THE MOLECULAR AND BIOCHEMICAL BASIS OF NITROGEN TRANSFER BY THE MODEL ARBUSCULAR MYCORRHIZAL FUNGUS *RHIZOPHAGUS IRREGULARIS*

By

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ABSTRACT

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Plants can increase their effective root length and surface area by investing in symbioses with soil fungi, forming mycorrhizal associations. In mutualistic mode, the plant provides photosynthate (fixed C) to the fungus, whereas the fungus provides nutrients to the plant. Arbuscular mycorrhizal (AM) associations are found in 80% of vascular plant families and as a consequence, the AM symbiosis is of tremendous significance to life on this planet, in both natural and agricultural ecosystems. Work in recent years has substantially increased our understanding of nitrogen nutrition in the AM symbiosis. At the molecular level a working model for nitrogen uptake, metabolism, and transfer has emerged. In this dissertation, mechanisms and genes believed to be responsible for nitrogen flows in the AM symbiosis are described and open questions about the pathway and its regulation are highlighted. Molecular and biochemical experimental approaches were used to investigate these unresolved questions. A compartmented microcosm was developed for aseptic and leakage-free whole-plant mycorrhizal experiments. This was used to monitor S and N uptake by the fungal extraradical mycelium (ERM) and its transfer to host plants. Our results show rapid S and N transfer by ERM to the host plants. Using growth parameter

measurements, chlorophyll contents as well as ^{15}N labeling, we conclude that nitrogen transfer from an arbuscular mycorrhizal fungus confers growth benefits on the host plant under nitrogen limiting conditions and that the microcosm system developed will be useful for future work on AM nutrition and metabolism under physiologically relevant conditions. Isotopic labeling time course experiments using different ^{15}N and ^{13}C labeled substrates as well as expression analysis of the expression of key genes were performed using microcosms and an *in vitro* monoxenic culture system. The results demonstrated the operation of a new pathway of N transfer by AM fungi to the host via nitrate translocation from the extraradical mycelium to plant roots and shoots. The results also indicate that ornithine is made in the ERM via pyrroline-5-carboxylate and that some of it is broken down in the IRM to glutamate and in a lesser extent to putrescine. Labeling analysis strongly suggests that ornithine is also translocated from the intraradical mycelium to the ERM and is used to make arginine there. Changes in gene expression are consistent with the labeling data on n uptake, metabolism and movement. Gene expression analysis of glutamate dehydrogenase suggests a potential dissimilatory role in the IRM.

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Introduction and Literature review

Nitrogen transfer in the arbuscular mycorrhizal symbiosis

Introduction

The arbuscular mycorrhizal (AM) symbiosis is the most ancient and widespread plant-microbe mutualism among land plants. Arbuscular mycorrhizas are formed by the roots of over 80% of plant species with fungi of the *Glomeromycota*. Arbuscular mycorrhizal fungi are asexual, obligate symbionts (Smith and Read, 2008) that propagate by forming multinuclear vegetative underground spores and by the growth of the underground mycelium to colonize additional roots. After spore germination, hyphal growth is associated with nuclear divisions as well as the migration of nuclei from the spore (Bianciotto and Bonfante, 1992; Becard and pfeffer, 1993; Bianciotto *et al.* 1995). The hyphae use carbohydrate and lipid reserves during pre-symbiotic growth (Becard *et al.*, 1991; Bago *et al*., 1999). In the absence of a host root, the growth of the mycelia ceases within several days, and spores are capable of multiple germination events. Presymbiotic hyphae respond to signaling molecules in plant root exudates that stimulate branching (Giovannetti *et al.*,1993; Buee et al. 2000). Akiyama *et al.,* (2005) identified strigolactones in plant root exudates as increasing germ tube metabolism and growth. Strogolactone production is stimulated when plants experience mineral deficiency. Hyphal contact with the root is usually followed by adhesion of the hyphal surface and after 2-3 days, the formation of appressoria followed by root penetration and formation of arbuscules around 2 days later (Brunndrett et al., 1985; Becard and Fortin, 1988; Peterson and Bonfante, 1994). Once the AM symbiosis has been established, the fungal growth proceeds in both the soil (extraradical mycelium) and in the root (intraradical mycelium).

AM fungi (AMF) have so far proven to be unculturable axenically and appear in nature to be unable to complete their life cycle without forming a symbiosis with plant host roots (Smith and Read, 2008). AMF take up photosynthetically fixed carbon from the plant and convert it into triacylglycerols, which are the main nutritional form of carbon stored and translocated by the fungus for growth and sporulation and utilized during germination (Bago *et al.* 2000). The intraradical mycelium imports glucose from roots and converts it into trehalose and glycogen and then to triacylglycerol (Shachar-Hill *et al.,* 1995; Pfeffer *et al.*, 1999). Lipid bodies transport C from the intraradical mycelium (IRM) to extraradical mycielum (ERM) and are utilized for mycelial growth (Bago *et al.,* 2002; Lammers *et al.,* 2001)

Morphology and anatomy

Details of fungal interactions with plant cells and tissues of the root were first described by Gallaud (1905) in which he indicated that AM roots can contain a wide variety of structures. Gallaud (1905) categorized them into four distinct structural classes; Arum, Paris, hepatic and orchid types. The most common forms are found in AM roots: the Paris-type and Arum-type. In Paris-type the cortical colonization of the root is characterized by extensive development of intracellular coiled hyphae which spread from cell to cell. Paris-type occurs in many families of pteridophytes, gymnoserms and angiosperems (Smith and Smith 1997). By contrast, Arum-type is often described in the fast-growing roots systems of crop plants (Brundrett *et al.,* 1990). In these associations the fungus spread rapidly in the root cortex by intercellular hyphae which extend along well-developed intercellular air spaces. Side braches penetrate the cell wall of cortical cells to produce highly branched arbuscules. These arbscules

invaginate root cortical cells without penetrating the host cell membrane and form a periplasmic space between the root cell and the arbuscular membrane. This periplasmic space is the site of nutrient transfer between the fungus and the plant (Bago, 2000; VanAarle *et al.,* 2005)

Phylogeny

It was believed, partly on the basis of their asexual life cycle, that AM fungi are most closely related to zygomycota but the use of DNA sequences forced a re-evaluation their relationship (Smith and read, 2008). Based on small subunit (SSU) rRNA gene sequences, morphological and ecological characteristics, AM fungi are now regarded as separate from all other major fungal groups in a monophyletic clade. Consequently they were placed into a new phylum, the *Glomeromycota* (Schußler *et al.*, 2001). About 240 species of glomeromycotan fungi have been described based on their spore morphology and molecular phylogenetic data (Schüßler & Walker, 2010; Redecker *et al*., 2013). Alves de silva *et al*., (2006) analyzed the large subunit (LSU) ribosomal RNA (rRNA) for AM fungal taxonomy and their data indicated that *Archaeosporaceae* are a basal group in *Glomeromycota*, *Acaulosporaceae* and *Gigasporaceae* belong to the same clade, while Glomeraceae are polyphyletic. large subunit of RNA polymerase II (RPB1; Redecker and Raab 2006; Stockinger *et al*., 2014) and β-tubulin gene (Msiska and Morton 2009) were phylogentically analyzed too. The β-tubulin gene phylogeny was similar to the 18S (LSU) rRNA gene phylogeny at the family and species level, but not at the order level (Msiska and Morton 2009) while sequences of the large subunit of RNA polymerase II. Based on all of these studies, arbuscular mycorrhizal fungi are divided into ten families in the phylum

Glomeromycota, order *Glomerales*. Those families are *Gigasporaceae, Glomeracea, Acaulosporaceae, Diversispora, Paralglomaceae, Geosiphoaceae, Ambisporaceae, Eutrophosphosporaceae, and Arcaesporaceae.*

Glomus is the largest genus within the phylum with more than 70 species (Redecker and Raab 2006). Several of the *Glomus* species, most frequently *Glomus intraradices*, are commonly studied (Smith & Read, 2008). Based on a molecular analysis of ribosomal DNA and a re-evaluation of an early description of this species, it was recently renamed *Rhizophagus irregularis (*Krüger *et al.,* 2012) despite the misleading "root eater" title. Tisserant *et al*., (2013) assembled and annotated the genome of *Rhizophagus irregularis from high throughput DNA sequencing i*n association with transcriptome data*. This study* provides insight into the capabilities of this fungus and points to genes involved in mycorrhizal symbioses.

Plant benefits from AM symbioses

Association with arbuscular mycorrhizal fungi (AMF) is often beneficial to plants by improving their access to nutrients (Smith and Smith, 2011). AMF also enhance pest and disease suppression and improve drought tolerance (Smith and Read, 2008). Among the benefits that AM fungi provide to host plants, improved phosphorus nutrition has received the most attention. Previous studies, however, indicated that AM fungi may also be important for a wide variety of nutrients (Smith and Smith, 2011). AM fungi enhance the uptake of nitrogen (Govindarajulu *et al*., 2005), sulfur (Allen & Shachar-Hill, 2009), zinc (Seres *et al*., 2006), copper (Toler *et al*., 2005) and iron (Kim *et al*., 2010) . Hart and Forsythe, (2012) showed that the identity of AM fungi can influence the uptake

of many nutrients but that the magnitude and direction of the nutrient-derived growth response is also affected by host plant characteristics and soil nutrient status.

Phosphorous uptake and transfer

AM fungi improve the phosphorous nutrition of host plants (Bolan, 1991). The extraradical mycelium (ERM) explores a larger soil volume than is possible for roots and reaches zones and soil pores that the lateral roots cannot access. The small hyphal diameter (typically several microns) leads to an increased P absorbing surface area (Marschner and Dell, 1994) and estimates of ERM levels around host plants indicate that these reach several meters of hyphal length per gram of soil (Jakobsen *et al.,* 1992). In AM fungi, polyphosphates (polyP) are formed after P uptake thus lowering internal inorganic P concentrations. Organic acids and phosphatases are released by the ERM which increase the availability of P from organic and inorganic sources. In the extraradical hyphae, N is transported as arginine (Govindarajulu *et al*., 2005; Tian *et al*., 2010) which may be bound to polyphosphate and therefore be coupled to Pi translocation (Jin *et al*., 2005). Several studies have shown that AM specific root phosphate transporters are induced in the roots of plants colonized by AM fungi (Javot *et al* , 2007; Gomez *et al*., 2009; Nagy *et al*., 2009). It was suggested that inorganic phosphate delivery to cortical cells was necessary for sustaining the symbiosis because in *Medicago truncatula* mutants affected in the AM-specific inorganic phosphate (Pi) transporter 4 gene, the arbuscules accumulated polyphosphate and prematurely degenerated (Javot *et al*., 2007). Increasing carbohydrate availability stimulates P uptake by the ERM to and translocation to the mycorrhizal roots as well as altering the

metabolic and spatial distribution of P within the fungus (Bücking and Shachar-Hill, 2005). Previous studies suggested that there is a cross-talk between P and N nutrition (Blanke et al., 2005, Bonneau et al, 2013). It was found that low P and N fertilization induced a physiological state of plants favorable for AM symbiosis despite their higher P status (Bonneau et al, 2013).

Sulfur uptake and transfer

Previous studies have reported the effects of AM colonization on the uptake of S (Gray and Gerdemann, 1973; Rhodes and Gerdemann, 1978). Gray and Gerdemann (1973) showed that mycorrhizal colonization in clover (*Trifolium pratense*) and maize increased ^{35}S uptake compared to nonmycorrhizal plants. Furthermore, Rhodes and Gerdemann (1978) found that mycorrhizal colonization increased ^{35}S uptake in onion compared to nonmycorrhizal plants and they showed that the S uptake was heavily influenced by P nutritional benefits.

Allen and Shachar-Hill (2009) showed using *in vitro* cultures of transformed roots colonized by *Glomus intraradices* that the ERM takes up S in the form of sulfate and sulfur-containing amino acids and transfers it to mycorrhizal roots. They also showed that the root S contents were increased by 25% in a moderate (not growth-limiting) concentration of sulfate. 50% of $^{35}SO_4^{2}$ uptake from the fungal compartment were detected in the mycorrhizal roots. Similar quantities of ^{35}S were transferred to mycorrhizal roots whether $^{35}SO_4^{2-}$, [^{35}S]Cys, or [^{35}S]Met was supplied in the fungal compartment (Allen and Shachar-Hill, 2009). Sieh *et al*., (2013) studied the effect of mycorrhizal colonization on sulfur starvation responses in *M. truncatula* and they found

that colonization reduced S starvation when the plant's phosphate status is high, concluding that mycorrhizal sulfur transfer improves plant S nutrition.

Nitrogen uptake and transfer

Plant roots and the AM fungal ERM can absorb both nitrate and ammonium from soil (George *et al.,* 1995; Smith and Read, 2008) as well as soluble organic nitrogen which together dominate the soil N pool (Jin *et al*., 2005, McNeill and Unkovich, 2007). Nitrate and ammonium are mobile under most soil conditions but this can be restricted in dry soil (Tobar et al 1994, Tinker and Nye, 2000). These observations suggest a possible role for N uptake and transfer, a suggestion that has been strengthened by tracer studies and field observations.

Haines and Best (1976) found that colonization of *Liquidambar styraciflua* by the AM fungus *Glomus mosseae* retarded the leaching of ammonium and nitrate from soil suggesting that the AM fungi might be involved in N uptake. When N-15-labelled fertilizer was added to mycorrhizal or non mycorrhizal control pots, Azcon-Aguilar *et al.*, (1993) reported that the $^{14}N/^{15}N$ ratio was higher in AM onion plants than in uncolonized control plants, suggesting that AM fungi were able to access soil N that is less available to non mycorrhizal plants. Furthermore, Cliquet and Stewart (1993) demonstrated that ^{15}N translocation from roots to shoots through the xylem was higher in VAM plants compared with control plants. Under water-stressed conditions that affect the nitrate availability to roots, the 15N enrichment was four times higher in mycorrhizal than in non-mycorrhizal lettuce plants providing evidence of hyphal transport to the plant of N from labeled nitrate (Tobar *et al*., 1994). A mechanism of N transfer from the fungus to

the plant in the AM symbiosis was proposed (Bago *et al*., 2001) in which N taken up by the fungus is incorporated into amino acids, translocated from the ERM to the intraradical mycelium (IRM) as arginine, which is broken down to ammonium, that is released to the host root. This model has gained support from ^{15}N and ^{13}C isotopic labeling experiments, measurements of enzymatic activities (Govindarajulu *et al*., 2005; Jin *et al*., 2005, Cruz *et al*., 2007) and from gene expression data (Govindarajulu *et al*., 2005; Guether *et al*., 2009; Gomez *et al*., 2009; Tian et al 2010).

The mechanisms of nitrogen transfer and metabolism in the AM symbiosis

The pathway of nitrogen movement through the AM symbiosis

The current working model of N transfer from the fungi to the plant in the AM symbiosis was proposed by Bago *et al.* (2001) based on previous work that demonstrated fungal N uptake and metabolism and implicated amino acids in N handling (Johansen *et al*., 1996; Bago *et al*., 1996; 2000). This mechanism involves N uptake up by the fungi which is incorporated into amino acids, translocated from the extraradical mycelium (ERM) to the intraradical mycelium (IRM) as arginine (Arg), but transferred to the plant without C as inorganic N (Govindarajulu *et al.*, 2005; Jin *et al.*, 2005).

Gene expression and enzyme activities for plant and fungal proteins involved in the nitrogen metabolic pathway have been analyzed (**Table 1-1**). Most of the important enzymes and nitrate or ammonium transporters involved in nitrogen metabolism were identified from the AM fungus *Glomus intraradices* (Kaldorf *et al.* 1998; Govindarajulu *et al.*, 2005; Lopez-Pedrosa *et al.* 2006; Gomez *et al*., 2009; Tian *et al.*, 2010). Gene

expression at the transcriptional and post transcriptional levels and ^{15}N and ^{13}C labeling experiments support the proposed model (Govindarajulu *et al.*, 2005; Cruz *et al*., 2007; Tian *et al*., 2010). In **Figure 1-1**, the current model is illustrated, including the genes identified and the postulated regulation on the transcripts by the metabolites (Tian *et al.*, 2010).

The uptake of nitrogen by the ERM of the AM symbiosis

A high affinity ammonium transporter has been identified in *G. intraradices* and characterized by Lopez-Pedrosa *et al.* (2006), which is involved in the ammonium uptake of ERM. A putative high affinity nitrate transporter which is up-regulated in response to nitrate addition was identified in the ERM of the same AM fungus (Tian *et al.*, 2010). Plant and fungal uptake of macronutrient ions involves low affinity as well as high affinity transporters, and it is expected that low affinity transporters for NO_3^- and NH_4^+ will also be identified. AMF can also obtain N from decomposing organic materials and store it in the mycelium (Hodge & Fitter, 2010). Amino acids including Gly, Glu, Pro and Arg can be taken up by the ERM (Hawkins *et al*., 2000; Jin *et al*., 2005) and Arg, Gly, Gln and Ornithine can be taken up by germinating spores (Gachomo *et al.*, 2009). An amino acid permease *GmosAAP1* that can transport proline through a proton-coupled process has been characterized from *Glomus mosseae*; its expression is transcriptionally upregulated by external amino acids (Cappellazzo *et al.*, 2008). It is likely that AMF express permeases allowing the uptake of other amino acids such as Arg by the fungus from the environment. Indeed genome-scale analysis of an ectomycorrhizal fungus has highlighted the existence of many N transporters (Lucic *et al*., 2008). However the uptake rates of amino acids by the ERM measured in AM root

cultures are substantially lower than the rates for nitrate, ammonium and urea (Jin et al 2005, and this work). It also appears that most of the nitrogen taken up by AMF from organic patches in the soil is taken up without carbon (Hodge & Fitter, 2010), in agreement with earlier experiments on the fate of nitrogen and carbon supplied to mycorrhizal plants as labeled peptides (Persson *et al*., 2003).

The assimilation of N in the ERM and release of ammonium in the IRM.

Kaldorf *et al.* (1998) reported a partial sequence for a putative nitrate reductase from *G. intraradices*. The glutamine synthetase /glutamate synthase (GS/GOGAT) pathway is important in the assimilation of ammonium produced from nitrate or taken up directly from the soil. Activities for the GS/GOGAT pathway but not the alternative assimilatory NADP-dependent glutamate dehydrogenase (GDH) were reported by Cliquet and Stewart (1993) in mycorrhizal roots, and the application of a GOGAT inhibitor to extraradical mycelium reduced 15N assimilation. Breuninger *et al.* (2004) found that GS activity is upregulated in response to N addition in the ERM of *G. intraradices* and *G. mossae*. The glutamine synthetase from *G. intraradices* reported by Breuninger *et al.* was recently found to be one of a gene family with at least two members, and this isoform (named *GiGS1*, Tian et al 2010) has a high constitutive transcriptional level whereas another member (*GiGS2*) is actively up-regulated upon exposure to nitrate but has a lower constitutive expression level. This suggests different roles for the two enzymes at different environmental nitrogen levels. Additionally, both of them have low Km values for glutamate, which indicates that the GS/GOGAT pathway could operate even at low nitrogen levels (Tian *et al.*, 2010). Since both GS/GOTAT and NADP-GDH contribute to N assimilation in ectomycorrhizal fungi, more

direct measurements in AMF would be desirable to determine if and when NADP-GDH might be involved in the AM symbiosis, however the apparent absence of an NADP-dependent GDH in the sequence of the *Glomus* genome (Tisserant *et al*., 2013) and in transcript analyses so far (Tisserant *et al*., 2012& 2013).

Genes for all the reactions of the urea cycle have been identified in *G. intraradices*. Enzymatic activities associated with the synthesis of Arg, are up-regulated in the ERM after ammonium addition (Cruz *et al.*, 2007). Soon after this, fungal genes for breaking down Arg, including arginase (*GiCAR1*), urease (*GiURE*), and ornithine aminotransferase (*GiOAT1, 2*) are up-regulated in the colonized root tissues (Tian *et al*., 2010). Enzymatic activity of glutamine synthetase, arginase and urease were shown to be up-regulated in the colonized root in response to N addition to the ERM (Cruz *et al.,* 2007), and later gene expression analyses (Tian *et al*., 2010, Tisserant *et al.,* 2012) indicate that this activity is fungal.

The interface between fungal arbuscules and plant cortical cells is important for P transfer from the fungus to the plant (Harrison, 1999; Pumplin & Harrison, 2009**).** Localized gene expression analysis indicates that the periarbuscular membrane is also important for ammonium transfer from the fungus to the plant cells in arbuscular mycorrhiza (Javelle *et al*., 2003; Gomez *et al*., 2009). From *Lotus japonicus*, a mycorrhiza-inducible ammonium transporter *LjAMT2;2*, which is found located in the apoplastic interfacial compartment, was suggested to bind charged ammonium and release uncharged NH_3 into the plant cytoplasm (Guether *at al*., 2009). Furthermore, another mycorrhiza-inducible ammonium transporters *GmAMT4.1* from *Glycine max*

was also found to be located in the branch domain of periarbuscular membranes (Kobae *et al*., 2010).

What is the role of AM networks in the uptake and exchange of nitrogen among plants in ecosystems?

Movement of N from soil to plants via AMF in plant ecosystems

The discovery of an elaborate system for moving N from soil to plant is complemented by demonstrations under controlled experimental conditions of large N fluxes through the symbiosis. In different experiments, at least 21%, 30% and 50 % of the total N present in AM roots came from the fungal ERM in an *in vitro* mycorrhiza system where mycorrhizal roots had access to N both by direct uptake and via the fungus (Toussaint *et al.*, 2004). Tanaka and Yano (2005) found that 75 % of N measured in the leaves of mycorrhizal maize was taken up by the AM fungus' ERM. AMF can also obtain nitrogen from organic matter from the soil and transfer it to host plants in significant quantities (Leigh *et al.*, 2009; Hodge and Fitter, 2001; 2010).

However, by comparison with P, there are very few reports that directly demonstrate N-dependent enhancement of plant growth by AM colonization due to N transfer from the fungus. In a study by Tanaka and Yano (2005) divided compartments in which nitrogen was available in a compartment to which only the ERM had access were used. The presence or absence of an air gap to prevent diffusion between compartments significantly increased nitrogen contents in host plants when ammonium was used but did not significantly increase plant biomass. The authors concluded from this and 15N labeling results that the improved plant N content was due to direct N

transfer by the fungus from soil to plant. However the apparent absence of N transfer when nitrate was the form of N supplied, contradicts previous and subsequent reports (Jakobsen 1992, Johansen et al 1993, Tobar et al 1995, Jin et al 2005, this study) raising uncertainties about the experimental system used. Although the N contents of colonized plants have been observed to be higher than un-colonized plants in a range of other studies (reviewed in He *et al*., 2003; 2009), these have not distinguished N transfer by the fungus from increased uptake by the plant secondary to improved P status or other changes in the host and/or soil. Indeed other studies have indicated that the transfer of N does not always confer a net growth benefit to host plants. For example Reynolds *et al*. (2005) found that even at low N supply, AM colonization did not increase total N uptake.

