The C-economy, nutritional benefits and symbiotic performance of dual inoculated *Phaseolus vulgaris* (L.) plants, under variable nutrient conditions

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Abstract

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The tripartite symbiosis between *Phaseolus vulgaris*, arbuscular-mycorrhiza and the nodule bacteria, *Rhizobia* have been the focus of many studies ranging over a number of decades, however these studies have failed to answer certain questions relating the role of the symbionts in regard to host nutrition and the subsequent influence of these symbionts on the host C-economy. There is little doubt over the synergistic benefits involved in the dual inoculation of bean plants, as well as the resultant C-costs of maintaining the 2 symbionts, yet the specific contribution of the individual symbionts to the hosts overall nutrient and C-economy remain to be clarified. Thus the aim of this thesis is to help clarify these points by determining the symbiont induced photosynthetic, respiratory and nutritional changes taking place in the host. This was achieved by a series of experiments in which nodulated bean plants were split into two categories-those with and without AM colonized roots. These plants were then exposed to a range of growing conditions, including hi and low P, and a series of N treatments, ranging from zero N through to 3 mM NH_4^+. Under these differing nutrient conditions growth, photosynthetic, respiratory, nutrient and amino acid

responses were monitored, thus allowing for the determination of the symbionts influence on the host and the hosts reliance on the respective symbionts. Host reliance was noted most strongly under nutrient limiting conditions. Under low P treatment AM was the dominant symbiont as far as host C was concerned, allowing for the early establishment of the AM, thus ensuring the uptake of P for both host and nodule development. High P affected AM colonization to a greater extent than it did nodule dry weight and conversely the addition of NH_4^+ led to a greater decrease in nodule dry weight than it did AM colonization. In spite of this decline, AM benefited the host by improving host N nutrition and relieving N-feedback inhibition of the export amino acid asparagine on BNF. These AM induced benefits did come at a cost to the host though, the dual inoculated plants had higher below ground respiratory costs and subsequently higher photosynthetic rates to compensate for the increased demand for C. The higher photosynthetic rates associated with dual inoculation were as a result of symbiont induced sink stimulation and not due to the improved nutrition of the host, as shown by the photosynthetic and nutrient response ratios. However, the respiratory costs associated with the uptake of soil nutrients were lower in AM colonized roots, thus showing an increased efficiency in nutrient gain by AM colonized roots. This improvement in host N nutrition as a result of AM colonization, coupled with the lower respiratory costs of AM nutrition led to the conclusion that under certain growing conditions nodules can become redundant and possibly parasitic.

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DECLARATION

I declare that: 'The C-economy, nutritional benefits and symbiotic performance of dual inoculated *Phaseolus vulgaris* (L.) plants, under variable nutrient conditions' is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Peter E Mortimer

September 2010

Signed: . . . . . .

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I learnt a great deal more than just botany while writing this thesis, so I would like to thank those who contributed, both the good and the bad, to these life lessons. A special thanx to Alex, Maria and my family.

Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science.
Jules Henri Poincaré (1854-1912)

Table of contents

Chapter 1

Literature review

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Literature review

1.1 Introduction

The family of plants *Fabaceae* is the third largest of the plant families and consists of about 18 000 species, ranging from annual herbs to large trees (Udvardi *et al.,* 2005). Many of the species are of commercial value and *Fabaceae* is second only to the grains as a food source for humans (Graham and Vance 2003). The value of legumes as a crop plant lies in the ability of many of legumes to, via the symbiotic association with rhizobia, fix free nitrogen (N) into an organic form. This process supplies both the legumes and the subsequent crops with a renewable source of N (Frey and Schuepp 1992; Udvardi *et al.*, 2005).

Rhizobia bacteria are classified into various genera including *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium* and *Mesorhizobium* (Lodwig and Poole 2003; Denison and Kiers 2004a), collectively known as rhizobium. Rhizobia are free-living bacteria capable of living independently in the soil or in nodules that develop on the roots of legumes (Denison and Kiers 2004b). Inside these nodules rhizobia are able to fix atmospheric N, via the enzyme nitrogenase, into mineral N. Part of the N fixed by the rhizobia is exchanged with the host for photosynthetically fixed carbon (C) (Lodwig and Poole 2003).

However, there is often a third party involved in the Legume-rhizobia symbiosis, Legume roots can also be colonized by arbuscular mycorrhizal fungi (AMF). AMF belong to the phylum *Glomeromycota*, order *Glomales* and form a symbiotic

relationship with about 80% of all plants (Smith and Read 1997; Schussler *et al.,* 2001). The key function of arbuscular mycorrhizae (AM) is the uptake of P from the soil and the subsequent exchange of this for host derived C. Thus the two symbionts are competing for the same source of C (Harris *et al.,* 1985).

Despite the C drain imposed by the symbionts both the rhizobia and the AMF can improve the nutrition and growth of their host plants. However, especially under nutrient limiting conditions, the combined inoculation of both rhizobia and AMF enhances plant growth to a greater extent than singular inoculation, as well as enhancing the degree of colonization by the respective symbionts (Daft and Elgiahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989).

Thus it is clear that in order to discuss the relationship between the legume and its two symbionts effectively, a number of different fields of study need to be considered. Initially I cover the basics of nutrition, focusing on the availability and role of both soil N and P. The plant is constantly in the process of accessing various sources of soil N and P and growth is often limited when it cannot do this effectively or the nutrients are in limited supply. I then move on to discussing the two symbionts in more detail. Describing how they access the respective nutrients which they provide for the plant, the costs involved of maintaining these relationships and when it is that the symbionts are most beneficial to the host plant. After which the effect of soil N on the nodulation process and the subsequent process of biological N fixation is discussed. The final topic of this literature review is that of C flux modeling. What the current models are and how they can be used to describe the symbiotic relationships.

1.2 Nitrogen

1.2.1 Available forms

For most plant species nitrogen (N) is the major nutrient limiting growth (Greenwood, 1982). It is available to the majority of plants in the form of soil N, as ammonium (NH_4) or nitrate (NO_3) and to a lesser extent amino acids. The NO_3 is taken up by the roots and reduced to NH_4 in a 2-step process. Initially NO_3 is reduced to NO_2 in the root via the enzyme nitrate reductase and then the NO_2 is further reduced to NH_4 via the enzyme nitrite reductase. The NH_4 is then assimilated into an organic form when coupled with a C skeleton via the glutamine synthase/glutamate synthase, glutamate-2-oxoglutarate amino-transferase cycle (GS/GOGAT cycle) (Lancien *et al.,* 2000; Miller and Cramer 2004; Foyer *et al.,* 2005).

Legumes however are capable of accessing an additional source of N, atmospheric N_2 via the process of biological N-fixation (BNF). Both free living and endosymbiotic bacteria are capable of BNF, which involves the conversion of N_2 gas into NH_4 (Vance 2002; Miller and Cramer 2004). Perhaps the most well known of the symbioses responsible for BNF is that between legumes and rhizobia (Graham and Vance 2000).

1.2.2 Soil N

The N available for plants in the soil is derived predominantly from the decomposition of organic matter (dead animals, plants and microbes) by soil microorganisms such as bacteria and fungi (Chapin *et al.,* 2002; Miller and Cramer 2004). The microbial breakdown of soil organic matter will result in either the

production of NH_4, a process known as ammonification, NO_3 via the process of nitrification or into free amino acids (Marschner 1995). However, the amino acids are usually rapidly scavenged by soil microorganisms and are seldom assimilated by the plant, making inorganic N the dominant form taken up by the plant (Owen and Jones 2001; Miller and Cramer 2004).

Whatever form it takes, N moves primarily by mass flow and diffusion in the soil, thus the transpirational pull of water towards the roots will also deliver N to the root system (De Willigen 1986; Miller and Cramer 2004). The rates of diffusion for N compounds vary according to both soil conditions such as soil moisture, soil buffer capacity, soil water viscosity, tortuosity and temperature as well as the size and charge of the N compound (Miller and Cramer 2004). NO_3 has the highest diffusion coefficient in soils, followed by NH_4 and then amino acids, thus NO_3 is the most readily available form of N to the plant. However, when NH_4 is available to the plant it will be taken up by the roots before NO_3 (Lee and Rudge 1986; Colmer and Bloom 1998). The inverse is that the forms of N with the higher diffusion co-efficiencies are also the forms of N most readily leached from the soil (Miller and Cramer 2004).

1.2.3 N uptake

Under N limiting conditions the plant tends to increase the allocation of resources below ground, causing a shift in the root:shoot ratio. Rufty *et al.,* (1988) found that when plants were exposed to low N conditions starch accumulated in the leaves and there was an increase in the amount of photosynthate transported to the roots. It is generally accepted that by increasing the root:shoot ratio in times of N deficiency, the plant enhances its below ground acquisition capacity (Brouwer 1981; Khamis and

Lamaze, 1990; Robinson, 1986; Rufty *et al.,* 1990). Furthermore, it has been found that the root system will proliferate in pockets of soil rich in N, thus maximizing on the N available to the plant (Drew and Saker 1975; Granato and Raper, 1989).

Once the root comes into contact with the soil N, there are various N transporters on the root for the uptake of N, depending on the form of N available to the plant. The genetic code for many types of N transporters have been identified and provide insight into the form and function of these transporters and the subsequent uptake of N by the root. NO_3 is actively transported across the epidermal and cortical plasma membranes of the root, independent of soil N concentrations (Glass *et al.,* 1992; Miller and Smith 1996; Zhen *et al.,* 1991). The uptake of N is coupled with the movement of 2 protons down an electrochemical potential gradient, thus being dependant on the H^+ -ATPase that maintains the H^+ gradient across the plasma membrane (McClure *et al.,* 1990; Meharg and Blatt, 1995; Miller and Smith, 1996). In addition there are both high and low affinity transport systems in the root for differing external NO_3 concentrations (Aslam *et al.,* 1992; Glass and Siddiqi 1995).

A number of NH_4 transport systems have been characterized in plant roots and are responsible for the uptake of NH_4 from the soil (Lauter *et al.,* 1996; Ludewig *et al.,* 2002; Ninnemann *et al.,* 1994; von Wirén *et al.,* 2000). Furthermore, it has been suggested that NH_3 can move across the plasma membrane, passively, through molecular channels such as aquaporins (Niemietz and Tyerman, 2000; Howitt and Udvardi 2000).

Gene families have been sequenced for the transport of a number of organic N

molecules, although their specific roles in the uptake of these compounds from the soil remains to be confirmed in many of the cases. Transport systems for the movement of amino acids, oligopeptides, urea, N-containing heterocyclic compounds, purines and oligopeptides have been characterized in plants (Desimone *et al.,* 2002; Li *et al.,* 2003; Liu and Tsay 2003; Gillissen *et al.,* 2000; Koh *et al.,* 2002; Steiner *et al.,* 1994). Specifically, it is known that transporters for the uptake of amino acids and auxins are found in the root systems of some plants (Miller and Cramer 2004; Popova *et al.,* 2003). However, considering the primary forms of N uptake from the soil are NO_3 and NH_4, it is likely that the expression of the genes coding for the uptake of organic N compounds would likely be in N limiting conditions.

1.2.4 Plant C-N interactions

The assimilation of inorganic N, ultimately in the form of NH_4 (due to the conversion of NO_3 to NH_4 in the plant) into an organic form is a key metabolic process in plants. NH_4 is toxic to plants in high concentrations and must therefore be rapidly assimilated by the plant. It is converted into the amino acids glutamine and glutamate via the GS/GOGAT cycle (Lancien *et al.,* 2000; Foyer *et al.,* 2005). Due to the assimilatory process using both energy and C, it is closely coupled with the C metabolism of the plant. The GS/GOGAT cycle requires ATP, C skeletons, 2-oxoglutarate and reduced ferredoxin or NADH, thus relying heavily on plant respiratory processes (Huppe and Turpin, 1994; Lancien *et al.,* 2000; Britto and Kronzucker 2005; Foyer *et al.,* 2005). The production of C skeletons and energy for the GS/GOGAT cycle can account for as much as 50% of the C found in some of the plant tissues (Huppe and Turpin 1994).

1.3 Phosphate

1.3.1 Available forms

Even though P occurs in high concentrations in the soil it is largely unavailable to plants due to its immobility (Van Tiehelen and Colpaert 2000). The two main forms in which P occur are organic (Po) and orthophosphate or inorganic P (Pi). For agricultural soils Po can make up as much as 70% of total soil P, yet it is not readily available for uptake by plants (Lambers *et al.,* 1998). The three forms in which Po is found in the soil are: soluble P in the soil solution; insoluble P adsorbed onto the surfaces of soil particles or as organic matter within the soil (Anderson 1980). The primary compounds in which Po is found in the soil are inisitol phosphate, glycerophosphate (phospholipids) and nucleic acids (Anderson 1980; Adams and Pate 1992). Po needs to be mineralized in order to be available to the plant (Horst *et al.,* 2001). This mineralization process is carried out by soil microbes, which feed off organic substances exuded by the plant (Richardson 1994) or by the action of acid phosphatases, which are secreted by the root (Adams and Pate 1992). These enzymes hydrolyze organic-phosphate containing compounds in the soil, releasing Pi into the soil and consequently making it available for uptake by the plant (Kroehler and Linkins 1991).

Although Pi makes up a much smaller component of soil P, it is readily available to plants for uptake. The 2 main forms of Pi in the soil are $H_2PO_4^-$ and HPO_4^{2-}, with an optimum pH lying between 4.5 and 5 for Pi uptake (Vance *et al.,* 2003). Pi can be found in soil solution, adsorbed onto the surfaces of soil particles or precipitated as discrete minerals. It is the Pi in the soil solution that constitutes the primary source of P for the plants (Bolan 1991). Pi precipitates out of solution as Fe and Al phosphates

in acidic soils and as Ca and Mg phosphates in alkaline soils, making it unavailable to the plant for uptake (Bolan 1991; Vance *et al.,* 2003). The form in which these phosphates are found and their availability to the plant is greatly influenced by soil pH, ionic strength, concentrations of both P and metals (Fe, Al, Ca) and the occurrence of other ions (Sample *et al.* 1980; Sanyal and DeDatta 1991; Hinsinger 2001).

1.3.2 P acquisition and uptake

The two main ways in which plants can come into contact with soil P are by root interception and by diffusion (Comerford, 1998; Hinsinger, 2001). Mass flow plays a role in the movement of other more mobile nutrients, but phosphate is bound too tightly to the surface of soil particles to move by mass flow (Bolan 1991).

Even when P is spatially available to the root, it is not necessarily available for uptake by the plant. Therefore in order to gain access to P, the plant secretes acidifying and chelating compounds (citric acid, malic acid, oxalic acid and piscidic acid) and phosphatases from the root into the rhizosphere (Marschner 1995; Comerford, 1998; Hinsinger, 2001). The subsequent acidification of the rhizosphere increases the solubility of P in alkaline soils and the chelating compounds (organic acids and phenolics) then bind to the cations that are bound to the phosphate groups, thus releasing the P for uptake by the plant (Marschner 1995).

The lack of bioavailable soil P can result in numerous anatomical, physiological and biochemical roots adaptations (Raghothama 1999). Some of the anatomical changes include root elongation and an increase in root hair number and length (Ma *et al.*

2001). Another anatomical change is the formation of proteoid (cluster) roots (Campbell and Sage 2002; Neumann and Römheld 1999). These are short, dense clusters of lateral roots known to form in certain Legume species (*Lupinus albus*) and members of the Proteaceae family. Cluster roots will form and proliferate in pockets of soil P, thus maximizing on available soil P.

Adaptations of a physiological and biochemical nature include the enhanced expression of phosphate transporters (Karthikeyan *et al.,* 2002) and the release of low molecular weight organic acids, phosphatases and protons into the rhizosphere (Johnson *et al.* 1994; Neumann and Römheld 1999). Kinetics studies have found that both high and low affinity transport systems exist in the roots for the uptake of P (Bieleski 1973; Smith *et al.,* 2000), with the high affinity system being expressed under P limiting conditions and the low affinity system being constitutively expressed (Raghothama 1999). The transport proteins responsible for the uptake of P from the soil are found in the younger root sections and root hairs, thus maximizing on the available soil P and avoiding depletion zones in the rhizosphere (Smith *et al.,* 2001).

1.3.3 Adaptations to low P

Plants have 2 ways of dealing with P limiting conditions, they either enhance the acquisition and uptake of P from the soil or they conserve the use of P internally (Lajtha and Harrison 1995; Horst *et al.,* 2001; Vance 2001; Vance 2003). Processes involved in the enhanced uptake of P from the soil include exudation of organic acids, the secretion of phosphatases, changes in root anatomy and root length, increase in number of root hairs and an upregulation in the expression of Pi transporters (Marschner *et al.,* 1986; Duff *et al.,* 1994; Schachtman *et al.,* 1998; Gilroy and Jones

2000; Lynch and Brown 2001). The production of acid phosphatase enzymes (APases) is a well-known response to P deficiency in plants, they function in 2 different ways to help the plant cope with P shortages. Firstly they are aid in the internal mobilization of P and when exuded into the rhizosphere they are known to release organic P from P-esters (Goldstein *et al.,* 1988; Lefebvre *et al.,* 1990; Duff *et al.,* 1991; del Pozo *et al.,* 1999; Baldwin *et al.,* 2001; Miller *et al.,* 2001).

And alternatively, processes resulting in a more efficient use of P by the plant include a lower growth rate, better growth per unit of P taken up, changes in C metabolism that bypass P requiring steps, remobilization of internal Pi and the use of alternative respiratory pathways (Schachtman *et al.,* 1998; Plaxton and Carswell, 1999; Raghothama, 1999; Uhde-Stone *et al.,* 2003 a, b).

1.3.4 Influence of low P on C metabolism

Plants roots lose a lot of C via exudation of organic compounds into the rhizosphere. This is an important process in that it allows the plant to control the conditions in the rhizosphere, thus aiding in the uptake of key nutrients and is involved in the signaling processes with soil organisms and allelopathic actions by the plant (Curl and Truelove 1986; Marschner *et al.,* 1986; Harrison 1997). The release of organic acids is a primary response to low soil P, in an attempt by the plant to access all available P in the rhizosphere. The amount of C lost in the form of malate and citrate under P deficient conditions can range from 10% to 25% of total plant dry weight (Vance *et al.,* 2003). The loss of C via the root will have a cascade effect on plant metabolism, requiring a increase in the TCA cycle for the production of C skeletons, enhanced photosynthesis for the capture of C and increased respiratory rates.

Apart from the improved acquisition of P from the soil plants also adapt to low P by being more conservative with internal pools of Pi. Due to the dependence of several enzymes in the glycolytic pathway on Pi or andenylates, metabolic processes may become impaired by a P shortage (Vance *et al.,* 2003). Alternative metabolic pathways are used that bypass Pi or adenylate requiring steps in an attempt to conserve the limited Pi available (Duff *et al.,* 1989; Mertens 1991; Theodorou *et al.,* 1992; Theodorou and Plaxton, 1996). Thus the plant is able to continue key processes, including those involved in the production of new C skeletons, ultimately to be released via the roots in an attempt to access more P from the soil. Further metabolic changes take place in the mitochondrial respiratory pathways when plants experience P stress. Nonphosphorylative pathways that bypass energy requiring steps are given priority in an attempt to reduce the internal use of Pi (Vance 2003).

Another plant response to low P nutrition is a switch to secondary metabolism resulting in a build up of secondary metabolites like flavonoids and indole alkaloids (Plaxton and Carswell 1999). Phenolic compounds are produced under P limiting conditions and are thought to be released into the soil in order to act as chelators and/or reductants to aid in the release of soil bound Pi. P deficiency is also known to induce the production of anthocyanins, a response most likely in aid of ameliorating the effects of photoinhibition of chloroplasts (Takahashi *et al.,* 1991). In general, secondary metabolism does not use as much Pi as primary metabolism, but can recycle internal P sources such as phosphate esters (Plaxton and Carswell 1999; Salano 2001).

1.4 Arbuscular mycorrhizae

1.4.1 Host benefits

Arbuscular mycorrhizae (AM) form a symbiotic association with plants, colonizing their root systems and developing a hyphal network extending out into the surrounding soil. This symbiosis results in a number of benefits for both host and symbiont. The plant supplies carbohydrates to the fungus in exchange for the benefits provided by the AMF. There is a range of benefits resulting from root colonization by the AMF, ultimately resulting in improved growth of the host plant.

It is generally accepted that the increase in the growth of plants colonized by AMF is attributable to increased P nutrition (Sanders & Tinker 1971; Smith 1982; Bolan 1991; Orcutt & Nilsen 2000). However, in addition the improved P nutrition of the host plant, AMF also offer other nutritional benefits for the host. Marschner and Dell (1994) showed that AMF acquired 80% of plant P, 25% of plant N, 10% of plant K, 25% of plant Zn and 60% of plant Cu, indicating the role that AM plays in the overall mineral nutrition of plants and not just P acquisition.

There are also a number of non-nutritional benefits associated with AM colonization. Karagiannidis and Nikolaou (2000) found that AM colonization protects plants from the influence of heavy metals such as Pb and Cd. The metals are taken up by the fungus and complexed with polyphosphate, thus preventing their transport to the host (Orcutt and Nilsen 2000). Bavaresco and Fogher (1996) reported that the AM colonization of grapevine roots, grown in calcerous soils, resulted in increased shoot growth compared to the non-mycorrhizal plants. AM colonization has also been shown to increase the ability of plants to grow in acidic soils (Howler *et al.*, 1987;

Clark 1997; Yano and Takaki 2005). Yano and Takaki (2005) found that under acidic conditions the root development and shoot growth of AM colonized *Ipomoea batatas* was enhanced compared to non-mycorrhizal plants.

