The Influence of Nitrogen Source on the Nutrition of Arbuscular Mycorrhizal Plants

Thomas James Thirkell

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University of York

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Abstract

Arbuscular mycorrhizal fungi (AMF) form a symbiosis with 70% of land plants and may contribute significantly to plant phosphorus (P) and nitrogen (N) acquisition in exchange for photosynthetic carbon. AMF mainly acquire N as simple, inorganic molecules such as ammonium and nitrate, but whether AMF display a preference for either source, or can increase plant N content is uncertain. Furthermore, soil nutrient availability, especially that of N, may determine the extent to which a fungus engages in nutrient-for-C trade with plants; further experimental validation of this suggestion is warranted. Experiments ranging from Petri plate microcosms to field trials were carried out to address these questions.

Plant N uptake via AMF was traced using stable isotope ^{15}N, having been added to hyphal-only compartments which allow access to AMF, while excluding plant roots. AMF species *Glomus aggregatum* and *Rhizophagus irregularis* were used individually to inoculate plants allowing comparisons between AMF isolates, while N uptake preferences were tested by providing ^{15}N as ammonium, nitrate or ammonium nitrate.

R. irregularis contribution to plant nutrient uptake was sufficient to increase host biomass in chapter 2, but limited evidence of AMF preference for nitrate or ammonium-N was seen in any experiment. N acquisition by AMF was highest in a Petri microcosm experiment when supplied as ammonium nitrate.

Functional diversity among AMF species was observed in terms of plant growth and nutrient uptake, and carbon acquisition from the plant: *G. aggregatum* was less beneficial to plant partners than *R. irregularis.* Experimental field-trial data suggests that soil nutrient levels influence both the community structure of AMF and the extent to which they engage in N-for-C trade with plant partners. These findings highlight the complexities and potential significance of the AM route for N uptake both in simplified experimental systems and full-scale commercial crop field trial plants.

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Author's declaration

I, Thomas James Thirkell declare that all the material contained within this thesis is a result of my own work and was written by myself. This work has not previously been presented for an award at this, or any other University. All sources are acknowledged as References.

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Chapter 1.
General Introduction

1.1. Rationale

The increased production of food crops over the past 100 years has allowed the global population to increase dramatically, and adding nutrients to the soil is essential in many agricultural systems. While the gains in crop output are undeniable, problems with the sustainability of modern agriculture are forcing a rethink in how land is managed. Soil erosion leads to losses of around 10 million acres of productive agricultural land per year (Montgomery, 2007), largely from poor management. Fertiliser application is partly responsible for this erosion, and increasing the efficiency of fertiliser application is a major target for making agriculture more sustainable. At present, only around 40 % of N fertiliser applied to arable land ends up in the crop plant (Sylvester-Bradley and Kindred, 2009), while much is lost to the environment through volatilisation or leaching in groundwater. A significant fraction is also acquired by soil microbes however, and immobilised in the soil. Certain microbes, such as arbuscular mycorrhizal fungi (AMF) are plant symbionts, and can transfer some of their acquired nutrient directly to their plant partners, including some of the most important crop plants. These fungi may be useful tool in agriculture and maximising the efficiency of crop nutrient uptake.

First it is important to consider the various forms of nutrients in the soils and the processes that control their subsequent availability for the crop. The various strategies plants have evolved to acquire these nutrients must also be considered. The situation is further complicated because the biotic and abiotic environments in which crop plants are grown are far removed from those in which the species and symbiosis evolved, and so their

relationship with soil microbes, for example may be shifted. This thesis focusses on one particular symbiosis, the arbuscular mycorrhizal (AM) symbiosis, formed between soil fungi of the Glomeromycota taxon and all major cereal crops, and how nitrogen (N) fertilisation might affect its function. It is first necessary to understand the essentials of crop nutrition with respect to this symbiosis, how plants may be nutrient limited and why the symbiosis is relevant to this. With the global population due to reach 10 billion and food production needing to double in the next 100 years, the need to understand how the most common crop plant symbiosis is affected by the most common agricultural practices is pressing.

1.2. Plant mineral nutrition

It became apparent at the end of the 19th century that certain mineral elements present in soils are required for plant growth and without which a plant cannot complete its lifecycle. It is now agreed that 14 elements constitute the list of essential mineral nutrients (Table 1.1) (Maathuis and Diatloff, 2013). The six nutrients that are constituents of organic molecules or required to maintain osmotic balance are needed in relative high quantities and are known as macronutrients (N, P, K, Ca, Mg, S). The remaining eight (Cl, B, Fe, Mn, Zn, Cu, Ni, Mo) are mostly required as constituents of enzymes and are therefore only needed in small amounts (Marschner, 2011). When plant demand for any of these nutrients exceeds supply in the soil, it can be said to be limiting to plant growth; by far the most likely nutrients to limit plant growth are N and P (Marschner, 2011). Although both these nutrients are present in soils in relatively high concentrations, fierce competition for N and relative immobility of P mean that uptake is often lower than required. Plant

strategies for acquisition of these nutrients include changes in root system architecture and symbioses with soil organisms.

1.2.1. Nitrogen as a plant nutrient

Depending on the tissue in question, around 1-5 % of the dry biomass of a plant is N (Marschner and Dell, 1994), and it is needed in the greatest quantity among all mineral nutrients (Table 1) and second only to carbon in terms of elemental abundance in plants. Nitrogen forms a part of all amino acids, peptides and proteins, as well as some nucleic acids and secondary metabolites and some signalling molecules (Maathuis and Diatloff, 2013). High content of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in plant shoots mean that the shoots are comparatively more N rich than roots (Maathuis and Diatloff, 2013). To understand N uptake by plants and why this may limit plant growth it is first necessary to discuss the forms of N in the soil and their availability to plants.

1.2.2. The Terrestrial N cycle

Forming 78 % by volume of the Earth's atmosphere, N abounds in all aerobic systems, but in its most common dimolecular form (N_2) it is inert and thus unavailable for uptake, metabolism or utilisation by almost all organisms. To become biologically available, the N must undergo fixation, where N_2 and hydrogen (H_2) are converted to ammonia (NH_3). The triple bond of N_2 is very strong and to break it uncatalysed requires significant amounts of energy; lightning is responsible for the fixation of around 10 Tg fixed nitrogen per year (Hill et al., 1980). The majority of N fixation is done by living organisms however, and prokaryotes known as the diazotrophs fix around 140 Tg N per year. This is performed using nitrogenase enzymes which catalyse the fixation of N_2 to NH_3. Some of this N is lost to the soil as NH_3 and may be acquired by other organisms.

While some diazotrophs such as *Azotobacter* bacteria are free-living in the soil, a subset of nitrogen-fixing bacteria known as *Rhizobia* are found living in symbiosis, contained within nodules which form on plant roots. Most species in the legume family Fabaceae and a few others form these symbioses, and allow significant increases in N fertility of soils in which they grow. In return for carbon, shelter, mineral nutrients and water, the carbon-fixing bacteria provide the plants with a source of N.

In the form of ammonium (NH_4^+), N is available for uptake by prokaryotes, plants and fungi. Some of this NH_4^+ is released to the soil as it leaks from the organisms, and more is released upon the death and decay of these.

Since human activity, especially the industrial evolution, N fixation has increased dramatically (Galloway 2003, Galloway 2005) and the N cycle has been significantly altered. Anthropogenic N fixation occurs as a result of combustion and by the Haber-Bosch process. The input of N to biologically available pools by biological N fixation at around 140 Tg per year compares to the 70 Tg per year which is fixed by anthropogenic activity (Bezdicek and Kennedy, 1988).

Biological fixation is one of the four principal reactions in the N-cycle (**Error! Reference s ource not found.**), in which N is metabolised through various forms by different organisms, and creating pools of N that are in constant flux both in time and space. Mineralisation, also known as ammonification, is another principal process in the N cycle by which NH_4^+ is formed. Complex organic matter is broken down into simpler organic forms by decomposers and saprotrophic organisms, before finally being reduced to NH_4^+, whereupon it becomes available for uptake by plants, fungi and soil microorganisms.

This NH_4^+ is often rapidly oxidised to nitrate (NO_3^-) by nitrifying bacteria such as *Nitrospira*, and Archaea (Leininger et al., 2006) in the third main reaction of the N cycle, nitrification. The extent to which nitrification takes place in aerobic soils is such that although almost all N enters the bio-available cycle as NH_4^+ or NH_3, by far the most common type of N in most soils is NO_3^- (Marschner, 2011). The exceptions to this generalisation are that in soils of low pH and reducing soil conditions promote NH_4^+ formation, while higher pH and aerobic soil will allow significant nitrification. The final main reaction of the N cycle is denitrification, where N is lost from the soil, after NO_3^- is reduced to N_2 by bacteria such as *Pseudomonas* and *Clostridium*. At this point, the N is once again in an inert form and is unavailable for uptake by anything except the diazotrophs. Denitrification is an anaerobic process and so is especially prevalent after waterlogging. Since nitrification is inhibited by anaerobic conditions, soils which are under water for long periods of time such as paddy fields do not lose significant amounts of N by denitrification as most of the N remains as NH_4^+.

1.2.3. Soil Nitrogen

The N cycle ensures a constant flux of N forms in aerobic soils, but the size of the pools from which N can be acquired by the plant are dynamic. Although variable with soil type and usage, around 95% of soil N is in complex organic form (Herbert, 1982) and as such is unavailable for uptake, and is not distributed homogenously throughout the soil, but is patchily spaced. Decay and decomposition of tissues or whole organisms and subsequent mineralisation of N are the processes by which N is returned to the soil in forms available to plants, and as such the temporal distribution of N is also heterogeneous.

As it is needed in such great quantities by all organisms and the pools of available N in the soils make up only a small fraction of the total (Marschner, 2011), N is very often limiting

to plant growth, and competition for its acquisition may be fierce. In unamended systems, the availability of N is controlled by soil microbes, which may be considered the gatekeepers of soil N (van der Heijden et al., 2008).

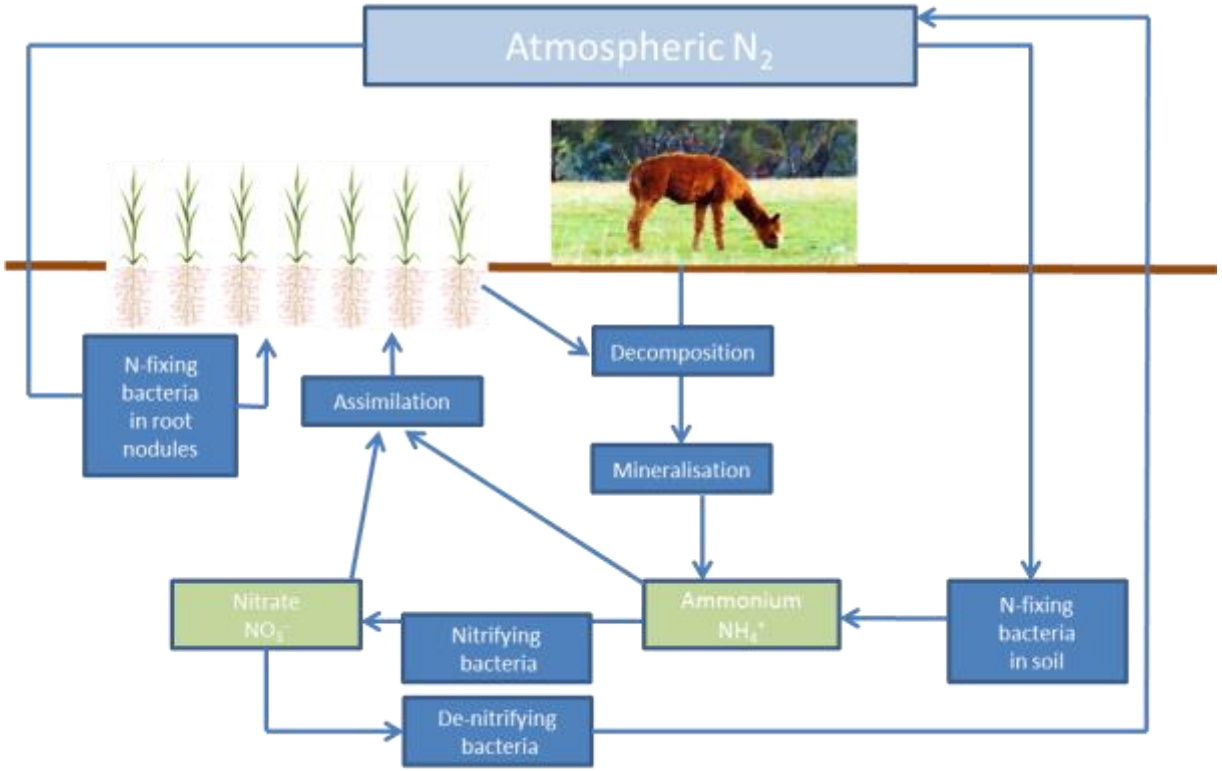

Figure 1.1. The Nitrogen (N) cycle. Atmospheric N_2 is fixed to ammonium (NH_4^+) by diazotrophs which are either free living or contained within nodules on plant roots. This makes the N biologically available for assimilation by plants and microbes. Decomposition and mineralisation release NH_4^+ from this organic matter returns NH_4^+ to the soil. Nitrifying bacteria convert a large proportion of $NH4^+$ into nitrate (NO_3^-) which may also be acquired by plants and microbes. Denitrifying bacteria reduce NO_3^- back to N_2.

1.2.4. Plant root N acquisition

Of the plant-available N in the soil, NO_3^- is the most mobile (Miller and Cramer, 2005) and the most abundant (Owen and Jones, 2001) and as such is the predominant form in which plants acquire N. The NO_3^- ion readily dissolves in soil solution and moves rapidly by mass flow and diffusion towards plant roots, drawn through the soil by the transpiration stream as water is lost through stomata aboveground (Tinker and Nye, 2000). At the root surface, low and high affinity nitrate transporters (Marschner, 2011) allow rapid N uptake from the soil solution.

Table 1.1. Average concentrations of mineral elements in dry biomass of plant shoots. The five most required nutrients, termed macroelements (in bold), are needed in significantly higher quantities than the others, known as microelements. Redrawn from Marschner 2011

Element	Chemical Symbol	mg g^{-1} plant DW
Nitrogen	**N**	**15**
Potassium	**K**	**10**
Calcium	**Ca**	**5**
Magnesium	**Mg**	**2**
Phosphorus	**P**	**2**
Sulfur	**S**	**1**
Chlorine	Cl	0.1
Iron	Fe	0.1
Manganese	Mn	0.05
Boron	B	0.02
Zinc	Zn	0.02
Copper	Cu	0.006
Nickel	Ni	0.0001
Molybdenum	Mo	0.0001

NH_4^+ ions are also soluble in water but adsorb to soil particles, meaning their movement through the soil is significantly reduced compared to NO_3^-, especially in dry soils (Tinker and Nye, 2000). Ammonium transporters are also found on plant roots (Forde and Clarkson, 1999). As discussed above, the relative abundance of NO_3^- or NH_4^+ in the soil

will depend on environmental conditions, and in response the prevalence of plant N transporters (NO_3^- vs NH_4^+) can vary accordingly (Hodge, 2004). There is more recent evidence that plants can acquire N directly from the soil as simple organic molecules such as amino acids (Svennerstam et al., 2011, Moran-Zuloaga et al., 2015) although probably not on scales to match that of inorganic uptake.

1.2.5. Phosphorus as a nutrient

As another essential mineral element, phosphorus (P) is also needed by plants to complete their lifecycle. Lipid membranes, DNA, and energy carrying ADP and ATP are all major ways in which plant P is used. Although not needed in comparable quantities to N, P is often the most growth-limiting nutrient (when N is not). As with soil N, relatively little of the P in soil can be acquired by plants. Organic P is seldom acquired by plants, but can make up 20-60% of total P (Conesa and Fardeau, 1982). In contrast to N entering the soil system via fixation by diazotrophs, P enters by the mineralisation and erosion of rocks by microbes and biogeochemical processes, and its availability is not limited by microbial action but by the soil chemistry of the most available form, orthophosphate (PO_4^{3-}).

1.2.6. Soil Phosphorus

Orthophosphate very readily forms complexes with metal cations in the soil which then bind to clay soil particles and are rendered immobile (Tinker and Nye, 2000). At acidic pH, the presence of Fe, Al, and Mn-P complexes may precipitate, while alkaline soils exhibit similar precipitation but with calcium or magnesium. A small proportion of the orthophosphate is more weakly sorbed to Fe, Mn, Al, Ca or Mg and is considered a labile pool. An even smaller fraction is in the soil solution, and it is this P which is available for plant uptake from the soil. As there is intense competition for this P and the equilibrium of labile and solution phase is so slow, P is very often limiting and its movement through the

soil is slow. The extent of this immobility is such that it led Conesa & Fardeau (1982), to remark that sufficient P resides in the top 50 cm of agricultural soils from previous additions to support cropping yield for a century or more without any P addition.

As discussed above, available soil P typically occurs at very low concentrations, usually in the range of 0.1 – 1.0 µM, far lower than P concentrations within roots (Marschner, 2011). High affinity transporters enable uptake from a sparse soluble P pool but cause a problem for the plant; the rates at which P is required to sustain plant growth are greater than the speed at which P moves through the soil. It is possible for the rate of uptake to outpace that of replacement from the bulk soil and so a depletion zone forms and further uptake is prevented, and the plant becomes P-limited. The depletion zone is sharply defined and may not extend more than a few millimetres into the soil, such is the effect of P immobility (Smith and Read, 2008).

1.2.7. Phosphorus acquisition

Plants have evolved several ways to increase their access to the P which is just beyond the reach of their roots. Changes to root system architecture are used widely, and plants grown in P-limited soils grow thinner roots which maximise the surface area of soil with which the roots come into contact and from which P uptake can take place. Cluster roots are a more extreme variation on this theme; very fine and densely branched root structures that form when P concentrations are very low. This clustering provides similarly highly increased surface area for P uptake. Cluster roots release exudative bursts of organic acids such as citric and malic acids which act as metal chelators to release labile P that is loosely sorbed to soil particles.

1.3. Mycorrhizas

While cluster roots are comparatively rare, the employment of symbiotic fungi to increase P uptake is all but ubiquitous. These symbioses are known as mycorrhizas (literally 'fungus root' from Greek, mykos = fungus, rhizo = root) and are found to occur in around 90 % of terrestrial plant species. There are various different kinds of mycorrhiza and, apart from a few exceptional types, they all exhibit the same premise; the plant provides photosynthetic carbon (C) to the fungus, which grows out from the root into the soil and acquires mineral nutrients and water, some of which it transfers to the plant. There are five types of mycorrhiza: ectomycorrhiza, arbutoid ectendomycorrhiza, ericoid, orchid and arbuscular mycorrhiza (Figure 1.2) (Selosse and Tacon, 1998): by far the most common being the arbuscular mycorrhiza (AM).

1.3.1 The Arbuscular Mycorrhizal symbiosis

Around 70% of terrestrial plant species form arbuscular mycorrhizal symbiosis with any of the 200 or so described morphospecies (Redecker and Raab, 2006) of soil fungi from the Glomeromycota phylum (Schussler et al., 2001). All cereal crops in western agriculture are capable of being colonised by AMF (Smith and Smith, 2011a). The fungus exists simultaneously in two separate environments; a portion inside the root, known as the intraradical mycelium (IRM), allows an exchange surfaces to form and nutrients to be transferred between plant and fungus, and the fungal hyphae outside the root known as the extraradical mycelium (ERM), which extends into the bulk soil, allowing more distal nutrient acquisition, and greatly increasing the surface area over which nutrient uptake can occur. Arbuscular mycorrhizal fungi (AMF) have been shown to be able to transfer P, N, S, Mg (Bucking and Kafle, 2015) as well as micronutrients Cu and Zn (Bolan, 1991) to their host plant. In addition to nutrient acquisition, AMF may aid plant defence against

pests and pathogens (Hooker and Black, 1995), improve drought tolerance (Davies et al., 1993) and even supress non-mycorrhizal competitors (Cameron, 2010). Plant carbon acquired by the fungal IRM is vital for the fungus and is thought to be its only significant source of C, making it an obligate biotroph (Jennings, 1995).

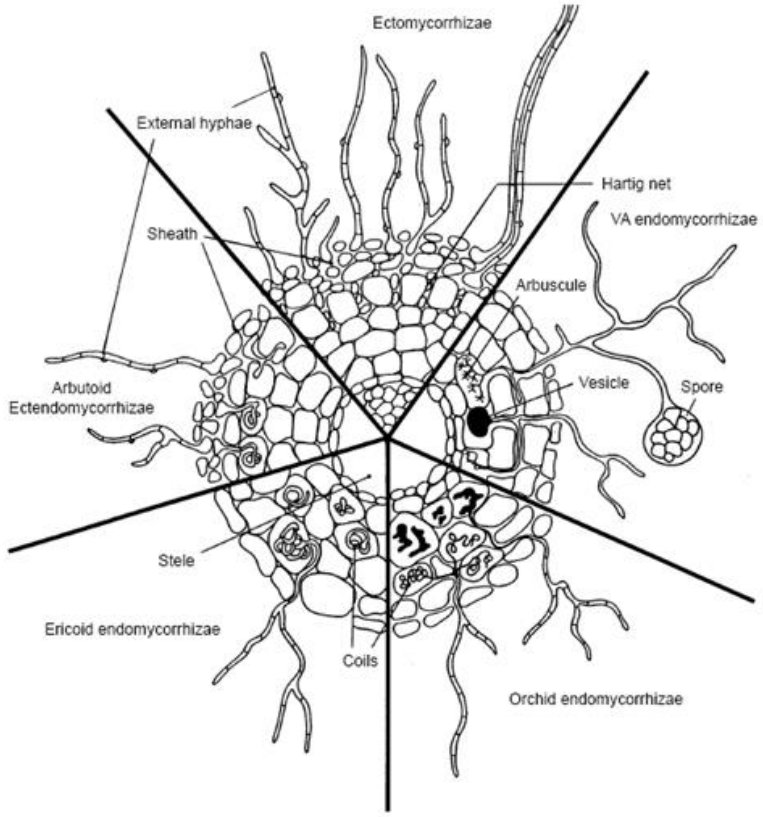

Figure 1.2. Defining fungal structures in roots colonised by the 5 types of mycorrhizas; orchid endomycorrhizas ericoid endomycorrhiza, arbutoid endectomycorrhiza, ectomycorrhizal and arbuscular mycorrhiza. (from Selosse & Tacon, 1998)

1.3.2. AMF physiology

The benefit of the AMF to plant nutrient acquisition is derived from the nature of the ERM and its ability to gain P which cannot be obtained by plant roots. The hyphal diameter of the ERM may be similar to that of root hairs (Gahoonia et al., 1997) but can extend far further into the soil, up to 25 cm (Jansa et al., 2003) compared to a few millimetres for root hairs (Gahoonia et al., 1997). The density of the ERM is also far

higher than that of roots, measured in one study as 2.6 m g^{-1} soil compared to 0.04 m g^{-1} for roots (Li et al., 2008). Such proliferation vastly increases the surface area of soil with which contact can be made and from which nutrient acquisition can occur. The improved uptake and translocation of P from beyond the rhizosphere afforded to the plant by the AMF is widely agreed to be the driving force behind the evolution of the symbiosis around 450 Mya (Simon et al., 1993), and its widespread occurrence in modern higher land plants.

The morphology of the IRM formed by AMF within roots of different fungal species show significant variation (Brundrett, 2004), but are defined by hyphal growth within and between cortical cells of the root, upon which highly branched, intracellular structures known as arbuscules (from Latin, arbor = tree) form (Brundrett, 2004). Further structures such as hyphal coils also exist and provide substantial interface between symbiont partners over which nutrient exchange can take place (Dickson, 2004, Dickson et al., 2007). Some AMF species also produce round, swollen structures known as vesicles which may act as fungal lipid stores within the roots (Brundrett, 2004).

The plasma membrane of plant cortical cells is not penetrated by the fungus, but by mutual control it becomes highly specialised, especially at the site of the arbuscule, where it forms a specialised structure known as the periarbuscular membrane (PAM) (Smith and Read, 2008). Phosphorus transporters (Pumplin and Harrison, 2009) located on the PAM indicate this to be a site of nutrient exchange from the fungus to the plant. Hexose transporters are expressed on intracellular hyphae and arbuscules and are thought to aid C acquisition by the AMF (Helber et al., 2011). Ammonium transporters have also been found to be expressed on the PAM too (Kobae et al., 2010, Guether et al., 2009), indicating a role for N uptake by the plant from the AMF.

Within a few centimetres of the same root, a plant may be colonised by multiple fungal species (Jansa et al., 2008), and the ERM of these fungi can colonise further plants creating a common mycelial network (CMN) in the soil. This may have significant implications for nutrient cycling and sink-source relations within the symbiosis (Walder and van der Heijden, 2015).

1.3.3. Multiple nutrient uptake pathways

Plants colonised by AMF have two nutrient uptake pathways (Bucking and Kafle, 2015): the direct pathway (DP), where low- and high-affinity nutrient transporters, or ion channels in the root epidermis or root hairs allow nutrient uptake from the rhizosphere (Gojon et al., 2009, Miller et al., 2009). Alternatively, the mycorrhizal pathway (MP), involves nutrient acquisition from the soil by transporters and ion channels of the ERM (Tisserant et al., 2012), after which the nutrients must be translocated through the ERM to the root, into the IRM, and then then released to the interface between the symbionts, where they may be acquired by inducible plant transporters (Koegel et al., 2013, Walder et al., 2016).

The conventional wisdom was that these two uptake pathways were additive and that they did not interact, but this view has widely been shown to be a simplification, as studies using isotopic P tracers have shown that the MP can dominate P supply to plants even when the total P content of an AM plant is not higher than in an non-mycorrhizal plant (Smith et al., 2003, Li et al., 2006). Further suggestion comes from the discovery that plant root phosphate transporters may be downregulated in colonised roots (Chiou et al., 2001, Grunwald et al., 2009), while AM-specific P transporters are upregulated in proximity to the hyphal interface (Harrison et al., 2002, Xu et al., 2007). The relationship between P uptake by the DP and MP is more complex than previously thought and remains unresolved.

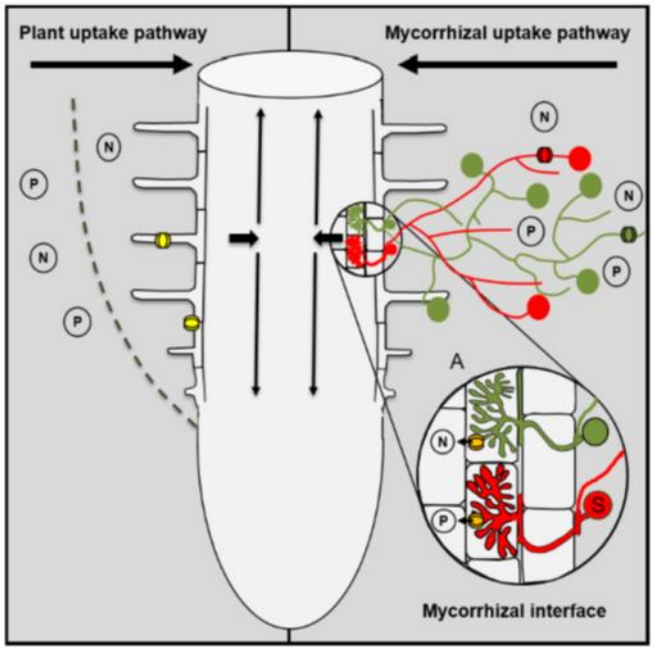

Figure 1.2. The comparison of the direct pathway (DP) and mycorrhizal pathway (MP) of nutrient uptake in a plant colonised by and arbuscular mycorrhizal fungus. DP (shown on the left of the root), where and the root transporters (yellow) allow uptake from the rhizosphere and around which a depletion zone of low nutrient concentration may form (dotted line). The MP (on the right) shows AMF proliferation beyond the rhizosphere to acquire distal N and P which is unavailable for uptake by the DP. From Bucking and Khafle, 2015

1.3.4. Arbuscular Mycorrhizal N uptake

Due to high the higher mobility of inorganic N compared to P in the soil, it was thought that the MP would not contribute to plant N acquisition, offering no advantage over the DP, as the depletion zone for N is far larger and less sharply defined as it is for P (Tinker and Nye, 2000). As NH_4^+ in soil moves more slowly than NO_3^- and may be bound to soil particles (Miller and Cramer, 2005), the MP may represent a mechanism for N uptake. Recent understanding of transporter activities supports this suggestion. Plant NH_4^+ transporters may also be downregulated in AM roots (Kobae et al., 2010) while AM-induced transporters may be upregulated by fungal colonisation (Guether et al., 2009, Gomez et al., 2009). These changes therefore strongly suggest a role in plant N uptake via the MP. Furthermore, the high affinity NH_4^+ transporter found in the ERM of AMF

(Perez-Tienda et al., 2012) suggests NH_4^+ uptake may be able to acquire N at lower concentrations in the soil than roots. Similarly, the AM plant may be able to improve a plant's ability to compete with microbes for N (Averill et al., 2014). The fine hyphal diameter and high abundance compared to roots may also help AMF exploit smaller microsites in the soil and in this way, acquire inorganic N before competing microbes or plants. The relative contributions of the MP and DP to N uptake have received relatively little experimental attention (Bucking and Kafle, 2015), and the question of how important the MP may be for N uptake in plants needs further resolution.

N transfer from AMF to plants has been reported for a relatively long time but due to the focus on P in the AM symbiosis, it was not considered to be a significant phenomenon. Studies have shown however than AMF can transfer to the plant significant quantities of N having acquired it from inorganic (Hawkins et al., 2000, Fellbaum et al., 2012) and organic sources (Leigh et al., 2009, Hodge and Fitter, 2010). Whether the MP could contribute enough N to increase total N content of partner plants is not well understood, and further investigation is needed to determine the conditions in which a plant might benefit from AM uptake of N.

N uptake by AMF probably takes place predominantly by NO_3^- (Hawkins et al., 2000, Tobar et al., 1994a) and NH_4^+ (Ames et al., 1983, Frey and Schuepp, 1993, Govindarajulu et al., 2005). NH_4^+ uptake is thought to be preferable as any NO_3^- acquired must be reduced to NH_4^+ before incorporation in the GS/GOGAT cycle (Toussaint et al., 2004, Jin et al., 2005), after which it is assimilated into arginine (Govindarajulu et al., 2005). It is thought that arginine is then transported through the ERM to the IRM, where it is catabolised and released into the plant-fungal interface as NH_4^+ and can then be acquired by the plant (Govindarajulu et al., 2005). Small scale acquisition of amino acids may occur (Whiteside et al., 2012a, Whiteside et al., 2012b), but not in significant quantities or when it can be avoided (Talbot and Treseder, 2010). The inability of AMF to acquire large

amount of organic nutrition is probably what forced them to be obligate symbionts (Smith and Read, 2008).

Although models of N uptake suggest that NH_4^+ should be energetically cheaper to acquire than NO_3^- , experimental evidence for this preference is not definitive. ERM transporters for NO_3^- and NH_4^+ have been identified by studies investigating transcriptome of AMF (Tisserant et al., 2012), suggesting uptake of both ions is possible, supporting the model of Govindarajulu et al. (2005). In addition, expression of high-affinity transporters has been found in the ERM for NH_4^+ (Lopez-Pedrosa et al., 2006) and NO_3^- (Tian et al., 2010). Experimental measurement of ^{15}N uptake by plants via AMF have shown that greater N acquisition may follow N added as NO_3^- (Hawkins and George, 2001; Ngwene et al., 2013) or NH_4^+ (Johansen et al., 1996; Tanaka and Yano, 2005). Clearly, further experimental evidence into AMF preference for N source is required to approach resolution of these questions.

1.4. Control of AMF nutrient trade

High functional diversity between AMF isolates has been reported both for the amount of P transferred (Avio et al., 2006) and the growth response of the plant following AMF colonisation (Klironomos, 2003). There is now evidence that AMF isolates also show diversity in the degree to which they engage in C-for-N trade with the plant (Mensah et al., 2015), as some plant N acquisition is decreased by colonisation, some is improved (Saia et al., 2014), and some unaffected (Hawkins and George, 1999).

The range of responses that AMF may have on plant growth and nutrient uptake has given rise to a number of explanatory models or conceptual frameworks. First, the biological markets model (Kiers et al., 2011), which arose from work on inoculated monoxenic root

cultures, suggests that nutrient trade is governed by rules akin to a biological market. According to the model, nutrient trade (e.g. C-for-P, C-for-N) is done on strict terms under the control of the plant, and because of which, 'cheating' fungi attempting to obtain C for no mineral nutrient cost are excluded. The model predicts that exchange should take place on a 'reciprocal rewards' basis. Such a market system certainly cannot occur in all scenarios however, as mycoheterotrophic plants gain all their nutrition from AMF. Work on common mycelial networks (CMN), where multiple plants are colonised (and thereby connected) by fungal symbionts (Walder et al., 2016) has shown that reciprocal rewards do not always occur. Instead of reciprocal rewards, Walder and van der Heijden (2015) propose a less strictly-controlled system where sink strength of the plant and fungus, competition for resources and the functional diversity between plant and fungal partners drives the symbiosis. The debate as to which model best suits is still very much a contentious issue (Kiers et al., 2016, van der Heijden and Walder, 2016) and further work is needed to quantify flow of nutrients between partners to determine the extent to which these models fit the observed trends.

A further model by Johnson (2010) suggests that the relative availability of soil N and P will govern the extent to which the plant receives a nutritional benefit from the symbiosis, or whether the fungus acts more as a parasite, and reduces plant performance (Johnson et al., 1997). This 'trade balance model' (TBM) states that colonisation by AMF will be most beneficial for both partners when there is limiting P availability, and luxury amounts of N. In this case, plant P uptake by the MP will be strongly beneficial as the DP would be insufficient, and luxury N supply means that neither plant photosynthesis (hence C supply to the fungal partner) or fungal growth will be limited, allowing positive feedback of nutrient trade to establish in the system to both partners' benefit. Conversely, the worst scenario for both partners would be high-P and low-N, in which case plant photosynthesis may be N-limited, and P uptake by the MP is likely to offer little advantage over the DP.

Under these circumstances N transfer from the fungus to the plant is unlikely to occur as the fungus (like the plant) has a very high N demand (Hodge and Fitter, 2010) and will satisfy its own needs for N before attempting C-for-N trade with the plant (Fitter, 2006). Unfortunately for the plant, the fungus may still acquire apoplast hexose sugars with high affinity transporters (Helber et al., 2011), thus depriving the roots of C without contributing N or P. Limited testing of the TBM has taken place since its publication, except for Johnson et al. (2015) who showed that fertilisation of grassland ecosystems using inorganic N was beneficial for plant and mycorrhizal growth. The contribution of the MP to the plants' nutrient acquisition could not be quantified in that study however, and the model needs further experimental testing using different combinations of plant, fungus and nutrient addition.

Strong preference for N source may well influence the amount of N which is acquired by the AMF and affect whether they are N limited or N sated. The trade balance model, the biological market model and the model of Walder and van der Heijden (2015) all suggest that N satiety in the AMF is required for the mutualistic function of the symbiosis. N source may then be a further mechanism by which the symbiosis is controlled and the extent to which nutrient trade is mutualistic or if the AMF acts more parasitically.

