* sp., and/or soil drenched with a commercial plant growth-promoting rhizobacteria (PGPR) against *Pythium myriotylum* damping-off of tomato.

	'Mountain Spring'	**'Celebrity'**
Treatment Effect	**P – value[a]**	
Beauveria	0.2239	0.1127
Herbage	0.0012	0.0010
PGPR	0.6934	0.7142
Pathogen	<0.0001	<0.0001
*Beauveria**Herbage	0.6411	0.8110
*Beauveria**PGPR	0.7946	0.9528
*Beauveria**Pathogen	0.1133	0.5538
Herbage*PGPR	0.1794	0.4995
Herbage*Pathogen	0.2771	0.0630
PGPR*Pathogen	0.2395	0.7410
*Beauveria**Herbage*PGPR	0.6088	0.9954
*Beauveria**Herbage*Pathogen	0.8223	0.3457
*Beauveria**PGPR*Pathogen	0.3458	0.7130

[a] Values <0.05 are significant.

Table II.3. Achieved P–values for treatment effects on disease index of tomato seedlings grown in soil amended with one of three *Monarda* sp. varieties from seed treated with *Beauveria* sp., and/or soil drenched with a commercial plant growth-promoting rhizobacteria (PGPR) against *Pythium myriotylum* damping-off of tomato.

	'Mountain Spring'	**'Celebrity'**
Treatment Effect	**P – value[a]**	
Beauveria	0.1902	0.0276
Herbage	0.2190	0.0020
PGPR	0.6637	0.3671
Pathogen	<0.0001	<0.0001
*Beauveria**Herbage	0.7517	0.8899
*Beauveria**PGPR	0.7668	0.8823
*Beauveria**Pathogen	0.0645	0.1571
Herbage*PGPR	0.6931	0.4371
Herbage*Pathogen	0.8181	0.0413
PGPR*Pathogen	0.2211	0.8734
*Beauveria**Herbage*PGPR	0.8161	0.8890
*Beauveria**Herbage*Pathogen	0.4560	0.7753
*Beauveria**PGPR*Pathogen	0.3433	0.6619

[a]Values <0.05 are significant.

Table II.4. Achieved P–values for treatment effects on survival (%) of tomato seedlings grown in soil amended with one of three *Monarda* sp. varieties from seed treated with *Beauveria* sp., and/or soil drenched with a commercial plant growth-promoting rhizobacteria (PGPR) against *Pythium myriotylum* damping-off of tomato.

	'Mountain Spring'	**'Celebrity'**
Treatment Effect	**P – value[a]**	
Beauveria	0.0505	0.0353
Herbage	0.8771	0.0059
PGPR	1.0000	0.5534
Pathogen	<0.0001	<0.0001
*Beauveria**Herbage	0.8239	0.7838
*Beauveria**PGPR	0.5649	0.8601
*Beauveria**Pathogen	0.0335	0.3033
Herbage*PGPR	0.3507	0.4881
Herbage*Pathogen	0.6493	0.1031
PGPR*Pathogen	0.2303	0.4234
*Beauveria**Herbage*PGPR	0.6448	0.8466
*Beauveria**Herbage*Pathogen	0.8664	0.5734
*Beauveria**PGPR*Pathogen	0.3297	0.5521

[a]Values <0.05 are significant.

Table II.5. Stem diameter of 'Celebrity' tomato seedlings treated with herbage (dried leaves and flowers) and pathogen ($P = 0.1605$).

Herbage	*Pythium myriotylum*	Stem diameter (mm)
None	No pathogen	1.985 ± 0.1548 a
'Cerise'	No pathogen	1.698 ± 0.1548 ab
'Puerto Purification'	No pathogen	1.905 ± 0.1548 a
'Violet Queen'	No pathogen	1.566 ± 0.1548 bc
None	Pathogen	0.901 ± 0.1548 d
'Cerise'	Pathogen	0.980 ± 0.1548 d
'Puerto Purification'	Pathogen	1.306 ± 0.1548 c
'Violet Queen'	Pathogen	0.844 ± 0.1548 d

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.6. Stem diameter of 'Mountain Spring' tomato seedlings treated with herbage (dried leaves and flowers) and pathogen ($P = 0.5500$).

