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Fungal endophytes enhance growth and production of natural products in *Echinacea purpurea* (Moench.)

A Thesis Presented for the
Master of Science
Degree
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Richard James Gualandi, Jr.
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Abstract

Echinacea purpurea is a native herbaceous perennial with substantial economic value for its medicinal and ornamental qualities. Arbuscular mycorrhizae are symbiotic fungi that form relationships with plant roots and are known to enhance growth in the host. Mycorrhizae and other fungal endophytes often affect stress resistance and secondary metabolism in the host, as well as the ecology of other endophytes in the plant. A newly emerging paradigm in sustainable biotechnique is the targeted use of fungal endophytes to enhance growth and secondary metabolism in crops. Many of the therapeutic compounds in *E. purpurea* could be affected by fungal colonization. In this research the effects of inoculation of *Echinacea purpurea* with two classes of fungal endophytes: the arbuscular mycorrhizal fungi *Glomus intraradices* and *Gigaspora margarita* and the entomopathogenic endophyte *Beauveria bassiana* were evaluated . Endophyte colonization and impacts on plant growth and phytochemistry were tested in multiple greenhouse experiments. Arbuscular mycorrhizae and *B. bassiana* effectively colonized *E. purpurea* with some significant interactive effects. Consistent, substantial, and significant increases in all growth parameters were observed in mycorrhizal plants; mycorrhizal plants produced up to four times the biomass of controls in 12 weeks. Broad spectrum changes in fertilization were necessary to produce mycorrhizal and nonmycorrhizal samples of equal size, and severely nutrient-limited mycorrhizal *E. purpurea* seedlings maintained growth rates comparable to well fertilized samples. Treatment with *B. bassiana* had minor and inconsistent effects on some plant growth parameters, and there were significant interactive effects with arbuscular mycorrhizae. Phytochemical concentrations in all metabolite classes tested responded significantly to inoculation with both classes of fungal endophytes. Changes were observed in

various pigments, caffeic acid derivatives, alkylamides, and terpenes. Many of the affected compounds have important roles in metabolism or have bioactive value as natural products. When considered from a net production perspective (concentration X dry weight), compared to controls, plants inoculated with endophytes produced as much as 30 times the content of some compounds in 12 weeks. This work effectively demonstrates that fungal endophytes can enhance the bioactivity of plant tissues and the production of natural products in *E. purpurea*.

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Chapter I

Introduction

Echinacea is a genus of herbaceous perennial plants in the family Asteraceae. Species are distributed across a wide range of North America extending from Alberta, Canada south to the Gulf of Mexico and west to the foot hills of the Rocky Mountains (Flagel et al., 2008). Economically, the genus has substantial value with multiple species marketed as medicinal herbs, ornamentals and cut-flowers (Blumenthal, 2005). Native Americans and early North American settlers depended on *Echinacea* to treat multiple ailments (Gilmore, 1913). *Echinacea* is still commonly used in multiple parts of the world; new properties continue to be identified.

A growing body of data supports the folk medicine claims that extracts of *Echinacea* have real therapeutic value. The exact mechanisms and full range of benefits are not fully understood; however, modern research is revealing new insight into the full range of potential properties and the mode of action in the human body (Blumenthal et al., 2003; Shah, 2007). *Echinacea* extracts are most well known for their immune-stimulatory properties; however, extracts also have anti-viral, anti-bacterial, anti-inflammatory, antioxidative and anti-cancer properties (Barrett, 2003; Pellati et al., 2004; Matthias et al., 2005; Senchina et al., 2006; Chicca, 2007). However, research opportunities exist to explore the production of active compounds in the plant and potential new properties and applications.

Plants often harbor microsymbionts known as endophytes; “endo” meaning “in” and “phyte” meaning “plant”. These organisms cause no damage or disease symptoms and can

confer various degrees of benefit to the host (Ownley et al., 2008b; Gunatilaka, 2006). Endophytic organisms play an important role in many facets of plant growth and development. The foundations of the mechanisms that allow these relationships have evolutionary origins and exist in the genomes of both organisms. Complex changes in gene expression, morphology and biochemistry take place in both partners, leading to altered growth and development patterns that allow the symbiosis to function (Armstrong and Peterson, 2002; Balestrinini and Bonfante, 2005, Strack et al., 2003). The plant host can benefit in various ways. Enhanced growth, nutrient use efficiency, stress tolerance and disease resistance have all been demonstrated (Augé, 2001; Clay, 1990; Clay and Holah, 1999; Kageyama et al., 2008; Redman et al, 2002; Smith and Gianinazzi-Pearson, 1988; Rudgers et al., 2009). Some of these benefits are a direct result of altered biochemistry in the plant or bioactive compounds produced by the endophyte (Bultman et al., 2004; Dehne, 1986; Smith and Gianinazzi-Pearson, 1988; Morandi et al., 1984; Allen et al., 1980; Fester et al., 2002; Nemec and Lund, 1990). The various benefits endophytes can offer crops, and the compounds produced by plants and their endophytes, could have potential applications in agriculture, horticulture, biotechnology and the natural products industry.

Many fungi exist as endophytes and can be found across broad habitat types in most plant species, and can illicit various morphologic and chemical changes in the host (Arnold, 2007; Giminez, 2007; Vega, 2008). A better understanding of the range of species-specific interactions and their effects on plant growth and metabolism may lead to the development of production schemes utilizing fungal endophytes. Targeted, applied use has the potential to produce better yielding, more resilient crops with increased concentrations of desirable natural

products. Effective systems have the potential for broad usefulness in organic and alternative production and could reduce the need for agrochemical pesticides and fertilizers in conventional systems.

Echinacea purpurea can serve as good model plants for studying the effects of fungal endophyte colonization on secondary metabolism. It has significant economic importance, a well documented chemical profile, and some of its therapeutic chemicals are known to be affected by endophytic fungi colonization (Araim et al., 2009; Bauer and Wagner, 1991; Lata et al., 2003).

Growing demand for *Echinacea* herbal products is spurring interest in novel and more efficient production methods (Rai et al., 2001). Some work has been done developing and selecting improved varieties which could be propagated in large volume using micro-propagation. Arbuscular mycorrhizae can enhance acclimation rates of tissue cultured plantlets, subsequent growth of *Echinacea* plantlets (Lata et al., 2003), and plants grown from seed (Araim et al., 2009), and likely even vegetative propagation. Arbuscular mycorrhizae has been shown to alter phytochemistry in *E. purpurea,* and other endophytes are well known to have the same effect in a diverse range of other plant species (Araim et al., 2009; Kapulniki et al., 1996; Peipp et al., 1997; Strack et al., 2003; Toussaint et al., 2007; Zhi-lin et al., 2007).

Further research with fungal endophytes in *Echinacea* could lead to more productive and efficient production schemes as well as a better understanding of the dynamics of environmental influence on phytochemical production in plants. This study seeks to explore the potential of inoculation of *Echinacea purpurea* with two classes of fungal endophytes as part of a commercially viable greenhouse production scheme. The primary focus of this investigation is

the efficacy and effect of endophyte inoculation on growth and phytochemistry in *Echinacea purpurea*.

The potential economic value inherent in demonstrating a natural method to increase production efficiency and therapeutic potency in *E. purpurea* warrants investigation. This research could offer new insight and approaches useful to reaching those goals. The perspective gained from this research could also offer a more complete look into the potential role that fungal endophytes play in the regulation of growth and metabolism in *E. purpurea* and other plant species.

This work seeks to explore the potential use of endophytes in plant production, from the broader context of a new paradigm. Endophyte inoculation as a natural method to stimulate desirable effects in crops could lead to more efficient, resilient, productive, potent and ultimately valuable crops. If explored and maximized, the benefits could lead to less dependency on synthetic inputs in conventional systems, and improved crop yields and stress resistance in all systems. Organic systems could benefit since nutrient availability is often a limiting factor, and biological controls are considered an important and effective strategy for pest and disease management. Due to the high demand for organic herbal products, and the potential for increasing the content of desirable natural products in plant tissues, the natural products industry could greatly benefit as well.

Novel bioactive fungal compounds, with desirable properties, could also theoretically be produced in plants inoculated with specific endophytes. Some endophytes produce many of the same desirable compounds that their host plants do (Tan and Zou, 2001). Isolation of such fungi

and *in vitro* or *in planta* growth in proxy host species could relieve demand and collection pressures for threatened or endangered species.

If desirable uses for these fungi can be identified and applied efficiently it has the cumulative potential to address many relevant modern and future social and ecological issues such as increasing demand for food, pollution and dependency from overuse of synthetic chemicals, mineral shortages, and endangered species conservation.

This work could never address all considerations and concerns related to such a broad application of a complicated biological relationship. However, establishing the potential usefulness of such an approach through testing inoculation methods and physiological effects of various endophytes in plant species would be the necessary first step towards that end. This work seeks to contribute to the body of knowledge related to the subject.

1. Hypothesis and Research Objectives.

Central research question:

Will inoculation with fungal endophytes lead to colonization and alter growth and phytochemical profiles in *Echinacea purpurea*?

Hypothesis:

Successful inoculation with fungal endophytes will result in altered growth and phytochemical profile in *Echinacea purpurea*.

Research objectives:

1. Determine if inoculation of *Echinacea purpurea* with two classes of fungal endophytes results in successful colonization and if interactive effects exist.
2. Determine if inoculation with fungal endophytes alters growth and development in *Echinacea purpurea.*
3. Determine if inoculation with fungal endophytes alters phytochemical profile in *Echinacea purpurea.*

Chapter II

Review of Related Literature

1. The genus Echinacea.

1.1. Taxonomy. Species in the genus *Echinacea* (Family Asteraceae) are distributed across much of North America extending from Alberta, Canada south to the Gulf of Mexico and west to the foothills of the Rocky Mountains (Flagel et al., 2008). *Echinacea purpurea* and *E. angustifolia* have the broadest ranges (Ault, 1999).

Rudbeckia purpurea (later renamed *Echinacea purpurea)* was first described by Linneaus in the 18th century (Ault, 2007). Taxonomy of the genus was in a state of flux until genus descriptions were formally accepted by the 1959 Botanical Congress (McGregor, 1968). Based on morphological characteristics, McKeown (1999) recognized nine species, some with multiple varieties: *E. angustifolia* DC. var. *angustifolia; E. angustifolia* DC. var. *strigosa* McGregor; *E. atrorubens* Nutt.; *E. laevigata* (Boynton and Beadle) Blake; *E. pallida* (Nutt.) Nutt.; *E. paradoxa* (Norton) Britton var. *neglecta* McGregor; *E. paradoxa* (Norton) Britton var. *paradoxa* McGregor*; E. purpurea* (L.) Moench; *E. sanguinea* Nutt.; *E. simulata* McGregor; and *E. tennesseensis* (Beadle) Small.

Based on taxonomic studies using multivariate data analysis, cpDNA restriction site variation and random amplified polymorphic DNA (RAPD) techniques, broader clads within the genus have been proposed (Binns et al., 2004*;* Kapteyn et. al., 2002*;* Leinert et al, 1998; Urbatsch and Jansen, 1995). The suggested revised taxonomy places several of the variants within larger clads under *E. pallida* and *E. atrorubens,* as varieties rather than distinct species,

with *E. laevigata* and *E. purpurea* remaining distinct (Binns et al., 2002a). A dendrogram developed based on lipophilic metabolic profiling was more consistent with the taxonomy based on morphology than the taxonomy proposed based on the RAPD technique (Wu et al. 2009).

All species of *Echinacea* are herbaceous perennials with upright flower stalks that emerge from basal rosettes. Size ranges from 0.6–to 0.9m. Foliage can be cordate, oblong, or lanceolate (Armitage, 1997; Greenfield and Davis, 2004) depending on species and growth stage. Most species have taproots with the exception of *E. purpurea* which has a more fibrous root system. Cone-shaped flower heads range from 3.5 to 18 cm in diameter and are composed of disk and ray flowers with radiating colorful ligules. Often incorrectly described as petals, ligules range in color from purple to pink, yellow or white and can be found drooping, outstretched or upright (Ault, 1999; Armitage, 1997).

1.2. Agronomic value and production. The genus has substantial economic value based on its use as herbal medicine and for ornamental plantings. Three species are presently marketed as medicinal herbs: *E. angustifolia, E. purpurea,* and *E. pallida*; however, others may have potential as well. *Echinacea purpurea* is a popular ornamental and cut-flower species (Armitage, 1997; Bauer and Wagner, 1990; Valo, 1995). In 2005, U.S. sales of *Echinacea* herbal products exceeded $23 million (Blumenthal, 2005); ornamental sales are not well documented, but the plant is popular and commonly available.

Native American Indian tribes used *Echinacea* sp. to treat a diverse group of ailments including colds, burns, snake bites and more (Gilmore, 1913). Early European settlers also used and exported *Echinacea* to Europe where it had a myriad of applications (Ault, 2007).

Echinacea extract is still commonly used for its therapeutic value, especially in North America and Europe.

Although *Echinacea* is grown commercially around the world in various climates, European countries are the leading producers (Galambosi, 2004). Many propagation methods are used, e.g., seeds, division, and stem cuttings (Armitage, 1997; Choffe et al., 2000a; Choffe et al., 2000b; Smith-Jochum and Albrict, 1988), but tissue culture is becoming increasingly common as the number of cultivated varieties increases. Field, nursery, and greenhouse production schemes are typically used, depending upon the end product (Ault, 2007), but most commercial growing operations use seed production. Crops grown for the medicinal market are typically field grown, while ornamentals are often container grown for easy sale. Seeds are direct sown in spring or fall or started in greenhouses prior to planting out. Plants grown for herbal products are typically harvested 3-4 years after planting (Ault, 2007; Greenfield and Davis, 2004). All *Echinacea* species require full sun to part shade and deep well drained, neutral to alkaline soils (Mordalsky, 1994; Foster, 1991; Galambosi et al., 1994). Most species are considered exceptionally drought tolerant, especially tap rooted species (Galambosi, 2004).

Echinacea species have few pest or pathogen problems that cause serious economic losses. However, some diseases can significantly reduce plant growth and quality. Aster Yellows, Rhizoctonia or Pythium damping-off; Botrytis blight; Fusarium crown and root rot; Sclerotina stem rot, and Alternaria leaf spot can all cause serious damage or death (Chang et al., 1999; Chang et al., 2000a; Chang et al., 2000b; Greenfield and Davis, 2004; Hwang et al., 2001; Galambosi, 2004). Although insect pest problems are few, whiteflies can be problematic in

greenhouse crops (personal observations). When grown in the field, weed control is important since *Echinacea* is easily outcompeted until well established (Greenfield and Davis, 2004).

1.3. Phytochemistry. Many studies support the claims that extracts of *Echinacea* have therapeutic properties. The mechanisms are not fully understood, but synergistic effects among compounds are believed to play a role. Modern biomedical research has validated the historical role of *Echinacea* for treating multiple ailments (Blumenthal et al., 2003; Shah, 2007). Some documented therapeutic actions of *Echinacea* extracts include: immuno-stimulatory and regulatory; anti-microbial; anti-inflammatory; anti-oxidative; and anti-cancer (Barrett, 2003; Pellati et al., 2004; Matthias et al., 2005; Senchina et al., 2006; Chicca, 2007). Although many active compounds have been identified, their mode of action, synergistic effects, biosynthesis and role in plant physiology are not fully understood. Phytochemicals produced by *Echinacea* and their distribution in the plant have been well documented. Three primary groups considered to be therapeutic are: 1) polyphenolic derivatives of caffeic acid; 2) lipophillic alkylamides; and 3) polysaccharides (Goel et al., 2002; Bone, 1998; Hsieh, 2009). The alkylamide and phenolic acids are often used as standard marker compounds to establish potency and for species verification of *Echinacea* used to produce herbal products (Galambosi, 2004).

1.3.1. *Polyphenolic acids*. Polyphenolic acids, polar compounds with more than one phenol molecule, are widespread in the plant kingdom. In the plant, they have important roles in environmental adaptation and plant defense (Luzzatto et al., 2007). They have multiple bioactive properties (anti-microbial, anti-oxidant, anti-allergenic, anti-tumor, anti-inflammatory

and radioprotective properties) and function as phytoestrogens (Barret, 2003; Bone, 1998; Speroni et al., 2002; Kono et al., 1997; Samorodov et al., 1996).

Several caffeic acid derivatives (CADs) have been isolated from *Echinacea* including echinacoside, cynarin, chlorogenic acid, caftaric acid, and cichoric acid (Figure 2.1) (Luo et al., 2006; Begeron et al., 2000). Activities of these compounds include antimicrobial (viral, bacterial, and fungal), antioxidant, stimulation of the immune system, and reduction of blood pressure, tremors, and pain (Bauer and Wagner, 1991; Samorodov et al., 1996). The CADs are generally abundant and distributed throughout the plant; however, concentrations vary with species, tissue, growth stage and environmental conditions (Araim et al., 2009; Bauer et al., 1988; Binns, 2002; Qu et al., 2005; Stuart and Wills, 2000).

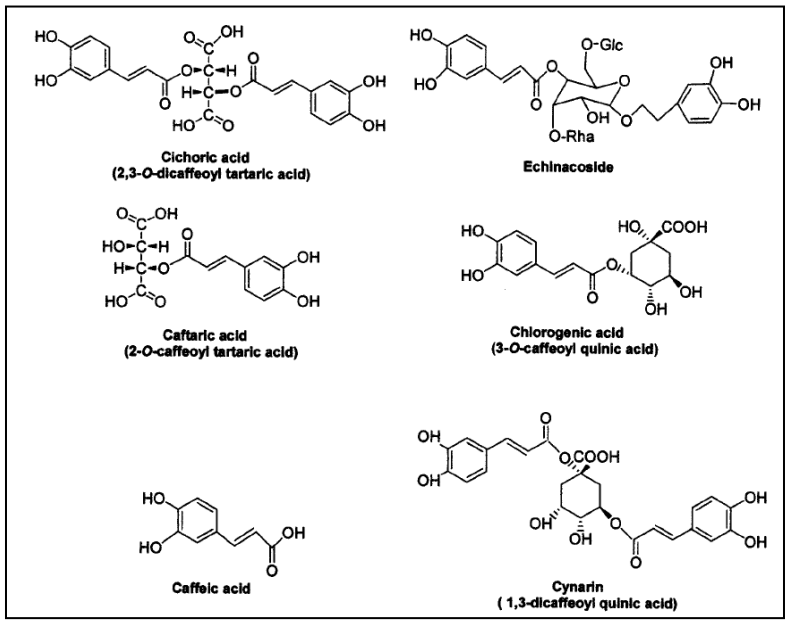


Figure 2.1. Polyphenolic fractions from *Echinacea* species. Diagram from Binns et al. (2002). Used with permission (Elsevier publishing).

In *E. purpurea,* cichoric acid content can range from 0.6-2.1% in roots and flowers with substantially less in leaves and stems (Bauer, 1998). UV light levels increased levels of CADs in hairy root cultures of *Echinacea* (Bilal et al., 2007). Biosynthesis of CADs is linked to the early steps of the shikimate pathway and may be regulated by phenylalanine ammonia lyase (PAL). PAL activity is influenced by various environmental (Bilal et al., 2007; Dixon and Paiva, 1995) and biological factors including colonization by endophytic fungi (Harrison and Dixon, 1993).

1.3.2. *Lipophyllic Alkylamides.* The lipophylic alkylamides are unsaturated fatty acid chains of carboxylic acid with attached amide groups (Bauer and Remiger, 1989). Neither biosynthesis nor the biological significance of these compounds in the plant have been fully elucidated. Synthesis of some of these compounds has been achieved through common intermediates. Alkylation of a silylated diacetylene anion has been determined to be the critical step (Wu et al., 2004).

At least 20 alkyamides are produced by *Echinacea* sp. (Bauer et al. 1998, 1999; Harborne and Williams, 2004) (Figure 2.2) although their distribution varies with species, organ and age (Leinert, et al., 1998; Qu et al., 2005; Wu et al. 2004). Alkylamide concentrations are highest in roots, developing flowers, and seeds and are lowest in stems and leaves (Qu et al., 2005). Seasonal reductions in roots are correlated with increased levels in flowers (Stuart and Wills, 2000) indicating dynamic movement within the plant. Environmental and genetic variability also affect the production and accumulation of these compounds (Smith-Jochum and Davis, 1991). Alkylamide production can be induced in *E. pallida* roots with methyl jasmonate

(Binns et al., 2001), a signaling compound with elevated concentrations in plants colonized with mycorrhizae (Hause et al., 2002).

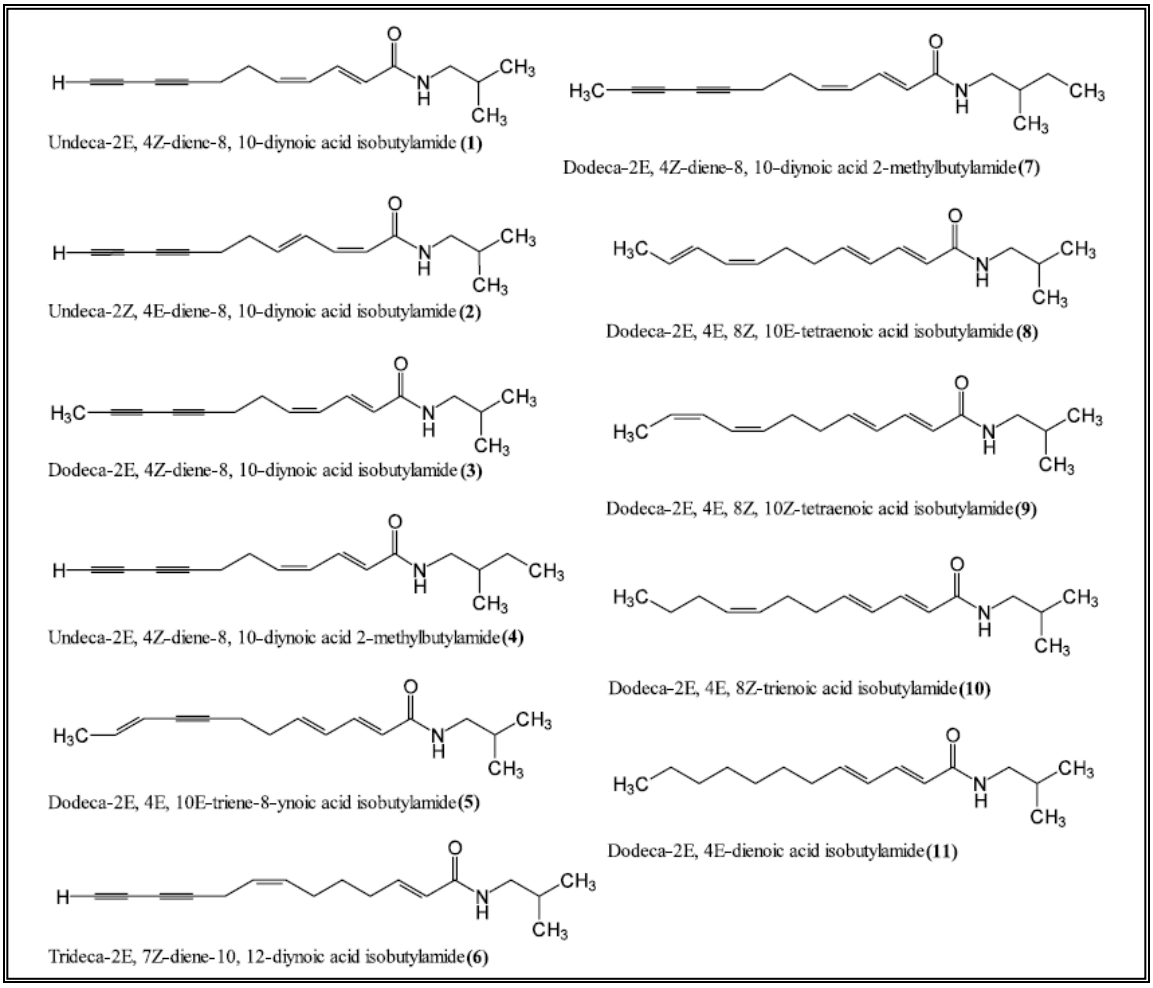


Figure 2.2. Liphophylic alkylamides from *Echinacea* species. Numbering system as devised by Bauer and Remiger (1989). Diagram from Hudaib et al. (2002). Used with permission (Elsevier publishing).

Alkylamides are often used as standard marker compounds in *Echinacea* products and are major contributors to their bioactivity (Barett, 2003; Woelkart and Bauer, 2007). They stimulate immune function by binding directly to a cannabinoid receptor in the brain, related to immune function, known as CB2 (Raduner et al., 2006). The CB2 receptor is related to immune system macrophage (Goel et al., 2002) and T-cell activity (Sasagawa et al, 2006). All alkylamides do not have the same CB2 binding affinities (Raduner et al., 2006) and differences in the number and placement of the double and triple bonds, along a fatty acid chain, affect receptor binding and other bioactivity (Matthias et al., 2007, Chen et al., 2005). Some of these compounds also have anesthetic (Bauer and Wagner, 1991), anti-inflammatory (Chen et al. 2005), antimicrobial, allelopathic, insecticidal and anticancer properties (Bauer and Wagner, 1991; Barret, 2003; Piechowski, 2006).

1.3.3. *Polysaccharides.* Polysaccharide fractions in *Echinacea* are believed to have nonspecific immune-enhancing, tissue-regenerating and possibly anti-viral properties (Berman et al., 1998; Enbergs and Woestman, 1986; Newall et al., 1996). Therapeutic value has been established for three polysaccharides; two are fructogalactoxyloglucans, and one is an arabinogalactan (Wagner et al., 1988; Wagner and Proksch, 1987). Although the exact mechanisms are not understood, these polysaccharides have immune stimulatory properties (Bauer and Wagner, 1991) and other therapeutic actions. Increased macrophage activity (Ying et al., 2005) and tissue regeneration as well as reduced inflammation (Tubaro et al., 1987) have all been reported. Recent studies have demonstrated that endophytic bacteria likely play a significant role in the accumulation of these significant polysaccharides in many plant species (Strobel, 2003; Sun et al., 2006).

Polysaccharide fractions have been isolated from root and aerial tissues and are often primary cell wall components (Wagner et al., 1995; Harborne and Williams, 2004). Although they are not known to play a major role in plant defense, increased synthesis of some polysaccharides (e.g., xyloglucans, arabinogalactans) has been observed at the peri-arbuscular interface within mycorrhizal plant cells (Balestrini et al., 1994; Perotto et al., 1994; Bonfante and Perotto, 1995).

1.3.4. *Other Phytochemicals.* Several other classes of compounds of interest have been identified in *Echinacea* including alkaloids, flavonoids, carotenoids, and essential oils. (Bauer et al., 1998a). Over 70 volatile compounds including several terpenes, aldehydes, alcohols, hydrocarbons, ketones and more have been isolated from root and aerial tissue of species of *Echinacea* (Mazza and Cottrell, 1999); some of those may have value to the natural products industry. The specific therapeutic value of these other compounds in *Echinacea* sp. has not been well studied to date; however, they could prove to be important since synergistic effects among compounds are thought to contribute to *Echinacea*'s full mode of action (Dalby-Brown et al., 2005).

2. Echinacea purpurea.

2.1. Taxonomy. *Echinacea purpurea*, commonly known as Purple Coneflower, has a natural range that extends from Texas to the Midwest and southeast regions of the United States (Greenfield and Davis, 2004; Radford, 1968), but it is more widely distributed because it was exported to many regions of the world for medicinal and ornamental purposes.

Being evolutionarily distinct from the other species, it is slightly different culturally and morphologically from the other *Echinacea* species. Morphologically, *E. purpurea* can be distinguished easily by its lack of a taproot and 10-20cm long broadly lanceolate, ovate to cordate-shaped, sharply pointed juvenile basal foliage. Flower stalks have smaller lanceolate sharply pointed foliage, typically stand 0.6-1.2m tall and emerge in June to August (Greenfield and Davis, 2004; Armitage, 1997).

2.2. Culture. *Echinacea purpurea* is more adaptable than the other species; it is better able to tolerate a wider range of conditions (Balambosi, 1993; Greenfield and Davis, 2004). Although it requires slightly moister conditions than the other species for optimal growth, well drained soils are still important (Galambosi et al, 1994). *Echinacea purpurea* displays several effective drought avoidance strategies (Chapman and Augé, 1994; Greenfield and Davis, 2004); however, it is still less drought tolerant than most other *Echinacea* species. It prefers slightly lower pH than other *Echinacea* species with optimum soils in the 5.5 to 7 pH range (Cech, 2002). Tolerant of 50% shade, *E. purpurea* is more typical of woodland habitats than other species (Dey, 2000; Foster, 1991).

2.3. Agronomic value and production. The largest producers of *E. purpurea* are located in the U.S., Canada, Germany, Austria, New Zealand and Switzerland (Greenfield and Davis, 2004; Galambosi, 2004). Crops are produced for ornamental plantings, cut-flowers, and herbal products (e.g., tinctures, ointments, creams, lotions, and toothpastes) (Adam, 2008; Galambosi, 2004). Combined ornamental and herbal sales make *E. purpurea* a substantial economic

commodity, and the market for *E. purpurea* has remained strong even though prices have varied in recent years. In 2001, 195,000 kg of *E. purpurea* was sold; and prices ranged from $3.00-$5.00/kg of dried root and $1.00-$2.20/kg of dried herbage (Adam, 2002).

Fresh *E. purpurea* seeds do not need stratification or priming to germinate, however a 7-28 day cold stratification at 5 ºC and/or 3-9 day priming with 50 mMol $K_2HPO_4 + KH_2PO_4$ at 16 º C improves germination rates, especially in suboptimal conditions (Beattie and Berghage, 1997; Brachter et al., 1993; Dina et al, 1991; Samfield et al., 1990; Shalabi et al., 1997). Light is also required for germination, so seeds should be sown on or just below the soil surface (Greenfield and Davis, 2004). Ideal conditions for germination are 20-25 ºC, and germination typically takes 10-20 days (Brachter et al., 1993). Prepared seed beds or containers are often used, and transplants can be planted to the field in late spring or early summer. Greenhouse production of *E. purpurea* seedlings, prior to planting, can enhance establishment in field crops as opposed to direct seeding (Smith-Jocum an Albricht, 1988).

Field crops are often grown for 2-4 years with aerial portions harvested in fall for the first few years. Once deemed mature, the entire root system is lifted for harvest after the onset of dormancy. Roots are cleaned and processed quickly to optimize the phytochemical content (Cech, 2002; Greenfield and Davis, 2004; Galambosi, 2004) and dried on racks or in ovens; temperature and duration are important since some bioactive compounds are quickly degraded at high or low temperatures (Keinhanen and Julkunen-Titto, 1996; Stuart and Wills, 2000a).

2.4. Phytochemistry. In mature *E. purpurea* plants, 70% of total plant alkylamides content is found in the roots, while percentages in flowers, stems and leaves are 20%, 10%, and 1%

respectively (Stuart and Wills, 2000). Alkylamides 8 and 9 (Baueré, 1989) predominate in *E. purpurea*, but account for a larger percentage of total alkylamide content in flowers than in roots (Qu, et al, 2005). Seedlings often have high concentrations of these compounds which decrease throughout the first growing season (Qu et al., 2005, Stuart and Wills, 2000b) possibly indicating that the plants may be utilizing allelopathic potential of these compounds (Piechowski et al., 2006; Viles and Reese, 1996).

3. <u>Endophytes.</u>

Endophytes are organisms that live inside the plant (Gimenez, 2007). There is some debate over where the boundaries of this definition should exist (Schulz and Boyle, 2005), but the broad definition is not in question. Generally, an endophytic relationship refers to a mutualistic relationship with a positive impact on the fitness of both organisms (Lewis, 1985); however, antagonisms in species-specific interactions have been demonstrated (Saikkonen et al., 2004, 2006). In some cases, the nature of the interaction varies depending on environmental conditions (Gimenez et al., 2007; Rodriguez et al., 2009; Ownley et al., 2010).

Plants can serve as hosts to a large and diverse group of endophytic organisms including bacteria, fungi, and algae (Cimino and Delwiche, 2002; Hurek and Reihold-Hureh, 2003). Important examples include *Rhizobium* bacteria in legume roots and the mycorrhizal fungi that colonize most land plants. Cumulatively, these symbiotic organisms have a profound effect on global bio-productivity, nutrient and gas cycling and geologic progression (Rodriguez et al.,

2009; Dalton et al., 2004; Heckman et al., 2001; Simon et. al 1993; Raich and Schlesinger, 1992).

These endophytic relationships can exert specific and broad selective pressures that significantly influence fitness, ecology, and evolution of plants and consequently, most other organisms (Brundrett, 2004; Clay and Holah, 1999; Omacini et al., 2001; Saikkonen et al., 2004). Complex changes in gene expression, morphology and physiology in both host and endophyte underlie these relationships (Cooper, 1984; Harley and Smith, 1983; Armstrong and Peterson, 2002; Strack et al., 2003; Kapulnik et al,. 1993; Peipp et al., 1997; Rodriquez et al., 2009; Toussaint et al., 2007; Yaun et al., 2009; Shi et al, 2009; Zhi-lin et al. 2007). Common symbiosis signaling pathways in plants are believed to play a role in symbiosis with beneficial microbes, but it remains unclear exactly how these mechanisms operate and if they apply to all beneficial microbe associations (Gutjahr et al., 2008; Oldroyd et al., 2009).

3.1. Fungal Endophytes. Fungal endophytes from several families and orders have been isolated from nearly every species of vascular plant and some algae (Cimino and Delwiche, 2002; Tan and Zou, 2001). Most species are classified in the Phyla Ascomycota and Glomeromycota (Arnold et al., 2007; Rodriguez et al., 2008). These fungi have evolved to inhabit the apoplastic and symplastic regions of plant tissues (Saikkonen et al., 1998) without causing visible harm or sign of disease in the host (Giminez et al., 2007).

3.1.1. *Ecology.* Fungal endophyte species are found across diverse habitats in the majority of plant species (Vega, 2008). They can reside in root, stem, leaf or multiple tissues (Carrol, 1988; Stone et al.. 2000; Yuan et al., 2009) and exhibit a wide range of functional diversity and life

histories (Arnold and Lutzoni, 2007; Rodriguez et al., 2009). Fungal endophytes alter the content of several important nutrients in tissues and can play an important role in plant defense (Araim et al., 2009; Garg et al., 2006; Gimenez, 2007; Rodriguez et al., 2009; Shokri and Maadi, 2009; Strack et al., 2003; 2008 Yuan et al. 2009). It has been suggested that nearly all plants in their natural habitat live in symbiosis with some kind of symbiotic fungal partner (Petrini, 1986).

From the broadest view, two classifications of mutualistic fungal endophytes have been identified: constitutive mutualists and inducible mutualists (Carrol, 1988). Constitutive mutualists are characterized by systemic infection and vertical transfer through direct infection of seeds. Inducible mutualists exhibit horizontal transfer, high taxonomic and host diversity, and can broadly colonize all plants in an eco-system (Yuan et al., 2009). Various other classification systems and groupings have been proposed; however, ongoing molecular studies are prompting constant reevaluation of the true relationships among endophytic fungi (Crozier et al., 2006; Yuan et al., 2009).

Although conventionally considered mutually beneficial, these relationships may be more accurately described as balanced antagonisms or conditional mutualisms with the details being highly dependent on species-specific interactions and environmental factors (Freeman and Rodriquez, 1993; Redman et al., 2001; Shultz and Boyle, 2005). This continuum has led to the speculations that pathogenic species originated as endophytes or that endophytes evolved from pathogens (Carrol, 1998; Giminez etal., 2007; Remy et al. 1994; Rodriguez et al., 2009; Krings et al., 2007; Redecker et al., 2000; Saikkonen et al., 2004). It seem likely that speciation in both directions is possible given changing environments and evolving hosts.

The combined influence of environment as well as biotic and abiotic factors affect the colonization and ecology of endophytes in the host plant (Tan and Zou, 2001). Host ranges can be broad or specific and infection frequencies can be as high as 90-100% especially in high stress environments (Rodriguez et.al, 2009). It is clear that the ecology governing these relationships is very complex. Fungal species typically adapt to colonize either specific or diverse tissue types, and most plants exist in association with multiple endophytes, each playing a role in plant metabolism (Schultz and Boyle, 2005). Colonization of endophytic fungi in physically distinct parts of the plant can affect the colonization rates and patterns of endophytes in other parts of the plant (Antunes et al. 2008; Arnold, 2007; Gamboa et al., 2001; Lodge et al., 1996). Some plants can harbor hundreds of species at once and these species may change across the native range of the plant (Rodriguez et al., 2009; Tan and Zou, 2001) making understanding of the broad ecology of fungal endophytes challenging.

3.1.2. *Host benefits*. Fungal endophytes, and their effects on plant metabolism, play a large role in phenotypic plasticity and environmental adaptability and likely played a unique role in selection and speciation in many host species (Rudgers et al., 2009). In general, endophytes derive nutritional resources and protection from external biotic and abiotic stresses (Clay, 1988, 1990, Clay and Holah, 1999; Kageyama et al., 2008; Redman et al, 2002; Smith and Gianinazzi-Pearson, 1988), and the plant gains various symbiosis-induced competitive advantages (e.g., improved resource availability and use efficiency, increased growth and biomass production, enhanced regulation of metabolism and gene expression, and improved defense against herbivores and pathogens), which increase overall fitness (Augé, 2001; Gimenez et al., 2007; Rodriguez et al., 2009; Genre and Bonfante, 1998; Yaun et al., 2009). Regardless of the degree

of specific benefit to either partner, it can be considered a true endophytic relationship if a sustainable equilibrium is reached and maintained between both the fungi and plant (Giminez et al., 2007). In nature, the balance between host and any specific endophyte is also impacted by the complex ecology among the multiple endophytes and the plant and environmental conditions (Antunes et al., 2008; Rodriguez et.al, 2009; Keenan et al, 2008).

Evidence for endophyte-stimulated Induced Systemic Resistance (ISR) has been documented in many host species (Giminezet al, 2007; Ownley et al., 2008b, 2010; Vega et al., 2008; Zhi-lin et al., 2007). New insight that some entomopathogenic fungi also exist as endophytes (Bing and Lewis, 1993) raises new questions about symbiotic ecology and the expansive role fungal endophytes play in the broader ecology.

3.1.2.1. *Secondary Metabolites*. Many changes in phytochemistry have been observed in response to colonization with endophytes. They can induce changes in plant metabolism leading to enhanced production of bioactive compounds or the fungi can produce them themselves (Zhi-lin et al., 2007). Concentrations of a wide array of plant phytochemical classes can be altered. Alkaloids, polyphenols, lignins, flavonoids, volatile terpenoids, peptides, phyto-sterols, indole derivitives, amines and amides can be altered with endophyte infection in some plants (Strobel., 2003; Tan and Zou, 2001; Yue, et al., 2000; Zhi-lin et al., 2007). Novel fungal-derived chemicals can also accumulate in the plant that can have antibiotic, anti-cancer, anti-viral, antioxidant, anti-diabetic, immune stimulatory and suppressive and insecticidal properties (Strobel, 2004). Examples include but are not limited to ammonia, hydrogen cyanide, alklypyrones, alcohols, esters, ketones, lipids, and enzymes (Vega et al., 2008). This pool of novel chemicals is becoming an important source of new medicines and natural products (Tan

and Zou, 2001). Intentional inoculation with endophytes for enhanced production of plant and/or fungal chemicals could represent a new paradigm in medicinal plant and natural product production (Zhi-lin et al., 2007).

Some compounds produced by endophytes are identical to plant-derived compounds (Strobel, 2003; Tan and Zou, 2001). Taxol, a bioactive chemical found in *Taxus* sp., has also been isolated from an endophytic species commonly found in *Taxus* species. It has been hypothesized that this could indicate that gene transfer and recombination may have occurred during the evolution of the symbionts (Tan and Zou, 2001). If this is applicable in other endophytic relationships, alternatives to rare or endangered plants that are being over harvested for their medicinal potential may be developed thus protecting indigenous populations.

The dark septate endophytes (DSE) were once mistaken for mycorrhizae, because they so often co-exist (Arnold, 2007). The DSE are characterized by asymptomatic colonization of roots, darkly pigmented and highly melanized hyphae, and microsclerotia (Kageyama et al., 2008). The heavily melanized tissue is thought to act as a protective physical barrier to other microbes and possibly herbivores; unique fungal secondary metabolites may create a chemical deterrent (Jumpponen, 2001; Jumpponen and Trappe, 1998).

Great diversity in nature and the complexity of the functional ecology of these relationships offers vast opportunities for ecology, agronomy, horticulture, and biotechnology research. Applied technologies have great potential; however, more research will be required to better understand these natural relationships and the potential uses. Because of the importance to the research described in this document, two types of endophytic fungi will be discussed in

greater detail: mycorrhizae and *Beauveria bassiana*, an entomopathogen that also colonizes plant tissues.

3.2. Mycorrhiza. Mycorrhizal (*myco* = fungus; *rhiza* = root) fungi have co-evolved with their plant hosts to produce highly specialized and unique associations with plant roots (Armstrong and Peterson, 2002; Brundrette, 2004). This co-evolution dates back as much as 450-500 million years to the Ordovician period and likely played a crucial role in transition of plants from an aquatic to a terrestrial life style (Simon et. al, 1993; Remy et al., 1994).