The extent and significance of N movement through the AM symbiosis under natural conditions is even less clear - partly because of methodological difficulties. Recently, the finding that mycorrhizal fungi discriminate against ^{15}N during nitrogen transfer from soil to host plant has shown a potential application to track N flow in N-limited ecosystems. The discrimination against ^{15}N by mycorrhizal fungi during nitrogen transfer will result in relatively enriched ^{14}N in plants rather than ^{15}N, while enriched ^{15}N rather than ^{14}N in mycorrhizal fungi. Accordingly, the lower ^{15}N:^{14}N in mycorrhizal plants compared with non-mycorrhizal plants suggests the contribution of N transferred from fungi to host plants (Hobbie & Macko, 2000; Hobbie & Colpaert, 2003; Hobbie *et al*., 2005). In addition, N transfer from fungi to host plants could be quantified by using ^{15}N:^{14}N values for host plants, mycorrhizal fungi, and soil. However, even though this approach has proved applicable for evaluating nitrogen transfer from fungus to ecto-

mycorrhizal plants in N-limited ecosystems (Hobbie & Colpaert, 2003; Hobbie *et al*., 2005; Hobbie & Hobbie, 2008), no reliable estimates of the contribution of AMF to plant N contents have yet been made using this approach. This is partly due to uncertainties of interpretation of such analyses and partly because the ^{15}N abundance in AM plants is closer to background than in nitrogen fixing or ectomycorrhizal symbioses. Further improvements for the analysis and interpretation of isotopic discrimination results in model AM systems and natural settings may enable estimates of the extent of N movement from AMF to plants in a range of ecosystem types.

Colonization by AMF depends on the nutrient status of host plants

Indirect evidence for AMF having a role in N uptake under natural conditions comes from the effect of soil N levels on colonization and growth of AMF. As reported by Bago et al. (2004), the AM fungal morphological and developmental changes subjected to different nutritional conditions, especially nitrogen status, as a strategy to exploit the substrate efficiently. Low nutrient status including low nitrogen in the soil induce the development and growth of AMF (Yoneyama *et al*., 2007), and AM colonization rates have been shown to be related to N availability in natural and disturbed ecosystems (Egerton-Warburton & Allen, 2000; Jackson *et al*., 2001; Jia *et al*., 2004; Blanke *et al.*, 2005). For example, Blanke *et al.* (2005) reported a negative relationship between percentage root colonization by AMF and both tissue N concentration and N:P ratio in *Artemisia vulgaris* growing in high P soils and Johnson *et al*., (2003) found that N fertilization lowered AM colonization in grassland sites with low N:P but not at sites with low P. This inverse relationship between AM colonization rates and nutrient levels is well documented for P in the AM symbiosis and is also consistent

with the finding that development of N_2-fixing nodules is reduced when N levels in the soil are high (**Streeter**, 1985). This similarity is consistent with a beneficial role for AMF when N is limiting. Although significant, the effects of N deposition on colonization are generally not so marked as for elevated CO_2 or P addition (Constable *et al*., 2001; Treseder 2004; Gamper *et al.*, 2005).

In an *in vitro* mycorrhizal symbiosis, Olsson et al. (2005; 2010) found that increased availability of N or P to host roots reduced carbon allocation to the fungus and concluded that negative impacts of N high nutrient level on AM abundance are caused by reduced C allocation from the plant when plant requirements are met by direct uptake. However no colonization rates were reported in that work and it should be followed up in more representative mycorrhizas. Other evidence suggests that the effects of soil N levels on the colonization of AMF could result in part from the change of fungal morphology which is influenced by the form and availability of inorganic nitrogen (Bago *et al*., 1996). Furthermore both root exudates and the growth and metabolism of rhizospheric microorganisms which influence AM colonization are also likely to vary with soil N (Gryndler *et al.*, 2009). Thus colonization rates may be due to both direct and indirect effects on the plant and fungal partners making it unclear whether the correlation between colonization and N availability is related to a beneficial role for AMF in plant N nutrition. Direct investigation of the importance of plant N status on the regulation of the symbiosis through analyses of exudate composition and plant defense gene expression in response to AMF would be valuable. More recently the turnover rate of arbuscules, which is accelerated in plants lacking the AM-specific P transporter, was

shown to return to normal when N levels were limiting to plant growth (Javot *et al.,* 2011)

Thus isotopic discrimination analyses, the effects of soil N levels on colonization, and the increased N contents of AM plants all point towards a significant role for the symbiosis in N uptake by plants. However none of these lines of evidence is sufficient to demonstrate unambiguously that N movement through the symbiosis provides a direct growth benefit to host plants, especially under natural conditions. It is interesting in this context that work on P nutrition has shown that a large fraction of plant P can be taken up through AM fungal partners, even in cases when there is no plant growth enhancement or even net increase in P uptake (Smith *et al*., 2003). This may be the case for N more frequently than for P because the higher mobility of nitrate than phosphate generally gives both partners access to soil N, where P depletion zones can more easily restrict direct access to P by roots.

N exchange between plants through mycorrhizal networks and its ecological significance

Transfer of N among different plants via AMF has been reported in experimental systems indicating that AMF can play an important role in N transfer between plants, especially from legumes to non-legumes (Bethlenfalvay *et al.,* 1991; Frey & Schuepp, 1993; Johansen & Jensen, 1996). Recent work using ^{15}N labeling and natural abundance measurements has reported modest nitrogen transfer between plants via common arbuscular mycorrhizal networks. For example Jalonen *et al*. (2009) deduced that 2.5% of the total N of grass was transferred by the common mycorrhizal network

from neighboring leguminous trees. Such studies (reviewed by He *et al*., 2003; 2009), have resulted in estimates of nitrogen transfer between plants through mycorrhizal networks, (especially from N fixing legumes to non-legumes) that range widely, between 0% and 80% of N in recipient plants coming from donor plants. AMF probably also influence the allocation of N at the community level through effects on rhizospheric functioning; plant growth, uptake and release of N; sequestration of N in soil organic matter (Rillig *et al*., 2001); soil structure, affecting N mobility (Rillig, 2004; Wilson *et al.*, 2009); as well as transport through common mycorrhizal networks. The relative importance of many of these factors in natural, disturbed and agricultural settings has been reviewed by He *et al*. (2003). Because of the challenges of assigning the relative importance of different mechanisms and the wide variation among findings no clear consensus has yet emerged about the occurrence or significant direct plant-to-plant N transfer via AMF in natural settings.

Interactions between AMF and symbiotic N_2-fixing microbes and their contribution to N accumulation in host plants

Nitrogen fixing bacteria colonizing mycorrhizal plants play important roles in N metabolism and movement in the symbiosis (Spriggs and Dakora, 2009). **Figure 1-2** illustrates the network of nitrogen movement and recycling in ecosystems from a mycorrhizal perspective. It has been found that the N_2-fixing activity of the bacteria can be improved when they are inoculated together with AMF because of the more available nutrients for bacteria by AMF in mycorrhizal than non-mycorrhizal plants (Barea *et al*., 1980; 2005). Indeed, AMF have been found in root nodules (Scheublin *et al*., 2004)

making it possible that nutrients are transferred between the symbionts without being translocated within the plant vasculature.

Nitrogen transferred from symbionts to plants is believed to be predominantly in the form of ammonium in both N_2-fixing bacteria and AMF (Day *et al.*, 2001; Rosendahl *et al.*, 2001; Govindarajulu et al., 2005, Jin et al 2005), although uncertain amounts of Alanine and/or other amino acids can also be exported by bacteroids (Waters *et al*., 1998). Photosynthate in the form of sucrose is the major form of carbon translocated from source leaves to symbiotic roots, although neither AMF nor N-fixing bacteria utilize this directly. Sucrose synthase seems to be involved in making C available to the micro-symbiont in both arbuscular mycorrhizas and nodules - being activated in both tissues (Hohnjec *et al.*, 2003) - although the AM IRM takes up hexose (Shachar-Hill *et al.,* 1995, Solaiman and Saito, 1997) whereas bacteroids take up organic acids produced by their host cells. Thus N and C exchange with host plants by the two symbioses have common metabolic intermediates, making it tempting to speculate about the relative costs for plants of obtaining N from one or the other. The metabolic cost of converting N_2 to ammonium is one and a half reducing equivalents and eight ATP's per N atom versus four reducing equivalents for nitrate reduction and none for ammonium taken up directly by AMF. Adding the costs of assimilating and then releasing ammonium in AMF in the pathway described earlier and estimating the additional costs of long distance translocation, appear to make N acquisition via nodules significantly less expensive than via arbuscular mycorrhizas. However, a complete accounting should include the respective costs of development and maintenance of the two symbioses, which is much harder to do (Leake *et al*., 2004; Kaschuk *et al*., 2009).

Conclusions and perspectives

The transformation of inorganic nitrogen taken up from the soil into organic form by the fungus and its translocation within the mycelium as arginine and subsequent conversion to ammonium followed by the release of this inorganic N in colonized root tissues seems to constitute the main pathway of nitrogen movement through the AM symbiosis. Many of the molecular mechanisms involved in this pathway have been identified in recent years lending support to the current model and providing detailed information on genes and proteins involved. Future work in this area may be expected to address significant remaining questions about the regulation, undetermined components of the metabolism and transport machinery, and possible additional routes of N movement in both plant and fungal partners. The work of this thesis contributes to this effort.

AM fungi can increase the uptake of N by host plants from the soil under natural and perturbed conditions and transfer from AMF can account for significant proportions of N in plants in controlled model systems. Plant growth may be improved by this N transfer although the extent to which this N-transfer-dependent growth enhancement occurs outside the laboratory or greenhouse is unclear. Natural abundance isotope fractionation studies indicate that some N is acquired by plants from AMF and it is to be hoped that experts in this methodology will be able to obtain quantitative estimates of N transfer. The effect of elevated soil N levels on suppressing AM colonization rates is also indicative of a beneficial role for the symbiosis in plant N acquisition. This implies control by the plant host through C allocation, for which there is some support or via altered defense and/or signaling mechanisms which have not apparently been explored.

The AM symbiosis can increase the exchange of N between plants and common AM mycelial networks have been shown to contribute to this flow. The extent of this AM mediated exchange seems to be quite variable and its ecological implications in natural, agricultural and disturbed systems remain to be determined. More detailed analyses that go beyond demonstrating exchange to quantify net transfer would be valuable in this context. The interaction between AMF and N_2-fixing bacteria can increase both N fixation and colonization by AM fungi (Stancheva *et al*., 2008), and the interactions in the tripartite association may play a role in the N cycle.

The uptake of N through AMF from the soil to the host plants and the exchange of N between plants via the mycorrhizal networks have potential implications for the application of AMF in sustainable agriculture. Horticultural and on-farm experiments have shown that substantial benefits in yields can be realized from inoculation with AMF (Johansson *et al*. 2004, Artursson *et al.*, 2006). The extent to which N transfer is important in these cases has been little studied, and it is generally believed that P movement is more important. It may be that more attention to this question would increase the range of settings where AMF are usefully applied. The interaction of AMF and beneficial soil bacteria (both N fixing and non-N fixing) contributes to plant fitness and soil quality, which has been argued to be important for a sustainable agricultural development and ecosystems (Jeffries *et al*., 2003). Exploration of different combinations of AMF and bacterial inocula in greenhouse and on-farm experiments show promise for increased productivity (Zaidi *et al*., 2003). Optimal combinations will depend on the particular soil, crop, and cultivation methods (Jeffries *et al*., 2003) and

finding them is likely to benefit from investment in systematically identifying AMF strains in a wide range of different environments.

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Figure Legends and Figures

Figure 1-1. Working model of N transport and metabolism in the AM symbiosis. Inorganic N and amino acids are taken up by the fungal ERM, the nitrogen is then incorporated into Arg in the urea cycle which is translocated to the fungal IRM in colonized root tissues. Arg is broken down to release ammonium which is exported from the fungus and imported by the host into the root cortical cells. In addition, other forms of nitrogen may be transferred from the ERM to the IRM and transferred to or exchanged with the host. **Red circles** refer to genes identified and measured at the transcriptional level in mycorrhizal fungus (*GmosAAP1* (Cappellazzo *et al*., 2008), *GintAMT1* (López-Pedrosa *et al*., 2006)**,** *NT*, *GS1*, *GS2*, *GluS*, *CPS*, *ASS*, *AL*, *OAT1*, *OAT2*, *ODC*, *CAR1* and *URE* (Tian *et al*., 2010)) and host plant (*LjAMT2;2* (Guether *et al*., 2009) and *DcGS* (Higashi *et al*., 1998)); **Red circles with orange rectangle** refer to enzymatic activities detected in ERM and colonized root tissues (Cruz *et al.*, 2007); **Purple arrows** refer to the pathway steps whose activity has been supported by evidence from labeling studies; **Black solid lines** refer to steps in the pathway with published experimental support **black dashed lines** refer to possible additional processes. **Blue dashed arrows** refer to possible regulation of gene expression by N metabolites (Tian *et al*., 2010); The question marks represent the uncertain fate of Glu and Putrescine produced in IRM part.

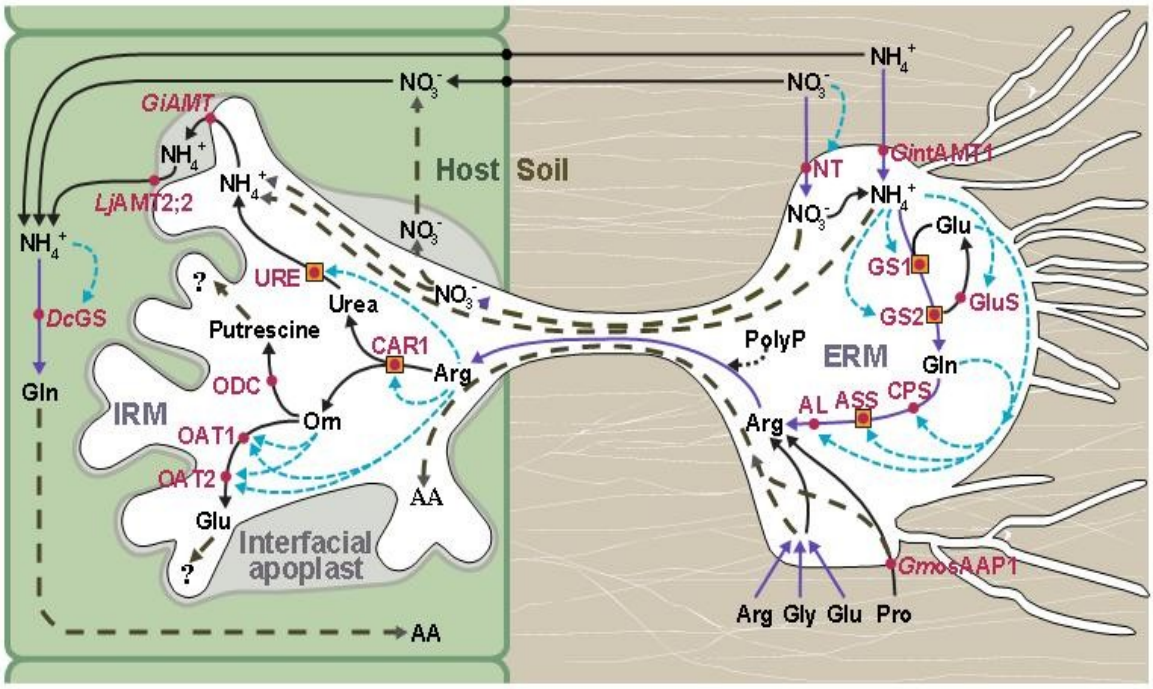

Host
Soil
GiAMT
LjAMT2;2
DcGS
Gln
URE
Urea
Putrescine
CAR1
Arg
ODC
Om
OAT1
OAT2
Glu
IRM
Interfacial apoplast
AA
NT
GintAMT1
GS1
GS2
Glu
GluS
Gln
PolyP
ERM
AL
ASS
CPS
Arg
GmosAAP1
Arg Gly Glu Pro

Figure 1-2. Interactions among arbuscular mycorrhizal fungi (AMF), host plants, and N_2-fixing bacteria and other soil microbes in nitrogen cycling. AMF take up inorganic nitrogen from the soil through the ERM, assimilate and translocate it into colonized roots where it is transferred to the host. Nitrogen fixed by symbiotic N_2-fixing bacteria in root nodules and nitrogen taken up directly by the plant root itself from the soil also contribute to plant nitrogen nutrition. N_2-fixing and non-N_2-fixing plants are connected by common mycorrhizal networks, allowing exchange and net movement of N between plants.

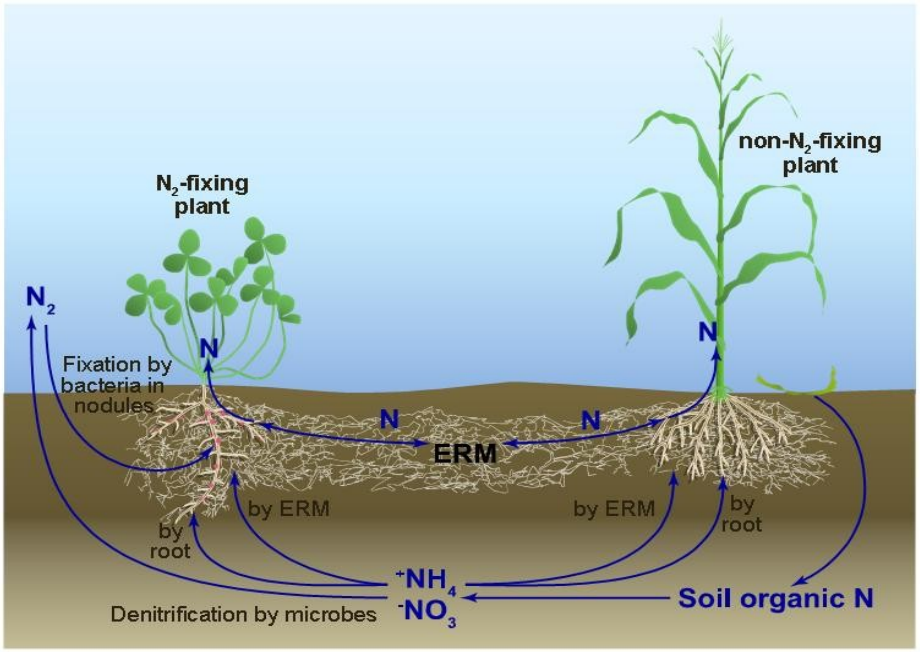

non-N2-fixing plant
N2-fixing plant
N2
N
Fixation by bacteria in nodules
N
N
ERM
N
by ERM
by ERM
by root
by root
+NH4
-NO3
Denitrification by microbes
Soil organic N

Table 1-1. Nitrogen metabolic genes identified in the arbuscular mycorrhizal symbiosis.

Genes reported	Potential function for N transfer in the AM symbiosis
Fungal Amino acid permease (*GmosAAP1* from *G. mosseae*)	Amino acid acquisition by fungus from the soil. Cappellazzo *et al.*, (2008) characterized a permease that facilitates the uptake of proline and is induced in the presence of several N sources.
Fungal Nitrate transporter (*GiNT* from *G. intraradices*)	Nitrate uptake from the soil. Transcript levels upregulated by nitrate addition to the ERM (Tian *et al*., 2010).
Fungal Ammonium transporters (*GiAMT* and *GintAMT1* from *G. intraradices*)	Ammonium uptake from soil and transfer to host. Two fungal AMT ammonium transporters reported: one is expressed more highly in the IRM (*GiAMT*, Govindarajulu *et al*., 2005) the other (*GiAMT1*, López-Pedrosa *et al*., 2006) is induced in the ERM in response to NH_4^+.
Plant Ammonium Transporters (*MtAMT* from *M. truncatula*; *LjAMT2;2* from *Lotus japonicus*; *GmAMT4.1* from *Glycine max*)	Ammonium/ammonia uptake by plant from the host/fungus interface. Plant AMT transporters are induced in mycorrhizal roots of three species (*MtAMT*, Gomez *et al*., 2009; *LjAMT2;*2, Guether *et al*., 2009; *GmAMT4.1*, Kobae *et al*., 2010). *LjAMT2;*2 and *GmAMT4.1* were localized to the periarbuscular membrane of arbusculated cortical cells.
Fungal (*G. intraradices*) and Plant (*Zea Mays*) Nitrate Reductases.	Nitrate assimilation in mycorrhizal roots. The mRNA level of maize NR was lower in roots and shoots of mycorrhizal plants than in noncolonized controls, and fungal NR transcripts were localized in the IRM.

	Suggests that the fungal nitrate reductase assimilated nitrate in AM roots (Kaldorf *et al*., 1998).
Fungal Glutamine synthetase (*GmGln1* from *G. mosseae*; *GiGln1* (same as *GiGS1*) and *GiGS2* from *G. intraradices*) and glutamate synthase (*GiGluS* from *G. intraradices*)	N assimilation in the ERM. Breuninger *et al.* (2004) identified GS homologs (*GiGln1* and *GmGln1*) in two fungal species expressed in all AM tissues. Activity was elevated after N addition to the ERM. Govindarajulu *et al*. (2005) reported induction of the G intraradices gene (*GiGS1*) in the ERM in response to N. Gomez et al. (2009) reported the expression of *GiGS1* in mycorrhizal roots. Tian et al. (2010) found a second *GS* (*GiGS2*) in *G. intraradices* and showed that the two GiGS genes are differently upregulated by N addition to the ERM and have different kinetic properties. A GOGAT gene (*GiGluS*) was upregulated in the ERM (Tian *et al*., 2010).
Plant (*Daucus carota*) Glutamine synthetase genes	Assimilation by plant of fungal-derived N. One of three *D. carota GS*'s reported by Higashi et al. (1998) is upregulated following the import of N into mycorrhizal roots by the fungus (Tian *et al*., 2010).
Fungal Arginine synthesis: Carbamoyl-phosphate synthase glutamine chain (*GiCPS*); Argininosuccinate synthase (*GiASS*); Arginosuccinate lyase (GiAL); (from *G.*	Arginine synthesis in the ERM. Gomez et al. (2009) reported the expression of *GiASS* in mycorrhizal roots. Tian et al. (2010) showed that these arginine synthesis genes are induced in the ERM but not in the IRM after N addition to the ERM.

intraradices)	
Fungal Arginine breakdown Arginase (*GiCAR1*); Urease accessory protein (*GiUAP*); Urease (*GiURE*); Ornithine aminotransferases (*GiOAT1* and *GiOAT2*); Ornithine decarboxylase (*GiODC*); (from *G. intraradices*)	N release in the IRM. Govindarajulu *et al*., (2005) reported higher expression of *GiUAP* in the IRM than the ERM. Gomez et al. (2009) showed transcription of *G. intraradices* arginase, and GiOAT1 in cortical cells of mycorrhizal *M. truncatula*. Tian et al. (2010) reported the upregulation of *GiCAR1*, *GiURE*, *GiOAT1*, *GiOAT2* and *GiODC* in the IRM but not ERM after N addition to the ERM.