AM colonization also benefits the host plant by increasing the host's resistance to soil-borne pathogens in a number of different ways. Improved nutrition, primarily P but possibly Zn and Cu, aid in the suppression of root pathogens (Marschner 1995; Orcutt and Nilsen 2000). Increased production of phenolics and isoflavonoids is thought to also increase the host's resistance to infection (Orcutt and Nilsen 2000). The resulting lignification and suberization of the root due to AM colonization lowers the risk of infection by root pathogens (Dehne and Schonbeck 1975; Yedidia *et al.,* 1999). Another mechanism by which AMF can protect the host against pathogenic fungal infection is by competing for the same infection sites on the host's roots. Thus AM colonization limits the number of sites available for pathogenic fungal infection and lowers the host susceptibility to colonization by these fungi (Waschkies 1994; Vigo *et al.,* 2000).

Both the fungal species and the host plant species will determine the extent of the symbiotic benefits (Karagiannidis *et al.,* 1997; Scheublin *et al.,* 2004). The species of fungus found in the soil can differ from one location to the next and is usually determined by the soil characteristics (pH, soil moisture, particle size) and the vegetation cover (Nappi *et al.,* 1985; Shubert and Cravero 1985). Certain species of fungi will be dominant in a soil that is covered by a specific host plant.

1.4.2 Parasitism

When the plants are growing in conditions where nutrients are freely available, they may not rely on the fungus for the supply of nutrients. Thus the fungus may have a negative effect on the growth of the host plant, due to the C drain imposed (Johnson *et al.,* 1997).

To determine at what stage the AM becomes parasitic is a difficult task and would be dependant on the actual definitions of parasitism used. In its simplest form, if the resultant benefits of colonization do not out weigh the costs incurred then the AMF could be considered parasitic. However it is not easy to quantify such a cost-benefit relationship. One way of trying to determine this was by subtracting the nutrient concentration of the AM plant with the nutrient concentration of a non-AM plant (Jones and Smith 2004). A positive outcome for the calculation would imply a non-parasitic relationship. Although this is difficult to draw a conclusion from because there are other benefits due to colonization, hence the need to be clear on what aspects of parasitism you are trying to define. Perhaps a more accurate means of determining parasitism is by evaluating plant growth as a whole. In a study done by Smith *et al.,* (2003), looking at various plant-fungus combinations, a couple of the combinations resulted in a growth depression of the plant, indicating a parasitic response to AM colonization. Thus, to determine whether there has been a parasitic response is difficult and often the cause of much debate. However, it would be shortsighted to ignore the possibility of parasitism in plant-mycorrhizal relationships.

1.4.3 The C-cost of arbuscular mycorrhizae

AMF are dependent on the host plant as a C source and therefore act as a C sink. There is conflicting evidence as to whether or not the percentage colonization of the root by AMF is related to the soluble carbohydrate content of the root. Pearson and Schweiger (1993) found that colonization was negatively correlated with the soluble carbohydrate content of the root, whilst Thompson *et al.,* (1990) found a positive correlation. This may be because the conflicting experiments were carried out during different developmental stages of colonization. The three stages of colonization are the lag phase, the phase of rapid development and the plateau phase (Smith and Read 1997). Pearson and Schweiger (1993) carried out their experimental work towards the end of the phase of rapid development, when the colonization period starts to decline and therefore is a subsequent decline in the demand for C by the fungus. Thompson *et al.,* (1990) experimented during the end of the lag phase and the start of the phase of rapid development, when the demand for C is high. It appears that the process of colonization does depend on carbohydrates from the root during the initial phases and then reaches equilibrium as the process of colonization comes to an end and a stable symbiotic relationship develops.

Once established, the fungus acts as a sink for photosynthate from the host plant. It has been estimated that the fungus receives between 10% and 23% of the plant's photosynthetically fixed carbon (Snellgrove *et al.,* 1982; Koch and Johnson 1984; Kucey and Paul 1982; Jakobsen and Rosendahl 1990). Black *et al.,* (2000) showed that mycorrhizal plants have a higher photosynthetic rate than non-mycorrhizal plants. This may be because of either an increased level of phosphate in the leaves due to the mycorrhizae (Azcon *et al.,* 1992; Black *et al.,* 2000) or because the AMF acts as a

carbon sink (Snellgrove *et al.,* 1982; Kucey and Paul 1982; Koch and Johnson 1984; Jakobsen and Rosendahl 1990). Both explanations have been found to be true, but under different conditions and for different plants. Therefore it may result from a combination of both, depending on the growing conditions and the developmental stage of both the fungus and the plant.

The carbon taken up by the fungus is incorporated into the growth and development of new fungal structures and spores. More than 90% of the root can be colonized by an AMF (Motosugi *et al.,* 2002) and the fungus can constitute up to 20% of the root dry mass (Harris and Paul 1987). Respiration of colonized roots was found to be between 6.6% and 16.5% (depending on fungal species) higher than non-colonized roots in cucumber plants (Pearson and Jakobsen 1993). The increased respiration rate contributes to the sink effect of the fungus and indicates that colonized roots have a higher metabolic activity than non-colonized roots.

There are three main ways that organic C is lost from the host via the fungus. Firstly, via the loss of sloughed off fungal material, secondly through the release of fungal spores into the soil and thirdly via the exudation of organic acids and phosphatase enzymes by the fungus. Fungal mycelia are constantly being replaced because of older material either breaking off as the root pushes through the soil or dying and being released into the soil. Bethlenfalvay *et al.,* (1982) found that as much as 88% of the fungal biomass was external of the root for soybean, similarly Olsson and Johansen (2000) found that 70% of the fungal biomass was external mycelium on cucumber roots. This will account for a large portion of C lost into the soil considering that at some stage the external hyphae will be released into the soil. The

release of spores from the external mycelium accounts for a high percentage of lost organic C. In a study done by Sieverding *et al.,* (1989) it was estimated that 919 kg ha^{-1} of plant C went into the production of spores, which are subsequently released into the soil by the fungus. Furlan and Fortin (1977) found spore production was influenced by the amount of C that is available to the fungus. The third means of organic C loss is through the exudation of organic acids and enzyme phosphatases by the fungal hyphae in order to aid in the uptake of nutrients such as phosphate. However, the main body of evidence supporting this has been found in ectomycorrhizae (Bolan *et al.,* 1987). Although the release of organic acids is not thought to be the primary means of P uptake (Bolan 1991), it does constitute a loss of organic C from the host.

1.4.4 P uptake and supply

AM hyphae can extend out into the soil by about 25 cm, thus exploring a much greater area of soil than the roots would (Smith and Read 1997). The P taken up by the hyphae is then transported along the hyphae and supplied to the host. A number of genes have been identified that code for P transporters in the hyphae, including a high affinity transporter likely to be used in the uptake of P from the soil (Harrison and van Buuren 1995; Maldonado-Mendoza *et al.,* 2001). These transporters closely resemble the P transporters found in plants. Once the P has been taken up into the hyphae it is complexed into polyphosphates and transported to the arbuscules. The arbuscules form the periarbuscular membrane, the site where P is transported across to the host (Smith *et al.,* 2003).

1.5 Nodules

1.5.1 General concepts of the legume – rhizobium symbiosis

The effective management of N in the environment forms one of the cornerstones of sustainable agriculture. This management process usually involves at least some use of biologically fixed N_2 because N from this source is used directly by the plant, and so is less susceptible to volatilization, denitrification and leaching. Symbioses involving leguminous plants and species of rhizobium and *Bradyrhizobium* bacteria form an integral part of this process. In agricultural settings, roughly 80% of this biologically fixed N_2 comes from this type of symbiotic relationship. N fixing bacteria in concert with legumes fix atmospheric nitrogen, which is then available to the infected plant. Worldwide, legumes are grown on approximately 250 Mha and they fix about 90 billion tons of N_2 per year (Carling *et al.,* 1978; Bécard *et al.,* 1997; Graham and Vance 2000).

A mutualistic symbiotic relationship is established when legumes and rhizobium bacteria interact. Nitrogen-fixing bacteria in concert with legumes fix atmospheric nitrogen, which is then made available to the infected plant (Carling *et al.,* 1978; Bécard *et al.,* 1997). Legume nodules can be classified into two groups. Determinate nodules have no meristem, are usually spherical in shape, the infected cells lack vacuoles and they generally export ureides. Indeterminate nodules have a meristem and, because of their continuing growth, are generally cylindrical in shape. In addition, indeterminate nodules have vacuolated infected cells and export amino acids - mostly in the form of asparagine (Streeter 1991).

In functional nodules, the bacteria-infected central zone of the nodule is surrounded by layers of uninfected cells that occupy a region of the nodule referred to as the nodule cortex. The vascular tissue within the nodule cortex contains phloem and xylem surrounded by vascular endodermis. These tissues are continuous with similar tissues in the subtending root. In some nodules, especially those that produce ureides as the end product of N_2 fixation, the central zone contains both infected and uninfected cells. The uninfected cells are thought to play a role in ureide synthesis (Layzell and Atkins 1990).

Within the infected cells, the symbiotic bacteria or bacteroids occupy enclosures, known as symbiosomes, surrounded by a plant-derived membrane called a peribacteroid or symbiosome membrane. The bacteroids differ from the free-living bacteria in that they are larger and express a complement of genes that are not expressed in the free-living form. Plant organelles, including mitochondria, plastids and peroxisomes, tend to be localized near gas-filled intercellular spaces that form a network throughout the entire central zone and are thought to play a role in providing a low-resistance diffusion pathway for O_2 supply to, and H_2 and CO_2 removal from, the metabolically active cells within the central zone (Layzell and Atkins 1990).

1.5.2 Biological N fixation

Atmospheric N_2 is reduced by the microbial enzyme nitrogenase expressed by the rhizobium bacteria during the N fixation process. The nitrogenase enzyme requires anaerobic conditions in order to function properly. In order to maintain this anaerobic environment the plant produces leghemoglobin, an O_2 binding heme protein that scavenges any free O_2. Biological N fixation is energetically expensive, the nitrogenase

complex reduces 16 molecules of ATP to ADP whilst fixing 1 molecule of N_2 (Vance 1990; Layzell *et al.,* 1990):

$$N_2^- + 16\,ATP + 8\,H^+ + 8\,e^- = 2\,NH_4^+ + H_2 + 16\,ADP + 16\,Pi$$

The NH_4 that is produced is then released through the peribacteroid membrane and used in the plant cells for the synthesis of the amino acid glutamine (Kennedy 1966a,b; Lodwig and Poole 2003). The glutamine produced is then converted to either asparagine or to purine derivates known as ureides, depending on the legume species (Logwig and Poole 2003). Legumes such as clover, pea and alfalfa forming indeterminate nodules mainly produce asparagines (Vance 2000). The second group of legumes, forming determinate nodules, (*Phaseolus* and soybean) produce ureides such as allantoin and allantoic acid (Schubert 1986; Atkins and Smith 2000).

The assimilation of NH_4 requires C skeletons, derived mainly from C-4 acids produced by the TCA cycle. However, any consumption of acids of the TCA cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate, and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a build-up of acetyl-CoA and the input of the TCA cycle would stop. Thus an alternative source of oxaloacetate is required as oxaloacetate is the carbon skeleton used in asparagine production by the bacteroid. The overall stoichiometry for N assimilation in the nodule requires one molecule of oxaloacetate to be converted to one molecule of asparagine per N_2 molecule fixed. One possible source for the required oxaloacetate is the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPc). The PEPc in roots is located in the cytoplasm, as are the root and nodule enzymes for ammonia assimilation. In addition, it has been shown that PEPc is present in the root nodule of broad bean, and that these nodules take up CO_2, the substrate used by PEPc (Christeller *et al., 1977*).

1.5.3 Metabolism and C-costs of N fixation

The high turnover of carbon in the nodule is reflected by a substantial requirement for newly fixed carbon continuously provided by the shoot, and by about a two-fold higher respiration rate per unit dry weight of nodulated compared with non-nodulated legume roots. The carbon costs of N fixation vary with host species, bacterial strain and plant development. At certain stages of the growth period nodules may consume as much as 50% of plant photosynthate and about half of this is respired as CO_2. However, between 25% and 30% of the respired CO_2 can be reassimilated by the nodules via PEPc. This provides up to 25% of the carbon needed for malate and aspartate synthesis, both required for the assimilation of NH_3 and export to the host plant. On a fresh weight basis, PEPc activity is between 20 times (pea) and 1000 times (soybean) higher than in the roots (Marschner 1988; Schulze *et al.,* 1998; Vance *et al.,* 1983).

1.5.4 The effects of P deficiency on N fixation

The rhizobium -legume symbiosis is based on the exchange of nutrients between the symbionts. Besides the exchange of C and N, other nutrients are important for this plant-microbe association to function optimally. Studies with soybean have consistently shown a positive response to Pi fertilization. Whole plant N; plant dry matter; nodule number; nodule dry weight and nitrogenase activity were found to increase as a result of P_i fertilization. The process of N fixation requires a relatively large amount of P in the form of ATP, thus forming strong P sinks. Al-Niemi *et al.,* (1998) found that nodules will receive P from the host plant but nodular P will not be supplied to the host, resulting in nodular tissue having a 3 fold higher P concentration than surrounding plant tissues (Vadez *et al.,* 1997).

The ability of inorganic phosphate (P_i) to limit N fixation of legumes has received considerable attention by various researchers (Jakobsen 1985; Israel 1987; Sa and Israel 1991; Al-Niemi *et al.,* 1997, 1998; Tang *et al.,* 2001; Le Roux *et al.,* 2006). Under prolonged P deprivation, the impairment of N fixation by P deficiency can be brought about by various mechanisms. P deficiency can indirectly limit N fixation through an N-supply feedback mechanism, as indicated by the high concentration of asparagine in the phloem of P deprived white clover plants (Almeida *et al.,* 2000; Høgh-Jensen *et al.,* 2002). It was also proposed that P deficiency eventually reduces plant growth, with a subsequent reduction in N demand and thus a reduction in N fixation (Høgh-Jensen *et al.,* 2002). A reduction in P will also lead to a reduction in photosynthesis, thereby decreasing the amount of available photosynthate, evidence points to photosynthetic C being a key regulatory feature of N fixation (Robson *et al.,* 1985; Jakobsen, 1985; Sa and Israel 1991).

1.5.5 Respiration in symbiotic root systems

Nodules have an extremely high demand for energy, the N fixation process alone consumes at least 16 mol ATP for every N_2 molecule converted into NH_3 (Ljones and Burris, 1972). The nodules rely on aerobic respiration for the provision of this energy, resulting in the nodular tissue having about a 4 fold greater respiratory O_2 consumption rate than the surrounding root tissue. However, in contrast to this high O_2 demand, O_2 has a strong irreversible inhibitory effect on the nitrogenase enzyme, therefore the nodules need to regulate their O_2 economy very carefully. This is done in three ways: The production of leghemoglobin to bind free O_2, thus maintaining a steady flux of O_2 to the bacteria, yet providing extremely low, non-toxic concentrations of O_2; A high

rate of O_2 consumption and a variable diffusion barrier which controls the amount of O_2 entering the area of the nodules containing the bacterial cells (Appleby 1984; Witty *et al.,* 1986; Hunt *et al.,* 1987; Layzell and Atkins 1990; Denison, 1992). Thus the nodules are able to function normally in a low O_2 environment, yet sustain relatively high respiration rates.

1.5.6 C and N budgets in symbiotic root systems

In legumes, the phloem provides the nodule with reduced carbon in the form of sucrose, whereas the xylem removes the products of N fixation as either ureides (allantoin and allantoic acid) or as the amide asparagine. Sucrose provided to the nodules is not only a source of C for growth, but provides oxidizable substrates needed for plant and bacteroid respiration. In addition, sucrose provides the C skeletons required in the synthesis of asparagine or ureides. Evidence indicates that C4 acids, principally malate, are synthesized in the cytosol of the infected cell and transported across the plant symbiosome membrane and the bacterial plasma membrane where they are metabolized by the bacterial TCA cycle. PEPc is a key enzyme in the infected cell, together with malate dehydrogenase, it generates the malate for the bacteria, and in nodules that export asparagine, it provides the 4-C skeleton for asparagine synthesis (Layzell *et al.,* 1990).

Pi deficiency has been shown to stimulate the activity of PEPc in leaves and nonphotosynthetic PEPc in legume roots. In addition to supplying anaplerotic C to replenish TCA-cycle intermediates, elevated PEPc caused by Pi limitation may be a response to increased demands for pyruvate and/or a greater need for Pi recycling. PEPc in the roots of Pi-deficient plants provides as much as 25% of the C for citrate and

34% of the C for malate for exudation (Johnson *et al.,* 1996; Vance and Stade 1984). PEPc clearly plays a key role in amino acid biosynthesis, this is especially true in nodules of amide-exporting plants (McClure *et al.,* 1983).

Although sinks and sources for C and N respond in concert to increased supply of photosynthetic products, there is evidence that N accumulation lags C accumulation as photosynthesis increases. It was found that the quantity of N fixed by clover could be greatly increased, if the photosynthetic activities of the plant were accelerated (Wilson *et al.*, 1933).

Although quantitative estimates of the spatial and temporal relationships between C and N economy are undoubtedly more informative, indirect evidence - based on manipulations of the plant or its environment - provides some insight into the competitive restraints under which the nodules must function, and of the potential of various symbioses when relieved of these restraints. Thus, manipulations - which impair photosynthesis (e.g. defoliation or decreased light intensity) - invariably decrease the rate or duration of N fixation, whilst those which promote photosynthesis (e.g. supplementary light) typically lead to increased fixation activity. The short-term effect of altering C supply is on fixation efficiency (the rate of N fixation per unit nodule dry weight), whilst long term changes in C supply cause a decrease in nodule mass per plant, in the rate of nodule growth, in the onset of senescence and in the rate of degeneration of the nodule population. However, such 'manipulation' studies are complicated by endogenous control mechanisms, which allow the magnitude of N fixation to reflect, if not always meet, the demands of the host plant. Whether or not roots and nodules can benefit directly from CO_2 enrichment of the soil is debatable. The

relative magnitude of the effect of CO_2 enrichment depends on when and for how long the supplementary CO_2 is provided, on where the plants are grown and on the fixation activity of control plants as determined by available soil N concentration. Nevertheless, the available evidence suggests that CO_2 enrichment of grain legume shoots increases overall plant growth, which allows vegetative plants to produce larger populations of nodules (Minchin *et al.,* 1981). These larger nodule populations senesce later into the reproductive period, or are replenished by continued initiation and growth of new nodules (Minchin *et al.,* 1981).

N fixation requires a steady supply of photosynthates, however there are also other endogenous mechanisms that can regulate the rate of fixation. An analysis of the data from atmospheric CO_2 enrichment studies suggests that the resultant improvement in fixation rate is often due to increases in overall plant growth rather than an immediate increase in nodule efficiency. C and N budgets for nodulated roots suggest that 40-50% of total photosynthate is consumed by below-ground organs during vegetative growth and 63-64% of this is used in respiration, 16-22% is used in growth and 14-20% in the production and export of organic nitrogenous products of fixation (Minchin *et al.,* 1981; Gordon *et al.,* 1997).

The nodule and its subtending root system represent one of the strongest sinks, receiving an estimated 15-30% of the net photosynthate of the plant. This supply of photosynthate transported via the phloem is used for energy-yielding substrates and carbon skeletons to support (a) the growth and maintenance of the nodule tissue; (b) the energy-consuming reactions associated with the reduction of N_2 in the endophyte and

the assimilation of the NH_4 produced in the host cytosol; and (c) the synthesis of N-containing organic compounds for export from the nodule (Schubert 1986).

Inorganic phosphate (P_i) is known to regulate bio-energetic processes in plants by being one of the substrates for photo- and oxidative phosphorylation. Lack of P_i has been found to decrease the levels of ATP and ADP, as well as the adenylate energy charge in leaves and roots. In some plants, P_i limitation has been found to decrease photosynthetic activity. During prolonged Pi limitation, a decreased activity of the cytochrome pathway and an increased participation of the cyanide-resistant pathway were observed. The activity of the alternative, non-phosphorylating pathway allows the functioning of the Krebs cycle and operation of mitochondrial electron transfer chain with limited ATP production and thereby may contribute to the survival of P_i-deficient plants (Juszczuk *et al.,* 1997; Mikulska *et al.,* 1998).

Furthermore, because Pi is necessary for nucleotide synthesis, severe Pi deficiency can result in decreased total RNA biosynthesis. In addition, Pi deficiency has been found to induce a specific RNase in some plants. This RNase functions to liberate Pi from RNA, facilitating its remobilization. A decrease in total RNA concentration due to Pi deficiency may be the result of decreased biosynthesis, increased degradation or a combination of both (Johnson *et al.,* 1996). Johnson *et al.,* (1996) also found that although the concentration of total RNA was reduced by Pi deficiency, roots continued to have enhanced expression of PEPc mRNA, which suggests preferential synthesis and/or stability of PEPc mRNA. In addition, they found that increased PEPc specific activity was related directly to an increase in PEPc mRNA and PEPc enzyme, providing

strong evidence that this non-photosynthetic PEPc is in part under transcriptional regulation.

Increased PEPc activity in plants has been associated with increases in internal cellular pH or a high demand for C skeletons, such as during amino acid biosynthesis, N assimilation, and exudation of organic acids. It has been suggested that changes in cytosolic pH may modulate PEPc activity by directly or indirectly regulating its phosphorylation status. The phosphorylation status of PEPc in soybean root nodules appears to be modulated by photosynthate transported from shoots. In legumes, root nodule C_3 non-photosynthetic PEPc is also regulated by transcriptional and translational events, as well as by phosphorylation. Phosphorylation of root nodule PEPc reduces the sensitivity of the enzyme to malate inhibition. This is a fundamental characteristic of an enzyme that functions in an environment in which malate synthesis is high, such as in root nodules. Enhanced synthesis of organic acids is dependent on continued high PEPc activity (Johnson *et al.* 1996).

