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**ISOFLAVONOID INDUCTION AND  
NITROGEN ASSIMILATION IN AM COLONIZED  
RED CLOVER**

**DEBBIE PHELPS**

Thesis submitted to the  
Faculty of Graduate and Postdoctoral Studies  
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## ABSTRACT

This study postulates that the symbiosis between red clover, *Trifolium pratense* L., and the arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* Schenck & Smith, enhances secondary phenolic metabolism and nitrogen assimilation. To test this hypothesis, the four objectives set forth were to determine the impact of AM colonization and/or rhizobial inoculation in red clover on the: 1) growth parameters; 2) nitrogen assimilation, via the activity of glutamine synthetase (GS, E.C. 6.3.1.2), total N content and soluble protein concentration; 3) contents of isoflavonoids (e.g. genistein, daidzein, biochanin A, and formononetin); and 4) contents of these four isoflavonoids over time and with a hormonal factor, jasmonic acid (JA). To accomplish these objectives, four greenhouse experiments were performed. The overall results indicate that the colonized red clover plants, with 22% to 37% of AM root colonization, had enhanced shoot height as well as increased shoot and root dry masses. Co-inoculation with *Rhizobium* enhanced the growth parameters more than inoculation with either microsymbiont alone. The concentrations of micronutrients significantly increased in red clover with AM colonization and/or rhizobial inoculation, while the macronutrients did not vary to any great degree. The GS activity in the shoots and roots of red clover increased with AM colonization, indicating that AM fungi are able to take up and/or assimilate N forms. However, co-inoculation with *Rhizobium* showed that GS was no longer the main N assimilating pathway. A dual N assimilation pathway may likely occur. Growing red clover with AM fungi and/or *Rhizobium* significantly enhanced the isoflavonoid contents in both the shoots and roots. In addition, the JA treatment approximately doubled the concentrations of formononetin, biochanin A and genistein in the shoots. Over time, the

isoflavonoid concentrations increased in the shoots and decreased in the roots, suggesting their translocation. Overall, these results support the hypothesis that AM colonization enhances GS activity and the isoflavonoid contents. The findings of this thesis support the idea of using AM symbioses to promote a natural method of harnessing phytomedicinal compounds such as the isoflavonoids.

## RÉSUMÉ

La présente étude postule que la symbiose entre le trèfle rouge, *Trifolium pratense* L., et le champignon arbusculaire mycorhizien (AM), *Glomus intraradices* Schenck & Smith, stimule le métabolisme secondaire phénolique en plus d'augmenter l'assimilation azotée. Afin de vérifier cette hypothèse, les quatre objectifs suivants ont été établis afin de déterminer l'impact de la colonisation AM et/ou l'inoculation rhizobiale dans le trèfle rouge sur: 1) les paramètres de la croissance; 2) le métabolisme azoté, via l'activité de la glutamine synthétase (GS, E.C. 6.3.1.2) et les contenus en azote total et en protéines solubles; 3) les teneurs en isoflavonoïdes -génistéine, daidzéine, biochanine A et formononétine - et 4) le contenu de ces quatre isoflavonoïdes en fonction du temps et d'un facteur hormonal, l'acide jasmonique (JA). Pour atteindre ces objectifs, quatre expériences en serre ont été réalisées. Les résultats, dans l'ensemble, indiquent que les plants de trèfle rouge, avec une colonisation mycorhizienne de 22% à 37%, avaient des parties aériennes plus grandes ainsi que des masses sèches des parties racinaires et aériennes plus élevées. La co-inoculation avec *Rhizobium* a accru les paramètres de croissance du trèfle rouge plus que l'inoculation avec seulement l'un ou l'autre des microsymbiontes. Les concentrations de microéléments minéraux ont augmenté significativement dans le trèfle rouge avec la colonisation AM et/ou l'inoculation rhizobiale, alors que celles de macroéléments étaient relativement constantes. L'activité de la GS a augmenté dans les feuilles et les racines du trèfle rouge avec la colonisation AM, suggérant que les champignons AM sont aptes à absorber et/ou assimiler des formes azotées. Cependant, la co-inoculation avec *Rhizobium* a montré que la GS n'était plus la principale voie d'assimilation de l'azote. Une double assimilation de l'azote apparaît

vraisemblable. La croissance du trèfle rouge avec le champignon AM et/ou *Rhizobium* a conduit à des augmentations significatives dans les teneurs en isoflavonoïdes dans les parties aériennes et racinaires. De plus, le traitement avec le JA a fait doubler, ou presque, les concentrations de la formononétine, de la biochanine A et de la génistéine dans les parties aériennes. Avec le temps de croissance, les concentrations en isoflavonoïdes ont augmenté dans les parties aériennes et diminué dans les racines, suggérant ainsi leur translocation. Dans l'ensemble, nos données supportent l'hypothèse que la colonisation AM augmente l'activité de la GS et les teneurs en isoflavonoïdes. Les résultats de cette thèse soutiennent la proposition d'utiliser les symbioses AM afin de développer des méthodes naturelles dans la production de composés phytomédicinaux, dont les isoflavonoïdes du trèfle rouge.

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## List of Abbreviations

AM	Arbuscular mycorrhizal
ANOVA	Analysis of Variance
AS	Asparagine synthetase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C/N	Carbon to Nitrogen ratio
CFU	Bacteria colony forming units
CHI	Chalcone isomerase
CHS	Chalcone synthase
D	Dalton
dH <sub>2</sub> O	Distilled water
DM	Dry mass
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
DW	Dry weight
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
<i>K<sub>m</sub></i>	Michaelis-Menten constant
M-	Treatment without mycorrhizae
M+	Treatment with mycorrhizae
MW	Molecular weight
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced form)
NiR	Nitrite reductase
NR	Nitrate reductase
NS	Not significant
OD	Optical density
P	Phosphorus
PAL	Phenylalanine ammonia-lyase
PVLG	Polyvinyl-alcohol-lactic acid-glycerol
R-	Treatment without <i>Rhizobium</i>
R+	Treatment with <i>Rhizobium</i>
SE	Standard error
T	Time
TCA	Trichloroacetic acid
TCD	Thermal conductivity detector

# CHAPTER 1

## INTRODUCTION

Red clover, *Trifolium pratense* L., Fabaceae, is an important phytomedicinal species. Four isoflavonoids (e.g. biochanin A, formononetin, genistein, and daidzein) produced in red clover have been shown to have estrogenic, anti-cancer, and antioxidant properties (Widyarini *et al.*, 2001). A number of studies have investigated the isoflavones daidzein and genistein in soybean (Boue *et al.*, 2001; Kronenberg and Fugh-Berman, 2002; Setchell, 1998). Since soy supplements have shown promise in relieving some menopausal and other symptoms, the demand for supplements rich in isoflavones has recently increased (Bingham *et al.*, 1998; Krenn *et al.*, 2002). Although red clover is one of the richest plant sources of total isoflavones, few studies have investigated them (Ganora, 2002). Moreover, only a few studies have investigated the importance of microsymbionts in the agricultural area of botanical supplements. This thesis makes a significant contribution to our understanding of the effect of rhizobial and fungal symbioses on phytoestrogen expression in red clover.

### 1.1 Mycorrhizal Symbiosis

The symbiotic association between mycorrhizal fungi and plant roots improves the survival and growth of most plants in natural communities (Ibijbijen *et al.*, 1996; Smith and Read, 1997). The word mycorrhiza comes from the Greek words *mukês* and *rhiza* meaning, respectively, fungus and root. Since the discovery of mycorrhizal fungi in 1885, their associations with plants have been well characterized and documented. By

some estimates, mycorrhizal fungi are thought to colonize the roots of 85-90% of the approximately 230,000 species of gymnosperms and angiosperms (Charest *et al.*, 1997; Raina *et al.*, 2000).

Anton de Bary (1887) was the first to use the term symbiosis, the living together of differently named organisms. He considered two classes of symbiosis – parasitic, in which one organism benefits to the detriment of other, and mutualistic, in which all the organisms involved are believed to derive benefits (Raina *et al.*, 2000). The symbiotic relationship between fungus and root is described as mutualistic, where the host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon compounds (Smith and Read, 1997; van der Heijden and Sanders, 2002).

### *1.1.1 Mycorrhizal Associations*

There are two major types of mycorrhizal associations: ectomycorrhizae and endomycorrhizae (Fig. 1.1). Ectomycorrhizae are characteristic of certain groups of trees or shrubs, which dominate boreal, temperate and many sub-tropical forests (Raina *et al.*, 2000). Basidiomycetes and some Ascomycetes are predominantly involved in ectomycorrhizal associations. These associations have a well-defined external fungal sheath around the root. The hyphae grow between the cells of the root cortex forming a structure called the Hartig net (Smith and Read, 1997).

Endomycorrhizae, in which the fungi belong to the class Zygomycetes, are by far more common, occurring in about 80% of all vascular plants (Smith and Read, 1997). A characteristic feature of the endomycorrhizae is the intracellular penetration of root cortical cells by the symbiotic fungi and formation of minute, highly branched, tree-like structures called arbuscules, or swellings called vesicles (Gupta *et al.*, 2000). Endomycorrhizae are commonly referred to as vesicular arbuscular mycorrhizae. The endomycorrhizae also comprise the orchid and ericoid mycorrhizas, forming coils within the cell walls.

Endomycorrhizae of the Order Glomales is subdivided in two suborders, Glomineae and Gigasporineae (Fig. 1.2). Glomineae suborder has two families, Glomaceae to which the genera *Glomus* (used in the present study) and *Sclerocystis* belong, and Acaulosporaceae which include the genera *Acaulospora* and *Entrophospora* (Gupta *et al.*, 2000).

Another group, the ectendomycorrhizae are characterized by the presence of both morphological structures found in ecto- and endomycorrhizae and are characteristics of forest shrubs and small trees (Gupta *et al.*, 2000).

### 1.1.2 Evolution of Mycorrhizae

The mycorrhizal association has evolved as a survival mechanism for both the fungi and higher plants, allowing each to survive in environments of low or high temperatures, poor nutrient soils, periodic drought, diseases, and other natural stresses (Gupta *et al.*, 2000).

The arbuscular mycorrhizal (AM) symbiosis originated at least 400 million years ago

Fig. 1.1 Different types of Mycorrhizae (Raina *et al.*, 2000)

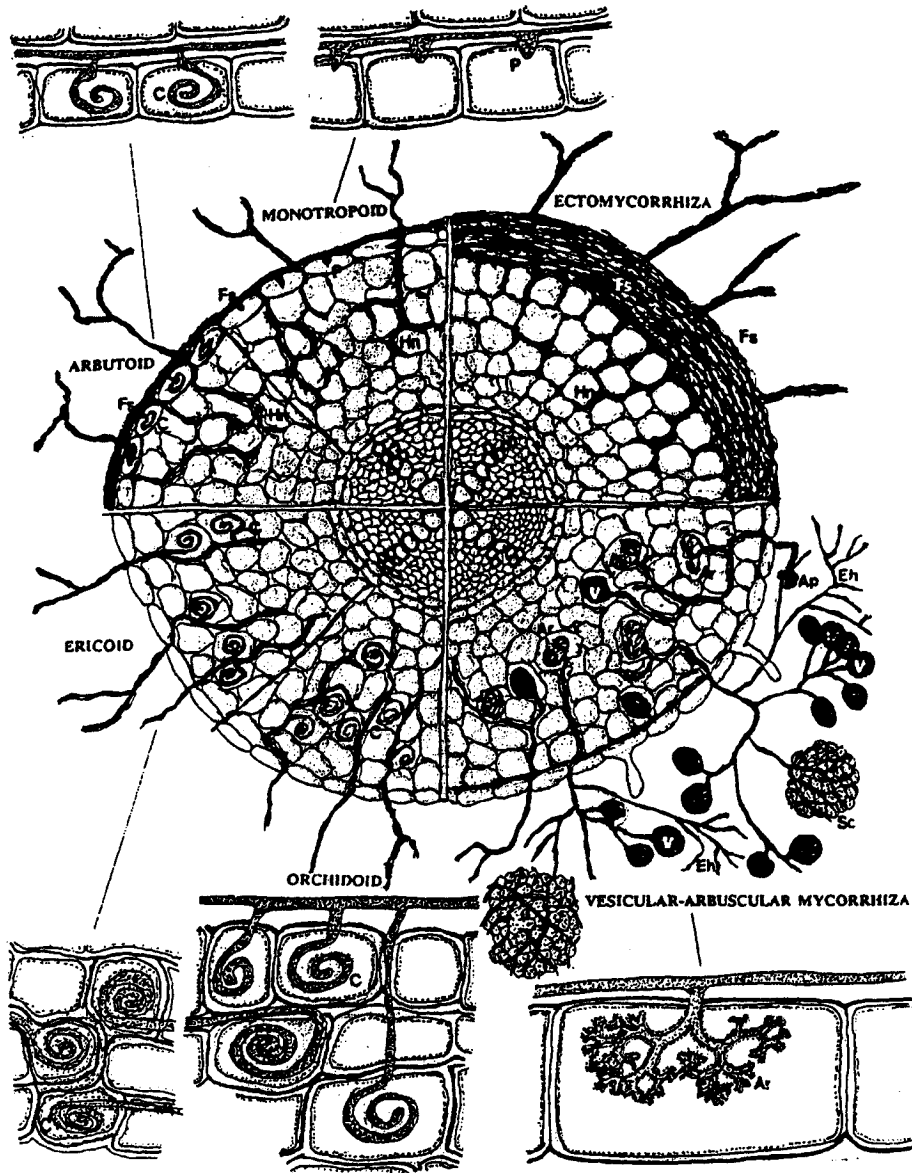
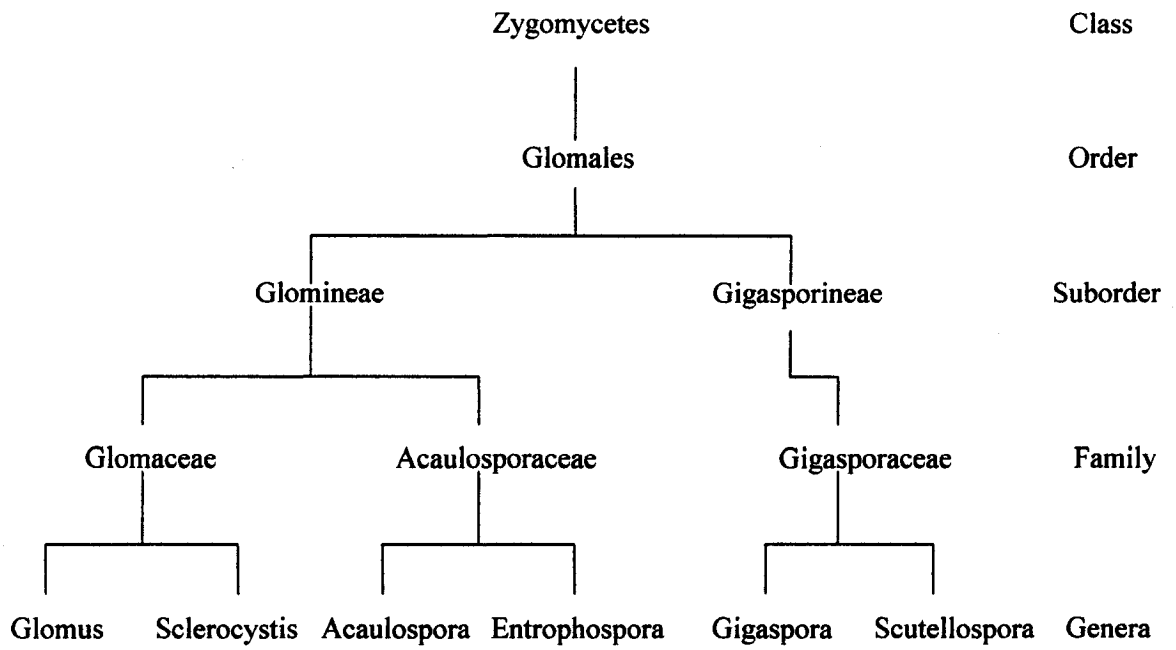


Fig. 1.2 Classification of the Order Glomales (Gupta *et al.*, 2000)



(Devonian period), and over time has become an obligatory association for the endomycorrhizal fungi (Jakobsen *et al.*, 2002; Raina *et al.*, 2000). The evolution of the mycorrhizal fungi has primarily occurred with terrestrial plants and the complexity and diversification of both taxa have occurred simultaneously (Pirozynski and Malloch, 1975).

### *1.1.3 Mycorrhizal Colonization*

Colonization of roots by mycorrhizal fungi (Fig. 1.3) can arise from three sources of inoculum – spores, pre-colonized root fragments, and hyphae. Most commonly, the hyphal network in the soil, together with root fragments, is the main means by which plants become colonized even when significant spore populations are also present (Smith and Read, 1997).

The interaction between both partners begins before the fungus makes physical contact with the plant root. Root exudates and volatiles exuded by the host root enhance spore germination and hyphal morphogenesis (Giovannetti *et al.*, 1994; Phillips, 1992; Vierheilig *et al.*, 1998). Specifically, flavonoids released by plants have been found to stimulate hyphal growth and branching near the root, facilitating fungal contact with the roots of the host plant (Harrier, 2000; Mandelbaum and Piché, 2000; Smith and Read, 1997). Root penetrations occur when one or more hyphae form appressoria (hyphal swellings) between adjacent epidermal cells. The hyphae then enter the hypodermis and branch in the outer root cortex (Smith and Read, 1997). Arbuscules (sites of nutrient

exchange between the two partners) grow within the cortical cells and vesicles (lipid containing bodies) develop to accumulate storage products (Harrier, 2000). The lifespan of an arbuscule is relatively short, only being a few days, after which it collapses and is ingested by the cell, leaving the cell undamaged and capable of hosting another arbuscule (Harrier, 2000). Extensive growth of external mycelium then ensues.

The AM colonization has been shown to play an important role in plant mineral nutrition, especially phosphorus (Johansen and Jensen, 1996; Villegas and Fortin, 2001) and nitrogen (Azcon-Aguilar *et al.*, 1993; Smith and Read, 1997; Toussaint *et al.*, 2004). The symbiotic relationship has also been well characterized for its contribution to plant recovery following physico-chemical stresses such as cold (Paradis *et al.*, 1995) or drought (Subramanian and Charest, 1998 and 1999). AM colonization can also increase the tolerance of root pathogens, and enhance the stability of soils (Dodd, 2000).

## **1.2 Rhizobia**

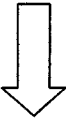
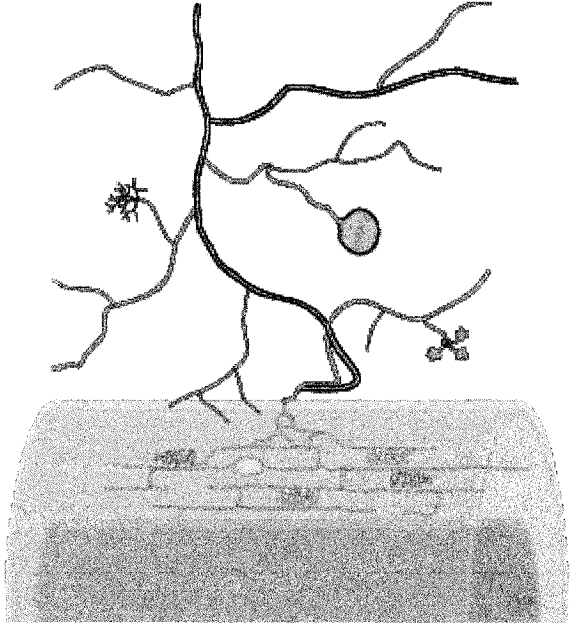
Rhizobia are bacteria that form nitrogen-fixing nodules on legumes. Under nitrogen (N) limiting conditions, the three rhizobial genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia, convert atmospheric nitrogen into ammonia, which is used by plants as a source of N (van Rhijn and Vanderleyden, 1995).

**Fig. 1.3**      **Components of vesicular-arbuscular mycorrhizal association**

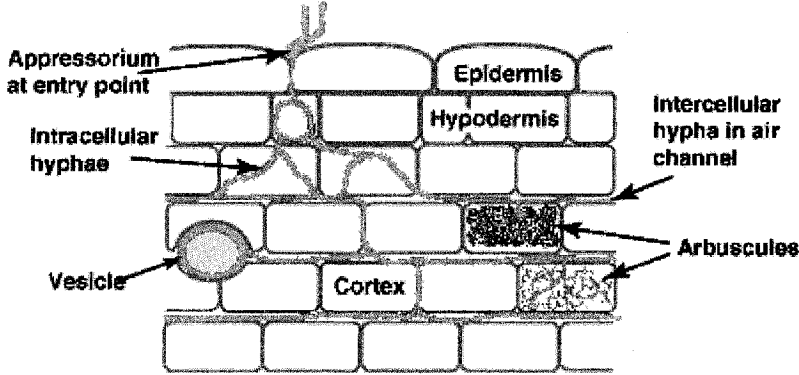
- A**      **Hyphae and spores produced by mycorrhizal fungi in soil**
  
- B**      **Structures formed by AM fungi in colonized roots:  
arbuscules, vesicles, and intracellular hyphae**

**(Brundrett *et al.*, 1996)**

A. External mycelium in soil



B. Mycorrhizal structures in roots



The symbiosis between legumes and rhizobia is not obligatory. However, under N-limiting conditions, the symbionts seek out one another through an elaborate exchange of signals. Flavonoids and isoflavonoids of legumes act in the interaction with rhizobia as specific inducers of bacterial nodulation genes, the *nod* genes - plant genes specific to nodulation (Xie *et al.*, 1995). Specifically, these attractants activate the rhizobial NodD protein, which then induces transcription of the other *nod* genes (van Rhijn and Vanderleyden, 1995). These *nod* genes are involved in the synthesis of Nod factors, a family of lipo-oligosaccharide signal molecules. Nod factors stimulate various responses on host plants, such as root hair deformation, expression of early nodulin genes, mitosis in the root cortex, and the formation of nodule-like structures (Fisher and Long, 1992; Xie *et al.*, 1995).

Root nodules differ in appearance and structure, a trait determined by the host legume. Determinate nodules, such as those that occur in soybean, are round, and have no pronounced meristematic (host cells undergoing active division) region. By contrast, the indeterminate nodules (as in the present study) are elongated with a pronounced meristematic region (Graham and Swenson, 2003). Regions of active atmospheric nitrogen (N<sub>2</sub>) fixation often appear red or pink due to leghemoglobin. Leghemoglobin stores enough oxygen to support nodule respiration but sufficiently low to avoid inactivation of the nitrogenase system (Kawashima *et al.*, 2001). The ammonia produced through N<sub>2</sub> fixation is exported to the host cell, and converted via glutamine, glutamate, and/or aspartate to asparagine. Asparagine is then exported to the shoot (Graham and Swenson, 2003).

There are similarities between mycorrhizal and rhizobial symbioses. Flavonoids and isoflavonoids, metabolites of the phenylpropanoid pathway, are exuded by the roots of the host plant and appear to play a role as early signals for both microsymbionts (Xie *et al.*, 1995). Both the rhizobial and fungal microsymbionts improve the mineral nutrition of the host plant. The nitrogenase of rhizobia fixes  $N_2$  in the nodules (van Rhijn and Vanderleyden, 1995), and fungal hyphae facilitate the uptake of ions, mainly phosphate, in mycorrhizal roots (Johansen and Jensen, 1996; Villegas and Fortin, 2001). When both P and N are limiting factors, AM fungi and rhizobia have been shown to act synergistically since a combined inoculation enhances plant growth and development more than inoculation with either microsymbiont alone (Naqvi and Mukerji, 1998; Xie *et al.*, 1995).

### **1.3 Nitrogen Assimilation**

Nitrogen is the primary limiting element for plant growth in most terrestrial ecosystems (Aerts 2002; Hodge *et al.*, 2001). In the soil, nitrate ( $NO_3^-$ ) is the main form of N available to plants (Oaks, 1994), but ammonium ( $NH_4^+$ ) is also widespread in acidic soils. Once  $NO_3^-$  is absorbed by the plant, it can be assimilated in the root, transported to the shoot, or stored in the vacuole of root or shoot cells (Fernandes and Rossiello, 1995). Nitrogen assimilation includes uptake of nitrate ( $NO_3^-$ ), its reduction to nitrite ( $NO_2^-$ ), the conversion of  $NO_2^-$  to ammonium ( $NH_4^+$ ), and the incorporation of  $NH_4^+$  into amino acids (Subramanian and Charest, 1998). The  $NO_3^-$  entering the plant cell is assimilated in a series of steps (Fig. 1.4) involving the action of four major enzymes: nitrate reductase

(NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT).