3.2.1. *Ecology.* Mycorrhizal fungi establish and maintain beneficial symbioses with plant roots and can be found in 80-90% of diverse terrestrial plant species (e.g., gymnosperms, angiosperms, pteridophyte, and bryophytes) (Stewart and Press, 1990; Bonfante-Fasolo, 1987; Smith and Read, 1997). These associations have been observed in temperate and tropical rain forests, deserts, grasslands, the arctic and even aquatic environments (Strack et al., 2003). Due to the wide global distribution and drastic impact on plant productivity, mycorrhizal associations have a profound influence on the biosphere; they impact global photosynthetic rates, mineral and gas cycling and ecosystem diversity. Improved soil health, fertility and structure can also be attributed to abundant mycorrhizae in native soils (Hargreaves et al., 2008; Pringle and Bever, 2008). Taken as a whole, these relationships could be argued to be the most significant symbioses on earth due to their cumulative global impact.

Plant species have varying degrees of dependency on their fungal partners. Mycorrhizal associations allow plants to grow in conditions or niches on the extreme fringes or outside of their normal range (Manjunath and Habte, 1991). Many orchids are totally dependent on

mycorrhizae to attain nutrients immediately after germination during the protocorm stage (McCormick et al., 2006). Other plants, such as many achloropholous angiosperms, rely solely on a mycorrhizal partner for nutrition throughout their life cycle (Furman, 1971).

3.2.2. *Taxonomy.* Two broad groups of mycorrhizae are recognized: ectomycorrhizae and endomycorrhizae. Ectomycorrhizae create a dense fungal sheath around the outer surface of the root with extensive hyphal growth in surrounding soil. Internal hyphae may colonize the apoplastic or extra-cellular region of the root epidermis or cortex; however, they do not penetrate root cortical cells (Brundrette, 2004). They are typically associated with many woody species and play a major ecological role in many forest eco-systems (Brundrette, 2004). Fungi that form ectomycorrhizal relationships are taxonomically diverse and are classified in three phyla (Ascomycota, Basidiomycota, and Zygomycota).

Endomycorrhizae colonize the cortex region of plant roots and do penetrate root cortical cells (Brundrette 2003, 2004). Endomycorrhizae also colonize surrounding soil with fungal hyphae. The remainder of this document will focus on one important group of endomycorrhizae - the arbuscular mycorrhizae (AM). The AM are formed by fungi in the Phylum Glomeromycota (Schussler et al., 2001) and are characterized by the presence of arbuscules; highly branched hyphae that develop inside of plant root cortical cells. There are approximately 150 identified species of AM fungi that infect an estimated 230,000 species of angiosperms, including many important agricultural and horticultural crops (Koide and Schreiner, 1992).

3.2.3. *Establishment of arbuscular mycorrhizae.* Highly coordinated changes in morphology, physiology and gene expression in both partners lead to and maintain the AM symbiotic relationship (Garg et al., 2006; Balestrini and Bonfante, 2005; Genre and Bonfante, 1998;

Armstrong and Peterson, 2002). Colonization by AM fungi is divided into three basic stages: pre-colonization, colonization, and mature symbiosis.

3.2.3.1. ***Pre-colonization*****.** Organic acids and carbohydrates in root exudates hasten hyphal branching and growth in surrounding soil encouraging contact with roots (Akiyama, 2007). Once in contact with the root surface, fungal hyphae swell to create appressoria at the site of epidermal penetration. A host-derived intracellular pre-penetration apparatus (PPA) composed of cytoskeleton and endoplasmic reticulum-derived materials are produced in response to undefined fungal signaling mechanisms (Genre et al., 2008). The formation of the PPA also marks initiation of expression of symbiosis-specific plant genes; these are related to cell cytoskeleton formation and expansin proteins (Siciliano et al., 2007).

3.2.3.2. ***Colonization.*** Fungal hyphae producing several plant cell wall degrading enzymes push through the epidermis following the course determined by the plant-derived PPA (Garg et al., 2006; Genre et al., 2008). The colonization stage begins after hyphae enter the PPA and proliferate in the root cortex. The plant is generally believed to play the dominant role in regulating of the interaction, but recent work suggests that the fungal partner may down-regulate plant defense genes during this phase. These “mycofactors” induce molecular and cellular responses in the host and can induce symbiosis-specific genes in the host plant (Kosuta et al., 2003). Once inside of the root, hyphae typically spread intracellulary in the cortex prior to cellular penetration and arbuscule formation (Armstrong and Peterson, 2002). Upon fungal penetration of the cortex cell, the plant and fungi begin a series of anatomical and molecular changes that allow a highly specialized interface to form (Smith and Gianinazzi-Pearson, 1988).

3.2.3.3. ***Mature symbiosis.*** Arbuscule formation is the defining feature of the AM symbiosis and represents the beginning of the mature symbiosis stage. Some species of AM fungi also produce reproductive spores and storage vesicles during this stage (Brundrette, 2004). Often formed in the late phases of the symbiosis, vesicles can be formed intra- or intercellularly and are lipid-rich storage organs (Smith and Read, 1997). During arbuscule development, a plant-derived peri-arbuscular membrane is formed in very close association with the fine arbuscule branches (Armstrong and Peterson, 2002; Hause and Fester, 2005; Stack et al., 2003) resulting in drastic physical changes to the cell membrane, cytoskeleton and organelle arrangement (Armstrong and Peterson, 2002; Strack et al., 2003). Although derived from plant cytoskeleton and membrane components, formation appears to be regulated, in part, by the fungal partner as well as the host (Armstrong and Peterson, 2002; Hause and Fester, 2005; Stack et al., 2003). Formation begins with invagination of the plant cell wall during hyphal penetration and is facilitated by cytoskeleton components (Armstrong and Peterson, 2002). The cell wall material grows around the developing arbuscule creating an extracellular compartment that accommodates the arbuscule but maintains separation with the plant cell cytoplasm (Balestrini and Bonfante, 2005; Hause and Fester, 2005). Formation of this peri-arbuscular membrane can increase plant cell membrane surface area by 300-400 % (Strack et al., 2003). As this interface grows, unique apoplastic material is generated between the newly formed membrane and the surface of the developing fungal cells (Balestrini and Bonfante, 2005).

3.2.3.3.1. Cell Structure. Peri-arbuscular interface formation is preceded by, and closely associated with the rearrangement of the cell cytoskeleton and organelles (Bonfante and Perotto, 1995). The two primary cytoskeletal structural elements, tubulin microtubules and actin

microfilaments, become intimately associated with peri-arbuscular membrane (Armstrong and Peterson, 2002). Since microfilaments play a role in cell wall synthesis and cell cycle functioning (Sarka et al., 2009), it is not surprising that they are present in the highly specialized peri-arbuscular membrane. In uncolonized cells, actin micro-filaments are abundant and randomly arranged around the cell periphery; fine bundles of microfilaments are concentrated near the plasma membrane and connect to thick bundles that extend into the interior of the cell and associate with the nucleus. Microfilaments in mycorrhizal cells drastically alter distribution; they are in greatest abundance near the developing peri-arbuscular membrane and scarce near the outer plasma membrane. Fine arbuscule branches are densely wrapped with a network of thin microfilaments while their presence on arbuscule trunks is scarce. Microfilament bundles can still be seen in close association with the nucleus, however in mycorrhizal cells, they are extending from the dense network covering arbuscule branches rather than near the outer portion of the cell. During arbuscule collapse and degradation, microfilaments cover the entire arbuscule in a single mat and then begin to reorganize back to the typical pattern of arrangement seen in uncolonized cells (Armstrong and Peterson, 2002; Genre and Bonfante, 1998; Smith and Gianinazzi-Pearson, 1988).

Microtubule patterns also reorganize in response to mycorrhizal symbiosis. Although still closely associated with the fungal arbuscule, the pattern of distribution is looser and less closely associated with individual branches (Armstrong and Peterson, 2002). Microtubule bundles are in a transverse pattern across the arbuscule and cytoplasm, connecting arbuscular branches together, to the cell wall or nucleus, and running along the arbuscule trunk. Some microtubules accumulate in dense concentrations during arbuscule senescence but soon return

to the distribution pattern typical of uncolonized cells (Armstrong and Peterson, 2002; Genre and Bonfante, 1998; Gianinazzi-Pearson, 1988).

Although mycorrhizal cells are typically larger than non-AM cells, mature arbuscules occupy a large portion of the interior space (Balestrini and Bonfante, 2005). This requires cytoskeleton mediated changes to the location, shape and number of organelles in the cell (Bonfante and Perotto, 1995). The nucleus migrates toward the center of the arbuscule, undergoes hypertrophy and exhibits increased chromatin dispersion (Smith and Giananazzi-Pearson, 1988; Hause and Fester, 2005). Increases in the volume of cytoplasm and the number of organelles are characteristic of AM roots cells and reflect increased metabolic rates in colonized cells (Balestrini and Bonfante, 2005). Plastids, mitochondria, and endoplasmic reticulum can be observed in a network-like structure on developing arbuscules. Numbers of plastids are especially high in AM cells and can be found encircling the developing arbuscule and the nucleus in high numbers (Fester et al., 2002).

3.2.3.3.2. Peri-arbuscular interface. The apoplastic space between fungus and plant cell walls is only 80-100nm thick (Hause and Fester, 2005); however, it effectively separates the two organisms completely. Materials pass across this peri-arbuscular interface by mechanisms that are not fully understood, however passive movement and active movement facilitated by specialized glucose, phosphorus, and nitrogen membrane-bound transporter proteins has been observed (Hause and Fester, 2005).

The plant-derived peri-arbuscular membrane contains typical plant cell wall molecules (e.g., cellulose, non-esterfied homogalacturonans, β-1,4-glugans, xyloglucans, arabinogalactan proteins and hydroxyproline rich proteins) (Balestrini and Bonfante, 2005). It tends to be less

dense near the fine arbuscules branches and denser near the base and around collapsing arbuscules (Balestrini and Bonfante, 2005, Hause and Fester, 2005). Although influenced by arbuscule development, the enzymatic machinery is plant derived and presumably controlled by the plant (Bonfante-Falso, 1987). Several fungal-derived cell wall degrading enzymes are believed to play a role, along with plant derived α-expansin proteins, in peri-abuscular interface formation (Strack et al., 2003, Smith and Gianinazzi-Pearson, 1988).

The specialized structure of this interface and the production of symbiosis-specific membrane-bound protein transporters allow for highly efficient material transfer. Both the fungal and plant symbionts maintain membrane-bound enzyme systems and proteins (Hause and Fester, 2005). Interestingly, both symbionts have an abundance of symbiosis-specific membrane bound ATPase proteins at the interface. Their presence and increased activity at this interface seems to be a critical part of driving the bi-directional transfer of resources (Hause and Fester, 2005). Hydrogen ions are discharged in to this interface space creating a proton motive force that allows for high volumes of active nutrient transport by membrane bound transport proteins (Guttenberger, 2000). Phosphorus transporters are found in abundance along this interface and allow for direct and efficient transfer of P from fungi to plant (Maldonado-Mendez et al., 2001). These symbiosis-specific phosphate transporters become more abundant during symbiosis while the normal plant phosphorus transporters are utilized to a much smaller degree (Smith et al., 2003).

Transcripts for special nitrogen transport proteins and nitrogen reductase have been identified in abundance at the interface (Hildebrandt et al., 2002). Special glucose and sucrose/fructose transporters are also present which facilitate the passage of sugars to the fungus

(Hause and Fester, 2005; Strack et al., 2003). Interestingly enough, these are critical for survival of the fungus because the AM fungi are unable to acquire carbon on their own (Douds et al., 2000). Each symbiont also maintains their own membrane-bound enzyme systems that relate to production and maintenance of their tissues. (Smith, 1988)

3.2.3.3.3. Gene expression. Expression of many genes during AM symbiosis leads to the drastic changes observed in plant cells. The role of gene expression in the formation of the peri-arbuscular interface is not fully understood; however, cytoskeleton-related genes for actin, α-tubulin, and β-tubulin are up-regulated upon infection. These genes likely play a role in the many organizational changes observed in plant cells prior to and during fungal penetration. The β-tubulin gene also remains up-regulated in later stages of colonization, suggesting a more active role in cytoskeleton and organelle reorganization during and after the accommodation process (Genre and Bonfante, 1998; Genre et al., 2005).

Genes for the α-expansin proteins are upregulated during infection, which affect cell wall loosening and expansion by disrupting hydrogen bonding between the cellulose fibers. Expansin proteins are expressed in highly specific cell types and locations and accumulate specifically at the peri-arbuscular interface (Balestrini and Bonfante, 2005). This accumulation may play an important role in cell wall loosening during fungal penetration and the subsequent enlargement of the plant cell to accommodate the developing arbuscule.

The expression of phosphorus transporter genes is altered in mycorrhizal plants. Genes for symbiosis-specific phosphorus transporters are up-regulated while typical plant phosphate transporter genes are down-regulated. (Harrison and VanBurren, 1995; Smith et al., 2003) Interestingly, this seems to indicate that the plant favors the symbiosis phosphorus uptake

system. The extent of altered gene activity is not fully understood and more genes are believed to be involved, yet more research is necessary to reveal the specifics.

3.2.4. *Benefits of arbuscular mycorrhizae*. Arbuscular mycorrhizal associations can be characterized as inducible, mutualistic symbioses involving bi-directional transfer of resources (Carrol, 1988; Smith and Gianinazzi-Pearson, 1988). The plant receives minerals from the fungi in return for carbon products from photosynthesis, lipids and protection (Strack et al., 2003; Garg et al., 2006). The fungus is an obligate partner, while most plants are considered facultative (Smith and Gianinazzi-Pearson, 1988). Benefits of AM to the plant host are numerous. Growth and photosynthetic rates increase with mycorrhizal colonization in some species (Araim, 2009; Fan et al., 2008), and improved water relations offer a greater degree of drought tolerance and environmental stress resistance (Augé, 2001). Arbuscular mycorrhizal plants often have enhanced resistance to biotic and other abiotic challenges (Bayat et al., 2009; Elsen et al., 2001; Peipp et al, 1997; Toussaint et al., 2007). The combined benefit to the plant leads to more vigorous, productive, adaptable and competitive individuals.

The symbiosis can; however, "cost" the host plant as much as 20% of its photosynthetically-fixed carbon (Graham, 2000). Carbon is delivered in the form of hexose and sucrose, and the sugars are converted, by the fungus, to the fungal carbohydrates, trehalose and glycogen, for use or storage (Strack et al., 2003).

Arbuscular mycorrhizal plants have increased mineral and water uptake. External fungal hyphae extend beyond the root into the soil effectively scavenging soil resources which are channeled directly to the plant root allowing access to a greater pool of resources (St. John and Coleman, 1983; Garg et al., 2006). The nutritional status of AM plants is drastically improved,

especially in conditions where soil resources are limited (Garg et al., 2006). Plants grown in nutrient limited conditions usually support higher AM fungi populations (Garg et al., 2006; Johnson, 1993). When soils minerals are limited, roots exudates often contain higher amounts of carbohydrates, strigolactones, and hyphal branching factors which attract AM fungal hyphae and help quickly establish the symbiosis (Akiyama, 2007; Giovanetti et al., 1996; Johnson, 1993). In soils with ample fertilization, AM fungi typically produce fewer hyphae and arbuscules in host roots and more vesicles suggesting that the fungi retains more of the collected soil resources yielding fewer nutritional benefits to the plant host (Johnson, 1993). Highly fertile soils also tend to select for AM fungal species that are considered "inferior mutualists" (Johnson, 1993).

Phosphorus nutrition is closely related to the rate of root exudation of compounds that encourage mycorrhizal colonization with phosphorus-limited plants exuding greater amounts of these necessary factors (Bucher et al., 2009). Improved phosphorus nutrition in host plants is considered one of the most important benefits of AM symbiosis (Harrison, 1999); it is the mineral element that is most often limiting to plant growth (Vance et al., 2003). Crop yield on an estimated 30-40% of the world's arable land is limited by phosphorus availability (Runge-Metzger, 1995). Phosphorus rapidly becomes unavailable in soils as it readily forms insoluble complexes with various cations in acidic conditions; especially aluminum and iron (Vance et al., 2003). Up to one half of soil phosphorus can be bound up in organic matter from deposited plant residues and other soil organisms. This organic phosphorus must be mineralized by microbes and released into solution as orthophosphate (Pi) before it can be taken up by plants (Garg et al, 2006). Phosphate also diffuses very slowly in soil, and a phosphorus depletion zone

often develops around roots (Marschner, 1995; Jungk, 2001). As a result of these factors, many soils have ample amounts of phosphorus; however, little is available for uptake by plants (Vance et al., 2003). To overcome this, plants have evolved multiple strategies to acquire and release Pi from the soil (Vance et al. 2003; Hammond et al. 2004; Raghothama, 2005). Mycorrhizal symbiosis may be one of the most elegant and effective of these strategies. Increased growth of plants in phosphorus deficient soils can be as much as nineteen-fold when in symbiosis with AM fungi (Haymann and Mosse, 1971). Phosphorus nutrition also has a profound impact on N_2 fixation of rhizobial bacteria in legume roots because root nodules are high volume phosphorus sinks (Robson et al., 1981). Fixation rates of N can increase as much as three times in mycorrhizal legumes (Sa and Israel, 1991). Nitrogen can be transferred from legumes to nonlegumes via mycorrhizal hyphal bridges (Hammel et al., 1991). Presumably a transfer of N among other mycorrhizal plants could occur; this raises the question if other nutrients are shared among mycorrhizal plants and to what degree.

Although phosphorus is considered one of the most important minerals that AM fungi offer the plant host, several other important minerals are supplied. Nitrogen, calcium, potassium, iron, sulfur, manganese, zinc and copper accumulate in greater amounts in mycorrhizal plants (Araim et al., 2009; Bethlenfalvay et al. 1998, Clarkson, 1985; Gerdemann, 1975; Manjunath and Habte, 1988; Tinker and Gilden, 1983); the delivery mechanisms are not fully understood. An abundance of symbiosis-specific plant and fungal transporters have been identified in mycorrhizal cells for K^+, Pi, NH_4, Cu, Zn, and organic acids (Hause and Fester, 2005, Strack et al., 2003). Presumably these play a role in the increased nutrient status observed in mycorrhizal plants. Enzymes that may play a crucial role in improving phosphorus and

nitrogen nutrition have also been identified in higher amounts in mycorrhizal plants; phosphatases, aminotransferases, glutamine synthatases, glutamate synthase, urease, and aspargine synthetase can all be more active in mycorrhizal plants (Yadav et al., 2005). Lead can be accumulated differently in mycorrhizal plants in response to soil Pb levels; uptake is increased when soil lead levels are low and decreased where levels are high (Malcova et al., 2003). Collectively, this suggests a very dynamic system of environmental monitoring and uptake adjustment which may also be at play with other mineral nutrients, pollutants, phytotoxins, and allelopathic chemicals. Sources of some minerals, that are normally unavailable for plant use, are accessible by fungal enzymes and subsequently made available to plants via mycorrhizae. Organic sources of phosphorus, nitrogen and sulfur are mineralized and supplied to mycorrhizal plants (Allen and Shachar-Hill, 2009; Bucking & Shachar-Hill 2005; Clarkson, 1985; Marschner and Dell, 1994,; Schimel and Bennet, 2004). Organic sources of nitrogen previously believed to be unavailable to plants (e.g., amino acids, proteins) can be made available to plants by mycorrhizae (Smith and Read, 1997; Schimel and Bennet, 2004). Sulfur can accumulate as much as 25% more in mycorrhizal vs. nonmycorhizal plants (Allen and Shachar-Hill, 2009).

More research will be necessary to fully understand the details of these interactions, but it is clear that the mycorrhizal status of any particular plant can profoundly impact responses to environmental conditions. Increased work with AM symbiosis has revealed new insights; however, many details remain unclear regarding signaling, resource transfer mechanisms, and effects on plant primary and secondary metabolism.

3.2.5. *Secondary metabolites in arbuscular mycorrhizal plants*. A wide range of metabolite classes can be altered in AM plants although the pattern of production and accumulation varies among species interactions. Plant hormones and secondary metabolites are believed to play a key role in regulation of AM symbiosis, although the molecular and physiological mechanisms are not fully understood. Mycorrhizal associations can alter production and accumulation of many primary and secondary metabolites (Armstrong and Peterson, 2002; Strack et al., 2003; Kapulniki et al., 1993; Peipp et al., 1997; Toussaint et al., 2007; Zhi-lin et al., 2007; Lata et al., 2003). Increased production of cytokinins (Allen et al., 1980), abscisic acid (Dannenberg et al., 1992), ethylene (Dehne, 1986), and jasmonic acid (Hause et al., 2002) can lead to a range of downstream morphological and phytochemical changes in hosts.

Increase in the levels of some antimicrobial flavonoid, phenolic, and phytoalexin compounds increase in plants colonized with AM fungi (Toussaint et al., 2007; Bonfante and Perotto, 1995). Sesquiterpenoid volatile compounds increased in leaves of AM citrus trees (Nemec and Lund, 1990); essential oils are also in greater abundance in mycorrhizal mint (*Mentha* sp.) and basil (*Ocimum* basilicum) when compared to uncolonized specimens (Sirohi and Singh, 1983; Toussaint et al., 2007). Transcripts for enzymes involved in the phenyl-propanoid and methyl-erythritol phosphate (MEP) pathways increase in mycorrhizal cells (Harrison and Dixon, 1993) likely leading to increased production of polyphenolics.

When *Echinacea* plants in tissue culture were inoculated with mycorrhizae, concentrations of some phenolic compounds were altered. Chlorogenic and cichoric acid both increased significantly in shoots of mycorrhizal plants, but no difference was observed between roots with and without mycorrhizae. Levels of echinacoside were not significantly different in

roots and were decreased in shoots (Lata et al., 2003). Inoculation with *Glomus intraradices* significantly increased levels of several polyphenolic caffeic acid derivatives (CAD) in roots of *E. purpurea*. Although there were no significant changes in levels of CADs in leaves, there was a significant increase in total phenolic acids in foliage (Araim, 2009).

Carotenoid biosynthesis were increased in AM roots (Akiyama, 2007; Fester et al., 2002), and chlorophyll levels were higher in some mycorrhizal plants (Tsang and Maun, 1999). Several apocarotenoid cyclohexane derivatives, originating from carotenoid precursors, (Akiyama, 2007; Walter et al., 2000) accumulated in significantly higher amounts in AM plants; these may play a role in regulation of the symbiosis (Peipp, 1997; Klinger et al., 1995). Mycorradicin, which accumulates in AM roots, is responsible for the yellow color often associated with AM roots (Akiyama, 2007; Schleiman et al., 2006).

Mycorrhizal infection increases resistance to pathogens and insects in many plant species (Garcia-Garrido and Ocampo, 2002; Rabin and Pacovsky, 1985). Observed increases in jasmonic acid levels in mycorrhizal plants may suggest that Induced Systemic Resistance (ISR) mechanisms are stimulated during infection. Increased jasmonic acid levels can induce genes involved in plant defense (Wasternack and Hause, 2002) which could affect other secondary metabolic pathways.

3.2.6. *Glomus intraradices.* Mycorrhizae induced by *Glomus intraradices* (Phylum Glomeromycota; Order Glomerales; Suborder Glomineae ; Family Glomaceae) have both arbuscules and vesicles and are considered vesicular arbuscular mycorrhizal (VAM) (Walker and Trapp, 1993). *G. intraradices* has a broad host range and has been used extensively as a model organism for mycorrhizal research. Relative to other AM fungi species, it demands a

higher carbon allocation from the plant host. It produces hyphae and spores in greater abundance when nutrition is not limited (Johnson, 1993). The *G. intraradices* genome is smaller than other AM fungi, and typically, the fungus exist in a haploid form (Hijri and Sanders, 2004).

Primary spores are 40-140 µm wide and white or yellowish brown in color. Smaller secondary spores, 20-30 µm wide, are sometimes produced on hyphae from germinating spores prior to root infection (Chabot et al., 1992). Spores are globose to elliptical and have three distinct external layers. Spores can be, but are not always, separated from subtending hyphae by a distinct septate plug. Hyphae are cylindrical with widths ranging from 11-18 µm and wall thicknesses between 3.2-6.4 µm and can be seen coiling in some cells (Chabot et al., 1992). Hyphal walls are also composed of three layers that are continuous with spore wall layers. Numerous finely branched arbuscules (15-20 µm), on thick (2-3 µm) hyphal trunks, develop throughout colonized roots. Numerous spores (50-100 µm) and vesicles (40-60 µm) can be seen to aggregate intracellularly near the entry sites of mature infections. Vesicle may form intra- and intercellularly (Biermann and Linerman, 1983).

Typical colonization pattern for *G. intraradices* begins with a radial mycelium (5mm in diameter) developing around the germinating spore. Hyphae colonize the root cortex intracellularly before entering cells and establishing arbuscules. Once resource transfer between symbionts begins, vesicle and spores develop, proliferate, and spread the symbiosis.

3.2.7. *Gigaspora margarita.* *Gigaspora margarita* (Phylum Glomeromycota) is an AM fungus in the Suborder Gigasporineae and Family Gigasporaceae (Walker and Trapp, 1993). Its host range has not been as well investigated as *G. intraradices* but *G. margarita* can infect multiple plant species and has been isolated in plants from around the globe including North America,

New Zealand and South Africa (Becker and Hall, 1976). Unlike *G. intraradices*, *G. margarita* exerts a relatively low carbon demand from the plant and produces hyphae and spores in greater abundance when nutrition is limited (Johnson, 1993). Active acid phosphatase and other enzymes, possibly related to nutrient availability, have been found in hyphae of *G. margarita* (Saito, 1995). Also, *G. margarita* can harbor an intracellular bacterium *Burkholderia* sp. (Ruiz-Lozano and Bonfante, 2000).

Spores are 280-460 μm, white and cream or dark yellow with a warty exterior borne terminally on a sporogenous cell. They are globose or subglobulose and have three distinct external layers with a combined wall thickness of 5-24 μm (Becker and Hall, 1976). Spores are typically separated from subtending hyphae by a septate plug. Hyphae are 34-47 μm wide with 3-9 μm thick walls often exhibiting flattened knob-like protrusions up to 16 μm wide near entry points and cortical cells. Densely branched arbuscules (15-30 μm) on swollen hyphal trunks (3-5 μm) develop in colonized root cortex cells. Vesicles (22-35 μm) typically are not observed extraradically, but have been observed in tight bundles of up to 20 on coiled hyphae in soil (Becker and Hall, 1976). Unique tubular vesicles, related to cytoplasmic streaming, have been observed inside of hyphae (Saito et al., 2004; Uetake et al, 2002); however, both are often absent. Unique axillary cells are also sometimes observed in bundles in soil (Bentivenga and Morton, 1995).

Germination of *G. margarita* often takes place near the warty protrusions on spores, and the resulting germ tube is subject to geotropism (Watrud et al, 1978). Hyphal growth progresses very slowly until plant roots are encountered. If spores germinate and no plant factors are encountered, they will cease growth and begin again when conditions are favorable. This can

happen up to ten to twenty times before spore reserves are depleted (Becard and Piche, 1989). Upon entry into the root, thinner hyphae (3-9 μm) colonize the root cortex intracellularly eventually entering cells and establishing arbuscules. Once resource transfer between symbionts begins, spores develop proliferating and spreading the symbiosis (Becard and Piche, 1989).

3.2.8. *Echinacea and Mycorrhizae.* Species of *Echinacea* will associate with multiple AM fungal species. Growth rate and lateral root development increased significantly, and the highest colonization rate is with a *Glomus* species, although there was also successful infection by several species of *Gigaspora* (Lata et al., 2003) and others. *Echinacea purpurea* colonized with *G. intraradices* had increased root and shoot mass, leaf nutrient content, and concentration of several proteins and secondary metabolites (Araim, 2009). Arbuscular mycorrhizae also improved survival rates, growth and development in acclimated tissue culture-produced plantlets of *E. pallida* (Lata et al., 2003).

3.3. *Beauveria bassiana.* Around 700 species from 90 genera, of entomopathogenic fungi exist, including *Acremonium, Beauveria, Cladosporium, Clonostachys,* and *Isaria* which have also been shown to be endophytic in plants (Vega, 2008; Vega et al., 2008). Twelve have been researched as biocontrol agents (Faria and Wraight, 2007; Vega 2008; Vega et al., 2008). Most are members of the Order Hypocreales, which includes species that produce multiple toxigenic secondary metabolites (White et al., 2003). These fungi can exist as soil-inhabiting saprotrophs, mycotrophs, necrotrophs, entomopathogens, endophytes, or may use multiple strategies (Ownley et al., 2010). Entomopathogenic species often impart a degree of bioprotection to the host plant (Giminez et al., 2007; Goettel et al., 2005; Ownley et al., 2008b). The plant host is

protected from pathogens and herbivores using a variety of mechanisms that can be species specific. Endophyte-produced bioactive compounds, competition for resources, induced systemic response, and direct parasitism have all been identified as sources of enhanced resistance (Arnold and Lewis, 2005; Ownley and Windham, 2007; Rudgers et al., 2007; Schulz and Boyle, 2005; Saikkonen et al., 2006; Vega et al., 2008). Targeted use of these endophytes offers potential to induce specific desirable responses in host crops.

3.3.1. *Taxonomy.* *Beauveria bassiana* (Phylum Ascomycota: Order Hypocreales) was originally placed in the Family Clavicipitaceae (Arnold and Lutzoni, 2007; Sung et al., 2007), but has recently been moved to Family Cordycipitaceae (Ownley et al., 2010). Considered an inducible mutualist (Carrol, 1988), *B. bassiana* is the anamorph stage of *Cordyceps bassiana* (Quesada-Moraga et al., 2006; Sung et al., 2007), an important traditional Chinese medicine, but the two forms are rarely seen together in nature.

3.3.2. *Ecology.* Although first recognized as an insect pathogen, *B. bassiana* can exist endophytically in many wild and cultivated plant species (Vega, 2008). *Beauveria bassiana* colonization of several herbaceous and woody speciescan imparted a degree of bio-protection to the hosts (Gomez-Vidal et al., 2006; Posada and Vega, 2005; Quesada-Moraga et al., 2006; Ownley et al., 2008b, Ownley et al., 2010).

More is known about the role of *B. bassiana* as an insect pathogen than as an endophyte in plants, but recent interest has begun to spur new research into the subject. The ecology of this organism is complex and not well understood. It is ubiquitous in soils and can exist in multiple phases infecting members of multiple kingdoms (Bing and Lewis, 1993).

3.3.3. *Colonization of the plant.* *Beauveria bassiana* colonizes the plant through the epidermal tissue. Conidial suspensions applied to roots, leaves, petioles and seeds has resulted in successful colonization in multiple plant hosts (Gomez-Vidal et al., 2006; Posada and Vega, 2005, 2006; Quesada-Moraga et al., 2006; Ownley et al, 2008b, Wagner and Lewis, 2000). A typical colonization pattern begins with conidia germination and germ tube formation; the germ tube can penetrate the epidermis immediately or propagate into a surface mycelium as an epiphyte prior to penetration (Posada and Vega, 2006; Quesada-Moraga et al., 2006; Wagner and Lewis, 2000). Appressoria do not form at the penetration site; however, a total breach of the cuticle, related to a distortion in surrounding cell wall structures, allows passage (Quesada-Moraga et al., 2006). Signaling and mechanics of this penetration process are not fully understood. Initial stages of colonization seem to primarily involve inter- and intracellular regions of parenchyma tissues with appressorial formation at the surface of cells prior to entry. In well colonized plants, hyphae in xylem vessels facilitate systemic translocation throughout the plant; *B. bassiana* has been isolated from root, leaf, stem, and cotyledon tissues distant from the site of inoculation as much as one year later (Gomez-Vidal et al., 2006; Posada and Vega, 2005, 2006; Quesada-Moraga et al., 2006, Ownleyet al., 2008b). In some cases, *B. bassiana* emerged on the surface of distant tissues, existed as an epiphyte, and sporulated (Posada and Vega, 2005).

Colonization of plants by *B. bassiana* can be determined by surface sterilizing sections of plant tissue and plating onto selective media. Detection of fruiting bodies can take 6-8 weeks or more; however, hyphal growth can be seen in 10-12 days (Ownley et al, 2008a). Other methods including PCR amplification and detection using PCR amplicons have been developed

to identify the presence of *B. bassiana* in plant tissues (Griffin, 2007; Quesada-Moraga et al., 2006; White et al., 1999).

3.3.4. *Phytochemistry.* *Beauveria bassiana* is known to produce several compounds with known bioactivity *in vitro* and *in vivo* (Zimmerman, 2007; Strasser et al., 2000; Vey et al., 2001). The nutritional, molecular and physiological nature of this association in plants has not been extensively studied; however, improved resistance to challenges from pests and pathogens have been observed in plants colonized by *B. bassiana* (Ownley, 2008b). Increased deterrence and resistance have been linked to increased production and accumulation of several classes of secondary metabolites, although it is unclear whether these are plant or fungal-derived compounds, or a combination (Vega, 2008). Enhanced resistance in parts of the plant that are not colonized indicated that an ISR response was stimulated during colonization by these fungi (Griffin et al., 2006). Some of the metabolites involved in ISR are related to chemicals of therapeutic interest in *Echinacea purpurea.*

Beauvericin, a cyclic hexadepsipeptide produced by *B. bassiana* has antimicrobial, insecticidal, cytotoxic, ionophoric, apoptotic and immunosuppressive activities (Hammil et al., 1969; Dombrink-Kurtzman, 2003; Ojcius et al., 1991) and has been evaluated for potential insecticidal and medicinal properties (Gupta et al., 1995; Klaric et al., 2007). Beauvericin was also a potent inhibitor of cholesterol acyltransferase (Tomoda et al., 1992), and it increased ion permeability in biological membranes by forming complexes with calcium, sodium, or potassium cations and/or lipid membrane-bound cation-selective channels, likely affecting ionic homeostasis (Ojcius et al., 1991; Kouri et al., 2003).

3.3.5. *Biological Control.* The safety of *B. bassiana* as a biocontrol organism was evaluated based on impacts to nontarget insects and mammals including humans, and no safety concerns were identified (Zimmerman, 2007). Increasing levels of interest and new research are leading to new possibilities in various agricultural systems (Ownley et al., 2008b). Inoculation with *B. bassiana* offers a novel organic and environmentally friendly method of reducing pest pressure and increasing the levels of natural products in economically important crops. Corn leaves inoculated with *B. bassiana* had effective and sustained biocontrol against the European corn borer (Wagner and Lewis, 2000). Control of leaf hoppers on rice and tea crops has also been successful (Hussey and Tinsley, 1981). A product known as "Boverin" developed from *B. bassiana* has been successfully and extensively used as a biocontrol agent for Colorado potato beetle and coddling moth across thousands of hectares in Russia (Ferron, 1981).

Innoculation with *B. bassiana* has also displayed effectiveness in controlling various soilborne and foliar pathogens in many plant species (Renwick et al., 1991) including *Fusarium* (Reisenzein and Tiefenbrunner, 1997), *Rhizoctonia* (Lee et al. 1999; Ownley et al., 2008b), and *Pythium* (Vesely and Koubova, 1994). Intentional inoculation has been achieved with various methods, but the highest infection rates were observed using a seed coating of conidia prior to germination (Ownley et al., 2008a; Quesada-Moraga et al., 2006). This is likely due to the easy infection of young tender seedling tissues that lack well developed cuticles and the fact that germinating seedlings may not have developed environmentally- or developmentally-induced resistance mechanisms. Since it is a soil inhabiting fungus, seedling infection by *B. bassiana* may occur in nature.

CH. III

Experimental Design and Methods

1. Organisms.

1.1. Plants. Prior to cultivation and before experimentation, benches were washed with a bleach solution (10%). Tools and other materials used during preparation and planting were washed with detergent (Generic brand dish soap) and the same bleach solution. All plants were grown in calcined montmorillonite clay medium (Turface® Proleague, Turface Athletics, Buffalo Grove, IL); in a greenhouse fitted with an environmental monitoring system (Priva North America, Inc., Ontario, Canada). Heat and cooling were moderated by radiant floor heat and a multi-stage cooling system utilizing passive and fan forced ventilation and evaporative cooling. Heating and cooling mechanisms were initiated at 18.3 ºC and 21.1 ºC respectively. Artificial light was provided with multiple high intensity discharge high pressure sodium lamps set to provide a 16 h photoperiod. Shade cloth was maintained at 50%. Plants were watered as needed.

Fertilizer applications were made using a fertilizer injector (Dosa-tron®, Clearwater, FL) set at a 1:100 ratio. All plants were fertilized with 150 mg/L Peter's™ 15-0-15 water soluble fertilizer (Scott's, Marysville, OH) weekly unless otherwise stated. Phosphorus rates were determined by species and treatment. For *Sorghum bicolor* plants, potassium phosphate levels were either low phosphorus (0.6 mM KH_2PO_4) (CAS# 7778-77-0: Fisher Scientific, Waltham, MA) for AM plants or high phosphorus (1.2 mM KH_2PO_4) for nonmycorrhizal *Sorghum* plants applied weekly unless otherwise stated. *Echinacea purpurea* plants received

either low phosphate (0.8 mM KH_2PO_4) or high phosphorus (3.0 mM KH_2PO_4). Micromax micronutrient solution (Scott's, Marysville, OH) was applied every 4 weeks.

Safer® brand (Lititz, PA) insecticidal soap and pyrethrum aerosol (Prescription Treatment, St. Louis, MO) were used as needed to control aphid and whitefly populations.

1.1.1. *Sorghum bicolor.* *Sorghum bicolor* 'DK39Y' (Monsanto Corp., St. Louis, MO) was used as a propagation host for mycorrhizal fungi. Surface sterilized seeds were planted approximately 60 mm below the surface of the media infested with or without mycorrhizal fungi. Medium was kept moist until germination and watered as needed. Plants were periodically cut back to 5-10 cm above the soil level to rejuvenate foliage and encourage vigor.

1.1.2. *Echinacea purpurea*. Seeds of *E. purpurea* (Johnny's Selected Seed, Winslow, ME) were placed on the surface of the growth medium and kept moist. Seed germination was recorded, and multiple growth parameters (height, stem diameter, and size of largest leaf) were measured at regular intervals. After 12 weeks, plants were harvested, weight, other growth parameters and mycorrhizal colonization were measured; tissues were then analyzed for several types of phytochemicals (e.g., phenolic acids, alkylamides, sesquiterpenes, carotenoids, and chlorophylls).

1.2. Beneficial fungi.

1.2.1. *Arbuscular Mycorrhizae.* Cultures of *Glomus intraradices* (Isolate IA509) (*Gi*) and *Gigaspora margarita* (Isolate NC175) (*Gm*), originally from The International Culture Collection of Vesicular Arbuscular Mycorrhizae Fungi (INVAM) (Morgantown, WV), were provided by Dr. Robert Augé (University of Tennessee, Knoxville, TN). All experiments were

conducted in a glass greenhouse (The University of Tennessee, Knoxville, TN). Mycorrhizal cultures were grown and maintained on roots of actively growing *S. bicolor.* Non-mycorrhizal control cultures were grown without mycorrhizal inoculum. New mycorrhizal and nonmycorrhizal cultures were reestablished, several times per year.

Inoculum was harvested by cutting *S. bicolor* plants below the crown and finely chopping roots and media. Equal amounts of inoculum harvested from *Gi* and *Gm* cultures were combined for a dual culture inoculum. Plastic mesh screening was placed over the drain holes of plastic pots (2 L) which were then partially filled (75%) with Turface®. 150 ml of the nonmycorrhizal or mycorrhizal inoculum was added and the pot was filled to within 2.54 cm. (1 in.) of the lip with fresh media.

To minimize cross contamination of cultures, nonmycorrhizal cultures were prepared prior to mycorrhizal cultures, and tools, containers and gloves were sterilized with bleach solution (10%) and detergent (Generic brand dish soap) between treatments.

In order to standardize other soil microflora, a filtrate solution that excluded mycorrhizal propagules but contained bacteria was prepared immediately prior to inoculation and applied to each pot. Mycorrhizal inoculum (50 mL) was mixed into distilled water (400 mL), and the suspension was then filtered through a vacuum filtrate apparatus with a 25µm filter (Fisher Scientific, Waltham, MA). The procedure was repeated until enough filtrate was obtained to treat all pots. The primary filtrate solution was filtered a second time. Filtrate (50 mL) was applied to each pot and watered. Surface-sterilized *S. bicolor* seed were planted, covered with fresh medium and watered.

1.2.2. *Beauveria bassiana*. The isolate of *B. bassiana* (Isolate - Bb 11-98) (Provided by Dr. Bonnie H. Ownley, University of Tennessee) used in this research was originally isolated from a click beetle (Coleoptera: Elateridae) collected in Scott County, TN and identified by Dr. Roberto Pereira (University of Florida). It was used to infect tomato seedlings and then re-isolated from conidia collected from the seedlings. Stock cultures were grown on Sabouraud's dextrose agar (SDA) (Difco, Becton, Dickenson & Co., Sparks, MD) at room temperature. Conidia were collected from mature *B. bassiana* cultures (after approximately 4 weeks growth) that were dried in a Class II Biosafety cabinet (Labconco Corporation, Kansas City, MO); conidia were scraped from the surface, collected in a glass vial, and stored at 4°C.

Conidia were used to coat *E. purpurea* seed planted in Experiment 1 according to methods described in Ownley et al. (2008a). Seed were surface-sterilized in a bleach solution (10%) with one drop of detergent (Generic brand dish soap), agitated for 15 min and rinsed two times in sterile water. Seed were dried in a biosafety cabinet (Environmental Air Control Inc., Hagerstown, MD). Conidia were suspended in a methyl cellulose suspension (2% wt/v) and applied evenly to the surface of *E. purpurea* seeds. Seeds were dried for 16 h in a biosafety cabinet, turning after the first 30-60 minutes to prevent the seeds from sticking together.