1.5.7 Effect of Soil N on nodulation and N fixation

The presence of N in the soil and the form in which the soil N occurs can influence the formation and activity of root nodules. Small amounts of soil N can have a positive effect on the formation and development of nodules by ensuring a positive growth response in the plant and its roots (Patriarca *et al.,* 2002). However, the continued presence of soil N, primarily NO_3 is known to decrease both nodulation and rhizobial nitrate reductase activity. Many studies have shown the negative effect of NO_3 on the nodulation process, the presence of NO_3 in the soil was found to decrease the number of nodules produced and led to a decrease in the efficiency of N fixation

(Champigny *et al.,* 1985; Minchin *et al.,* 1986; Giannakis *et al.,* 1988; Alcantar-Gonzales *et al.,* 1988; Serrano and Chamber, 1990; Arrese- Igor *et al.,* 1997; Chamber-Perez *et al.,* 1997;). The effect of NH_4 on the N fixation process is less clear, although it appears that NH_4 does not have much of a negative influence on N fixation, most likely due to it not inhibiting nitrate reductase as NO_3 does. Thus the formation and function of nodules is regulated on many levels and influenced by a number of factors, including the presence, concentration and form of soil N.

1.6 C flux modeling/equations

1.6.1 AM efficiency and C-flux modeling

The transfer of fixed C from the host to the symbiont has a direct effect on the host plant and thus it would be of importance to be able to quantify this process. Koide and Elliott (1989) described this relationship mathematically using various models. They developed models describing both the gross benefit of the mycorrhizal colonization and the net benefit of colonization.

Gross benefit was defined as the difference between the quantity of gross C assimilation (mole C) in mycorrhizal and non-mycorrhizal plants over a given period of time (Koide and Elliott 1989):

$\Delta A^g_m - \Delta A^g_{nm}$

Where ΔA^g_m and ΔA^g_{nm} is the gross C assimilation of the mycorrhizal and non-mycorrhizal plants during that time interval respectively.

The net benefit of colonization for the same time period was described as the difference between mycorrhizal and non-mycorrhizal C accumulation (moles C) in the whole plant over the given time period (Koide and Elliott 1989):

$\Delta C^{w}_{m} - \Delta C^{w}_{nm}$

Where ΔC^{w}_{m} and ΔC^{w}_{nm} represent the amount of C accumulated in the mycorrhizal and non-mycorrhizal plants over the given time period.

Koide and Elliott (1989) also described the efficiency of the relationship in terms of P acquisition, P utilization and below ground C utilization. The efficiency of the P acquisition was defined as:

$$\frac{\Delta P^{w}}{\Delta C^{b}}$$

Where ΔP^{w} is the total P that has accumulated in the plant during the given time interval and ΔC^{b} is the total below ground C expenditure over the same time period (Koide and Elliott 1989). This describes the efficiency of the relationship in terms of the amount of P taken up compared to the amount of C used for the uptake of P. C^{b} can be calculated as follows (Koide and Elliott 1989):

$C^{b} = C^{r} + C^{o} + C^{n}$

Where C^{r} is the C that is allocated to the root tissue, C^{o} is the C lost via root below ground respiration and C^{n} is the non-respiratory, below ground C loss.

The efficiency of P utilization was defined by the following equation (Koide and Elliott 1989):

$$\frac{\Delta C^{w}}{\Delta P^{w}}$$

Where ΔC^w is the total amount of C accumulated, in the whole plant, over the same period (Koide and Elliott 1989). This efficiency can be applied to any of the respective plant components.

The final model proposed by Koide and Elliott (1989) was used to define the efficiency of below ground C utilization and was expressed as the ratio ΔC^w: ΔC^b. This ratio is the product of the previous two models:

$$\frac{\Delta C^w}{\Delta C^b} = \frac{\Delta C^w}{\Delta P^w} \times \frac{\Delta P^w}{\Delta C^b}$$

Koide and Elliott (1989) defined C^b (see above) as the total below ground C expenditure, which included all the C in the living tissue of the root system and the C lost from the root, via exudation, leaching, respiration, cell death and direct transport to the fungus. Jones *et al.,* (1991) went one step further and formulated two models that defined C^b in terms of the factors influencing the changes in C^b. The first model expressed C^b as a function of the C fixed via photosynthesis:

$$C_{b(Pn)} = Pn \frac{\%C_{BG}}{100 - \%C_{SR}} t$$

Where $C_{b(Pn)}$ is the amount of photosynthetically fixed C that is allocated below ground in a given period of time. Pn is the net photosynthetic rate as mmol C s^{-1} for the whole shoot system; $\%C_{BG}$ is the percentage of the total fixed C which is allocated below ground, over a given period of time; $\%C_{SR}$ is the percentage of the fixed C which was released via respiration in the shoot and *t* is the length of the daily light period, measured in seconds. The term 100-$\%C_{SR}$ represents the total amount of C left after respiration.

Their second model expressed C^b as a function of the change in shoot mass, which will give an indication of C fluxes within the shoot:

$$\Delta C_{b(Wt)} = \Delta W_s \frac{\%C_{BG}}{\%C_{ST}}$$

Where ΔW_s is the mean increase in shoot weight over a given time period and $\%C_{ST}$ and $\%C_{BG}$ are the mean percentages of the C fixed and allocated to the shoot tissue and to the below ground components respectively.

The work of Koide and Elliott (1989) forms the backbone of mycorrhizal efficiency modeling, but they never tested their models experimentally. Therefore they have not defined the influencing factors that affected each of the parameters involved in the different models. The models proposed by Jones *et al.*, (1991) elaborated on those of Koide and Elliott (1989) by defining C^b as a function of the influencing factors, not just its components.

However, the expression of C^b in terms of photosynthetically fixed C can be misleading. It assumes that photosynthetic C is the only source of C available to the plant. It does not include structural and non-structural C that is already stored in the plant, which may be used and transported below ground, or anywhere else in the plant for that matter. Similarly the expression of C^b in terms of the changes shoot mass assumes that the shoots are the only structures that will have an influence on below ground C, again ignoring other, pre-existing sources of C within the plant. This also neglects to take into account that VAM and non-VAM plants may allocate

photosynthetic C in different proportions to different organs (Smith 1980; Koide 1985).

1.6.2 Tissue construction cost and below ground respiration

Williams *et al.,* (1987) proposed a model that can be used to determine the construction cost of various tissues within a plant. They defined construction cost as the amount of glucose required to provide C skeletons, reductant and ATP for synthesizing the organic compounds in a tissue via standard biochemical pathways. They calculated tissue construction cost as:

$$C_w = \{(0.06968 \times \Delta H_c - 0.065)(1 - A) + \frac{kN}{14.0067} \times \frac{180.15}{24}\} \frac{1}{E_G}$$

Where C_w is the construction cost of the tissue (g glucose gDW^{-1}) and ΔH_c is the ash-free heat of combustion of the sample (kJ g^{-1}). *A* is the ash content of the sample (g ash gDW^{-1}); k is the reduction state of the N substrate (NO_3 was used, therefore k is +5) and E_g is the deviation of growth efficiency from 100%. E_G represents the fraction of the construction cost that provides reductant that is not incorporated into biomass. Williams *et al.*, (1987) determined the value of E_g to be 0.89.

Peng *et al.,* (1993) slightly modified this equation and converted the g glucose into mmol C:

$$C_w = \{(0.06968 \times \Delta H_c - 0.065)(1 - A) + \frac{kN}{14.0067} \times \frac{180.15}{24}\} \frac{1}{0.89} \times \frac{6000}{180}$$

The units of construction cost are now mmol C gDW^{-1}. However, the tissue construction cost equation was further modified by Mortimer *et al.,* (2005):

$Cw = [C + kN/14 \times 180/24] (1/0.89)(6000/180)$

Where Cw is the construction cost of the tissue (mmolC/gDW), C is the carbon concentration (mmolC/g), *k* is the reduction state of the nitrogen substrate and N is the organic nitrogen content of the tissue (g/g DW) (Williams *et al.,* 1987). The constant *(1/0.89)* represents the fraction of the construction cost which provides reductant that is not incorporated into biomass (Williams *et al.,* 1987, Peng *et al.,* 1993) and *(6000/180)* converts units of g glucose/g DW to mmolC/g DW.

Peng *et al.,* (1993) use the construction cost to determine the growth respiration, which was defined as the respired C associated with the biosynthesis of new tissue:

$R_{G(t)}=C_t-\Delta W_c$

Where $R_{G(t)}$ is the growth respiration (μmol CO_2 d^{-1}) ; C_t (μmol CO_2 d^{-1}) is the C required for daily construction of new tissue. C_t was calculated by multiplying the root growth rate (ΔW_w, mgDW d^{-1}) by tissue construction cost (C_w). ΔW_c (μmol d^{-1}) is the change in root C content and was calculated by multiplying the root C content and the root growth rate (ΔW_w, mgDW d^{-1}).

1.7 Conclusion

The length of this review chapter gives testament to the fact that this is a field of study that has received much attention. The reason for this is most likely due to the importance of legumes in modern day agriculture, primarily in nutrient poor soils, and the role that the respective symbionts play in improving the growth and production of

these crop plants. The synergistic effects of dual inoculation have been well documented, although there are some areas that remain to be elucidated. There has been a shortage of research into the individual contributions of the two symbionts within the dual symbiosis and the subsequent effect on the plant physiology in terms of respiration and photosynthesis. It also remains to be seen whether or not the dual symbiosis is always beneficial to the host plant or only under certain growing conditions. This can be determined by evaluating the costs and benefits of the respective symbionts in legumes grown under both nutrient limiting conditions and favourable conditions. Once the specific contributions and roles of the two symbionts are known it can also be determined if one symbiont is dominant over the other in terms of sink strength. The allocation of host resources, especially during the early stages of both host and symbiont development, will offer competitive advantages in favour of the recipient. I trust that this thesis further aids in the understanding of these topics and helps to fill in some of the gaps regarding the tripartite symbiosis between the legumes nodules and mycorrhizae.

Chapter 2

General Introduction

Chapter 2

General introduction

2.1 Introduction

The legumes ability to form tripartite associations with arbuscular mycorrhizal fungi (AMF) and rhizobia gives them access to sources of P and N that would normally not be available to the plant. Rhizobia are able to fix atmospheric N and convert it into an organic form that is subsequently made available to the host (Lodwig and Poole 2003). Whilst the arbuscular mycorrhizal (AM) symbiosis is not only more efficient in the uptake of P from the soil it can also access pockets of soil P that would ordinarily not be available to the host. This allows for improved plant growth, especially in nutrient poor soils. The ability to form these associations makes legumes valuable as a crop plant as it provides both the legumes and the subsequent crops with a renewable source of N and the ability to grow in low P soils (Frey and Schuepp 1992; Udvardi *et al.,* 2005).

However, these nutrients provided to the host plant come at a cost, in exchange the host plant supplies the two symbionts with photosynthetically derived sugars (Vessey and Layzell 1987; Smith and Read 1997; Vance 2002). In spite of the C costs of maintaining a dual symbiosis, the cumulative benefits of dual inoculation are greater than those of singular inoculation to both the host plant and to the respective symbionts (Daft and Elgiahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989). However the legume is able to balance these costs by increasing its photosynthetic rate, thus producing more sugars for the growth and maintenance of both itself and the two symbionts.

2.2 Symbiotic P and N nutrition

One of the main functions of AM is the provision of soil P to the host plant, therefore under low soil P conditions the dependency of the host on the AMF increases (Smith and Read 1997). Fredeen and Terry (1988) found that AM colonized Legumes growing under low soil P had higher shoot P as well as greater shoot and nodule dry weights. This indicates the role that AM play in both the P nutrition and the growth of the host as well as the nodules. The improvement in host growth is attributed to an increase in the production of photosynthate by the host (Fredeen and Terry 1988; Jia *et al.,* 2004).

Rhizobia, found in the root nodules, play a crucial role in the N nutrition of their legume hosts. The bacteria are able to provide the host with organic N, which is derived from atmospheric N_2. Atmospheric N is fixed into an organic form, with the aid of the enzyme nitrogenase, which is consequently exchanged with the host for C (Thorneley 1992; Lodwig and Poole 2003). Nodules are strong P sinks and the process of N-fixation is energy intensive, resulting in the nodules requiring more energy and P than the host roots (Sa and Israel 1991; Al-Niemi *et al.,* 1998; Almeida *et al.,* 2000). Vadez *et al.,* (1997) reported that nodules had a 3-fold greater concentration of P than other plant tissues, which gives an indication of the nodular sink strength for P. It has also been reported that a deficiency of P can lead to a reduction in both nodulation and symbiotic N fixation (Othman *et al.,* 1991; Drevon and Hartwig 1997). Alternatively, P availability has been found to increase the ratio of nodule:total plant mass, nodule mass appears to be more influenced by the availability of P than nodule number (Othman *et al.,* 1991, Almeida *et al.,* 2000). This

is confirmed by the work of Olivera *et al.,* (2004), who reported that an increase in the P supplied to host plants led to a 4-fold increase in nodule mass. This dependency on P by the nodule bacteria will also create a strengthened dependency on AMF by the host in order to supply the high amounts of P required by the rhizobia.

Although legumes rely on the N contribution of the rhizobia for growth and development, the plant can access other sources of N. Numerous studies have shown that AM play an important role in the uptake of N and the subsequent supply of N to the host plant (Marschner and Dell 1994; Constable *et al.,* 2001; Toussaint *et al.,* 2004; Govindarajulu *et al.,* 2005). AM have also been found to have an indirect effect on the N nutrition of legumes. Mycorrhizal legumes, including *Phaseolus vulgaris*, have been reported to have a greater number of nodules, increased nodular weight and improved N-fixation rates, thereby enhancing the N nutrition of the host (Carling *et al.,* 1978; Kawai and Yamamoto 1986; Luis and Lim 1988, Vejsadova *et al.,* 1993; Goss and Varennes 2002; Mortimer *et al.,* 2008).

2.3 C-costs of the dual symbiosis

In exchange for the nutrients provided the two symbionts require C from the host. Thus, both the AMF and the rhizobial bacteria act as C sinks, competing for the same source of host C (Harris *et al.,* 1985). The combined drain of host photosynthate can be substantial, with the AMF receiving between 10 and 23% of host photosynthate (Snellgrove *et al.,* 1982; Koch and Johnson 1984; Kucey and Paul 1982a; Jakobsen and Rosendahl 1990) and the nodule between 6 and 30% (Kucey and Paul 1981; Kucey and Paul 1982b; Harris *et al.,* 1985; Provorov and Tikhonovich 2003). Photosynthate availability has been shown to influence both symbiotic N fixation as

well as nodule number, thus, factors influencing photosynthesis will influence N fixation (Bethlenfalvay and Phillips 1977; Murphy 1986; Atkins *et al.,* 1989; Sussanna and Hartwig 1996; Schortemeyer *et al.,* 1999). Therefore, due to the demand for plant derived carbohydrates, when soil N and P are not limiting factors for host growth, a growth depression is often observed in the host as well as a drop in the nodular dry weight and a decrease in percentage AM colonization (Fredeen and Terry 1988; Peng 1993).

2.4 Synergistic effect of dual inoculation

Despite this C drain imposed by the symbionts, both the rhizobia and the AMF can improve the nutrition and growth of their host plants. The positive effects are most clear under nutrient limiting conditions, especially low soil P, which can limit the P intensive N fixation process. It is well established that the combined inoculation of both rhizobia and AMF enhances plant growth to a greater extent than singular inoculation, as well as enhancing the degree of colonization by the respective symbionts (Daft and Elgiahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989). It was reported by Nwoko and Sanginga (1999) that the percentage AM colonization of host plants increased 57% due to inoculation with *Bradyrhizobium* and dual inoculation resulted in an increase in nodular weight. Toro *et al.,* (1998) found that AM colonized lucerne plants had higher rates of N fixation than non-AM plants and it was reported by Jia *et al.,* (2004) that the synergistic or additive effects of dual inoculation with both AM and rhizobia led to increased photosynthetic rates as well as greater plant productivity.

The host plant compensates for the C drain of the two symbionts by increasing photosynthate production. This is achieved by an increase in leaf P due to the AM symbiosis as well as by an increase in not only the rate of photosynthesis but also the specific leaf area and the rate of leaf expansion of the host plants (Harris *et al.,* 1985; Fredeen and Terry 1987; Jia *et al.,* 2004). Harris *et al.,* (1985) found that nodulated AM soybeans had a 47% increase in CO_2 fixation, resulting from improved leaf P, starch mobilization and an increase in the specific leaf area of the host. Similarly, Jia *et al.,* (2004) have shown that plants with rhizobia and AMF have higher photosynthetic rates per unit leaf area. Alternatively, the results of work done by Fredeen and Terry (1988) indicate that the improved photosynthate production was a result of an increase in the rate of expansion of the leaf surface and not due to an increase in the rate of photosynthesis or an increase in photosynthesis on a leaf area basis. Thus it is clear that the plant is able to compensate for the photosynthate demand of the two symbionts using a number of different mechanisms, possibly varying according to plant species and growing conditions.

2.5 Conclusion

In summation, we know that when exposed to nutrient stressed conditions, legumes are more reliant on AM and nodule bacteria and that these symbionts provide much needed nutrients for the plant. However, the benefits provided by the symbionts come at a cost to the host in the form of C, primarily derived from photosynthesis. The benefits of dual inoculation with AM and nodule bacteria has an enhanced cumulative effect on host nutrition and growth, despite the high costs of maintaining two symbionts. Thus both the AM and nodules rely on the same source of C and will therefore compete with one another for this C.

In spite of all the research done on this tripartite symbiosis there are important areas of this complex relationship that remain unknown or speculative at best. In order to fully understand the dynamics between these three organisms a number of questions will need to be answered. Most of the work on the tripartite symbiosis has focused on the cumulative effects of both the AM and nodules on the host and neglected to determine the relative contributions of the symbionts to both the host and each other. In addition to the specific contributions of each symbiont it is also important to know the specific costs maintaining the symbionts. Thus allowing for an accurate cost-benefit analysis to be done on each symbiont as well as the cumulative costs of the dual symbiosis. Furthermore it has yet to be determined which symbiont is most beneficial to the host and if this changes under different conditions. This will clarify if the specific roles of the symbionts overlap and if one symbiont is dominant over the other. This knowledge could even go as far as to show whether or not any of the symbionts tend towards the parasitic end of the spectrum, having a negative effect on the host growth. For example, under conditions of high P, the plants dependence on AM is diminished, therefore the AM can have a negative effect on host growth, draining C that could have been utilized in both the N-fixation process as well as for host growth. Similarly under conditions where N is freely available in the soil the host may not be so reliant on the high energy process of N fixation, especially if the AMF can also help provide N to the host.

2.6 Aim: Respiratory and photosynthetic C costs of tripartite legume N-nutrition under P deficiency

Thus it is clear that there are definite gaps in our knowledge regarding the intricate relationship between the host legume, the AM and the nodules. The aims of this thesis will be to fill in these gaps by determining the photosynthetic and respiratory costs of symbiont growth and nutrition, the relative contribution of each symbiont to host nutrition and determine mycorrhizal and nodular dependency of the host under conditions of N and P stress as well as N and P abundance. This will give us further insight into how these organisms co-exist and the roles each play in aiding each others ability to grow and function optimally in an ever-changing environment.

Chapter 3

The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris* (L.)

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Chapter 3

The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris* (L.)

3.1 Summary

In the tripartite symbiosis between nodulated legume roots and arbuscular mycorrhizal fungi (AMF), symbiont sink strength may depend upon developmental stage and the nutrient benefits to the host plant. The cost-benefits of the tripartite symbiosis were investigated in terms of C-economy and nutrition. Nodulated *Phaseolus vulgaris* seedlings with and without arbuscular mycorrhizae (AM), were hydroponically grown under high (2 mM) and low (1μM) P conditions in an N-free Long Ashton nutrient solution. Plants were sequentially harvested at 17, 24 and 31 days after emergence. At each harvest, measurements for biomass, N_2 fixation, photosynthesis, root respiration, calculated C and nutritional economy, were taken. Nodular growth was suppressed by the early development of AM colonization. This coincided with higher photosynthetic and respiratory rates in AM plants. These effects were most pronounced under low P when AM colonization peaked. Once AM levels reached the plateau phase, the efficiency of P nutrition increased. This was followed by improved nodular and host growth and enhanced N_2 fixation. This indicates that the AM was the dominant symbiont for host C in the tripartite symbiosis, due to its rapid development and subsequent role in supplying P more effectively to both host and nodules.

Key words: Tripartite; legume; arbuscular mycorrhiza; C-sink; phosphate; N_2 fixation

3.2 Introduction

The availability of soil phosphate (P) is the most limiting factor in legume growth and biological N-fixation (Toro *et al.,* 1998; Vance 2001). Low P soils will limit legume growth to a greater extent than low N soils due to the fact that the legume can utilize both atmospheric N and soil N. One mechanism of overcoming this limitation is the formation of mycorrhizae, a symbiotic relationship between the legume and an arbuscular mycorrhizal fungus (AMF) (Barea *et al.,* 1989; Barea *et al.,* 1992; Barea *et al.,* 2005a, b). It is well established that arbuscular mycorrhizae (AM) are able to benefit host nutrition and subsequently their growth, primarily through the enhanced uptake of P (Jakobsen *et al.,* 1994; Smith and Read 1997). In addition to the P requirements of the host legume, nodules require relatively high amounts of P, needing more P and energy than the plant roots (Sa and Israel 1991; Al-Niemi *et al.,* 1998; Almeida *et al.,* 2000), thus creating a further demand on host P and subsequently a stronger host reliance on AM.

Vadez *et al.,* (1997) reported that nodules require up to 3 times more P than the surrounding root tissue, indicating the high demand for P by the nodules. Thus a P deficiency can lead to a reduction in both nodulation and symbiotic N-fixation (Othman *et al.,* 1991, Drevon and Hartwig 1997). The dependency of the nodules on P is further confirmed by the work of Olivera *et al.,* (2004), who reported that an increase in the P supplied to host plants led to a 4-fold increase in nodule mass. Therefore the improved P nutrition resulting from AM colonization would result in improved nodulation and N-fixation, thus improving host performance.

