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NITROGEN TRANSFER AND ASSIMILATION IN A MYCORRHIZAL *IN VITRO* ROOT CULTURE SYSTEM

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Abstract

The present study aimed to determine the effect of arbuscular mycorrhizal (AM) symbiosis on plant nitrogen (N) nutrition. Previous studies had shown, mainly from soil experiments, the ability of AM fungi in their N uptake and transfer to host plants. These results led us to postulate that AM fungi contribute to enhance the activity of N-assimilating enzymes in host roots, and possess their own N-metabolic system. To investigate this, N key-enzyme activities were examined in Ri T-DNA carrot (*Daucus carota* L.) roots, grown into an *in vitro* system, with or without the AM fungus, *Glomus intraradices* Schenck & Smith. Such a system allowed us to grow and maintain an experimental model of intact roots-AM fungus, in a controlled and pathogen-free environment. Total carbon (C) and N contents had been quantified by Elemental Analysis in AM and non-AM roots and in the mycelium. By Mass Spectrometry, the uptake and transfer of labelled nitrogen (^{15}N) from the fungal mycelium to AM roots, as well as in the non-AM roots, were also determined. This was done in order to evaluate and discriminate between the two inorganic N forms, NH_4^+ and/or NO_3^-, both used as substrates by the N-enzymatic systems. Finally, some of the N-assimilating enzymes were characterized by immunodetection (Western blot). The results showed that AM colonization led to significant increase in the activity of glutamate dehydrogenase (GDH) in AM roots, but not of glutamine synthetase (GS), nor of nitrate reductase (NR). However, the addition of NH_4NO_3, as substrate, in the hyphal compartment only -not directly available to AM roots- induced, in these, the same level of GS, GDH and NR activities as in non-AM roots which had direct access to the N source, and significantly higher enzyme activities than in roots which received a control water treatment. The total

N content in non-AM roots was significantly higher than in AM roots but similar to the N level in extraradical hyphae. The lower total C content in AM roots compared to the fungus, could be the result of carbohydrates transferred to the latter, and may relate to their increased GDH activity. The N labelling experiment indicated the uptake and transfer of N from the extraradical hyphae to host roots. The isotopic form of $^{15}NH_4NO_3$ was shown to be taken up more efficiently than of $NH_4{}^{15}NO_3$ by the AM fungus when supplied to the hyphal compartment only. This suggests a clear preference for ammonium over nitrate. The immunodetection analysis revealed the same GS and GDH isoforms in both AM and non-AM roots. Interestingly, the AM fungus, *Glomus intraradices*, showed a different isoform of GS than in Ri T-DNA carrot roots, colonized or not. This is the first time such findings have been reported. Moreover, our overall results emphasize the active role of AM fungi in the uptake, transfer and assimilation of N in their symbiotic association with host roots. In general, the stated hypotheses have been supported by our overall results. Finally, the *in vitro* system, used in this research, has proven to be a keen and useful tool for short- or long-term studies on the physiology of the AM fungus-host root symbiosis.

Résumé

La présente étude visait à déterminer les effets de la symbiose mycorhizienne arbusculaire (MA) sur la nutrition azotée des plantes. Des études antérieures, principalement des expériences en sol, avaient montré la capacité des champignons MA à absorber et transférer l'azote (N) aux plantes-hôtes. À partir de ces résultats, nous avons postulé que les champignons MA contribuent à accroître l'activité des enzymes-clés assimilatrices de l'azote dans les racines-hôtes, et qu'ils possèdent leur propre système du métabolisme de l'azote. L'activité de ces enzymes-clés a donc été étudiée dans des racines transformées (*Ri T-DNA*) de carottes (*Daucus carota* L.), cultivées dans un système *in vitro*, avec ou sans champignon MA, le *Glomus intraradices* Schenck et Smith. Un tel système nous a permis de faire croître et de maintenir un modèle expérimental 'racines intactes-champignon MA, dans un environnement contrôlé et libre de tout organisme pathogène. Les teneurs en carbone (C) et en azote totaux ont été quantifiées par Analyse Élémentaire dans les racines MA et non-MA et dans le mycélium. Par Spectrométrie de Masse, l'absorption et le transfert d'azote marqué (^{15}N) du mycélium fongique aux racines MA, ainsi que dans les racines non-MA a aussi été déterminé. Cette expérience-ci a été réalisée dans le but de quantifier et de discriminer entre les deux formes d'azote inorganique, NH_4^+ et/ou NO_3^-, toutes deux utilisées comme substrats par les systèmes enzymatiques de l'azote. De plus, certaines enzymes assimilatrices d'azote ont été caractérisées par immunodétection (*Western blot*). Nos résultats montrent que la colonisation MA a contribué significativement à augmenter l'activité de la glutamate déshydrogénase (GDH) dans les racines MA, mais pas celle de la glutamine synthétase (GS), ni celle de la nitrate réductase (NR). Cependant, l'addition

de NH_4NO_3, comme substrat, uniquement dans le compartiment des hyphes -non directement accessible aux racines MA- a induit, chez celles-ci, des activités enzymatiques similaires de la GS, de la GDH et de la NR, à celles mesurées dans les racines non-MA, lesquelles avaient un accès direct au substrat azoté, et significativement plus élevées que dans les racines-contrôles n'ayant reçu que de l'eau. Le contenu en azote total dans les racines non-MA était significativement plus élevé que dans les racines MA, mais similaire à celui des hyphes extra-racinaires. La teneur plus basse en C total dans les racines MA que dans le champignon, peut résulter du transfert d'hydrates de carbone des racines vers son partenaire fongique, et être reliée à leur activité accrue de la GDH. L'expérience sur l'azote marqué a montré une absorption et le transfert de l'azote des hyphes vers les racines-hôtes. La forme isotopique $^{15}NH_4NO_3$ a été absorbée et transférée plus efficacement que le $NH_4{}^{15}NO_3$ par le champignon MA alors que l'azote n'était fourni que dans le compartiment des hyphes. Ceci suggère une nette préférence pour l'ammonium par rapport au nitrate. L'analyse par immunodétection a révélé les mêmes isoformes de la GS et de la GDH tant dans les racines MA que non-MA. Fait intéressant, le champignon MA, *G. intraradices*, a montré une isoforme différente de la GS, par rapport à celle des racines transformées de carotte, colonisées ou non. Cette étude-ci, la première à révéler de tels résultats, a mis l'emphase sur le rôle actif du champignon MA dans l'absorption, le transfert et l'assimilation de l'azote dans son association avec des racines-hôtes. De l'ensemble de nos résultats, la plupart de nos hypothèses énoncées s'avèrent assez justes. Enfin, le système *in vitro* s'est révélé être un outil de pointe, utile et efficace dans l'étude, à court ou à long terme, de la physiologie de la symbiose champignon MA-racines hôtes.

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I would like to dedicate this work to my late little brother who has been with me in thoughts and spirit, I love you Marc-Olivier.

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List of Abbreviations

α-kg	Alpha-ketoglutarate
AM	Arbuscular mycorrhizal
ANOVA	Analysis of Variance
APE	Atom percent excess
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Carbon
DAOM	Department Agriculture Ottawa Mycology
dH_2O	Deionized water
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DW	Dry weight
EC	Enzyme Commission
EDTA	Ethylenediamine tetra acetic acid
F	Fresh
FAD	Flavin adenine dinucleotide
Fd	Ferredoxin
g	Gravitational force
GDH	Glutamate dehydrogenase
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HC	Hyphal compartment
HCl	Hydrochloric acid
HEPES	N-2-hydroxypiperazine-N-2-ethanosulfonic acid
hrs	Hours
INRA	Institut National de Recherche en Agronomie
kDa	kilo Dalton
M	Molar
mg	Milligram
Mo	Molybdenum
Myc	Mycorrhizal fungus
MW	Modified White's medium
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced form)
NED	N-1 naphthyethylene-diamine-dihydrochloride
NiR	Nitrite reductase
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nitrate reductase
OD	Optical density
P	Phosphorus
PVLG	Polyvinyl-alcohol-lactic acid-glycerol
RC	Root compartment

Ri	Root inducing
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
sp	Species
T-DNA	Transferred deoxyribonucleic acid
TCA	Tricholoroacetic acid
w/v	Weight / volume

Chapter 1. Introduction

Nitrogen (N) is a key element of many compounds and structures of plant cells, and one of the most limiting factors in terrestrial ecosystems (Hodge *et al.*, 2000). In agricultural production, requirements in N are especially important. Therefore, chemical fertilizers have been intensively used to increase crop productivity, which, consequently, has resulted in increased soil and water pollution. However, as our knowledge increases in environmental sciences as well as in plant physiology, new environmentally-friendly approaches are being developed in order to reduce the input of such fertilizers in soils. For instance, mycorrhizal symbiosis has proved to play an important role in plant mineral nutrition, especially with phosphorus (P) (Johansen & Jensen, 1996; Villegas & Fortin, 2001) and N (Azcon-Aguilar *et al.*, 1993; Frey & Schuepp, 1993; Tobar *et al.*, 1994a). Moreover, arbuscular mycorrhizal (AM) fungi have been shown to benefit host plants by increasing their tolerance to biotic stresses (*e.g.* pathogens; Benhamou *et al.*, 1994; Filion *et al.*, 1999; St-Arnaud *et al.*, 1994; 1995; St-Arnaud *et al.*, 1997), and abiotic stress factors (*e.g.* drought; Subramanian *et al.*, 1995; Tobar *et al.*, 1994b). Therefore, it would be beneficial to have a better understanding of the mechanisms involved in these roles, particularly in N transfer and assimilation between AM fungi and host roots. The present study was designed to look at some physiological aspects of AM symbiosis in plant N nutrition. Previous studies have shown the ability of AM fungi in the uptake and transfer of N to host plants in soil experiments (Johansen *et al.*, 1996; Subramanian & Charest, 1998). These results led us to postulate that AM fungi could indeed enhance the activity of N-assimilating enzymes in host roots and that the fungus itself might possess such enzymes. To investigate this, we looked at N-assimilating enzyme activities in Ri T-DNA

carrot roots (*Daucus carota* L.) grown under *in vitro* conditions (St-Arnaud *et al.*, 1996), with or without the AM fungus, *Glomus intraradices* Schenck & Smith. We also examined the transfer of labelled N from the fungus mycelium to host roots and determined the preference between inorganic N forms, NH_4^+ and NO_3^-. Finally, we characterized different N-assimilating enzymes by immunodetection (Western blot).

1.1. Mycorrhizal symbiosis

In order to withstand the diverse climatic and edaphic conditions, terrestrial plants have developed several strategies. One of the most successful strategies is the capacity of the root systems to establish a number of symbiotic relationships with mycorrhizal fungi (Gianinazzi-Pearson, 1996). The word mycorrhiza (Gr. "*mukês*", fungus; and "*rhiza*", roots) was first used by Frank (1885) to illustrate the close association, or "symbiosis", between plants and fungi.

Mycorrhizal fungi form two major groups: (1) the ectomycorrhizae, which are mostly associated with Gymnosperms and some woody Angiosperms, and which develop extracellular hyphae in the root cortex (Hartig net) from a mycelial sheath covering the surface of short lateral roots (Gianinazzi-Pearson, 1996; Smith & Read, 1997); and (2) the endomycorrhizae, where the hyphae grow extra and intracellularly, and form either hyphal coils, vesicles or arbuscules within cell walls of most herbaceous plant species and some tree species. The endomycorrhizae also comprise the orchid and ericoid mycorrhizas. Another group, the ectendomycorrizae are characterized by the

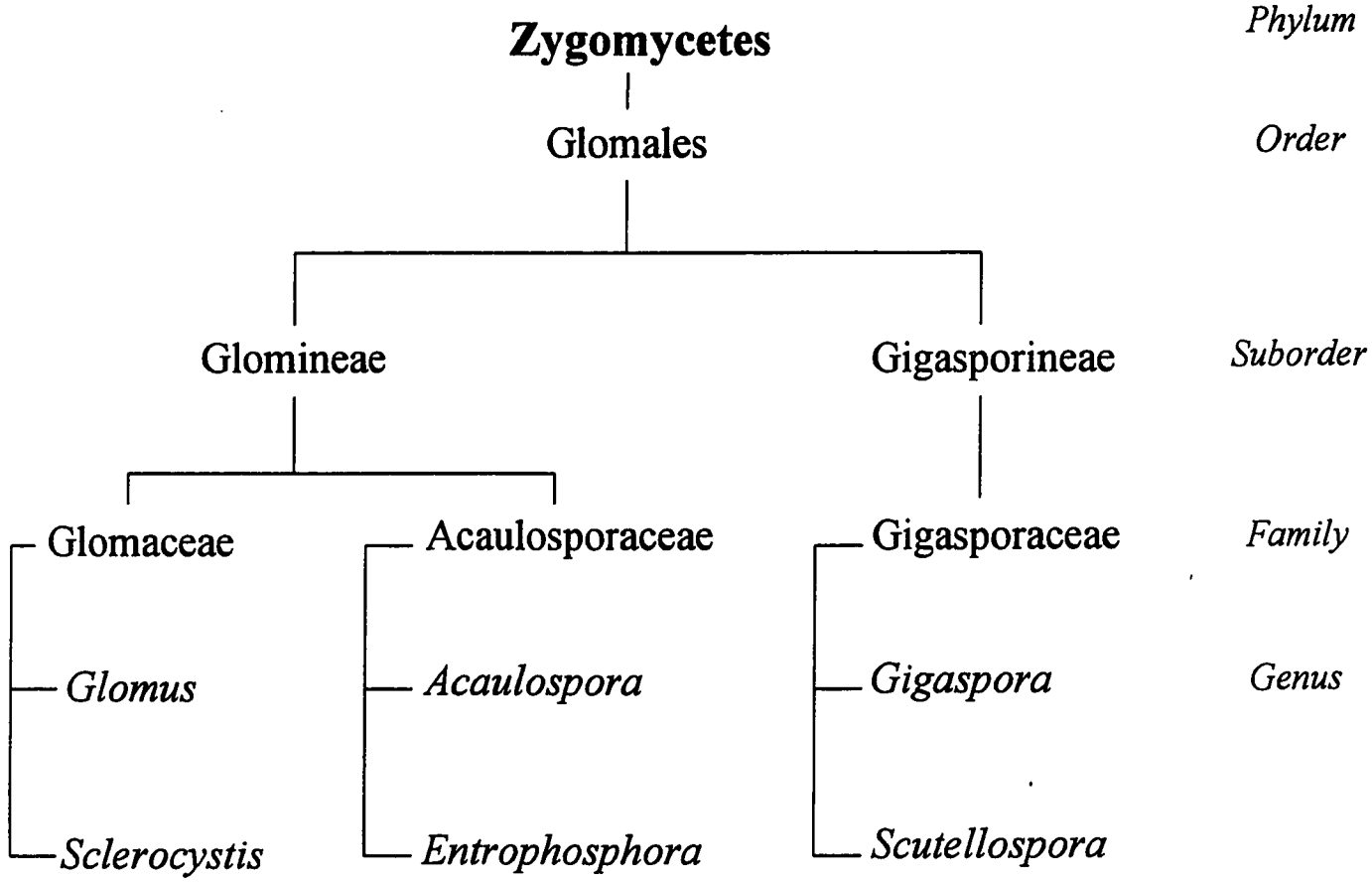
simultaneous presence of morphological structures found in ecto- and endomycorrhizae (Smith & Read, 1997).

1.1.1. Arbuscular mycorrhizal symbiosis

Among the different types of endomycorrhizae, AM fungi are symbionts responsible for the most prevalent plant root symbiosis (Simon *et al.*, 1993). This symbiosis is known to form associations with the majority of vascular plants in different ecosystems (Smith & Read, 1997). Indeed, about 85% of vascular plants have the capacity to establish AM symbiosis (Dalpé, 1995). This particular association is formed between host plant roots and Zygomycete fungi belonging to the order Glomales. The Glomales, recently revised by Morton & Benny (1990), comprise two suborders, the Gigasporineae and the Glomineae. The latter includes the Glomaceae family, which has two major genera: *Glomus* and *Sclerocystis* (Fig. 1.1). The genus used in the present study, *Glomus*, comprises the largest number of identified species (77 spp) (Smith & Read, 1997), and is found in a wide variety of soil and climatic conditions.

The AM symbiosis is recognized to be very ancient (Pyrozynski & Dalpé, 1989). Molecular studies (Simon *et al.*, 1993), as well as fossil evidence (Remy *et al.*, 1994; Wilkinson, 2001), put the date of their appearance back to 460 million years ago. It appears that the AM association evolved with terrestrial plants and may have been crucial for their colonization of land (Redecker *et al.*, 2000; Simon *et al.*, 1993).

Figure 1.1. Classification of the Order Glomales (Morton & Benny, 1990)


Zygomycetes
Phylum
Glomales
Order
Glomineae
Gigasporineae
Suborder
Glomaceae
Acaulosporaceae
Gigasporaceae
Family
Glomus
Acaulospora
Gigaspora
Genus
Sclerocystis
Entrophosphora
Scutellospora

This symbiotic association is now well known for its broad range of functions such as plant defence (Filion *et al.*, 1999; St-Arnaud *et al.*, 1997), nutrient uptake (Harrier, 2001; Tobar *et al.*, 1994a), drought resistance (Subramanian & Charest, 1998; Tobar *et al.*, 1994b), and soil aggregation stability (Hodge, 2000).