Subsamples of the coated seed were tested for the rate of surface colonization using dilution plating (Becker et al., 1996). Seed (10) were rinsed in a test tube containing sterile water (10 ml) and agitated for 10-15 min. A series of 10-fold dilutions were prepared from the original rinse, and 0.1 ml aliquots from the resulting solutions were spread evenly across the surface (Becker et al., 1996) of a *B. bassiana* selective medium (Shimazu and Sato, 1996). All seeds for treatments without *B. bassiana* treatments were coated with an equivalent amount of

methyl cellulose solution with no suspended conidia. Treated seeds were planted as described above.

2. <u>Phytochemical analyses.</u> Composite samples were prepared from equal amounts of fresh plant tissue from three *E. purp*urea plants grown in a single pot. Samples were stored at -20 °C in plastic bags between harvest, sample collection and chemical analysis. Root tissues were dried in paper bags in an oven with unrestricted air flow (Precision Model 6530; Thermo Fisher Scientific; Waltham, MA) at 50 °C for 24 h. Once dried, the crown and thick upper portions of roots attached to the crown were removed; the remainder of the root system was ground in a Wiley Mill to pass a 40-mesh screen. Fresh leaf tissue was collected, from the apex of mature leaves from each of the three plants in a pot at the time of harvest. Tissues were frozen; then ground to a fine powder in liquid nitrogen (sesquiterpenes), lyophilized at a constant temperature of -30°C for at least 140 h in a lyophilizer and then ground (chlorophylls and carotenoids), or prepared as described for the root tissue. All solvents were HPLC grade.

2.1. Chlorophylls and Carotenoids. Chlorophylls (A and B) and carotenoids (antheraxanthin, violaxanthin, neoxanthin, lutein, zeaxanthin, and β-carotene) were extracted using a four stage extraction and analyzed with High Performance Liquid Chromatography (HPLC) (Kopsell et al., 2004; Khachik et al., 1986).

2.1.1. <u>*Tissue Extraction*</u>. A subsample of freeze-dried tissue (0.10 g) was rehydrated in 0.8 mL of reverse osmosis water at room temperature for 20 minutes. The internal standard, ethyl-β-apo-8´-apo-carotenoate (CAS# 1107-26-2, Sigma Chemical Co., St. Louis, MO) (0.8 ml) was added to determine extraction efficiency tetrahydrofuran (THF) (2.5 ml) (CAS# 1109-99-9,

Fisher Scientific, Waltham, MA), stabilized with 2,6-di-*tert*-butyl-4-methoxyphenol (BHT) (25 mg/L) (CAS# 128-37-0, Fisher Scientific, Waltham, MA) was added; tubes were vortexed for 3 to 5 seconds. Sample were then ground in Potter-Elvehjem tissue grinding tubes (Kontes, Vineland, NJ) with 20 insertions of a glass pestle attached to a drill press rotating at 540 rpm; tube was immersed in ice to dissipate excess heat. Tubes were centrifuged for 5 min at 500 *g*. Supernatant was removed, without disturbing the pellet and stored on ice. Pellet was suspended in THF (2.0 ml), then vortexed, ground, and centrifuged as before; this procedure was repeated three times, and the resulting supernatant fractions were combined. By the fourth extraction, the extracted supernatant was nearly colorless. The pooled supernatant was reduced to 0.5 mL under nitrogen (N-EVAP 111; Organomation Inc., Berlin, MA), and methanol (MeOH: CAS# 67-56-1) was added to a final volume of 5 mL. Extract was filtered a polytetrafluorotheylene (PTF) filter with a pore size of 0.2 μm (Econofilter PTFE 25/20; Agilent Technologies, Wilmington, DE) into a 2 mL amber glass vial (Fisher Scientific, Waltham, MA).

2.1.2. *High-Performance Liquid Chromatography* (HPLC). Methods used for HPLC separation and detection followed those described by Kopsell et al. (2007). A 1200 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA) fitted with an analytical scale polymeric RP-C_{30} column (250 x 4.6 mm i.d., 5 μm) and a 10 x 4.0 mm i.d. guard cartridge and holder (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, PA) was used for chlorophyll and carotenoid extractions. Separation was isocratic with methyl *tert*-butyl ethanol (MTBE: CAS# 1634-04-4), 88.9% MeOH, and 0.1% triethylamine (TEA: CAS# 121-44-8) (11.8:88) (v/v/v) as the mobile phase. Flow rate was 1.0 mL/min, with a total run time of 53 min. A 2 min equilibration period was allowed prior to the next injection. Injection size was

10 µL and detection wavelengths were 453 nm (carotenoids, chlorophyll *b*, and internal standard) and 652 nm (chlorophyll *a*). Data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies).

2.1.3. Compound identification and quantification. Compounds were identified based on retention times and spectral data of authentic standards (ChromaDex Inc., Irvine, CA). Concentration of the external pigment standard was determined spectrophotometrically (Davies and Köst, 1998).

An extraction efficiency of 52% was calculated based on the recovery of the internal standard. Data were converted to mg/g dry weight adjusted for extraction efficiency. An adjusted recovery based on extraction efficiency was calculated:

$$\textbf{Adjusted Recovery} = \frac{\text{mg/g Dry Weight}}{\text{Recovery factor (0.52)}}$$

2.2. Phenolic Caffeic acid Derivatives. Concentrations of polyphenolic caffeic acid derivatives (CAD) (cynarin, chlorogenic acid, cichoric acid and caftaric acid) were determined following a methanol extraction and reverse phase HPLC separation [Institute for Nutraceutical Advancement (INA), Method 106.000 (www.nsf.org); Wagner and Farnsworth (1991)]. All polyphenolic acid extractions and analyses were performed at The North Carolina Bionetwork Biobusiness Center's Natural Product Laboratory (Enka, NC).

2.2.1. *Tissue Extraction.* Root or leaf tissue samples (0.150 g) and ethanol:water (60:40) (25 ml) in 50 ml centrifuge tubes (Fisher Scientific, Waltham, MA), were shaken at room

temperature for 15 min (Model E24: New Brunswick Scientific, Edison, NJ) . Tubes were then centrifuged (Sorvall; Thermo Scientific, Waltham, MA) at 3000 g and filtered though a 0.45 µm PTFE filter (Millipore Corp., Billerica, MA) into an HPLC vial.

2.2.2. *HPLC.* Samples were injected into a Dionex ICS-3000 HPLC unit equipped with a variable wave length detector (Dionex Corp., Sunnyvale, CA) fitted with a Cosmosil™ 5C18-AR-II analytical column (150× 4.6 mm i.d., 5.0 μm) (Nacalai USA inc., San Diego, CA) maintained at 35 ºC. Mobile phase was a gradient elution consisting of 0.1% Phosphoric acid (A) (CAS# 7664-38-2) in water and acetonitrile (B) (CAS# 75-05-8). Elution gradient was as follows: 0.1 min- (90% A : 10% B); 13 min-(78% A : 22% B); 14min- (60% A : 40% B).

Flow rate was 1.5 mL/min, with a sample run time of 14.5 min. Injection size was 5 µl. Eluted compounds were detected at 330 nm. Collected data were processed using Chromeleon analytical Software (Dionex corp., Sunnyvale, CA). Samples were quantified by comparison of peak areas to pre-established standard curves of commercially available internal standards (Sigma Chemical Co., St Louis, MO); linear equations based on least square regression of each external standard. Concentration of compounds was reported in µg/ml were collected as raw data.

2.3. Alkylamides. Concentrations of alkylamide compounds in roots were evaluated following organic solvent extraction and gas chromatography-mass spectrometry (GC/MS) (Leinert et al., 1998).

2.3.1. *Tissue extraction.* Root tissues (100 mg dried root) were extracted in hexane (1 ml), (CAS# 110-54-3: Fisher Scientific; Waltham, MA) in 20 ml glass vials (Fisher Scientific,

Waltham, MA) with Teflon caps. Internal standard (1-eicosene) (CAS# 3452-07-1: MP Biomedicals; Solon, OH) was added to the solvent [0.2805 % (w/v)]. The mixture was shaken (150 rpm) at room temperature on an orbital shaker (Model 3590; Labline Instruments, Inc.; Melrose Park, IL) for 24 h, then filtered through a 0.45µm filter (Pall Corporation; Port Washington, NY) into 2 ml borosilicate glass vials (National Scientific; Rockwood, TN) for gas chromatography-mass spectrometry (GC/MS) analysis.

2.3.2. ***GC/MS Analysis.*** An Agilent Model 6850 GC paired with an Agilent Model 5973 mass selective detector (Agilent Technologies, Palo Alto, CA) fitted with a 5% phenyl methyl siloxane capillary column (30 m x 0.25 mm i.d. with a 0.25µm film thickness) (HP-5MS; Agilent Technologies, Palo Alto, CA) was used for GC/MS analysis. One µl of the hexane extract was injected as a splitless injection; injector temperature was 250 °C and pressure was 15.13 psi. Helium flow rate was 1.4 ml/min. Oven temperature began at 100° C and was held for 5 min, followed by an initial temperature increase of 10 °C per min to 200 °C with a 2-min hold, and a second temperature increase of 3 °C per min to a maximum temperature of 250 °C; maximum temperature was held for 5 mins. Total run time was 39.7 min. Mass selective detector inlet temperature was 300 °C with an EM voltage of 1905.5 volts.

2.3.3. ***Compund Identification and Quantification.*** The compounds (undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (Alkylamide 2) (Retention time: 19.05 min), dodeca-2E,4(Z-diene-8,10 diynoic acid isobutylamide (Alkylamide 3) (Retention time: 24.23 min), and dodeca-2E,4E,8Z,10[E/Z]-tetraenoic acid isobutylamide (Alkylamides 8/9 [#8: 10E + #9: 10Z]) (Retention time: 26.62 min.), were identified by comparison of retention times and mass spectra with published data (Bauer and Remiger, 1988; Bauer and Remiger, 1989) (Figures 4.18, 4.19.

4.20). Alkylamides 8 and 9 are detected as a single peak; the data represents combined concentration of the two. Quantification was based upon comparison with the internal standard peak. Relative concentration (RC) was determined by dividing total peak area of each compound by the area of the internal standard and converting to percent of internal standard.

2.4. Sesquiterpenes. Sesquiterpenoid compounds were evaluated following organic solvent extraction and GC/MS (Chen et al., 2003).

2.4.1. *Tissue extraction*. Sample (200 mg) was placed into 20-ml glass vials with Teflon caps (Fisher Scientific, Waltham, MA) and mixed with HPLC grade ethyl acetate (CAS# 141-78-6: Fisher Scientific, Waltham, MA) (1 ml) containing 1-octanal (CAS# 124-13-0: Aldrich Chemical St. Louis, MO)(0.003% w.v) added as an internal standard. Mixture was shaken at room temperature (Lab-Line Orbital Shaker Model 3590; Labline Instruments, Inc.; Melrose Park, IL) (150 rpm) for 2 hr, then filtered through a nylon membrane (45µm pore; 4 mm diameter) syringe filter (Gelman Laboratory, Port Washington, NY) into 2-ml glass vials.

2.4.2. *GC/MS Protocol.* GC/MS methods were the same as for alkylamides except that injector pressure was 11.05 psi, and the oven temperature profile was altered. Oven temperature was held at 40 °C for 3 min and then increased at a rate of 5 °C per min to a final maximum temperature of 240 °C. Total run time was 49.89 min.

2.4.3. *Compound identification and quantification.* Compounds were identified by comparison to mass spectra (Figures 4.22, 4.23, 4,24) in the National Institute of Standards and Technology (NIST) database. RC values were calculated as described for alkamide phytochemical analysis.

3. Study 1.

3.1. Objectives. The objectives were to compare frequency of colonization of *E. purpurea* by *B. bassiana* in nonmycorrhizal and mycorrhizal *E. purpurea* and to determine effects of beneficial fungi on growth, nutrient use efficiency and phytochemistry in *Echinacea purpurea*.

3.2. Design. The experiment, had six treatments with 15 replications (6 x 15) spatially separated on five adjacent greenhouse benches in a standard randomized complete block design (RCBD). The experiment had two trials. Trial 1 was conducted from 4/20/2009 to 8/7/2009 and Trial 2 from 8/1/2009 to 11/10/2009. In the two trials, environmental conditions varied due to seasonal differences, but all procedures and applications were consistent (Table 3.1, Appendix 1).

3.3. Treatments.

3.3.1. *Mycorrhizae*. For the experiment 120 nonmycorrhizal cultures and 60 mycorrhizal cultures were prepared prior to Trial 1 as described above. Prior to Trial 2, new inoculum and filtrate was added before planting.

3.3.2. *Beauveria bassiana.* The *E. pupurea* seed were coated with *B. bassiana* conidia in methyl-cellulose, at the rate of10^7 conidia, or with methyl-cellulose alone (no conidia) as described above.

3.3.3. *Fertilization*. Two phosphate rates were used: low phosphorus (0.6 mM KH_2PO_4) for mycorrhizal plants or high phosphorus (1.2 mM KH_2PO_4) for nonmycorrhizal treatments. These were applied in the regular weekly fertigation treatments.

3.3.4. *Treatment Designations.* The following designations were used for the six treatment combinations:

Treatment 1- **Control (no beneficial fungi and low phosphorus)** (Con)

Treatment 2- ***B. bassiana* (*B. bassiana* only and low phosphorus)** (Bb)

Treatment 3- **High P (no beneficial fungi and high phosphorus)** (HP)

Treatment 4- ***B. bassiana:* high P (*B. bassiana* only and high phosphorus)** (HPBb)

Treatment 5- **AM: (*G. intraradices, G. margarita* dual culture with low phosophorus)** (AM)

Treatment 6- **AM X *B. bassiana: (G. intraradices, G. margarita* dual culture with low phosophorus + *B. bassiana*)** (AMBb*)*

3.4. Echinacea. Five *E. purpurea* seeds coated with *B. bassiana* conidia or only methyl-cellulose were direct seeded into the prepared mycorrhizal and nonmycorrhizal cultures. Seeds were planted with sterilized tweezers, and approximately 1/2 of the seed was left exposed to light. After seeding, pots were covered with clear plastic sheeting and kept moist with a seedling mist nozzle. After one week, when the first signs of germination were observed, the plastic was removed; pots were randomly arranged into blocks, and seedlings were kept moist with frequent misting. The plants were grown for 12 weeks after onset of germination. At 2 weeks after germination, pots were thinned to three seedlings per pot. These seedlings were used to determine infection rates with *B. bassiana.* Watering was slowly transitioned from frequent gentle misting to normal applications with a hose mounted water break over the first 2 weeks of growth. Fertigation commenced at this time.

For the remainder of the experiment, plants were watered as needed, and fertilizer applications were made weekly as described above. Micronutrient solution was applied at 6 and 10 weeks post germination. All samples were harvested and processed at 12 weeks post germination. Greenhouse environmental conditions were recorded using a Hobo brand 2800DP light and temperature sensor and logger (Onset Computer Corporation, Bourne, MA). For mean temperatures and light data see Table 3.1 (Appendix 1).

3.5. Data collected.

3.5.1. *Beauveria bassiana colonization*. In order to verify that *B. bassiana* can endophytically colonize *E. purpurea* and to test host affinity, a random sampling of 2-week-old seedlings from each treatment was assayed for colonization. Seedlings were cleaned of soil debris and surface sterilized in 95% ethyl alcohol (1 min), followed by 20% chlorine bleach solution ($NaCLO_3$) (3 min), followed by a second dip in 95% ethyl alcohol (1 min). Samples were air dried in a biosafety cabinet and plated onto selective media (Doberski and Tribe, 1980). Presence of *B. bassiana* was confirmed by the presence of mycelia and conidia emerging from the plant tissues, after 8-12 weeks. Percent colonization was determined as the percentage of seedlings infected with *B. bassiana*.

3.5.2. *Arbuscular Mycorrhizae colonization*. Rates of AM fungi colonization in roots were determined after harvest, using histology techniques and light microscospy based on techniques originally described in Phillips and Hayman (1970). Fresh root samples (ca. 100 mg) were collected from each pot at harvest. Roots were then placed into plastic histology cassettes for staining. Cassettes were placed into a beaker and submerged in a 10% KOH solution (CAS#

1310-58-3, Fisher Scientific, Waltham, MA) and brought to a simmer. Samples were simmered for 5 min, but not allowed to boil. The KOH was removed, and a 2% hydrochloric acid solution (HCL) was added; samples were maintained at room temperature for 1.5 h. The HCL solution was removed and samples were stained in 0.05% Trypan Blue solution (CAS# 72-57-1: Mallininckrudt, inc., Hazelwood, MO) solution for 1 h. Samples were destained in a lactoglycerol solution (Glycerol-CAS# 56-81-5, Acrose, Geel, Belgium; Lactic acid-CAS 7732-18-5, Fisher Scientific, Waltham, MA) for a minimum of 48 h. Roots from each sample were mounted in lactoglycerol solution, covered with a cover slide and viewed with a light microscope (Fisher Scientific, Waltham, MA) at 200x power.

Percent colonization values for each sample were determined using methods described in McGonicle et al. (1990). Standardized counts were made by moving the microscope field of view in a grid-like pattern across each slide. Each visual intersection with a root was scored as a positive or negative count based on visual identification of AM fungal structures in the root cortex. A standard percent colonization value for each sample was determined based on the number of positive counts out of 100. Hyphae, arbuscules and vesicles were also counted if seen intersecting the vertical reticle line.

3.5.3. *Growth*. Two weeks after the onset of germination, germination rates were determined, and plants were thinned to three plants per pot. Number of leaves, overall plant height, and the size of the largest leaf was measured for each plant at 4, 6 and 8 weeks. For all parameters at all times, average values from the three plants in each replicate were calculated and used for statistical analysis.

Any visible leaf, not counting cotyledons, was counted if observed. Plant height was measured from the surface of the media to the highest portion of the plant as it stood naturally. The length of the largest leaf on the plant was measured from its base to its apex. Growth measurements were reported to the nearest millimeter.

At 12 weeks, plants were harvested, rinsed clean and patted dry. Small root samples (as described above) were removed from each sample, labeled, and put aside for mycorrhizae colonization analysis. The number of leaves, number of actively growing shoots, plant height, leaf size, crown caliper, and fresh root and shoot weight were measured and recorded. Any visible leaf was counted. If a developing side shoot had produced a leaf that was counted in the leaf count, the side shoot was counted as an actively growing shoot. Size of the largest leaf on the plant was measured from base to apex. Final plant height was measured from the cotyledon scar, on the plant crown, to the tip of the largest leaf. The caliper of the crown, at the cotyledon scar, and the caliper of side shoots were measured using a digital caliper to the nearest 0.1 mm. If multiple shoots were counted for the plant, they were measured with the caliper, and the values were summed with the crown caliper to determine a total caliper value for that plant. Root tissue was separated from shoot tissue, at the cotyledon scar. Plants were placed in plastic bags and frozen between the time of harvest and phytochemical analysis and drying.

3.5.4. *Phytochemical analysis.* Fresh samples were collected for analysis of volatile compounds and carotenoid and chlorophylls as described above. For the sesquiterpene analysis, 300 mg of fresh leaf tissue (100 mg/plant) was collected from each pot. Because the chemical analysis requires fresh tissue, this tissue could not be reincorporated into the dry weight values. An additional 900 mg of leaf tissue (300 mg from each plant) was collected from five samples of

each treatment (one representing each greenhouse bench) and freeze-dried for analysis of carotenoids and chlorophylls. The final dry weight of these samples was recorded after freeze drying and reincorporated into the final foliage dry weight values for the respective samples. The remaining dry weights of root and foliage tissues were measured to the nearest 0.1 g

3.6. Statistical analysis. All statistical analysis was performed using Statistical Analysis Software (SAS, Cary, NC). Analyses, other than the standard t-test, were performed using program code contained in the "DANDA" macro for SAS designed by Dr. Arnold Saxton [University of Tennessee Statistical Design and Analysis Web Guide (http://dawg.utk.edu/)].

3.6.1. ***Percent colonization.*** Data for *Beauvera bassiana* colonization were compared using a standard t-tests to a significance level of $P \leq 0.05$. Data was verified for normality and confirmed for equal variance using a Satterthwaite test for unequal variance.

3.6.2. ***All other parameters.*** Arbuscular mycorrhizae colonization, all growth parameters and phytochemical content data was compared using mixed model Analysis of Variance (ANOVA) and mean separation procedures to determine significant differences to the $P=0.05$ level. For parameters with Shapiro-Wilke values below 0.90, or standard deviation values not within a fivefold difference, log or power transformations were performed prior to analysis (Table 3.2, Appendix 1).

Growth data analysis was based on average sample values (as described above) from all 15 blocks established in the randomized complete block. For phytochemical comparisons, the same ANOVA and mean separation procedures were used, however only one block from each greenhouse bench was used giving a total of five blocks analyzed.

3.7. Data Reporting. Data for endopyte colonization is reported as the least square mean (LSM) percentages of all samples tested. All growth data is represented as the least square mean in the unit used to collect the data.

Due to concentration variations among trials, likely due to storage time, the phytochemical concentration and content data is presented graphically as relative to control treatment LSM. Graphs of the total concentration of chlorophyll and xanthophyll data are presented as LSM in mg/g dry weight. All tables are presented in the units used during statistical analysis. Statistical significance was established using the actual data output from the various analytical instrumentation. Relative content (RC) was derived from the least square means (LSM) of the data output from SAS. The following formula was used:

$$\textbf{Relative Content} = \frac{\text{Treatment 2-6 LSM}}{\text{Control LSM}}$$

In this scheme, control treatment values are determined to be 1. Represented standard errors (SE) for RC were determined by calculating the ratio of the original standard error to the original LSM for each treatment. This ratio was then used to determine the equivalent represented SE for the expressed RC value using the following equation:

$$\textbf{Relative Content SE} = \frac{\text{Treatment LSM}}{(\text{Treatment LSM SE}) * (\text{Relative Content})}$$

P-values and mean separation for treatments are represented as output from SAS prior to making the relative content conversion.

4. Study 2.

4.1. Objectives. The objective of Study 2 was to compare phytochemical content among mycorrhizal and nonmycorrhizal plants of equivalent size and physiology.

4.2. Design. The experiment had two treatments with 10 replications for a total of 20 samples. In order to ensure that statistically similarly samples were obtained, 10 samples of each treatment were produced, and the five for each treatment that that were not statistically different from the five plants in the other treatment were chosen for phytochemical analysis. Samples, greenhouse culture (with the exception of fertilization) and data collection were the same as in Study 1. Two trials were conducted. Trial 1 was conducted from 11/1/2009 to 2/5/2010, and Trial 2 from 11/14/2009 to 2/19/2010. See Table 1 (Appendix 1) for environmental conditions.

4.3. Fertilization. Nutrient applications were adjusted throughout the growing period in response to growth, with the goal of achieving plants of statistically similar mass. Fertilizer applications were recorded to compare nutrient uptake and use efficiency in *E. purpurea* with and without mycorrhizal inoculum.

4.3.1. *Treatments.*

4.3.1.1. *Mycorrhizae*. A set of 20 mycorrhizal (AM) and 20 nonmycorrhizal (NM) cultures were prepared and grown in the greenhouse as described in Study 1. Colonization of *S. bicolor* inoculum was verified prior to the study.

4.3.1.2. *Fertilization*. Fertilizer applications were made, using the same materials and concentrations described for Study 1. Application frequency was adjusted in the AM treatment with an intentional bias towards limiting growth in the AM plants to match growth in NM plants. NM samples were fertilized as described in Study 1. Fertilizer applications began for both treatments at 2 week post germination. In the first week, plants received the applications for the control and AM treatments described in Study 1. After the first week, NM plants continued to receive a 150 mg/L solution of Peters® 15-0-15 and a 3.0 mM solution of potassium phosphate weekly, but AM plants received 150 mg/L solution of Peters® 15-0-15 and a 0.8 mM solution of potassium phosphate every other week. In response to accelerating growth of AM plants towards the end of the growing period, the last application of Peters® 15-0-15, applied to AM plants at 11 weeks, was reduced to 75 mg/L See Table 3.2 (Appendix 1) for application history. Trial 2 received the same regime.

4.3.2. *Treatment Designations.*

Treatment 1- **Nonmycorrhizal** (NM)

Treatment 2- **Mycorrhizal** (AM)

4.4. Echinacea. Planting of *E. purpurea* seed, and establishment of seedlings, followed the same procedures and time frames as described for Study 1. Plants were arranged adjacently on a single greenhouse bench and watered by hand or automated drip irrigation. Regardless of irrigation method, all samples from both treatments were watered consistently at the same time with equivalent volumes of water throughout the trial.

All other greenhouse culture activities were consistent with those in Study 1. The experiment was repeated (Trial 2). Greenhouse environmental conditions were recorded as described for Study 1.

4.5. Data Collection. After 12 weeks of growth, plants were harvested and tissues processed according to methods as in Study 1 with one exception. Phytochemical samples for phenolic acids, from Trial 2 of this study, were not frozen prior to drying and analysis. Total sample dry weights were determined, and the treatment groups (n=10) were determined to be statistically similar. The five plants closest to the mean weight value for each treatment were then analyzed for phytochemical content.

4.6. Statistical analysis. Multistep statistical analysis was used to select five samples, from each treatment, that were statistically the most similar. In order to establish statistical similarity among both treatment groups, the experimental data was analyzed as a completely random design (CRD) using SAS (Cary, NC) software. Source code from the "DANDA" macro (Dr. Arnold Saxton, University of Tennessee) was included in all analyses. Average whole plant dry weight was used as the critical growth factor. First, all 10 samples from AM and NM treatment

groups were determined to be not different at a significance level of P=0.05. All data was verified for normality and acceptable range of standard deviations as expressed for Study 1. Once statistical similarity of all samples from the two treatments was established, data from the two treatments were pooled and a combined mean value for the entire trial was determined. Five samples from each treatment, that had mean dry weights closest to the trial mean, were selected for phytochemical analysis. This reduced set of 10 samples (5 from each treatment) was again analyzed to verify statistical similarity of the selected AM and NM samples to the significance level of P=0.05. All samples were determined to be statistically similar at each step in the process.

4.7. Data Reporting. Colonization, growth and phytochemical data are reported as described for Study 1.

5. <u>Other experiments.</u>

5.1. *Echinacea purpurea* seedling heat tolerance. Seedlings damaged during the failed initial attempt to to create Trial 2 of Study 1 were evaluated for their survival rates after the unexpected "heat event" (See Table 4.33). Pot culture preparation, seedling germination and greenhouse culture were as described for Study 1. No fertilizer applications were made prior to the heat event. Seeds were planted on May 5, 2009, germination was counted on May, 15, 2009 and the heat event occurred on May 16, 2009. Counts of surviving seedlings were made on May 19, 2009.

5.2. *Beauveria bassiana* colonization at 15 weeks. After counting for survival following the heat event, the surviving seedlings were transplanted to individual 10.3 cm (4 in.) plastic pots and planted into a Turface Proleague (Turface Athletics, Buffalo Grove, IL) / Terragreen (Oil-Dri Corp. of America, Chicago, IL) (1:1, v:v) mixture and grown on for an additional 14 weeks. Fetilization materials and regimes remained as described for mycorhizal and nonmycorrhizal plants in Study 1 and plants were watered by hand as needed. Plants were harvest on August 26, 2009 and assayed for the presence of *Beauveria bassiana*.

The analysis was as described for Study 1 with a few differences. Plants were harvested, cleaned and lightly, but thoroughly scrubbed with a mild detergent (generic brand dish soap) concentrating on the crown region. Leaves and roots were removed approximately 4-5 cm above and below the cotyledon scar, and samples were sterilized as described for Study 1. The crown was then sectioned vertically in half and the two cut surfaces placed down on the culture medium. Observations were made and recorded after 8-12 weeks.

5.3. Statistical analysis. For *B. bassiana* colonization at 15 weeks, standard t-tests were used to determine statistical significance at P=0.05 as described in Study 1. For *E. purpurea* heat tolerance, each treatment was compared to each other treatment and mean separations were deduced.

Chapter IV

Results

1. Study 1.

1.1. Endophyte Colonization. (See Appendix 1, Tables 4.1, 4.2 for data and *P* values)

1.1.1. *Beauveria bassiana* (Bb). In all experiments, no seedlings from treatments without *B. bassiana* were colonized by the entomopathogen. Colonization of seedlings grown from seed treated with *B. bassiana* planted in media that did not contain mycorrhizal fungi, culled from Trial 1, was not different from colonization of plants treated with both endophytes (AMBb) (P=0.28). In Trial 2, colonization by *B. bassiana* was decreased in plants treated with both endophytes (AMBb) (P=0.032); 74% of seedlings grown from *B. bassiana*-treated seed in medium that did not contain mycorrhizal fungi were colonized, but only 33% of the seedlings grown in co-culture with mycorrhizal fungi in the AMBb treatment were colonized (Figure 4.1).

1.1.2. *Arbuscular Mycorrhizae.* In both trials AM fungal colonization of *E. purpurea*, at 12 weeks exceeded 95% (Figure 4.2). In Trial 1 mycorrhizal colonization was significantly less in the AM treatment than in the AMBb treatment (P=0.044); however, no differences were detected in Trial 2. Numbers of AM fungal hyphae, vesicles, and arbuscules were also not significantly different between AM and AMBb treatments in either trial (Figure 4.3).

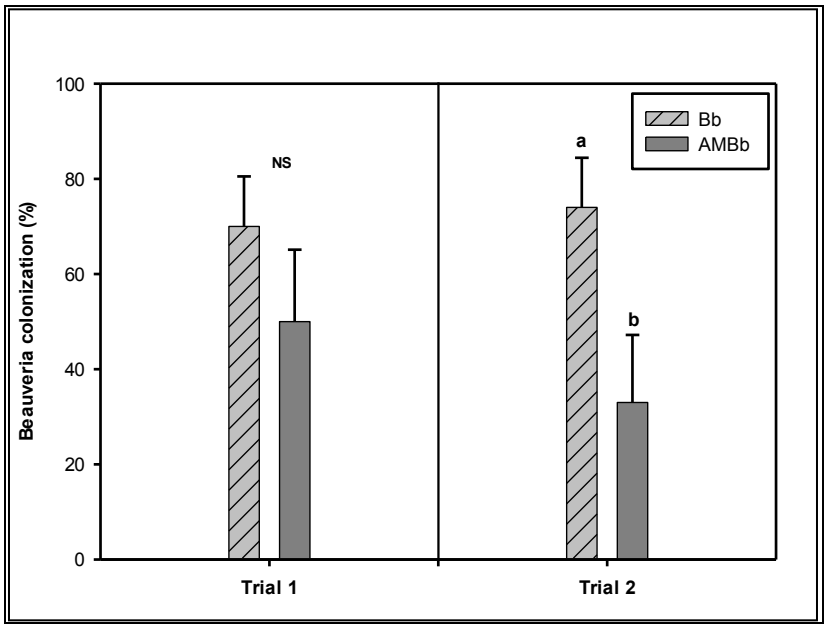


Figure 4.1. Perecentage of plants colonized by *Beauveria bassiana* in 2-week-old *Echinacea purpurea* seedlings treated with *B. bassiana* (Bb) and plants treated with arbuscular mycorrhizal fungi and *Beauveria bassiana* (AMBb). See Table 4.1 (Appendix 1) for data and *P* values. NS=Not significant.

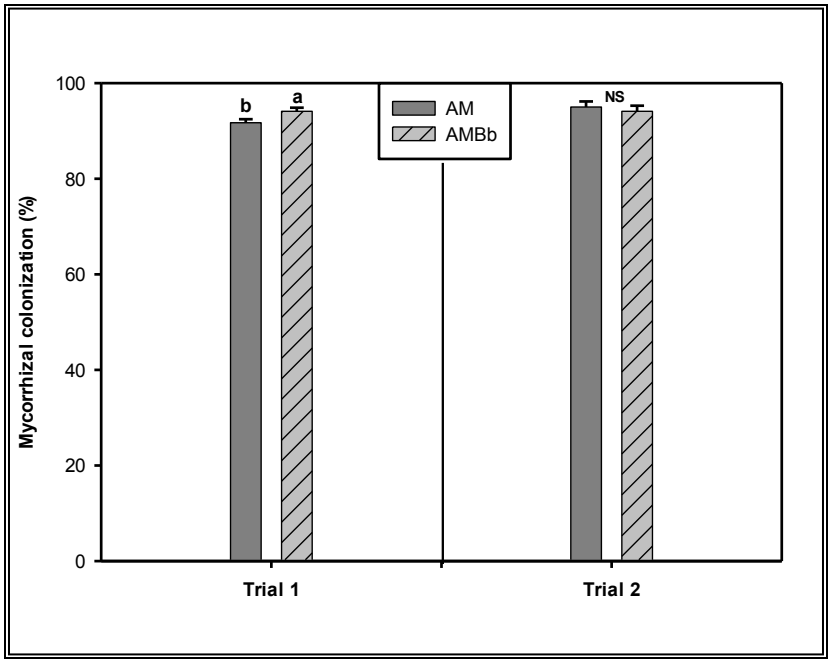


Figure 4.2. Colonization by mycorrhizal fungi in roots of 12-week-old *Echinacea purpurea* plants treated with arbuscular mycorrhizal fungi (AM) and plants treated with arbuscular mycorrhizal fungi and *Beauveria bassiana* (AMBb). See Table 4.2 (Appendix 1) for data and *P* values.

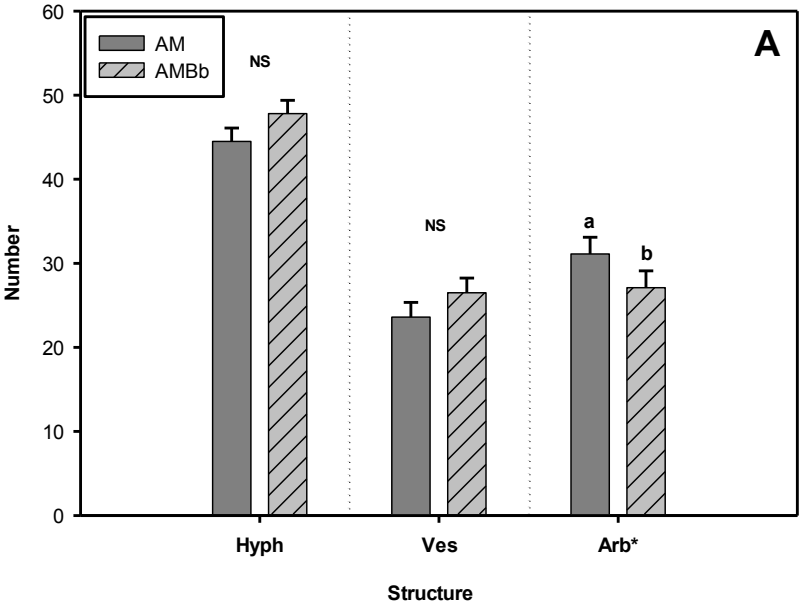


Figure 4.3. Number of hyphae (Hyph), vesicles (Ves), and arbuscules (Arb) in roots of -week-old *Echinacea purpurea* plants treated with arbuscular mycorrhizal fungi (AM) and plants treated with arbuscular mycorrhizal fungi and *Beauveria bassiana* (AMBb) from Trial 1 (Panel A) and Trial 2 (Panel B). See table 3 (appendix 1) for P=values. NS=Not significant. See Table 4.2 (Appendix 1) for data and *P* values.

1.2. Growth parameters. (See Appendix 1, Tables 4.3, 4.4, 4.5, 4.6, 4.7 for data and *P* values)

All growth measurements were greater for plants in AM and AMBb treatments than in all other plants in the study ($P < 0.0001$). For most parameters measured, plants grown from *B.bassiana*-treated seed did not differ from the corresponding no *Beauveria* control (e.g., Bb = control; HPBb = HP; AM = AMBb).

1.3.1. *Seed Germination.* No differences were observed in any treatments in either trial (Trial 1: $P=0.227$, Trial 2: $P=0.932$). Germination percentage for all trials was 75%. (Appendix 1, Table 4.3)

1.3.2. *Leaf Number.* The number of leaves was not different between low (control and Bb) and high phosphorus (HP and HPBb) treatments at 4 weeks, but high phosphorus treatments produced more leaves at all other times in both trials (Figure 4.4). By 12 weeks, *E. purpurea* plants in AM and AMBb treatments produced an average of 19-20 leaves, and low phosphorus treatments averaged 6-7 in both trials.

1.3.3. *Leaf Size.* The size of the largest leaf followed a similar trend as in Trial 1, but in Trial 2, leaves in the high phosphorus treatments were larger than those in the low phosphorus treatments even at 4 weeks (Figure 4.5). By 12 weeks, the mean size of the largest leaf of *E. purpurea* plants in the AM trratment was larger than plants in AMBb treatment in Trial 1, but not in Trial 2. In Trial 1, at 12 weeks, Bb plants had larger leaves than control plants, but plants in the HP treatment had larger leaves than those in HPBb. However, in Trial 2 these differences were not present.

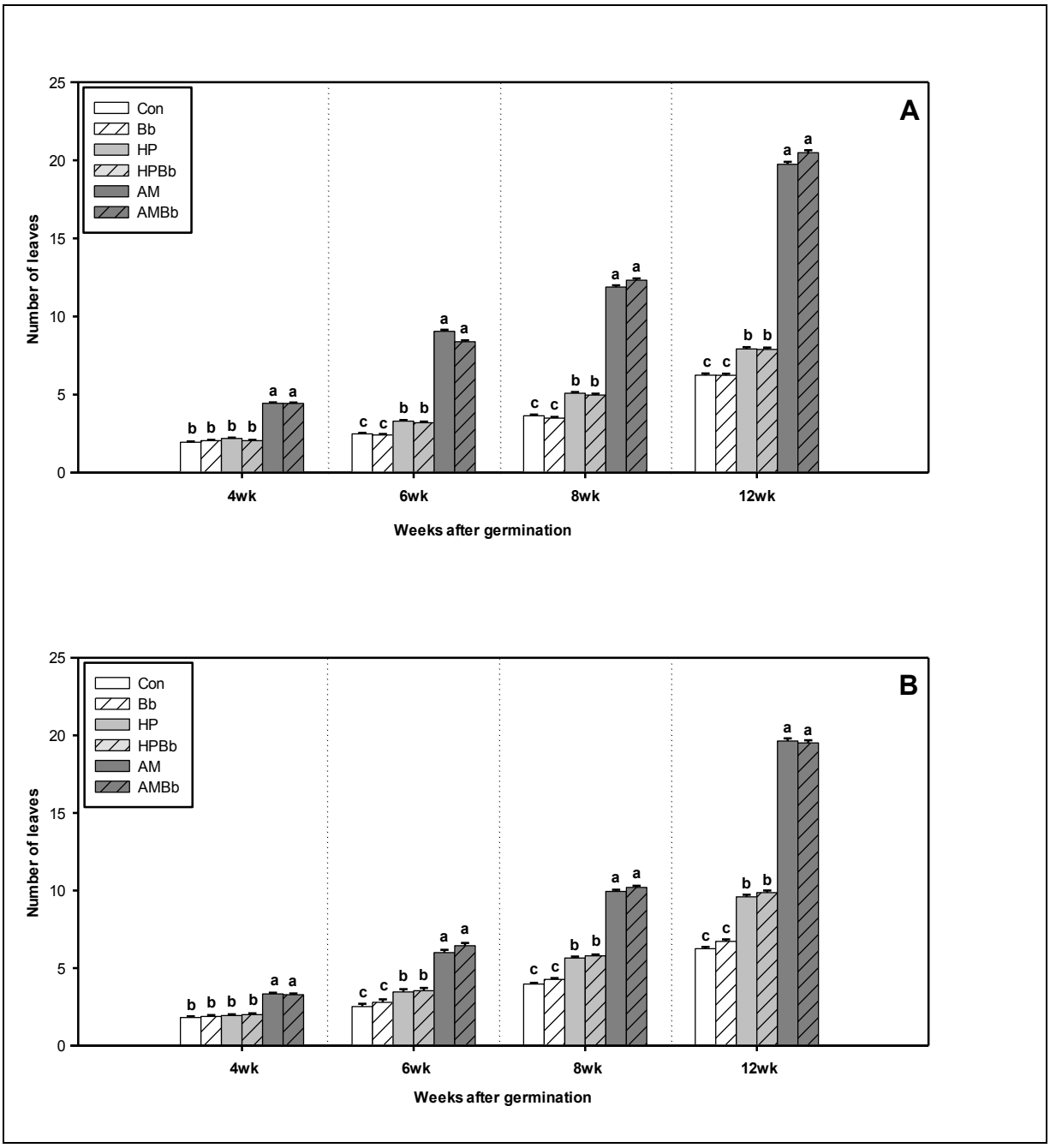


Figure 4.4. Number of leaves on *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B) at intervals during the 12 week growing period. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.3 (Appendix 1) for data. *P*<0.0001.