It has been well documented that the synergistic effects of the combined application of rhizobia and AMF enhances plant growth to a greater extent than singular inoculation, as well as enhancing the degree of colonization by the respective symbionts (Daft and Elgiahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989). Fredeen and Terry (1988) found that AM colonized legumes growing under low soil P had higher shoot P as well as greater shoot and nodule dry weights. Furthermore, they also found that under high soil P conditions AM colonization had no significant effect on host growth and nodule weight was decreased. These findings indicate the effect of the AM sink on nodule development and the role of AM in the P nutrition of the host.

The dependence of the host plant on the symbionts is balanced by the costs, in terms of photosynthetically derived C, of maintaining these symbiotic interactions. The two symbionts act as C-sinks and compete for the same source of C from the host (Harris *et al.,* 1985). The combined drain of host C can be substantial, with the AMF receiving between 10 and 23% of host photosynthate (Snellgrove *et al.,* 1982; Koch and Johnson 1984; Kucey and Paul 1982; Jakobsen and Rosendahl 1990) and the nodule between 6 and 30% (Kucey and Paul 1981; Kucey and Paul 1982; Harris *et al.,* 1985; Provorov and Tikhonovich 2003). However not only does the relative C-sink strengths of the two symbionts need to be elucidated, the cumulative drain on host C by the two symbionts remains to be clarified.

Previous studies have had a singular approach in determining symbiont sink strength and C use, however this gives no indication as to which symbiont is the dominant sink and what the cumulative effect of the two symbionts is on host C economy. The sink

strengths of the respective symbionts might be reliant on their developmental stage or the interdependence of the symbionts on each other. Therefore, the aim of this paper is to investigate the relative sink strengths of each symbiont under various P-supply levels, in order to establish the interdependence between microbial symbionts as well as with their host.

3.3 Materials and Methods

3.3.1 Plant growth and AM inoculation

The seeds of *Phaseolus vulgaris* (var. contender), were inoculated with a commercially available, genus-specific rhizobial inoculum containing *Rhizobium leguminosarum* biovar phaseoli bacteria (StimuPlant cc, Zwavelpoort, 0036). The *Phaseolus vulgaris* seeds were coated with a paste of 2g of inoculum per 100 seeds. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in seedling trays containing vermiculite.

The AM treatments were inoculated with *Glomus etunicatum* (Becker and Gerdemann) (accession number J100092, Moss Herbarium, University of the Witwatersrand) and the control plants received a filtered inoculum solution, which was prepared by filtering the inoculum through a 37 µm mesh, which removed all fungal material. The AM treatment received 50g mycorrhizal inoculum per seedling tray (100 seeds). The inoculum was mixed with the vermiculite in the seedling tray just below the level which the seeds where planted at. Seedling trays were watered daily with the aid of timer controlled overhead sprayers. Seeds were germinated in an east-facing glasshouse at the Cape Peninsula University of Technology, Cape Town,

South Africa. The range of midday irradiances were between 580 and 620 μmol $m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 20/15 °C and 35/75%, respectively. Once the primary leaves were fully expanded, at 10 days after emergence (dae), the seedlings were transferred to 22-liter hydroponics tanks under the same glasshouse conditions. During transplanting cotyledons were removed to reduce reliance on the cotyledonary reserves. The tanks contained a N-free Long Ashton nutrient solution (Hewitt, 1966) modified for high (2mM) and low (1μM) P and pH maintained at 5.8. Solutions were changed every 7 days and the pH corrected daily. The base of the seedling's stems were wrapped with foam rubber and inserted through holes in the lids of the tanks, allowing the roots to hang freely in the solution below. Each tank was supplied with an air supply line, which bubbled ambient air.

3.3.2 Harvesting and nutrient analysis

Harvest intervals occurred at 17, 24 and 31 days after emergence (dae). Upon harvesting the plants (n=6) were separated into roots, nodules, stems and leaves. Sub-samples of root segments were stored in 50% ethanol in order to determine percentage AM fungal colonization at a later stage. The harvested material was then placed in a drying-oven, at 80 °C, for two days and dry weights were recorded. The dried plant material was milled using a 0.5 mm mesh (Arthur H Thomas, California, USA). The milled samples were analyzed for their respective C, N and P concentrations, by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyzer with suitable standards (BemLab, De Beers Rd, Somerset West, South Africa).

3.3.3 Determination of percentage AM colonization

Roots were cut into 1cm segments and rinsed and cleared with 20% KOH for 3 days at room temperature. The KOH was rinsed off and the segments acidified with 1% HCL overnight. Thereafter the roots were stained with 0.05% (w/v) aniline blue and left overnight. The roots were then destained in a 1% HCL/glycerol mix. Root segments were placed on slides and the colonization components were determined according to the method of Brundrett *et al.,* (1994).

3.3.4 Photosynthesis

The youngest fully expanded center leaflet, of the trifoliate leaves for each plant was used for the photosynthetic determinations. Light-response curves were used to determine the irradiance (1100 $\mu mol.m^{-2}.s^{-1}$) at which to conduct the photosynthetic rates. Readings were taken at midday, using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA England).

3.3.5 Root oxygen consumption

O_2 consumption of nodulated root segments (root segments containing 4-6 nodules) was measured with a Clark type polarographic oxygen electrode system (Hansatech Instruments, King's Lynn, England). Nodulated root segments were measured in 20 ml chambers at 20°C. Fresh hydroponic nutrient solution was used to suspend the nodulated root material.

3.3.6 Calculations of □ N15

The δ15N analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as δ = 1000‰

$[R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et al.,* (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5mm mesh (Arthur H Thomas, California, USA). Between 2.100mg and 2.200mg of each sample was weighed into 8mm by 5mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

The δ15N natural abundance of the legumes was corrected for the seed N, according to Boddey *et al.*, (1995):

δ15N enrichment (Seed corrected) = ((plant N X $\delta 15N_{plant}$) – (seed N X Ps X $\delta 15N_{seed}$)/ (plant N – seed N))

Where plant N and seed N represent the respective N concentrations of the plant and seed, δ15N plant and δ15N seed represent the respective δ15N values of the plant and seed and Ps is the proportion of the seed N that was assimilated by the legume.

The seed corrected δ15N values were used to determine the percentage N derived from the atmosphere (NDFA). NDFA was calculated according to Shearer and Kohl (1986):

$\%NDFA=100 * ((\delta 15N_{reference\ plant} - \delta 15N_{legume})/(\delta 15N_{reference\ plant} - B))$

Where *B* is the δ15N natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N-free culture, according to Shearer and Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76 ‰.

3.3.7 Carbon and nutrient cost calculations

Construction costs ($mmolC.g^{-1}$ dw) were calculated according to Mortimer *et al.*, (2005), modified from the equation used by Peng *et al.*, (1993):

$Cw = [C + kN/14 \times 180/24] (1/0.89)(6000/180)$

Where *Cw* is the construction cost of the tissue (mmolC/gDW), *C* is the carbon concentration (mmolC/g), *k* is the reduction state of the N substrate (k=-3 for NH_3) and *N* is the organic nitrogen content of the tissue (g/g DW) (Williams *et al.*, 1987). The constant *(1/0.89)* represents the fraction of the construction cost which provides reductant that is not incorporated into biomass (Williams *et al.*, 1987, Peng *et al.*, 1993) and *(6000/180)* converts units of g glucose/g DW to mmolC/g DW.

Specific P absorption rate (SPAR) ($mgP.g^{-1}$ root $dw.d^{-1}$) is the calculation of the net P absorption rate per unit root dw (Nielson *et al.*, 2001):

$SPAR = (M_2 - M_1)/(t_2 - t_1) \times (\log_e R_2 - \log_e R_1) / (R_2 - R_1)$

Where M is the P content per plant and R is the root DW.

Specific P utilization rate (SPUR) ($g\ dw.mg^{-1}P.d^{-1}$) is a measure of the dw gained for the P taken up by the plant (Nielson *et al.*, 2001):

$SPUR = (W_2 - W_1) / (t_2 - t_1) \times (\log_e M_2 - \log_e M_1) / (M_2 - M_1)$

Where M is the P content of the plant and W is the plant dw.

The Specific Nitrogen utilization rate (SNUR) was adapted from the above equations to include N instead of P.

Growth respiration Rg(t) (μmol $CO_2.d^{-1}$) is the daily growth respiration for the plant (Peng *et al.*, 1993):

$Rg(t) = Ct - \Delta Wc$

Ct (μmol CO_2 day^{-1}) is the C required for daily construction of new tissue. Ct was calculated by multiplying the root growth rate (gDW day^{-1}) by tissue construction cost (Cw). ΔWc (μmol C day^{-1}) is the change in root C content and was calculated by multiplying the root C content and the root growth rate.

3.3.8 Statistical analysis

The percentage data were arcsine transformed (Zar, 1999). The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (SuperAnova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the ANOVA revealed significant differences between treatments the means (n=6) were separated using a *post hoc* Student Newman Kuehls (SNK), multiple range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

3.4 Results

3.4.1 Biomass

The non-inoculated plants remained non-mycorrhizal for the duration of the experiment. The percentage colonization of the AM plants peaked (97%) at 17 days after emergence (dae), followed by a decline in the level of colonization (Fig 1). The plants exposed to low P maintained greater levels of AM colonization (Fig 1). The decline in AM colonization coincided with the increase in the dry weights of the AM plants relative to the non-AM plants especially under low P (Table 1).

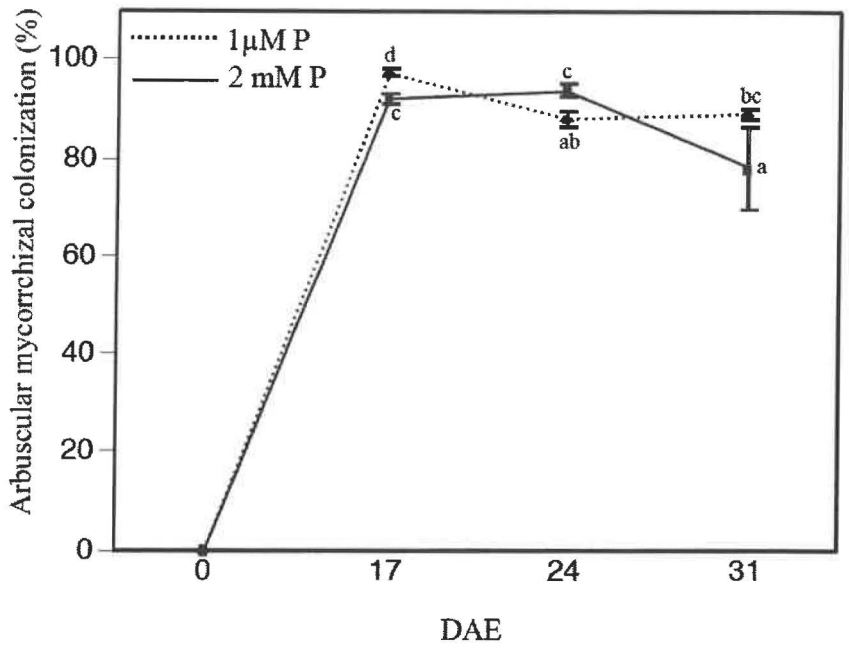

Figure 1. Arbuscular mycorrhizal colonization (%) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence (dae). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

At 17 dae there were no differences in the shoot and total plant dry weights between treatments however, from 24 dae the AM plants had greater dry weights (Table 1). The greater plant dry weights resulted from the greater shoot mass for the AM plants because the root dry weights of the AM plants were consistently lower for the duration of the experiment (Table 1). The non-AM plants had greater root:shoot ratios than the double symbiotic plants (Table 1).

Table 1. Biomass and nutrient concentration of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

Parameters	Low Phosphate						High Phosphate					
	17 Days		24 Days		31 Days		17 Days		24 Days		31 Days	
Dry weights	+AM	-AM	+AM	-AM	+AM	-AM	+AM	-AM	+AM	-AM	+AM	-AM
Plant (g)	0.57bcde[f]	0.57bcde[f]	0.60de	0.45a	0.91[j]	0.81[i]	0.66[f]	0.61ef	0.52bc	0.57cde	0.79[h]	0.75gh[i]
Roots (g)	0.10bc	0.12d	0.11c	0.13eg	0.17h	0.21[i]	0.15gh	0.15gh	0.09ab	0.15fg	0.13deg	0.17h
Shoot (g)	0.46c	0.46c	0.49c	0.30a	0.73g	0.64f	0.48c	0.46c	0.38b	0.49c	0.53e	0.60d
Root:Shoot	0.21a	0.26c	0.22b	0.37g	0.21ab	0.31e	0.32ef	0.27d	0.32ef	0.21ab	0.32ef	0.27d
Plant Nutrients												
P (mmol.g^{-1} dw)	0.17c	0.18c	0.14b	0.15b	0.11a	0.11a	0.54e	0.60f	0.47d	0.48d	0.46d	0.47d
N (mmol.g^{-1} dw)	2.66k	2.60jk	2.11g	1.91ef	1.86de	1.77c	2.42h	2.51ij	1.96g	2.08g	1.47b	1.41a

Nodules were first visible at 17 dae and the AM plants had greater nodule dry weights (Fig 2). By the end of the experiment the AM plants had a 7.2 fold increase in total nodule dry weight (Fig 2).

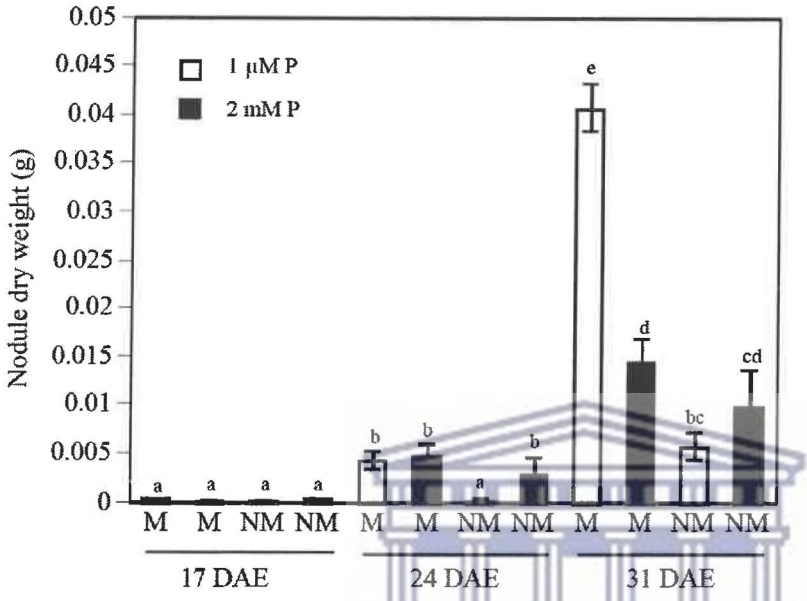

Figure 2. Nodule dry weight of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (M) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (NM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence (dae). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

3.4.2 Nutrition

The N concentration (mmolN. g^{-1} dw) of the P stressed AM plants followed a similar pattern of development as the nodules, initially there were no differences in plant N between treatments, but from 24 dae the low P AM plants had greater total N concentrations (Table 1). This concurs with the higher % NDFA values, which are indicative of increased biological N_2 fixation (Fig 3).

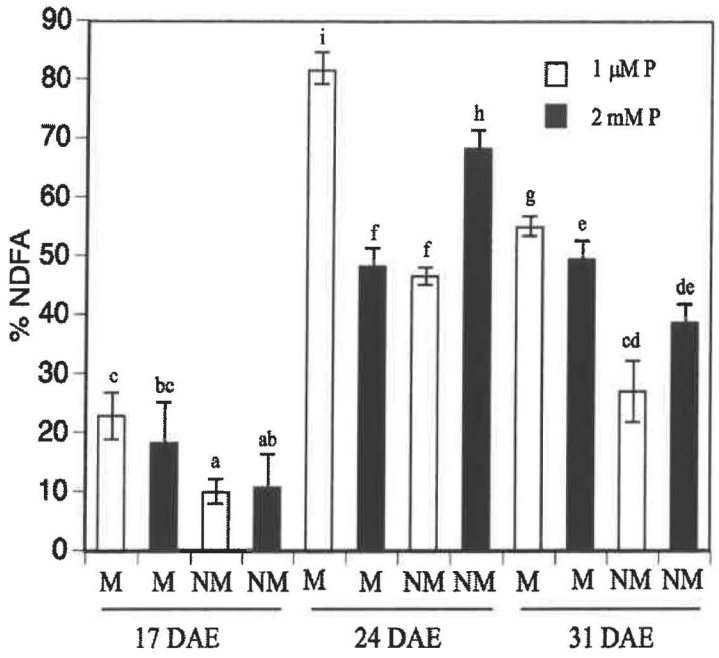

Figure 3. Percentage nitrogen derived from atmosphere (% NDFA) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (M) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (NM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence (DAE). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

A positive relationship was found between the plant N concentration and mycorrhizal development, as evidenced by the correlation between Plant N and percentage AM colonization (R^2=76) (Fig 4). In addition to the greater total N in the P stressed AM plants, a higher N utilization rate was also maintained (Fig 5a). In spite of the lack of differences in the plant P concentrations between treatments the AM plants under low P had greater P absorption and utilization rates (Fig 5 b,c).

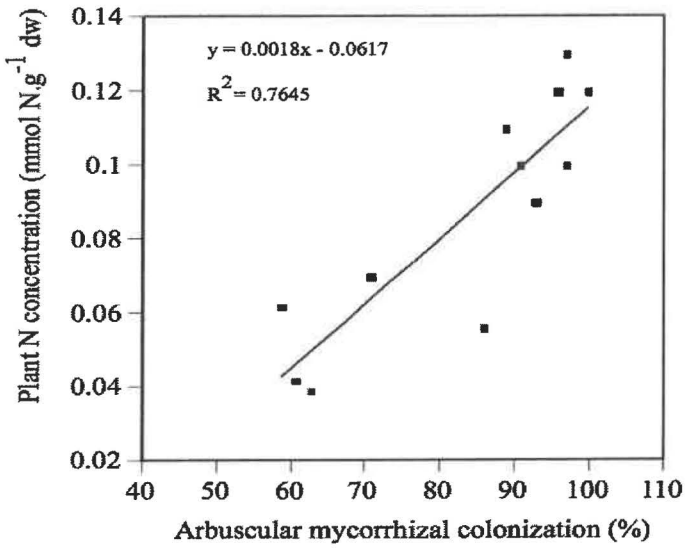

Figure 4. Correlation between the plant N concentration and arbuscular mycorrhizal colonization of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular myccorhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at either 1 µM P or 2 mM P.

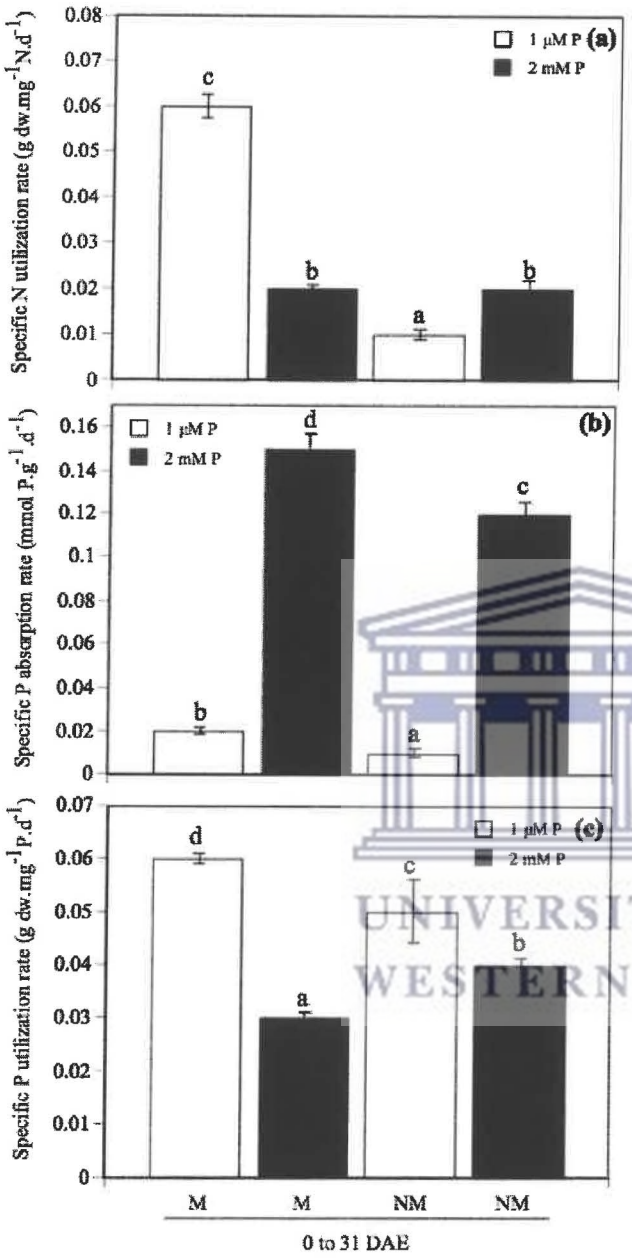

Figure 5. The **(a)** specific N utilization rates (SNUR), **(b)** specific P absorption rates (SPAR) and **(c)** specific P utilization rates (SPUR) of *Phaseolus vulgaris* (*L.*). Plants were either colonized (M) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (NM). Plants were grown at either 1 µM P or 2 mM P and harvested 31 days after emergence (dae). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

3.4.3 Construction costs and respiration

The sink effect was more pronounced in plants with the double symbiosis and to a greater extent under low P conditions. This is shown by the greater growth respiration of low P AM plants in comparison to the high P AM plants (Fig 7b). The sink effect of the AM colonization at low P was consistently evidenced by the higher photosynthetic rates of host plants (Fig 6) for the duration of the experiment.