Chapter 2

Nitrogen uptake and transfer in aseptic whole plant-mycorrhizal culture system

Abstract

Arbuscular mycorrhizal (AM) fungi can increase the uptake of N by their host plants and play a significant role in the transformation and movement of N in plant communities. It is widely assumed that N transfer to host plant roots, which has been observed in tracer experiments, can improve host plant growth, but no unambiguous

demonstration of this has been reported. To test whether N uptake via an AM fungus can enhance plant growth and fitness, a compartmented microcosm was developed for aseptic and leakage free whole-plant mycorrhizal experiments. This was used to monitor S uptake by the fungal extraradical mycelium (ERM) and its transfer to host plants. Our results indicated fast S transfer by ERM to the host plants as indicated by high ^{35}S DPM in roots and shoots. To assess the contribution of the AM fungal mycelium to plant nitrogen nutrition, N was added to the fungal compartment of mycorrhizal plants. Controls were used to ensure that N transfer was responsible for any benefits (mycorrhizal plants with P but without N addition to the fungal compartment) and that transfer was entirely via the fungus (non-mycorrhizal plants with N added to the empty compartment). Phenotypical characteristics, chlorophyll contents, and ^{15}N labeling were used to investigate N transfer fro the fungus and nutritional benefits to the plant. Mycorrhizal plants with N available to the fungal ERM had higher weight, longer shoot length, higher number of fruits as well as higher chlorophyll content than the controls which showed N deficiency symptoms. Furthermore, mycorrhizal plants with N had high levels of, and fractional ^{15}N labeling in, N metabolites (glutamine, glutamate, pyrroline-5-carboxylate, ornithine and arginine) in both roots and shoots while the control plants showed no labeling and low level of those metabolites. We conclude that Nitrogen transfer from an arbuscular mycorrhizal fungus confers growth benefits on the host plant under nitrogen limiting conditions and that the microcosm system developed will be useful for future work on AM nutrition and metabolism under physiologically relevant conditions.

Introduction:

Plant-microbial mutualistic associations play an important role in global nutrient cycles as well as in the ecology and physiology of plants (Read and Perez-Moreno, 2003). Plants growth depends on mineral nutrients whose availability frequently limits plant growth and fitness. Phosphorous and nitrogen availability most commonly limits plant growth in ecosystems (Reich *et al*., 2006). Hayman and

Mosse, (1971) first documented that the arbuscular mycorrhizal (AM) symbiosis can increase plant growth in P-deficient soils dramatically.

Plants gain several benefits from arbuscular mycorrhizal fungi, for example AMF take up and transfer to their host nutrients including phosphorus, nitrogen (Govindarajulu et al., 2005), sulfur (Allen and Shachar-Hill, 2009), and zinc (Clark & Zeto, 2000), improve drought resistance (George *et al*., 1992), protection from pathogens has also been demonstrated (Newsham *et al*., 1995). In return plants translocate photosynthetically fixed carbon to the fungal symbiont (Smith and Read, 2008). While P is generally believed to be the most important nutrient taken up by plants via AM networks, other macronutrients can be moved in large amounts via AM fungi. Thus after it was shown that mycorrhizal colonization increased plant ^{35}S uptake compared to nonmycorrhizal plants (Rhodes and Gerdemann, 1978; Gray and Gerdemann, 1973), Allen and Shachar-Hill, (2009) reported that 50% of $^{35}SO_4^{2}$ uptake by the fungus was detected in the mycorrhizal roots of an in vitro mycorrhizal system. Similar quantities of ^{35}S were transferred to mycorrhizal roots whether $^{35}SO_4^{2-}$, [^{35}S]Cys, or [^{35}S]Met was supplied in the fungal compartment (Allen and Shachar-Hill, 2009).

The AM fungal hyphae take up N from the soil in different forms and transfer it to the plant, however nitrogen is mainly captured by the AM extraradical mycelium in inorganic form (i.e. as nitrate or ammonium) (Bago *et al.*, 1996; Hawkins et al., 2000; Azcon *et al*., 2001; Hodge *et al*., 2001; Vazquez *et al.,* 2001). Although nitrogen transfer from AM fungi to host plant has been demonstrated (Ames *et al.*, 1983; Johansen *et al.*, 1993; Hawkins *et al.,* 2000; Azcon *et al*., 2001; Hodge *et al*., 2001; Vazquez *et al.,* 2001) and a working model of the metabolism and transport processes has been proposed (Bago *et al*., 2001) and supported (Jin *et al*., 2005;

Cruz *et al*., 2007; Tian *et al.*, 2010), nitrogen transfer has never been rigorously demonstrated to confer a growth benefit. Indeed much of the work in recent years has been on the molecular mechanisms and on ecological aspects in the field. A monoxenic culture system consisting of transformed roots in symbiosis with the mycorrhizal fungus *Glomus intraradices* has been used to study the transfer and metabolism of nitrogen (Govindarajulu *et al*., 2005; Jin *et al*., 2005; Tian *et al.,* 2010). This transformed root system was used because it is aseptic and provides easy access to the ERM. However, it is not a whole plant system, which casts doubt on the relevance of the plant metabolic results obtained. Unlike phosphate, which is immobile in soils (Gahoonia and Nielsen, 1991) inorganic nitrogen is mobile in most soils which makes it hard to determine the role of nitrogen transfer via AM fungi on plant growth and reproduction.

In order to test whether nitrogen transfer confers a nutritional benefit in plants, an aseptic and leakage –free system is required which prevents diffusion between compartments and which minimizes physical restrictions on plant growth that are usually seen in closed systems. Such a system should allow rapid and efficient fungal mycelial growth into a fungal compartment separated from the growth zone of the colonized plant roots. This is important so that nutrients can be made available to the fungal extraradical mycelia (ERM) without the plant having access to them other than the fungus.

Previous studies reported compartmented pot systems based on the same concept of having a root and fungal compartments (Ames *et al*., 1983; Frey and Schuepp, 1992; Ma¨der *et al*., 1993; Schweiger and Jakobsen, 2000; Smith *et al*., 2000; Jansa *et al.,* 2003; Smith *et al.*, 2003). Several researchers have used different variants of the pot system to demonstrate nutrient transfer in arbuscular

mycorrhizal symbionts; Johansen *et al., (*1993*)* used containers divided by a fine nylon mesh into a root compartment (RC) and a root-free hyphal compartment (HC) to demonstrate phosphorous nutrient transfer between Subterranean clover (*Trifolium subterraneum* L. cv. Nuba) and *Glomus intraradices.* Nylon mesh was used also in a container system to separate the main soil compartment from the fungal compartment in experiments studying P acquisition by two AM fungi and its transfer to host plants (Smith et al., 2000). Hodge et al., (2001) found that the arbuscular mycorrhizal symbiosis can enhance the decomposition of, and increase nitrogen capture from, complex organic material in soil. They grew plants of *Plantago lanceolata* inoculated with the mycorrhizal fungus *Glomus hoi* in microcosms in which the compartments were separated by a double layer of 20-um mesh, which was permeable to hyphae but not roots. In studying nitrogen and phosphorous transfer and regulation, Fellbaum *et al.,* (2014) used a double membrane with an air gap (two sheets of 50-µm nylon mesh with a wire spiral between then) to prevent the diffusion of nutrients from the fungal compartment (FC) to the root compartment (RC), but allow fungal hyphae to cross from the RCs into the FCs has been used.

All of these system have some drawbacks such as lack of control over the presence and growth of other microorganisms which could influence element bio-availability, especially when dealing with organic compounds, and whose levels and behavior is likely to be influenced by the fungal ERM. In other systems without complete isolation via an uninterrupted air gap, diffusion of mobile nutrients or tracers allows direct uptake by the roots due to leakage into the root compartment caused by diffusion or mass-flow due to transpiration. Another system type was used by Dupre´ de Boulois *et al.* (2006), an arbuscular mycorrhizal–plant (AM–P) in vitro

culture system. In this system the root compartment and hyphal compartments consist of a bi-compartmented Petri plate and a shoot compartment consists of a 50 ml Falcon tube and a membrane filter fixed onto the shoot compartment to allow gas exchange. This system is aseptic but the drawbacks are media drying and substantial physical restrictions on plant growth. Our aim in this study was to overcome the limitations of previous systems by developing an aseptic, leakage-free system that allows longer and more physiologically relevant experiments in a re-usable, inexpensive microcosm of modest footprint, with separate reservoirs for supplying nutrients and water independently to the two compartments with sufficient capacity for multi-week long experiments.

Here we report such a system. This system was used to investigate growth parameters, chlorophyll content and nitrogen metabolite ^{15}N labeling and levels when N was made available to the fungal ERM at N levels that limited plant growth and reproduction. We observed that nitrogen taken up by the fungal extraradical mycelium and transferred to the plant accounts for much of the nitrogen entering the plant under N-limited conditions and confers growth and reproductive benefits.

Experimental procedures:

Chemicals and reagents

Gelzan, (MP biomedical, Solon, OH) was used for solidification of M media. Radioactive labeled sulfate was obtained as $Na_2\,^{35}SO_4$ fom MP biomedicals, (Solon, OH). 15N Labeled nitrate was obtained as K $^{15}NO_3$ from (MP biomedicals, (Solon, OH)).

Seed Sterilization

M. truncatula seeds were scarified with enough concentrated sulfuric acid to cover for 5-10 minutes. After that, seeds were rinsed with sterile water 4-5 times. Surface sterilization was done in concentrated Clorox for 2 minutes . then seeds were rinsed 8 times.

Growth condition

Seeds were germinated at 22.5 °C for 48 hours on the M-media (Fortin *et al*., 2002) after cold treatment at 4 °C for 36 hours. After that, *M. truncatula* seedlings and old fragments of *Daucus carrota* roots (Ri T-DNA transformed) colonized with *Rhizophagus irregularis* were transfer to root compartments filled with bacto:perlite (2:1) soils in the two-compartment system (Figure 2-1). On the other hand, the fungal compartment was fill with medium grain sand. This system was constructed using 50µm woven double mesh glued to metal frame with air gap to separate the two compartments and so it was preventing the crossing of roots, but allowed fungal crossover into the fungal compartment (RCs). The lower compartments were used as reservoir to supply both compartments with 0.5x Hoagland's solution (Hoagland and Arnon ,1950) . Linen Cotton rope wicks were used for capillary transfer of Hoagland's solution to both compartments.

The plants were grown in a growth chamber under the following conditions:

16 h photoperiod, 22.5°C, photosynthetically active radiation of 200 µmol m^{-2} s^{-1}, and 30% humidity.

Experimental design

Three experiments were conducted to demonstrate the role of arbuscular mycorrhizal in nutrition transfer. I used our whole plant two compartments system to test the transfer of $^{35}SO_4$ from AM fungi to the host plants. After growing plants for 5 weeks, plants were deprived from sulfur for a week then 100µci of $^{35}SO_4$ was added directly to the fungal compartment for a week. Plant leaves were sampled 1, 3, 5, 7 days after addition of $^{35}SO_4$. Then plant shoots and roots were collected to measure radioactivity. Two controls were used, mycorrhizal colonized plant with no $^{35}SO_4$ added and non-mycorrhhizal plant with $^{35}SO_4$ added to FC to test leakage. Five replicates was used for each.

The second experiment was conducted to demonstrate the nutritional benefit of arbuscular mycorrhizal colonization on N transfer. After growing plants for 5 weeks, plants were deprived from N for 4days then 10mM KNO_3 was added to the reservoir of fungal compartment for two weeks. Plant shoots and roots were collected for growth parameter and chlorophyll measurements. Two controls were used, mycorrhizal colonized plant with no N added and non-mycorrhizal plant with 10mM KNO_3 added to FC to test leakage. Ten replicates were used for each.

The third experiment was done to test the uptake and transfer of ^{15}N in the whole plant system. After growing plants for 5 weeks, plants were deprived from N for 4 days then 10mM K $^{15}NO_3$ was added to the reservoir of fungal compartment for two weeks. Plant shoots and roots were collected for N metabolite analysis. Two controls were used, mycorrhizal colonized plant with no ^{15}N added and non-mycorrhizal plant with 10mM K $^{15}NO_3$ added to FC reservoir to test leakage. Ten replicates were used for each.

Testing the diffusion between compartments and the capillary transfer

In order to make sure that there was no diffusion of nutrients from the FC into the RC. $^{35}SO_4$ was added to FC reservoir, Aliquots of the soil and the reservoir solution of the RC were collected (3, 5, 7 days after adding $^{35}SO_4$). Aliquots of the sand of FC were collected too to investigate the rate of nutrients transfer using Linen Cotton rope wicks. the ^{35}S content was extracted from all samples and measured by liquid scintillation counting.

Extraction and measurement of $^{35}SO_4$ from mycorrhizal plants

Plant leaves were sampled 1, 3, 5, 7 days after addition of $^{35}SO_4$. Also plant roots and shoots were sampled after 7 days . 100mg of the leaves of each time point as well as 100 mg of each plants roots and shoots were ground in a mortar and pestle with a pinch of acid washed sand and extracted three times with a mixture of cold methanol : water (70 : 20). then the solution were vortexed for 5 min. While keeping particulates suspended, a 1-mL aliquot of the solution was transferred to a microcentrifuge tube and centrifuged. Then 0.5 mL of supernatant solution was scintillation counted after adding to 5 mL of BioSafe II (MP Biomedicals) scintillation cocktail.

Analysis of mycorrhizal colonization and ERM crossing to the FC

Fraction colonization were checked for plants 1,2,3 and 4 weeks after transferring the seedlings with the spores to the system. 30 root fragments of each time point were stained with trypan blue and the percentage root length colonized by *Rhizophagus irregularis* was estimated using the gridline intersect method (Newman,

1966). In order to check crossing, ERM from the FCs of 5 week old plants were collected, cleared in KOH, and stained with Trypan blue.

Growth parameters and Chlorophyll content measurements

Experimental plants were collected two weeks after adding KNO_3 to the FC reservoir . the two control plants were collected. The plants were weighed and the length of the shoots were measured. The fruits were collected and counted. Leaves' Chlorophyll was extracted using 80% acetone and quantified according to Ni *et al*., (2009).

Extraction, isolation and quantification ^{15}N metabolites

300 mg of each plants roots and shoots were ground in a mortar and pestle with a pinch of acid washed sand and extracted three times with a mixture of methanol : chloroform : water (12 : 5 : 3, v/v/v). Methylene chloride and water were added to the extraction solution to facilitate the separation of chloroform and the methanol–water phases. The methanol–water phase containing the amino acids (AAs) was collected and evaporated in a rotary evaporator at 50°C, and the residues containing the AAs were dissolved in 1 ml of 0.01 M HCl and loaded onto a cation exchange column (0.3 ml of DOWEX 50 X8-200– hydrogen form; Sigma-Aldrich, St Louis, MO, USA), which was previously washed with 1 M NH4OH, deionized H2O and 1 M HCl, and followed by deionized H2O. The neutral compounds, principally carbohydrates washed off the column with 5 ml of water and the free were eluted with 5 ml of 1 M NH4OH (Bengtsson & Odham, 1979). This eluent was collected and dried then resuspended in 70 µl of milliq water. N metabolite levels and labeling were measured using Liquid chromatography (LC)-MS analyses. LC-MS was carried out using a Quattro micro (Waters) mass spectrometer system bundled with an

electrospray ionization source and a Shimadzu HPLC system. Isotopomers of glutamate, glutamine, pyrroline-5-carboxylate, ornithine, and arginine were separated using a Waters Symmetry C18 (100 × 2.1 mm, 3 μm) column with 1 mM perflurohetanoic acid in a water/acetonitrile gradient at ambient temperature and a flow rate at 0.3 mL min^{-1}.

Results:

To establish conditions for studying N transfer between the AM fungus and the host plants, a whole plant mycorrhizal two-compartment culture system was developed (Figure 2-1). The final model has three compartments; the top compartment allow room for shoot growth and is separated from the outside atmosphere using a 0.22uM pore size *easy-breathe* membrane across the entire top of the microcosm to allow

gas exchange, including water vapor, and to maintain the sterility of the system. The central compartment contains soil or other solid medium for root and fungal growth. This is divided into two; a soil and a sand sub-compartment separated by two layers of metal woven mesh (Dutch weave 40 µm exclusion size) that are glued with autoclavable epoxy to a metal frame. The divider is fixed vertically in the middle of the central compartment using autoclavable silicone rubber. This creates a robust air gap of ~1.5mm and a window area for hyphal penetration that spans most of the area of the divider. A seedling is inoculated with *Rhizophagus irregularis* spores and grown in the soil compartment. The woven mesh was found to allow the fungal ERM to cross from plant to fungal compartment at high frequency while preventing plant roots from doing so. By sticking two stiff mesh sheets to a metal frame to form the divider, the air gap consistently prevented diffusion of nutrients between compartments, which occurs for diffusible nutrients or tracers when single mesh barriers are used. To create air gaps, flexible double mesh barriers were tested with smaller area dividers to prevent the mesh sheets from touching. These had low hyphal connectivities between compartments. Using a course mesh as a spacer between the root-excluding mesh layers can facilitate diffusion between compartments by forming a continuous diffusion pathway. The design shown in figure 2-1 prevents leakage between compartments as indicated by the absence of $^{35}SO_4^{2-}$ in the plant compartment when this diffusible tracer was added to the distal compartment of non-mycorrhizal plants (Figure 2-2A). The third, lower compartment contains liquid reservoirs to supply the root and fungal compartments with water and nutrients via wicks. The reservoirs in the lower compartment compartment was separated by sticking a 50-100mL glass beaker to the floor using autoclavable silicone gel under the fungal compartment (FC) while the rest of the chamber was

used as a root compartment (RC) reservoir. In order to supply each compartment of nutrients from reservoirs, different kinds of ropes were tested as wicks. Some of them allowed to little capillary transfer capacity, resulting in water stress for the plants while other materials allowed too much transfer saturating the RC and FC and causing diffusional tracer leakage between compartments and excessive soil moisture that reduced plant growth. Cotton rope wicks of the clothes-line type were found to provide efficient but not excessive transfer of water and nutrients as indicated by rapid $^{35}SO_4^{2-}$ movement into the fungal (sand) compartment (Figure 2-2A) without leakage into the root compartment or soil water saturation. In order to eliminate the complications arising from the uptake and metabolism of nitrogen, carbon, or other tracers by other microorganisms, the maintenance of aseptic conditions over several weeks was required. By selection of appropriate materials for wicks dividers and adhesives, the microcosms can be autoclaved after assembly, with the *easy breath* membranes being added under sterile conditions (laminar flow hood). The sterility of the system was investigated using periodic sampling of all compartments and inoculation onto enrichment media (trypticase soy agar and potato dextrose agar) for bacteria and fungi. This culture system was found to be reproducibly aseptic with no growth of any microbes seen on the rich media throughout the time frame of the experiments.

The level of colonization per root length of *Medicago truncatula* plants (Figure 2-3) reached approximately 90% for internal hyphae and 62% for with arbuscules and vesicles in four weeks old plants. These high and reproducible colonization levels and establishment rate (most plants were substantially colonized within a week) allows observations on nutrient exchange between the fungal mycelium and the

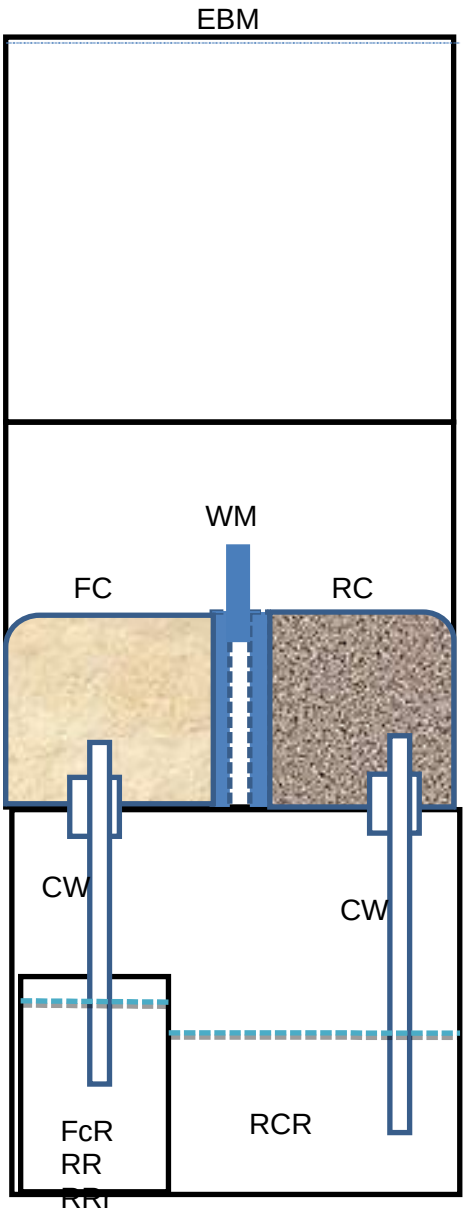
plant roots to be interpreted without uncertainties due to variable or slow colonization rates.

We used the culture system described above to monitor S uptake by the fungal extraradical mycelium and its transfer to host plants. By supplying $^{35}SO_4$ to FC, It was found that leaves had measurable ^{35}S counts after one day and reaching very high counts after 7 days (Figure 2-2B). Furthermore, ^{35}S DPM were measured in whole plant roots and shoots after 7 days and compared with two controls (mycorrhizal plants with no $^{35}SO_4$ added to the FC and non-mycorrhizal plants with $^{35}SO_4$ provided to the FC) (Figure 2-2C) . High ^{35}S DPM of roots and shoots were found. However, plant shoots had higher ^{35}S DPM than roots. The two controls' roots and shoots were showing no counts (comparable with the background counts) proving that this culture system had no leakage between compartments.

By adding nitrogen to the fungal compartment reservoir (FCR) of the mycorrhizal plants, it was found that nitrogen transfer confers growth benefit as plants were larger and greener with multiple stems compared with the other two controls (mycorrhizal plants with nutrients but no nitrogen added to the FCR and non-mycorrhizal plants with N provided to the FCR, see Figure 2-4). In the two controls, plants stopped growing and senesced. Shoots dried dry and had low numbers of leaves as many dehisced and most of the residual leaves were visibly chlorotic (a symptom of nitrogen deficiency, Figure 2-4). Chlorophyll contents of Mycorrhizal plants (2-5) with nitrogen supplied to the FCR was significantly higher than for plants from the two control treatments. In the two controls no nitrogen was available to the plant through the fungus and this presumably led to chlorophyll degradation, and/or reduced biosynthesis (Figure 2-5A) Mycorrhizal plants with nitrogen added to their fungal compartment reservoirs had significantly longer

shoots, higher biomasses and higher numbers of fruits compared with the two control treatments (Figure 2-5 B,C, and D). Mycorrhizal plants with nitrogen added to the fungal compartment reservoir had significantly higher levels of the soluble nitrogen-containing metabolites glutamate, glutamine, pyrroline-5- carboxylate, ornithine and arginine as well as high ^{15}N percentage labeling in both root and shoot tissues. This confirms that nitrogen transfer from the fungal compartment via the fungal mycelium drives substantial fluxes through N metabolism (Figure 2-6). The control plants showed low levels of N metabolite and no detectable 15N labeling, consistent with the ^{35}S results that indicated no significant nutrient movement between compartments not connected by fungal mycelium. Mycorrhizal plants with P and other nutrients but not N supplied to the FCR had similar N metabolite levels and growth parameters to non-mycorrhizal plants. Thus, the nutritional benefit to plants is due to direct fungal-mediated N transfer. The high percentage labeling of intermediary metabolites in shoots as well as roots demonstrates directly, in a way that the use of transformed roots cannot, that N transfer to mycorrhizal roots benefits the N status of the whole plant.

