FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

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FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

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GRADE / DEGREE

Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

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Phytochemical induction in *Echinacea purpurea* (L.) Moench

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University Of Ottawa
In partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton

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ISBN: 978-0-494-32432-5
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ISBN: 978-0-494-32432-5

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Abstract

Purple coneflower, *Echinacea purpurea* (L.) Moench, is an important phytomedicinal species, producing phenolics and alkamides which have medicinal properties. This study examined the contribution of arbuscular mycorrhizal (AM) colonization on the physiology and biochemistry of *E. purpurea*, alone or in combination with jasmonic acid (JA), and over time. It was hypothesized that AM colonization enhances growth, nitrogen assimilation and secondary metabolism in *E. purpurea*. It was also hypothesized that JA application alone, or in combination with AM colonization, and growth time increases the phytochemical content. To verify these hypotheses, three greenhouse experiments were performed with *E. purpurea*, with or without AM fungus, *Glomus intraradices* Schenck & Smith, and harvested after (1) 13 weeks of growth; (2) 13 weeks and JA treatment; and (3) five different growth periods starting from week four to fifteen. The overall results indicated that AM colonization significantly increased the mass of shoots and roots, the concentration of proteins in roots and most of the phenolics. Results also suggested that the effects of AM colonization and /or JA application are similar on the induction of phenolics. Furthermore, time was a main factor on the enhancement of the phytochemical content, of the alkamides found to be dominant in roots, and phenolics in shoots. Optimizing the growth by AM colonization represents an organic method of enhancing *E. purpurea* yield and phytochemical content.

Résumé

L'échinacée 'pourpre', *Echinacea purpurea* (L.) Moench, est une espèce phytomédicinale de grande importance, produisant des phénols et alcamides aux propriétés anti-pathogènes. Cette étude visait à déterminer l'effet de la colonisation arbusculaire mycorhizienne (AM), seule ou en interaction avec l'acide jasmonique (JA), ainsi qu'en fonction du temps, sur la physiologie et la biochimie des plantes d'*E. purpurea*. Il a été postulé que la colonisation AM augmente la croissance, l'assimilation de l'azote ainsi que le métabolisme secondaire chez *E. purpurea*. Une autre hypothèse voulait que l'application JA seule ou combinée à la colonisation AM, ainsi que le temps de croissance accroissent les teneurs en composés phytochimiques. Afin de vérifier ces hypothèses, trois expériences en serre ont été réalisées impliquant *E. purpurea*, avec ou sans inoculation du champignon AM, *Glomus intraradices* Schenck & Smith, et les récoltes effectuées suivant (1) 13 semaines de croissance, (2) 13 semaines avec traitement JA, et (3) en cinq temps depuis la 4ème jusqu'à la 15ème semaine de croissance. Les résultats, dans leur ensemble, indiquent que la colonisation AM a significativement accru la masse des parties aériennes et racinaires, la concentration des protéines dans les racines ainsi que la majorité des composés phénoliques. Les résultats suggèrent aussi que la colonisation AM ou le traitement JA ont des effets semblables sur l'accroissement des composés phénoliques. De plus, le temps de croissance a été un facteur important sur l'augmentation des composés phytochimiques, les alcamides étant dominants dans les racines, les phénols dans les feuilles. L'optimisation de la croissance d'*E. purpurea* par la colonisation AM représente une méthode naturelle dans la production de composés phytomédicinaux.

Acknowledgements

I would like to take this opportunity to convey my sincere appreciation to my supervisor Dre. Christiane Charest, for her invaluable guidance, encouragement, enthusiasm, and patience during the past three years of my study, which made this work not only possible, but also immensely rewarding. My deepest gratitude to my committee members, Dr. John T. Arnason from the University of Ottawa and Dr. Susan Aitken from Carleton University for their valuable contribution, advices and encouragement.

Thanks and best wishes must also be extended to my lab mates, past and present, for their friendship, support and encouragement: Julie Parent, Debbie Phelps, Agnès Lichota, and finally my special thanks must go to Patrick Audet, for his support, continued help with statistical analyses and with any technical help I needed, and to Keomany Ker for her friendship, help and advice during the years of my study.

Special thanks should also go to Dr. Arnason for his continued support, encouragement and scientific advice, and to his entire lab: Rosalie Awad, Cory Harris, Erin Lamont, Renée Leduc, Martha Rae Mullally, Vicky Filion, and finally big thanks to Ammar Saleem and Andrew Burt for their help with HPLC analysis.

I would also like to thank Ms. Huguette Allard, the Greenhouse Supervisor, for her help, support and friendship, and to everyone who helped me during the past three years.

Finally, I would like to thank my big family in Iraq for their support, love and kindness, and to my husband Dr. Helmi Hussien for his constant encouragement and support, and to my three kids; Nawar, Bassam and Bashar for their love. Lastly, I dedicate this thesis to the spirit of my late father, who is always my example in honesty and hard working.

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List of Abbreviations

AM	Arbuscular mycorrhizal
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Chl	Chlorophyll
dH_2O	Distilled water
DM	Dry mass
DMSO	Dimethylsulfoxyde
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
GDH	Glutamate dehydrogenase
GH	Gutamylhydroxamate
GOGAT	Glutamate synthase
GS	Glutamine synthetase
H-	Treatment without jasmonic acid
H+	Treatment with jasmonic acid
HPLC	High pressure liquid chromatography
JA	Jasmonic acid
kD	Kilo Daltons
M-	Treatment without mycorrhizae
M+	Treatment with mycorrhizae
MW	Molecular weight
N	Nitrogen
$NADH_2$	Nicotinamide adenine dinucleotide (reduced form)
NH_4^+	Ammonium
NiR	Nitrite reductase
NR	Nitrate reductase
OD	Optical density
P	Phosphorus
PVLG	Polyvinyl-alcohol-lactic acid-glycerol
SE	Standard error
T	Time
Wks	Weeks

Chapter 1

General Introduction

Echinacea purpurea (L.) Moench, Asteraceae, is a native North American plant species with a long history of use as a phytomedicine in both the Indigenous and European traditions (Barrett 2003; Binns *et al.* 2002a; Percival 2000). Preparations from *E. purpurea* (common name: purple coneflower) are among the most widely used herbal medicines (Barrett 2003). The literatures suggests that *Echinacea* preparations are useful in the treatment of upper respiratory and herpes infections, cancer, skin disease, ulcer, and a number of *Candida* species (Block and Mead 2003), but the most widespread use, is the treatment of acute upper respiratory infection (Barrett 2003). Echinacea has been reported to be the second best selling product in the North American botanical market and represents 8.5 % of the total sales in 2005 (Blumenthal 2005). There are more than 800 echinacea containing products on the market in North America (Barrett 2003; Block and Mead 2003).

Many studies have reported beneficial effects of mycorrhizal fungi on plant growth, nutrient uptake, and tolerance to environmental stressful factors (Audet and Charest 2006, 2007a, b; Azcón *et al.* 2003; Smith and Read 1997; Subramanian and Charest 1998, 1999). However, little is yet known about the potential of these fungi to affect plant secondary metabolic pathways (Copetta *et al.* 2006). The present study investigated the effect of arbuscular mycorrhizal (AM) colonization on the production of phytochemicals in purple coneflower. To our knowledge, this is the first study that investigates the impact of AM fungus on the primary and secondary metabolism in plants of *Echinacea purpurea*.

1.1 Mycorrhizal fungi

The word mycorrhiza comes from Greek words *muke* and *rhiza* meaning fungus and root, respectively. It was first used by Frank in1877, as a neutral term that did not imply parasitism (Smith and Read 1997). The term symbiosis was first defined by de Bary in1887, as "the living together of differently named organisms" (Raina *et al.* 2000). De Bary considered two classes of association that can either be mutualistic implying that all organisms involved are believed to derive benefits, or parasitic when one organism benefits to the detriment of the other (Raina *et al.* 2000). A mycorrhiza is a symbiotic, non-pathogenic association between a root and a fungus, both under natural or cultivated conditions. For the fungus, the primary benefit from this symbiosis is the availability of readily useable carbon. For the host plant, the potential benefits include increased nutrient uptake, enhanced growth and yield (Azcón *et al.* 2003; Eom *et al.* 2000; Raina *et al.* 2000; Smith and Read 1997), or resistance to environmental stress factors, such as salinity (Al-Karaki 2000), drought (Augé 2001; Subramanian and Charest 1998, 1999), chilling (Paradis *et al.* 1995), mineral deficiencies (Smith and Read 1997), and pathogens (St-Arnaud *et al.* 1997). Mycorrhizal fungi are thought to colonize the roots of 85-90% of the approximately 230,000 species of vascular plants (Charest *et al.* 1997; Raina *et al.* 2000).

1.1.1 Classification of mycorrhizae

The most commonly accepted classification of mycorrhizae is the one proposed by Harley and Smith (1985), who divided mycorrhizae into seven categories on the basis of their morphology and physiological features: (i) ectomycorrhizae, (ii) endomycorrhizae, (iii) ectendomycorrhizae, (iv) arbutoid ectendomycorrhizae, (v) monotropoid mycorrhizae, (vi) ericoid endomycorrhizae, (vii) orchidoid endomycorrhizae (Fig. 1.1), the most common being the first two, the ecto and endomycorrhizae. Ectomycorrhizae are characteristically associated with temperate and boreal forest trees, and the fungal species being among the Basidiomycetes and Ascomycetes (Smith and Read 1997). The ectomycorrhizal fungi colonize the cortical region of the host roots, without penetrating cortical cells. The main features of ectomycorrhizae are the formation within the root of a hyphal network known as the Hartig net around cortical cells and a thick layer of hyphal mat on the root surface known as sheath or mantle (Habte 2000; Smith and Read 1997).

The endomycorrhizae are the most widely distributed associations in plants. About 80% of all terrestrial plant species form symbiosis with endomycorrhizal fungi (Quilambo 2003; Smith and Read 1997). The arbuscular mycorrhizal (AM) symbiosis is one of the endomycorrhizal types and is characterized by the arbuscular formation (Smith and Read 1997). The characteristic features of the AM symbiosis are the intracellular penetration of root cortical cells by the fungi and the formation of highly branched, tree-like structures called arbuscules, or round to oval thick walled structures called vesicles (Gupta *et al.* 2000). The AM fungi belong to the phylum Zygomycetes, of the order Glomales, subdivided in two suborders, the Glomineae and Gigasporineae. The AM fungus used in this study, *Glomus intraradices*, belongs to the Glomineae suborder, Glomaceae family (Gupta *et al.* 2000).

Figure 1.1 Types of mycorrhizae (Raina *et al.* 2000).

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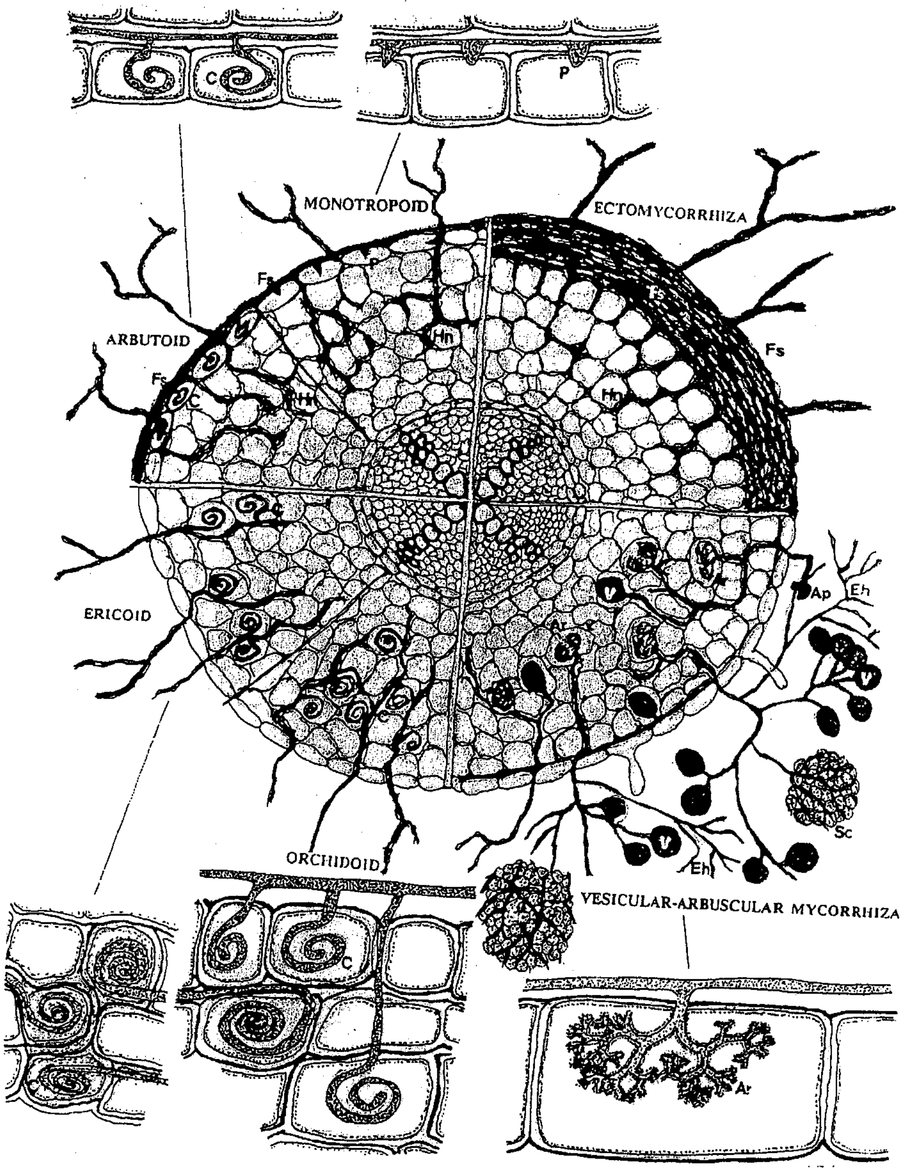
C
P
MONOTROPOID
ECTOMYCORRHIZA
ARBUTOID
Fs
Hn
C
Fs
Fs
Hn
ERICOID
Ap
Eh
V
Ar
V
Sc
Eh
ORCHIDOID
VESICULAR-ARBUSCULAR MYCORRHIZA
C
Ar

1.1.2 Evolution of arbuscular mycorrhizae

The AM fungi have a long evolutionary history and have been dated as early as the Early Devonian, 410-360 million years ago (Raina *et al.* 2000). The colonization of land by the ancestors of the vascular plants clearly seems to have been facilitated by the origin of symbiotic associations between plants and fungi. The arbuscular mycorrhizal fungi have a very ancient origin indicated both by the fossil record and DNA sequences of living members of AM fungi (Smith and Read 1997). The number of plants colonized by AM fungi is very large; herbaceous plants, shrubs and trees of temperate and tropical habitats may all form AM associations (Smith and Read 1997).

1.1.3 Mycorrhizal colonization

Colonization of roots by mycorrhizal fungi can arise from different sources including spores, colonized root fragments or hyphae. In many habitats, the hyphal soil network, together with root fragments, are the main means by which plants become colonized, even when significant spore populations are present (Smith and Read 1997).

Spore germination and limited hyphal growth may occur in the absence of the plant root; however, plant root exudates and volatiles, including CO_2, stimulate spore germination (Fig. 1.2) (Harrison 1998, 2005; Marsh and Schultze 2001). Flavonoid compounds, the major root exudates of some plant species, have been found to stimulate growth and branching of the fungal hyphae, then facilitating their contact with host roots (Harrison 1998; Smith and Read 1997). Following germination, the fungus uses its triacylglyceride and glycogen reserves to support growth and hyphal germ tube to extend a few centimeters from the spore. Once the hypha reaches the vicinity of a root, its growth increases substantially and the hypha undergoes an intense branching which maximizes the chance of contact with the root (Harrison 2005). Upon contact with the root epidermis, hyphal tips swell and form appressoria followed by the root penetration and proliferation of intraradical hyphae, then cortical cells are subsequently penetrated and arbuscules develop (Marsh and Schultze 2001).

Arbuscules are considered the major site of exchange between the fungus and host plant; they are short-lived (~ 7 days) and begin to collapse after a few days, but hyphae and vesicles can remain in roots for months or years (Fig. 1.3) (Smith and Read 1997). Vesicles develop to accumulate storage products in many AM associations, and they are initiated soon after the first arbuscules, but continue to develop when the arbuscules senesce. Vesicles rich in lipids are hyphal swellings in the root cortex and develop in either inter- or intracellular positions (Raina *et al.* 2000; Smith and Read 1997).

Figure 1.2 Stages of arbuscular mycorrhizal development

(Marsh and Schultze 2001).