The AM fungal species, as obligate biotrophs, depend on host roots to complete their life cycle (Harrier, 2001). The process of AM colonization (Fig. 1.2) involves different steps, each characterized by specific structures (Gianinazzi-Pearson, 1996; Harrier, 2001). It is generally believed that the recognition process of a host plant with an AM fungus initiates before they come into physical contact (Gianinazzi-Pearson, 1996). Then, spore germination is induced by root exudates that contain signalling molecules (Koide & Schreiner, 1992), or organic acids produced by helper bacteria which promote mycorrhizal development (Garbaye, 1994). The active plant signalling molecules have not yet been identified (Gianinazzi-Pearson, 1996), although it is known that certain phenolics, *e.g.* flavonoids, in root exudates enhance root colonization by AM fungi (Pinior *et al.*, 1999; Vierheilig *et al.*, 1998). This recognition process, usually starting with spore germination, leads to the formation of a germ tube that will grow towards the root. At this point, hyphal differentiation occurs and, immediately after contacting the host root, the fungus will form appressoria which indicate that recognition occurs at this stage (Giovannetti *et al.*, 1994). The next steps involve the hyphal penetration into the root cortex, the intercellular growth and proliferation of the mycelium, the arbuscules formation and then, the formation of hyphal swellings called vesicles (Gianinazzi-Pearson, 1996; Harrier, 2001).

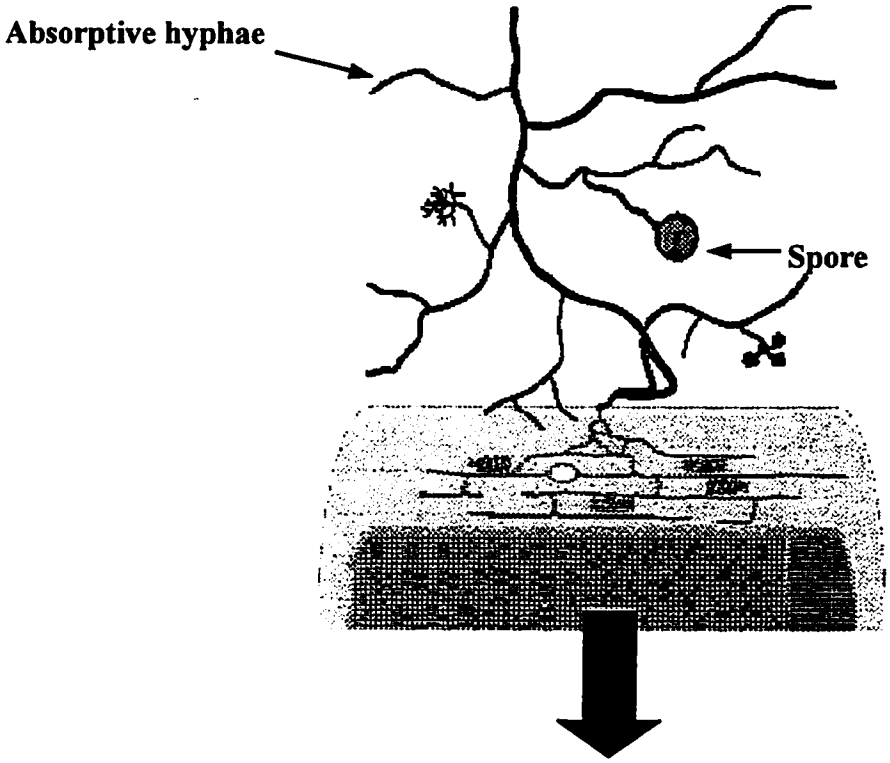
Figure 1.2. Components of vesicular-arbuscular association

(Brundrett *et al.*, 1996)

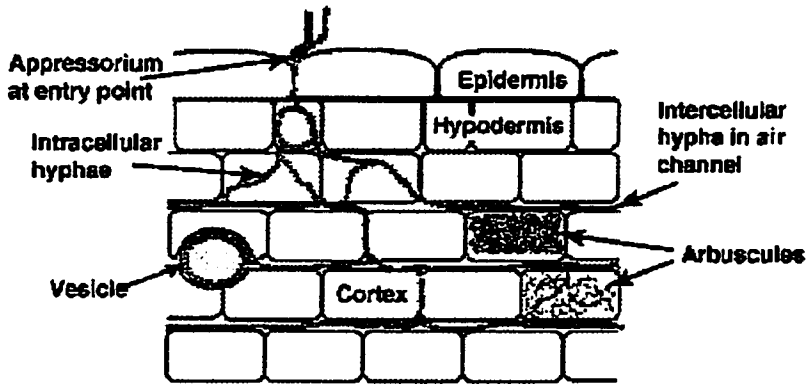
A Hyphae and spores produced by mycorrhizal fungi in soil

B Structures formed in colonized roots (vesicles, arbuscules, intracellular hyphae)

A. External mycelium in soil



B. Mycorrhizal structures in roots



The arbuscules establish within the plant cells a large surface area of contact that plays a key role in the bidirectional nutrient transfer between both symbiotic partners (Smith & Smith, 1990). These arbuscules only live for four to 15 days, after which they begin to senesce (Alexander *et al.*, 1988). The vesicles are structures that contain lipids, which serve as a nutrient storage for the fungus.

1.2. Nitrogen uptake and assimilation

Nitrogen is one of the most important minerals for all living plants, the primary limiting nutrient in most terrestrial ecosystems (Hodge *et al.*, 2000), and one of the most limiting factors for plant growth (Fernandes & Pereyra Rossiello, 1995). Ammonium (NH_4^+) and nitrate (NO_3^-) are the major inorganic forms of N found in most edaphic conditions; organic N-compounds are also found in soils. Mineralization of organic nitrogen to NH_4^+ and its nitrification to NO_3^- in soils make inorganic N available for plants (Hodge *et al.*, 2000). Absorption of these two elements by roots of land plants is the principal way that N enters the food chain (Morot-Gaudry, 1997). Plants acquire N from two main sources: (1) the soil, through fertilizer, manure and/or mineralization of organic matter; and (2) the atmosphere, through bacterial symbiotic N_2 fixation for only few plant species (Vance, 1997). Soil-derived N, generally in the form of NO_3^- and atmospheric N_2 (for N_2 fixing organisms), must be reduced to NH_4^+ to become available for amino acid and protein synthesis. Some of that NH_4^+ is lost from the N cycle by reactions which convert it to NO_2^- and then to N_2 gas again or to NO_3^- (Oaks & Hirel, 1985).

In agricultural soils, it is believed that NO_3^- (oxidized form) is the predominant form of N (Campbell, 1988; Subramanian & Charest, 1999); hence its reduction and assimilation is of great biological importance (Oaks, 1994). The assimilation of NO_3^- occurs mostly in the leaves of herbaceous plants and in the root systems of ligneous plants (Morot-Gaudry, 1997). In natural soils, N is present as NO_3^- and/or NH_4^+ and in organic compounds such as amino acids, whereas NH_4^+ usually dominates in forest ecosystems, particularly in conifer stands where soils are mostly acidic (Kreuzwieser *et al.*, 2000). NH_4^+, which can be readily assimilated by roots, is, in fact, the easiest and cheapest way to acquire N (Morot-Gaudry, 1997). However, NH_4^+ usually comprises only a small part of mineral N in soils of temperate regions as it is rapidly oxidized to NO_3^- by nitrifying microorganisms (Bray, 1983; Morot-Gaudry, 1997).

1.3. The role of AM symbiosis in N nutrition

After colonization by AM fungi, roots maintain their general metabolic activities; however, the nutrient metabolism of host roots may be affected (George, 2000). Important advances in research on AM symbiosis have increased the emphasis on the organization and function of the external mycelium and its role in nutrient mobilization (Smith & Read, 1997). Phosphorus is often the key element for increased growth or fitness of mycorrhizal plants as it is transported in large amount into hyphae (George, 2000). Therefore, a number of studies investigated the contribution of AM symbiosis in P uptake and transfer (George *et al.*, 1992; Kytoviita & Arnebrant, 2000; Smith *et al.*, 1994).

While the contribution of AM symbiosis is clear concerning P, it is still the subject of some discussion in N transfer. It is recognized that the highly developed hyphal network of AM fungi is of importance in plant N uptake, and that it plays a key role in plant N status. Transfer of N from the fungus to the host plant has been demonstrated in ecto- (Martin *et al.*, 1994) and ericoid mycorrhizas (Smith *et al.*, 1994), and is likely to occur in AM associations. *In vivo* studies have shown that AM colonization improves N uptake in *Zea mays* – *G. intraradices* (Subramanian & Charest, 1997; 1998; 1999) and *Lactuca sativa* – *Glomus mosseae* symbioses (Tobar *et al.*, 1994b); it relates also indirectly to N_2 fixation increases in *Hedysarum coronarium* – *Rhizobium / G. mosseae* symbiosis (Barea *et al.*, 1987). Other studies have reported that hyphae can absorb and transport inorganic N taken from the soil and direct it to the roots of a host plant (Hogberg *et al.*, 1994; Johansen *et al.*, 1996; Subramanian & Charest, 1998; 1999).

Despite the growing body of literature on N transfer in AM symbiosis (Frey & Schuepp, 1993; Hawkins & George, 1999; Johansen *et al.*, 1992; 1993; 1994), few studies have looked at the N metabolic pathway (Bago *et al.*, 2001; Hawkins *et al.*, 2000; Pfeffer *et al.*, 2001), and even fewer have investigated the key enzymes of N metabolism in this symbiosis (Johansen *et al.*, 1996; Subramanian & Charest, 1998; 1999). Barea *et al.* (1987) and Azcon *et al.* (1992) have shown increased GS (Glutamine synthetase) and NR (Nitrate redutase) activities in mycorrhizal roots of *H. Coronarium* and *Lactuca sativa*, respectively, grown in pot systems. Using a compartmented box system, which allows the separation of AM fungus from host roots, Subramanian & Charest (1999) have reported higher GS, GOGAT (Glutamate syntase) and NR activities in *Zea mays* colonized with *G.*

intraradices, suggesting that the AM fungus assimilates both NO_3^- and NH_4^+. A number of ^{15}N studies have also used the compartmented system to show the fungal contribution in plant N nutrition (Frey & Schuepp, 1993; Johansen *et al.*, 1992; 1993; 1994; Subramanian & Charest, 1999).

One major drawback occurring in the pot system though, is the risk of contamination with other microorganisms, which can hamper the experimental characterization of N metabolism in AM symbiosis (Johansen *et al.*, 1996). Therefore, current experimental work concerning N metabolism has been carried out with *in vitro* systems. Using this experimental tool, Bago *et al.* (1996) obtained evidence of an uptake of NO_3^- by the extraradical hyphae of *G. intraradices*. Villegas *et al.* (1996) have also reported a preference for NH_4^+ with *D. carota* roots grown *in vitro* with the AM fungus *G. intraradices*. Using an *in vitro* system, a recent model on N assimilation pathway in AM symbiosis has been proposed from NMR studies (Pfeffer *et al.*, 2001). This model suggests that the catabolic arm of the urea cycle coupled with urease operate in the intraradical mycelium to release NH_4^+ in the root.

It was also shown that ectomycorrhizal symbioses enhance acquisition of N by plants, mostly that of NH_4^+ (Finlay *et al.*, 1988). Using ^{15}N-NMR to follow the N assimilation pathway in the free-living ectomycorrhizal fungus *Laccaria bicolor*, Martin *et al.* (1994) have shown that NH_4^+ is predominantly incorporated via the GS/GOGAT system with a smaller proportion being metabolized via GDH. However, at lower extracellular NH_4^+, the contribution of GDH in its assimilation was increased. Likewise, it has been

suggested that the AM fungus might possess its own GS-GOGAT system (Johansen *et al.*, 1996; Kaldorf *et al.*, 1994; Subramanian & Charest, 1999), and possibly its own NR complex (Kaldorf *et al.*, 1994; Kaldorf *et al.*, 1998), in the spores and arbuscules of *Glomus* sp, respectively. Yet, such mechanisms involving N-assimilating enzymes are not as well defined in AM as in ectomycorrhizal symbiosis, and further investigations need to be done.

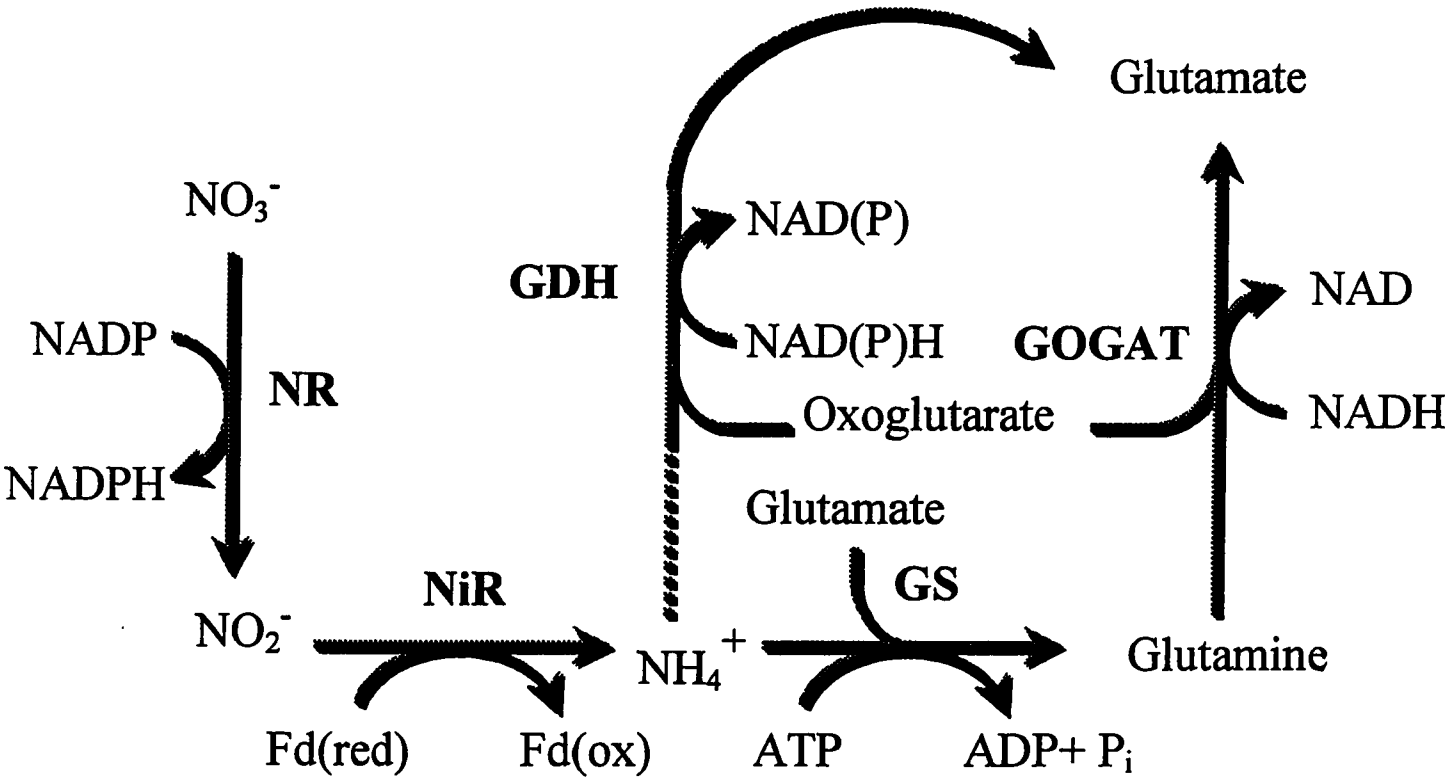
1.4. Enzymes in N metabolism

Assimilation of NO_3^- in higher plants involves, after its uptake, its reduction to NO_2^-, which is converted to NH_4^+, then incorporated into amino acids. The N entering the plant is assimilated through a series of steps involving four main enzymatic systems (Fig. 1.3): nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT). Although the role of glutamate dehydrogenase (GDH) in N assimilation has aroused controversy, its involvement has not been experimentally excluded (Bago *et al.*, 2001; Oaks, 1994; Oaks & Hirel, 1985; Robinson *et al.*, 1991).

Nitrate reductase (NR)

NR is a key assimilatory enzyme which acts by regulating the reduction of NO_3^- to NO_2^- in higher plants (Bray, 1983), and represents the rate-limiting step (Campbell, 1988). It occurs as three forms: NADH-NR (EC 1.6.6.1), the most common form in higher plants and algae; NADPH-NR (EC 1.6.6.2), also found in all plant tissues, particularly in roots; and NADPH-NR (1.6.6.3), the characteristic isoform in fungi (Morot-Gaudry, 1997; Oaks & Hirel, 1985; Vance, 1997).

Figure 1.3. Nitrogen assimilation pathway in higher plants.