A



B

Figure 2-1 Whole plant-mycorrhizal two-compartment culture system. A) Diagrammatic representation of the culture system which is composed of two compartments; root (RC) and fungal compartments (FC). RC and FC are separated by double woven mesh (WM) glued to metal thus air gap is created between the two layers of mesh. Upper compartment is sealed with easy breathe membrane (EBM) and lower compartments are used as reservoirs; root compartment reservoir (RCR) and fungal compartment reservoir (FCR). Nutrients was transferred using using Linen Cotton wicks (CW). B) Four weeks old *Medicago truncatula plant growing in the system .*

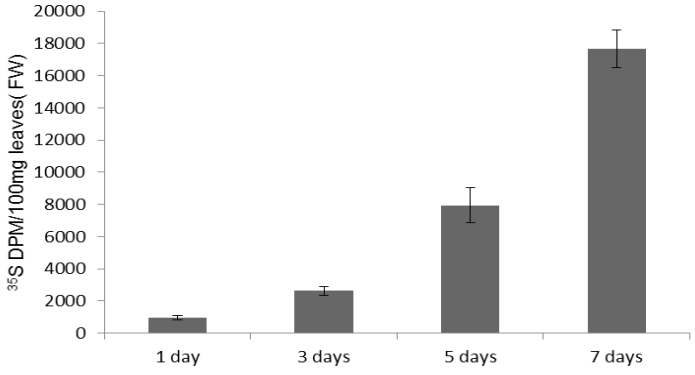
Figure 2-2 DPM of ^{35}S in the whole plant system A) DPM of ^{35}S in root compartment (RC) soil, reservoir (RCR) in the two-compartment system at the days 1, 3, 5, and 7 days after applying to applying $^{35}SO_4$ to fungal compartment reservoir (FCR) to investigate diffusion between compartments. DPM of $^{35}SO_4$ in fungal compartment (FC) sand were measured to investigate the rate of transfer of nutrients by the cotton wicks. The right panel is the same figure as the left but with lower maximum limits to show smaller values. . Means and standard error of means of three replicates.

B) DPM of ^{35}S in leaves at 1, 3, 5, and 7 days after applying $^{35}SO_4$ to Fungal compartment reservoir (FC). Means and standard error of means of five replicates.

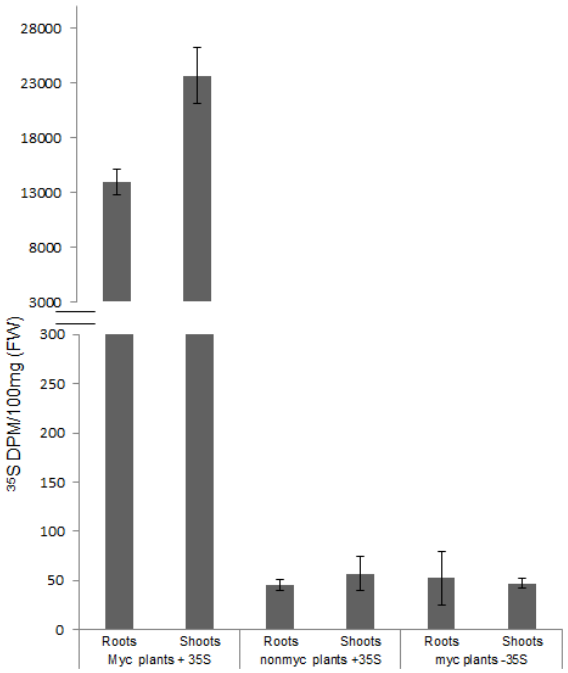
C) DPM of ^{35}S whole plant shoots and roots at 7 days after applying $^{35}SO_4$ to Fungal compartment. Means and standard error of means of five replicates.

A

B


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
35S DPM/100mg leaves(FW)
1 day
3 days
5 days
7 days

C


28000
23000
18000
13000
8000
3000
300
250
200
150
100
50
0
35S DPM/100mg (FW)
Roots
Shoots
Myc plants + 35S
Roots
Shoots
nonmyc plants +35S
Roots
Shoots
myc plants -35S

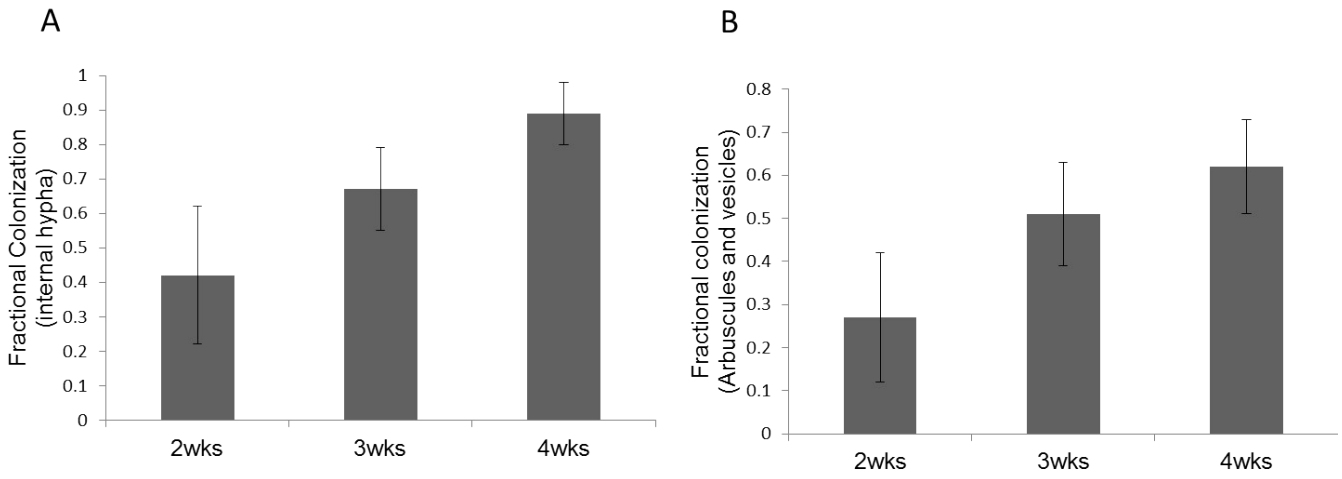


Figure 2-3 Colonization of *Medicago truncatula* by *Rhizophagus irregularis.* Plants were raised in the two-compartments system under low phosphate and in the presence of old *Daucus carota* colonized roots. Values are fraction of root length associated with (A) internal hyphae (B) arbuscules *and vesicles*. Means and standard error of means of three plants

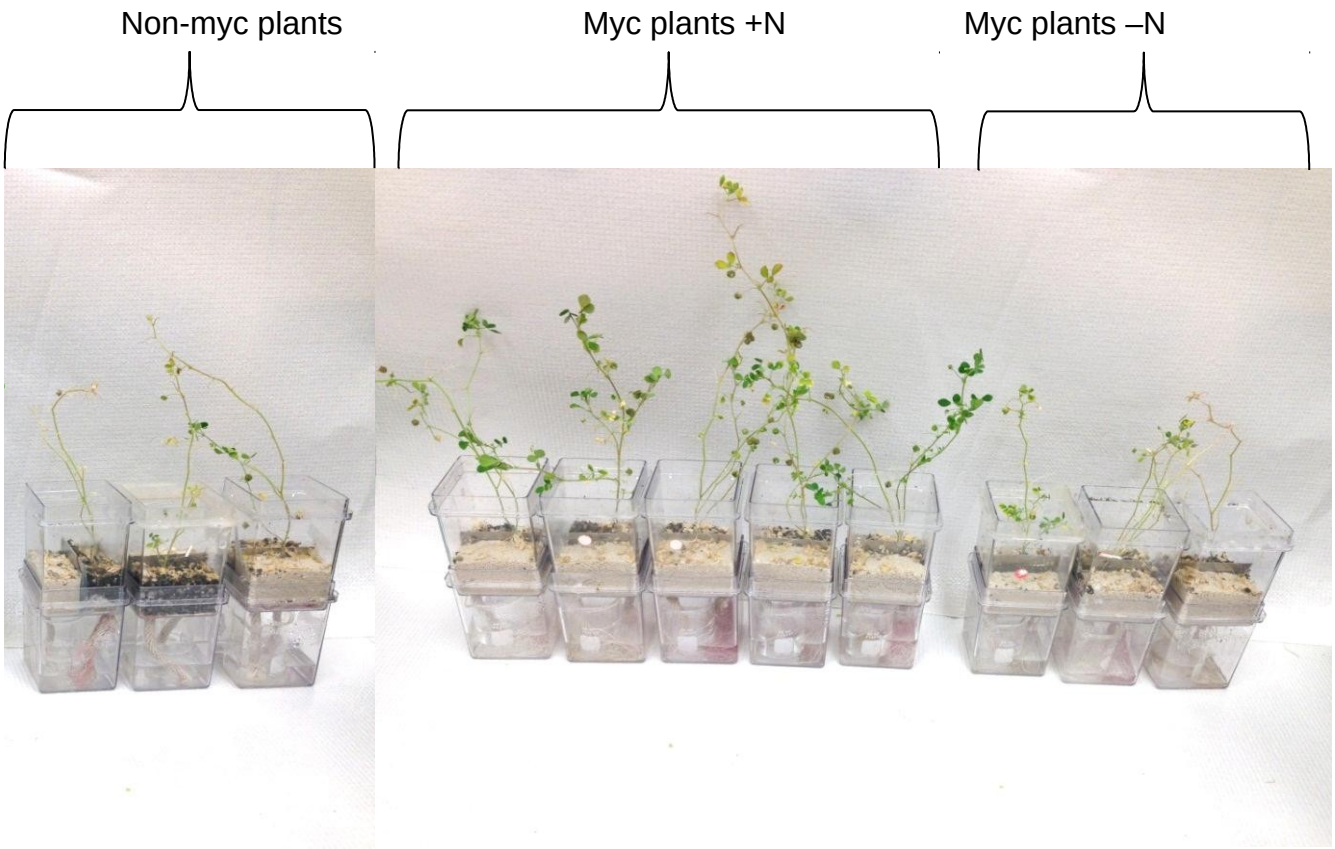


Figure 2-4 Seven weeks old *Medicago trancatula* plants growing in the two-compartment system. Mycorrhizal plants with KNO_3 (Myc plants +N) added to the fungal compartment reservoir for two weeks. Two controls were used; non-mycorrhizal (non-myc) plant with KNO_3 added to FCR to check for leakage and Mycorrhizal plants with no N added (Myc –N)

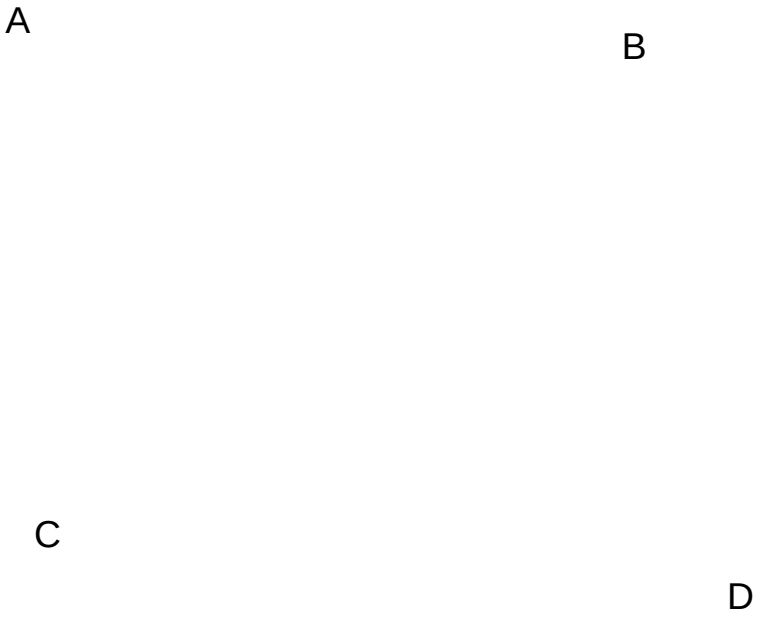


Figure 2-5 Total chlorophyll content (A) and growth parameters (number of fruits , fresh weight and shoot length) measurements(B, C, D) of myc plants +N and the two controls; non-mycorrhizal (non-myc) plant and mycorrhizal plants with no N added (Myc –N). Means and standard error of means of ten replicates

A

C

Figure 2-6 Free N metabolites' concentrations and ^{15}N labeling in the mycorrhizal plants with 10mM $K^{15}NO_3$ (Myc + ^{15}N) added to the fungal compartment reservoir (FCR) for two weeks. Two controls; non-mycorrhizal (non-myc) plant and mycorrhizal plants with no N added (Myc –N). N metabolites are glutamine (A), Glutamate (B), Pyrroline-5-carboxylate (C), ornithine (D) and arginine (E). Means and standard error of means of ten replicates

A

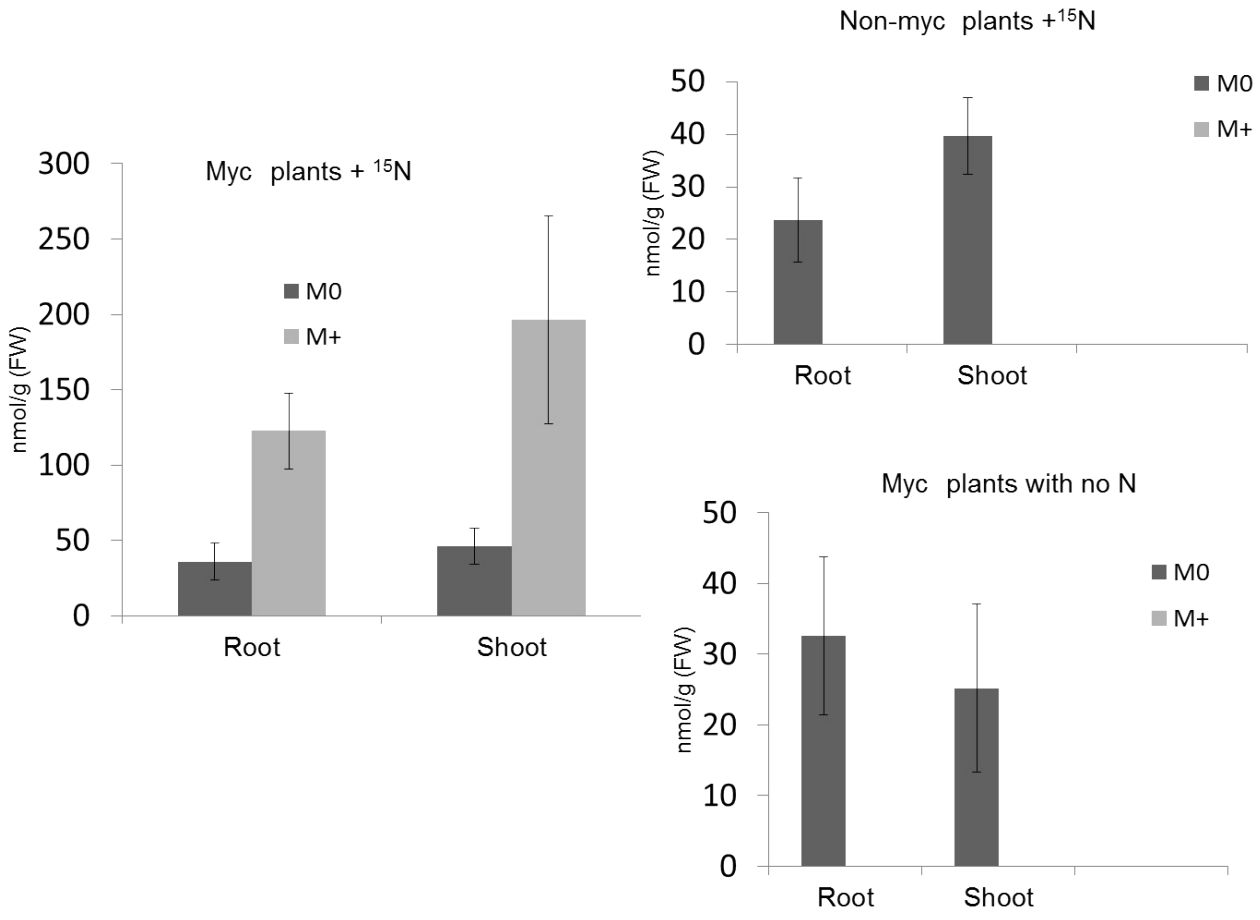

Glutamine
Myc plants + 15N
nmol/g (FW)
300
250
200
150
100
50
0
M0
M+
Root
Shoot
Non-myc plants +15N
50
40
30
20
10
0
Myc plants with no N

Figure 2-6 (cont'd)

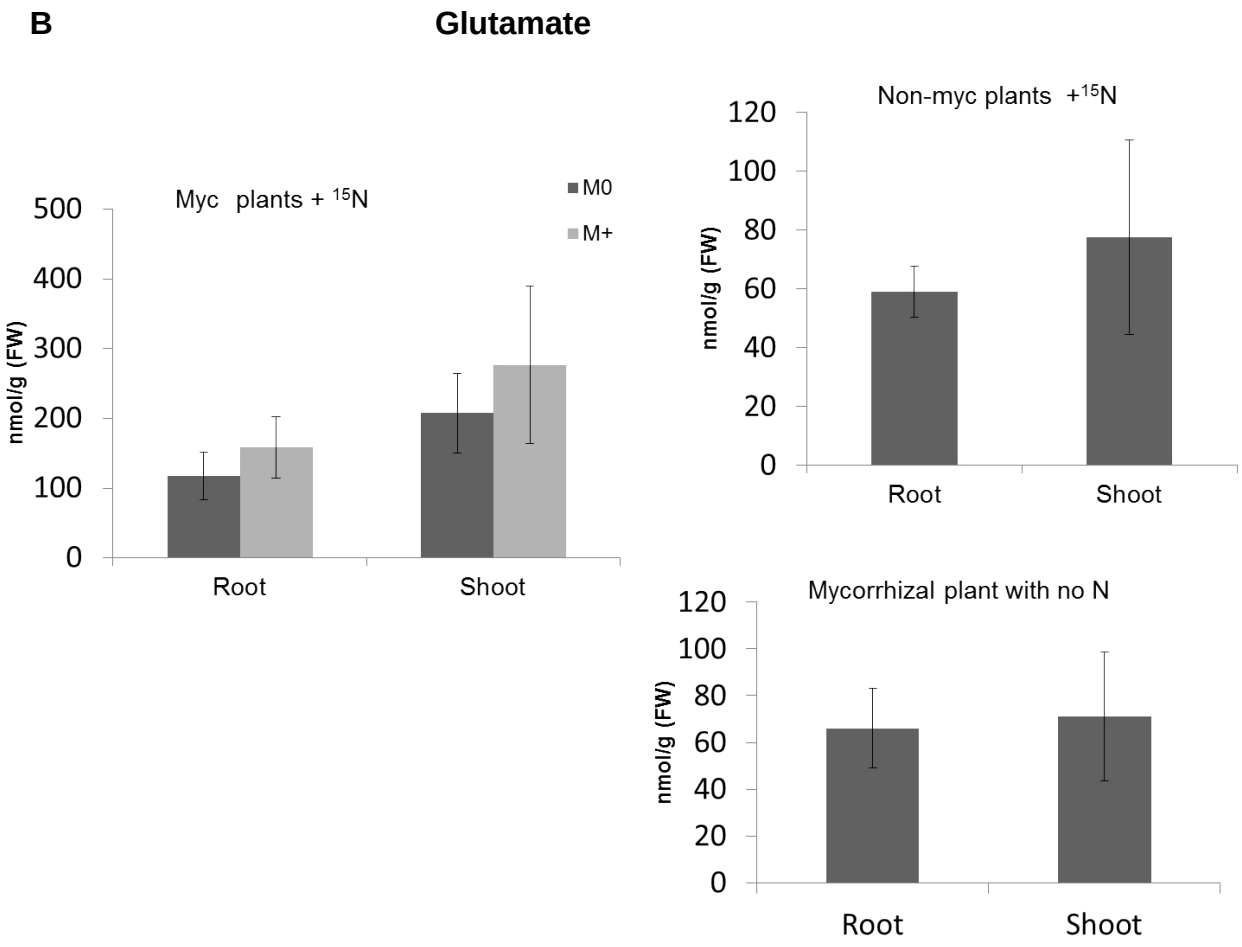


Figure 2-6 (cont'd)

C

Pyrroline-5-Carboxylate

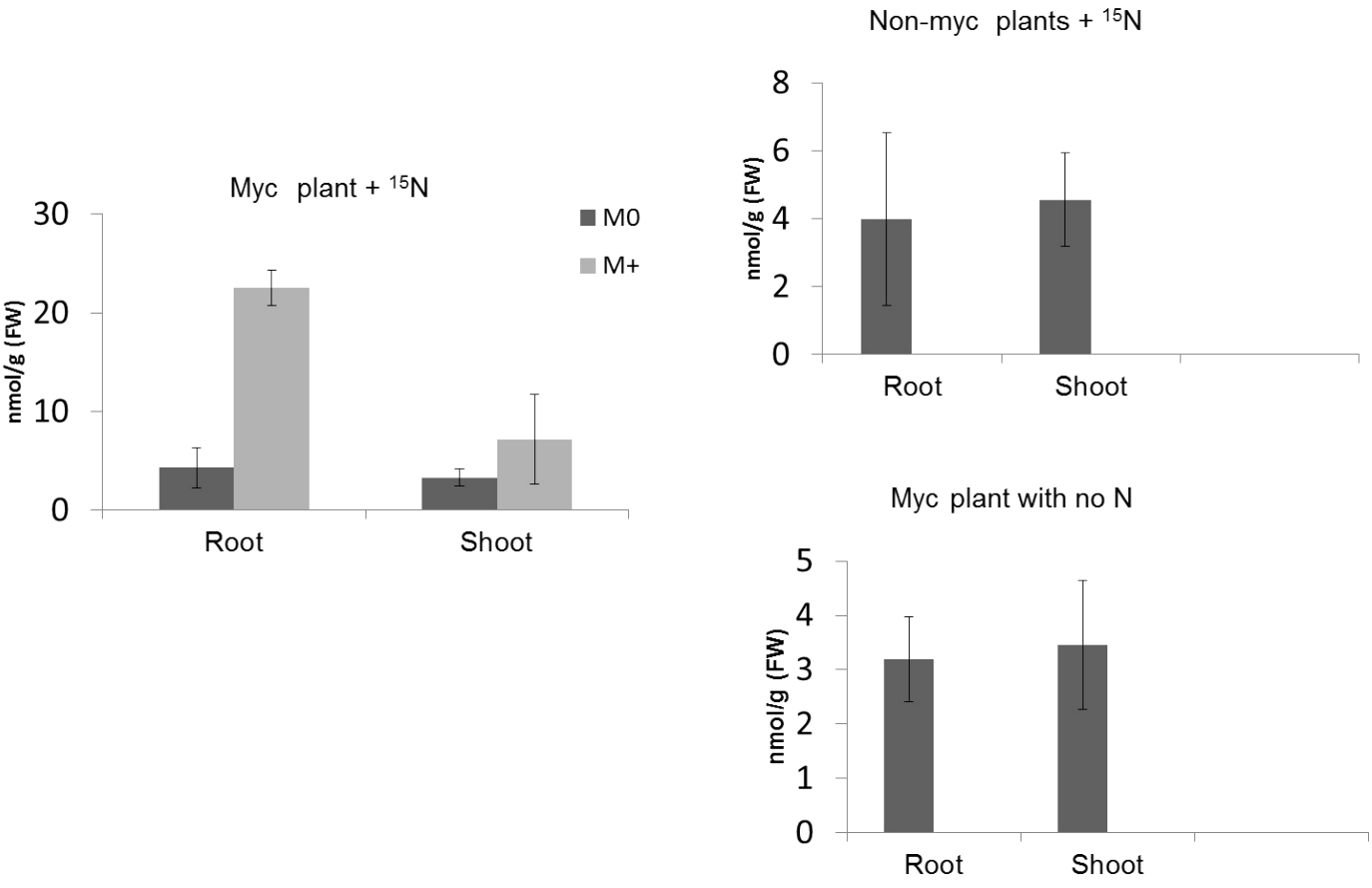


Figure 2-6 (cont'd)