1.5. Crop plants and AMF

Nutrient trade in the AM symbiosis, specifically that of N, is more than a mere curiosity in plant biology, as almost all arable crops (with the notable exception of the Brassicaceae) form AMs (Smith and Read, 2008). Inoculum for AMF is found in abundance in temperate soils, especially at northern latitudes (Figure 1.3). Sowing arable crops will mean roots almost certainly become colonised to some extent; it is exceptional for plants which are capable of being mycorrhizal not to be mycorrhizal, unless excluded by

environmental conditions (Smith and Smith, 2011b). Considering AMF can comprise 20 % or more of the microbial biomass in soils (Leake et al., 2004), they are doubtless important in nutrient cycling in these systems (Hodge and Storer, 2015).

1.5.1. AMF for sustainable agriculture

By changing the physical, chemical, and biological aspects of soil, modern agricultural practice reduces the potential for AMF mycelia to establish (Helgason et al., 1998), and the AMF communities may be altered from what might be expected in an unmanaged habitat (Daniell et al., 2001). Functional diversity in AMF species, as mentioned above, may mean that such shifts in fungal community brought about by management practice may further alter symbiotic function of AMF in agricultural systems.

Furthermore, modern cultivars of agricultural plants with which the AMF engage in symbiosis are the result of thousands of years of cultivation, and are radically different from the ancestors from which they have been developed (Diamond, 2002). Changes to the source sink relationships in these modern plants, particularly nutrient uptake and senescence (Thomas et al., 2009), may influence the symbiotic function of the fungus. This may in part explain why cereal crops often suffer growth depression following colonisation by AMF while wild grasses may benefit (Smith and Smith, 2011b). Further work is needed to determine the roles of agricultural practice may have in AM plant nutrient dynamics.

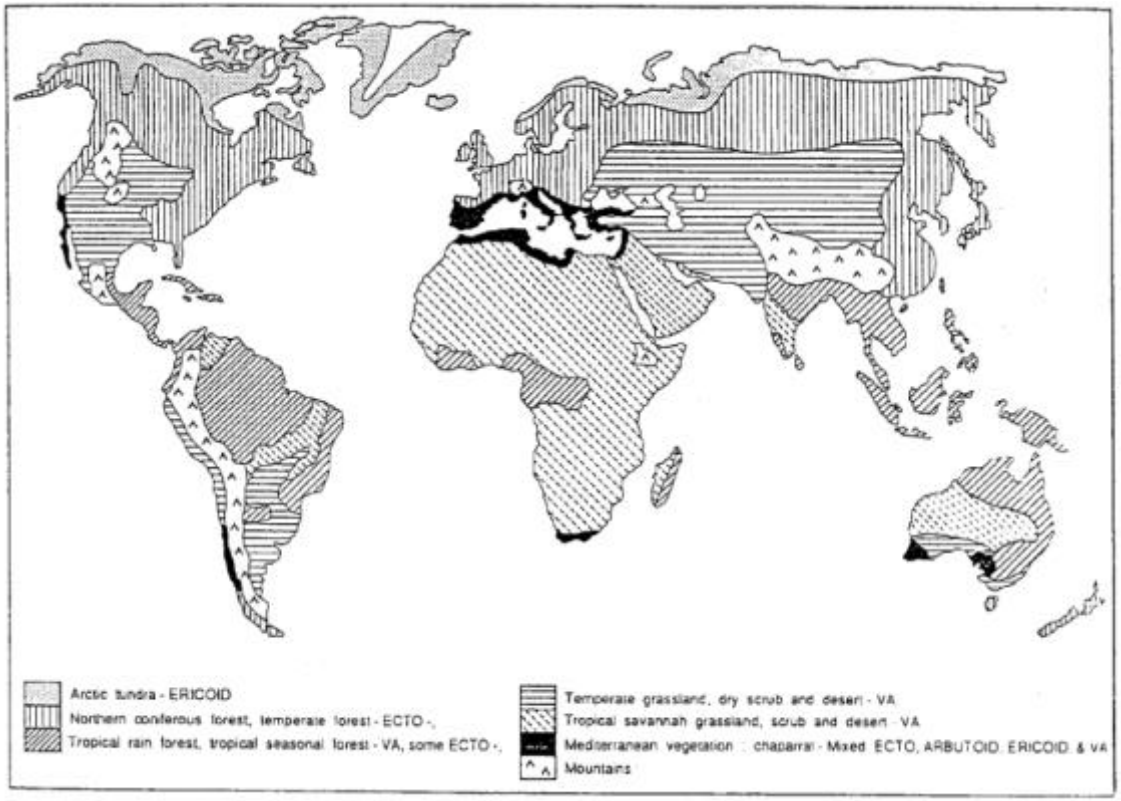

Figure 1.3. The distribution of major terrestrial biomes around the world, and the dominant mycorrhizal type associated with the system. Note that land which would have been forested in 'northern coniferous forest' has largely been cultivated for the production of cereal crops, and now AMF will dominate in these areas. From Read (1991).

1.6. AMF uptake of fertiliser N

Fertilisation of agricultural soils has taken place for as long as agriculture itself (Smil, 2001), and originally would involve the addition of manure, compost, blood, bones and any other nutrient-rich detritus available. Fertilisation with organic matter is still carried out on a significant scale (Smil, 2001), using manure, effluent, compost and seaweed-based fertilisers. AMF are known to be able to acquire N from organic matter patches and transfer substantial amounts to their partner plants (Leigh et al., 2009, Hodge and Fitter, 2010, Nuccio et al., 2013), but to do so in quantities sufficient to increase total plant N uptake or to bring about growth increases is not commonly reported. As decomposing organic matter releases NH_4^+, which is thought to be the preferred source of N for AMF (Jin et al., 2012), it should be possible for organic matter addition to bring about such benefits for plant nutrition and growth.

Modern fertiliser application is a very different process, notably using industrially produced fertilisers. Around 200 Mt of inorganic fertiliser alone is applied to agricultural land around the world each year (FAOSTAT, 2015), of which around a half is N fertilizer. The majority of inorganic N added contains or evolves, NH_4^+ and/or NO_3^- to the soil (Table 1.2). The choice of which fertiliser to use for a particular application may be complex. Ammoniacal fertilisers tend to reduce soil pH (Fox and Hoffman, 1981) and so are suitable for alkaline soils, while NO_3^- fertilisers may raise soil pH and so may be better on neutral / acid soils (Tagliavini et al., 1995). The high mobility of NO_3^- means that excess application may result in leaching from the soils into water courses, where it may cause environmental health problems such as eutrophication (Rivett et al., 2008), and if in drinking water, can cause human health concerns (Croll and Hayes, 1988). Addition of NH_4^+ evolving fertilisers may allow significant losses due to volatilisation of NH_3.

Table 1.2 Most commonly used forms of N fertiliser in modern western agriculture. Redrawn from Smil (2001).

Compound	Formula	% N by mass	N form evolved
Ammonia	NH_3	82.4	NH_4^+
Ammonium Nitrate	NH_4NO_3	35	NH_4^+ NO_3^-
Ammonium Sulphate	NH_4SO_4	21.2	NH_4^+
Calcium nitrate	$CaNO_3$	15.5	NO_3^-
Urea	$CO(NH_2)_2$	45	NH_4^+

The decision of which fertiliser to apply to crop plants rarely (if ever) takes into account the effect it may have on the soil microbial community, including AMF. However, N fertilisation may have significant implications not only for AMF community structure but also its function, including the extent to which symbiotic nutrient exchange occurs.

1.7. Studying AM symbioses

As obligate biotrophs, AMF cannot be cultured to complete their lifecycle in isolation, and as such their nutrient dynamics can only be considered when colonising a plant root. Nutritional benefits afforded to the plant by the AMF have been demonstrated in a number of ways, mostly with the aim to exclude any nutrient uptake gained by the MP. Isotope tracers including ^{15}N, ^{13}C and ^{14}C, ^{32}P and ^{33}P have been used extensively to quantify

nutrient transfer (Smith and Read, 2008, Smith and Smith, 2011b). Many studies use uninoculated plants in microcosm studies (Hodge et al., 2001), using compartmented microcosms where hyphae may cross a fine mesh barrier to reach a compartment containing nutrients to which the roots have no access, or adding fungicide to field soil and comparing nutrient uptake in plants with those on untreated sites.

At the finer scale, T-DNA transformed roots may been grown monoxenically (Cranenbrouck et al., 2005) so that any other organisms are excluded, and the more mechanistic details of nutrient trade, molecular and genetic processes might be elucidated. Neither end of the scale of study is ideal, as mechanistic studies necessarily exclude many of the biotic and abiotic interactions the AMF might encounter, while such interactions may cloud interpretation of data from field scale studies (Read, 2003). A combined approach of microcosm and field studies using the same isolates of AMF may allow some complementarity between designs and clearer patterns to be seen in nutrient exchange and symbiotic function.

1.8. Aims and hypotheses

N uptake via the MP to plants has been repeatedly demonstrated and is almost certain to take place at more than incidental levels, as AM specific transporters for NH_4^+ are known to exist in colonised root cells. AMF are known to be able to acquire N from organic and inorganic sources but uptake is thought to occur predominantly as NH_4^+ and NO_3^-, although low levels of organic N uptake may occur. Whether a significant preference for N source is exhibited by the AMF is unclear. Studies using different experimental approaches and AMF isolates mean that comparisons that find different sources of N to be preferred are difficult to compare (Hawkins and George, 1999, Tanaka and Yano, 2005). Various models have been proposed to describe regulating nutrient trade but different

research groups are far from reaching consensus on which best describes the control of mycorrhizal function in AM systems.

Questions remain unanswered as to which N source is preferred by AMF, how N addition levels affect nutrient trade between partners, how N addition affects microbial communities in soils and whether trends seen in microcosm experiments translate to field-scale experiments. Possible interactions between these factors have been identified and are in need of investigation.

Using a combination of experiments of different scales and levels of complexity, from Petri plates to field studies, and using a range of analytical techniques including isotope tracers, elemental analysis and molecular tools, the following hypotheses were investigated to determine the influence of N source on the nutrition of arbuscular mycorrhizal plants:

1. N uptake from organic matter by the MP will contribute sufficient N to a partner plant so that total N content and biomass increase, provided the P content of the growing substrate is limiting to plant growth.
2. AMF acquisition of N will be higher when provided as NH_4^+ over NO_3^- as this should represent an energetically more attractive N form for the fungus than NO_3^- As such, greater amounts of ERM will be observed when fungi receive NH_4^+ than NO_3^-. As a consequence, AMF are more likely to be sated for N when receiving NH_4^+ and N transfer to the plant will be higher than from AMF receiving NO_3^-.
3. N transfer to a plant partner takes place by reciprocal rewards, and a plant receiving more N from an AMF will repay with more C.
4. The structure and function of AMF in a field setting will be influenced by the interactive effects of N application rate and form.

Chapter 2.
Resolving the 'nitrogen paradox' of arbuscular mycorrhizas: fertilization with organic matter brings considerable benefits for plant nutrition and growth

2.1 Introduction

The arbuscular mycorrhizal (AM) association is the most common type of mycorrhizal symbiosis and forms between c. two-thirds of all land plant species and soil fungi in the phylum Glomeromycota. The fungus receives photosynthetically fixed carbon (C) while, in return, the fungus confers a number of benefits to its associated host plant, the most well-established being that of increased acquisition of phosphorus (P) (Smith and Read, 2008). More recently, however, there has been renewed interest in the ability of arbuscular mycorrhizal fungi (AMF) to supply nitrogen (N) to their associated host plant and the implications this may have for N cycling (reviewed by Hodge and Storer (2015)).

While it has been shown that AMF can transfer N to their associated host (Ames et al., 1983, Hodge et al., 2001, Barrett et al., 2011) significant doubts remain as to the ecological relevance of such a AMF-N uptake pathway (Read, 1991, Smith and Smith, 2011b). In particular, regarding the exact mechanism of N transfer and, more importantly, the amounts of N transferred via the AMF compared to the N requirements of the plant (Smith and Smith, 2011b). Although results from root organ culture studies suggest values of up to 50 % of root N may be acquired via the AMF route (Govindarajulu et al., 2005), ideal as these systems are for unpicking mechanisms involved in nutrient exchange, it may be unwise to infer much about whole plant nutrient dynamics. Source-sink relationships, for example, are undoubtedly unrealistic given the growth conditions employed (Smith

and Smith, 2015). More realistic experiments, using whole plants and adding N as organic matter patches, have shown that AMF contribution to plant N uptake can be as high as 15-20% (Leigh et al., 2009, Barrett et al., 2014). Although this may suggest a significant nutritional contribution to the plant, the total plant N content (Leigh et al., 2009, Hodge, 2001, Hodge et al., 2000a), and plant biomass (Herman et al., 2012, Hodge et al., 2001) is usually unaffected. In some cases, the plant may even suffer a reduction in biomass (Reynolds et al., 2005), implying providing N fertilization to N-limited symbioses may be deleterious.

Johnson (2010) proposed the 'trade balance model' to explain the apparent 'nitrogen paradox', where nitrogen fertilisation of AM plants causes apparent mycorrhizal parasitism of partner plants. Fundamentally, the model states that the relative supply of C from the plant and availability of N and P in the soil determines the extent to which the AM-route for N uptake is mutually beneficial. The model suggests that fertilization with N is only beneficial if the plant is limited by P and will therefore benefit from providing C to the roots and mycorrhizal fungi.

Positive growth responses to N fertilization have been shown in plants receiving inorganic N inputs (Johnson et al., 2015), but corroborating evidence for a mycorrhiza-mediated plant growth response after being fertilized with organic N is lacking. Addressing this knowledge gap is now pressing, given the ecological role of AMs in nitrogen cycling (Hodge, 2014, Hodge and Storer, 2015) and the nature of soil N. Most rhizosphere N is bound in complex, organic material (Bremner, 1949, Stevenson, 1994) and only a small, ephemeral pool of inorganic nitrogen exists at any given time, and inorganic N turnover in soil is rapid (Jackson et al., 1989). The integrity of the trade balance model in systems fertilized with organic N is thus far untested.

Organic N fertilisation is receiving increased attention in both research and agriculture with the adoption of more sustainable agricultural practices not only in Europe but across the world (Matson et al., 1997). Inorganic N fertilization may reduce mycorrhizal inoculum potential of agricultural soil (Liu et al., 2012) increase pathogen severity (Huber, 1981 cited in Matson et al. (1997)), and boost greenhouse gas fluxes from agricultural soils (McSwiney and Robertson, 2005). Combining organic N fertilisation and the mycorrhizal symbioses may be useful in negating some of these problems and increasing assimilation of fertilizer N into plants which is currently limited to around 40-60% in crop plants to which inorganic N is applied (Huber and Watson, 1974, Paustian et al., 1995).

Given the abundance of AMF in temperate soils and the range of plant species they may colonise (Smith and Read, 2008), it is surprising that we do not comprehensively know which forms of soil nitrogen can be utilised by the fungus. It is well established that AMF acquire inorganic N as NH_4^+ and NO_3^- (Govindarajulu et al., 2005, Leigh et al., 2011, Johnson, 2010) , which represent the most abundant available inorganic sources in the hyphosphere (Tinker and Nye, 2000). How commonly AMF utilise organic N directly is less well known. Experiments have shown that AMF may be capable of direct glycine uptake (Hawkins et al., 2000, Whiteside et al., 2012b, Hodge, 2001) and Cappallazzo et al. (2008) identified an amino acid permease in *Glomus mosseae*, a mechanism by which an AMF may acquire organic N directly from soil substrates. Similarly, Belmondo *et al*. (2014) show evidence for potential uptake of organic N by a dipeptide transporter in the extraradical mycelium of *Rhizophagus irregularis*. However, AMF seem not to acquire organic N exclusively or indeed preferentially, as ^{13}C enrichment is usually not detected in AM hyphae or plant tissue following hyphal access to ^{13}C:^{15}N dual labelled organic matter (Hodge and Fitter, 2010, Nuccio et al., 2013).

By their very nature, complex organic matter patches contain a mixture of organic and inorganic sources of N. Both inorganic N and the simplest organic N components are

likely to be relatively labile and more easily mobile in the soil than larger organic, nitrogenous constituents (Nemeth et al., 1987). In microcosm experiments with separate root and hyphal compartments, the potential for the N-rich, labile fraction from organic matter patches to leach from one compartment to another presents uncertainty. This is compounded as there remains in the literature a lack of patch analysis to show the relative composition of the patch (organic vs. inorganic N).

In this experiment a patch of ^{15}N labelled algal material was used in order that the amount of N acquired by the plant from the patch could be measured. Algae was used owing to its low C:N ratio of 7:1, representing a rich N source. Compartmented microcosms were employed to investigate the effect of a discrete zone or 'patch' of N-rich organic matter to a sand and clay growth medium of low-N and low-P availability. Mycorrhizal plants were contained in one compartment while AMF hyphae were permitted access to a second compartment containing the algal patch. Control microcosms in which the AMF could not access the patch were included in order that N movement via mass flow and diffusion could be determined.

2.2 Materials and Methods

2.2.1 Microcosm Design

Microcosm units were constructed by fastening together two polypropylene boxes, adapted from Hodge & Fitter (2010). The plant compartment measured 7 x 14 x 16 cm, and the patch compartment 14 x 14 x 16 cm. A window cut in the abutting sides of the boxes created an aperture (4 x 6 cm) that was covered with a double-ply mesh barrier. The 'AMA' (Arbuscular Mycorrhizal Access) units used a 20 μm mesh barrier, which prevented root access but allowed AMF hyphal access (John Stanier and Co., Whitefield,

Manchester, UK). The 'NAMA' (No Arbuscular Mycorrhizal Access) units used a 0.45 µm mesh (Anachem, Bedfordshire, UK) that prevented the access of both roots and AMF hyphae to the patch compartment. This 0.45 µm mesh barrier does not retard the diffusion of solutes from the patch compartment to the plant compartment, but the AMF mycelium cannot encounter the organic matter patch directly in NAMA microcosms. Into the bottom of each compartment, four holes were drilled and covered with 20 µm mesh to permit drainage. Both compartments of the microcosms were filled with a 1:1 (v/v) mix of silica sand and AgSorb® (a calcinated, attapulgite clay soil conditioner, Oil-Dri, Cambridgeshire, UK (formerly TerraGreen®; see Hodge *et al*.(2000a)). Both sand and AgSorb® were washed 3 times in de-ionized water prior to mixing, in order to minimize mobile mineral ions in the growth medium. Within the patch compartment of the microcosm units, the organic matter was contained inside a PVC pipe of diameter 2 cm and height 7 cm, which has two windows cut into the sides, creating two apertures each with dimensions 4 cm (H) x 1 cm (W). These apertures were covered in the same 20 µm mesh as detailed above (and see Field et al. (2012)). Such a setup ensures a uniform patch size across all microcosms, permits AMF hyphal access and allows easy placement of the organic matter patch (Figure 2.1).

Plantago lanceolata L. was selected as the host plant owing to its ability to become highly colonised by AMF. Seeds of *P. lanceolata* (Emorsgate Wild seeds, Nottingham, UK) were surface sterilised in a 5% (w/v) calcium hypochlorite solution, after which they were germinated on filter paper in a sterile Petri dish. At week 0, 2-week-old seedlings were transferred into each plant compartment, 4 cm from the mesh aperture (3 seedlings were planted into each microcosm, subsequently thinned to a single seedling at week 2). The plant compartment was watered daily, as required, with de-ionized water.

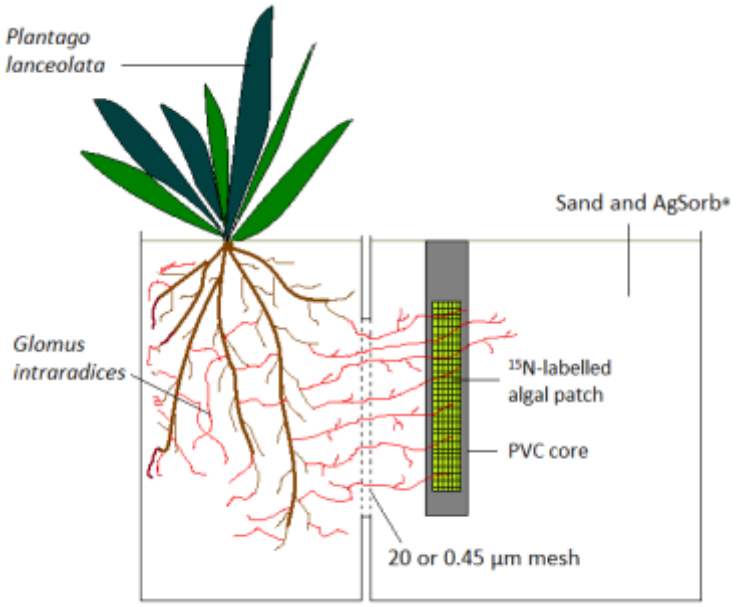

Figure 2.1 Diagram of the microcosm design. An individual *Plantago lanceolata* plant, colonised by *Glomus intraradices* was contained within one compartment of the microcosm, and a ^{15}N-labelled organic matter patch was placed into an adjoining patch compartment. Patches were contained within a PVC core, and retained within mesh sides which allow arbuscular mycorrhizal fungal (AMF) hyphal entry. Half of the microcosm units contained a 0.45 µm mesh rather than a 20 µm mesh, to prevent the roots and the AMF hyphae crossing from the plant compartment to the patch compartment. This allowed for any mass flow and diffusion of ^{15}N from the patch across the barrier to be accounted for, rather than genuine transfer via the AMF hyphae.

Except for an aperture through which the plants grew, microcosms were enveloped in aluminium foil to reduce the influx of contaminating organisms. The microcosms were planted on August 30th, 2013, and maintained in a heated, lit glasshouse and re-randomised weekly to avoid environmental artefacts. From planting to harvesting, the experiment ran for 23 weeks.

2.2.2 AMF Inoculum

Into the plant compartment, 50 g of *Glomus intraradices* inoculum comprising macerated *P. lanceolata* and *Trifolium repens* L roots inoculated with *G. intraradices* (isolate BB-E; Biorhize, Dijon, France) and growth medium (sand and AgSorb® mix described above)

was added. The mycorrhizal inoculum was 9 months old when added to microcosms. While we acknowledge that changes in the nomenclature of AMF species have been recommended (see Redecker *et al*., (2006)), here, we retain the previous name '*G. intraradices*', given that the exact phylogenetic position of this particular isolate is uncertain.

2.2.3 Nutrient Addition

Each week, the plant compartment of each microcosm received 50 ml of a low-N and low-P nutrient solution (Leigh et al., 2009) containing 2.5 mmol l^{-1} N as NH_4NO_3 and 0.034 mmol l^{-1} P as NaH_2PO_4. The pH of this nutrient solution was adjusted to 7.0 with KOH. The plant compartment also received 0.25 g l^{-1} bone meal (Vitax, Leicestershire, UK), a complex N and P source which encourages AM establishment (Hodge and Fitter, 2010). Bone meal was added only once, at the start of the experiment. Over the course of the experiment, the nutrient solution added to the plant compartment provided 112 mg N and 2.16 mg P. The bone meal provided 23 mg N and 58 mg P. The patch compartment received no further nutrient additions after the patches had been placed.

2.2.4 Patch Material

After 16 weeks of plant growth in the microcosms, patches of organic litter were added to the patch compartment, 6 cm away from the mesh aperture between compartments. Each patch contained 0.075 g of 98 Atom% ^{15}N-labelled algae (obtained from Sigma-Aldrich, St Louis, MO, USA) in a matrix of 0.8 g homogenised algal matter (*Chlorella variabilis* - PinkSun Essentials and Organics, Clayton, Yorkshire, UK). The patch contained 59 mg N (8.85 mg of which was ^{15}N), 26 mg P, 413 mg C, and the C:N ratio of the organic matter patch was 7:1.

The organic patch was mixed into 20 g of the silica sand: AgSorb® mix, which was then placed into the PVC pipe, filling the bottom 5 cm. The remaining 2 cm of PVC core was filled with the sand: AgSorb® growth medium only. The PVC pipe was placed into the patch compartment to a depth of 7 cm, such that the top of the core was flush with the level of the growth medium in which it sat.

Labile nitrogen as ammonium or nitrate in the algal patch was quantified by spectrophotometer (CECIL 100 spectrophotometer, Spectronic Analytical Instruments, Leeds, UK) and calculation from standard curve, created using standards containing 10 mg N l^{-1} made from NH_4Cl and KNO_3. Briefly, 0.2 g algal material was mixed with 10 ml de-ionised water and incubated for 60 minutes at 50 °C. This preparation was then centrifuged at 5,000 *g* for 15 minutes, after which the supernatant was decanted. Labile nitrate was measured as detailed in Cataldo *et al*. (1975). Briefly, a 0.2 ml aliquot of supernatant was placed in a 50 ml Erlenmeyer flask, to which 0.8 ml 5% (w/v) salicylic acid in > 96 % (v/v) H_2SO_4 was added. After cooling to 20 °C the flask received 19 ml of 2 M NaOH to raise the pH above 12. Absorbance was measured at 410 nm, after samples had cooled to 20 °C. Labile ammonium quantification required the use of the solutions 'A' and 'B', with details of preparation given below. A 0.05 ml aliquot of supernatant was mixed with 1 ml 'solution A', 0.25 ml 'solution B' and 2.5 ml de-ionised H_2O. Both solutions 'A' and 'B' were prepared using de-ionised water. Solution A contained 20 g trisodium citrate dihydrate, 17 g salicylic acid, 5 g NaOH, and 0.2 g sodium nitroprusside, made up to 500 ml. Solution B, also made up to 500 ml, contained 5 g NaOH and 0.4 g dichlorosyonurate. Absorbance was measured by spectrophotometer at 650 nm.

2.2.5 Harvest and Analysis

At 23 weeks after planting, the systems were destructively harvested. The *P. lanceolata* was separated into shoots and roots, and dried at 80°C for 48 hours. A subsample of the

extracted roots was retained to assess root length colonisation by the AMF. After drying, root and shoot material was ground and homogenized in a ball mill (Retsch MM400, Retsch GmbH, Haan, Germany), for analysis by Isotope Ratio Mass Spectrometry (PDZ 2020, Sercon Ltd, Crewe, UK).

Phosphorus content was measured using X-Ray fluorescence spectrometry (XRF). Briefly, dried plant material was milled and homogenised as described above, before being pressed into a pellet and analysed with a portable X-ray fluorescence spectrometer (Reidinger et al., 2012)

Mycorrhizal roots were stained using the method of Kormanik & McGraw (1982). Roots were cleared in 10% (w/v) KOH, acidified in 1% (v/v) HCl, stained with acid fuchsin and then stored in destain solution (lactic acid, glycerol, distilled H_2O 10:1:1). All procedures were incubated at 20°C, as per the 'no heating' variation of the method, detailed by Kormanik & McGraw (1982).

To quantify AMF extraradical mycelium, 5 g samples of growth medium were taken from the plant compartment, from within the PVC pipe containing the organic matter patch, and from the 'bulk' growth medium (i.e. within the patch compartment but outside the PVC core). Hyphal extraction was carried out by the modified membrane filter technique of Staddon *et al* (1999), and hyphal length assessed using the gridline intercept method from which hyphal length densities were then calculated (Hodge, 2003).

2.2.6 Statistical Analysis

All data were analysed using SPSS 21 (IBM SPSS Inc. Armonk, NY, USA). Before proceeding with statistical analysis, data were tested using Levene's test for equality of variance and Kolmogorov-Smirnov and Shapiro-Wilk tests or normality. Where these tests

were not satisfied, data were log-transformed. Percentage data were converted to a proportion and then square root-arcsine transformed before analysis. Data for HLD in bulk vs plant compartments within individual microcosm units were analysed using Paired-Sample T-Tests as they represent portions of the same AMF mycelium, while all other data were analysed using Independent-Samples T-Test.

2.3 Results

Total plant dry weight increased substantially when AMF hyphae were allowed access to the patch compartment (3.44 ± 0.21 g with access versus 2.09 ± 0.23 g without access, $T_{1,37} = 4.33$, $P < 0.001$). This increase in plant dry weight was driven by an increase in both the shoot and root mass, which increased by 62% and 73% respectively compared with those plants whose AMF partner was not permitted access to the organic patch (Fig. 2.2). There was however, no significant difference in the root weight ratio (RWR; ratio of root dry weight to total plant dry weight) between any treatments, suggesting that allocation of biomass between roots and shoots did not change as a result of the plants' AMF partner having access to the organic material substrate.

Allowing AMF hyphal access to the patch greatly increased the plant uptake of ^{15}N, measured both in the shoot and the root of the partner plants (Table 2.1). In total, plants with AMF access to the patch contained 1.10 ± 0.25 mg ^{15}N compared with 0.34 ± 0.12 mg ($T_{1,37} = 4.91$, $P < 0.001$) in the plants whose AMF partner was denied access to the patch. The presence of ^{15}N in the No AMF Access microcosm plants (Table 1) is ascribed to the mass flow and diffusion of ^{15}N-containing molecules through the 0.45 µm mesh from the patch to the plant compartment. Such a difference in plant ^{15}N content between AMF Access and No AMF Access microcosms highlights the important role AMF can play in nutrient acquisition from nutrient-rich areas placed at significant distances beyond

the rhizosphere. Similarly, the contribution made by patch N to overall plant N was greatly increased when plants had AMF access to the patch (Fig. 2.3): 18 ± 3%, compared to 9 ± 1% ($T_{1,37} = 3.57$, $P = 0.001$). *P. lanceolata* benefitted greatly from this AMF contribution acquired from the patch as demonstrated by the 68% increase in shoot N content when AMF had access to the patch compartment, corroborated by an 80% increase in root N content (Table 2.1). Thus, total N in the whole plant was increased 76%. Although total plant N content increased, plant N concentration was not significantly different between the two AMF access treatments.

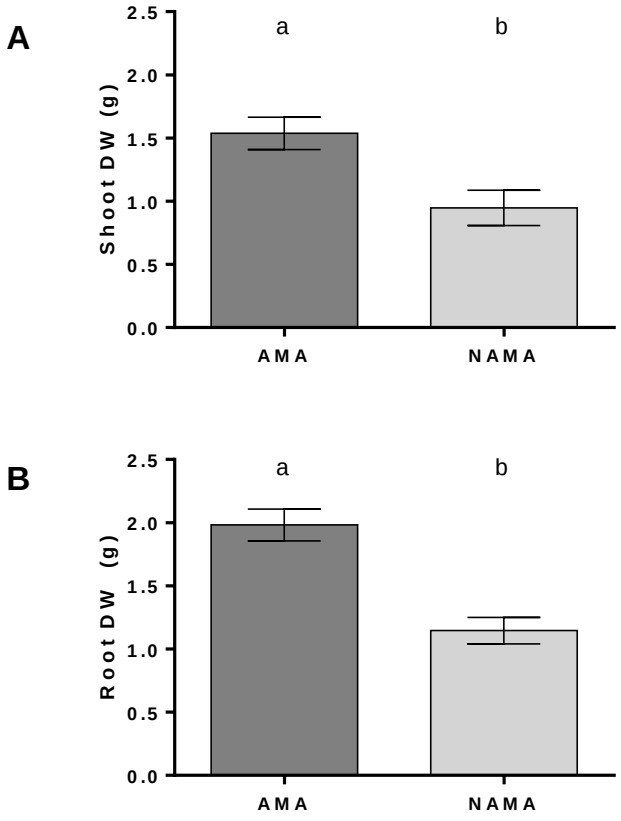

Figure 2.2. Dry weight (DW) (g) of *Plantago lanceolata* colonised by *Glomus intraradices* in Arbuscular Mycorrhizal fungal Access (AMA) and No Arbuscular Mycorrhizal fungal Access (NAMA) microcosms. A) Allowing arbuscular mycorrhizal fungal hyphal access to the patch compartment resulted in a significant increase in shoot DW (P = 0.001). B) Plant root DW was significantly greater for AMA plants than for NAMA (P < 0.001). Data shown are means ± S.E., n = 19. Different letters above bars indicated significantly different means (P < 0.05).

Table 2.1. The consequence of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* hyphae being permitted access to the patch on *Plantago lanceolata* nutrient acquisition. Data presented are values per plant, for microcosms allowing AMF Access (AMA) versus No AMF Access to the patch (NAMA), measured 16 weeks after patch addition. Allowing AMF access to the organic matter patch allowed the plant greater uptake of ^{15}N, phosphorus (P) and nitrogen (N). Data were analysed by Independent-samples T Test, and data shown are means (n = 19 for N measurements; n = 17 for P measurements) ± S.E.

	Shoot ^{15}N content (mg)	Root ^{15}N content (mg)	Shoot N content (mg)	Root N content (mg)	Shoot P content (mg)	Root P content (mg)
AMA	0.57 ± 0.15	0.53 ± 0.11	11.43 ± 1.11	10.53 ± 0.69	9.92 ± 0.93	3.48 ± 0.33
NAMA	0.20 ± 0.08	0.14 ± 0.04	6.82 ± 1.46	5.84 ± 0.69	5.12 ± 0.59	1.48 ± 0.14
Test statistics	$T_{1,37}$ P	$T_{1,37}$ P	$T_{1,37}$ P	$T_{1,37}$ P	$T_{1,34}$ P	$T_{1,34}$ P
	3.99 < 0.001	5.96 < 0.001	3.77 < 0.001	5.02 < 0.001	4.98 < 0.001	5.83 < 0.001

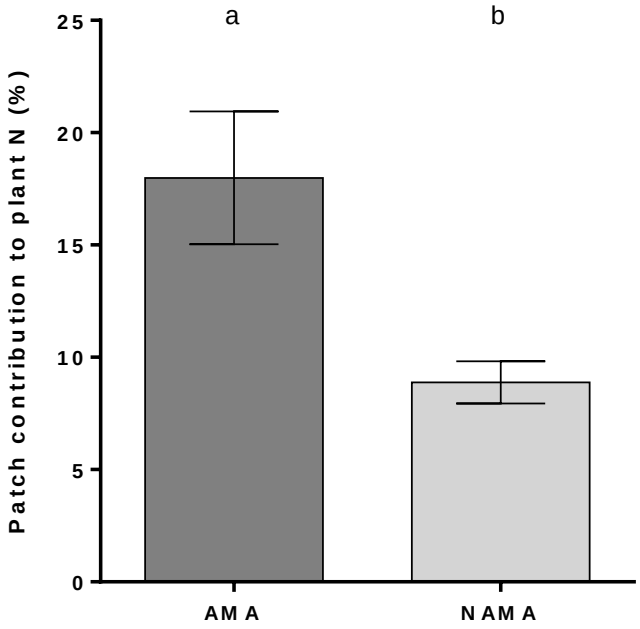

Figure 2.3. The proportion of plant N that was derived from the organic patch was greater in plants with Arbuscular Mycorrhizal fungal Access (AMA) to the patch than in those plants with No Arbuscular Mycorrhizal fungal Access (NAMA) to the patch (P = 0.001). Data shown are means ± S.E., n = 19. Different letters above bars indicated significantly different means (P < 0.05).

The proportion of the patch N acquired by the plant increased from 4% to 12% when AMF were permitted access to the patch compartment ($T_{1,37}$ = 4.98, P < 0.001), suggesting that the AMF were adept at exploiting a newly available patch of organic matter and transferring the N acquired to their plant partner. Allowing AMF hyphal access to the patch resulted in 4.69 mg extra N in the roots and 4.61 mg in the shoots which greatly outweighs the 0.001124 mg of ammonium-N and 0.0003976 mg nitrate-N extractable from the patch.

Root P concentration increased by 28% when AMF had access to the patch ($T_{1,34}$ = 3.31, P = 0.002), but the shoot P concentration was not affected by allowing AMF access to the patch. The increase in root P concentration was not substantial enough to change the total plant P concentration ($T_{1,34}$ = 0.16, P = 0.88), but combined with an increased root mass, root P content increased by 135%. Similarly, despite no increase

in P concentration, shoot P content was 94% greater in AMF access plants than in no AMF access plants (Table 1). Plants with AMF access to the patch had marginally higher N:P ratio (total plant N content / total plant P content) than plants with no patch access although this was only weakly significant ($T_{1,34} = 1.98$, P = 0.060). Mean AMA plant N:P was 2.18 ± 0.15, compared with mean NAMA plant N:P of 1.84 ± 0.10.