Glutamate
NO_3^-
NAD(P)
GDH
NADP
NR
NAD(P)H
GOGAT
NAD
NADPH
Oxoglutarate
NADH
Glutamate
NiR
GS
NO_2^-
NH_4^+
Glutamine
Fd(red)
Fd(ox)
ATP
ADP+ P_i

NR is a large holoprotein complex containing FAD (flavin adenine dinucleotide), and a haem structure with molybdenum (Mo), as a cofactor (Vance, 1997). NR is a homodimer with a native molecular weight of 200-230 kDa (Morot-Gaudry, 1997; Vance, 1997). The NR mechanism implies the donation of two electrons by NADPH to the acceptor FAD. Electrons then flow through to the Mo domain, where they are donated to NO_3^- for its reduction to NO_2^- (Vance, 1997). Just like the leaf NR isozyme, root NR is substrate inducible and located in the cytosol (Bray, 1983; Oaks, 1994; Oaks & Hirel, 1985). Addition of NO_3^- results in increases of NR synthesis and activity, both in roots and shoots (Oaks & Hirel, 1985). Finally, NR is regulated by light, N source, sucrose, and plant status (Sivasankar & Oaks, 1995; 1996).

Nitrite reductase (NiR)

The next enzyme in the sequence is NiR (EC 1.7.7.1) that catalyzes the reduction of NO_2^- to NH_4^+ (Bray, 1983). Like NR, NiR is substrate (NO_2^-) inducible (Oaks, 1994; Oaks & Hirel, 1985). NiR, a monomer of 60-64 kDa, is localized in the chloroplasts of leaves, and plastids of roots (Morot-Gaudry, 1997; Oaks, 1994; Vance, 1997). The NiR mechanism involves ferredoxin (Fd), which serves as the electron donor and NADPH, as reducing power (Bray, 1983; Oaks & Hirel, 1985; Vance, 1997). Nitrite rarely accumulates under normal conditions, since NiR is consistently present at much higher levels than NR.

Glutamine synthetase (GS) / Glutamate synthase (GOGAT)

The third enzyme in the N assimilation pathway is GS (EC 6.3.1.2.) that concomitantly works with glutamate synthase (GOGAT), both being the key N-assimilating enzymes incorporating NH_4^+ into amino acids in plants (Morot-Gaudry, 1997). GS catalyzes the formation of glutamine from glutamate with the simultaneous cleavage of ATP (adenosine triphosphate) to ADP (adenosine diphosphate) using Mg^{2+} as a cofactor (Bray, 1983; Oaks, 1994; Oaks & Hirel, 1985). GS is found in both chloroplasts (GS2 isoform) and cytosol (GS1 isoform) in leaves, only in its cytosolic form in roots (Morot-Gaudry, 1997; Oaks & Hirel, 1985). GS, a holoenzyme of 320-400 kDa, is comprised of eight subunits ranging from 38 to 46 kDa (Morot-Gaudry, 1997; Vance, 1997). According to Vance (1997), GS comprises up to 1 to 2% of the total soluble protein content in actively assimilating N organs.

Glutamate synthase (GOGAT), closely related to GS, is the fourth enzyme that catalyzes the amino group transfer from glutamine and α-ketoglutarate to form two glutamate molecules. GOGAT is localized in chloroplasts of leaves and plastids of roots (Morot-Gaudry, 1997; Oaks, 1994; Oaks & Hirel, 1985; Vance, 1997). The discovery of the GS/GOGAT cycle in higher plants changed the emphasis from a primary role of GDH to this cycle in N assimilation (Bray, 1983).

Glutamate dehydrogenase (GDH)

GDH (EC 1.4.1.2) plays a key role in N metabolism in fungi and acts at branch points between N and carbon (C) metabolism (Ahmad & Hellebust, 1991). GDH converts α-

ketoglutarate (α-kg) to glutamate in the presence of NADH or NADPH and NH_4^+ (Oaks & Hirel, 1985). This enzyme is mainly located in mitochondria in both leaves and roots of higher plants (Oaks & Hirel, 1985); low levels of GDH may be found in chloroplasts (Bray, 1983). The native enzyme is of 252 kDa and consists of six 42.5 kDa subunits (Loulakakis & Roubelakis-Angelakis, 1990). There are two main factors opposing the fact that GDH plays a crucial role in NH_4^+ assimilation: (1) the mitochondrial location of GDH suggests a function in the oxidation and deamination from glutamine to α-kg in TCA cycle, under physiological conditions; and (2) kinetics studies have shown that GDH has a low affinity for NH_4^+ (high K_m), which is inconsistent with an assimilatory role (Bray, 1983). Conversely, the K_m of GDH was shown to be biphasic and depended on the concentration of NH_4^+, this suggesting that the K_m argument is not clearly valid (Oaks & Hirel, 1985).

1.5. Ri T-DNA roots

Chilton *et al.* (1982) were among the first researchers to produce Ri T-DNA root cultures by infecting plant roots with a soil microorganism, *Agrobacterium rhizogenes*, which contains genes that affect plant development and physiology through genetic transformation (Tepfer, 1989). *Agrobacterium rhizogenes* is, as its name indicates, a bacterium of the Rhizobiaceae family and, since its discovery (Smith & Townsend, 1907), has been studied for its morphogenic effects on plants as a pathogen (Tepfer, 1989). When this bacterium infects a wound, it inserts into the plant cells a portion of a large plasmid termed Ri (root-inducing). The bacterial DNA incorporated into the plant nuclear genome is called T-DNA (transferred DNA) and is also designated Ri for its

morphological effects (Tepfer, 1989). The bacterial genome, which possesses genes encoding for cytokinins and auxins, allows the roots to grow without having to develop a shoot structure. Ri T-DNA can be seen as a genetic signal that ensures the emission and maintenance of chemical and morphological reply signals, which constitute the transformed phenotype (Tepfer, 1989).

Mycorrhizal root culture was first performed by Mosse & Hepper (1975) using an *in vitro* system with dual culture of spores and excised roots of clover. Later on, Mugnier & Mosse (1987) developed a method using Ri T-DNA transformed carrot roots as the hosts for the AM fungi, *Glomus mosseae* (Nicolson & Gerdemann) and *Gigaspora margarita* (Becker & Hall). Bécard & Fortin (1988) developed a minimal medium on which *G. margarita* could complete its life cycle when grown with transformed carrot roots. The understanding of the biology and physiology of AM fungi has been greatly improved by the use of these *in vitro* techniques (Bécard & Piché, 1990; Chabot *et al.*, 1992; Declerck *et al.*, 1996; Filion *et al.*, 1999; Fortin *et al.*, 2002; St-Arnaud *et al.*, 1995b)

Because of the obligatory biotrophic relationship the AM fungi form with host plants, their biological studies have been difficult (Chabot *et al.*, 1992; St-Arnaud *et al.*, 1996). The *in vitro* system of axenic root culture, developed by Bécard & Fortin (1988), was further modified by St-Arnaud *et al.* (1995b, 1996) into a compartmented system that isolates the extraradical mycelium from the host roots. This system gives the advantage to maintain an aseptic and controlled environment free of other organisms. Therefore, it appears that the production of propagules under aseptic conditions remains the most promising way to

produce pathogen-free AM inocula (Fortin *et al.*, 1996; St-Arnaud *et al.*, 1996). Using this system, Shachar-Hill *et al.* (1997) and Hawkins *et al.* (2000) reported a mobilization and transfer of organic N. However, to our knowledge, no studies on the activities of N-assimilating enzymes have yet been reported using this dual-culture system.

1.6. Hypotheses and objectives

The first hypothesis of the present study is that AM colonization increases the activity of N-assimilating enzymes in Ri T-DNA carrot roots under *in vitro* conditions. Secondly, it was postulated that AM fungi transfer N to host roots within this system. To test these hypotheses, the specific objectives of this study were as follows: 1) to determine the effect of AM symbiosis on glutamine synthetase (GS), glutamate dehydrogenase (GDH) and nitrate reductase (NR) under *in vitro* culture conditions; 2) to investigate the contribution of the extraradical mycelium in $^{15}NO_3$ and $^{15}NH_4$ transfer to the host roots; and 3) to characterize the N-assimilating enzymes in Ri T-DNA roots and AM fungus by immunodetection (Western blot).

Chapter 2. Materials and Methods

2.1. Ri T-DNA transformed carrot roots and fungal culture

Transformed carrot (*D. carota* L.) roots used in the present study were prepared originally by Bécard & Fortin (1988) as follows: carrots were washed, peeled, soaked in 95% (v/v) ethanol for 10 s, surface sterilized in 1% NaCl for 15 min, and rinsed in sterile distilled water before being sectioned transversely into 5mm thick slices. The slices were then immediately placed on 1% water agar in Petri dishes and inoculated with the A_4 *Agrobacterium rhizogenes* strain on the distal face of the sections (Bécard & Fortin, 1988). A loopful of bacterial suspension taken from a 2-day-old culture was used as inoculum. Three weeks later, a few transformed roots proliferating on the inoculated sections were aseptically excised and transferred into Petri dishes containing modified White's medium supplemented with 500 mg L^{-1} of carbenicillin. Three successive subcultures were necessary to free the transformed roots of bacteria. The authors then took one root apex from the final subculture was excised and grown on fresh minimal medium (M medium) (see Appendix 1) (Bécard & Fortin, 1988), and solidified with 0.4% (w/v) gellan gum (ICN Biochemical Inc., Cleveland, Ohio 44128). Spores of *Glomus intraradices* Schenck & Smith (DAOM 181602) were originally provided by Dr Valentin Furlan (Agriculture Canada, Sainte-Foy, QC), and primary colonization was initially done by St-Arnaud *et al.* (1995) by placing 10-15 spores of *G. intraradices* near the apex of a single transformed root in the same dish. In the present study, transformed carrot roots (colonized or not with *G. intraradices*) were grown as described by St-Arnaud *et al.* (1996). Stock cultures were kept by inoculating AM roots in fresh media regularly.

2.2. Preparation and culture of the experimental units

One- or two-compartments 100 × 15 mm Petri dishes were used as experimental units. Non-compartmented dishes were filled with 25 mL of the modified M medium (Bécard & Fortin, 1988). Compartmented Petri dishes were filled with 20 mL of the modified M medium in the root (proximal) compartment and 20 mL of the same medium without sucrose in the hyphal (distal) compartment (St-Arnaud *et al.*, 1996). Mycorrhizal roots were inoculated in the proximal compartment containing sucrose. Petri dishes were incubated in the dark at 25°C. Compartmented dishes were placed upside down at a 45° angle in order to minimize roots crossing into the hyphal compartment. Eight weeks were allowed for the mycelium to cross the central wall and to heavily colonize the hyphal compartment. Petri dishes were examined regularly and roots were trimmed as required to prevent their crossing into the hyphal compartment. Eight-week-old Petri dishes with vigorous roots and densely colonized hyphal compartments (in the AM treatment) were selected for the first two experiments described further. Non-AM roots were inoculated in one-compartment Petri dishes containing 25 mL of the modified M medium. These were also incubated upside down in the dark at 25°C.

2.3. Substrate induced enzyme activity

2.3.1. First experiment

This first experiment was designed in order to evaluate if a nitrogenous substrate would induce any enzyme activity compared to a water treatment. This experiment consisted of a factorial design with two factors: with or without N substrate treatment, and AM or non-AM inoculation treatment. There were three replicates of each treatment

combination. In order to have the required root biomass to conduct the enzyme assays, each replicate was composed of ten 8-week-old compartmented Petri dishes (AM) or five 8-week-old non-compartmented Petri dishes (non-AM).

A NH_4NO_3 solution was applied evenly on the medium of the hyphal compartment of *G. intraradices* inoculated Petri dishes, while non-AM roots received it directly in the root compartment. The concentration of NH_4NO_3 solution for AM and non-AM treatments was adjusted in order to obtain a final concentration of 2 mM NH_4NO_3 in the medium. Control treatments received sterile distilled water. After 24 hrs, both AM and non-AM roots were separated from the gellan gel by solubilization of the medium in 10 mM sodium citrate buffer, pH 6·0 at 30 °C (Doner & Bécard, 1991), washed in dH_2O and immediately freeze-dried in 50 mL polyethylene tubes for 24 hrs (Lyph-lock 6, LABONCO serial #180662), before being assayed for GS and GDH and soluble protein analyses.

2.3.1.1. Enzyme assays

Glutamine synthetase (GS; EC 6.3.1.2)

GS activity was determined by the synthetase assay as described by Robinson *et al.* (1991). Freeze-dried roots (50 mg) were ground on ice in a 7 mL Potter vase with 2.0 – 2.5 mL extraction buffer. The GS buffer, pH 8.0, contained 25 mM Tris-HCl, 1 mM ethylenediamine tetra acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM mercaptoethanol, 1 mM reduced glutathione, 10 mM MgSO4, 5 mM glutamate, 2% PVP, and 0.01% Triton. The extract was centrifuged at 10 320 g for 25 min at 4°C. The supernatant was

kept to proceed with the enzyme assays. The GS reaction mixture contained 15 µmol ATP, 20 µmol $MgSO_4$, 5 µmol hydroxylamine, 60 µmol L-glutamate, and 37.75 µmol Tris-HCl (final volume 0.75 mL at final pH 7.6). The reaction was started by adding 0.25 mL of the enzyme extract and was stopped after 30 min by the addition of 0.75 mL ferric chloride reagent {4 mL $FeCl_3$ 10%, 1 mL trichloroacetic acid (TCA) 24% and 0.5 mL HCl 6 M in 6.5 mL dH_2O}. After centrifugation for 5 min at 2000 g 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 enzyme extract to prevent any reaction from occuring. GS was expressed as γ-glutamylhydroxamate produced g^{-1} dry weight (DW) h^{-1} using a standard curve prepared from different concentrations (0 – 3.0 µmol) of γ-glutamylhydroxamate (see Appendix 2).

Glutamate dehydrogenase (GDH, EC 1.4.1.2)

GDH was assayed using a combined method from Robinson *et al.* (1991) and Ahmad *et al.* (1990). The same extraction buffer as described above for GS was used for GDH. The reaction mixture, pH 8.2, contained 150 µmol NH_4Cl, 1 µmol $CaCl_2$, 0.3 µmol NADH, 20 µmol 2-oxoglutarate and 100 µmol Tris-HCl (final volume 1 mL). The reaction was started with the addition of 0.2 mL of the enzyme extract to 1 mL of the reaction mixture. The GDH activity was read at 340 nm and expressed as NADH oxidized g^{-1} DW h^{-1}. The blank, made for each extract, contained all of the above compounds except NADH.

Nitrate reductase (NR; EC 1.6.6.1)

The method used for NR was adapted from Sivasankar & Oaks (1995). Freeze-dried roots (50 mg) were ground on ice with an extraction buffer, pH 8.5, containing 25 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 20 µM flavin adenine dinucleotide (FAD), 1% (w/v) bovine serum albumin (BSA) and 10 µM chymostatin (dissolved in dimethyl sulfoxide, DMSO) in order to stabilize NR activity in roots. The assay mixture contained 0.2 mL of 0.65 M N-2-hydroxypiperazine-N-2-ethanesulfonic acid (HEPES) buffer, pH 7.0, 0.2 mL of 0.1 M KNO_3 and 0.1 mL enzyme extract. This mixture was brought to 1.4 mL with dH_2O and the reaction was started by adding 0.1 mL of NADH (3.6 mg/mL in 0.04 M KH_2PO_4, pH 7.0), and then incubated at 25°C for 15 min. The reaction was stopped by adding 1 mL of 1% (w/v) sulfanilamide in 1 M HCl, and 1 mL of 0.01% (w/v) N-1 naphthylethylene-diamine-dihydrochloride (NED) in dH_2O. The NR activity was measured at 540 nm, 30 min after the reaction was stopped. The blank, made for each extract, contained all the compounds except that the reaction stopping-agent was added before the enzyme extracts. The NR activity was expressed as µmol NO_2^- produced g^{-1} DW h^{-1}.

2.3.2. Second experiment

The second experiment consisted of a complete randomized block design with five blocks and one factor, *i.e.* with or without AM inoculation. There were two replicates of each treatment within each block. In order to have the required root biomass to conduct the enzymatic assays, each replicate was composed of ten compartmented Petri dishes (AM) or five non-compartmented Petri dishes (non-AM), as described previously.

This experiment was designed to compare the activity of GS, GDH and NR in AM and non-AM roots. To do so, we applied NH_4NO_3 in the hyphal compartment of AM and directly on the roots of non-AM treatments as described above. After 24 hrs of incubation, roots were harvested and GS, GDH and NR activities were measured as described previously.

Protein concentrations and Total C and N contents

Protein concentrations were used to calculate the specific activity of each enzyme for both the first and the second experiments. Soluble proteins were determined according to Bradford (1976) in all the extracted samples of GS, GDH and NR. Each protein extract (20 µL) was placed in a test tube containing 200 µL Bio-Rad dye reagent and 780 µL of dH_2O, and the solutions were homogenized by manual inversion. The Bio-Rad protein assay is a dye-binding (Coomassie Blue) assay in which a differential color change occurs in response to various concentrations of protein in the extracts. The ODs were read at 595 nm and the protein concentrations measured from the BSA standard curve (0 – 6 mg/mL) (see Appendix 3). The specific enzyme activity was calculated by dividing the activity of a particular enzyme by its total soluble protein concentration. Total C and N contents (%) in AM and non-AM roots, and in the fungal material were measured using an Elemental Analyzer (CE instruments, EA 1110 CHN, Milan, Italy) in the G.G. Hatch Isotope Laboratories (University of Ottawa).