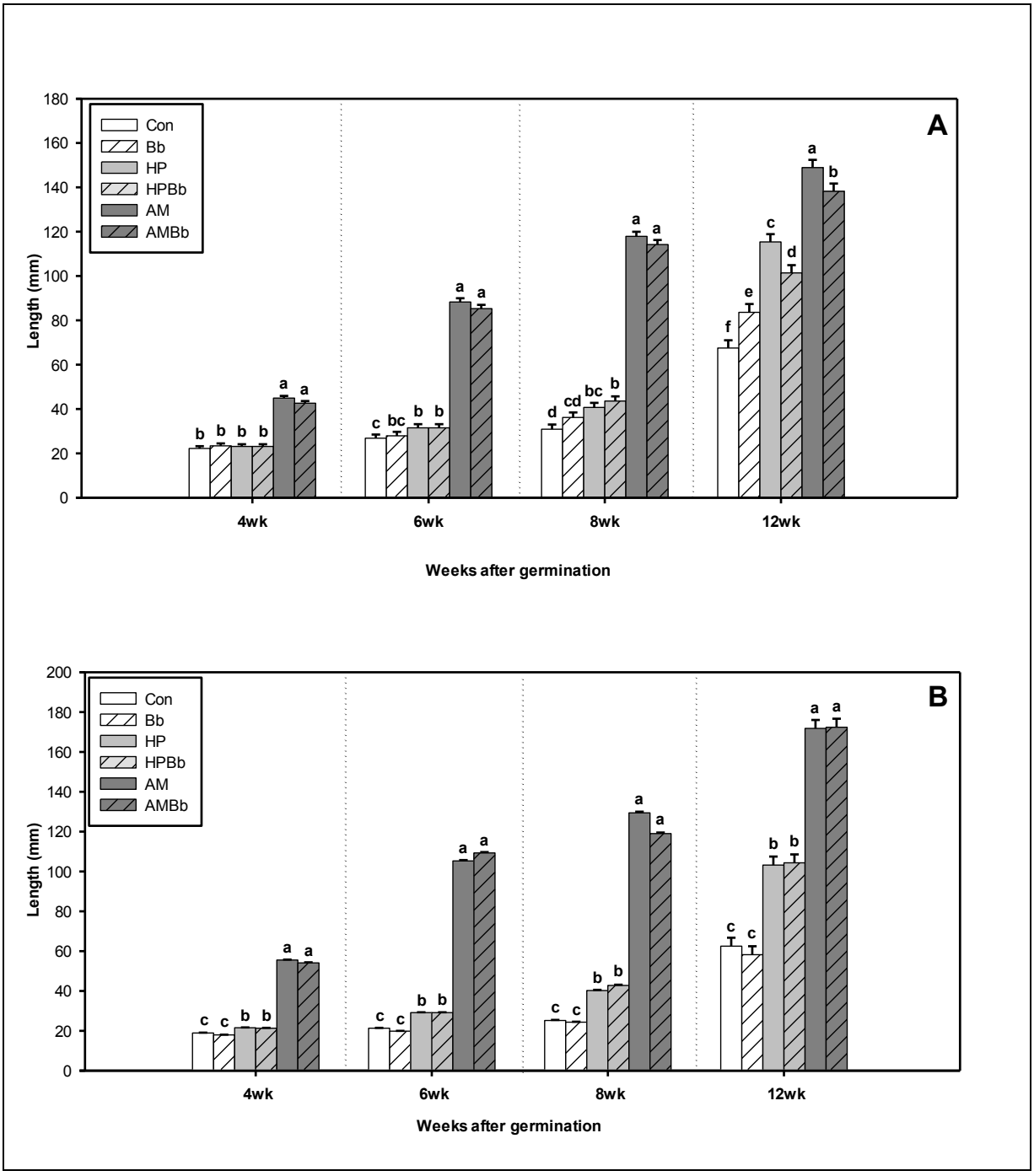


Figure 4.5. Length of the largest leaf on *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B) at intervals during a 12 week growing period. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.4 (appendix 1) for data. *P*<0.0001.

1.3.4. ***Plant Height.*** In both trials, at all intervals, AM plants were more than twice the height of control plants (Figure 4.6). Up to 8 weeks, heights of plants in the high phosphorus treatments were more similar to those in low phosphorus treatments than to plants colonized by mycorrhizal fungi. By 12 weeks, in Trial 1, height of plants treated with high phosphorus were closer to AM treatments than to plants treated with low phosphorus and in Trial 2, with mean values approximately mid-way between low phosphorus and mycorrhizal plants.

1.3.5. ***Crown caliper.*** In both trials**,** plants treated with myorrhizal fungi had crowns larger than twice the size of all other treatments (Figure 4.7) ($P<0.0001$). In Trial 1, plants in Treatment AMBb had smaller crowns than those in treatment AM however; there was no difference in Trial 2. In both trials, plants treated with high phosphorus had larger crown that those treated with low phosphorus.

1.3.6. ***Number of shoots.*** In both trials**,** plants in the AM and AMBb treatments produced more shoots than all other treatments (Figure 4.8) ($P<0.0001$). Plants treated with both endophytes (AMBb) did not differ in Trial 1, however, in Trial 2 plants treated with only AM fungi had fewer shoot. In Trial 1, the high phosphorus treatment, without Bb, had more shoots than low phosphorus treatments, but all other high phosphorus treated plants were not different from all low phosphorus treatments.

1.3.7. ***Dry Weight.*** Plant dry weight, at 12 weeks, (root + shoot) increased approximately 3.5-fold (Trial 1) and 5.5-fold (Trial 2) in AM and AMBb treatments when compared to NM treatments receiving high phosporous (Figure 4.9) ($P<0.0001$). A greater than 10-fold increase was observed when comparing the AM and AMBb treatments to low phosphorus treatments

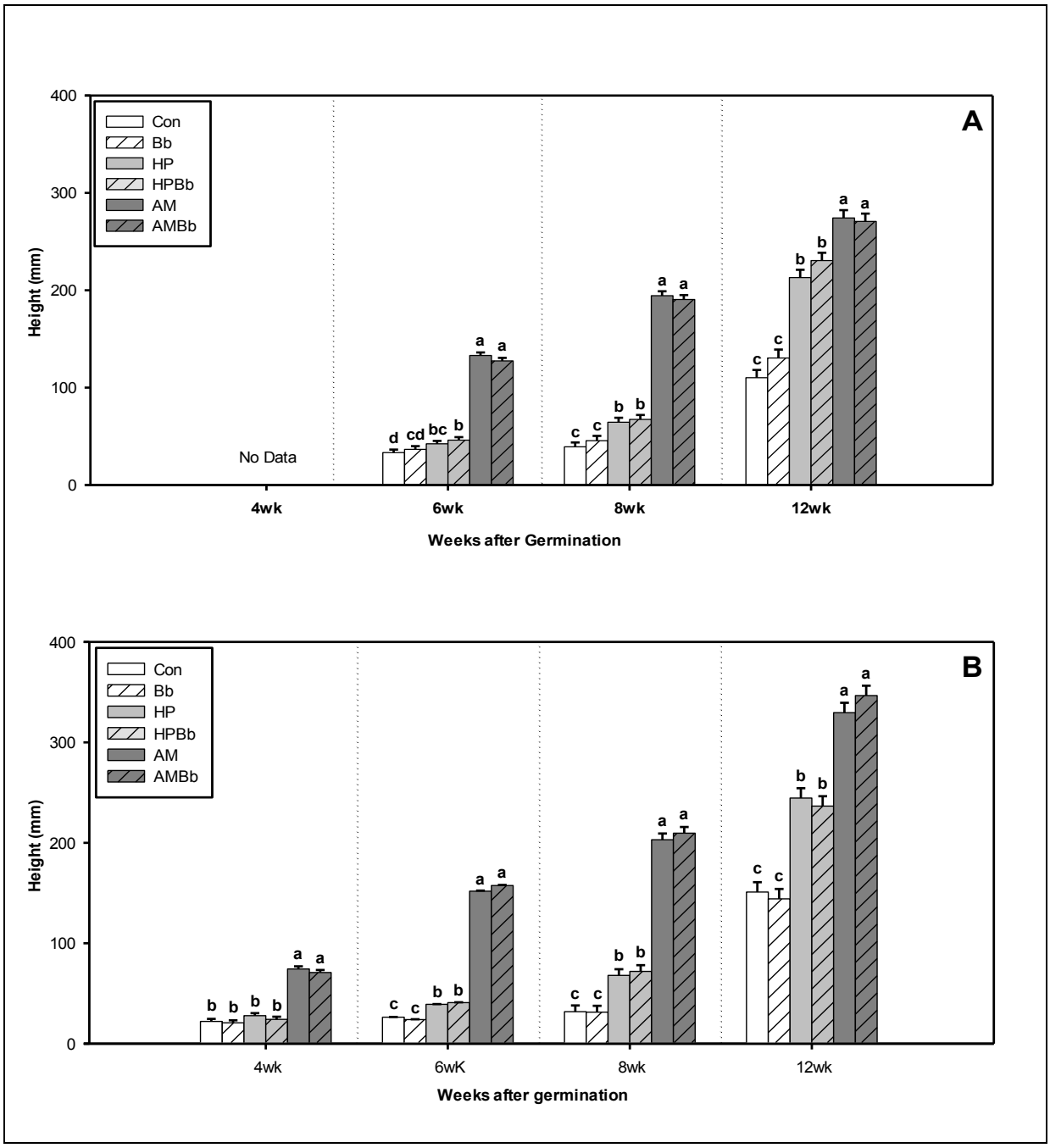


Figure 4.6. Height of *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B) at intervals during a 12 week growing period. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.5 (Appendix 1) for data. *P*<0.0001.

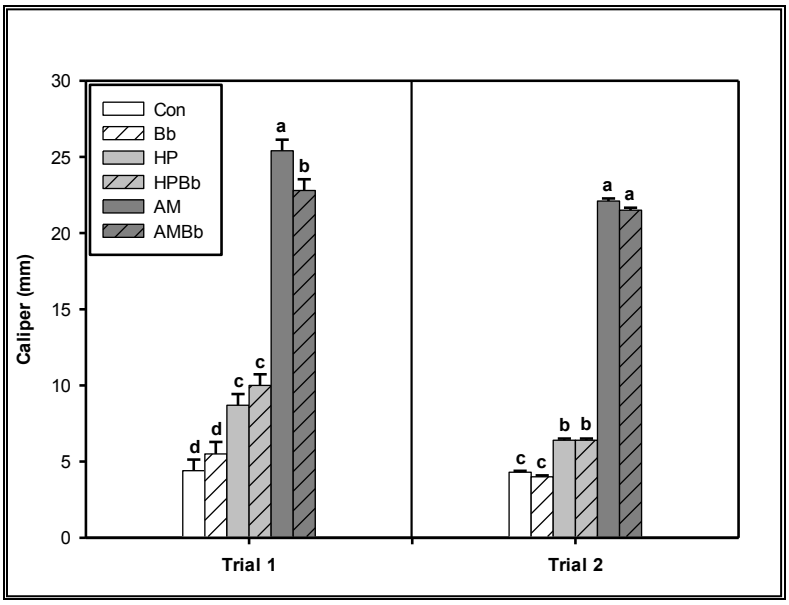


Figure 4.7. Crown caliper of 12-week-old *Echinacea purpurea* plants from Trial 1 and Trial 2. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.6 (Appendix 1) for data. *P*<0.0001.

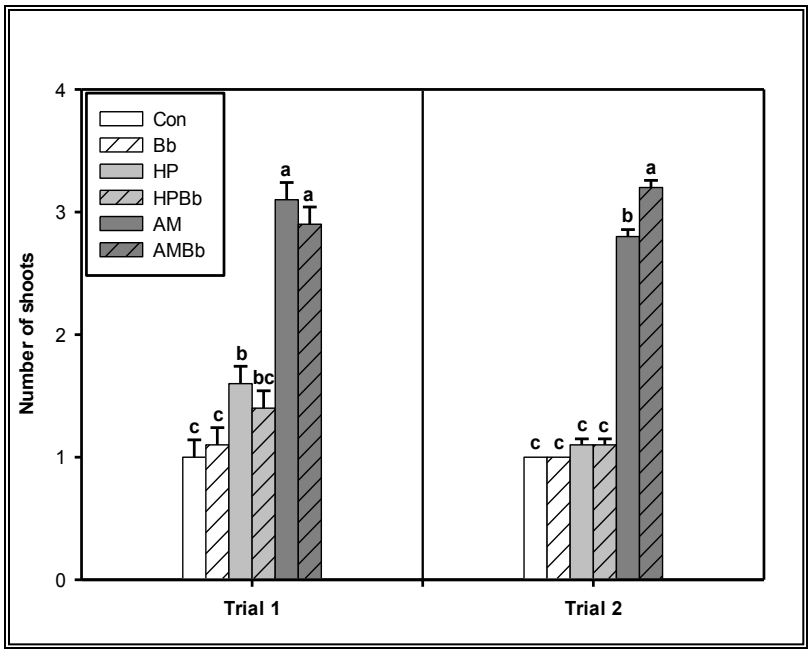


Figure 4.8. Number of growing shoots on 12-week-old *Echinacea purpurea* plants from Trial 1 and Trial 2. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.6 (Appendix 1) for data. *P*<0.0001.

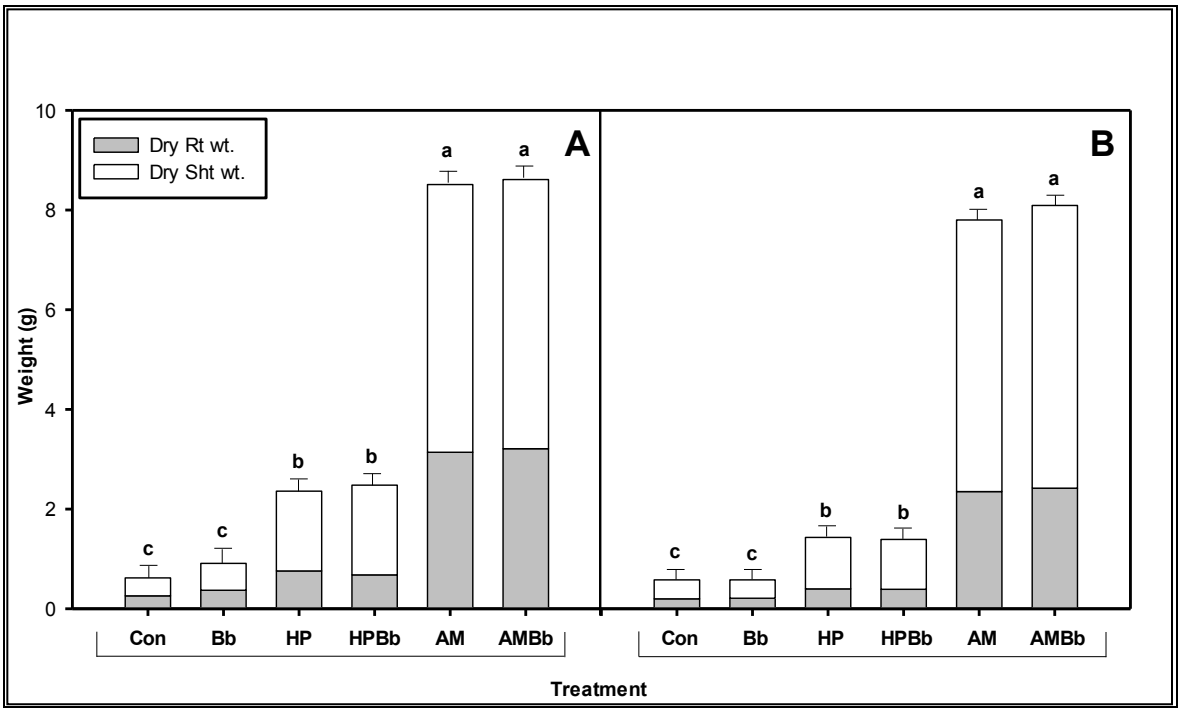


Figure 4.9. Whole plant dry weight of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.7 (Appendix 1) for data, root and shoot weight, mean separation, and *P* values.

Root:shoot ratios were greater for treatments receiving low phosphorus than all other treatments (Table 4.7, Appendix 1).

1.3. Phytochemistry. See Appendix 1, Tables 4.8, 4.9, 4.5, 4.6, 4.7 for data and *P* values. Figures for total chlorophylls and total xanthophylls are expressed as absolute concentration in mg/g dry weight. All other figures are expressed as relative to the low phosphorus control treatment. Content data are: the absolute concentration (mg/g) multiplied by the tissue biomass, and are expressed as relative to the low phosphorus control treatment.

1.3.1. *Chlorophyll.* Chlorophylls A and B were identified in all extracts, but there was no consistent pattern between Trials 1 and 2 except that concentration of chlorophyll A was always at least twice the concentration of chlorophyll B. Representative chromatograms are shown in Figure 4.10.

1.3.1.1. *Concentration.* In Trial 1, total chlorophyll concentration (chlorophyll A+B) was less in treatments Bb, AM and AMBb than in the other treatments (P=0.0008) (Figure 4.11); however in Trial 2, all plants treated with high phosphorus (HP and HPBb) and those treated with both endophtes (AMBb) were greater. Compared to controls, total chlorophyll concentration in high phosphorus treatments were not different in Trial 1, but were higher in Trial 2 (P=0.0015). Plants treated with low phosphorus and Bb (Bb) had reduced total chlorophyll concentration in Trial 1, but not in Trial 2.

Relative to control (low phosphorus, no fungi treatment), chlorophyll A levels decreased in the Bb, and AM, and AMBb treatments (P=0.0008) (Figure 4.12). In Trial 2 all plants treated with high phosphorus had significantly higher chlorophyll A concentrations (P=0.0084). Chlorophyll B levels were increased in plants in treatment HPBb, but decreased in all treatments with mycorrhizae (AM and AMBb) (P=0.003) in Trial 1. In Trial 2, plants in Treatment AM had the lowest concentration of chlorophyll B (P<0.0001).

Compared to controls, chlorophyll A:B ratios in Trial 1 were decreased in treatment Bb (P=0.021). In Trial 2, no treatments differed from the control treatent, but plants in Bb treatment had lower chlorophyll A:B ratios than all plants treated with high phosphorous or mycorrhizal fungi (P=0.049).

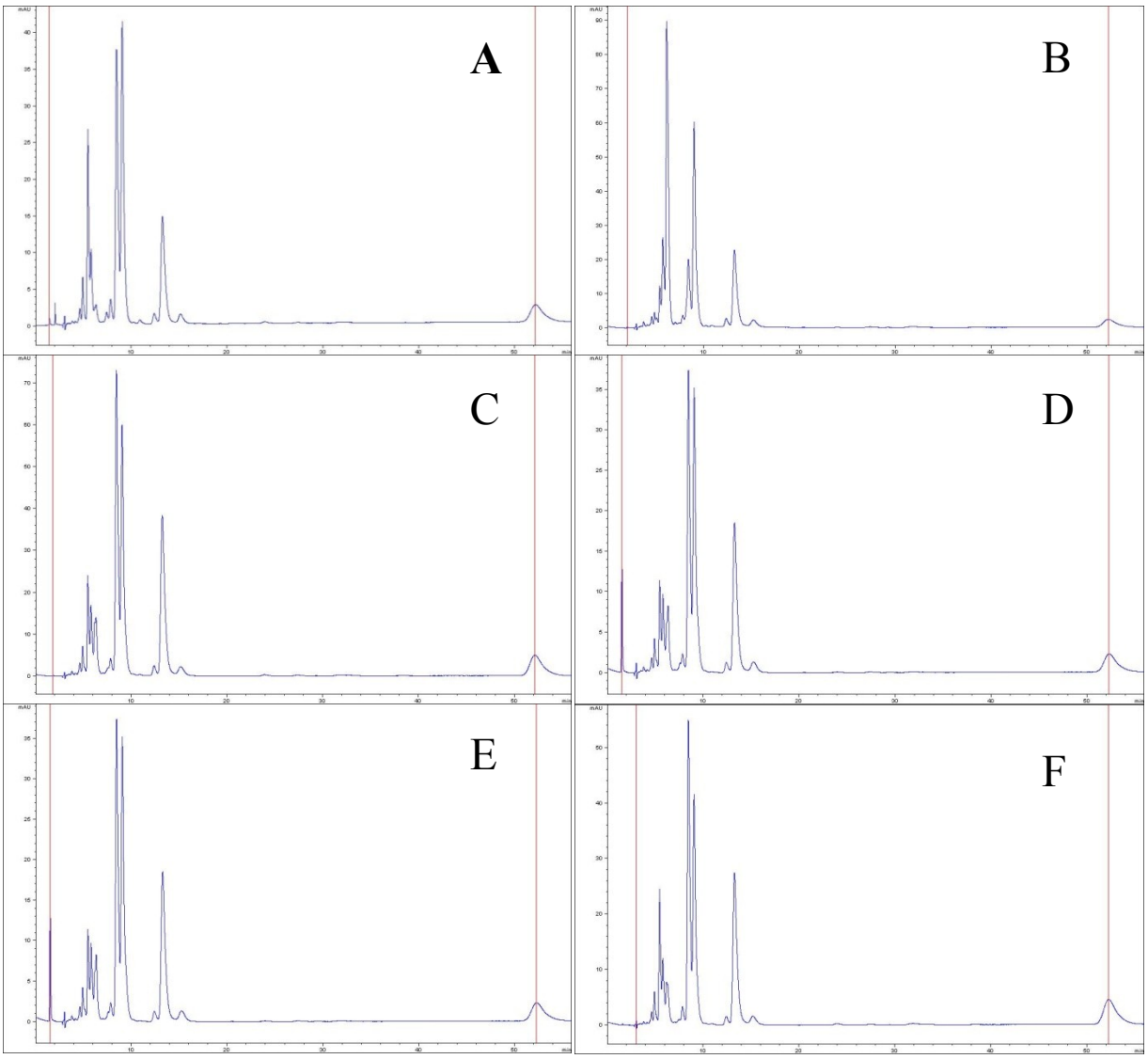


Figure 4.10. Representitive HPLC chromatograms identifying chlorophylls carotenoids and xanthophylls from leaves of *E. purpurea* plants from treatments: control (Con) (Panel A), *Beauveria bassiana* (Bb) (Panel B), high phosphorus (HP) (Panel C), high phosphorus and *B. bassiana* (HPBb) (Panel D), arbuscular mycorrhizal fungi (AM) (Panel E), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb) (Panel F). See Table 4.21 for HPLC retention times.

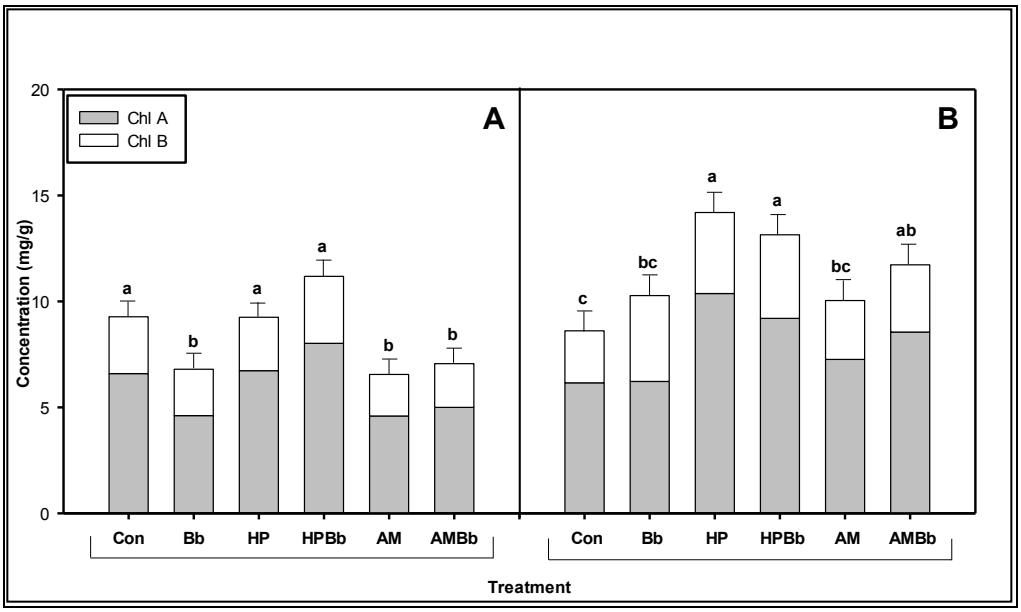


Figure 4.11. Total chlorophyll [chlorophyll A + chlorophyll B] concentration in leaves of 12-week-old *Echinacea purpurea* leaves from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.8 (Appendix 1) for data and *P* values.

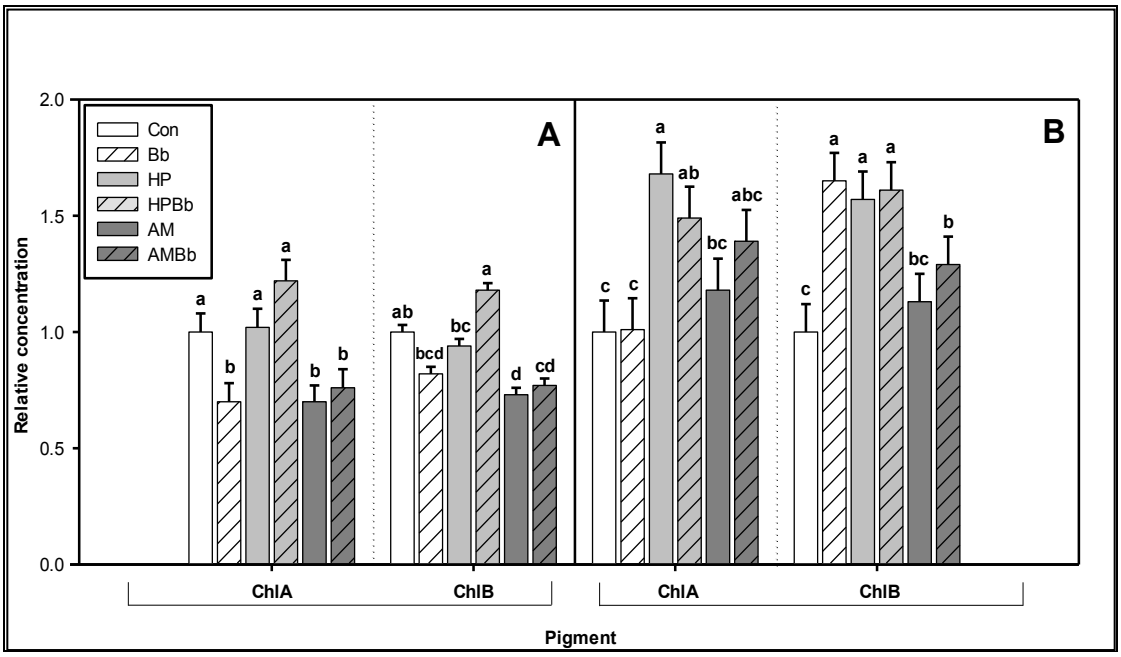


Figure 4.12. Relative concentration of chlorophyll A and chlorophyll B in leaves of 12-week-old *Echinacea purpurea* leaves from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.8 (Appendix 1) for data and *P* values.

1.3.1.2. C*ontent.* In both trials, all plants in treatments AM and AMBb contained at least 1.9 times more chlorophyll than all plants in high phosphorus treatments and at least 7.6 times more compared to plants in low phosphorus treatments ($P < 0.0001$) (Table 4.9, Appendix 1). All plants treated with high phosphorus contained more chlorophyll than plants treated with low phosphorus.

1.3.2. *Xanthophylls.* Three xanthophylls were identified in leaf extracts from all treatments. Plants treated with high phosphorus or mycorrhizae had the highest concentrations of xanthophylls in Trial 1; this pattern was generally true in Trial 2.

1.3.2.1. *Concentration.* Relative to control plants, plants in high phosphorus and mycorrhizae treatments had higher concentrations of zeazanthin (Trial l; $P<0.00001$), antheraxanthin (Trial 1: $P< 0.0001$) (Trial 2; $P=0.067$), and violaxanthin (Trial 1: $P<0.0001$) (Figure 4.13). In Trial 2, Bb plants contained higher zeanthin levels, while AM plants had a lower concentration ($P<0.0001$). AMBb-treated plants had lower levels of violaxanthin than all high phosphorus treated plants; concentrations in AM plants were less than HP plants, but were not different from HPBb. In Trial 2, violaxanthin concentration was less in the Bb and AM treatments ($P=0.013$).

Compared to control, total xanthophyll concentration (zeaxanthin + antheraxanthin + violaxanthin) in Trial 1 was reduced in all treatments ($P=<0.0001$) (Figure 4.14). Plants in the AMBb treatment had the lowest concentrations. Plants treated with both endophytes (AMBb) had lower levels than plants treated with AM fungi. In Trial 2, total xanthophylls levels were lower in Bb and AM treatments, but not in treatment AMBb ($P= 0.011$).

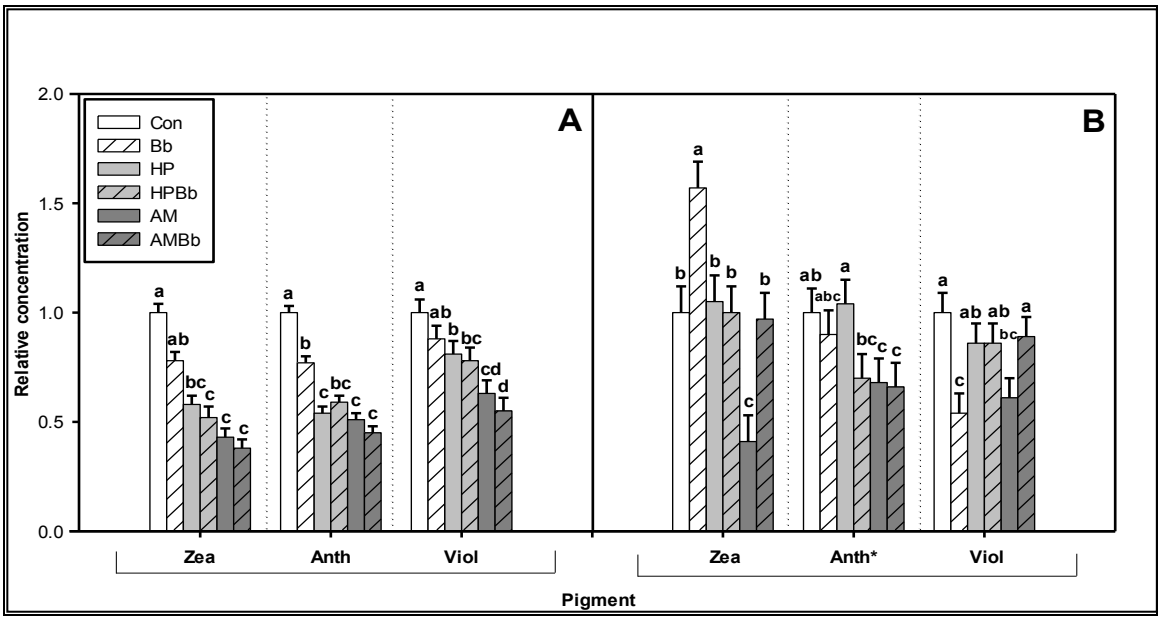


Figure 4.13. Relative concentration of zeaxanthin (Zea), antheraxanthin (Anth), and violaxanthin (Viol) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). *=P<0.1. See Table 4.10 (Appendix 1) for data and *P* values.

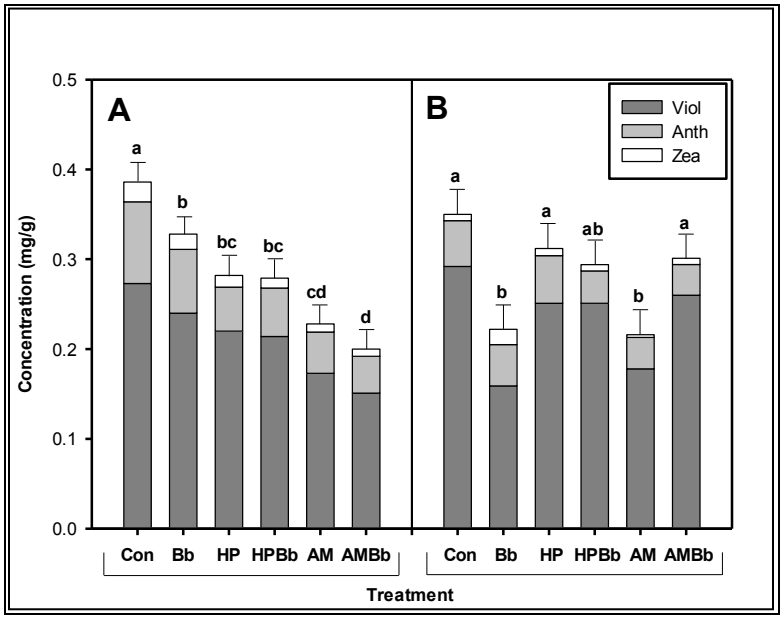


Figure 4.14. Xanthophyll concentration [violaxanthin (Viol) + antheraxanthin (Anth) + zeaxanthin (Zea)] in leaves of 12-week-old *Echinacea purpurea* from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.10 (Appendix 1) for data and *P* values.

1.3.2.2. *Content.* In Trial 1, total leaf content of zaexanthin, antheraxanthin, voliaxanthin, were highest in plants treated with mycorrhizae, intermediate in those treated with high phosphorus, and lowest in plants treated with low phosphorus ($P \leq 0.0001$). Combined totals for the three xanthophylls (zeaxanthin + antheraxanthin + violaxanthin) in Trial 1, followed the same pattern as plants treated with AM fungi; containing at least 2.1 times the amount in high phosphorus treated plants, and 5.3 times the amount in low-phosphorus-treated plants ($P \leq 0.0001$).

In Trial 2, zeaxanthin and antheraxanthin content followed similar patterns to those observed in Trial 1; plants in treatments AM and AMBb were greater than all other treatments, however plants treated with both endophytes (AMBb) contained more violaxanthin than plants treated with only mycorrhizal fungi (AM) ($P \leq 0.0001$). Total combined content of the three xanthophylls (zeaxanthin + antheraxanthin + violaxanthin) in Trial 2, was greater in AMBb-treated plants than any other treatment ($P < 0.0001$).

1.3.3. *Other carotenoids.* The effect of treatment on β-carotene and lutein was not consistent between trials.

1.3.3.1. *Concentration.* In Trial 1, β-carotene and lutein concentrations were not different in any treatments (Figure 4.15). Neoxanthin levels were lower in plants treated with HP, AM, and AMBb, but plants treated with HP and Bb were not different ($P = 0.012$).

In Trial 2, significant differences were observed in levels of β-carotene, lutein, and neoxanthin ($P \leq 0.0001$). β-carotene concentrations were higher in all high phosphorus and mycorrhizae-treated plants, while lutein concentration was higher only in plants treated with

high phosphorus. Neoxanthin levels were higher in plants treated with Bb or with high phosphorus.

1.3.3.2. *Content.* Content levels for β-carotene, lutein, and neoxanthin followed identical patterns in both trials (Figure 4.16). Levels were highest in plants treated with mycorrhizae, intermediate in plants treated with high phosphorus, and lowest levels in plants treated with low phosphorus ($P \leq 0.0001$). In all cases, the amount produced in all plants treated with mycorrhizae was at least twice as much produced by high phosphorus treated plants; β-carotene levels in Trial 2 were 10 times control. Compared to low phosphorus treated plants, mycorrhizal plants produced at least 6 times the amount of all three carotenoids studied; β-carotene levels in Trial 2 were 25 times the amount in control plants.

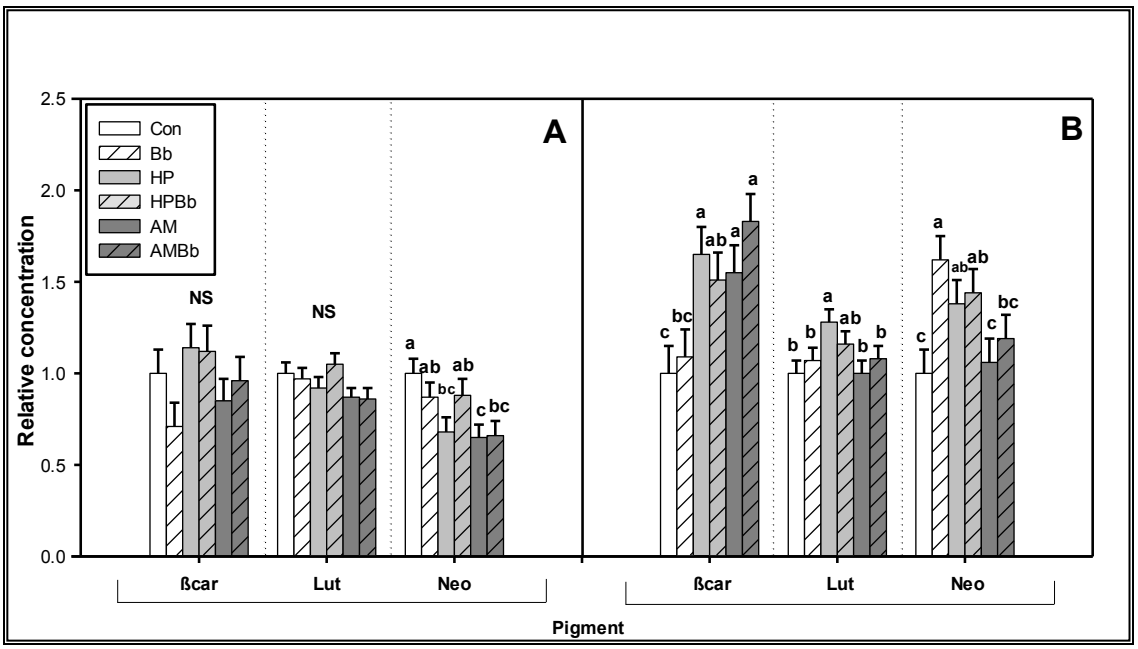


Figure 4.15. Relative concentration of beta-carotene (βcar), lutein (Lut), and neoxanthin (Neo) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). NS=Not significant. See Table 4.12 (Appendix 1) for data and *P* values.

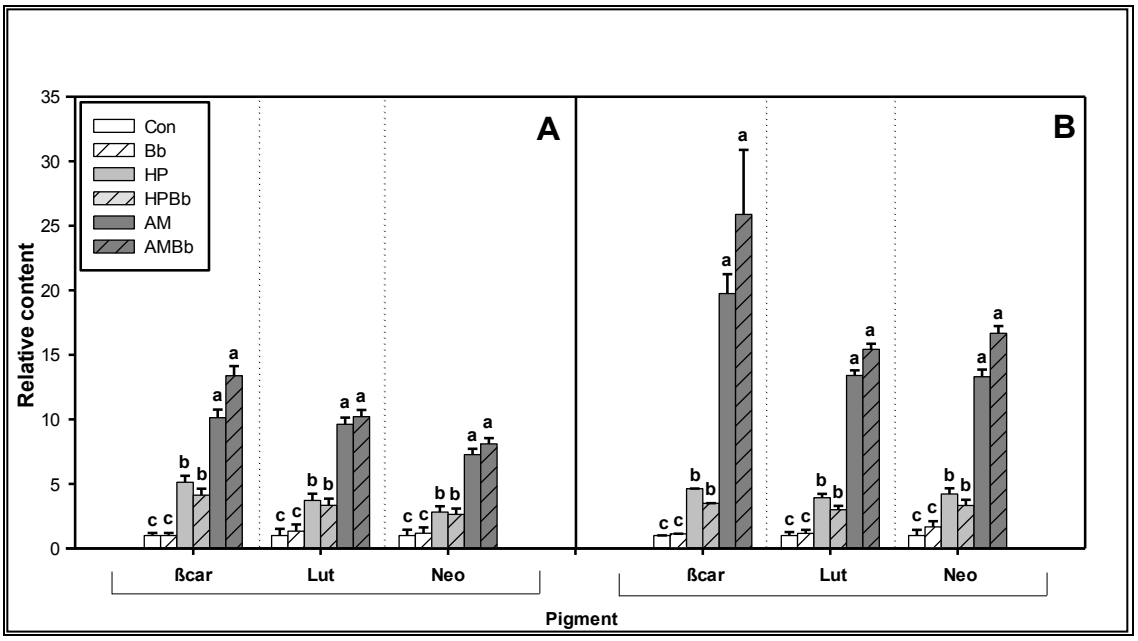


Figure 4.16. Relative content (concentration * dry weight) of beta-carotene (βcar), lutein (Lut), and neoxanthin (Neo) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). NS=Not significant. SeeTable 4.12 (Appendix 1) for data and *P* values.

1.3.4. *Phenolic acids.* Two phenolic acids (caftaric and cichoric) were found in all extracts. Cynarin was identified in the all leaf extracts but not in all root extracts; conversely, chlorogenic acid was identified in all root extracts but not in all leaf extracts. Representative chromatograms are shown in Figure 4.17.

1.3.4.1. *Concentration in leaves*. Cynarin concentration was not significantly different in any treatment from Trial 1 or Trial 2 (Figure 4.18).

In Trial 1, compared to controls, caftaric acid levels were highest in AM plants; HPBb plants had low levels ($P<0.0001$). In Trial 2, caftaric acid levels were low in plants in treatments HPBb, AM, and AMBb treatments ($P<0.0001$).

Cichoric acid concentration in leaves was increased (*P*=0.062) in AM plants in Trial 1. In Trial 2, levels were lower than control in HP plants. (*P*=0.002).

1.3.4.2. *Concentration in roots.* Concentration of caftariccaftaric acid was not significantly different in roots from any treatment or trial (Figure 4.19). Chlorogenic acid concentration was not different from control in any treatment in Trial1 but was reduced in roots from plants in HPBb, AM, and AMBb treatments in Trial 2 (*P*=0.003).

Concentrations of cichoric acid concentrations did not differ from control in any treatment. In Trial1 all mycorrhizal plants had higher concentrations than plants in HP treatment (*P*=0.033). No significant differences were observed in Trial 2.

1.3.4.3. *Whole Plant content.* In Trial 1, the total content of caftaric acid and cichoric acid produced per plant was higher all mycorrhizae (AM and AMBb)-treated plants (Figure 4.20) High phosphorus-treated plants did not differ from low phosphorus treated plants.

