THE INFLUENCE OF NITROGEN, PHOSPHATE AND MICROBIAL ASSOCIATIONS ON PHOTOSYNTHESIS, RESPIRATION AND GROWTH IN *VICIA FABA* L.

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The Influence of Nitrogen, Phosphate and Microbial Associations on Photosynthesis, Respiration and Growth in *Vicia faba* L.

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the requirements for the degree of Doctor of Philosophy.

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DECLARATION

I declare that this dissertation to be entirely my own unaided work. It is being submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg, South Africa. It has not been previously submitted for any other degree or examination in any other university.

Signature:_____

Yinsuo Jia

Date:_____**day of** _____**, 2009.**

ABSTRACT

This project represents the development of a comprehensive description of growth in *Vicia faba L*. Particular attention has been paid to the impact in the tripartite legume-*Rhizobium*-AMF on the growth. The development of the description was divided into two parts. The first one was made up of (i) different N supply (0, 10, 25, 50, 100, 250 and 500ppm soil N) with normal P supply and (ii) different N supply (0, 10, 25, 50, 100, 250 and 500ppm soil N) with two phosphorus (0.05 and 1.6 mmol P) concentration applied and the *Vicia faba* seeds were planted pots filled with autoclaved river sand in order to non-producing the nodules just like normal cereal crops. As leaf nitrogen concentration (N_L) increased, the quantum yield efficiency (α)，carboxylation efficiency (C_e), photon saturated net photosynthetic rats (P_{Nmax}) were converged onto a maximum asymptotic value，the C_i value fell to an asymptotic minimum. A monotonic decline in the steady-state value of R_f occurred with increasing N supply. Specific leaf area (δ_L) increased with increasing N supply or with increasing N_L. An increase in P supply was consistently associated with an increase in N accumulation and N productivity in terms of biomass and leaf area production. Furthermore, P increased the photosynthetic N use efficiency in terms of P_{max} and α. An increase in P was also associated with an increase in C_e and a decrease in $C_{i.}$. Under variable daily meteorological conditions, the values for $N_{L,}$ specific leaf phosphorus content (P_L), specific leaf phosphorus content (P_L), specific leaf area (δ_L), root mass fraction (R_f), P_{Nmax} and α remained constant for a given N supply during the stage of steady-state exponential growth. This study tests the hypothesis that P supply positively affects both N demand and photosynthetic NUE by influencing the upper limit of the asymptotic values for P_{max}, C_e, and the lower limit for C_i in response to increasing N. The short-term photosynthetic responses to the increasing concentrations of CO_2 were observed to be co-limited by both N and P supply. These findings support the proposal that the N:P supply ratio controls the plant photosynthetic capacity in response to elevated CO_2 concentrations. Also, the short-term photosynthetic responses to the increasing concentrations of CO_2 were observed to be co-limited by both N and P supply.

The second part is tripartite symbiosis experiment with the concentration of nitrate-N for (a) the low N (LN, 10ppm N) and the high N treatments (HN, 250ppm N) without any microbial symbiotic associations;; (b) two different N supply rates plus AMF association, LNM and HNM; (c) two different N supply rates plus *Rhizobium* association, LNR and HNR; and (d) two different N supply rates plus both AMF and *Rhizobium* symbiotic associations: LNMR and HNMR. All treatments received a low level of phosphorus supply with 0.05mg P L^{-1} (1.61 mM NaH_2PO_4).

AMF promoted biomass production and photosynthetic rates by increasing the ratio of P to N accumulation. An increase in P was consistently associated with an increase in N accumulation and N productivity, expressed in terms of biomass and leaf area. Photosynthetic N use efficiency, irrespective of the inorganic source of N (e.g. NO_3^- or N_2), was enhanced by increased P supply due to AMF. The presence of *Rhizobium* resulted in a significant decline in AMF colonization levels irrespective of N supply. Without *Rhizobium*, AMF colonization levels were higher in low N treatments. Presence or absence of AMF did not have a significant effect on nodule mass but high N with or without AMF led to a significant decline in nodule biomass. Plants with the *Rhizobium* and AMF symbiotic associations had higher photosynthetic rates per unit leaf area and increased plant productivity.

The plants colonized with both microbial symbionts had significantly higher total biomasses, leaf areas, the whole plant photosynthesis and respiration rates than plants with only one or no microbial symbionts. Similarly, plants with both microbial symbionts also had significantly higher growth yield (Y_g) values than all the other treatments. Maintenance respiration rates were also highest in plants with two microbial symbionts. In low N plants colonized by both microbial symbionts there was evidence of compensatory increases in the photosynthetic rates in response to the carbon sink demands of the microbial symbionts. It was shown that the plant potential photosynthetic capacity exceeds the carbon demand of the *Rhizobium*–AMF symbiotic associations.