2.4. Labelled nitrogen experiment

Growth conditions

Ri T-DNA transformed carrot roots were grown on the M medium as described before. Both AM and non-AM roots were grown in compartmented Petri dishes containing 20 mL of the M medium in both compartments. In the hyphal compartment, sucrose was omitted from the medium. These Petri dishes were incubated upside down at 25°C for 12 weeks at a 45° angle in a complete randomized block design with three blocks and two factors: mycorrhizal inoculation treatment randomized among the main plots, and labelled N substrate treatment randomized among the subplots.

2.4.1. Nitrogen labelling

A preliminary experiment was done to determine if a solution of 1 atom % ^{15}N (Isotech Inc., A Matheson, USA Company, Miamisburg, OH) at a final concentration of 2 mM was sufficient to obtain measurable values by Mass Spectrometer analysis (Delta Plus, Finningan Mat, Bremen, Germany; G.G. Hatch Isotope Laboratories, University of Ottawa). Results from this first set of experiments showed that these levels (atom % and concentration) were not high enough (all the values were close to zero). Therefore, the final ^{15}N substrate consisted of ammonium nitrate (NH_4NO_3) with the isotope (5 atom % ^{15}N) labelled either on ammonium ($^{15}NH_4NO_3$) or nitrate ($NH_4{}^{15}NO_3$). After a 12 week growth period, both AM and non-AM roots received one of the four following labelled N substrate treatments: 1) $^{15}NH_4NO_3$ applied in the hyphal compartment, 2) $^{15}NH_4NO_3$ applied in the root compartment, 3) $NH_4{}^{15}NO_3$ applied in the hyphal compartment, and 4) $NH_4{}^{15}NO_3$ applied in the root compartment. For each of the treatments labelled N had a

final concentration of 4mM N in the medium. Control treatments consisted of AM and non-AM roots with dH_2O as substrate.

After 24 hrs of incubation, the roots and the fungal material were harvested and freeze-dried for 24 hrs before being analyzed in a Mass Spectrometer for ^{15}N atom % excess (APE). These analyses have been done by Dr. Paul Middlestead, G.G. Hatch Isotope Laboratories (University of Ottawa).

2.5. Western blot analysis

The AM and non-AM extracts from the second substrate induced experiment were used for the Western blot analysis. Root (AM and non-AM) and fungal samples from the labelled N experiment have also been analysed. Other extracts from fresh root and fungal tissues were also used for this analysis. The proteins in the extracts were separated by SDS-PAGE (20% polyacrylamide) according to Laemmli (1974) using Bio-Rad's Mini-Protean II kit. We used a 10% resolving gel and a 3% stacking gel to do the electrophoretical separation. In each of the wells of our gels, we loaded 20 µL of the protein extracts and 4 µL of sample buffer. The concentration of proteins in each extract was about 0.5 µg/µL, except for the fungal extracts for which protein concentrations were only of 0.25 µg/µL due to limiting biomass. The sample buffer consisted of 2 µL mercaptoethanol, 2 µL Bromophenol Blue and 2 µL SDS used to stain and denature the proteins, respectively. Before loading the proteins into the gel wells, all samples were pre-treated at 90 °C for 5 min. In each gel, one well was loaded with a pre-stained standard sample (Bio-Rad). This standard comprised six different protein standards with

a molecular weight ranging from 106 to 20.9 kDa. This allowed us to identify our protein samples knowing their approximate molecular weight. Gel electrophoresis was performed for approximately one hour in a running buffer containing Tris-Base, glycine, and 0.1% SDS, pH ~8.3. For each sample, duplicates were done in order to obtain two gels: one for denaturated protein staining with 0.1% Coomassie Blue, and the other for the transfer on nitrocellulose membrane. The staining allowed us to verify whether the electrophoresis of the proteins was well done.

The electrophoretical transfer to a nitrocellulose membrane (Protran pore size 0.45 µm, Schleicher & Schuell, Keene, USA) was done as described by Towbin *et al.* (1979). The transfer was done overnight at 4°C in a buffer, pH ~8.3, containing Tris-base, glycine and MeOH. We then sent our membranes to Ms. Elisa Carrayol in the laboratory of Dr. Bertrand Hirel, Unité de la Nutrition Azotée des Plantes (Institut National de la Recherche Agronomique, Versailles, France) for the immunodetection of enzymes. Immunodetection was performed using anti-GS-2 immunoglobulin Gs raised in rabbits against the GS-2 of tobacco (Hirel *et al.*, 1984), and using rabbit antibodies raised against the NADH-GDH isoenzyme 1 purified from grapevine (Loulakakis & Roubelakis-Angelakis, 1990). Because of the lack of the antibodies for NR, immunodetection could not be performed for this particular enzyme.

2.6. Root colonization

For the enzymatic experiments and the labelled N experiment, three mycorrhizal Petri dishes were randomly selected in order to determine the mycorrhizal colonization. The

AM-colonized roots were separated from the gellan gel by solubilization in citrate buffer as described previously. Roots were then cut into 1 cm segments and stained with acid Fuchsin according to the method of Kormanik & McGraw (1982). First, roots were washed thoroughly in dH_2O, heated in 10% KOH in an autoclave for 7 min and washed again in tap water to remove the excess of KOH. Roots were then acidified in 1% HCl for a minimum of 45 min, stained overnight with a 0.2% acid Fuchsin solution (0.2% acid Fuschin in 1000 mL destaining solution: 2000 mL lactic acid; 156 mL glycerol; 156 mL water), then destained with the destaining solution to remove colorant excess. The roots were mounted on slides in polyvinyl-alcohol-lactic acid-glycerol (PVLG) medium (Dalpé, 1993). A total of one hundred root segments were examined at 100X under a compound microscope (Nikon Eclipse 800) for the presence of arbuscules, vesicles, or hyphae, in the laboratory of Dr. Yolande Dalpé (Agriculture and Agri-Food Canada). Pictures were taken using a digital camera (Nikon CoolPix 950). Mycorrhizal colonization was estimated as the percentage of the total root segments containing at least one of the fungal structures (Dalpé, 1993).

2.7. Statistical analyses

One-, Two-, or Three- Way Analyses of Variance (ANOVAs) were performed on all variables measured (SYSTAT 9 ®, SPSS 1999). The "a posteriori" comparison mean tests between the treatments were done using Tukey's studentized range tests at 5% level of significance. All data were verified for normality and, when needed, transformed by natural logarithm to normalize skewed distributions before statistical analysis. When sample size was not too low and all statistical assumptions met, parametric One-, Two- or

Three-way ANOVAs were performed on data. When needed, rank transformation was performed in order to do the proper analysis. When the sample sizes were low and the statistical assumptions not met, non-parametric Kruskal-Wallis analyses were done. Blocks were used as one of the factors in the second enzyme experiment and ^{15}N experiment in order to take into account the variability of the experimental design.

Chapter 3. Results

3.1. Mycorrhizal colonization

For the first and the second experiments, the root mycorrhizal colonization (Fig. 3.1) levels were similar, 22% and 24% respectively, after 8 weeks of growth. In the labelled N experiment, colonization increased to 34% after 12 weeks of growth.

3.2. Substrate induced enzyme activity

3.2.1. First experiment

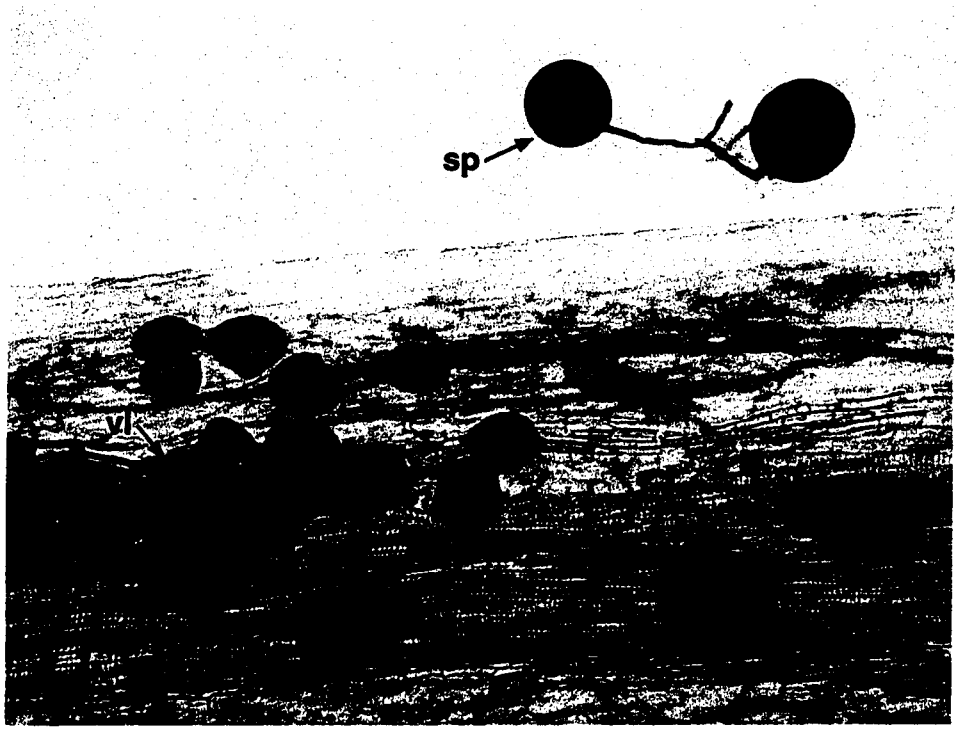
This first experiment allowed us to verify if the N substrate induced the enzyme activity in both AM and non-AM carrot roots (Table 3.1A and 3.1B). There were significant effects for both mycorrhizal (P = 0.05) and substrate (P = 0.000) treatments on GS activity, but the interaction was not significant (Table 3.2). Even though there was no significant interaction, we analyzed each treatment separately. Due to the small sample size, a one-way ANOVA on ranked data of GS activity was performed for each mycorrhizal treatment. A significant increase in GS activity was induced when the N substrate (NH_4NO_3) was added both in AM (P = 0.018) and non-AM (P = 0.004) roots, compared to the control water treatment (see Appendices 4 and 5). A Two-way ANOVA analysis on the GDH activity showed a significant (P = 0.000) effect induced by the N treatment (Table 3.3) for both AM (P = 0.015) and non-AM roots (P = 0.003) compared to the water treatment (see Appendix 6). The statistical results from the specific activities for both enzymes were mostly the same as those obtained for their enzyme activities (see Appendices 7 to 13). Marginally significant (P = 0.077), the GDH specific activity tended to increase in AM compared to non-AM roots. The N treatment significantly

Figure 3.1. Mycorrhizal structures observed for colonization of transformed carrot roots (100X)

A Chlamydospore (*sp*) and vesicles (*vl*) of *Glomus intraradices*

B Arbuscules (*ar*) and extra- or intraradical hyphae (*eh*, *ih*)

A



B

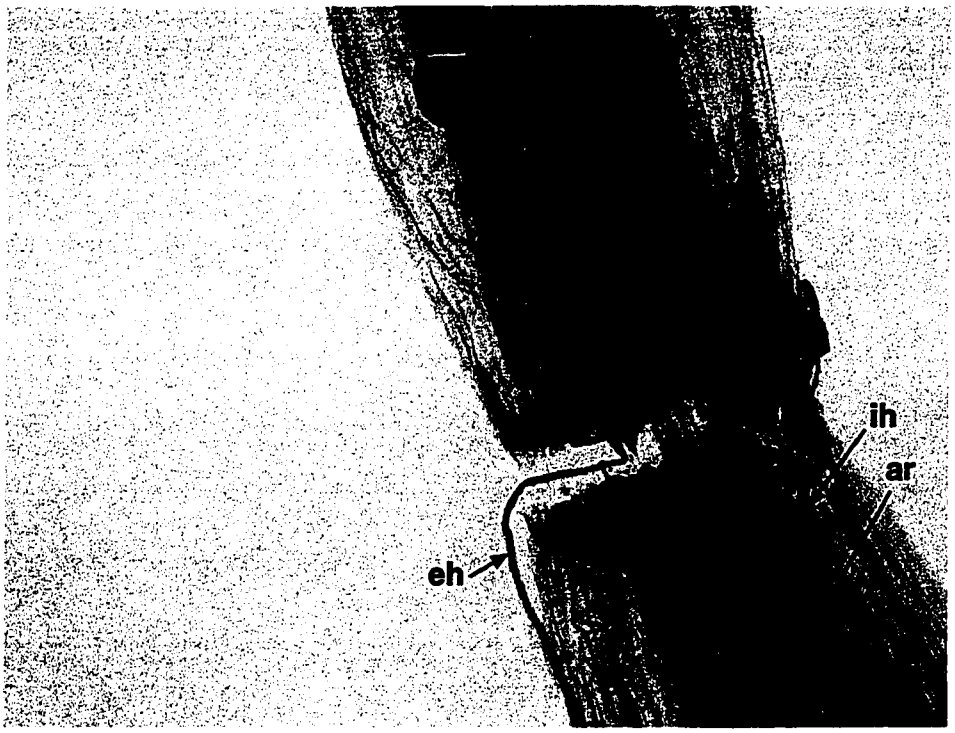


Table 3.1. **A)** Glutamine synthetase (GS) and **B)** Glutamate dehydrogenase (GDH) activities (GS μmol γ-glutamylhydroxamate g^{-1} DW h^{-1}; GDH μmol NADH oxidized g^{-1} DW h^{-1}), and specific activities (μmol mg^{-1} proteins h^{-1}) in carrot roots with (AM) or without (non-AM) colonization, and with nitrogen (NH_4NO_3) or control (H_2O) treatment.

A

[1]Mycorrhizal Treatment	GS Activity		[2]GS specific Activity	
	NH_4NO_3	H_2O	NH_4NO_3	H_2O
AM	[3]170 a, x (23)	55 a, y (8)	27 a, x (12)	8 a, y (1)
Non-AM	147 a, x (23)	[4]4 b, y (4)	16 a, x (5)	0.3 b, y (0.3)

B

[1]Mycorrhizal Treatment	GDH Activity		[2]GDH specific Activity	
	NH_4NO_3	H_2O	NH_4NO_3	H_2O
AM	[3]188 a, x (40)	16 a, y (16)	26 a, x (6)	2 a, y (2)
Non-AM	120 a, x (40)	[4]0.1 b, y (0.1)	14 a, x (8)	0.01 b, y (0.01)

($n = 3$; SE in parentheses)

[1]AM roots were grown in compartmented Petri dishes where the extraradical mycelium was allowed to grow in a distinct compartment and received the NH_4NO_3 or H_2O-control treatment. Non-AM roots were grown in non-compartmented Petri dishes and exposed directly to the N or H_2O-control treatment.

[2]Specific activity was calculated by taking each enzyme activity divided by its respective protein concentration. The protein concentrations are shown in Appendix 5.

[3]Within each column (a, b) and each row (x, y), different letters indicate significant differences according to Tukey's test ($P < 0.05$). Separate statistical analyses were done for GS and GDH activities.

[4]Enzyme activity almost not detected, therefore the smallest value possible was indicated.

Table 3.2. Two-way ANOVA on ranked data of GS activity for the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	108.00	1	108.00	48.000	0.000
Mycorrhizal	12.00	1	12.00	5.333	0.050
Substrate*Mycorrhizal	3.00	1	3.00	1.333	0.282

SS = sum of squares, df = degrees of freedom, MS = mean square, F = F distribution, p = probability, $R^2 = 0.872$

Table 3.3. Two-way ANOVA on ranked data of GDH activity for the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	108.00	1	108.00	44.690	0.000
Mycorrhizal	5.33	1	5.33	2.207	0.176
Substrate*Mycorrhizal	0.33	1	0.33	0.138	0.720

Symbols as in Table 3.2.

$R^2 = 0.855$

increased the GS specific activity for both AM (P = 0.055) and non-AM roots (P = 0.023) (see Appendices 9 and 10). As for GS, the GDH specific activity was significantly increased by the N treatment (see Appendix 11) in both AM (P = 0.017) and non-AM (P = 0.012) roots compared to the water treatment (see Appendices 12 and 13).

3.2.2. Second experiment

The GS and NR activities (Figs. 3.2 and 3.3) were similar between the AM roots, which received NH_4NO_3 in the hyphal compartment only, and the non-AM roots which were exposed directly to the N substrate as shown by the Two-way ANOVA analyses (Tables 3.4 and 3.5). In contrast, the GDH activity was significantly (P = 0.058) higher in AM roots, when supplied with NH_4NO_3 in the hyphal compartment only, than in non-AM roots (Fig. 3.4, Table 3.6). The specific activities (Table 3.7) for GS and NR were not significantly affected by the mycorrhizal treatment (see Appendices 14 and 15), except for GDH where a significant (P = 0.000) effect was found (see Appendix 16). However, when taking a closer look at these specific enzyme activities, an interesting pattern was found. In AM roots, the specific activities of GDH and NR were, respectively, 15 and 3 times higher than that of GS (Table 3.7). GDH specific activity was 5 times higher than that of NR in AM roots. In non-AM roots, the GDH specific activity was ten times higher than that of GS and equivalent to that of NR.