Although low levels of fungal hyphae (0.01 ± 0.01 m g^{-1} DW) were found in the organic matter patches where AMF were denied access, hyphal length densities (HLD) were significantly greater ($T_{1,37} = 18.67$, $P < 0.001$) in the treatments that permitted AMF hyphal access to the organic matter patch (1.54 ± 0.19 m g^{-1} DW). Hyphal growth in the plant compartment was 21 % greater when the AMF partner was denied access to the patch compartment than when access was permitted (Fig. 2.4). In AMA microcosms, hyphal proliferation in the bulk growth medium was significantly greater than in the plant compartments ($T_{1,18} = 4.94$, $P < 0.001$) suggesting that the C supply from the plant was limited and that the fungus was optimising distribution of its hyphal network; into the patch compartment instead of the plant compartment. Calculating total hyphal length (by extrapolating from the HLD in compartments, assuming equal distribution of hyphae within compartments) shows that the AMA microcosms supported in excess of three times the hyphae seen in the NAMA microcosms (Fig. 2.4). The higher HLD in the NAMA plant compartments suggests that this was not due to reaching a maximum attainable density in this compartment, and supports the notion of limited C supply to the AMF mycelium.

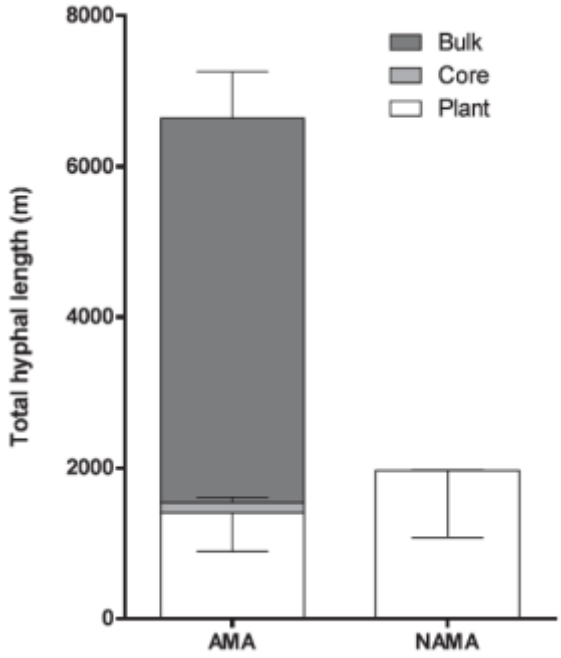

Figure 2.4. Total hyphal length, extrapolated from hyphal length density (HLD) measurements. HLD was calculated from growth medium in the PVC core, from the plant compartment and the bulk growth medium that surrounded the core. Plants with Arbuscular Mycorrhizal fungal Access (AMA) supported substantially more total hyphal length than those plants with No Arbuscular Mycorrhizal Access (NAMA), despite plant compartment hyphal length being higher in NAMA microcosms than AMA. Total hyphal length in the bulk growth medium surrounding the core was higher than in the plant compartment when AMF hyphae were permitted access to the compartment containing the patch ($T_{1, 18} = 4.94$, $P < 0.001$). (Data shown are means ± S.E., n = 19).

^{15}N content in the growth medium outside the patch did not change between treatments, even in the plant compartment, suggesting that ^{15}N lost from the patch was either lost as volatile constituents to the atmosphere, or that the AMF was very successful at acquiring N from the patch. Unfortunately, it was not possible to quantify the root length colonisation due to disintegration of the root material during the clearing process.

2.4 Discussion

We show for the first time that both total N content and total dry weight of plants increased as a result of allowing AMF access to an organic matter patch. Our data show that an organic N source can elicit the 'strong mutualism' scenario predicted by the 'trade balance model' of Johnson (2010), whereby both plant and fungi benefit from the addition of a rich N source in a P-limited system. Previous work has shown mutual benefit, but only following inorganic N addition (Johnson et al., 2015). Here, *G. intraradices* was adept at acquiring N from patches of algal material and transferring a significant fraction of patch N to the partner plant. The quantity of nitrogen transferred here, and the increased contribution made by the ERM in AMA microcosms support the argument that AMF can be a significant conduit for plant N uptake, a position not universally supported in the literature (Reynolds et al., 2005). Although AM uptake of N from organic matter patches has been reported, (Leigh et al., 2009, Barrett et al., 2014), previous studies generally have not displayed increased total plant N, as we show here, and a concurrent increase in plant biomass as found in this study is unprecedented. The increase of ^{15}N in the plant shoots (Table 2.1) is noteworthy as it indicates genuine transfer of patch N to the plant via the AMF, whereas it cannot be determined what proportion of ^{15}N in roots remains in the intraradical mycelium of the AMF. We assumed no fractionation of ^{15}N and ^{14}N during uptake by the fungus or transfer to the plant. Ectomycorrhizal fungi may transfer ^{14}N preferentially to a partner plant, so the mycelium becomes relatively ^{15}N enriched (Hobbie and Colpaert, 2003). Were such a phenomenon to have taken place here, our calculations for N transfer would be underestimating the contribution made by the AMF to plant N.

The increased contribution of patch N to the plants' total N when AMF were allowed access the patch was similar to that shown by Leigh *et al.*(2009), suggesting that the AMF was at least as able to exploit algal patches as *Lolium perenne* patches, as used by Leigh *et*

al.(2009). Where that study showed increased plant N concentration however, we saw increased total N content and plant mass, but no difference in N concentration between treatments. Differences in patch composition may explain different responses of the AM plant, despite using the same plant and fungal symbiont species. The low C:N ratio of our patch compared to that used by Leigh *et al.* (2009) makes our patch more N-rich, and should therefore allow more rapid loss of the N it contained (Hodge et al., 2000a). Rapid efflux of N from the patch is suggested by the reasonably high level of ^{15}N detected in the plant tissue from NAMA microcosms (Table 2.1). Movement of labile N sources by mass flow and diffusion across the 0.45 µm membrane from patch to plant compartment is implicated, but higher N and P levels in AMA treatments confirm the importance of AMF mediated nutrient transfer. Although it remained inside the PVC tube, the algal powder settled and mixed with the sand and AgSorb® during the course of the experiment, and became inseparable from the latter by the time the microcosms were harvested. As such, the retrieval of the patch at the end of the experiment was not possible. This prevented patch analysis to determine the extent of decomposition.

The contribution of patch N to total plant N varies among different studies using similar experimental systems: from < 7 % (Hodge and Fitter, 2010, Barrett et al., 2011, Herman et al., 2012) to > 15 % (Leigh et al., 2009, Barrett et al., 2014). Some of these differences can be explained by variation among different AMF symbionts (Leigh et al., 2009, Barrett et al., 2014). However, the AMF may also benefit the plant from acquiring 'extra' N from sources other than the patch (Herman et al., 2012). Increased P uptake by AMA plants is perhaps expected, given the amount of P present in the patch and that AMs probably evolved to improve the uptake of immobile ions, such as phosphate, from soil beyond the rhizosphere (Smith and Read, 2008). In this study, the N:P ratios were remarkably low, but not without precedent for forbs (Maloney and Lamberti, 1995), and indicate that the plants were severely N-limited. The increase in N:P ratio in AMA plants compared with

NAMA plants suggests that the AMF reduced the extreme N limitation the plants were experiencing and in so doing facilitated growth benefits for the plant. Leigh *et al.*(2009) showed no difference in N:P between AMA and NAMA plants, suggesting that the AMF in that case did less to lift the plant from N-limitation, and offering an explanation as to why no growth response was observed there.

Increased P content in AMA plants suggests that the patch represented a significant source of P for the fungus (Barrett et al., 2014). Cavagnaro *et al*. (2005) demonstrated that *G. intraradices* proliferated in high P patches, while reducing P uptake from low P areas. Hyphal proliferation in the high-N, high-P patch compartment and reduced AMF growth in the plant compartment in AMA microcosms (Fig. 2.4) suggests the fungus behaved similarly here.

Reduced hyphal length in the plant compartment of AMA microcosms may suggest C limitation, as previous studies with similar experimental design (Hodge et al., 2001) showed increased growth of AMF in plant and patch compartments. Here, the fungus may have been unable to obtain enough C from the plant to maintain the hyphal biomass in the plant compartment when it was also supporting a mycelium in the patch compartment, and thus co-ordinated its hyphal growth for the greatest benefit - the mineral nutrition from the patch, a phenomenon that is well documented in root allocation (Drew, 1975).

Previously, experimental evidence for strong mutual benefit of AMs was obtained only by inorganic N addition to mycorrhizal plants. Our findings demonstrate that AMF can provide considerable benefit to plant N and P nutrition following the addition of organic matter, followed by substantial increases in biomass, both for plant and fungus.

Chapter 3.
Nitrogen source preference of arbuscular mycorrhizal plants across different scales: Petri plates to glasshouse microcosms

3.1. Introduction

Arbuscular mycorrhizal fungi (AMF) are soil fungi which can form symbiotic relationship with up to two-thirds of higher land plants (Schussler et al., 2001). Inside the cortical cells of the host plant's root, the fungal mycelium proliferates and forms hyphal coils and arbuscules, highly branched structures characteristic of the symbiosis (Smith and Smith, 2011b). This interface between plant and fungus facilitates the trade of resources between partners; mineral nutrients such as nitrogen (N) and phosphorus (P) and water from the fungus are exchanged for photosynthetic carbon (C) from the plant. Estimates vary as to the amount of C the plant apportions to the fungus, but allocation of 10 % (Bryla and Eissenstat, 2005), or even 22 % (Wright et al., 1998) of the plant's fixed C have been reported. As obligate symbionts, the fungi rely on this C supply from the plant as their sole source of C (Smith and Read, 2008).

By proliferating out into the soil from the root, the mycelium of the fungus vastly increases the surface area over which mineral nutrients and water can be acquired, after which some may be transferred to the plant. This increased surface area is perhaps most beneficial for P uptake. Phosphate ions (PO_4^{3-}), the most bioavailable form of P in the soil, very readily react with metal cations and precipitate, whereby the P becomes insoluble and may be adsorbed to soil particles. As a result, very little P moves by mass flow and diffusion is greatly impaired (Tinker and Nye, 2000). Consequently, the uptake of P by plant roots outpaces the diffusion of P to the rhizosphere and a depletion zone is

established (Hinsinger, 2001). By increasing the soil volume encountered, the mycelium of the AMF gains access to a much larger P pool.

It has largely been suggested that uptake of N via the AM route is of no advantage to plants over direct uptake (Hawkins et al., 2000), as movement of N through the soil is more rapid than that of P. As such, it is posited that the depletion zone nitrate or ammonium (the most readily-acquired forms of N to AMF) around the root would extend so far beyond the rhizosphere that the ERM of AMF should offer no benefit over direct root uptake (Smith and Smith, 2011b).

Both nitrate- and ammonium-N move freely in soil solution and therefore can be transported by mass flow to the plant root from distal bulk soil. This said, ammonium movement through soil can be slowed by adsorption to clay particles (Mortland and Wolcott, 1965) and in dry soils, mass flow of ammonium and nitrate may become impeded (Tinker and Nye, 2000). Given that plant demand for N can be around 10 times that of P (Fitter, 2005), and N is the most commonly limiting plant nutrient in many ecosystems (Maathuis and Diatloff, 2013), C-for-N trade between plant and fungus may be mutually beneficial. A model proposed by Fitter (2006) suggests that the AMF will transfer N or P if it can elicit a C flux from the plant. While the physiological and ecological relevance of N uptake via AMF is still disputed (Smith and Smith, 2011b), it has been repeatedly demonstrated that plants can acquire substantial amounts of N through the AM route from inorganic (Ames et al., 1983) and organic sources (Hodge and Fitter, 2010, Thirkell et al., 2016), and that this route can contribute significantly to plant N acquisition (Leigh et al., 2009) and growth (Thirkell et al., 2016). Furthermore, the amount of N transferred to the plant may be correlated to the amount of carbon which is apportioned to the fungus by the plant (Fellbaum et al., 2012).

Although inorganic N uptake by AMF from decomposing organic sources is widely known, and uptake of amino acids and other simple organic molecules has been reported (Whiteside et al., 2012a, Whiteside et al., 2012b) there is no consensus that the uptake of intact organic N sources makes up any significant fraction of the N intake of AMF (Hodge and Storer, 2015). It has been suggested that AMF uptake of organic N is more energetically expensive and so is only done *in extremis*, when the fungi themselves are N limited (Talbot and Treseder, 2010). Current opinion is that the predominant forms of N acquired by AMF are simple, inorganic, ions, namely ammonium (NH_4^+) and nitrate (NO_3^-). Improved understanding of N dynamics within the arbuscular mycorrhizal symbiosis may help to improve the uptake efficiency of N added to arable systems. At the same time, recent emphasis on the importance of soil health as a part of sustainable agriculture has seen a move towards improving our understanding of the potential role of AMF in crop plant nutrient cycling (Johansson et al., 2004).

A variety of nitrogen fertilisers are applied to crop plants, including nitrate, ammonium, urea, and numerous organic fertilisers including slurry, manure and seaweed derivatives. Of these sources, nitrate and ammonium are by far the most commonly added forms of N to arable crop systems in the UK (DEFRA, 2015), as well as being the most likely candidates for N uptake by AMF. The question is pressing, given the vast majority of crops – wheat, maize, barley, as well as many salad crops – cucumber, tomato, onion and others, form arbuscular mycorrhizas (Smith and Read, 2008).

A model put forward by Govindarajulu et al. (2005) suggests that NH_4^+ should be the most likely candidate for N uptake, as this is energetically less expensive than nitrate uptake. As N assimilation in AMF takes place following the incorporation of NH_4^+ into the GS/GOGAT cycle, (Smith et al., 1985, Toussaint et al., 2004) NO_3^- must first be reduced to NH_4^+ by nitrate reductase and then nitrite reductase (Govindarajulu et al., 2005). Recent discovery of a transporter implicated in NH_4^+ uptake by the ERM from the

hyphosphere lends support to the suggestion (Lopez-Pedrosa et al., 2006). By contrast, it has been suggested that the available pool of NH_4^+ in the soil is relatively small compared to that of NO_3^-, owing to the faster movement of NO_3^- compared to NH_4^+ (Mortland and Wolcott, 1965), and the nitrification by bacteria that oxidise NH_4^+ to NO_3^- before it can be acquired by AMF or plants (Barber, 1985).

Numerous microcosm studies using whole plants have demonstrated uptake of both NO_3^- (Tobar et al., 1994a) and NH_4^+ (Ames et al., 1983, Johansen et al., 1994, Tobar et al., 1994b, Jin et al., 2005) by the AM route following addition of ^{15}N as NO_3^- or NH_4^+ to a 'hyphal compartment' into which only AMF hyphae can infiltrate from an adjoining 'root compartment', by passing through a fine nylon mesh screen (Ames et al., 1983, Frey and Schuepp, 1993, Johansen et al., 1993, Johansen et al., 1994). Johansen et al. (1993) showed similar hyphal transfer of ^{15}N to mycorrhizal plants when supplied as NO_3^- and NH_4^+, although each plant only received one N source or the other.

Experimental comparison of N uptake following addition of both NH_4^+ and NO_3^- to a single mycelium is comparatively rare; only two studies have added both sources of N to each microcosm unit. In both cases, half of the experimental units received ^{15}N ammonium and ^{14}N nitrate, while the other half received ^{15}N nitrate, and ^{14}N ammonium.

Tanaka and Yano (2005), used a split hyphal compartment made from an agar gel rather than soil, which contained ammonium on one side and nitrate on the other. The experimental plants obtained 10 times more ^{15}N when it was supplied as ammonium than nitrate (Tanaka and Yano, 2005). While agar allows simple placement of a source of N, it does not represent a realistic substrate from which hyphae may acquire N, but the higher uptake of ammonium fits with current understanding given the experimental conditions. Uptake of ^{15}N nitrate was indicated by ^{15}N enrichment of cell walls of extraradical hyphae of the AMF (*Glomus aggregatum*) (Tanaka and Yano, 2005). The only other study using

simultaneous nitrate and ammonium addition found the opposite trend; plant uptake of ^{15}N was highest following addition as NO_3^- (Ngwene et al., 2013), but that ERM was more highly ^{15}N enriched when following labelled NH_4^+ addition.

It is suggested here that the difference in substrate of the hyphal compartment (soil vs. agar) has caused the contradictory results (between Ngwene (2013) and Tanaka and Yano (2005)) rather than the selection of two different, but closely related AMF species.

This chapter reports on two experiments designed to test both whether i) AM exhibit a preference for uptake of nitrate or ammonium and ii) whether this trend depends on the scale of the experiment and the substrate used i.e. from Petri dish to whole plant and adding the N into soil or as a section of agar gel.

Where both ammonium- and nitrate-^{15}N are added to an agar substrate accessible to hyphae, we would expect to observe higher uptake of ammonium, as the movement of neither NH_4^+ nor NO_3^- should be hindered, while AMF ammonium acquisition should be higher. A Petri dish experiment (Experiment 1) was carried out using AMF *Glomus aggregatum*, with either $K^{15}NO_3$, $^{15}NH_4Cl$ or $^{15}NH_4{}^{15}NO_3$ added to a hyphal only compartment after which ^{15}N uptake into transformed carrot root was determined by isotope ratio mass spectrometry. Control microcosms used non-AM carrot roots, with no access to the hyphal compartment.

Experiment 2 used whole plants (*Allium cepa* L., onion) and AMF species *Rhizophagus irregularis* and *Glomus aggregatum* in a soil-based system, into which discrete zones for ammonium and nitrate addition were placed. Each microcosm received both N sources, while ^{15}N labels were supplied as ammonium in half of microcosms and nitrate in the other. Control microcosms used non-mycorrhizal plants. A $^{14}CO_2$ tracer study was carried out within the main experiment to test whether increased N acquisition from the

ammonium or nitrate zone can be correlated with increased C 'investment' by the plant in hyphae.

Experiment 2 was predicted to show higher transfer of ^{15}N from both N sources to the plant when colonised by AMF compared to non-mycorrhizal plants. This benefit to the plants' ^{15}N uptake was predicted to be greatest when $^{15}NH_4^+$ was added, as the fungus should 'prefer' to acquire NH_4^+ from the soil over NO_3^-, and the fungus should alleviate the slow movement of NH_4^+ through the soil to a greater extent than that of NO_3^-. It is predicted that ammonium cores of the mycorrhizal treatments should receive the highest import of ^{14}C, as the plant and fungus apportion more C to the area of the growth medium from which it obtains more N.

3.2. Materials and Methods

3.2.1. Experiment 1 (Petri plates)

3.2.1.1. Experimental setup

Experiment 1 used 90 mm Petri plates with a single dividing barrier in the centre, giving two equal sized compartments. One, the 'root' compartment was filled to within 1 mm of the top of the central barrier, with a modified Strullu-Romand (MSR) medium (Cranenbrouck *et al* 2005). Into the hyphal compartment, a minimal media was added, to within 2 mm of the top of the barrier. This media contained only the gelling agent (Gelzan, Sigma-Aldrich) and a $1/10^{th}$ recommended quantity of N, added as NH_4NO_3.

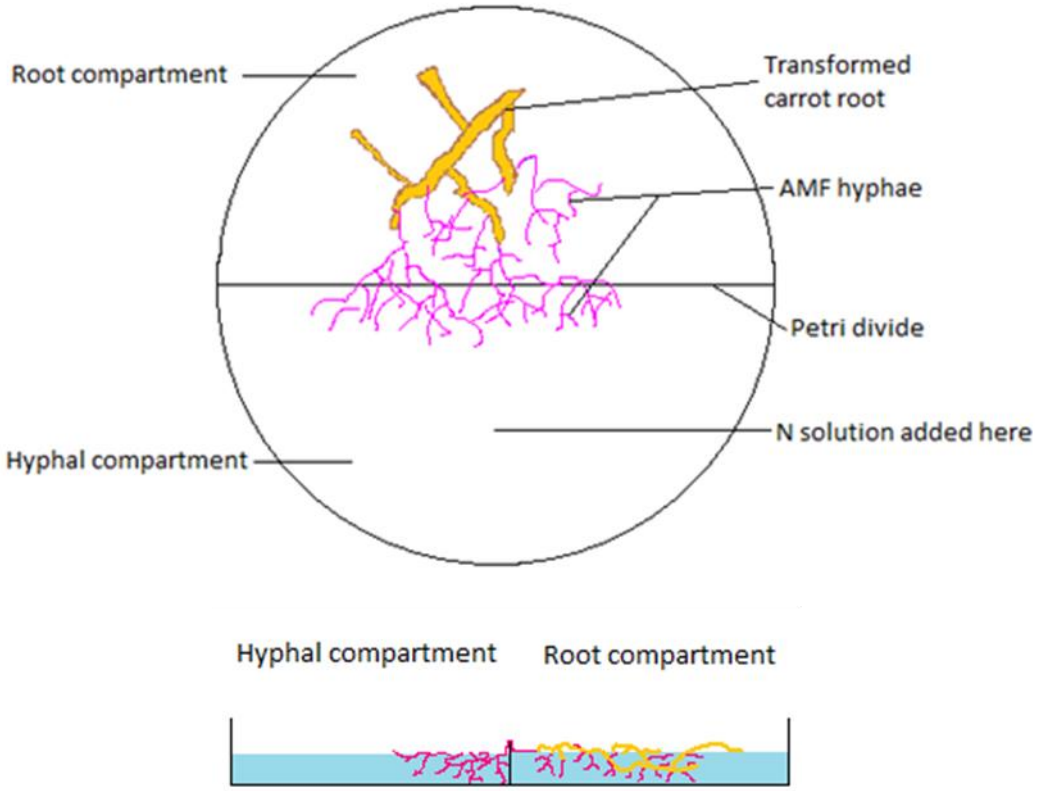

Figure 3.1. Petri plate systems containing T-RNA transformed carrot root, *Daucus carota*, colonised by *Glomus aggregatum*. Using a plate with a central divide allowed designation of a root compartment and hyphal compartment, so ^{15}N added to the hyphal compartment can only be transferred to the root compartment via the extraradical hyphae of the fungus.

3.2.1.2. Arbuscular Mycorrhizal Inoculum

AMF inoculum was obtained from axenic culture stocks, whereby AMF are grown on T-RNA transformed roots of *Daucus carota* L.(carrot) (Fortin *et al* 2002). These combined (root + AMF) stocks are grown on the same MSR media as used for the root compartment described above, and maintained in darkness at 22 °C. Sections of agar were selected for excision and replacement into experimental Petri plates. Sections were suitable if they contained a length of living root with no fewer than 3 branch points visible. These sections were excised in a laminar flow hood to maintain sterility. Dishes were sealed with Parafilm® and placed into a lidded box and maintained at 22 °C.

3.2.1.3. ^{15}N addition treatments

After hyphae were seen in sufficient plates to be crossing the barrier, the plates were organised into groups for ^{15}N addition. Each group received as close as possible to the same number of plates in which there was slightly more or slightly less than the mean hyphal ingrowth into the hyphal compartment. This was to ensure that the amount of hyphae in the hyphal compartment was as close to the same between N treatment groups as possible. In a laminar flow hood, each Petri plate received 0.015 mg ^{15}N, added as 0.25 mL of a 4 mM solution, added by pipette to the centre of the hyphal compartment. Additions were as follows: $^{15}NH_4Cl$ (99 atom%) to the ammonium group, $K^{15}NO_3$ (99 atom%) to the nitrate group, and $^{15}NH_4{}^{15}NO_3$ (50 atom%) to the ammonium nitrate group. Plates were lidded, placed carefully into an incubator at 22 °C and left in the dark.

3.2.1.4. Harvest and Analysis

After 1 day, plates were transferred back to the laminar flow hood, and a small (1 to 2 cm) section of root was removed from the root compartment, at the edge of the plate furthest from the central barrier. Plates were lidded again and returned to the incubator. Removed root samples were oven dried at 70 °C for 48 hours and stored for further analysis. Seven days after isotope addition, plates were opened and a scalpel blade run along the central barrier on the root compartment side, to sever all hyphae crossing the central barrier.

The 5 mm x 90 mm section of media abutting the central barrier of the hyphal compartment was removed using a scalpel. This section was transferred to a polypropylene tube containing 30 mL of a 6 mM citrate buffer to dissolve the Gelzan®. Tubes were placed in a water bath at 35 °C for 12 hours.

All material was removed from the root compartment and roots pulled from the media, and then washed under distilled water, after which they were dried for 48 hours at 70 °C.

All root samples were homogenised in a Yellowline analytical blender (IKA, Boutersem, Belgium).

The slice of media taken for quantification of extraradical hyphae crossing the barrier had dissolved entirely after 12 hours in the buffer. The 30 mL sample was vacuum filtered through a 0.45 µm nitrocellulose membrane filter (Sartorius Stedim, Gottingen, Germany). Hyphae were then stained using a 0.01% solution of acid fuchsin, left to dry for 1 hour and then mounted onto microscope slides using Histomount™ (National Diagnostics, Hessle, UK). Extraradical hyphal length was then quantified using the gridline intersection method of Hodge (2003).

N and ^{15}N content of the carrot root was determined by isotope ratio mass spectrometry (IRMS) of homogenised tissue. Root colonisation was confirmed by microscopic analysis, following staining with "Chlorazol black E" stain, in the protocol described below (3.2.2.6.).

3.2.1.5. Statistical Analysis

Statistical analysis was carried out in the "RStudio 3.1.0" interface of the R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Non-parametric tests were performed on data as they failed to satisfy Shapiro-Wilk and Kolmogorov-Smirnov tests for normally and Levene's test of homogeneity of variance. One sample Wilcoxon signed rank tests were carried out on each combination of nitrogen label type and time since label addition to determine whether ^{15}N content differed from zero. Wilcoxon rank sum tests were carried out to determine whether independent groups means differed i.e. among different N type (i.e. nitrate vs ammonium vs ammonium nitrate) at each time point. To test the difference in uptake of ^{15}N within individual dishes at 1 day and 7 days

after label addition, Wilcoxon signed rank tests were carried out, as these samples are paired.

3.2.2. Experiment 2 (*Allium cepa* microcosms)

3.2.2.1. Experimental setup

Commercially available onion (*Allium cepa* L.) seeds (variety "Tokyo Long White", Unwins seeds, Huntingdon, UK) were surface sterilised in 5% (w/v) calcium hypochlorite solution before being germinated on moist Whatman Number 1 filter paper (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) in a Petri dish. One-week-old seedlings were then placed singly into pots measuring 15 x 15 x 15 cm, filled with 1.5 litres of growth medium. For this, a twice-autoclaved (121 °C for a total of 40 minutes) mixture of soil, washed silica sand and polypropylene beads at a ratio of 5 : 3 : 2 was created. The propylene beads were added to assist in retrieving hyphae from the substrate at the end of the experiment, although this later proved to be unfeasible. The soil was obtained from Spen Farm (co-ordinates 53°52'05.4"N 1°19'36.3"W), an experimental farm employing a mixed arable rotation, owned and managed by the University of Leeds, UK. The field chosen had been used the previous year for wheat and the previous season, spring barley. The soil is characterised as a "clayey-silty-loam and the parent material mostly consists of dolostone" by the UK soil observatory (UKSO). Soil was taken from between 5 and 30 cm below the surface of the field site, one week before planting and allowed to partially air dry, after which it was incorporated into the mix to be autoclaved.

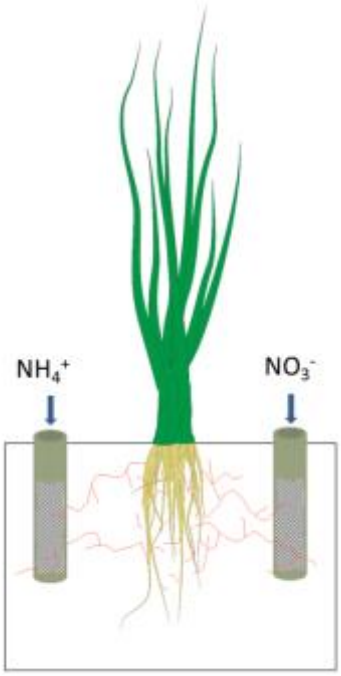

Figure 3.2. Diagram of experimental design. Onion plants (*Allium cepa* L), either colonised by AMF *G. aggregatum*, or *R. irregularis* or Non-AM, are grown individually in a microcosm system with a sand and soil substrate. In two corners of each pot, lengths of PVC piping, with mesh covered windows are inserted. Into one core, ammonium-type Long Ashton Solution (LAS) is added, and nitrate-LAS added to the other. A stable isotope ^{15}N label is added to each pot: as a single dose of $^{15}NH_4^+$-LAS in half of the microcosms, and $^{15}NO_3^-$-LAS in the other half. As such, plants receive the same amount of ^{15}N, but in varied form, and receive the same amount of N overall as NH_4^+ or NO_3^-, with the only difference between treatments being the N form used to provide the isotope.

3.2.2.2. Arbuscular Mycorrhizal Fungi

AMF inoculum was obtained from the same stock cultures used in Experiment 1 (3.2.1.2). Combined stock cultures were 6 months old when used, an age at which the root and the AM fungus had proliferated across the Petri dish and the fungus had begun to sporulate. Depending upon the treatment group, plants were inoculated either with *Rhizophagus irregularis*, *Glomus aggregatum* or, in the case of the non-AMF group, with a double autoclaved mixture of the two. The inoculum consisted of a homogenised mix of transformed carrot root culture, including extraradical hyphae, spores and root fragments, all known to perform as potential inoculum. Each pot received an equal volume of

inoculum, and fungal stock plates were chosen as to have the same quantity of root, hyphae and spores. The inoculum was mixed into the growth medium before planting, such that it was homogenously distributed through the pot.

3.2.2.3. Nutrient addition

N addition zones were established by inserting into the soil 10 cm lengths of PVC pipe of diameter 1.5 cm into which apertures had been cut, and then covered with a 20 µm mesh (Johnson et al., 2001). Such a mesh barrier allows the ingrowth of AMF hyphae but not plant roots, while allowing a clearly demarcated zone into which the mineral N could be added. Each plant pot contained two such PVC pipe cores, set towards the corners of the pot, allowing each plant to receive both ammonium and nitrate nutrient solutions, one to each core. To half of the microcosms, ^{15}N ammonium was added to one core, and ^{14}N nitrate to the other. In the other half of the microcosms, ^{15}N nitrate was added to one core, and ^{14}N ammonium to the other. Thus, all plants received the same amount of total N, the same amount of ^{15}N and an equal proportion of nitrate and ammonium.

Nitrogen was added in the form of Long Ashtons nutrient solution (Hewitt & Smith 1975), which can be prepared to a 'nitrate type' or an 'ammonium type'. Used at the recommended concentration, Long Ashton solution contains 0.17g N L^{-1}. Cores each received 3 mL of Long Ashtons solution daily, containing 0.51 mg N (6 mL and 1.02 mg total N per pot), thus over the course of the experiment, nutrient solution N contributed [0.51 mg x 2 cores x 126 days] 128.52 mg N. Long Ashton solution added to the ammonium core also contained the nitrification inhibitor 'N-Serve' (2-chloro-6 (trichloromethyl) pyridine) (Sigma Aldrich, St Louis, Missouri, USA) added at 5 µg mL^{-1} to prevent nitrification of the ammonium added in nutrient solution.

One week before harvesting, plants received a single dose (3 mL) of Long Ashton solution, containing 655.7112 µg ^{15}N, added to ammonium (^{15}N ammonium chloride, $^{15}NH_4Cl$, from MP Biomedicals LLC, Santa Ana, California, USA) or nitrate (^{15}N potassium nitrate, $K^{15}NO_3$, from Sercon Ltd, Crewe, UK) cores respectively.

3.2.2.4. ^{14}C labelling

To determine the reciprocity of the exchange of nutrients in the symbiosis (C-for-N), a radioisotope tracer experiment was employed, specifically to determine the extent of carbon flow through the plant, to the AMF and out into the AMF extraradical mycelium. From each AMF treatment (i.e. *R. irregularis*, *G. aggregatum* or non-AMF), four plants receiving ^{15}N ammonium were used in the ^{14}C tracer experiment. The ^{14}C tracer experiment therefore tested differences in C acquisition between the AMF, and not any effect between N-type. Plants were enclosed into a double bag system allowing the headspace of gas above the shoots to be separated from the headspace above the roots and hyphae (Fig. 3.3). Shortly after mid-day, 8 µL of $NaH^{14}CO_3$ (MP Biomedicals LLC, Santa Ana, California, USA), representing 0.5918 MBq specific activity was added to the shoot compartment of each system, and this compartment was then closed. The outer compartment was then closed.

Using a hypodermic needle to penetrate the bags, 1.5 mL 10% (v/v) lactic acid was added to the cuvette, thus liberating $^{14}CO_2$. Double-sided tape between the two bags formed an airtight seal to prevent any flow of gas between compartments. Following the liberation of ^{14}C into the shoot compartment, detection of ^{14}C in the headspace above the roots and hyphae implies shoot fixation by photosynthesis and flow through the phloem to the roots, after which it is respired by the roots and AMF, percolates through the soil and is detectable in the headspace above the root and hyphae headspace. The excess ^{14}C detected

in the AMF cores compared to non-AMF cores represents the amount of C that has been translocated to the extraradical mycelium.

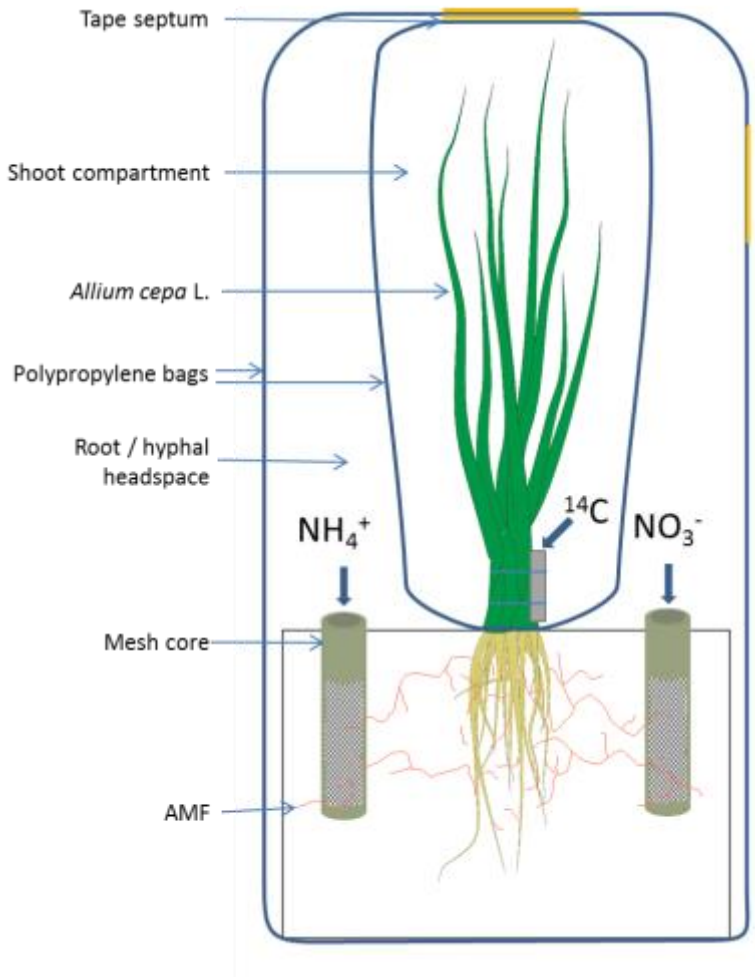

Figure 3.3 Diagram of ^{14}C labelling chamber of onion plants. Shoots were enclosed in a zipped polypropylene bag, after a scintillation vial containing $NaH^{14}CO_3$ was taped to the stem. The entire plant, including pot, was then sealed in another bag. Double-sided tape was used to create a septum between the inside of the shoot compartment and the outside of the whole system, to allow injection of lactic acid to the scintillation vial without interfering with the root/headspace compartment.