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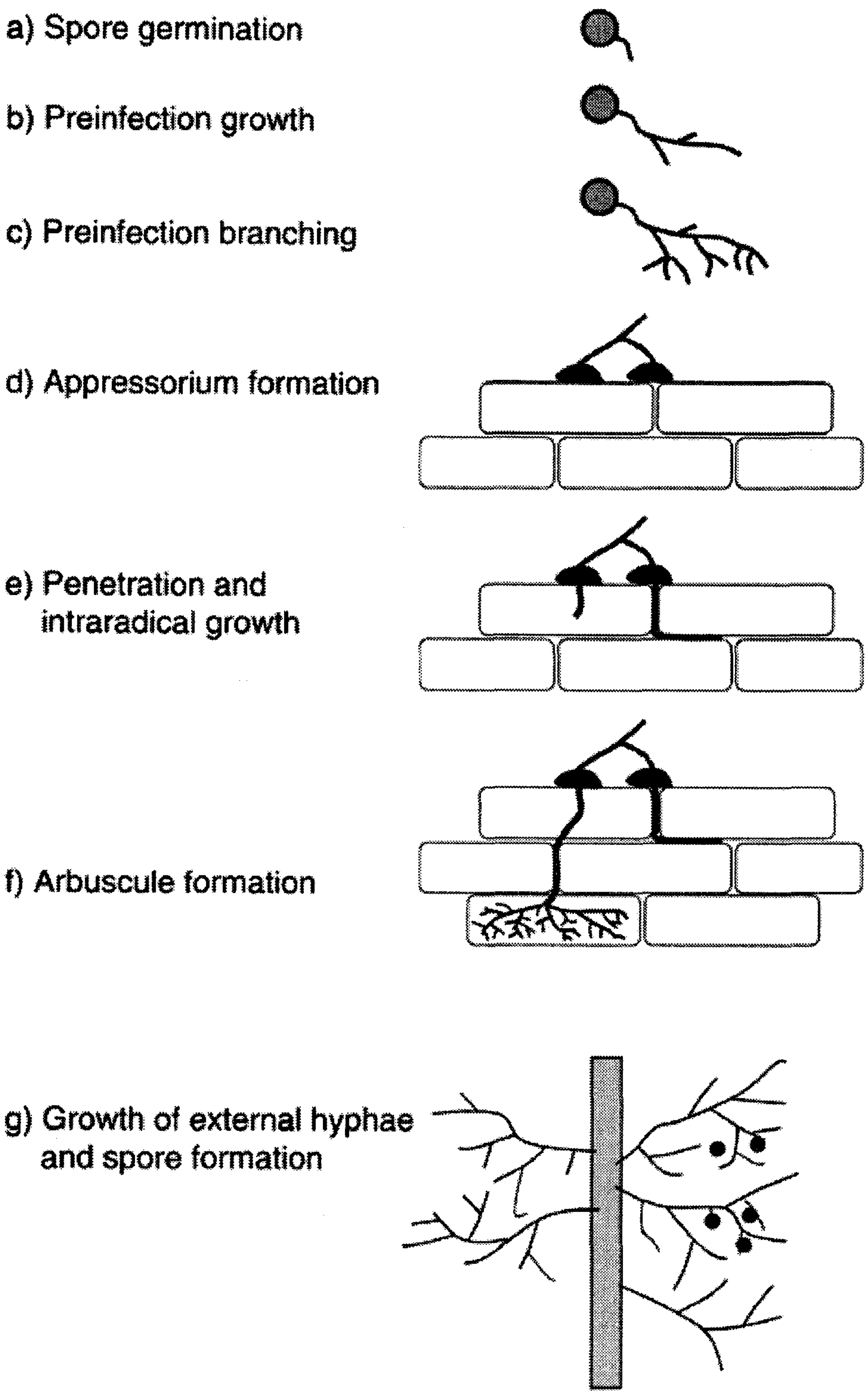
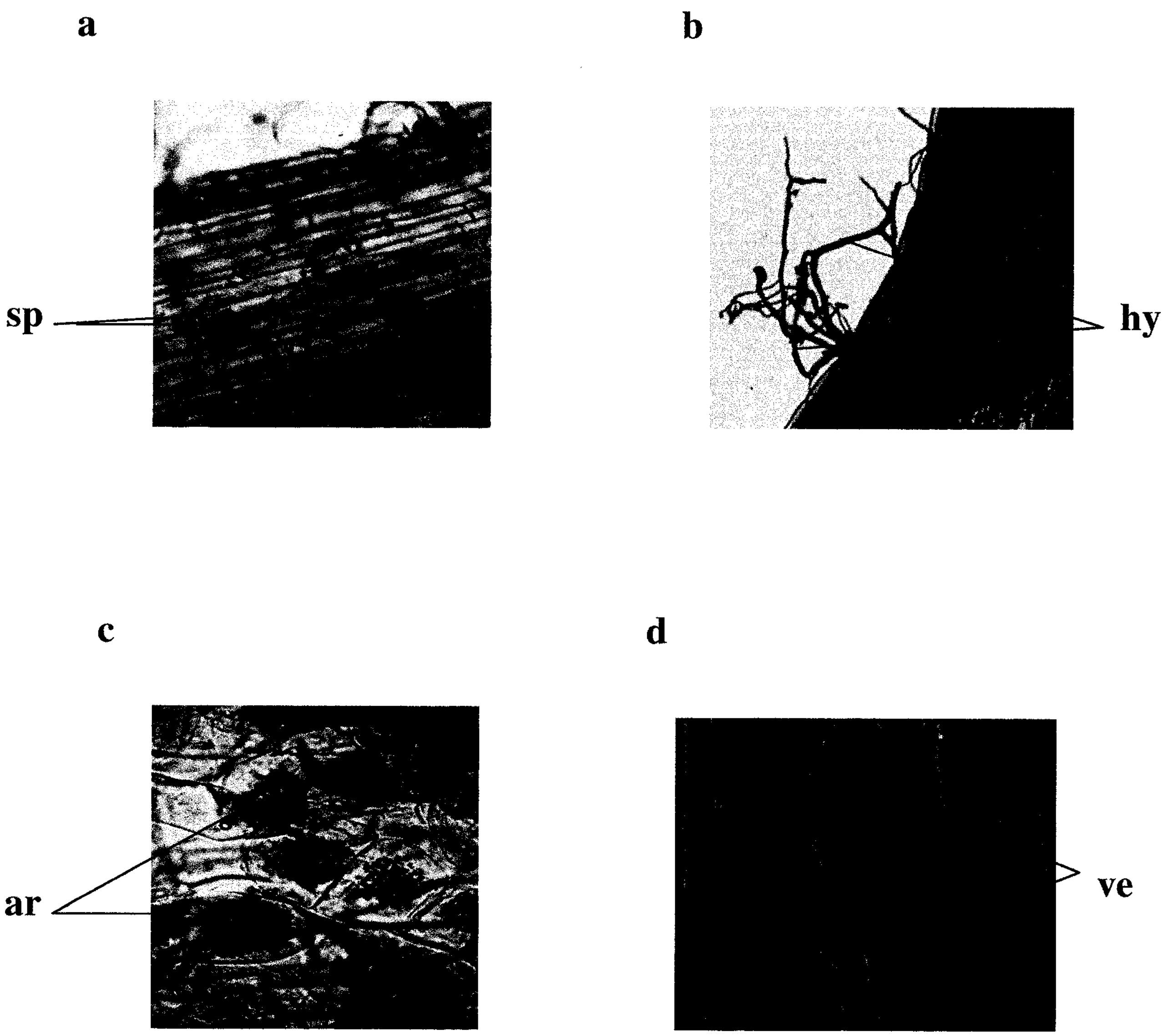

a) Spore germination
b) Preinfection growth
c) Preinfection branching
d) Appressorium formation
e) Penetration and intraradical growth
f) Arbuscule formation
g) Growth of external hyphae and spore formation

Figure 1.3 Colonization structures of *Glomus intraradices* in roots of *Echinacea purpurea* including: (a) spores (**sp**) 100 X, (b) hyphae (**hy**) 100 X, (c) arbuscules (**ar**) 400 X, and (d) vesicles (**ve**) 400 X.

(Pictures taken by Ghada Araim)


a
b
sp
hy
c
d
ar
ve

1.2 Nitrogen metabolism

1.2.1 Nitrogen assimilation

Nitrogen (N) content in plants is about ten times greater than other macronutrients (e.g. P). Major N input derives from organic matter and biological N fixation for a few plant species. The two most important sources of inorganic N for the majority of plant species are nitrate (NO_3^-) and ammonium (NH_4^+) (Smith and Read 1997). NO_3^- is the predominant nitrogen source in agricultural soils, and NH_4^+ is generally present at low concentrations in acidic soils (Smith and Read 1997). Nitrogen assimilation includes uptake of nitrate (NO_3^-), its reduction to nitrite (NO_2^-), the conversion of NO_2^- to ammonium (NH_4^+), then its incorporation into amino acids (Subramanian and Charest 1998) (Fig. 1.4).

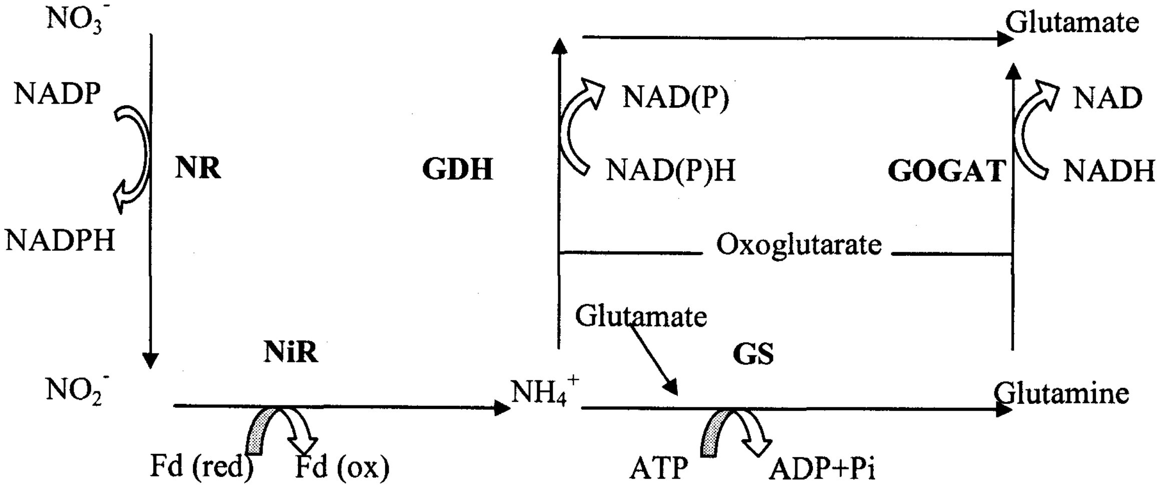
1.2.2 Nitrogen-assimilating enzymes

Glutamine synthetase (GS, E.C.6.3.1.2)

Glutamine synthetase catalyses the formation of glutamine from ammonium and glutamate at the expense of ATP hydrolysis (Oaks and Hirel 1985). It is commonly found in the cells of root and shoot, however its activity is generally higher in shoot than root (Lea *et al.* 1990). In shoots, GS is present in the cytosol and chloroplasts with two isoenzymes GS1 and GS2 of 310 to 350 kD, and in root, it is located in the cytosol only with GS1 (Carvalho *et al.* 2003; Oaks and Hirel 1985). The high affinity of GS for NH_4^+ together with high concentration of this enzyme, ensures that free NH_4^+ concentration is kept below toxic levels (Hopkins and Huner 2004; Miflin and Habash 2002).

Figure 1.4 Schematic diagram of nitrogen assimilation pathway in higher plants. *Abbreviations*: NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase.

(Oaks and Hirel 1985; Subramanian 1998).


NO3-
NADP
NR
NADPH
NO2-
NiR
Fd (red)
Fd (ox)
NH4+
GDH
NAD(P)
NAD(P)H
Glutamate
GOGAT
NAD
NADH
Oxoglutarate
Glutamate
GS
Glutamine
ATP
ADP+Pi

1.2.3 Role of mycorrhizal fungi in nitrogen assimilation

There is increasing evidence of a major contribution by AM fungi to plant N uptake and assimilation (Faure *et al.* 1998; Smith and Gianinazzi-Pearson 1988). Smith and Read (1997) reported that GS activity increased in mycorrhizal root systems. Azcón and Tobar (1998) reported increased NR and GS activities in AM onion plants. Subramanian and Charest (1998, 1999) reported higher NR, GS and GOGAT activities in AM maize plants especially under drought conditions. Toussaint *et al.* (2004) reported, under *in vitro* conditions, higher GDH activity and similar GS or NR activities in colonized Ri T-DNA carrot roots. In general, most of the studies showed enhanced activities of N-assimilatory enzymes and levels of amino acids in mycorrhizal plants (Smith and Read 1997; Subramanian and Charest 1998, 1999). Mycorrhizal root colonization plays a key role in plant health by increasing acquisition of immobile mineral nutrients, particularly phosphorus (Marsh and Schultze 2001; Smith and Read 1997). Although the role of mycorrhizal fungi in phosphorus nutrition of their hosts is well documented, the significance of these fungi with respect to nitrogen nutrition has been less studied until now (Faure *et al.* 1998; Toussaint *et al.* 2004).

1.3 Secondary metabolites

Plants produce a remarkably diverse array of over 100,000 compounds, known as secondary metabolites (SM), with no known direct function in plant growth and development, but have a direct effect on plant defense (Dixon 2001). These compounds have received much attention due to their medicinal value (Dixon 2001). Although these compounds hold a great deal of potential use for many human ailments, they are often extracted only in trace amounts from plants considered as medicinal. Plant SMs are divided into three major biosynthetic groups: terpenes, phenols, and alkaloids; as well as many minor groups (Hopkins and Huner 2004).

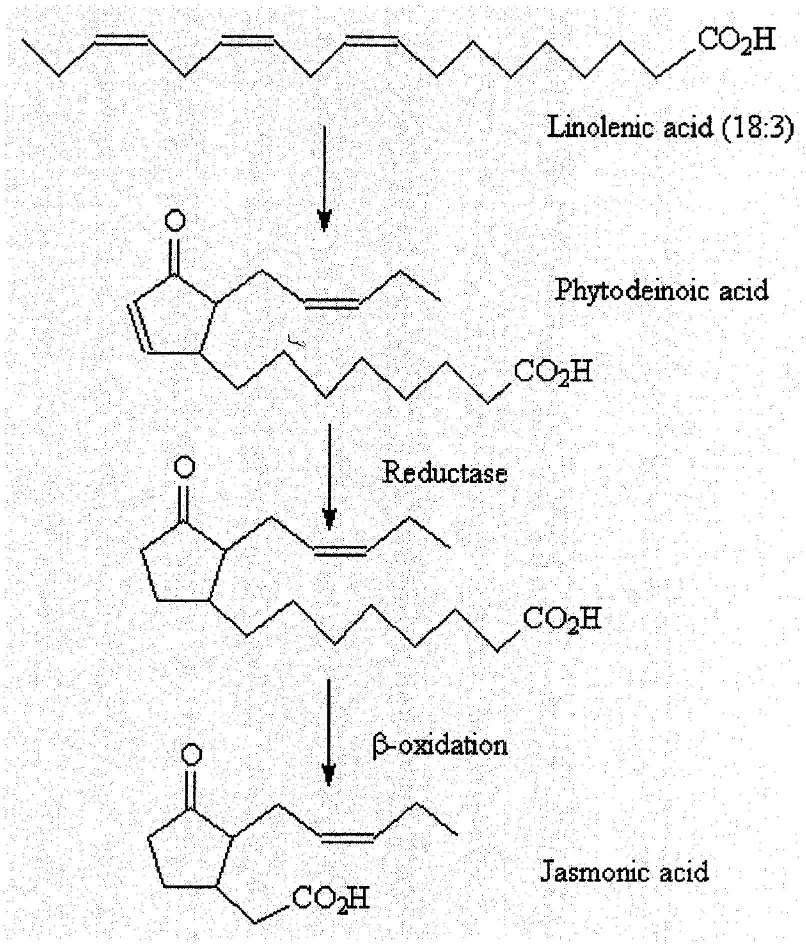
Defense is not the only role of the SMs in plants. Other functions include attraction of pollinators, protection from UV light, structural support, temporary nutrient storage, and hormonal regulation (Herms and Mattson 1992). They also may act as signaling agents in plant-plant, plant-herbivore, and plant-microbe relationships (Herms and Mattson 1992; Wink 1999). Many SMs are synthesized in the cytoplasm or organelles, but stored in the vacuole (Wink 1999). It is well established that SM profiles vary with time, space and developmental stage (Wink 1999). Secondary metabolites have also been used as a taxonomic tool in plant systematics, however SM patterns are not unambiguous systematic markers (Wink 1999).

1.4 Jasmonic acid

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (Me-JA), are plant growth regulators widely distributed within the plant kingdom (Ulloa *et al.* 2002). JA is biosynthesized through the octadecanoid pathway (Fig. 1.5), in which the precursor, α-linolenic acid, is converted by lipoxygenase to the intermediate 12-oxo-phytodienoic acid, then converted to JA through the action of a reductase and three rounds of β-oxidation (Vick and Zimmerman 1984). Jasmonate has been proposed to be a key signaling compound in the process of elicitation leading to accumulation of secondary metabolites (Dixon *et al.*1994; Li and Barz 2006; Walker *et al.* 2002). It was shown that levels of endogenous JA increased in plants upon wounding, or colonization by mycorrhizal fungi (Binns *et al.* 2001; Hause *et al.* 2002; Ryan 2000). When jasmonate solutions were exogenously applied to plant tissues, they were shown to exert opposite effects either inhibitory or promotive in growth and development, and induce secondary metabolism in different plant species (Maier *et al.* 2000; Walker *et al.* 2002; Ulloa *et al.* 2002).

Figure 1.5 Metabolic scheme of jasmonic acid biosynthesis

(Cited in Sept. 2001: http://www.chm.bris.ac.uk/motm/jasmine/jasmineh.htm)


CO_2H
Linolenic acid (18:3)
Phytodeinoic acid
CO_2H
Reductase
CO_2H
β-oxidation
CO_2H
Jasmonic acid
O
O
O

1.5 Echinacea

Purple coneflower, *Echinacea purpurea* (L.) Moench, (Fig. 1.6), belongs to the Asteraceae family (Awang and Kindack 1991; Binns 2001). The first mention of *Echinacea* dates from 1762 (Clayton in the Flora Virginica), and it has been recorded regularly in medicinal literatures for over 200 years (Awang and Kindack 1991; Barrett 2003). The genus *Echinacea* gets its name from the Greek *echinaos*, meaning hedgehog or sea-urchin, descriptive of the plants' conical spiny seed heads (Awang and Kindack 1991). *Echinacea* has been recently reclassified into four species and eight varieties using morphometric assessment of this genus (Binns *et al.* 2002c), but the McGregor taxonomy remains the most widely-used taxonomy in current literature. According to the McGregor taxonomy, *Echinacea* is classified as nine species and two varieties using morphological characters and chromosome numbers (Binns *et al.* 2002c; McGregor 1968). Of the nine species, three are widely cultivated and traded for medicinal and horticultural purposes; *E. purpurea, E. pallida* and *E. angustifulia*. The most predominant is *E. purpurea*, which is characterized by erect main stems up to two meters in height, alternate leaves on long stalks and solitary spiny and purplish bracts (Barrett 2003).

Echinacea plants are native to North America, with wild populations ranging from the US Great Plains north to the Canadian prairies, east to the Appalachian uplands and the southeastern coastal plains (Binns *et al.* 2002a; McGregor 1968). Traditionally, the First Nations used various *Echinacea* preparations extensively for a variety of

Figure 1.6 *Echinacea purpurea* (L.) Moench.

(Picture taken by Ghada Araim)

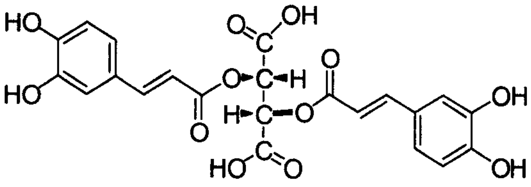
ailments including colds, toothaches, throats aches, burns, and snakebites (Awang and Kindack 1991; Duke 1999).

Preparations of *E. purpurea* are among the most widely used herbal medicines for their effects on the immune system (Barrett 2003; Sun *et al.* 2001). *In vitro* studies have demonstrated in animals and humans the ability of various *E. purpurea* extracts to enhance the activities of various immune cells such as macrophages, monocytes and natural killer cells (Barrett 2003; Bone 2004). These extracts have been attributed to significant antifungal activities in a series of *in vitro* experiments against *Saccharomyces cerevisiae* and a number of *Candida* species, including *C. albicans*, the most common fungal cause of human skin diseases (Binns *et al.* 2000; Barrett 2003).

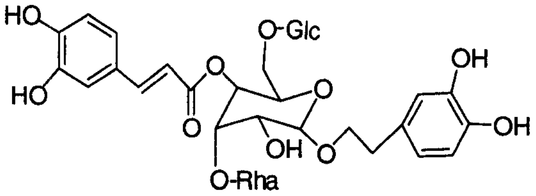
The secondary chemistry of commercial *Echinacea* species has been well characterized and it is the primary method of botanical identification for marketed Echinacea plant materials. *Echinacea* contains three major groups of compounds; caffeic acid conjugates (phenolics), alkamides and polysaccharides (Binns *et al.* 2002a; Barrett 2003; Duke 1999; Matthias *et al.* 2004; Miller and Yu 2004). Phenolics and alkamides have been reported to be active and to benefit the immune system by their immune-stimulating and anti-inflammatory activities (Fig. 1.7).