Herbage	*Pythium myriotylum*	Stem diameter (mm)
None	No pathogen	1.789 ± 0.1601 a
'Cerise'	No pathogen	1.473 ± 0.1601 b
'Puerto Purification'	No pathogen	1.685 ± 0.1601 ab
'Violet Queen'	No pathogen	1.763 ± 0.1601 a
None	Pathogen	1.124 ± 0.1601 c
'Cerise'	Pathogen	1.006 ± 0.1601 c
'Puerto Purification'	Pathogen	1.979 ± 0.1601 c
'Violet Queen'	Pathogen	1.107 ± 0.1601 c

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.7. Shoot height of 'Celebrity' tomato seedlings treated with herbage (dried leaves and flowers) and pathogen ($P = 0.0630$).

Herbage	***Pythium myriotylum***	**Stem diameter (mm)**
None	No pathogen	11.689 ± 0.7319 a
'Cerise'	No pathogen	9.254 ± 0.7319 b
'Puerto Purification'	No pathogen	10.199 ± 0.7319 ab
'Violet Queen'	No pathogen	8.550 ± 0.7319 b
None	Pathogen	4.761 ± 0.7319 cd
'Cerise'	Pathogen	4.776 ± 0.7319 cd
'Puerto Purification'	Pathogen	6.340 ± 0.7319 c
'Violet Queen'	Pathogen	3.825 ± 0.7319 d

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.8. Shoot height of 'Mountain Spring' tomato seedlings treated with herbage (dried leaves and flowers) and pathogen ($P = 0.2771$).

Herbage	***Pythium myriotylum***	**Stem diameter (mm)**
None	No pathogen	12.324 ± 0.8085 a
'Cerise'	No pathogen	9.188 ± 0.8085 b
'Puerto Purification'	No pathogen	10.086 ± 0.8085 b
'Violet Queen'	No pathogen	10.754 ± 0.8085 ab
None	Pathogen	7.128 ± 0.8085 c
'Cerise'	Pathogen	5.672 ± 0.8085 c
'Puerto Purification'	Pathogen	6.906 ± 0.8085 c
'Violet Queen'	Pathogen	6.306 ± 0.8085 c

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.9. Disease index of 'Celebrity' tomato seedlings treated with *Beauveria* and with or without the pathogen ($P = 0.1571$).

Beauveria	***Pythium myriotylum***	**Disease index**
None	No pathogen	1.979 ± 0.2645 d
Bb 11-98	No pathogen	2.823 ± 0.2645 c
BotaniGard	No pathogen	2.542 ± 0.2645 cd
None	Pathogen	4.323 ± 0.2645 b
Bb 11-98	Pathogen	4.427 ± 0.2645 ab
BotaniGard	Pathogen	5.000 ± 0.2645 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.10. Disease index of 'Celebrity' tomato seedlings treated with herbage (dried leaves and flowers) and *Beauveria* ($P = 0.8899$).

Beauveria	**Herbage**	**Disease index**
None	None	3.208 ± 0.3572 cd
Bb 11-98	None	3.583 ± 0.3572 abc
BotaniGard	None	3.500 ± 0.3572 bcd
None	'Cerise'	3.146 ± 0.3572 cd
Bb 11-98	'Cerise'	3.292 ± 0.3572 cd
BotaniGard	'Cerise'	3.458 ± 0.3572 bcd
None	'Puerto Purification'	2.625 ± 0.3572 d
Bb 11-98	'Puerto Purification'	3.229 ± 0.3572 cd
BotaniGard	'Puerto Purification'	3.667 ± 0.3572 abc
None	'Violet Queen'	3.625 ± 0.3572 abc
Bb 11-98	'Violet Queen'	4.396 ± 0.3572 ab
BotaniGard	'Violet Queen'	4.458 ± 0.3572 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.11. Survival (%) of 'Celebrity' tomato seedlings treated with *Beauveria* and pathogen ($P = 0.3033$).