Total C and N contents

The total N content (%) was significantly higher (P = 0.02) in non-AM roots and in the fungal material than in AM roots (Fig. 3.5 and see Appendix17). Total N uptake was

significantly higher (P = 0.019, see Appendix 18) in non-AM roots (0.026 mg N/mg DW) compared to the AM roots and fungal material taken together (0.021 mg N/mg DW). By calculation, 21% of the total N uptake in AM roots came from the extraradical mycelium. The C content (%) was similar in AM and non-AM roots but significantly higher ($P \leq 0.05$) in the extraradical mycelium (Fig. 3.6 and see Appendix 19). However, total C content was significantly higher (P = 0.021, see Appendix 20) in both AM roots and fungal material together (0.477 mg C/mg DW) compared to non-AM roots alone (0.432 mg C/mg DW). It was also found that 6% of the total C content in the extraradical mycelium came from the AM roots. As for the C/N ratio, it was 16.8 for non-AM roots, and 22.6 for both AM roots and the fungal mycelium.

Neither the N treatment in both experiments, nor the water treatment in the first experiment did significantly affect the protein. Therefore, no significant difference in protein concentrations was found in regard to AM or non-AM treatment. This was reflected by the fact that specific activities did not differ from the activities found for each enzyme in terms of statistical differences (Table 3.13, see Appendix 21).

3.3. Labelled nitrogen experiment

Because the ^{15}N enrichment data from the hyphal compartments were almost all close to zero, these were analyzed separately from the ones of the root compartments. $NH_4{}^{15}NO_3$ levels were not significantly different between AM and non-AM roots in the hyphal compartment (Fig. 3.7 A). When $^{15}NH_4NO_3$ was applied in the hyphal compartment, AM roots showed higher APE than non-AM roots (P = 0.05).

Figure 3.2. Glutamine synthetase (GS) activity (μmol γ-glutamylhydroxamate g^{-1} DM h^{-1}), in AM and non-AM carrot roots. Means (n=10) and standard error bars are shown. Same letters indicate no significant differences according to Tukey's test ($P > 0.05$).

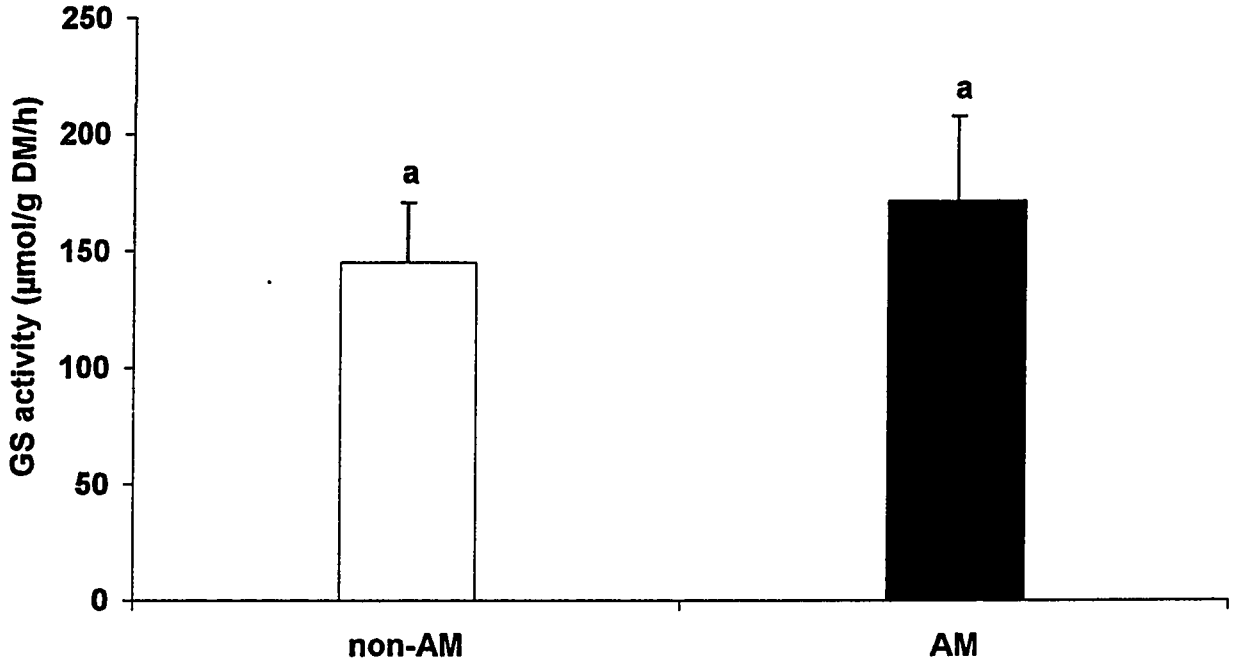

250
200
150
100
50
0
GS activity (µmol/g DM/h)
a
a
non-AM
AM

Figure 3.3. Nitrate reductase (NR) activity (μmol NO_2^- g^{-1} DM h^{-1}), in AM and non-AM roots. Means (n=10) and standard error bars are shown. Same letters indicate no significant differences according to Tukey's test ($P > 0.05$).

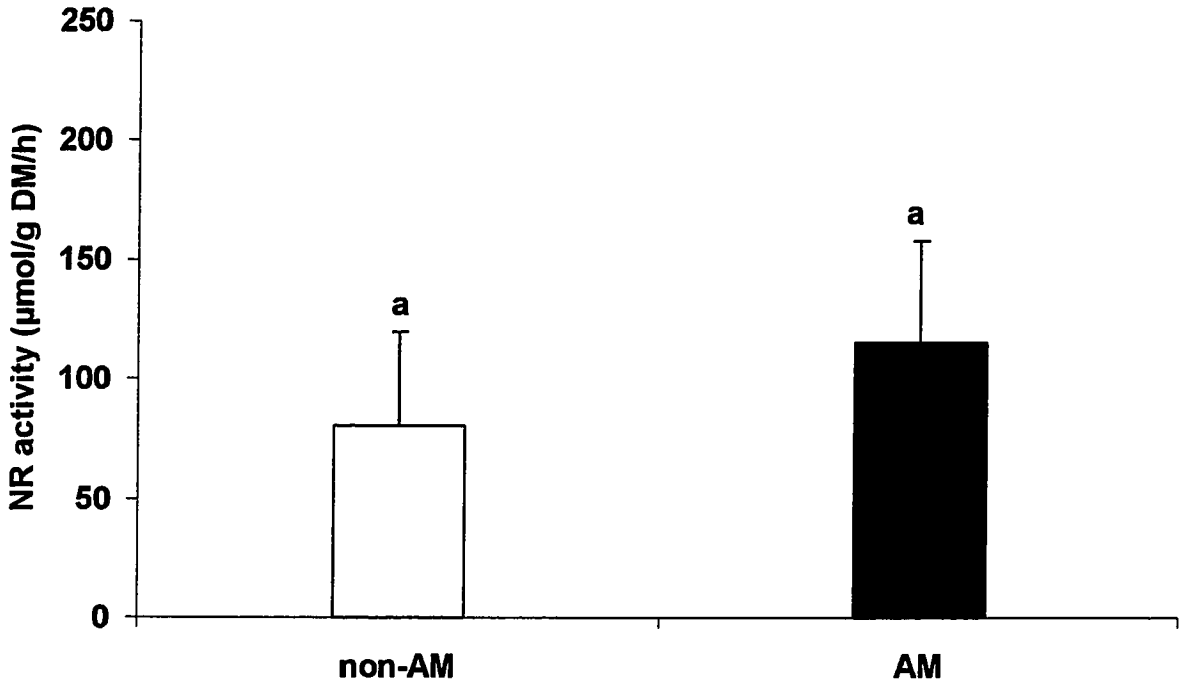

250
200
150
100
50
0
NR activity (µmol/g DM/h)
a
a
non-AM
AM

Figure 3.4. Glutamate dehydrogenase (GDH) activity (μmol NADH oxidized g^{-1} DM h^{-1}), in AM and non-AM roots. Means (n=10) and standard error bars are shown. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

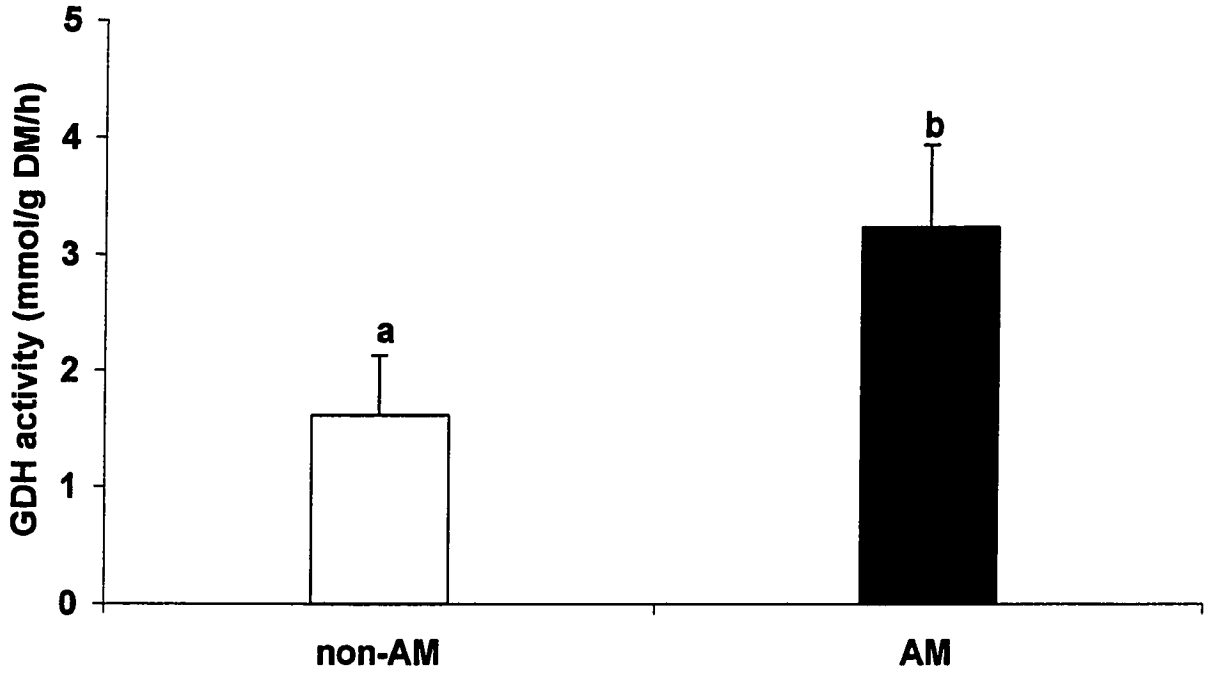

GDH activity (mmol/g DM/h)
5
4
3
2
1
0
a
b
non-AM
AM

Table 3.4. Two-way ANOVA on GS activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	107700.460	4	26925.115	4.499	0.025
Mycorrhizal	3553.778	1	3553.778	1.264	0.324
Block*Mycorrhizal	11248.223	4	2812.056	0.470	0.757

Symbols as in Table 3.2.

$R^2 = 0.672$

Table 3.5. Two-way ANOVA on NR activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	125039.410	4	31259.853	0.787	0.151
Mycorrhizal	5996.531	1	5996.531	2.137	0.425
Block*Mycorrhizal	30474.495	4	7618.624	0.521	0.723

Symbols as in Table 3.2.

$R^2 = 0.525$

Table 3.6. Two-way ANOVA on GDH activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	36.994	4	9.248	3.848	0.038
Mycorrhizal	12.993	1	12.993	6.960	0.058
Block*Mycorrhizal	7.467	4	1.867	0.777	0.565

Symbols as in Table 3.2.

$R^2 = 0.705$

Table 3.7. Specific activities for NR, GS and GDH (μmol mg^{-1} proteins h^{-1}), and protein concentrations (mg g^{-1} DW) in carrot roots with (AM) or without (non-AM) colonization for the second experiment.

Mycorrhizal	Specific activity			Proteins	
Treatment	NR	GS	GDH	NR	GS/GDH
AM	[1]24.3 [a] (12.8)	7.8 [a] (2.4)	116.1 [a] (30.9)	4.3 [a] (0.9)	29.1 [a] (3.8)
Non-AM	59.9 [a] (42.2)	4.6 [a] (0.8)	46.3 [b] (15.6)	4.5 [a] (1.1)	33.6 [a] (3.9)

(Means, n = 10; SE in parentheses)

[1]Within each column, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Figure 3.5. Total N content (%) in carrot roots with (AM) or without (non-AM) colonization, and in the extraradical mycelium (Fungus). Means (n=4) and standard error bars are shown. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

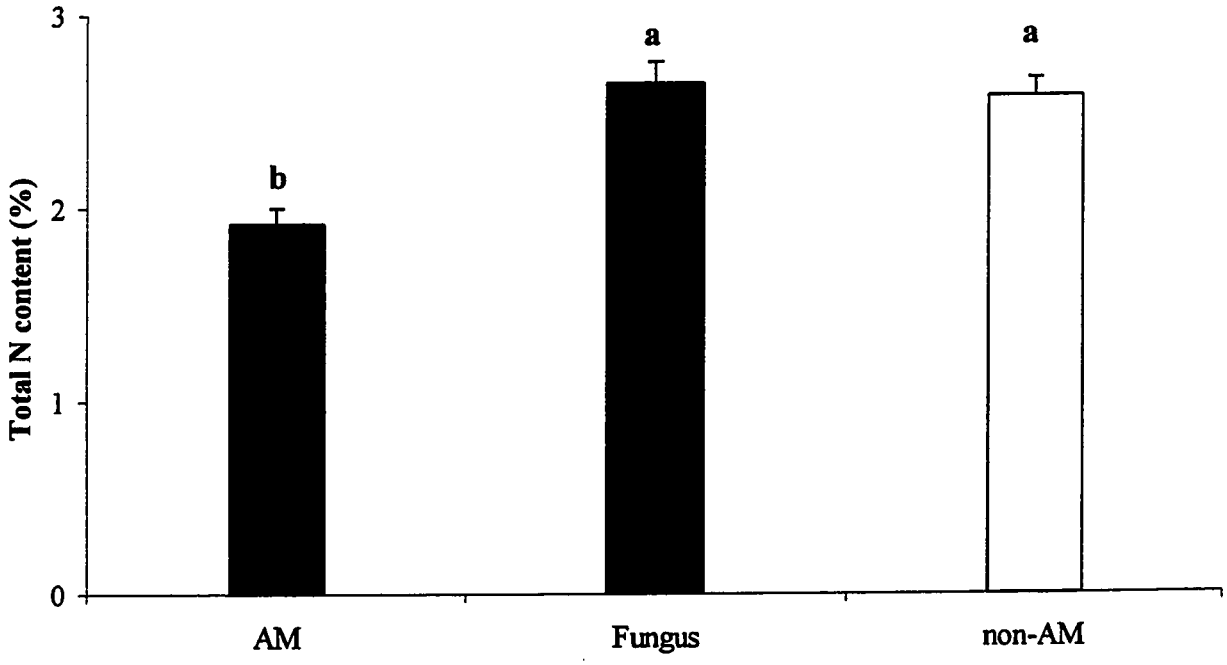
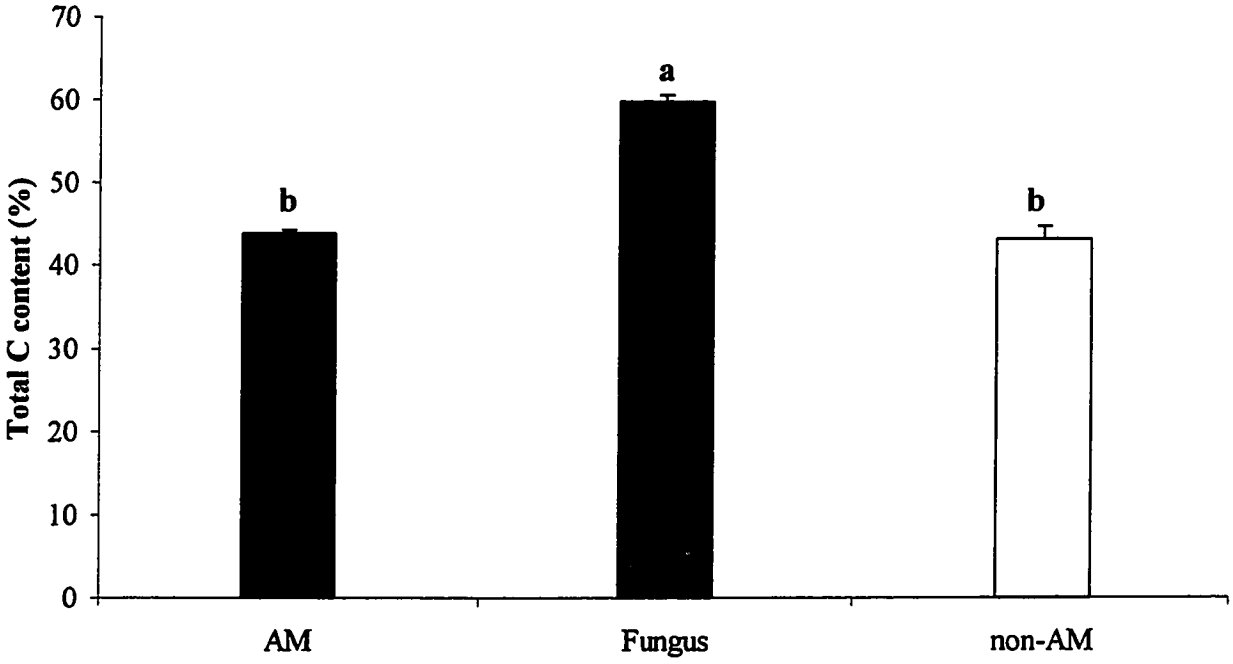

Total N content (%)
3
2
1
0
b
a
a
AM
Fungus
non-AM

Figure 3.6. Total C content (%) in carrot roots with (AM) or without (non-AM) colonization, and in the extraradical mycelium (Fungus). Means (n=4) and standard error bars are shown. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).