### *1.3.1 Enzymes involved in N assimilation*

#### *Nitrate Reductase (NR)*

Nitrate reductase (NR) catalyses the reduction of  $\text{NO}_3^-$  to nitrite ( $\text{NO}_2^-$ ), the first enzymatic, and rate-controlling step of the nitrate assimilation pathway (Oaks and Hirel, 1985; Wray and Fido, 1990). In higher plants, NR is NADH-specific, whereas in fungi the enzyme shows a preference for NADPH as the electron donor (Ahmad and Hellebust, 1991).

#### *Nitrite Reductase (NiR)*

Nitrite reductase (NiR) mediates the reduction of  $\text{NO}_2^-$  to  $\text{NH}_4^+$ . Reduced ferredoxin is the natural electron donor and NiR is located in the chloroplasts of leaves and plastids of roots (Fernandes and Rossiello, 1995).

#### *Glutamine Synthetase (GS)*

Glutamine synthetase (GS) catalyses the formation of glutamine from ammonium and glutamate, at the expense of ATP hydrolysis (Ahmad and Hellebust, 1991; Oaks and Hirel, 1985). It is distributed throughout the plant, however, the activity of the enzyme is higher in the shoot than in the root (Lea *et al.*, 1990). In the shoot, GS is present in the

chloroplasts and cytosol, and in the root, it is located only in the cytosol (Oaks and Hirel, 1985). Because of its abundance, location and low  $K_m$  (concentration of substrate needed to reach half-maximum velocity of the catalytic reaction) for ammonia, the reaction mediated by GS is a major route for the assimilation of ammonia in both leaves and roots (Oaks and Hirel, 1985). The GS protein has a MW of 350 kD (Sivasankar and Oaks, 1996).

#### *Glutamate Synthase (GOGAT)*

Glutamate synthase (GOGAT) catalyses the transfer of glutamine formed by GS to 2-oxoglutarate to yield two molecules of glutamate (Sivasankar and Oaks, 1996). One of the glutamate molecules can then be cycled back as a substrate for the GS reaction. This is the GS-GOGAT cycle as defined by Lea and Mifflin (1974, cited by Sivasankar and Oaks, 1996), and is now widely accepted as the major route of ammonium assimilation in higher plants (Lea *et al.*, 1990; Robinson *et al.*, 1991).

#### *Glutamate Dehydrogenase (GDH)*

Prior to 1970, it was assumed that ammonia was directly incorporated into glutamate via glutamate dehydrogenase (GDH) (Lea *et al.*, 1990). However, GDH has a high  $K_m$  for ammonia, and cannot compete with GS for the available  $\text{NH}_4^+$  (Oaks and Hirel, 1985).

### *Asparagine Synthetase (AS)*

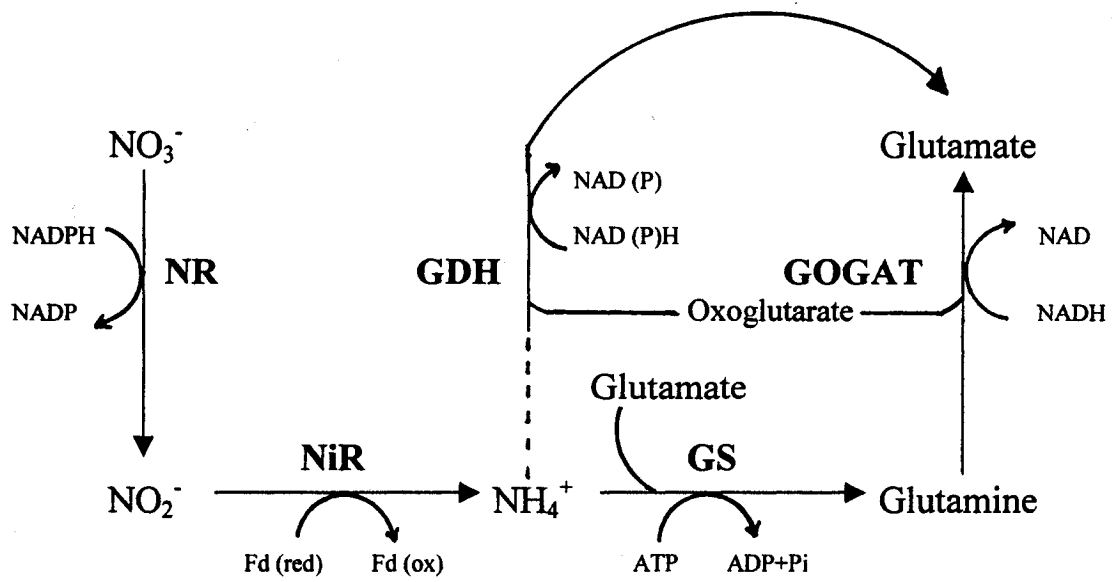
Asparagine synthetase (AS) catalyses the formation of asparagine from glutamine and aspartate, at the expense of ATP hydrolysis. However, AS can also use ammonium instead of glutamine when the level of ammonium is high (Carvalho *et al.*, 2003; Sivasankar and Oaks, 1996). The AS isoforms are found in the cytosol of leaf and root cells and in nitrogen-fixing nodules.

#### *1.3.2 Role of mycorrhizal fungi in nitrogen assimilation*

Mycorrhizal root colonization promotes increased plant nutrient uptake and growth as the hyphae growing beyond the rhizosphere considerably increase the absorptive surface area of the roots (Frey and Schuepp, 1993). Thus, activities of AM mycelium in the soil result in greater efficiency of nutrient absorption (Subramanian and Charest, 1999). Radioisotopic and mesh-compartmented studies have shown that mycorrhizal associations assist the host plant to assimilate greater amounts of soil N than non-mycorrhizal plants (Ames *et al.*, 1984; Frey and Schuepp, 1993; Johansen *et al.*, 1992; Mader *et al.*, 2000; Subramanian and Charest, 1999; Tobar *et al.*, 1994). Specifically, these studies have revealed that the extraradical mycelium of AM fungi can derive radiolabeled <sup>15</sup>N from the soil. In addition, Subramanian and Charest (1998 and 1999) showed that AM colonization of maize stimulated activities of key enzymes involved in N assimilation such as NR, GS, and GOGAT, especially under drought conditions.

**Fig. 1.4 Nitrogen assimilation pathway in higher plants**

**(Oaks and Hirel, 1985)**



Most of the nitrogen found in soils is present in an organic form, often occurring in complex molecules (Hodge *et al.*, 2001). Some plants can directly take up these organic compounds while others take up organic N sources by association with specialist mycorrhizal fungi. This mycorrhizal shortcut in the N cycle has been shown mostly for ectomycorrhizae (about 5% of plant species, all woody), and ericoid mycorrhizae (1% of plant species) (Frey and Schuepp, 1993; Hodge *et al.*, 2001), and AM associations under *in vitro* culture system (Hawkins *et al.*, 2000).

The AM fungi increase uptake of inorganic and immobile resources. The two most important sources of inorganic N for plants and AM fungi are nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) ions (Smith and Read, 1997). Nitrate, however, is mobile in moist soils and diffuses rapidly to the roots. Consequently, a mycorrhizal effect is not expected for this ion, except in dry soils when mobility is reduced (Hodge *et al.*, 2000; Smith and Read, 1997; Subramanian and Charest, 1998, 1999). In contrast,  $\text{NH}_4^+$  is relatively non-mobile, so that even in moist soils depletion zones develop. Mycorrhizal fungi are therefore important in increasing the rate of uptake of this less mobile ion (Hodge *et al.*, 2001; Toussaint *et al.*, 2004).

#### **1.4 Secondary Metabolites**

Plants produce a large, diverse array of organic compounds that appear to have no direct function in growth and development. These substances are known as secondary metabolites. Studies of these substances were pioneered by the early 19<sup>th</sup> and 20<sup>th</sup>

century organic chemists who were interested in these substances because of their importance as medicinal drugs, dyes, flavours, fragrances, insecticides, and poisons (Wink, 1999).

Secondary metabolites also have important ecological functions in plants. These compounds serve as chemical defense against predators, herbivores, microbes or competing plants (Gianinazzi-Pearson *et al.*, 1996; Wink, 1999), and may act as signaling agents in plant-plant, plant-herbivore, and plant-microbe relationships (Mandelbaum and Piché, 2000; Wink, 1999).

#### *1.4.1 Isoflavonoids*

Plant secondary metabolites are divided into three chemically distinct groups: terpenes, phenolics, and nitrogen-containing compounds (alkaloids). One of the largest classes of plant phenolics are the flavonoids (Markham, 1982). The basic carbon skeleton of a flavonoid contains 15 carbons with two aromatic rings connected by a three-carbon bridge (Markham, 1982). These are produced via the phenylpropanoid pathway (Fig. 1.5) catalyzed by chalcone synthase (Dixon *et al.*, 1995; Lambais and Mehdy, 1995). Isoflavonoids are biogenetically related to flavonoids but have a rearranged C<sub>15</sub> skeleton. The isoflavones such as daidzein, formononetin, genistein, and biochanin A, are extremely common and are among the most abundant of the natural isoflavonoid derivatives (Dewick, 1982).

Daidzein (4,7-dihydroxyisoflavone) is insoluble in water and has a molecular formula  $C_{15}H_{10}O_4$  and a MW of 254.24 D. Formononetin (7-hydroxy-4-methoxyisoflavone), known to stimulate germination of AM spores (Elmer, 2002), has a molecular formula  $C_{16}H_{12}O_4$  and a MW of 268.26 D. Genistein (4,5,7-trihydroxyisoflavone) has a molecular formula  $C_{15}H_{10}O_5$  and a MW of 270.24 D. And biochanin A (5,7-dihydroxy-4-methoxyisoflavone), also insoluble in water, has a molecular formula  $C_{16}H_{12}O_5$  and a MW of 284.26 D (Dewick, 1982). Formononetin and biochanin A differ from daidzein and genistein in that they have methoxy groups instead of hydroxyl groups on their aromatic rings.

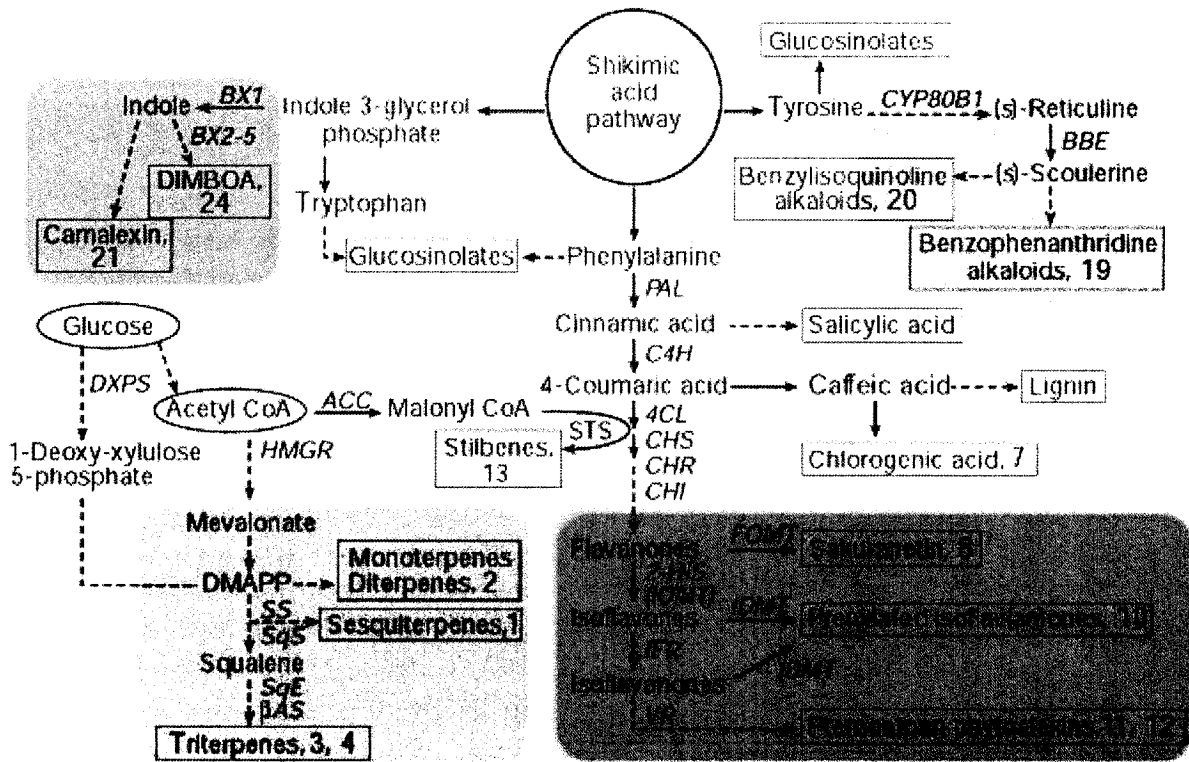
#### 1.4.2 Isoflavonoids and microorganisms

Several studies have observed plant defense-gene expressions in response to AM colonization (Mohr *et al.*, 1998). In most cases, an induction of defense-gene responses (in the roots) was detected at early stages of root colonization followed by suppression at later stages of the symbiosis (Bonfante and Perotto, 1995; Fries *et al.*, 1998; Gianinazzi-Pearson *et al.*, 1996; Kapulnik *et al.*, 1996; Lambais and Mehdy, 1995; Volpin *et al.*, 1994). Defense genes include: encoding enzymes that degrade fungal walls, such as chitinases and glucanases, enzymes involved in the biosynthesis of phytoalexins like phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) (the first enzyme of the flavonoid biosynthetic pathway), and chalcone isomerase (CHI) (Bonfante and Perotto, 1995). The suppression of defense-gene responses coincides with extensive fungal growth within the roots, and the establishment of mycorrhiza (Gianinazzi-Pearson *et al.*,

Fig. 1.5 Biosynthesis of isoflavones from L-phenylalanine.

Pathways leading to flavonoids/isoflavonoids are in blue, alkaloids in pink, indole derivatives in green, and isoprenoids in orange. BBE, berberine bridge enzyme; PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHR, chalcone (polyketide) reductase; CHI, chalcone isomerase; STS, stilbene synthase; FOMT, flavanone 7-*O*-methyltransferase; 2-HIS, 2-hydroxyisoflavanone synthase ('isoflavone synthase'); IOMT, isoflavone 4'-*O*-methyltransferase; IDMT, isoflavone or isoflavanone dimethylallyl transferase; IFR, isoflavone reductase; VR, vestitone reductase; BX1, indole-3-glycerol phosphate lyase; BX2-5, four consecutive cytochrome P450 enzymes of DIMBOA biosynthesis; DXPS, 1-deoxy-xylulose 5-phosphate synthase; ACC, acetyl CoA carboxylase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; SS, sesquiterpene synthase; SqS, squalene synthase; SqE, squalene epoxidase;  $\beta$ AS,  $\beta$ -amyrin synthase.

(Dixon, 2001)



1996). Interestingly, it was also found that inoculation of legumes with nodulating bacteria also suppresses the constitutive synthesis of isoflavonoids (Edwards *et al.*, 1997; Tiller *et al.*, 1994).

In contrast to the above studies, a consistent increase in formononetin levels have been found in AM inoculated roots (Kapulnik, 1996; Volpin *et al.*, 1994, 1995). In addition, Mohr *et al.* (1998) found an increase in CSH mRNA at later stages of mycorrhizal root colonization, while Peipp *et al.* (1996) found a continuous accumulation of the cyclohexenone derivatives with the establishment of a functional barley mycorrhiza. Devi and Reddy (2002) reported that mycorrhizal and *Rhizobium* inoculation resulted in a significant increase in the quantities of phenolic compounds in roots and shoots of groundnut plants, as compared to uninoculated plants. The AM-host plant relationships have also been reported to have deleterious effects upon pathogenic microorganisms (Habte *et al.*, 1999; St-Arnaud *et al.*, 1994) and foliar feeding insects (Gange and West, 1994).

Historically, the presence of isoflavonoids has been associated most closely with pathogenic events in legumes (Dakora and Phillips, 1996). Numerous studies have demonstrated that in pathogenic interactions, distinct changes in plant gene expression take place (Mohr *et al.*, 1998). Jasmonic acid (JA) and its derivatives are hormonal regulators involved in plant responses to abiotic and biotic stresses. In plants, JA is synthesized from linolenic acid, which is released from membrane lipids and then

converted to JA by a series of enzymatic steps. Levels of JA increase upon wounding and eventually activate genes involved in plant defense such as those coding for proteinase inhibitors (Hause *et al.*, 2002). Treatments with JA also affect secondary metabolism in plants (Tebayashi *et al.*, 2000).

## 1.5 Red clover

Red clover, *Trifolium pratense* L., Fabaceae (Fig. 1.6), is an important forage legume grown in temperate regions throughout the world. It originated in southeastern Europe, and was introduced into the United States by European colonists in the late 1600's (Taylor and Quesenberry, 1996). Red clover is a herbaceous, short lived perennial plant. Stems develop from the crown and may vary in height up to 80 centimeters at maturity (Taylor and Quesenberry, 1996). Stems and leaves are densely hairy and leaves are arranged alternately on the stems. The first true leaf is unifoliate with succeeding leaves being trifoliate. Individual leaflets are marked with a shape resembling a whitish V. Flower heads have 75-125 individual pink-violet flowers (Taylor and Quesenberry, 1996). Red clover grows in a wide range of soil and environmental conditions, but not in excessively wet, acidic or shallow soils (Frame *et al.*, 1998). The optimal growth conditions are a pH range of 6.0 - 7.5 and temperature range of 20°C - 25°C (Frame *et al.*, 1998). Red clover is extensively grown for pasturage and hay for livestock and poultry, and has historically been used for treatments such as bronchitis, coughs, eczema, and acne (Duke, 1983).

### 1.5.1 Red clover and its medicinal properties

Clinical trials demonstrating an increased risk of breast cancer, heart attacks, and strokes among women due to hormone replacement therapy (Rossouw *et al.*, 2002) have increased interest in other therapies for menopausal symptoms. Phytoestrogens are one of the possible alternatives.

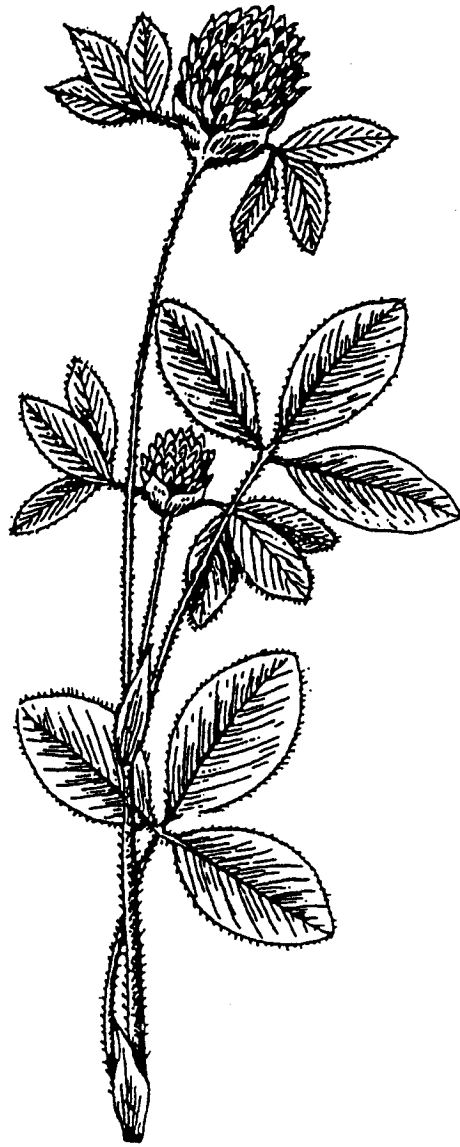
Isoflavones represent one of the three major classes of phytoestrogens, the others being the coumestans and ligans (Aliza and Zecharia, 2002). Phytoestrogens are estrogen-like plant compounds that act similarly to the hormone estrogen, although they are weaker than estrogen itself (Setchell, 1998). Isoflavones have structural similarities to estradiol, the main female hormone, which allows them to interact with estrogen receptors in human cells (Cos *et al.*, 2003; Setchell, 1998). These similarities include polarity, MW, and the distance between the two hydroxyl groups (Ganora, 2002a; Setchell, 1998).

Of the approximately 1000 different isoflavones found in plants, the four isoflavones with strong estrogenic, anti-cancer, and antioxidant properties that are of particular importance to humans are daidzein, formononetin, genistein, and biochanin A (SoyLiving, 2001; Widyarini *et al.*, 2001). All four isoflavones act independently and complement each other.

Red clover contains mainly formononetin and biochanin A glycosides (one or more of the flavonoid hydroxyl groups is or are bound to a sugar), and smaller amounts of daidzen and genistein glycosides (Markham, 1982). In humans, the glycosides are hydrolyzed

Fig. 1.6 Diagram of red clover, *Trifolium pratense* L.

(Marie-Victorin, 1990)



and formononetin and biochanin A are metabolized to daidzein and genistein (Krenn *et al.*, 2002). Approximately two percent of the total dry mass of red clover leaves consists of isoflavones (SoyLiving, 2001). This is about ten times the percentage found in soybean (SoyLiving, 2001). Thus, red clover is an inexpensive and easily available source for the production of isoflavone-rich supplements.

Studies of red clover isoflavones in reducing menopausal symptoms are few and results have varied. Studies by Kronenberg and Fugh-Berman (2002) and Tice *et al.* (2003) showed that red clover isoflavonoids have no significant effect in reducing menopausal symptoms, especially hot flashes. The biological relevance and potency of red clover isoflavones have not yet been well characterized (Aliza and Zecharia, 2002). However, controlled clinical trials have clearly shown that phytoestrogens from red clover help to maintain proper bone density in menopausal women, as well as relieving hot flashes, night sweats (van de Weijer and Barentsen, 2002), and increase blood levels of HDL (good) cholesterol (SoyLiving, 2001).

## 1.6 Hypothesis and Objectives

Red clover was selected for this study for both its ability to host mycorrhizal fungi and its growing interest in the phytomedicinal field for producing isoflavonoids with estrogenic, anti-cancer, and antioxidant properties.

Hypothesis: The symbiosis between red clover, *Trifolium pratense* L., and the mycorrhizal fungus, *Glomus intraradices* Schenck & Smith, enhances nitrogen assimilation and secondary phenolic metabolism.

To test this hypothesis, the objectives of this study were to determine the impact of AM colonization and/or rhizobial inoculation (1<sup>st</sup> exp. only) in red clover on the:

- 1 Growth parameters;
- 2 Nitrogen assimilation, via the activity of glutamine synthetase (GS), and soluble protein content;
- 3 Contents of isoflavonoids, e.g. genistein, daidzein, biochanin A, and formononetin;
- 4 Contents of these four isoflavonoids over time and by induction with a hormonal factor, jasmonic acid (JA).

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Red clover and fungal culture

Four greenhouse experiments were performed in this study. Red clover, *Trifolium pratense* L., variety Iroquois (Richters, Goodwood, ON, Canada), was inoculated, or not, with *Glomus intraradices* Schenck & Smith (DAOM 181602) 1) and/or *Rhizobium leguminosarum* bv. *trifolii* (ATCC 14480), 2) with or without *G. intraradices*, 3) treated or not with jasmonic acid (JA, Sigma-Aldrich, Oakville, ON, Canada), and 4) harvested over time (every 2 weeks for 10 weeks).

#### 2.2 Preparation for germination

Seeds were all surface sterilized twice with 25 mL NaOCl (Clorox bleach) and 75 mL dH<sub>2</sub>O for 5 min on a rotary shaker. Seeds were then rinsed twice with 100 mL dH<sub>2</sub>O.

Percentage germination was determined before the first and second experiments. Twenty seeds were grown in two Petri dishes (10 seeds/dish) lined with Whatman #1 filter paper. The Petri dishes were kept in the dark and moistened daily with dH<sub>2</sub>O. After one week, the number of seeds that germinated was recorded (an average of 70%).

#### 2.3 Preparation of *Rhizobium*

Red clover was pre-inoculated with *R. leguminosarum* bv. *trifolii* (ATCC 14480) in the first experiment. The *Rhizobium* was cultured on an agar plate. One colony from this plate was placed into a 50 mL growth medium that consisted of 0.5g K<sub>2</sub>HPO<sub>4</sub>, 0.2g

MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1g NaCl, 0.4g Yeast Extract, 5 g Mannitol, 15g Agar, and 1 L dH<sub>2</sub>O. The bacterial culture was grown at room temperature on a rotary shaker until the medium turned misty white (approximately 48 hrs). Half of the plants were inoculated with the *Rhizobium* culture (2 mL/ compartment) or given a control treatment with only the medium. Bacteria colony forming units (CFU's) were counted ( $6.9 \times 10^9$  bacteria/mL) using the dilution plate technique.