D

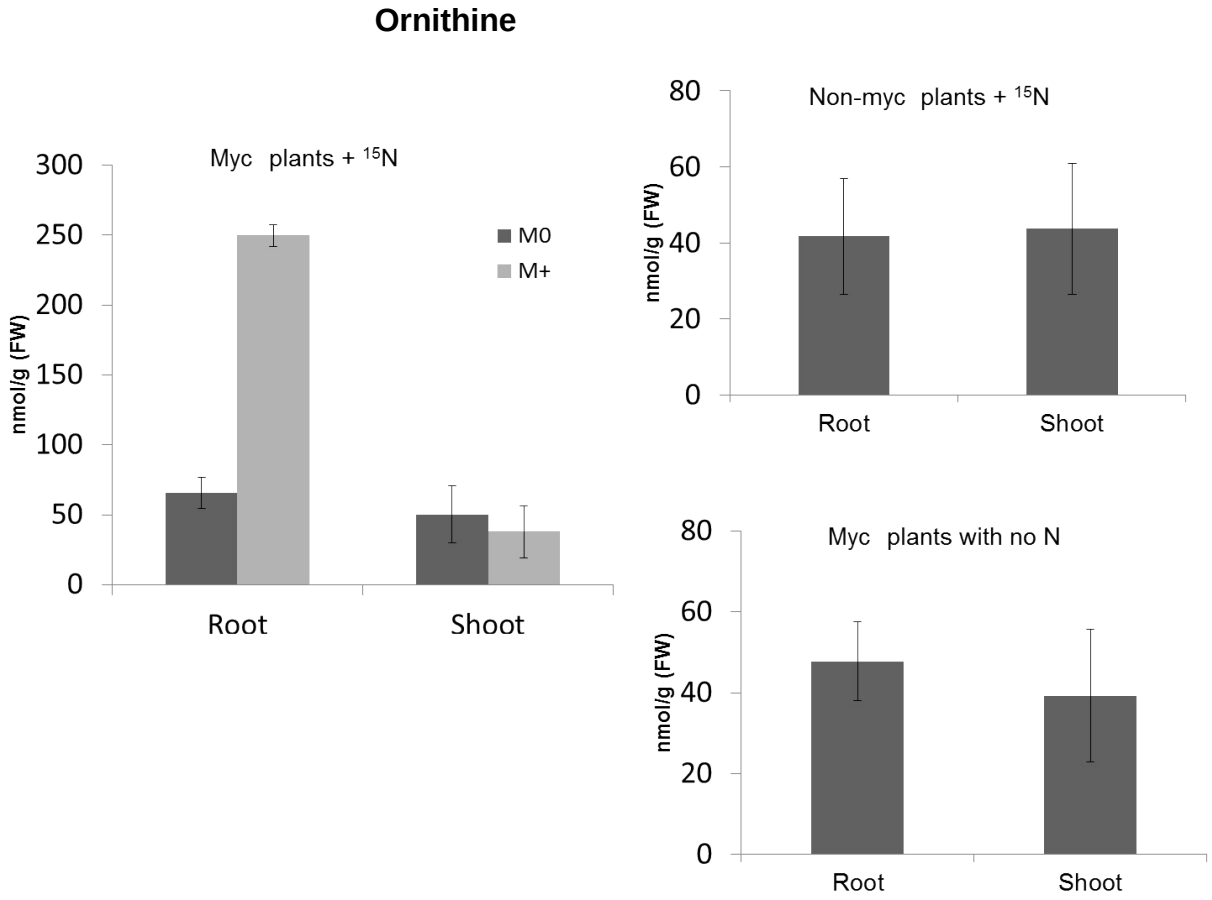
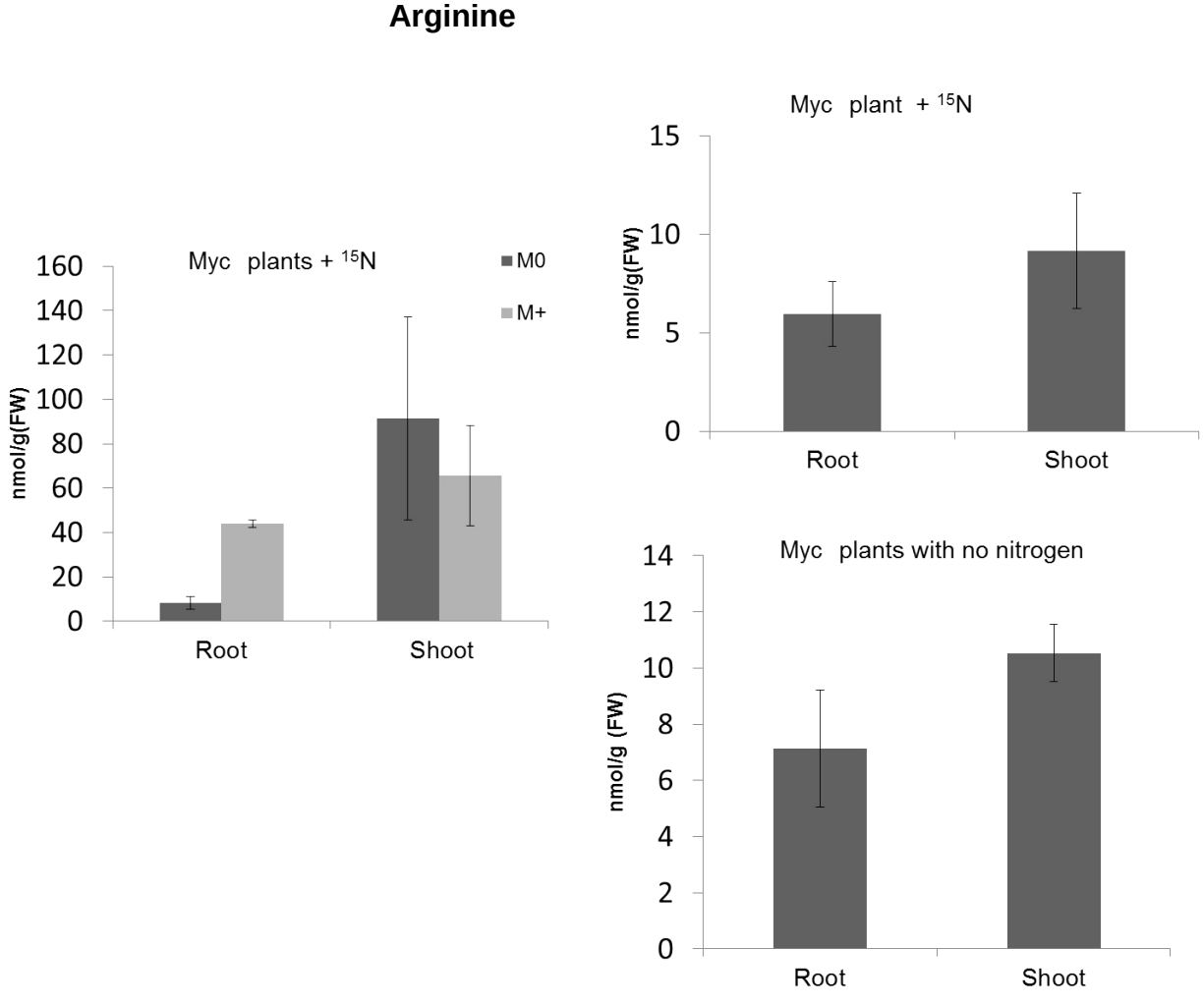


Figure 2-6 (cont'd)

E



Discussion

Many of the detailed studies of mycorrhizal metabolism and nutrient transfer (e.g. Bago et al., 1996, Jin et al., 2005, Govindarajulu *et al*., 2005) were done using transformed roots cultured on bicompartmental petri plates as the model mycorrhizal system (St. Arnaud et al., 1996). This system was used because of ease of handling, maintenance of sterility, the advantages of defined media and ease of ERM tissue isolation. However, these studies were not investigating nutrient transfer in whole-plant mycorrhizal systems which are likely to have different physiological characteristics and regulatory dynamics.

The studies of N nutrition and metabolism in the AM symbiosis that have been reported using whole plants were not conducted under aseptic conditions (eg Johansen *et al.,* 1993*,* Hodge *et al., 2001;* Fellbaum *et al., 2014).* Although such studies have been important in establishing for example the contribution of AM fungi to the mobilization of soil N (Tobar et al 1995, Hodge et al 2001) and are physiologically substantially more realistic than transformed root systems, the impractibility of maintaining sterility complicates the interpretation of metabolic and translocation experimental results. In this study, we developed a sterile and leakage-free (Figure 2-2A) whole plant two-compartment culture system (Figure 2-1) allowing the exclusion of other microbes and avoidance of diffusion between compartments. Low cost, easy to use, reprodicible and autoclavable, these microcosms make feasible investigations of mycorrhizal transport, gene expression, metabolicms and nutrition that have hitherto been challenging or impossible. In particular the sterility of this system will enable the study of the role of organic C, P, N, and S compounds in AM symbiosis. The culture system provided *Medicago trancatula* plants with optimized levels of nutrients and

water, including low phosphorous levels to stimulate colonization. Within two weeks of seedling planting, the plant were associated with higher levels of colonization and numbers of arbuscules (Figure 2-3) than is commonly observed, and significantly, also with well developed external hyphal mycelia of *Rhizophagus irregularis.* This allows experiments on uptake and transfer to be conducted on plants that, while mature, are not senescent or growth-limited by physical space constraints.

The uptake and transfer of $^{35}SO_4^{2-}$ by the fungal partner to the host plant was demonstrated in the microcosms (Figure 2-2B) with ^{35}S detected in the leaves of host plants after one day. Shoots had higher ^{35}S DPM than roots (Figure 2-2C) which is consistent with the fact that plants transport sulfate to the aerial parts, where the majority is stored as a vacuolar sulfate pool or metabolized in the reductive sulfur assimilation (Kataoka *et al*.,2004).

We conclude that sulfate is transferred by *Rhizophagus irregularis* to host plants, as has been reported for AM transformed roots (Allen and Shachar-Hill, 2009). Allen and Shachar-Hill (2009) demonstrated the transfer of sulfate by *Rhizophagus irregularis* (formerly *Glomus intraradices*) to transformed roots and found that the fungus can uptake and transfer reduced forms of S at rates comparable to sulfate. Since 95% of S is in organic form in soil (Tabatabai, 1986; Scherer, 2001), this observation points to a wider role for AM fungi for S plant nutrition in nature. This example also highlights the need for aseptic whole plant AM experiments to determine whether this capacity may be significant for S nutrition of AM plants.

It has been reported that AM fungi are able to substantially increase the uptake of N by host plant roots (Ames *et al.*, 1983; Johansen *et al.*, 1993; Bago *et al.*, 1996; Johansen *et al.*, 1996; Tobar *et al*., 2004; Hodge *et al.*, 2010). Nevertheless, the literature lacks a demonstration of growth or reproductive benefits to plants of N transfer f AM fungi. The microcosm system developed in this study provides sufficient room for plant growth with minimal physical restriction, sufficient gas exchange, and high rates of hyphal crossing to examine this important question. We observed that N transfer by AM fungi conferred growth and reproductive benefits to the host plants.

After supplying the fungal compartment with N, the mycorrhizal plants appeared healthier, greener and larger than the controls (Figure 2-4). Mycorrhizal plants with N had longer shoots, higher weights and chlorophyll contents than controls (Figure 2-5). Control plants showed N deficiency symptoms. N deficiency in plants results in a breakdown of chlorophyll (Gaude *et al*., 2007)and also affects the abundance of thylakoid membranes in chloroplasts (Malavolta et al., 2004). Furthermore, N deficiency causes severe consequences for N and C metabolism (Wang et al., 2003).

^{15}N labeling and concentrations of N metabolites (glutamine, glutamate, pyrroline-5-carboxylate, ornithine, and arginine) showing that N taken up by the ERM in the FC arrives in significant amounts at the roots and shoots of host plants (Figure 2-6). Control plants have low levels of N metabolites and no 15N labeling proving that there is no diffusion between compartments. it has been reported that N deficiency affects the abundance of amino acids in plants (Scheible *et al*., 2004).

The high abundance of N metabolites and high ^{15}N percentage labeling in mycorrhizal plants after adding $^{15}NO_3$ coincides with N transfer model in which Inorganic N is taken up by the fungal ERM, and assimilated by GS-GOGAT system rising the levels of glutamate and glutamine then nitrogen is incorporated into arginine in the urea cycle which is translocated to the fungal IRM in colonized root tissues the then broken down into ornithine and urea that is in turn is broken down to release ammonium which is exported from the fungus and imported by the host into the root cortical cells. Plants then assimilate ammonium to produce its own free amino acids (Tian *et al*., 2010). So the ^{15}N labeled metabolites in the root are in fungal IRM and plant roots. The presence of high levels ^{15}N labeled metabolites in shoots indicated that a significant amount of N being transferred to the host plants.

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Chapter 3

Evidence for additional pathways in the nitrogen transfer network of arbuscular mycorrhizas

Abstract:

In recent years our understanding of nitrogen nutrition in the AM symbiosis has substantially increased. Work at the ecological level has highlighted interactions among soil nitrogen, mycorrhizal fungi, and plant communities; and at the molecular level a working model for nitrogen uptake, metabolism, and transfer has been established. However significant questions remain about potential additional N fluxes through the metabolic network, including about nitrate transport and the origins and fate of ornithine. In ^{15}N labeling experiments with cultured mycorrhizal transformed roots as well as with mycorrhizal plant microcosms, we observed that nitrate taken up by the AM fungal extraradical mycelium (ERM) is translocated to the intraradical mycelium (IRM) and transferred to the host roots. Labeled nitrate was measured in substantial quantities in shoots, showing translocation within the plant after transfer from the fungus. After providing 15N labeled nitrate or $13C_{U6}$ arginine to the fungal ERM, the levels and labeling of metabolic intermediates as well as the expression of fungal N metabolism genes were measured over a 3 day time course in the ERM and colonized roots. The results are consistent with ornithine biosynthesis in the ERM via pyrroline-5-carboxylate and indicate that that some ornithine is broken down in the IRM to glutamate and to a lesser extent to putrescine. The timing of ornithine labeling in the IRM and ERM indicates that a significant proportion of ornithine in the ERM is derived from arginine breakdown in the IRM and translocated back to ERM where it is recycled to make arginine. The results extend the working model of the N metabolic and transport network in ways that point to significantly greater flexibility and

energetic efficiency by the fungal partner than appeared possible in the network structure.

Introduction:

More than 80% of land plant species are mycorrhizal. Of the different mycorrhizal types, the arbuscular mycorrhizal symbiosis is the predominant one (Wang & Qiu, 2006). Arbuscular mycorrhizal fungi (AMF) take up and transfer to their hosts nutrients including P, N, S, and Zn (Clark & Zeto, 2000; Hamel, 2004; He et al 2005). In return AMF depend on fixed carbon received from host roots, which can consume up to 20% of photosynthate (Jakobsen & Rosendahl, 1990).

Nitrogen availability limits plant growth in many ecosystems **(**Reich *et al*., 2006; Jackson *et al*., 2008; Marschner, 1995). The arbuscular mycorrhiza creates a well distributed and extensive absorption network, which has been shown to be able to substantially increase the uptake of N by host plant roots (Ames *et al.*, 1983; Johansen *et al.*, 1993; Bago *et al.*, 1996; Johansen *et al.*, 1996; Tobar *et al*., 2004; Hodge *et al.*, 2010). In the current model for N transfer in the symbiosis (Bago *et al*., 2001, Govindarajulu et al. 2005), nitrogen taken up by the ERM is assimilated via the GS/GOGAT pathway and used to synthesize arginine, which is translocated to the intraradical mycelium (IRM), and broken down to ammonium, which is released to the host inside the colonized root without nutritionally significant amounts of carbon (see figure 1 in chapter 1). Since it was proposed by Bago et al. (2001) substantial biochemical and molecular biological evidence has accumulated to support this scheme (Govindarajulu et al 2005, Cruz et al., 2007; Tian *et al*., 2010). However, important questions remain to be answered about the mechanisms and regulation of nitrogen handling and about N transfer from the fungus to the host plant.

In the current model, arginine translocation is responsible for all or almost all N movement from ERM to IRM (Bago *et al*., 2001, Tian *et al*., 2010)), but a possible role for nitrate in translocation is unknown. Kaldorf et al. (1998) reported that the expression of plant nitrate reductase is lower in mycorrhizal than non-mycorrhizal maize plants, suggesting that N transfer is predominantly not in this form. However, Faure et al. (1998) reported that nitrate reductase is increased in leaves of mycorrhizal plants independently of P status, and this was interpreted as indicating that nitrate is transferred from fungus to plant. Direct assessment of nitrate movement within the fungus and/or between IRM and the plant would be valuable in settling this question, and has not been addressed in previous studies that tracked 15N and 13C labeling using GCMS methods that do not detect inorganic nitrogen molecules. Likewise, the role if any of ammonium in the translocation of N within the fungus is not known although it can accumulate to millimolar levels in some fungi (Jennings, 1995), and Chalot et al. (2006) suggested that ammonium might be taken up into vesicles within the mycorrhizal fungi and released to the host by exocytosis.

Although AM as well as ectomycorrhizal fungi have the enzymes of the GS/GOGAT pathway for N assimilation (Chalot *et al*., 1994; Johansen *et al*., 1996; Tian et al. 2010) the role, if any, of Glutamate dehydrogenase (GDH) in nitrogen assimilation in the ERM of the arbuscular mycorrhizal is still unknown. In *Tilia platyphyllos-Tuber borchii* ectomycorrhizae, N assimilation in the mycelium is the combined function of NADPH dependent GDH and GS (Pierleoni *et al*., 2001). However, Morel et al. (2005) found that NADP-GDH is dispensable for ammonium assimilation by ECM fungi. The expression of a putative GDH gene from G. intraradices was reported (Govindarajulu *et*

al., 2005) although this now appears not to be a GDH (Tian and Shachar-Hill unpublished) and another gene was annotated with this function (Tisserant et al. 2012, 2013) but none of the published data provide evidence for its function.

The breakdown of ornithine (a product of Arginine hydrolysis) in the IRM yields glutamate, apparently via ornithine aminotransferase, and ornithine may also be converted to putrescine via ornithine decarboxylase, but the importance and fates of these products is unclear. Glutamate in the IRM might be broken down to release ammonium or metabolized by amino-transferase reactions to produce other amino acids. Also the origin of ornithine in ERM is still not well understood, although enzymes that can be involved in its synthesis, including ornithine amino-transferase and other urea cycle enzymes are upregulated in the ERM upon N addition. Specifically, since carbon is not transferred to the plant upon arginine breakdown (Jin et al 2005) in the IRM, the carbon skeletons may return to the ERM for re-use in ornithine and arginine or their precursors.

The expression of N transport and metabolism genes in fungi is commonly regulated by the levels of nitrogenous metabolites such as Arg, Gln and Orn (nitrogen metabolite control, Hinnebusch, 1988; ter Schure *et al*., 2000) and the levels of amino acids in presymbiotic AMF tissue are increased substantially in the presence of inorganic N (Gachomo *et al*., 2009). However *G. intraradices* (now *Rhizophagus irregularis*), which is the primary model AM fungus in molecular genetic and biochemical studies, does not down-regulate the genes for Arg synthesis in the ERM when intracellular Arg levels are high, as occurs for example in *S. cerevisiae* suggesting either that Arg is sequestered, or perhaps that host demands for N are communicated to

maintain N flow. So that studying the gene expression of the key genes of the pathway coupled with ^{15}N labeling can help us to understand how the genes involved in nitrogen metabolism and transport are being regulated (Jin et al 2010).

In this study, isotopic labeling time course experiments using different ^{15}N and ^{13}C substrates were performed and the levels and labeling patterns of putrescine, pyrroline-5- carboxylates and nitrate in addition to the amino acids glutamate, glutamine, ornithine and arginine were measured. The identification and expression time courses of several new N metabolic enzyme transcripts were measured over the same time course. The results extend our understanding of the N transfer network, including the origins and fate of key intermediates and shed light on its regulation.

Experimental procedures:

Chemicals and reagents

Gelzan, (MP biomedical, Solon, OH) was used for solidification of M media. ^{13}C-argiinine and ^{15}N Labeled potassium nitrate were obtained from MP biomedicals, (Solon, OH)).

Spore material and mycorrhizal in vitro growth conditions

The spore material of *Rhizophagus irregularis* (DAOM 181602) was purchased from Premier Tech Biotechnologies in units of 10^6 and was stored at 4°C until further use. Ri T-DNA-transformed carrot (*Daucus carota* clone DCI) roots were grown at 25°C in modified medium (Bécard and Fortin, 1988) with 3.5 g L^{-1} Phytagel (Sigma) using the

split-plate method of St-Arnaud et al. (1996). The roots and fungus were allowed to proliferate on both sides of bicompartmented petri plates at 25°C until the fungal ERM was well developed (approximately 6 weeks). The colonized roots and media in each compartment were transferred to empty compartments of new plates in which the other compartment contained new medium with. the fungal ERM typically grew over the barrier within 2 week of the transfer, colonizing the empty compartment. Root growth over the barrier after transplantation was prevented by pruning.

Isotopic labeling in colonzied Ri T-DNA-transformed carrot (*Daucus carota* clone DCl) roots culture system

^{15}N labeling

The medium of the root compartment was modified to limit the nitrogen concentration to 1 mM. The ERM was allowed to cross over the divider into the fungal compartment that contained modified medium with no N or Sucrose added and only 2 g L^{-1} Phytagel. After 2-3 weeks, the ERM were supplied with 4 mM KNO_3 for gene expression studies or $K^{15}NO_3$ to determine the labeling percentage of the free amino acids; glutamate, and $^{15}NO_3$ in the IRM part and plant tissues. The colonized roots and ERM samples were collected after 0, 2, 4, 8, 16, 24, and 72 h, rinsed with sterilized water, and immediately frozen in liquid N and stored at −80°C

^{13}C labeling

0.5mM arginine $^{13}C_6$ was supplied to ERM. ERM and AM roots samples were collected after 0, 3, 6, 12, 24, and 48 h for metabolite analysis. rinsed with sterilized water, and immediately frozen in liquid N and stored at −80°C

^{15}N labeling for $^{15}NO_3$ measurements in whole plant-mycorrhizal system

Medicago truncatula plants were grown for 5 weeks in two-compartment culture system (growth condition and the culture system mentioned in the methods of chapter 2) after that plants were deprived from N for 4 days then 10mM K $^{15}NO_3$ was added to the reservoir of fungal compartment for two weeks. Plant shoots and roots were collected for N metabolite analysis. Two controls were used, mycorrhizal colonized plant with no ^{15}N added and non-mycorrhizal plant with 10mM K $^{15}NO_3$ added to FC reservoir to test leakage. Ten replicates were used for each.