Gas samples were taken from the headspace above the roots and hyphae at 1 hour after $^{14}CO_2$ liberation, and at hourly intervals thereafter. Samples were injected through SubaSeals (Sigma-Aldrich, St Louis, Missouri, USA) into evacuated scintillation vials filled with 1: 1 (v/v) Carbosorb: Permafluor scintillation cocktail (both from Perkin Elmer, Waltham, Massachusetts, USA). $^{14}CO_2$ was quantified by scintillation counting (Packard

Tri-Carb 3100TR; Perkin Elmer, Waltham, Massachusetts, USA). The peak flux $^{14}CO_2$ from the headspace above the root and hyphae indicates the peak flow of fixed C from the plant to the AMF mycelium, as it is respired by the roots and fungus. At the peak flux of respired $^{14}CO_2$ into the root headspace, the bag systems were vented and the microcosms destructively harvested.

3.2.2.5. Growth chamber

Plants were maintained in a heated, lit greenhouse (Arthur Willis Environment Centre, University of Sheffield, UK) and watered with distilled water as required. Day time temperatures were maintained at 18 °C and night time temperatures at 15°C. The experiment was set up on 16th March 2015 and destructively harvested on the 25th July 2015, when the plants were 18 weeks old.

3.2.2.6. Harvest and Analysis

Following destructive harvest, plants were separated into above and belowground parts and oven-dried at 80 °C for 72 hours, then homogenised first in a table top blender, then in a ball mill (Retsch MM400, Retsch GmbH, Haan, Germany).

Plant N and ^{15}N content were both determined by use of an isotope ratio mass spectrometry, IRMS (PDZ 2020, Sercon Ltd, Crewe, UK). To quantify ^{14}C contained within the plant samples, 50 mg of the oven-dried tissue were weighed into Combusto-Cones (Perkin Elmer, Waltham, Massachusetts, USA), then oxidised in a sample oxidiser (Model 307 Packard, Isotech, Perkin Elmer, Waltham, Massachusetts, USA) with the $^{14}CO_2$ trapped in the same mix of Carbosorb and Permafluor as before, and similarly, scintillation was counted (Packard Tri-Carb 3100TR; Perkin Elmer, Waltham, Massachusetts, USA).

Using a protocol modified from Staddon *et al* (1990), samples of growth medium were taken from the cores to quantify the proliferation of extraradical AMF hyphae into zones of different N type. Briefly, samples of known mass (between 5-10g) were suspended in 500 mL of water and stirred vigorously on a magnetic stirrer plate in order to agitate the hyphae away from soil particles. From this 500 mL, 200 mL was removed to another beaker on a magnetic stirrer. 10 mL aliquots of this were vacuum filtered as described previously (3.2.1.4.), and extraradical hyphae quantified.

The extent of root length colonisation (RLC) by the AMF was determined microscopically, following the staining of fungal structures using 'Chlorazol Black E' stain, a method adapted from Brundrett (1984). Briefly, washed and air dried roots were 'cleared' by immersion in 10% (w/v) KOH at 70 °C for 25 minutes, followed by acidification in 1% HCl at 25 °C for 10 minutes. After this, roots were immersed in Chlorazol Black E stain, kept at 70 °C for 20 minutes, after which they were placed into a 50/50 mix of glycerol and distilled water, stored at 25 °C for 18 hours to allow excess stain to be removed. Stained roots were then mounted onto microscope slides using Histomount™, as before.

Plant tissue phosphorus (P) concentration was determined by spectrophotometer assay, following sulphuric acid and hydrogen peroxide digest. Plant root and shoot samples of known weight (50 mg ±5 mg) were heated in a dry block heater (Grant Instruments, Shrepreth, UK) to 365 °C in 1 mL of 3.3 % (v/v) solution of salicylic acid in 96 % (v/v) sulfuric acid for 15 minutes. Once samples had cooled to 25 °C, 5 mL of 30 % (v/v) hydrogen peroxide was added, at which point the samples turned colourless. Samples were again left to cool to 25 °C. A 0.5 mL sample of this digest product was transferred to a 4 mL spectrophotometry cuvette, together with 0.2 mL 0.1 M L-ascorbic acid ($C_6H_8O_6$), 0.5 mL of 3.44 M NaOH to neutralise acidity, and 0.5 mL of a developer solution. The developer was prepared by dissolving 4.8 g of ammonium molybdate

($(NH_4)_6Mo_7O_{24}.4H_2O$) and 0.1 g antimony potassium tartrate ($C_6H_4O_7SbK$) in 500 mL 2 M H_2SO_4. The volume of sample in the cuvette was made up to 3.8 mL, and left to stand for 45 minutes after which the absorbance was measured at a wavelength of 882 nm using a Cecil 1020 spectrophotometer (Cecil Instruments Ltd. Cambridge, UK). Sample P concentration was calculated from a calibration curve, constructed using known concentrations of sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$).

3.2.2.7. Statistical Analysis

Data were analysed using the "R 3.1.0" statistical package, via the "RStudio" integrated development environment. (R foundation for Statistical Computing, Vienna, Austria). Data were checked for homogeneity of variance by Levene's test and for normality by Shapiro-Wilk and Kolmogorov-Smirnov.

Data for HLD within each microcosm unit (i.e. comparison between fungal biomass in the ammonium core vs. nitrate core) were tested using dependent samples T-test, as these cores contain HLD which constitute fractions of the same mycelium. Similarly, data for ^{14}C enrichment of core contents between ammonium and nitrate cores were tested using dependent samples T-Test. Comparisons of mycelium growth (HLD) between AMF species were carried out using Independent Samples T-tests as the samples are from different microcosm units and are therefore unpaired. Root length colonisation, growth medium ^{14}C enrichment, and all measures of plant nutrition and growth between AMF isolates was tested by analysis of variance.

Based on the Akaike information criterion (AIC) indication, a better model fit for testing ^{15}N enrichment data was achieved by pooling the mycorrhizal treatments together and then testing between mycorrhizal and non-mycorrhizal plants.

3.3. Results.

3.3.1. Experiment 1 - Petri dish microcosm

At 7 days after isotope addition, the Petri plate systems which received ammonium nitrate had root ^{15}N content that was significantly higher than in the ammonium group ($W = 0$, $p = 0.029$) and almost significantly higher than the nitrate group ($W = 0$, $p = 0.057$). After 7 days, the ^{15}N content of roots in the nitrate and ammonium treatment groups did not differ from zero (Figure 3.).

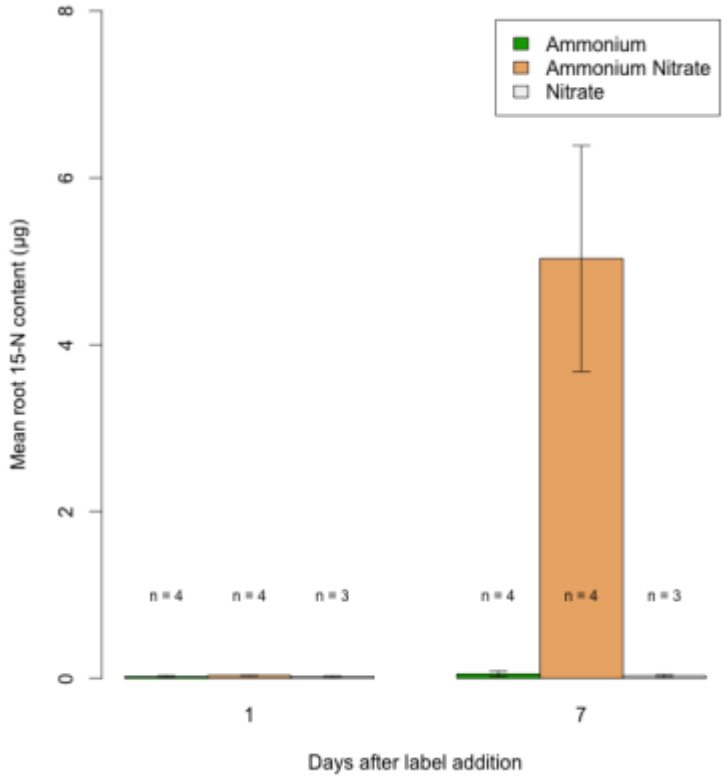

Figure 3.4. Carrot root uptake of ^{15}N label from three sources, measured by IRMS. After 1 day, no N treatment induced a root ^{15}N content higher than zero. After 7 days, roots which had received ammonium nitrate showed significantly higher enrichment of ^{15}N than either roots which had received labelled nitrate or ammonium alone. Data presented are means ± SEM.

In addition, the ammonium nitrate group roots harvested 7 days after label addition contained significantly more ^{15}N than ammonium nitrate group roots sampled 1 day after label addition ($W = 0$, $p = 0.029$). This suggests that that significant uptake of the label did not immediate following addition, but was gradual. Ammonium nitrate-fed roots were significantly more enriched than the control group zero ($t = 3.7177$, df = 3, $p = 0.017$).

The ^{15}N content of the roots 1 day after adding the labelled N was not statistically significantly different from zero in any treatment group. The value of zero was calculated from the ^{15}N value in the control Petri plates, in which non-AMF roots had no access to the hyphal compartment. Hyphal length density, calculated from the band of agar medium removed from the dish did not differ significantly between treatments, as dishes were chosen with equal proliferation of ERM into the hyphal compartment a priority. Root length colonisation (RLC) also did not differ among treatments.

3.3.2. Experiment 2 – *Allium cepa* microcosms

Both *Glomus aggregatum* and *Rhizophagus irregularis* were able to colonise onion plants, as shown by microscopic inspection of stained roots. There was no detectable colonisation of plants in the non-AMF treatment.

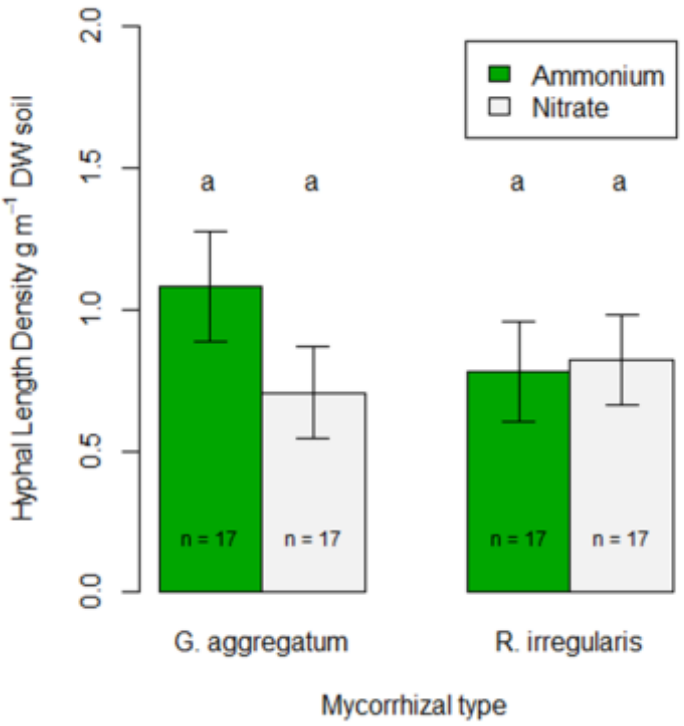

Figure 3.5. Hyphal Length Density (HLD) in PVC cores inserted into experimental pots. The proliferation of hyphae in the cores was no different between *Glomus aggregatum* and *Rhizophagus irregularis*. Neither fungus displayed a propensity to grow into the cores fed with ammonium or those fed with nitrate nutrient solutions. Data are means ± SE.

Root length colonisation (RLC) was statistically significant between the mycorrhizal treatment groups ($F_{(2, 48)} = 69.53$, $p < 0.001$). A post-hoc test (Tukey's honestly significant differences (HSD)) showed that mean RLC of *G. aggregatum* (32.76% ± SE 2.79) was significantly higher than that of *R. irregularis* (19.18% ± 1.97), ($F_{(1, 48)} = 13.65$). Root length colonisation is the percentage of the root length that contains AMF.

Neither AMF species showed a preference to grow into and proliferate within the core containing ammonium or nitrate, calculated as the hyphal length density (HLD, Figure 3.). Despite the significantly different root colonisation by AMF shown above, the hyphal growth into the two PVC cores did not differ between AMF species. For this between-

species comparison of AMF, HLD was calculated as a mean of ammonium core and nitrate core for each microcosm unit.

Following the addition of the radiolabel tracer, ^{14}C enrichment (above cores from unlabelled microcosm2s) was detected in mesh cores from all treatment groups. Radioactivity data presented are total Bq of the substrate within the mesh core per gram of plant aboveground DW. In no treatment group (*G. aggregatum*, *R. irregularis* or non-AM) was ^{14}C enrichment higher in one mesh core or the other, indicating no preferential allocation of C fixed in the labelling photoperiod to the zone of ammonium or nitrate. After this, mean core radioactivity was generated for each microcosm so that mycorrhizal treatment groups could be compared more robustly. Radioactivity of cores varied by AMF treatment ($F_{2,6} = 15.71$, $p = 0.0041$). Mean radioactivity was lowest in the non-AM group (0.437 Bq ± 0.04), and not significantly higher in the *R. irregularis* cores (0.789 Bq ± 0.09). Post hoc analysis by Tukey HSD test showed cores from *G. aggregatum* contained significantly more ^{14}C (2.709 Bq ± 0.525) than either those from *R. irregularis* or the non-AM groups (Figure 3.), a trend not mirrored in the hyphal length density distributed within the cores (Figure 3.).

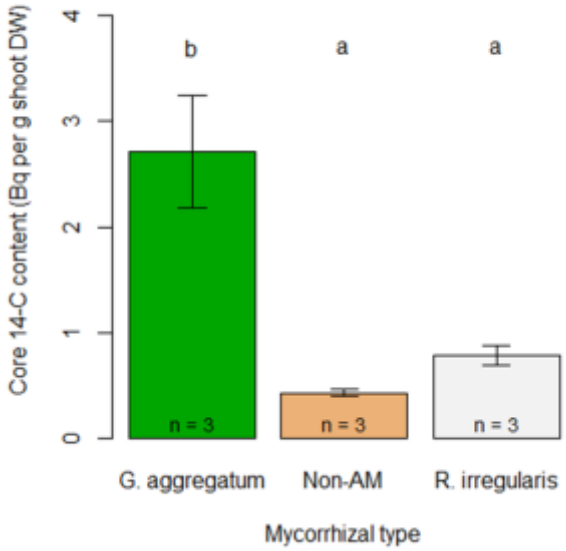

Figure 3.6. Radioactivity in the mesh cores, indicative of transfer of photosynthetically fixed ^{14}C to the zones of N addition. Mean core radioactivity was calculated for each microcosm, as data for ammonium core and nitrate core were not found to differ. *G. aggregatum* transferred significantly more C to the mesh cores than was detected either in the *R. irregularis* cores or the non-AM cores. Data are means ± SEM.

While total plant content of ^{15}N did not differ between the two AMF treatments, pooling the AMF data and testing against the non-AMF treatment indicated that N source was important; an ANOVA yielded significant variation between groups $F_{(3, 47)} = 4.73$, $p = 0.0058$. A Tukey HSD post-hoc test showed that non-mycorrhizal plants provided with ^{15}N as nitrate obtained significantly more ^{15}N than mycorrhizal plants which received ^{15}N ammonium ($p = 0.0030$) (Figure 3.).

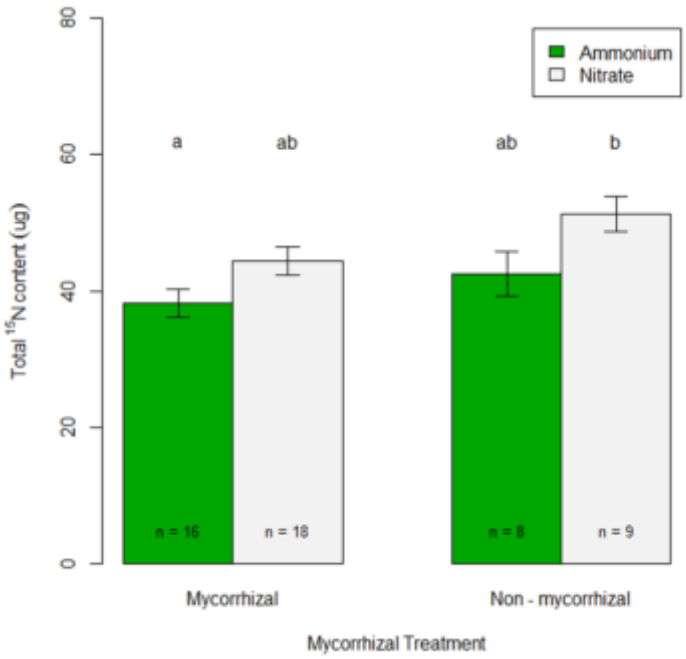

Figure 3.7 Plant total ^{15}N content. Mycorrhizal plants fed with ammonium-^{15}N had significantly less ^{15}N than non-mycorrhizal plants fed with nitrate-^{15}N. Data are means ± SE.

^{15}N content in shoots depended on both the AM status of the plant and the source of ^{15}N supplied ($F_{(3,47)} = 5.249$, $p = 0.0033$). Shoot ^{15}N content of non-AM plants did not differ between nitrogen treatments, but in the AM plants, it was significantly lower when added as ammonium. Non-AM plants receiving nitrate-^{15}N had significantly higher shoot ^{15}N content than AM plants receiving ammonium ^{15}N (Figure 3.). Shoot acquisition of the ^{15}N added to the cores ranged from 5.83 % (± 0.32 %) in the mycorrhizal ammonium group to 7.82 % (± 0.43 %) in the non-AM nitrate group.

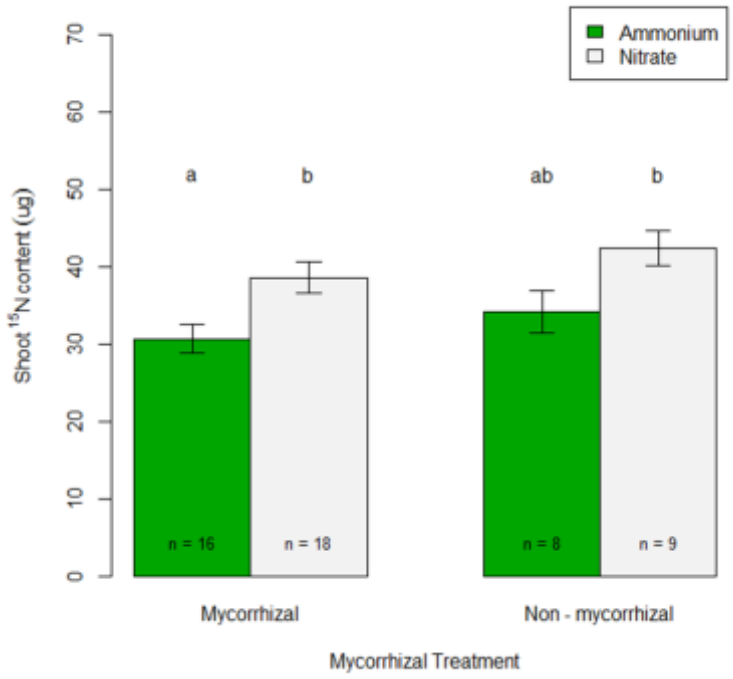

Figure 3.8 Shoot ^{15}N content was influenced by both nitrogen source and mycorrhizal treatment ($F_{3,47}$ = 5.249, p = 0.0033). Mycorrhizal plant shoots contain significantly more ^{15}N when provided with ^{15}N-nitrate than with ^{15}N-ammonium. Non-mycorrhizal plants' given ^{15}N-nitrate also have significantly higher shoot ^{15}N content than mycorrhizal plants given ^{15}N-ammonium. Data are means ± SE.

Similarly, root ^{15}N content showed significant variation between treatment groups ($F_{(3,47)}$ = 3.23, p = 0.031) which was due to mycorrhizal plants provided with ^{15}N as nitrate contained significantly less ^{15}N than non-mycorrhizal plants which received ^{15}N as nitrate (p = 0.040) (Figure 3.).

Hyphal length density (HLD) in the core was plotted against ^{15}N content to determine whether more fungus in the core resulted in more ^{15}N acquisition by the plant. Including data only from *G. aggregatum* and *R. irregularis* (i.e. excluding the non-mycorrhizal treatment), shoot ^{15}N content was negatively correlated with the HLD in the core to which the isotope was added ($t_{(32)}$ = -2.7663, p = 0.0093), (Figure 3). If those cores which contained no HLD are excluded from this analysis, so that all microcosms compared

contained some AMF, there was a similar negative correlation (t = -2.216, df = 26, p = 0.036) (Figure 3). These data corroborate the notion that the uptake of ^{15}N from the PVC cores was hindered by the fungi rather than facilitated. It is likely that in a low-N substrate, the fungi would not transfer N to the plant, as they had not satisfied their own N demand. It is possible that aboveground tissue biomass of the plant and by proxy, the potential strength of the transpiration stream that can be generated, was a determinant of the ^{15}N acquisition by the plant; indeed, aboveground DW and ^{15}N acquisition by the plant correlate strongly (Figure 3).

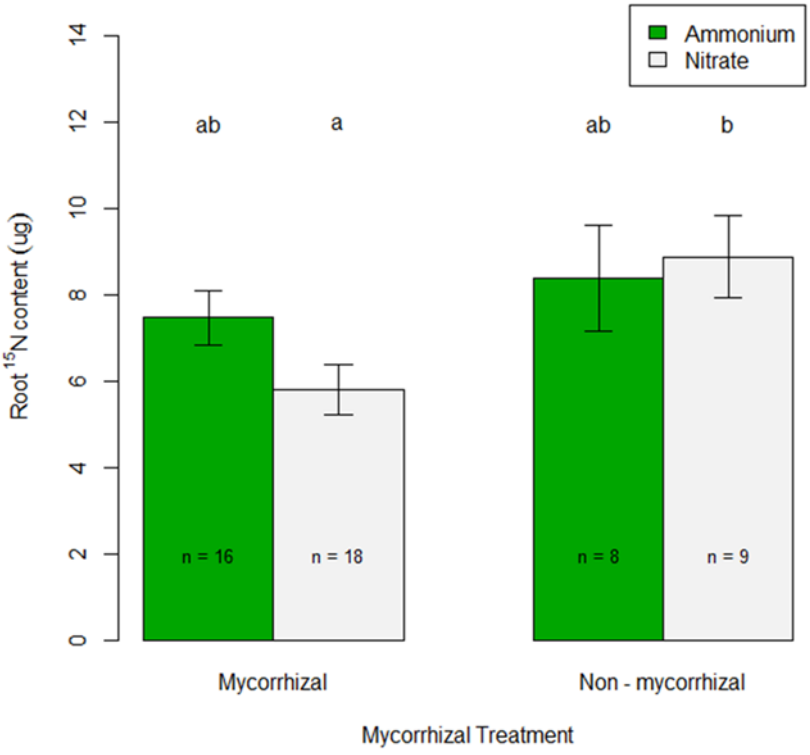

Figure 3.9. Root ^{15}N content. Mycorrhizal plants fed with nitrate-^{15}N had significantly less root ^{15}N content than non-mycorrhizal plants fed with nitrate-^{15}N Tukey HSD test, p = 0.040). Data are means ± SE

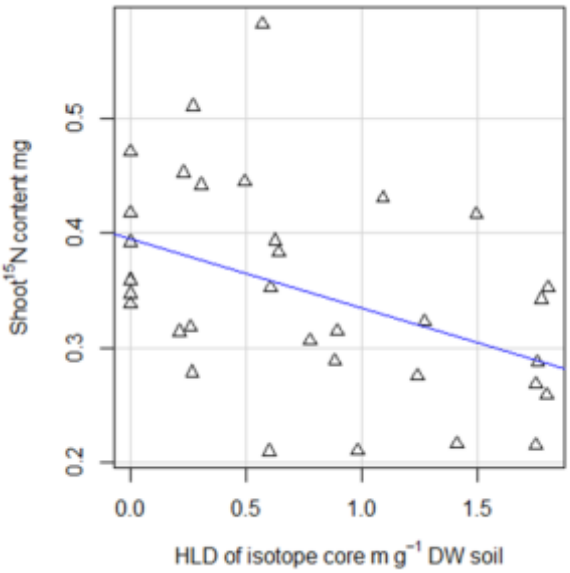

Figure 3.10. Shoot ^{15}N content plotted against HLD of AMF in the microcosm core to which the ^{15}N was added. Data are pooled from both species of AMF and both sources of ^{15}N, ammonium and nitrate. Hyphal proliferation within the core resulted in decreased shoot acquisition of ^{15}N ($t_{(32)}$ = -2.7663, p = 0.0093).

Across all treatment groups, the distribution of ^{15}N between shoot and root was even and as such ^{15}N concentration was not different between shoots and roots. Differences in ^{15}N content therefore may reflect the differences in DW.

Root dry weight (DW) was 24 % higher in the non- mycorrhizal plants compared to (F (1, 49) = 4.456, p = 0.039). Shoot DW was no different between the treatment groups. Overall, the difference in root DW between mycorrhizal plants and non-mycorrhizal plants was not substantial enough to make the total DW of the plants different between treatments. Similarly, the root weight ratio (proportion of the plant DW that is root DW) did not differ between treatments.

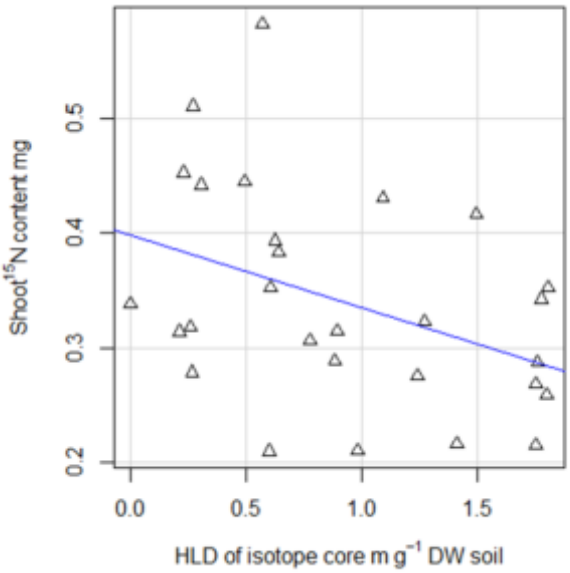

Figure 3.11. Shoot ^{15}N content plotted against HLD of AMF in the microcosm core to which the ^{15}N was added. Data are pooled from both species of AMF and both sources of ^{15}N, ammonium and nitrate. Cores in which HLD was zero have been excluded from this plot. Hyphal proliferation within the core resulted in decreased shoot acquisition of ^{15}N (t = -2.216, df = 26, p = 0.036).

Root N content was significantly higher in mycorrhizal plants than non-mycorrhizal plants $F_{(1, 49)} = 4.812$, $p = 0.033$). Neither the N content of shoots nor the total plant N content differed between mycorrhizal and non-mycorrhizal plants. N content of the roots did not differ between the two mycorrhizal fungal treatments. Mean concentration of N in roots (mg N g^{-1} DW) did not differ between *G. aggregatum* (11.14 ± 1.22), *R. irregularis* (12.25 ± 0.84) and the non-mycorrhizal plants (12.37 ± 1.00). Similarly, mean N concentration in shoots (mg N g^{-1} DW) did not differ between treatments.

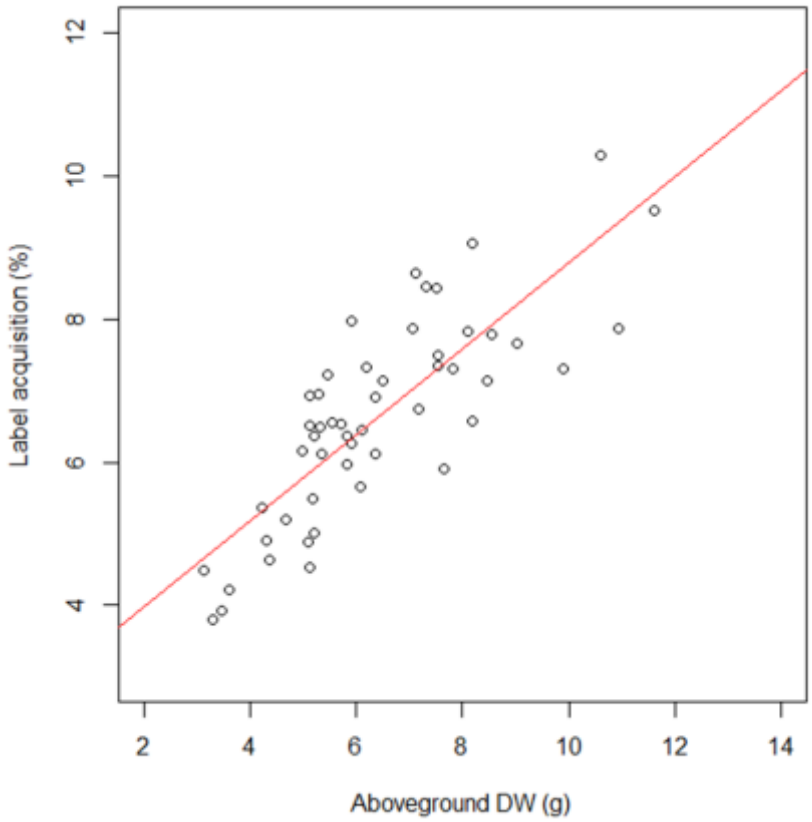

Figure 3.12. There is a strong positive correlation between the aboveground dry weight of onion plants and the relative acquisition of the ^{15}N label, calculated as the amount of ^{15}N in aboveground biomass expressed as a percentage of the total ^{15}N added to the mesh core ($t = 9.7383$, $df = 49$, $p < 0.001$).

Root phosphorus content of non-mycorrhizal plants was 30% higher than in mycorrhizal plants ($F_{(1,22)} = 4.518$, $p = 0.04501$), suggesting that inoculation was deleterious to total P uptake. Similarly, mean shoot P was higher in non-mycorrhizal (25.03 mg ± 1.35 mg) than mycorrhizal plants (17.94 mg ± 1.02 mg), ($F_{(1, 22)} = 16.67$, $p = 0.00049$). Combined, mean total P content of non-mycorrhizal plants was 36 % higher than in mycorrhizal plants ($F_{(1,22)} = 16.77$, $p = 0.00048$). The concentration of P in plant tissue did not differ between treatment groups, either in roots or shoots.

3.4. Discussion

Using a number of experimental approaches, this study was carried out to shed light on the relative importance of ammonium and nitrate as sources of N for the AM symbiosis. Using hyphal access compartments which excluded roots, and comparing against non-AM controls, ^{15}N uptake from these compartments and detection in plants was used as an indication of fungal preference for N source. In addition, the second experiment allowed the investigation of what relationship existed between the source of N acquisition, and the amount of C transferred to the sites of application of these N sources.

Experiment 1 shows that the AMF were engaging in N transfer to the roots, and it is presumed that the root was providing the AMF with C. Without isotopic analysis, we could not tell what C crossed to the ERM. The data from Experiment 2 suggests that the fungus did not engage in C-for-N trade with the plant, as the general trend shows more enrichment of ^{15}N in those plants which were in the non-AM treatment. This suggests that the fungus acted to hinder plant N uptake from the zones of N addition. A lack of mycorrhizal response to inorganic N addition is not unheard of (Smith and Smith, 2011b).

While higher uptake of ammonium N was predicted from the Petri plate experiment, surprisingly little ^{15}N acquisition was measured in roots unless the N was added as ammonium nitrate (Figure 3.), and even then, only 7 days after addition. While confirming the AMF isolate used was capable of acquiring N and transferring it to the plant, more ^{15}N transfer to the root might have been expected from the other treatments, knowing that AMF have previously been shown to be able to acquire N when provided solely as nitrate (Johansen et al., 1993) and ammonium (Ames et al., 1983, Johansen et al., 1992). While AMF are sometimes known not to contribute to plant N, and the nutrient environment in which the AM symbiosis obtains nutrients may determine the extent to which C-for-N (or C-for-P) trade takes place (Johnson, 2010), the presence of ^{15}N in the roots of the

ammonium nitrate group suggest that experimental system was capable of eliciting an N flux from AMF to shoot. The explanation for lack of ^{15}N flux in ammonium and nitrate plates may be that the fungus was unable to maintain electrochemical ion balance with the media (Jin et al., 2005), and thus further uptake was prevented. Before treatment addition, the media contained only a very low concentration of NH_4NO_3, and no pH buffer. As the AMF is thought to release H^+ when taking up NH_4^+ and OH- when taking up NO_3^- (Govindarajulu et al., 2005, Smith and Smith, 2011b), the pH of the media would be altered (Bago et al., 1996). As such, after treatment addition there may have been limited capacity for simultaneous anion and cation uptake in the ammonium and nitrate groups, without risking significant changes to hyphosphere pH, a problem not faced in the ammonium nitrate group. It was not possible to detect a change in hyphosphere pH in the agar here, as the method by which agar can be liquefied for measurement involves soaking in copious volumes of citrate buffer. As the ammonium nitrate group received a dual labelled isotope source ($^{15}NH_4{}^{15}NO_3$), it cannot be determined whether the ^{15}N present in the root had originated from the ammonium, the nitrate, or some from both.

The apparent inability of the AMF to acquire substantial amounts of N from the ammonium or nitrate-only agar has important implications. First, that the AMF were able to acquire two sources of N simultaneously, but were unable to acquire K^+ or Cl^- (in nitrate and ammonium groups, respectively) to balance the charge associated with ionic-N uptake. A further implication of this is that AMF uptake of mineral nutrients may be limited by the composition of the soil solution in which they exist, and that an absence of suitable ions which can be exchanged may represent a problem for fungal nutrition. In such a scenario, a fungus may not be able to acquire further nutrients until some exogenous force such a wetting event or nutrient uptake or efflux by another organism in the proximity (Cui and Caldwell, 1997).

Hyphal length densities of onion plants (Experiment 2) were within the ranges reported by other pot-based studies (Smith and Smith, 2011b), and the lack of difference between the two AMF species suggest that fungal proliferation was no different. In spite of this, the data from ^{14}C labelling experiment suggests that the *G. aggregatum* obtained significantly more ^{14}C from the plant than *R. irregularis* could. As HLD was no different between the AMF species, a number of possible explanations present themselves: higher hyphal respiration (Heinemeyer et al., 2006) or turnover (Staddon et al., 1999) by the *G. aggregatum* would result in increased release of C (and thereby ^{14}C) to the hyphosphere, without increased HLD. Alternatively, fungal ERM in the mesh cores may have been relatively C-enriched in the *G. aggregatum* compared to the *R. irregularis.* AMF are known to be able to acquire significant amounts of C from their hosts (Grimoldi et al., 2006) and are a major source of soil carbon (Rillig et al., 2001). Clearly, the ^{14}C data demonstrate that considering HLD data alone may not give a complete representation of the C cost of an AMF to a plant, and the balance of trade of the whole mycorrhizal system.