Figure 1.7 Characteristic phenolics and alkamides in *Echinacea purpurea* (L.) Moench.

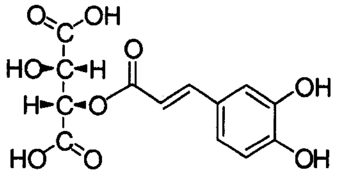
(Bauer *et al.* 1988)



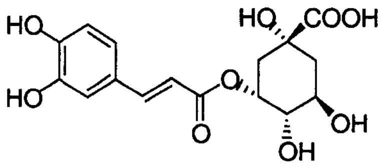
Cichoric acid
(2,3-*O*-dicaffeoyl tartaric acid)



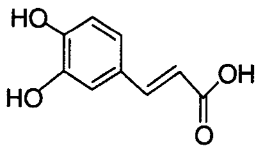
Echinacoside



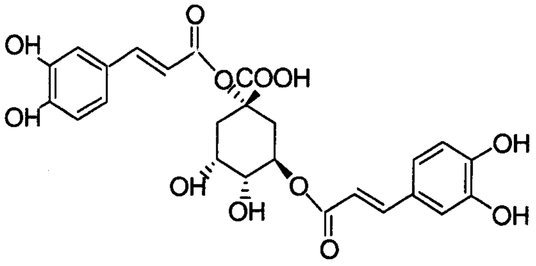
Caftaric acid
(2-*O*-caffeoyl tartaric acid)



Chlorogenic acid
(3-*O*-caffeoyl quinic acid)



Caffeic acid



Cynarin
(1,3-dicaffeoyl quinic acid)

8

9

Alkamides 8/9

1.6 Rationale, Objectives and Hypotheses

Purple coneflower is recognized as an economically important species as it is used both as a modern and traditional medicinal plant against a variety of ailments. As with many other plant species, the phytomedicinal compounds, or secondary metabolites, are synthesized as a response to herbivory and physico-chemical stressors. Moreover, the arbuscular mycorrhizal fungi are known to benefit plants under biotic and abiotic stress conditions, including pathogens, drought, and nutrient deficiency or toxicity. Incorporating all of these components, this collection of studies examined the physiology and phytochemistry of *Echinacea purpurea* (L.) Moench, colonized with the AM fungus, *Glomus intraradices* Schenck & Smith, alone, and in combination with a simulated herbivory factor, the jasmonic acid, and over time.

In our first study (Chapter 2), we investigated the impact of AM colonization on the plant growth parameters (mass, height, root / shoot ratio), the primary metabolism by the analysis of glutamine synthetase activity, and protein and mineral content, and the secondary metabolism by the determination of phytochemicals (cynarin, echinacoside and caffeic, cichoric, caftaric and chlorogenic acids and alkamides). Deriving from the known general role of AM symbiosis, it was hypothesized that AM fungal colonization would enhance all of the above parameters as compared to non-AM plants.

The second study (Chapter 3) examined the effects of JA application, AM colonization, and the combination of both, on the plant physiology and phytochemical production. It was hypothesized that JA application alone, or in combination with AM colonization, increases the phytochemical content.

The third study (Chapter 4) determined the effect of AM colonization over time on the growth parameters and the production of phytochemicals. It was hypothesized that the synthesis of the phytochemical content increases with time due to a growth effect.

Chapter 2

Effect of AM colonization on primary and secondary metabolism in *Echinacea purpurea* (L.) Moench

2.1 Introduction

Colonization of roots by arbuscular mycorrhizal (AM) fungi has been shown to increase productivity of several crops such as maize, sorghum, soybean and potato (Hayman 1980; Koide and Mosse 2004; Subramanian and Charest 1997). This symbiosis has also been shown to improve the survival and growth of the majority of plant species in natural communities (Smith and Read 1997). The AM fungi could be used as bio-fertilizer tools to improve nutrient cycling and crop productivity, and as such to reduce chemical fertilizer input, and environmental costs (Podeszfinski *et al.* 2002; Harrison 2005). In this way, the use of mycorrhizae can be seen as an important alternative strategy in the context of sustainable agriculture and development (Podeszfinski *et al.* 2002; Subramanian 1997; Toussaint *et al.* 2004).

The most prominent contribution of AM fungi to plant growth relates to an increased uptake of immobile nutrients, especially phosphorus (P), by extraradical hyphae (Bolan 1991; George *et al.* 1995; Gerdemann 1968; Smith and Read 1997). Extraradical hyphae reach beyond the root depletion zone, and translocate macro and micronutrients from the soil to root cortex (Neumann and George 2005; Smith and Gianinazzi-Pearson 1988; Smith and Read 1997; Smith *et al.* 2003).

Few studies have looked at the nitrogen (N) metabolic pathway or investigated the key enzymes of N metabolism in AM plants (Faure *et al.* 1998; Ruiz-Lozano and Azcón 1996; Subramanian and Charest 1998, 1999; Toussaint *et al.* 2004). In the studies available, AM colonization was shown to increase the activity of N-assimilatory enzymes, especially nitrate reductase (NR) and glutamine synthetase (GS), and the synthesis of amino acids in AM compared to non-AM plants (Faure *et al.* 1998; Smith and Read 1997; Subramanian and Charest 1998, 1999).

Echinacea, commonly known as purple coneflower, is a perennial plant species in the Asteraceae family. Of the nine species of *Echinacea* in the widely used McGregor taxonomy, three are the most common and the most studied; *E. purpurea*, *E. pallida* and *E. angustifulia*, the most predominant being *E. purpurea*. *E. purpurea* has the ability to enhance the activity of various immune cells, to stimulate lymphocytes and natural killer cells, and to treat acute upper respiratory infection (Barrett 2003).

Echinacea contains three major groups of secondary compounds that have received much attention due to their medicinal value. These compounds are the caffeic acid conjugates, alkamides and polysaccharides (Barrett 2003; Binns 2001; Duke 1999; Miller and Yu 2004). Caffeic acid conjugates (phenolics) are an important group of medicinally active compounds in *Echinacea* (Li 1998), and include caftaric, caffeic, chlorogenic and cichoric acids, and cynarin and echinacoside (Fig. 1.7). The major alkamides in *E. purpurea* are the tetraenes, dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamides 8+9 (Binns *et al.* 2002b) (Fig. 1.7). The immunostimulant effects of *Echinacea* have been connected with the polysaccharide fraction. Furthermore, the antiviral, antibacterial and antifungal properties have been attributed to the phenolic (especially echinacoside) and alkamide fractions (EMEA 1999).

Phytochemical defenses have been shown to increase in response to fungal infection or AM symbiosis (Binns *et al.* 2001; Fester *et al.* 1999; Maier *et al.* 1999; Peipp *et al.* 1997). Binns *et al.* (2001) reported that the phytochemical defenses in *E. pallida* increased and varied in response to wounding or fungal infection. Other studies showed that inoculation by *Glomus intraradices* induced the accumulation of secondary compounds in the roots of barley and wheat (Fester *et al.* 1999; Peipp *et al.* 1997), and tobacco (Maier *et al.* 1999).

Fontenla *et al.* (2001) reported that the Asteraceae is among the few families in which both mycotrophic and non-mycotrophic plant species are found, and that AM colonization was recorded in 14 out of 22 species examined in this family. Only a few studies have focused on the effect of AM association on *Echinacea*. Lata *et al.* (2003) showed, under *in vitro* conditions, that AM inoculation enhanced the survival rate, growth and phenolic content in the shoots of *E. pallida*.

2.2 Hypotheses and objectives

This study aimed to determine the effect of AM colonization on the metabolism of *Echinacea purpurea.* It was hypothesized that AM colonization enhances the growth, and the primary and secondary metabolism in *E. purpurea.* To test this, the objectives were to determine the impact of AM colonization in *E. purpurea* on physiological parameters: mass, height, root/shoot ratio and mineral content, the activity of glutamine synthetase (GS); and the content of phenolics (cynarin, echinacoside and caffeic, cichoric, caftaric and chlorogenic acids) and alkamides.

2.3 Materials and methods

A factorial (1 plant sp. X 2 myc. treatments X 12 replicates) greenhouse experiment was performed including *Echinacea purpurea* (L.) Moench, grown with or without the AM fungus, *Glomus intraradices* Schenck & Smith.

2.3.1 Sowing conditions

Echinacea seeds (Florabanda Farm, ON, Canada, 2004) and pots (12.5 X 12.5 X 15 cm) were surface sterilized with NaOCl 5% (Chlorox bleach) before planting. The soil consisting of a sand:soil mixture (1:1, v/v), had been autoclaved for 20 min at 121°C and 15 PSI. Pots were filled with 1000 mL of soil mixture, then 500 mL of the inoculum or control substrate, and topped with the soil mixture. Eight seeds were sown ~ 1 cm deep in the soil mixture, and then (after ~ 2 weeks) thinned to one plant per pot. In total, 24 pots (12 pots per treatment with one plant per pot) were prepared. The germination rate was 43%. The AM inoculum consisted of *G. intraradices* (DAOM 181602), or a non-AM control substrate that contained filtrates of the rhizosphere microflora while excluding fungal spores (Mycorhize Pro, Premier Tech, Rivière-du-Loup, QC, Canada).

2.3.2 Greenhouse conditions

Plants were grown in the greenhouse of the Center for Advanced Research in Environmental Genomics (CAREG), University of Ottawa, for 13 weeks at 27/23°C (day/night), 40% R.H., and a 16 h-photoperiod, under natural day-light and high-pressure sodium (HPS) lamps (PL Light Systems, Beamsville, ON, Canada). The average light intensity (408 μmol s^{-1} m^{-2}) was measured using a light meter attached to a quantum sensor (LiCor L.I.-250A and LiCor L.I.-190SA, Lincoln, NE, USA).

2.3.3 Watering and fertilization

Plants were watered with dH_2O as required, and fertilized after the third week following germination with 20 mL of ½ ammonium nitrate type Long Ashton Nutrient Solution (LANS, Hewitt and Smith 1975), once a week for two weeks (the 4th and 5th wks). Then the plants were fertilized with the full LANS (50 mL at the 6th wk, and 100mL until harvest). The nutrient solution contained 2.0 mM K_2SO_4, 4.0 mM $CaCl_2$ anhydride, 1.5 mM $MgSO_4.7H_2O$, 1.5 mM $NaH_2PO_4.H_2O$, 5.0 mM NH_4NO_3, 0.01 mM $MnSO_4.4H_2O$, 1.0 μM $CuSO_4.5H_2O$, 1.0 μM $ZnSO_4.7H_2O$, 0.05 mM H_3BO_3, 0.09 mM NaCl, 0.5 μM $Na_2MoO_4.2H_2O$ and 5.7 ppm EDTA-Fe.

2.3.4 Harvest

Plants, harvested after 13 wks of growth, were separated into shoots and roots at the basal knot from which the shoot height was measured. Roots were rinsed in water to remove excess substrate, and three root replicates were used to determine the AM colonization level. After the fresh mass was measured, fresh roots and shoots were frozen with liquid nitrogen and stored at -80°C until freeze-drying for 72 h, at -50°C, under vapor pressure at 3.7 x 10^{-1} mbar (Uni-trap Model 10-100, Virtis Inc., Gardiner, N.Y., U.S.). The dry mass was recorded after removing the samples from the freeze-dryer.

2.3.5 Root colonization

The fresh roots were washed, patted dry, placed in beakers with 2.5% KOH, and heated at 90° C on a hot plate for 20 min, then washed with distilled water for 2-3 times to remove excess KOH and lower the pH (Dalpé, 1993). Roots were transferred to a 1% HCl solution for 45 min at room temperature. These acidified roots were transferred to an Aniline Blue 0.02% staining solution (0.5 g aniline blue, 500 mL glycerol, 450 mL dH_2O and 50 mL 1% HCl), heated on a hot plate for 10 min at 90° C, then placed in a discoloring solution (500 mL glycerol, 450 mL dH_2O and 50 mL 1% HCl). Ten 1 cm-root segments were lined up per slide. A polyvinyl-alcohol-lactic acid-glycerol solution (PVLG; 8.33 g PVA, 50 mL dH_2O, 50mL lactic acid and 50 mL glycerol) was used as a preservative and slides were sealed with clear nail polish. Root segments were examined at 40X, 100X and 400X magnifications via a compound microscope. Mycorrhizal colonization percentage was estimated as the total of root segments containing at least one of the fungal structures (hyphae, vesicles, arbuscules or spores). Fifty root segments (10/slide) were observed for each of the three replicates.

2.3.6 Mineral analyses

For the P, K, Ca, Mg, Mn and Cu analyses, approximately 2 g (per replicate) of dry shoot or root material were ground and sent for mineral analyses via Atomic Absorption Spectrometry, at the "Laboratoire de Chimie Organique et Inorganique, Direction de la recherche forestière", MRN, QC, Canada. A sulfuric acid (H_2SO_4) digestion on hot plate was carried out, followed by analysis of these elements using Atomic Plasma Emission Spectroscopy.

2.3.7 Chlorophyll extraction and determination

Chlorophyll (chl) was determined according to the method of Bruinsma (1963). Fresh leaf material (1g) was immersed in tube containing 100 mL of 95% ethanol and covered with aluminum foil, and kept in the dark at room temperature until complete leaf discoloration. The optical densities (ODs) were read at 645 and 663 nm, and concentrations determined as follows:

$\mathrm{Chl}a = (\mathrm{OD}_{663} \times 12.7) - (\mathrm{OD}_{645} \times 2.7)$

$\mathrm{Chl}b = (\mathrm{OD}_{645} \times 22.9) - (\mathrm{OD}_{663} \times 4.7)$

$\mathrm{Chl\ total} = \mathrm{Chl}a + \mathrm{Chl}b$

2.3.8 Enzyme activity and protein concentration analyses

Glutamine synthetase (GS, E. C. 6.3.1.2)

The GS activity was determined by the synthetase assay as described by Robinson *et al.* (1991) and Toussaint *et al.* (2004). Freeze-dried shoots or roots (0.2 g) were ground with mortar and pestle over ice, with sand, 2% PVP, and 10 mL of extraction buffer, pH 8.0, that contained 25 mM Tris-HCl, 1 mM EDTA-diNa salt, 1 mM dithiothreitol (DTT), 1 mM reduced glutathione, 10 mM $MgSO_4$, 5 mM glutamate, and 0.01% Triton. After centrifugation at 11 591 g for 25 min at 4° C, each of the supernatants was used for the GS and soluble protein assays. The GS reaction was initiated by the addition of 0.25 mL of enzyme extract to 0.75 mL of reaction mixture. This mixture, pH 7.6, contained 2 mM ATP, 2.6 mM $MgSO_4$, 0.7 mM hydroxylamine, 8 mM L-glutamate, and 5 mM Tris-HCl. After 30 min at room temperature, the reaction was terminated by adding 0.75 mL of ferric chloride reagent (4 mL $FeCl_3$ 10%, 1 mL trichloroacetic acid 24% and 0.5 mL HCl 6M in 6.5 mL dH_2O). After centrifugation for 2 min at 2000 g, the OD was read at 540 nm. Blanks, prepared for each extract, contained the same compounds with the exception that the $FeCl_3$ solution was added before the extract to prevent any enzyme reaction to occur. The GS activity was expressed as γ-glutamylhydroxamate (GH) produced g^{-1} DM h^{-1}, using a 0-3 μmol γ-GH standard curve. The GS specific activity of each sample was calculated by dividing its GS activity by its protein concentration.

Protein concentration

Soluble proteins were determined according to the micro-method of Bradford (1976). Each protein extract, 20 μL, was combined with 780 μL dH_2O and 200μL Bio-

Rad dye reagent and mixed by manual inversion. The OD was then read at 595 nm, and the protein concentration measured from a Bovine Serum Albumine (BSA) standard curve (0 - 1.2 mg mL^{-1}).

2.3.9 HPLC analyses of the phytochemicals

Sample preparation

Phenolics and alkamides were determined by using a method adapted from Bergeron *et al.* (2000). Each freeze-dried sample (0.1 g) was ground, then 2 mL of 70% ethanol were added and put in an ultrasonic water bath for 15 min. After centrifugation at 180 g for 5 min at 21° C, the supernatant was collected and put in a new centrifuge tube. 1 mL of ethanol 70% was added to the residue, and then put in the ultrasonic water bath for 15 min and re-centrifuged as before. After each centrifugation, the supernatant was collected and combined with the first one. The total volume of each extract was adjusted to 4 mL with ethanol 70%. An extract aliquote (1 mL) was filtered through a 0.2 μm PTFE (polytetrafluoroethylene) membrane, prior to injection of 1μL into the HPLC system.

Instrumentation and chromatography

The extracts were analyzed using an Agilent model 1100, HPLC system consisting of G1313A Autosampler (Module 1100), with 100 μL loop, G1311A Quaternary pump, G1315A Photodiode array detector (Module 168), G1322A In-line solvent degasser, Solvent delivery system (Module 126), and chemstation software (version 9.1). Separations were achieved on an YMC ODS-AM Spherical 3 μm 120A, 2.0 mm x 100 mm cartridge (Waters, Mississauga, ON, Canada), at 47°C. A solvent gradient system based on solvent A: 0.05% trifluoroacetic acid (TFA) in water, B: 0.05% TFA in acetonitrile (MeCN), at a flow rate of 0.4 mL min^{-1}, permitted separation and quantification of phenolics and alkamides.

Standard curves were generated from pure standards (ChromaDex Inc. California). Elution times for the hydrophilic compounds (phenolics) were from 3.3 to 8.8 min, and for lipophilic compounds (alkamides) at 14.1 min. Peak identities from extracts were confirmed by relative retention times compared to the standards. Quantifications were performed at 210 nm, 260 nm and 326 nm (Binns 2001). The limits of detection (LOD) of phenolics and alkamides were in low micro gram range (Harris *et al.* 2007).

2.3.10 Statistical analyses

One-way Analyses of Variance (ANOVAs) were performed on all the parameters, using S-Plus (S-Plus 6.2, Insightful Corp. 2003), and mean comparisons were done by Student's *t* test. All the data were verified for the assumptions of normality and homoscedasticity. When needed, log or square-root transformations were performed. When statistical assumptions were not met, non-parametric Kruskall-Wallis or non-parametric Wilkcox analyses were done (Zar 1999).

2.4 Results

2.4.1 Mycorrhizal colonization

The AM colonization levels in roots of *E. purpurea* were almost 100% (data not shown). A root was considered colonized when one of the fungal structures was present. Among all the colonized roots, most of the colonization structures were present, e.g. hyphae, arbuscules and vesicles (Fig. 1.3). Some of these roots contained all of these structures, mostly hyphae and arbuscules, others having only one structure. There was no colonization in the non-AM plants.