Extraction and isolation of ^{15}N and ^{13}C metabolites

300 mg of AM roots (or plant roots and shoots in whole plant system) were ground in a mortar and pestle with a pinch of acid washed sand and extracted three times with a mixture of methanol : chloroform : water (12 : 5 : 3, v/v/v). Methylene chloride and water were added to the extraction solution to facilitate the separation of chloroform and the methanol–water phases. The methanol–water phase containing the amino acids (AAs) was collected and evaporated in a rotary evaporator at 50°C, and the residues containing the AAs and N metabolites were dissolved in 1 ml of 0.01 M HCl. On the other hand lyophilized fungal mycelium was pulverized with two 3-mm stainless steel beads using a bead mill (Retsch MM301). 0.2 mL of cold methanol:water (70:30) was added to aid in disruption. The samples were shaken at 30 Hz for 4 min, and 2-μL samples were analyzed by dissecting microscope to ensure that hyphae and any spores had been broken. After disruption, 0.8 mL of cold methanol:water (70:30) was added, and the sample was vortexed for 5 min. Samples were then centrifuged and

supernatants collected. The cold aqueous methanol extraction was repeated twice more using 1 mL each time and the supernatants pooled. Then the methanol-water mixer were evaporated in a rotary evaporator at 50°C, and the residues containing the AAs and N metabolites were dissolved in 1 ml of 0.01 M HCl.

ERM and AM root samples in 1 ml of 0.01 M HCl were loaded onto a cation exchange column (0.3 ml of DOWEX 50 X8-200– hydrogen form; Sigma-Aldrich, St Louis, MO, USA), which was previously washed with 1 M NH4OH, deionized H2O and 1 M HCl, and followed by deionized H2O. after loading the sample, the column were washed 5 times with water so the neural compounds and anions with be eluted so that they were collected for nitrate measurements and the free amino acids were eluted with 5 ml of 1 M NH4OH (Bengtsson & Odham, 1979). This eluent was collected and dried then resuspended in 70 µl of milliq water.

Liquid chromatography-mass spectrometry (LC-MS)

N metabolite levels and labeling were measured using Liquid chromatography (LC)-MS analyses. LC-MS was carried out using a Quattro micro (Waters) mass spectrometer system bundled with an electrospray ionization source and a Shimadzu HPLC system. Isotopomers of glutamate, glutamine, pyrroline-5-carboxylate, ornithine, arginine and putrescine were separated using a Waters Symmetry C18 (100 × 2.1 mm, 3 μm) column with 1 mM perflurohetanoic acid in a water/acetonitrile gradient at ambient temperature and a flow rate at 0.3 mL min^{-1}.

NO_3 and $^{15}NO_3$ were separated and quantified using 3200 Q-TRAP. NO_3 and $^{15}NO_3$ were separated using ZIC®-pHILIC column (50x2.1 mm, 5 µm) with gradient of water and acetonitrile at ambient temperature and a flow rate at 0.3 mL min^{-1}

RNA Extraction and putative gene Fragment Isolation

Sequences of nitrite reductase, ornithine transcarbomylase, pyrroline-5-carboxylate dehydrogenase, and glutamate dehydrogenase gene fragments were identified from EST sequences obtained by high throughput RNAseq performed at MSU (Tian et al 2010). To confirm their identity total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) from *Glomus intraradices* germinating spore tissue that was disrupted using a bead mill, followed by DNA removal using RNase-free DNase (Turbo DNA-free; Ambion). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Primer sets were developed from EST and designed using primer3 website (http://frodo.wi.mit.edu/primer3/), PCR was performed and the products were separated by and isolated from agarose gel using the QIAquick Gel Extraction kit (Qiagen) and sequenced at the MSU RTSF on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were compared with the Glomus intraradices genomic data base to confirm identity.

Quantitative Real-Time PCR Measurements

Mycorrhizal split plates were grown until the fungal compartment was approximately one-half colonized. To the fungal compartment, 1 ml of sterile KNO_3 solution was applied to give a final concentration of 4mM mM KNO_3. Plates were incubated for 0, 2, 4, 8, 16, 24 and 72h before tissue from 9-12 plates was collected and immediately

frozen in liquid N. RNA was extracted and converted to cDNA as described above. The initial quantitative real-time PCR (qRT-PCR) reaction mixture containing primers at a concentration of 300 nM and 1 ng of cDNA template. The PCR reactions were monitored using an ABI Prism 7900 HT Sequence Detection system (Applied Biosystems) with the following cycling program: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Power SYBR Green 2-Step Master mix (Applied Biosystems) was used for all real-time PCR assays. The $\Delta\Delta C_T$, and comparative C_T, methods were utilized for the determination of relative gene expression (Livak and Schmittgen, 2001). The expression of an S4 ribosomal protein was used to normalize relative gene expression data as described by Govindarajulu et al. (2005). Primers of the four genes above and previously used primers (Table 3-1) and the closest homology of the newly identified genes are listed in table 3-2

Results

Nitrate movement through the AM symbiosis

The fungal extraradical mycelium (ERM) of transformed carrot roots was exposed to 4 mM $K^{15}NO_3$ in the fungal compartment of split plate *in vitro* mycorrhizal cultures (Fortin et al 1996) so that the ERM but not the roots had access to labeled nitrate. The levels of labeled and unlabeled nitrate ($^{15}NO_3^-$ and $^{14}NO_3^-$) in the mycorrhizal roots were measured over the next 3d using LC mass spectrometry and the percentage labeling was calculated by dividing the concentration of ^{15}N nitrate by the total concentration of labeled plus unlabeled nitrate. The percentage labeling of $^{15}NO_3$ increased rapidly in extracts of AM roots reaching approximately 20% within 8h (Figure 3-1). The

percentage labeling of $^{15}NO_3$ reached 50% after 24h with no significant increase between 24 and 72h (Figure 3-1). The mycorrhizal carrot roots contained 1-2% fungal biomass, so that it is uncertain whether the labeled nitrate remained in the fungal tissue or was transferred to plant cells.

To assess transfer of nitrate from the fungus to the host plant, an aseptic microcosm (see chapter 2) system was used to perform a similar experiments with mycorrhizal *Medicago truncatula* plants. Levels of $^{15}NO_3$ and $^{14}NO_3$ were measured in root and shoot tissues after the ERM in the fungal compartment was exposed to 10mM $K^{15}NO_3$ for two weeks. The mycorrhizal plants showed high $^{15}NO_3$ concentrations in both roots and shoots (Figure 3-2). The control plants (non-mycorrhizal plants with ^{15}N added to the second compartment or mycorrhizal plants with no 15N added) showed no $^{15}NO_3$ labeling and low levels of $^{14}NO_3$ – consistent with the N deprivation pretreatment.

N Movement through the AM Symbiosis

The levels and labeling of the free N metabolites glutamine, glutamate, ornithine, arginine, pyrroline-5-carboxylate, and putrescine in the ERM and AM roots were measured using LCMS after the ERM was exposed to 4 mM $K^{15}NO_3$. The fractional labeling of the free N metabolites were calculated by dividing the concentration of each ^{15}N-labeled metabolite by the total concentration of labeled plus unlabeled forms of the same metabolite. Fractional labeling levels are shown as a function of time after ^{15}N addition in Figure 3-3. Significant labeling levels were observed in free N metabolites within 2 h after ^{15}N addition, and within 8 h about 40% of the arginine molecules were ^{15}N labeled in the ERM. Pyrroline-5-carboxylate, glutamate, glutamine, ornithine also

became rapidly labeled within the ERM, consistent with their serving as precursors for Arginine biosynthesis. After 24h, more than 50% of glutamate, glutamine, ornithine and arginine molecules were labeled within the ERM. On the other hand, about 25% of pyrroline-5-carboxylate molecules were labeled after 24h within the ERM and the labeling levels rose in a hyperbolic manner indicating saturation of the labeling levels significantly below full labeling. No ^{15}N labeling of putrescine was detectable in the ERM. After 72 hours more than 80% labeling of the amino acid molecules contained one or more 15N atoms.

In the colonized roots (IRM plus plant cells), the labeling of metabolites showed a lag of ~2h and was slower than in the ERM over the first 8h. The fraction of arginine molecules labeled reached approximately 25% after 8 h, and rose to about 90% by 72h. 50% of ornithine molecules were labeled within the colonized roots 16h. The other metabolites showed similar patterns of 15N fractional labeling in the AM roots. The ^{15}N fractional labeling of arginine and ornithine and other metabolites is consistent with arginine was transport from ERM to the IRM and its breakdown there via arginase. ^{15}N Putrescine was detectable in IRM after 8h and reaching more than 25% fractional labeling after 72h (Figure 3-3).

In order to study the origin and fate of the metabolites in the ERM, ^{15}N isotopomers (chemically identical molecules containing different numbers of 15N atoms in different positions) of glutamate, ornithine and arginine were measured using intact molecular ions and fragment ions to obtain the number of 15N atoms and information on positional labeling from mass spectra. M+1 isotopomers were detectable first and higher mass isotopomer levels increased after that. M+2 ornithine molecules were detected at

significant levels after 8h while the fully labeled M+4 arginine mass isomer was detectable after 4hs (Figure 3-4) .

^{13}C labeling in the ERM and AM roots

The levels and ^{13}C labeling of free N metabolites (glutamate, pyrroline-5-carboxylate, glutamine, ornithine, arginine and putrescine) in the ERM and AM roots of mycorrhizal transformed carrot roots were measured after the ERM was exposed to 0.5 mM $^{13}C_6$ arginine. $^{13}C_6$ arginine was found in the ERM within 3h (Figure 3-5E). Arginine $^{13}C_5$ was detected in the ERM at 12h and then rapidly increased. On the other hand, glutamine $^{13}C_5$, glutamate $^{13}C_5$, putrescine $^{13}C_4$ and pyrroline-5-carboxylate $^{13}C_5$ were not detectable at any time in the ERM. Ornithine $^{13}C_5$ was detectable in the ERM at 12h and then rapidly increased (Figure 3-5C).

In the AM roots, arginine $^{13}C_6$ appeared first at 3h and its level rapidly increased till 12h. No significant increase of arginine $^{13}C_6$ levels were observed between 12 and 24h, levels then doubled between 24 and 48h. Arginine $^{13}C_5$ was detected at significant levels only at 24h and its level rose between 24 and 48h (Figure 3-5E). Glutamine was not detectable at any time. Ornithine $^{13}C_5$ was detectable in AM roots within 3h while glutamate $^{13}C_5$, and pyrroline-5-carboxylate $^{13}C_5$ were detectable in AM roots after 6h and putrescine $^{13}C_4$ was detectable after 12h (Figure 3-5).

Gene identification

Based on sequence data previously deposited in public databases (GenBank), sequences from *Rhizophagus irregularis* for 4 putative enzymes of N metabolism were identified. Partial CDSs of nitrite reductase (NiR), ornitinine transcrabomylase (OTC),

pyrroline-5-carboxylate dehydrogenase (P5CD) and glutamate dehydrogenase (GDH) were obtained that show high sequence similarities to known genes involved in N uptake and metabolism in fungi and bacteria (Table 3-2).

Nitrite reductase was identified with 83% similarity at the amino acid level to the *Laccaria bicolor* NADPH-nitrite reductase (EC 1.7.1.4), an **assimilatory nitrite reductase** which catalyzes the NADPH-dependent formation of ammonia from nitrite. Ornithine carbamoyltransferase was identified with 81% similarity to the *Cryptococcus gattii* (WM276) Ornithine carbamoyltransferase, mitochondrial precursor (OTCase; EC 2.1.3.3) which catalyze catalyzes the reaction between carbamoyl phosphate and ornithine to form citrulline and phosphate. Pyrroline-5-carboxylate dehydrogenase was identified with 63% similarity to *Cryptococcus neoformans var. neoformans JEC21* 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) which catalyze the reversible conversion of pyrroline-5-carboxylate to glutamate. Furthermore, glutamate dehydrogenase was identified with 63% similarity with glutamate dehydrogenase $NAD(P)^+$ (EC 1.4.1.3) of *Sphingobacterium spiritivorum* (ATCC 33861) Which catalyzes the reversible oxidative deamination of glutamate to alpha-ketoglutarate and ammonia.

Gene Expression in Response to N Addition

Based on the current model and previous results (Cruz *et al*., 2007; Tian *et al*., 2010) we hypothesized that the expression of the genes involved in N movement and metabolism from soil through the fungus and into the host is temporally and spatially coordinated with the flux of N. The transcriptional levels for 8 genes from *G.*

intraradices (4 of them identified here, the others identified and functionally confirmed previously) were measured in ERM and IRM tissues by quantitative real-time PCR.

The transcript levels of the putative assimilatory nitrite reductase (NiR) and ornithine transcarbomylase (OTC) increased strongly in the ERM beginning by 2h after 4 mM KNO_3 was added to the fungal compartment. NiR and OTC were highly upregulated after 4h of nitrate addition. By contrast ornithine aminotransferase 1 (OAT1) and pyrroline 5 carboxylase (P5CD) were modestly upregulated after 4h in the ERM. Arginase (CAR1), ornithine aminotransferase 2 (OAT2), glutamate dehydrogenase (GDH), and ornithine decarboxylase (ODC) transcript levels in the ERM were little affected by the supply of nitrate. By contrast, the expression of CAR1, OAT1, OAT2, P5CD and GDH was substantially up-regulated in the IRM within 24 h. The transcript level of ODC increased modestly in the IRM by 24h. NiR transcript level was low in the IRM and not upregulated over the time course.

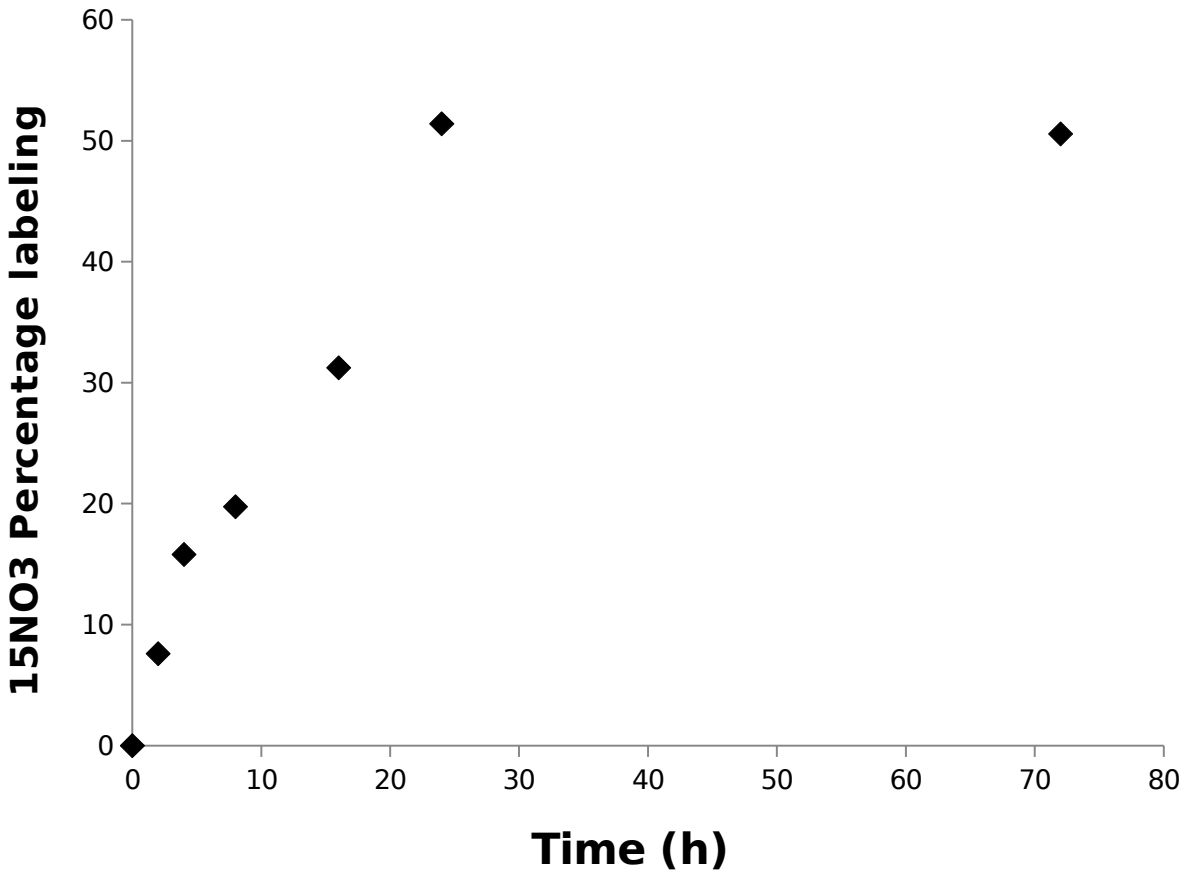


Figure 3-1 Time course of ^{15}N percentage labeling of Nitrate in transformed carrot (*Daucus carrota*) roots colonized by *G. intraradices* after supplying 4 mM $K^{15}NO_3$ to the fungal ERM compartment in divided petri dishes. Symbols show the means and standard errors of the means of three replicates.

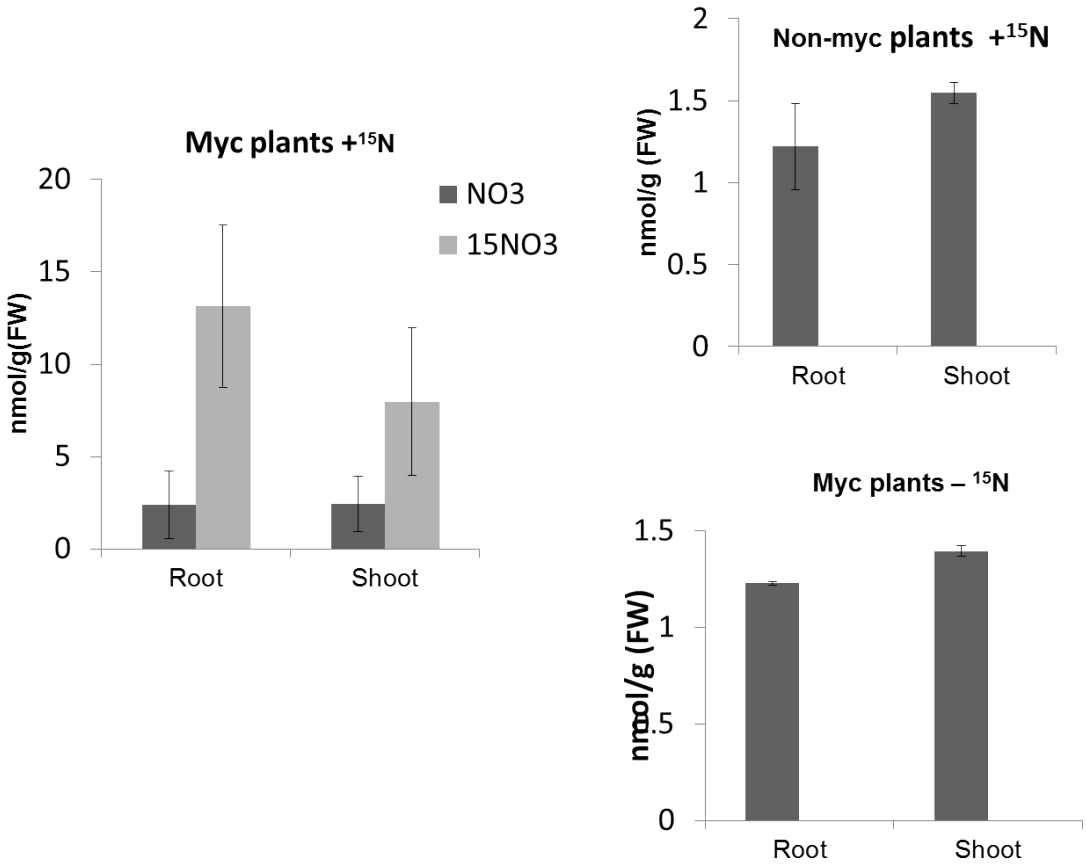
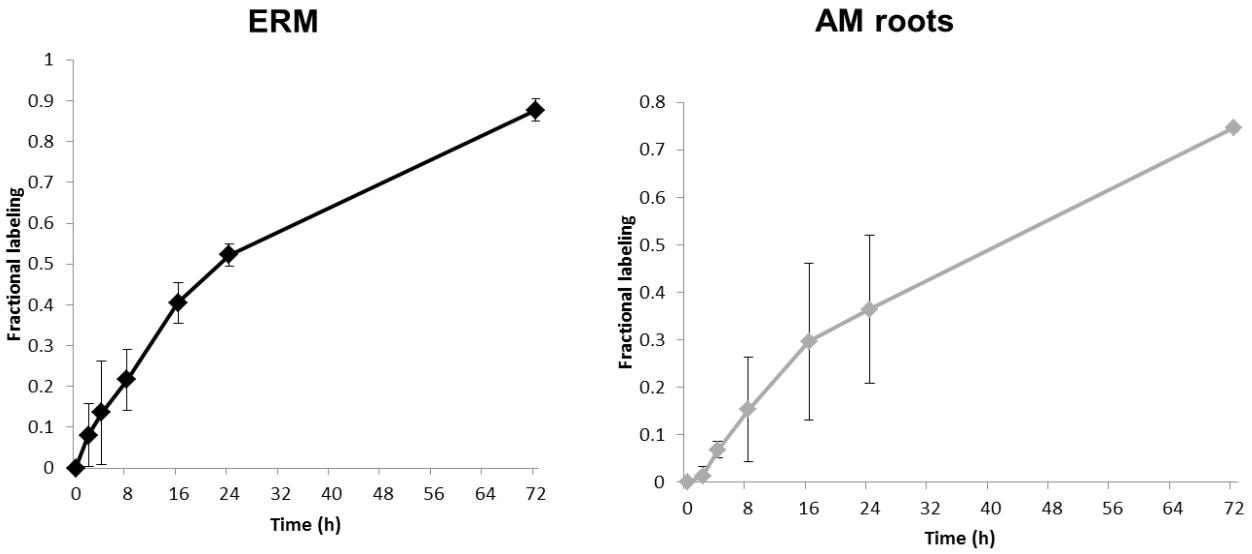


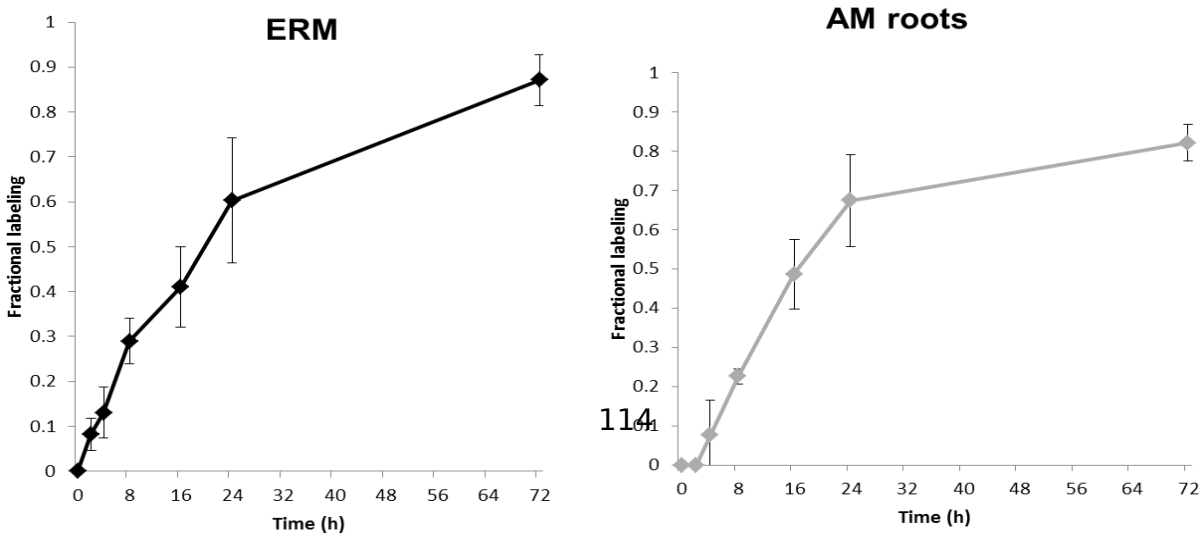
Figure 3-2. The concentrations of $^{15}NO_3^-$ (15NO3, light grey bars) and $^{14}NO_3^-$ (NO3, dark grey bars) in the roots and shoots of mycorrhizal *M. truncatula* plants grown in microcosms (see Ch. 2 for microcosm description). Measurements were made by LCMS using internal and external standards for quantification. **Myc + ^{15}N:** 10mM K^{15}NO3 was supplied to the fungal compartment reservoir (FCR) for two weeks before tissue collection and analysis. **Non-myc plants +N15:** non-mycorrhizal plants supplied with 10mM 15N nitrate in the FCR reservoir – controlling for non-mycorrhizal N levels and for non-fungally mediated N label movement. **Myc plants –^{15}N:** mycorrhizal plants with no 15N added – controlling for N deprived N levels in myc plants and for natural abundance isotope levels. Results are the means and standard errors of five biological replicates.