Zones of ammonium and nitrate addition did not show different HLD or ^{14}C however, suggesting that apportioning of C from the AMF, and by extension, the plant, was not determined by the type of N that was supplied. It must be concluded that the AMF found no predisposition to proliferate in a zone of ammonium or nitrate N addition over the course of the experiment, in the way that has been demonstrated for zones of organic N addition (Hodge and Fitter, 2010). Both nitrate and ammonium could have moved rapidly away from the zone of addition (Tinker and Nye, 2000) so what started as an N 'hotspot' may not have remained so for very long after the addition of N solution. Rapid movement of nitrate and ammonium out of the mesh cores to other areas of the substrate would give a more even concentration of inorganic N across the microcosm. This may explain why AMF proliferation was not observed, as has been in organic matter patches (Hodge et al., 2001, Leigh et al., 2009, Thirkell et al., 2016), which will last much longer, more

gradually releasing N (Hodge et al., 2000b). Combined, our data on ^{14}C labelling and HLD support the notion that there was no ammonium-nitrate split in allocation of fungal C resources. The HLD tests for a long-term pattern, as the experiment ran for 18 weeks, we might expect to see more growth into one core or another if the fungus displayed a preference. The ^{14}C labelling gives us a snapshot of nutrient dynamics within 1 day. Neither suggests that ammonium or nitrate was 'preferred' by the fungus. Importantly, it has been demonstrated that the C allocation from a plant to a discreet zone of soil can depend more on the identity of the AMF with which the plant is associated than the type of N that is provided at that zone.

The 'reciprocal rewards' notion that AMF-plant exchange is regulated by a fair trade of C-for-mineral nutrients (Kiers et al., 2011, Fellbaum et al., 2012) is perhaps the most hotly debated subject in the literature at present. Neither the ^{15}N or ^{14}C data here support it. We observe most ^{14}C allocation to mesh cores by *G. aggregatum*, not to one or other of the N sources. If the N-for-C were tightly regulated by reciprocal rewards, more ^{15}N would be expected in plants colonised by *G. aggregatum*, but this is not borne out in the data. In fact, we saw that AMF behaved no differently from one another in terms of ^{15}N transfer, or lack thereof, to the plant.

It is possible that *G. aggregatum* provided significant P to the plant, and the ^{14}C detected in the mesh cores was not specifically a 'reward' for the zones of N addition but was a part of a broader allocation of C to the mycelium of *G. aggregatum* as a whole. Although plant total P content was not increased by *G. aggregatum*, it cannot be said from that alone that the AMF did not provide a significant proportion of the plant P. Previous studies have shown a negative mycorrhizal growth response, even though the AM pathway provides 100% of the plant's P (Smith et al., 2003, Smith et al., 2004) so to say that the fungus was not contributing to plant P nutrition purely based on reduced plant P content is not

possible. As no P in the experiment was radiolabelled, it is not possible to track uptake by the AM route vs by direct root uptake.

Future microcosm studies of this nature would benefit from triple labelling of C, N and P, in concert with gene expression for transporters for both N and P in the roots of plants. Together, these measures should aid the elucidation of the mechanism of the nutrient flux. Using that approach, studies at different soil nutrient levels may be of benefit, so as test the Trade Balance Model (Johnson, 2010), to new levels of rigour i.e. relative contribution of the direct route and the mycorrhizal route for N and P uptake, and beyond the current limits of total plant nutrient quantity.

While it seems AMF hindered N capture by the plant rather than improved it, inferences can be drawn from the ^{15}N isotopic enrichment of the onion tissue. It could be argued that root ^{15}N data and shoot ^{15}N point towards a preference for the AMF to acquire ammonium over nitrate. In non-mycorrhizal plants, the shoot ^{15}N content does not differ between nitrate and ammonium systems (Figure 3.). By contrast, mycorrhizal plant shoots have significantly more ^{15}N when supplied as nitrate than ammonium. This would be the expected trend if AMF had a preference for ammonium, as they would leave the substrate relatively depleted in ammonium compared to nitrate, allowing more nitrate for direct uptake by roots. Remembering that root ^{15}N content includes IRM fungal ^{15}N too, and that AMF have a lower C:N ratio than plants (Hodge and Fitter, 2010), we see that AM nitrate plants do not have more ^{15}N than AM ammonium; in fact the trend has moved towards the ammonium roots being more enriched (although this is not statistically significant).

This gives further evidence that the AMF has taken ^{15}N ammonium from the substrate but has not yet passed it to the plant, or it would lead to relatively enriched shoots also. If this speculation is correct, it must be true that the IRM fungi is significantly enriched in ^{15}N, as root mass was less in AM plants. This would be the same trend as seen by Tanaka and

Yano (2005), who reported significant enrichment of ^{15}N in intraradical hyphae after addition of labelled ammonium to a hyphal compartment.

Although predicted to improve plant uptake of ^{15}N and N, and to do so to a greater extent for ammonium than nitrate, we find precisely the opposite occurs when considering shoot ^{15}N content (Figure 3., Figure 3, Figure 3). Across all measures of ^{15}N, the mycorrhizal plants acquired either an equal or lesser proportion of the ^{15}N pulse than the non-mycorrhizal plants, suggesting that the fungus was not operating to the benefit of the plant, at least in terms N acquisition, as has been demonstrated by other studies (Ames et al., 1983, Leigh et al., 2009, Thirkell et al., 2016). Plant aboveground biomass may have been a far greater determinant in ^{15}N acquisition, as shown by the correlation in (Figure 3).

Neutral or negative responses of plant nutrient content or biomass in response to colonisation by AMF is common (Klironomos, 2003), so its occurrence here is not entirely surprising. It seems very likely that the substrate here was N-limited, and as such the fungus satisfied its own demand before it would transfer any N to the plant. Such a scenario is predicted by the trade balance model (Johnson, 2010) which states that a low-N, low-P soil environment is likely to promote 'limited mutualism', in which C-for-N trade between plant and fungus is not favoured, as observed here.

Chapter 4.
Response to N source may be mediated by AMF symbionts in some, but not all barley cultivars

4.1 Introduction

In terms of the quantity produced and area cultivated, barley is the 4th most popular cereal in the world, and is a versatile crop; while primarily used as a fodder crop for livestock, barley is also used for malt in the brewing process (FAOSTAT, 2015). 'Winter' barley varieties are sown in the autumn and seedlings establish before a dormancy period over winter. This dormancy is broken in spring and growth continues, and the plant completes its lifecycle for harvest in autumn. 'Spring' barley varieties are sown in February-March, have a shorter growing season, and are also harvested that autumn. Barley ear physiology can be categorised into two types - depending on the number of fertile rows of spikelets around the rachis, barley is classed as either 2-row or 6-row barley, that is to say that 2 or 6 parallel rows of grain form along the ear (Komatsuda et al., 2007). Conventionally, two-row barley has the low protein content desirable in malt for brewing (Baethgen et al., 1995), while 6-row's higher protein content makes it suited for feedstock (Shewry, 2007).

Barley was domesticated in Eurasia around 13,000 years ago (Badr et al., 2000), from a wild ancestor *Hordeum vulgare* subsp. *spontanuem* L. , widely distributed in the 'fertile crescent' in Southern-central Eurasia (Salamini et al., 2002). Since then, various cultivars have been developed, many of which are well-suited to the climate of northern Eurasia. At these latitudes the majority of higher land plant species, including barley, form a symbiosis with arbuscular mycorrhizal fungi (AMF) (Read, 1991). Fungi from the Glomeromycota taxon (Schussler et al., 2001) are a type of soil fungus which grow into

the roots of around two thirds of higher plant species (Schussler et al., 2001), and in so doing facilitates a trade of nutrients (Smith and Read, 2008).

The fungus receives photosynthetic carbon from the plant, which in return acquires mineral nutrients from the fungus, having been obtained from the soil. The fungus is an obligate symbiont, relying on the plant as its only source of carbon. From the plant's perspective the main advantage of the symbiosis is its ability to aid uptake of immobile P from the soil (Smith and Smith, 2011b), by reaching out further into soil than the roots, and by massively increasing the surface area of soil exploited (Li et al., 2008)). While increased P nutrition may be the main benefit AMF bring to plants, they have also been shown to improve N, Cu, Zn nutrition (Marschner and Dell, 1994, Jones and Smith, 2004), as well as to provide improved drought tolerance (Auge, 2001) and pest and pathogen defence (Cameron et al., 2013).

Root colonisation by AMF is all-but ubiquitous in the grass family (Poaceae) to which barley belongs (Roumet et al., 2006) but this is not to say that all plants benefit equally from colonisation. Pairings of plant and AMF can result in greatly varied results in terms of plant performance, as colonisation may be greatly beneficial, neutral or negative for plant nutrient uptake and growth (Klironomos, 2003), deemed their 'mycorrhizal growth response' (MGR) (Hetrick et al., 1992). With respect to a plant's response to mycorrhizal colonisation, it has been suggested that a continuum between mutualism and parasitism exists (Johnson et al., 1997, Klironomos, 2003), and on which (for given environmental conditions) each pairing of plant and AMF species/cultivar could be placed. This is an over-simplification however, as external factors such as soil nutrient conditions may influence the degree to which colonisation is beneficial (Johnson, 2010, Johnson et al., 2015).

Like many grasses, barley shows a range of MGRs between different cultivars and in different environments (Jensen, 1984, Zhu et al., 2003, Castellanos-Morales et al., 2011), and is perhaps more often negatively than positively affected (Smith and Smith, 2011b) by colonisation. The influence of mycorrhizal colonisation on crop plant nutrition and growth cannot be overlooked given the ubiquitous distribution of AMF spores and inoculum through soils in temperate biomes (Smith and Read, 2008). Such a distribution of spores of the Glomermycota means that if a plant is capable of being colonised, and the environmental conditions allow, it is overwhelmingly likely to be colonised.

In addition to facilitating plant nutrient acquisition and by virtue of their abundance, AMF have a significant influence on nutrient dynamics within soil (Bender et al., 2015). Being N-rich (Hodge and Fitter, 2010), having rapid hyphal turnover (Staddon et al., 2003, Godbold et al., 2006) and being capable of moving mineral nutrients through their mycelium considerable distances to the plant; from 8 cm (Rhodes and Gerdemann, 1975) or even 25 cm (Jansa et al., 2003), AMF cannot be ignored when considering crop plant nutrition.

AMF have been promoted as a tool for sustainable agriculture (Johansson et al., 2004, Barea, 2015), as they have been shown to improve soil structure (Bearden and Petersen, 2000, Auge, 2001), which can in turn improve water holding capacity (Auge et al., 2001) and nutrient retention of soils (van der Heijden, 2010). Before advocating AMF as a 'silver bullet' solution for problems of modern agricultural systems however, it must be remembered how stark the contrast is between the environment in which the barley-AM symbiosis has evolved, and the present-day agricultural systems in which it is observed today.

The reliance of modern agricultural output on the addition of synthesized, inorganic fertilizer (especially nitrogen) cannot be overstated. Current application of fertilizer in the

developed world amounts to millions of tons per year (FAOSTAT, 2015), allowing significant yield increases but not without changing soil chemistry in the process (Marschner, 2011). Fertilizer input into agricultural soils leads to greatly increased concentrations of ammonium (NH_4^+), nitrate (NO_3^-) and other mineral nutrients such as K^+, Mg^+, Cl^- (Marschner, 2011). Fertilizer application is tailored not only to the target crop, but also the soil type, climate and season (DEFRA, 2013). While improved crop nutrition is the ultimate goal, the choice of which fertilizer to apply to crops is also influenced by the cost, availability and environmental considerations of particular fertilizer types (DEFRA, 2013).

Extensive research carried out over the last 100 years and more has shown that (for any given crop receiving a given quantity of N), fertilizers are not equal in their efficacy at improving growth, nutrition and yield (Silvertown et al., 2006). Many plant species experience 'NH_4^+ toxicity' when fed with ammoniacal fertilisers alone (Marschner, 2011). It has become apparent that barley plants provided with N exclusively as ammonium often perform worse than those provided solely with nitrate or a combination of ammonium and nitrate (Britto and Kronzucker, 2002). Ali et al. (2001) and Lopes et al. (2004) found that barley (varieties 'Arivat' and 'Graphic', respectively) given an ammonium nitrate nutrient solution performed better than those given either N source exclusively, while Soares and Lewis (1986) found that the barley variety 'Clipper' plants performed best when fed NO_3^-.

An additional level of complexity is added as the AMF themselves show varied responses to N fertilization (Toljander et al., 2008, Verbruggen et al., 2010, Daniell et al., 2001). AMF are known to be able to acquire NH_4^+ (Tobar et al., 1994b) and NO_3^- (Bago et al., 1996), from the hyphosphere and transport a fraction to the plant (Govindarajulu et al., 2005), and like their plant hosts, AMF have been shown to be influenced by the N source provided. Chambers et al. (1980) showed that colonisation by AMF was reduced in plants

fed only NH_4^+ while Hawkins and George (1999) showed that extraradical hyphal growth was reduced compared to nitrate-fed counterparts.

Given plants' varied tolerance or 'preference' for different N sources (Marschner, 2011), that plants are almost universally colonised by AMF (Smith and Smith, 2011a), and that these AMF can certainly be influenced by N source (Daniell et al., 2001), there is considerable scope for interaction between AMF type, N source and plant variety in terms of nutrient uptake and growth. However, there is currently a paucity of such experimental comparisons in the literature.

Thus, this study aimed to address the following hypotheses: i) NH_4^+ should be the worst source of N for barley and AMF growth, due to NH_4^+ toxicity; ii) AMF species will perform differently in response to N treatment and plant host identity, given the variability between the treatment groups, and AMF response variability.

A fully factorial, greenhouse-based microcosm study was designed to investigate the interactive effect of AMF species, N source and barley variety. Two AMF species *Rhizophagus irregularis* (Schenck and Smith isolate 09) and *Glomus aggregatum* (Schenck and Smith isolate 0165) were used, along with non-AM controls, and two barley (*Hordeum vulgare* L.) cultivars 'Maris Otter' and 'KWS Meridian' (hereafter, Meridian). To examine the effect of N source, plants were fed Long Ashton Nutrient Solution (LAS) (Smith et al., 1983) that was prepared to contain equal quantities of either ammonium-N, nitrate-N or ammonium nitrate-N.

4.2. Materials and Methods

4.2.1. Experimental design

The effects of inoculation by arbuscular mycorrhizal fungal species, *Rhizophagus irregularis* and *Glomus aggregatum*, on the growth of two barley (*Hordeum vulgare* L.) varieties, Maris Otter and Meridian were investigated. The influence of N source to the plants was investigated, by providing the plants with nutrient solution containing NH_4^+, NO_3^- or NH_4NO_3. Barley plants were inoculated with either *G. aggregatum* or *R. irregularis*, or were given sterilised inoculum to act as non-AM control treatment (Figure 4.). Inoculum was obtained from monoxenic, transformed root cultures, described below.

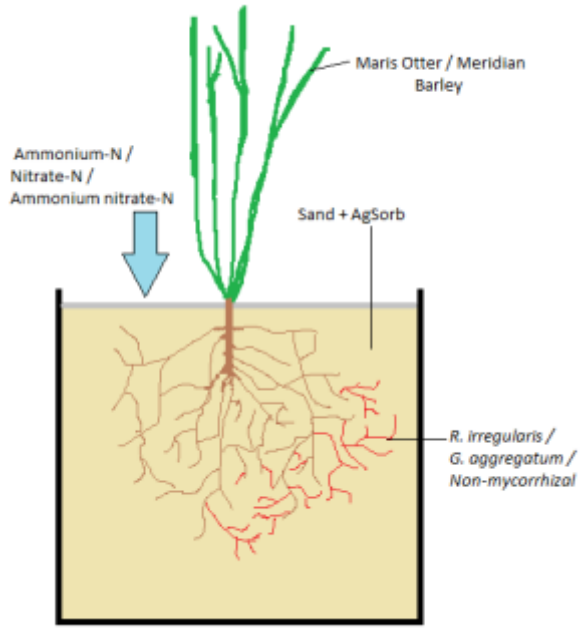

Figure 4.1. Barley (*Hordeum vulgare* L. cv. "Meridian' and 'Maris Otter') plants were grown individually in a sand/TerraGreen substrate. Plants were colonised by AMF species *R. irregularis* or *G. aggregatum*, or were non-AM controls. Systems received equal quantities of Long Ashtons nutrient solution prepared to provide N either solely as ammonium (NH_4^+) or nitrate (NO_3^-), or in combination, as ammonium nitrate (NH_4NO_3).

Using a fully factorial design, there were 18 treatment groups, each with 7 replicate plants, making 126 plants in total. Combinations of barley variety, mycorrhizal inoculum and N source were as follows:

Table 4.1. Treatment combinations. A fully factorial design was used, comprising two barley varieties- (Maris Otter, Meridian); three AMF treatments – (*Rhizophagus irregularis, Glomus aggregatum*, non-AM); and three nitrogen (N) addition sources – nitrate (NO_3^-), ammonium (NH_4^+), ammonium nitrate (NH_4NO_3). This arrangement gives 18 treatment groups.

Barley variety	AMF inoculum	Nitrogen Source
Meridian	*R. irregularis*	Ammonium
Meridian	*R. irregularis*	Nitrate
Meridian	*R. irregularis*	Ammonium Nitrate
Meridian	*R. irregularis*	Ammonium
Meridian	*R. irregularis*	Nitrate
Meridian	*R. irregularis*	Ammonium Nitrate
Meridian	*G. aggregatum*	Ammonium
Meridian	*G. aggregatum*	Nitrate
Meridian	*G. aggregatum*	Ammonium Nitrate
Maris Otter	*G. aggregatum*	Ammonium
Maris Otter	*G. aggregatum*	Nitrate
Maris Otter	*G. aggregatum*	Ammonium Nitrate
Maris Otter	Non-Mycorrhizal	Ammonium
Maris Otter	Non-Mycorrhizal	Nitrate
Maris Otter	Non-Mycorrhizal	Ammonium Nitrate
Maris Otter	Non-Mycorrhizal	Ammonium
Maris Otter	Non-Mycorrhizal	Nitrate
Maris Otter	Non-Mycorrhizal	Ammonium Nitrate

4.2.2. Experimental conditions

Each plant was grown in a 15 x 15 x 15 cm polypropylene pot, resting in a saucer to collect any excess water and nutrient solution added. Plants were grown in a 1:1 (v/v) mixture of silica sand and TerraGreen® (a calcinated, attapulgite clay soil conditioner - Oil-Dri, Cambridgeshire, UK), which had been washed 3 times in deionised water to remove labile minerals, and then autoclaved to sterilise any residual microbial inoculum.

To inoculate plants in the AM treatment groups, 15 g of the stock culture (see 4.2.3. AMF Inoculum) was homogenised with a hand blender and mixed thoroughly into the growth medium immediately prior to planting, so that the distribution of mycelium and colonised root fragments was equal throughout each pot. For those pots in the non-AM treatments, the same quantity of twice-autoclaved inoculum (121 °C, for a total of 44 minutes) was included.

Week-old seedlings, prepared (see 4.2.5. Seed preparation) were planted October 14th 2015, and the experiment ran for 20 weeks. Plants were destructively harvested in the week commencing February 29th 2016, by which point the barley had reached growth stage 39 on the BBCH cereal development stage scale. By the growth stage, the plants had produced all the tillers that would develop, and are in the stem elongation phase. This represents the period of most rapid N uptake in the barley lifecycle (YARA, 2015). Throughout the experiment, plants were maintained in a heated, lit greenhouse with a day-time temperature maintained at 18 °C (± 2.1 °C), and a night-time temperature of 15 °C (± 1.5 °C). Additional lighting was provided throughout the duration of the experiment by 400 W halogen lamps, to extend the photoperiod to 16 hours per day. Watering was carried out daily as needed, and nutrients provided by addition of 'Long Ashtons' nutrient solution. Plants were randomised fortnightly to ameliorate any environmental gradient of the glasshouse conditions.

4.2.3. AMF Inoculum

Inoculum for both *G. aggregatum* and *R. irregularis* were obtained by propagation of stocks kindly donated from the lab of Toby Kiers, Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit, Amsterdam. The Kiers group stock of *R. irregularis* was created using spores obtained from Schenck & Smith, isolate 09 collected from Southwest Spain by Mycovitro S.L. Biotechnología ecológica,

Granada, Spain, and *G. aggregatum* from Schenck & Smith; isolate 0165 collected from the Long Term Mycorrhizal Research Site, University of Guelph, Canada.

Inoculum was prepared by growing the AMF mycelium in monoxenic culture utilising Ri T-DNA transformed *Daucus carota* L. (carrot) root stocks, as Becard and Fortin (1988). Propagation of the transformed root on a low mineral, modified Strullu-Romand media (MSR, as in Declerck et al. (1998) from the original medium of Strullu and Romand (1986)) and subsequent inoculation with spores of AMF allows the production of significant quantities of both AMF mycelium and spores (Diop et al., 1994). Stocks are prepared by excising a section of colonised, transformed root from one Petri dish, and placing it into another dish of MSR medium. Plates are then sealed with Parafilm M® (Bemis Flexible Packaging, Oshkosh, WI, USA), and maintained in the dark at 22 °C. After 3-5 months, roots and AMF will have grown to cover the dish, and sporulation will have occurred, providing ample inoculum for the experimental plants.

4.2.4. Barley varieties

Two winter barley varieties were chosen for this experiment: Maris Otter is characterised as a 2-row, malting barley, preferred for use in brewing for its low N content. By contrast, Meridian is a 6-row barley that is preferred for use as animal feed, owing to its relatively high protein, and therefore N, content. Seed was kindly provided by ADAS, having been obtained from Robin Appel (Waltham Chase, Hampshire, UK) and KWS UK Ltd (Thriplow, Hertfordshire, UK) respectively.

4.2.5. Seed preparation

As is standard for commercially available seed of winter varieties of arable crops, both Maris Otter and KWS Meridian seeds are sold coated in a substantial amount of a

combined insecticide and fungicide, (in this case RedigoDeter, Bayer, Newbury, UK). The fungicidal ingredient, prothioconazole prevents ergosterol synthesis in the fungus, thereby inhibiting proper plasma membrane formation (Parker et al., 2013). Therefore, before embarking on the full experimental programme, it was necessary to ensure that the seed treatment would not impair the development of arbuscular mycorrhizas within the greenhouse-based experiment.

To that end, a trial was set up to confirm that the plants were capable of forming mycorrhizas despite the fungicidal coating of the seeds. As far as possible, the seed treatment was removed prior to germination. A 50 mL sample of seed was transferred to a 1000 mL Erlenmeyer flask and suspended in 500 mL of deionised water to which a few drops of surfactant Tween80 (Acros Organics BVBA Janssen Pharmaceuticalaan, Geel, Belgium) had been added, to improve wetting of the seeds. The suspended seeds were mixed for 30 minutes by the use of a magnetic stirrer plate, to agitate the seeds and dissolve away the RedigoDeter seed coating. Owing to the vivid pink colour of the treatment, it was easily noticeable when it began to dissolve into the water. After 30 minutes the water was discarded, and replaced with deionised water, and the stirring rinse repeated. In total, each 50 mL sample of seeds was washed in this way 4 times, after which only very few seeds retained traces of Redigo Deter. These individuals were discarded at this stage.

Seeds (with no visible traces of RedigoDeter) were placed onto moist Whatman No. 1 filter paper (GE LifeSciences, Little Chalfont, UK), inside 90 mm Petri dishes, wetted with deionised water, and left in a 20 °C incubator to germinate. After 1 week, germinated seeds were moved to seedling trays containing washed and autoclaved silica sand, to which a 10 g sample of AMF inoculum described above had been mixed until distributed homogenously. Seedlings were maintained in a heated, lit glasshouse for 6 weeks, watered

daily and had weekly additions of 50 mL half-strength 'ammonium nitrate type' Long Ashtons nutrient solution, detailed below (see 4.2.6. Nutrient Addition).

After 6 weeks, plants were destructively harvested, and roots were cleaned of sand in tap water. To confirm AM colonisation, roots were stained using the ink + vinegar method described below. Mycorrhizal colonisation was confirmed by inspection of stained roots at 200x magnification.

After confirmation of colonisation by AMF, the rinsing protocol outlined above was deemed sufficient to allow the experiment to continue with the seed selected.

4.2.6. Nutrient Addition

Mineral nutrition was provided by addition of Long Ashtons nutrient solution (LAS), which can be made to different protocols to mean that N can be supplied as NH_4^+, NO_3^- or NH_4NO_3. While the form of nitrogen changed, alterations to other mineral constituents of the solution were minimal. Long Ashtons solutions were prepared as per the protocol of Smith et al. (1983) and contained 170 mg N L^{-1}. For the first 2 weeks, 100 mL half-strength LAS was added to each microcosm pot. In weeks 4-5, 100 mL full-strength LAS was added once per week. From week 6 to week 20, 100 mL full-strength LAS was added twice weekly. In total, plants received 578 mg N. The concentration of P in the solution was reduced from the original protocol (41 mg L^{-1}) to 20.5 mg L^{-1}. Over the course of the experiment, each pot therefore received 73.2 mg P.

4.2.7. Harvest and Analysis

Plants were destructively harvested in the week commencing February 29th, 2016. At harvest, a sub-sample of growth medium was taken for quantification of extraradical

hyphal mycelium, detailed below, (see section 4.2.7. Harvest and Analysis). Roots were washed of growth medium and a small sub-sample was taken for determination of root length colonisation (RLC) and arbuscule and vesicle frequency, also detailed below. The remaining root samples were dried in at 70 °C for 72 hrs, after which they were weighed. Tiller number of the plants were recorded, as this is an important determinant of the total grain yield (YARA, 2015). Shoots were then dried at 70 °C for 72 hrs and then weighed. Following drying, individual root and shoot samples were homogenised first in a kitchen blender (Morphy Richards, Mexborough, South Yorkshire, UK) and then in a Retsch MM 400 mill (Retsch GmbH, Haan, Germany).

Sub-samples of homogenised shoot and root samples were pelleted using a Specac 10,000 kg manual press (Specac Ltd., Orpington, UK) and P concentration was determined by x-ray fluorescence (XRF) using a Thermo Scientific Niton XL3t900 GOLDD Analyzer (Thermo Scientific, Winchester, UK), following the protocol of Reidlinger et al., (2012).

Shoot and root tissue N was determined by CN analysis. Known weights (15 mg ± 5 mg) of homogenised sample were analysed with a Vario EL Cube CNHS analyser (Elementar isoprime, Elementar Analysensysteme GmbH, Hanau, Germany).

Root length colonisation was determined microscopically following staining using the protocol of Vierheilig et al (1998). Briefly, washed roots were ‘cleared’ in 10 % (w/v) KOH at 70° C for 20 minutes, after which they were acidified for 10 minutes at 25 °C in 1 % HCl. Roots were then transferred to a staining solution, prepared with Pelikan ‘Brilliant Black’ ink (Pelikan Vertriebsgesellschaft mbH & Co. KG), de-ionised water and acetic acid in a ratio of 1:1:18, where they remained at 25 °C for 20 minutes. After removal from the stain, roots were rinsed in de-ionised water and immersed in a 50 % (v/v) glycerol solution for 24 hours to remove excess stain, after which they were mounted onto microscope slides in the same 50 % glycerol.

Extraradical mycelium was extracted from samples of growth medium collected at harvest, using the method of Staddon et al. (1999), as detailed in Chapter 3, section 2.2.6. Soil hyphal length was quantified using the gridline intercept method and subsequent scaling calculations, as Hodge (2003).

4.2.8. Statistical Analysis

Data analysis was performed using the "R 3.1.0" statistical package, through the "RStudio" integrated development environment. (R foundation for Statistical Computing, Vienna, Austria). Prior to analysis, data were checked using Levene's test and by Shapiro-Wilk and Kolmogorov-Smirnov for normality.

Criteria of plant and/or fungal performance (e.g. shoot N concentration) were treated as response variables, while explanatory variables were barley cultivar (Meridian, Maris Otter), mycorrhizal inoculum (*G. aggregatum, R. irregularis*, Non-AM) and nitrogen application type (ammonium, nitrate, ammonium nitrate). Three-way ANOVA was used to test AMF colonisation and hyphal length density among barley cultivars, nitrogen addition types and AMF inoculation treatments. Akaike information criterion (AIC) suggested that for subsequent analysis, the data were better analysed by two-way ANOVA, with each barley cultivar tested separately. As such, each ANOVA used explanatory variables of nitrogen addition type and AMF inoculum treatment.

4.3. Results

Both Maris Otter and Meridian were colonised by *G. aggregatum* and *R. irregularis*, while non-AM controls had no detectable mycorrhizal colonisation. In the two AMF treatments, root length colonisation did not differ between N sources, AMF species or between barley varieties; and colonisation was reasonably consistent and not significantly

different between treatment groups. Mean colonisation ranged from 26.87 % (± 5.14 %) in Meridian colonised by *R. irregularis*, fed ammonium-N; to 54.42 % ± 9.44 % in Meridian, colonised by *R. irregularis*, fed nitrate-N (Figure 4.2).

Indeed, none of the fungal parameters, i.e. RLC, arbuscule or vesicle frequency, or hyphal length density, were influenced by the N source added. There was also no difference between in vesicle frequency between AMF treatment groups, or between the barley varieties.

There was a significant interaction between barley variety and AM inoculum type in arbuscule frequency ($F_{3,80} = 4.36$, $p < 0.01$), although there was no influence of the main effects individually. Meridian showed higher arbuscule frequency when colonised by *G. aggregatum* (13.14 % ± 2.10 %) than by *R. irregularis* (4.62 % ± 1.19 %).

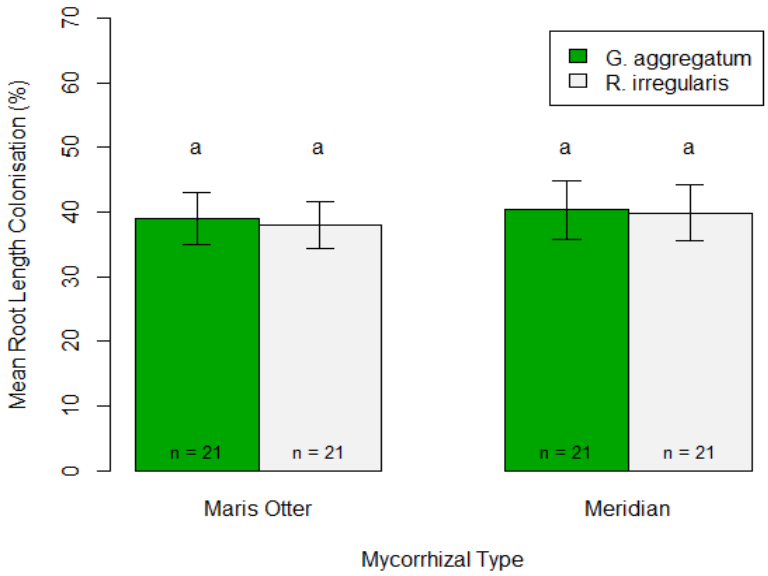

Figure 4.2. The barley cultivars Maris Otter and Meridian showed remarkably similar root length colonisation, and there was no difference between groups inoculated with *Rhizophagus irregularis* and *Glomus aggregatum*. Non-AM controls not colonised by AMF. Nitrogen treatment did not influence root length colonisation. Data shown are means ± standard error, n = 21. Green bars - *G. aggregatum*, grey bars - *R. irregularis*.

All subsequent analysis was carried out *within* each barley cultivar, comparing the AMF treatments (*R. irregularis* vs. *G. aggregatum* vs. non-AM), and the N source treatments (NH_4^+ vs. NO_3^- vs NH_4NO_3), in a fully factorial experiment. Comparison of tissue DW, nutrition etc between barley cultivars would not represent meaningful comparison.

Shoot N content in Maris Otter was significantly influenced the by N source ($F_{2,54} = 5.61$, $p < 0.01$). Post-hoc analysis by Tukey Honest Significant Difference test (TukeyHSD) showed that NH_4^+ -fed plants had lower N content than those fed either NO_3^- ($p = 0.026$) or NH_4NO_3 ($p = 0.014$). Reduced N content was driven by a significantly lower N concentration in shoots of NH_4^+ -fed plants compared to NO_3^- -fed or NH_4NO_3-fed ($F_{2, 54} = 7.99$, $p < 0.001$). Shoot N concentration was sufficiently lower in NH_4^+ -fed plants that total plant N content ($F_{2,54} = 4.98$, $p = 0.011$) and the N:P ratios were reduced in the shoot ($F_{2,54} = 10.68$, $p < 0.001$), and in the plant overall ($F_{2,54} = 5.574$, $p < 0.01$). Neither the concentration nor the content of N in Maris Otter roots were influenced by changes in N source or AM inoculum treatments. By contrast, the P content and concentration of Maris Otter did not vary between treatments, either in shoots or roots.

Despite significant nutritional gain in terms of N, dry weight (DW) did not differ between treatments for shoots or roots of Maris Otter. Root DW was not different between NH_4^+ fed Maris Otter, (4.59 g ± 0.18 g) those fed NH_4NO_3 (5.30 g ± 0.34 g) or NO_3^- (5.47 g ± 0.29 g). Root-weight-ratio (*i.e.* the proportion of the total plant DW that is root DW) was significantly lower in the ammonium-fed plants, however ($F_{2,54} = 3.66$, $p = 0.03$).

Tiller number in Maris Otter was significantly influenced by both AMF treatment ($F_{2,54} = 8.02$, $p < 0.001$) and N source ($F_{2,54} = 5.96$, $p < 0.01$) although there was no interaction. Plants colonised by *G. aggregatum* had significantly fewer tillers (11.85 ± 0.47) than those colonised by *R. irregularis* (14.61 ± 0.57) ($p < 0.001$) (see Figure 4.4). Nitrate-fed

plants had significantly fewer tillers (12.00 ± 0.39) than ammonium fed plants (14.38 ± 0.63) ($p < 0.01$).

Data in Tables 4.2 and 4.3 show the results of ANOVA performed for N and P nutrition data of Meridian, between AMF inoculum treatments and N source treatments, respectively. With one exception (shoot N:P ratio, addressed later), there was no significant interaction between AMF inoculum treatment and N source in Meridian. The data provide evidence for mycorrhiza-dependent nutrient acquisition by Meridian barley. Plants colonised by *R. irregularis* seemed to perform best, and had N and P nutrition that was either better than, or not significantly different from, *G. aggregatum* or Non-AM groups.

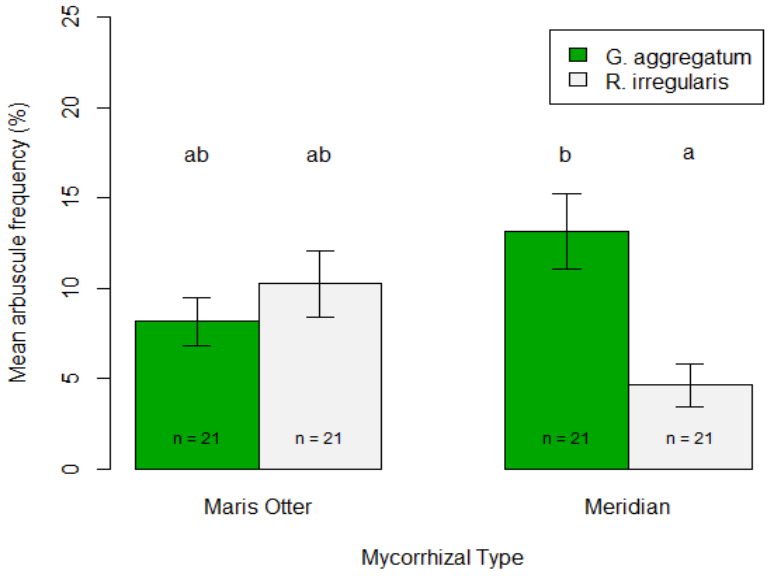

Figure 4.3. Mean arbuscule frequency (the percentage of root intersections observed to contain arbuscules) in Meridian was significantly higher when roots were inoculated with *Glomus aggregatum* than when inoculated with *Rhizophagus irregularis* (Tukey HSD, $p = 0.004$). By contrast, arbuscule frequency in Maris Otter did not differ between AMF species. Arbuscule frequency did not differ between nitrogen treatments. Data shown are means ± standard error.