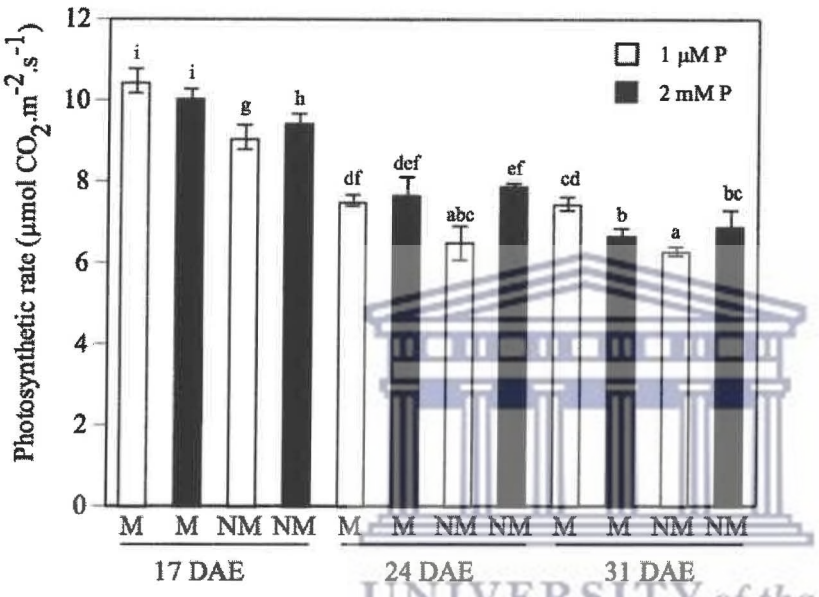

Figure 6. The photosynthetic rates of *Phaseolus vulgaris* (*L.*). Plants were either colonized (M) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (NM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence (DAE). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

Coinciding with this, were the higher construction costs (Fig 7a), enhanced root growth respiration (Fig 7b) and higher O_2 consumption rates (Fig 7c) of the AM plants throughout the growth periods. In the high P plants at 31 dae, there was a greater decline in the level of AM colonization than at low P (Fig 1). In spite of this decline in AM colonization, there were no differences in the photosynthetic rates (Fig 6) or in the O_2 consumption rates (Fig 7c) between AM and non-AM plants at high P.

However, under high P conditions, the AM plants had greater construction costs than non-AM plants (Fig 7a).

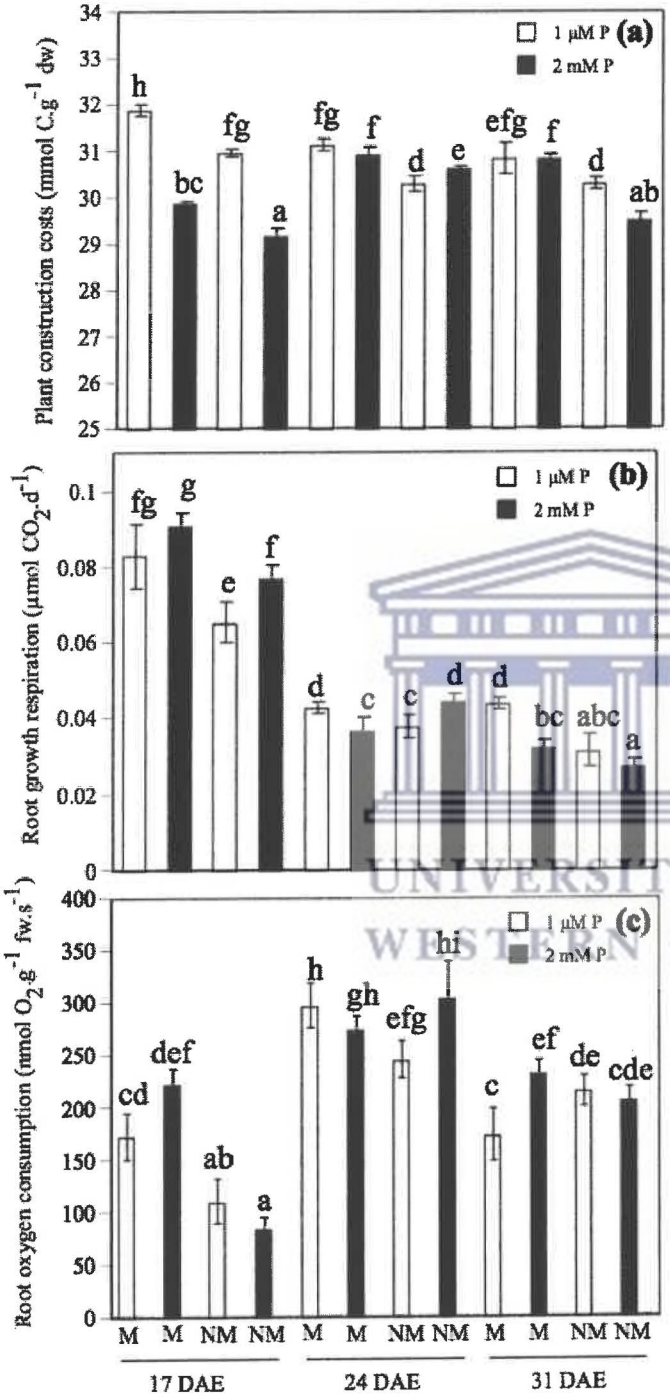

Figure 7. The **(a)** construction costs, **(b)** growth respiration and **(c)** O_2 consumption rates of *Phaseolus vulgaris* (*L.*). Plants were either colonized (M) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (NM). Plants were grown at either 1 µM P or 2 mM P and harvested at 17, 24 and 31 days after emergence (DAE). Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

3.5 Discussion

AM was the primary below-ground sink of host C resources in the dual symbiosis with root-nodules, which resulted in the delayed onset of nodular growth. This allowed for AM establishment and the subsequent enhancement of P nutrition, which benefited nodular and host development.

Following the peak at 17 dae, the decline in the percentage AM colonization (24 dae) coincided with the increase in nodule mass, these changes were most pronounced under low P conditions. This increase in nodule development was delayed due to host C being used to support the high levels of AM colonization, confirmed by the higher construction costs and growth respiration of these plants. Once AM colonization reached the plateau phase, more C was available for nodule growth (Harris *et al.,* 1985). Coupled with this argument is that nodules require relatively high amounts of P for normal growth and maintenance (Sa and Israel 1991; Al-Niemi *et al.,* 1998; Almeida *et al.,* 2000), thus under low P conditions the host and nodules would rely on AM-derived P during the AM plateau phase. Therefore, initial AM development up to 17 dae took preference over the formation of nodules, so that the plateau phase of AM was reached and the subsequent benefits of P nutrition accrued. This would concur with previous studies, where P nutrition was enhanced once the plateau phase of AM development was reached (Mortimer *et al.,* 2005).

In the current study, this AM induced response was most pronounced under the low P treatment primarily due to the improved P nutrition of AM roots, evidenced by the greater specific P absorption and utilization compared to non-mycorrhizal plants. These effects of P on host growth and nutrition are confirmed by previous studies

showing improved P nutrition and growth of AM plants (Sanders and Tinker 1971; Smith 1982; Bolan 1991; Orcutt and Nilsen 2000). Confirmation for the preference of AM development over nodular growth (0-17dae) is apparent in the difference between colonization levels at low and high P and the fungal effect on the dual symbiotic below ground sink strength. This sink effect is evidenced by the higher photosynthetic rates and root oxygen consumption rates of the double symbiotic roots at low P than at high P, for the period of 0-17 dae. This is consistent with the work of Valentine and Kleinert (2007) who found that mycorrhizal plants had increased respiration resulting from the higher levels of root colonization associated with low P conditions.

The synergistic effects were most pronounced under low P conditions due to the key role of AM in the tripartite symbiosis. These synergistic effects on nodule development resulted in greater biological N-fixation by the nodules, providing the host with more N than their non-AM counterparts. This is consistent with previous studies showing enhanced levels of colonization and nodulation resulting from the synergistic effect of dual inoculation, as well as greater nodular dry weights (Daft and Elgiahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989; Nwoko and Sanginga 1999). In addition, Catford *et al.,* (2003) found that the tripartite symbiotic partners may autoregulate the development of the various symbionts. The nutritional benefits of the double symbiosis that resulted in greater host growth under low P are in agreement with the work of Gavito *et al.,* (2000). Gavito *et al.,* (2000) proposed that the improved nutrition of the mycorrhizal legumes led to enhanced N-fixation, thereby resulting in the dual symbiotic plants having greater growth.

The cumulative sink effect imposes a considerable drain on host C reserves, as evidenced by the increased oxygen consumption and growth respiration of the dual symbiotic plants. The increased demand for host C by the two symbionts resulted in the host plants having higher photosynthetic rates, which concurs with the findings of Jia *et al.,* (2004) for *Vicia faba.* Although Jia *et al.,* (2004) attributed the higher photosynthetic rates to improved N and P nutrition, no evidence was presented for respiratory costs driving below ground sink stimulation of photosynthesis. In this regard, previous studies have found that colonization of host roots by AM led to increased levels of below ground respiration (Peng *et al.,* 1993; Valentine and Kleinert 2007). Therefore the addition of a further symbiont, such as nodule-producing Rhizobium, should lead to an even greater respiratory demand on the host. From the current study, further evidence for the greater C-consumption by the dual symbiosis is the higher construction costs of these plants. This means that the hosts require more C for every gram of tissue produced, thus further increasing the photosynthetic and respiratory C costs. This is consistent with the work done by Peng *et al.,* (1993) who found that mycorrhizal plants had higher construction costs than their non-mycorrhizal counterparts.

The greater impact of AM over host C allocation belowground was most pronounced under P deficient conditions, when both host and nodular P would be limiting. Under these conditions the additional C costs of the dual symbiosis were clearly for symbiont metabolism and their subsequent nutrient uptake. These findings indicate a more prominent role for the AM in this tripartite symbiosis, because of its role in supplying P more effectively to both host and nodules. However, the prominence of the AM in this tripartite symbiosis would be dependant on the symbiotic species

involved, due to their high functional diversities. The potential value of these findings to crop production is that the dual symbiosis would benefit subsistence farming in soils that are low in N and P.

Chapter 4

Arbuscular mycorrhiza can maintain nodule function during external NH_4^+ supply in *Phaseolus vulgaris* (L.)

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Chapter 4

Arbuscular mycorrhiza can maintain nodule function during external NH_4^+ supply in *Phaseolus vulgaris* (L.)

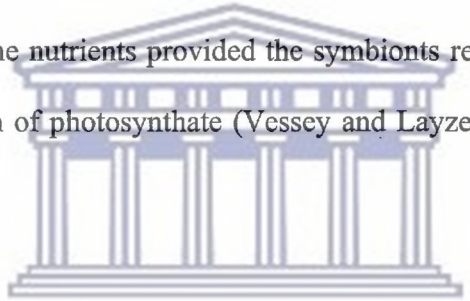
4.1 Summary

The synergistic benefits of the dual inoculation of legumes with nodule bacteria and arbuscular mycorrhizae (AM) are well established, however the effect of an external NH_4^+ supply on this tripartite relationship is less clear. This effect of NH_4^+ supply was investigated in the legume host, as well as the effects on the function of both the legume host and the respective symbionts. Nodulated *Phaseolus vulgaris* seedlings with and without AM, were grown in a sand medium with either 0 N, 1 mM or 3 mM NH_4^+. Plants were harvested at 30 days after emergence and measurements were taken for biomass, N_2 fixation, photosynthesis, asparagine content, construction cost calculations and N nutrition. The addition of NH_4^+ led to a decline in the percentage AM colonization and nodule dry weights, although AM colonization was affected to a lesser extent. NH_4^+ supply also resulted in a decrease in the reliance on biological nitrogen fixation (BNF), but in the presence of AM higher levels of NH_4^+ uptake were maintained. The inhibitory effects of NH_4^+ on nodule function can be reduced by AM presence at moderate levels of NH_4^+ (1mM), by improving nodule growth or relieving asparagine induced inhibition of BNF.

Key words: Nodules; arbuscular mycorrhiza; NH_4^+; photosynthesis; asparagine

4.2 Introduction

The tripartite symbiosis formed between legumes, mycorrhizae and nodule bacteria are known to benefit both the host as well as the respective symbionts. It is well established that this 3-way relationship benefits the host to a greater extent than singular inoculation with either symbiont, resulting in improved nutrition and growth of the host plant (Carling *et al.,* 1978; Kawai and Yamamoto 1986; Luis and Lim 1988, Vesjsadova *et al.,* 1993; Goss and Varennes 2002; Mortimer *et al.,* 2008). The benefits of the two symbionts are amplified under nutrient limiting conditions, where the P supplied by the AM and the N supplied by the nodules aid the plant in growing in these stressed conditions (Fredeen and Terry 1988; Smith and Read 1997). However, in exchange for the nutrients provided the symbionts require C, which the legume provides in the form of photosynthate (Vessey and Layzell 1987; Smith and Read 1997; Vance 2002).



Thus the two symbionts act as C sinks, drawing host photosynthate below ground, draining a large portion of the host' C budget (Harris *et al.,* 1985). Therefore, unless the legume is able to photosynthesize at a faster rate, the demand for C by the symbionts could have a negative effect on host growth. The subsequent increase in the rate of photosynthesis due to either singular or dual inoculation of legume roots has been well documented (Harris *et al.,* 1985; Brown and Bethlenfalvay 1987; Jia *et al.,* 2004; Mortimer *et al.,* 2008). In the study by Brown and Bethlenfalvay (1987) it was shown that rhizobia increased the rate of photosynthesis by 5% and AM by 17%. This increase in photosynthesis is made possible by the improved nutrition resulting from the symbioses (Harris *et al.,* 1985; Fredeen and Terry 1987; Jia *et al.,* 2004).

However, when N and P are found in sufficient quantities in the soil, the legume does not rely as heavily upon the symbionts for the acquisition of these nutrients. Furthermore the presence of N in the soil has been shown to have a negative effect on both nodulation and N fixation, although certain rhizobial strains are more sensitive to soil N than others (Awonaike *et al.,* 1980; Senaratne *et al.,* 1987; Müller *et al.,* 1993). Similarly the presence of N in the soil has been linked to a decrease in the percentage AM colonization of plant roots (Chambers *et al.,* 1980; Johnson *et al.,* 1984; Azcon *et al.,* 1992; Valentine *et al.,* 2001, 2002; Valentine and Kleinert 2006).

The effects of NO_3 on nodule development have been studied and are well known, as well as the ability of AM to assimilate soil N and provide this to the host. However, the effects of supplemental NH_4^+ on nodules and the role of AM under these conditions are less clear. We aim to provide some clarity on these issues by exposing the dual symbiotic plants to an NH_4^+ gradient and monitoring the growth responses of both the symbionts and the host as well as the photosynthetic response of the host to the changing NH_4^+ concentrations.

4.3 Materials and Methods

4.3.1 Seed Inoculation and Plant growth

The seeds of *Phaseolus vulgaris* (var. contender), were inoculated with a rhizobial inoculum containing *Rhizobium leguminosarum* biovar *phaseoli bacteria* (StimuPlant cc, Zwavelpoort, 0036) and germinated in vermiculite. The *Phaseolus vulgaris* seeds were coated with a paste of 2g of inoculum per 100 seeds. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in seedling trays containing vermiculite.

The AM treatments were inoculated with *Glomus etunicatum* (Becker & Gerdemann) (AmphiGro, Grahamstown, RSA) and the control plants received a filtered inoculum solution, which was prepared by filtering the inoculum through a 37μm mesh, which removed all fungal material. The AM treatment received 50g mycorrhizal inoculum per seedling tray (100 seeds). The inoculum was mixed with the vermiculite in the seedling tray just below the level which the seeds where planted at.

Seedling trays were watered daily with the aid of timer controlled overhead sprayers. Seeds were germinated in an east-facing glasshouse at the University of the Western Cape, Cape Town, South Africa. The range of midday irradiances were between 570 and 650 μmol $m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 23/16 °C and 30/70%, respectively. At ten days after emergence (dae) the seedlings were transferred to pots containing river sand. Once in the pots the plants were separated into treatments and received, once a week, Long Ashton nutrient solution (Hewitt, 1966) modified for the respective treatments and pH was maintained at 5.8. In addition the pots were watered with distilled water once a week. The treatments were divided into AM and non-AM, both receiving 3 levels of N nutrition: 0 mM NH_4^+; 1mM NH_4^+ and 3Mm NH_4^+. The NH_4 used was supplied as NH_4Cl^- and no NO_3 was added to the nutrient solution.

4.3.2 Photosynthesis

At 30 dae the youngest fully expanded leaf for each plant was used for the photosynthetic determinations. Readings were taken using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA, England).

4.3.3 Harvesting and nutrient analysis

Harvesting of the legumes took place at 30 dae, after the gas exchange readings had been taken. Upon harvesting the plants were separated into roots (and nodules), stems and leaves. Sub-samples of root segments were stored in 50% ethanol in order to determine percentage AM fungal colonization at a later stage. The harvested material was then placed in a drying-oven, at 80 °C, for two days and dry weights were recorded. The dried plant material was milled using a 0.5 mm mesh (Arthur H Thomas, California, USA).

4.3.4 Determination of percentage AM colonization

Roots were cut into 1cm segments and rinsed and cleared with 20% KOH for 3 days at room temperature. The KOH was rinsed off and the segments acidified with 1% HCL overnight. Thereafter the roots were stained with 0.05% (w/v) aniline blue and left overnight. The roots were then destained in a 1% HCL/glycerol mix. Root segments were placed on slides and the colonization components were determined according to the method of Brundrett *et al.,* (1994).

4.3.5 Determination of □ N15

The δ15N analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as δ = 1000‰ [$R_{sample}/R_{standard}$], where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et al.,* (1989). Between 2.100mg and 2.200mg of each sample was weighed into 8mm by 5mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen,

Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

The δ15N natural abundance of the legumes was corrected for the seed N, according to Boddey *et al.,* (1995):

δ15N enrichment (Seed corrected) = ((plant N X $\delta 15N_{plant}$) – (seed N X Ps X $\delta 15N_{seed}$)/ (plant N – seed N))

Where plant N and seed N represent the respective N concentrations of the plant and seed, δ15N plant and δ15N seed represent the respective δ15N values of the plant and seed and Ps is the proportion of the seed N that was assimilated by the legume.

The seed corrected δ15N values were used to determine the percentage N derived from the atmosphere (NDFA). % NDFA was calculated according to Shearer & Kohl (1986):

$$\%NDFA=100 * ((\delta 15N_{reference\ plant} - \delta 15N_{legume})/(\delta 15N_{reference\ plant} - B))$$

Where *B* is the δ15N natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N-free culture, according to Shearer & Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76 ‰.

4.3.6 Asparagine determination

Asparagine concentrations were commercially analyzed (Central Analytical Facilities, University of Stellenbosch, RSA) using a Waters API Quattro Micro. Nodule tissue of 100mg were hydrolyzed and subjected to EZ:Faast analysis. Asparagine levels are estimated as Apartic acid concentrations, because HCl hydrolysis causes the Asparagine to be deaminated to Aspartic acid.

4.3.7 Construction costs

Construction costs, Cw (mmolC.g^{-1} dw), were calculated according to Mortimer *et al.,* (2005), modified from the equation used by Peng *et al.,* (1993):

$Cw = [C + kN/14 \times 180/24] (1/0.89)(6000/180)$

Where *Cw* is the construction cost of the tissue (mmolC/gDW), *C* is the carbon concentration (mmolC/g), *k* is the reduction state of the N substrate (k=-3 for NH_3) and *N* is the organic nitrogen content of the tissue (g/g DW) (Williams *et al.,* 1987). The constant *(1/0.89)* represents the fraction of the construction cost which provides reductant that is not incorporated into biomass (Williams *et al.,* 1987, Peng *et al.,* 1993) and *(6000/180)* converts units of g glucose/g DW to mmolC/g DW.

4.3.8 Statistical analysis

There were 6 replicates for each treatment. The percentage data were arcsine transformed (Zar, 1999). The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (SuperAnova,). Where the ANOVA revealed significant differences between treatments the means were separated using a *post hoc* Student Newman Kuehls (SNK), multiple range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

4.4 Results

4.4.1 Biomass

The addition of NH_4^+ resulted in a decline in the percentage AM colonization (Figure 1), which was greatest in the plants that received no supplemental NH_4^+ and lowest for those receiving 3mM NH_4^+. The plants in the non-AM treatments remained non-mycorrhizal for the duration of the experiment.

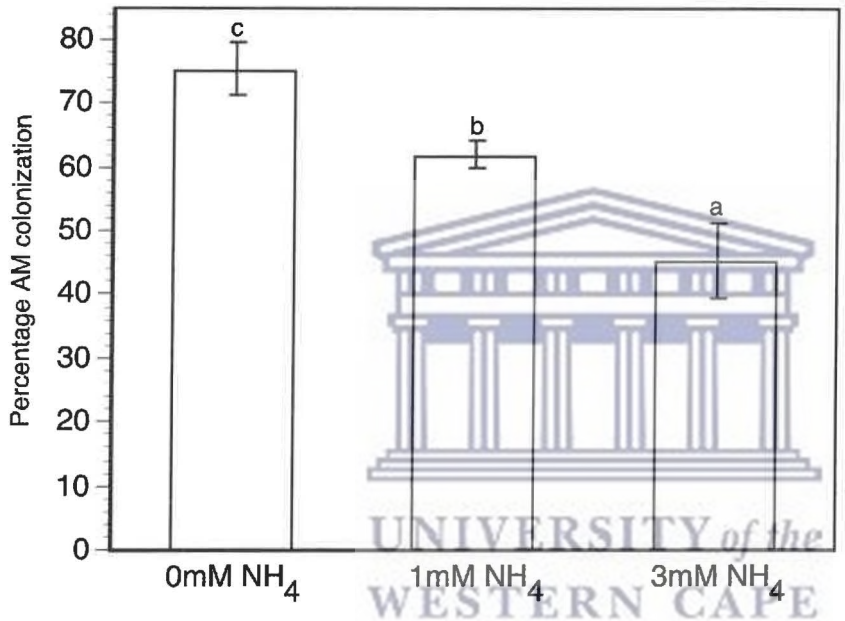


Figure 1. Percentage mycorrhizal colonization of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

The addition of NH_4^+ also caused a drop in nodule dry weight (dw) (Figure 2), the greatest effect being seen at 3 mM NH_4^+. However, the addition of NH_4^+ appeared to have a greater effect on the nodules than the AM, evidenced by the higher percentage drop in nodule dw than the percentage decline in percentage AM colonization (Figure 3).