Figure 3-3 N movement from the ERM to the IRM in mycorrhizal transformed carrot roots based on the timing of labeling in N metabolites in the ERM and AM roots after the addition of 4 mM $K^{15}NO_3$ to the fungal ERM. The fractional labeling of N metabolite is expressed as the level of ^{15}N labeled molecules divided by the total concentration (both labeled and unlabeled). Fractional labeling of A) Glutamate, B) Pyrroline-5-carboxylate, C) Glutamine, D) Ornithine, E) Arginine in ERM and AM roots, and F) Putrescine in AM roots only as ^{15}N-labeled putrescine was not detectable in ERM. Means and standard error of the means of three replicates.

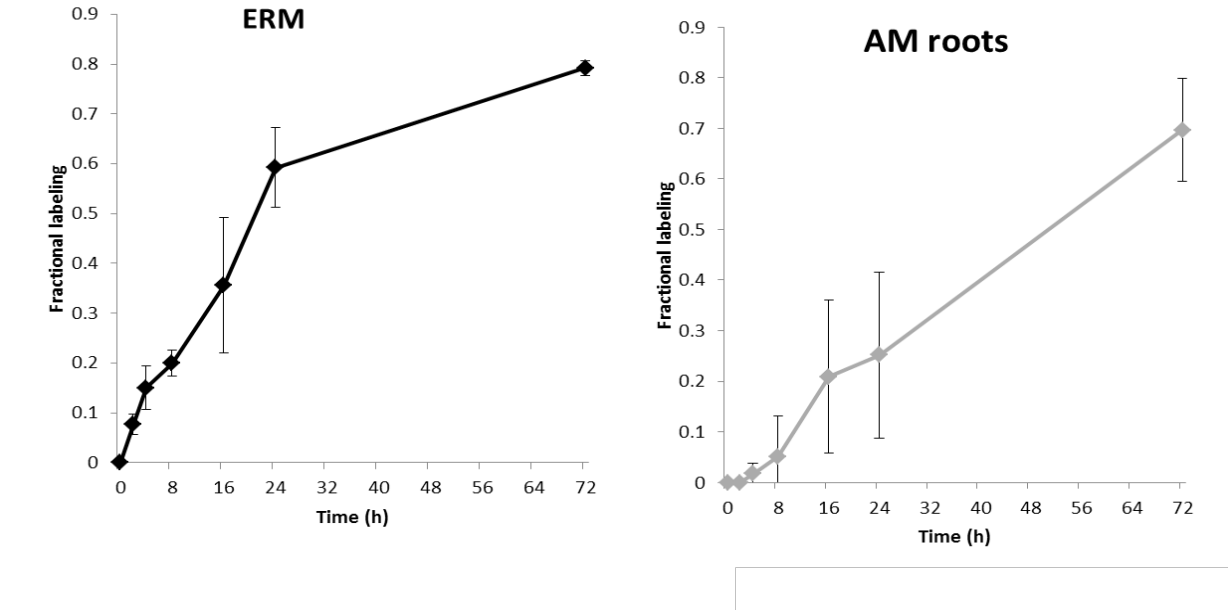
A **^{15}N-Glutamate**



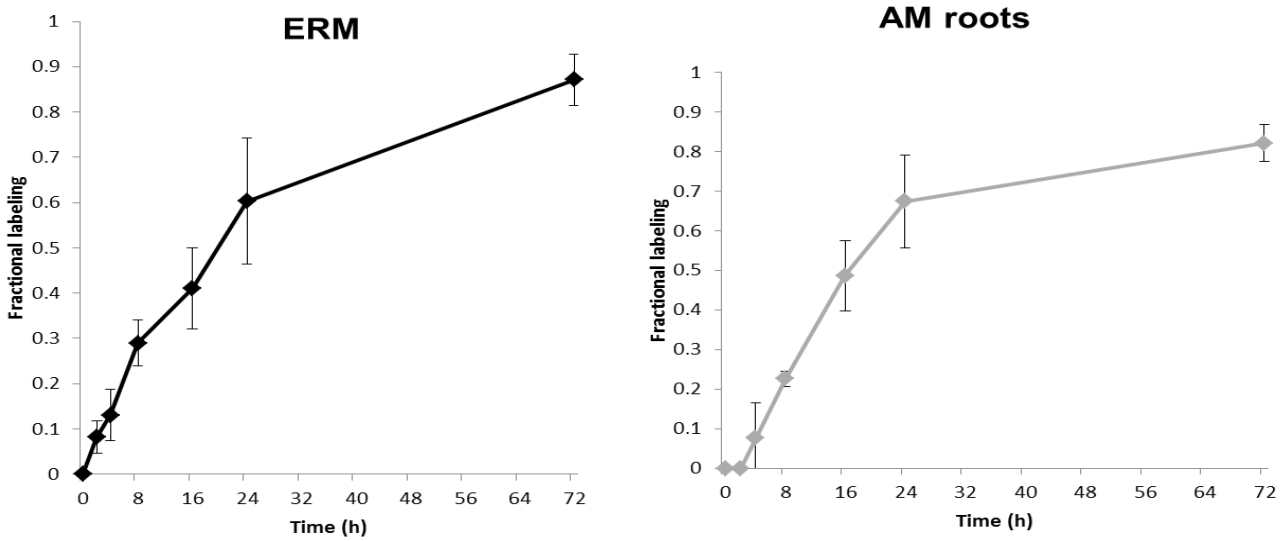
B **^{15}N-Pyrroline-5-carboxylate**



C 15N Glutamine



D 15N – Ornithine



E 15N Arginine

F 15N Putrescine

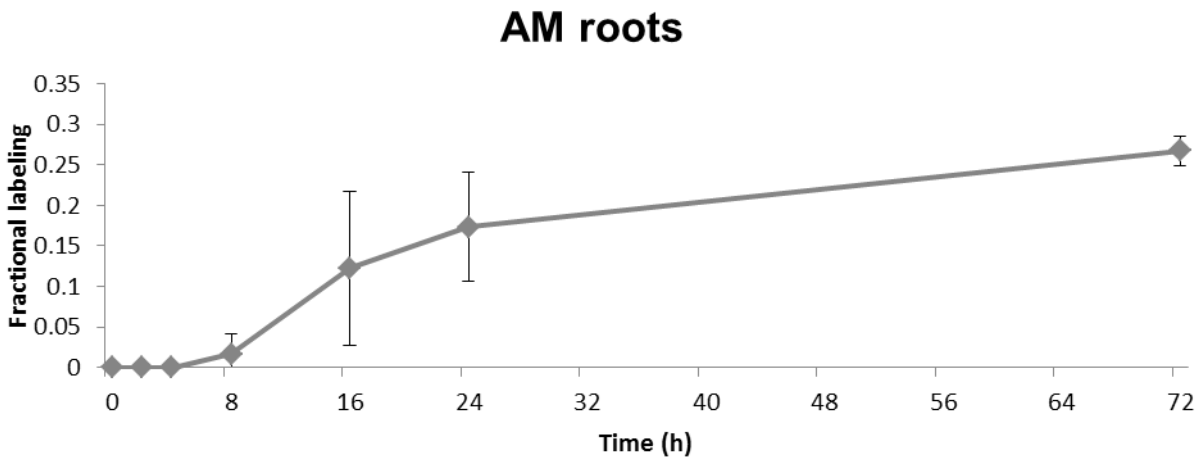
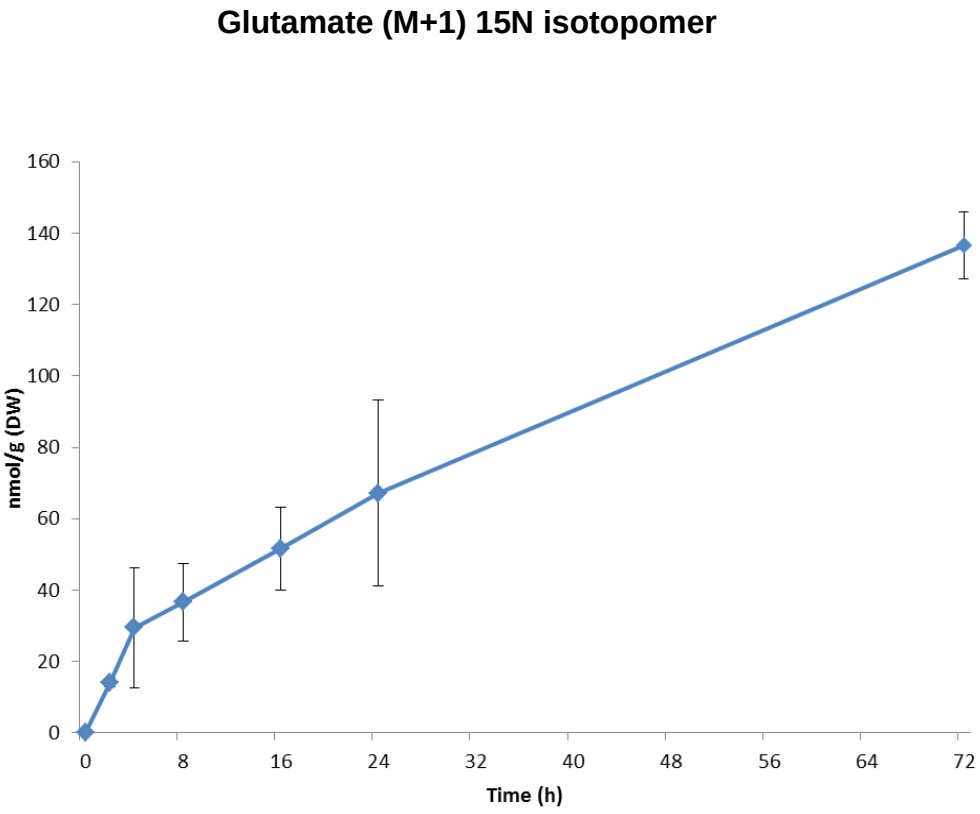
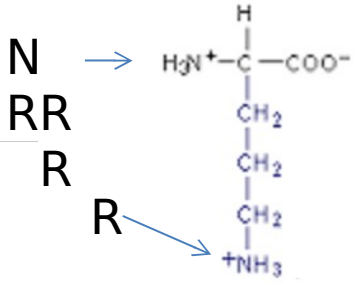


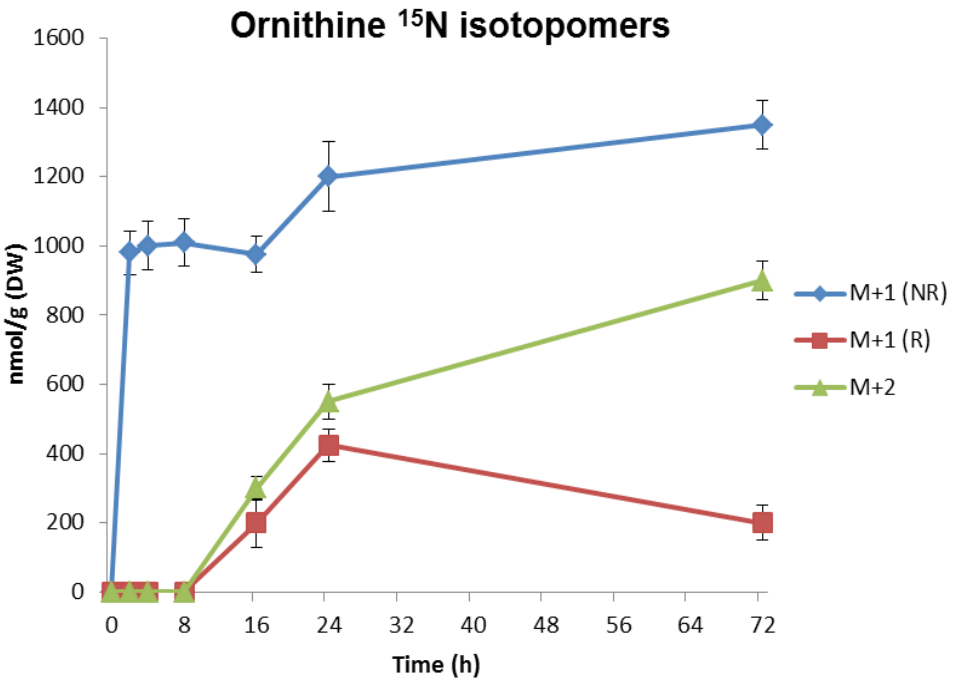
Figure 3-4 Time course of the free N metabolites ^{15}N-Isotopomers (mass isomers) in the ERM after the addition of 4 mM $K^{15}NO_3$ to the fungal ERM. M+1, singly labeled, M+2 doubly labeled, etc. position of label within a molecule is indicated with inset chemical structures. The levels of ^{15}N isotopomers of A) Glutamate B) Ornithine and C) Arginine. Means and standard errors of means of three replicates.

A



B


N
RR
R
R
H
H3N+
C
COO−
CH2
CH2
CH2
+NH3


Ornithine 15N isotopomers
nmol/g (DW)
1600
1400
1200
1000
800
600
400
200
0
0
8
16
24
32
40
48
56
64
72
Time (h)
M+1 (NR)
M+1 (R)
M+2

C **Arginine 15N isotopomer**

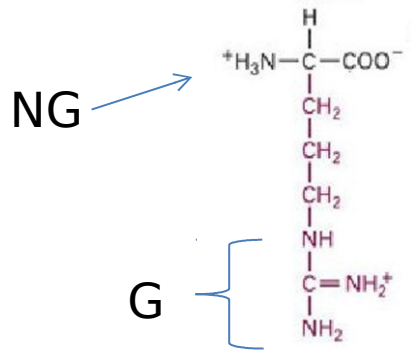
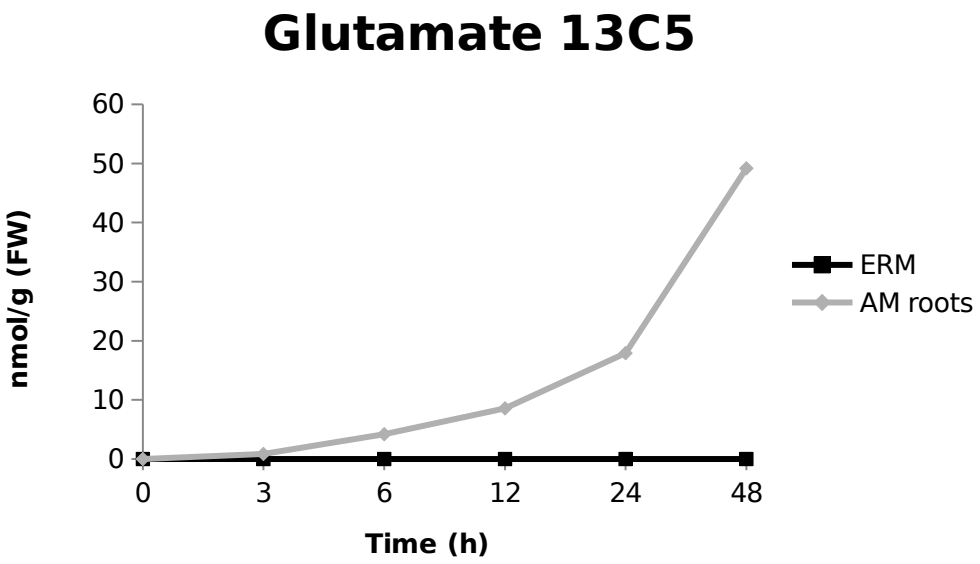
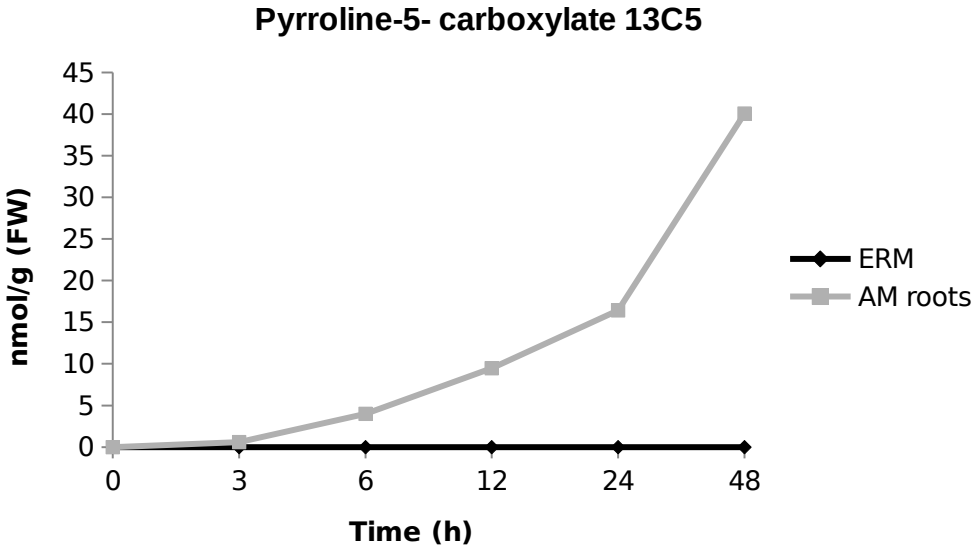


Figure 3-5 Timing course of ^{13}C labeling of N metabolites in the ERM and the mycorrhizal roots after the addition of 0.5 mM $^{13}C_6$ arginine to the fungal ERM compartment. Levels of: A) Glutamate $^{13}C_5$, B) Pyrroline-5-carboxylate $^{13}C_5$, C) Ornithine $^{13}C_5$, D) Arginine $^{13}C6$ and Arginine $^{13}C_5$, and E) Putrescine $^{13}C_4$. Symbols show the means and standard errors of the means of three replicates.

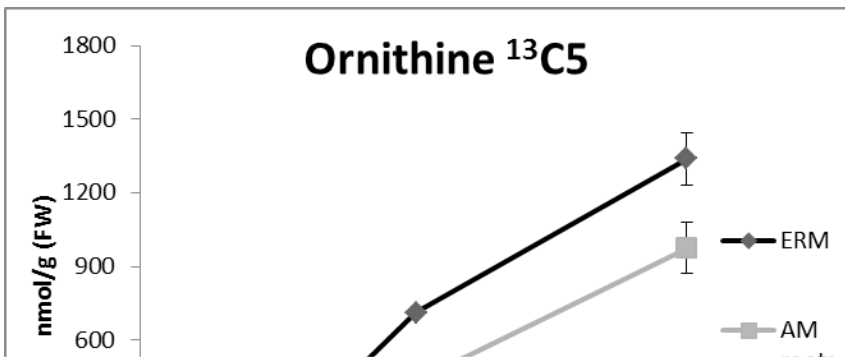
A

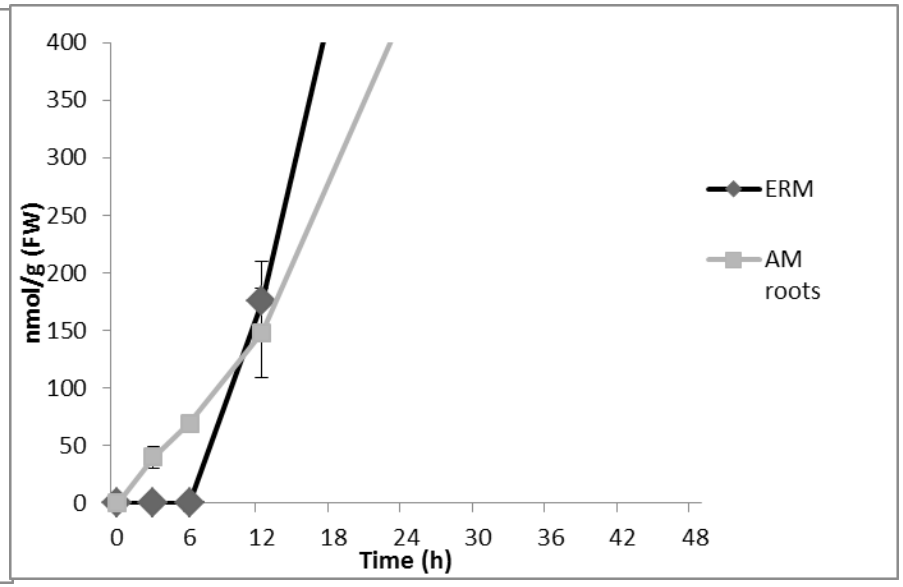


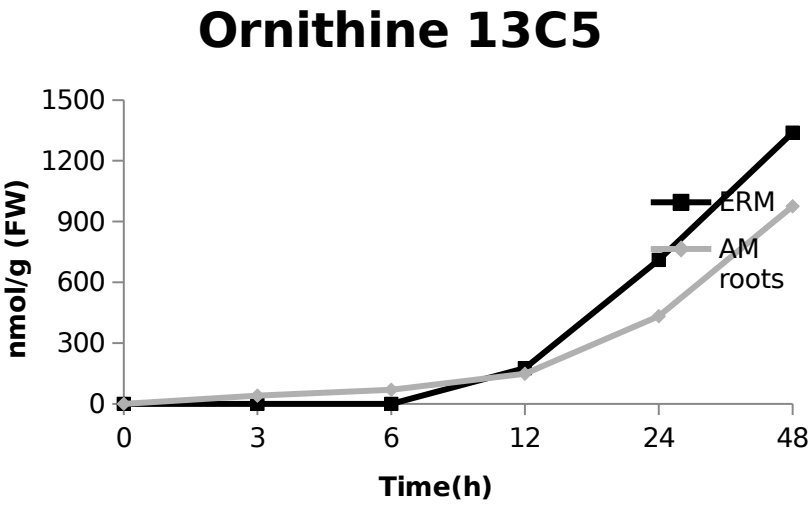
B



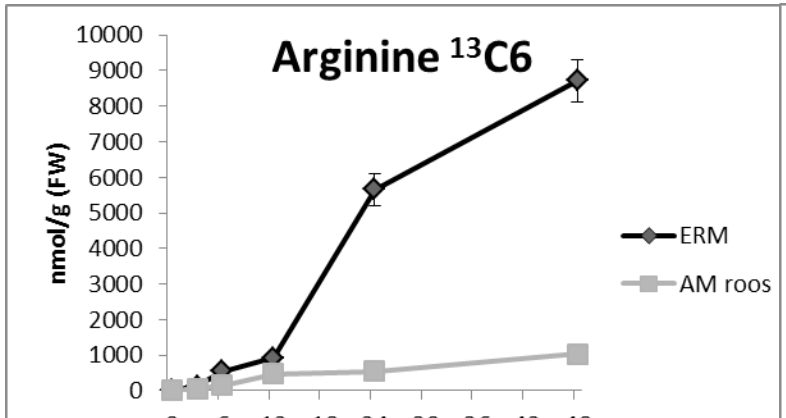
C

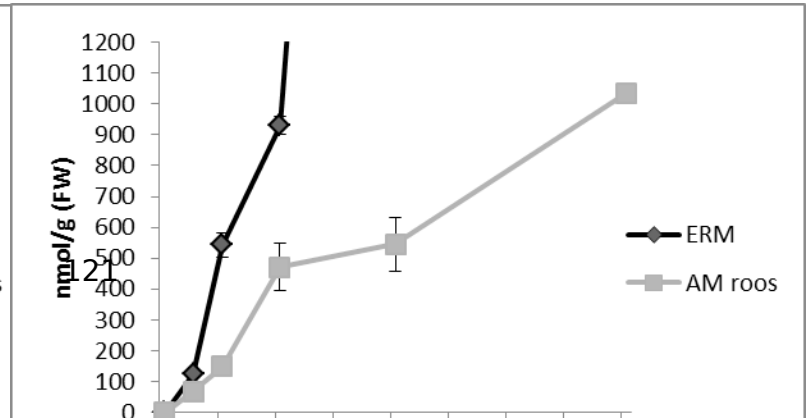

Ornithine ^{13}C5
nmol/g (FW)
1800
1500
1200
900
600
ERM
AM


nmol/g (FW)
400
350
300
250
200
150
100
50
0
0
6
12
18
24
30
36
42
48
Time (h)
ERM
AM roots