1.3.5. *Alkylamides.* Since standards are not available, and alkylamides from *E. purpurea* are not in the NIST standards database, three alkylamides were identified in this experiment by comparison to published mass spectra (Figures 4.21, 4.22, 4.23). There were also several other components that may be alkylamides, but these could not be identified confidently based on published spectra. Figure 4.24 shows representative chromatographs.

1.3.5.1. *Concentration in roots.* Alkylamides 8/9 was not different among treatments in either trial (Figure 4.25) In Trial 1, concentration of Alkylamide 3 was more than twice in plants in HPBb treatment than the concentration in the control (*P*=0.019); concentrations were not different in Trial 2. In Trial 1, relative concentration of Alkylamide 2 was greater than control in all plants treated with *B. bassiana* (Bb, HPBb, AMBb) (*P*=0.041).

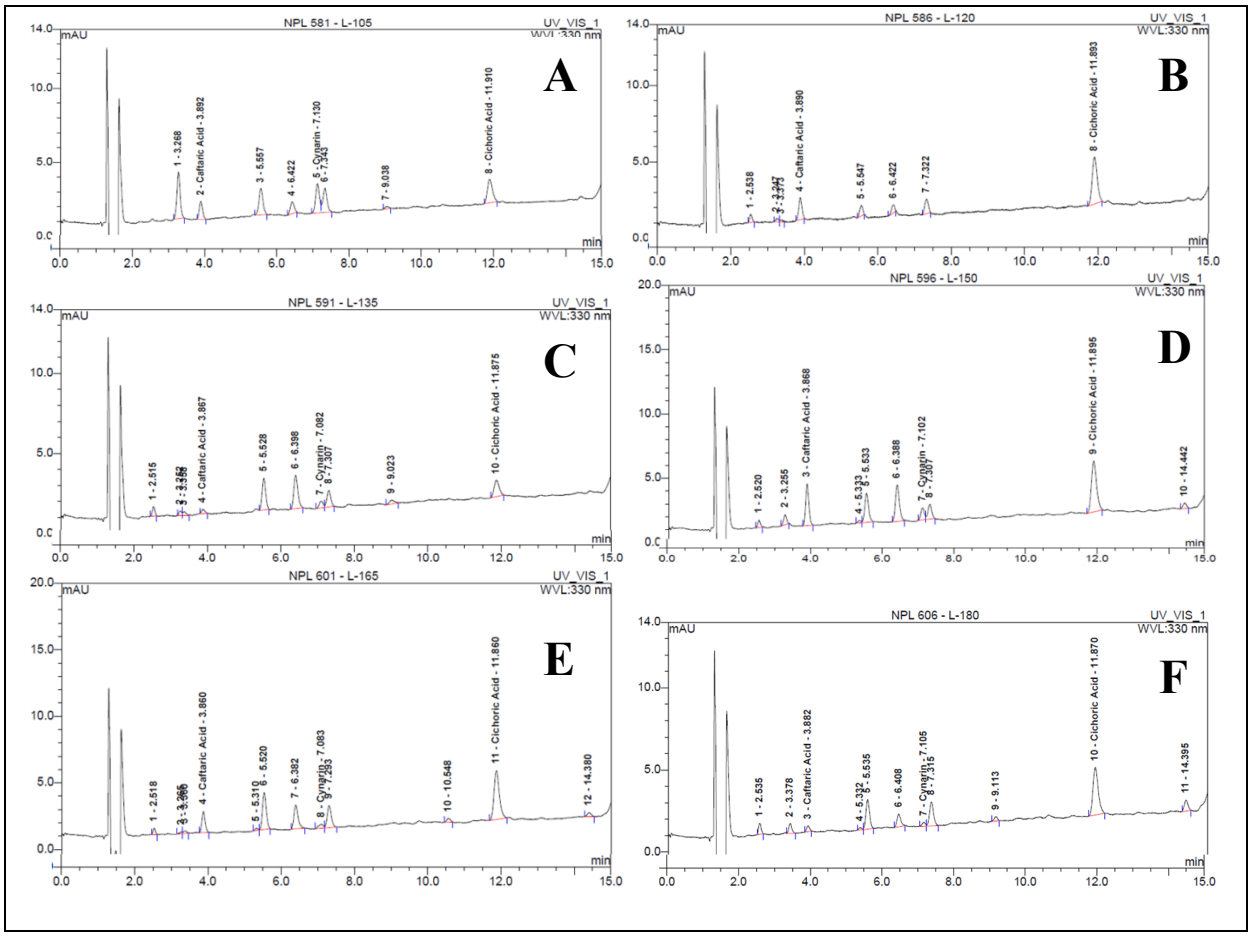


Figure 4.17. Representitive HPLC chromatograms identifying phenolic acids from leaves of *E. purpurea* plants from treatments: control (Con)(Panel A), *Beauveria bassiana* (Bb) (Panel B), high phosphorus (HP) (Panel C), high phosphorus and *B. bassiana* (HPBb) (Panel D), arbuscular mycorrhizal fungi (AM) (Panel E), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb) (Panel F). See Table 4.31 for HPLC retention times.

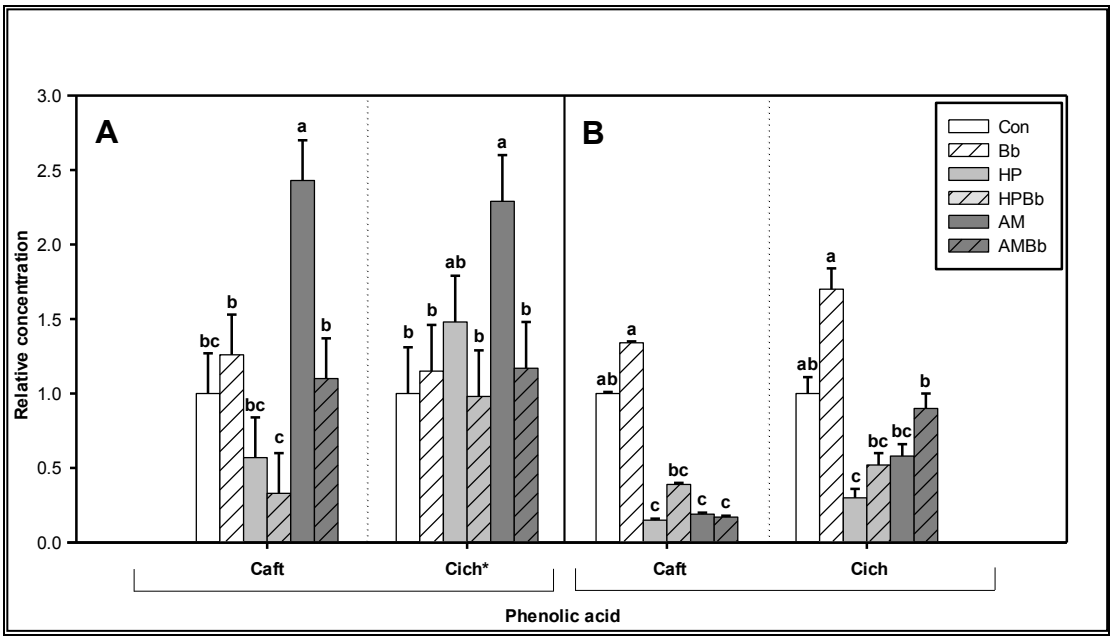


Figure 4.18. Relative concentration of caftaric acid (Caft) and cichoric acid (Cich) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). NS=Not significant; *=P<0.1. See Table 4.28 (appendix 1) for data and *P* values.

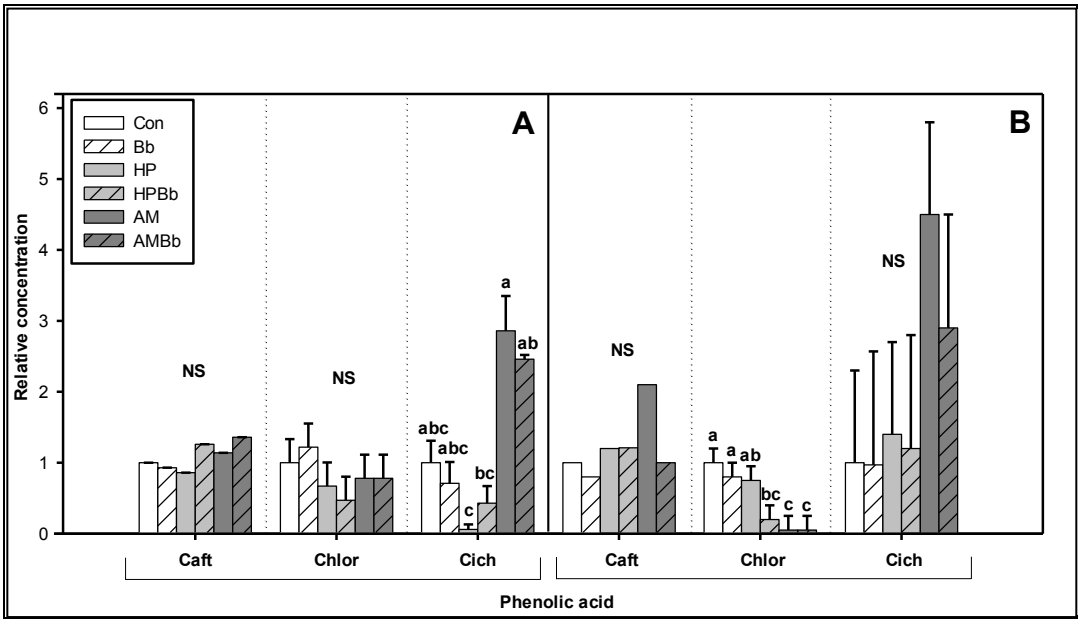


Figure 4.19. Relative concentration of caftaric acid (Caft), chlorogenic acid (Chlor) and cichoric acid (Cich) in roots of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizae (AM), and arbuscular mycorrhizae and *B. bassiana* (AMBb). NS=Not significant. See Table 4.29 (Appendix 1) for data and *P* values.

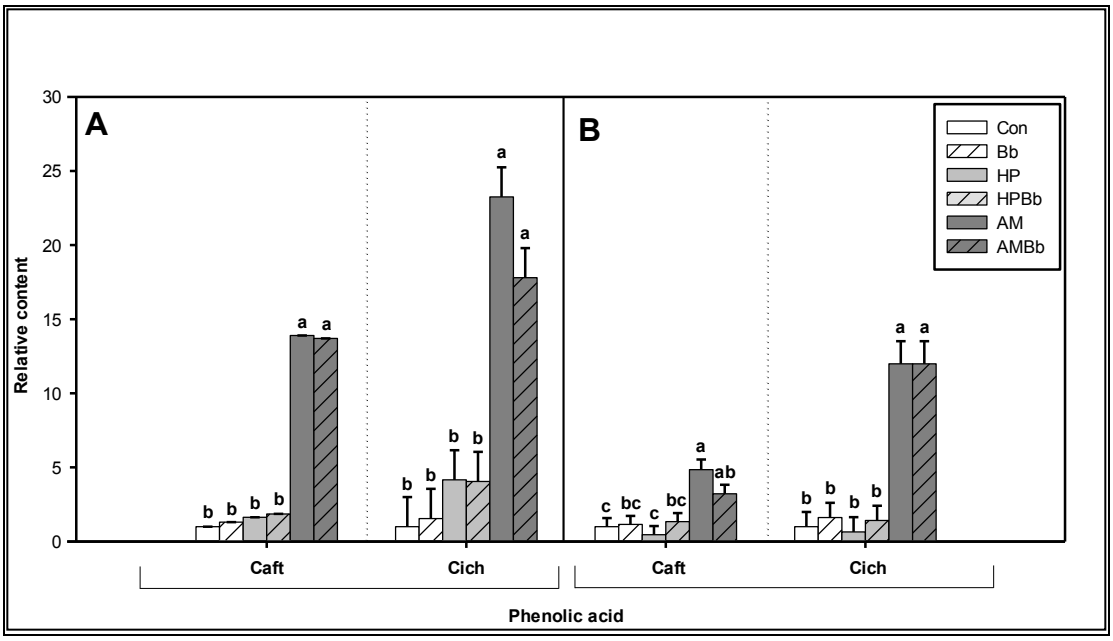


Figure 4.20. Whole plant (root + shoot) relative content (concentration * dry weight) of caftaric acid (Caft), and cichoric acid (Cich) in 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.30 (Appendix 1) for data. *P*<0.0001.

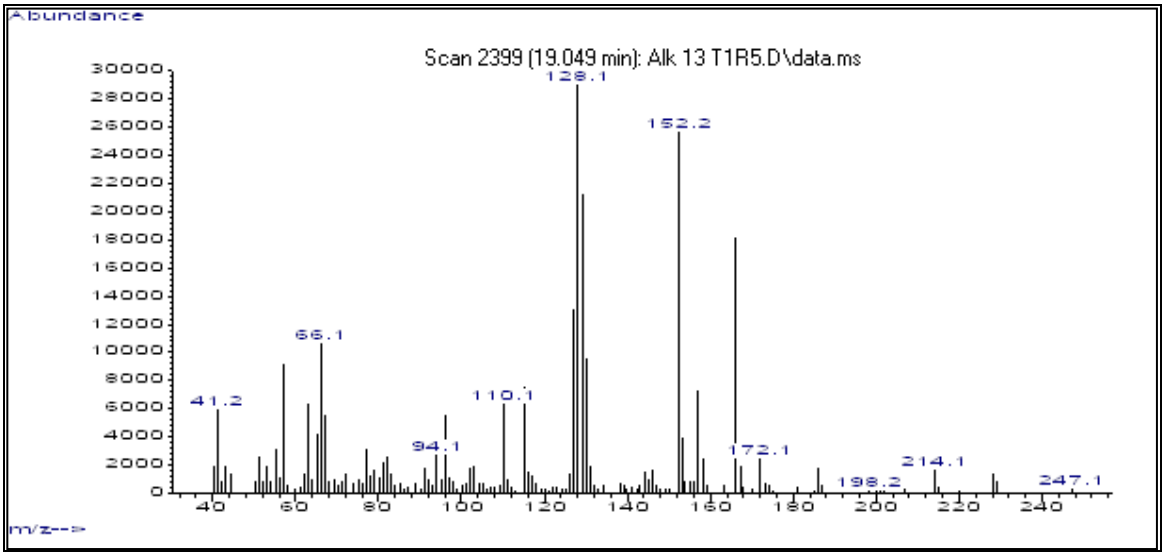


Figure 4.21. Mass spectrum of Alkylamide #2 (Bauer and Remiger, 1988) detected in roots of 12-week-old *Echinacea purpurea* plants.

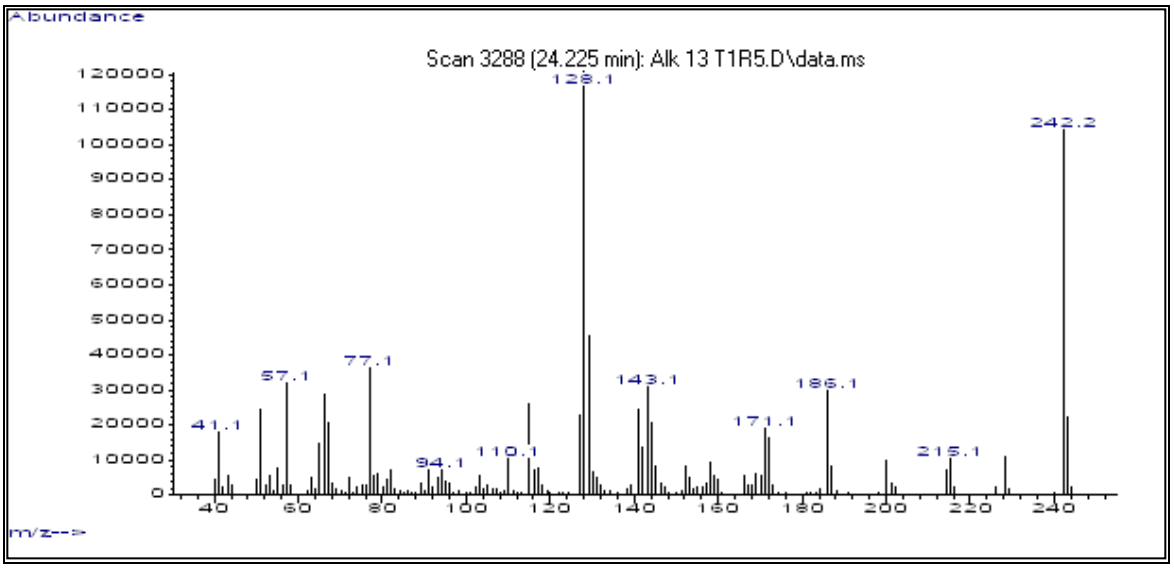


Figure 4.22. Mass spectrum of Alkylamide #3 (Bauer and Remiger, 1988) detected in roots of 12-week-old *Echinacea purpurea* plants.

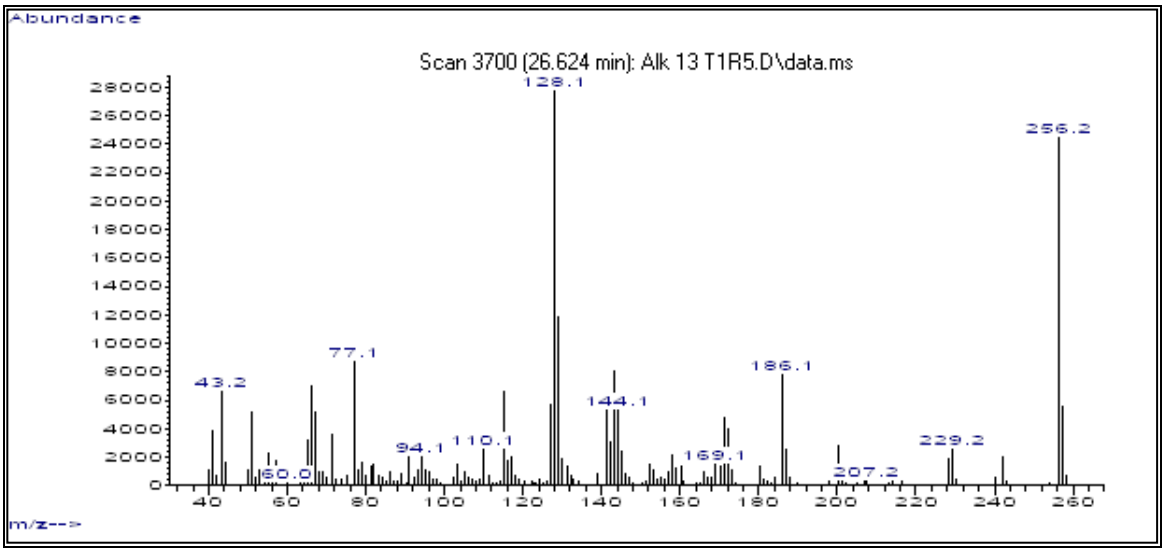


Figure 4.23. Mass spectrum of a mixture of alkylamide isomers # 8/9 (8 + 9) (Bauer and Remiger, 1988) detected in roots of 12-week-old *Echinacea purpurea* plants.

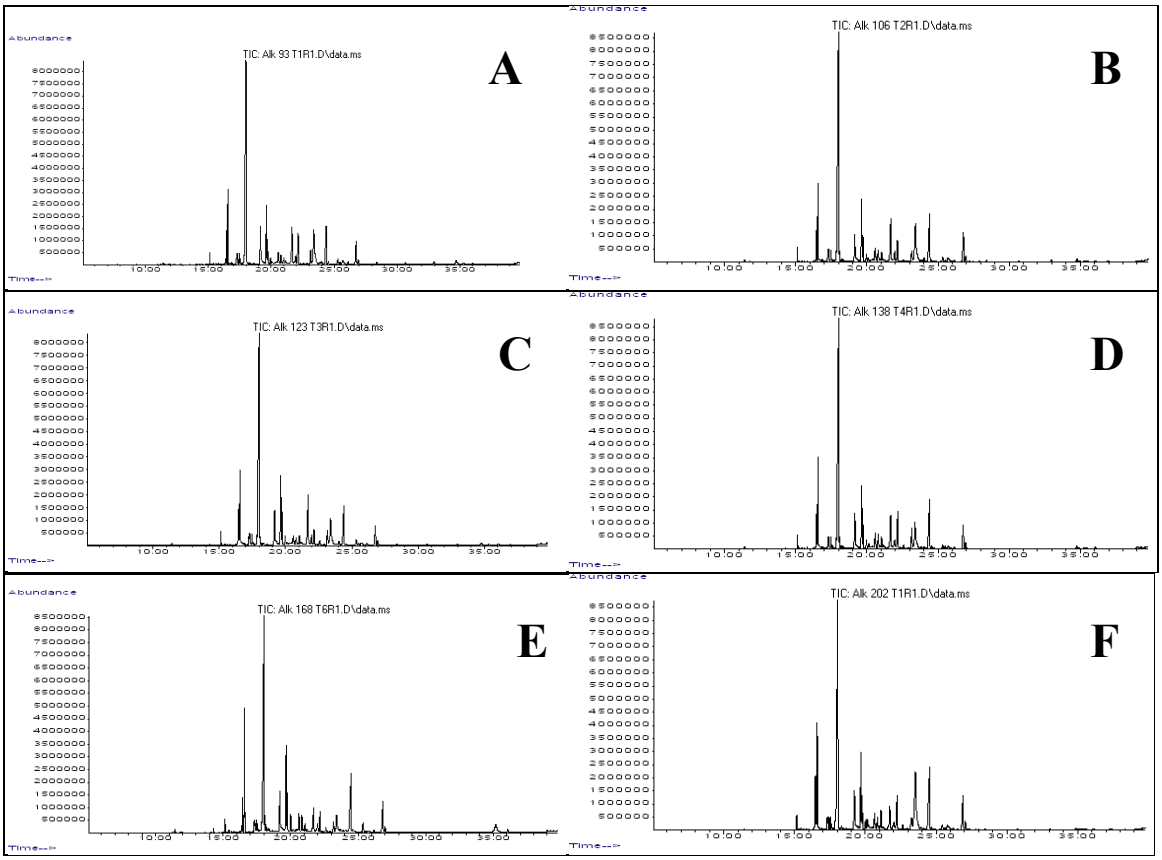


Figure 4.24. Representitive GC chromatograms identifying alkylamides in roots of *E. purpurea* plants from treatments: control (Con)(Panel A), *Beauveria bassiana* (Bb) (Panel B), high phosphorus (HP) (Panel C), high phosphorus and *B. bassiana* (HPBb) (Panel D), arbuscular mycorrhizal fungi (AM) (Panel E), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb) (Panel F). See Table 4.32 (Appendix 1) for GC retention times.

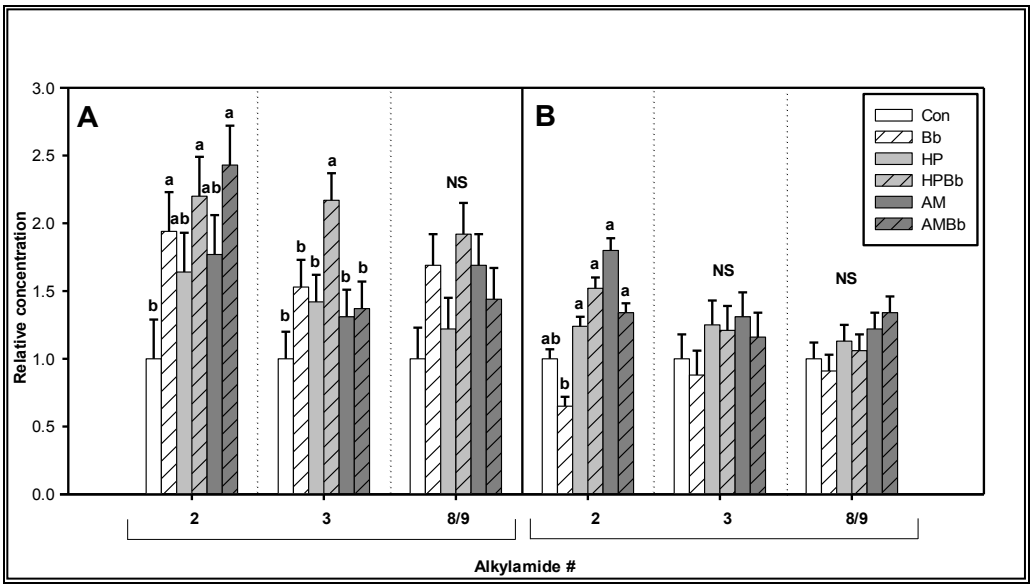


Figure 4.25. Relative concentration of Alkylamides # 2, 3, and 8/9 (Bauer and Remiger, 1989) in roots of 12-week-old *Echinacea purpurea* plants Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). NS=Not significant. See Table 4.18 (Appendix 1) for data and *P* values.

Plants in treatments HPBb and AMBb were not different from plants in HP or AM treatments. In Trial 2, no treatments had concentrations of Alkylamide 2 that were different from controls, but levels in Bb treatments were lower than plants treated with high phosphorus or mycorrhizae (P=0.036).

1.3.5.2. *Content.* In both trials, all plants treated with mycorrhize had 2.9 to 31 times the amount of the individual alkylamides found in the control (P <0.0001) (Figure 4.26). In Trial 1, for all three alkylamides, content in HPBb-treated plants was higher than in control plants, but content in HPBb treatments were not different from in HP or Bb treatments.

1.3.6. ***Sesquiterpenes.* Three sesquiterpenes (β-caryophyllene, α-humulene, and Germacrene-D) were identified in all leaf extracts. Mass spectra are shown in Figures 4.27, 4.28, 4.29.

Representitive chromatograms are shown in Figure 4.30. Concentration was different among treatments in Trial 1 but not in Trial 2.

1.3.6.1. *Concentration in leaves*. In Trial 1, β-carophyllene concentrations were greater than controls for all plants treated with high phosphorus or mycorrhizae (P=0.003), while in Trial 2, only the mycorrhizae-treated plants had elevated levels (P =0.035) (Figure 4.31). Concentrations of α-humulene were also higher in plants treated with high phosphorus (or mycorrhizae than in control (P <.001) in Trial 1, but there were no differences in Trial 2.

Germacrene-D concentrations followed similar patterns; concentration of Germacrene-D was greater than control in all plants treated with high phosphorus or mycorrhizae (P <0.001) in Trial1. There were no differences in Trial 2.

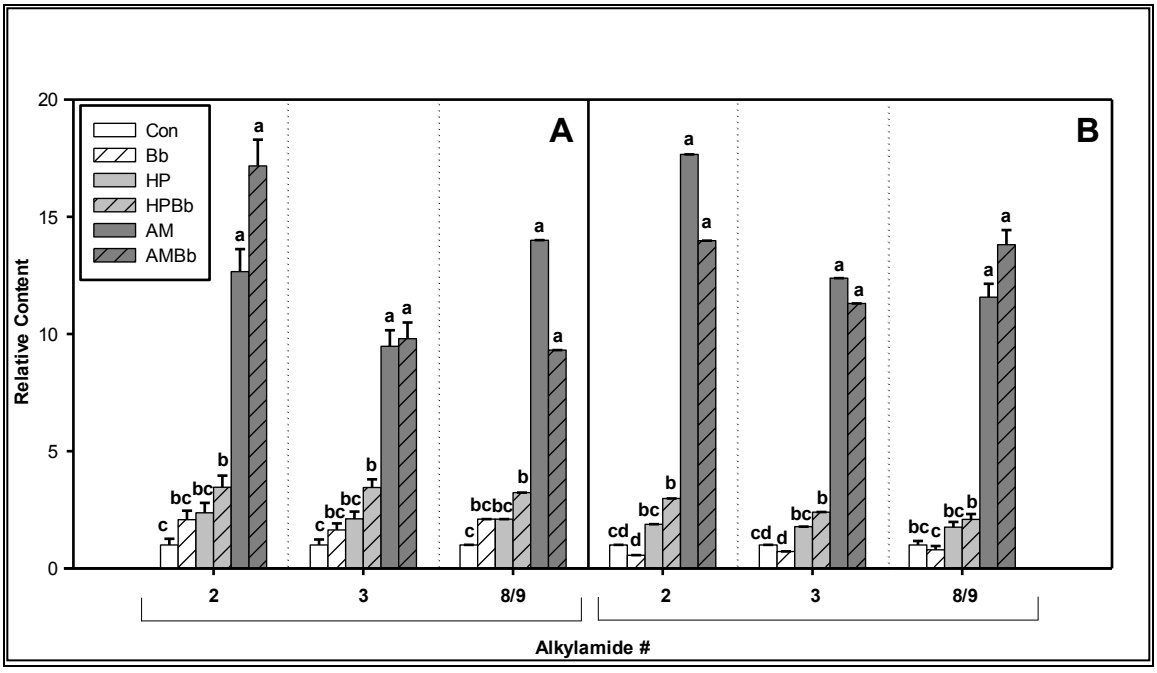


Figure 4.26. Relative content (concentration * dry weight) of alkylamides # 2, 3, and 8/9 (Bauer and Remiger, 1989) in roots of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.19 (Appendix 1) for data. P<0.0001.

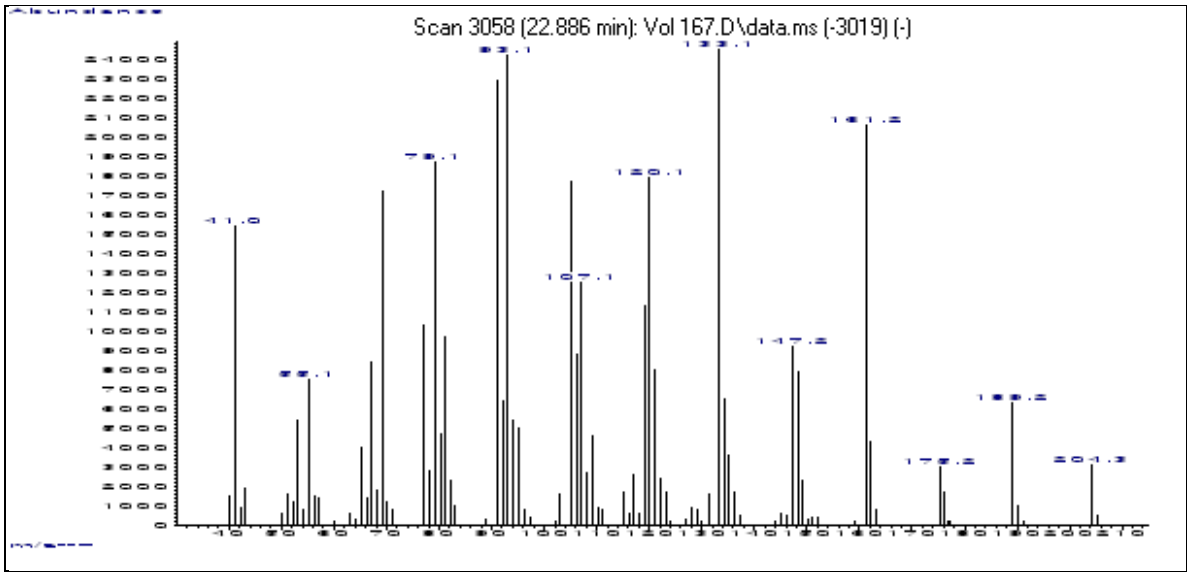


Figure 4.27. Mass spectrum of β-carophyllene detected in leaves of 12-week-old *Echinacea purpurea* plants.

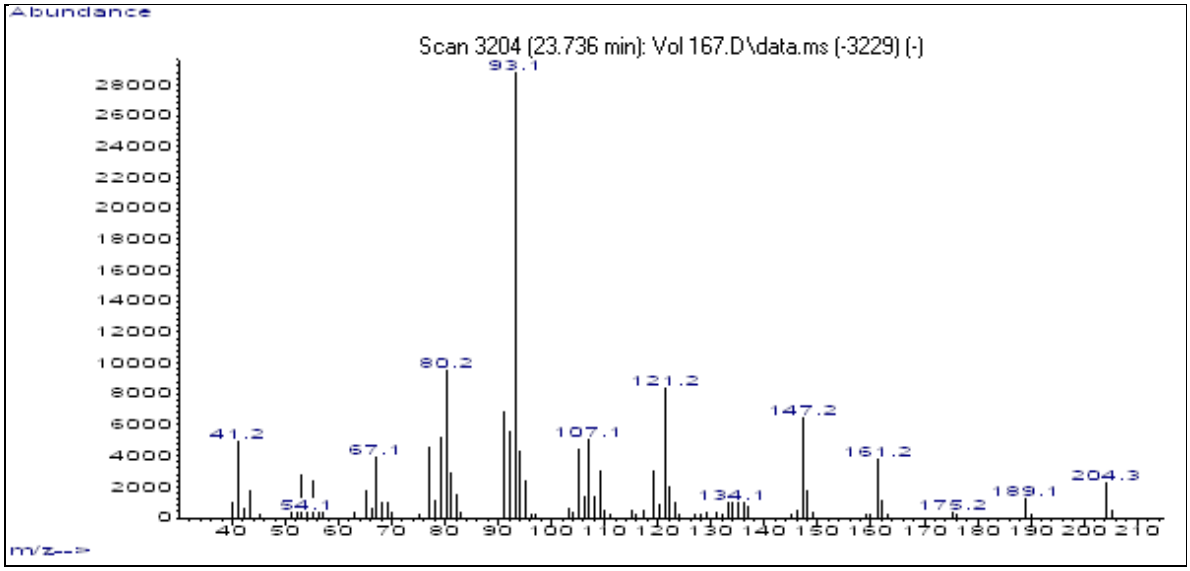


Figure 4.28. Mass spectrum of α-humulene detected in leaves of 12-week-old *Echinacea purpurea* plants.

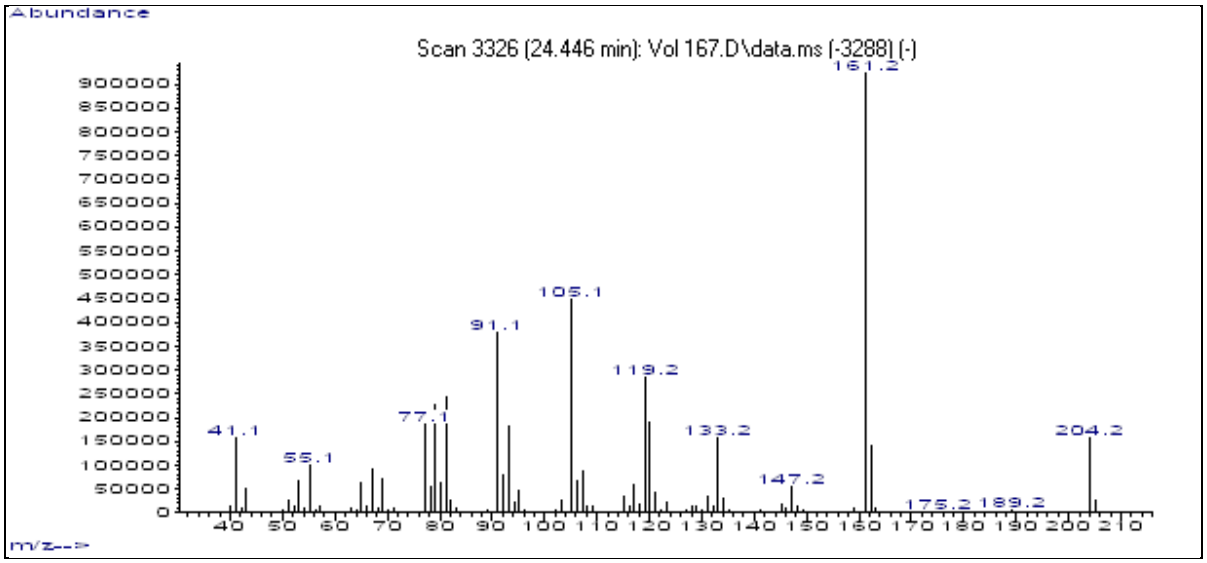


Figure 4.29. Mass spectrum of germacrene-D detected in leaves of 12-week-old *Echinacea purpurea* plants.

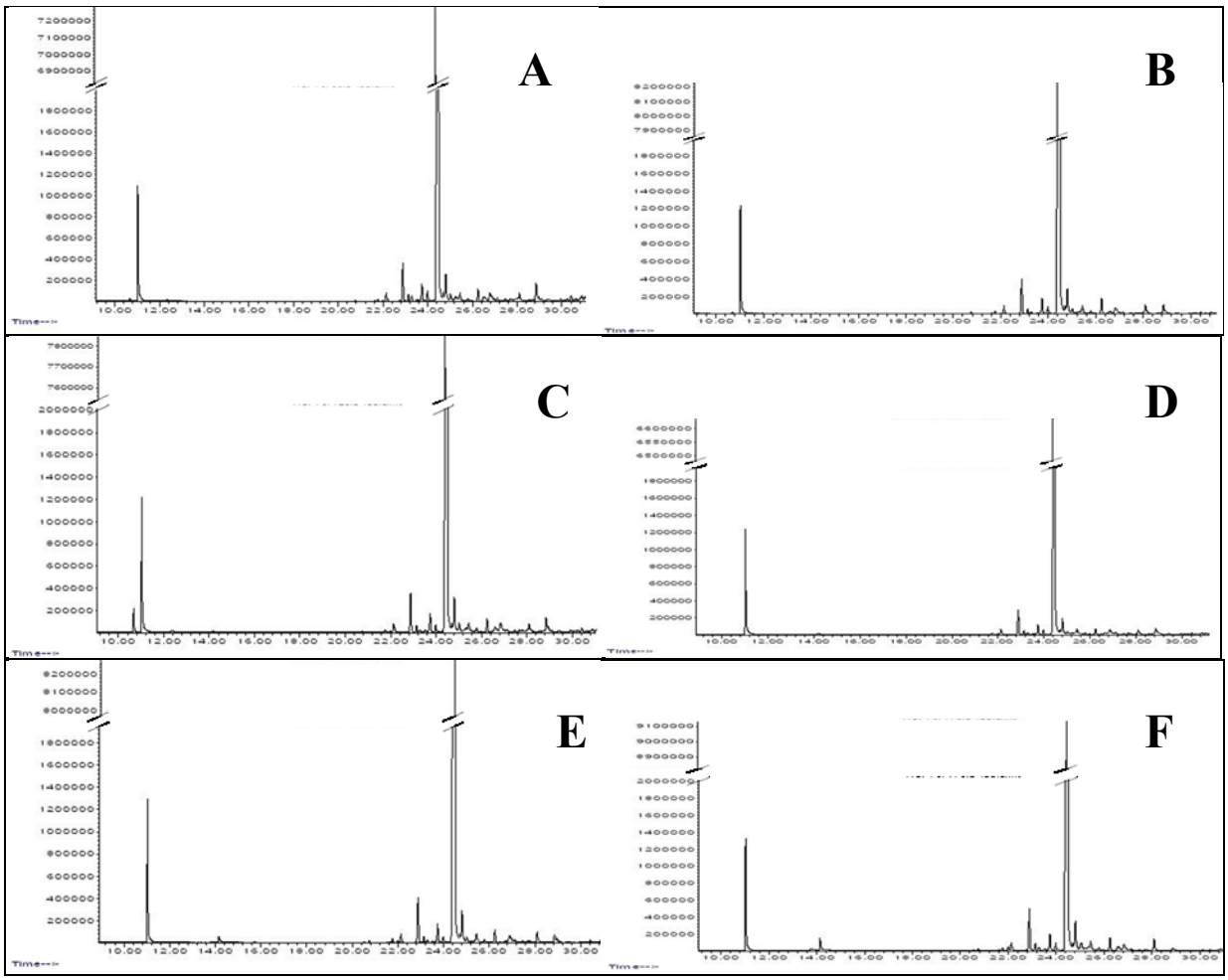


Figure 4.30. Representitive GC chromatograms identifying sesquiterpenes in leaves of *E. purpurea* plants from treatments: control (Con)(Panel A), *Beauveria bassiana* (Bb) (Panel B), high phosphorus (HP) (Panel C), high phosphorus and *B. bassiana* (HPBb) (Panel D), arbuscular mycorrhizal fungi (AM) (Panel E), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb) (Panel F). See Table 4.32 (Appendix 1) for GC retention times.

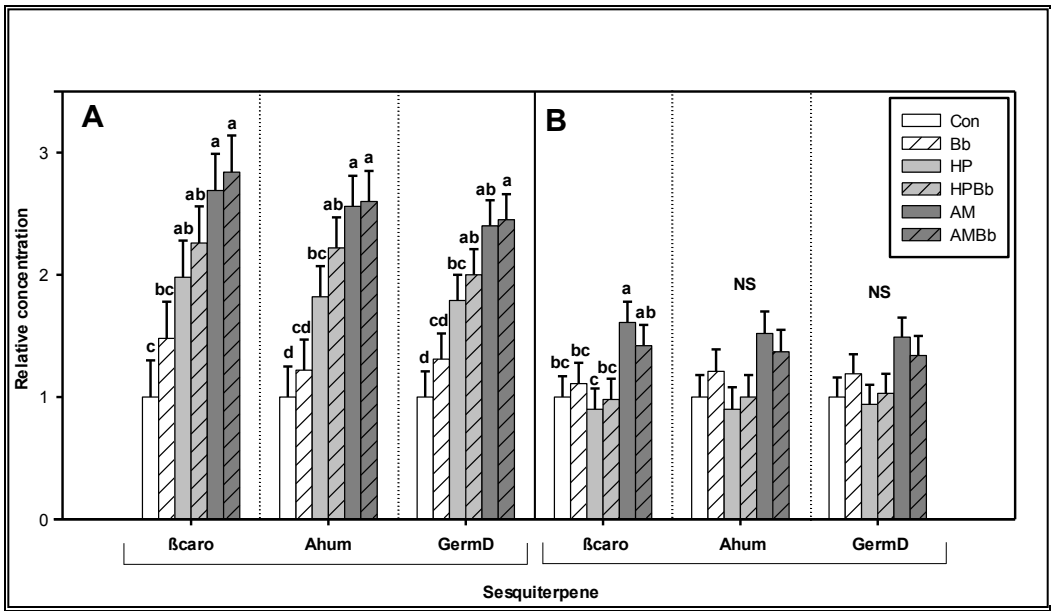


Figure 4.31. Relative concentration of beta-carophyllene (βcaro), alpha-humulene (Ahum), and germacrene-D (GermD) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). NS=Not significant. See Table 4.20 (Appendix 1) for data and *P* values.