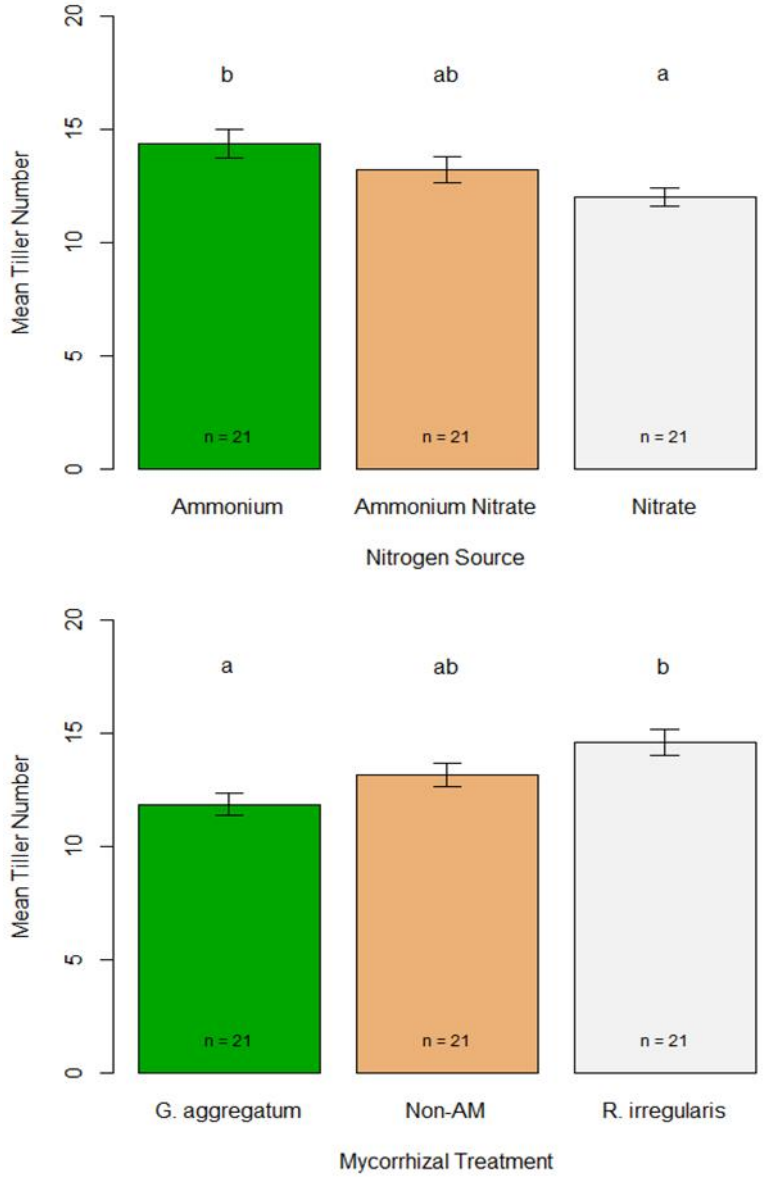

Figure 4.4. Tiller number of Maris Otter was influenced both by mycorrhizal inoculum ($F_{2,54}$ = 8.02, p < 0.001) and by nitrogen source ($F_{2,54}$ = 5.96, p < 0.01), but there was no interaction between these factors. Plants receiving ammonium Long Ashton solution (LAS) had an average of 14.4 tillers, (± 0.6) compared to 12.0 (± 0.4) in those receiving nitrate LAS (TukeyHSD, p < 0.01). Between AMF treatments, tiller number was greatest in those plants inoculated with *R. irregularis* (14.6 ± 0.6), and significantly lower in the *G. aggregatum* group (11.9 ± 0.5) (TukeyHSD, p < 0.001). Data shown are means ± standard error.

The *R. irregularis* group had shoot N content 15 % higher and total N content 13% higher than those colonised by *G. aggregatum*, but not higher than Non-AM controls (Table 4.2).

Root N content showed a different trend, and was 16 % higher in *G. aggregatum* plants and 22 % higher in *R. irregularis* plants than in Non-AM control.

Improved shoot N nutrition of plants in the *R. irregularis* colonised plants meant that the N:P ratio of the whole plant was significantly higher than in the non-AM plants, but not significantly higher than those colonised by *G. aggregatum*. While both AMF inocula may have benefitted root N acquisition compared to non-AM controls, shoot N content was significantly lower in *G. aggregatum*-inoculated plants than non-AM controls, although shoot N concentration did not significantly differ. Total P content was higher in *R. irregularis* than in non-AM controls and the *G. aggregatum* plants, driven by the increase in shoot P concentration and content (Table 4.2).

The source of N provided to Meridian barley had dramatically different effects on N and P nutrition of the plant. Concerning N content and concentration, Meridian plants receiving NH_4NO_3 were always at an advantage over, or no different from, plants fed only NO_3^- or NH_4^+ (Table 4.2). This trend is despite all groups receiving the same quantity of N overall.

Plant P uptake was greatest when plants received ammonium, and either reduced or not significantly different when fed ammonium nitrate or nitrate (Table 4.). Similarly, nitrate-fed plants were either no different or lower in P than plants fed ammonium nitrate.

As might be expected from these patterns of N and P acquisition, the ratio of N:P content depended upon the N source supplied, both in roots ($F_{2,54} = 8.51$, $p < 0.001$), and in plants overall ($F_{2,54} = 40.96$, $p < 0.001$). In both cases, N:P was highest in the nitrate group and lowest in the ammonium group.

Table 4.2. Nitrogen (N) and phosphorus (P) concentration and content data for Meridian barley under different mycorrhizal treatments. Plants colonised by *Glomus aggregatum* generally acquired less N than those colonised by either *Rhizophagus irregularis* or the non-AM controls while *R. irregularis* was not different from non-AM controls. Plants colonised by *G. aggregatum* also performed worst in terms of P acquisition. Data are means ± standard error, n = 21

	G. aggregatum	*R. irregularis*	Non-AM	ANOVA output
N concentration (mg g^{-1} DW)				
Shoot	17.99 ± 0.59 ^{a}	18.97 ± 0.50 ^{a}	18.93 ± 0.49 ^{a}	NSD
Root	8.60 ± 0.30 ^{b}	8.20 ± 0.30 ^{ab}	7.55 ± 0.21 ^{a}	$F_{2,54} = 5.84$, $p = 0.023$
N content (mg)				
Shoot	106.15 ± 4.11 ^{a}	122.79 ± 4.02 ^{b}	121.35 ± 4.08 ^{b}	$F_{2,54} = 6.99$, $p = 0.002$
Root	40.56 ± 1.00 ^{b}	42.60 ± 1.05 ^{b}	34.84 ± 1.03 ^{a}	$F_{2,54} = 15.99$, $p < 0.001$
Total plant	146.71 ± 4.34 ^{a}	165.39 ± 4.27 ^{b}	156.19 ± 4.29 ^{ab}	$F_{2,54} = 6.17$, $p = 0.004$
P concentration (mg g^{-1} DW)				
Shoot	2.70 ± 0.09 ^{a}	2.93 ± 0.07 ^{b}	2.68 ± 0.08 ^{a}	$F_{2,54} = 4.75$, $p = 0.013$
Root	1.54 ± 0.08 ^{a}	1.53 ± 0.09 ^{a}	1.37 ± 0.10 ^{a}	NSD
P content (mg)				
Shoot	16.06 ± 0.77 ^{a}	18.93 ± 0.63 ^{b}	17.17 ± 0.67 ^{ab}	$F_{2,54} = 7.84$, $p = 0.001$
Root	7.43 ± 0.51 ^{ab}	8.07 ± 0.52 ^{b}	6.41 ± 0.54 ^{a}	$F_{2,54} = 3.20$, $p = 0.048$
Total plant	23.50 ± 1.18 ^{a}	27.00 ± 0.81 ^{b}	23.58 ± 0.94 ^{a}	$F_{2,54} = 6.88$, $p = 0.002$

Table 4.3. N and P nutrition data for Meridian under different N source treatment groups. Plants received equal quantities of N, provided either as ammonium (NH_4^+), nitrate (NO_3^-) or ammonium nitrate (NH_4NO_3). Plants receiving NH_4^+ or NO_3^- were at a disadvantage for N capture compared to those fed NH_4NO_3. P acquisition however, was highest in plants receiving NH_4^+, and lowest in those receiving NO_3^-. Data are means ± standard error, n = 21.

	Ammonium	Ammonium Nitrate	Nitrate	ANOVA output
N concentration (mg g^{-1} DW)				
Shoot	17.30 ± 0.56 [a]	19.65 ± 0.39 [b]	18.95 ± 0.49 [b]	$F_{2,54} = 4.04$, $p = 0.023$
Root	7. 86 ± 0.26 [a]	8.46 ± 0.25 [a]	8.02 ± 0.33 [a]	NSD
N content (mg)				
Shoot	107.64 ± 4.27 [a]	130.64 ± 3.97 [b]	112.00 ± 3.05 [a]	$F_{2,54} = 12.28$, $p < 0.001$
Root	39.03 ± 1.30 [a]	39.70 ± 1.19 [a]	39.50 ± 1.19 [a]	NSD
Total	146.67 ± 4.35 [a]	169.83 ± 4.20 [b]	151.50 ± 3.61 [a]	$F_{2,54} = 10.24$, $p < 0.001$
P concentration (mg g^{-1} DW)				
Shoot	3.06 ± 0.07 [c]	2.78 ± 0.07 [b]	2.46 ±0.06 [a]	$F_{2,54} = 22.58$, $p < 0.001$
Root	1.78 ± 0.91 [b]	1.35 ± 0.07 [a]	1.30 ± 0.07 [a]	$F_{2,54} = 11.30$, $p < 0.001$
P content (mg)				
Shoot	18.96 ± 0.54 [b]	18.58 ± 0.69 [b]	14.61 ± 0.55 [a]	$F_{2,54} = 21.68$, $p < 0.001$
Root	8.98 ± 0.57 [b]	6.39 ± 0.40 [a]	6.54 ± 0.43 [a]	$F_{2,54} = 9.70$, $p < 0.001$
Total	27.94 ± 0.89 [c]	24.99 ± 0.85 [b]	21.16 ± 0.81 [a]	$F_{2,54} = 19.95$, $p < 0.001$

A significant interaction between N source and AMF treatment was evident in N:P ratio of shoots in Meridian ($F_{4,54} = 5.70$, $p < 0.001$; see Figure 4.5). Plants colonised by *G. aggregatum* had either the highest or lowest N:P ratio, depending on the N source provided; plants receiving NH_4^+ had the lowest mean N:P (4.91 ± 0.08) and NO_3^- , the highest (8.33 ± 0.40). N:P ratios did not differ between N treatments in plants colonised by *R. irregularis*. Non-AM plants showed higher N:P when N was supplied as NO_3^- than as NH_4^+.

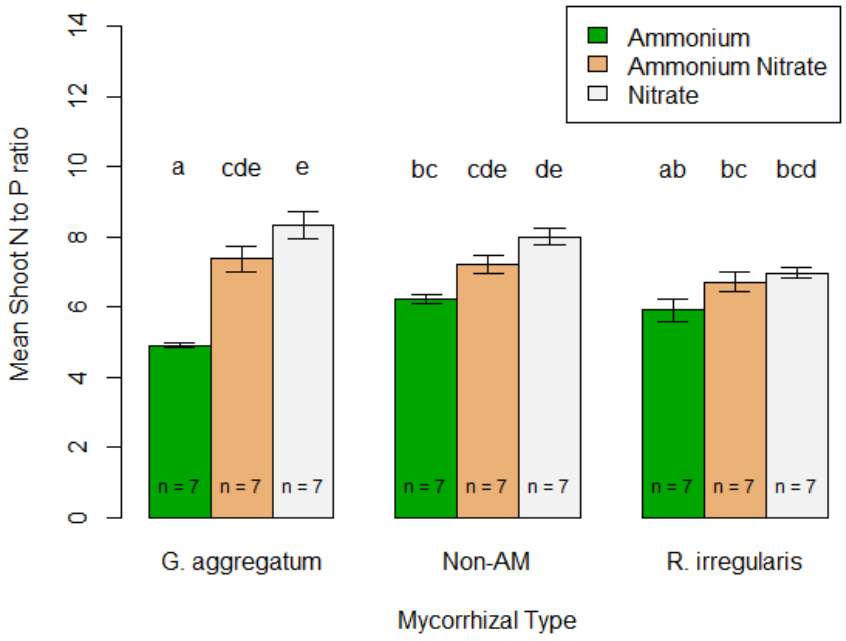

Figure 4.5. There were significant effects on Meridian shoot N:P ratio from N source ($F_{2,54} = 4.07$, $p = 0.02$) and AMF treatment ($F_{2,54} = 48.26$, $p < 0.001$) and there was a significant interaction between these factors ($F_{4,54} = 5.70$, $p < 0.001$). Bar colour denotes N source: green - NH_4^+, peach - NH_4NO_3, grey - NO_3^-. Plants which were colonised by *Glomus aggregatum* and received NH_4^+ had lower shoot N:P than those fed NO_3^- (TukeyHSD, $p < 0.001$) and NH_4NO_3 (TukeyHSD, $p < 0.001$). In the Non-AM controls, NH_4^+ -fed plants had lower N:P than those in the NO_3^- group (TukeyHSD, $p = 0.001$). In the *Rhizophagus irregularis*-colonised plants, N treatment had no effect. Data shown are means ± standard error, n = 7.

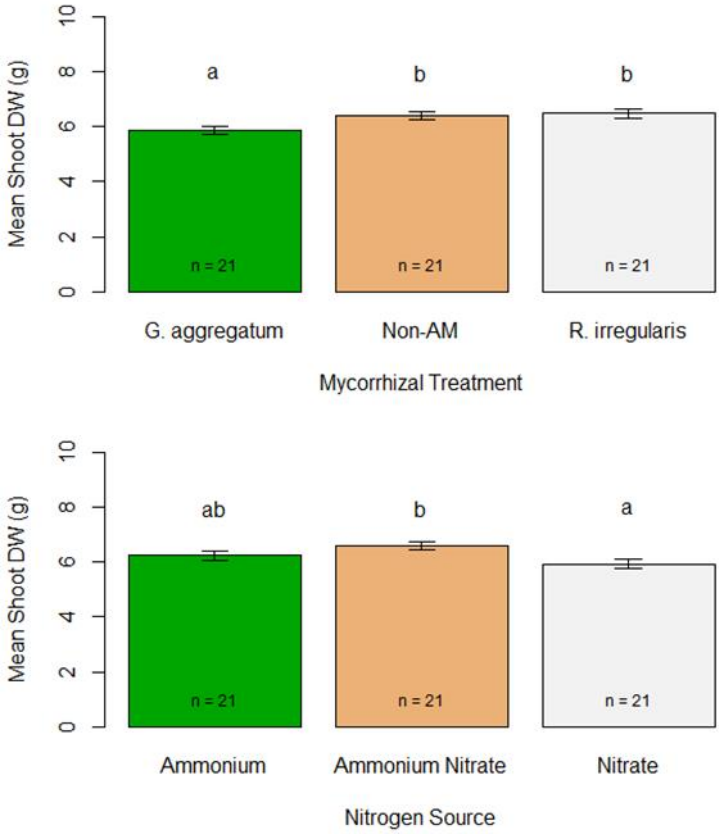

Figure 4.6 . Shoot dry weight (DW) of Meridian barley was influenced by **a)** AMF treatment ($F_{2,54}$ = 5.03, p < 0.001). *Glomus aggregatum*-inoculated plants had significantly lower shoot DW than those inoculated either *Rhizophagus irregularis* (TukeyHSD, p = 0.013) or those in the Non-AM control group (TukeyHSD, p = 0.033). **b)** N source also affected shoot DW of Meridian barley and N source ($F_{2,54}$ = 5.04, p < 0.001). Greatest shoot DW was achieved by plants receiving NH_4NO_3 LAS, having significantly higher DW than those in the NO_3^- group (TukeyHSD, p < 0.01), but no different from the NH_4^+ group. Data are means ± SE, n = 21.

Shoot DW was influenced by nitrogen source ($F_{2,54}$ = 5.03, p < 0.01) and AMF ($F_{2,54}$ = 5.04, p < 0.01) (Figure 4.6). Greatest shoot DW was achieved by plants receiving NH_4NO_3 as the N source. Similarly, total DW was greatest in the plants colonised by *R. irregularis* plants, which had significantly higher mass than those colonised by *G. aggregatum* ($F_{2,54}$ = 3.48, p = 0.038, TukeyHSD, p = 0.041). N source did not significantly influence the total DW, and root DW was not affected by any treatment. Tiller number in Meridian did not differ between N sources and AMF treatments.

4.4. Discussion

It is clear from the results that the relationship between barley variety, the identity of AMF and the source of N provided is complex, even in simplified microcosm systems of the kind employed in this study. The barley varieties show greatly different responsiveness to the N and AMF treatments. While Meridian showed significant variation between treatment groups in almost every growth or nutritional factor measured, Maris Otter was largely unresponsive to treatments. In Meridian, there was some evidence that the extent to which N source influence nutrient uptake is determined by the identity of the AMF symbiont.

It is obvious that barley N acquisition was lowest when given ammonium in isolation. This trend is apparent in both barley varieties and all AMF treatments. The first hypothesis, that NH_4^+ would be the worst N source for barley and AMF has been corroborated. Reduced N uptake and a decreased root weight ratio (RWR) compared to NH_4NO_3 and NO_3^- groups, Maris Otter in the NH_4^+ group shows signs of suffering mild 'NH_4^+ toxicity'. Reduced plant N acquisition and growth associated with the addition of N exclusively as NH_4^+ has been shown previously in multiple plant species (Cramer and Lewis, 1993, Falkengren-Grerup, 1995), and numerous barley varieties (Ali et al., 2001, Lopes et al., 2004). N uptake by Meridian and Maris Otter was indeed lowest in those plants fed NH_4^+, compared to those which received NO_3^- or NH_4NO_3, despite all treatments receiving the same amount of N in LAS, albeit in different forms. It appears that Maris Otter and Meridian responded differently to the N treatments however.

There are many potential mechanisms behind NH_4^+ toxicity (Britto and Kronzucker, 2002), and without specifically testing for each, it is difficult to ascribe a cause to the reduced growth in Maris Otter when fed NH_4^+. Suggested mechanisms include C drain from roots due to forced assimilation of NH_4^+ into amino acids (Cramer and Lewis, 1993).

If this is occurring in the Maris Otter of the NH_4^+ group, the AMF must be able to retain their C supply, as HLD was not reduced in these plants. Other potential mechanisms for NH_4^+ toxicity include: reduced cation uptake, e.g. K^+, Mg^{2+}, Ca^{2+} (Gloser and Gloser, 2000); a direct ion effect of the NH_4^+ (Chambers et al., 1980, Kronzucker et al., 1999); a cytokinin deficiency, production of which has been shown to be nitrate dependent (Chen et al., 1998, Walch-Liu et al., 2000); or a lack of nitrate for regulation of N assimilation by the GS/GOGAT cycle (Redinbaugh and Campbell, 1993). While it is possible that H^+ efflux associated with NH_4^+ uptake (Findenegg, 1987, Goodchild and Givan, 1990) brought about a reduction in pH to such an extent that growth was inhibited, NH_4^+ toxicity has previously been shown to occur independently of pH (Pill and Lambeth, 1977, Blacquiere et al., 1987, 1988).

It is somewhat surprising, given the decreased N nutrition, that NH_4^+ fed plants had more tillers than the other N groups, as tillering usually correlates with N uptake (YARA, 2015). Tillering may also be a sign of stress in plants (YARA, 2015), a more likely explanation here, given that the shoot DW of these NH_4^+ fed plants was no different from those receiving other N sources. It is likely that a number of these tillers would be lost before anthesis as the plant prioritised its N into fewer healthy tillers (YARA, 2015).

By contrast, it seems Meridian was not suffering NH_4^+ toxicity, at least the reduced nutrition of NH_4^+-fed plants compared to NH_4NO_3 and NO_3^- was less pronounced. Although N acquisition was lowest in the NH_4^+ group, the absence of typical symptoms implicated in 'NH_4^+ toxicity' such as leaf chlorosis (Britto and Kronzucker, 2002) further suggest that Meridian barley was not suffering this condition. In fact, NH_4NO_3 was the best source of N for overall plant N acquisition in Meridian, probably because the presence of NO_3^- negates those NH_4^+ toxicity effects brought about NO_3^- absence and similarly, plants provided exclusively with NO_3^--N have shown to have limited ability to form nitrate reductase (Cox and Reisenauer, 1973), and thus NO_3^- assimilation is limited.

Previous work has found that barley plants provided with NH_4NO_3 had higher biomass (Ali et al., 2001) and were more photosynthetically capable (Lopes et al., 2004) than those provided with either NH_4^+ or NO_3^- in isolation.

The increase in P uptake in Meridian by the plants receiving NH_4^+ corroborates trends which have been shown before (Gahoonia et al., 1992, Ortas et al., 1996, Zhang et al., 2004). Perhaps the most parsimonious explanation is that P uptake was increased by virtue of H^+ efflux from plant and AMF following uptake of NH_4^+. Increased efflux of H^+ to the rhizosphere may increase plant P uptake in a number of ways. First, it can increase the solubility of inorganic P (Pi), as the H^+ replaces Pi on exchange surfaces in the soil (Hinsinger, 2001), forcing it into the soil solution and making it available for uptake. Second, as Pi uptake is thought to occur by symport with H^+ (Schachtman et al., 1998), the uptake of NH_4^+ from the rhizosphere will provide the counterpart needed for Pi uptake. Third, Pi may have higher solubility in lower pH (Maathuis and Diatloff, 2013), meaning the available pool for uptake is larger following H^+ efflux. Unfortunately, pH measurements to corroborate these assertions could not be taken, and so decreased pH in the NH_4^+-fed plants are speculative.

A complicated picture emerges where providing N as NH_4^+ in isolation is sub-optimal for N uptake for both barley cultivars, and is best for P uptake in one but not the other cultivar. For DW or N content in Meridian, NH_4NO_3 is the best source of N, while for meridian P, NH_4^+ is isolation is best. From the perspective of grain yield, N acquisition is the chief concern during tillering and shortly thereafter (YARA, 2015), rather than P nutrition, although P deficiency should obviously be avoided. In the later growth stages, N uptake from the soil reduces dramatically in cereals (Mae et al., 1985) and grain filling is achieved by redistribution of N from other parts of the plant (Mae et al., 1985). As these plants were not followed to harvest, it cannot be known how yield would be affected, but it can be speculated that poor N uptake up to the end of this experiment may have been

reflected in poorer yield of the ammonium-fed plants, especially in the relatively N-rich Meridian. From the data collected however, NH_4NO_3 can be said to be the best N source for Maris Otter and Meridian.

Differences between barley cultivars have been reported before in terms of P acquisition (Gahoonia et al., 1997), and it is well known that plant identity in the AM symbiosis can influence the degree to which AMF inoculation elicits a growth response (Johnson et al., 1997, Klironomos, 2003). The fact that P uptake by Maris Otter was not influenced by N treatment, and that mycorrhizal treatments had almost no effect on Maris Otter either, indicate that it may be more resilient to changes in its biotic and abiotic environments than Meridian. This is especially interesting given the relative ages of the cultivars. Meridian is a more modern variety than Maris Otter (1990s vs. 1960s, respectively), and as such, the development of higher yielding characteristics in Meridian may have inadvertently reduced its tolerance to environmental variation, a growing problem not just in barley (Dawson et al., 2015) but also in cereals more generally (McCouch, 2013).

It is clear that *G. aggregatum* was the less beneficial of the two AMF symbionts , supporting the second hypothesis that there would be variation between the AMF species, and that to some extent the barley conforms to the suggestion of being generally negatively affected by colonisation by AMF (Grace et al., 2009, Smith and Smith, 2011b). The negative impacts of *G. aggregatum* are not as pronounced as have been reported for some AM interactions (Hobbie and Colpaert, 2003). Indeed, plants colonised by *R. irregularis* were generally not different from non-AM plants. Barley is known to show varied response to AMF inoculation. Baon et al. (1993) found that P uptake efficiency of 6 cultivars colonised by the AMF *Glomus etunicatum* was lower than non-AM controls, but higher in 2 cultivars. Unfortunately, limited comparison is possible as Baon et al. (1993) used a different AMF isolate from those chosen here, and there was also no cross-over with barley cultivar either.

Compared to *R. irregularis*, *G. aggregatum* was less beneficial to the Meridian, shown by reduced N and P nutrition and depressed DW values. Higher root N concentration in Meridian colonised by *G. aggregatum* (Table 4.2) might suggest increased N acquisition by the plant, but is probably a reflection of increased fungal tissue in the root, indicated by higher arbuscule frequency, rather than evidence of AMF transfer of N to the root. AMF are known to be N-rich relative to plants (Hodge and Fitter, 2010), and have been shown to accumulate N in intraradical hyphae, having acquired it from the rhizosphere (Tanaka and Yano, 2005). This accumulation suggests that the *G. aggregatum* may have been able to compete with the plant for, and retain within hyphae, NH_4^+ N added to the systems, better than *R. irregularis* was, and that it was less beneficial symbiont than *R. irregularis*, as far as its colonisation of Meridian is concerned. In the Maris Otter, however, *G. aggregatum*-colonised plants were no different from the Non-AM plants.

The combination of the three main trends which emerge from the Meridian data (i.e. that i) NH_4^+ is the worst N source for N acquisition, ii) NH_4^+ is best N source for P acquisition, and iii) *G. aggregatum* is a less beneficial symbiont than *R. irregularis*), lead to a very interesting suggestion; the Meridian barley's response to changes in N source are different depending on the identity of the AMF symbiont.

The significant interaction between N source and AMF treatment in shoot N:P ratio of Meridian (Figure 4.5) seems to suggest that *G. aggregatum* amplifies the changes in N and P acquisition brought about by different N sources, while *R. irregularis* may act to dampen them. As there was not a significant interaction between AMF group and N source in either N or P nutrition individually, it is probable that the magnitude of the effect is too low to be noticed on these alone, but when considered together, the trend becomes apparent.

As isotope tracers were not employed here, it is not possible to say what fraction of N and P nutrition was acquired by the direct route compared to the mycorrhizal pathway (Smith and Smith, 2011b), so it cannot be said which fungus was contributing more to plant N and P acquisition. A high contribution of the mycorrhizal pathway is not always associated with improved nutrition compared to Non-AM (Smith et al., 2003), and can even be seen in plants with a negative MGR (Munkvold et al., 2004).

It is fully appreciated how far removed these experimental systems were from the reality of agricultural production. For reasons of logistics and to make it possible for trends to be identified and putatively explained, experiments under controlled conditions are necessarily pared down to give a simplified representation of the environment in which these symbioses exist (Read, 1997). The most obvious simplifications are growing plants in monoculture and inoculating plants with an isolated AMF species or deliberately not allowing them to be colonised. In agricultural systems, a dense monoculture is used, where plants are linked by common mycelial networks of multiple AMF species, (Smith and Read, 2008) not to mention colonised other endophytes and pathogens, fungal or otherwise. Under multiple AMF colonisation, plant performance may depend on the symbiont identities and interactions between them (Jansa et al., 2008). While the complexity of the symbiosis in field systems is formidable, field trials are needed given the prevalence, diversity and abundance of AMF in agricultural systems, in order that patterns emerging from microcosm studies can be scrutinised.

4.4.1. Conclusions

It has been demonstrated that two barley varieties differ in their response to the two AMF isolates studied and the inorganic N form provided. The older variety, Maris Otter showed remarkable resilience to changes in symbiont and N source, as the nutrition and growth was barely affected, while the more modern Meridian showed variability in N and P

acquisition by N source and AMF treatments. In Meridian but not Maris Otter, it is apparent that the degree to which a N source affects plant nutrition and growth may depend on the AMF symbiont with which it is associated, a trend not previously reported, as far as can be determined.

Chapter 5.
Discrete communities of arbuscular mycorrhizal fungi associate with barley under different nitrogen addition rates, with concurrent increases in the mycorrhizal transfer of N to barley

5.1 Introduction

The application of inorganic fertilizer to arable crop land takes place on an enormous scale in western agriculture and has allowed the productivity of land to increase dramatically. Such a nutrient input is not without cost, and problems such nutrient runoff, leaching, pollution of groundwater and eutrophication are well known. Another, albeit lesser studied consequence of nitrogen addition to crop fields is the impact it may have on the structure and function of the belowground microbial community, and how this affects plant nutrient acquisition.

One such group of soil microbes form the most common symbiosis in terrestrial ecosystems: the arbuscular mycorrhizal fungi (AMF). In temperate climates, the majority of plant species form an association with these soil fungi, allowing the trade of mineral nutrients such as phosphorus (P), nitrogen (N), copper (Cu) and zinc (Zn) obtained from the soil in exchange for photosynthetic carbon (C) from the plant (Smith and Read, 2008). While the principal benefit to the plant is increased P uptake, it has also been shown that the mycorrhizal pathway (MP) can contribute significantly to plant N capture too (Leigh et al., 2009). AMF colonise all of the most important cereal crop species (Sawers et al., 2008), can receive as much as 20% of the plants' carbon (Pearson and Jakobsen, 1993, Hamel, 2004) and given their substantial abundance (up to 30% of soil microbial biomass

(Leake et al., 2004)), their role in nutrient cycling cannot be underestimated (Hodge and Storer, 2015).

A great deal of work has been done on the role of AMF in N dynamics of the AM symbiosis, but the overwhelming majority of these have been in greatly simplified systems with single plants inoculated with a single isolate of AMF, and grown in greenhouse conditions in sand-based substrates or transformed root stocks in Petri plate microcosms (Cranenbrouck et al., 2005). Given the significance AMF may have in N cycling, surprisingly little data using isotopic N tracer have been collected from field trials of crop plants. This chapter reports on experiments carried out the impact of contrasting N fertilization rates on a commercial barley trial, using a combination of microscopic analysis, community analysis by terminal restriction fragment length polymorphism analysis (TRFLP) and stable isotope (^{15}N) tracer application.

Numerous bodies of evidence allow us to make predictions about the effects of N fertilizer rate on AMF community structure and N cycling in agricultural systems. Application of fertilizer N to an arable crop may directly affect AMF cycling of N in two ways; by changing the composition of the AMF community and/or by changing the behaviour of the members of that community. It is well known that process which disrupt the soil structure, such as ploughing and tilling are deleterious not only to AMF mycelial abundance (Boddington and Dodd, 2000), but also to the AMF community diversity in soil (Jansa et al., 2002), and subsequently in roots (Peyret-Guzzon et al., 2016, Helgason et al., 1998). N fertilisation has similarly been shown to alter AMF community structure in the soil of arable systems. N fertilization has been shown to reduce AMF community complexity in the roots of wheat roots (Wu et al., 2011) and in grazed steppe habitats (Chen et al., 2014) Indeed, (Avio et al., 2013) showed that N fertilisation regime was the most important factor in determining AMF community structure in soil.

By a combination of chemical and physical processes of modern agriculture, the community of AMF inoculum in the soil may be shifted away from that in unmanaged soil. The AMF community observed in roots is not a simple mirroring of the soil inoculum potential however, and may differ between plant species (Vandenkoornhuyse et al., 2003) and even cultivars of the same species (Mao et al., 2014) growing in any particular soil. Moreover, the communities which establish within the roots are not simply random assemblages, but driven by plant identity (Davison et al., 2011). Subsequent shifts in N cycling in the symbiosis may then occur due to shifts in community composition, as plant N benefit from AMF has been shown to depend on fungal identity (Mensah et al., 2015).

As well as changing plant N uptake via AMF by shifting the AMF community in the roots to one more or less likely to engage in mutualistic trade of mineral nutrients for photosynthetic C, the addition of N may also change the 'behaviour' of AMF isolates/species which have colonised the root, and thereby increase nutrient flux (Johnson et al., 2015). The trade balance model proposed by (Johnson, 2010) and supported by other studies e.g. Johnson et al. (2015) and Puschel et al. (2016) suggests that high-N, low-P systems are likely to promote the greatest AMF benefit to plants, including maximised N transfer from the fungus. In agricultural settings, the increased N added to the soil may encourage more N to be acquired via the MP rather than by roots directly (the direct pathway, DP), either by altering the behaviour of the fungi already in the roots, or by shifting to a more beneficial community, or a combination of both of these. As yet, these predictions based on the model of Johnson (2010) have not been tested in an agricultural setting.

Our ignorance of AM uptake of N in arable crop plants is not limited to nutrient quantity but also N type. It is unclear whether N forms are equally acquired when applied to AM plants, if there is a difference in the extent to which N is transferred from the AMF to the plant, or which results in the greatest N capture by the plant overall. It is known however

that AMF can acquire N and pass it to plants if it is applied as ammonium-N (NH_4^+) (Frey and Schuepp, 1993, Johansen et al., 1993) or nitrate-N (NO_3^-) (Bago et al., 1996). These represent overwhelmingly the majority of N that will be available to AMF and plants in agricultural systems, as the most commonly used fertilizers contain NH_4^+ or NO_3^-, and N present in unamended soil is principally NO_3^- and NH_4^+ (Marschner, 2011).

Evidence from Petri plate studies and models subsequently derived from these indicate that NH_4^+ should be the preferred source of N for AMF uptake but that NO_3^- and indeed organic N sources may also be acquired (Govindarajulu et al., 2005). There is a conflict in the literature of experiments using whole plants, as to whether greater N acquisition via the AM route occurs after addition as NH_4^+ (Tanaka and Yano, 2005) or as NO_3^- (Hawkins and George, 2001). Contrasting results may be due to differences in designs between these two greenhouse microcosm studies, neither of which truly represents how agricultural crop plants are grown. There is a pressing need for data on N dynamics in a realistic field setting of arable systems to determine the importance of the AM route in crop N nutrition.

A series of experiments was carried out on an established field trial investigating the effect of contrasting N addition rates on the yield of different barley cultivars. Stable isotope (^{15}N) tracers were used to quantify N transfer to barley shoots via AMF. It was hypothesised that i) barley root colonisation should decrease in barley cultivars at the higher N addition, as N fertilization has been shown to be deleterious to colonisation; ii) AMF community shifts (measured by TRFLP) should be apparent between the cultivars and between the N rates; iii) more N should be taken up by plants via the AM route under the higher N rate, as per the trade balance model predictions; iv) More N should be passed on to the plants when added to the soil as NH_4^+ compared to NO_3^-.

5.2. Methods

5.2.1. Background

The experiments detailed in this chapter were carried out within the confines of a far larger field trial, designed and implemented at Sancton, East Riding of Yorkshire (co-ordinates 53°51'10.2"N 0°35'29.1"W), by ADAS (Pendeford, Wolverhampton, UK). The main ADAS trial was set up to test how barley yield compares between 6 application rates of ammonium nitrate (NH_4NO_3) fertiliser (Nitram, CF Fertiliser, Ince, Cheshire, UK) ranging from 0 – 300 kg Ha^{-1}. Six barley cultivars, all winter barley, were used in the main trial: SY Volume, SY Venture, Pastoral, KWS Cassia, KWS Meridian and Maris Otter. The soil at the trial site comprises a silty rendzina, with a significant proportion of chalk fragments (UKSO, 2016).