2.4.2 Physiological parameters

The biomass of AM plants was significantly ($p < 0.01$ and $p < 0.05$), by approximately 3 times higher, compared to non-AM plants (Table 2.1), without any significant differences for root/shoot ratio. The AM shoot ($p < 0.05$), root ($p = 0.06$) and total plant lengths ($p < 0.05$), by approximately 1.5 times, higher than non-AM plants. The number of leaves of the AM plants was also significantly ($p < 0.05$), by approximately 2.3 times, higher than non-AM plants (Table 2.1).

2.4.3 Mineral concentration and content

The P concentration tended to increase ($p = 0.06$) in the shoots of AM plants (Table 2.2). There were no significant differences for the other mineral concentrations in shoots or roots. For the content in shoots (Table 2.3), the AM treatment significantly ($p < 0.05$) increased the content of P and Cu, by approximately 1.5 and 1.2 times higher, respectively, or tended to increase the content of Mg ($p = 0.06$). AM plants had significantly ($p < 0.05$) increased P content, by approximately 1.2 times, compared to non-AM plants. There were no significant differences in the other mineral contents in roots or the whole plants.

Table 2.1 Physiological parameters in roots and shoots of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (n=6), (SEs), and *t* test results are shown.

Plant organ	Treatment	Fresh mass (g)	Dry mass (g)	Plant length (cm)	Root/shoot ratio	# Leaves
Shoots	M-	8.1^{b} (2.8)	1.5^{b} (0.5)	23^{b} (5)	0.37 (0.17)	5.6^{b} (1.1)
	M+	23.8^{a} (4.5)	4.6^{a} (1.0)	35.6^{a} (1.4)	0.40 (0.03)	12.8^{a} (2.7)
	t test	**	*	*	NS	*
Roots	M-	4.9^{b} (1.8)	0.6^{b} (0.3)	18.6^{b} (3.3)		
	M+	16.7^{a} (4.4)	2.0^{a} (0.6)	24.8^{a} (1.3)		
	t test	**	*	*MS*		
Total	M-	13^{b} (4)	2.1^{b} (0.7)	41.6^{b} (6.2)		
	M+	40.5^{a} (8.9)	6.6^{a} (1.5)	60.4^{a} (1.4)		
	t test	**	*	*		

*p<0.05; **p<0.01; '*MS*' marginally not significant (0.05<p<0.1); 'NS' not significant.

Table 2.2 Mineral concentrations (mg g^{-1} DM) in shoots and roots of *E. purpurea*, without (M-) or with (M+) AM colonization. Means (n=3), (SEs) and *t* test results are shown.

Plant organ	Treatment	P	Ca	K	Mg	Cu	Mn
Shoots	M-	3.07^{b} (0.10)	25.4 (1.0)	25.3 (2.8)	11.7 (0.5)	0.02 (0.00)	0.30 (0.07)
	M+	3.66^{a} (0.21)	24.4 (2.1)	25.6 (2.4)	12.1 (0.7)	0.02 (0.00)	0.25 (0.03)
	t test	*MS*	NS	NS	NS	NS	NS
Roots	M-	4.05 (0.10)	6.55 (0.08)	30.3 (2.3)	8.21 (1.04)	0.02 (0.00)	0.06 (0.02)
	M+	4.11 (0.40)	6.37 (0.49)	29.0 (2.8)	7.80 (1.13)	0.02 (0.00)	0.07 (0.01)
	t test	NS	NS	NS	NS	NS	NS

'*MS*' marginally not significant (0.05<p<0.1); 'NS' not significant.

Table 2.3 Mineral contents in shoots (mg shoot^{-1} DM), roots (mg root^{-1} DM) and the whole plants (mg plant $^{-1}$ DM) of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (n=3), (SEs) and *t* test results are shown.

Plant organ	Treatment	P	Ca	K	Mg	Cu	Mn
Shoots	M-	17.0 ^{b} (1.7)	142 (15)	139 (13)	65.0 ^{b} (5.6)	0.11^{b} (0.00)	1.71 (0.56)
	M+	25.0 ^{a} (1.7)	166 (14)	175 (19)	83.7 ^{a} (4.0)	0.14 ^{a} (0.00)	1.74 (0.24)
	t test	*	NS	NS	*MS*	*	NS
Roots	M-	22.6 (3.6)	36.3 (4.7)	167 (25)	47.3 (11.5)	0.11 (0.01)	0.30 (0.04)
	M+	21.1 (1.1)	33.8 (6.1)	149 (7)	40.1 (4.6)	0.10 (0.01)	0.35 (0.00)
	t test	NS	NS	NS	NS	NS	NS
Total	M-	39.6 ^{b} (1.8)	178 (11)	306 (31)	112 (6)	0.22 (0.01)	2.01 (0.61)
	M+	46.1 ^{a} (1.2)	200 (15)	324 (12)	124 (5)	0.23 (0.01)	2.09 (0.24)
	t test	*	NS	NS	NS	NS	NS

* $p<0.05$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant

2.4.4 Chlorophyll concentration

There were no significant differences in the concentrations of chlorophylls *a*, *b*, and total, between the treatments (Table 2.4).

Table 2.4 Leaf chlorophyll concentration (mg g^{-1} FM) of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (n= 3), (SEs) and *t* test results are shown.

Treatment	Chl *a*	Chl *b*	Chl total
M-	1.32 (0.15)	0.87 (0.06)	2.20 (0.20)
M+	1.24 (0.10)	0.97 (0.09)	2.22 (0.14)
t test	NS	NS	NS

'NS' not significant.

2.4.5 Glutamine synthetase (GS) activity and soluble protein concentration

In the AM roots (Table 2.5), the protein concentration was slightly higher ($p = 0.06$) than non-AM roots, while there were no significant differences in shoots. There were no significant differences in the GS or specific GS activities, neither in shoots or roots.

Table 2.5 Protein concentration (mg proteins g^{-1} DM), GS activity (μmol γGH g^{-1} DM h^{-1}) and specific GS activity (μ mol γ GH mg^{-1} protein h^{-1}) in shoots and roots of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (shoots n= 6, roots n= 5), (SEs) and *t* test results are shown.

Plant organ	Treatment	Proteins	GS activity	Specific GS activity
Shoots	M-	30.42 (2.86)	37.95 (2.11)	1.24 (0.16)
	M+	24.13 (2.41)	40.51 (9.06)	1.67 (0.50)
	t test	NS	NS	NS
Roots	M-	23.86^{b} (1.96)	31.57 (6.56)	1.32 (0.33)
	M+	31.52^{a} (2.61)	33.90 (9.58)	1.08 (0.23)
	t test	*MS*	NS	NS

'*MS*' marginally not significant ($0.05<p<0.1$); NS' not significant.

2.4.6 Phytochemical responses to AM colonization

Phenolics and alkamides

In roots (Table 2.6), the AM treatment significantly increased the concentrations of cichoric ($p < 0.05$), caftaric ($p < 0.001$), and chlorogenic ($p < 0.01$) acids, and cynarin ($p < 0.05$), by 1.5, 1.7, 2.6 and 1.3 times, respectively. There were no significant differences in the concentration of alkamides in roots. In shoots, the AM treatment had no significant effects on phytochemical concentrations. However, it tended to decrease ($p = 0.08$), by 1.2 times, the concentration of caftaric acid.

For phytochemical content in shoots (Table 2.7), the AM treatment significantly ($p < 0.05$) increased the content of cichoric and caftaric acids, by 2.8 and 2 times, respectively, or tended to increase the content of caffeic acid ($p = 0.07$), by 2.5 times. In roots, the AM treatment significantly increased the content of caftaric ($p < 0.05$) and chlorogenic ($p < 0.01$) acids, by 3.4 and 4.8 times, respectively, or tended to increase, both by 3 times, the content of cichoric acid ($p = 0.08$) and cynarin ($p = 0.07$).

In the whole plant, the AM treatment significantly ($p < 0.05$) increased the content of caftaric and chlorogenic acids, by 2.2 and 4.3 times, respectively, and tended to increase ($p = 0.07$) the content of cichoric acid by 2.9 times. The AM treatment also tended to increase ($p = 0.07$), by 2.2 times, the content of alkamides in roots.

Table 2.6 Phytochemical concentrations (mg g^{-1} DM) in shoots and roots of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (shoots n= 5-6, roots n= 3-5), (SEs) and *t* test results are shown.

Plant organ	Treatment	Cichoric acid	Caftaric acid	Chlorogenic acid	Caffeic acid	Cynarin	Echina-coside	Alkam-ides
Shoots	M-	20.2 (2.3)	11.3 ^{a} (0.8)	0.3 (0.1)	1.0 (0.3)	0.2 (0.1)	0.5 (0.3)	
	M+	21.3 (1.8)	9.3 ^{b} (0.7)	0.2 (0.1)	0.7 (0.1)	0.3 (0.1)	0.4 (0.2)	
	t test	NS	*MS*	NS	NS	NS	NS	
Roots	M-	11.3 ^{b} (1.3)	2.9 ^{b} (0.3)	0.16 ^{b} (0.02)	0.6 (0.2)	0.13 ^{b} (0.01)		1.8 (0.4)
	M+	16.6 ^{a} (1.6)	4.9 ^{a} (0.3)	0.42 ^{a} (0.07)	0.7 (0.1)	0.17 ^{a} (0.01)		2.2 (0.1)
	t test	*	***	**	NS	*		NS

* p<0.05; ** p<0.01; *** p<0.001; '*MS*' marginally not significant (0.05<p<0.1); 'NS' not significant.

Table 2.7 Phytochemical contents in shoots (mg shoot^{-1} DM), roots (mg root^{-1} DM) and the whole plants (mg plant $^{-1}$ DM) of *E. purpurea,* without (M-) or with (M+) AM colonization. Means (shoots n= 5-6, roots n= 3-5, total n= 3-5), (SEs) and *t* test results are shown.

Plant organ	Treatment	Cichoric acid	Caftaric acid	Chlorogenic acid	Caffeic acid	Cynarin	Echinacoside	Alkamides
Shoots	M-	36.5^{b} (10.5)	20.8^{b} (5.7)	0.41 (0.06)	1.35^{b} (0.58)	0.31 (0.06)	0.41 (0.12)	
	M+	104^{a} (29)	41.6^{a} (6.2)	1.69 (1.21)	3.44^{a} (1.26)	1.74 (1.26)	2.72 (1.64)	
	t test	*	*	NS	*MS*	NS	NS	
Roots	M-	13.6^{b} (4.1)	3.2^{b} (0.7)	0.18^{b} (0.02)	0.79 (0.23)	0.14^{b} (0.02)		2.2^{b} (0.3)
	M+	41^{a} (16)	11^{a} (4)	0.87^{a} (0.17)	1.82 (0.77)	0.43^{a} (0.15)		4.9^{a} (1.5)
	t test	*MS*	*	**	NS	*MS*		*MS*
Total	M-	50.1^{b} (9.3)	24^{b} (6)	0.59^{b} (0.07)	2.14 (1.02)	0.45 (0.07)		
	M+	145^{a} (48)	52.6^{a} (9.3)	2.56^{a} (1.34)	5.26 (2.15)	2.17 (1.63)		
	t test	*MS*	*	*	NS	NS		

* $p<0.05$; ** $p<0.01$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant.

Total phytochemical (phenolic and alkamide) content

The AM treatment significantly ($p < 0.05$) increased the total content of phenolics in the shoots, by 2.6 times (Table 2.8, and Figs. 2.1 and 2.2), or tended to increase the total content of phenolics and phytochemicals in roots ($p = 0.07$), and the whole plant ($p = 0.06$), both by approximately 3 times.

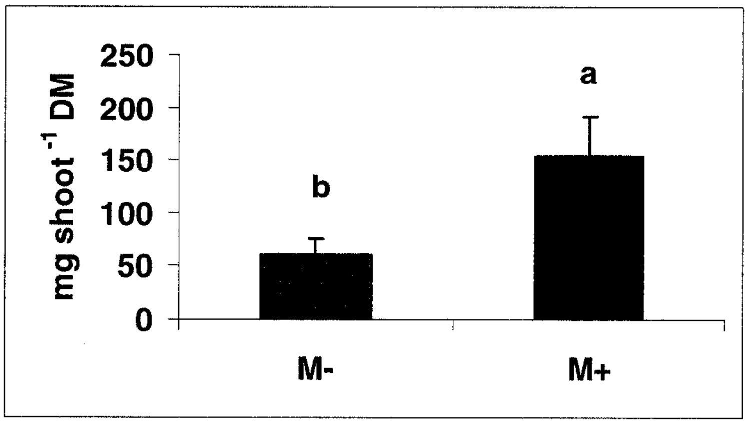
Table 2.8 *t* test results for the total phenolic and phytochemical contents in shoots, roots and the whole plants of *E. purpurea,* without or with AM colonization.

Plant organ	Total phenolics	Total phytochemicals
Shoots	*	
Roots	*MS*	*MS*
Total	*MS*	*MS*

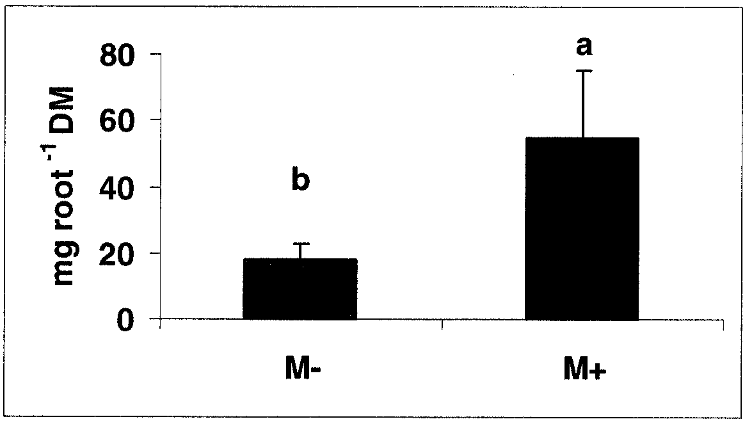
* $p<0.05$; '*MS*' marginally not significant ($0.05<p<0.1$).
Alkamides in shoots were found in traces only.

Figure 2.1 Total phenolic contents in (a) shoots (mg shoot $^{-1}$ DM), (b) roots (mg root $^{-1}$ DM), and (c) the whole plants (mg plant $^{-1}$ DM) of *E. purpurea*, without (M-) or with (M+) AM colonization. Means (shoot n= 5; roots n= 3; and whole plants n= 3) and SEs are shown.

a


mg shoot⁻¹ DM
250
200
150
100
50
0
b
a
M-
M+

b


mg root⁻¹ DM
80
60
40
20
0
b
a
M-
M+

c

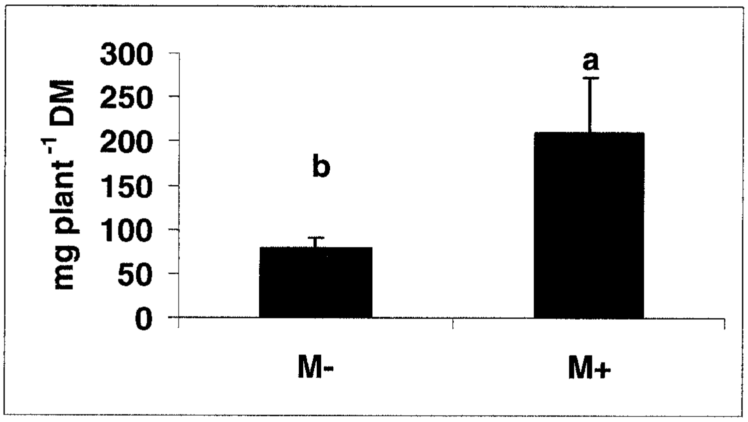
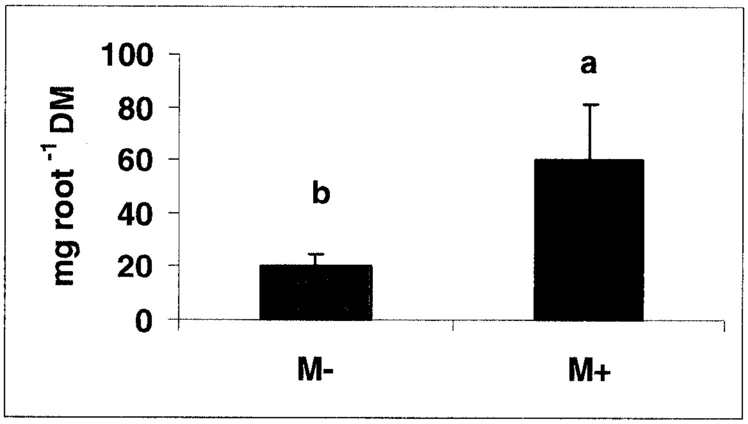
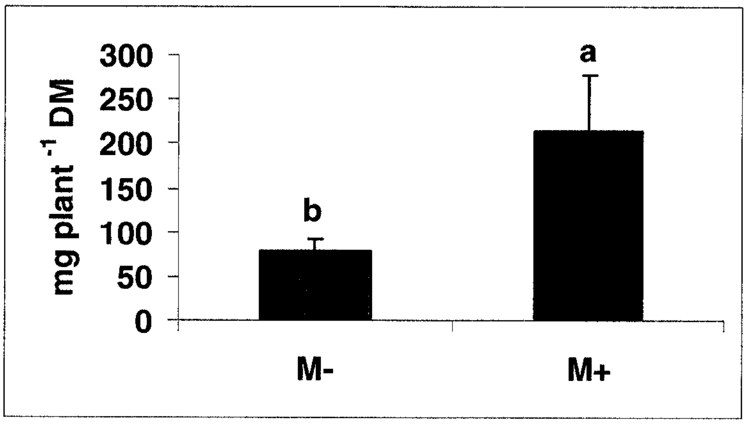

mg plant⁻¹ DM
300
250
200
150
100
50
0
b
a
M-
M+

Figure 2.2 Total phytochemical contents in (a) roots (mg root $^{-1}$ DM), and (b) the whole plants (mg plant $^{-1}$ DM) of *E. purpurea*, without (M-) or with (M+) AM colonization. Means (n= 3) and SEs are shown.

a


100
80
60
40
20
0
mg root -1 DM
b
a
M-
M+

b


300
250
200
150
100
50
0
mg plant -1 DM
b
a
M-
M+

2.5 Discussion

The AM root colonization observed in *Echinacea purpurea* with *Glomus intraradices* reached almost 100%, and most of the colonization structures were present, e.g., hyphae, arbuscules and vesicles. Lata *et al.* (2003) showed in *E. pallida*, under *in vitro* conditions, different levels of root colonization between four AM fungal species, these varying between *Entrophospora colombiana* (29%), *Scutellospora fulgida* (20%), *Glomus mosseae* (77%) and *Gigaspora ramisporophora* (30%). This suggests a preference of some *Echinacea* species for the genus Glomus. Eom *et al.* (2000) and Husband *et al.* (2002) reported that some AM fungal species are not randomly distributed, exhibit a preference for particular hosts, and may differ in their own growth response to different plant species.