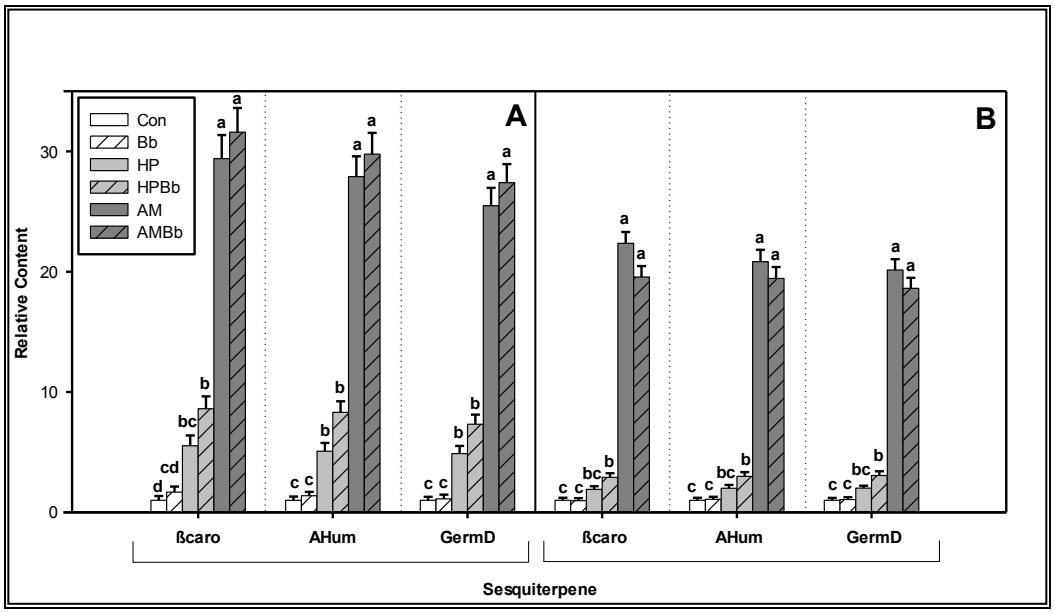


Figure 4.32. Relative content (concentration * dry weight) of beta-carophyllene (βcaro), alpha-humulene (Ahum), and germacrene-D (GermD) in roots of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb). See Table 4.21 (Appendix 1) for data. *P*<0.0001.

1.3.6.2. ***Leaf content.*** All plants treated with mycorrhize had at least 3.5 times the amount of the individual sesquiterpenes, compared to plants treated with high phosphorus and at least 18.6 times the amount in plants treated with low phosphorus ($P<0.0001$) (Figure 4.33).

2.1. Endophyte Colonization. See Appendix 1, Table 4.23 for data and *P* values.

2.1.1. ***Arbuscular Mycorrhizae*** **(AM).** Colonization by AM fungi at 12 weeks was 67 and 72.6% for Trials 1 and 2, respectively (Figure 4.33). Numbers of hyphae, vesicles, and arbuscules was slightly greater in Trial 2 than in Trial1 (Figures 4.34).

2.2. Growth. Statistical methods for plant selection resulted in treatments that were not different in plant size.

2.2.1. ***Dry Weight.*** In both trials, mycorrhizae treatment did not affect dry weight of the plant; there was no difference in dry weight of plants in AM and NM treatments (Figures 4.35 and 4.36).

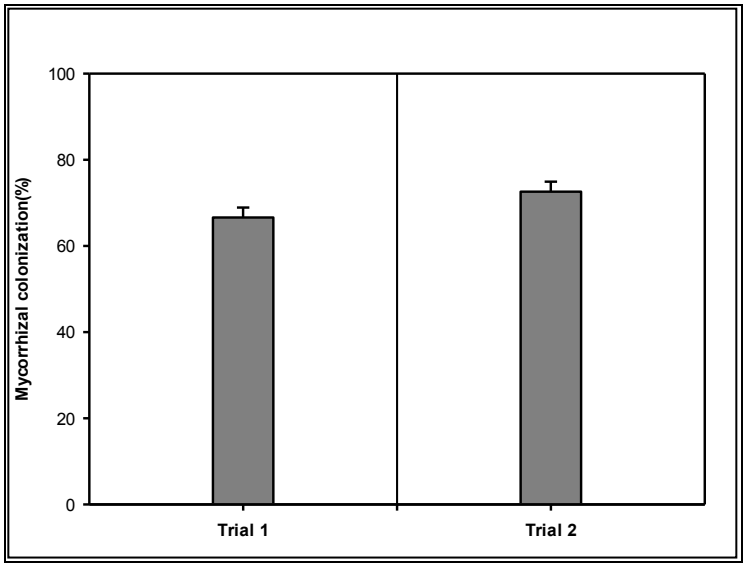


Figure 4.33. Study 2. Colonization of arbuscular mycorrhizal fungi in roots of 12-week-old *Echinacea purpurea* roots. See Table 4.22 (Appendix 1) for data.

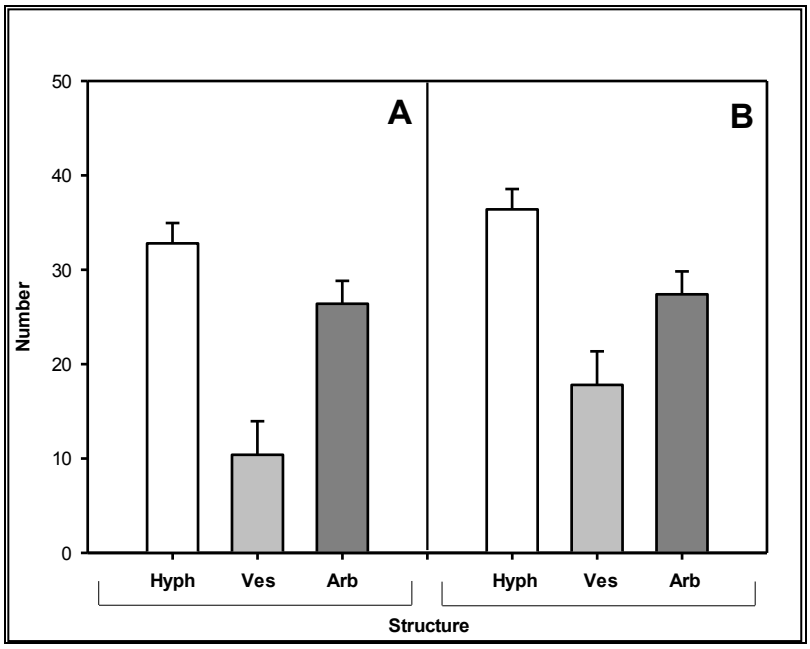


Figure 4.34. Study 2. Number of hyphae (Hyph), vesicles (Ves), and arbuscules (Arb) in roots of 12-week-old *Echinacea purpurea* plants treated with arbuscular mycorrhizal fungi from Trial 1 (Panel A) and Trial 2 (Panel B). See Table 4.22 (Appendix 1) for data.

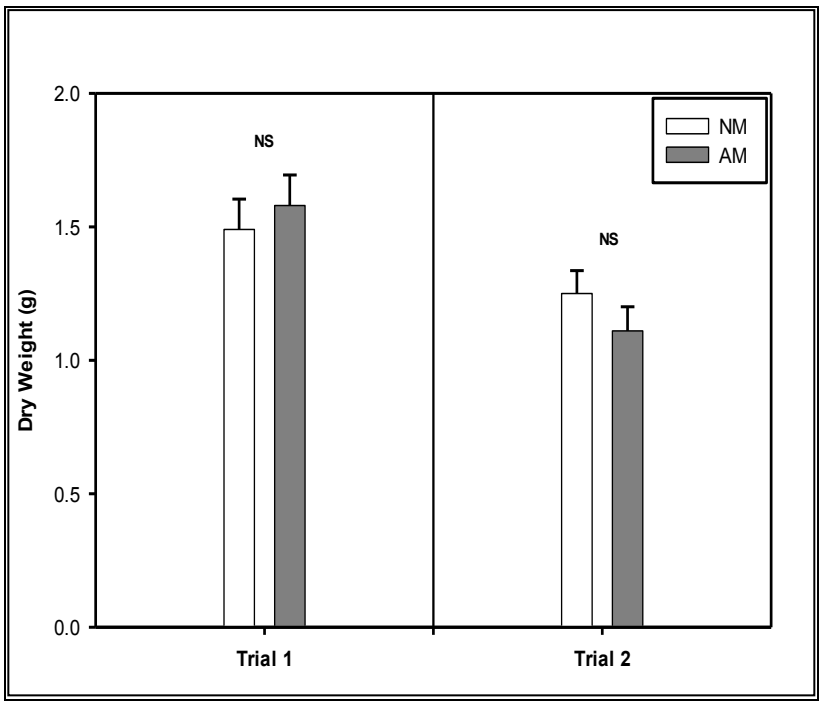


Figure 4.35. Study 2. Whole plant dry weight of 12 week old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B) representing the whole treatment group (n=10). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). Not significant (NS). See table 4.23 (appendix 1) for data and *P*=values.

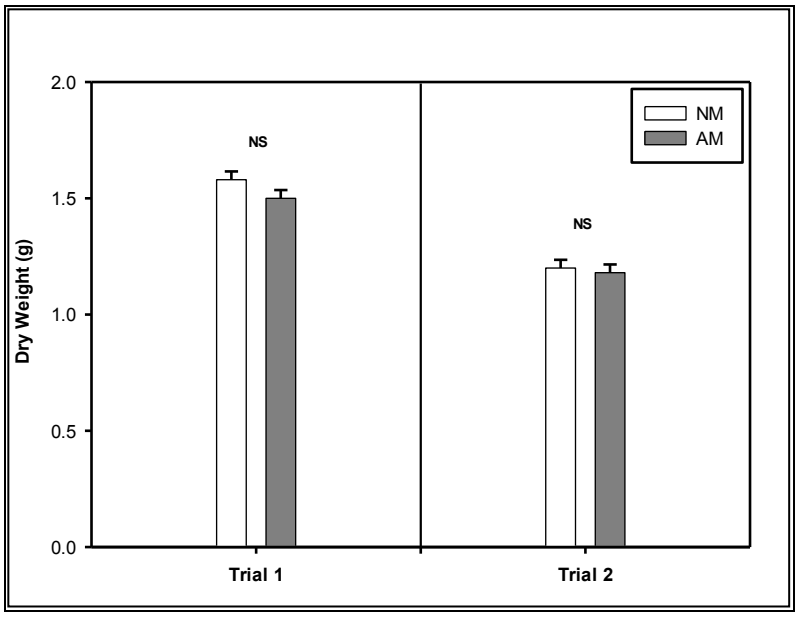


Figure 4.36. Study 2. Whole plant dry weight of a subset of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B) used for phytochemical analysis (n=5). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant . See Table 4.23 (Appendix 1) for data and *P* values.

2.2.2. *Fertilizer applications.* In Trial 1, more than twice the amount of 15-0-15 and 7.5 times the amount of potassium phosphate were applied to NM plants (Table 3.3, Appendix 1). In Trial 2, twice the amount of 15-0-15 was applied and potassium phosphate applications were as described in Trial 1.

2.3. Phytochemistry. See Appendix 1, Tables 4.24, 4.25, 4.26, 4.27, 4.28, 4.29, 4.30 for data and *P* values. There were no differences between AM and NM treatments for most phytochemicals [chlorophylls (Figures 4.37, 4.38), carotenoids (Figure 4.39), alkylamides (Figure 4.40), and sesquiterpenes (Figure 4.41)]. There were treatment differences in the concentration of two phytochemicals classes (xanthophylls and phenolic acids).

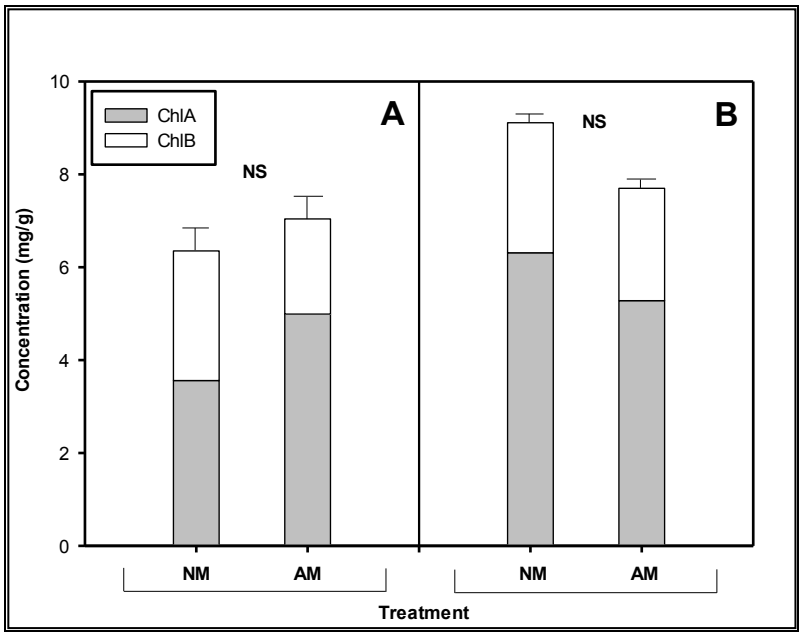


Figure 4.37. Study 2. Total chlorophyll [chlorophyll A + chlorophyll B] concentration in leaves of 12-week-old *Echinacea purpurea* from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant. See Table 4.24 (Appendix 1) for data and *P* values.

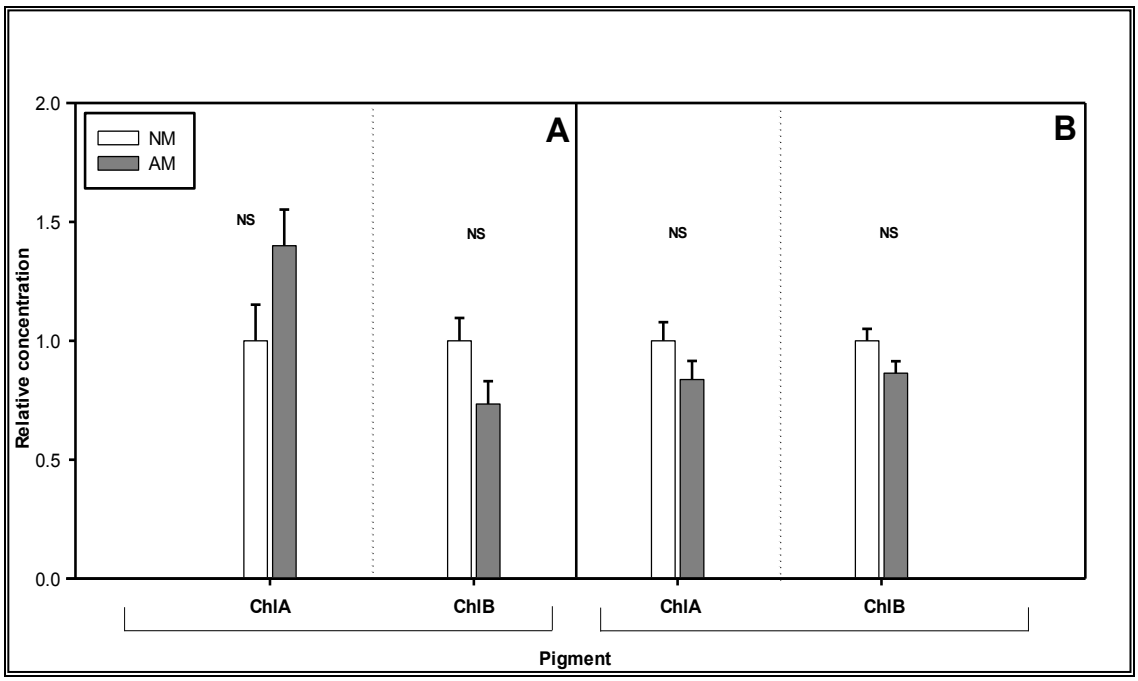


Figure 4.38. Study 2. Relative concentration of chlorophyll A and chlorophyll B in leaves of 12-week-old *Echinacea purpurea* from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant . See Table 4.24 (Appendix 1) for data and *P* values.

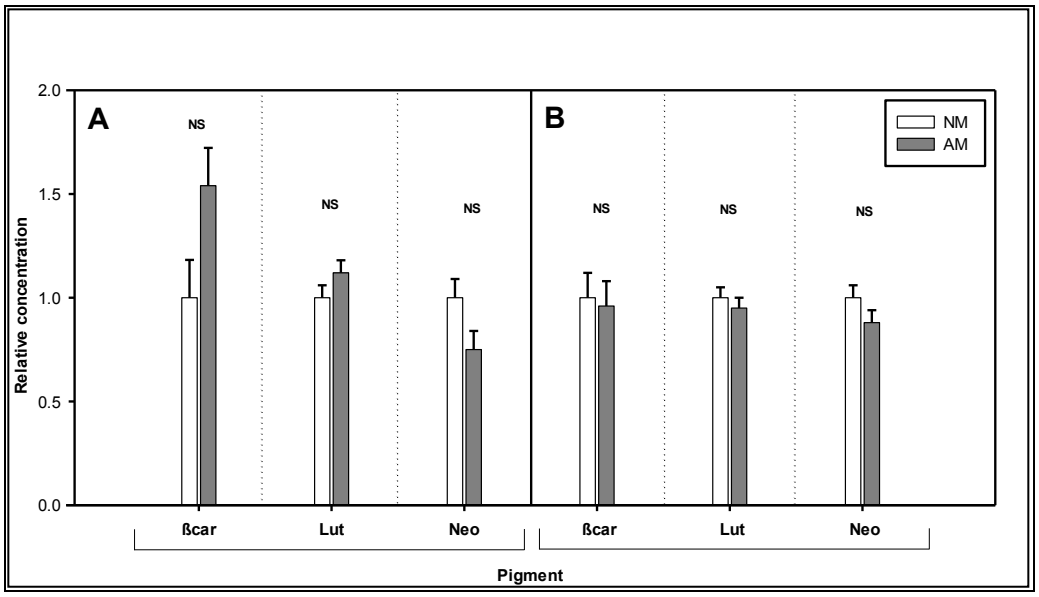


Figure 4.39. Study 2. Relative concentration of beta-carotene (βcar), lutien (Lut), and neoxanthin (Neo) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant. See Table 4.25 (Appendix 1) for data and *P* values.

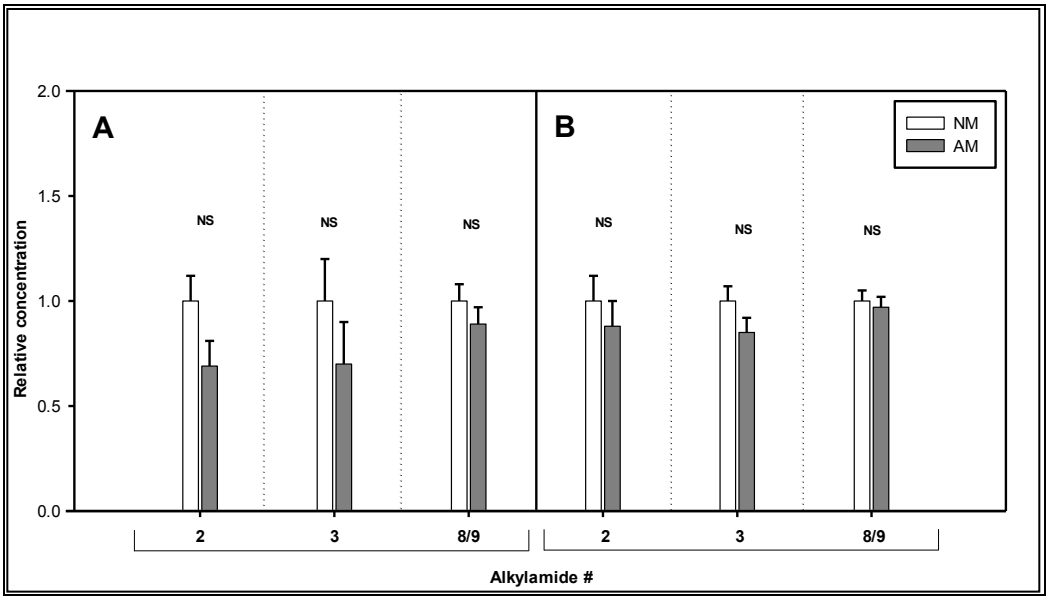


Figure 4.40. Study 2. Relative concentration of Alkylamides # 2, 3, and 8/9 (Bauer and Remiger, 1989) in roots of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant. See Table 4.26 (Appendix 1) for data and *P* values.

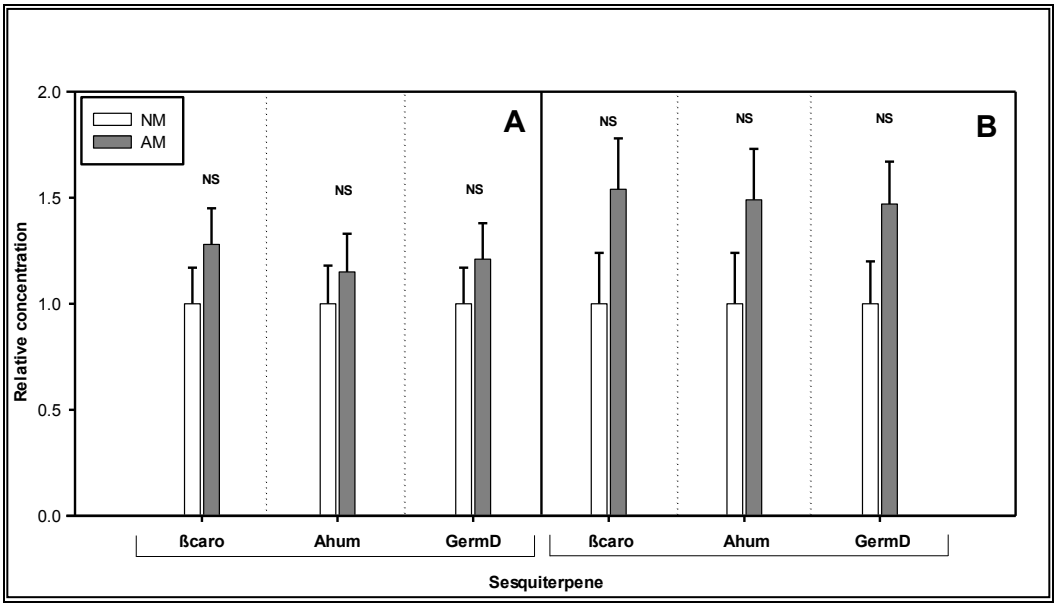


Figure 4.41. Study 2. Relative concentration of β-caryophyllene (βcaro), α-humulene (Ahum), and germacrene-D (GermD) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant . See table 4.30 (Appendix 1) for data and *P* values.

2.3.1. ***Xanthophylls.***

2.3.1.1. *Leaf concentration*. Total xanthophyll concentrations (zeaxanthin + antheraxanthin + violaxanthin) was increased in AM fungi-treated plants (AM) in Trial1 (*P*=0.0009) but was not different in Trial 2 (Figure 4.42). This was driven by a significant increase in violaxanthin concentration which was nearly three times higher in mycorrhizal plants (AM) in Trial 1 (*P*=0.0002), however there were no differences in Trial 2 (Figure 4.43).

2.3.2. ***Phenolic acids.***

2.3.2.1. *Leaf concentration.* In Trial1, concentrations of caftaric acid (*P*=0.025) and cichoric acid (*P*=0.051) were greater in AM plants than in NM plants; there were no differences in Trial 2 (Figure 4.44).

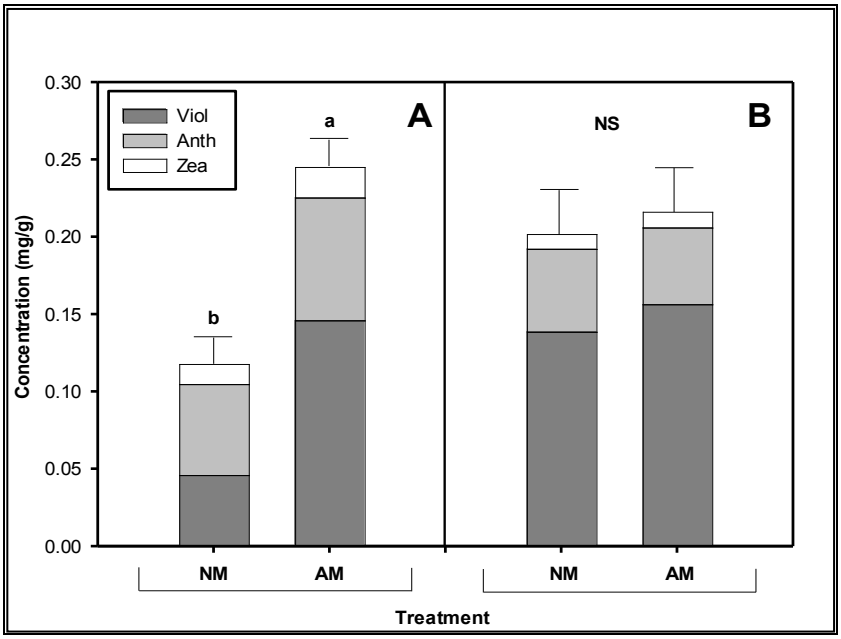


Figure 4.42. Study 2. Total xanthophyll concentration [violaxanthin (Viol) + antheraxanthin (Anth) + zeaxanthin (Zea)] in leaves of 12-week-old *Echinacea purpurea* from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant. See Table 4.27 (Appendix 1) for data and *P* values.

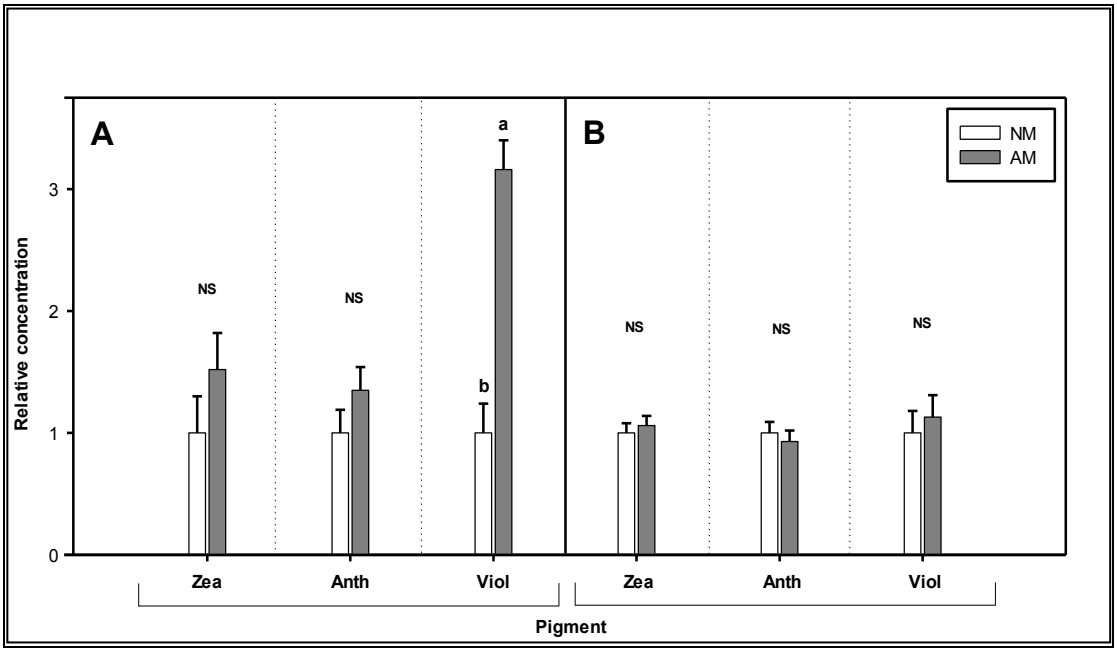


Figure 4.43. Study 2. Relative concentration of zeaxanthin (Zea), antheraxanthin (Anth), and violaxanthin (Zea) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). Not significant (NS). See Table 4.27 (Appendix 1) for data and *P* values.

2.3.2.2. *Root concentration.* In both trials, caftaric acid concentration in roots increased in plants treated with AM fungi (P=0.034, P=0.008) (Figure 4.45).

Chlorogenic acid levels significantly decreased in Trial 1; but there were no differences in Trial 2.

3. <u>Other experiments.</u> See Tables 4.33, 4.34 for data and P values, Appendix 1.

3.1. *Echinacea purpurea* seedling heat tolerance. The recorded temperature spike, in ambient air temperature in the greenhouse topped 60°C (140°F) (Figure 4.46) and temperatures above 37.8°C (100°F) were sustained for over 6 hours (Table 4.33). Temperatures under the clear plastic were likely higher, but could not be measured.

Seedlings treated with *B. bassiana* (Bb) had higher survival rates than controls (Con) and plants also inoculated with both endophytes (AMBb), however these differences were not significant (Figure 4.47). Both mycorrhizal treatements (AM and AMBb) showed highly significant (P<0.0001) reductions in survival compared to nonmycorrhizal seedlings (Con and Bb), with reductions as high as 52.8%.

3.2. *Beauveria bassiana* colonization at 15 weeks. *E. purpurea* were still colonized by *B. bassiana* at 15 weeks of age (Figure 4.48). Percentage of plant colonization at 15 weeks was similar to colonization at 2 weeks (Study 1). At 15 weeks, 62% of plants treated with only *B. bassiana* were infected. Arbuscular mycorrhizae did significantly reduce colonization again by 42% confirming the significant effects observed in Study 1.

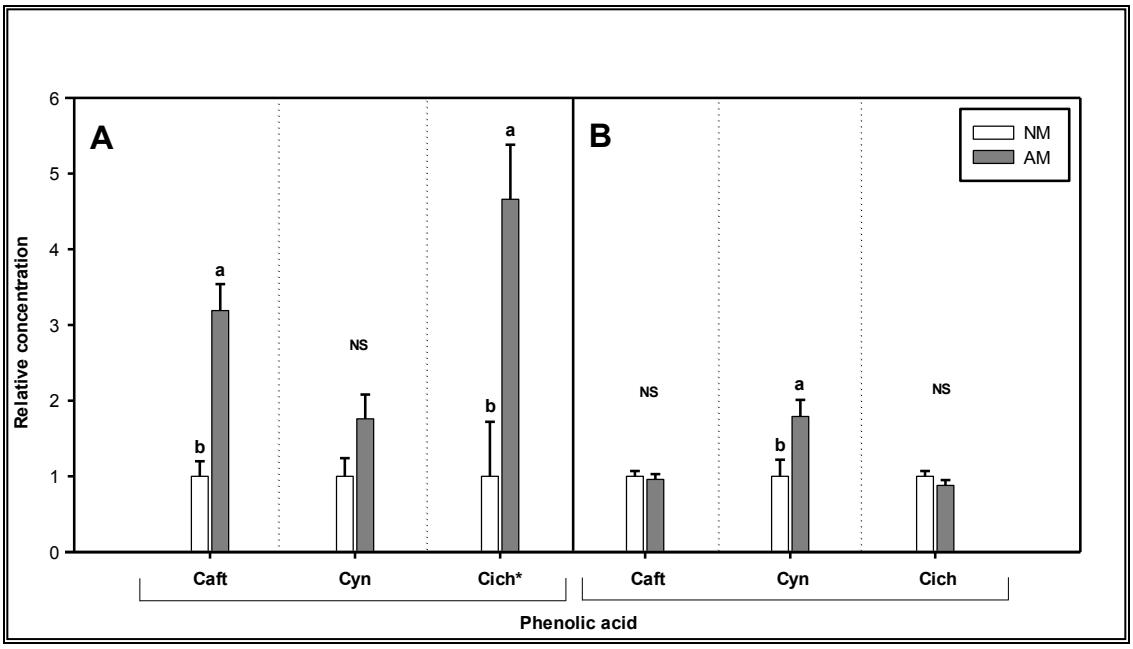


Figure 4.44. Study 2. Relative concentration of caftaric acid (Caft), cynarin (Cyn), and cichoric acid (Chic) in leaves of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). NS=Not significant; *=P<0.1. See Table 4.28 (Appendix 1) for data and *P* values.

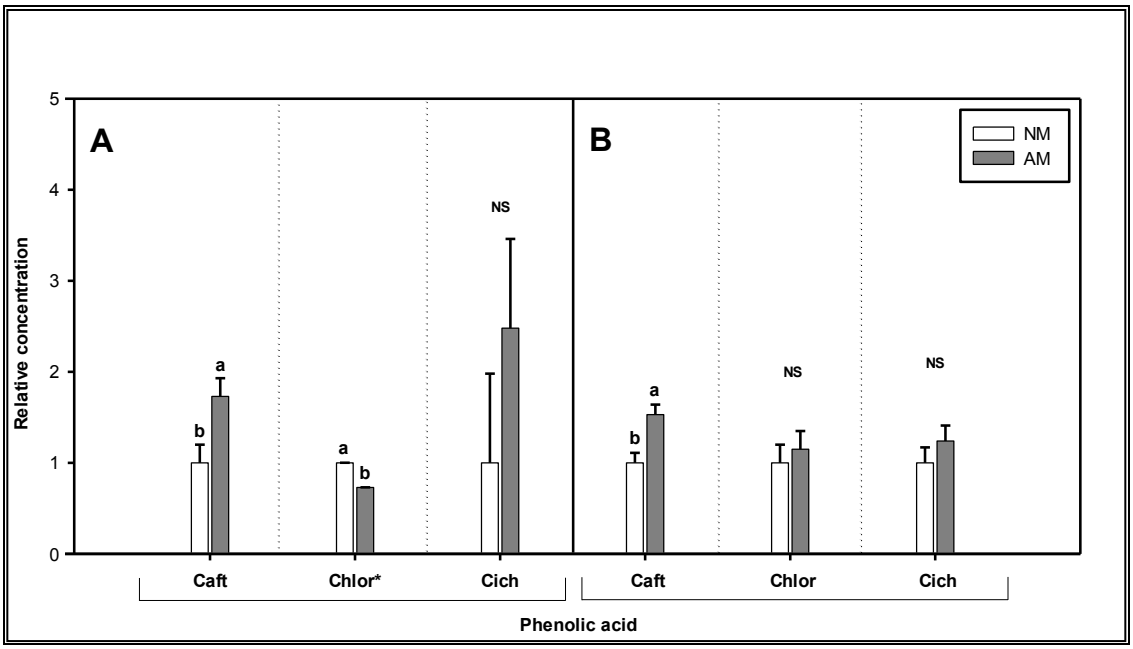


Figure 4.45. Study 2. Relative concentration of caftaric acid (Caft), chlorogenic acid (Chlor), and cichoric acid (Cich) in roots of 12-week-old *Echinacea purpurea* plants from Trial 1 (Panel A) and Trial 2 (Panel B). Treatments are arbuscular mycorrhizal fungi (AM) and no arbuscular mycorrhizal fungi (NM). Not significant (NS); (*)={P<0.1}. See Table 4.29 (Appendix 1) for data and *P* values.

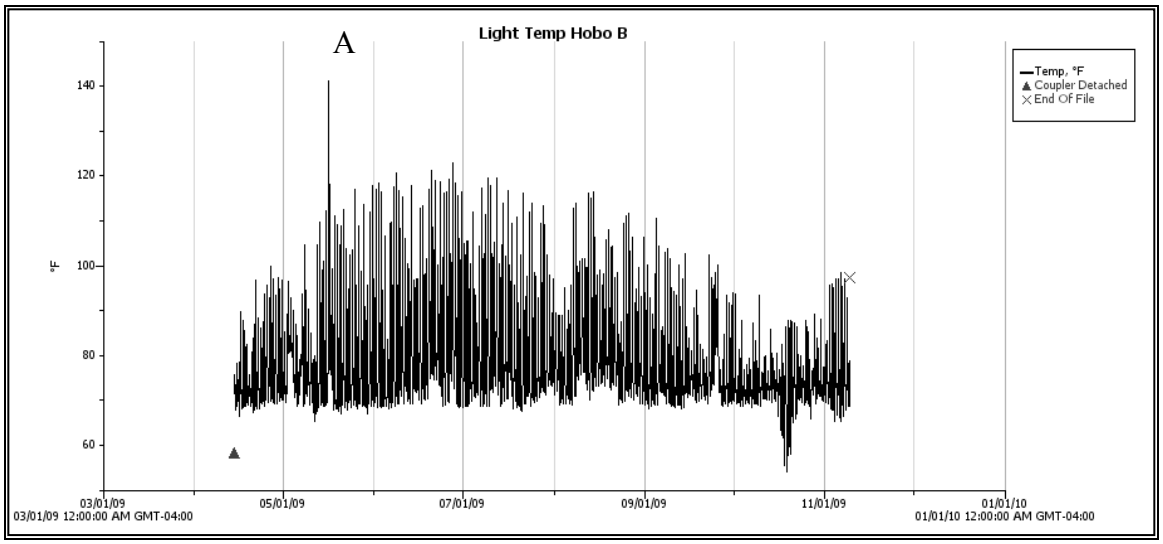


Figure 4.46. Graph showing unexpected greenhouse temperature spike (A) on May 16, 2009. Data documented by a Hobo brand environmental sensor sitting on a central greenhouse bench.

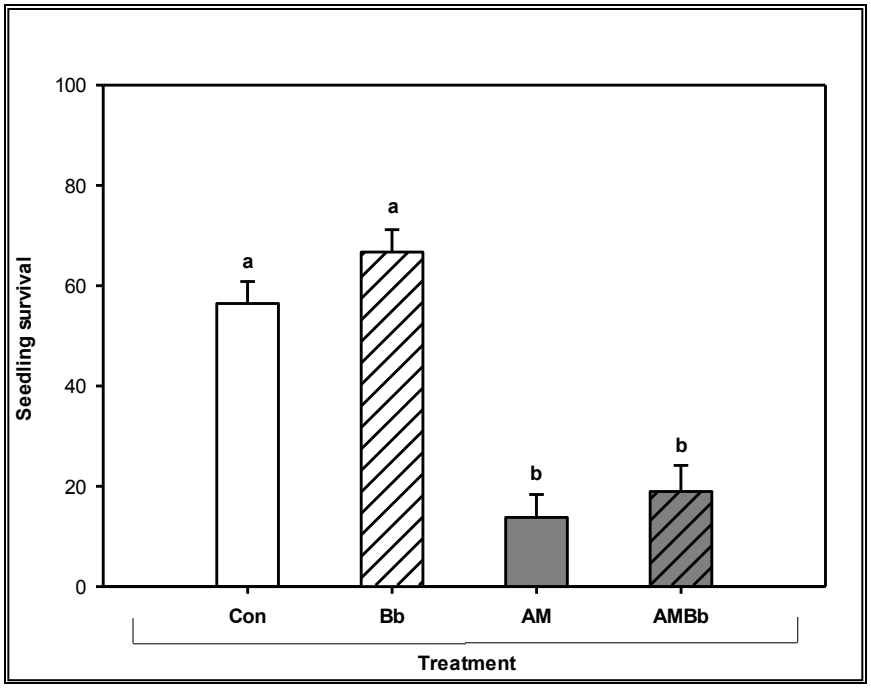


Figure 4.47. Survival of 2-week-old *Echinacea purpurea* seedlings exposed to an unexpected heat event on May 16, 2009. Data reported as percentage of seedlings survived. Treatments are control (Con), *Beauveria bassiana* (Bb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *Beauveria bassiana* (AMBb). See Appendix 1, Table 4.33 data and *P* values.

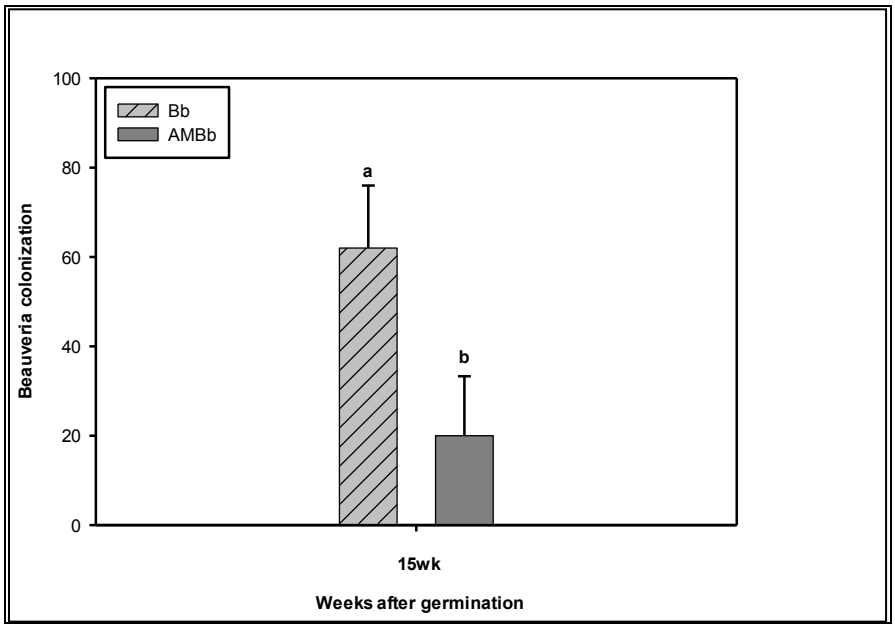


Figure 4.48. Colonization of *Beauveria bassiana* in 15-week-old *Echinacea purpurea* seedlings treated with *B. bassiana* (Bb) and plants treated with arbuscular mycorrhizal fungi and *Beauveria bassiana* (AMBb). See Table 4.34 (Appendix 1) for data and *P* values.