#### **2.4 Planting conditions**

All trays, pots, and compartments in all four experiments were surface sterilized with Liquinox before planting. Tray, compartment, and pot dimensions were 7200 cm<sup>3</sup>, 198 cm<sup>3</sup>, and 812 cm<sup>3</sup>, respectively.

##### *First Experiment*

A two-way factorial design was performed with red clover inoculated, or not, with *G. intraradices* and/or *R. leguminosarum* bv. *trifolii*. Seeds were planted in trays containing 16 compartments. Nine seeds were planted in each compartment. Ten trays per treatment (M-R-, M-R+, M+R-, M+R+; M: mycorrhizal inoculated + or not - ; R: rhizobial inoculated + or not - ) were planted for a total of 40 trays. Treatments growing with *Rhizobium* received 2 mL of *Rhizobium* culture per compartment 4 days after seeds were sown.

### *Second Experiment*

A one-way experimental design was performed with red clover inoculated, or not, with *G. intraradices*. Seeds were planted in trays containing 12 compartments per tray with 9 seeds in each compartment. Ten trays per treatment (M- or M+) were planted for a total of 20 trays.

### *Third Experiment*

A two-way factorial design was performed with red clover inoculated, or not, with *G. intraradices*, and sprayed, or not, with a jasmonic acid (JA) solution (20  $\mu$ L/100 mL dH<sub>2</sub>O) and dimethylsulfoxide (DMSO, 20  $\mu$ L/100 mL dH<sub>2</sub>O). In total, 15 pots per treatment (M-H-, M-H+, M+H-, M+H+; H: treated + or not - with JA) with 9 seeds per pot were sown. Plants were sprayed two weeks after germination with approximately 50 mL of the JA solution twice a week for 7 weeks. Control plants were sprayed with dH<sub>2</sub>O and DMSO only.

### *Fourth Experiment*

A one-way experimental design was performed with red clover inoculated, or not, with *G. intraradices*. Seeds were planted in trays containing 6 compartments per tray with 9 seeds in each compartment. Five trays per treatment (M+ or M-) were planted for a total of 10 trays. Two compartments from each tray were harvested every other week (starting on week four, ending on week eight). The results presented at week 10 are those from the second experiment.

## 2.5 Soil Medium

In all four experiments, the soil (pH 6.7) consisted of a 1:1 ratio of sand and vermiculite (Ritchie Feed & Seed, Ottawa, ON, Canada). Before planting, both substrates were autoclaved separately for 20 min at 121°C, and 15 PSI. To determine the soil pH, 20 mL of sand and 20mL of vermiculite were mixed with 80mL of dH<sub>2</sub>O and 15mL of ½ Long Ashton nutrient solution (described below). After 30 min, when the sand and vermiculite had settled, the pH was read.

In the first, second, and fourth experiments, compartments were filled with 50 mL vermiculite and 50 mL sand. Then approximately 15 mL of mycorrhizal inoculum or non-mycorrhizal control substrate was added to each compartment. Twenty mL of vermiculite was added to top up each compartment. In the third experiment, pots were filled with 200 mL vermiculite and 200 mL sand. 100 mL mycorrhizal inoculum or non-mycorrhizal control substrate was added to each pot, and 100 mL of vermiculite was added to top up each pot. Non-mycorrhizal treatments were prepared first to prevent contamination.

The mycorrhizal inoculum consisted of *G. intraradices* Schenck & Smith (DAOM 181602) (Mycorhize Pro, Premier Tech, Rivière-du-Loup, QC, Canada). Premier Tech also supplied the non-mycorrhizal control substrate that contained filtrates of the rhizosphere microflora while excluding fungal spores. This control substrate was added to non-mycorrhizal compartments in the same manner as the mycorrhizal inoculum. Mycorhize Pro inoculum was stored at 4°C.

In the first, second, and fourth experiments, all compartments were fertilized with 15 mL of ½ Long Ashton nutrient solution (Hewitt, 1966), once a week. The pots in the third experiment were fertilized with 50 mL once a week. The nutrient solution consisted of 200 mg/L KNO<sub>3</sub>; 175 mg/L K<sub>2</sub>SO<sub>4</sub>; 450 mg/L Ca(NO<sub>3</sub>)<sub>2</sub> · 4H<sub>2</sub>O; 100 mg/L NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O; 250 mg/L MgSO<sub>4</sub> · 7H<sub>2</sub>O; 1.125 mg/L MnSO<sub>4</sub> · 4H<sub>2</sub>O; 0.125 mg/L CuSO<sub>4</sub> · 5H<sub>2</sub>O; 0.15 mg/L ZnSO<sub>4</sub> · 7H<sub>2</sub>O; 1.5 mg/L H<sub>3</sub>BO<sub>3</sub>; 2.5 mg/L NaCl; 0.88 g/L (NH<sub>4</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub> · 4H<sub>2</sub>O (used 0.025 ml/L); and 5.5 g/L EDTA-Fe (used 2mL/L).

## **2.6 Greenhouse conditions**

All plants were grown in the greenhouse at 20 °C and 24 °C (min. nightly temp. and max. day temp., respectively) with daily watering and a 16 h photoperiod. The combined natural and metal halide light conditions were measured using a LiCor L.I.-189 light meter. Average measurements were 1156 μmol/m<sup>2</sup>/s in the light and 183 μmol/m<sup>2</sup>/s in the shade. The locations of the trays and pots in the greenhouse were rotated weekly.

## **2.7 Harvest**

For the first experiment, seeds were planted on March 6<sup>th</sup> (M-R-, and M-R+) and 7<sup>th</sup> (M+R-, M+R+) and harvested between May 27 and May 30, 2002, after 12 weeks of growth. For the second experiment, seeds were planted on January 9<sup>th</sup> (M-) and 10<sup>th</sup> (M+) and harvested on March 20<sup>th</sup> 2003, after 10 weeks of growth. For the third experiment, seeds were planted on February 20<sup>th</sup> (M-H-, M-H+, M+H-, M+H+) and harvested on April 10<sup>th</sup> 2003, after 7 weeks of growth. Finally, for the fourth experiment,

seeds were planted on January 9<sup>th</sup> (M-) and 10<sup>th</sup> (M+) and harvested every two weeks starting on the fourth week for 10 weeks.

For all experiments, the roots of the red clover plants were rinsed in water, to remove excess substrate. Subsequently, the roots were separated from the shoots. The heights of the plants were measured (the tallest plant in each compartment or pot) from the basal knot to the top of the stem, and the fresh mass of the roots and shoots were taken separately. The root section from the first compartment or pot of each tray was used to determine mycorrhizal colonization. In the first experiment, an additional compartment from each tray was used to determine nodulation. These roots were stored in glycerol until bacterial counts were performed under a dissecting microscope.

Fresh roots and shoots were frozen with liquid nitrogen and stored in a -80°C freezer until freeze-dried. This frozen material was lyophilized for 48 hrs at -33 °C, vapor pressure  $3.7 \times 10^{-1}$  mbar in a freeze dryer (Uni-trap Model 10-100, Virtis Inc., Gardiner, N.Y., U.S.). The dry masses were subsequently measured for root and shoot sections.

## **2.8 Root colonization**

Fresh roots were washed, patted dry, and placed in a beaker containing 2.5% KOH. Roots were then heated on a hot plate at 90°C for 20 minutes. After passing the roots under distilled tap water 2-3 times to remove excess KOH and to lower the pH, the roots were then transferred to a 1% HCl solution and left at room temperature for 45 min. The acidified roots were then transferred to an Aniline Blue staining solution (0.5g aniline

blue, 500mL glycerol, 450mL dH<sub>2</sub>O and 50mL 1% HCl) and heated on a hot plate for 5 min. Roots were then placed in a discoloring solution (500mL glycerol, 450mL dH<sub>2</sub>O and 50mL 1%HCl) (Dalpé, 1993). Ten 2 cm-root segments were lined up per slide. A polyvinyl-alcohol-lactic acid-glycerol medium (PVLG) (8.33g PVA, 50mL dH<sub>2</sub>O, 50mL lactic acid, 50mL glycerol) was used as a preservative and the slide was sealed with clear nail polish.

Percentage colonization was determined by counting mycorrhizal structures on the root segments with a light microscope at 100x and 400x magnification. A root section was considered mycorrhizal if it contained any of the structures associated with *G. intraradices* - hyphae, vesicles, arbuscules or spores. One compartment per tray per treatment was used to determine colonization. Twenty root sections were observed for each compartment (two slides).

## **2.9 Enzyme Activity and Protein Concentration Analyses**

### **2.9.1 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). Freeze-dried roots or shoots (0.2g) were ground over ice with sand, 2% PVP, and 5 mL of extraction buffer. The GS buffer, pH 8.0, contained 25 mM Tris, 1 mM EDTA-disodium salt, 1 mM DTT, 1 mM reduced glutathione, 10 mM MgSO<sub>4</sub>, 5 mM glutamate, and 0.01% Triton. The extract was centrifuged at 11 951g for 25 min at 4°C. The reaction was initiated by the addition of 0.25 mL enzyme extract with 0.75 mL reaction mixture. The GS reaction mixture, pH 7.6, contained 15 μmol ATP, 20 μmol MgSO<sub>4</sub>, 5

$\mu\text{mol}$  hydroxylamine,  $60 \mu\text{mol}$  L-glutamate, and  $37.75 \mu\text{mol}$  Tris. After 30 min at room temperature, the reaction was terminated by adding 0.75 mL ferric chloride reagent (4 mL  $\text{FeCl}_3$  10%, 1 mL trichloroacetic acid TCA 24%, and 0.5 mL HCl 6 M in 6.5 mL  $\text{dH}_2\text{O}$ ). After centrifugation for 2 min at 2000g the optical density (O.D.) was measured at 540 nm. The blank, made for each extract, contained all of the above compounds except that the ferric chloride solution was added before the extract to prevent any enzyme reaction. GS was expressed as  $\gamma$ -glutamylhydroxamate (GH) produced  $\text{g}^{-1}$  dry mass  $\text{h}^{-1}$  using a standard curve prepared from different concentrations (0 – 3  $\mu\text{mol}$ ) of  $\gamma$ -GH. The specific GS activity was calculated by dividing the activity of GS by its total soluble protein concentration.

### *2.9.2 Protein concentration*

Soluble proteins were determined according to the micro-method of Bradford (1976). This method involves the binding of Coomassie Brilliant Blue dye to proteins in the extract, causing a differential colour change. The O.D. was read at 595 nm. Each protein extract, 20  $\mu\text{L}$ , was combined with 780  $\mu\text{L}$   $\text{dH}_2\text{O}$  and 200  $\mu\text{L}$  Bio-Rad dye reagent and homogenized by manual inversion. The protein concentrations were measured from the BSA standard curve (0 – 1.2 mg/mL).

## **2.10 HPLC Analyses of Isoflavonoids**

### *2.10.1 Sample preparation*

Isoflavonoids were determined according to the Laboratory method of Dr. J.T. Arnason, University of Ottawa. Dried samples (roots and shoots) were milled to a powder (1 mm)

using a Wiley mill. A portion of each powdered sample (~0.5g) was incubated in 10 mL 99% ethanol plus 3 mL 12 N HCl for 2 hours at 90°C. The acid was partially neutralized by addition of 8 mL of 4 N NaOH, followed by centrifugation and collection of the supernatants. The pellet was re-extracted with 20 mL 99% ethanol, which was pooled with the acidic ethanol fraction. After centrifugation and collection of the supernatants, the extract (1mL) was filtered through a 0.22  $\mu\text{m}$  PTFE membrane prior to injection of 2  $\mu\text{L}$  into the HPLC system.

### *2.10.2 Instrumentation*

The root and shoot extracts were analyzed using a Hewlett-Packard Series 1100 HPLC system (Palo Alto, CA, U.S.) consisting of G1313A Autosampler with 100  $\mu\text{L}$  loop, G1311A Quaternary pump, G1315A Photodiode array detector, G1322A In-line solvent degasser, and ChemStation software (Rev A.08.03). Separations were achieved on a YMC ODS-AM Spherical 3  $\mu\text{m}$  120A, 2.0 mm x 50 mm cartridge (Waters, Mississauga, ON, Canada) at a temperature of 50°C. A solvent gradient system based on solvent A: 0.05% formic acid (pH 3.0) and B: acetonitrile at a flow rate of 0.5 mL / min permitted separation and quantification of the four isoflavonoids.

### *2.10.3 HPLC Analyses*

Standard curves were generated from injection of pure standards (Fluka Inc., Buchs, S.G., Switzerland). Elution times were 5.2, 6.3, 7.3, and 8.7 minutes for daidzein, genistein, formononetin, and biochanin-A, respectively. Peak identities in samples were confirmed

by relative retention times versus the standards. Quantifications were performed at an absorbance of 248 nm.

## **2.11 Mineral Analyses**

### *2.11.1 Carbon and Nitrogen Analyses*

Total C and N contents (%) were analyzed in the G.G. Hatch Isotope Laboratories, University of Ottawa. Approximately 3 mg of dried root or shoot material were sealed in a tin capsule and dropped into a combustion chamber of the elemental analyzer (EA 1100 CHNS, Isomass Scientific, Calgary, AB, Canada) where the samples were combusted at 1800°C. The resulting gases were reduced to N<sub>2</sub> and CO<sub>2</sub>, separated by a gas chromatographic column, and detected by a thermal conductivity detector (TCD), which was then manipulated by data handling software (CE Eager 200).

### *2.11.2 Other Mineral Analyses*

For the analysis of macroelements K, Ca, P, and Mg and microelements Fe, Cu, Mn, and Zn, approximately 5 g per sample of dry root or shoot material were analyzed and performed in Accutest Laboratories Ltd., Ottawa.

Two digestions were carried out. For the macroelements, a wet sulfuric acid digestion on a hot plate, followed by analysis on an ICP/AES (Vista Pro, Spectroflame, Analytical Instruments, 1994) was performed. For the microelements, the dry plant material was reduced to a dry ash, which was then dissolved in acid, and analyzed on the ICP/AES.

## **2.12 Statistical Analyses**

One – or Two-Way Analyses of Variance (ANOVA's) were performed on all parameters using Systat 7 (SPSS, 1997). All data were verified for the assumptions of normality, homogeneity of variances, and independence. When needed, log or square-root transformations were performed. When the statistical assumptions were not met, non-parametric Kruskal-Wallis analyses were done. For all parameters, Tukey's studentized range distributions at the 5 % level of significance were also performed.

## CHAPTER 3

### RESULTS

#### 3.1 Experiments 1&2: AM Colonization with or without *Rhizobium*

##### 3.1.1 Mycorrhizal Colonization and Nodulation

###### *First Experiment*

Mycorrhizal (AM) colonization levels were 32.5% and 22% in treatments M+R- and M+R+, respectively, after 12 weeks of growth. There was no colonization in the non-AM treatments (M-R- and M-R+).

All plants were nodulated with *Rhizobium*; however, non-preinoculated treatments (M-R- and M+R-) were nodulated with approximately half the number of nodules as compared to the preinoculated treatments (M-R+ and M+R+). The numbers of active nodules (+/- standard error) were: M-R- (55.8 +/- 6.95), M+R- (41.8 +/- 10.8), M-R+ (107.7 +/- 11.4), and M+R+ (116.5 +/- 17.4).

###### *Second Experiment*

The AM colonization was 37% in the second experiment after 10 weeks of growth. As in the first experiment, no AM colonization was observed in the non-AM plants.

##### 3.1.2 Physiological Data

###### *Shoot Height*

###### *First Experiment*

The mycorrhizal ( $P < 0.001$ ) and rhizobial ( $P < 0.001$ ) treatments and their interaction ( $P < 0.001$ ) significantly increased the shoot height of the red clover plants. However,

according to the Tukey's test, only the shoot height of the M-R- control plants were significantly lower than the other three treatments by approximately 1.2 times (Fig. 3.1).

### *Second Experiment*

In the second experiment, shoot height was significantly ( $P < 0.001$ ) higher (by approximately 1.2 times) in the M+ plants compared to the M- plants with a difference of approximately 2.5 cm (Fig. 3.2).

## ***Biomass***

### *First Experiment*

Mycorrhizal ( $P < 0.001$ ) and rhizobial ( $P < 0.001$ ) treatments significantly increased both shoot and root dry masses. There were also significant interactions between mycorrhizal and rhizobial treatments in shoots ( $P = 0.001$ ) and roots ( $P = 0.007$ ) (Fig. 3.1). According to the Tukey's test, the combined inoculation with mycorrhizae and rhizobia significantly enhanced (by approximately 1.5 times) the root and shoot dry masses more than inoculation with either microsymbiont alone.

### *Second Experiment*

Both shoot and root dry masses were significantly ( $P < 0.001$ ) higher in the M+ plants compared to the M- plants by approximately 1.4 and 1.3 times, respectively (Fig. 3.2).

Fig. 3.1      Shoot height, shoot dry mass, and root dry mass (n = 160) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .

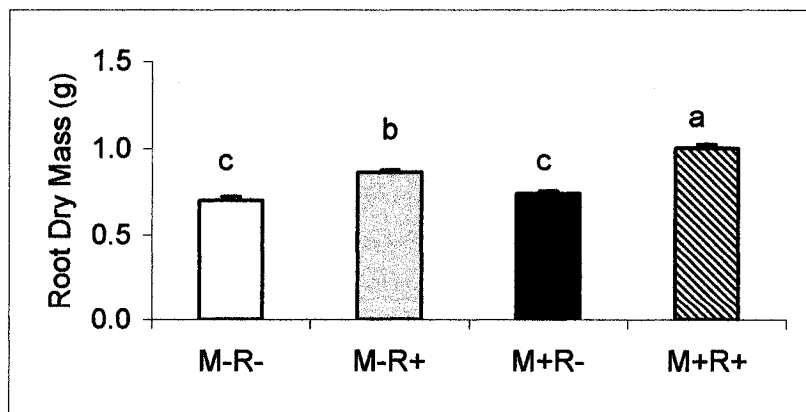
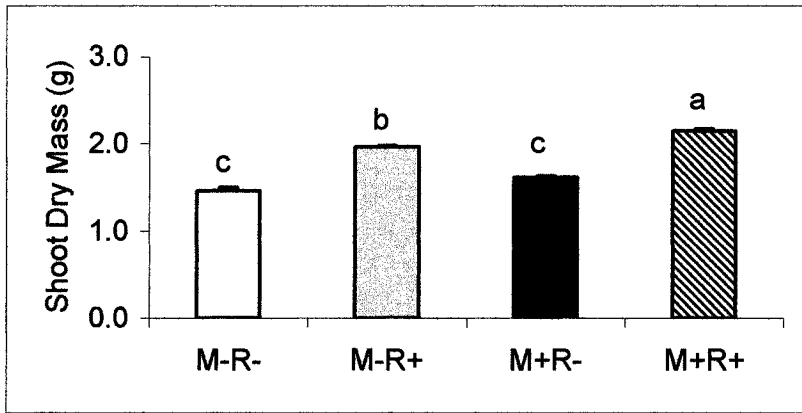
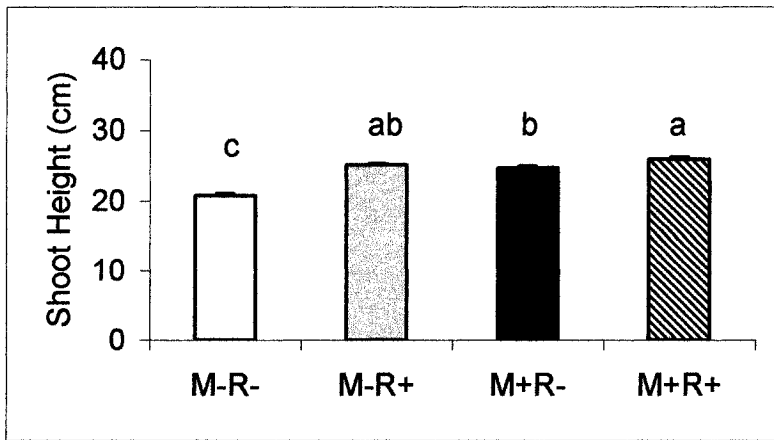
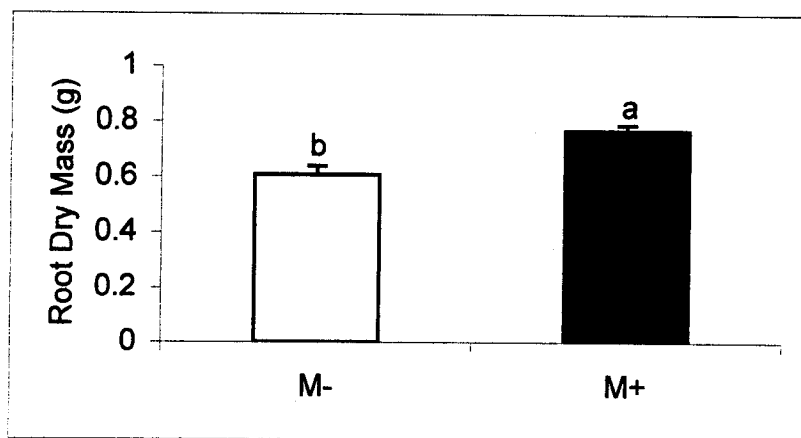
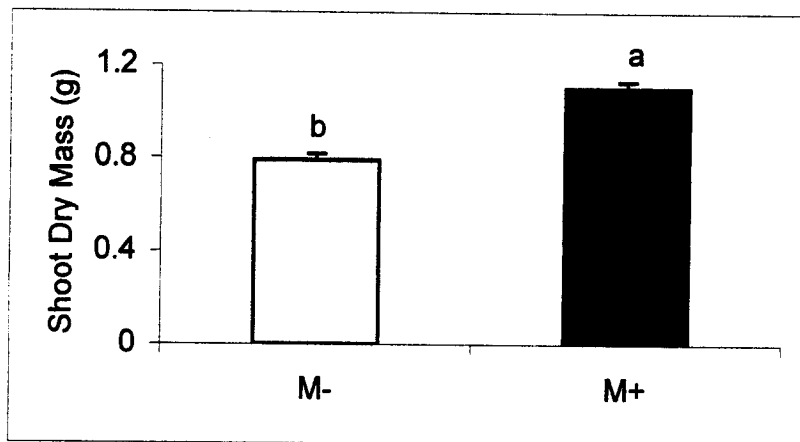
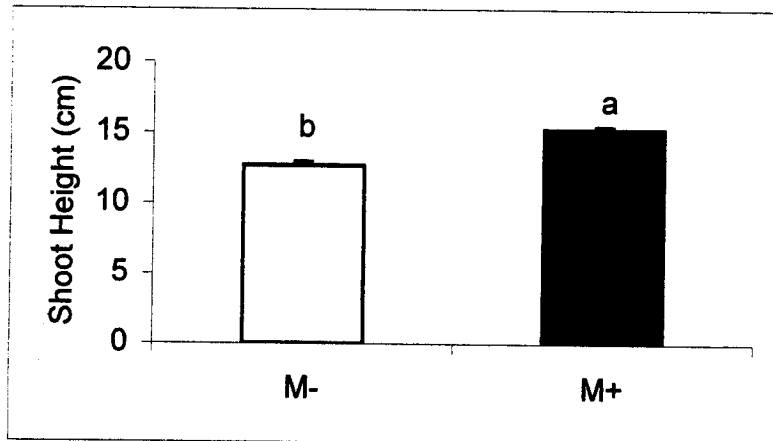


Fig. 3.2      Shoot height, shoot dry mass, and root dry mass (n = 24 to 79) of red clover with (M+) or without (M-) AM colonization. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .



### 3.1.3 Glutamine Synthetase (GS)

#### *First Experiment*

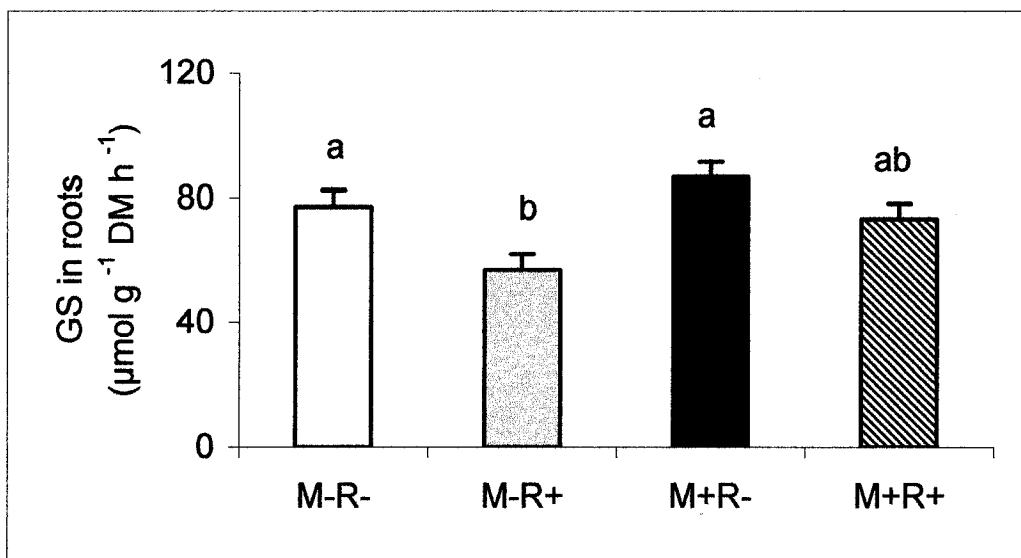
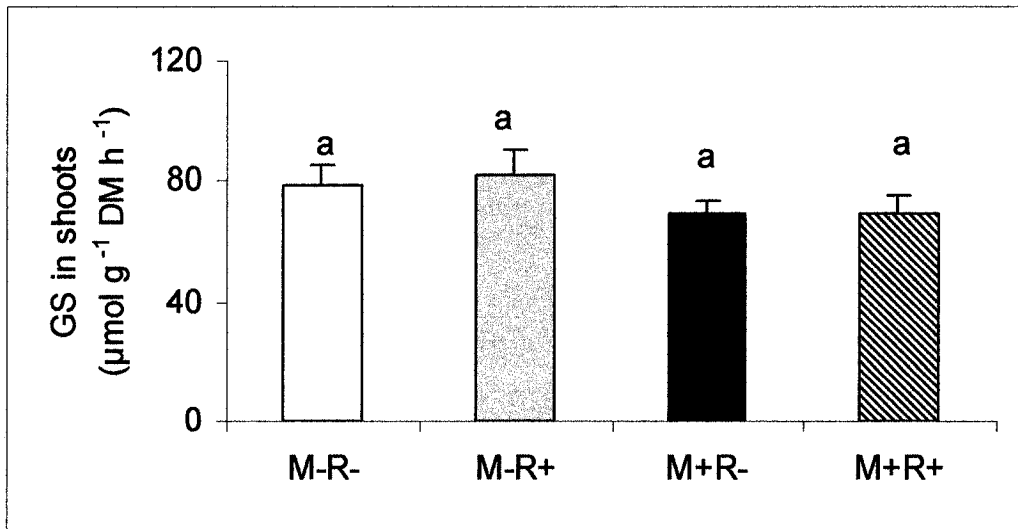
Mycorrhizal ( $P = 0.115$ ) and rhizobial ( $P = 0.778$ ) treatments had no significant effect on GS activity in the shoots of red clover plants (Fig. 3.3). In contrast, the mycorrhizal ( $P = 0.015$ ) and rhizobial ( $P = 0.002$ ) treatments had significant effects on GS activity in the roots. Tukey's test indicated that the mycorrhizal treatment enhanced GS activity while pre-inoculation with *Rhizobium* tended to decrease it (Fig. 3.3).

Mycorrhizal ( $P = 0.0083$ ) and rhizobial ( $P = 0.03$ ) treatments had significant effects on the specific activity of GS in the shoots (Table 3.1), but there was no significant interaction ( $P = 0.14$ ). The GS specific activity was the highest in the M-R- treatment; however, according to the Tukey's test, only significantly higher than the M+R- and M+R+ treatments. In the roots, the rhizobial treatment ( $P < 0.001$ ) significantly decreased the specific activity of GS (Table 3.1).

#### *Second Experiment*

The GS activity was significantly higher in the M+ plants compared to the M- plants in both shoots ( $P < 0.001$ ) and roots ( $P < 0.001$ ) (Fig. 3.4). In the shoots, GS activity was approximately 1.7 times higher in the M+ than the M- treatment. In the roots, GS activity was approximately 9 times higher in the M+ than the M- treatment.

Fig. 3.3      Glutamine synthetase (GS) activity (n = 10) in shoots (top) and roots (bottom) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .



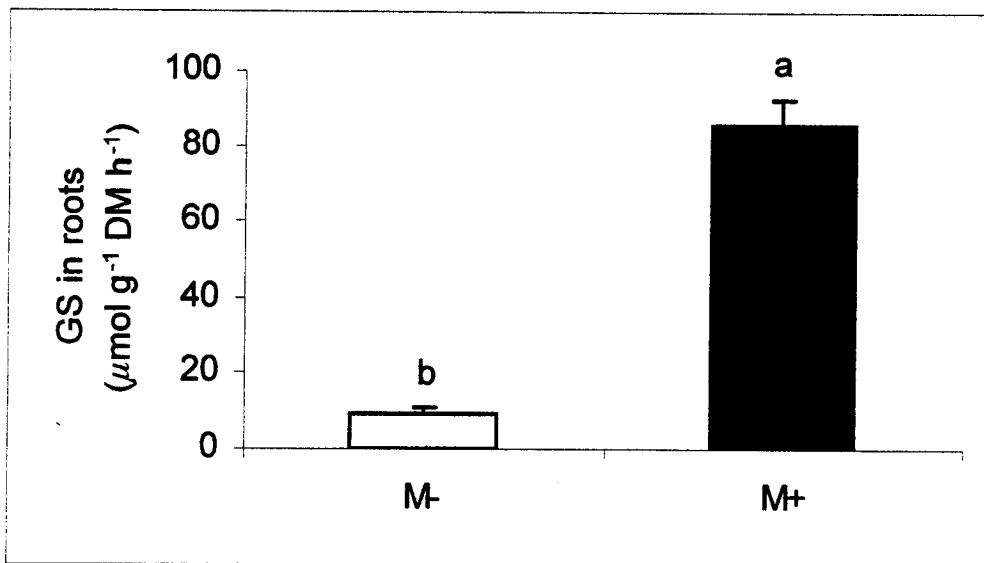
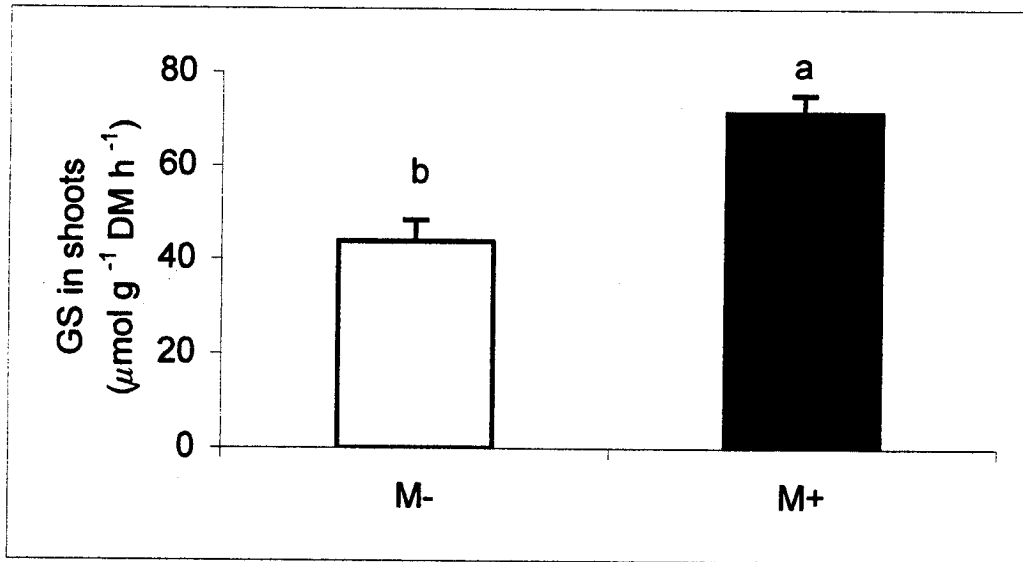
**Table 3.1** Specific activity for GS ( $\mu\text{mol } \gamma \text{ GH mg}^{-1} \text{ proteins h}^{-1}$ ) in the roots and shoots of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*.

	M-R-	M-R+	M+R-	M+R+
Shoots	1.84 <sup>a</sup> (0.15)	1.36 <sup>ab</sup> (0.16)	1.29 <sup>b</sup> (0.07)	1.20 <sup>b</sup> (0.11)
Roots	4.73 <sup>a</sup> (0.33)	3.15 <sup>c</sup> (0.29)	4.26 <sup>ab</sup> (0.24)	3.37 <sup>bc</sup> (0.23)

Means, n = 10 (SE in parentheses)

Within each row, different letters indicate significant differences according to Tukey's test at  $P < 0.05$ .

Fig. 3.4      Glutamine synthetase (GS) activity (n = 5 to 8) in shoots (top) and roots (bottom) of red clover with (M+) or without (M-) AM colonization. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .



**Table 3.2** Specific activity for GS ( $\mu\text{mol } \gamma \text{ GH mg}^{-1} \text{ proteins h}^{-1}$ ) in the roots and shoots of red clover with (M+) or without (M-) AM colonization.

	M-	M+
Shoots	3.52 <sup>a</sup> (0.41)	1.51 <sup>b</sup> (0.07)
Roots	2.33 <sup>b</sup> (0.23)	5.38 <sup>a</sup> (0.60)

Means, n = 5 to 8 (SE in parentheses)

Within each row, different letters indicate significant differences according to Tukey's test at  $P < 0.05$ .

Mycorrhizal colonization had a significant effect on the GS specific activity in both shoots ( $P < 0.001$ ) and roots ( $P < 0.001$ ) (Table 3.2). The Tukey's test indicated that in the shoots, the M- plants had a higher GS specific activity than the M+ plants. By contrast, in the roots, the M+ plants had a higher specific activity than in the M- plants.

### 3.1.4 Soluble Protein Concentration

#### *First Experiment*

The soluble protein concentration (Fig. 3.5) in the shoots of red clover was significantly enhanced in the rhizobial treatment compared to the non-rhizobial treatment ( $P < 0.001$ ). There was also a significant interaction ( $P = 0.032$ ) with the mycorrhizal treatment. However, according to the Tukey's test, only the protein concentration of the M-R-control treatment was significantly lower than the other three treatments. In the roots, AM colonization significantly ( $P = 0.007$ ) enhanced the soluble protein concentration. There was no effect of *Rhizobium* ( $P = 0.242$ ); nor was there an interaction ( $P = 0.918$ ). Tukey's test indicated that only the M+R+ treatment was significantly ( $P = 0.033$ ) higher than the M-R- treatment, by approximately 1.3 times ( $\sim 5 \text{ mg g}^{-1} \text{ DM}$ ).

#### *Second Experiment*

Both the shoot and root protein concentrations were significantly ( $P < 0.001$ ) higher in the M+ plants compared to the M- plants (Fig. 3.6). In the shoots, the protein concentrations of the M+ plants were approximately 3.7 times higher (a difference of  $\sim 34 \text{ mg g}^{-1} \text{ DM}$ ) than the M-plants. In the roots, the M+ plants had approximately 4.6 times higher protein concentrations (a difference of  $\sim 13 \text{ mg g}^{-1} \text{ DM}$ ) than the M- plants.

Fig. 3.5 Soluble protein concentrations (n = 10) in shoots (top) and roots (bottom) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .

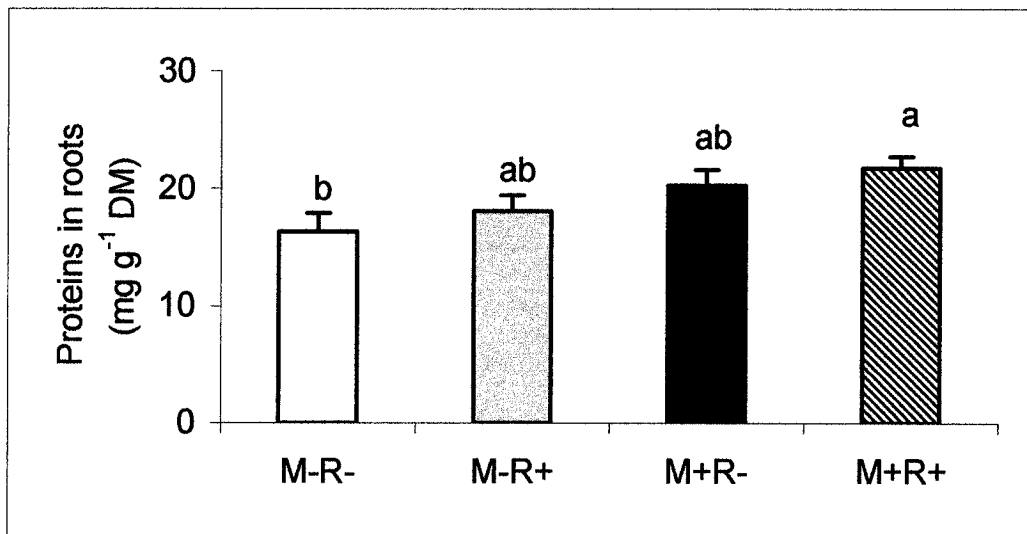
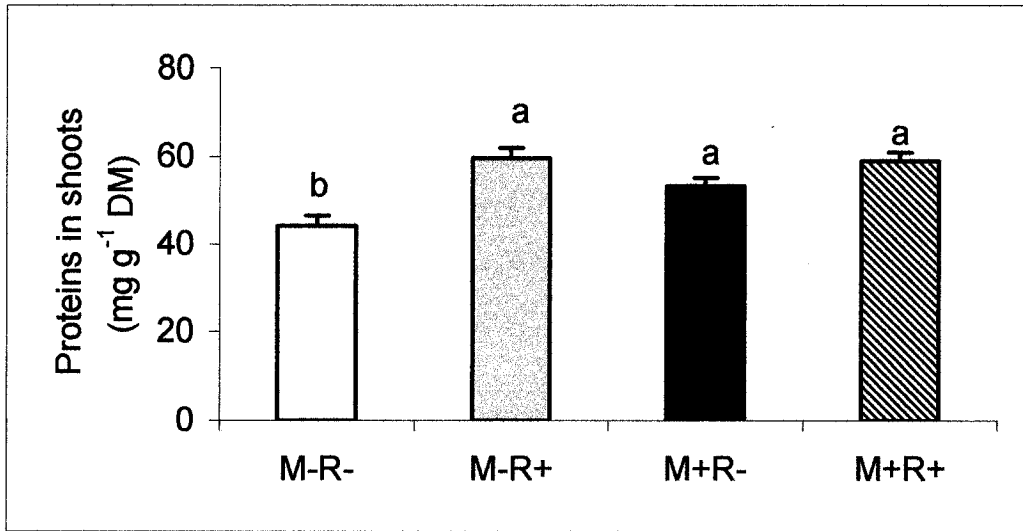
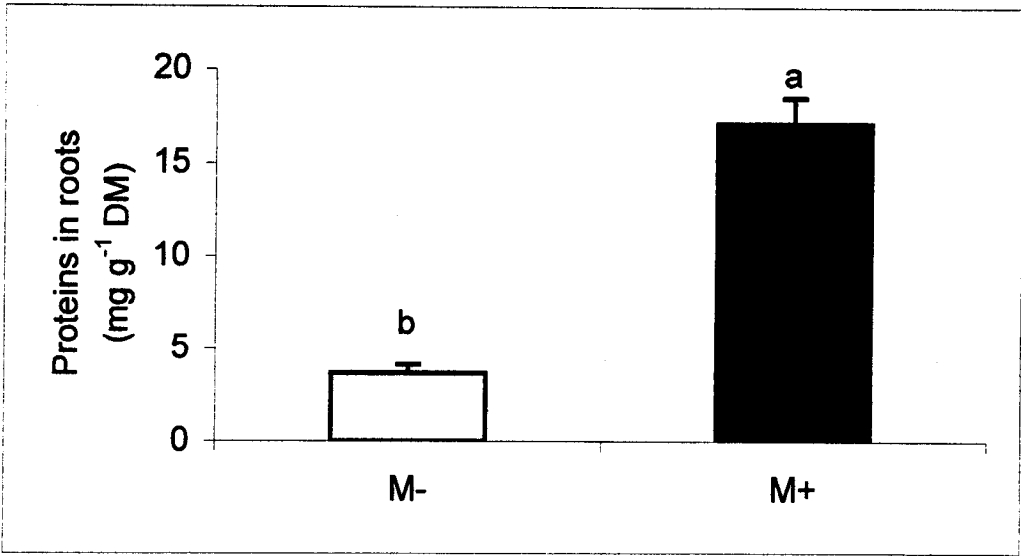
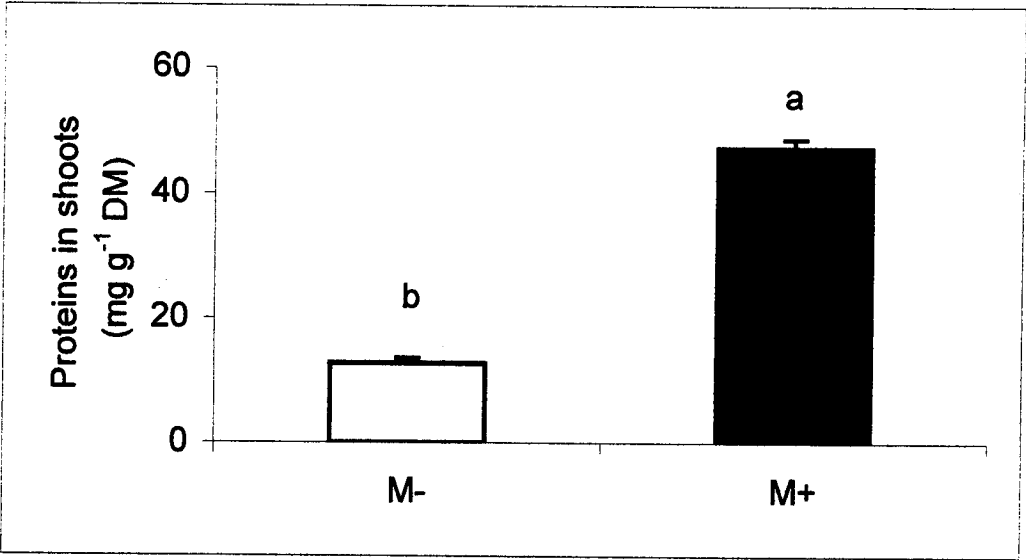


Fig. 3.6 Soluble protein concentrations (n = 5 to 8) in shoots (top) and roots (bottom) of mycorrhizal (M+) and non-mycorrhizal (M-) plants of red clover. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .



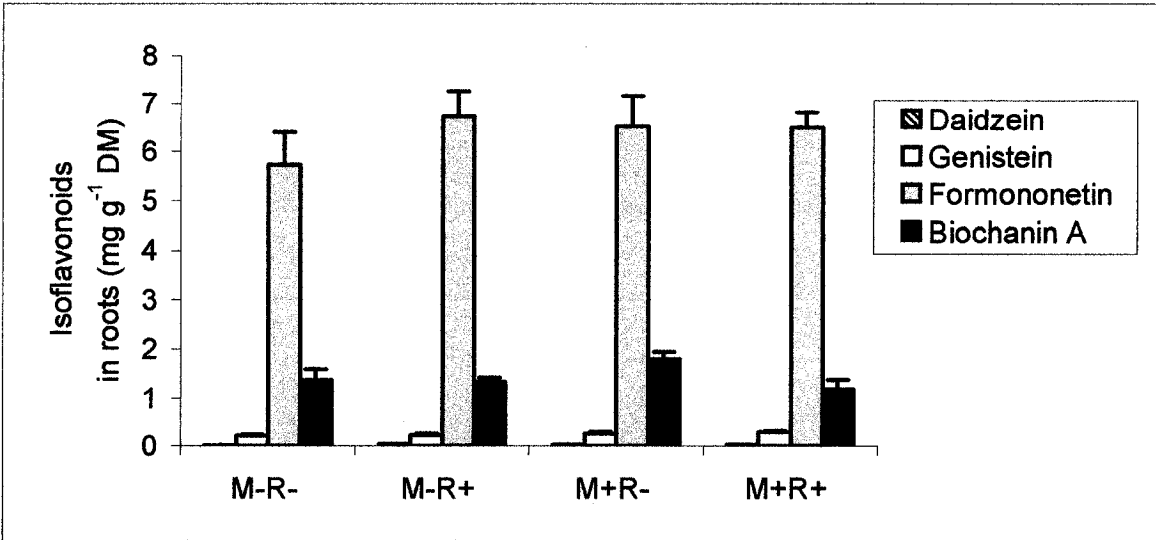
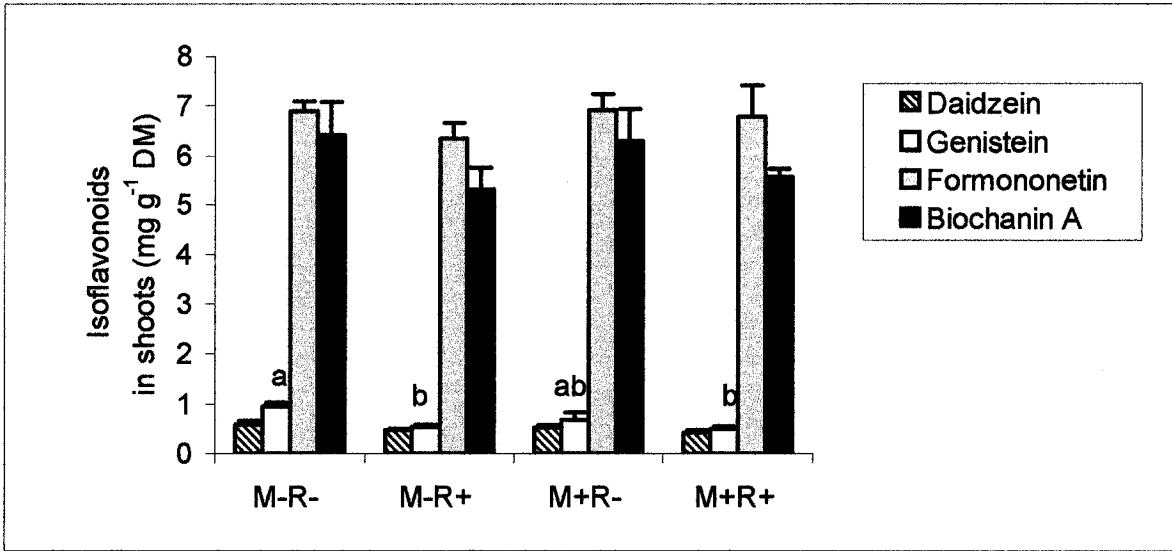
### 3.1.5 Isoflavonoid Analyses

#### *First Experiment*

Mycorrhizal and rhizobial treatments had no significant effect on any of the isoflavonoid concentrations ( $\text{mg g}^{-1}$  DM) in the shoots or roots of red clover plants after 12 weeks of growth (Fig. 3.7). The only exception was the concentration of genistein in the shoots, in which the rhizobial treatment had a significant effect ( $P = 0.003$ ). According to the Tukey's test, red clover pre-inoculated with *Rhizobium* (M-R+ and M+R+) only had significantly lower concentrations of genistein than the M-R- control. In both the shoots and roots, formononetin had the highest concentration followed by biochanin A, genistein and daidzein, respectively. Overall, isoflavonoid concentrations were 1.7 times higher in the shoots compared to the roots.

In contrast, for the total isoflavonoid contents (calculated for the total shoot or root DM), the rhizobial treatment had a significant effect ( $P = 0.0016$ ) on the formononetin content ( $\mu\text{g shoot}^{-1}$  DM) in the shoots of red clover (Table 3.3). There was also a marginally significant effect of mycorrhiza ( $P = 0.0532$ ), without any significant interaction ( $P = 0.532$ ). According to the Tukey's test, *Rhizobium* enhanced the formononetin content compared to the non-rhizobial treatments. AM colonization ( $P = 0.041$ ) and *Rhizobium* ( $P = 0.002$ ) also significantly increased the total isoflavonoid content. According to the Tukey's test, the M+R+ treatment was significantly higher than the M+R- (~1.2 times) and the M-R- (~1.3 times) treatments.

Fig. 3.7 Concentration of isoflavonoids ( $\text{mg g}^{-1}$  DM) ( $n = 5$ ) in shoots (top) and roots (bottom) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Statistical analyses were done for each isoflavonoid separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ . Bars without letters indicate no significant differences between the treatments.