Our results showed in *E. purpurea* plants significant increases in all growth parameters with AM root colonization. This agrees with the study of Lata *et al.* (2003) who showed, under *in vitro* conditions, significant increases in growth and development of *E. pallida* when colonized with four AM fungal species. Increased plant growth with AM colonization is believed to result from a greater efficiency of nutrient absorption (Smith and Read 1997), and water uptake, especially under drought conditions (Subramanian and Charest 1998, 1999). Chen *et al.* (2003) indicated that it is the uptake of the slowly diffusing mineral P, through the extraradical mycelium, that enhanced shoot yield in red clover.

Several studies have shown that AM plants are more efficient in taking up soil P than non-AM plants (George *et al.* 1995; Hayman 1980; Smith and Gianinazzi-Pearson 1988; Smith *et al.* 2003). Our results showed significant increases in the content of P and Cu in the shoots of AM plants. These results correspond to the studies of Audet and Charest (2006), Hayman (1980), and Smith and Gianinazzi-Pearson (1988), who indicated that mycorrhizal benefits are not only limited to improve P uptake, but also other minerals such as Zn and Cu. Other studies showed the contribution of AM symbiosis to enhance biomass and mineral uptake in different plant species (Azcón *et al.* 2003; Chen *et al.* 2003; Phelps 2004; Toussaint *et al.* 2004).

We did not detect any significant effect of AM colonization on GS activity in the shoots or roots of *E. purpurea*. These results are in contrast with those of Subramanian and Charest (1998, 1999) who showed an increased GS activity in maize roots colonized by *G. intraradices*. However, the marginally non significant increase of proteins in the AM roots of *E. purpurea* is in accordance with Subramanian and Charest (1998) and Phelps (2004) who showed increased protein concentrations in AM colonized maize and red clover plants, respectively. However, as our study showed increases of root and shoot mass, and of root protein concentration without any significant differences in GS activity, suggests that another N-assimilating pathway may have occurred. To pinpoint which pathway would be the most prominent, further analyses would need to be done. This is consistent with the study of Toussaint *et al.* (2004) who reported, under *in vitro* conditions, a significantly higher GDH activity in AM than non-AM carrot roots, and similar GS or NR activities.

In this study on *E. purpurea*, the highest phenolic levels were detected in shoots, and the highest levels of alkamides in roots. These results agree with Binns *et al.* (2002b) who reported that phenolics are predominantly located in shoots, whereas alkamides are mostly translocated to roots, in all the *Echinacea* species studied. In *E. purpurea,* the major constituents were cichoric and caftaric acids, while the minor components were chlorogenic acid and cynarin (Binns *et al.* 2002 a,b; Miller and Yu 2004). The fact that echinacoside was absent in roots of *E. purpurea*, agrees with the studies of Binns *et al.* (2002a, 2002b). In our study, cynarin was present in shoots and roots at low concentrations, a result that is in contrast with Binns *et al.* (2002 b) who reported the lack of cynarin in the young *E. purpurea* roots. This phytochemical variability among *Echinacea* species may be attributed in part to genetic adaptation (Binns *et al.* 2002a). These authors suggested that different *Echinacea* species produced offspring that varied significantly in their phytochemical defenses according to the geographical source (Binns 2001). Also, Binns *et al.* (2002a) indicated that the seed source, within a single species, influence the overall level of phytochemicals. In addition, Wills and Stuart (2000) highlighted the effect of handling and storage of freshly harvested and dried *E. purpurea* plants on the concentrations of alkamides and cichoric acid. These authors reported that storage conditions have a major effect on retention of these phytochemicals, the optimal conditions being in darkness, at low temperature and relative humidity.

In the present study, the AM colonized roots had significantly increased concentrations of most of the phenolics, especially cynarin and cichoric, caftaric and chlorogenic acids. In addition, due to the increased biomass with AM colonization, the content of these phenolics was significantly increased in roots or shoots of *E. purpurea*. These results correspond to the studies of Lata *et al.* (2003), Fester *et al.* (1999), and Maier *et al.* (1999), who indicated that AM colonization induces the synthesis of secondary compounds (phenolics, cyclohexenone derivatives and blumenin) in roots or shoots of *E. pallida*, barley and wheat, and tobacco, respectively. Toussaint *et al.* (2007) investigated the effect of different AM species on the phytochemical concentrations in shoots of sweet basil. They showed increased concentration of rosmarinic and caffeic acids with *G. caledonium*, and of caffeic acid by *G. mosseae*, without any differences with *G. intraradices*. Copetta *et al.* (2006) reported that *Gigaspora rosea* was the most useful fungus among different fungus species on the growth and essential oil production in sweet basil plant.

In summary, our results showed that the AM colonization significantly increased the shoot and root mass, and most of the phenolics in *E. purpurea*. Increasing the growth of medicinal plants under organic conditions is one of the most important requirements in the natural health product market. From our findings, and to examine the capacity to induce phytochemical production through the use of jasmonic acid, it appeared important to further study the effect of AM colonization in combination with the application of jasmonic acid on the induction of phytochemicals in *E. purpurea*.

Chapter 3

The induction of phytochemicals with AM colonization & Jasmonic acid in *Echinacea purpurea* (L.) Moench

3.1 Introduction

Methyl jasmonate (Me-JA) was first described as the fragrant constituent of the essential oil in *Jasminum grandiflorum* L. (Demole *et al.* 1962 cited in Ulloa *et al.* 2002). The interest in this compound first centered on its fragrant properties that display a strong lemon-like odor. It has become an important ingredient in the perfume industry (Farmer and Ryan 1990; Vick and Zimmerman 1984). More recently, plant physiologists described jasmonic acid (JA) as a plant growth inhibitor, and Me-JA as a senescence-promoting factor; subsequently both compounds were shown to inhibit growth and promote senescence (Ueda and Kato 1980; Vick and Zimmerman 1984). JA is synthesized from an unsaturated fatty acid, linolenic acid, which is released from membrane lipids and then converted to JA by the octadecanoid pathway (Farmer and Ryan 1992; Menke *et al.* 1999; O'Donnell *et al.* 2003).

JA and its volatile methyl ester (Me-JA) are involved in plant development and plant resistance to abiotic and biotic stress factors (Dixon *et al.* 1994; Hause *et al.* 2002; Pena-Cortes *et al.* 2005; Phelps 2004). It was shown that levels of JA increased upon plant wounding (Ryan 2000), or in roots colonized by mycorrhizal fungi (Hause *et al.* 2002; Isayenkov *et al.* 2005), followed by activation of genes involved in plant defense responses such as those coding for proteinase inhibitors or storage proteins (Hause *et al.* 2002; Pena-Cortes *et al.* 2005).

Induced resistance to herbivores (insects) or pathogens (bacteria and fungi) in plants is mediated by the JA pathway (Hause *et al.* 2002; Pena-Cortes *et al.* 2005). Jasmonic acid and its methyl ester have been shown to induce secondary metabolism in a wide range of plant species (Binns *et al.* 2001; Dixon *et al.* 1994; Hause *et al.* 2002; Li and Barz 2006; Peipp *et al.* 1997; Phelps 2004). Hause *et al.* (2002) and Peipp *et al.* (1997) indicated that the increase of secondary compounds occurring in AM barley roots can be induced by JA in non-AM roots. Binns *et al.* (2001) showed that Me-JA induces production of secondary metabolites (alkamides) in *Echinacea pallida* seedlings. Li and Barz (2006) showed, under *in vitro* conditions, that Me-JA is effective in the formation and accumulation of phenolics in *E. purpurea*. Also, Phelps (2004) showed that Me-JA application approximately doubled the isoflavonoid concentration in red clover shoots.

The synthesis of defense compounds is constrained by the availability of environmental resources and the internal trade-off in their allocation between growth and defense (Herms and Mattson 1992). In general, defense allocations increase with environmental stress factors (Chapin *et al.* 1987). In nutrient-rich environments, plants place a priority on growth processes, thus reducing the availability for carbon based secondary defense compounds, e.g. terpenes and phenolics (Bazzaz *et al.* 1987; Kainulainen *et al.* 1996). In contrast, increasing the allocation of resources to chemical and structural defenses resulted in decreasing plant growth (Herms and Mattson 1992).

According to these authors, there is a trade-off between growth and defense as primary and secondary metabolic pathways share common precursors and intermediates. At least 60% of total plant biomass is composed of molecules that give rise to certain amino acid precursors of protein, phenolics, and alkaloids. As such, phenolic and alkaloid syntheses compete with growth for common substrates (Herms and Mattson 1992). Plants of *Pinus sylvestris* L. with well developed mycorrhizas were shown to allocate more carbon to shoot growth and antiherbivore defense due to better water and nutrient uptake (Kainulainen *et al.* 1996).

3.2 Hypothesis and objective

The objective was to determine the effect of AM colonization or JA application alone, and the combination of both on the physiology and content of phenolics and alkamides in plants of *Echinacea purpurea*. It was hypothesized that JA application alone or combined with AM colonization increases the phytochemical content in *E. purpurea*.

3.3 Materials and methods

The detailed methods of growth conditions, root colonization, and HPLC analyses are presented in Chapter 2. Only the changes that were brought here are indicated below.

3.3.1 Sowing conditions

Echinacea seeds (Wildflower Farm, ON, Canada, 2005) put in an aerated container, were incubated (Conviron Incubator, Controlled Environments Limited, Manitoba, Canada) at 5°C and constant light (172 μmol s^{-1} m^{-2}), for two weeks. Then, seeds were put in a beaker with dH_2O for 10 days (water was changed every day), in the greenhouse at 27/23°C (day/night) and a 16-hour photoperiod. The average light intensity was 234 μmol s^{-1} m^{-2}. Pots (12.5 X 12.5 X 15 cm) were filled with vermiculite, where seeds were sown ~ 1 cm deep, and sprayed each day with dH_2O (all pots were stand in water). The germination rate was 86%. Germinated plantlets were transplanted in surface sterilized pots (12.5 X 12.5 X 15 cm), filled with 1000 mL of Promix (Ritchie's Feed and Seed, Ottawa, ON.), and 500 mL of the inoculum or control substrate, and topped with Promix.

3.3.2 Growth conditions and treatments

A factorial (1 plant sp. X 2 M X 2 H X 6 replicates) greenhouse experiment was performed, including *E. purpurea* (L.) Moench, grown with or without the AM fungus, *G. intraradices* Schenck & Smith, and sprayed, or not, with a 100 ppm JA final solution (20 μL/100 mL dH_2O) or a dimethylsulfoxyde (DMSO) solution (20 μL/100 mL dH_2O) as control. In total, 24 pots (with one plant per pot) were prepared. Plants were sprayed eight weeks after germination with 100 mL of the JA or DMSO control solutions once a week for 5 weeks. The JA treated plants were left for 30 min outside the greenhouse to prevent any contamination of the control plants. Plants were grown for 13 weeks after germination.

3.3.3 Statistical analyses

Two way Analyses of Variance (ANOVAs) were performed on all parameters using S-Plus (S-Plus 6.2, Insightful Corp. 2003). Mean comparison analyses were done by Tukey's studentized range test at the 5% level of significance. All data were verified for the assumptions of normality and homoscedasticity. When needed, log or square-root transformations were performed. When the statistical assumptions were not met, non-parametric Kruskall-Wallis analyses were done.

3.4 Results

3.4.1 Mycorrhizal colonization

The AM root colonization levels for *E. purpurea* plants were almost 100% (data not shown). A root was considered colonized with the presence of at least one of any of the fungal structures. All the colonization structures were present, hyphae, arbuscules, vesicles, and spores among all the colonized roots. There was no colonization in the non-AM plants.

3.4.2 Physiological parameters

Fresh and dry mass

According to ANOVA, the AM treatment was significant ($p < 0.05$) on the shoot fresh mass (Table 3.1), and the JA application was marginally non significant ($p = 0.07$)

on the root fresh mass. The AM treatment also significantly increased ($p < 0.01$) the shoot dry mass, especially when JA was applied, by 1.5 times higher (Table 3.1).

Shoot length and number of leaves

The shoot length did not vary among the treatments (Table 3.1). However, the AM colonization significantly increased ($p < 0.01$) the number of leaves, with or without the JA treatment (M+H+ and M+H-), by 1.8 and 1.7 times, respectively.

Table 3.1 Growth parameters of shoots and roots of *E. purpurea* without (M-) or with (M+) AM colonization and/or JA treatment (H). Means (n=6), (SEs) and ANOVA results are shown.

Plant organ	Treatment	FM(g)	DM(g)	Shoot length (cm)	# Leaves
Shoots	M-H-	26	5.7^{ab}	32	11^{b}
		(2)	(0.5)	(2)	(1)
	M+H-	30	6.8^{a}	32	19^{a}
		(1)	(0.2)	(2)	(2)
	M-H+	23	4.7^{b}	31	10^{b}
		(3)	(0.6)	(1)	(2)
	M+H+	29	6.9^{a}	29	18^{a}
		(3)	(0.4)	(2)	(3)
ANOVA:					
M		*	**	NS	**
H		NS	NS	NS	NS
M x H		NS	NS	NS	NS
Roots	M-H-	41	5.4		
		(4)	(0.5)		
	M+H-	45	6.1		
		(4)	(0.5)		
	M-H+	34	4.0		
		(4)	(0.4)		
	M+H+	36	5.4		
		(4)	(0.9)		
ANOVA:					
M		NS	NS		
H		*MS*	*MS*		
M x H		NS	NS		

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test (no letters: $p>0.05$).
* $p<0.05$; ** $p<0.01$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant.

3.4.3 Effect of AM and JA on the phytochemistry of *Echinacea purpurea*

Phenolic and alkamide concentrations in plant material

In shoots (Table 3.2), the JA treatment significantly (ANOVA) increased or tended to increase the concentrations of caftaric ($p < 0.001$) and cichoric ($p < 0.01$) acids, and cynarin ($p = 0.06$), and decreased the chlorogenic acid ($p < 0.01$) concentration. The AM treatment had no significant effect, but the interactions of AM and JA treatments were highly significant ($p < 0.01$) for cichoric acid and cynarin, and significant ($p < 0.05$) for caftaric acid and echinacoside. The Tukey's test indicated that the concentrations of cichoric and caftaric acids, and of cynarin and echinacoside, were significantly higher, by approximately 1.8 times, in non-AM with JA (M-H+) compared to non-AM without JA (M-H-) treated plants. The chlorogenic acid concentrations, in both AM and non-AM plants without JA (M+H- and M-H-), were significantly higher, by 5 and 5.5 times, respectively, than AM with JA treated plants (M+H+).

In roots (Table 3.2), also according to ANOVA, the AM treatment significantly increased the concentration of cynarin ($p < 0.01$) and tended to decrease the concentration of chlorogenic acid ($p = 0.08$), while the JA treatment tended to increase ($p = 0.08$) it. Tukey's test indicated that the concentration of chlorogenic acid in the non-AM plants with JA treatment (M-H+) was significantly higher, by 2.5 times, than in AM plants without JA (M+H-). Also, the concentrations of cynarin in JA treated plants were significantly higher, by 7.1 times, in AM (M+H+) than non-AM (M-H+) plants. There was no significant effect of AM or JA treatment on the concentrations of alkamides.

Table 3.2 Phytochemical concentrations in shoots and roots of *E. purpurea* without (M-) or with (M+) AM colonization and/or JA treatment (H). Means (n=6), (SEs) and ANOVA results are shown.

Plant organ	Treatment	Cichoric[1]	Caftaric[1]	Chlorogenic[1]	Caffeic[2]	Cynarin[2]	Echinacoside[1]	Alkamides[1]
Shoots	M-H-	17.1^{b} (1.4)	6.5^{b} (0.5)	2.33^{a} (0.85)	12 (2)	78^{b} (15)	0.51^{b} (0.04)	
	M+H-	24.8^{ab} (2.4)	8.6^{ab} (0.8)	2.16^{a} (0.50)	11 (2)	112^{ab} (6)	0.67^{ab} (0.10)	
	M-H+	29.3^{a} (2.3)	11.0^{a} (0.5)	1.07^{ab} (0.33)	13 (2)	145^{a} (21)	0.84^{a} (0.13)	
	M+H+	25.2^{ab} (2.7)	10.2^{a} (0.6)	0.42^{b} (0.05)	9.8 (2.2)	103^{ab} (10)	0.59^{ab} (0.05)	
ANOVA:								
M		NS	NS	NS	NS	NS	NS	
H		**	***	**	NS	*MS*	NS	
MxH		**	*	NS	NS	**	*	
Roots	M-H-	13.9 (1.7)	4.2 (0.2)	0.99^{ab} (0.26)	---	57^{ab} (36)		2.5 (0.5)
	M+H-	12.3 (2.0)	3.5 (0.5)	0.49^{b} (0.06)	---	118^{ab} (13)		5.1 (2.1)
	M-H+	11.6 (1.9)	3.8 (0.5)	1.21^{a} (0.22)	---	19^{b} (19)		3.1 (0.5)
	M+H+	12.3 (1.5)	4.2 (0.3)	0.99^{ab} (0.15)	---	135^{a} (14)		4.2 (0.9)
ANOVA:								
M		NS	NS	*MS*		**		NS
H		NS	NS	*MS*		NS		NS
MxH		NS	NS	NS		NS		NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test (no letters: $p>0.05$).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant; '---' trace.

[1]: mg g^{-1} DM; [2]: µg g^{-1} DM.

Phenolic and alkamide contents in plant material

In shoots (Table 3.3), the AM treatment (ANOVA) significantly ($p < 0.05$) increased the content of cichoric and caftaric acids, whereas the JA application ($p < 0.01$) decreased the content of chlorogenic acid, and tended to increase ($p = 0.07$) the content of caftaric acid. Tukey's test indicated that the content of caftaric acid in the AM with JA treated plants (M+H+) was significantly higher, by 1.7 times, than the non-AM without JA plants (M-H-). The content of chlorogenic acid of the AM with JA plants (M+H+) was significantly lower, by 5 times, compared to the AM without JA plants (M+H-).

In roots (Table 3.3), according to ANOVA, the AM treatment significantly ($p < 0.01$) increased the content of cynarin. Tukey's test indicated that the content of cynarin in AM plants with or without JA (M+H+ and M+H-) was higher, by 7.5 and 6.8 times, respectively, than non-AM plants with JA (M-H+). There were no effects of AM colonization or JA application on the content of the other phenolics. The AM treatment tended to increase ($p = 0.07$) the content of alkamides without any effect of the JA treatment.

Table 3.3 Phytochemical contents in shoots (µg or mg shoot^{-1} DM) and roots (µg or mg root^{-1} DM) of *E. purpurea* without (M-) or with (M+) AM colonization and/or JA (H) treatment. Means (n=6), (SEs) and ANOVA results are shown.