CH. V

Discussion

Due to its popularity as a medicinal plant, alternative production schemes of *E. purpurea* are in demand. Although the greenhouse production scheme used in this research differed from commercial production schemes, it allowed the production of plants consistent with those in commercial production (Jeanine Davis, personal communication). In these studies, greenhouse production continued year round, and environmental variables were continuously monitored. Also, because of the focus on mycorrhizae, plant nutrition, and root chemistry, typical greenhouse growing media were not used. Turface® Brand Proleague was used because it is an effective mycorrhizal culture medium, lacks organic matter, and allows for easy harvest and cleaning of roots. Fertigation schemes were varied to compare physiologically similar samples and to quantify differences in nutrient uptake efficiency in mycorrhizal and nonmycorrhizal *E. purpurea* plants. To minimize genetic variability, the experimental design incorporated high numbers of treatment replication and composite sampling.

The scheme generally resulted in high quality *E. purpurea* plants, but there was one notable exception. While still under plastic (e.g., less than 2 weeks from seedling emergence) seedlings intended for Trial 2 of Study 1 were exposed to high temperatures during a mechanical failure of greenhouse cooling mechanisms. Plants in Trial 1 (2.5 weeks old at the time) showed no physical damage so that trial was allowed to continue. All other experiments produced healthy, vigorous plants.

Environmental conditions changed between trials in both studies; there were greater differences between trials in Study 1 because the interval was greater (ca. 3.5 months) than for

Study 2 (ca. 2 weeks); this resulted in increased seasonal differences. Both mean temperature and light levels were markedly higher in Trial 1 (26.26°C; 17773.4 lm/m^2) than Trial 2 (24.67°C; 12032.9 lm/m^2). In Study 2, mean temperatures were 23.91°C and 22.09°C, and mean lumens per square meter were 10972.7 and 10842.5 for Trials 1 and 2, respectively.

Observations of growth, in the first several weeks of Study 1, raised concerns about differences in growth rates among the mycorrhizal and nonmycorrhizal plants. A series of experiments were done to explore several possible reasons for differences among treatments (e.g., allelopathy from *Sorghum* tissues, nutrient deficiency, soluble salt levels, moisture during germination, methyl cellulose seed coating and media properties). No obvious problems were identified, and growth in the mycorrhizal treatments was consistently enhanced compared to those in all other treatments. Sorghum residues incorporated in the media reduced growth slightly compared to controls, but did not recreate the observed differences. This was consistent with known effects of AM fungi colonization in other plants and the one other report on *E. purpurea* and *G. intraradices* (Araim et al., 2009). Since this pattern was independently observed and the effect could not be replicated with other applied factors, it was concluded that the experimental observations in Trial 1 were valid. Trial 2 of Study 1 was commenced and yielded equivalent results solidifying the conclusion that AM fungi were responsible for the effect.

Because plant size can alter many aspects of primary and secondary metabolism, Study 2 was devised to compare the concentration of secondary metabolites in mycorrhizal and nonmycorrhizal plants of the same size and age. To achieve this goal, mineral applications were limited in the mycorrhizal plants not increased in nonmycorrhizal plants; this strategy proved to

be an effective method for generating plants of equal size. Collectively, the full body of data paints a picture of the interactions between fungal endophytes and *E. purpurea*. First, *B. bassiana* effectively colonized *E. purpurea* endophytically, for at least 15 weeks after seed germination. In some trials, colonization by *B. bassiana* was decreased in plants treated with both *B. bassiana* and the mycorrhizal fungi. To my knowledge this is the first ever such report. Colonization of of *E. purpurea* by fungal endophytes elicited significant responses in multiple growth parameters. *B. bassiana* influenced leaf size, crown caliper and the number of growing shoots at 12 weeks after germination and may offer a degree of improved heat tolerance in developing seedlings. The other class of endophytes used in this study, the AM fungi, increased plant growth rates; this difference could not be overcome with enhanced phosphate fertility (Study 1). Even severely nutrient limited (the reduced fertility in AM treatments) mycorrhizal *E. purpurea* plants were able to maintain growth rates equivalent to nonmycorrhizal plants with ample fertilization (Study 2).

Concentration and content of multiple secondary metabolites were altered with endophyte inoculation. Colonization by *B. bassiana* affected concentrations of compounds in multiple metabolite classes. Arbuscular mycorrhizae also affected concentration of compounds with diverse biochemical origins; however, many of the changes proved to be directly related to plant size (Study 2). Increases in the concentration of some xanthophylls pigments and phenolic acids in leaves of mycorrhizal plants; however, this could not be definitively linked to plant size and may be the result of other systemic mechanisms.

1. <u>Endophyte Colonization.</u>

Successful colonization of *E. purpurea* with both classes of endophytes alone and in dual culture was consistently achieved. In most *B. bassiana*-infected 2-week-old seedlings, the endophyte emerged from the crown region; however, in a few samples, the endophyte was cultured from leaf or cotyledon tissues. Also, *B. bassiana* emerged from the crown region of 15-week-old *E. purpurea* plants indicating that it would persist in our 12-week-old experimental plants.

In preliminary trials with *Echinacea* and AM fungi, arbuscules were present and there were positive effects in growth as early as two weeks after germination. The 12-week-old *E. purpurea* plants were highly colonized; large regions of the root cortex were densely packed with hyphae, arbuscules and vesicles.

When both endophytes were used in treatments, colonization by *B. bassiana* was consistently lower than when no AM fungi were introduce; 70% of plants culled from the nonmycorrizal treatments (Bb and HPBb) in Trial 1, and 74% in Trial 2, were colonized with *B. bassiana*. In mycorrhizae-treated plants (AMBb), percentages of *B. bassiana* colonization were lower in both Trial 1 and Trial 2 (50% and 33%, respectively). In 15-week-old *E. purpurea* plants colonization by *B. bassiana* followed similar patterns of colonization, but rates were overall slightly lower than rates observed in Study 1. Colonization rates could not be measured in test plants since the plant tissues needed for the *B. bassiana* test was used for phytochemical analysis. Colonization of 12-week-old plants were assumed to be similar to those of the culled samples; however, based on data obtained in a separate study from seed treated at

the same time as the seed used in Studies 1 and 2, actual colonization rates might have been lower. This is important, as it relates to observed changes in phytochemistry, since effects would likely have been more statistically evident if all plants in the *B. bassiana* treatments were known to be colonized. This is particularly important to consider in the dual endophyte treatments (AMBb). Endophytic relationships are highly dependent on genotypic interactions (host and endophytes), environmental conditions, and ecology of the diverse population of multiple endophytes in the plant (Antunes et al. 2008; Arnold, 2007; Gamboa et al., 2001; Lodge et al., 1996). Several mechanisms could be responsible or contributing to the reduced colonization of *B. bassiana* when AM fungi are also introduced. Seedlings assayed for *B.bassiana* had not received fertilizer applications prior to harvest and assay, so factors related to fertility can be ruled out as being the source of the reduced colonization of *B. bassiana* in dual culture.

One possible mechanism is direct competition for available plant-derived resources. Although AM fungi are solely dependent on the host for carbon, they do have the ability to scavenge resources from the soil, but it is unknown exactly what materials and in what proportions they are allocated to the plant or incorporated into fungal tissues. On the other hand, *B. bassiana*, is limited by the resources available inside the host plant system. The peri-arbuscular interface represents a high carbon sink due to increased metabolic activity in mycorrhizal root cells and highly efficient glucose transport mechanisms (Dehne, 1986, Hause and Fester, 2005). This could lead to a disproportionate appropriation of host resources offering a distinct competitive advantage based solely on uptake efficiency. From this perspective, AM fungi could be considered to have a competitive advantage compared to *B. bassiana.*

The affinity of *E. purpurea* for AM symbiosis and the magnitude of observed benefits in our experiments suggest a degree of dependency for optimal growth in some conditions. This raises the question whether host preference for mycorrhizae could be influencing *B. bassiana* colonization. Considered from a cost-benefit perspective, it seems reasonable that a preferential affinity for mycorrhizae compared to other endophytes could exist. Genes for symbiosis-specific nutrient transporters that are expressed preferentially, in mycorrhizal plants (Harrison, 1999; Smith et al., 2003) are one type of genetic selection mechanism known to exist in arbuscular mycorrhizal symbiosis. Other similar undescribed specific genetic responses could affect other aspects of host metabolism leading to *de facto* preferential host response for mycorrhizae.

Induced systemic resistance (ISR) is likely to be involved in the observed reduction of colonization by *B. bassiana*. Enhanced abiotic and biotic resistance has been observed in mycorrhizal plants and is attributed to a range of molecular and hormonal signaling mechanisms that elicit physical and biochemical changes in host tissues (Bayat et al., 2009; Elsen et al., 2001; Peipp et al, 1997; Toussaint et al., 2007). These reactions are complex and highly variable, but many defense-related compounds (including phenols) are known to be involved (Morandi et al., 1984). Increased concentrations of caftaric and cichoric acid in leaf tissues of mycorrhizal plants in Study 2 support the triggering of an SAR- or ISR-like response since these compounds are biosynthetically-related to the shikimic and phenylpropanoid pathways (Prasad et al., 2006).

In contrast, colonization of 12-week-old plants by AM fungi was mostly unaffected by inoculation with *B. bassiana.* In Trial 1 of Study 1 mycorrhizal colonization was statistically

higher when *B. bassiana* was introduced; however, the difference was slight (2.4%). It is difficult to determine if the reduction in mycorrhizal colonization without *B. bassiana* truly represents an important effect since only a 2.4% change resulted in statistical significance. The small change could be due to chance related to the particular roots chosen to test, the individual intersections observed under the microscope, or the unavoidable subjectivity of the counting method (Mcgonigle et al., 1990).

No attempt was made to quantify the relative abundance of the two arbuscular mycorrhizae species: *Glomus intraradices* and *Gigaspora margarita*; however, the overall colonization and the distribution of mycorrhizal structures were different in the two studies. Number of vesicles observed in Study 2 was substantially decreased from what was observed in Study 1, and the numbers of fertilizer applications for mycorrhizal plants were lower in Study 2. Taken together, there may have been greater colonization in Study 1 by *G. intraradices,* a species that produces vesicles and is known to perform better when minerals are not limited (Johnson, 1993). In Study 2, colonization may have been predominantly *G. margarita* which does not produce vesicles and tends to perform better in nutrient-limited conditions (Johnson, 1993).

Mycorrhizal colonization rates in Study 1 were approximately 20-30 % higher than mycorrhizal plants in Study 2, suggesting a link to nutrition. Typically, nutrient-limited conditions tend to encourage mycorrhizal colonization. Much of the literature focuses on studies with one or a few minerals [particularly phosphorus (Akiyama, 2002)], but some studies have shown lower colonization when other minerals like magnesium are reduced (Gryndler et al., 1991). Our experiment limited applications of a broad spectrum of minerals (Study 2), so

although phosphorus was limited, so were other important minerals that could affect overall host productivity and secondary metabolism possibly reducing mycorrhizal colonization.

Finally, the effect of inoculation methods could be contributing to our results. It is difficult to predict whether the same patterns of response would be observed if a different inoculation method was used. The rate of *B. bassiana* conidia on the seed coating, in our experiments, was at the optimal levels for infection as determined by Ownley et al. (2008a). The impact of other methods on colonization is unknown. A smaller volume of introduced mycorhizal inoculum could affect the speed and rate of seedling colonization potentially yielding different results as well. Despite these uncertainties, this work has demonstrated the ability of arbuscular mycorrhizal colonization to impede colonization of *B. bassiana* under some circumstances.

2. Growth.

Significant changes in growth were observed in response to endophyte inocolulation and increased applications of potassium phosphate. Although *B. bassiana* generally did not have major impacts on growth, changes were observed in some parameters. In one trial in Study 1, leaf size was significantly different in all treatments with *B. bassiana* (Bb, HPBb, AMBb) when compared to corresponding controls (Con, HP, AM). Leaf size was both increased and reduced depending on conditions. Crown caliper and number of shoots were also significantly affected by *B. bassiana.*

Many endophytes can produce or stimulate the production of multiple plant hormones including cytokinins, giberellins, and absisic acid which could affect host growth (Armstrong and Peterson, 2002; Kapulniki et al., 1993; Peipp et al., 1997; Smith and Gianinazzi-Pearson, 1988; Strack et al., 2003; Toussaint et al., 2007; Zhi-lin et al., 2007; Lata et al., 2003). Compared to plants inoculated with only mycorrhizae, significnat changes in caliper and shoot number were associated with *B. bassiana*; this may suggest that a cumulative or competitive effect based on available host resources rather than a systemic hormonal basis.

Arbuscular mycorrhizae had a significant positive effect on all growth parameters. Although increasing potassium phosphate applications by 3.5 times (Con vs. HP) did produce a measureable positive response in growth, growth was far short of what was obsered in mycorrhizal samples. Araim et al. (2009) also observed results similar to ours with *E. purpurea* grown in a "sand/soil (1:1, v/v)" (unspecified) mixture treated with *G. intraradices*. Their experiment used a Long Ashton Nutrient Solution (LANS) (Hewitt and Smith, 1975) with mineral concentrations comparable to our high phosphorus treatment for both non mycorrhizal and mycorrhizal plants. All of their dry weight results, at 13 weeks after seeding, were within 7 percentage points of our high phosphorus and mycorrhizal treatments. This validates our conclusion that growth of *E. purpurea* increased in response to AM fungal colonization. This phenomenon has also observed in other *Echinacea* species (Lata et al., 2003; Personal observations).

The combination of media and synthetic mineral nutrition used in these studies represented a unique environment, and it is doubtful that such dramatic results would occur in all conditions. Turface could have bound particular minerals, early in plant development, or

limited their availability, as it is montmorillinite clay with relatively high CEC values and some inherent fertility (Figge et al., 1995) including nitrogen, phosohorous, potassium, calcium, and magnesium (Bugbee and Elliot, 1998). Potassium levels are high compared to many greenhouse growing media and field soils. Since potassium is high in the fertilizer applications in this study (in the 15-0-15 fertilizer and potassium phosphate applications), it is possible that high levels of potassium cations in the media impeded uptake of other important cations, such as calcium and magnesium. Yield of *E. purpurea* decreased when potassium applications were extremely high in field experiments (Shalaby et al., 1997). Phosphorus quickly binds to clay soils becoming immobile and unavailable until the saturation point is reached. Turface's rapid drying properties also have the potential to limit water and nutrient uptake and availability (Norikane et al., 2002). Despite these potential sources of growth inhibition, many other plant species have been grown in Turface media without these large differences in plant size (Robert Augé: personal communication), again leading to the conclusion that the observed effects are directly related to the impact of AM symbiosis on *E. purpurea* nutrition.

Informal trials with *Echinacea* in potting media did not yield such obvious differences, but the sterility and nutritional properties of the growing media were not known. These large differences will also not likely be seen in field plantings with biologically healthy soils.

In our studies, AM symbiosis had a profound impact on *E. pupurea* nutrition and subsequent growth. Mycorrhizae can greatly increase the surface area of host root systems leading to greater physical access to soil resources. This is partricularly significant for phosphorus, which is largely immobile in the soil, but increasing root system surface area also allows greater access to all soils resources. Undoubtedly this offers benefits to mycorrhizal *E.*

purpurea plants, but other species grown in this culture system have not benefitted to the same degree. For instance, the *Sorghum bicolor* plants which were produced as mycorrhizal pot cultures for our experiments only required a doubling of potassium phosphate concentration in non mycorrhizal plants to match growth in mycorrhizal plants (0.6 mM vs. 1.2 mM). The benefits in growth observed in *E. purpurea* (Study 1 and Study 2) suggest other mechanisms.

One possible explanation is that *E. purpurea* has a very inefficient phosphorus uptake mechanism and is unable to utilize available and/or unavailable phosphorus. The AM symbiosis-specific phosphorus transport channels are highly efficient and if the inherent genetic mechanisms in *E. purpurea* are inefficient or slow to develop, the benefits from mycorrhizal symbiosis could account for the enhanced growth. However, our high phosphorus treatments with three and a half times the concentration of phosphorus did not come close to reproducing the same results as mycorrhizal colonization. This suggests either a high degree of dependency on AM symbiosis for phosphorus uptake in developing *E. purpurea* seedlings, or that the uptake of other minerals is also being affected. Our data makes a strong case for the theory that colonization with AM fungi is affecting the uptake of a complex of minerals.

In Study 2, in mycorrhizal plants, applications of nitrogen, calcium, boron, copper, iron, manganese, molybdenum, and zinc were reduced by 2.2 times, potassium was reduced by 9.7 times (reduction in 15-0-15 + KH2PO4), and phosphorus was reduced by 7.5 times, yet mycorrhizal plants yielded statistically similar dry weights as the nonmycorrhizal controls. It is interesting to note that despite severely limiting broad spectrum nutrition in the mycorrhizal plants, they did not exhibit any obvious deficiency symptoms.

Although these experiments cannot fully elucidate which minerals are being supplied to *E. purpurea* by AM fungi and in what proportions, they do demonstrate that multiple minerals are likely involved. Many minerals can accumulate in higher amounts in mycorrhizal plants (Bethlenfalvay et al. 1998, Gerdemann, 1975; Manjunath and Habte, 1988; Shokri and Maadi, 2009; Tinker and Gilden, 1983). Araim et al. (2009) found increases in phosphorus, copper, and magnesium in *E. purpurea* inoculated with *G. intraradices.* The presence of nitrogen transport proteins at the periarbuscular interface makes it likely that nitrogen is also being supplied which could contribute to observed increases in growth.

Considered from an ecological point of view, even small changes in growth rate and habit, could have larger significance related to competitive fitness, and species distribution. Given the evolutionary roots of the mycorrhizal symbiosis and the huge potential for positive impacts on growth in *Echinacea*, it seems likely that it has important ecological significance in natural habitats.

3. <u>Phytochemistry.</u>

Compounds from multiple metabolite classes can be altered in response to increased potassium phosphate fertilization and inoculation with fungal endophytes; however, these responses can be variable. Many of the compounds considered have physiological significance or bioactive properties which could impact habitat interactions and therapeutic potency of *E. purpurea* products.

3.1. Fertility. Phosphorus and potassium fertilization was responsible for significant changes in concentration of chlorophylls, xanthophylls, carotenoids, phenolic acids, and sesquiterpenes. Interestingly, all significant changes in response to phosphorus fertilization were observed in leaf tissue with no significant changes in roots.

Significant changes in chlorophyll A, chlorophyll B and total chlorophyll were observed in response to increased phosphate fertility. Positive and negative responses in carotenoid and xanthophyll concentrations were observed varying with environmental conditions. Other carotenoids differed in the response to potassium phosphate. Concentrations of multiple phenolic acids were altered in some conditions in both leaves and roots. Alkylamide concentrations did not respond to potassium phosphate, but all three sesquiterpenes tested increased significantly in Study 1.

Effects of treatments on chlorophyll, carotenoid, phenolic acid, terpene, and alkylamide concentrations were variable among the trials; however, there were similar patterns for a few compounds. In each trial from both studies, a unique yet nearly identical pattern of variation was seen in all three sesquiterpenes compounds. This is likely due to the influence of a common sesquiterpene synthesis intermediary molecule known as E,E-Farnesyl diphosphate (*E,E*-FPP) . This farnysl diphosphate (FPP) variant is acted on by several terpene synthase (TPSs) enzymes, controlled by multiple TPS genes, to produce different sesquiterpenes (Figure 5.1). A single TPS enzyme acts to produce B-carophyllene and α-humulene, while germacrene-D is produced by a seperate TPS (Chen et al., 2003; Yaun et al., 2008). This suggests that the consistent patterning is the result of altered activity of multiple enzyme systems or more likely that the abundance of the common intermediary molecule (*E,E*)-FPP is being influenced. This could

have particular relavence as it relates to phosphorus fertility since the molecule has a diphosphate group (Feng Chen, personal communication). Xanthophylls and sesquiterpenes responded to potassium phosphate only in Trial 1, while chlorophylls, carotenoids, and phenolics responded in Trial 2. The inconsistencies suggest that other environmental factors are interacting with phosphorus and potassium fertility to mediate the effect.

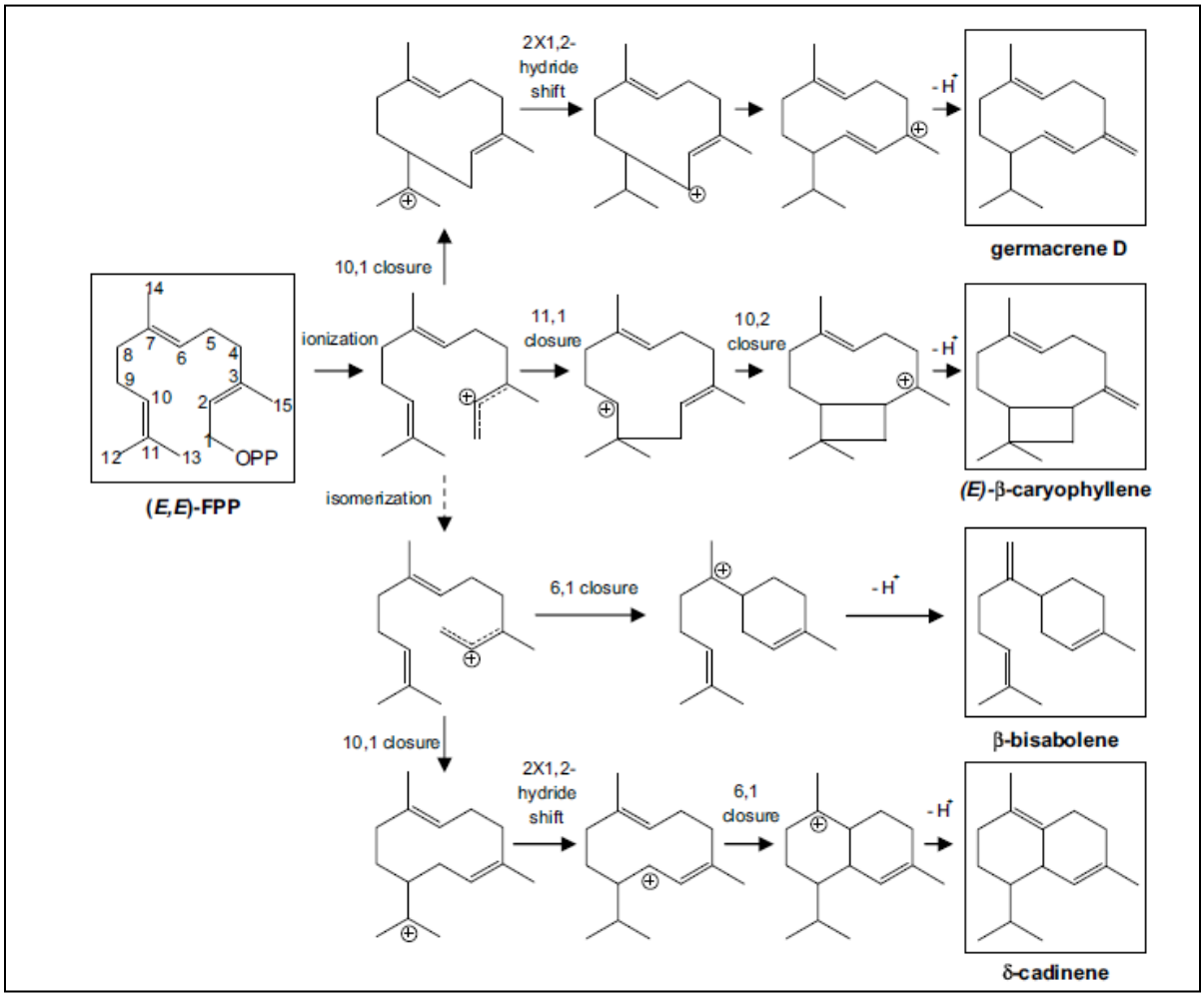


Figure 5.1. Biosynthesis pathways for major sesquiterpenes: germacrene D, β-caryophyllene, β-bisabolene, and δ-cadinene. α-humulene is synthesized through the same pathway as β-caryophyllene. Figure from Chen et al. (2009). Used with permission (Elsevier publishing).

Concentrations of chlorophyll and carotenoids and xanthophylls are influenced by fertilization, light intensity and other environmental factors (Kopsell et al., 2004; Kopsell et al., 2007; Kopsell and Kopsell, 2006; Lesfrud et al., 2006). Light intensity can affect phenolic acid production in *E. purpurea* hairy root cultures (Abbasi et al, 2007), so the differences in light intensity may have contributed to the difference between trials in Study 1. Temperature differences between the trials might have also been a factor but the difference was small (2.4 °C).

Phosphorus and potassium play many important roles in overall plant metabolism [e.g., nucleic acid synthesis, protein synthesis, photo-synthesis, glycolysis, respiration, membrane synthesis, enzyme activity, signaling, carbohydrate metabolism (Evans and Edwards, 2001, Vance et al., 2003, Wadleigh, 1949). With such diverse and significant roles in plant physiology, applications of phosphorus and potassium could have a myriad of downstream direct effects related to secondary metabolism. The vast array of potential effects and the possible influence of environmental factors make speculating on the source of this effect very difficult.

3.2. Endophytes.

Beauveria bassiana altered the concentration of many secondary metabolites tested; although results were variable, some patterns emerged in Study 1.

3.2.1. Beauveria bassiana. Treatment with *B. bassiana* resulted in both increased and decreased levels of some pigments, phenolics, and alkylamides; however variablilty existed among fertility regimes and trials. The observed differences among trials suggest environmental

interactions influenced the outcome of our treatments. The lack of consistent response in the low (Con and Bb) and high phosphorus (HP and HPBb) treatments, demonstrates that phosphorus and potassium fertility can influence the outcome of the *B. bassiana* symbiosis. Generally, *B. bassiana* caused more changes in low phosphorus (Con vs. Bb) treatments, compared to high phosphorus treatments (HP vs. HPBb). This suggests that improved phosphorus and potassium fertility influenced the mutualism. Changes in phytochemistry may be related to resource use and availability in the host tissues, and *B. bassiana* may be depleting or fortifying *in planta* levels of important minerals leading to downstream changes in metabolism. Phosphorus and potassium have important roles in cell metabolism across kingdoms and would be necessary for healthy plant and fungal growth. A competitive effect could alter availability, of these minerals, in host tissues and affect metabolism. *Beauveria bassiana* would be a net consumer of plant resources, however many nonmycorrhizal endophytes have been shown to increase levels of some important nutrients in plant tissues (Rodriguez et al., 2009), so other nutritional effects could be occurring as well.

An induced systemic response also seems a likely source of some of the observed changes in phytochemistry. Many endophytes have been shown to produce or stimulate the production of phytohormones and other signaling molecules which can affect aspects of host gene expression and metabolism leading to enhanced biotic resistance (Smith and Gianinazzi-Pearson, 1988). The changes induced by *B.bassiana* in phenolic acids and alkylamides support this theory since both are known to be induced by jasmonic acid and related to plant defense (Chicca et al., 2007; Dehne, 1982; Strack et al., 2003). However; the lack of change in

sesquiterpene concentrations, some of which are also known to be induced by jasmonates (Boland et al., 1995), does not support this idea.

3.2.2. Arbuscular mycorrhizae (AM). Colonization by AM fungi also produced significant changes in the concentration of multiple metabolites (Study 1), but many of those could be traced back to the impact of plant size likely driven by enhanced nutrient uptake in mycorrhizal *E. purpurea* plants (Study 2). Variability among the trials existed again indicating environmental influence on the outcome of arbuscular mycorrhizae inoculation. In the further discussion, AM treatments, in Study 1 (AM and AMBb), will be considered "optimal fertilization", so changes that are not reproduced in Study 2, are likely attributed to the impact of enhanced nutrient uptake.

Various changes were observed which varied with condition. Alkylamide concentration did tend to increase with mycorrhizal colonization, however no significant changes were observed in any trial of either study.

In Study 1, plants innoculated with AM fungi had consistently higher concentrations of β-caryophyllene, α-humulene, and germacrene-D, although α-humulene and germacrene-D were not significantly different in Trial 2 of Study 1. Despite the lack of statistical significance, in some conditions, the consistency of the positive response in sesquiterpene concentration can offer some insight into the interaction.

When results from Study 1 and 2 are compared, many of the changes in *E. purpurea* phytochemistry, related to mycorrhizal colonization, seem to be related to the effects of enhanced nutrient uptake. Any number of minerals, known to be supplied in some mycorrhizal relationships, could produce changes in phytochemistry. These would include, but may not be

limited to, phosphorus, nitrogen, calcium, potassium, iron, sulfur, manganese, zinc and copper; these can lead to changes in protein synthesis and enzyme activity in a variety of host systems (Clarkson 1985; Evans and Edwards, 2001; Hause and Fester, 2005; Yadav et al., 2005).

Based on the significant changes observed in phenolic acids in both Study 1 and Study 2 and the increasing trend of sesquiterpene concentration, nutritional factors cannot fully explain these changes, and this suggests a systemic response is being stimulated in mycorrhizal *E. purpurea* plants. Changes observed in spatially distinct leaf tissues of mycorrhizal plants indicate a systemic mechanism that is likely related to jasmonic acid mediated signaling. Jasmonate synthesis, in mycorrhizal plants, has been linked to induced systemic resistence (ISR) responses mediated by increased activity of the enzyme phenylalanine ammonium lyase (PAL) (Conrath et al., 2002). This enzyme plays a intermediate role in regulating phenolic acid synthesis (Wen et al, 2008); caftaric and cichoric acid are known to have antimicrobial properties (Dalby-Brown et al., 2005; Samorodov et al., 1996). Other signaling mechanisms such as salycilic acid or hormonal signals could be contributing as well, but less literature is available and potential links have not been well explored.

3.2.3. Both endophytes. In most cases, when both *B. bassiana* and arbuscular mycorrhizae were introduced (AMBb), phytochemical levels were consistent to those in treatment with only arbuscular mycorrhizae with a few notable exceptions. Significant changes in some pigments and phenolics were observed.

Multiple mechanisms could be contributing to the interactive effects when *E. pupurea* plants are inoculated with both endophytes. Many biotic and abiotic factors contribute to the outcome of endophytic relationships and could likely be contributing to the observed effects.

The influence of another endophyte, in a distinct plant tissue, involves yet another biotic variable which complicates speculation about potential causes. Although this work cannot address causal mechanisms, it does demonstrate existence of significant interactive effects of fungal endophytes on phytochemistry in *E. purpurea.*

It is likely that no one particular mechanism would account for any observed effect. The observed responses in phytochemistry could be part of the symbiosis process or a secondary result of it. The complex ecology of endophytic relationships and the vast number of factors involved in their regulation, make it likely that environmental and genotypic variation produces a unique synergy of changes which can vary widely in downstream response.

When the data from Study 1 is considered from a natural products production point of view (the total amount of harvestable natural product produced i.e. concentration × tissue dry weight), the economic benefits endophytes could offer become clearer. Increasing potassium phosphate fertilization produced plants that contained as much as a 5 times the amount of some natural products. In contrast, plants with arbuscular mycorrhizae (AM and AMBb) produced many more times the amount of every compound considered with the same inputs (Con.Vs. AM). These changes were driven primarily by increased plant biomass, however enhanced concentration in tissues contributed. Colonization by *B. bassiana* did result in some changes in content relative to mycorrhizal plants when both were introduced, however none proved to be significant. Values ranged from 5 to 30 times the content depending on the compound. β-carotene content was increased as much as 25 times, and lutein as much as 15 times, when both endophytes were present (AMBb). Both are important dietary carotenoids with important health benefits associated with eye health, vitamin A synthesis, and have antioxidant and potential

anti-cancer effects (Kopsell and Kopsell, 2006). Caftaric and cichoric acid content were increased by as much as almost 15 and 23 times in mycorrhizal plants; both of these have important bioactive and health promoting properties including anti-viral, anti-inflammatory, antioxidant, anti-tumor, and immune stimulatory effects (Dalby-Brown et al., 2005; Samorodov et al., 1996).

All three sesquiterpenes were produced in greater amounts in mycorrhizal plants. β-caryophyllene and α-humulene content were increased by approximately 30 times when both endophytes were present. These compounds have important anti-inflammatory, immune stimulatory and potential anti carcinogenic properties as well as other bioactivities (Oesch and Gertsch, 2009; Rostelien et al., 2000). Interestingly, β-caryophyllene is common across the plant kingdom and has been shown to stimulate the same cannabinoid receptor (CB2) in the human brain that is believed to be targeted by many of the alkylamides in *Echinacea* (Gertsch et al, 2008; Oesch and Gertsch, 2009). Germacrene-D content also increased by comparable amount and is known to have insecticidal and possibly antimicrobial properties (Arimura et al., 2004).

4. Future work.

A lot of valuable data was collected during the course of these experiments; which was generally successful at addressing the original set of research objectives, however some aspects could be improved in future work. The fact that colonization for *B. bassiana* and AM fungi were determined at different times and that only estimated colonization rates could be

determined for *B. bassiana* creates some uncertainties. The use of a dual culture AM fungi innoculum (*Glomus intraradices* and *Gigaspora margarita*) does not allow any conclusions to be drawn concerning the impact of either species and further complicates speculation concerning the mechanisms behind the observed effects. Future work should have separate experiments that address each class of endophyte individually which would help alleviate conflicts with sampling methods and minimize variables.

It is unclear if the observed benefits in *E. purpurea* growth would be realized in other conditions. It seems likely that the special properties of the root zone created in Turface media, or some other aspect of our fertility regime amplified the effects. It could be related to physical properties of the media, our nutrient sources or how mycorrhizae performs in the Turface profile, but it is clear that in this condition, AM symbiosis has a profound positive impact on growth and nutrient use efficiency. Experiments considering media composition, mineral sources, and water applications could be useful to determine the factors that are producing the enhanced growth in mycorrhizal *E. purpurea.*

Assaying larger numbers of samples for a larger group of compounds could help to minimize variability further and help identify broader patterns of response.

Although much more work would be necessary to fully understand the causes of the observed effects, I believe that these experiments were successful at demonstrating the potential impact that fungal endophytes can have in *E. purpurea.*

5. Potential significance.

The potential ecological and economic implications of the observed changes induced by fungal endophytes cannot be overlooked. The potential impacts on growth, stress resistance, and phytochemistry could influence overall fitness of individuals in certain environments, thus influencing range tolerances and speciation. The degree of potential nutritional benefits related to AM symbiosis suggests that mycorrhizal species distribution may be closely related to *Echinacea* species distribution. With the vast number of endophytic fungal species and highly complex ecology, the full ecological impact would be very challenging to estimate.

Despite the ecological complexity, in more controlled environments, these relationships could represent a unique tool to enhance growth, increase nutrient use efficiency and induce beneficial metabolic responses in crops. Optimal benefits in growth from inoculation of *E. purpurea* with arbuscular mycorrhizae are most likely limited to environments where native or other mycorrhizal populations are not present (Bethlenfalvay and Lindermann, 1992). Organic systems also may not stand to gain such drastic benefits because organic soils already support healthy mycorrhizal populations (Mader et al., 2000). Despite this, as a tool to speed early development and lower input costs, in greenhouse or nursery production it appears to have great potential.

This work has also demonstrated that both from potency and volume standpoint, fungal endophytes can enhance the production of natural product in *E. purpurea*. The combined benefits of lowering costs and raising production could offer opportunities for more profits in the horticulture and natural products industry.

Whether other plant species will respond in similar way to these or other endophytes remains to be seen; however, this work demonstrates that targeted application of fungal endophytes in crops has the potential to elicit specific and beneficial changes. With the coming reality of mineral shortages, higher demand for food, chemical pollution and climate change, agricultural science will be forced to consider other more sustainable approaches to crop production. I believe this work demonstrates that fungal endophytes represent an innovative and sustainable approach to incorporate into a more sustainable future agriculture.

Chapter V

Concluding Remarks

As a learning experience, this project proved to be a challenging and satisfying experience. It offered the opportunity to explore diverse facets of plant biology and scientific methodology. As a research endeavor, it yielded data which contributes to the broad understanding of plant interactions with endophytic fungi in the environment and their significance in *Echinacea pupurea.* This work also demonstrates the potential benefits, of endophyte inoculation, as an applied biotechnique, in an economically important model plant system.

Echinacea purpurea seedlings respond to arbuscular mycorrhizae with notably enhanced growth and nutrient use efficiency under some circumstances. Production schemes often begin in the greenhouse. Even if these benefits are most profound only during seedling development, the potential cost reductions and increases in growth rate could equate to substantial economic benefit in large scale production. Both the ornamental and herbal markets could stand to benefit.

Both endopytes caused significant changes in phytochemistry in *E. purpurea* including some compounds with value in the natural products industry. In this system, the combined influence of altered tissue concentration and increased biomass resulted in substantially increased amounts of natural product produced. Drastic increases in biomass primarily drove this effect.

Undoubtedly, with the full diversity of endophytic fungi in nature, other species may illicit similar effects in *E. purpurea.* This work also could not address the long term implications in a mult-year *E. purpurea* crop or how other environmental condition would affect

the outcome. More research would be necessary to select combinations of species that could be applied commercially to enhance growth and production of natural products in the most efficient way. It is also unknown how other host species would respond to endophytes and how various endophtes might interact with each other.

Despite the need for more development, I believe this work demonstrates the potential for endophyte inoculation as a viable approach to enhance agricultural and specialty crop production. Endophytes can offer many other benefits to the host as well that could not be considered in the scope of this project, but other work has shown the potential for enhanced biotic and abiotic stress resistance which also could reduce input costs and dependency associated with agricultural chemicals.

Many challenges will continue to present themselves as man moves into an uncertain future of food shortages, mineral scarcity, and environmental pollution. Unsustainable agricultural practices, on which we have depended, will need to be reevaluated and sustainable alternatives developed and adopted. Whether or not the urgency is fully appreciated now, it will be critically clear to future generations. A multi-faceted approach will be necessary to address these challenges, but endophytes offer one promising new sustainable biotechnology to develop and incorporate into a new paradigm of future agriculture. This work contributes to a foundation that can be built upon to further that vision.

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APPENDIX 1

Table 3.1. Greenhouse environmental conditions in Study 1 and Study 2.

	Trial 1		Trial 2	
Study	Temp.	Lum/ m^2	Temp.	Lum/ m^2
1	26.26°C	17773.4	24.67°C	12032.9
2	23.91°C	10972.7	22.09°C	10842.5

Mean values shown.

Table 3.2. Applications of Peters® 15-0-15, potassium phosphate (KH2PO4), and micronutrient solution (Micro) applied to both trials of Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM). All treatments received 150ppm solutions of 15-0-15 (unless otherwise stated), NM plants received 3.0mM KH_2PO_4 and AM plants received 0.8mM KH_2PO_4.

Week	15-0-15		KH_2PO_4		Micro	
	NM	AM	NM	AM	NM	AM
Preplant					X	X
1						
2	X	X	X	X		
3	X		X			
4	X	X	X	X		
5	X		X			
6	X	X	X	X	X	X
7	X		X			
8	X	X	X	X		
9	X		X			
10	X	X	X	X	X	X
11	X		X			
12	X	X*	X	X		

X indicates application of the given fertilizer on the given week. X*= ½ strength.

Table 4.1. *Beauveria bassiana* colonization of 2-week-old Echinacea purpurea plants treated with B. bassiana (Bb) and *B. bassiana* and arbuscular mycorrhizal fungi (AMBb) in Study 1.

	Trial 1	Trial 2
Trt	Colonization {P=.28}	Colonization {P=.032}
Bb	70[11] -	74[10] a
AMBb	50[15] -	33[14] b

Mean values shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.2. Arbuscular Mycorrhizae Colonization (Col) and number of Hyphae (Hyp.), vesicles (Ves.), and arbuscules (Arb.) in Study 1.

	Trial 1				Trial 2			
Trt	**Col.** {P=0.044}	**Hyp.** {P=0.13}	**Ves.** {P=0.25}	**Arb.** {P=0.076}*	**Col.** {P=0.57}	**Hyp.** {P=0.76}	**Ves.** {P=0.87}	**Arb.** {P=0.38}
AM	**91.7**[.75] **b**	**44.5** [1.59] **-**	**23.6** [1.74]-	**31.1** [2.00] **a**	**95.0**[1.16] **-**	**34.7**[2.49] -	**42.5**[3.92] -	**31.7**[1.72] **-**
AMBb	**94.1**[.75] **a**	**47.8** [1.59] **-**	**26.5** [1.74]-	**27.1** [2.00] **b**	**94.1**[1.16] **-**	**35.7**[2.49] -	**41.5**[3.92] -	**33.8**[1.72] **-**

Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.3. Germination percentages in Study 1 and Study 2 counted 3 weeks after planting. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1	Trial 2
Trt	**Germ %** {P=0.227}	**Germ %** {P=0.932}
Con	**69**[3.46] **-**	**69**[5.06] **-**
Bb	**77**[3.46] **-**	**73**[5.06] **-**
HP	**73**[3.46] **-**	**80**[5.06] **-**
HPBb	**68**[3.46] **-**	**76**[5.06] **-**
AM	**69**[3.46] **-**	**79**[5.06] **-**
AMBb	**72**[3.46] **-**	**80**[5.06] **-**

Mean values shown. [Standard Error], (-) = Not significant.