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LIST OF ABBREVATIONS

AC%	Percentage of arbuscular colonization
AMF	Arbuscular mycorrhizal fungi
c	a maintenance coefficient
C_a	ambient CO_2 concentration
CE (C_e)	carboxylation efficiency
C_i	intercellular CO_2 concentration
D	total whole plant daytime CO_2 influx (g CO_2 12 h^{-1} $plant^{-1}$)
δ_L	specific leaf area
DM	plant dry mass
E_a	content of activated Ribulose-1,5-bisphosphate carboxylase/oxygenase
h	leaf thickness
HC%	Percentage of hyphae colonization
HN	the high N (17.86 mM KNO_3 or 250 ppm N) treatment
HNM	the high N with AMF colonization treatment
HNMR	the high N with AMF and *Rhizobium* colonization treatment
HNR	the high N with *Rhizobium* colonization treatment
k_{cat}	Ribulose-1,5-bisphosphate carboxylase/oxygenase turnover rate k_{cat}^{app}
k_{cat}^{app}	P-dependent apparent catalytic constant for Ribulose-1,5-bisphosphate carboxylase/oxygenase
Km_{appCO2}	apparent Km for CO_2 at varying O_2 concentrations
K_N	Michaelis constant for N

K_N^{app}	apparent specificity constant for N
K_{NP}	kinetic coefficient
K_P	Michaelis constant for P
LN	the low N (0.71 mM KNO_3 or 10 ppm N) treatment
LNM	the low N with AMF colonization treatment
LNMR	the low N with AMF and *Rhizobium* colonization treatment
LNR	the low N with *Rhizobium* colonization treatment
m	maintenance respiration coefficient (mg CO_2 (g DM) $^{-1}$ d $^{-1}$)
M_r	the maintenance respiration
N	nitrogen
N_c	tissue nitrogen concentration
N_d	total whole plantCO_2 efflux after prolonged dark exposure (gCO_2 12h^{-1} plant $^{-1}$)
N_L (SLN)	specific leaf nitrogen content (g N m^{-2})
N_{plt}	total plant elemental N
N_r	total night time whole plant CO_2 efflux (g CO_2 12 h^{-1} plant $^{-1}$)
N_s	nitrogen supply concentration
N_{soil}	total soil 12 h dark CO_2 efflux (g CO_2 12 h^{-1}plant $^{-1}$)
N_t	total elemental N accumulated by the plant
NUE	nitrogen use efficiency
P	phosphorus
P_G	gross photosynthetic rate
Pi	inorganic orthophosphate
P_L (SLP)	specific leaf phosphorus content (g P m^{-2})
P_{max} (A_{max})	photon saturated rate of photosynthesis (µmol CO_2 m^{-2} s^{-1})
P_N	net photosynthetic rate (µmol CO_2 m^{-2} s^{-1})
P_{Nmax}	the saturated net photosynthetic rate (µmol CO_2 m^{-2} s^{-1})
PPFD	photosynthetic photon flux density
PUE	phosphate use efficiency
Qt	quantum
R	root dry mass (g)

R_f	root mass fraction;
R_G	root growth respiration
r_m	mesophyll resistance (s cm^{-1})
RuBPCO	Ribulose-1, 5-bisphosphate carboxylase / oxygenase
TP	Triose-phosphate
T_1	Plants were harvested at 54 d (4 October) after planting
T_2	Plants were harvested at63 d (13 October) after planting
VC%	Percentage of vesicular colonization
V_{max}	the maximum velocity of Ribulose-1, 5-bisphosphate Carboxylase/oxygenase activity
W	total plants dry mass (g)
Wc	substrate carbon
Wn	substrate nitrogen
Y_g	growth yield efficiency (g CO_2 (g CO_2) $^{-1}$)
Y_{max}	maximum growth yield efficiency
α/(QYE)	quantum yield efficiency (µmol CO_2 µmol $quanta^{-1}$)
Γ	The CO_2 compensation point
δ_G	specific growth rate
δ_L	specific leaf area (m^2 Kg^{-1})
τ	the carboxylation constant
μ_w	specific growth rate

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of the relationship among the legume, N and CO_2

The major elemental constituents of plant biomass are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulphur (S), calcium (Ca), potassium (K) and magnesium (Mg). Most of the plant biomass is formed from the first three elements (C: 45%, O: 45%, H: 6%). The remaining six elements constitute the macronutrients. Of these, it is generally the supply of N, P and K which often limits plant growth. Nitrogen is a major constituent of proteins, nucleic acids and chlorophyll and its elemental content on a dry mass basis when N supply is not limiting can be as high as 1.5 %. Nitrogen is a major component of the cellular catalytic machinery and is thus the most fundamental element limiting biomass production (Ågren 1985).