Plant organ	Treatment	Cichoric^{1}	Caftaric^{1}	Chlorogenic^{1}	Caffeic^{2}	Cynarin^{2}	Echinacoside^{1}	Alkamides^{1}
Shoots	M-H-	102	39.3 ^{b}	14.9 ^{ab}	77	476	3.0	
		(15)	(6.5)	(6.1)	(15)	(115)	(0.3)	
	M+H-	168	57.9 ^{ab}	14.5 ^{a}	75	759	4.6	
		(17)	(5.4)	(3.4)	(11)	(29)	(0.7)	
	M-H+	148	54.8 ^{ab}	5.4 ^{ab}	66	736	4.4	
		(31)	(9.0)	(2.3)	(11)	(200)	(1.2)	
	M+H+	175	68.4 ^{a}	2.9 ^{b}	68	703	4.0	
		(28)	(5.7)	(0.5)	(17)	(96)	(0.4)	
ANOVA:								
M		*	*	NS	NS	NS	NS	
H		NS	*MS*	**	NS	NS	NS	
MxH		NS	NS	NS	NS	NS	NS	
Roots	M-H-	77	22.9	5.6		365 ^{ab}		13.9
		(12)	(2.7)	(1.6)		(231)		(3.0)
	M+H-	71	19.8	2.9		663 ^{a}		29.2
		(14)	(3.8)	(0.5)		(77)		(12.7)
	M-H+	49	15.2	5.1		98 ^{b}		12.5
		(12)	(2.4)	(1.3)		(98)		(2.4)
	M+H+	67	21.4	5.3		735 ^{a}		21.4
		(17)	(3.3)	(1.3)		(165)		(6.0)
ANOVA:								
M		NS	NS	NS		**		*MS*
H		NS	NS	NS		NS		NS
MxH		NS	NS	NS		NS		NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test (no letters: $p>0.05$).

* $p<0.05$; ** $p<0.01$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant.

^{1}: mg plant^{-1} DM; ^{2}: µg plant^{-1} DM.

Content of phenolics and alkamides in the whole plant

According to ANOVA (Table 3.4), the AM treatment significantly or tended to increase the contents of cynarin ($p < 0.01$), caftaric acid ($p < 0.05$), cichoric acid ($p = 0.08$) and alkamides ($p = 0.07$) in the whole plant. The JA application significantly ($p < 0.01$) decreased the content of chlorogenic acid without any significant differences according to Tukey's test.

Table 3.4 Phytochemical contents in the whole plant (S + R) of *E. purpurea* without (M-) or with (M+) AM colonization and/or JA (H) treatment. Means (n=6), (SEs) and ANOVA results are shown.

Treatment	Cichoric^1	Caftaric^1	Chlorogenic^1	Caffeic^2	Cynarin^2	Echina-coside^1	Alkam-ides^1
M-H-	179	62.2	20.5	77	841	3.00	13.9
	(21)	(8.0)	(5.8)	(15)	(260)	(0.38)	(3.0)
M+H-	239	77.7	17.4	75	1422	4.57	29.2
	(25)	(7.6)	(3.3)	(11)	(100)	(0.72)	(12.7)
M-H+	197	70	10.5	66	834	4.38	12.5
	(34)	(10)	(2.4)	(11)	(290)	(1.24)	(2.4)
M+H+	242	89.8	8.2	68	1438	4.00	21.4
	(33)	(7.4)	(1.2)	(17)	(210)	(0.46)	(6.0)
ANOVA:							
M	*MS*	*	NS	NS	**	NS	*MS*
H	NS	NS	**	NS	NS	NS	NS
MxH	NS	NS	NS	NS	NS	NS	NS

When no letters are shown as $p>0.05$ according to Tukey's Test.
* $p<0.05$; ** $p<0.01$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant.
^{1}: mg plant $^{-1}$ DM; ^{2}: μg plant $^{-1}$ DM.

Total phytochemical contents

According to ANOVA (Table 3.5), the AM treatment tended to increase ($p = 0.07$) the content of total phytochemicals in the shoots and the whole plant, without any effect of JA treatment.

Table 3.5 Total phytochemical contents in shoots (mg shoot^{-1} DM), roots (mg root^{-1} DM) and the whole plant (mg plant^{-1} DM) of *E. purpurea* without (M-) or with (M+) AM colonization and/or JA (H) treatment. Means (n=6), (SEs) and ANOVA are shown.

Treatment	Shoots	Roots	Total
M-H-	160 (27)	119 (18)	279 (36)
M+H-	246 (18)	123 (26)	369 (41)
M-H+	214 (43)	81 (14)	295 (47)
M+H+	251 (34)	115 (21)	366 (42)
ANOVA:			
M	*MS*	NS	*MS*
H	NS	NS	NS
MxH	NS	NS	NS

When no letters are shown as $p>0.05$ according to Tukey's Test.
'*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant.

3.5 Discussion

The AM colonization by *Glomus intraradices* measured in the roots of *Echinacea purpurea* with/or without the JA treatment, was almost 100%. All the colonization structures were present, e.g. hyphae, arbuscules, vesicles and spores. In response to JA application, other studies indicated increases in root colonization levels of spruce (Regvar *et al.* 1997; Regvar and Gogala 1996), or decreases in papaya, cucumber, and red clover (Ludwig-Muller *et al.* 2002; Meixner *et al.* 2005; Phelps 2004).

Several studies have shown in a number of plant species such as *E. pallida*, tomato, red clover, and potato, that JA treatment inhibits plant growth (Binns *et al.* 2001; Farmer and Ryan 1990; Karban *et al.* 1997; Phelps 2004; Ulloa *et al.* 2002). In the present study, our JA treatment (0.44 μM) reduced the fresh and dry mass of roots, without any effect on shoot mass, which was significantly increased by the AM treatment. Our results correspond to those of Ludwig-Muller *et al.* (2002) who showed that a 0.5 μM JA application on *Carica papaya* L., resulted in a slight reduction of root fresh mass. An experiment on *Solanum tuberosum* L., by Ulloa *et al.* (2002), resulted in stem elongation and leaf growth at JA concentrations of 1 and 10μM, with a simultaneous decrease in root length. At higher JA concentrations of 50μM, an inhibition of stem and leaf growth, and a strong impedment of root development resulted; and at 250μM JA, a stunted growth was due to a severe hormone-induced stress. Ulloa *et al.* (2002) suggested that the inhibition of plant growth by JA did not result from cell division reduction, but more likely from inhibition of cell wall polysaccharide synthesis.

In the present study, JA application approximately doubled the concentration of cynarin, echinacoside, and cichoric and caftaric acids of *E. purpurea* in the shoots of non-AM plants. These findings correspond to the study of Hause *et al.* (2002), who indicated in barley that the accumulation of secondary compounds in AM roots is also inducible by JA application in non-AM roots. Pozo *et al.* (2005) reported that application of Me-JA to different plant species such as parsley, rice, and tobacco, induces the enhancement of phenylpropanoids and lipid peroxidation. Binns *et al.* (2001) and Phelps (2004) reported, respectively, an induction of alkamides in roots of *E. pallida*, and isoflavonoids in shoots of red clover, in response to JA application. Tebayashi *et al.* (2000) showed that the accumulation of clovamide (caffeoyl) is increased by JA application in roots of 5-day-old red clover plantlets.

A number of studies have shown that the association of roots with mycorrhizal fungi leads to marked increases of JA levels (Hause *et al.* 2002; Meixner *et al.* 2005; Stumpe *et al.* 2005). Hause *et al.* (2002) showed in barley that root colonization by *G. intraradices* induces the level of endogenous JA, and that AM colonization of barley roots is accompanied by a 5-fold elevation of the JA level. Meixner *et al.* (2005) also reported in soybean that root colonization by *G. mosseae* strongly induces the accumulation of endogenous JA level. Stumpe *et al.* (2005) showed in *Medicago truncatula* a three times higher level of JA in roots colonized by *G. intraradices* than non-AM roots.

In the present study, there were no significant differences in the concentrations of some of the phenolics in shoots between AM and JA treated plants, and they were both higher than the control. This may indicate that these phenolics have been induced by AM colonization, JA application, or both. The level of endogenous JA may have also been enhanced in response to AM colonization, then leading to the increase of phenolics.

In summary, showing that the application of exogenous jasmonate resulted in an increase production of phenolics in *E. purpurea* could be a promising finding. This also suggests that the effect of endo- or exogenous jasmonate could be the same on the phenolic concentrations in AM and non-AM plants. Optimizing the production of phytochemicals in *E. purpurea* could be done by AM colonization and/or JA application. These results, indicating similar effects from AM or JA treatment on the phenolic synthesis, support our hypothesis that JA application alone or in combination with AM colonization increases the production of secondary metabolites. From these results, we further studied over time the synthesis of phytochemicals in *E. purpurea*.

Chapter 4

Induction of phytochemicals in AM colonized *Echinacea purpurea* (L.) Moench over time

4.1 Introduction

Echinacea purpurea is cultivated widely throughout North America and Europe, not only for its beauty, but for its medicinal properties (Barrett 2003). It is one of the top three phytomedicinal plant species used to treat colds (Barrett, 2003; Binns 2001), and for which there is an increasing demand for highest quality and quantity of its products (Bergeron *et al.* 2000). The profile of secondary metabolites may vary with time, space, and plant developmental stage (Percival 2000; Wink 1999). In *Echinacea spp,* few studies have yet reported the time effect on phytochemical production (Binns *et al.* 2002b; Gengaihi *et al.* 1998).

Phenolics, the most widely studied secondary compounds found in all terrestrial plants, have been associated extensively with chemical defence against microbes or defense-like responses to mycorrhizal fungi (Devi and Reddy 2002; Riipi *et al.* 2002). Other studies reported that certain phenolic compounds also have ecological functions such as UV protection and regulation of nutrient cycling (Keinanen *et al.* 1999; Morandi 1996; Riipi *et al.* 2002).

Many studies reported a trade-off between growth and defence (Bazzaz *et al.* 1987; Vrieling *et al.* 1996), whereas in other studies no such trade-off was found (Briggs and Schultz 1990; Mutikainen and Walls 1995; Riipi *et al.* 2002). The chemical defence responses of plants are well documented, and are thought to be metabolically costly (Bazzaz *et al.* 1987). However, Mutikainen and Walls (1995) reported that the cost of defence is not detected in all plant species because the chemical defence production is controlled by resource availability, and as such sensitive to environmental conditions, especially in stressful environments.

Allen (2001) showed that the gradual build-up of phenolics can decrease fungal growth. Phenolic bonds to cell walls, then increasing cell wall strength, were shown to prevent pathogen invasion (Grandmaison *et al.* 1993; Morandi 1996). The AM fungi, by inducing the accumulation of anti-microbial phenolics (flavonoids and isoflavonoids), protect to some extent the mycorrhizal roots from pathogens (Morandi 1996).

In general, root colonization increases over time (Joner and Leyval 2001), but the composition of AM fungi may change over space and time (Husband *et al.* 2002). The fungus then either retreats or is eliminated from older roots, which retain only xylem transport structures in place of living cortical tissues (Allen 2001). Root colonization development can be affected by the level of available nutrients, which could be reduced by root age (Wilson 1984), history of host plant (Husband *et al.* 2002), and soil factors such as pH, nutrient content, moisture and temperature which influence sporal distribution (Husband *et al.* 2002).

The community of AM fungi around the rhizosphere varies widely in response to soil depth, time and host plant (Husband *et al.* 2002; Jakobsen and Nielsen 1983; Simpson and Daft 1990). Percentage colonization fluctuates, AM development shows a lag phase, a period of increasing colonization, and finally a period where the colonization level remains stable. Simpson and Daft (1990) reported that spore production increases with time and reaches a maximum sporal numbers with the host plant maximum growth rate.

4.2 Hypothesis and objectives

This study aimed to determine at different growth times the impact of AM colonization on the physiological parameters and the production of phytochemicals in *Echinacea purpurea*. It was hypothesized that time increases growth and phytochemical content in *E. purpurea*. To test this hypothesis, the objectives were to determine the impact of AM colonization on the biomass, shoot length, number of leaves, and the concentration and content of phenolics and alkamides in *E. purpurea* over time.

4.3 Material and methods

The detailed methods of growth conditions, root colonization, HPLC and statistical analyses are presented in Chapters 2 and 3. Only the changes that were brought here are indicated thereafter.

4.3.1 Sowing conditions

Echinacea seeds (Wildflower Farm, ON, Canada, 2005) were sown as in Chapter 3, except that seeds were put immediately in dH_2O for 10 days in the greenhouse. The average light intensity was 171 μmol s^{-1} m^{-2}. The germination rate was 19%.

4.3.2 Growth conditions and treatments

A factorial (1 plant sp. X 2 M X 5 T X 7 replicates) greenhouse experiment was performed including *Echinacea purpurea* (L.) Moench, grown with or without *Glomus intraradices* Schenck & Smith, and harvested after 4, 6, 9, 12, and 15 weeks after germination. In total, 75 pots (7-8 pots per treatment with 4 plants / pot for the first harvest, 2 plants / pot for the second harvest, and one plant / pot for the subsequent harvests) were prepared.

4.4 Results

4.4.1 Mycorrhizal colonization

Over time, the AM colonization percentages (Table 4.1) increased between 29% to 99%, with some variation. All the colonization structures e.g. hyphae, arbuscules, vesicles, and spores, were observed. There was no colonization in the non-AM plants.

Table 4.1 Colonization and structure percentages (%) in roots of *E. purpurea* over time. Means (n= 5 / plant, 150 root segments / treatment).

Time (weeks)	Colonization	Hyphae	Vesicles	Arbuscules	Spores
4	43	39	8	16	0
6	29	28	7	15	0.6
9	59	55	16	23	1.3
12	99	97	52	25	13
15	82	69	5	23	7

4.4.2 Physiological parameters

According to ANOVA, the growth period significantly increased ($p < 0.001$) the fresh and dry masses (shoot, root, and total), root/shoot ratio, and number of leaves (Tables 4.2.1, 4.2.2, and 4.2.3). The Tukey's test indicated that all these parameters were significantly increased over time from week 4 to 15 in both AM and non-AM treatments. The AM treatment also had significant effects ($p < 0.05$) on all these parameters, and a marginally non-significant effect on shoot length ($p = 0.06$), except shoot dry mass ($p > 0.05$). Even non significant according to the Tukey's test, most of these parameters, over time, tended to be higher in AM than non-AM treatment; shoot fresh mass 9.2 and 9.3 times higher, root fresh mass 21 and 14, total fresh mass 13 and 11, shoot dry mass 15 and 16, root dry mass 45 and 35, total dry mass 18 and 19, root/shoot ratio 3 and 2, shoot length 2.6 and 2.8, and number of leaves 5 and 4, respectively.

Table 4.2.1 Fresh mass (g) of shoots, roots and total plant of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n= 4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Shoots	Roots	Total
4	M-	2.9^{c} (0.2)	1.8^{d} (0.3)	4.7^{e} (0.4)
	M+	3.7^{c} (0.2)	1.9^{d} (0.3)	5.6^{de} (0.4)
6	M-	6.6^{bc} (0.6)	3.6^{cd} (0.5)	10.2^{cd} (1.0)
	M+	9.6^{bc} (1.9)	5.2^{bc} (1.1)	14.8^{bc} (2.9)
9	M-	12.8^{b} (1.6)	10.0^{b} (0.8)	22.8^{b} (2.3)
	M+	15.2^{b} (1.1)	10.8^{b} (1.6)	26.0^{b} (2.1)
12	M-	27.4^{a} (1.8)	19.7^{a} (1.9)	47.1^{a} (2.7)
	M+	29.3^{a} (3.1)	24.9^{a} (2.1)	54.2^{a} (5.0)
15	M-	26.9^{a} (3.8)	26.0^{a} (5.0)	52.9^{a} (8.8)
	M+	34.1^{a} (3.3)	40.1^{a} (8.2)	74.2^{a} (9.7)
	ANOVA:			
	M	**	*	***
	T	***	***	***
	MxT	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.
*$p<0.05$; **$p<0.01$; ***$p<0.001$; 'NS' not significant

Table 4.2.2 Dry mass (g) in shoots (S), roots (R), total plant and R/S ratio of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n=4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Shoots	Roots	Total	R/S
4	M-	0.38^{d} (0.02)	0.08^{e} (0.01)	0.46^{d} (0.01)	0.21^{c} (0.03)
	M+	0.44^{d} (0.02)	0.09^{e} (0.01)	0.53^{d} (0.03)	0.20^{c} (0.02)
6	M-	0.88^{c} (0.12)	0.17^{de} (0.06)	1.05^{c} (0.17)	0.19^{c} (0.03)
	M+	1.38^{bc} (0.28)	0.40^{d} (0.08)	1.78^{bc} (0.33)	0.28^{bc} (0.06)
9	M-	2.28^{b} (0.29)	1.02^{cd} (0.14)	3.30^{b} (0.43)	0.44^{ab} (0.01)
	M+	2.60^{b} (0.20)	1.12^{c} (0.16)	3.72^{b} (0.29)	0.43^{ab} (0.05)
12	M-	5.20^{a} (0.27)	1.84^{bc} (0.22)	7.04^{a} (0.40)	0.35^{b} (0.03)
	M+	5.86^{a} (0.92)	2.66^{ab} (0.46)	8.52^{a} (1.35)	0.45^{ab} (0.02)
15	M-	6.05^{a} (1.05)	2.80^{ab} (0.40)	8.85^{a} (1.40)	0.46^{ab} (0.02)
	M+	6.45^{a} (0.78)	4.02^{a} (0.75)	10.47^{a} (1.43)	0.62^{a} (0.09)
	ANOVA:				
	M	NS	**	**	*
	T	***	***	***	***
	MxT	NS	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.
*$p<0.05$; **$p<0.01$; ***$p<0.001$; 'NS' not significant

Table 4.2.3 Shoot length and number of leaves of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n=6-8), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Shoot length (cm)	# Leaves
4	M-	12.8 ^{e} (1.4)	3.2 ^{e} (0.2)
	M+	15.6 ^{de} (0.4)	3.1 ^{e} (0.1)
6	M-	23.6 ^{cd} (1.5)	4.5 ^{de} (0.3)
	M+	27.8 ^{bc} (1.9)	5.7 ^{cd} (0.5)
9	M-	31.9 ^{ac} (2.3)	6.6 ^{bc} (0.9)
	M+	39.4 ^{ab} (4.6)	8.8 ^{bc} (1.4)
12	M-	42.0 ^{a} (2.6)	13.0 ^{ab} (1.8)
	M+	36.4 ^{ab} (1.5)	14.6 ^{a} (2.2)
15	M-	36.1 ^{ab} (2.5)	11.5 ^{ab} (0.7)
	M+	40.4 ^{a} (4.4)	15.8 ^{a} (1.0)
	ANOVA:		
	M	*MS*	**
	T	***	***
	MxT	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.

**$p<0.01$; ***$p<0.001$; '*MS*' marginally not significant ($0.05<p<0.1$); 'NS' not significant

4.4.3 Phytochemicals (Phenolics & Alkamides)

Concentrations

In shoots (Table 4.3), according to ANOVA, time significantly increased ($p < 0.001$) the concentrations of cichoric acid and echinacoside, and decreased ($p < 0.05$) the concentrations of chlorogenic and caffeic acids, without any significant effects on the concentrations of caftaric acid and cynarin, neither any significant effect of the AM treatment. The time also significantly decreased ($p < 0.001$) the concentrations of alkamides in both AM and non-AM shoots, by 3.4 and 5.4 times lower, respectively.