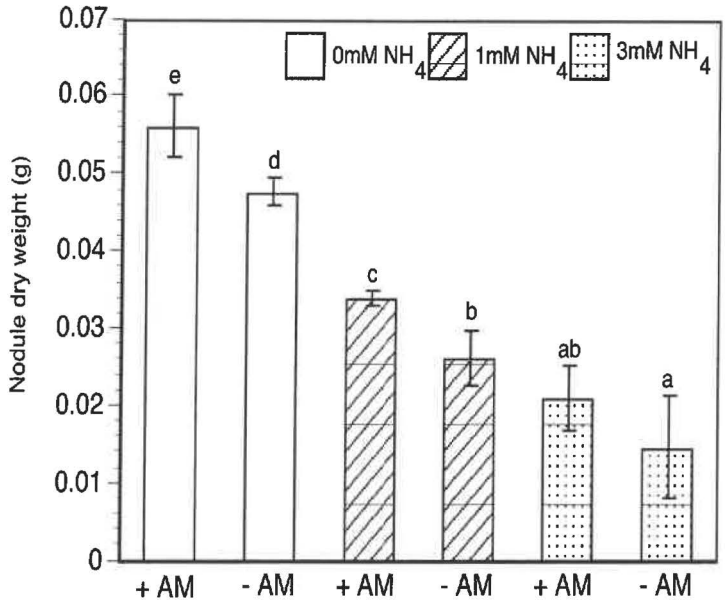


Figure 2. Nodule dry weight (g) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

The addition of NH_4^+ also had an effect on host dw (Table 1). It resulted in a drop on root dw, although the roots in the 3 mM NH_4^+ treatment were larger than those of the 1 mM treatment and the AM roots were generally smaller than the non-AM roots (Table 1). There was an opposite effect on the shoot dw of the plants receiving NH_4^+. Non-AM, NH_4^+ fed plants had increasing shoot dw with the increase in supplied N, however the increase in shoot dw of AM plants leveled off at 1 mM NH_4^+ and differences between the AM and non-AM shoot dw were only apparent in the 0 N and 1 mM NH_4^+ treatments (Table 1).

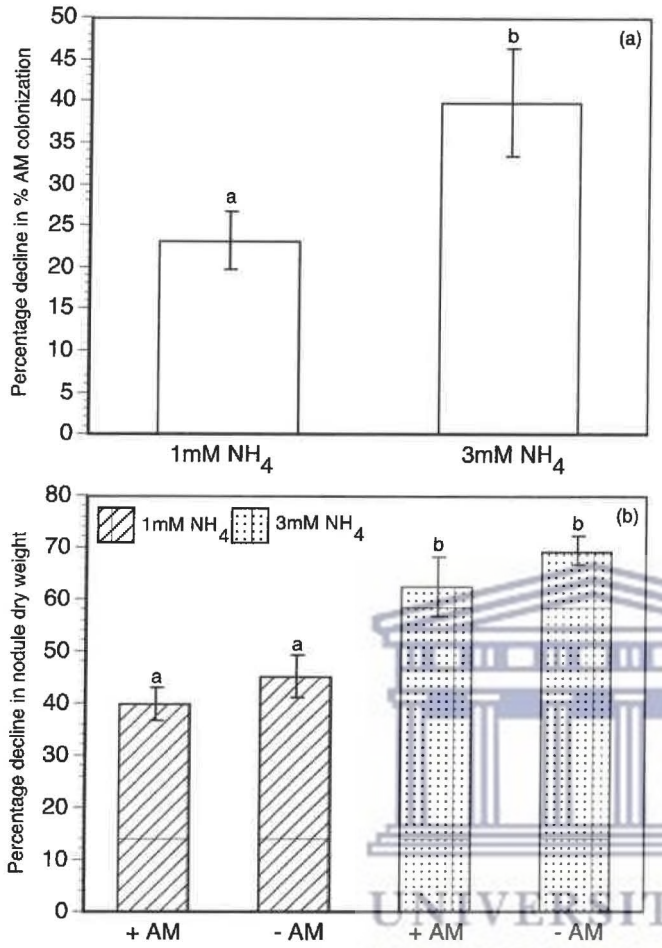


Figure 3. Percentage decline in the percentage mycorrhizal colonization (a) and Percentage decline in nodule dry weight (b) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

Table 1. Biomass parameters (g) and nitrogen concentrations (mmol/g) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

Dry weights (g)	0 mM NH_4^+		1 mM NH_4^+		3 mM NH_4^+	
	+AM	-AM	+AM	-AM	+AM	-AM
Plant	0.894 b	0.749 a	0.977 c	0.853 b	1.073 d	0.991 cd
Roots	0.159 c	0.198 d	0.109 a	0.141 bc	0.136 b	0.154 c
Shoot	0.735 bc	0.551 a	0.868 cd	0.712 b	0.937 d	0.838 cd
Nitrogen (mmol.g)						
Plant	1.65 b	1.349 a	1.826 c	1.712 bc	2.083 d	1.886 c
Roots	1.223 b	1.017 a	1.428 d	1.359 c	1.411 cd	1.366 cd
Shoot	1.685 b	1.492 a	2.082 c	1.795 b	2.094 c	1.964 bc

4.4.2 Nutrition

The percentage N derived from the atmosphere (NDFA), which represents the amount of N gained via biological N fixation (BNF), was greatest when no N was supplied, with the AM plants deriving the most N (Figure 4). However the addition of NH_4^+ resulted in a decline in NDFA, although no differences between AM and non-AM plants were found at 3 mM NH_4^+ (Figure 4).

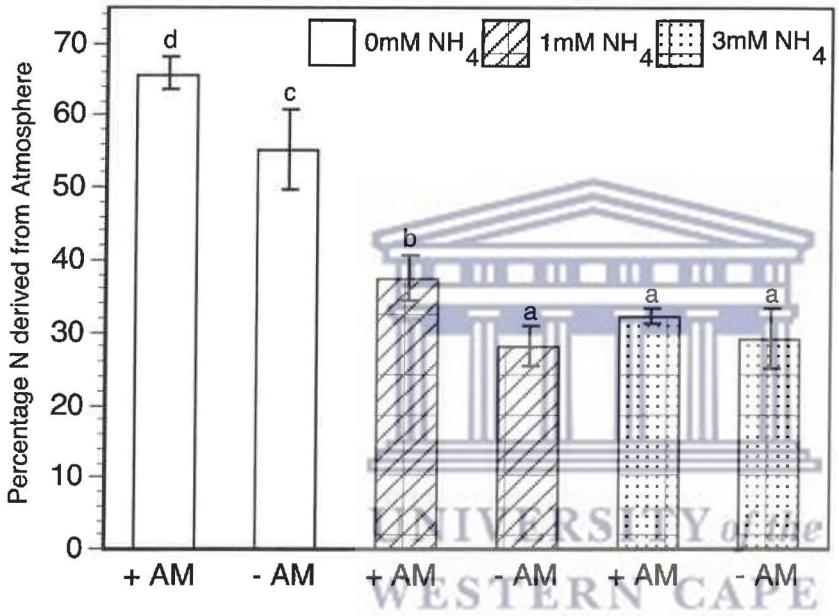


Figure 4. Percentage N derived from the atmosphere of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

N supply improved the N nutrition of the plants, with the AM plants having higher concentrations of N for all treatments. In the roots the positive effects of the N supply leveled off after 1 mM NH_4^+, however the AM roots had greater N concentrations at 1 mM NH_4^+ but no difference in root N was found at 3 mM NH_4^+(Table 1). In a similar fashion shoot N concentrations were higher with the addition of NH_4^+ but leveled off

after 1 mM NH_4^+ (Table 1).

4.4.3 Photosynthesis and construction costs

There was an increase in the rates of photosynthesis when NH_4^+ was added to the different treatments, however this increase leveled off after 1 mM NH_4^+(Figure 5a). The presence of AM also led to increased photosynthetic rates across all treatments (Figure 5a).

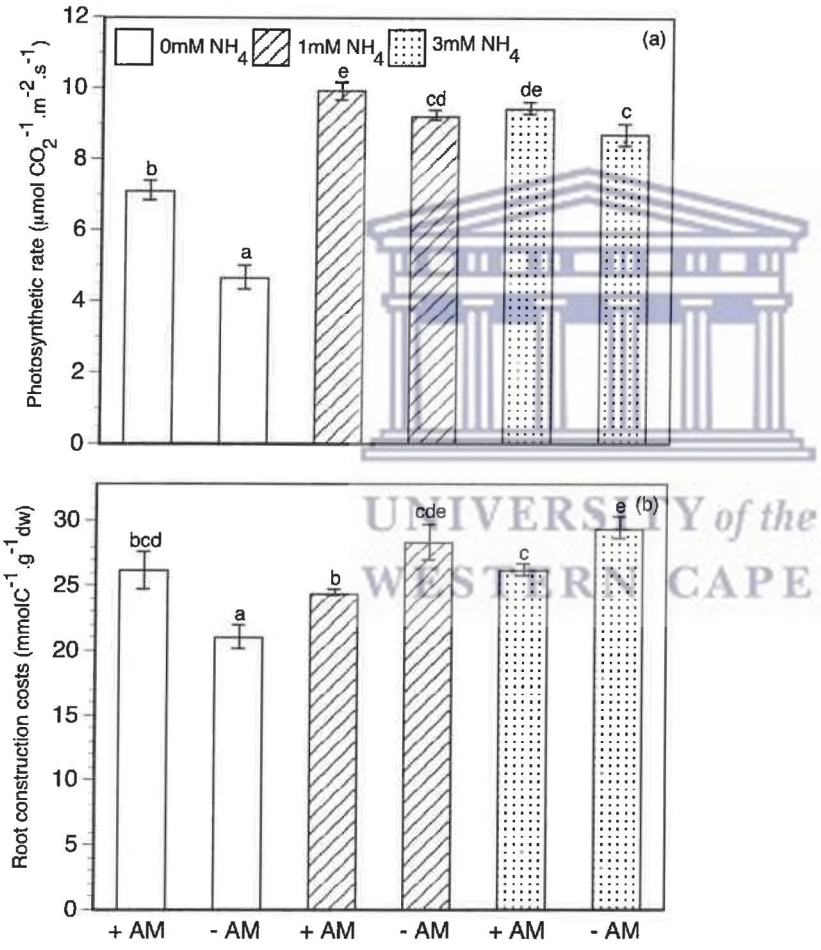


Figure 5. Photosynthetic rate (mmol $CO_2^{-1}.m^{-2}.s^{-1}$) (a) and root construction costs (mmol $C^{-1}.g^{-1}dw$) (b) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

In the absence of external N supply the construction costs of the AM roots were

higher than those of the non-AM roots, however the opposite was true once NH_4^+ was added. The addition of NH_4^+ led to the AM roots having lower construction costs than the non-AM roots (Figure 5b).

4.4.4 Asparagine content

The asparagine content between AM and non-AM plants were unaffected in the absence of external NH_4^+ supply (Figure 6). However, with the addition of NH_4^+ the non-AM plants maintained higher levels of asparagine in both the 1 mM and 3 mM treatments (Figure 6).

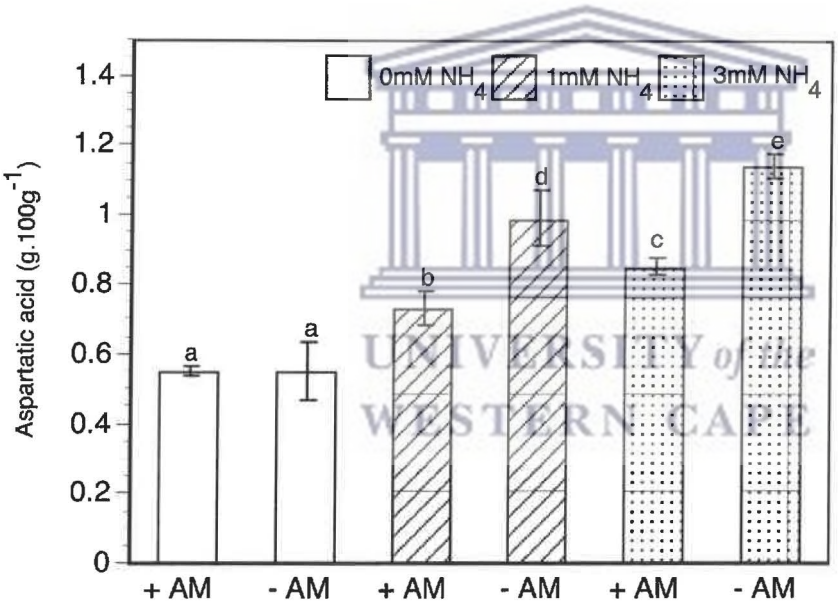


Figure 6. Aspartic acid concentration (g/100g) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

4.5 Discussion

In the dual symbiotic legumes, NH_4^+ supply inhibited nodules to a greater extent than arbuscular mycorrhizal (AM) symbionts, and the AM appeared to maintain nodule

function under moderate NH_4^+ supply.

Although the slower nodule development during supplemental NH_4^+ feeding led to a decline in biological N fixation (BNF), the decline in BNF leveled off after 1mM NH_4^+ (Nutman 1956; Malik *et al.,* 1987; Luciñski *et al.,* 2002). However, the NH_4^+ fed plants N maintained higher levels of N than those relying solely on BNF for N supply. This was particularly evident in the AM colonized plants, which had higher levels of both root N and shoot N. In spite of AM colonization being reduced by NH_4^+ supply, the increases in N concentration and biomass of the AM host plants, suggest that AM roots can cope better with external NH_4^+ supply than nodules, especially at 1mM NH_4^+ supply. Furthermore, this may also indicate that AM symbionts can reduce the inhibitory effects of 1mM NH_4^+ supply on nodules.

This AM induced improvement in plant N nutrition has been well documented in previous studies (Marschner & Dell 1994; Constable *et al.,* 2001; Govindarajulu *et al.,* 2005; Valentine & Kleinert 2006; Mortimer *et al.,* 2009). Not only did the presence of AM improve N uptake in the 1mM NH_4^+ and 3mM NH_4^+ treatments, but AM also led to an increase in BNF in the absence of N supply. The AM increase in NH_4^+ uptake is a known benefit of AM colonization (Valentine and Kleinert 2006;) and the resultant increase in BNF with AM colonization is in accordance with previous studies showing the benefits of the dual inoculation of legumes (Toro *et al.,* 1998; Mortimer *et al.,* 2008, 2009). In the present study, the AM-induced increase of BNF during 1mM NH_4^+ supply may be related to two major reasons, the nodule biomass and the accumulation of asparagine. Firstly, the improved nodule growth in the presence of AM concurs with previous findings on the dual symbiosis of legume roots with AM and Rhizobial nodules (Mortimer *et al.,* 2008, 2009). In this regard,

the lower root construction costs of AM plants during NH_4^+ supply, may suggest that the lower investment into AM structures (Mortimer *et al.*, 2008, 2009) can make more C available for nodule growth. Secondly, it is known that BNF can be regulated by N-feedback inhibition (Hartwig 1998) and that asparagine is one of the major amino acid that can induce this (Almeida *et al.* 2000). Therefore, the lower accumulation of the asparagine derivative, aspartic acid may have relieved the feedback inhibition on BNF, compared to non-AM NH_4^+ fed roots.

The plant dry weights were greatest in the AM colonized plants that received NH_4^+, although an increase in the NH_4^+ concentration above 1 mM NH_4^+ did not further benefit these plants. The AM colonized plants also had greater photosynthetic rates than the non-AM plants, thus coupled with lower root construction costs than their non-AM counterparts these plants had more C available for plant growth. The higher construction costs of the non-AM roots correspond with the increased root dry weights of these when exposed to an external N supply, possibly due to the higher cost of producing root tissue as opposed to fungal tissue (Harley 1989).

The cost associated with these AM benefits to host is an increase in photosynthetic rate. This increase in photosynthesis is required to maintain the high C demand placed on the host by the growth and maintenance of the two symbionts and the assimilation of NH_4^+ (Kucey & Paul 1981; Kucey & Paul 1982 (a); Kucey & Paul 1982 (b); Snellgrove *et al.,* 1982; Koch & Johnson 1984; Jakobsen and Rosendahl 1990; Harris *et al.,* 1985; Provorov & Tikhonovich 2003). Furthermore, the photosynthetic rates of the NH_4^+ fed plants were higher than the plants receiving no supplemental N. This increased photosynthetic rate may have resulted from the root sink stimulation of the

higher C costs associated with the root metabolism of NH_4^+ (Valentine and Kleinert 2006) and the increase in shoot N concentrations (Smith *et al.,* 1989; Dennis and Turpin *et al.,* 1997).

In conclusion, nodules were more negatively affected than AM by the additional NH_4^+ supply, and the inhibitory effects of NH_4^+ in nodule function can be reduced by AM presence at moderate levels of NH_4^+ (1mM).

Chapter 5

Arbuscular mycorrhizae affect the N and C economy of nodulated *Phaseolus vulgaris* (L.) during NH_4^+ nutrition

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Chapter 5

Arbuscular mycorrhizae affect the N and C economy of nodulated *Phaseolus vulgaris* (L.) during NH_4^+ nutrition

5.1 Summary

In the symbiosis between nodulated legume roots and arbuscular mycorrhizal fungi (AMF), the C and N economy can be influenced by the source of N supply from either AM-derived NH_4^+ uptake or nodule-derived biological nitrogen fixation (BNF). This relationship was investigated in terms of NH_4^+ supply and BNF by the two symbionts. Nodulated *Phaseolus vulgaris* seedlings with and without arbuscular mycorrhizae (AM), were hydroponically grown with either 0 N or 1 mM NH_4^+ supply. Plants were harvested at 30 days after emergence and measurements were taken for biomass, N_2 fixation, photosynthesis, CO_2 and O_2 root respiration, calculated C and N economy. AM roots had higher NH_4^+ uptake and this was associated with the suppression of BNF and nodule growth. The higher NH_4^+ uptake in AM roots occurred with lower root maintenance respiration, compared to when N was derived from BNF. There was also an increase in the below-ground sink strength of NH_4^+ fed AM roots compared to NH_4^+ fed non-AM roots, as evidenced by the increases in root CO_2 and O_2 respiration and photosynthetic stimulation. These results indicate that although the AM root had higher total below-ground respiratory costs during NH_4^+ nutrition, there were lower respiratory C costs associated with N derived from AM symbionts in comparison to N from BNF.

Key words: Nodules; arbuscular mycorrhiza; NH_4^+; N metabolism; C economy

5.2 Introduction

Legumes are able to form symbiotic relationships with both rhizobial bacteria and arbuscular mycorrhizal fungi (AMF). The rhizobia provide N and the arbuscular mycorrhizae (AM) provide P in exchange for photosynthetically derived C from the host (Vessey and Layzell 1987; Smith and Read 1997; Vance 2002). N and P are the two most limiting nutrients for plant growth, therefore the enhanced nutrition due to the respective symbionts can result in the improved growth and development of the legume, especially under nutrient limiting conditions (Marschner 1995; Runge-Metzger 1995; von Uexküll and Mutert 1995).

The two symbionts require C from the host in return for the nutrients supplied. The C drain imposed by the respective symbionts can amount to a relatively large portion of the host C budget (Harris *et al.,* 1985, Mortimer *et al.,* 2008). The AMF can receive between 10 and 23% of host photosynthate (Snellgrove *et al.,* 1982; Koch and Johnson 1984; Kucey and Paul 1981; Jakobsen and Rosendahl 1990) and the nodule between 6 and 30% (Kucey and Paul 1981; Kucey and Paul 1982; Harris *et al.,* 1985; Provorov and Tikhonovich 2003).

Although legumes rely on the N contribution of the Rhizobia for growth and development, the plant can access other sources of N. Numerous studies have shown that arbuscular mycorrhizae (AM) play an important role in the uptake of N and the subsequent supply of N to the host plant (Marschner and Dell 1994; Constable *et al.,* 2001; Toussaint *et al.,* 2004; Govindarajulu *et al.,* 2005). AM have also been found to have an indirect effect on the N nutrition of legumes. In this regard, AM legumes have been reported to show a greater number of nodules, increased nodular weight

and improved N-fixation rates, thereby enhancing the N nutrition of the host, these findings were often exacerbated by low P environments (Carling *et al.,* 1978; Kawai and Yamamoto 1986; Luis and Lim 1988, Vesjsadova *et al.,* 1993; Goss and Varennes 2002; Mortimer *et al.,* 2008).

The positive effect of AM on the growth and performance of legumes and rhizobia has been established, especially under nutrient limiting conditions (Carling *et al.,* 1978; Kawai and Yamamoto 1986; Luis and Lim 1988, Vejsadova *et al.,* 1993; Goss and Varennes 2002; Mortimer *et al.,* 2008). However, the specific contribution of AM to the N economy of the host legume remains to be elucidated. Although many studies have shown the synergistic effects of AM colonization, thus indicating the indirect role of AM on the N nutrition of the legume, this study aims to make clear the specific role that each symbiont plays in both the C and N economies of the host legume, under nutrient limiting conditions.

5.3 Materials and Methods

5.3.1 Plant growth and AM inoculation

The legume *Phaseolus vulgaris* (common bean, var. contender) was selected for the trial due to its short lifecycle and ability to be readily colonized by both AMF and nodule bacteria. The *Phaseolus vulgaris* seeds were inoculated with a rhizobial inoculum containing *Rhizobium leguminosarum* biovar *phaseoli* bacteria (STIMUPLANT CC: Reg. No. CK89/04756/23; PO Box 11446, Brooklyn 0011, South Africa) and germinated in vermiculite. The *Phaseolus vulgaris* seeds were coated with a paste of 2g of inoculum per 100 seeds. The seeds were spread out, away

from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in seedling trays containing vermiculite.