Ornithine 13C5
nmol/g (FW)
1500
1200
900
600
300
0
0
3
6
12
24
48
Time(h)
ERM
AM roots

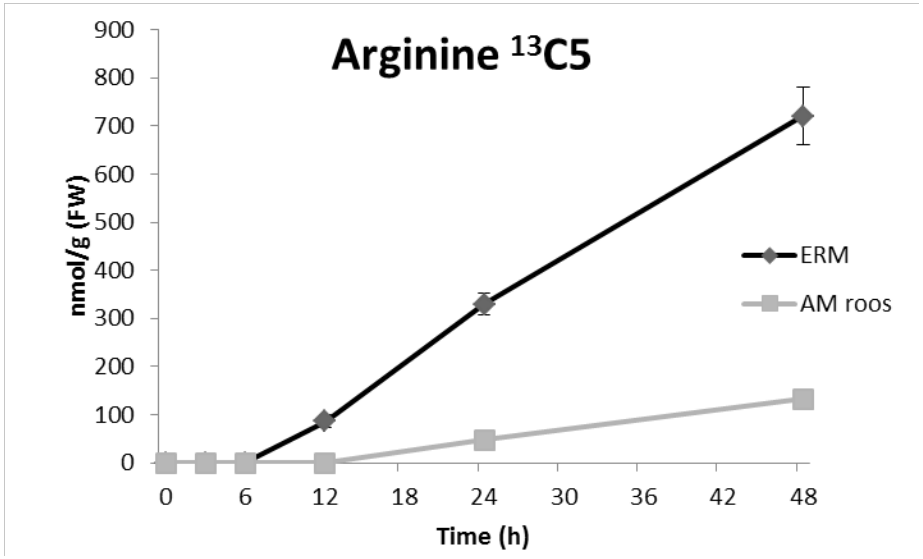
D


Arginine ^{13}C6
nmol/g (FW)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ERM
AM roos


nmol/g (FW)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
ERM
AM roos

Time (h)

Time (h)


Arginine ^{13}C5
nmol/g (FW)
900
800
700
600
500
400
300
200
100
0
0
6
12
18
24
30
36
42
48
Time (h)
ERM
AM roos

E

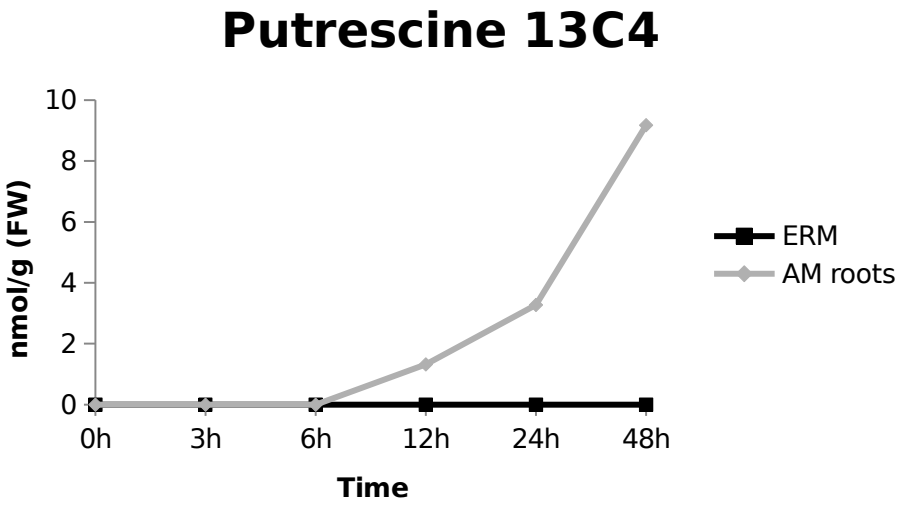

Putrescine 13C4
10
8
6
4
2
0
nmol/g (FW)
0h
3h
6h
12h
24h
48h
Time
ERM
AM roots

Figure 3-6 The expression of primary N metabolic and transport genes in the arbuscular mycorrhizal symbiosis after the addition of 4 mM KNO_3 to the fungal ERM. Bars are as follows: 2 h (white bars), 4 h (gray bars), 8 h (hatched bars), 24 h (black bars), and 72 h (striped bars). Gene expression was measured by quantitative real-time PCR. A and B, ERM (A) and IRM (B) with fungal ribosomal protein S4 gene expression as the reference. Means and standard errors of means of three biological replicates.

A

B

Table 3-1 Primers for real time PCR

Primer	Forward (5'-3')	Reverse (5'-3')	Reference
Nitrite reductase (GiNiR)	CCAGCTATACGCGTCAATTTT	AGGCGTAATTTCACCTCCAG	This study
Ornithine transcarbomylase (OTC)	GCTCAACGTATAAAAGATTTTGCTG	CATTAGTGCATCAATAACGGCTA	This study
1-Pyrroline-5-carboxylate dehydrogenase (P5CD)	TTAAGACCGGTCCTCCTGAA	AAACTTGGGCTTCCTGCTTT	This study
Glutamate dehydrogenase (GDH)	TTCCCTTTACACCATAATACACACC	CTGCGCCAGATATGGGTACT	This study
Ornithine aminotransferase 1 (GiOAT1)	GGTTCGAGCGGATATTGTCATAC	AGGACTGCTGATATTGGGTAAACG	Tian *et al*., (2010)
Ornithine aminotransferase 2 (GiOAT2)	CGGGTAAGATGCTTTGTCAAGA	GCCTGAAAGTGCTTTACCAAGTATAAC	Tian *et al*., (2010)
S4 ribosomal protein (GiSR4)	AAGCCGCCTACGTGTCGTT	AACAGGTGGTAGAAATATGGGAAG	Govindarajulu *et al*., (2005)
Arginase (GiCAR1)	TGATGCGGTGAATCCTAAGAGA	GATCAAGTGCATCAACGTCAAAG	Tian *et al*., (2010)
Ornithine decarboxylase (GiODC)	TTGATTGCGTTACCAAAAATGG	TCGAAATACAACCAGTCACCAAGA	Tian *et al*., (2010)

Table 3-2 New nitrogen metabolic genes identified in *Rhizophagus irregularis*

Gene name	**Amplicon size (bp)**	**Closest homolog**
Nitrite reductase (NiR)	154	XP_001887050.1 (*Laccaria bicolor*) Identity= 83%
Glutamate dehydrogenase (GDH)	180	ZP_07083347.1 (*Sphingobacterium spiritivorum*), Identity= 64%
Ornithine transcarbomylase (OTC)	205	XP_003192971.1 (*Cryptococcus gattii*) Identity= 81%
Pyrroline-5-carboxylate dehydrogenase (P5CD)	**484**	XP_572420.1 (*Cryptococcus neoformans* var. *neoformans*) Identity= 63%

Discussion

The current working model of N transfer from the fungi to the plant in the AM symbiosis was proposed by Bago *et al.* (2001) based on previous work that demonstrated fungal N uptake and metabolism and implicated amino acids in N handling (Johansen *et al*., 1996; Bago *et al*., 1996; 2000). Supporting evidence for the model (Govindarajulu *et al*., 2005, Cruz *et at.,* 2007, Jin *et al*., 2005, Tian *et al*., 2010) has led to its widespread acceptance (Smith and Read 2008, Parniske *et al*., 2009, He *et al.,* 2011). However the network is incomplete on the question of the fate of carbon translocated from ERM to IRM in the form of arginine and the potential remains for other forms of N besides arginine to be involved in N movement from ERM to the IRM and roots.

The detection of high 15N percentage labeling of nitrate in AM carrot roots after 15N nitrate was supplied to the ERM (Figure 3-1) indicates that not all the nitrate taken up by the ERM is reduced and assimilated there. Because total nitrate levels are not high, it may be that the nitrate translocated to the colonized roots remains in the IRM. To address this question we conducted gene expression analysis of the fungal putative assimilatory nitrite reductase in the colonized transformed roots and ERM as well as a 15N nitrate labeling experiment using the whole plant microcosm described in Chapter 2. NiR expression is upregulated in the ERM but not IRM in response to nitrate addition (Figure 3-6) suggesting that the nitrate that reaches the IRM is not converted to ammonia. Tian et al., (2010) reported that other genes involved in N assimilation, such as GS/GOGAT were not upregulated in the IRM. The results of the whole plant experiment showed that the roots and shoots both had high 15N nitrate labeling levels while control treatments showed no labeling (Figure 3-2). We conclude that nitrate also

contributes to N transfer by AM fungi to host plants so that arginine synthesis and translocation within the fungus followed by its breakdown and the transfer of ammonium to the plant is not the only route for N transfer in AM symbiosis.

After supplying $K^{15}NO3$ to the ERM, rapid ^{15}N labeling was found in N metabolites in the ERM and in the AM roots (Figure 3-3). This is consistent with previous observations of amino acid labeling by Tian *et al*. (2010). The increase of the ornithine ^{15}N fractional labeling in the ERM and in the mycorrhizal roots coincided with the increase of labeled arginine (Figure 3-3 C,D), which is consistent with their close location in the metabolic network. These results are consistent with previous observations (Jin et al 2008) and with the synthesis of arginine in ERM and its breakdown in the IRM as previously proposed (Bago et al 2001).

Neither the data of figs 3-1 - 3-3, nor previous studies provide a complete picture of the origins of ornithine in the ERM or its fate in the IRM (Govindarajulu *et al*., 2005; Jin *et al*., 2005; Cruz *et al*., 2007). Ornithine released during arginine breakdown in the IRM might be stored, transferred to the host, broken down or translocated to the ERM. Govindarajulu *et al.,* (2005) reported that ornithine was neither stored in substantial quantities in the IRM nor is transferred to the host in detectable amounts. Breakdown of ornithine in the IRM with the release of ammonium would seem to be more efficient for N transfer to the host compared with its translocation back to the ERM, which would return half of the N in arginine to its original location. However the simultaneous de novo synthesis of ornithine in the ERM and its catabolism in the IRM also consumes energy and carbon.

The presence of 15N labeled pyrroline-5-carboxylate as well as 15N glutamate in the ERM (Figure 3-3) is consistent with *de novo* ornithine biosynthesis, since pyrroline-5-carboylate is the intermediate metabolite for the formation of ornithine from glutamate via P5CD. Gene expression analysis of OAT1 and P5CD showed 3 fold increases in gene expression for these genes 4h after adding nitrate to the ERM. Both of those enzymes catalyze the reversible interconversion of ornithine and glutamate. However OAT1 and P5CD were not highly upregulated in the ERM compared with the IRM (Figure 3-6). Enzyme activity analyses will be required to determine which of the routes is more active.

The absence of detectable levels of ^{15}N putrescine in the ERM is consistent with the gene expression analysis of ODC which was not up regulated in the ERM after N addition. By contrast, significant levels of 15N putrescine were found in the IRM later during the time course, which coincided with an approximately 3 fold ODC gene expression increase in IRM 24h after nitrate addition to the ERM (Figure 3-6). Significant amounts of 15N glutamate and 15N pyrroline-5-carboxylate were found in the IRM coinciding with the up regulation of OAT1, OAT2 and P5CD. Base on the levels and fractional labeling of putrescine, pyrroline-5 carboxylate and glutamate as well as OAT1, OAT2 and P5CD gene expression analysis, we conclude that significant amounts of ornithine are converted to glutamate and that lower quantities are converted to putrescine in the IRM.

The levels of ^{15}N isotopomers of glutamate, ornithine and arginine were measured in the ERM after adding $K^{15}NO_3$ to the ERM (Figure 3-4). The increase in M+1 (Non R group N(NR) ornithine in the ERM coincided with the increase in M+1 ^{15}N-glutamate is

consistent with the formation of ornithine from glutamate. M+2 ornithine levels increased with the increase of ^{15}N arginine isotopomers containing 2 or more 15N atoms and these increased more rapidly when 15N arginine M+4 levels rise. Application of $^{13}C_6$ arginine to the ERM resulted in detectable level of arginine $^{13}C_6$ within 3h while ornithine $^{13}C_5$ was detectable in the ERM only after 12 h (Figure 3-5). The absence of ornithine $^{13}C_5$ before 12h and the appearance of ornithine $^{13}C_5$ in AM roots indicate that arginine was broken down in the IRM but not in the ERM (consistent with low arginase expression there). The absence in the ERM of ^{13}C labeled N metabolites other than ornithine indicates that ornithine is not broken down in the ERM.

Several observations are consistent with translocation of ornithine from IRM to ERM. The appearance of $^{13}C_5$ ornithine in the ERM is substantially delayed compared to the IRM. This could be due to either the return of $^{13}C_5$ ornithine from IRM to ERM or to a delayed induction of arginase in the ERM. The total levels of arginine, ornithine and other amino acids in the ERM are not affected by arginine uptake (which is much slower than the uptake of inorganic N substrates) so that a delayed induction of arginase activity in the ERM is improbable as an explanation. Indeed arginase expression and activity were not induced in the ERM by N addition, (this study, Tian et al 2010, and Cruz et al 2007). $^{13}C_5$ arginine, which is made from $^{13}C_5$ ornithine, was detected in the ERM much later than $^{13}C_6$ arginine, which is consistent with the delayed appearance of $^{13}C_5$ ornithine in the ERM as a precursor not a product of $^{13}C_5$ arginine. The appearance of $^{13}C_5$ arginine in ERM preceded its appearance in the IRM, consistent with the unidirectional translocation of arginine from ERM to IRM (Figure 3-5). The fractional labeling of ornithine in the IRM is higher as well as rising faster than that in the ERM

(since carbon is not transferred to the plant, ornithine in the plant cells is unlabeled so that the measured fractional labeling of ornithine in the AM roots represents a still higher labeling level in the IRM). This is necessary if ornithine in the IRM is the precursor of ornithine in the ERM. The presence of glutamate $^{13}C_5$, pyrroline-5-carboxylate $^{13}C_5$ and putrescine $^{13}C_4$ indicated that some ornithine is being converted to them in the IRM of AM roots. The absence in the ERM of ^{13}C labeled glutamate, glutamine, Pyrroline-5-carbocylate or putrescine despite their accumulation in the AM roots indicates that none of these metabolites returns to the ERM from the IRM.

The possible role of glutamate dehydrogenase in N movement is still unknown. Cliquet and Stewart (1993) reported the activities of the GS/GOGAT pathway but not assimilatory NADP-dependent glutamate dehydrogenase (GDH) in mycorrhizal roots, and the application of a GOGAT inhibitor to extraradical mycelium reduced 15N assimilation. A putative GDH gene was identified and it is most probably **dissimilatory** GDH. The gene expression of GDH in slightly upregulated in ERM and and highly unregulated in IRM indicating that GDH break down glutamate to release more ammonium to the host in IRM.

Within 2h of nitrate addition to the ERM the expression of the OTC transcript was upregulated in the ERM, coinciding with the building up of arginine since OTC is part of urea cycle. On the other hand OTC was not upregulated in the IRM which is consistent with the previously reported down regulation of other arginine biosynthesis genes in the IRM (Tian et al., 2010). The up regulation in the IRM of the expression of fungal genes involved in arginine and ornithine breakdown (Arginase: CAR1, OAT1, OAT2 as well as P5CD and ODC) coincides with the arrival of ^{15}N label and a rise in arginine levels within

AM roots. Thus, it is likely that this is the signal for regulatory gene expression in the IRM.

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Chapter 4

Conclusions and future research

Conclusions

Nitrogen nutrition has been a topic of considerable interest and growing importance in mycorrhizal research, AM fungi can increase the uptake of N by host plants from the soil under natural and perturbed conditions and transfer from AMF can account for significant proportions of N in plants in controlled model systems. However, our knowledge of the nutritional importance of, as well as of the enzymes and transporters involved in, N transfer is still limited. Furthermore, important questions remain to be answered about the mechanism and regulation of nitrogen handling and its transfer from fungus to the host plant. In this study, I highlighted recent findings about nitrogen transfer and metabolism in arbuscular mycorrhizal symbiosis and then report my findings concerning the remaining unanswered questions. We developed a sterile and leakage-free whole plant two-compartment culture system allowing the exclusion of other microbes and the avoidance of nutrient diffusion between compartments. Low cost, easy to use, reproducible and autoclavable, these microcosms make feasible investigations of mycorrhizal transport, gene expression, metabolisms and nutrition that have hitherto been challenging or impossible. The uptake and transfer of $^{35}SO_4^{2-}$ by the fungal partner to the host plant was demonstrated in the microcosms. Based on our data we conclude that sulfate is transferred by *Rhizophagus irregularis* to host plants leaves within a day as has been reported for AM transformed roots (Allen and Shachar-Hill, 2009). Using this microcosm to study the role of N transfer by AM fungi to host plant indicated that N transfer by AM fungi conferred growth and reproductive benefits to the host plants.

^{15}N isotopic labeling results showed increased levels of N metabolites with high ^{15}N percentage labeling in mycorrhizal plants after adding $^{15}NO_3$. Labeling time course data are consistent with the current N transfer model in which inorganic N is taken up by the fungal ERM, assimilated via the GS-GOGAT pathway raising the levels of glutamate and glutamine. Nitrogen is then incorporated into arginine via enzymes of the the urea cycle and is translocated to the fungal IRM in colonized root tissues and then broken down into ornithine and urea that is in turn is broken down to release ammonium that is exported from the fungus and imported by the host into the root cortical cells. The presence of high levels ^{15}N labeled metabolites in shoots indicated that a significant amount of N being transferred to the host plants. One of the major questions in N transfer in the AM symbiosis was the role, if any, of nitrate translocation. Based on ^{15}N labeling experiments in microcosms (described in chapter 2) and monoxenic culture system (mycorrhizal transformed roots) we conclude that arginine translocation is not the only pathway for N movement and transfer in AM symbiosis and that nitrate is also directly transferred by the AM fungus to host plants. Gene expression of a putative fungal nitrite reductase indicated that a significant amount of nitrate was assimilated in the ERM but not IRM which is consistent with the labeling and the revised N transfer model.

Investigating the origin and fate of ornithine was another objective of this study. The presence of ^{15}N labeled pyrroline-5-carboxylate as well as ^{15}N-glutamate in the ERM suggests a role for these metabolites in making ornithine there since pyrroline-5-carboxylate is a biosynthetic intermediate between glutamate and ornithine. This conclusion was also supported by the gene expression analysis of OAT1 and P5CD

which showed a 3-fold increase in gene expression 4h after adding nitrate to ERM. The presence of ^{15}N glutamate, ^{15}N pyrroline-5-carboxylate and low levels of ^{15}N putrescine in 15N labeling experiments as well as of glutamate $^{13}C_5$, pyrroline-5-carboxylate $^{13}C_5$ and putrescine $^{13}C_4$ in the IRM after labeling the ERM with arginine $^{13}C_6$ coincided with the upregulation of OAT1, OAT2 and P5CD. This suggests that ornithine is converted to glutamate and to a lesser extent to putrescine but that a significant proportion is translocated back to the ERM. Together with the translocation and transfer of nitrate, this additional flux in the N transfer network provides increased flexibility and potential for improved efficiency. The expression of a putative GDH gene is slightly upregulated in the ERM and highly upregulated in IRM indicating that GDH may play a dissimilatory role in breaking down glutamate to release more ammonium to the host.

Future research

In this dissertation, I report experimental data on the metabolism and transfer of N from the fungus to the host plant in the AM symbiosis and its role plant growth. We extended our understanding of the N transfer network, including the origins and fate of key intermediates and shed light on its regulation. However there are some unresolved questions that need investigation. The way that arginine is transferred from the ERM to the IRM is still not well understood. Passive diffusion is too slow for the long distances from ERM to IRM. It has been found that arginine is bound to polyphosphate in ectomycorrhizal symbiosis (Martin, 1985), and polyphosphate is believed to move in vesicles or tubular vacuoles (Dürr *et al*., 1979). Accordingly, where polyphosphate from and how arginine is bond with polyphosphate are the key points for illuminating the transfer of arginine.

Rhizophagus irregularis does not down-regulate the genes for Arginine synthesis in the ERM when intracellular arginine levels are high, as occurs for example in *S. cerevisiae* suggesting either that arginine is sequestered, or perhaps that host demands for N are communicated to maintain N flow. So How the genes involved in nitrogen metabolism and transport are regulated need further studies.

Studying the enzymatic activity of the putative genes which were identified so far, shall confirm their role in N transfer. Enzyme assay of the glutamate dehydrogenase will clarify its role in N transfer as well as its specificity and regulation. On the other hand, The possibility of transforming *Rhizophagus irregularis* shall lead to a better understanding of the role of genes in nutrients transfer through studying knockout mutants.

Cross talk between C and N as well as N and P are not well understood and need further investigation. Furthermore, the incorporation of *Medicago truncatula* mutants impaired in N assimilation pathway genes would help reveal the extent to which AM fungi contribute to plant N metabolism and the possibility of plant-to-plant N transfer.

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