In roots (Table 4.4), the time also significantly increased ($p < 0.001$) the concentrations of cichoric, caftaric, chlorogenic and caffeic acids, and cynarin (ANOVA). The Tukey's test indicated that some of these phenolics were significantly increased over time in both AM and non-AM roots such as cichoric acid 27.8 and 175 times higher, caftaric acid 12.5 and 6.5, and cynarin 4.7 and 11.7, respectively, between week 4 to 15. There was no significant effect of the AM treatment on the concentrations of phenolics, except of chlorogenic and caffeic acids ($p < 0.05$), but the concentration of cichoric acid at week 12 was significantly higher, by 1.5 times, in AM than non-AM roots. According to ANOVA, the time had a significant effect ($p < 0.01$) on the concentrations of alkamides, however without any significant trends according to the Tukey's test.

Table 4.3 Phytochemical concentrations in shoots of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n= 4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Cichoric^{1}	Caftaric^{1}	Chlorogenic+ Caffeic^{1}	Cynarin^{2}	Echinacoside^{1}	Alkamides^{1}
4	M-	11.7 ^{b} (0.7)	8.9 (0.5)	0.51 (0.09)	235 (36)	0.18 ^{b} (0.02)	1.3 ^{a} (0.1)
	M+	10.9 ^{b} (2.5)	8.2 (1.2)	0.35 (0.05)	168 (54)	0.22 ^{b} (0.02)	1.0 ^{a} (0.2)
6	M-	22.3 ^{ab} (5.8)	9.6 (0.9)	0.45 (0.08)	140 (21)	0.27 ^{ab} (0.03)	0.24 ^{b} (0.04)
	M+	21.4 ^{ab} (2.9)	8.9 (0.6)	0.53 (0.06)	138 (20)	0.24 ^{ab} (0.02)	0.29 ^{b} (0.08)
9	M-	26.8 ^{ab} (2.7)	10.7 (2.7)	0.27 (0.03)	146 (18)	0.27 ^{ab} (0.08)	---
	M+	29.9 ^{a} (1.7)	8.0 (3.4)	0.30 (0.03)	146 (14)	0.34 ^{ab} (0.1)	---
12	M-	33.2 ^{a} (2.3)	11.1 (0.6)	0.39 (0.11)	120 (10)	0.38 ^{ab} (0.03)	---
	M+	33.3 ^{a} (5.5)	9.5 (0.9)	0.25 (0.03)	147 (27)	0.49 ^{a} (0.09)	---
15	M-	30.7 ^{a} (5.3)	8.5 (0.8)	0.39 (0.11)	161 (36)	0.39 ^{ab} (0.04)	---
	M+	34.9 ^{a} (3.5)	9.8 (0.07)	0.46 (0.23)	124 (17)	0.47 ^{a} (0.09)	---
	ANOVA:						
	M	NS	NS	NS	NS	NS	NS
	T	***	NS	*	NS	***	***
	MxT	NS	NS	NS	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test (no letters: $p>0.05$).

* $p<0.05$; ***$p<0.001$; 'NS' not significant

^{1}: mg g^{-1} DM; ^{2}: µg g^{-1} DM; '---' trace.

Table 4.4 Phytochemical concentrations in roots of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n= 4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Cichoric[1]	Caftaric[1]	Chlorogenic+ Caffeic[1]	Cynarin[2]	Alkam-ides[1]
4	M-	0.1^{c} (0.2)	0.6^{d} (0.2)	0.7^{ab} (0.1)	20.9^{c} (1.1)	2.5^{ab} (0.2)
	M+	0.6^{c} (0.1)	0.4^{d} (0.1)	0.4^{b} (0.1)	41.7^{c} (5.7)	2.9^{ab} (0.2)
6	M-	3.5^{bc} (0.7)	2.1^{bc} (0.3)	1.1^{ab} (0.3)	112^{b} (27)	3.2^{a} (0.2)
	M+	3.8^{bc} (1.3)	1.8^{c} (0.6)	0.9^{ab} (0.2)	95^{bc} (23)	3.2^{a} (0.5)
9	M-	7.6^{bc} (1.4)	0.9^{cd} (0.6)	1.8^{a} (0.3)	198^{ab} (32)	2.7^{ab} (0.8)
	M+	5.3^{bc} (0.9)	---	1.2^{ab} (0.2)	127^{ab} (10)	2.3^{ab} (0.1)
12	M-	9.5^{b} (1.9)	2.6^{ab} (0.4)	1.8^{a} (0.4)	154^{ab} (27)	1.9^{b} (0.2)
	M+	14.4^{a} (2.4)	3.6^{ab} (0.4)	1.7^{a} (0.2)	202^{ab} (37)	2.4^{ab} (0.2)
15	M-	17.5^{a} (1.6)	3.9^{ab} (0.2)	1.7^{a} (0.2)	245^{a} (22)	2.0^{ab} (0.2)
	M+	16.7^{a} (2.2)	5^{a} (1)	0.8^{ab} (0.2)	197^{ab} (29)	2.3^{ab} (0.6)
	ANOVA:					
	M	NS	NS	*	NS	NS
	T	***	***	***	***	**
	MxT	NS	NS	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.
* $p<0.05$; **$p<0.01$; ***$p<0.001$; ‘NS’ not significant
[1]: mg g^{-1} DM; [2]: µg g^{-1} DM; ‘---’ trace.

Contents

In shoots (Table 4.5), according to ANOVA, the time significantly increased ($p < 0.001$) the contents of cichoric, caftaric, chlorogenic and caffeic acids, and of cynarin and echinacoside. The Tukey's test indicated that phenolic contents were significantly increased over time in shoots of both AM and non-AM treatments such as cichoric 49 and 40 times higher, caftaric 18 and 15, chlorogenic and caffeic acids 19 and 13, cynarin 11 and 10.6, and echinacoside 30 and 34, respectively. The time significantly decreased ($p < 0.01$) the contents of alkamides in shoots of non-AM plants, by 2.6 times.

Similarly, in roots (Table 4.6), the time significantly increased ($p < 0.001$) the contents of all the phenolics of both AM and non-AM plants. These increases were approximately of 1300 and 500 times higher for cichoric acid, 660 and 190 for caftaric acid, 70 and 90 for chlorogenic and caffeic acids, and 220 and 420 for cynarin, respectively. Time ($p < 0.001$) significantly increased the content of alkamides in both AM and non-AM roots, by 36 times higher. At week 12, the contents of alkamides were significantly ($p < 0.05$) higher, by 1.7 times, in AM than non-AM roots.

Table 4.5 Phytochemical contents in shoots of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n= 4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Cichoric^{1}	Caftaric^{1}	Chlorogenic+ Caffeic^{1}	Cynarin^{2}	Echina-coside^{1}	Alkam-ides^{1}
4	M-	4.4 ^{d} (0.1)	3.4 ^{bc} (0.2)	0.18 ^{d} (0.02)	88.1 ^{d} (12.6)	0.07 ^{c} (0.01)	0.49 ^{a} (0.02)
	M+	4.8 ^{d} (1.1)	3.6 ^{bc} (0.6)	0.15 ^{d} (0.02)	74.1 ^{d} (23.0)	0.10 ^{c} (0.01)	0.43 ^{ab} (0.10)
6	M-	19.2 ^{cd} (6.8)	7.9 ^{b} (1.7)	0.4 ^{cd} (0.1)	109 ^{cd} (15)	0.21 ^{bc} (0.03)	0.19 ^{b} (0.04)
	M+	29 ^{c} (8)	12.5 ^{b} (3.2)	0.7 ^{c} (0.1)	200 ^{cd} (66)	0.35 ^{b} (0.09)	0.36 ^{ab} (0.08)
9	M-	59.5 ^{bc} (7.8)	22 ^{b} (6)	0.6 ^{c} (0.1)	313 ^{bc} (25)	0.66 ^{b} (0.21)	---
	M+	78 ^{b} (8.4)	20 ^{b} (8)	0.8 ^{bc} (0.1)	373 ^{ab} (26)	0.81 ^{b} (0.19)	---
12	M-	175 ^{a} (21)	57.7 ^{a} (4.3)	2.2 ^{a} (0.7)	634 ^{ab} (77)	2.05 ^{a} (0.25)	---
	M+	199 ^{a} (48)	53 ^{a} (6)	1.4 ^{ab} (0.1)	940 ^{a} (293)	3.01 ^{a} (0.78)	---
15	M-	176 ^{a} (25)	50.2 ^{a} (6.4)	2.3 ^{a} (0.7)	936 ^{a} (201)	2.39 ^{a} (0.40)	---
	M+	233 ^{a} (49)	63.1 ^{a} (7.3)	2.8 ^{a} (1.4)	824 ^{a} (170)	3.01 ^{a} (0.56)	---
	ANOVA:						
	M	NS	NS	NS	NS	NS	NS
	T	***	***	***	***	***	**
	MxT	NS	NS	NS	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.

**p<0.01; ***p<0.001; 'NS' not significant

^{1}: mg shoot^{-1} DM; ^{2}: µg shoot^{-1} DM; '---' trace.

Table 4.6 Phytochemical contents in roots of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n= 4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Cichoric[1]	Caftaric[1]	Chlorogenic+ Caffeic[1]	Cynarin[2]	Alkam-ides[1]
4	M-	0.09^{f} (0.03)	0.06^{d} (0.02)	0.05^{c} (0.01)	1.7^{e} (0.2)	0.20^{d} (0.03)
	M+	0.05^{f} (0.02)	0.03^{d} (0.01)	0.04^{c} (0.01)	3.6^{e} (0.6)	0.25^{cd} (0.05)
6	M-	0.8^{ef} (0.3)	0.5^{cd} (0.2)	0.3^{c} (0.2)	23.4^{de} (11.7)	0.67^{cd} (0.23)
	M+	1.6^{de} (0.7)	0.8^{c} (0.3)	0.4^{c} (0.2)	39.2^{d} (11.9)	1.3^{c} (0.4)
9	M-	8.3^{c} (1.9)	0.9^{c} (0.6)	1.9^{ab} (0.6)	214^{c} (50)	2.7^{b} (0.6)
	M+	6.1^{cd} (1.5)	---	1.3^{b} (0.2)	140^{c} (19)	2.6^{b} (0.5)
12	M-	18.6^{bc} (5.3)	4.9^{b} (1.1)	3.7^{a} (1.1)	295^{bc} (75)	3.6^{b} (0.6)
	M+	42^{ab} (14)	9.7^{ab} (2.1)	4.7^{a} (1.4)	583^{ab} (197)	6.4^{a} (1.2)
15	M-	51^{ab} (12)	11.2^{a} (1.9)	4.7^{a} (1.1)	711^{a} (150)	5.4^{a} (0.6)
	M+	65^{a} (14)	19.9^{a} (5.2)	2.8^{ab} (0.2)	786^{a} (192)	9.1^{a} (3.1)
	ANOVA:					
	M	NS	NS	NS	NS	*
	T	***	***	***	***	***
	MxT	NS	*	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.
* $p<0.05$; ***$p<0.001$; 'NS' not significant
[1]: mg root^{-1} DM; [2]: µg root^{-1} DM; '---' trace.

Content of phenolics and alkamides in the whole plant

After combining the content in shoots and roots all together, ANOVA indicated that time significantly ($p < 0.001$) increased all the phenolics and alkamides in both AM and non-AM plants (Table 4.7). The AM treatment had a significant effect ($p < 0.01$) on the content of alkamides, however without any significant trends according to Tukey's test. The contents of phenolics and alkamides were significantly increased over time in both AM and non-AM plants. These increases were approximately of 60 and 50 times higher for cichoric acid, 23 and 18 for caftaric acid, 29 and 30 for chlorogenic and caffeic acids, 20 and 18 for cynarin, 30 and 34 for echinacoside, and 13 and 8 for alkamides, respectively.

Total phenolic contents in shoots, roots and whole plant

The total phenolic contents in shoots, roots, and whole plant (Fig. 4.1 and Table 4.8) were significantly increased ($p < 0.001$) with time in both AM and non-AM plants, without any significant effects of AM treatment.

Table 4.7 Phytochemical contents (mg plant^{-1} DM) in the whole plant (S+R) of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n=4-5), (SEs) and ANOVA results are shown.

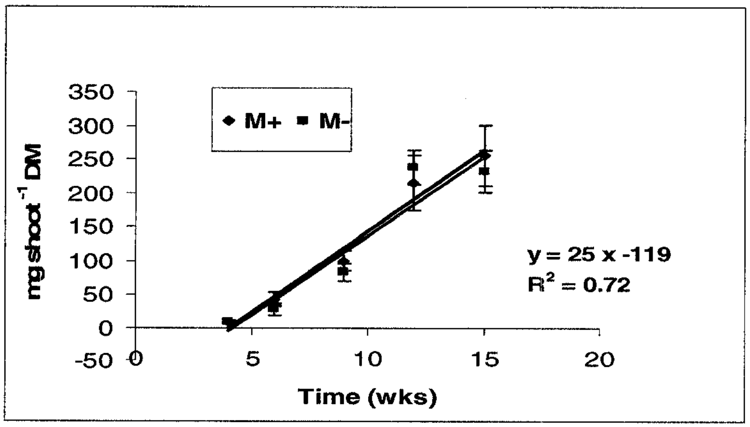
Time (weeks)	Treatment	Cichoric acid	Caftaric acid	Chlorogenic+ Caffeic acids	Cynarin	Echina-coside	Alkamides
4	M-	4.5 ^{d}	3.5 ^{b}	0.23 ^{c}	0.09 ^{cd}	0.07 ^{d}	0.69 ^{c}
		(0.2)	(0.3)	(0.04)	(0.02)	(0.01)	(0.04)
	M+	4.9 ^{d}	3.6 ^{b}	0.19 ^{c}	0.08 ^{d}	0.10 ^{cd}	0.68 ^{c}
		(1.1)	(0.5)	(0.03)	(0.03)	(0.01)	(0.14)
6	M-	20 ^{cd}	8.4 ^{b}	0.7 ^{c}	0.13 ^{cd}	0.21 ^{bd}	0.89 ^{c}
		(7)	(1.8)	(0.2)	(0.01)	(0.03)	(0.12)
	M+	30.6 ^{cd}	13.3 ^{b}	1.1 ^{c}	0.24 ^{bc}	0.35 ^{bc}	1.7 ^{bc}
		(7.7)	(3.2)	(0.1)	(0.07)	(0.09)	(0.4)
9	M-	67.8 ^{bc}	23.9 ^{b}	2.5 ^{bc}	0.53 ^{b}	0.66 ^{bc}	2.7 ^{b}
		(9.3)	(6.3)	(0.6)	(0.06)	(0.21)	(0.6)
	M+	84.1 ^{b}	20.0 ^{b}	2.1 ^{bc}	0.51 ^{b}	0.81 ^{b}	2.7 ^{b}
		(9.3)	(8.4)	(0.2)	(0.04)	(0.19)	(0.5)
12	M-	194 ^{a}	62.6 ^{a}	5.9 ^{ab}	0.93 ^{a}	2.05 ^{a}	3.6 ^{ab}
		(23)	(3.6)	(1.6)	(0.11)	(0.25)	(0.6)
	M+	241 ^{a}	62.7 ^{a}	6.1 ^{ab}	1.52 ^{a}	3.01 ^{a}	6.5 ^{a}
		(54)	(7.5)	(1.4)	(0.41)	(0.78)	(1.1)
15	M-	227 ^{a}	61.4 ^{a}	7.0 ^{a}	1.65 ^{a}	2.38 ^{a}	5.4 ^{ab}
		(33)	(7.9)	(1.2)	(0.31)	(0.40)	(0.6)
	M+	298 ^{a}	83.0 ^{a}	5.6 ^{ab}	1.61 ^{a}	3.01 ^{a}	9.1 ^{a}
		(63)	(12.5)	(1.3)	(0.32)	(0.56)	(3.1)
	ANOVA:						
	M	NS	NS	NS	NS	NS	**
	T	***	***	***	***	***	***
	MxT	NS	NS	NS	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.

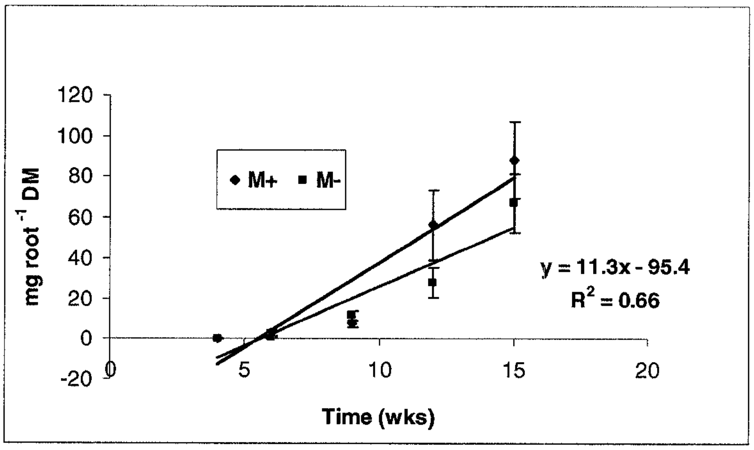
**p<0.01; ***p<0.001; 'NS' not significant

Figure 4.1 Total phenolic contents in (a) shoots (mg shoot^{-1} DM), (b) roots (mg root^{-1} DM), and (c) the whole plant (mg plant $^{-1}$ DM) of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n=4-5) and SEs are shown.

a


350
300
250
200
150
100
50
0
-50
0
5
10
15
20
mg shoot $^{-1}$ DM
Time (wks)
◆ M+ ■ M-
y = 25 x -119
$R^2 = 0.72$

b


120
100
80
60
40
20
0
-20
0
5
10
15
20
mg root $^{-1}$ DM
Time (wks)
◆ M+ ■ M-
y = 11.3x - 95.4
$R^2 = 0.66$

c

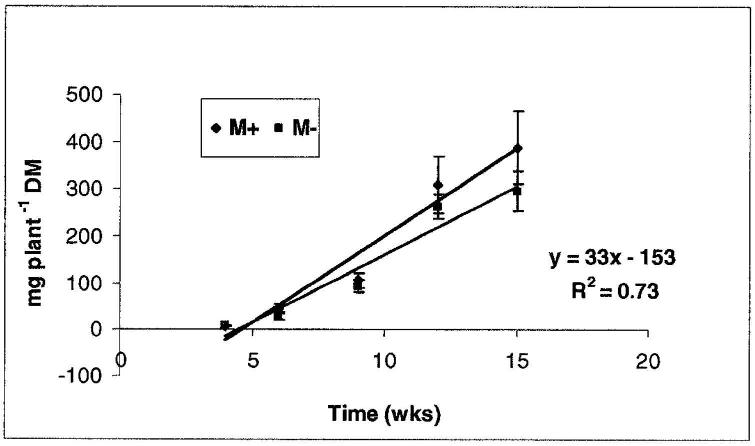

500
400
300
200
100
0
-100
0
5
10
15
20
mg plant $^{-1}$ DM
Time (wks)
◆ M+ ■ M-
y = 33x - 153
$R^2 = 0.73$

Table 4.8 ANOVA results for the total phenolic contents in shoots, roots, and the whole plant of *E. purpurea* over time (T), without or with AM colonization (M).