Table 4.4. Number of leaves on *Echinacea purpurea* plants, counted at intervals, in Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}
Con	**1.81**[0.08] **b**	**2.52**[0.18] **c**	**3.97**[0.08] **c**	**6.25**[0.08] **c**	**1.95**[0.05] **b**	**2.48**[0.07] **c**	**3.46**[0.07] **c**	**6.24**[0.01] **c**
Bb	**1.88**[0.08] **b**	**2.79**[0.19] **c**	**4.27**[0.09] **c**	**6.72**[0.09] **c**	**2.05**[0.05] **b**	**2.41**[0.07] **c**	**3.49**[0.07] **c**	**6.23**[0.01] **c**
HP	**1.94**[0.08] **b**	**3.46**[0.18] **b**	**5.65**[0.09] **b**	**9.59**[0.09] **b**	**2.19**[0.05] **b**	**3.30**[0.07] **b**	**5.08**[0.07] **b**	**7.92**[0.01] **b**
HPBb	**2.00**[0.08] **b**	**3.54**[0.18] **b**	**5.79**[0.09] **b**	**9.89**[0.09] **b**	**2.05**[0.05] **b**	**3.20**[0.07] **b**	**4.97**[0.07] **b**	**7.89**[0.01] **b**
AM	**3.33**[0.08] **a**	**6.01**[0.18] **a**	**9.94**[0.10] **a**	**19.63**[0.10] **a**	**4.44**[0.06] **a**	**9.05**[0.09] **a**	**11.88**[0.09] **a**	**19.74**[0.16] **a**
AMBb	**3.29**[0.08] **a**	**6.44**[0.18] **a**	**10.20**[0.10] **a**	**19.51**[0.10] **a**	**4.43**[0.06] **a**	**8.38**[0.09] **a**	**12.32**[0.09] **a**	**20.48**[0.16] **a**

Least square means shown. [Standard Error].

Table 4.5. Size of the largest leaf on *Echinacea purpurea* plants, measured at intervals, in Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}
Con	**22.2**[1.00] **b**	**26.8**[1.67] **c**	**30.9**[2.08] **d**	**67.5**[3.50] **f**	**18.9**[0.16] **c**	**21.3**[0.18] **c**	**25.2**[0.26] **c**	**62.5**[4.24] **c**
Bb	**23.4**[1.07] **b**	**27.9**[1.79] **bc**	**36.2**[1.79] **cd**	**83.6**[3.76] **e**	**17.9**[0.16] **c**	**19.8**[0.18] **c**	**24.3**[0.25] **c**	**58.2**[4.24] **c**
HP	**23.1**[1.00] **b**	**31.5**[1.67] **b**	**40.7**[2.08] **bc**	**115.4**[3.50] **c**	**21.5**[0.17] **b**	**29.1**[0.18] **b**	**40.2**[0.32] **b**	**103.2**[4.24] **b**
HPBb	**23.1**[1.00] **b**	**31.5**[1.67] **b**	**43.6**[2.08] **b**	**101.4**[3.50] **d**	**21.3**[0.17] **b**	**29.1**[0.21] **b**	**42.8**[0.33] **b**	**104.3**[4.24] **b**
AM	**44.9**[1.00] **a**	**88.3**[1.67] **a**	**117.9**[2.08] **a**	**148.9**[3.50] **a**	**55.5**[0.26] **a**	**105.3**[0.38] **a**	**129.4**[0.55] **a**	**171.8**[4.24] **a**
AMBb	**42.6**[1.00] **a**	**85.3**[1.67] **a**	**114.2**[2.08] **a**	**138.2**[3.50] **b**	**54.1**[0.25] **a**	**109.3**[0.39] **a**	**119.0**[0.53] **a**	**172.4**[4.24] **a**

Data is (mm). Least square means shown. [Standard Error].

Table 4.6. Height of the largest leaf on *Echinacea purpurea* plants, measured at intervals, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}	**4wk** {P<0.0001}	**6wk** {P<0.0001}	**8wk** {P<0.0001}	**12wk** {P<0.0001}
Con	ND	**33.2**[3.05] **d**	**39.1**[3.05] **c**	**110.1**[8.01] **c**	**22.1**[2.56] **b**	**26.3**[0.27] **c**	**26.3**[0.27] **c**	**150.9**[9.84] **c**
Bb	ND	**36.5**[3.28] **cd**	**45.4**[3.28] **c**	**130.4**[8.60] **c**	**20.7**[2.56] **b**	**24.0**[0.26] **c**	**24.0**[0.26] **c**	**144.2**[9.84] **c**
HP	ND	**42.2**[3.05] **bc**	**64.4**[3.05] **b**	**213.0**[8.01] **b**	**27.8**[2.56] **b**	**39.1**[1.32] **b**	**39.1**[1.32] **b**	**244.5**[9.84] **b**
HPBb	ND	**46.0**[3.05] **b**	**67.3**[3.05] **b**	**230.4**[8.01] **b**	**24.2**[2.56] **b**	**40.9**[0.33] **b**	**40.9**[0.33] **b**	**236.5**[9.84] **b**
AM	ND	**132.9**[3.05] **a**	**194.4**[4.55] **a**	**274.2**[8.01] **a**	**74.4**[2.56] **a**	**151.8**[0.62] **a**	**151.8**[0.62] **a**	**329.5**[9.84] **a**
AMBb	ND	**127.4**[3.05] **a**	**190.5**[4.55] **a**	**270.7**[8.01] **a**	**70.8**[2.56] **a**	**157.4**[0.63] **a**	**157.4**[0.63] **a**	**346.6**[9.84] **a**

Data is (mm). Least square means shown. [Standard Error].

Table 4.7. Dry weight and root to shoot ratios (Rt./Sht.) of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**Root** {P<.0001}	**Shoot** {P<.0001}	**Whole** {P<.0001}	**Rt. / Sht.** {P<.0001}	**Root** {P<.0001}	**Shoot** {P<.0001}	**Whole** {P<.0001}	**Rt. / Sht.** {P<.0001}
Con	**0.26**[0.10] **c**	**0.36** [0.15] **c**	**0.62** [0.23] **c**	**0.80** [.034] **a**	**0.20**[0.03] **c**	**0.38**[0.1] **c**	**0.58**[0.17] **c**	**0.57**[0.02] **a**
Bb	**0.37**[0.10] **c**	**0.54** [0.15] **c**	**0.91** [0.23] **c**	**0.78** [.035] **a**	**0.21**[0.03] **c**	**0.37**[0.1] **c**	**0.59**[0.17] **c**	**0.59**[0.02] **a**
HP	**0.76**[0.10] **b**	**1.60** [0.15] **b**	**2.36** [0.23] **b**	**0.47** [.034] **bc**	**0.40**[0.03] **b**	**1.03**[0.1] **b**	**1.44**[0.17] **b**	**0.40**[0.02] **b**
HPBb	**0.68**[0.10] **b**	**1.83** [0.15] **b**	**2.51** [0.23] **b**	**0.38** [.034] **c**	**0.39**[0.03] **b**	**1.01**[0.1] **b**	**1.40**[0.17] **b**	**0.40**[0.02] **b**
AM	**3.14**[0.10] **a**	**5.37** [0.15] **a**	**8.51** [0.23] **a**	**0.58** [.034] **b**	**2.35**[0.04] **a**	**5.45**[0.1] **a**	**7.81**[0.17] **a**	**0.43**[0.02] **b**
AMBb	**3.21**[0.10] a	**5.40** [0.15] **a**	**8.61** [0.23] **a**	**0.60** [.034] **b**	**2.41**[0.04] **a**	**5.67**[0.1] **a**	**8.11**[0.17] **a**	**0.43**[0.02] **b**

Data is (g). Least square means shown. [Standard Error].

Table 4.8. Crown caliper (Cal.) and number of shoots (Sht. #) of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1		Trial 2	
Trt	**Cal.** {P<0.0001}	**Sht. #** {P<0.0001}	**Cal.** {P<0.0001}	**Sht. #** {P<0.0001}
Con	**4.35**[0.731] **d**	**1.00**[0.000] **c**	**4.29**[0.096] **c**	**1.00**[0.040] **c**
Bb	**5.15**[0.731] **d**	**1.07**[0.160] **c**	**4.01**[0.094] **c**	**1.00**[0.040] **c**
HP	**8.65**[0.731] **c**	**1.63**[0.154] **b**	**6.42**[0.107] **b**	**1.06**[0.040] **c**
HPBb	**9.95**[0.731] **c**	**1.37**[0.154] **bc**	**6.37**[0.107] **b**	**1.13**[0.040] **c**
AM	**25.35**[0.731] **a**	**3.10**[0.154] **a**	**22.10**[0.170] **a**	**3.10**[0.046] **a**
AMBb	**22.77**[0.731] **b**	**2.86**[0.154] **a**	**21.48**[0.168] **b**	**2.86**[0.048] **a**

Caliper data is (mm). Least square means shown. [Standard Error].

Table 4.9. Concentration of chlorophyll A (Chl A), chlorophyll B (Chly B), total chlorophyll (Total Chl), and chlorophyll A:B ratios (Chl A:B) in leaves of 12-week-old *Echinacea purpurea* plant, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**Chl A** {P=0.0008}	**Chl B** {P=0.003}	**Total Chl** {P=0.0008}	**Chl A :B** {P=0.021}	**Chl A** {P=0.0084}	**Chl B** {P<0.0001}	**Total Chl** {P=0.0015}	**Chl A:B** {P=0.049}
Con	**6.59**[0.52] **a**	**2.68**[0.08] **ab**	**9.27**[0.69] **a**	**2.43**[0.12] **ab**	**6.16**[0.83] **c**	**2.45**[0.03] **c**	**8.60**[0.96] **c**	**2.50**[0.21] **ab**
Bb	**4.61**[0.52] **b**	**2.19**[0.08] **bcd**	**6.80**[0.69] **b**	**2.10**[0.12] **c**	**6.22**[0.83] **c**	**4.05**[0.03] **a**	**10.27**[0.96] **bc**	**2.18**[0.21] **b**
HP	**6.73**[0.52] **a**	**2.52**[0.08] **bc**	**9.26**[0.69] **a**	**2.64**[0.12] **a**	**10.37**[0.83] **a**	**3.82**[0.03] **a**	**14.19**[0.96] **a**	**2.73**[0.21] **a**
HPBb	**8.02**[0.59] **a**	**3.16**[0.09] **a**	**11.21**[0.77] **a**	**2.60**[0.12] **ab**	**9.20**[0.83] **ab**	**3.94**[0.03] **a**	**13.14**[0.96] **a**	**2.63**[0.21] **a**
AM	**4.59**[0.48] **b**	**1.96**[0.07] **d**	**6.57**[0.63] **b**	**2.27**[0.12] **bc**	**7.26**[0.83] **bc**	**2.78**[0.03] **bc**	**10.05**[0.96] **bc**	**2.61**[0.21] **a**
AMBb	**5.00**[0.52] **b**	**2.06**[0.07] **cb**	**7.06**[0.69] **b**	**2.43**[0.12] **ab**	**8.55**[0.83] **abc**	**3.17**[0.03] **b**	**11.72**[0.96] **ab**	**2.71**[0.21] **a**

Data is (mg/g dry weight). Least square means shown. [Standard Error].

Table 4.10. Content of chlorophyll A (Chl A), chlorophyll B (Chly B), and total chlorophyll (Total Chl) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**Chl A** { P<0.0001}	**Chl B** {P<0.0001}	**Total Chl** {P<0.0001}	**Chl A** {P<0.0001}	**Chl B** {P<0.0001}	**Total Chl** {P<0.0001}
Con	**2.95**[0.24] **c**	**1.19**[0.69] **c**	**4.14**[2.91] **c**	**2.41**[0.31] **c**	**0.99**[0.14] **c**	**3.33**[0.33] **c**
Bb	**2.81**[0.24] **c**	**1.31**[0.69] **c**	**4.12**[2.91] **c**	**2.67**[0.56] **c**	**1.69**[0.16] **c**	**4.38**[0.36] **c**
HP	**12.07**[0.24] **b**	**4.51**[0.69] **b**	**16.59**[2.91] **b**	**12.30**[0.32] **b**	**4.57**[0.21] **b**	**16.80**[0.62] **b**
HPBb	**11.08**[0.24] **b**	**4.18**[0.69] **b**	**15.26**[2.91] **b**	**9.20**[0.49] **b**	**3.86**[0.20] **b**	**13.07**[0.55] **b**
AM	**21.91**[0. 24] **a**	**9.48**[0.69] **a**	**31.39**[2.91] **a**	**38.77**[0.94] **a**	**14.97**[0.34] **a**	**53.72**[1.07] **a**
AMBb	**26.78**[0.24] **a**	**10.99**[0.69] **a**	**37.77**[2.91] **a**	**49.70**[1.06] **a**	**18.38**[0.37] **a**	**68.08**[1.19] **a**

Data is (mg/plant). Least square means shown. [Standard Error].

Table 4.11. Concentration of zeaxanthin (Zea), antheraxanthin, (Anth), violaxanthin (Viol), and their combined concentrations (ZAV Total) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**Zea** {P<0.0001}	**Anth** {P<0.0001}	**Viol** {P<0.0001}	**ZAV Total** {P<0.0001}	**Zea** {P<0.0001}	**Anth** {P=0.067}*	**Viol** {P=0.013}	**ZAV Total** {P=0.011}
Con	**0.022**[.001] **a**	**0.091**[.003] **a**	**0.273**[.016] **a**	**0.386**[.021] **a**	**0.007**[.001] **b**	**0.051**[.005] **ab**	**0.291**[.026] **a**	**0.350**[.027] **a**
Bb	**0.017**[.001] **ab**	**0.071**[.003] **b**	**0.240**[.016] **ab**	**0.328**[.021] **b**	**0.011**[.001] **a**	**0.046**[.005] **abc**	**0.159**[.026] **c**	**0.216**[.027] **b**
HP	**0.013**[.001] **bc**	**0.049**[.003] **c**	**0.221**[.016] **b**	**0.282**[.021] **bc**	**0.008**[.001] **b**	**0.053**[.005] **a**	**0.251**[.026] **ab**	**0.311**[.027] **a**
HPBb	**0.011**[.001] **c**	**0.054**[.003] **bc**	**0.214**[.018] **bc**	**0.279**[.023] **bc**	**0.007**[.001] **b**	**0.036**[.005] **bc**	**0.251**[.026] **ab**	**0.294**[.027] **a**
AM	**0.009**[.001] **c**	**0.046**[.002] **c**	**0.173**[.015] **cd**	**0.228**[.020] **cd**	**0.003**[.001] **c**	**0.035**[.005] **c**	**0.178**[.026] **bc**	**0.216**[.027] **bc**
AMBb	**0.008**[.001] **c**	**0.041**[.003] **c**	**0.151**[.016] **d**	**0.201**[.021] **d**	**0.007**[.001] **b**	**0.034**[.005] **c**	**0.260**[.026] **a**	**0.301**[.027] **ab**

Data is (mg/g dry weight). Least square means shown. [Standard Error]. (*) =P<0.10.

Table 4.12. Content of zeaxanthin (Zea), antheraxanthin, (Anth), violaxanthin (Viol), and their combined concentrations (ZAV Total) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1				Trial 2			
Trt	**Zea** {P<0.0001}	**Anth** {P<0.0001}	**Viol** {P<0.0001}	**ZAV Total** {P<0.0001}	**Zea** {P<0.0001}	**Anth** {P<0.0001}	**Viol** {P<0.0001}	**ZAV Total** {P<0.0001}
Con	**0.010**[.004] **c**	**0.042**[.004] **c**	**0.127**[.060] **d**	**0.179**[.072] **c**	**0.003**[.000] **d**	**0.021**[.015] **b**	**0.120**[.037] **d**	**0.143**[.037] **d**
Bb	**0.010**[.004] **c**	**0.042**[.004] **c**	**0.146**[.060] **cd**	**0.198**[.072] **c**	**0.005**[.000] **c**	**0.022**[.015] **b**	**0.071**[.036] **d**	**0.098**[.036] **d**
HP	**0.022**[.004] **b**	**0.085**[.004] **b**	**0.400**[.060] **b**	**0.507**[.072] **b**	**0.009**[.000] **b**	**0.065**[.015] **b**	**0.310**[.038] **c**	**0.383**[.039] **c**
HPBb	**0.016**[.004] **bc**	**0.083**[.004] **b**	**0.303**[.060] **bc**	**0.402**[.072] **b**	**0.007**[.000] **b**	**0.038**[.015] **b**	**0.287**[.038] **c**	**0.331**[.038] **c**
AM	**0.046**[.004] **a**	**0.021**[.004] **a**	**0.858**[.060] **a**	**1.113**[.072] **a**	**0.029**[.000] **a**	**0.190**[.015] **a**	**0.961**[.043] **b**	**1.171**[.045] **b**
AMBb	**0.044**[.004] **a**	**0.022**[.004] **a**	**0.804**[.060] **a**	**1.067**[.072] **a**	**0.042**[.000] **a**	**0.196**[.015] **a**	**1.514**[.047] **a**	**1.753**[.049] **a**

Data is (mg/plant). Least square means shown. [Standard Error].

Table 4.13. Concentration of β-carotene (β car), Lutein (Lut), and neoxanthin (Neoxan) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**β Car** {P=0.16}	**Lut** {P=0.17}	**Neoxan** {P=0.012}	**β Car** {P=0.005}	**Lut** {P=0.027}	**Neoxan** {P=0.001}
Con	**0.209**[0.12] **-**	**0.654**[0.04] **-**	**0.254**[0.02] **a**	**0.194**[0.03] **c**	**0.744**[0.05] **b**	**0.219**[0.03] **c**
Bb	**0.149**[0.12] **-**	**0.637**[0.04] **-**	**0.222**[0.02] **ab**	**0.211**[0.03] **bc**	**0.796**[0.05] **b**	**0.354**[0.03] **a**
HP	**0.238**[0.12] **-**	**0.599**[0.04] **-**	**0.172**[0.02] **bc**	**0.321**[0.03] **a**	**0.955**[0.05] **a**	**0.303**[0.03] **ab**
HPBb	**0.244**[0.13] **-**	**0.689**[0.04] **-**	**0.225**[0.02] **ab**	**0.294**[0.03] **ab**	**0.860**[0.05] **ab**	**0.316**[0.03] **ab**
AM	**0.178**[0.11] **-**	**0.568**[0.03] **-**	**0.165**[0.02] **c**	**0.300**[0.03] **a**	**0.741**[0.05] **a**	**0.232**[0.03] **c**
AMBb	**0.201**[0.12] **-**	**0.560**[0.04] **-**	**0.167**[0.02] **bc**	**0.355**[0.03] **a**	**0.800**[0.05] **a**	**0.261**[0.03] **bc**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (-) = Not significant.

Table 4.14. Content of β-carotene (β car), Lutein (Lut), and neoxanthin (Neoxan) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**β-Car** {P<0.0001}	**Lut** {P<0.0001}	**Neoxan** {P<0.0001}	**β-Car** {P<0.0001}	**Lut** {P<0.0001}	**Neoxan** {P<0.0001}
Con	**0.08**[0.16] **c**	**0.29**[0.15] **c**	**0.11**[0.05] **c**	**0.08**[0.00] **c**	**0.30**[0.08] **c**	**0.09**[0.04] **c**
Bb	**0.08**[0.16] **c**	**0.39**[0.15] **c**	**0.13**[0.05] **c**	**0.09**[0.00] **c**	**0.35**[0.08] **c**	**0.15**[0.04] **c**
HP	**0.41**[0.04] **b**	**1.08**[0.15] **b**	**0.31**[0.05] **b**	**0.37**[0.00] **b**	**1.18**[0.09] **b**	**0.38**[0.04] **b**
HPBb	**0.33**[0.04] **b**	**0.97**[0.15] **b**	**0.29**[0.05] **b**	**0.28**[0.00] **b**	**0.90**[0.09] **b**	**0.32**[0.04] **b**
AM	**0.81**[0.05] **a**	**2.79**[0.15] **a**	**0.80**[0.05] **a**	**1.58**[0.12] **a**	**4.02**[0.12] **a**	**1.26**[0.05] **a**
AMBb	**1.07**[0.06] **a**	**2.96**[0.15] **a**	**0.89**[0.05] **a**	**2.07**[0.40] **a**	**4.63**[0.13] **a**	**1.50**[0.05] **a**

Data is (mg/plant). Least square means shown. [Standard Error].

Table 4.15. Concentration of cafteric acid (Caft), Cynarin (Cyn), and Cichoric acid (Cich) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**Caft** {P<0.001}	**Cyn** {P=0.185}	**Cich** {P=0.062}*	**Caft** {P<0.001}	**Cyn** {P=0.763}	**Cich** {P=0.002}
Con	**0.034**[.009] **bc**	**0.022**[.026] -	**0.052**[.016] **b**	**0.163**[<.0001] **ab**	**0.036**[.007] **-**	**0.215**[.024] **ab**
Bb	**0.043**[.009] **b**	**0.097**[.026] -	**0.060**[.016] **b**	**0.220**[<.0001] **a**	**0.017**[.005] **-**	**0.366**[.031] **a**
HP	**0.019**[.009] **bc**	**0.013**[.026] -	**0.077**[.016] **ab**	**0.024**[<.0001] **c**	**0.018**[.005] **-**	**0.064**[.013] **c**
HPBb	**0.011**[.009] **c**	**0.021**[.026] -	**0.051**[.016] **b**	**0.063**[<.0001] **bc**	**0.026**[.006] **-**	**0.112**[017] **bc**
AM	**0.082**[.009] **a**	**0.060**[.026] -	**0.119**[.016] **a**	**0.031**[<.0001] **c**	**0.027**[.006] **-**	**0.125**[.018] **bc**
AMBb	**0.042**[.009] **b**	**0.019**[.026] -	**0.061**[.016] **b**	**0.027**[<.0001] **c**	**0.015**[.004] **-**	**0.192**[.022] **b**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.16. Concentration of cafteric acid (Caft), Chlorogenic acid (Chlor), and Cichoric acid (Cich) in roots of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**Caft** {P=0.23}	**Chlor** {P=0.68}	**Chic** {P=0.033}	**Caft** {P=0.17}	**Chlor** {P=0.003}	**Chic** {P=0.44}
Con	**0.014**[<.0001] **-**	**0.009**[.003] -	**0.007**[.016] **abc**	**0.015**[<.01] **-**	**0.020**[.007] **a**	**0.010**[.013] **-**
Bb	**0.013**[<.0001] **-**	**0.011**[.003] -	**0.005**[.016] **abc**	**0.012**[<.01] **-**	**0.016** [.005] **a**	**0.010**[.016] **-**
HP	**0.012**[<.0001] **-**	**0.006**[.003] -	**0.0004**[.016] **c**	**0.018**[<.01] **-**	**0.015** [.005] **ab**	**0.014**[.013] **-**
HPBb	**0.018**[<.0001] **-**	**0.004**[.003] -	**0.003**[.016] **bc**	**0.017**[<.01] **-**	**0.004** [.006] **bc**	**0.012**[.016] **-**
AM	**0.016**[<.0001] **-**	**0.007**[.003] -	**0.020**[.016] **a**	**0.031**[<.01] **-**	**0.0002** [.006] **c**	**0.045**[.013] **-**
AMBb	**0.019**[<.0001] **-**	**0.007**[.003] -	**0.017**[.016] **ab**	**0.014**[<.01] **-**	**0.0002** [.004] **c**	**0.029**[.016] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (-) = Not significant.

Table 4.17. Content of cafteric acid (Caft), and Cichoric acid (Cich) in whole plants (roots + shoots) of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1		Trial 2	
Trt	**Caft** {P<0.0001}	**Chic** {P<0.0001}	**Caft** {P=0.003}	**Chic** {P<0.0001}
Con	**0.036**[<.00001] **b**	**0.038**[.076] **b**	**0.132**[.077] **c**	**0.141**[.140] **b**
Bb	**0.047**[<.00001] **b**	**0.059**[.077] **b**	**0.152**[.077] **bc**	**0.229**[.146] **b**
HP	**0.059**[<.00001] **b**	**0.158**[.080] **b**	**0.060**[.076] **c**	**0.092**[.137] **b**
HPBb	**0.067**[<.00001] **b**	**0.154**[.089] **b**	**0.177**[.078] **bc**	**0.200**[.144] **b**
AM	**0.500** [<.00001] **a**	**0.936**[.096] **a**	**0.640**[.086] **a**	**1.694**[.215] **a**
AMBb	**0.493**[<.00001] **a**	**0.677**[.096] **a**	**0.425**[.082] **ab**	**1.688**[.215] **a**

Data is (mg/plant). Least square means shown. [Standard Error].

Table 4.18. Concentration of Alkylamides # 2 (Alk2), 3 (Alk3) and the isomers 8/9 (8 + 9) (Alk8/9) in roots of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**Alk2** {P=0.041}	**Alk3** {P=0.019}	**Alk8/9** {P=0.101}	**Alk2** {P=0.036}	**Alk3** {P=0.34}	**Alk8/9** {P=0.21}
Con	**4.56**[1.34] **b**	**12.29**[2.42] **b**	**4.13**[.962] **-**	**10.58**[.697] **ab**	**20.42**[3.63] **-**	**9.60**[1.41] **-**
Bb	**8.86**[1.34] **a**	**18.75**[2.42] **b**	**7.02**[.962] **-**	**6.85**[.584] **b**	**17.09** [3.63] **-**	**8.76**[1.41] **-**
HP	**7.46**[1.34] **ab**	**17.46**[2.42] **b**	**5.04**[.962] **-**	**13.16**[.765] **a**	**25.53** [3.63] **-**	**10.84**[1.41] **-**
HPBb	**10.03**[1.34] **a**	**26.03**[2.42] **a**	**7.91**[.962] **-**	**16.05**[.835] **a**	**24.76** [3.63] **-**	**10.22**[1.41] **-**
AM	**8.05**[1.34] **ab**	**16.11**[2.42] **b**	**7.00**[.962] **-**	**19.04**[.901] **a**	**26.82** [3.63] **-**	**11.74**[1.41] **-**
AMBb	**11.06**[1.34] **a**	**16.88**[2.42] **b**	**5.94**[.962] **-**	**14.22**[.791] **a**	**23.61** [3.63] **-**	**12.90**[1.41] **-**

Data is (% Internal Standard). Least square means shown. [Standard Error], (-) = Not significant.

Table 4.19. Content of Alkylamides # 2 (Alk2), 3 (Alk3) and the isomers 8/9 (8 + 9) (Alk8/9) in roots of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**Alk2** {P<0.0001}	**Alk3** {P<0.0001}	**Alk8/9** {P<0.0001}	**Alk2** {P<0.0001}	**Alk3** {P<0.0001}	**Alk8/9** {P<0.0001}
Con	**0.050**[.014] **c**	**0.133**[.029] **c**	**0.039**[<.0001] **c**	**0.064**[<.0001] **cd**	**0.122**[<.0001] **cd**	**0.058**[.010] **bc**
Bb	**0.105**[.020] **bc**	**0.218**[.037] **bc**	**0.080**[<.0001] **bc**	**0.036**[<.0001] **d**	**0.088**[<.0001] **d**	**0.046**[.009] **c**
HP	**0.120**[.021] **bc**	**0.278**[.042] **bc**	**0.080**[<.0001] **bc**	**0.120**[<.0001] **bc**	**0.217**[<.0001] **bc**	**0.102**[.013] **bc**
HPBb	**0.173**[.026] **b**	**0.459**[.054] **b**	**0.126**[<.0001] **b**	**0.191**[<.0001] **b**	**0.294**[<.0001] **b**	**0.121**[.014] **b**
AM	**0.633**[.049] **a**	**1.263**[.089] **a**	**0.546**[<.0001] **a**	**1.128**[.036] **a**	**1.508**[.116] **a**	**0.671**[.033] **a**
AMBb	**0.859**[.057] **a**	**1.303**[.090] **a**	**0.363**[<.0001] **a**	**0.895**[.013] **a**	**1.378**[.077] **a**	**0.801**[.036] **a**

Data is (Mg/plant: relative to Internal Standard). Least square means shown. [Standard Error]

Table 4.20. Concentration of β-carophyllene (β-Caro), α-humulene (α-Hum), and germacrene-D (Germ-D) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**β-Caro** {P=0.003}	**α-Hum** {P=<0.001}	**Germ-D** {P=<0.001}	**β-Caro** {P=0.035}	**α-Hum** {P=<0.126}	**Germ-D** {P=<0.118}
Con	**0.218**[.065] **c**	**0.100**[.025] **d**	**6.68**[1.40] **d**	**0.310**[.051] **bc**	**0.134**[.024] **-**	**9.60**[1.41] **-**
Bb	**0.322**[.065] **bc**	**0.122**[.025] **cd**	**8.78**[1.40] **cd**	**0.344** [.051] **bc**	**0.162** [.024] **-**	**8.76**[1.41] **-**
HP	**0.432**[.065] **ab**	**0.182**[.025] **bc**	**11.94**[1.40] **bc**	**0.278** [.051] **c**	**0.120** [.024] **-**	**10.84**[1.41] **-**
HPBb	**0.492**[.065] **ab**	**0.222**[.025] **ab**	**13.39**[1.40] **ab**	**0.304** [.051] **bc**	**0.134** [.024] **-**	**10.22**[1.41] **-**
AM	**0.586**[.065] **a**	**0.256**[.025] **a**	**16.02**[1.40] **ab**	**0.500** [.051] **a**	**0.204** [.024] **-**	**11.74**[1.41] **-**
AMBb	**0.602**[.065] **a**	**0.260**[.025] **a**	**16.38**[1.40] **a**	**0.440** [.051] **ab**	**0.184** [.024] **-**	**12.90**[1.41] **-**

Data is (% Internal Standard). Least square means shown. [Standard Error]

Table 4.21. Content of β-carophyllene (β-Caro), α-humulene (α-Hum), and germacrene-D (Germ-D) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are control (Con), *Beauveria bassiana* (Bb), high phosphorus (HP), high phosphorus and *B. bassiana* (HPBb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb).

	Trial 1			Trial 2		
Trt	**β-Car** {P<0.0001}	**α-Hum** {P<0.0001}	**Germ-D** {P<0.0001}	**β -Car** {P<0.0001}	**α -Hum** {P<0.0001}	**Germ-D** {P<0.0001}
Con	**0.028**[.010] **d**	**0.013**[.004] **c**	**0.889**[.262] **c**	**0.034**[.007] **c**	**0.014**[.003] **c**	**1.017**[.205] **c**
Bb	**0.047**[.013] **cd**	**0.018**[.005] **c**	**1.296**[.316] **c**	**0.033**[.007] **c**	**0.016**[.004] **c**	**1.075**[.211] **c**
HP	**0.154**[.024] **bc**	**0.066**[.009] **b**	**4.336**[.578] **b**	**0.065** [.009] **bc**	**0.028**[.003] **bc**	**2.049**[.291] **bc**
HPBb	**0.241**[.029] **b**	**0.108**[.012] **b**	**6.511**[.708] **b**	**0.099** [.012] **b**	**0.044**[.005] **b**	**3.124**[.359] **b**
AM	**0.824**[.055] **a**	**0.360**[.022] **a**	**22.651**[1.32] **a**	**0.738**[.032] **a**	**0.300**[.014] **a**	**20.540**[.921] **a**
AMBb	**0.885**[.057] **a**	**0.387**[.023] **a**	**24.391**[1.37] **a**	**0.666**[.031] **a**	**0.279**[.014] **a**	**18.990**[.885] **a**

Data is (Mg/plant: relative to Internal Standard). Least square means shown. [Standard Error]

Table 4.22. Arbuscular Mycorrhizae Colonization (Col) and number of Hyphae (Hyp.), vesicles (Ves.), and arbuscules (Arb.) in Study 2.

	Trial 1			
Trt	**Col.**	**Hyp.**	**Ves.**	**Arb.**
AM	**66.6**	**32.8**	**10.4**	**26.4**
	Trial 2			
AM	**72.6**	**36.4**	**17.8**	**27.4**

Mean values shown.

Table 4.23. Dry weight of 12-week-old *Echinacea purpurea* plants from entire treatment groups (Whole Trt) and subsamples used for phytochemical analysis (Phytochem), from Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1		Trial 2	
Trt	**Whole Trt** {P=0.59}	**Phytochem.** {P=0.14}	**Whole Trt** {P=0.29}	**Phytochem.** {P=0.69}
NM	**1.49**[.11] **-**	**1.58** [.03] **-**	**1.25**[.09] **-**	**1.20**[.03] **-**
AM	**1.58**[.11] **-**	**1.50** [.03] **-**	**1.11**[.09] **-**	**1.18**[.03] **-**

Data is (g). Least square means shown. [Standard Error], (-) = Not significant.

Table 4.24. Concentration of chlorophyll A (Chl A), chlorophyll B (Chly B), total chlorophyll (Total Chl), and chlorophyll A:B ratios (Chl A:B) in leaves of 12-week-old *Echinacea purpurea* plant, from Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1				Trial 2			
Trt	**Chl A** {P=0.099}*	**Chl B** {P=0.088}*	**Total Chl** {P=0.357}	**Chl A:B** {P=0.029}	**Chl A** {P=0.18}	**Chl B** {P=0.088}*	**Total Chl** {P=0.11}	**Chl A:B** {P=0.84}
NM	**3.56**[.540] **a**	**2.79**[.268] **a**	**6.35**[.495] **-**	**1.45**[.262] **b**	**6.31**[.494] **-**	**2.80**[.139] **a**	**9.03**[.181] **-**	**2.25**[.171] **-**
AM	**4.99**[.540] **a**	**2.05**[.268] **a**	**7.03**[.495] **-**	**2.43**[.262] **a**	**5.28**[.494] **-**	**2.42**[.139] **a**	**7.66**[.168] **-**	**2.20**[.171] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.25. Concentration of β-carotene (β car), Lutein (Lut), and neoxanthin (Neoxan) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1			Trial 2		
Trt	**β-Car** {P=0.070} *	**Lut** {P=0.22}	**Neoxan** {P=0.087}*	**β-Car** {P=0.84}	**Lut** {P=0.38}	**Neoxan** {P=0.19}
NM	**0.17**[.031] **a**	**0.60**[.04] **-**	**0.26**[.024] **a**	**0.23**[.05] **-**	**0.68**[.15] **-**	**0.26**[.015] **-**
AM	**0.27**[.031] **a**	**0.67**[.04] **-**	**0.20**[.024] **a**	**0.24**[.06] **-**	**0.64**[.15] **-**	**0.23**[.015] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.26. Concentration of Alkylamides # 2 (Alk2), 3 (Alk3) and the isomers 8/9 (8 + 9) (Alk8/9) in roots of 12-week-old *Echinacea purpurea* plants, from Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1			Trial 2		
Trt	**Alk2** {P=.099}*	**Alk3** {P=.16}	**Alk8/9** {P=.64}	**Alk2** {P=.49}	**Alk3** {P=.15}	**Alk8/9** {P=.62}
NM	**10.85**[1.27] **a**	**20.97**[1.60] **-**	**8.77**[.733] **-**	**14.81**[.327] **b**	**28.05** [1.90] **-**	**12.05**[.547] **-**
AM	**7.51**[1.27] **a**	**14.84**[1.35] **-**	**7.81**[.692] **-**	**13.00**[.327] **a**	**23.79** [1.90] **-**	**11.65**[.547] **-**

Data is (% Internal Standard). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.27. Content of zeaxanthin (Zea), antheraxanthin, (Anth), violaxanthin (Viol), and their combined concentrations (ZAV Total) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1				Trial 2			
Trt	**Zea** {P=0.25}	**Anth** {P=0.24}	**Viol** {P=0.0002}	**ZAV Total** {P=0.0009}	**Zea** {P=0.61}	**Anth** {P=0.22}	**Viol** {P=0.64}	**ZAV Total** {P=0.73}
NM	**0.013**[.004] **-**	**0.059**[.011] **-**	**0.046**[.011] **b**	**0.118**[.018] **b**	**0.010**[.0008] **-**	**0.054**[.005] **-**	**0.139**[.026] **-**	**0.202**[.029] **-**
AM	**0.020**[.004] **-**	**0.079**[.011] **-**	**0.146**[.011] **a**	**0.245**[.018] **a**	**0.010**[.0008] **-**	**0.050**[.005] **-**	**0.157**[.026] **-**	**0.216**[.029] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (-) = Not significant.

Table 4.28. Concentration of cafteric acid (Caft), Cynarin (Cyn), and Cichoric acid (Cich) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 2. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1			Trial 2		
Trt	**Caft** {P=0.025}	**Cyn** {P=0.36}	**Chic** {P=0.062}*	**Caft** {P=0.708}	**Cyn** {P=0.040}	**Chic** {P=0.71}
NM	**0.051**[.010] **b**	**0.021**[.005] -	**0.148**[.106] **b**	**4.65**[.316] **-**	**0.034** [.008] **b**	**25.20**[1.84] **-**
AM	**0.166**[.019] **a**	**0.037**[.007] -	**0.690**[.106] **a**	**4.48**[.316] **-**	**0.061** [.008] **a**	**26.22**[1.84] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.29. Concentration of cafteric acid (Caft), Chlorogenic acid (Chlor), and Cichoric acid (Cich) in roots of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1			Trial 2		
Trt	**Caft** {P=0.034}	**Chlor** {P=0.069}*	**Cich** {P=0.051}*	**Caft** {P=0.008}	**Chlor** {P=0.61}	**Cich** {P=0.35}
NM	**0.009**[.002] **b**	**0.011**[<.001] **a**	**0.008**[.005] **a**	**3.04**[.327] **b**	**0.129** [.026] **-**	**9.45**[1.65] **-**
AM	**0.015**[.002] **a**	**0.008**[<.001] **b**	**0.019**[.005] **a**	**4.65**[.327] **a**	**0.148** [.026] **-**	**11.74**[1.65] **-**

Data is (mg/g dry weight). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.30. Concentration of β-carophyllene (β-Caro), α-humulene (α-Hum), and germacrene-D (Germ-D) in leaves of 12-week-old *Echinacea purpurea* plants, from Study 1. Treatments are: no mycorrhizal fungi (NM) and mycorrhizal fungi (AM).

	Trial 1			Trial 2		
Trt	**βCar** {P=.29}	**αHum** {P=.59}	**GermD** {P=.41}	**βCar** {P=.14}	**αHum** {P=.18}	**GermD** {P=.14}
NM	**0.106**[.018] **-**	**0.047**[.009] -	**3.64**[.613] **-**	**0.101**[.024] **-**	**0.038** [.009] **-**	**3.42**[.680] **-**
AM	**0.135**[.018] **-**	**0.054**[.009] -	**4.38**[.613] **-**	**0.156**[.024] **-**	**0.057** [.009] **-**	**5.01**[.680] **-**

Data is (% Internal Standard). Least square means shown. [Standard Error], (*) =P<0.10, (-) = Not significant.

Table 4.31. HPLC retention times of compounds tested and internal standards (Int. Std.) used.

Compound	Retention time (Min.)
Ethyl-β-apo-8´-apo-carotenoate (Int. Std.)	**19.5**
Chlorophyll A	**13.3**
Chlorophyll B	**8.4**
β-carotene	**52.3**
Lutein	**9.0**
Neoxanthin	**5.8**
Zeaxanthin	**10.9**
Antheraxanthin	**7.4**
Violaxanthin	**5.4**
Cafteric acid	**3.9**
Cynarin	**7.1**
Chlorogenic acid	**4.5**
Cichoric acid	**11.9**

Table 4.32. GC retention times of compounds tested and internal standards (Int. Std.) used.

Compound	Retention time (min.)
1-eicosene (Int. Std.)	18.0
Alkylamide # 2	19.1
Alkylamide #3	24.2
Alkylamides # 8/9	26.6
1-octanal (Int. Std.)	11.0
β-Caryophyllene	22.9
α-Humulene	23.7
Germacrene-D	24.4

Table 4.33. Survival rates of 2-week-old *Echinacea purpurea* seedlings exposed to an unexpected heat event on May 16, 2009. Treatments are control (Con), *Beauveria bassiana* (Bb), arbuscular mycorrhizal fungi (AM), and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb.

Trt	Survival	% of total	*P* values
Con	70/124	56.45[4.47] a	Con vs. Bb (P=0.1061), Con vs. AM and AMBb (P<0.0001)
Bb	76/114	66.68[4.35] a	Bb vs. AM and AMBb (P<0.0001)
AM	8/58	13.79[4.57] b	AM vs. AMBb (P=0.4560),
AMBb	11/58	18.97[5.19] b	

Mean % shown. [Standard Error]

Table 4.34. *Beauveria bassiana* colonization of 15-week-old *Echinacea purpurea* plants treated with *Beauveria bassiana*(Bb) and arbuscular mycorrhizal fungi and *B. bassiana* (AMBb) in Study 2.

	15wk
Trt	Colonization {P=.044}
Bb	62[14] a
AMBb	20[13] b

Mean values shown. [Standard Error],

VITA

Richard James Gualandi Jr. was born on September 1, 1977 in Charleston, SC. Graduated highscholl in 1995 and through various work and family experiences gained a love for plants. This progressed into much time spent in the fiels and classromm studying and learing about the plant biology and the green industry.

At the time of this thesis he was living near Asheville, NC with his wife Michelle, two dogs, two cats and a lot of plants. His future goals include continuing in the field of plant research and education, and continuing the pursuit of personal interests including ceramics, bonsai, and sustainable living.