Beauveria	***Pythium myriotylum***	**Survival (%)**
None	No pathogen	90.6 ± 4.9216 a
Bb 11-98	No pathogen	79.2 ± 4.9216 a
BotaniGard	No pathogen	83.3 ± 4.9216 a
None	Pathogen	58.3 ± 4.9216 b
Bb 11-98	Pathogen	52.1 ± 4.9216 bc
BotaniGard	Pathogen	41.7 ± 4.9216 c

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.12. Survival (%) of 'Celebrity' tomato seedlings treated with herbage (dried leaves and flowers) and *Beauveria* ($P = 0.7838$).

Beauveria	**Herbage**	**Survival (%)**
None	None	72.9 ± 6.8559 ab
Bb 11-98	None	62.5 ± 6.8559 bcd
BotaniGard	None	66.7 ± 6.8559 abcd
None	'Cerise'	70.8 ± 6.8559 abc
Bb 11-98	'Cerise'	70.8 ± 6.8559 abc
BotaniGard	'Cerise'	66.7 ± 6.8559 abcd
None	'Puerto Purification'	85.4 ± 6.8559 a
Bb 11-98	'Puerto Purification'	77.1 ± 6.8559 ab
BotaniGard	'Puerto Purification'	66.7 ± 6.8559 abcd
None	'Violet Queen'	68.8 ± 6.8559 abcd
Bb 11-98	'Violet Queen'	52.1 ± 6.8559 cd
BotaniGard	'Violet Queen'	50.0 ± 6.8559 d

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Table II.13. Survival (%) of 'Celebrity' tomato seedlings treated with herbage (ground leaves and flowers) and with or without the pathogen ($P = 0.1031$).

Herbage	***Pythium myriotylum***	**Survival (%)**
None	No pathogen	91.7 ± 5.6405 a
'Cerise'	No pathogen	87.5 ± 5.6405 a
'Puerto Purification'	No pathogen	88.9 ± 5.6405 a
'Violet Queen'	No pathogen	69.4 ± 5.6405 b
None	Pathogen	43.1 ± 5.6405 d
'Cerise'	Pathogen	51.4 ± 5.6405 cd
'Puerto Purification'	Pathogen	63.9 ± 5.6405 bc
'Violet Queen'	Pathogen	44.4 ± 5.6405 d

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Figure II.14. Disease index of 'Celebrity' tomato seedlings treated with *Beauveria*, herbage (dried leaves and flowers), and with or without the pathogen ($P = 0.7753$).

Beauveria	**Herbage**	**Pathogen**	**Disease index**
None	None	No pathogen	1.5 ± 0.4928 i
Bb 11-98	None	No pathogen	2.2 ± 0.4928 ghi
BotaniGard	None	No pathogen	2.0 ± 0.4928 hi
None	'Cerise'	No pathogen	1.8 ± 0.4928 hi
Bb 11-98	'Cerise'	No pathogen	2.6 ± 0.4928 fghi
BotaniGard	'Cerise'	No pathogen	2.3 ± 0.4928 ghi
None	'Puerto Purification'	No pathogen	1.9 ± 0.4928 hi
Bb 11-98	'Puerto Purification'	No pathogen	2.5 ± 0.4928 ghi
BotaniGard	'Puerto Purification'	No pathogen	2.8 ± 0.4928 defghi
None	'Violet Queen'	No pathogen	2.7 ± 0.4928 efghi
Bb 11-98	'Violet Queen'	No pathogen	4.0 ± 0.4928 bcd
BotaniGard	'Violet Queen'	No pathogen	3.1 ± 0.4928 defgh
None	None	Pathogen	4.8 ± 0.4928 ab
Bb 11-98	None	Pathogen	5.0 ± 0.4928 ab
BotaniGard	None	Pathogen	5.0 ± 0.4928 ab
None	'Cerise'	Pathogen	4.5 ± 0.4928 abc
Bb 11-98	'Cerise'	Pathogen	4.0 ± 0.4928 bcdef
BotaniGard	'Cerise'	Pathogen	4.6 ± 0.4928 abc
None	'Puerto Purification'	Pathogen	3.4 ± 0.4928 cdefg
Bb 11-98	'Puerto Purification'	Pathogen	4.0 ± 0.4928 bcde
BotaniGard	'Puerto Purification'	Pathogen	4.5 ± 0.4928 abc
None	'Violet Queen'	Pathogen	4.6 ± 0.4928 abc
Bb 11-98	'Violet Queen'	Pathogen	4.8 ± 0.4928 ab
BotaniGard	'Violet Queen'	Pathogen	5.8 ± 0.4928 a

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

Figure II.15. Survival (%) of 'Celebrity' tomato seedlings treated with *Beauveria*, herbage (dried leaves and flowers), and with or without the pathogen ($P = 0.05734$).