70
60
50
40
30
20
10
0
Total C content (%)
a
b
b
AM
Fungus
non-AM

The Three-way ANOVA for the root compartment data showed a significant effect for the N treatment ($P \leq 0.05$), but not for the mycorrhizal treatment (Table 3.8). However, a significant interaction ($P \leq 0.05$) between the two treatments was found. Because of this significant interaction, separate analyses for each of the N and the mycorrhizal treatments were done. Due to the small sample size, non-parametric Kruskal-Wallis analyses were performed on these data. When $NH_4{}^{15}NO_3$ was applied in the root compartment, the ^{15}N enrichment was higher in non-AM roots compared to AM roots (Fig. 3.7 B, see Appendix 22). In fact, non-AM roots had a two-fold higher enrichment compared to AM roots when supplied directly with $NH_4{}^{15}NO_3$. The ^{15}N enrichment data were the highest when supplied directly to the roots with $^{15}NH_4NO_3$ for both AM and non-AM roots, which showed similar values (see Appendix 23). Furthermore, both AM and non-AM roots showed a two to five-fold enrichment level ($P = 0.05$) when treated with $^{15}NH_4NO_3$ compared to $NH_4{}^{15}NO_3$ (see Appendices 24 and 25).

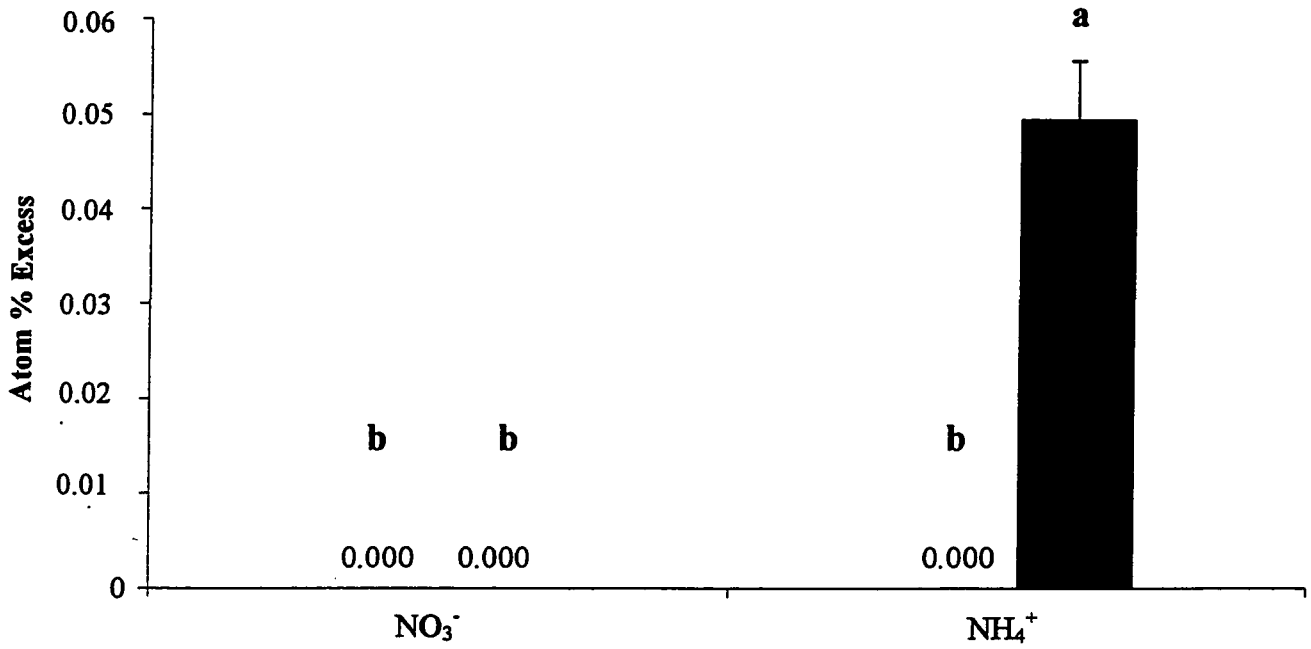
3.4. Western blot analysis

Western blot analysis indicated that the AM and non-AM roots as well as the fungal material possess some of the main enzymes involved in N metabolism. In AM and non-AM roots, GS was detected as a single band with a molecular weight of about 45 kDa (Fig. 3.8). This form of GS was probably that of GS1, the cytosolic form. In the fungal material, GS was present as a different isoform than that present in AM or non-AM roots (Fig. 3.8); its molecular weight ranged between 35.6 and 45 kDa. The AM and non-AM roots also showed two different forms of GDH (Figs. 3.9 and 3.10) which could correspond to NADH- and NADPH-GDH. The NADH-GDH that has a molecular weight

of 42.5 kDa corresponds closely to one of the bands we found out in our analysis. The other band has a molecular weight close to 50.8 kDa. The enzyme extracts from the second substrate induced experiment also revealed the presence of GDH (Fig. 3.10).

Figure 3.7. Atom % excess in AM and non-AM roots with ^{15}N applied in both hyphal (A) and root (B) compartments (HC and RC, respectively). Means (n=3) and standard error bars are shown. Separate statistical analyses were done for data of graph A from graph B. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

A **Application to hyphal compartment (HC)**



B **Application to root compartment (RC)**

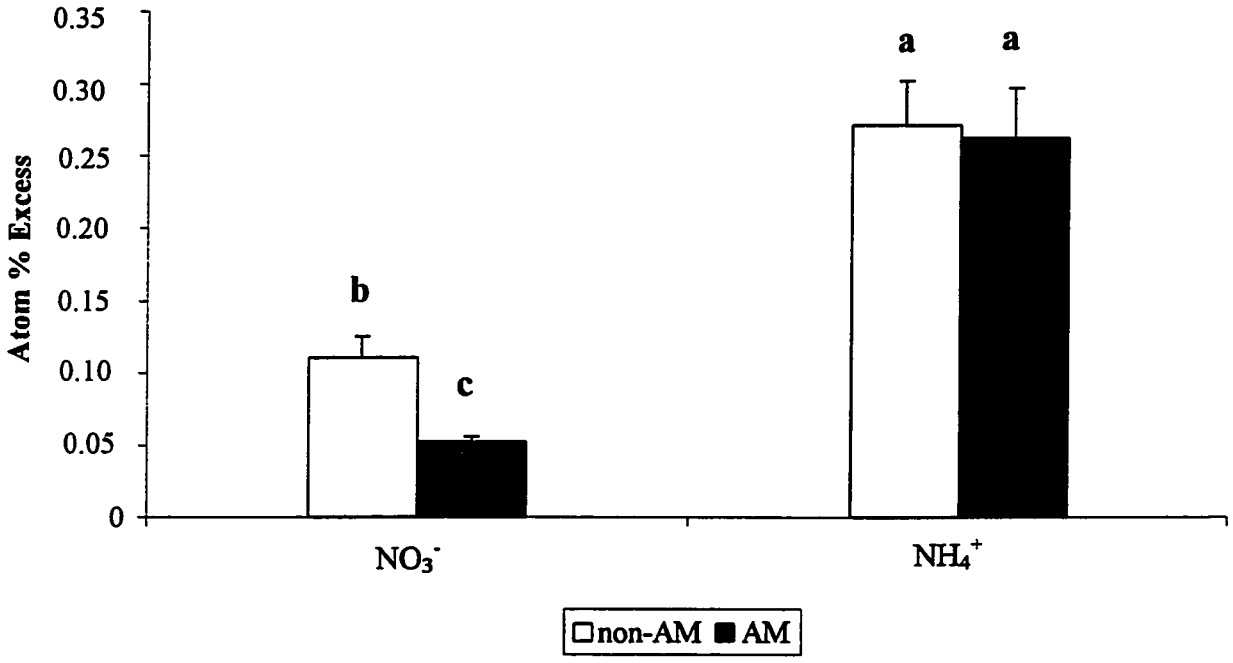


Table 3.8. Three-way ANOVA on ^{15}N enrichment levels when applied in the root compartment for the labelled N experiment.

Source of variation	SS	df	MS	F	p
Block	0.047	2	0.024	82.521	0.012
Mycorrhizal	0.024	1	0.024	2.369	0.264
Block*Mycorrhizal	0.021	2	0.010	36.056	0.027
Nitrogen	0.769	1	0.769	38.962	0.025
Block*Nitrogen	0.039	2	0.020	68.881	0.014
Nitrogen*Mycorrhizal	0.013	1	0.013	46.525	0.021

Symbols as in Table 3.2.

$R^2 = 0.999$

Figure 3.8. Western blot analysis of GS from AM and non-AM carrot roots, and fungal mycelium (Myc) extracts. AM and non-AM roots have a same isoform of GS with a molecular weight of 45 kDa, whereas the fungal mycelium shows a different isoform of approximately 40 kDa.

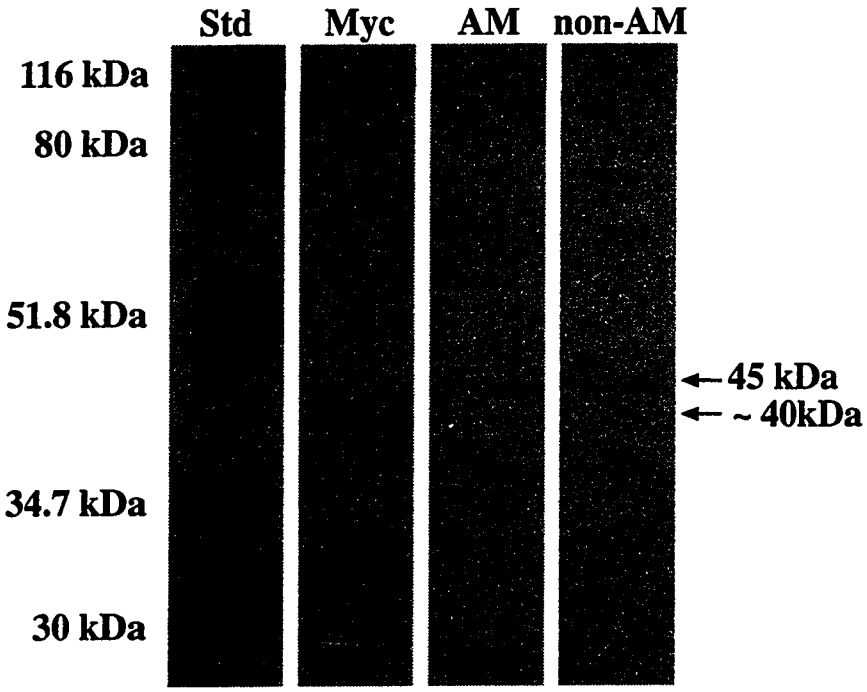

Std
Myc
AM
non-AM
116 kDa
80 kDa
51.8 kDa
34.7 kDa
30 kDa
45 kDa
~ 40kDa

Figure 3.9. Western blot analysis of GDH from AM and non-AM root extracts from the labelled N experiment. HC and RC represent the hyphal and root compartments, respectively, whereas F represents fresh extract. There is two isoforms of GDH in both AM and non-AM roots. One of these isoforms appears to be NADH-GDH with a molecular weight of 43 kDa. The other isoform has a molecular weight of approximately 60 kDa.

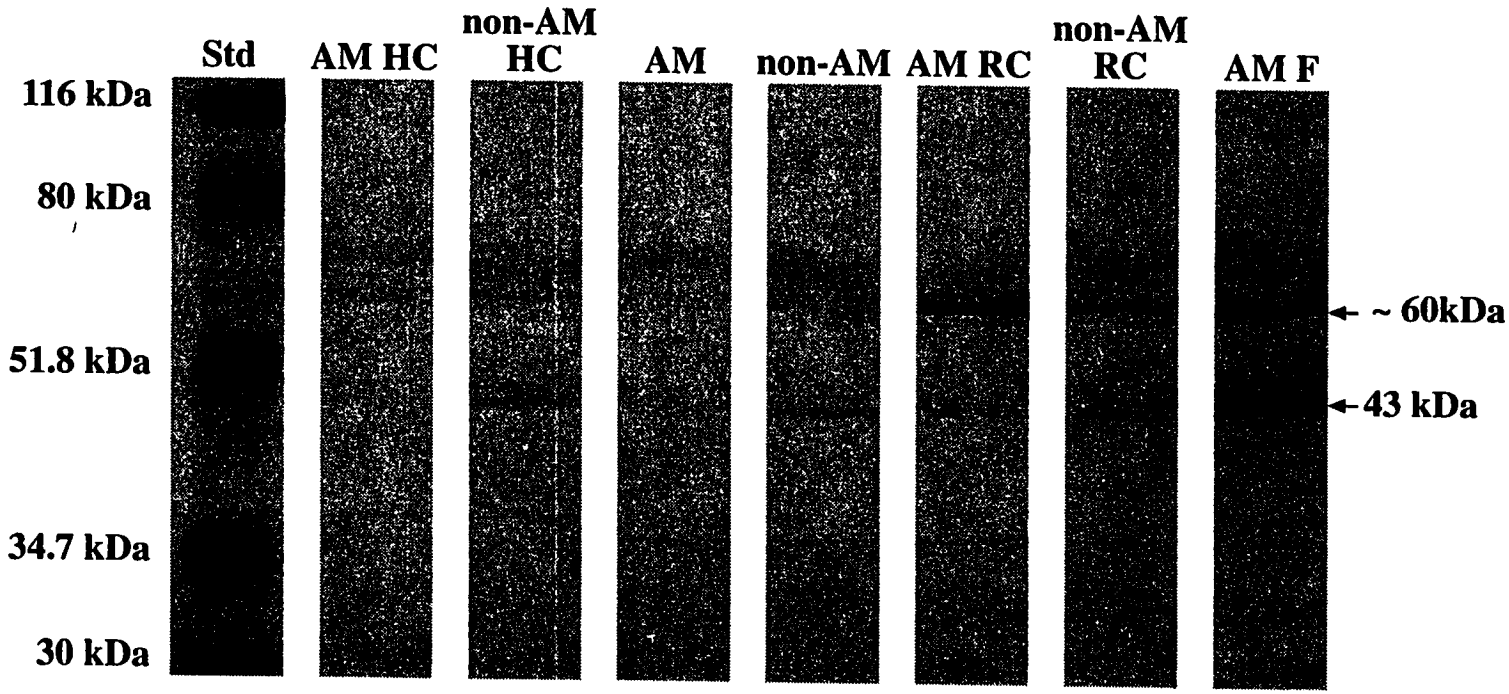
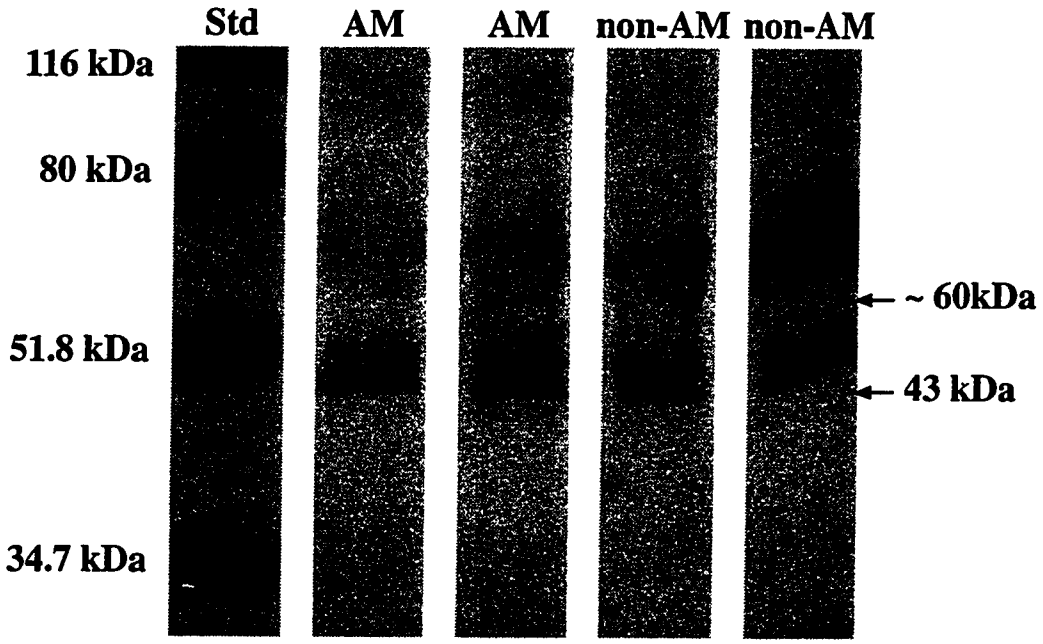

Std
AM HC
non-AM HC
AM
non-AM
AM RC
non-AM RC
AM F
116 kDa
80 kDa
51.8 kDa
34.7 kDa
30 kDa
~ 60kDa
43 kDa

Figure 3.10. Western blot analysis of GDH from AM and non-AM root extracts from the second substrate induced experiment. There is two isoforms of GDH in both AM and non-AM roots. One of these isoforms appears to be NADH-GDH with a molecular weight of 43 kDa. The other isoform has a molecular weight of approximately 50 kDa.