The AM treatments were inoculated with a commercial strain (AmphiGro CC, Grahamstown 6140, South Africa) of *Glomus etunicatum* (Becker and Gerdemann). This specific inoculum was chosen due to the known relationship between the host legume and this mycorrhizal strain (Pacovsky *et al.,* 1991; Mortimer *et al.,* 2008). The control plants received a filtered inoculum solution, which was prepared by filtering the inoculum through a 37µm mesh, which removed all fungal material. The AM treatment received 50g mycorrhizal inoculum (10 viable spores/g) per seedling tray (100 seeds). The inoculum was mixed with the vermiculite in the seedling tray just below the level which the seeds where planted at.

Seedling trays were watered daily with the aid of timer controlled overhead sprayers. Seeds were germinated in an east-facing glasshouse at the University of the Western Cape, Cape Town, South Africa. The range of midday irradiances were between 570 and 650 µmol $m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 23/16 °C and 30/70%, respectively. At ten days after emergence (dae) the seedlings were transferred to pots containing commercial quartz sand with a grain size of 0.2-0.5 mm (Consol Glass, Minerals Division, Silica Road, Athlone Industria-2, Western Cape, South Africa). On a weekly basis, the plants were watered with Long Ashton nutrient solution, modified for the respective treatments. The modification of the Long Ashton nutrient solution entailed a low (1µM) P and 0 N or 1mM NH_4Cl with the pH at 5.8.

5.3.2 Harvesting and nutrient analysis

Harvesting of the legumes took place at 30 days after emergence (dae). Upon harvesting the plants were separated into roots, nodules, stems and leaves. Sub-samples of root segments were stored in 50% ethanol in order to determine percentage AM fungal colonization at a later stage. The harvested material was then placed in a drying-oven, at 80 °C, for two days and dry weights were recorded. The dried plant material was milled using a 0.5 mm mesh (Arthur H Thomas, California, USA). The milled samples were analyzed for their respective C and N concentrations, by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyzer with suitable standards (BemLab, De Beers Rd, Somerset West, South Africa).

5.3.3 AM colonization

Roots were cut into 1cm segments and rinsed and cleared with 20% KOH for 3 days at room temperature. The KOH was rinsed off and the segments acidified with 1% HCL overnight. Thereafter the roots were stained with 0.05% (w/v) aniline blue and left overnight. The roots were then destained in a 1% HCL/glycerol mix. Root segments were placed on slides and the colonization components were determined according to the method of Brundrett *et al.,* (1994).

5.3.4 Photosynthesis

The youngest fully expanded leaf for each plant was used for the photosynthetic determinations. Light-response curves were used to determine the irradiance (1200 $\mu mol.m^{-2}.s^{-1}$) at which to conduct the photosynthetic rates. The photosynthetic

readings were taken at midday, using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA, England).

5.3.5 Root respiration

O_2 consumption of roots and nodules were measured with a Clark type polarographic oxygen electrode system (Hansatech Instruments, King's Lynn, England). Plants were carefully removed from the sand medium and placed in the same nutrient solution that the plants were supplied with. Nodulated root segments were excised from the plant and placed in the chamber of the oxygen electrode system, containing the same nutrient solution. Root segments and whole nodules were measured in 20 ml chambers at a 20°C.

CO_2 release was measured in whole root systems, using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA, England). The analyzer used was the same system used for the photosynthesis measurements, but for total root respiration an adaptable soil hood was used. Maintenance respiration (Rm), largely representing the costs associated with nutrition, particularly nitrogen (Van der Werf *et al.* 1988; Martinez *et al.* 2002), calculated from total root respiration, according Martinez *et al.* 2002.

5.3.6 Cost and efficiency calculations

Construction costs, Cw (mmolC.g^{-1} dw), were calculated according to Mortimer *et al.*, (2005), modified from the equation used by Peng *et al.*, (1993):

$Cw = [C + kN/14 \times 180/24] (1/0.89)(6000/180)$

Where *Cw* is the construction cost of the tissue (mmolC/gDW), *C* is the carbon concentration (mmolC/g), *k* is the reduction state of the N substrate (k=-3 for NH_3) and *N* is the organic nitrogen content of the tissue (g/g DW) (Williams *et al.,* 1987). The constant *(1/0.89)* represents the fraction of the construction cost which provides reductant that is not incorporated into biomass (Williams *et al.,* 1987, Peng *et al.,* 1993) and *(6000/180)* converts units of g glucose/g DW to mmolC/g DW.

Growth respiration, Rg(t) (μmol $CO_2.d^{-1}$), is the daily growth respiration for the plant (Peng *et al.,* 1993):

$Rg(t) = Ct - \Delta Wc$

Ct (μmol CO_2 day^{-1}) is the C required for daily construction of new tissue. Ct was calculated by multiplying the root growth rate (gDW day^{-1}) by tissue construction cost (Cw). ΔWc (μmol C day^{-1}) is the change in root C content and was calculated by multiplying the root C content and the root growth rate.

N incorporation rate, N_{inc} (mmolN.d^{-1}), is the amount of N incorporated into the plant on a daily basis:

$N_{inc} = N_{plant}/gDW/d$

Where N_{plant} is the total N concentration for the plant, gDW is the dry weight of the root system and d is the amount of days.

5.3.7 Calculations of □ N15

The δ15N analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as δ = 1000‰ [$R_{sample}/R_{standard}$], where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et al.,* (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5mm mesh (Arthur H Thomas,

California, USA). Between 2.1mg and 2.2mg of each sample was weighed into 8mm by 5mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard- $(NH_4)_2SO_4$.

The δ15N natural abundance of the legumes was corrected for the seed N, according to Boddey *et al.,* (1995):

δ15N enrichment (Seed corrected) = ((plant N X $\delta 15N_{plant}$) – (seed N X Ps X $\delta 15N_{seed}$)/ (plant N – seed N))

Where plant N and seed N represent the respective N concentrations of the plant and seed, δ15N plant and δ15N seed represent the respective δ15N values of the plant and seed and Ps is the proportion of the seed N that was assimilated by the legume.

The seed corrected δ15N values were used to determine the percentage N derived from the atmosphere (NDFA). % NDFA was calculated according to Shearer and Kohl (1986):

$\%NDFA=100 * ((\delta 15N_{reference\ plant} - \delta 15N_{legume})/(\delta 15N_{reference\ plant} - B))$

Where *B* is the δ15N natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N-free culture, according to

Shearer and Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76 ‰.

5.3.8 Statistical analysis

There were 6 replicates for each treatment. The percentage data were arcsine transformed (Zar, 1999). The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (SuperAnova,). Where the ANOVA revealed significant differences between treatments the means were separated using a *post hoc* Student Newman Kuehls (SNK), multiple range test ($P \leq 0.05$). Letters of the alphabet were used to separate the differences among treatments, where different letters indicate significant differences and the same letter denoting no differences.

5.4 Results

5.4.1 Biomass

The application of NH_4^+ lowered the percentage arbuscular mycorrhizal (AM) colonization (non-AM plants remained AM free for the duration of the experiment) (Table 1) and resulted in a drop in nodule dry weight (DW) (Table 1). However with NH_4^+ supply, the percentage decline in AM colonization was 16% compared to 53% of nodular decline.

Table 1. Biomass parameters and percentage AM colonization of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N or 1 mM NH_4Cl and were harvested 30 days after emergence. Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

	1 mM N		0 mM N	
Dry weights (g)	+AM	-AM	+AM	-AM
Plant	1.26 b	1.01 a	1.23 b	1.17 a
Roots	0.42 b	0.35 a	0.39 b	0.49 c
Shoot	0.84 b	0.66 a	0.83 b	0.68 a
Nodule	0.031 b	0.022 a	0.066 d	0.046 c
AM colonization (%)	61 b	0 a	73 c	0 a

Despite this decline in nodule DW, AM colonized plants had improved nodular DW relative to non-AM plants (Table 1). Total plant DW was not affected by the addition of NH_4^+ in AM and non-AM plants (Table 1).

5.4.2 Nutrition

The application of NH_4^+ led to increased root N (Figure 1c) and resulted in a decrease in the %NDFA (Figure 1a). The supply of NH_4^+ led to a decline in the %NDFA, although AM colonization increased the %NDFA when no NH_4^+ was added (Figure 1a). Furthermore, the AM colonization rate coincided with improved N incorporation rates, irrespective of N source (Figure 1b).

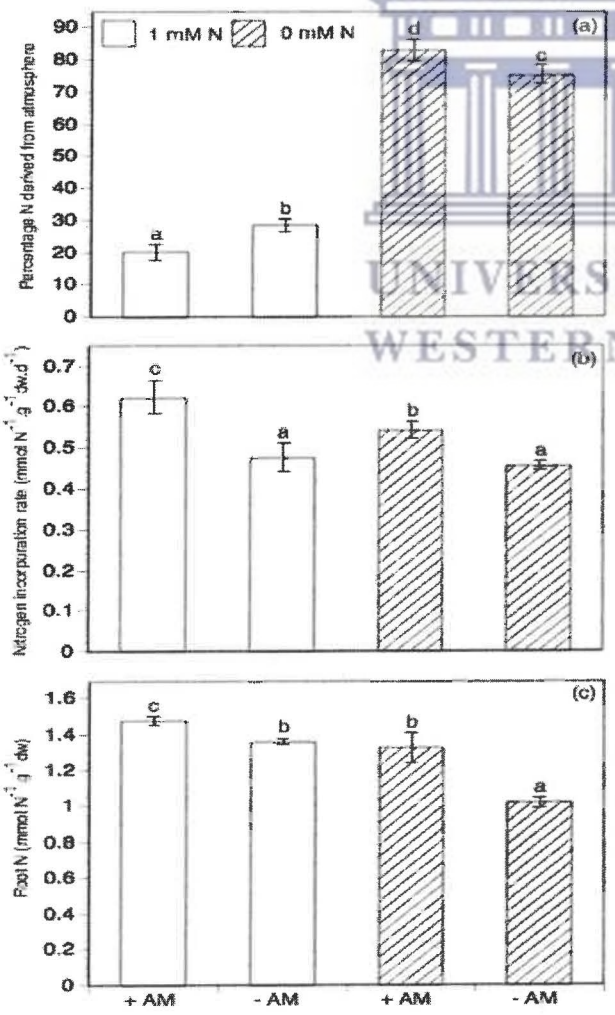


Figure 1. Percentage N derived from the atmosphere (a), N incorporation rate (mmol $N^{-1}.g^{-1}dw.d^{-1}$) (b) and root N concentration (mmol $N^{-1}.g^{-1}dw$) (c) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N or 1 mM NH_4Cl and were harvested 30 days after emergence. Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

5.4.3 Photosynthetic and respiratory C-costs

The addition of NH_4^+ in the dual symbiosis resulted in raised levels of photosynthesis, compared to the non-AM plants, whilst no differences were found in AM and non-AM plants that were NH_4^+ free (Figure 2a).

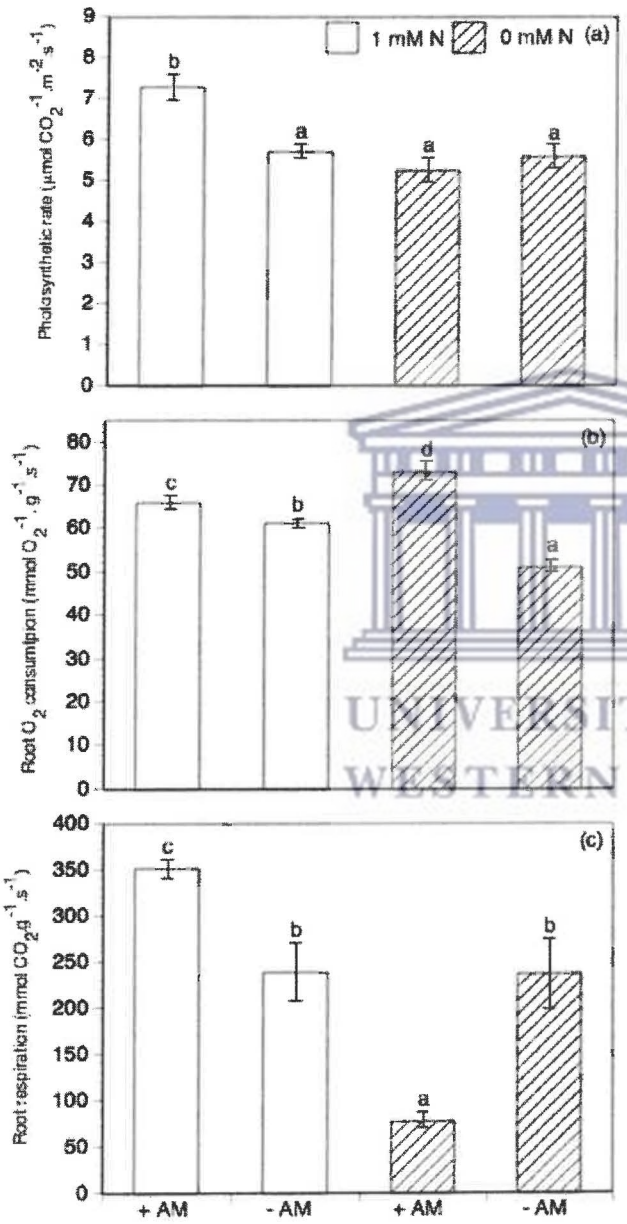


Figure 2. Photosynthetic rate (mmol $CO_2^{-1}.m^{-2}.s^{-1}$)(a), root oxygen consumption (mmol $O_2^{-1}.g^{-1}.s^{-1}$) (b) and root respiration (mmol $CO_2^{-1}.g^{-1}.s^{-1}$) (c) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N or 1 mM NH_4Cl and were harvested 30 days after emergence. Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

The root O_2 consumption was higher in the AM plants compared to non-AM plants which was most pronounced in the absence of NH_4^+ supply (Figure 2b). However

with NH_4^+ supply, the AM roots had higher CO_2 respiration rates (Figure 2c), compared to non-AM roots. In the absence of NH_4^+ the AM roots showed a decline in respiratory CO_2 release rates, relative to the non-AM roots. Maintenance respiration (Figure 3a), the component of root respiration attributed to nutrient acquisition and assimilation, was higher in the AM plants when NH_4^+ was absent and showed a decrease in AM plants during NH_4^+ supply.

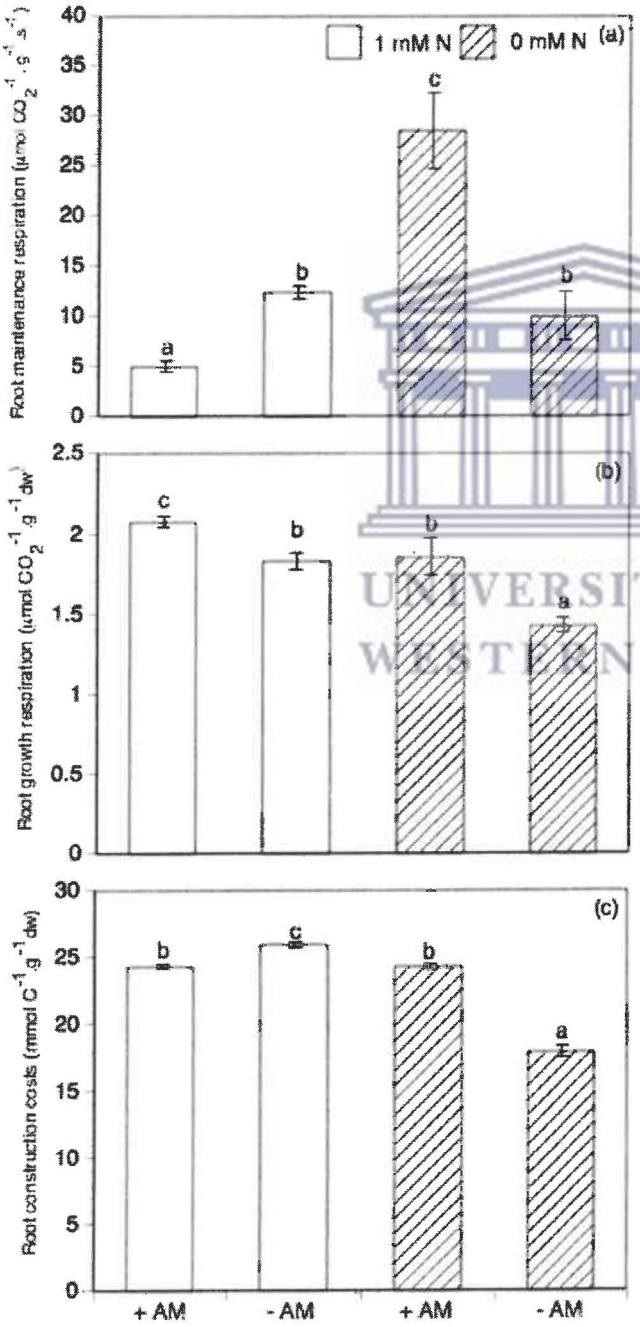


Figure 3. Root maintenance respiration (mmol $CO_2^{-1}.g^{-1}.s^{-1}$) (a), root growth respiration (mmol $CO_2^{-1}.g^{-1}dw$) (b) and root construction costs (mmol $C^{-1}.g^{-1}dw$) (c) of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N or 1 mM NH_4Cl and were harvested 30 days after emergence. Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

The dual symbiosis resulted in both higher growth respiration rates (Figure 3b) in the nodulated roots as well as an increase in the ratio of below ground to above ground growth respiration (Figure 3b). Furthermore, both below ground to above ground growth respiration (Figure 4) were exacerbated by the addition of NH_4^+. In addition, the AM roots which received NH_4^+ had lower construction costs than their non-AM counterparts (Figure 3c).

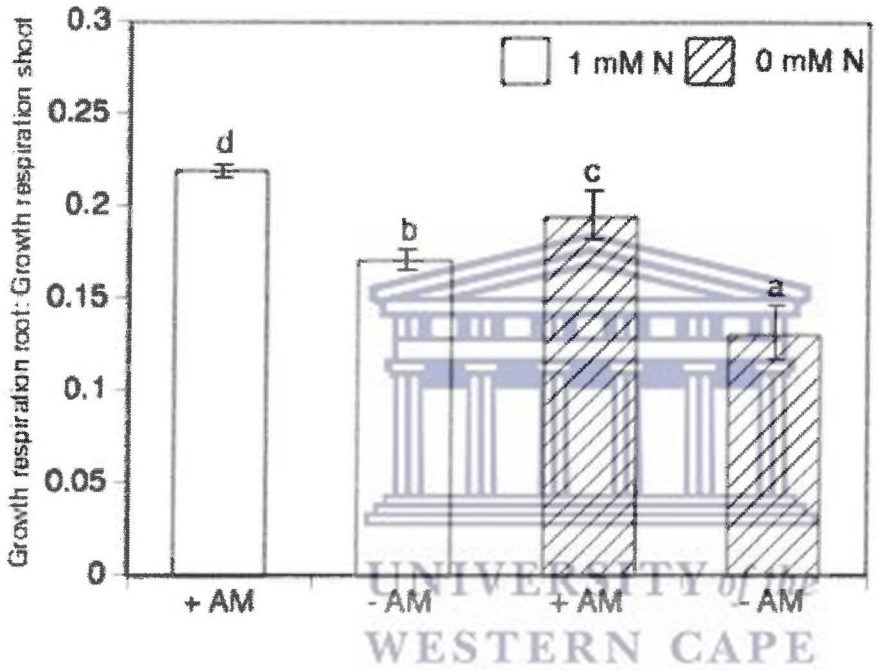


Figure 4. Ratio of root growth respiration: shoot growth respiration of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized (+AM) with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker and Gerdemann) or remained uncolonized (-AM). Plants were grown at 1 µM P and received either 0 N or 1 mM NH_4Cl and were harvested 30 days after emergence. Error bars represent the standard error of the mean (n = 6). Different letters indicate significant differences among the treatments, and are ranked from the lowest values ($P \leq 0.05$).

5.5 Discussion

Nodulated legumes that were colonized by arbuscular mycorrhizal (AM) fungi, became less reliant on biological nitrogen fixation (BNF) when exposed to an external source of NH_4^+.

The decline in the percentage AM colonization as a result of NH_4^+ nutrition has been

well documented (Chambers *et al.,* 1980; Johnson *et al.,* 1984; Azcon *et al.,* 1992; Valentine *et al.,* 2001, 2002; Valentine and Kleinert 2006) and the high root-C costs of NH_4^+ uptake and incorporation into amino acids (Oaks and Hirel 1985; Schweitzer and Erismann 1985; Arnozis *et al.,* 1988; Vourinen and Kaiser 1997) has been attributed as a possible cause of this decline in AM colonization (Valentine and Kleinert 2006). NH_4^+ nutrition further resulted in lower nodule dry weights, but the effect was less pronounced in AM plants. This concurs with previous reports and may be a result of the improved P nutrition of these plants (Daft and El-Giahmi 1974; Cluett and Boucher 1983; Kawai and Yamamoto 1986; Pacovsky *et al.,* 1986; Chaturvedi and Singh 1989; Fredeen and Terry 1988; Mortimer *et al.,* 2008). Since nodules are known to be P sinks (Le Roux *et al.,* 2006), an increase in P nutrition can improve nodule growth, metabolism and N_2 fixation (Le Roux *et al.,* 2008).