The ADAS trial used plots measuring 12 m x 1.5 m, clustered in groups of 6 by N application rate, with each variety represented once per cluster (see Figure 5.1). Each N application rate was applied to 3 clusters, of 6 varieties, meaning 18 clusters in total, with a combined area of 1944 m^2. Experimental clusters of N application rates were separated to each side by buffer zones 6 m wide, and at each end by buffer zones 3 m long.

Owing to the logistical challenges of sampling the entire trial, the experimental work presented here is gathered from a maximum of 2 of the N application rates (60 kg Ha^{-1} (N rate 2), and 280 kg Ha^{-1}(N rate 5)), and 2 of the barley cultivars: KWS Meridian (KWS UK Ltd, Thriplow, Hertfordshire, UK), a 6-row feedstock barley; and Maris Otter (Robin Appel, Waltham Chase, Hampshire, UK), a 2-row malting barley, giving 4 treatment groups, with replicates per treatment. (Figure 5.1)

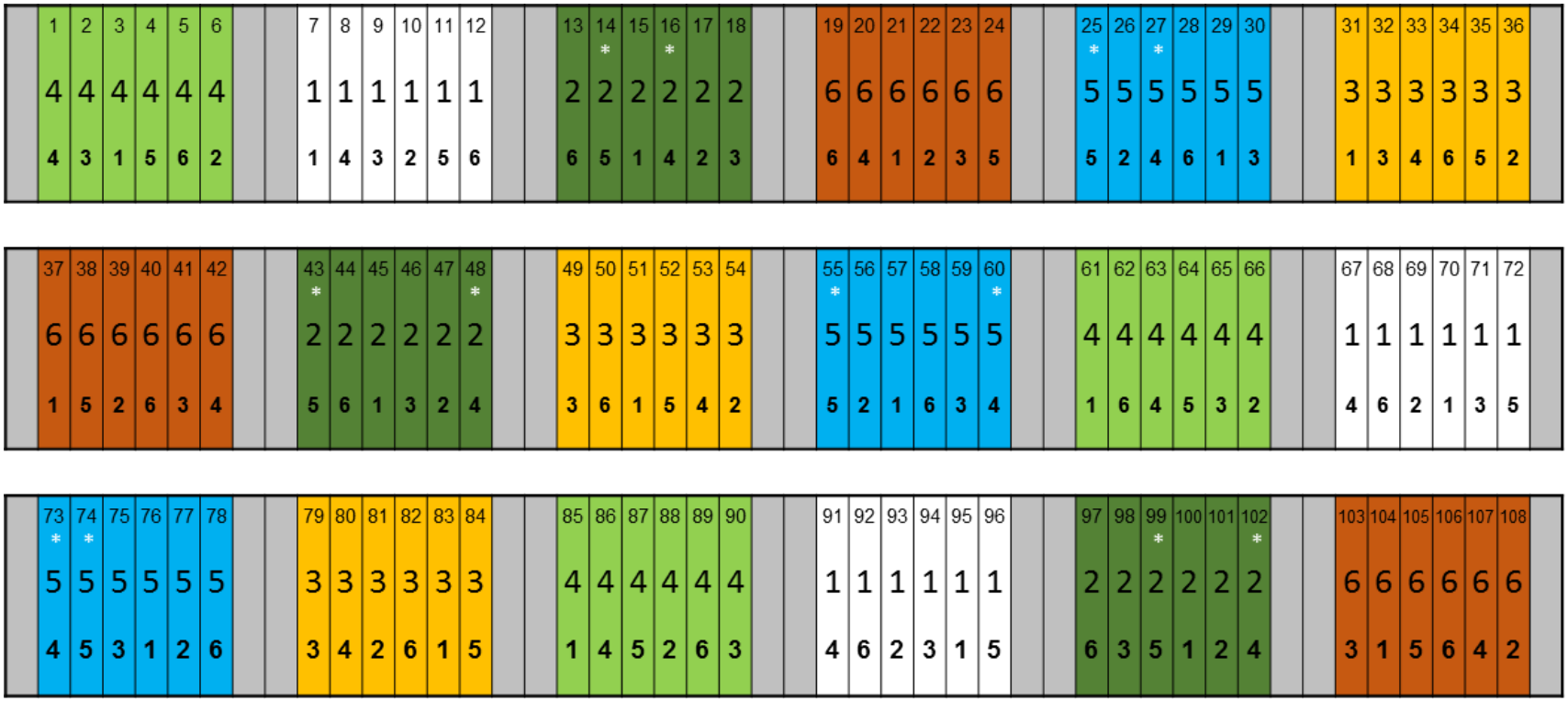

Figure 5.1 ADAS experiment established at Sancton, East Riding of Yorkshire. Six Barley (*Hordeum vulgare* L.) cultivars were planted at the trial site, and received one of 6 N addition rates, ranging from 0 to 300 kg Ha^{-1}. Each combination of barley cultivar and N rate was replicated 3 times. Each plot has 3 numbers, denoting: plot identity, N addition rate and barley cultivar, reading top to bottom. Nitrogen addition rate '2' represents 60 kg Ha^{-1} and '5' is 280 kg Ha^{-1}. Plot colours also represent N addition rate. Meridian barley is denoted by '4' and Maris Otter by '5'. Asterisks (*) represent plots from which root samples were taken for TRFLP analysis, samples were taken for root length colonisation and to which ^{15}N tracer was added.

5.2.2 Microscopic quantification of AMF in roots soil extracts

First, AMF colonisation of both barley varieties was confirmed and then quantified by staining of roots collected from the trial plots. Roots were collected from between 5 and 15 cm below the surface and any attached soil was washed from them. After clearing in 10 % (w/v) KOH for 20 minutes at 70 °C, roots were rinsed in de-ionised water, acidified in 1 % (v/v) HCl at 25 °C for 10 minutes and then stained in Trypan Blue at 25 °C for 20 minutes. Roots were then rinsed again in de-ionised water before being left in a 50% (v/v) glycerol solution for 24 hours, before being mounted onto microscope slides to allow quantification of root length colonisation (RLC) using the gridline intersect method of McGonigle et al. (1990).

Extraradical hyphal quantity in the plots was determined using an adapted method of Staddon et al. (1999). Soil samples were collected from under roots of barley plants in the plots and refrigerated during transport back to the lab for analysis. Hyphal extraction took place within 6 hours of collection to minimise loss due to degradation / decomposition. Samples of known mass (5-10 g) were suspended in 500 mL of de-ionised water and agitated with a magnetic stirring plate in order to free the hyphae from soil particles. From this 500 mL mixture, 200 mL was decanted to a smaller beaker on a magnetic stirrer. From this, 10 mL aliquots were removed and vacuum filtered through 0.45 µm nylon mesh (Anachem, Bedfordshire, UK) and hyphal length density (HLD) was quantified using the gridline intersect method of Staddon et al. (1999).

5.2.3 Isotopic N Labelling

The AMF contribution to barley N uptake was investigated by adding ^{15}N as either NH_4^+ or NO_3^-, into mesh-walled cores, into which AMF hyphae could access but plant roots

could not, or (as controls for diffusion and mass flow of the added N) cores into which neither AMF hyphae or roots could access.

Adapting a method from Johnson et al. (2001), 85 mm lengths of PVC tubing (internal diameter 13 mm, external diameter 16 mm) with 2 windows cut in the sides of the lower 2/3 of the tube so that 50 % of the side area was open, were wrapped in a 20 µm nylon mesh (John Stanier and Co., Whitefield, Manchester, UK). The open lower ends of the tubes were covered with the same size mesh. Control cores, which allowed diffusion and mass flow of solutes but prevent hyphal ingrowth, were covered with 0.45 µm mesh to prevent root and hyphal ingrowth. Cores were filled with a 1/1 (v/v) mixture of silica sand and TerraGreen (calcinated attapulgite clay, Oil-Dri, Cambridgeshire, UK), which had been sterilised by autoclaving (121 °C for 44 minutes), providing a uniform substrate into which the ^{15}N solutions could be added.

Each of these 'cores' was then placed inside another, slightly larger core, assembled in the same manner (internal diameter 18, external diameter 21, length 75 mm). Such a 'core in a core' design allows the placement of zones of defined and uniform size into the soil, to which ^{15}N label solutions can be added. A small (approx. 1 mm) air gap is made between the external mesh wall of one core, and the internal mesh wall of the larger core which should reduce the rapid diffusion of N from the site of addition, which has been a problem in studies where ^{15}N has been added (Smith and Smith, 2011b). Diffusion and mass flow are unlikely to be prevented entirely, as the pressure of soil on the sides of the core may push the mesh together so that the two layers of mesh make contact. In any case, the system provides a more stable labelling zone than systems using only one core, where only one mesh layer may be easily damaged (Johnson et al., 2001).

Each of the 12 trial plots received 4 treatment cores:

- AMF Access, $^{15}NH_4^+$
- No AMF Access, $^{15}NH_4^+$
- AMF Access, $^{15}NO_3^-$
- No AMF Access, $^{15}NO_3^-$

Cores were placed centrally within trial plots, and spaced 3 m (Figure 5.2) apart to avoid contamination of ^{15}N from neighbouring cores. Placement of cores took place 8 weeks before label addition, to allow hyphal ingrowth from the bulk soil. A piece of tape was placed over the top of cores to minimise contamination. This tape was taken off to permit N addition and then replaced. Isotopic ^{15}N was added in the form of Long Ashton nutrient Solution (LAS) (Smith et al., 1983), which can be prepared variously to provide N as NH_4^+ or NO_3^- in equimolar concentrations. The LAS was made to the standard protocols except N being 300% the original concentrations. Each core received 5 mL of LAS, containing 0.683 mg ^{15}N.

After 7 days, the nearest plant to the core was cut at ground level and removed, dried at 70 °C for 48 hours and homogenised first in a kitchen blender (Morphy Richards, Mexborough, South Yorkshire, UK) and then a ball mill (MM400 Ball Mill, Retsch GmbH, Haan, Germany). Homogenised shoot samples of known mass (3 mg ± 0.5 mg) were used to quantify ^{15}N and N content, performed by isotope ratio mass spectrometry (IRMS) (PDZ 2020, Sercon Ltd, Crewe, UK.

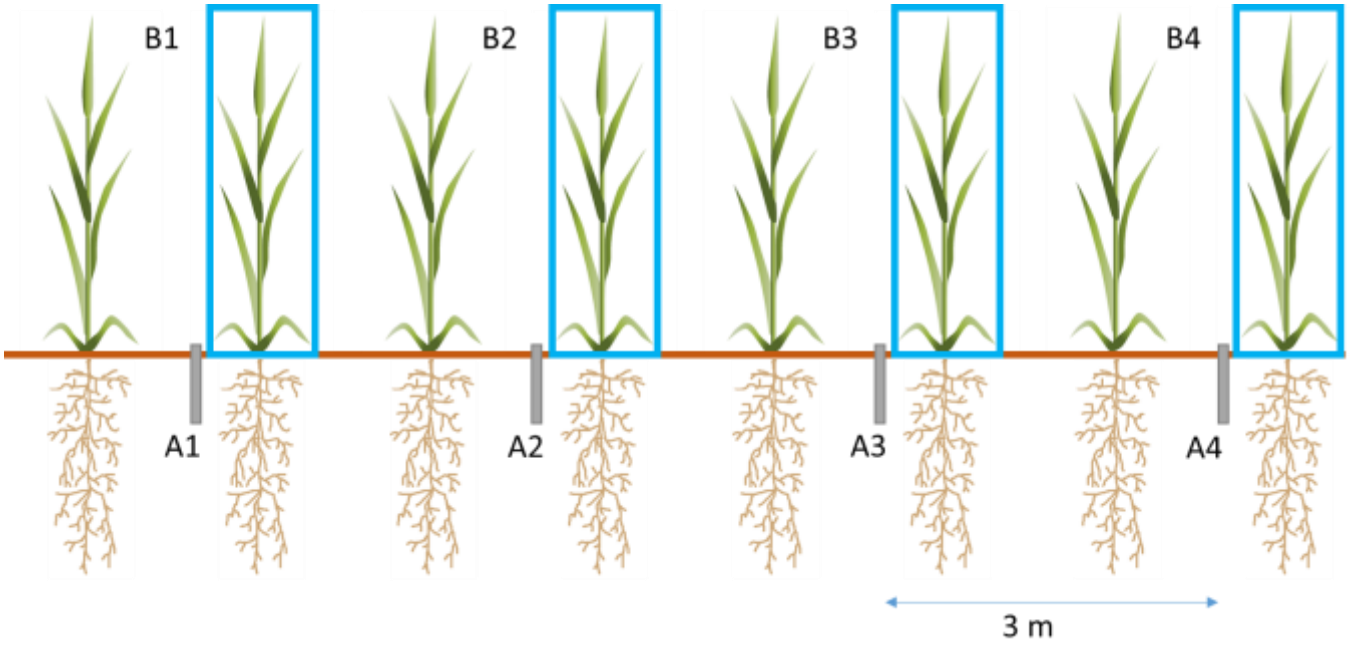

Figure 5.2. Diagram of ^{15}N addition experiment. PVC cores were inserted adjacent to barley (*Hordeum vulgare* L). plants, four cores per plot, spaced 3 m apart. Cores were organised as follows A1 – AMF Access + Ammonium (NH_4^+); A2 - No AMF Access + Nitrate (NO_3^-); A3 - AMF Access + NO_3^-; A4 - No AMF Access + NH_4^+ Each core received 0.683 mg ^{15}N added as Long Ashtons Nutrient Solution. Plant shoots closest to the core (B1-4) were removed, then dried and homogenised for N analysis. Blue boxes (B1-4) represent shoot samples taken. All aboveground material from the plant closest to the inserted mesh core.

Further shoot samples of known mass (15 mg ± 3 mg) were used for quantification of P. Homogenised shoot material was formed into a pellet using a hand press (Specac, Orpington, UK) and then P concentration measured by X-Ray fluorescence (XRF), using a Thermo Niton XL3t900 GOLDD Analyzer (Thermo Scientific, Winchester, UK), using a method developed by (Reidinger et al., 2012).

5.2.4 AMF community analysis

AMF community analysis was performed by Terminal Restriction Fragment Length Polymorphism (TRFLP). Samples were taken from all those plots used in the ^{15}N study, plus those from the Low-N group, meaning 12 plots in all (Figure 5.1).

Root samples were collected from experimental plots of the treatments indicated above, transported to the lab, washed to remove as much soil as possible without damaging the roots, dried and then frozen to -20 °C, all within 2 hours. Approximately 6 months later, frozen root samples were homogenised (while still frozen) using a Retsch MM400 mill (Retsch GmbH, Haan, Germany), in sterile 2.5 mL polypropylene tubes containing 3 sterile, 2 mm steel ball bearings. AMF DNA was extracted from homogenised roots using the 'MO BIO PowerPlant® Pro DNA Isolation Kit' (MO BIO Laboratories Inc., Carlsbad, California, USA), following the protocol provided by the manufacturer.

Partial small subunit (SSU) ribosomal DNA fragments (approx. 550 base pairs) were amplified by Polymerase Chain Reaction (PCR) using a universal eukaryotic primers AML1 and AML2 (Lee et al., 2008) (Table 5.1) and Qiagen MasterMix Kit (Qiagen, Manchester, UK) added to the DNA extract (Table 5.). Primers were diluted from 100pmol supplied to 10 pmol before use to create Primer mix (Table 5.1). PCR reaction mixes were made up as detailed in Table 5.. PCR cycles used were as follows: 95° C for 15 minutes; followed by 35 repeat cycles of: 94 °C for 30 seconds, 58 °C for 90 seconds,

72 °C for 60 seconds; then 72 °C for 10 minutes, after which the product was held at 4 °C until ready for digestion.

Gel electrophoresis was used in order to check the correct functioning of the PCR. The gel was prepared using 180 mL TBE buffer (ThermoFisher Scientific), 2.52 g Agarose, and 9 µL Ethidium bromide. PCR product was mixed with a loading dye (6X loading dye, ThermoFisher Scientific), with 1 µL loading dye: 5 µL PCR product loaded into each well. Inspection of fluorescence image suggested suitable DNA amplification, and that PCR product was suitable for the next step, restriction digest.

Table 5.1. Protocol for mixing 25 µL Primer mix, sufficient for 6 PCR reaction samples (with 1 µL spare, see Table 2). AML1 and AML2 were provided at 100 pmol, and then diluted to 10 pmol before use in this mix. Reagents was mixed in a 2.5 mL polypropylene tube and gently vortex mixed.

Reagent	Volume (µL)
AML1	1
AML2	1
Low TE Buffer	23

Table 5.2. Protocol for volumes required for each 12 µL PCR reaction, i.e. each experimental sample. Qiagen Mastermix was used as obtained from supplier and Primer mix was that prepared as per the protocol in Table 1. Reagents were mixed in a 2.5 µL polypropylene tube and gently vortex mixed.

Reagent	Volume (µL)
Qiagen Master Mix	6
Primer mix	4
DNA extract	2

Restriction digest of the PCR product was then carried out, using 1 µL of the digest mix (detailed in Table 5.3), which contained H_2O, HPY-CH4III restriction enzyme

(ThermoFisher Scientific, Paisley, UK) and a KAPA Hi-Fi buffer (Kapa Biosystems, London, UK). To the 1 µL digest mix, 4 µL of PCR product was added.

Table 5.3. Protocol for mixing reaction digest, as required for one sample.

Reagent	Volume (µL)
H_2O	0.75
HPYCH4III Restriction Enzyme	0.1
Kapa Hi-Fi Buffer	0.15

Reagents were added to a PCR plate and centrifuged briefly (centrifuge allowed to spin up to 2,000 RPM before turning off), to mix. The plate was then placed into a PCR block and heated to 37 °C for two hours to perform the restriction digest, heated to 65 °C to denature the enzyme, and then placed immediately on ice. A 2 µL aliquot of the digest product was diluted to 10 % by mixing with 18 µL H_2O in a fresh PCR plate, and mixed by brief centrifuging as before, ready for sequencing preparation.

A master mix of formamide and LIZ1200 marker (Genescan, ThermoFisher Scientific, Paisley, UK) was prepared as detailed in Table 5.4. An aliquot of 9 µL of the formamide master mix was put into each plate of a high profile (AB0600) 96-well plate, along with 1 µL of the diluted digest product. The plate was then sealed with a rubber septum, and then samples denatured at 94 °C for 5 minutes, after which the plate was placed immediately onto ice. The plate was then sequenced using a Hitachi 3730 DNA Analyser (Applied Biosystems, ThermoFisher Scientific, Paisley, UK).

Table 5.4. LIZ1200 marker and formamide proportions in preparation for sequencing. As each digest samples require 9 µL of master mix, the preparation listed here is sufficient for 100 samples. Master mix was briefly centrifuged up to 2,000 RPM.

Reagent	Volume (µL)
LIZ1200 marker	8.33
Formamide	891.67

GeneMapper software v.4.0 software (Applied Biosciences Inc., ThermoFisher Scientific) was used to determine peak area and quantity from the 3730 DNA Analyser output. Peaks with fluorescence greater than 100 units in height, and with fragment lengths greater than 100 base pairs were analysed, using bin widths of 5 base pairs. Peak area has been shown to be more appropriate than peak height (Sipos et al., 2007, Cotton et al., 2014), as longer fragments may have longer retention time and thus a lower peak height. Terminal restriction fragments which represent less than 5% of the total output from TRFLP were eliminated as noise, following convention (Dumbrell et al., 2010b, Cotton et al., 2014). Data were transferred to a Microsoft Excel file, such that they were suitable for analysis using SIMCA-P software (MKS Data Analytics Solutions, Crewe, UK). Principal Components Analysis (PCA) of TRFLP peak area data showed some clustering of treatment groups (Figure 5.3), indicating that further analysis was appropriate.

5.2.5. Statistical Analysis

Data analysis was performed using the "R 3.1.0" statistical package, through the "RStudio" integrated development environment. (R foundation for Statistical Computing, Vienna, Austria). Data were tested for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. Levene's test was used to confirm homogeneity of variance.

Data for root length colonisation, hyphal length density, barley N concentration and biomass were tested by two-way ANOVA, using N addition rate and barley variety as explanatory variables. As two additional explanatory variables were added in the trial for ^{15}N uptake (N addition type, ammonium / nitrate; AM treatment, access / no access), and the small number of replicates in the ADAS field trial, it was not possible to test these factors and the N addition rate and barley cultivar at once. As such, data were split into barley cultivar and nitrogen application rate for the ^{15}N data, and tested by two-way ANOVA. Here, ^{15}N enrichment was the response variable, while N type and AMF access treatment were the explanatory variables. Hyphal length density in the cores was tested in the same manner.

TRFLP peak area data was analysed by orthogonal partial least squares discriminant analysis (OPLS-DA), which indicated that OTU operational taxonomic units (OTUs) 9, 10, 12 and 13 (as identified in GeneMapper), showed clustering dependent upon treatment. Two-way ANOVA was performed on peak area of these 4 OTUs, to determine what, if any, differences were evident in the community composition of AMF colonising roots of the two barley varieties, and if these differences were affected by N application rates.

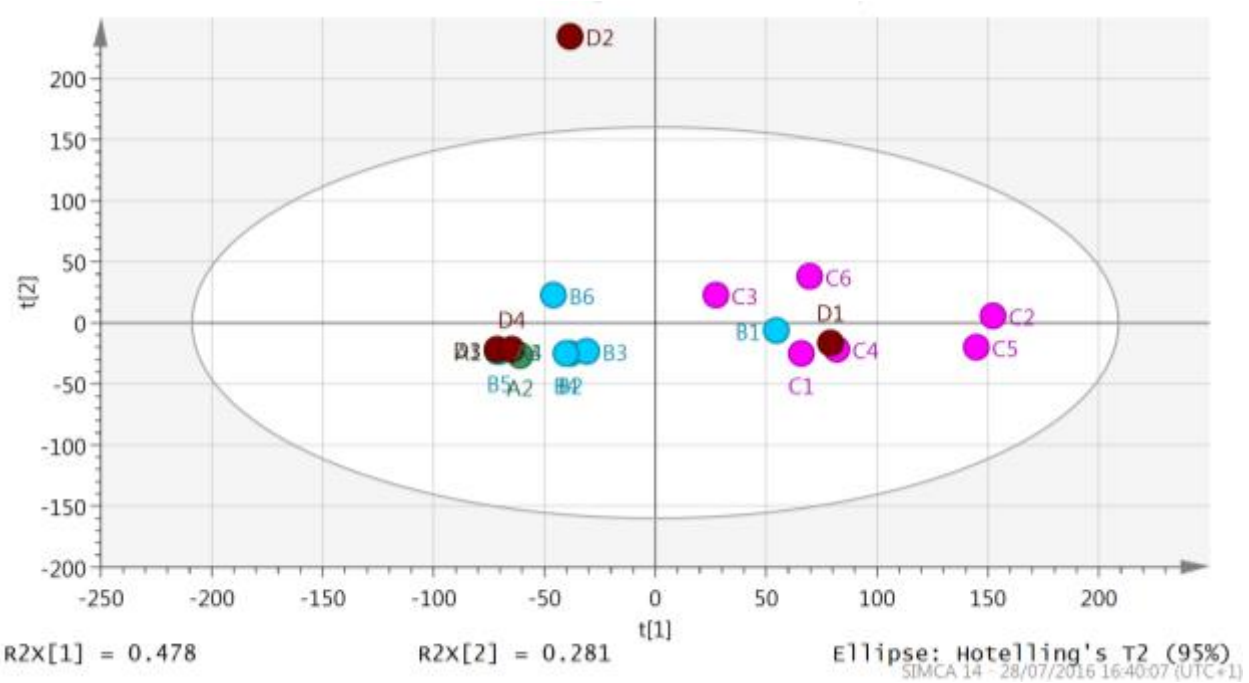

Figure 5.3. Principal components analysis (PCA) of peak area of terminal restriction fragments of arbuscular mycorrhizal fungal Operational Taxonomic Units (OTUs), as determined by terminal restriction fragment length polymorphism (TRFLP). Treatment groups of barley cultivar and nitrogen application rate are represented by coloured dots (A – High-N Meridian barley; B – Low-N Meridian barley; C – High-N Maris Otter barley, D Low-N Maris Otter). Clustering of lettered treatment groups indicated further analysis was justified.

5.3 Results

All plant roots from the Sancton field trial site that were stained and inspected microscopically were found to be colonised by AMF, indicating a substantial inoculum potential of the Yorkshire wold soil at the trial site. While all treatment groups sampled in this experiment (Meridian, Maris Otter, High-N and Low-N) were found to host AMF (Figure 5.4), colonisation was not significantly different between treatments groups, indicating that N rate and barley variety did not determine the quantity of the AMF present in roots.

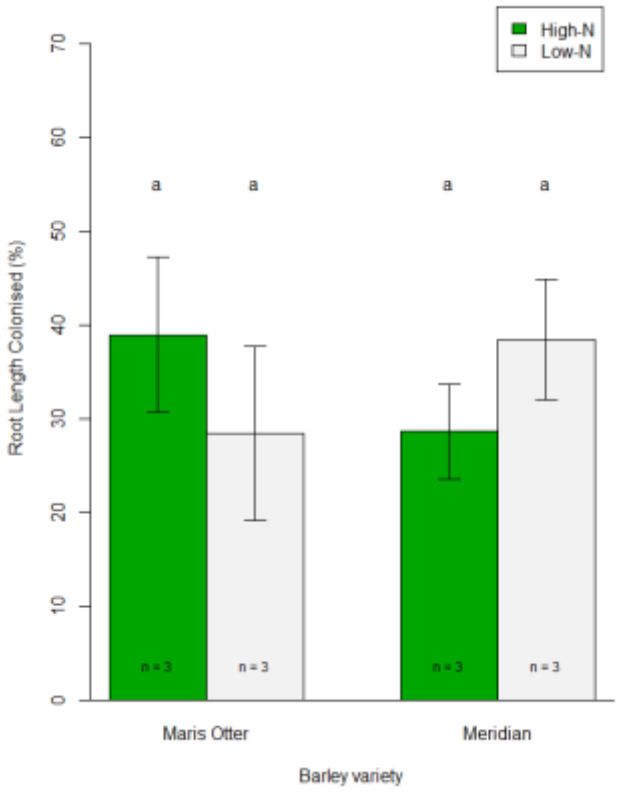

Figure 5.4. Percentage root length colonisation, as determined by Trypan Blue staining. All inspected plants were colonised by arbuscular mycorrhizal fungi (AMF) Mean colonisation ranged from 28.5 % in Maris Otter in Low N, to 38.0 % in Meridian Low-N, but no groups were significantly different. Bars sharing the same letter were not significantly different. Green bars represent High-N data; grey bars represent Low-N treatment. Data shown are means ± SEM, N = 3.

Similarly, the extraradical hyphal densities, as measured from extracts from the PVC cores of the ^{15}N labelling experiment, were not shown to be statistically significantly different between treatment groups, ranging from 2.44 m g^{-1} DW soil under Meridian barley to 2.54 m g^{-1} DW soil under the Maris Otter barley.

In both Meridian and Maris Otter, the N content of barley shoots was strongly and significantly affected by the rate at which N was added to the trial plots ($F_{1,8}$ = 74.55, p < 0.001) (Figure 5.5). High-N plots supported ~ 60 % higher shoot N content than Low-N plots of both barley cultivars.

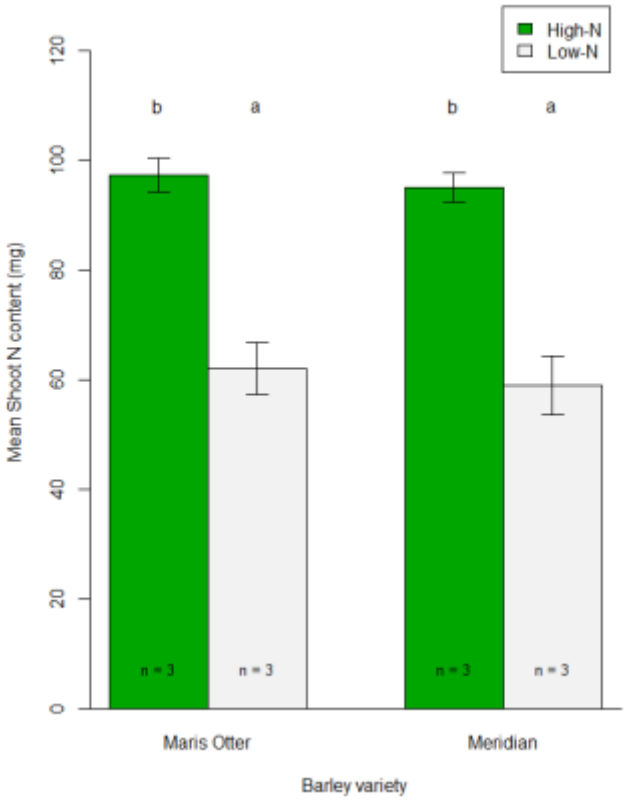

Figure 5.5. Shoot N content was significantly reduced in those plots receiving the lower N application rate for both Maris Otter and Meridian barley ($F_{1,8}$ = 74.55, p < 0.001). Barley varieties did not differ in N shoot content. Green bars represent High-N plot data; Grey bars represent Low-N treatment. Data shown are means ± SEM. N = 3.

Eliciting this difference in N content was a closely corresponding increase in N concentration of barley shoots between the High-N and Low-N treatments ($F_{1,8} = 84.28$, $p < 0.001$). Mean shoot N concentration was 9.30 mg g^{-1} DW in Low-N blocks of Maris Otter, and 14.75 mg g^{-1} DW in the High-N. Meridian showed a very similar trend, as N concentration increased from 9.57 mg g^{-1} DW in Low-N plots to 14.38 mg g^{-1} DW in the High N. Shoot N concentration and content did not differ between the two cultivars tested. Shoot DW did not differ between the varieties or the N addition rates. Shoot acquisition of ^{15}N added to mesh cores was significantly improved by allowing AMF access into cores, but only in those to which NO_3^- was added, and only in the High-N plots of Meridian barley. Comparing shoot ^{15}N concentration (µg ^{15}N g^{-1} shoot DW) accounts for the potential influence that different DW may have on transpirational sink strength of plants. When this is considered, the same trend is apparent in Meridian barley; plants in proximity to cores with AMF access to $^{15}NO_3^-$ (Figure 5.6) obtain significantly more ^{15}N than those with no access to NO_3^- , or those in proximity to cores to which $^{15}NH_4^+$ was added.

In Low-N plots of Maris Otter and Meridian, and High-N plots of Maris Otter, shoot ^{15}N content (mg ^{15}N) did not differ between any of the four treatments added i.e. NH_4^+/NO_3^- and Access/ No Access treatments, indicating no benefit of adding one type of nitrogen over the other, or in allowing or preventing access to the core. Moreover, mean shoot ^{15}N content did not differ between these plots, indicating similar acquisition of ^{15}N from the N-patches in these treatments.

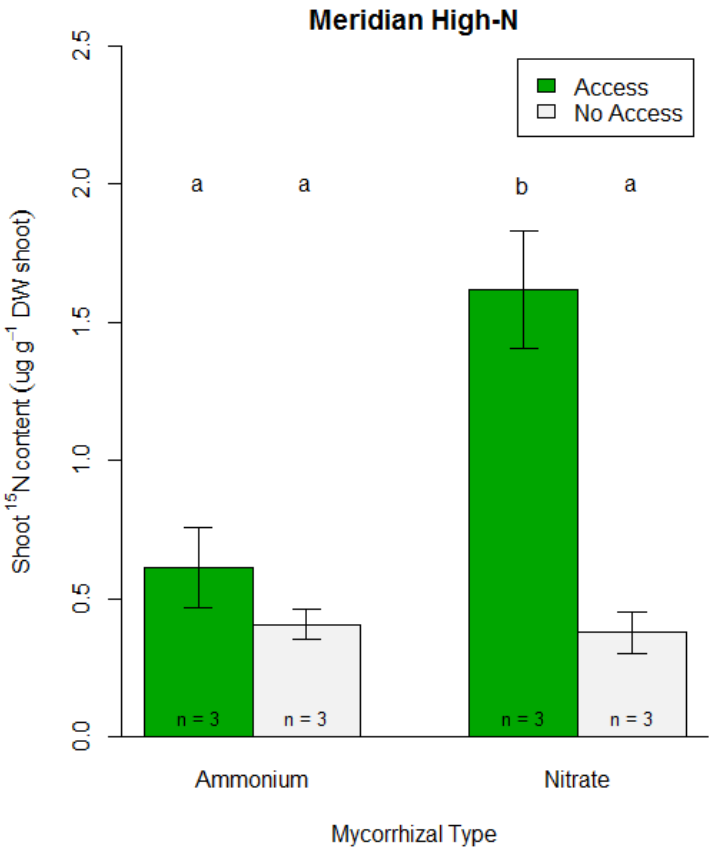

Figure 5.6. Shoot ^{15}N content in Meridian barley was significantly highest in plants which had AMF access to the core to which ^{15}N-nitrate was added. These plants were significantly more ^{15}N enriched than those in closest proximity to ammonium-N cores and those which received nitrate-N but had no AMF access. Green bars represent plants with AMF access; grey bars represent no AMF access. Data shown are means ± SEM, n = 3.

While RLC and ERM data suggest no overall change in AMF quantity, TRFLP analysis suggests that the structure of the AMF community within the roots of the barley plants was not the same between varieties, and was influenced by N rate. Specifically, significant variation in response to N application rate was found in OTU 9 ($F_{1,16}$ = 5.97, p = 0.026). OTU 9 was not present in High-N plots of Maris Otter (Figure 5.7A), but was found in the other three treatment groups. OTU 10 responded significantly to N application rate ($F_{1, 16}$ = 18.38, $p < 0.001$) and showed a significant interaction was found between barley variety and N rate ($F_{1,16}$ = 17.03, $p < 0.001$). OTU 10 was not present in High N plots of Meridian barley (Figure 5.7B)

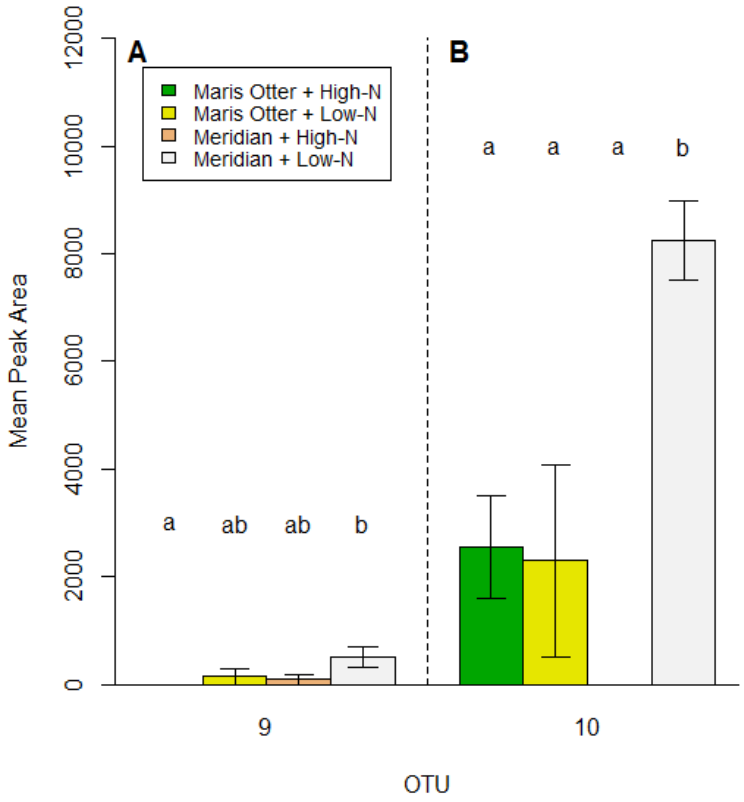

Figure 5.7. Results from separate analysis of variance (ANOVA) carried out on operational taxonomic units (OTUs) 9 and 10, as identified by terminal restriction fragment length polymorphism (TRFLP). Abundance of OUT-9 was significantly lower in Meridian Low-N plots than Maris Otter High-N plots. OUT-10 abundance was significantly higher in Meridian Low-N than in Meridian High-N or either Maris Otter treatment. Data shown are means ± SEM. N = 3.