Std
AM
AM
non-AM
non-AM
116 kDa
80 kDa
51.8 kDa
34.7 kDa
~ 60kDa
43 kDa

Chapter 4. Discussion

4.1. Mycorrhizal colonization

After eight or twelve weeks of growth, *Glomus intraradices* was well established in the *in vitro* system; *i.e.*, the mycorrhizal fungus was covering the entire M medium surface in the Petri dishes. These observations are in accordance with those of St-Arnaud *et al.* (1994, 1996) who observed a vigorous growth of the fungal mycelium in the hyphal compartment after eight weeks of incubation. Therefore, any mycorrhizal effect found in our experiments could be truly interpreted as a direct contribution of the fungus. Non-AM Petri dishes (controls) were not colonized at all. Our mycorrhizal colonization levels (22 – 34%) in *D. carota* roots are comparable to those observed in other studies using an *in vitro* system. Jolicoeur *et al.* (1999) obtained colonization levels ranging from 25 to 50% in Ri T-DNA carrot roots colonized with *G. intraradices* in liquid M medium after 12 weeks of growth. After five months, Declerck *et al.* (1996) obtained 76 – 88% colonization levels in transformed carrot roots inoculated with *G. versiforme* and grown in the modified Strullu-Romand (MSR) medium. On the other hand, after five months, Chabot *et al.* (1992) obtained 10% colonization with *G. intraradices* in Ri T-DNA carrot roots grown on a modified White's medium (MW).

Our colonization levels, obtained *in vitro*, are closely equivalent to those obtained in soil experiments although slight differences might be observed. For instance, Subramanian & Charest (1999) obtained a colonization level of 50% after 56 days with *Zea mays* colonized with *G. intraradices* in a soil experiment. Likely, after six weeks of growth, Hawkins *et al.* (2000) obtained 66% root colonization in wheat (*Triticum aestivum* L.)

colonized with *G. intraradices*. Therefore, our results proved to be comparable to those obtained in soil experiments taking into account the different types of AM fungi and growth conditions.

4.2. Substrate induced enzyme activity

4.2.1. First experiment

Transformed carrot roots colonized with *G. intraradices* showed similar activities for GS and GDH compared to non-colonized roots. The fact that non-AM roots had direct access to the N substrate, while only mycorrhizal hyphae were in contact with it for the AM roots, suggests that the extraradical mycelium was transporting N to the host roots under these *in vitro* conditions. This hyphal N transfer may have led to the increased activities of key N-assimilating enzymes (GS and GDH) in AM roots, when compared to control water treatment. These results support the hypothesis that when NH_4^+ is supplied, it might increase the activity of GS or the expression of GS genes as shown for different plant species such as soybean, rice and maize (Morot-Gaudry, 1997). Our findings correspond to other studies which showed the mobilization and transport of N in soils from a hyphal compartment to a root compartment for a number of host plants – AM fungal species combinations (Ames *et al.*, 1983; Frey & Schuepp, 1993; Hawkins & George, 1999; Hawkins *et al.*, 2000; Johansen *et al.*, 1992; 1993; Johansen *et al.*, 1996; Subramanian & Charest, 1999; Tobar *et al.*, 1994a). In addition, AM carrot roots showed some enzyme activity when supplied only with water, whereas non-AM roots did not show any enzyme activity over the same treatment. This supports the hypothesis that the AM fungus possesses its own N-assimilating enzyme system, as suggested by Subramanian &

Charest (1999) and Johansen *et al.* (1996), and therefore would have added to the activity found in AM roots that received water only. Smith *et al.* (1985) suggested a similar idea regarding GS activity, that an increased assimilation of NH_4^+ via GS in AM roots might be partly the result of the fungal contribution. In contrast, Vézina *et al.* (1989) reported that in the case of ectomycorrhizal symbiosis, the relatively low amount of fungal biomass present might not be sufficient to affect the activity of the root system.

4.2.2. Second experiment

In the second experiment, AM roots showed a significant increase in GDH activity compared to non-AM roots. The same trend was observed for GS and NR activities, although not significant. This is in accordance with the results obtained by Faure *et al.* (1998) where GS and NR activities were not significantly increased in AM roots of *Lolium perenne* L. colonized with *G. fasciculatum* Thax. *Sensu* Gerdermann. In contrast, Subramanian & Charest (1998) showed that maize roots colonized with *G. intraradices* had two to four times higher NR and GS activities, respectively, compared to non-AM roots. Barea *et al.* (1987) and Azcon *et al.* (1992) had also shown increased GS and NR activities in AM roots of *H. coronarium* and *Lactuca sativa*, respectively. Cliquet & Stewart (1993) indicated increased NR and GS activities in maize roots colonized with *G. fasciculatum*. Smith *et al.* (1985) also found a significant increase in GS activity in roots of *Trifolium subterraneum* L. colonized with *G. intraradices* and therefore concluded that AM fungi are able to assimilate NH_4^+. In our *in vitro* system, enhanced GDH activity in AM roots might suggest a preference for NH_4^+ as the nitrogen source over NO_3^-, which would be in accordance to Smith *et al.* (1985). This could explain the relatively low NR

activities compared to those of GS and GDH in AM roots. However, Brunner *et al.* (2000) obtained lower root NR activities with Norway spruce (*Picea abies* L.) seedlings colonized with ectomycorrhizal fungi, *Hebeloma crustuliniforme* or *Laccaria bicolor*, compared to non-colonized seedlings. They suggested that this was a reflection of a better uptake of NO_3^- by the extraradical mycelium and since the fungus itself had a high NR activity, this may have reduced the need of the seedlings for enhancing NR.

Our results are consistent with the fact that NH_4^+ is a more easily assimilated N form than NO_3^+ and energetically more efficient (Johansen *et al.*, 1996; Villegas *et al.*, 1996). Recently, Hawkins *et al.* (2000) have shown, from an *in vitro* experiment, that excised hyphae of *G. mosseae* better acquired ^{15}N when supplied as $(^{15}NH_4)_2SO_4$ or $^{15}NH_4NO_3$ than when supplied as $K^{15}NO_3$ or $NH_4{}^{15}NO_3$. This also coincides with our findings of higher GDH activity in AM roots, which could be the result of a greater uptake of NH_4^+ over NO_3^-. Robinson *et al.* (1991) suggested that GDH plays an important role in reassimilating NH_4^+ under stressed conditions, especially under low levels of C source. They also showed in carrot cell suspension cultures that GDH activity increases under low sugar levels. *D. carota* mycorrhizal roots were probably out of available nutrients after eight weeks under the *in vitro* conditions, therefore out of a C source; this could possibly explain the enhanced GDH activity. In the same way, this could have affected NR activity, which seems to respond to addition of sucrose as stated by Sivasankar & Oaks (1996). Jolicoeur *et al.* (1999) showed that carrot roots, under *in vitro* conditions, hydrolysed all the sucrose contained in the medium in 25 days, but that glucose and fructose then became available for the roots without being limiting, but still at low

concentrations. Therefore, it is possible to presume that the high values of GDH activity might correlate with a need for C skeletons in AM roots which would have led to a greater uptake of NH_4^+. The same idea has also been proposed by Lancien *et al.* (2000) to explain the catabolic role of GDH during C-limiting conditions in order to replenish the TCA cycle. However, these suggestions have been criticized by Fricke & Pahlich (1992) who stated that changes in the apparent deamination reaction of GDH were independent of the N or sucrose starvation.

It is recognized that most fungi utilize NH_4^+ as a primary source of N (Chambers *et al.*, 1980) and that GDH is the primary NH_4^+ assimilating enzyme (Martin *et al.*, 1987). Thus, the mycorrhizal fungus could have contributed to higher values in GDH activity in AM roots. GDH still seems to have an unclear role in NH_4^+ assimilation in higher plant and little is known about its function in AM fungi. Its function seems to be clearer in ectomycorrhizal fungus where it is believed to play an important role in primary NH_4^+ assimilation. In a study about the involvement of GDH and GS in ammonia assimilation, Martin *et al.* (1988) indicated a pivotal role for the GS activity in *Cenococcum geophilum* Fr., an ectomycorrhizal fungus. Consequently, they suggested that the primary assimilation of NH_4^+ in the rapid phase of growth of the fungus differs from the assimilation pathways working in its stationary phase. Martin *et al.* (1994) also supported these findings with *Laccaria bicolor*, suggesting that when the mycelium was in the stationary phase, both GS and GDH pathways were simultaneously assimilating N. They also suggested that the intracellular concentration of NH_4^+ controls the synthesis of GDH. In fact, GDH would be more active in the latest phase whereas GS would contribute more

than GDH in the rapid phase of growth (Genetet *et al.*, 1984). Correspondingly, Quoreshi *et al.* (1995) stated that in ectomycorrhizal fungi, both the external N source and the fungal growth status have a major influence on the participation of GS and NADPH-GDH pathways. In the same vein, these observations might be relevant to AM fungi as well, considering the fact that the carrot roots in our *in vitro* system might have been at the stationary phase (slow growth). It is then possible to assume that GDH might have been predominant, thus having higher activity compared to GS. Further studies would be needed to answer such a question, and to which extent this hypothesis could be true in AM fungi is still unknown. Some might argue that GDH cannot compete with GS because of its low affinity (high K_m) for NH_4^+ (Lea & Miflin, 1974); but as mentioned before, this is not a valid argument given the fact that the two enzymes are spatially separated within the cell, and the fact that the K_m of GDH is biphasic and depends on the concentration of NH_4^+ (Oaks & Hirel, 1985).

Finally, in our experiment, GDH specific activity in AM roots was 15 times higher than that of GS. This corresponds closely to the results obtained by Grotjohann *et al.* (2000) who showed a 12-fold increase of specific GDH activity compared to GS in the ectomycorrhizal fungus *Suillus bovines*. This could be yet another piece of evidence supporting the idea of the active role of GDH in N assimilation under C limiting conditions.

Total C and N contents

In the present study, the total N content (%) in non-AM roots was similar to that of the fungal mycelium but significantly higher than in AM roots. However, when combined, the total N uptake in AM roots and fungal mycelium was higher than in non-AM roots. This could be explained by the fact that both non-AM roots and fungal mycelium were in direct contact with the N substrate and, therefore, able to directly assimilate it. It is possible that a 24-hr period was not sufficient to allow the total transfer of N from the extraradical hyphae to the host roots. This could explain the lower total N values in AM compared to non-AM roots. Although not significantly higher than in non-AM roots, the N content in our colonized roots was similar to the one obtained by Barea *et al.* (1987) in *Hedysarum coronarium* plants colonized with *G. mosseae* (2.27% N content). The C content was significantly higher in the fungal mycelium than in the AM or non-AM roots. This is consistent with the fact that mycorrhizal fungi act as a C sink in the symbiosis (Rygiewicz & Andersen, 1994). Moreover, our results suggest that under *in vitro* conditions, approximately 6% of the total C found in the AM fungus derives from the host roots. This would also be consistent with the fact that the total carbohydrate cost for the host plant to support AM fungal colonization is estimated at approximately 10% (Fitter, 1991; Rygiewicz & Andersen, 1994; Tinker *et al.*, 1994). As a result, even though the mycorrhizal fungus improved the nutrition of the carrot roots, it might not have resulted into higher growth rate of AM roots compared to non-AM roots, given that the C penalty from host roots to the fungus could not be outweighed by a photosynthetic C gain (Tinker *et al.*, 1994). In addition, our results on C content seem to be in accordance with the high GDH activity measured in the AM roots. The low C content in AM roots

compared to the fungus material could be due to the transfer of carbohydrates from the roots to the fungus and it is likely that GDH activity had to be higher in order to provide C skeletons to host roots. As stated by Lancien *et al.* (2000), the direction of the GDH reaction would depend on the N and C source.

When trying to discern the respective role of the AM fungus and the host root in N and C uptake (Lancien *et al.*, 2000), it has become more evident over the years that plant C and N metabolisms are tightly coordinated, and moreover, plant N dynamics are coupled to other nutrient interactions (Kytoviita & Arnebrant, 2000). Therefore, it is of foremost importance to analyze concomitantly the contribution of both metabolisms. In this regard, excellent models of C and/or N flow in mycorrhizal symbiosis as well as in higher plants have recently been provided to elucidate the uptake and metabolic pathways of these two major compounds (Bago *et al.*, 2000; 2001; Lancien *et al.*, 2000; Pfeffer *et al.*, 1999; Pfeffer *et al.*, 2001).

4.3. Labelled nitrogen experiment

Results from the labelled N experiment clearly showed its uptake and transfer from extraradical hyphae to host roots. Since $^{15}NH_4NO_3$ was taken up more efficiently than $NH_4{}^{15}NO_3^-$ by the AM fungus when supplied to the hyphal compartment, this suggests a preference for ammonium over nitrate. These observations are in accordance with other studies that found similar results (Johansen *et al.*, 1996; Villegas *et al.*, 1996) and could imply that *G. intraradices* favours the uptake of NH_4^+. In contrast, results obtained by Bago *et al.* (1996) pointed towards an active uptake of NO_3^- by the extraradical mycelium

of *G. intraradices* grown *in vitro* with tomato (*Lycopersicon esculentum* L.) roots. This distinction from our findings could be the result of the difference in host root species for the AM symbiosis. From our study, non-AM roots also showed a preference for NH_4^+ over NO_3^- but, surprisingly, more NO_3^- was taken up compared to AM roots when N was supplied directly to the roots. However, looking back at the specific enzyme activities in the second experiment, these results may not be so surprising. In fact, specific enzyme activities showed that NR was 2.5 times higher in non-AM roots compared to AM roots. This corresponds to the ^{15}N enrichment result profiles, where non-AM roots showed a two-fold enrichment compared to AM roots when supplied directly with $NH_4{}^{15}NO_3$. This may indicate that when both NH_4^+ and NO_3^- are supplied to AM roots, the mycorrhizal roots will tend to first take up ammonium, and then nitrate. These results could also suggest that roots competed with root-near hyphae for the uptake of N or that hyphae did not contribute significantly to the uptake of NO_3^-, as mentioned by Hawkins *et al.* (2000). Furthermore, Oliver *et al.* (1983) suggested that the involvement of fungi in NO_3^- assimilation is rather small. This could be reflected in our results by a smaller response of the mycorrhizal fungus in NO_3^- uptake in the AM roots compared to non-AM roots. Barea *et al.* (1987) obtained similar results when they applied $(^{15}NH_4)_2SO_4$ to *Hedysarum coronarium* plants colonized with *G. mosseae*; the ^{15}N enrichment level they obtained was about 0.259 atom % excess, whereas our results indicated a 0.250 APE in AM roots which received $^{15}NH_4NO_3$. Once more, these results might suggest that NO_3^- is less likely to be assimilated than NH_4^+ in AM roots.

4.4. Western blot analysis

Our results showed a clear response of GDH to NH_4NO_3 substrate in AM and non-AM roots since this enzyme was detected by immunodetection in these extracts (second substrate induced experiment). Non-NH_4NO_3 fed roots (AM and non-AM) also showed the presence of GDH and GS. However, in the fungal extracts, only GS was detected as a different isoform compared to AM and non-AM roots. To our knowledge, this is the first time such findings have been reported in AM fungi. This could therefore emphasize the active role of AM fungi in N assimilation and metabolism in their symbiotic association with host roots. The isoforms of GS detected here seem to correspond to the cytosolic from (GS1), which is mostly found in root tissues.

The different bands obtained for GDH in both AM and non-AM roots might indicate the presence of the two major forms of GDH, NADH-GDH and NADPH-GDH. Indeed, one of the bands was NADH-GDH with a molecular weight of approximately 43 kDa as observed by Loulakakis *et al.* (1990) in root tissues of *Vitis vinifera* L. These two molecular forms have also been shown to be distinct in *Laccaria bicolor* (Ahmad *et al.*, 1990). Surprisingly, the AM fungus did not show any form of GDH, which was not quite expected. However, this could be explained by the low protein concentrations in the fungal material extracts. Therefore, the proteins of GDH might not have been detected by immunodetection considering the small amount of material used. On the other hand, GS was found in the AM fungus using the same protein concentrations in the extracts, which could imply that the main N-assimilating enzyme in *G. intraradices* could be GS. Unlike most ectomycorrhizal fungi, GDH could be less important in AM fungi in N metabolism.

Finally, the antibodies used to detect GDH might not have been quite specific enough to detect fungal enzymes such as GDH since these antibodies were raised against purified grapevine GDH. This could have affected the results obtained in this study.

Isoforms of GS and GOGAT were detected by Western blot in ectomycorrhizal fungi associated with *Fagus sp.* whereas fungal GDH was not revealed (Martin *et al.*, 1987); this is similar to our results in AM fungi. This could be the result of a non-specific antibody used in the immunodetection. As mentioned earlier, there are also two isoforms of GS (GS1 and GS2), which are regulated differently. GS2 seems to be induced by light and NH_4^+ whereas for GS1 there is no clear response to ammonium (Morot-Gaudry, 1997; Oaks, 1994). Oaks (1994) showed by means of SDS-PAGE, that there was a clear positive response to NH_4^+ (as well as both NH_4^+ and NO_3^-) of GS and GDH in roots of maize seedlings. In accordance to these results, our data also showed a response of GDH in AM and non-AM roots that were fed with NH_4NO_3.