**Table 3.3** Means ( $n = 5$ ) and standard errors (in parentheses) for isoflavonoid content in shoots ( $\mu\text{g shoot}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Shoots</i>					
M-R-	122.94 <sup>a</sup> (14.40)	197.94 <sup>a</sup> (13.03)	1428.3 <sup>b</sup> (41.5)	1330.8 <sup>a</sup> (136.8)	3080.0 <sup>b</sup> (142.2)
M-R+	134.34 <sup>a</sup> (6.74)	151.04 <sup>a</sup> (15.63)	1777.7 <sup>ab</sup> (85.8)	1490.7 <sup>a</sup> (120.8)	3553.9 <sup>ab</sup> (180.7)
M+R-	122.44 <sup>a</sup> (10.55)	157.42 <sup>a</sup> (32.83)	1589.9 <sup>b</sup> (77.1)	1447.6 <sup>a</sup> (149.7)	3317.4 <sup>b</sup> (158.6)
M+R+	131.27 <sup>a</sup> (18.10)	150.23 <sup>a</sup> (18.84)	2081.8 <sup>a</sup> (186.3)	1761.3 <sup>a</sup> (52.1)	4124.6 <sup>a</sup> (234.7)
ANOVA:	M (Mycorrhizal treatment), R (Rhizobial treatment)				
M	NS	NS	NS	NS	*
R	NS	NS	*	NS	*
M x R	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

**Table 3.4** Means (n = 5) and standard errors (in parentheses) for isoflavonoid content in roots ( $\mu\text{g root}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Roots</i>					
M-R-	2.20 <sup>b</sup> (0.45)	21.6 <sup>b</sup> (0.60)	574.4 <sup>b</sup> (67.8)	136.4 <sup>a</sup> (22.2)	734.7 <sup>b</sup> (83.2)
M-R+	4.81 <sup>a</sup> (0.49)	32.48 <sup>ab</sup> (2.72)	827.3 <sup>ab</sup> (67.7)	163.8 <sup>a</sup> (11.7)	1028.4 <sup>ab</sup> (79.0)
M+R-	3.37 <sup>ab</sup> (0.99)	27.76 <sup>ab</sup> (2.72)	690.3 <sup>b</sup> (68.1)	190.7 <sup>a</sup> (16.2)	912.1 <sup>ab</sup> (71.3)
M+R+	5.07 <sup>a</sup> (0.44)	40.72 <sup>a</sup> (5.79)	933.4 <sup>a</sup> (45.5)	169.4 <sup>a</sup> (28.6)	1148.7 <sup>a</sup> (70.4)
ANOVA:	M (Mycorrhizal treatment), R (Rhizobial treatment)				
M	NS	NS	NS	NS	NS
R	*	*	*	NS	*
M x R	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

**Table 3.5** Means (n = 5) and standard errors (in parentheses) for isoflavonoid content in roots and shoots combined ( $\mu\text{g plant}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
M-R-	125.15 <sup>a</sup> (14.23)	219.63 <sup>a</sup> (12.66)	2002.7 <sup>c</sup> (70.5)	1467.2 <sup>a</sup> (149.8)	3814.7 <sup>b</sup> (196.8)
M-R+	139.15 <sup>a</sup> (6.71)	183.52 <sup>a</sup> (15.92)	2605.1 <sup>ab</sup> (122.8)	1654.5 <sup>a</sup> (118.1)	4582.3 <sup>ab</sup> (208.8)
M+R-	125.81 <sup>a</sup> (10.32)	185.18 <sup>a</sup> (34.22)	2280.2 <sup>bc</sup> (66.5)	1638.3 <sup>a</sup> (148.6)	4229.5 <sup>b</sup> (181.3)
M+R+	136.35 <sup>a</sup> (17.91)	190.95 <sup>a</sup> (21.90)	3015.3 <sup>a</sup> (168.7)	1930.7 <sup>a</sup> (36.9)	5273.3 <sup>a</sup> (192.8)
ANOVA:	M (Mycorrhizal treatment), R (Rhizobial treatment)				
M	NS	NS	*	NS	*
R	NS	NS	*	NS	*
M x R	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

There was no significant effect of mycorrhiza or *Rhizobium* on any of the three other isoflavonoid contents in the shoots.

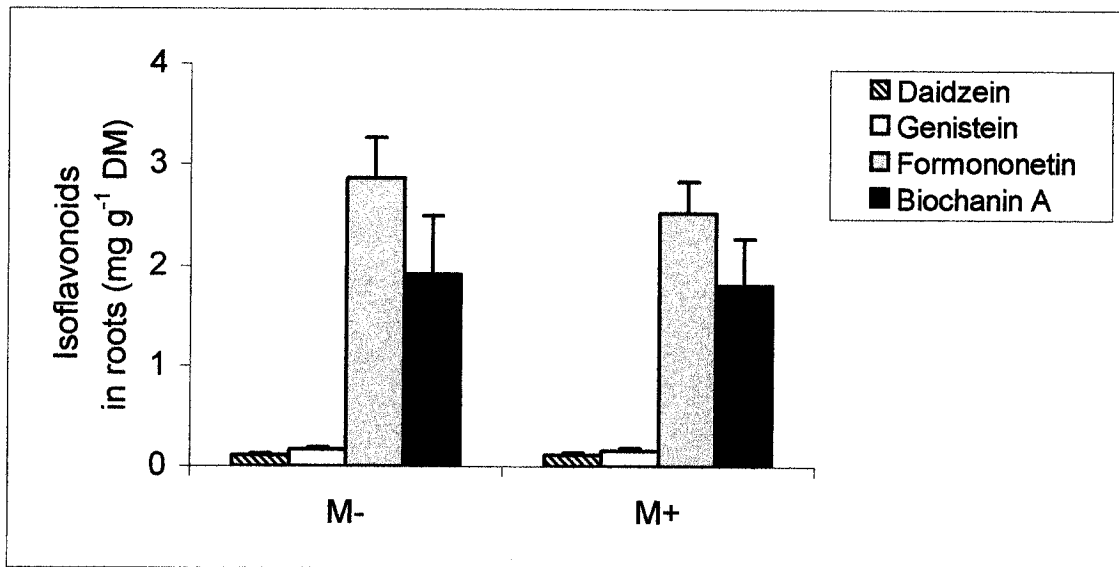
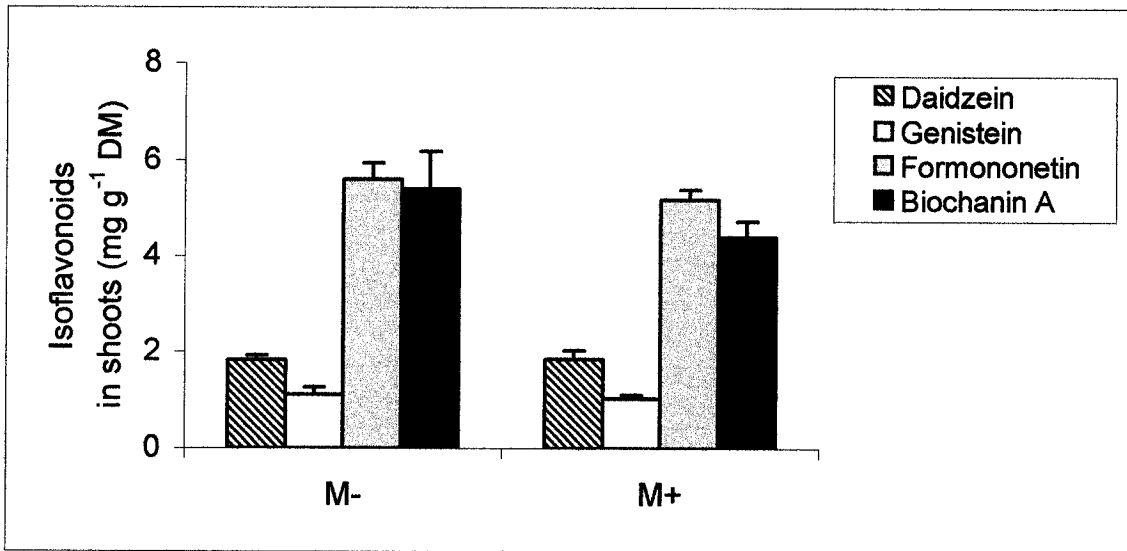
In the roots of red clover, the rhizobial treatment had significant effects on the isoflavonoid contents ( $\mu\text{g root}^{-1}$  DM) of daidzein ( $P = 0.003$ ), genistein ( $P = 0.005$ ), formononetin ( $P = 0.001$ ), and of the total isoflavonoid contents ( $P = 0.003$ ) (Table 3.4). There was no effect of mycorrhiza, nor any significant interaction. According to the Tukey's test, for genistein, only the treatment M+R+ was significantly higher than the M-R- control. In the case of formononetin, only M+R+ was significantly higher than M+R- and the M-R- control. For the daidzein content, M-R+ and M+R+ were both significantly higher than the M-R- control. For the total isoflavonoid content, only M+R+ was significantly higher than the M-R- control.

The isoflavonoid contents for the combined roots and shoots ( $\mu\text{g plant}^{-1}$  DM) of red clover are shown in Table 3.5. According to ANOVA, mycorrhizal ( $P < 0.01$ ) and rhizobial ( $P < 0.001$ ) treatments had significant effects on the formononetin and total isoflavonoid contents. The Tukey's test indicated that for formononetin, treatment M+R+ was 1.5 times higher than the M-R- control. For the total isoflavonoid content, treatment M+R+ was 1.4 times higher than the M-R- control.

### *Second Experiment*

In accordance with the first experiment, mycorrhiza had no significant effect on any of the isoflavonoid concentrations ( $\text{mg g}^{-1}$  DM) in red clover (Fig. 3.8). However, mycorrhiza did have significant effects on the shoot contents ( $\mu\text{g shoot}^{-1}$  DM) of daidzein ( $P = 0.006$ ),

Fig. 3.8 Concentration of isoflavonoids ( $\text{mg g}^{-1}$  DM) ( $n = 5$  or  $6$ ) in shoots (top) and roots (bottom) of mycorrhizal (M+) and non-mycorrhizal (M-) plants of red clover. Statistical analyses were done for each isoflavonoid separately. Means and SE's are shown and Tukey's test at 5% level indicated no significant differences between the treatments.



**Table 3.6** Means ( $n = 6$ ) and standard errors (in parentheses) for isoflavonoid content in shoots ( $\mu\text{g shoot}^{-1}$  DM), roots ( $\mu\text{g root}^{-1}$  DM), and shoots and roots combined ( $\mu\text{g plant}^{-1}$  DM) of mycorrhizal (M+) and non-mycorrhizal (M-) plants of red clover. Statistical analyses were done for roots and shoots separately. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Shoots</i>					
M-	208.80 <sup>b</sup> (13.50)	126.68 <sup>b</sup> (17.77)	643.10 <sup>b</sup> (39.24)	619.80 <sup>a</sup> (91.23)	1598.4 <sup>b</sup> (148.8)
M+	332.61 <sup>a</sup> (33.20)	187.41 <sup>a</sup> (12.52)	931.75 <sup>a</sup> (36.66)	792.07 <sup>a</sup> (56.72)	2243.8 <sup>a</sup> (39.6)
<i>Roots</i>					
M-	9.69 <sup>a</sup> (1.19)	14.80 <sup>a</sup> (2.01)	253.13 <sup>a</sup> (35.31)	169.10 <sup>a</sup> (51.32)	446.72 <sup>a</sup> (85.52)
M+	14.73 <sup>a</sup> (3.07)	20.39 <sup>a</sup> (2.65)	317.50 <sup>a</sup> (37.73)	226.04 <sup>a</sup> (57.43)	578.67 <sup>a</sup> (95.39)
<i>Shoots and Roots Combined</i>					
M-	218.49 <sup>b</sup> (14.09)	141.48 <sup>b</sup> (17.60)	896.23 <sup>b</sup> (34.19)	788.91 <sup>a</sup> (77.44)	2045.1 <sup>b</sup> (129.2)
M+	347.35 <sup>a</sup> (32.73)	207.81 <sup>a</sup> (13.07)	1249.2 <sup>a</sup> (39.8)	1018.1 <sup>a</sup> (91.5)	2822.5 <sup>a</sup> (91.3)

genistein ( $P = 0.01$ ), formononetin ( $P < 0.001$ ) and of the total isoflavonoid contents ( $P = 0.002$ ) (Table 3.6). According to the Tukey's test, mycorrhizal colonization significantly increased their contents by approximately 1.6, 1.5, 1.4, and 1.4 fold, respectively.

There was no significant effect of mycorrhiza on any of the isoflavonoid contents in the roots of red clover ( $\mu\text{g root}^{-1}$  DM) (Table 3.6). However, in the combined roots and shoots (Table 3.6), mycorrhiza significantly increased the contents ( $\mu\text{g plant}^{-1}$  DM) of daidzein ( $P = 0.004$ ) by 1.6 fold, genistein ( $P = 0.01$ ) by 1.5 fold, formononetin ( $P < 0.001$ ) by 1.4 fold, and the total isoflavonoid content ( $P = 0.001$ ) by almost 1.4 fold.

### **3.1.6 Mineral Analyses**

#### *First experiment*

Mycorrhizal and/or rhizobial treatments had significant effects ( $P < 0.05$ ) on Ca, Mg, K, Mn, and Zn in the shoots of red clover (Table 3.7). There were also significant interactions ( $P < 0.05$ ) for C, Mn and Zn. According to Tukey's test, mycorrhizal colonization increased the concentration of Mn and tended to increase the concentration of Ca (significantly increased in M+R- and tended to increase in M+R+). Mycorrhizal colonization decreased the concentration of Mg, which was significantly lower in the M+R+ treatment than the M-R- control, according to the Tukey's test. Shoots from the preinoculated rhizobial treatment tended to have lower levels of Mg and Zn. There was no significant difference for K between the treatments. For C, treatments M-R- and M+R- were marginally (0.8%) significantly different ( $P = 0.059$ ).

**Table 3.7** Means (n = 4 to 5) and standard errors (in parentheses) for nutrient concentrations in shoots of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	C (%)	N (%)	P (%)	Ca (%)	Mg (%)	K (%)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
<i>Shoots</i>										
M-R-	43.4 <sup>a</sup> (0.3)	2.87 <sup>a</sup> (0.11)	0.08 <sup>a</sup> (0.01)	0.97 <sup>bc</sup> (0.05)	0.56 <sup>a</sup> (0.04)	2.50 <sup>a</sup> (0.05)	11.2 <sup>a</sup> (1.7)	83.6 <sup>a</sup> (7.2)	49.8 <sup>c</sup> (3.6)	15.2 <sup>a</sup> (0.2)
M-R+	42.9 <sup>ab</sup> (0.2)	2.90 <sup>a</sup> (0.18)	0.08 <sup>a</sup> (0.01)	0.91 <sup>c</sup> (0.02)	0.45 <sup>ab</sup> (0.02)	2.21 <sup>a</sup> (0.10)	11.4 <sup>a</sup> (1.0)	90.0 <sup>a</sup> (7.2)	58.0 <sup>c</sup> (2.4)	11.0 <sup>b</sup> (0.5)
M+R-	42.6 <sup>b</sup> (0.2)	2.78 <sup>a</sup> (0.13)	0.10 <sup>a</sup> (0.01)	1.13 <sup>a</sup> (0.04)	0.45 <sup>ab</sup> (0.02)	2.31 <sup>a</sup> (0.05)	9.2 <sup>a</sup> (0.5)	85.8 <sup>a</sup> (6.0)	92.2 <sup>a</sup> (2.2)	11.8 <sup>b</sup> (0.6)
M+R+	43.2 <sup>ab</sup> (0.1)	3.05 <sup>a</sup> (0.13)	0.09 <sup>a</sup> (0.01)	1.07 <sup>ab</sup> (0.02)	0.40 <sup>b</sup> (0.01)	2.22 <sup>a</sup> (0.08)	9.0 <sup>a</sup> (0.3)	66.0 <sup>a</sup> (4.5)	79.6 <sup>b</sup> (2.7)	13.6 <sup>ab</sup> (2.3)

ANOVA: M (Mycorrhizal treatment), R (Rhizobial treatment)

M	NS	NS	NS	*	*	NS	NS	NS	*	NS
R	NS	NS	NS	NS	*	*	NS	NS	NS	*
M x R	*	NS	NS	NS	NS	NS	NS	NS	*	*

\*  $P < 0.05$ ; NS not significant

**Table 3.8** Means (n = 4 to 5) and standard errors (in parentheses) for nutrient concentrations in roots of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	C (%)	N (%)	P (%)	Ca (%)	Mg (%)	K (%)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
<i>Roots</i>										
M-R-	40.4 <sup>a</sup> (1.1)	2.21 <sup>a</sup> (0.04)	0.09 <sup>a</sup> (0.01)	0.30 <sup>a</sup> (0.01)	0.77 <sup>a</sup> (0.07)	1.67 <sup>a</sup> (0.13)	27.0 <sup>a</sup> (4.1)	398.0 <sup>a</sup> (48.2)	36.0 <sup>b</sup> (4.5)	23.7 <sup>a</sup> (3.3)
M-R+	40.4 <sup>a</sup> (1.8)	1.93 <sup>b</sup> (0.04)	0.11 <sup>a</sup> (0.01)	0.29 <sup>a</sup> (0.01)	0.89 <sup>a</sup> (0.12)	1.86 <sup>a</sup> (0.14)	24.3 <sup>a</sup> (0.9)	603.5 <sup>a</sup> (86.6)	61.5 <sup>b</sup> (6.6)	20.3 <sup>a</sup> (3.8)
M+R-	40.6 <sup>a</sup> (1.5)	1.71 <sup>c</sup> (0.03)	0.11 <sup>a</sup> (0.01)	0.25 <sup>a</sup> (0.02)	0.56 <sup>a</sup> (0.08)	2.10 <sup>a</sup> (0.33)	23.3 <sup>a</sup> (0.7)	753.0 <sup>a</sup> (287.9)	116.5 <sup>a</sup> (8.9)	19.3 <sup>a</sup> (3.5)
M+R+	41.9 <sup>a</sup> (1.08)	1.82 <sup>bc</sup> (0.05)	0.09 <sup>a</sup> (0.01)	0.23 <sup>a</sup> (0.02)	0.49 <sup>a</sup> (0.04)	1.90 <sup>a</sup> (0.16)	23.3 <sup>a</sup> (1.7)	651.2 <sup>a</sup> (220.5)	129.0 <sup>a</sup> (18.1)	22.0 <sup>a</sup> (3.0)
ANOVA:	M (Mycorrhizal treatment), R (Rhizobial treatment)									
M	NS	*	NS	*	*	NS	NS	NS	*	NS
R	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
M x R	NS	*	NS	NS	NS	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

In the roots (Table 3.8), mycorrhizal colonization had significant effects ( $P < 0.05$ ) on N, Ca, Mg, and Mn. There were no effects of *Rhizobium*. According to the Tukey's test, mycorrhizal colonization increased the concentration of Mn and tended to decrease the concentrations of N, Mg, and Ca.

The mineral levels were all in the expected range except for high Fe in the roots. On average, roots contained higher levels of Cu, Fe, Zn, and Mg, while shoots contained higher Ca and K levels. Carbon-nitrogen (C/N) ratios (Table 3.9) in the shoots did not vary significantly among the treatments. In the roots, mycorrhizal colonization significantly ( $P < 0.001$ ) increased the C/N ratios. There was no effect of *Rhizobium*. According to the Tukey's test, treatments M+R- and M+R+ had higher C/N ratios than the control M-R- treatment.

#### *Second experiment*

In the shoots of AM plants, both Fe ( $P = 0.021$ ) and Zn ( $P = 0.0004$ ) concentrations were significantly enhanced compared to the non-AM plants (Table 3.10). In the roots of AM plants, Mn concentration was significantly ( $P = 0.009$ ) enhanced. All the minerals were within the expected range, except for high Fe concentrations in the roots. Roots, on average, contained higher levels of Cu, Fe, and Mg while shoots contained higher levels of Ca, Mn and Zn. The C/N ratios did not vary significantly among the treatments in both the shoots and roots (Table 3.11).

**Table 3.9** Means (n = 5) and standard errors (in parentheses) for C/N ratio in shoots and roots of red clover with (M+) or without (M-) AM colonization and with (R+) or without (R-) *Rhizobium*. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Shoots	Roots
M-R-	15.21 <sup>a</sup> (0.50)	18.25 <sup>b</sup> (0.48)
M-R+	15.10 <sup>a</sup> (1.12)	20.94 <sup>ab</sup> (0.51)
M+R-	15.42 <sup>a</sup> (0.63)	23.75 <sup>a</sup> (1.07)
M+R+	14.28 <sup>a</sup> (0.76)	23.03 <sup>a</sup> (0.71)

ANOVA: M (Mycorrhizal treatment), R (Rhizobial treatment)

M	NS	*
R	NS	NS
M x R	NS	*

\*  $P < 0.05$ ; NS not significant

**Table 3.10** Means (n =2 to 3) and standard errors (in parentheses) for nutrient concentrations in shoots and roots of red clover with (M+) or without (M-) AM colonization. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	C (%)	N (%)	P (%)	Ca (%)	Mg (%)	K (%)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
<i>Shoots</i>										
M-	40.9 <sup>a</sup> (0.2)	1.58 <sup>a</sup> (0.09)	0.40 <sup>a</sup> (0.01)	1.01 <sup>a</sup> (0.01)	0.64 <sup>a</sup> (0.01)	2.60 <sup>a</sup> (0.03)	15.0 <sup>a</sup> (1.0)	91.0 <sup>b</sup> (4.3)	71.0 <sup>a</sup> (2.4)	22.0 <sup>b</sup> (0.3)
M+	40.3 <sup>a</sup> (0.5)	1.55 <sup>a</sup> (0.19)	0.44 <sup>a</sup> (0.02)	0.98 <sup>a</sup> (0.10)	0.66 <sup>a</sup> (0.03)	2.80 <sup>a</sup> (0.20)	18.0 <sup>a</sup> (0.9)	126.0 <sup>a</sup> (8.7)	96.0 <sup>a</sup> (10.0)	26.0 <sup>a</sup> (0.0)
<i>Roots</i>										
M-	35.4 <sup>a</sup> (2.7)	1.69 <sup>a</sup> (0.16)	0.36 <sup>a</sup> (0.10)	0.26 <sup>a</sup> (0.01)	0.89 <sup>a</sup> (0.22)	2.50 <sup>a</sup> (0.70)	21.0 <sup>a</sup> (1.0)	1122 <sup>a</sup> (157.9)	37.0 <sup>b</sup> (0.0)	15.0 <sup>a</sup> (1.9)
M+	36.3 <sup>a</sup> (2.3)	1.74 <sup>a</sup> (0.10)	0.41 <sup>a</sup> (0.02)	0.31 <sup>a</sup> (0.01)	0.96 <sup>a</sup> (0.16)	3.10 <sup>a</sup> (0.36)	22.0 <sup>a</sup> (1.6)	833.0 <sup>a</sup> (235.1)	47.0 <sup>a</sup> (1.3)	15.0 <sup>a</sup> (1.5)

**Table 3.11** Means ( $n = 3$ ) and standard errors (in parentheses) for C/N ratio in shoots and roots of red clover with (M+) or without (M-) AM colonization. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Shoots	Roots
M-	25.92 <sup>a</sup> (1.39)	20.99 <sup>a</sup> (0.50)
M+	26.71 <sup>a</sup> (3.27)	20.80 <sup>a</sup> (0.77)

## **3.2 Experiment 3: AM Colonization and Induction with Jasmonic Acid (JA)**

### **3.2.1 Mycorrhizal Colonization and Nodulation**

The AM colonization levels were 52.8% and 42.1% in treatments M+H- and M+H+, respectively, after 7 weeks of growth. There was no colonization in the non-AM treatments (M-H- and M-H+). Even though the red clover plants were not pre-inoculated with *Rhizobium*, in this experiment, some nodulation was observed in the non-mycorrhizal treatments (M-H- and M-H+) only.