	Shoot	Root	Total
M	NS	NS	NS
T	***	***	***
M x T	NS	NS	NS

***p<0.001; 'NS' not significant.

Total phytochemical contents

According to ANOVA, the total phytochemical contents (Table 4.9) were significantly increased ($p < 0.001$) with time in both AM and non-AM plants. These increases were approximately of 37 and 27 times higher in shoots, 245 and 183 in roots, and 47 and 34 in whole plants, respectively. Although the AM treatment was not significant, phytochemical contents tended to be slightly higher in AM than non-AM plants.

Table 4.9 Total phytochemical contents in shoots (mg shoot^{-1} DM), roots (mg root^{-1} DM) and the whole plant (mg plant^{-1} DM) of *E. purpurea* over time (T) without (M-) or with (M+) AM colonization. Means (n=4-5), (SEs) and ANOVA results are shown.

Time (weeks)	Treatment	Shoots	Roots	Total
4	M-	8.6^{d} (0.4)	0.4^{e} (0.1)	9.0^{d} (0.5)
	M+	8.1^{d} (1.6)	0.4^{e} (0.1)	8.5^{d} (1.7)
6	M-	28^{cd} (8)	1de (1)	29^{cd} (9)
	M+	43^{c} (11)	4^{cd} (1)	47^{c} (11)
9	M-	83^{bc} (13)	14^{bc} (3)	97^{bc} (14)
	M+	100^{b} (14)	10^{c} (2)	110^{b} (16)
12	M-	238^{a} (25)	31^{b} (8)	269^{a} (27)
	M+	258^{a} (53)	63^{a} (18)	321^{a} (62)
15	M-	232^{a} (31)	73^{a} (15)	305^{a} (42)
	M+	303^{a} (57)	98^{a} (22)	401^{a} (79)
	ANOVA:			
	M	NS	NS	NS
	T	***	***	***
	MxT	NS	NS	NS

Different letters within a column indicate significant differences at $p<0.05$ according to Tukey's Test.

***$p<0.001$; 'NS' not significant

4.5 Discussion

In this study, the mycorrhizal colonization increased from 43% at the 4th week to 82% at the 15th week of plant growth. These results correspond to those of other studies with a number of plant species (Allen 2001; Joner and Leyval 2001; Simpson and Daft 1990). Joner and Leyval (2001) found that colonization levels of *Glomus mosseae* in roots of subterranean clover and maize were 3% after three weeks, 17% and 5% after six weeks, and 33% and 15% after nine weeks of growth, respectively. Allen (2001) showed that root colonization varies rapidly with time for both Indian ricegrass and sagebrush. Simpson and Daft (1990) reported that root colonization levels by *G. clarum* in five plant species increased in general with some variability. In the present study, the highest spore percentage (13%) was noticed at the 12th week. These results correspond with those of Simpson and Daft (1990) who reported that spores of AM fungi are affected by the host plant growth stage. These authors also indicated that the maximum spore numbers in each of the five plant species occurred at their maximum growth rate.

The growth of *E. purpurea* was significantly increased with mycorrhizal colonization over time. This finding corresponds to the studies of Joner and Leyval (2001) and Phelps (2004) whom also showed an increased growth over time of subterranean clover and maize, and red clover when colonized by *G. mosseae* and *G. intraradices*, respectively. Our study also showed that almost all the phytochemical concentrations in roots and shoots of *E. purpurea* increased with plant growth. These results correspond to other studies (Devi and Reddy 2002; Keinanen *et al.* 1999) which reported increases of phenolics over time in shoots of groundnut (*Arachis hypogaea*) and silver birch (*Betula pendula*), respectively. Krishna and Bagyaraj (1984) reported in groundnut that phenolic contents increased rapidly in roots when colonized by *G. fasciculatum* from 30 to 60 days of growth.

Our results are in contrast with those of Binns *et al.* (2002b) who indicated that cichoric and caftaric acids, and cynarin, decreased in older roots of *E. purpurea*. This could result from the differences in plant phenological stages between the studies. In the study of Binns *et al.* (2002b), the plants were between 6 months and more than one year old, where as in our study, the plants were of 15 week old, at their maximum. Also, it could result from genetic variation within and among natural populations of *Echinacea* species (Binns 2001). Genetic variations were reported in other studies on different plant species. Keinanen *et al.* (1999) detected genetic differences in the phenolic chemistry in leaves of *Betula pendula.* Vrieling *et al.* (1996) reported in *Senecio jacobaea,* genetic variation in alkaloid content and relative growth rate.

The alkamide contents were significantly decreased in shoots, and increased in roots, over time. These results correspond to other studies, which indicated that roots of *Echinacea* generally accumulate more alkamides with age (Binns *et al.* 2002b; Gengaihi *et al.* 1998). Binns *et al.* (2002b) suggested that mature aerial tissues (> one year old) of wild *Echinacea* species, translocate their alkamides to roots or slowed down alkamide production over time. Thus, alkamides were predominantly located in roots (Binns *et al.* 2002b). Gengaihi *et al.* (1998) indicated in *E. purpurea*, that alkamide content decreased in shoots as the plant matured, but progressively accumulated in roots, reaching a maximum at the plant fruiting stage. These studies suggested that phenolics and alkamides are synthesized in both shoots and roots, but alkamides are translocated from shoots to roots, or diluted due to the growth factor.

Our results suggested that there was no trade-off between growth and defence. On the contrary, there was a positive correlation between them. These results agree with a number of studies (Briggs and Schultz 1990; Riipi *et al.* 2002; Vrieling *et al.* 1996). Briggs and Schultz (1990) reported that the growth of *Lotus corniculatus* is inexpensive with respect to defense compounds. Riipi *et al.* (2002) reported that accumulation of phenolics, in leaves of *Betula pubescens,* positively corresponds to leaf growth. The cost of different defence compounds may vary with the size, concentration, and construction costs of these compounds, as alkaloids or terpenes are almost twice as costly per gram as that of tannins or lignins (Bazzaz *et al.* 1987; Briggs and Schultz 1990).

In summary, the AM colonization enhanced plant growth over time. The time or growth was the significant factor for enhancement of the content of phytochemicals in *E. purpurea*. Alkamides were found to be dominant in roots, while phenolics were mostly located in shoots. These findings support our hypothesis that phytochemical content increases with plant growth.

General Conclusion

This research thesis investigated the contribution of AM colonization on the physiology and phytochemistry of *Echinacea purpurea*, a widely used medicinal plant species shown to have benefits ranging from the immune system stimulation to skin disease healing. To our knowledge, this is the first study that examined the effect of AM colonization on the primary and secondary metabolism of *E. purpurea*.

The AM colonization enhanced the biomass, the P and Cu contents, the contents of alkamides and most of the phenolics in *E. purpurea*. The highest levels of phenolics were detected in shoots, the alkamides in roots. The mass of roots and shoots of the AM plants and their root protein concentrations were significantly increased, without any significant change of the GS activity, compared to the non-AM plants. These results suggest that nitrogen assimilation may have been increased to some extent in the AM plants, however by another enzymatic pathway. These results support our hypotheses that AM colonization enhances the growth, and the primary and secondary metabolism of *E. purpurea*.

The AM and/or JA treatments approximately doubled the concentrations of some of the phenolics in the shoots of *E. purpurea*. These results point out that these phenolics might be induced by either AM colonization, JA application, or both. They also suggest that the level of endogenous JA may have been enhanced in response to AM colonization, then leading to phenolic increases. Therefore, the effect of endo- or exogenous jasmonate could have been the same on phenolic induction in AM and non-

AM plants. These results support our hypothesis that JA application alone or in combination with AM colonization increases the production of secondary metabolites.

The enhanced plant growth, over time, was the significant factor that has led to increased content of phytochemicals in *E. purpurea*. We showed that phenolics and alkamides are synthesized in both shoots and roots, and alkamides significantly decreased in shoots with plant growth. This may be due to the slowing down in the synthesis of alkamides in shoots, or to their translocation and accumulation in roots as the plant matures. These findings support our hypothesis that phytochemical content increases with plant growth.

There is increasing demand for high quality and quantity of *Echinacea* products. Optimizing the production of phytomedicines by AM fungi may open new avenues for natural health products industry, which requires plants growing under organic conditions. In addition, the perspective of using the AM fungi as biofertilizers to improve crop productivity and increase plant stress tolerance by reducing chemical fertilizer input is of ecological importance. Therefore, the use of AM fungi represents a natural alternative way of promoting the growth of important medicinal plant species.

Studying the effect of different AM fungal species on the growth and the quality and quantity of phytochemicals and even on the production of new phytomedicinal compounds in *E. purpurea* and other species, would be of high interest for future research.

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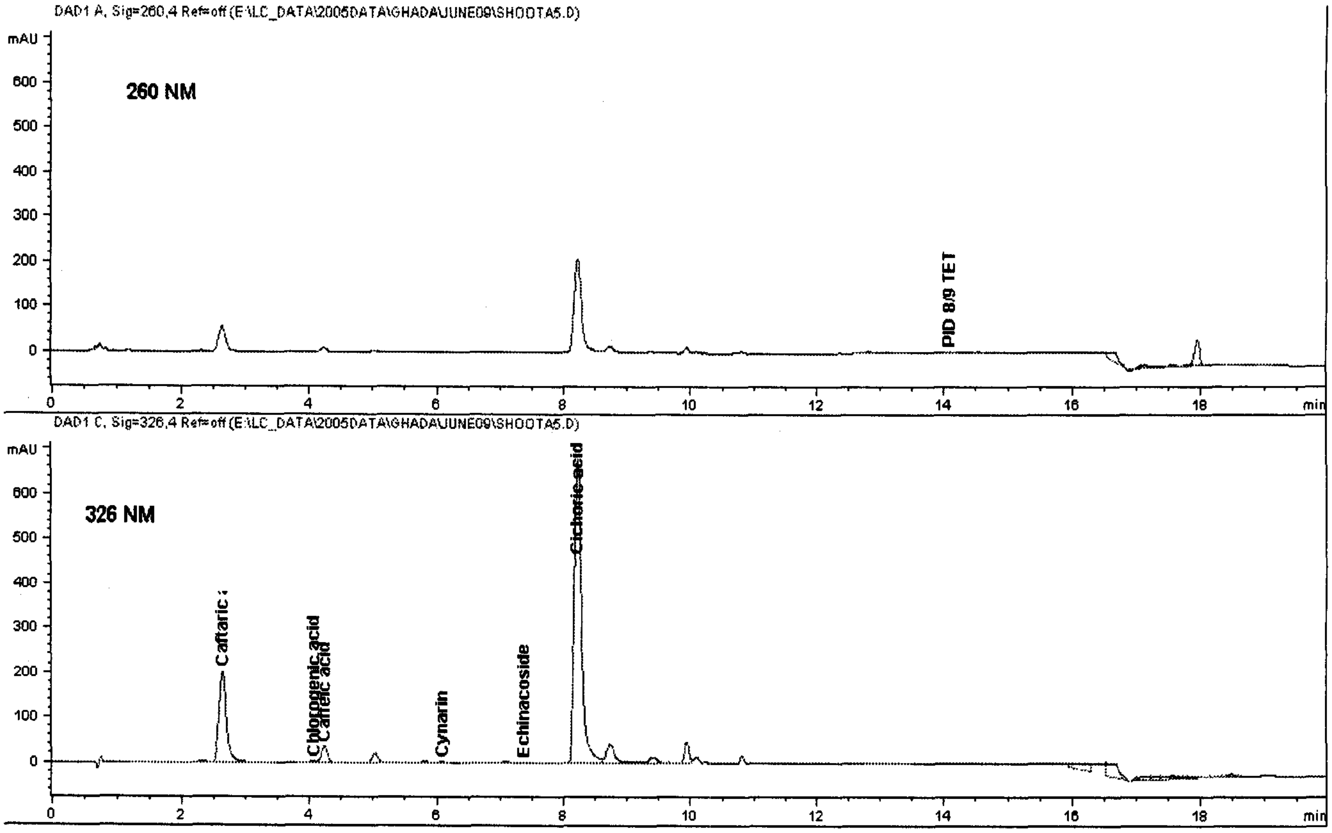
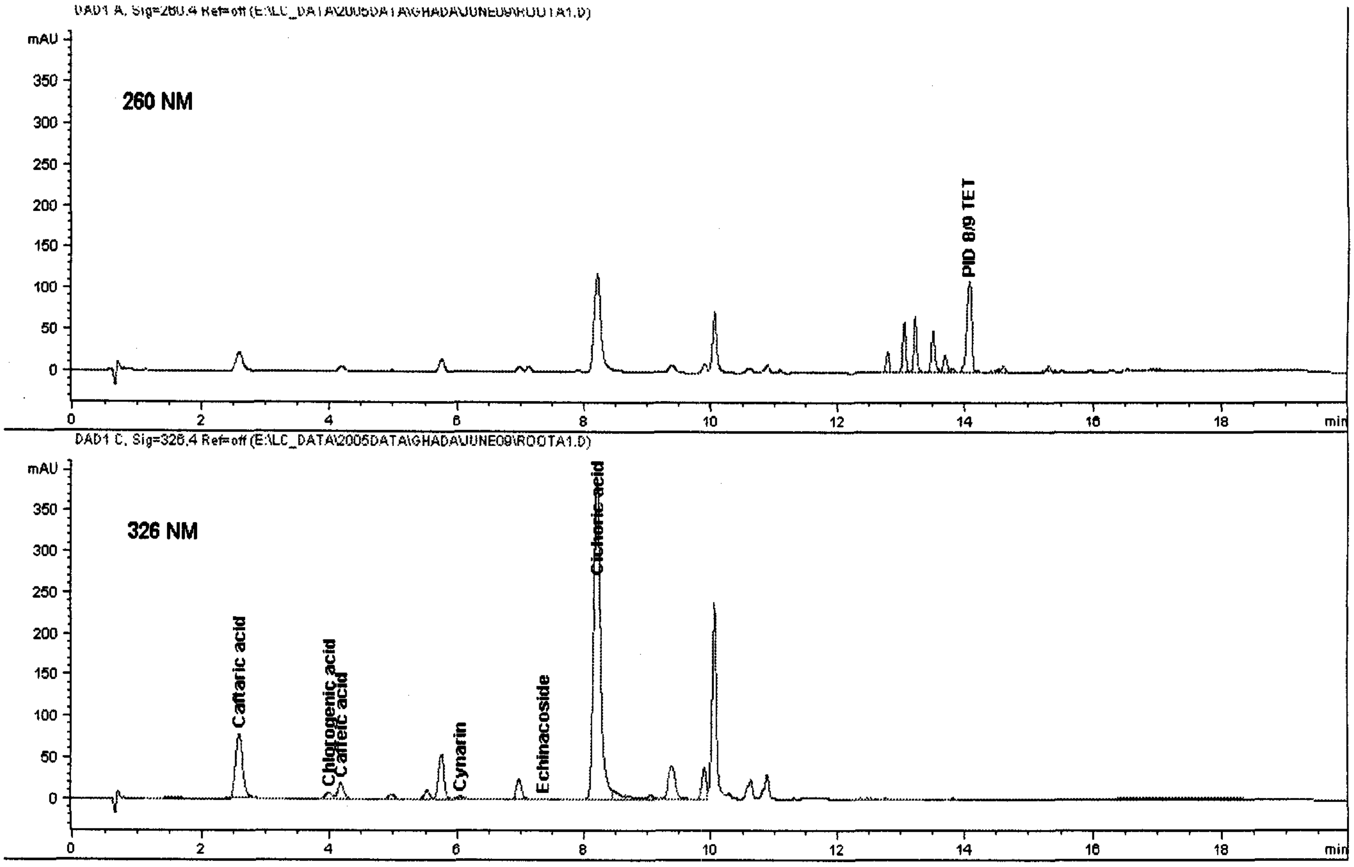
Appendix 1. Chromatogram of the phytochemicals under different wave lengths in *Echinacea purpurea* shoot.



Appendix 2. Chromatogram of the phytochemicals under different wave lengths in *Echinacea purpurea* root.



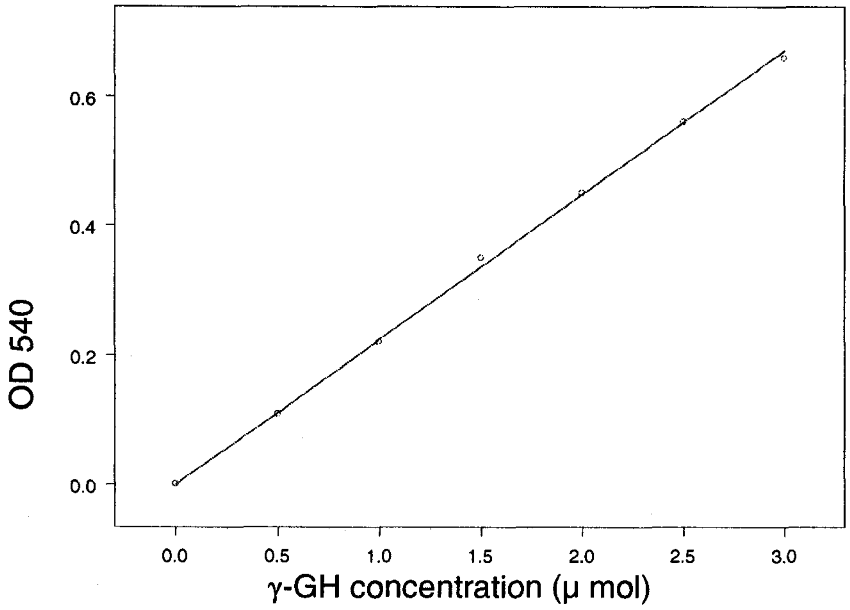
Appendix 3. A standard curve of γ-glutamylhydroxamate (γ-GH) concentration for the determination of glutamine synthetase (GS) activity.

γ – GH conc. (μmol)	OD $_{540}$
0.0	0.00
0.5	0.11
1.0	0.22
1.5	0.35
2.0	0.45
2.5	0.56
3.0	0.66

Regression output

Slope (m)	0.222
R squared	0.999
No. of observation	7
Intercept (b)	0.0036

$$Y = 0.222 (x) + 0.0036$$



Appendix 4. A standard curve of BSA (bovine serum albumin) for the determination of protein concentrations.

BSA conc. (mg mL^{-1})	OD $_{595}$
0.0	0.00
0.2	0.19
0.4	0.36
0.6	0.54
0.8	0.77
1.0	0.87
1.2	0.97
1.4	1.31

Regression output

Slope (m)	0.9385
R squared	0.996
No. of observation	8
Intercept (b)	- 0.0038

$Y = 0.9385 (x) - 0.0038$