Beauveria	**Herbage**	**Pathogen**	**Survival (%)**
None	None	No pathogen	95.8 ± 9.6210 a
Bb 11-98	None	No pathogen	87.5 ± 9.6210 ab
BotaniGard	None	No pathogen	91.7 ± 9.6210 a
None	'Cerise'	No pathogen	91.7 ± 9.6210 a
Bb 11-98	'Cerise'	No pathogen	83.3 ± 9.6210 abc
BotaniGard	'Cerise'	No pathogen	87.5 ± 9.6210 ab
None	'Puerto Purification'	No pathogen	95.8 ± 9.6210 a
Bb 11-98	'Puerto Purification'	No pathogen	91.7 ± 9.6210 a
BotaniGard	'Puerto Purification'	No pathogen	79.2 ± 9.6210 abcd
None	'Violet Queen'	No pathogen	79.2 ± 9.6210 abcd
Bb 11-98	'Violet Queen'	No pathogen	54.2 ± 9.6210 def
BotaniGard	'Violet Queen'	No pathogen	75.0 ± 9.6210 abcde
None	None	Pathogen	50.0 ± 9.6210 ab
Bb 11-98	None	Pathogen	37.5 ± 9.6210 fg
BotaniGard	None	Pathogen	41.7 ± 9.6210 fg
None	'Cerise'	Pathogen	50.0 ± 9.6210 efg
Bb 11-98	'Cerise'	Pathogen	58.3 ± 9.6210 cdef
BotaniGard	'Cerise'	Pathogen	45.8 ± 9.6210 fg
None	'Puerto Purification'	Pathogen	75.0 ± 9.6210 abcde
Bb 11-98	'Puerto Purification'	Pathogen	62.5 ± 9.6210 bcdef
BotaniGard	'Puerto Purification'	Pathogen	54.2 ± 9.6210 def
None	'Violet Queen'	Pathogen	58.3 ± 9.6210 cdef
Bb 11-98	'Violet Queen'	Pathogen	50.0 ± 9.6210 efg
BotaniGard	'Violet Queen'	Pathogen	25.0 ± 9.6210 g

[a] Values are least squares means ± the standard error of the mean.
[b] Values with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

APPENDIX III

Total Phenol Extraction and Analysis of Tomato Seedlings (*Lycopersicon esculentum* Mill. cv. Celebrity and Mountain Spring) from Experiments Testing Biological Control Efficacy of *Beauveria bassiana, Monarda* herbage, and PGPR Against *Pythium myriotylum*

Introduction

The function of secondary metabolites, or plant natural products, is to influence ecological interactions between the plant and its environment (Croteau et al., 2000). These compounds play a pivotal role in the protection of plants, attraction of pollinators and seed-dispersing animals, and influence competition among plant species (Rodríguez-Concepción and Boronat, 2002). Plant natural products generally belong to one of these chemical groups: alkaloids, terpenoids, and the phenylpropanoids. Phenolic compounds are formed through the shikimate or malonate/acetate pathways, and the group consists of about 8000 compounds (Croteau et al., 2000). These compounds, specifically salicylic acid, have been documented as precursors to systemic defense in plants otherwise known as systemic acquired resistance. Another phenolic compound, jasmonic acid, is believed to trigger ISR, or induced systemic resistance in plants that have plant growth-promoting rhizobacteria. Tomato roots colonized by the biological control *Pythium oligandrum* accumulated phenolic compounds during the first few hours after inoculation. This is significant because this oomycete invades in the same way as the pathogenic species (Le Floch et al., 2005). Treatment of tomato and hot pepper with fluorescent pseudomonads showed induction of defense-related enzymes, increased growth, and higher

accumulation of phenolics when challenged with *P. aphanidermatum* (Ramamoorthy, et al., 2002). It was therefore hypothesized that if *Beauveria bassiana*, *Monarda* herbage, or PGPR proved successful as biological controls in the system tested previously (Chapter 2), that the result would yield increased levels of total phenolics.