### **3.2.2 Physiological Data**

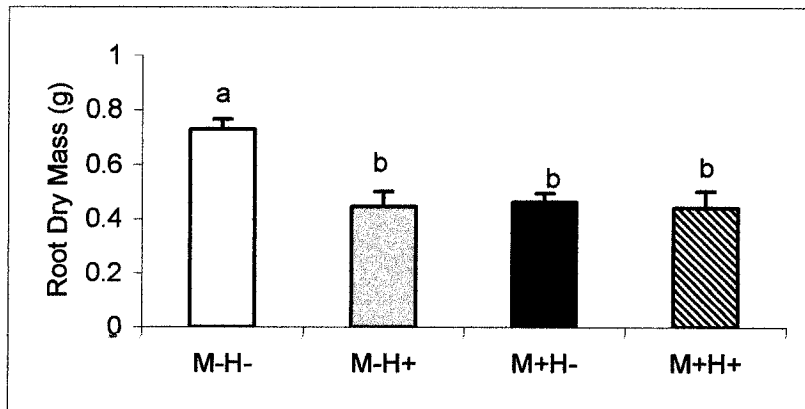
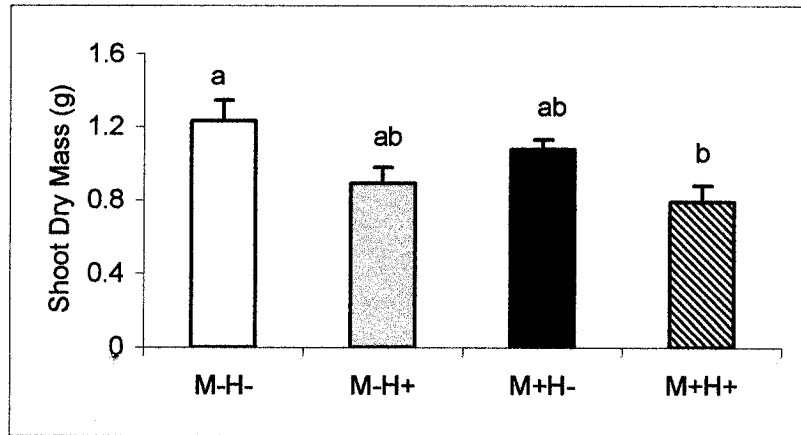
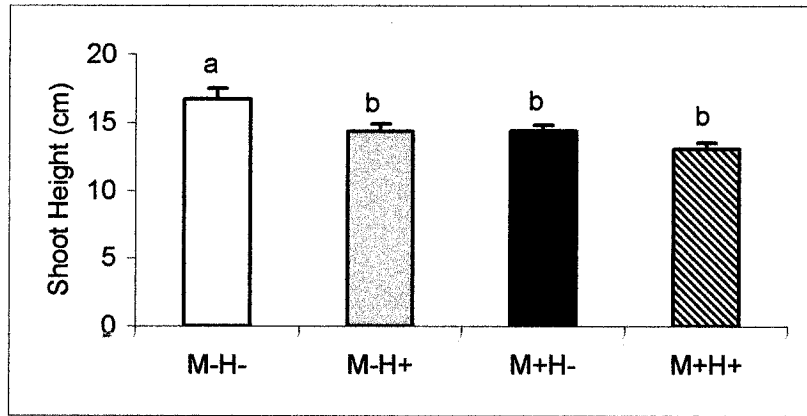
#### *Shoot Height*

Mycorrhizal ( $P = 0.002$ ) and JA induced ( $P = 0.002$ ) treatments had significant effects on shoot height without any significant interaction. Tukey's test indicated that the control plants (M-R-) were significantly (~1.3 times) taller than all the other treatments (Fig. 3.9).

#### *Biomass*

According to the ANOVA, induction of red clover with JA had a significant effect ( $P = 0.0015$ ) on shoot dry mass. There was no effect of AM colonization ( $P = 0.15$ ) or an interaction ( $P = 0.75$ ). Tukey's test showed that only the shoot dry mass of the control treatment (M-H-) was significantly higher (~1.6 times) than the one of the M+H+ treatment. Root dry mass was significantly affected by AM colonization ( $P = 0.01$ ) and JA induction ( $P = 0.006$ ), with a significant interaction ( $P = 0.01$ ). However, according

Fig. 3.9      Shoot height, shoot dry mass, and root dry mass ( $n = 7$  to  $14$ ) of red clover with (M+) or without (M-) AM colonization and with (H+) or without (H-) JA treatment. Statistical analyses were done for roots and shoots separately. Means and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .



to the Tukey's test, only the control treatment (M-H-) was significantly greater (~1.6 times) than the other three treatments (Fig. 3.9).

### 3.2.3 Isoflavonoid Analyses

Induction of red clover with JA had significant effects on formononetin ( $P < 0.001$ ), biochanin A ( $P < 0.001$ ) and genistein ( $P < 0.001$ ) concentrations in the shoots. There was no effect of AM colonization, or an interaction. The Tukey's test indicated that the isoflavonoid concentrations were approximately doubled in the JA induced treatments compared to the non-induced treatments (Fig. 3.10).

In the roots, ANOVA indicated that AM colonization ( $P = 0.048$ ) and JA induction ( $P = 0.004$ ) significantly affected the biochanin A concentration (Fig. 3.10). According to the Tukey's test, only M+H- was significantly higher (~1.4 times) than the other three treatments.

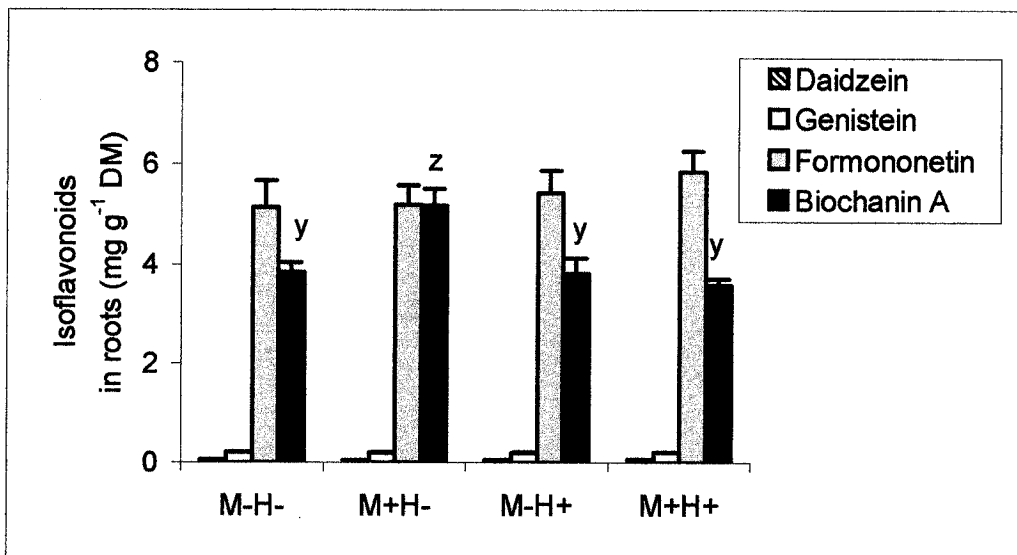
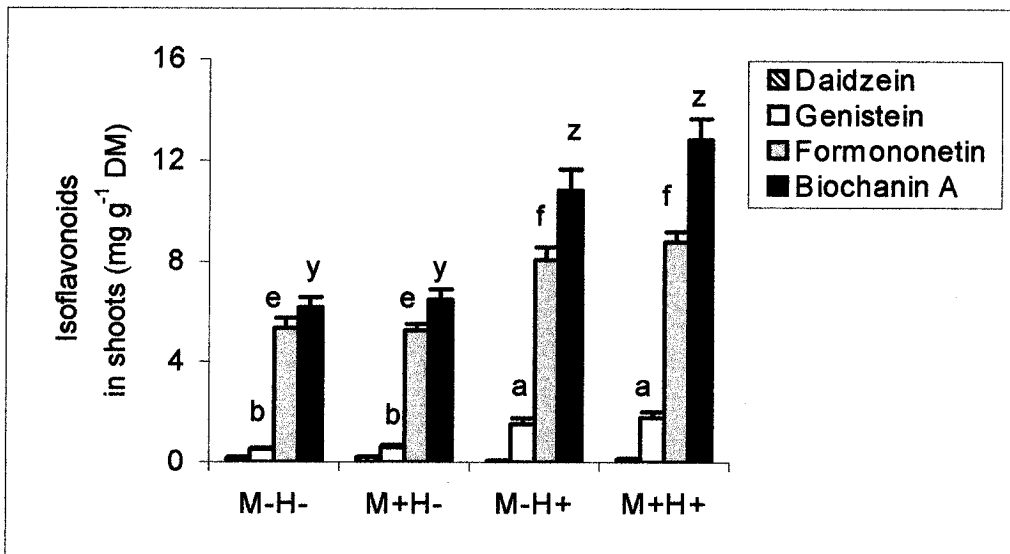
According to ANOVA, JA induction significantly increased the contents ( $\mu\text{g shoot}^{-1}$  DM) of genistein ( $P = 0.001$ ), biochanin A ( $P = 0.016$ ), and the total isoflavonoid contents ( $P = 0.029$ ) in the shoots of red clover (Table 3.12). There was no effect of mycorrhiza or any interaction. According to Tukey's test, only the content of genistein in the M+H+ treatment was significantly (~2.5 times) higher than the M-H- control.

In the roots, there were significant interactions between the mycorrhizal and JA induced treatments for the contents ( $\mu\text{g root}^{-1}$  DM) of daidzein ( $P = 0.001$ ), genistein ( $P < 0.001$ ),

formononetin ( $P = 0.012$ ), and the total isoflavonoid contents ( $P = 0.004$ ) (Table 3.13). There was no effect of mycorrhiza and only a significant effect of JA induction for biochanin A ( $P = 0.021$ ).

For the combined roots and shoots of red clover ( $\mu\text{g plant}^{-1}$  DM), JA induction significantly increased ( $P = 0.002$ ) the genistein content, but tended to decrease ( $P = 0.001$ ) the daidzein content (Table 3.14). According to the Tukey's test, for genistein, treatment M+H+ was significantly ( $\sim 2.2$  times) higher than the M-H- control. In the case of daidzein, M-H- and M+H- were significantly ( $\sim 3.8$  times) higher than the M-H+ treatment. There was no effect of mycorrhiza or JA induction on the formononetin, biochanin A, and total isoflavonoid contents.

Fig. 3.10 Concentration of isoflavonoids ( $\text{mg g}^{-1}$  DM) ( $n = 7$ ) in shoots (top) and roots (bottom) of red clover with (M+) or without (M-) AM colonization and with (H+) or without (H-) JA induction. Statistical analyses were done for each isoflavonoid separately. Means and SE's are shown and different letters [genistein (a-b), formononetin (e-f), and biochanin A (y-z)] refer to significant differences according to Tukey's test at  $P < 0.05$ . Bars without letters indicate no significant differences between the treatments.



**Table 3.12** Means (n = 7 to 8) and standard errors (in parentheses) for isoflavonoid content in shoots ( $\mu\text{g shoot}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (H+) or without (H-) JA induction. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Shoots</i>					
M-H-	30.76 <sup>ab</sup> (7.66)	100.85 <sup>b</sup> (11.29)	1078.6 <sup>a</sup> (86.2)	1257.4 <sup>a</sup> (111.3)	2467.7 <sup>a</sup> (156.5)
M+H-	35.94 <sup>a</sup> (6.57)	113.34 <sup>ab</sup> (17.22)	950.52 <sup>a</sup> (78.26)	1154.2 <sup>a</sup> (69.4)	2254.0 <sup>a</sup> (144.5)
M-H+	5.22 <sup>b</sup> (5.22)	222.31 <sup>ab</sup> (33.48)	1209.5 <sup>a</sup> (149.3)	1597.7 <sup>a</sup> (176.9)	3034.8 <sup>a</sup> (321.2)
M+H+	12.01 <sup>ab</sup> (7.91)	250.16 <sup>a</sup> (56.8)	1175.1 <sup>a</sup> (161.3)	1698.6 <sup>a</sup> (241.5)	3135.9 <sup>a</sup> (453.0)
ANOVA:	M (Mycorrhizal treatment), H (JA treatment)				
M	NS	NS	NS	NS	NS
H	*	*	NS	*	*
M x H	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

**Table 3.13** Means (n = 5 to 7) and standard errors (in parentheses) for isoflavonoid content in roots ( $\mu\text{g root}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (H+) or without (H-) JA induction. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Roots</i>					
M-H-	6.97 <sup>a</sup> (0.55)	24.50 <sup>a</sup> (1.42)	628.37 <sup>a</sup> (76.00)	470.59 <sup>a</sup> (38.14)	1130.4 <sup>a</sup> (109.5)
M+H-	3.33 <sup>b</sup> (0.34)	14.71 <sup>b</sup> (1.77)	396.36 <sup>a</sup> (28.59)	400.37 <sup>a</sup> (44.81)	814.77 <sup>ab</sup> (55.63)
M-H+	4.69 <sup>ab</sup> (0.88)	14.59 <sup>b</sup> (2.82)	407.23 <sup>a</sup> (78.94)	288.90 <sup>a</sup> (57.49)	715.42 <sup>b</sup> (83.38)
M+H+	6.70 <sup>a</sup> (0.98)	20.14 <sup>ab</sup> (1.96)	587.86 <sup>a</sup> (95.35)	354.86 <sup>a</sup> (49.90)	969.58 <sup>ab</sup> (105.97)
ANOVA:	M (Mycorrhizal treatment), H (JA treatment)				
M	NS	NS	NS	NS	NS
H	NS	NS	NS	*	NS
M x H	*	*	*	NS	*

\*  $P < 0.05$ ; NS not significant

**Table 3.14** Means (n = 5 to 7) and standard errors (in parentheses) for isoflavonoid content in roots and shoots combined ( $\mu\text{g plant}^{-1}$  DM) of red clover with (M+) or without (M-) AM colonization and with (H+) or without (H-) JA induction. Different letters within a column indicate significant differences according to Tukey's test at  $P < 0.05$ .

	Daidzein	Genistein	Formononetin	Biochanin A	Total
<i>Roots and Shoots Combined</i>					
M-H-	37.73 <sup>a</sup> (7.82)	125.35 <sup>b</sup> (11.77)	1707.0 <sup>a</sup> (99.2)	1728.0 <sup>a</sup> (108.7)	3598.1 <sup>a</sup> (178.2)
M+H-	39.27 <sup>a</sup> (6.57)	128.04 <sup>b</sup> (16.65)	1346.8 <sup>a</sup> (92.3)	1554.5 <sup>a</sup> (86.7)	3068.7 <sup>a</sup> (164.9)
M-H+	9.92 <sup>b</sup> (5.24)	236.91 <sup>ab</sup> (33.97)	1616.7 <sup>a</sup> (153.1)	1886.6 <sup>a</sup> (193.5)	3750.2 <sup>a</sup> (349.8)
M+H+	18.71 <sup>ab</sup> (7.82)	270.31 <sup>a</sup> (57.28)	1763.0 <sup>a</sup> (203.2)	2053.4 <sup>a</sup> (261.1)	4105.5 <sup>a</sup> (511.4)
ANOVA:	M (Mycorrhizal treatment), H (JA treatment)				
M	NS	NS	NS	NS	NS
H	*	*	NS	NS	NS
M x H	NS	NS	NS	NS	NS

\*  $P < 0.05$ ; NS not significant

### **3.3 Experiment 4: AM Colonization Over Time**

#### **3.3.1 Mycorrhizal Colonization**

Over time, AM colonization increased from 4% at four weeks, to 34% at six weeks, to 22% at eight weeks, and to 37% at ten weeks of growth. There was no AM colonization in the non-AM plants.

#### **3.3.2 Physiological Data**

According to the ANOVA, AM colonization ( $P < 0.05$ ) and time ( $P < 0.05$ ) had significant effects on shoot height, shoot dry mass, and root dry mass (Table 3.15). According to the Tukey's test, shoot height, shoot dry mass, and root dry mass were significantly higher in the M+ plants compared to the M- plants at week 10.

#### **3.3.3 Isoflavonoid Analyses**

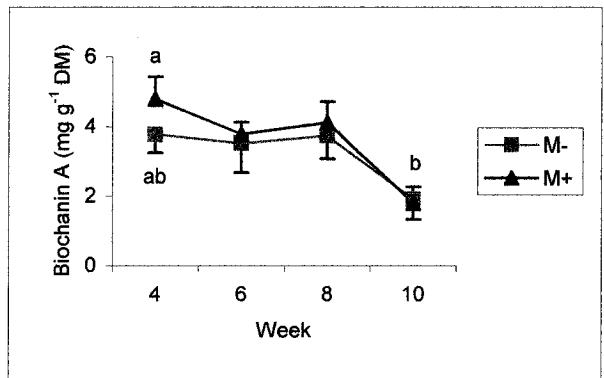
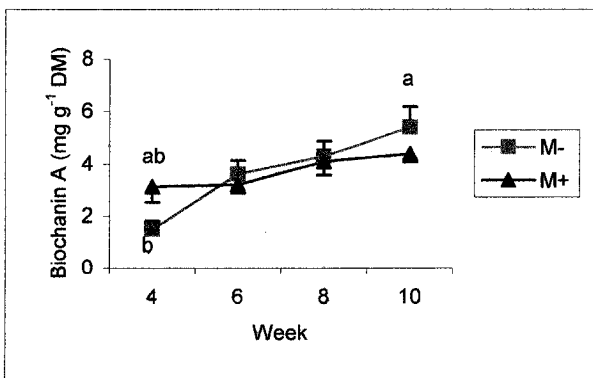
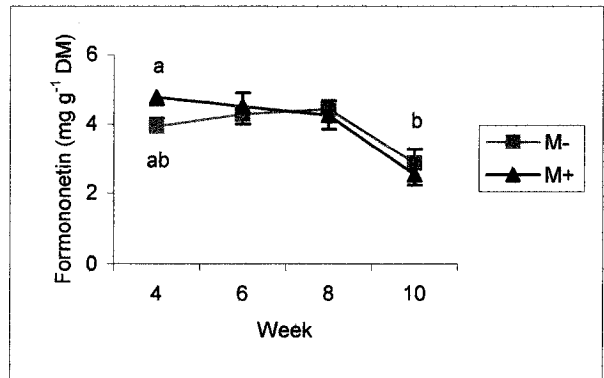
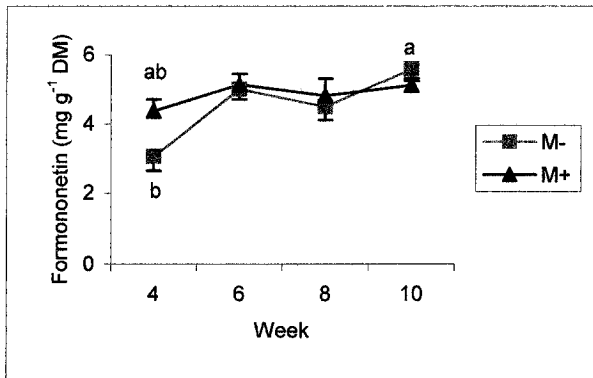
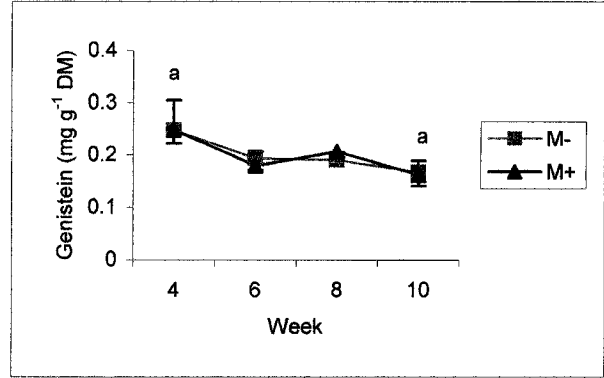
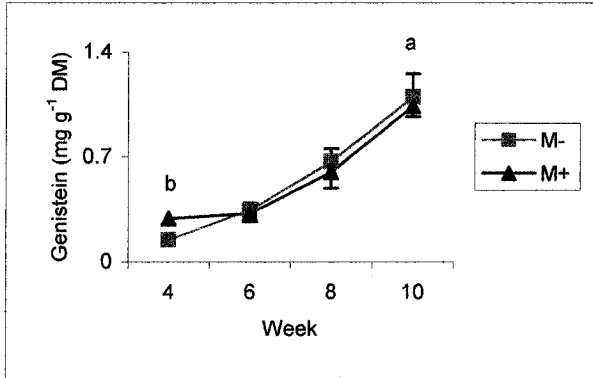
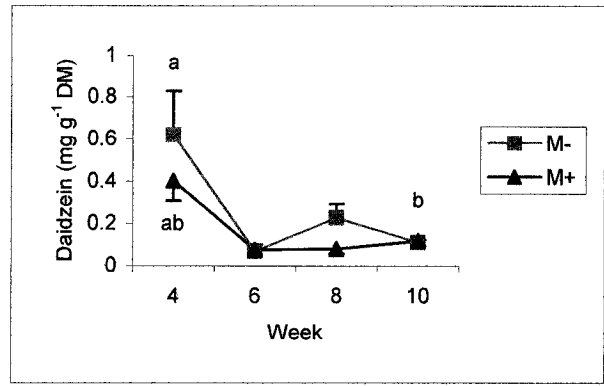
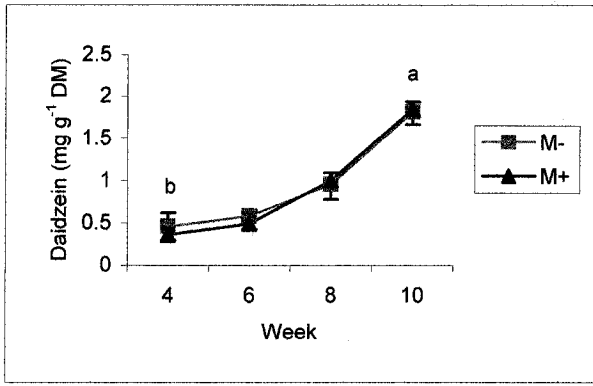
According to the ANOVA (Table 3.16), time had a significant effect ( $P < 0.001$ ) on all the isoflavonoid concentrations in the shoots and roots. There was no significant effect of mycorrhiza or any interaction. In the shoots (Fig. 3.11 left), according to the Tukey's test, daidzein and genistein concentrations significantly increased over time in both the M- and M+ treatments. The formononetin and biochanin A concentrations also increased over time, but, only significantly in the M- treatments.

**Table 3.15** Shoot height, shoot dry mass, and root dry mass (n = 10) of red clover with (M+) or without (M-) AM colonization after 4 to 10 weeks of growth. Means and SE's (in parentheses) are shown, and, within each column, different letters refer to significant differences according to Tukey's test at  $P < 0.05$ .

	Shoot Height (cm)	Shoot Dry Mass (g)	Root Dry Mass (g)
<i>4 Weeks</i>			
M-	8.19 <sup>e</sup> (0.21)	0.12 <sup>c</sup> (0.02)	0.06 <sup>c</sup> (0.01)
M+	8.95 <sup>de</sup> (0.53)	0.29 <sup>c</sup> (0.02)	0.23 <sup>c</sup> (0.02)
<i>6 Weeks</i>			
M-	11.01 <sup>cd</sup> (0.72)	0.58 <sup>b</sup> (0.05)	0.46 <sup>b</sup> (0.05)
M+	12.20 <sup>c</sup> (0.29)	0.59 <sup>b</sup> (0.05)	0.44 <sup>b</sup> (0.05)
<i>8 Weeks</i>			
M-	12.60 <sup>bc</sup> (0.33)	0.67 <sup>b</sup> (0.04)	0.48 <sup>b</sup> (0.02)
M+	14.75 <sup>ab</sup> (0.67)	0.73 <sup>b</sup> (0.03)	0.55 <sup>b</sup> (0.02)
<i>10 Weeks</i>			
M-	12.67 <sup>c</sup> (0.27)	0.79 <sup>b</sup> (0.02)	0.58 <sup>b</sup> (0.04)
M+	15.24 <sup>a</sup> (0.21)	1.03 <sup>a</sup> (0.04)	0.71 <sup>a</sup> (0.03)
ANOVA: M (Mycorrhizal treatment), T (Time)			
M	*	*	*
T	*	*	*
M x T	NS	*	NS

\* $P < 0.05$ ; NS not significant

Fig. 3.11 Concentration of isoflavonoids ( $\text{mg g}^{-1}$  DM) ( $n = 5$ ) in shoots (left) and roots (right) of red clover with (M+) or without (M-) AM colonization from 4 to 10 weeks. Statistical analyses for each isoflavonoid were done for roots and shoots separately. Mean and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ . Data points without letters indicate no significant differences between the treatments and overlapping points with a single set of letters indicate no significant differences between the M- and M+ treatments.



**Table 3.16** Levels of significance of ANOVA for the concentrations of isoflavonoids (mg g<sup>-1</sup> DM) in shoots and roots of red clover with (M+) or without (M-) AM colonization from 4 to 10 weeks (M mycorrhizal treatment; T time).