Although the supply of NH_4^+ resulted in the reduction of both nodule dry weights and AM colonization, the AM symbiont was affected to a lesser extent (16% reduction in AM colonization, compared to 53% reduction in nodule dry weight). Both symbionts impose a high C costs on the host (Mortimer *et al.* 2008, Le Roux *et al.* 2006, Le Roux *et al.* 2008), and with an external N-supply in the millimolar (mM) range, the host may have diverted the resources to its own uptake systems and depended less on the symbionts. The high root-C costs of NH_4^+ uptake and metabolism by the host roots (Oaks and Hirel 1985; Schweitzer and Erismann 1985; Arnozis *et al.,* 1988; Vourinen and Kaiser 1997) can therefore account for C being diverted away from symbiont development, as argued in previous work (Chambers *et al.,* 1980; Johnson *et al.,* 1984; Azcon *et al.,* 1992; Valentine *et al.,* 2001, 2002; Valentine and Kleinert 2006). In particular, host reliance on the N-supplying nodule symbiosis would

therefore be much less than the AM symbiosis, and this may account for the greater decline in nodule development under NH_4^+ supply.

In the dual symbiosis with legume roots, the possibility of host reliance on N supplied by the AM symbiosis may be associated with lower root respiratory C costs than BNF. This is comparable to previous reports of root N assimilation of an inorganic source (NO_3^-) having a lower C cost than N derived from BNF (Atkins 1984; Minchin and Witty 2005). However, this contention should take into account the complexity of AM root systems, where both the AM fungal tissue and host root tissue may contribute separately or synergistically to the lower root respiratory C costs for inorganic N assimilation. In this regard, the increases in N assimilation during inorganic N supply by AM fungal hyphae (Johansen *et al.* 1996), AM root systems (Smith *et al.* 1985; Cliquet and Steward 1993) is well- known, but may also be mediated by increased P supply from the AM root system (Oliver *et al.* 1983). Although the contributions of external hyphae and AM root systems (host root and fungal tissues) to N nutrition may be difficult to separate, the AM root respiratory cost of nutrient uptake and assimilation by both AM fungal and root tissue, can nonetheless be expressed as maintenance respiration (Van der Werf *et al.* 1988).

Maintenance respiration of roots is a component of the total root respiration and is an expression of the energy expense attributed to mineral nutrition, and not to growth of new tissue (Van der Werf *et al.* 1988, Peng *et al.* 1993, Martinez *et al.* 2002). In AM plants, the AM root maintenance respiration was the most enhanced in the absence of NH_4^+ nutrition, when most of plant N was derived from the atmosphere via BNF. However, in the presence of NH_4^+ the AM plants had a sharp decline in AM root

maintenance respiration, which coincided with the lowest percentage of N derived from atmosphere (%NDFA). This suggests that the costs of NH_4^+ based N nutrition from AM fungal hyphae or the AM colonized root systems, may impose a lower root respiratory cost than BNF from nodules. This would be in agreement with previous findings of inorganic N assimilation by roots systems having lower C costs compared to BNF of nodules (Atkins 1984; Minchin and Witty 2005).

The supply of NH_4^+ led to a reduction in BNF, which was most pronounced in the AM plants, despite the relatively greater nodule dry weights of these plants compared to the non-AM plants. This reduction in BNF may be the result of increased uptake of NH_4^+, which can suppress BNF. The increased uptake of NH_4^+ is evidenced by the higher N incorporation rates and by the higher root N concentrations of the NH_4^+ fed plants. Furthermore, the suppression of BNF is more pronounced in AM colonized roots as a result of AM root contribution to higher NH_4^+ uptake, which is in agreement with the work of Valentine and Kleinert (2006). The increased below ground C cost of NH_4^+ uptake by AM colonized roots is evident in the higher O_2 consumption, as previously found by Valentine and Kleinert (2006). It is likely that the majority of additional respiratory costs were AM root related and not from nodules, because BNF had declined.

During NH_4^+ supply, the decline in %NDFA of AM plants, was associated with enhanced respiratory O_2 and CO_2 fluxes from AM roots. These O_2 and CO_2 fluxes represent the total root respiration, where the other components such as growth of AM roots may have increased the respiratory sink strength. This concurs with the enhanced growth respiration during NH_4^+ supply of AM roots compared to non-AM

roots. The greater ratio of below ground to above ground growth respiration in NH_4^+ fed AM plants, supports the sink strength of below ground respiratory costs. The higher photosynthetic rates may have been due to sink stimulation, in order to sustain the demand for additional below ground C. These findings show that estimations of below-ground respiratory costs for tripartite roots, should be take into account the components associated with nutrient uptake and growth.

In conclusion, the complexity of AM root systems where both the AM fungal tissue and host root tissue may contribute separately or synergistically to NH_4^+ nutrition, should be considered with any interpretation of N nutrition in AM root systems. Although AM roots had higher total below-ground respiratory costs during NH_4^+ nutrition, there were lower respiratory C costs associated with N derived from AM roots in comparison to N from BNF. These findings illustrate the role of AM roots in host N nutrition, indicating a possible redundancy of nodular BNF in the presence of soil NH_4^+.

Chapter 6

Concluding chapter

Chapter 6

Concluding chapter

6.1 Introduction

The cost-benefit relationship between the legume host and its two symbionts, arbuscular mycorrhizal fungi (AMF) and nodule bacteria, has been well studied. This work has provided much insight into the synergistic benefits and additive costs of the two symbionts as well as the role that these organisms play in differing growing conditions. Furthermore new work is constantly being produced bringing new ways of interpreting and expressing the data that describes these relationships. Table 1 provides a summary of the majority of studies concerning the C economy and synergistic effects of this tripartite symbiosis. The studies have been broadly categorized according to what aspect of this relationship was being investigated. It is clear from the table the majority of work done in this field has looked at either the cumulative benefits of the dual inoculation or the additive effects of both symbionts on the host C-economy and nutrition. In light of the tabulated summary it is possible to put this thesis into context, filling in gaps concerning this 3-way relationship, specifically focusing on the role of the individual symbionts under varying nutrient conditions.

Table 1. Summary of research projects concerning the effects of the dual inoculation of legumes with regards to host nutrition and C-economy. The papers are in chronological order and a brief field of study and summary of findings is provided. The characters '0', '+' and '-' represent the influence that the symbionts had on the host or one another, showing no influence, a positive influence or a negative influence respectively.

	Authors	Field of study	Plant species	Outcome of study
1	Ross, 1971	Influence of nutrient supply	*Glycine max*	- AM colonization + Host nutrition

2	Pang and Paul, 1980	Synergistic effects of dual inoculation	*Vicia Faba*	+ Host nutrition + Host growth
3	Kucey and Paul, 1981	Sink strength of symbionts	*Vicia Faba*	- Host C + Host nutrition
4	Bethlenfalvay *et al.*, 1982a	Synergistic effects of dual inoculation	*Glycine max*	+ Host nutrition + Host growth
5	Bethlenfalvay *et* al., 1982b	Synergistic effects of dual inoculation C economy of dual inoculation	*Glycine max*	+ Host nutrition - Host C + Dual inoculation
6	Kucey and Paul, 1982a	Influence of nutrient supply	*Vicia faba*	+ Host nutrition + Host growth
7	Kucey and Paul, 1982b	Synergistic effects of dual inoculation	*Vicia faba*	+ Host nutrition - Host C
8	Kuo and Huang, 1982	Synergistic effects of dual inoculation	*Glycine max*	+ Dual inoculation + Host nutrition
9	Harris *et al.*, 1985	C economy of dual inoculation	*Glycine max*	+ Host growth - Host C
10	Barea *et al.*, 1987	Synergistic effects of dual inoculation	*Hedysarum* coronarium	+ Host nutrition + Host growth
11	Bethlenfalvay *et al.*, 1987	Synergistic effects of dual inoculation	*Glycine max*	+ Host growth + Host nutrition
12	Brown and Bethlenfalvay, 1987	Influence of nutrient supply C economy of dual inoculation	*Glycine max*	+ Host growth + Host nutrition - Host C
13	Badr El-din and Moawad, 1988	Synergistic effects of dual inoculation	*Lens culinaris, Vicia faba, Glycine*	+ Dual inoculation + Host growth + Host nutrition
14	Brown and Bethlenfalvay, 1988	Synergistic effects of dual inoculation C economy of dual inoculation	*Glycine max*	+ Host growth + Host nutrition - Host C

15	Barea *et al.*, 1989	Influence of nutrient supply	*Medicago sativa*	- AM colonization 0 Nutrient transfer
16	Eom *et al.*, 1994	Synergistic effects of dual inoculation	*Glycine soya*, *Cassia mimosoides*	+ Host growth + Host nutrition
17	Xie *et al.*, 1995	Synergistic effects of dual inoculation	*Glycine max*	+ AM colonization
18	Redecker *et al.*, 1997	Synergistic effects of dual inoculation	*Phaseolus vulgaris*	+ Host growth + Host nutrition
19	Wright *et al.*, 1998a	C economy of dual inoculation	*Trifolium repens*	- Host C + Dual inoculation
20	Wright *et al.*, 1998b	C economy of dual inoculation Synergistic effects of inoculation	*Trifolium repens*	- Host C + Host growth
21	Nwoko and Saginga, 1999	Synergistic effects of dual inoculation	*Glycine max*, *Lablab purpureus*, *Mucuna pruriens*	+ Host growth + Host nutrition
22	Gavito *et al.*, 2000	C economy of dual inoculation Synergistic effects of dual inoculation	*Pisum sativum*	+ Nutrition + Host growth
23	Xavier and Gamida, 2002	Synergistic effects of dual inoculation	*Lens culinaris*	+ Host growth + Host nutrition
24	Vazquez *et al.*, 2002	Influence of nutrient supply Synergistic effects of dual inoculation	*Medicago sativa*	- AM colonization 0 Host nutrition
25	Xavier and Gamida, 2003	Synergistic effects of dual inoculation	*Pisum sativum*	+ Host growth + Host nutrition
26	Jia *et al.*, 2004	Synergistic effects of dual inoculation C economy of dual inoculation	*Vicia faba*	+ Host growth + Host nutrition + Dual inoculation

27	Shockley *et al.*, 2004	Synergistic effects of dual inoculation	*Desmanthus illinoensis*, *Desmodium paniculatum*	+ Host growth + Host nutrition
28	Gamper *et al.*, 2005	C economy of dual inoculation Synergistic effects of dual inoculation	*Trifolium repens*	+ Host growth + Host nutrition
29	Atunes *et al.*, 2006	Synergistic effects of dual inoculation	*Glycine max*	0 N fixation
30	Atunes *et al.*, 2006	Effect of soil disturbance Synergistic effects of dual inoculation	*Glycine max*	+ Host growth + Nodulation + Dual inoculation
31	Chalk et al., 2006	Synergistic effects of dual inoculation	Review-numerous species	+ Host growth + Host nutrition
32	Lesueur and Sarr, 2008	Synergistic effects of dual inoculation	*Calliandra callothyrsus*	0 Host growth 0 Host nutrition
33	Kaschuk et al., 2009	C economy of dual inoculation	Review-numerous species	+ Host nutrition - Host C

6.2 Photosynthetic and nutrient response ratios

The recently published work by Kaschuk *et al.*, (2009) (Table 1), is a meta-analysis study comparing the photosynthetic and nutrient response ratios of legumes colonized by either nodules, AMF or both from a number of independent studies. This allows for the determination of whether the growth changes of the host were related to the symbiont-induced improvement of host nutrition or the increased photosynthetic activity as a result of symbiont sink stimulation. The photosynthetic and nutrient response ratios for this thesis are shown in Table 2, allowing further insight into the complex relationship between the symbionts and the legume under the different nutrient regimes. The higher photosynthetic response ratios found in the plants grown under low P (chapter 3) and either 0 N (chapter 4 & 5) or 1 mM N (chapter 4 & 5)

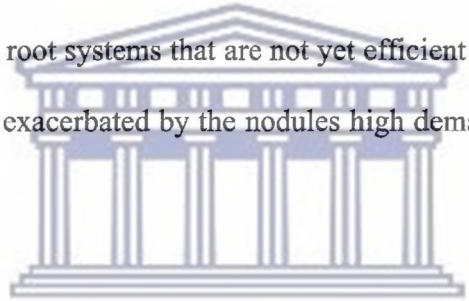
confirm the conclusions drawn in the research chapters of this thesis that the symbionts, primarily AM, were responsible for the drain in host C, resulting in higher photosynthetic rates. Comparatively, the plants grown under high P (chapter 3) or high N (chapter 4) conditions appear to be less reliant on the symbionts for changes in growth, as evidenced by the lack of differences in the photosynthetic and nutrient response ratios of these plants. These findings concur with those of previous studies, which found that under low nutrient conditions the rates of photosynthesis and the photosynthetic nutrient response ratios increased due to the reliance on the symbionts by the host (Harris *et al.,* 1985; Jia *et al.,* 2004; Kaschuk *et al.,* 2009). The lack of differences in the photosynthetic and nutrient response ratios of the plants grown under high nutrient conditions in is agreement with the work of Kaschuk *et al.,* (2009) and can be explained by the down regulation of the two symbionts under these favorable nutrient conditions (Schulze, 2004; Bittman *et al.,* 2006).

Table 2. The photosynthetic and nutrient response ratios of nodulated *Phaseolus vulgaris* (*L.*). Plants were either colonized with the arbuscular mycorrhizal fungus *Glomus etunicatum* (Becker & Gerdemann) or remained uncolonized. Plants were grown at low phosphate, 1μM P (LP) or high phosphate, 2mM P (HP) and received either 0 N, 1 mM NH_4Cl or 3 mM NH_4Cl. The plants were harvested 17, 24 and 31 days after emergence (dae). Values presented are the means (n=6) and the different letters indicate significant differences among the treatments for each row ($P \leq 0.05$).

	Response ratios		
Chapter 3	**Photosynthesis**	**Shoot N**	**Shoot P**
LP, 0 mM N 17 dae	1.15 fgh	1.05 def	0.96 abcde
LP, 0 mM N 24 dae	1.17 fgh	1.12 fgh	0.92 abcd
LP, 0 mM N 31 dae	1.19 gh	1.03 cdefg	1.03 cdefg
HP, 0 mM N 17 dae	1.07 efg	0.96 cd	0.88 abcd
HP, 0 mM N 24 dae	0.98 bcde	0.92 ab	0.98 cde
HP, 0 mM N 31 dae	0.97 bcd	1.03 def	0.95 abcd
Chapter 4 & 5			
LP, 0 mM N 31 dae	1.41 c	1.04 a	
LP, 1 mM N 31 dae	1.13 b	1.01 a	
LP, 3 mM N 31 dae	1.16 b	1.12 b	

6.3 Symbionts and nutrition

The positive contribution of the symbionts to host growth and nutrition under nutrient limiting conditions is well established. There have been many studies determining the synergistic benefits of the tripartite symbiosis (Table 1) and the results discussed in chapters 3, 4, and 5 only goes to confirm this. However the vast majority of previous work (Table 1) did not set out to determine the specific contribution of individual symbionts to the C economy and nutrition of the host. In chapter 3 it was concluded that the AM was the initial sink for host C (chapter 3: fig 7a, 7b), given preference over the development of the nodules. This is confirmed by the studies of Grant *et al.,* (2005) and Bittman *et al.,* (2006) who found that the early development of fungal networks are vital for young root systems that are not yet efficient in accessing soil P. This reliance would then be exacerbated by the nodules high demand for P (Le Roux *et al.,* 2006).



Further insight into the specific roles of the symbionts was provided in chapters 4 and 5, when the plants were grown with an external N supply. There was a drop in both AM colonization and nodule dry weight with the addition of N to the nutrients supplied (Chapter 4: fig 1, 2; chapter 5: table 1), however the percentage AM dropped to a lesser extent than the nodule dry weight. This may have been due to the continued reliance of the host on AM for P supply, however it may also have been due to the role of AM in host N nutrition. Past studies have shown that AM can play an important role in supplying the host with soil N, however the role of AM in the N nutrition of nodulated legumes has been neglected (Marschner and Dell 1994; Azcon *et al.,* 2001; Constable *et al.,* 2001; Toussaint *et al.,* 2004; Govindarajulu *et al.,* 2005). Evidence regarding the specific contribution of AM to host N nutrition was

presented in chapters 3 and 4, although it was however difficult to differentiate between the N supplied directly by the AMF and that which resulted from the improved P nutrition of the host, which would allow for enhanced N uptake by host roots (chapter4: table 1; chapter 5: fig 1). What is clear is that AM was responsible for the improvement of host N nutrition in a number of ways, both directly and indirectly and combinations thereof. Indirectly by improving the P nutrition of host roots (Oliver *et al.,* 1983), thus providing more ATP required for the N uptake processes in the root, and by improving the amount of N derived via BNF due to improved nodule P supply or the AM induced increase in nodule dry weights (Toro *et al.,* 1998; Valentine and Kleinert 2006; Mortimer *et al.,* 2008; Mortimer *et al.,* 2009). These findings are in agreement with the works of Barea *et al.,* (1987) and Redecker *et al.,* (1997), both of whom concluded that AM aid in the uptake of soil N as well as in the P-mediated improvement of BNF. The AM related improvement of BNF was further enhanced via the AM induced reduction in the export amino acid asparagine, which is known to inhibit BNF through N-feedback inhibition (chapter 4: fig 6) (Hartwig 1998; Almeida *et al.* 2000).

6.4 Symbiont costs

The nutritional benefits gained by the plant did however come at a cost, which is evident in the higher respiratory rates of the dual inoculated plants and the subsequent increase in the photosynthetic rates of these plants (chapter 3: fig 6, 7; chapter 4: fig 5a; chapter 5: fig 2). This is to be expected though and has been found in the past (Valentine and Kleinert, 2006), resulting from the host having to provide photosynthetic C to two additional organisms. In spite of the overall increase in below ground respiration in dual inoculated plants, the actual respiratory costs associated

with the uptake of N by the AM root systems was lower than the non-AM counterparts (chapter 5: fig 3), suggesting that the AM roots were more efficient at taking up soil N and imposed less of a C drain on host resources than the BNF process.

6.5 Nodule redundancy

This AM contribution to host N nutrition can result in a redundancy of the nodules. BNF is an energetically expensive process, thus if there is an alternative source of N to the plant, the nodules can become parasitic when the costs imposed by the nodules are outweighed by the benefits they provide (Denison and Kiers, 2004; Lodeiro *et al.,* 2004; Morgan *et al.,* 2005). These previous studies on the parasitism of nodule bacteria have focused on different types of bacteria involved, noting three different life strategies (Denison and Kiers, 2004). The first being mutualistic bacteria, fixing N_2 and supplying it to the host, the second being less mutualistic, it infects the host root but provides no nutritional benefits and the third is a non-symbiotic form of rhizobia that is free living in the soil. However, the results from chapter 5 appear to be novel in the suggestion that mutualistic rhizobia can display parasitic tendencies, depending on the amount of N nutrition available to the host plant, this is then further exacerbated by the active role of AM in host N nutrition.

6.6 Future work

Although we have gained insight into the specific roles of both AM and nodules in host nutrition, under differing nutrient conditions, many new questions regarding these topics have also been raised. These topics of future research can be grouped into three broad categories, those pertaining to nutrition, symbiotic costs and symbiont

functioning. An important issue was raised in chapter 5 regarding the role of AM and host N nutrition. Clarification is needed on whether or not AM is responsible for the direct uptake of N from the soil or if the improved host N nutrition is a result of the higher P levels found in host roots, allowing for the enhanced uptake of N by host roots. Another topic that requires attention is whether AM would continue to benefit the host plant in regards to N nutrition, under high P conditions. This would also address questions pertaining to the parasitic nature of AM under favourable host conditions. Furthermore, labeling of nutrients and the use of split root experiments would also show which nutrients are being taken up by the respective symbionts, and the ultimate distribution of these nutrients within the plant.

Future work delving into the costs associated with the symbionts should include studies determining the specific distribution of host photosynthate to the symbionts. Labeling experiments would be able to shown the exact portions of photosynthate going to the symbionts and at what stage of host development the respective symbionts gain more C in relation to one another. This would lead off from the conclusion of chapter 3, showing an early distribution of host C to AM, favoring AM development in the early stages of host growth. Further experiments also need to be carried out determining the different respiratory costs of the respective symbionts and colonized roots systems. This would enable the determination of exactly how much C is being used by the symbionts, under differing nutrient regimes and growth stages of the host and the symbionts.

The final category in which future work is discussed involves the actual functioning of the symbionts and their interactions with the root. For this a systems biology

approach would be more useful in gaining insight into the complex interactions betweens the organisms. The use of metabolic profiling studies and looking at the expression of different genes at various stages of host and symbiont development and under differing nutrient conditions could lead to more specific knowledge of how these organisms function and adapt to changing conditions. This coupled with enzyme assays determining which enzymes are involved in nutrient uptake and which are most active during the various processes involved in the growth and nutrition of the respective symbionts and the host. Thus providing answers not only to questions surrounding the functioning of the different organisms involved but also the role each organism plays in host nutrition. Questions such as whether or not the symbionts are directly involved in the uptake of nutrients and if the symbionts interact with each other or just the host.

In addition to these physical experiments meta-analysis studies such as that of Kaschuk *et al.,* (2009) should be carried out, combining information from a range of studies under a multitude of growing conditions. There is a wealth of information stored in past papers that remains to be accessed and compared with that of other studies.

6.7 Conclusion

In conclusion, this thesis has produced novel data regarding the C-economy and nutrition of *Phaseolus vulgaris* plants colonized with *Glomus etunicatum* and *Rhizobium leguminosarum*. Under nutrient limiting conditions the host plants were more reliant on the symbionts for nutrition and the subsequent C drain resulted in an increase in host photosynthesis. AM has also been shown to be the dominant

symbiont in the tripartite symbiosis, receiving more host C from an early stage of development and being able to provide both P and N to the host, providing the N at a lower cost than N derived from BNF. Furthermore the nodules have shown parasitic tendencies when the host has been exposed to an external source of N. From the data discussed in this thesis it is clear that the host plant is not always reliant on the symbionts that colonize its roots, yet the host still supports them, often at the expense of host growth. In spite of the costs incurred, the host continues to provide C to the symbionts, perhaps doing so to allow the plant a certain amount of plasticity to adapt and survive in a mutable environment that often requires an organism to adapt or die. Thus it may be a small price to pay supporting two symbionts that aren't always needed, yet providing security for an uncertain future.

Chapter 7

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