Phenol extraction

Phenols were extracted from frozen plant material from the experiments in chapter 2 using a modified method from Waterhouse (Slinkard et al., modified by Waterhouse, 2004). Samples were ground under liquid Nitrogen and approximately 500 mg placed in a plastic screw capped tube with 2.5 ml of 50% methanol. Samples were boiled for 2 hours with intermittent shaking at 90°C (Vinson et al., 1998). Each sample was filtered with a nylon filter (45 µm) and placed in a 1.5 ml microcentrifuge tube (Fisher Scientific, Fair Lawn, NJ) and stored at -80°C.

Phenol analysis

Gallic acid stock solution was 0.5% wt/v. in methanol. The calibration curve was then prepared by diluting the stock solution to give a final gallic acid equivalent (GAE) (mg/L) ranging from 0 to 632.66 (Table 1). Buffer solution (50 g anhydrous sodium carbonate in 200 ml deionized water) was brought to boiling then filtered through a Whatman No. 1 filter paper (Fisher Scientific, Fair Lawn, NJ) and water was added to 250 ml. In 1.5 ml polystyrene cuvettes (Fisher Scientific, Fair Lawn, NJ), aliquots (80 µl) of sample or standard, 1.52 ml deionized water and 100 µL of Folin-Ciocaltaeu's phenol reagent (Sigma-Aldrich, Inc., St. Louis, MO) were mixed well. After 30 seconds, but before 8 minutes, 300 µL of sodium carbonate buffer was added, and cuvette was

shaken. The solutions were incubated at 20°C for 2 h or 40°C for 30 min. Absorbance at 765 nm was determined by use of a (Shimadzu, Columbia, MD) using the reagent mix without standard or sample as reference.

Data analysis and discussion

Data was transformed and analyzed using PROC MIXED in Statistical Analysis System with an F-protected LSD of $P = 0.05$ (SAS Institute, Inc., Cary, NC). There was a significant increase in total phenols of 'Celebrity' seedlings when treated with herbage from 'Cerise' and 'Violet Queen' compared to no treatment and treatment with 'Puerto Purification' (data not shown). There was also an increase in total phenols of 'Celebrity' seedlings in the pathogen infested versus uninfested. The opposite was true for 'Mountain Spring,' which had higher total phenols in the uninfested control compared to the pathogen treatment. An accumulation of phenolic compounds was seen in tomato plants 3 h after inoculation with the biocontrol agent, *Pythium oligandrum* (Le Floch et al., 2005). However, in the previous analysis, phenols were measured in roots and a fresh weight was used for the calculation of phenolics. In the present study, total phenols were calculated in gallic acid equivalents in mg/l (Table 1) using a volumetric value. The value used in this calculation was a relative plant volume (shoot height x stem diameter). This was not a successful analysis because the phenol readings that were obtained were extremely small and unlikely to be a true representation of the total phenols in the plant. In the future, total phenol analysis should be calculated using a fresh weight, as the volumetric calculation did not prove successful.

Table III.1. Gallic acid equivalents for standards used to produce a standard curve in total phenol analysis of tomato (*Lycopersicon esculentum*) seedlings from experiment testing efficacy of soil amendment with one of three *Monarda* sp. varieties, seed treatment with *Beauveria* sp., and/or seed drench with a commercial plant growth-promoting rhizobacteria against *Pythium myriotylum* damping-off.

Amount from stock solution[a] (µl)	Gallic acid equivalent (mg/l)
0	0
100	4.52
200	9.04
300	13.56
400	18.08
500	22.60
1.0	45.20
2.0	90.38
3.0	135.57
5.0	225.95
7.5	338.92
10	451.90
12	542.28
14	632.66

[a] Stock solution: 500 mg monohydrated gallic acid dissolved in 10 ml ethanol, brought to volume in 100 ml volumetric flask.

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