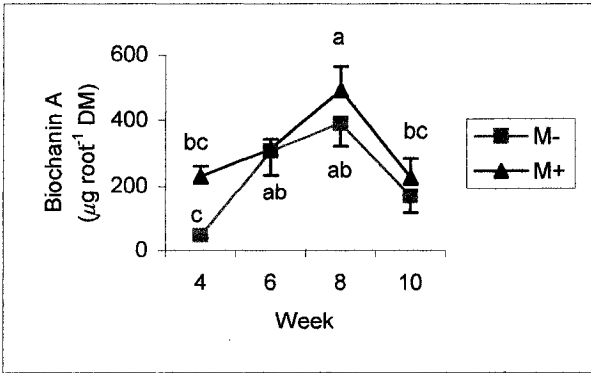
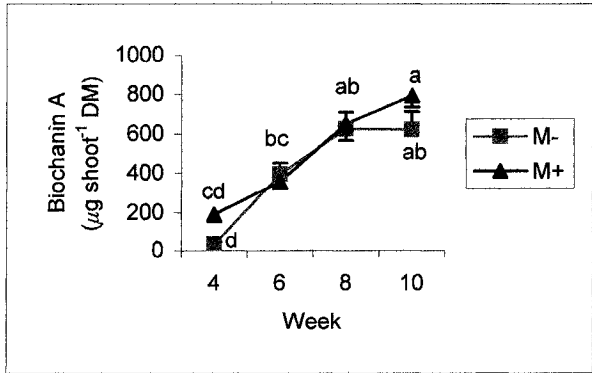
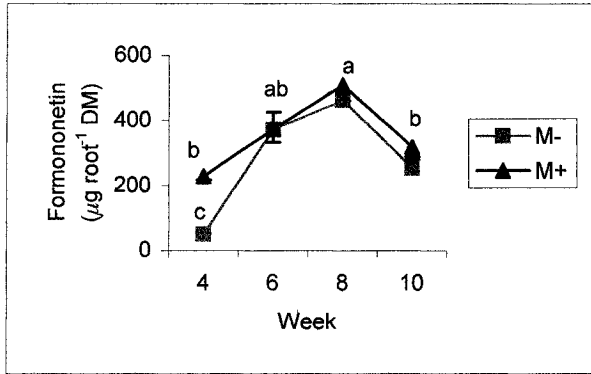
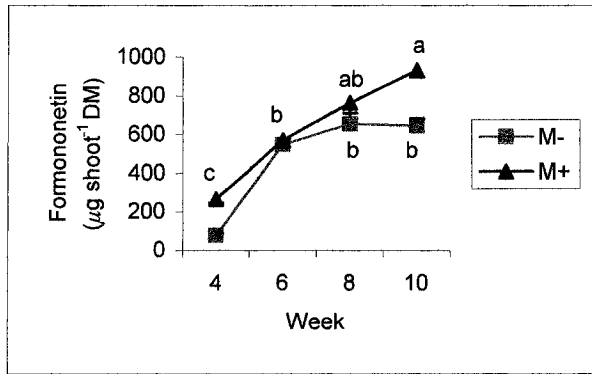
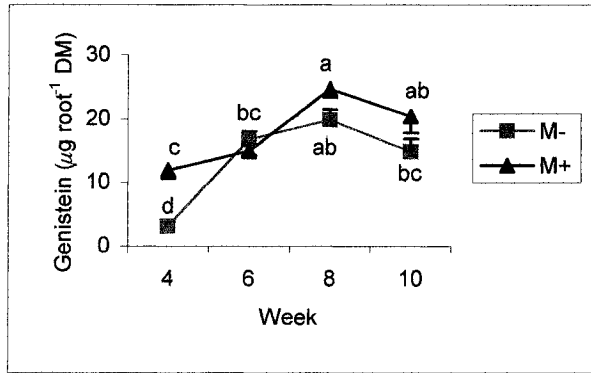
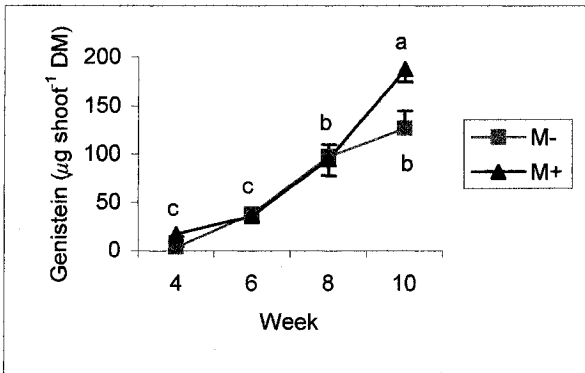
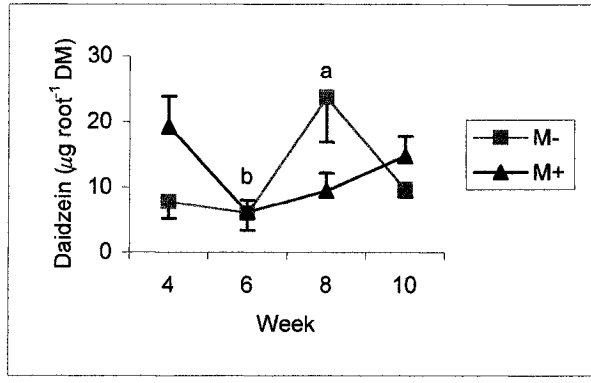
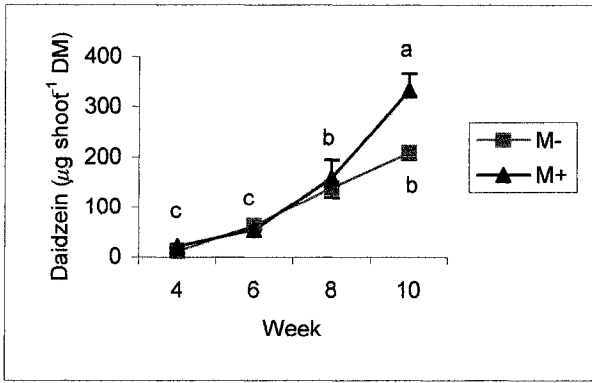
	M	T	M x T
<i>Shoots</i>			
Daidzein	NS	*	NS
Genistein	NS	*	NS
Formononetin	NS	*	NS
Biochanin A	NS	*	NS
<i>Roots</i>			
Daidzein	NS	*	NS
Genistein	NS	*	NS
Formononetin	NS	*	NS
Biochanin A	NS	*	NS

\*  $P < 0.05$ ; NS not significant

In the roots (Fig. 3.11 right), daidzein concentrations significantly decreased over time for the M- treatment only. There was no significant difference in the genistein concentration over time with or without mycorrhiza. The formononetin and biochanin A concentrations decreased over time, only significantly in the M+ treatments and especially between the 8<sup>th</sup> and 10<sup>th</sup> week of growth.

According to the ANOVA (Table 3.17), time had a significant effect ( $P < 0.001$ ) on all the isoflavonoid contents in the shoots ( $\mu\text{g shoot}^{-1}$  DM) and the roots ( $\mu\text{g root}^{-1}$  DM). Mycorrhiza also had a significant effect ( $P < 0.04$ ) on all the isoflavonoid contents in the shoots and roots except for biochanin A in the shoots and daidzein in the roots. In the shoots (Fig. 3.12 left), according to the Tukey's test, the isoflavonoid contents increased overtime in both the M+ and M- treatments. At week 10, the contents of the M+ treatments were higher by 1.6, 1.5, and 1.4 fold for daidzein, genistein, and formononetin, respectively, as compared to the M- treatments. In the roots (Fig. 3.12 right), the isoflavonoid contents increased up to week eight and subsequently decreased at week ten.

Fig. 3.12 Isoflavonoid content ( $n = 5$ ) in shoots ( $\mu\text{g shoot}^{-1}$  DM) (left) and roots ( $\mu\text{g root}^{-1}$  DM) (right) of red clover with (M+) or without (M-) AM colonization from 4 to 10 weeks. Statistical analyses for each isoflavonoid were done for roots and shoots separately. Mean and SE's are shown and different letters refer to significant differences according to Tukey's test at  $P < 0.05$ . Data points without letters indicate no significant differences between the treatments and overlapping points with a single set of letters indicate no significant differences between the M- and M+ treatments.



**Table 3.17** Levels of significance of ANOVA for isoflavonoid content ( $\mu\text{g shoot}^{-1}$  or  $\text{root}^{-1}$  DM) in shoots and roots of red clover with (M+) or without (M-) AM colonization from 4 to 10 weeks (M mycorrhizal treatment; T time).

	M	T	M x T
<i>Shoots</i>			
Daidzein	*	*	*
Genistein	*	*	*
Formononetin	*	*	*
Biochanin A	NS	*	NS
<i>Roots</i>			
Daidzein	NS	*	*
Genistein	*	*	*
Formononetin	*	*	NS
Biochanin A	*	*	NS

\*  $P < 0.05$ ; NS not significant

## CHAPTER 4

### DISCUSSION

#### 4.1 Experiments 1&2: AM Colonization with or without *Rhizobium*

##### 4.1.1 Mycorrhizal Colonization and Nodulation

The colonization levels of red clover, *Trifolium pratense* L., roots with *Glomus intraradices* were well established after 10 and 12 weeks of growth. The colonization levels (22 – 37%) are comparable with other studies on red clover. Bi *et al.* (2003) obtained colonization levels ranging from 14 - 38% in red clover roots colonized with *G. mosseae* after 6 weeks of growth. After 10 weeks, Arines *et al.* (1993) and Chen *et al.* (2003) obtained 45% and 50% colonization levels, respectively, in red clover roots inoculated with *G. mosseae*. Nadian *et al.* (1997) obtained 45% colonization with *G. intraradices* in *T. subterraneum* L. after 7 weeks of growth.

In the first experiment, the plant roots of all the treatments were nodulated with *Rhizobium*. However, those of the non-preinoculated treatments were nodulated with approximately half the number of nodules as compared to the preinoculated treatments. These results correspond to those of Edwards *et al.* (1997) who reported visible nodules in their non-preinoculated red clover plants. Arines *et al.* (1993), who grew red clover without any *Rhizobium* preinoculation, also showed after 10 weeks of growth that their plant roots were naturally inoculated.

Our results seem to indicate that arbuscular mycorrhizal (AM) colonization and nodulation are not dependent upon each other. These observations correspond to the

results of Xie *et al.* (1995) who found in soybean that a functional rhizobial symbiosis is not necessary for enhanced mycorrhizal formation.

#### **4.1.2 Physiological Data**

In the first experiment, the co-inoculation with *G. intraradices* and *Rhizobium* enhanced the shoot and root dry masses more than inoculation with either microsymbiont alone. Shoot height also increased with AM colonization and/or *Rhizobium* nodulation compared to the non co-inoculated control. These results correspond to the findings of El-Ghandour *et al.* (1996), who reported that fungal colonization and rhizobial inoculation either alone or in combination increased dry matter yield of faba bean as compared to uninoculated plants. Priya *et al.* (2001) indicated that co-inoculation with AM fungus and *Rhizobium* resulted in maximum plant growth of *Prosopis cineraria* L. In addition, Leopold and Hofner (1991) showed that in pot trials with Persian clover, maximum shoot yield occurred with the combined inoculation of AM fungi and *Rhizobium*. Goicoechea *et al.* (1997) showed that alfalfa plants have similar dry mass with AM colonization and/or *Rhizobium* under well-watered conditions; however, the dry mass of the drought-stressed non-inoculated plants was significantly lower than the dry mass of plants inoculated with AM fungi and/or *Rhizobium*. According to Xavier and Germida (2003) and Xie *et al.* (1995), the growth and productivity of legumes depend on the specific combination of AM fungi and rhizobia; thus, compatible microsymbionts result in growth and yield increases.

In the second experiment, in the absence of rhizobial preinoculation, AM colonization increased the shoot height, shoot dry mass and root dry mass of red clover. Chen *et al.* (2003) obtained similar results with red clover inoculated with *G. mosseae*, for which shoot and root dry masses were significantly increased by AM colonization. Bi *et al.* (2003) showed shoot yield to be the highest in plants of red clover inoculated with *G. mosseae*, but only with added phosphorous. A study by Nadian *et al.* (1997) also showed increased shoot dry mass of *T. subterraneum* inoculated with *G. intraradices*; however, all the plants were also nodulated with *Rhizobium*.

The improved plant growth with AM colonization is believed to result from greater efficiency of nutrient absorption by the AM mycelium (Smith and Read, 1997). The hyphae growing beyond the rhizosphere considerably increase the absorptive surface area of the root (Frey and Schuepp, 1993). Chen *et al.* (2003) stated that it was the uptake of the slowly diffusing mineral P, via the extramatrical mycelium, that enhanced their shoot yield. In our study, the average P concentration was higher, although not significantly, in the AM plants compared to non-AM plants.

#### **4.1.3 Glutamine Synthetase (GS)**

There are numerous reports on the combined inoculation of AM fungi and *Rhizobium* on nitrogen fixation. The majority of these studies have indicated that this combined inoculation improved nitrogen fixation (Singh, 1996; Ianson and Lindermann, 1993; Barea *et al.*, 1987). When nitrogen and phosphorus are limiting, AM fungi improve P uptake; the higher the P concentration in the plant benefits the bacterial symbiont and the

functioning of its nitrogenase, leading to increased N fixation (Fitter and Garbaye, 1994; Ibjibijen *et al.*, 1996). Very few studies, however, have investigated the effect on the combined inoculation on GS activity.

In the first experiment, after 12 weeks of growth, AM colonization and *Rhizobium* either alone or in combination had no significant effect on the GS activity in the shoots of red clover plants. In the roots, *Rhizobium* tended to decrease the GS activity, while AM colonization and the co-inoculation had no significant effect. The results of the present study correspond to the findings of Fateh (1994), in which leaves of flowering lentils showed a decrease in GS activity when inoculated with *Rhizobium*. Lynd and Ansman (1994, 1995 and 1997) found in nodule cytosol of *Cassia Chamaecrista fasciculata* L., *Lupinus albus* L., and *Astragalus cicer* L., no mycorrhizal effect on GS, GDH, and GOGAT activities.

These results are not in accordance with the results obtained by Basha and Vivekanandan (2002) where *Vigna mungo* L. inoculated with *Rhizobium* showed a significant increase in GS activity compared to the non-inoculated control plants. Smith *et al.* (1985), showing a two-fold increase in GS activity in roots of *T. subterraneum* inoculated with both *Rhizobium* and a mycorrhizal fungus, concluded that AM fungi are able to assimilate ammonium via GS.

According to Harrison *et al.* (2003), the reduction in GS activity is correlated with an increase in amino acid content in the nodules, primarily in asparagine. They

hypothesized that when GS becomes limiting, other enzymes (e.g. asparagine synthetase, AS) have the capacity to assimilate ammonium and may be important in controlling the flux of reduced N in temperate legumes. A recent study by Carvalho *et al.* (2003) supports this hypothesis. They found that GS activity negatively regulates the level of AS in root nodules of *Medicago truncatula* L. When the level of GS was reduced, the expression of AS (polypeptides and transcripts) was increased and vice versa. The regulation was specific for AS, as no changes were observed in the expression of five other key nitrogen and carbon assimilatory enzymes in nodules (NADH-GOGAT, aspartate aminotransferase, carbonic anhydrase, phosphoenolpyruvate carboxylase, and sucrose synthase). Moreover, AS can use ammonium instead of glutamine as a nitrogen donor when the level of ammonium is high (Oaks and Ross, 1984; Lam *et al.*, 1996).

An increase in AS expression is not maintained when GS is completely inhibited (Carvalho *et al.*, 2003). This suggests that maintaining partial GS activity is essential for maintaining the higher AS level (Carvalho *et al.*, 2003). Furthermore, as stated by these authors, the resulting pathway of dual ammonium assimilation via GS and AS would be energetically more favorable than ammonium assimilation through GS alone. They estimated 17% savings in energy requirements, and thus, an increase in plant productivity would result. Our results support this claim, as both the root biomass and shoot biomass increased as well as the protein concentrations but there was no significant effect on GS activity or there was a reduction of GS activity when colonized with *G. intraradices* and/or *Rhizobium*. Harrison *et al.* (2003) found minor increases in biomass following a specific reduction of nodule GS activity. Finally, according to Carvalho *et al.* (2003),

when GS was completely inhibited and the associated increase in AS was transient, there was an increase in GDH expression. Clearly, much work still needs to be done on studying the nitrogen assimilation pathway and understanding the consequences in relation to plant productivity and biomass.

In the second experiment, after 10 weeks of growth, AM shoots and roots showed a significant increase in GS activity compared to the non-AM shoots and roots. This increase in GS activity suggests that in the absence of *Rhizobium*, the dual ammonium assimilation pathway with AS is decreased. According to Carvalho *et al.* (2003), AS expression would decrease with increased GS activity. Our results from the second experiment are in accordance with those of Cliquet and Stewart (1993) where GS activity increased in both the shoots and roots of maize colonized with *G. fasciculatum* compared to the control plants. Azcón *et al.* (1992) found increased GS activity in *Lactuca sativa* L. colonized with *G. fasciculatum*. According to Subramanian and Charest (1998 and 1999), maize roots colonized with *G. intraradices* also had higher GS activity compared to non-AM roots.

These results support that AM fungi are able to assimilate ammonium via GS (Smith *et al.*, 1985), and that the AM association plays a major role in improving host plant N assimilation and nutritional status (Subramanian and Charest, 1998; Toussaint *et al.*, 2004). According to Lea *et al.* (1990), the enhanced P status of AM plants alters the activities of N-assimilating enzymes, especially GS which requires ATP. Oliver *et al.* (1983) showed increased NR activities in AM-colonized white clover, attributed to a P-

mediated mechanism. In contrast, Azcón and Tobar (1998) showed an increase in GS activities in roots and shoots of *Allium cepa* L. colonized with *G. fasciculatum* regardless of P status. Our data also suggest that AM association contributes to the increase in GS activity regardless of P status; as the P concentration did not significantly differ between the treatments.

The AM colonization assisted the host plant to maintain higher protein concentrations in both shoots and roots. Similarly, Arines *et al.* (1993) found a 2 to 6 fold increase in protein content in mycorrhizal red clover roots compared to the non-AM roots. Subramanian and Charest (1998) also found increased protein concentration by AM association in maize plants.

Finally, in the first and second experiments, the GS specific activity was the highest in the control treatments in both the shoots and roots of red clover. The only exception was in the second experiment, where the GS specific activity was higher in AM than non-AM roots. These results are consistent with our earlier findings that the co-inoculation of AM fungi and/or *Rhizobium*, is more efficient in producing proteins than in the control plants.

In summary, the present study suggests that when *Rhizobium* is present, a dual ammonium assimilation pathway may occur in mycorrhizal red clover plants. In the absence of *Rhizobium*, AM colonized plants have enhanced GS activity and the AM association plays a major role in improving host plant N assimilation. Future work of

interest would be to study the other enzymes associated with N assimilation, especially AS and GDH.

#### **4.1.4 Isoflavonoid Analyses**

Overall, the isoflavonoid analyses from the first and second experiments indicate that AM and/or rhizobial treatments had no significant effects on the concentration of isoflavonoids in red clover shoots and roots after 10 and 12 weeks of growth. These results correspond to the study of Codignola *et al.* (1989) who stated that AM fungi did not induce defense responses in host plants. Many studies have found that AM fungi and their host plants produce highly compatible interactions, where the plant defense responses are small (Bonfante and Perotto, 1995). Spanu *et al.* (1989) were one of the first to show the high compatibility between host plants and AM fungi. They showed in leek that chitinase, which degrades the fungal wall, was weakly activated in the early stages of colonization, but was then suppressed as root colonization by *G. versiforme* progressed.

In most cases, an induction of defense-gene responses (in the roots) was detected at early stages of root colonization followed by suppression at later stages of the symbiosis (Bonfante and Perotto, 1995; Fries *et al.*, 1998; Gianinazzi-Pearson *et al.*, 1996; Kapulnik *et al.*, 1996; Lambais and Mehdy, 1995; Volpin *et al.*, 1994). Lambais and Mehdy (1995) suggested that phytohormones (e.g. cytokinins), whose concentrations changed in AM roots, act as long distance signal molecules and modulate the expression of plant-defense-related genes. The behaviour of AM fungi is comparable to rhizobia, where

defense responses are not activated in effective nodule tissues, whereas they are elicited in ineffective *Rhizobium*-legume interactions (Bonfante and Perotto, 1995).

The fact, in this study, that the concentrations of isoflavonoids were maintained with fungal colonization and/or rhizobial inoculation may indicate that the partitioning of defense resources were also maintained. Ruehmann and Treutter (2003) indicated that partitioning of defense chemicals changed in plants with differing availability of nutrients, specifically N. Apple plantlets grown in high N-nutrition medium had reduced levels of total phenolic compounds in the leaves, whereas those grown in low N-nutrition had increased leaf total phenolic contents. Przemyslaw *et al.* (1993) obtained similar results with roots of white lupin. They found that a high concentration of N in their medium decreased the exudation of phenolics.

Due to an increased plant growth, some of the isoflavonoid contents found in red clover significantly increased with AM colonization and/or *Rhizobium*. Specifically, in the first experiment, the co-inoculation of AM fungi and *Rhizobium* significantly increased the formononetin content in the combined roots and shoots compared to the control. In the second experiment, AM colonization significantly increased the contents of daidzein, genistein and formononetin in the combined roots and shoots.

The isoflavonoid analyses from both experiments (1 & 2) suggest that the functional symbiotic roots and shoots containing mutualistic AM fungi and/or *Rhizobium* do not contain increased concentrations of isoflavonoids. However, studies have indicated that

both bacterial and fungal mutualistic symbionts trigger defense-like reactions in host plants during the establishment of the symbioses (Dakora and Phillips, 1996). According to Gianinazzi-Pearson *et al.* (1996), these defense-like reactions are, however, uncoordinated, weak, transient and/or very localized.

#### **4.1.5 Mineral Content**

The mineral concentrations in red clover did not vary to any great degree between the treatments. In the first experiment, in the shoots of red clover, Ca tended to increase with mycorrhizal colonization while Mg tended to decrease with mycorrhizal and/or rhizobial inoculation. In the mycorrhizal roots, the N, Mg and Ca concentrations tended to decrease. In the second experiment, there were no significant effects of AM colonization on any of the studied macronutrients in the shoots or roots. These results are unexpected, as plant mineral content usually increases with the addition of mycorrhizal fungi (Smith and Read, 1997). Hyphae growing beyond the rhizosphere increase the absorptive surface of the roots and enhance the uptake of mineral ions (Smith and Read, 1997). The effect is most pronounced when minerals are sparse in the soil environment. The most prominently researched of the elements enhanced by mycorrhizae is phosphorus (P). Numerous greenhouse and field experiments have shown that plants colonized by AM fungi are much more efficient in taking up soil P than non-AM plants (Asmah, 1995; Augé *et al.*, 1994; Koide and Kabir, 2000; Xiao-Lin *et al.*, 1997). Other macronutrients (N, K, Ca and Mg) have also been shown to increase during AM colonization (Subramanian and Charest, 1997), specifically when plants were grown in acidic soils, under severe mineral stress (Clark and Zeto, 1996, 2000; Lambais and Cardoso, 1993).

By contrast, the micronutrients (Mn, Zn, and Fe) significantly increased in AM and/or rhizobial treatments in the shoots and roots of red clover in both the first and second experiments. These results correspond to the ones of Chen *et al.* (2003) who found that AM colonization increased Zn absorption and accumulation in the roots of red clover. Liu *et al.* (2000) found that in soils with low P and low or no micronutrient addition, Zn and Fe contents were higher in AM than in non-AM plants of maize. Accordingly, AM fungi can increase plant uptake of soil trace nutrients when they are in short supply (Bi *et al.*, 2003). According to Azcón *et al.* (2003), a high availability of N and P in the soil reduced the content of macro (N, P, and K) and micronutrients (Mn and Zn) in AM compared to non-AM lettuce plants.

Al-Karaki and Al-Raddad (1997) found higher contents of P, Zn, Cu, Mn and Fe in mycorrhizal compared to non-mycorrhizal wheat plants and higher still under well-watered than drought-stressed conditions. In contrast, Sylvia *et al.* (1993) reported that colonization with *G. fasciculatum* increased the concentrations of P and Cu in both shoots and grains of field-grown maize under increasing intensities of drought stress. Subramanian and Charest (1997) reported mycorrhizal maize plants to have significantly higher uptake of N, P, K, Mg, Mn, and Zn into grain than non-mycorrhizal plants under drought conditions. Joner and Leyval (2001) stated that root density is crucial to control in pot experiments where plant uptake of nutrients through mycorrhiza is quantified. Mycorrhizal functioning depends on exploitation of non-rhizosphere soil by extraradical AM hyphae. If the plants are big and pots small, the soil is efficiently scavenged for

nutrients by the roots, and no additional effect can be expected from AM hyphal network (Joner and Leyval 2001).