5.4. Discussion

The results of this study provide a novel combination of microscopy, molecular analysis and isotope tracer techniques in a barley field trial setting that is identical to the scenario in which commercial barley is grown in the UK. The results demonstrate that higher N applications (280 kg Ha^{-1}) had no detectable impact on the quantity of AMF in plant roots or in the rhizosphere, but can influence community composition and the relative

significance of the AM-route for N uptake in agriculture, as demonstrated by ^{15}N tracer data.

Contrary to the first hypothesis, neither the RLC or the HLD were influenced by the rate of N addition. This is counter to the commonly reported trend of reduced AMF growth in high nutrient systems (Tawaraya, 2003, An et al., 2010, Chen et al., 2014), although this pattern is not always observed (Santos-Gonzalez et al., 2011). As the HLD quantification employed here used only hyphal length, an increase in hyphal biomass may have been missed, as hyphal diameter is known to vary considerably in AMF (Smith and Smith, 2011b). Determining hyphal biomass in the soil based on lipid fatty acid quantity (Olsson et al., 1995, Olsson, 1999, Balser et al., 2005) may have enabled more detailed comparison between the treatments but the method is not without limits. Doubts exist as to whether the method of lipid fatty acid profiling can be used to identify AMF accurately as some may not be truly unique to AMF, for example.

Differences in AMF abundance in the soil may have been missed if they were depth-dependent, as samples were only collected from 5-15 cm below the surface, while AMF can found 2 m or more below the surface (Zajicek et al., 1986, Oehl et al., 2005) although the effect of this may be limited as most AMF is found in the top 40 cm of the soil (Jakobsen and Nielsen, 1983). It is important to stress that the HLD detectable in the trial site may have shown more variation between treatments at a different time, as there is known to be a very strong seasonality in the abundance of mycelia in the soil (Daniell et al., 2001, Douds and Millner, 1999), and at another sampling period, a difference in the HLD of the fungi might have been observed.

While it has been suggested that spore collection and quantification may give a good indication of the AMF biomass and community composition, there are caveats to this: the seasonality of spore abundance is even more pronounced than that of hyphae (Douds and

Millner, 1999), and the link between spore presence or abundance is a very poor indicator of symbiotic function; as spores may persist from previous years' crops in the agricultural rotation, their presence in field soil cannot indicate symbiosis, even less can it suggest any mutualistic function.

The lack of observable differences in HLD and RLC highlights the usefulness of employing multiple methods concurrently, as analysis of TRFLP did show differences in the intraradical AMF community between treatments. The second hypothesis was supported by the TRFLP analysis; the combinations of N treatments and barley varieties appear to show the move towards hosting discrete communities of AMF. While AMF community structure has been shown to be influenced by N rate (Hendrix et al., 1995, Vandenkoornhuyse et al., 2003, Liu et al., 2012) and plant identity (Vandenkoornhuyse et al., 2002, 2003, Verbruggen et al., 2012, Mao et al., 2014), factorial data on N rate and plant identity is uncommon.

A combination of factors may explain why differences in N rate and variety are shown in the abundance of OTUs 9 and 10. A mixed rotation system, such as the one used at the field site will allow the creation of a spore bank in the soil of greater diversity than those found in a continuous monoculture regime (Oehl et al., 2004, Hijri et al., 2006) although if non-AM host plants (such as brassicas) are included in the rotation, the diversity of the AMF inoculum in the soil may be reduced (Hendrix et al., 1995). For any given soil with a particular cropping history, this spore bank represents the maximum possible diversity of AM symbioses which may establish, and a subsequent combination of biotic and abiotic factors then acts to limit the realised communities of AMF colonising roots. In this case the addition of a low or high rate of N fertiliser and the choice between two barley cultivars may dictate the community identity. Without sequencing DNA extracted from the soil directly, it is not possible to determine what proportion of the soil inoculum, if any, was present but did not colonise either barley cultivar here.

There are a number of ways in which the mycorrhizal community might have been affected by the addition of a higher N rate. Intensive agriculture, including high levels of inorganic N addition, can reduce the presence of *Acaulospora, Enterospora* (Oehl et al., 2004), *Scutellospora* (Jansa et al., 2002) and *Gigaspora* (Giovannetti et al., 1999). In such conditions, more robust fungi, often *Glomus* species, will then dominate the community (Sylvia and Schenck, 1983, Daniell et al., 2001, Oehl et al., 2004). Changes in N concentration in soil may limit the ability of groups of fungi to germinate, proliferate and colonise plants, thereby reducing the prevalence of these fungi in roots.

Alternatively, contrasting root morphology among barley cultivars (Hargreaves et al., 2009) and at different N concentrations (Drew, 1975, Grossman and Rice, 2012) may explain the shifts in AMF communities shown by the TRFLP, as the physiological niches provided in the roots differs between treatments. The influence of one or more of these factors is most likely to have brought about the changes in OTU peak area in the treatments, but possible effects of stochastic factors is also acknowledged (Dumbrell et al., 2010a).

As the field site has been under intense agricultural management for many years, it is very likely that the AMF community has a lower diversity than neighbouring, unmanaged soils (Helgason et al., 1998, Daniell et al., 2001). More dramatic shifts in TRFLP may have been observed between the treatment groups if the experiment had taken place on previously uncultivated soil (Chen et al., 2014, Sale et al., 2015). The results presented show how changes to the intraradical AMF community can be brought by short term changes in management.

Decreased diversity of AMF in soil and within the roots has been linked to decreased plant P uptake (van der Heijden et al., 1998) and plant growth (Johnson, 1993, Maherali and Klironomos, 2007). As AMF species /isolates may be functionally different (Johansen et

al., 1992, Francis and Read, 1995, Avio et al., 2006) there is a clear mechanism for AMF community shifts driven by N fertilisation to influence the N cycling in the system. Data from the ^{15}N tracer experiment supports this suggestion, as N contribution to the plant differs between treatments when differences in AMF community were also observed.

The isotope data suggests minimal N-for-C trade between soil fungi and Maris Otter, as ^{15}N uptake from the Access cores was no higher than from the Non-Access cores. While P-for-C trade was not considered here, it is possible that the nutritional benefit of Maris Otter from the AM route is minimal. Neither barely cultivar in the Low-N plots showed higher ^{15}N content in Access than Non-Access treatments, suggesting minimal advantage to the plant of AMF-route for N uptake, and supporting the third hypothesis (that more N should be acquired by plants via the AM-route in high-N conditions). These results agree in the context of the trade balance model of (Johnson, 2010) which states that the AMF will not transfer N to a partner plant until fungal N demand is satisfied. C-for-N trade in arbuscular mycorrhizas is known to be low or non-existent in Low-N (Hawkins and George, 1999, Candido et al., 2013).

Although the data presented here shows that the AMF Access treatments provided more N to neighbouring plants than Non-AMF Access treatment when supplied as NH_4^+. Previous experimental evidence for AMF preference of inorganic N forms is equivocal. Greater N transfer by AMF to plants when added as NH_4^+ was shown by Johansen et al. (1993) and Tanaka and Yano (2005), while Hawkins and George (2001) and Ngwene et al. (2013) show higher transfer after NO_3^- addition.

Higher uptake of NO_3^- than NH_4^+ is contrary to the predictions of the proposed model by Govindarajulu et al. (2005) which suggests NH_4^+ should be a less energetically expensive form of N, and is counter to the fourth hypothesis tested. Hyphal NH_4^+ uptake may be retarded by problems of charge balancing that are perhaps not encountered when N is

acquired as NO_3^- . Concurrent uptake of NO_3^- and cations (such as K^+, Ca^{2+}, Mg^{2+}, etc) from the soil may prevent deleterious changes in electrochemical potential across exchange surfaces. Meanwhile, excretion of H^+ with NH_4^+ uptake may shift soil pH to prevent further uptake, and concurrent NH_4^+ and anion uptake may not be possible. Alternatively, hyphal physiology may be acclimated to nitrate uptake (Garraway and Evans, 1984), as NO_3^- is the dominant N form in soils (Marschner, 2011) and were therefore unable to exploit the sudden availability of NH_4^+.

The data suggest that the importance of the AM-route for plant N acquisition depends not only on the background level of N in the soil, but also the identity of the barley cultivar and the form in which the N is added. These aspects of AM N nutrition, along with the detected shift in intraradical AMF community, are phenomena variously identified independently, but this study is the first to demonstrate them concurrently, with the added realistic setting of a commercial barley field trial.

While it is possible to say that the AM route contributed more to plant N acquisition in one treatment group (NO_3^-, Meridian, High-N) than any other, it is difficult to assess to what extent the overall impact of these treatment combinations might have on the AM symbiosis. Plant acquisition of N and P, as well as that of other minerals may be positively or negatively affected by AMF colonisation, or may not be affected, and factors other than nutrition are known to be influenced by AMF colonisation. The picture of AM-plant interactions is further complicated when we consider the effect of soil type, land management, nutrient amendment, and plant identity. While the ^{15}N data does not support the suggestion of Johnson (2003), that adding N selects less beneficial fungi, without data on P and further nutritional and physiological and other factors, it is not possible to state that any detected shifts in AMF communities were generally beneficial or not. Adding isotopic P and C labelling to trials of this kind may help resolve the impact of N fertilisation on the AM contribution to plant nutrition in arable context. Addition of

radiolabelled P to field sites may represent challenges, although is not without precedent (Schweiger et al., 2001), while stable isotope ^{13}C labelling could be used to trace C uptake by fungi.

5.4.1. Conclusions

Isotope tracer work in a field setting has shown that AMF can aid the movement of inorganic N from a site of addition to the shoots of barley plants. Despite no apparent differences in abundance of AMF either in the roots or in the soil, as determined by root staining & hyphal extraction and microscopic counting, TRFLP showed community shifts within roots, as two OTUs were reduced in frequency in High-N treatments compared to Low-N plots. The mechanism by which AMF communities are shifted by the addition of N is not clear, and needs further investigation. Nevertheless, a definite link can be drawn between inorganic fertilizer application rates, intraradical AMF community composition and the movement of N from the site of addition to a host plant's shoots via the extraradical mycelium of the fungus.

Chapter 6.
General Discussion

6.1. Summary of the initial aims

The transfer of nitrogen (N) from arbuscular mycorrhizal fungi (AMF) to plant partners has been observed for many years (Ames et al., 1983), but was until relatively recently thought to be inconsequential from the plants' perspective, representing insignificant quantities in the scale of total plant N acquisition. N uptake by the mycorrhizal pathway (MP) was therefore thought to be of minor, if any, ecological relevance. The discovery of AM-specific ammonium (NH_4^+) transporters expressed in arbusculated cells of colonised roots (Guether et al., 2009), and experimental evidence that the AM route can contribute up to 20% of plant N (Leigh et al., 2009) brought about a change in consensus. It is now widely agreed that AMF may under some circumstances transfer meaningful quantities of N to a partner plant (Smith and Smith, 2011b), but the exact nature of these circumstances is still not fully understood. Similarly, the circumstances under which colonisation by an AMF might bring a positive mycorrhizal growth response (MGR) (Hetrick et al., 1992) or increased phosphorus (P) uptake are also not fully understood (Smith and Read, 2008).

A number of models have been proposed to explain the functioning of nutrient trade in the arbuscular mycorrhizal symbiosis, e.g. reciprocal trade of mineral nutrients from the fungi for C from the plant (Kiers et al., 2011, Fellbaum et al., 2012), stoichiometry of N and P in soils (Johnson, 2010) and the model of Fitter (2006) which suggests that fungal loading of mineral nutrients into the plant cortex will elicit C relocation, in a mechanism analogous to root nutrient dynamics. Partly due to the complexity of the AM system in a field setting (Fitter, 2006), and the perceived primacy of P nutrition among benefits of the AM symbiosis, little testing of these models has taken place where the focus has been placed

on N nutrition. With N being the most-often limiting mineral nutrient to plant growth (Marschner, 2011), and the AM symbiosis possibly the most widespread in terrestrial ecosystems (Brachmann and Parniske, 2006), the circumstances under which AMF might benefit plant N nutrition undoubtedly deserve further attention.

Using a number of complementary approaches, the experiments detailed in this thesis aimed to address the following hypotheses:

1. N uptake from organic matter by the MP will contribute sufficient N to a partner plant so that total N content and biomass increase, provided the P content of the growing substrate is limiting to plant growth.
2. AMF acquisition of N will be higher when provided as NH_4^+ over NO_3^- as this should represent an energetically more attractive N form for the fungus than NO_3^- As such, greater amounts of ERM will be observed when fungi receive NH_4^+ than NO_3^-. As a consequence, AMF are more likely to be sated for N when receiving NH_4^+ and N transfer to the plant will be higher than from AMF receiving NO_3^-.
3. N transfer to a plant partner takes place by reciprocal rewards, and a plant receiving more N from an AMF will repay with more C.
4. The structure and function of the AMF community in a field setting will be influenced by the interactive effects of N application rate and type.

The main experimental approaches undertaken in this thesis, and the results obtained, are summarised in figure 6.1. The diagram acts as a visual aid to help the reader conceptualise the most important findings from chapters 2-5. It is hoped that fig. 6 gives an indication as to the breadth of the influence that AMF may have not only over the plant and its nutrition but also the nutrient cycling in the soil, as well as the impact that alterations to the soil chemistry (from fertiliser addition in this case) may have over the structure and function of the AMF community in soils.

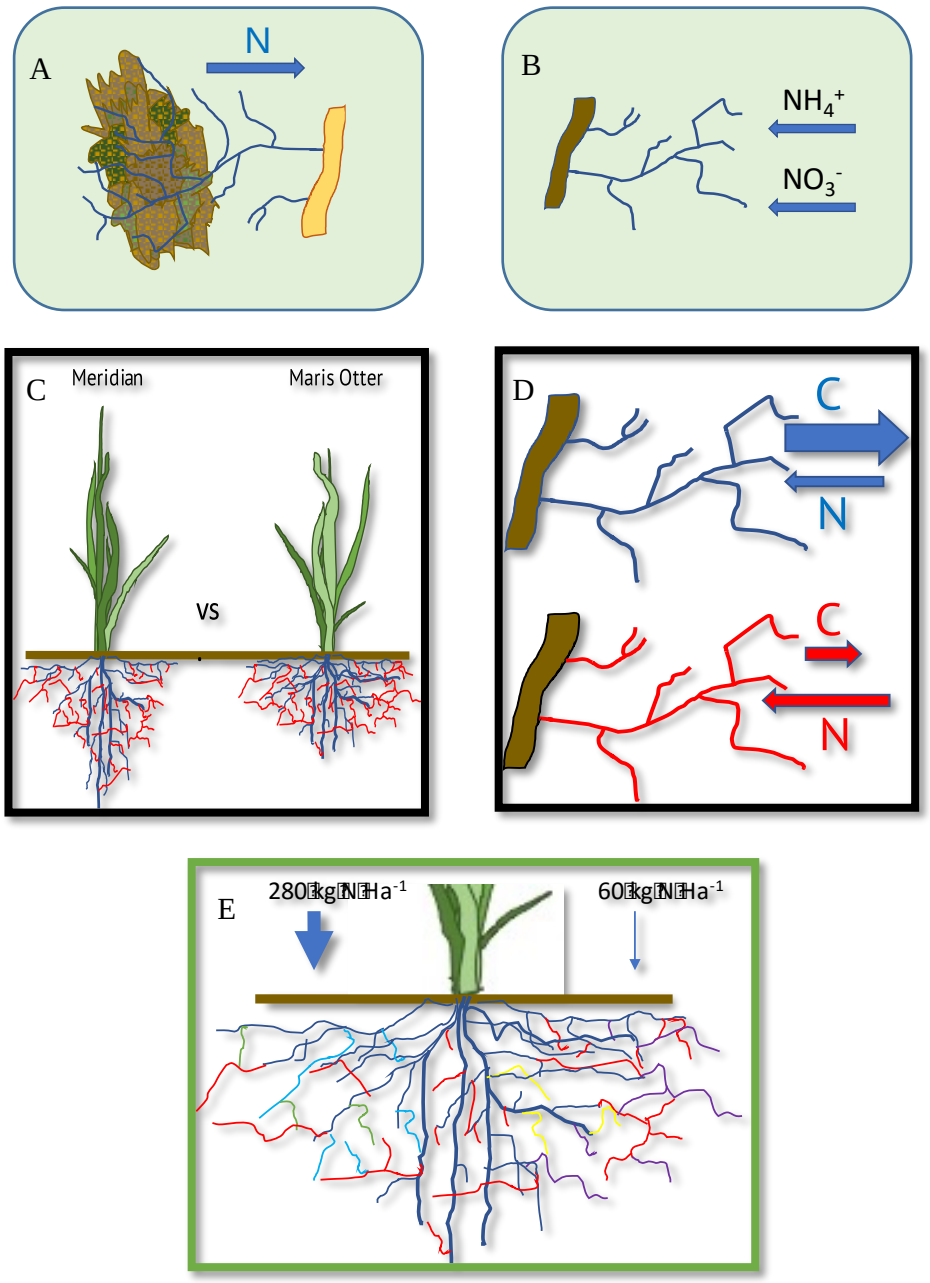

Figure 6.1. A conceptual diagram highlighting approaches and findings contained within the experimental chapters of this thesis, concerning arbuscular mycorrhizal fungi (AMF) on the nitrogen (N) nutrition of plants. **A** AMF were adept at transferring significant quantities of N from a patch or organic material, and improving plant N content and biomass. **B**. AMF preference for ammonium or nitrate was tested in a series of experiments (chapters 3, 4, 5), where no clear preference was exhibited. **C**. Functional diversity was demonstrated between two barley cultivars KWS Meridian and Maris Otter, as Meridian was largely more responsive to AMF than was Maris Otter, chapters 4 + 5. **D**. Functional diversity between two AMF isolates, *Glomus aggregatum* and *Rhizophagus irregularis* was demonstrated in terms of N transfer to the plant and the provision of plant C to the rhizosphere, and effects on plant growth and nutrition, in chapters 3 + 4. **E**. N addition rate to a barley crop trial was demonstrated to influence AMF community within barley roots and the extent to which plants received N via their AMF partners.

6.2. Organic matter fertilisation brings nutrition and growth benefits

Simultaneous increases in N content and plant DW as a result of AM hyphal have not been demonstrated before, although N uptake from organic matter has been demonstrated numerous times (Leigh et al., 2009, Hodge and Fitter, 2010, Barrett et al., 2011). The experiment in Chapter 2 was designed to test ideas proposed by the model of (Johnson, 2010), and provides support for hypothesis 1 at the start of this chapter. A Low-P growth substrate was used, into which an N-rich organic matter patch was added, and into to which only the ERM of AMF could access, while roots were excluded. As AMF hyphae may be capable of proliferating more rapidly than roots (Schnepf et al., 2008), and their diameter is far smaller (Abbott and Robson, 1985), they have the ability to navigate smaller soil niches. This gives them the opportunity to access small, N-rich microsites which may not be accessible to roots. Such exploitation of niches may represent an additional mechanism by which AMF can benefit N nutrition of their plant partner. It would be unwise to emphasise this experiment alone as evidence for ecologically significant N transfer from AMF to plants as conditions in which it was carried out vary wildly from those encountered in natural systems. It might be said however that a mechanism has been demonstrated by which levels of N uptake by the MP may occur that are significant in terms of plant nutrition. Further testing of N transfer by AMF from organic sources to plant partners in natural soil may provide more information on the extent to which this transfer might contribute to plant N nutrition in natural systems. High-P soils have been shown to reduce the benefits of the AMF for plants (Johnson et al., 1997), and the trade balance model (Johnson, 2010) makes similar predictions. According to these trends, highly P-adulterated soils would not allow AMF to improve N uptake in plants as demonstrated in Chapter 2. Soils which are nutrient-poor and are usually fertilised with organic matter may be a better field setting for trials looking to test the findings of Chapter 2.

6.3. Inorganic N source preference of AMF

Clear AMF preference for NH_4^+ uptake over NO_3^- was not demonstrated in the experiments presented here, in contrast to the second hypothesis. Current understanding of uptake of N into AMF states that prior to amino acid synthesis, NO_3^- must be reduced by nitrate dehydrogenase and then nitrite dehydrogenase to NH_4^+ and as such, where it is possible, NH_4^+ uptake should be preferable to NO_3^- (Govindarajulu et al., 2005).

Contrary to the second hypothesis, in no experiment where discrete zones received long-term addition of NH_4^+ or NO_3^- was there a difference in ERM growth detected, indicating that neither N source acted to encourage or discourage hyphal proliferation. The experiment in chapter 4 suggests that AMF are able to acquire N as both NH_4^+ and NO_3^-. It is presumed that the rates of nitrification of NH_4^+ and dissimilatory nitrate reduction to NH_4^+ were small, although they were not measured so it is not certain that the N applied remained in the form in which it was added. While ERM growth was assumed as a proxy for N uptake and content of AMF, it is possible that N uptake differed between zones of N addition but this was missed in analysis. Rapid translocation of N from these zones to other parts of the ERM is highly likely (Jin et al., 2012), so even N analysis of hyphal tissue N concentration here may not have been very enlightening. IRMS of the ERM for quantification of ^{15}N content may have given the clearest indication of preferential uptake, but it was not possible to extract sufficient hyphae to allow such analysis.

Data in Chapter 3 suggests that both species of AMF can acquire N both as NH_4^+ and NO_3^- but there might be a slight preference for NH_4^+, while Chapter 4 suggests that there is no effect of N source on fungal parameters. Chapter 5 shows that fungal N transfer to plants in the field was only higher in controls when added as NO_3^- . Suggestions that NH_4^+ should be preferred over NO_3^- (Perez-Tienda et al., 2012) assume that the fungus is presented with an equal choice between two sources of N, and post-uptake assimilation

costs are the factor which decide N preference. In reality this is unlikely to be the case and the source of fungal N acquisition will be influenced by numerous external factors. Primarily, the relative availability of N form will determine how N is acquired (Marschner, 2011). In soils where NO_3^- is present in far greater quantities than NH_4^+, organisms with high N demand such as AMF will benefit from NO_3^- acquisition, even if it incurs the cost of nitrate and nitrite reduction before the N can be assimilated. AMF expression of N transporters is likely to be adaptive to soil N forms so that available N can be acquired, similar to the trend observed in roots (Hodge, 2004).

Even in systems of greatly reduced complexity, the AMF must balance the electrochemical charge of NH_4^+ uptake with either excretion of H^+ (Perez-Tienda et al., 2012) or the simultaneous uptake of anions, so that the overall charge across the membrane is balanced. Data from the Petri plates in Chapter 2 suggest that the ability to balance charges within the fungus and in the hyphosphere is crucial for uptake.

Mineralisation, denitrification and other microbe-mediated reactions that determine the availability of N forms in the soil (Figure 1.1) will be influenced by various abiotic factors such as temperature, humidity, oxygen availability, pH and others (Nielsen and MacDonald, 1978). Differences in these abiotic factors may well explain the different perceived preferences for uptake by AMF and subsequent transfer to plants, if fungi had become acclimated to prevailing soil conditions.

It could be argued that stronger preference for N type might be elicited if AMF and plants were grown in soils dominated by one N source, such as waterlogged paddy fields where NH_4^+ dominates and NO_3^- supply is very low (Kamekawa et al., 1990, Sasaki et al., 2016). Similarly, soils which are low in Mo might force AMF propensity for NH_4^+ uptake, as Mo-limitation would prevent nitrate and nitrite reductase activity, as these enzymes require Mo (Campbell, 1999).

Differences in abiotic soil and substrate conditions may explain differential observations of AMF N source preference displayed by AMF in studies by (Hawkins and George, 2001, Tanaka and Yano, 2005, Ngwene et al., 2013), as well as functional diversity of AMF used (van der Heijden et al., 1998, Mensah et al., 2015) and the implication of using different plant species (Klironomos, 2003), are likely to promote different nutrient flux responses.

Higher Meridian barley acquisition of ^{15}N by the MP when supplied to High-N plots than to Low-N plots is indicative that the background soil N content is a determinant of the extent to which the AMF provide nutrients to the plants, as predicted by the trade balance model. (Johnson, 2010) The results in Chapter 2 also support the trade balance model.

6.4. Reciprocal C-for-N Trade between plant and AMF

Contrary to the third hypothesis, the data presented in Chapter 3 give no suggestion that trade of N and C took place between the plant and fungus by reciprocal rewards, as proposed by the biological market theory (Kiers et al., 2011, Fellbaum et al., 2012). Given the different levels of N delivery from the AMF to the plant (strong in Chapter 2, nil in Chapter 3, and partial in Chapter 4), it is clear that the dual labelling ($^{14}C/^{15}N$) experiment in Chapter 3 is not sufficient to determine whether a 'reciprocal rewards' mechanism governs C-for-N trade. Fellbaum et al. (2012) showed that C availability can trigger N supply, but whether traits of plant-fungal trade can be determined by these systems is under considerable doubt in the literature (Smith and Smith, 2011a, Smith and Smith, 2011b, van der Heijden and Walder, 2016). Reciprocal trade in C-for-P has been demonstrated in some systems (Field et al., 2012), but categorically refuted in others (Walder et al., 2012). This latter study is of note for taking into account the role of common mycelial networks (CMN) linking plants together; a phenomenon the authors

claim is inimical with the biological market theory. Certainly, the system used by Walder et al. (2012) is more similar to how plants grow in nature than that used by Fellbaum et al. (2012) The mechanism proposed by the biological market theory seems to suggest that both N and P could be traded reciprocally but it is not clear how a plant would 'reward' a fungus which provides N but not P, vice versa, or provides both nutrients. Experimental investigation with triple labelled (^{14}C, ^{33}P, ^{15}N) whole-plant systems may be a useful way to determine the extent to which plant-fungal nutrient trade occurs by reciprocal rewards. As the MP can deliver meaningful amounts of N and P to the plant, both should be considered together in further work. Using a factorial design with high and low N and P soil treatments may determine the extent to which the trade balance model (Johnson, 2010) and the reciprocal rewards model (Kiers et al., 2011) are congruous.

Rather than reciprocal trade of C and N, the ^{14}C labelling in Chapter 3 demonstrates functional diversity between AMF species. Despite contributing no more (or less) ^{15}N to onion plants, *G. aggregatum* acquired more C from the plant. This, together with the data from Chapter 4, makes it tempting to label *G. aggregatum* as a 'worse' symbiont than *R. irregularis*. This may be premature, as the effect of these fungi on the plant acquisition of other nutrients such as Zn and Cu is unknown, not to mention other potential benefits from AM colonisation such as reduced susceptibility to pests, pathogens (Zamioudis and Pieterse, 2012) and drought resistance (Birhane et al., 2015). Moreover, these experiments only used single isolates of AMF while plants in most soils will be colonised by multiple species of AMF (Clapp et al., 1995, Jansa et al., 2008), not to mention other fungal and bacterial endophytes (Gaiero et al., 2013, Mayerhofer et al., 2013). How well these trends can be translated from the greenhouse experiments to effects observed in the field are not clear.

6.5. The influence of N application on AMF community composition

While field studies have shown N fertilisation capable of altering the N dynamics in the AM symbiosis (Johnson et al., 2015) and the structure of the AMF community itself (Daniell et al., 2001, Vandenkoornhuyse et al., 2002), the simultaneous change of structure and function of the AMF community, as suggested by data in Chapter 5 is novel. These simultaneous changes also offer support for the fourth hypothesis. As terminal restriction fragment length polymorphism does not allow identification of AMF species (Osborn et al., 2000), it is not possible to say quantitatively how the community has changed, but we can identify that a shift has occurred. Further investigation to identify more precisely the members of the AMF communities under different agricultural management is certainly warranted. Denaturing gradient gel electrophoresis (DGGE) and quantification by quantitative PCR (q-PCR) may be a suitable next step forward (Schneider et al., 2015).

Shifts in AMF community structure may be significant for the plant, as AMF species are known to display significant functional diversity in plant uptake of soil N (Mensah et al., 2015), P (van der Heijden et al., 1998) and subsequently plant growth (Klironomos, 2003). AMF community shifts in field soil may affect not only the roles of the AMF in plant acquisition, as shown in chapter 5 with the ^{15}N, but also in the wider N dynamics of the soil. Kohl et al. (2016) showed that N leaching from soils as nitrate dissolved in water was reduced in soil with greater amounts of AMF mycelium. How far this is determined by the AMF function and how much by community identity is not clear.

Different C acquisition by the isolates of AMF in Chapter 3 suggest functional diversity in among the AMF community which may translate to significant differences in the C flux belowground in field systems where the AMF differ. As C flux from the plant to the AMF is known to be rapid (Johnson et al., 2002) and the fungus may acquire up to 20% of

colonised plants' C, there is significant potential for the management strategy of arable land to influence C flux to the soil by altering AMF community composition. The extent to which shifts in AMF community will affect carbon dynamics is uncertain however. Arable farming has been shown to reduce glomalin (an abundant soil proteinaceous substance of AMF origin) production compared to uncultivated land, (Preger et al., 2007), but increased carbon acquisition by fungi may boost glomalin production in some managed arable systems. This would suggest that the glomalin production in soils under different plant species or cultivars will depend on the identity of the AMF colonising the roots. It has been demonstrated variously that increased carbon deposition from roots and AMF hyphae (Bonkowski, 2004) may accelerate the decomposition of organic matter as C limitation of saprotrophs is relaxed (Kuzyakov, 2002, Cheng et al., 2012), thus depleting soil carbon. Conversely, soil fungal growth has been shown to increase C deposition in soils (Verbruggen et al., 2013). The fate of soil C may depend on its origin (Williams et al., 2006); glomalin is known to be partly recalcitrant and to its decomposition is known to vary (Steinberg and Rillig, 2003) and to respond to agricultural management (Rillig et al., 2003, Rillig, 2004). Wider models (Averill et al., 2014) suggest soils dominated by AMF allow sequestration of less C than those dominated by ectomycorrhizas. This seemingly does not take into account the underlying variability in AMF C acquisition highlighted in chapter 5, and further possible variation buy priming or sequestration.

Further investigation into the role of AMF in C cycling is certainly pressing, as soils are being degraded rapidly (Committee, 2016), and increased carbon sequestration in soils is being touted as a potential tool to help mitigate climate change (Lal, 2004). Carbon sequestration in agricultural soils might allow mitigation of 15% of fossil fuel C emissions (Lal, 2004). Glomalin production in in soils varies widely depending on soil type and the vegetation cover (Singh et al., 2013), and is known to be reduced in tilled agricultural systems compared to unmanaged grasslands (Wright et al., 2007, Treseder and Turner,

2007). Further investigation is needed into the influence of management practice on the impact of AMF and their role in soil C input and glomalin production.

There is certainly a great deal still to discover - the identification of fungal NH_4^+ transporters in arbusculated cells (Perez-Tienda et al., 2011) suggests NH_4^+ uptake from the interfacial apoplast by the fungus. The function of this transporter is not fully understood. It may represent a mechanism by which the fungus can recover N from leakage or an N acquisition strategy by the fungus if plant-derived NH_4^+ concentrations in the apoplast are high enough. Conversely it may be analogous to futile N cycling in roots (Britto et al., 2001, Coskun et al., 2013), where NH_4^+ efflux is performed alongside uncontrollably high NH_4^+ influx, in order to prevent NH_4^+ toxicity in roots. To the same end, fungal removal of NH_4^+ from the apoplast may prevent excessive NH_4^+ acquisition by the plant and thereby reduce the effects of NH_4^+ toxicity, one of which is depletion of root C (Bittsanszky et al., 2015); a phenomenon the AMF would wish to avoid. Foliar feeding plants with ^{15}N and detecting in hyphae of the ERM may shed some light on whether there is the capacity for large scale plant to fungus N transfer. The suggestion of aquaporins between symbionts (Giovannetti et al., 2012, Kikuchi et al., 2016) may be a very significant discovery, as they would allow luminal continuity between the plant and fungus. This would mean that control of nutrient and signal dynamics between plants and fungi was largely determined by source-sink relations (as in some parasitic plants (Cameron and Phoenix, 2013), and may give a mechanism by which signalling molecules may be loaded into CMNs to facilitate belowground communications between plants (Babikova et al., 2013).

6.6. Conclusions

Despite decades of focus on the role of AMF in the P nutrition of plants, the role of these fungi in plant N acquisition has been relatively neglected. It has been demonstrated by the experiments reported in this thesis that AMF can move significant quantities of N to a partner plant and this can benefit the total N acquisition, P acquisition and biomass of the plant (Chapter 2). The extent to which a similar effect would be seen outside the setting of a greenhouse experiment remains to be determined, but a mechanism has been identified by which the AMF may represent significant route of N uptake for the plant. As there is variation in the degree to which AMF isolates improve the growth of their partner plant (Klironomos, 2003), the same experimental design as used in Chapter 2 but with different plant and AMF species may have yielded different results. Extending this further into a field setting would test the effects of other AMF species, rhizosphere microbes and competing plants have on the trends observed.

The two isolates of AMF used in the experiments showed no clear, consistent preference for acquisition of inorganic N as NO_3^- or NH_4^+, probably because of functional differences in uptake and transporters, soil chemistry, and competition from other soil microbes affecting the realised niche in which they might acquire N. AMF may expend less energy in acquiring N as NH_4^+ but as the majority of N in soils is NO_3^- , and as AMF have a high N demand, it makes sense that the AMF are capable of acquiring N in numerous forms, and being able to adapt uptake depending on the forms of N available. It is likely that the demand for and benefit of N acquisition as NO_3^- outweighs the cost to the fungus. AMF are known to acquire N in organic forms, suggesting that N demand can be sufficiently high to justify inefficient uptake (Talbot and Treseder, 2010). Simultaneous addition of singly ^{15}N labelled (NO_3^- or NH_4^+) N may help resolve if AMF express a preference over uptake.

Shifts of AMF communities within roots due to changes in agricultural practices prompt further experimentation, as the potential effects of these shifts on the dynamics of N, P and C are significant. Complementary experimental designs using the same AMF inoculant and/or plant species and cultivars would improve the ability for wider patterns to be seen across scales of experiments. Read (2003) was somewhat disparaging of small scale experiments in greenhouses conditions as they bore little ecological relevance. Coupled with field trials however, the approach can be more useful than either individually. After identification of fungal isolates (by DNA sequencing) under contrasting agricultural regimes (such as different cultivars and N application rates), parallel microcosm experiments using these isolates may provide useful mechanistic information. Further field trials may provide agriculturally relevant conditions and environments in which these trends can be tested.

AMF are frequently cited as potential tools for improving the sustainability of agriculture (Jeffries and Barea, 2012, Pellegrino and Bedini, 2014) and the predicted depletion of rock P reserves (Koppelaar and Weikard, 2013), from which almost all P fertilizer is derived, has been a major driver in this renewed interest. Determining whether these fungi may be able to improve plant nutrition significantly in an agricultural system still needs to be proven however. The experiments in this thesis add weight to the arguments that AMF can deliver meaningful amounts of N to their host plant, and that N has a regulatory role in the degree to which the symbiosis is beneficial for plant nutrient acquisition. It is also apparent that significant functional diversity among AMF isolates in numerous traits (i.e. N transfer, C gain etc.) means deciding how 'mutualistic' any given AMF might be cannot as simple as measuring plant P uptake; N rate and form must also be considered when discussing the degree to which AMF are beneficial for plant nutrient acquisition.

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