4.5. Conclusion

Our study revealed that AM fungus can uptake and transfer inorganic N, for the most part in the form of NH_4^+, to Ri T-DNA carrot roots grown under our experimental *in vitro* conditions. These results are in accordance with several studies that have reported significant transfer of N from AM fungi to host plants in soil systems (Ames *et al.*, 1983; Frey & Schuepp, 1993; Ibijbijen *et al.*, 1996a; Johansen *et al.*, 1992; 1993; 1994; Johansen *et al.*, 1996; Mader *et al.*, 2000; Subramanian & Charest, 1999; Tobar *et al.*, 1994a). Other studies have also reported the uptake and transfer of amino acid (Cliquet *et*

al., 1997; Subramanian & Charest, 1998), and complex organic N (Hodge *et al.*, 2001; Ibijbijen *et al.*, 1996b) by AM fungi to host roots, highlighting the importance of such fungi in plant mineral nutrition. Furthermore, we found significantly higher GDH activity, but not of GS or NR, in AM roots compared to non-AM roots. This brings interesting information on the function of GDH in ammonium transfer in higher plants and particularly in AM symbiosis. It was also suggested that the lower NR activity in AM roots compared to that of GDH was correlated to the ^{15}N enrichment profiles where $^{15}NH_4NO_3$ was mostly taken up by AM roots compared to $NH_4{}^{15}NO_3$. Thus, most of the stated hypotheses have been supported by our results, except that not all of the N key-enzymes in AM roots showed a significant increase of their activity following the N transfer. Our findings also demonstrated that both AM and non-AM roots have the same N-assimilating enzymes isoforms (GDH and GS). Therefore, this would agree with the enzyme activities that we found in the substrate-induced experiment. Interestingly, the AM fungus *G. intraradices* seems to have a different isoform of GS compared to Ri T-DNA carrot roots (colonized or not). To our knowledge, this is the first time such findings have been reported. This could therefore emphasize the active role of AM fungi in N assimilation and metabolism in their symbiotic association with higher plants.

Different results reported in other studies, in terms of GS, NR or GDH activities in AM roots compared to non-AM roots, are probably the reflection of different symbiotic combinations (fungus species/host plant species), stage of growth (seedlings compared to full grown plants), levels of N concentration supplied to the mycorrhizal plants (Emmerton *et al.*, 2001), or growth conditions. However, even though growth conditions

might have an effect on the responses of AM plants compared to non-AM plants (measured by enzymatic activities or other response variables), we do believe that the *in vitro* system provides a suitable environment to study N metabolism. As a result, the *in vitro* system has proven to be a keen and useful tool for short- or long-term studies of the physiology of the AM fungus-host root symbiosis. Such a system allows the growth of intact roots-fungus complexes in a controlled and pathogen-free environment that is quite easy to manipulate and allows long-term experiments. It is probably one of the best biological tools for the study of particular key elements or metabolic pathways such as N metabolism in the AM symbiosis. The only limitation of the *in vitro* system is the amount of fungal tissue needed for enzyme analyses. This could therefore require a greater investment of time and material compared to whole plant culture systems, as mentioned by Hawkins *et al.* (2000). A solution to this problem could be the development of cultural methods using bioreactors that could produce large amounts of material. As stated by Pfeffer *et al.* (2001), the fact that AM fungus only represents a small portion of the tissue volume in AM roots grown *in vitro* has been a hindrance in studying this particular system.

In conclusion, recent advances in molecular biology as well as new techniques in physiology will surely prove to be of help in studies such as ours. As mentioned by Marschner & Dell (1994), utilization of mycorrhizal fungi will depend on progress in basic research. This will allow us to have a better understanding of the AM symbiosis and, more specifically, its function in N metabolism. Consequently, in a more practical way, it might prove to be useful in agriculture where scientists try to find new

environmentally-friendly approaches in order to reduce the chemical fertilizer input in soils. As emphasized by Paynel *et al.* (2001), because of increasing economic and environmental pressures, study of direct interspecific N transfer has received increasing attention in the last decade. Hence, the study of AM fungi has fundamental and practical issues because of its importance in the context of sustainable agriculture and horticulture, reforestation, biodiversity conservation, and ecosystem management.

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Appendix 1. Composition of the minimal (M) medium (Bécard & Fortin, 1988)

Product	**M medium ($mg\ l^{-1}$)**
$MgSO_4 . 7H_2O$	731
$Na_2SO_4 . 10H_2O$	-
KNO_3	80
KCl	65
$NaH_2PO_4 . 2H_2O$	-
KH_2PO_4	4.8
$Ca(NO_3)_2 . 4H_2O$	288
Sucrose	10 000
NaFeEDTA	8
Kl	0.75
$MnCl_2 . 4H_2O$	6
$ZnSO_4 . 7H_2O$	2.65
H_3BO_3	1.5
$CuSO_4 . 5H_2O$	0.13
$Na_2MoO_4 . 2H_2O$	0.0024
Glycine	3
Thiamine hydrochloride	0.1
Pyridoxine hydrochloride	0.1
Nicotinic acid	0.5
Myo inositol	50
Bacto Agar	10 000

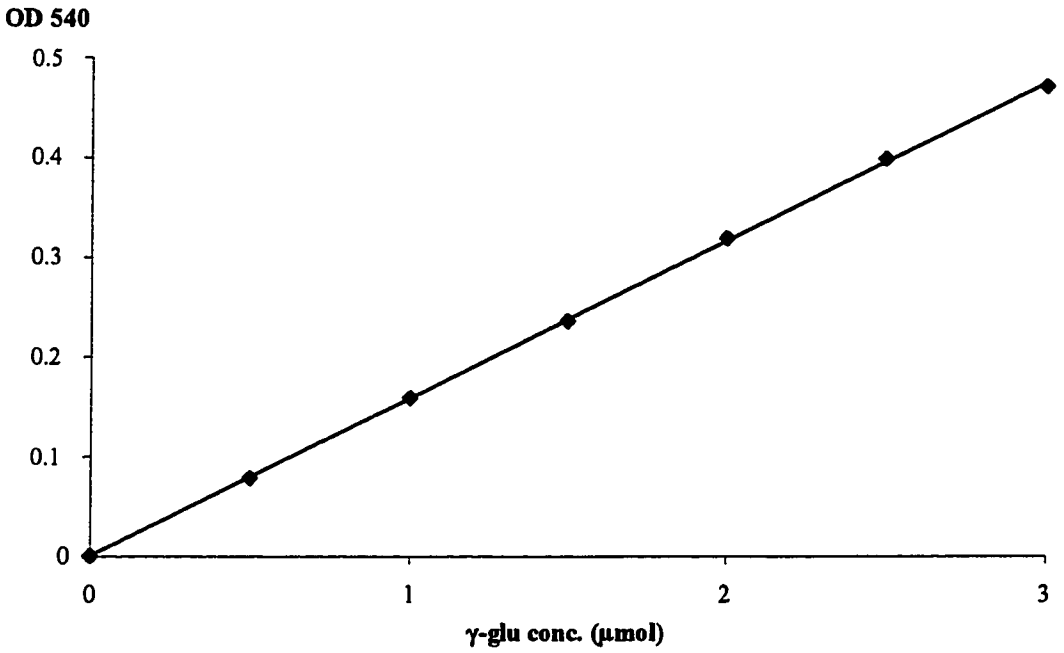
* The pH of the medium was adjusted to 5.5 before sterilization at 121 °C for 15 min.

Appendix 2. A standard curve of γ-glutamylhydroxamate (γ-glu.) concentrations for the determination of GS (glutamine synthetase) activity.

γ-glu. conc. *(μmol)*	OD_{540}
0.0	0.000
0.5	0.078
1.0	0.159
1.5	0.236
2.0	0.319
2.5	0.399
3.0	0.471

Regression output

Constant	0.000
R squared	1.000
No. of observations	7
X Coefficients	0.158
Std Err of	0.001

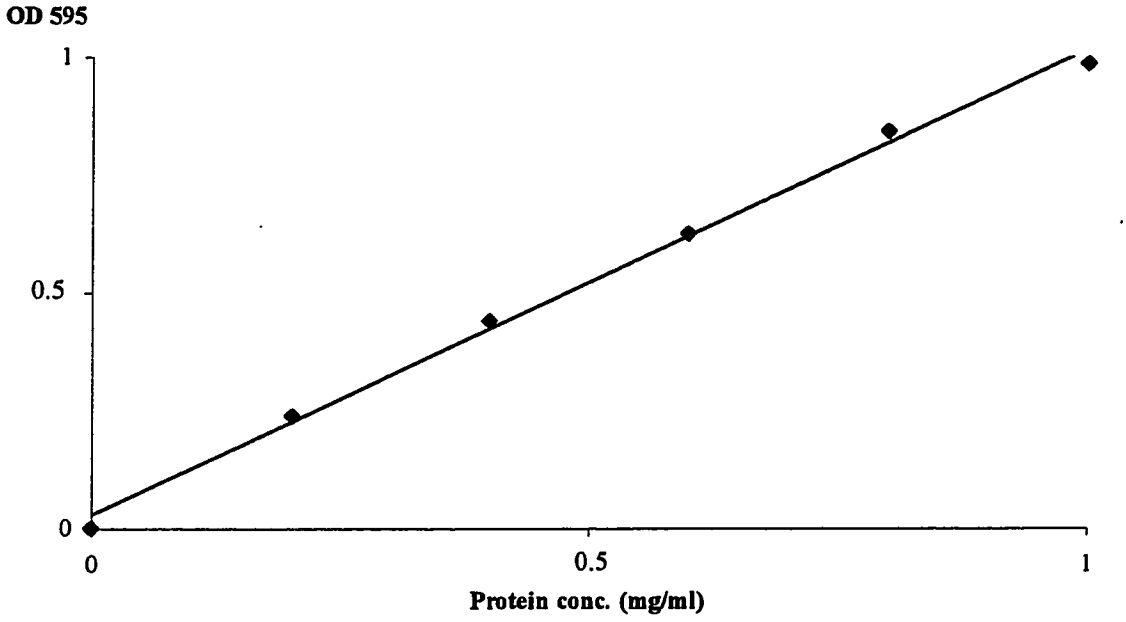


Appendix 3. A standard curve of BSA (bovine serum albumin) for the determination of protein concentrations.

Protein conc. (mg/ml)	OD_{595}
0.0	0.000
0.2	0.239
0.4	0.441
0.6	0.626
0.8	0.842
1.0	0.985

Regression output

Constant	0.028
R squared	0.996
No. of observations	6
X Coefficients	0.988
Std Err of	0.032



Appendix 4. One-way ANOVA on ranked data of GS activity in AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	37.5	1	37.5	15.000	0.018

Symbols as in Table 3.2.

$R^2 = 0.789$

Appendix 5. One-way ANOVA on ranked data of GS activity in non-AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	73.5	1	73.5	36.75	0.004

Symbols as in Table 3.2.

Non-AM: $R^2 = 0.902$

Appendix 6. One-way ANOVA on ranked data of GDH activity in AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	60.167	1	60.167	16.409	0.015

Symbols as in Table 3.2.

AM: $R^2 = 0.804$

Appendix 7. One-way ANOVA on ranked data of GDH activity in non-AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	48.167	1	48.167	41.286	0.003

Symbols as in Table 3.2.

$R^2 = 0.912$

Appendix 8. Two-way ANOVA on ranked data of GS specific activity for the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	85.33	1	85.33	20.078	0.002
Mycorrhizal	16.33	1	16.33	3.843	0.086
Substrate*Mycorrhizal	5.33	1	5.33	1.255	0.295

Symbols as in Table 3.2.

$R^2 = 0.759$

Appendix 9. One-way ANOVA on ranked data of GS specific activity in AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	24.000	1	24.000	7.200	0.055

Symbols as in Table 3.2.

$R^2 = 0.643$

Appendix 10. One-way ANOVA on ranked data of GS specific activity in non-AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	66.667	1	66.667	12.903	0.023

Symbols as in Table 3.2.

$R^2 = 0.763$

Appendix 11. Two-way ANOVA on ranked data of GDH specific activity for the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	96.33	1	96.33	33.029	0.000
Mycorrhizal	12.00	1	12.00	4.114	0.077
Substrate*Mycorrhizal	1.33	1	1.33	0.457	0.518

Symbols as in Table 3.2.

$R^2 = 0.825$

Appendix 12. One-way ANOVA on ranked data of GDH specific activity in AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	60.167	1	60.167	15.696	0.017

Symbols as in Table 3.2.

$R^2 = 0.797$

Appendix 13. One-way ANOVA on ranked data of GDH specific activity in non-AM roots for the substrate treatment in the first experiment.

Source of variation	SS	df	MS	F	p
Substrate	37.500	1	37.500	18.750	0.012

Symbols as in Table 3.2.

$R^2 = 0.824$

Appendix 14. Two-way ANOVA on ranked data of GS specific activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	475.000	4	118.750	8.247	0.003
Mycorrhizal	9.800	1	9.800	1.083	0.357
Block*Mycorrhizal	36.200	4	9.050	0.628	0.653

Symbols as in Table 3.2.

$R^2 = 0.783$

Appendix 15. Two-way ANOVA on ranked data of NR specific activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	107.500	4	26.875	0.974	0.464
Mycorrhizal	0.800	1	0.800	0.019	0.898
Block*Mycorrhizal	170.700	4	42.675	1.546	0.262

Symbols as in Table 3.2.

$R^2 = 0.503$

Appendix 16. Two-way ANOVA on ranked data of GDH specific activity for the second experiment.

Source of variation	SS	df	MS	F	p
Block	244.125	4	61.031	2.959	0.075
Mycorrhizal	198.450	1	198.450	250.016	0.000
Block*Mycorrhizal	3.175	4	0.794	0.038	0.997

Symbols as in Table 3.2.

$R^2 = 0.684$

Appendix 17. Non-parametric Kruskal-Wallis analysis on total N content (% N).

Source of variation	Count	Rank sum	p
AM	4	10	
Non-AM	4	37	0.020
Fungus	4	31	

p = probability assuming Chi-square distribution with 2 degrees of freedom

Kruskal-Wallis Test Statistic = 7.785

Appendix 18. Non-parametric Kruskal-Wallis analysis on total N content (mg N/mg DW).

Source of variation	Count	Rank sum	p
AM roots and Fungus	4	10	
Non-AM roots	4	26	0.019

p = probability assuming Chi-square approximation of 5.463 with 1 degree of freedom

Mann-Whitney U test statistic = 0.000

Appendix 19. Non-parametric Kruskal-Wallis analysis on total C content (% C).

Source of variation	Count	Rank sum	p
AM	4	18	
Non-AM	4	42	0.025
Fungus	4	18	

p = probability assuming Chi-square distribution with 2 degrees of freedom

Kruskal-Wallis test statistic = 7.385

Appendix 20. Non-parametric Kruskal-Wallis analysis on total C content (mg C/mg DW).

Source of variation	Count	Rank sum	p
AM roots and Fungus	4	26	
Non-AM roots	4	10	0.021

p = probability assuming Chi-square approximation of 5.333 with 1 degree of freedom

Mann-Whitney U test statistic = 16.000

Appendix 21. Protein concentrations for GS/GDH (mg g^{-1} dry weight) in carrot roots with (AM) or without (non-AM) arbuscular mycorrhizal colonization for the first experiment

Mycorrhizal treatment	**Proteins**	
	NH_4NO3	H_2O
AM	8.1 [a, y] (2.2)	7.3 [a, y] (1.6)
Non-AM	11.5 [a, y] (3.9)	13.2 [a, y] (2.8)

[1]Within each column, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Appendix 22. Non-parametric Kruskal-Wallis analysis on $NH_4{}^{15}NO_3$ enrichment levels when applied in the root compartment for the labelled N experiment.

Source of variation	Count	Rank sum	p
AM	3	6	
Non-AM	3	15	0.050

p = probability assuming Chi-square approximation of 3.857 with 1 degree of freedom

Mann-Whitney U test statistic = 0.000

Appendix 23. Non-parametric Kruskal-Wallis analysis on $^{15}NH_4NO_3$ enrichment levels when applied on the root compartment for the labelled N experiment.

Source of variation	Count	Rank sum	p
AM	3	10	
Non-AM	3	11	0.827

p = probability assuming Chi-square approximation of 0.048 with 1 degree of freedom

Mann-Whitney U test statistic = 4.000

Appendix 24. Non-parametric Kruskal-Wallis analysis on ^{15}N enrichment levels when applied on the root compartment for AM roots in the labelled N experiment.

Source of variation	Count	Rank sum	p
$^{15}NH_4NO_3$	3	15	
$NH_4{}^{15}NO_3$	3	6	0.05

p = probability assuming Chi-square approximation of 3.857 with 1 degree of freedom

Mann-Whitney U test statistic = 9.000

Appendix 25. Non-parametric Kruskal-Wallis analysis on ^{15}N enrichment levels when applied on the root compartment for non-AM roots in the labelled N experiment.

Source of variation	Count	Rank sum	p
$^{15}NH_4NO_3$	3	15	
$NH_4{}^{15}NO_3$	3	6	0.05

p = probability assuming Chi-square approximation of 3.857 with 1 degree of freedom

Mann-Whitney U test statistic = 9.000