## **4.2 Experiment 3: AM Colonization and Induction with Jasmonic Acid (JA)**

### **4.2.1 Mycorrhizal Colonization**

The colonization levels of red clover roots with *Glomus intraradices* after seven weeks of growth were quite high with (42.1%) or without (52.8%) jasmonic acid (JA) treatment. This trend for AM colonization to slightly decrease with JA treatment corresponds to some extent to the study of Ludwig-Muller *et al.* (2002) who found that JA greatly reduced AM root colonization in *Tropaeolum majus* L., *Carica papaya* L., and cucumber. Ludwig-Muller *et al.* (2002) also found that JA levels increased in cucumber roots during advanced stages of mycorrhizal colonization, and postulated that during the later stages JA is needed to maintain the homeostasis between plants and AM fungi. The application of JA during the early stages of mycorrhizal colonization may contribute to its later suppression (Ludwig-Muller *et al.*, 2002). In our study, JA was sprayed onto the leaves two weeks after germination; thus, this early application of JA may have contributed to the 10% difference in AM colonization between the JA and non-JA treatments, five weeks later.

My results with JA are in contrast to those of Regvar *et al.* (1996) who foliar-treated garlic plants with JA and found a promotion of AM colonization. However, the promotion of AM colonization was observed at low applied concentrations of JA (5  $\mu\text{mol/L}$ ) and was only applied once a week. In our study, JA was applied twice a week with a much higher concentration (918  $\mu\text{mol/L}$ ). Regvar *et al.* (1997) also reported that the first mycorrhizal contact of fungal hyphae with spruce seedling roots was significantly accelerated after treatment with 0.5  $\mu\text{mol/L}$  and 5  $\mu\text{mol/L}$  JA.

#### 4.2.2 Physiological Data

An alteration of plant growth was clearly visible in the JA treatment. The control treatment had significantly greater shoot height and root dry mass than all the other treatments. For the shoot dry mass, the control treatment was significantly higher than the mycorrhizal plus JA treatment. These results correspond with the study on *Solanum tuberosum* L. by Ulloa *et al.* (2002) who found that under high hormonal conditions, the inhibition of plant growth was due to the severe hormone-induced stress. The application of 50  $\mu$ M JA decreased stem and leaf growth, and strongly impeded root development, whereas 250  $\mu$ M JA produced stunted growth (Ulloa *et al.*, 2002). According to Ludwig-Muller *et al.* (2002), in *C. papaya*, a 5mmol/L JA application resulted in a drastic reduction, and with only 0.5mmol/L JA, in a slight reduction of root fresh mass. Similarly, in cucumber and *C. papaya*, a 5mmol/L JA application reduced shoot fresh mass (Ludwig-Muller *et al.*, 2002).

Ueda *et al.* (1995) postulated that JA inhibits the synthesis of cell wall polysaccharides, then resulting in JA-induced inhibition of plant growth. It was also reported by Ulloa *et al.* (2002) that chloroplasts from JA-treated plants are disorganized and do not contain starch granules. Moreover, JA is known to promote leaf senescence, which is characterized by chlorophyll degradation, increases in cellular respiration rate, as well as a reduction of photosynthetic activity (Ulloa *et al.*, 2002).

In the present study, it is also important to note that in contrast to the mycorrhizal plants, the non-mycorrhizal ones, with or without JA, had their roots naturally inoculated with

*Rhizobium*; thus probably playing a role in this increased plant growth. As for mycorrhizal colonization, it did not seem to have a positive effect on plant growth regardless of JA application. This contrasts with the study of Regvar *et al.* (1997) who reported that shoot dry mass of spruce seedlings were significantly increased by mycorrhizal colonization, irrespective of JA. In addition, a significant increase of mycorrhizal root dry mass was shown when JA was applied, the small concentration (5.0  $\mu\text{M}$ ) showing the most prominent effects.

#### **4.2.3 Isoflavonoid Analyses**

From the isoflavonoid analyses of the 3<sup>rd</sup> experiment, the JA treatment approximately doubled the concentrations of formononetin, biochanin A and genistein compared to the non-JA treatment, in the shoots of red clover plants. My findings correspond with a number of studies. For example, Yasufumi *et al.* (2001) reported that JA induced the accumulation of isoflavons in white lupin. Richard *et al.* (2000) showed that chalcone synthase (catalyzes the first step in the flavonoid biosynthesis) mRNA in spruce needles accumulates following JA application. Tebayashi *et al.* (2000) found in 5-day-old red clover roots, an induction of clovamide (a secondary metabolite found in red clover, for which no biological function was elucidated) following JA application. Lee *et al.* (1997) reported induction of two phenolic components in barley leaves in response to JA treatments.

The induction of isoflavonoids with JA application strongly contrasts with the low and transient expression of defense-like reactions from the establishment of mycorrhizal

fungi (Gianinazzi-Pearson *et al.*, 1996; Hirsch and Kapulnik, 1998; Mohr *et al.*, 1998). According to Mohr *et al.* (1998), symbionts and pathogens employ different strategies to cope with the plant's defense. Dakora and Phillips (1996) stated that during mutualistic interactions, exuded isoflavonoids come from excreted phytoanticipins (isoflavonoids stored in plants cells in anticipation of a pathogenic attack) as opposed to newly synthesized phytoalexins (formed in response to a pathogen attack). The low concentrations of phytoanticipins are released continuously as signals that attract and produce useful microbial communities while higher levels of the same compounds would help combat pathogens (Dakora and Phillips, 1996; Dixon, 2001). The regulatory events that control whether the molecules function as phytoalexins or phytoanticipins still remain to be elucidated (Dakora and Phillips, 1996).

Comparing the physiological parameters and the isoflavonoid concentrations, it seems, in the present study, that compensatory effects occurred between growth and defense. The JA treatment increased the isoflavonoid concentrations, but lowered plant growth. According to Herms and Mattson (1992), trade-offs between growth and defense are inevitable; primary and secondary metabolic pathways share common precursors and intermediates. Phenylalanine is the rate-limiting precursor for phenylpropanoid synthesis (flavonoids) and, at the same time, is an essential amino acid for protein synthesis (Herms and Mattson, 1992). As such, phenolic synthesis competes with growth for common substrates (Herms and Mattson, 1992).

Finally, in the roots of red clover plants, JA application and mycorrhizal colonization had no significant effect on the concentration of isoflavonoids, except for biochanin A. This significant effect may be due to methodology and cannot be interpreted as an effective induction effect. However, we should not reject the hypothesis that JA sprayed on the shoots has an impact on root metabolism.

### **4.3 Experiment 4: AM Colonization Over Time**

#### **4.3.1 Mycorrhizal Colonization**

Mycorrhizal colonization increased from 4% at four weeks, to 34% (mostly hyphae) at six weeks, to 22% at eight weeks, and to 37% at ten weeks of growth. These results are comparable to those of Joner and Leyval (2001) who found the colonization of *G. mosseae* in *Trifolium subterraneum* to be 3% at three weeks, 17% at six weeks and 33% at nine weeks of growth.

#### **4.3.2 Physiological Data**

The growth of red clover was significantly increased with mycorrhizal colonization after ten weeks of growth. This coincides with the increase in percentage of mycorrhizal colonization. Our results correspond to those of Joner and Leyval (2001) who found that after 6 weeks of growth with *G. mosseae*, subterranean clover and maize both had increased shoot and root dry masses.

#### **4.3.3 Isoflavonoid Analyses**

Time was the sole factor that influenced the isoflavonoid concentrations found in red clover. Generally, as time increased, the isoflavonoid concentrations seemed to increase in the shoots but decrease in the roots. These results strongly contrast those of Devi and Reddy (2002). Studying groundnut (*Arachis hypogaea* L.), these authors found that mycorrhizal and rhizobial inoculation significantly increased the concentration of phenolics in the roots and shoots compared to the uninoculated plants. Moreover, maximum total phenol content was recorded in roots and shoots at day 45 when

colonized with AM fungi and/or *Rhizobium*. Thus, the concentration of phenolics increased over time in both the shoots and the roots of groundnut and dual inoculation also increased the total phenol content.

The general accumulation of the isoflavonoid concentrations in the shoots of red clover over time coincides with the study of Briggs and Schultz (1990). They questioned the existence of a trade-off between growth and secondary metabolism based on their findings of a positive correlation between growth rate and the concentrations of secondary metabolites. As resource availability (light, water or nutrients) increased, photosynthesis also increased (Briggs and Schultz, 1990). The increased carbon assimilation creates the possibility for positive correlations between growth and secondary metabolism despite the competition for a common resource base (Briggs and Schultz, 1990). Moreover, in view of the isoflavonoids as phytoestrogens, their accumulation may reflect their defensive role as animal antifeedants (Edwards *et al.*, 1997).

The decreased isoflavonoid concentrations in the roots and increased concentrations in the shoots may also suggest the translocation of isoflavonoids. According to the study of Dakora and Phillipps (1996), analyses of alfalfa xylem sap showed that flavonoids are transported from roots to shoots. Chen *et al.* (2001) also provided evidence for phloem transport of glucosinolates (plant secondary metabolites found in the order Capparales) in *Arabidopsis sp.* Radiolabeled glucosinolates applied to the tip of leaves, were found in the phloem.

## GENERAL CONCLUSION

The main purpose of this study was to test the hypothesis that the symbiosis between red clover and the mycorrhizal fungus, *Glomus intraradices*, enhances nitrogen and a pathway of the secondary metabolism. To test this hypothesis, the objectives set forth were to determine the impact of AM colonization and/or rhizobial inoculation in red clover on the: 1) growth parameters, 2) nitrogen assimilation, via the activity of glutamine synthetase (GS) and soluble protein content, 3) contents of isoflavonoids (e.g. genistein, daidzein, biochanin A, and formononetin), and 4) contents of these four isoflavonoids over time and by induction with a hormonal factor, jasmonic acid (JA).

The AM colonization had beneficial effects on the growth parameters in red clover. The AM colonized plants had enhanced shoot height as well as increased shoot and root dry masses. In addition, co-inoculation with *Rhizobium* enhanced the physiological parameters more than inoculation with either microsymbiont alone. These results are in accordance with several studies that examined the beneficial effects of AM colonization on plant growth (Bi *et al.*, 2003; Chen *et al.*, 2003; El-Ghandour *et al.*, 1996; Goicoechea *et al.*, 1997; Leopold and Hofner, 1991; Priya *et al.*, 2001).

This study revealed that AM colonized red clover plants in the absence of *Rhizobium* preinoculation are able to assimilate ammonium via GS. Our findings correspond to the ones of Cliquet and Stewart (1993), Azcón *et al.* (1992), Subramanian and Charest (1998 and 1999), and Smith *et al.* (1985). These results support the importance of AM fungi in improving host plant N assimilation and nutritional status. However, our results also

revealed that when red clover was grown in the presence of *Rhizobium*, GS was no longer the main N assimilating pathway. Our results showed no significant effect on GS activity and even a decrease in GS activity in the presence of *Rhizobium* and/or mycorrhiza. A dual ammonium assimilation pathway may likely occur. The fact that in our study root and shoot biomasses increased as well as the protein concentrations, seems to support an alternative N assimilating pathway. To confirm which of the pathways are most prominent, other enzymes (e.g. AS, GDH) associated with N assimilation would need to be further examined.

Isoflavonoids serve many functions in addition to inhibiting pathogens. They also serve as chemo-attractants, promoters of microbial growth, signals within plants, and inducers of nodulation genes in *Rhizobium* (Dakora and Phillips, 1996). Isoflavonoids also represent one of the three major classes of phytoestrogens. From the results of this study, growing red clover with mycorrhizal fungi and/or *Rhizobium* had no significant effect on its concentration of isoflavonoids in the roots or shoots, after 10 weeks of growth. Thus, these microsymbionts did not induce defense-like reactions in red clover, and confirm the highly compatible interaction between AM fungi and *Rhizobium* with their host plant. However, because of an increased plant growth in AM and/or rhizobial plants, some of the isoflavonoid contents were significantly enhanced, promoting the use of AM fungi and *Rhizobium* to harness the isoflavonoids by natural means.

In contrast to the suppression of defense-like reactions in red clover from the establishment of AM fungal colonization, the JA treatment induced and enhanced the

concentration of isoflavonoids. Our results showed that JA induction approximately doubled the concentrations of formononetin, biochanin A and genistein in the shoots. Overtime, the isoflavonoid concentrations were also shown to increase in the shoots and decrease in the roots, demonstrating their possible translocation. These results clearly demonstrate the regulation of isoflavonoids in red clover. While symbiotic relationships elicit little or no defense reactions, there was a defense-like production of isoflavonoids with the JA application. Others have found similar results comparing AM colonization to plant-pathogen interactions (Gianinazzi-Pearson *et al.*, 1996; Hirsch and Kapulnik, 1998; Mohr *et al.*, 1998).

Mycorrhizal associations are extremely important in nature as they confer various advantages onto the host plant, including resisting environmental stresses and increased plant nutrient uptake. In light of this research, the study of AM fungi to promote a natural method to harness isoflavonoids for phytomedicinal purposes is promising, as the isoflavonoid contents increased due to increased plant growth. Future research would be to perform a field study to determine if mycorrhiza responsiveness differs under a more natural environment and to examine red clover with different symbiotic fungi.

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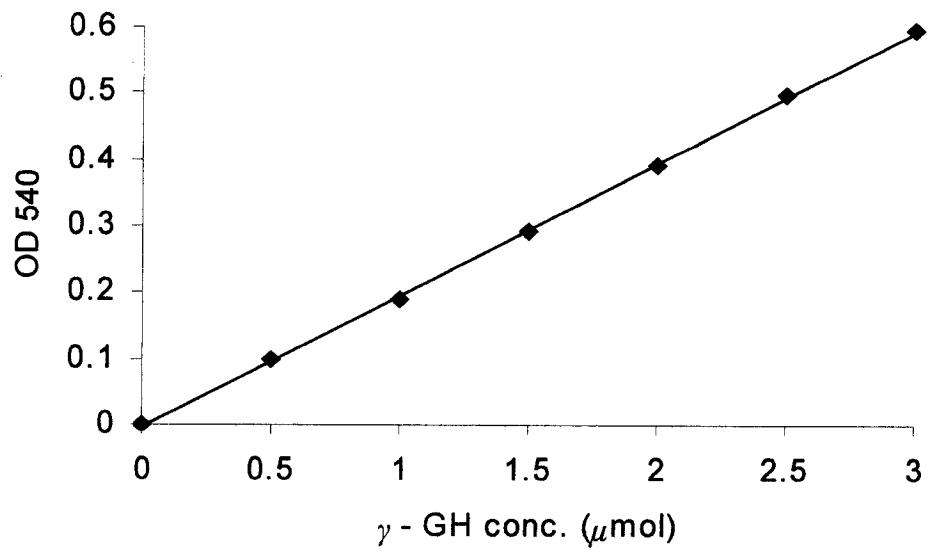
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**Appendix 1.** A standard curve of  $\gamma$  - glutamylhydroxamate ( $\gamma$  - GH) concentration for the determination of glutamine synthetase (GS) activity.

$\gamma$ - GH conc. ( $\mu\text{mol}$ )	OD <sub>540</sub>
0.0	0.000
0.5	0.099
1.0	0.192
1.5	0.294
2.0	0.391
2.5	0.496
3.0	0.597

**Regression output**

Constant	0.000
R squared	0.999
No. of observations	7
X Coefficients	0.199
Std Err of Coef.	0.081

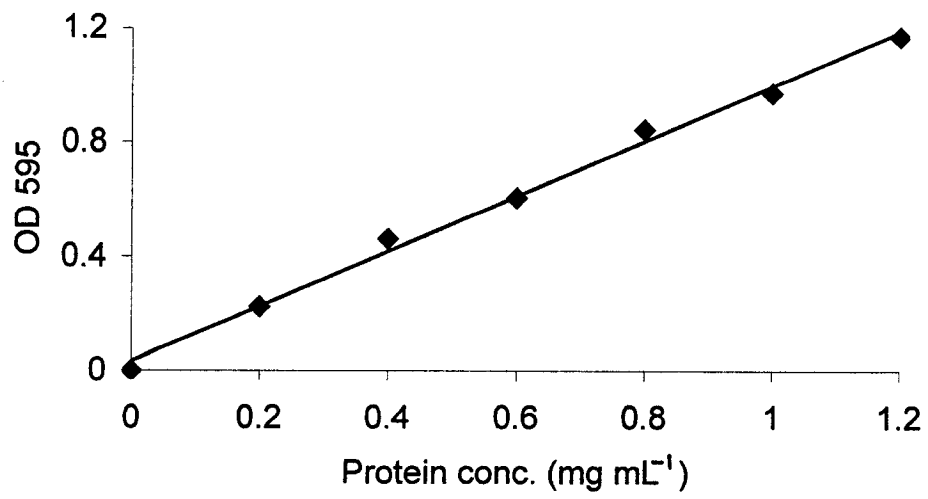


**Appendix 2.** A standard curve of BSA (bovine serum albumin) for the determination of protein concentrations.

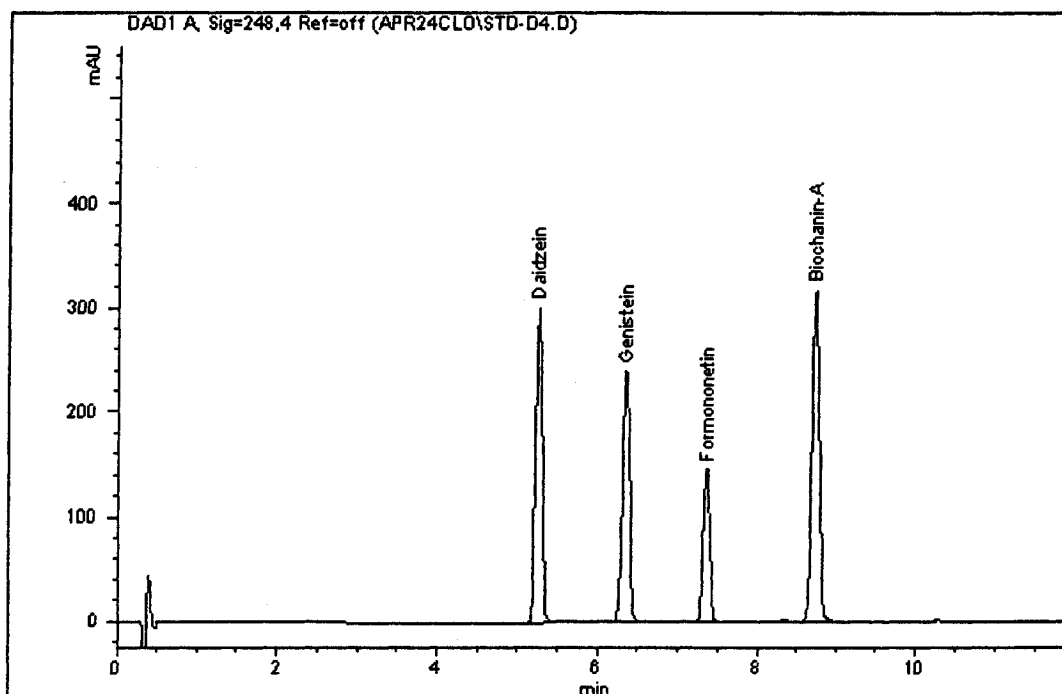
Protein conc. (mg mL <sup>-1</sup> )	OD <sub>595</sub>
0.0	0.000
0.2	0.226
0.4	0.463
0.6	0.605
0.8	0.843
1.0	0.971
1.2	1.171

**Regression output**

Constant	0.034
R squared	0.994
No. of observations	7
X Coefficients	0.961
Std Err of Coef.	0.157



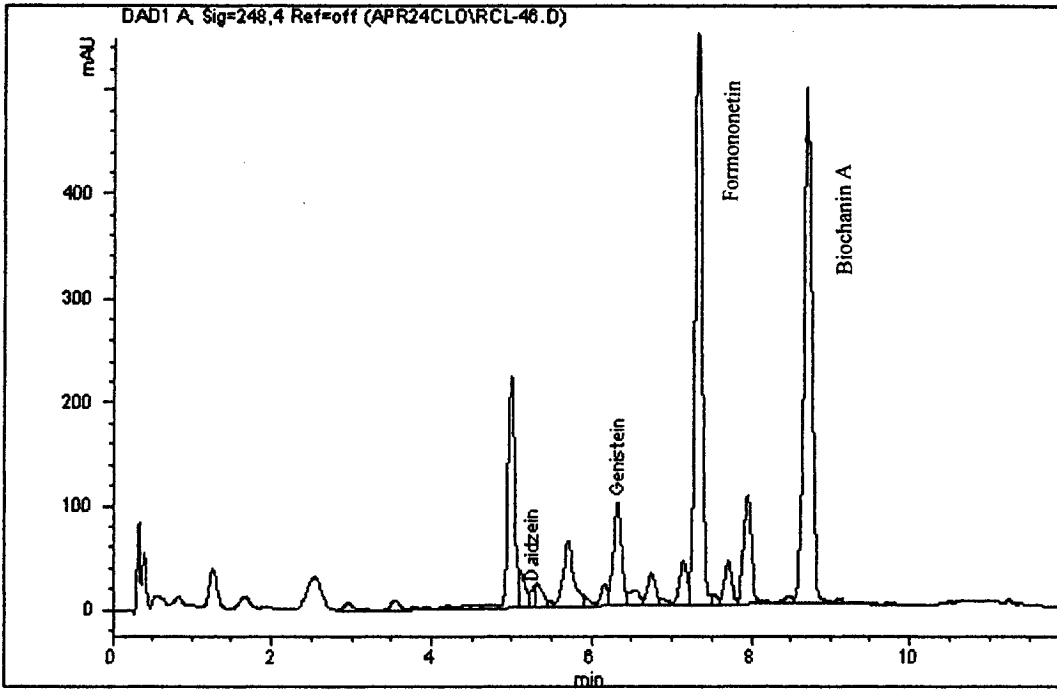
**Appendix 3. Chromatogram of pure standards: daidzein, genistein, formononetin, and biochanin A.**



Signal 1:DAD1 A, Sig=248,4 Ref=off

Ret Time (min)	Peak Area	Response Factor	Amount (µg/mL)	Compound Name
5.27	1719	0.038	64.8	Daidzein
6.35	1509	0.051	77.2	Genistein
7.36	877	0.040	34.8	Formononetin
8.74	2093	0.055	114.6	Biochanin-A

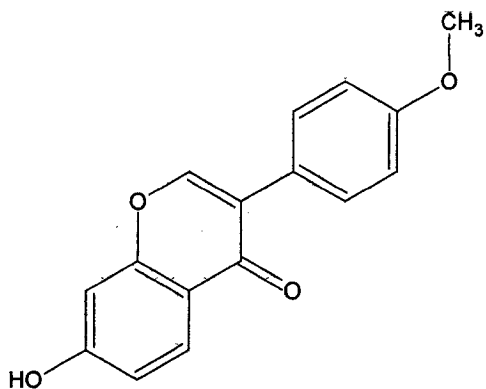
**Appendix 4.** Chromatogram of isoflavonoids: daidzein, genistein, formononetin, and biochanin A in AM colonized red clover root.



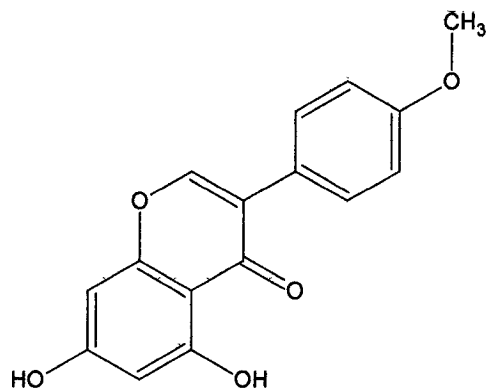
Signal 1:DAD1 A, Sig=248,4 Ref=off

Ret Time (min)	Peak Area	Response Factor	Amount (ug/mL)	Compound Name
5.27	70	0.039	2.7	Daidzein
6.33	699	0.052	36.5	Genistein
7.34	3383	0.037	124.0	Formononetin
8.72	3542	0.054	189.9	Biochanin-A

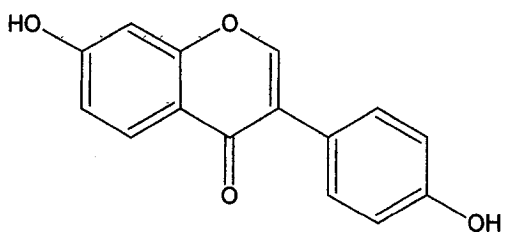
**Appendix 5.** Chemical structures of isoflavonoids: daidzein, genistein, formononetin, and biochanin-A.



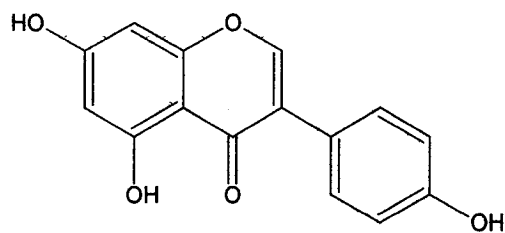
Formononetin



Biochanin A



Daidzein



Genistein