In general, the overall relationship between the photosynthetic rate and leaf nitrogen content (N_L) follows a curvilinear pattern, with the linear portion of the curve originating from a positive N_L-intercept (Schmitt and Edwards 1981, Sage and Pearcy 1987, Sinclair and Horie 1989, Meinzer and Zhu 1998). As N_L increases, the photosynthetic rate converges onto an asymptotic maximum value. This curvilinear relationship arises as a direct consequence of various rate limiting processes which play a role in setting the upper limit for the maximum possible values obtainable for photosynthetic parameters such as the photon-saturated net rate of photosynthesis

(P_{Nmax}) and the quantum yield efficiency (α) as N_L increases. For example, the maximum value for P_{Nmax} is directly proportional to the flux of CO_2 from the atmosphere. The rate of CO_2 diffusion into the leaf is directly proportional to the atmosphere-leaf CO_2 concentration gradient, the stomatal conductance, the mesophyll conductance, and the carboxylation efficiency of the leaf (Charles-Edwards 1978, Fisher *et al*. 1981, Evans and Caemmerer 1996, Katul *et al.* 2000). The carboxylation efficiency (C_e) is dependent on the concentration of the various enzyme catalytic sites available for the different reactions involved in photosynthetic metabolism. Theoretically the upper limit to the maximum value for P_{Nmax} which also determines the plant potential photosynthetic capacity is proportional to the number of active catalytic sites in chloroplasts that are involved in the reductive assimilation of CO_2. The total number of these catalytic sites involved in CO_2 assimilation is dependent on the proportion of the total N_L allocated to the chloroplasts.

The asymptotic relationship between the P_{Nmax} or α and N_L indicates that as the number of catalytic units increases, other processes become in turn rate limiting. As N_L increases, these processes will in turn fix the upper limit of the plant N efficiency for biomass production. In this respect, the rate of carbon dioxide diffusion into the leaf will be a factor limiting the plant N dependent capacity for biomass production (Evans and Caemmerer 1996, Katul *et al*. 2000). Other transport processes such as the rate of phosphate recycling between the chloroplast and cytosol also play a prominent role in fixing the upper limit of the plant capacity for biomass production (Cockburn *et al*.1967a, b, Usuda and Edwards 1982, Pradet and Raymond 1983, Mächler *et al*. 1984, Rao and Terry 1989, 1995, Rao *et al*. 1989, 1990).

If the concentration of nitrogen supply (N_s) to the roots remains constant while the plant exponential growth, then steady-state content of N within the plant can only be maintained if the capacity for N accumulation increases exponentially during plant growth (Hirose 1986, Garnier *et al*. 1989). In this context, the term balance exponential growth has been used to describe the situation where under constant conditions of nutrient supply, moisture availability, and saturating irradiances, all extensive variables (*e.g.* plant biomass and leaf area) increase exponentially at a constant growth specific growth rate (μ_w). Under these conditions all variables that are either ratios or rates (*e.g.* plant N concentration) remain constant (Thornley 1998). Under balanced exponential growth, it is expected that μ_w or specific photosynthetic rate would be determined by the plant steady-state N_c.

1.2 Overview of the relationship between the legume and P supply

Phosphate (P) is the other key element involved in biomass production. High specific rates of crop growth require P available in the form of HPO_4^{2-} and $H_2PO_4^-$ at soil contents greater than 10.0g P g^{-1} soil. Phosphorus requirement for plant growth ranges from 4 % to 10 % of the N accumulated by the plant. Its elemental content on a dry mass basis when P supply is not limiting approaches 0.2 %. Phosphate is a major component of sugar phosphates, nucleic acids, cell membranes and adenosine triphosphate (ATP), the latter constituting the energy currency of the cell. Many biosynthetic reactions have a high demand for P in the form of ATP. For example, 2 ATPs are required for the formation of each glycosidic bond in storage polysaccharides like starch and structural polysaccharides such as cellulose. Four ATP molecules are required for the formation of each peptide bond in protein synthesis (Miller and

Donahue 1990).

P is the third main macronutrients that it is often most limiting with regard to plant growth. Plant nutrient sufficiency levels for phosphorus in alfalfa on a percent dry mass basis ranges from 0.26 to 0.7 %. But higher P tissue concentrations result in toxic effects. It has been hypothesized that the responses of plant communities to global warming and elevated CO_2 will be influenced by leaf N:P ratios (Hedin 2004), which in turn will be dependent on N and P supply from the soil. This proposal has received support from experiments that show that increases in biomass production in plants acclimatized to elevated CO_2 (440 and 600 CO_2 μL L^{-1}) relative to control plants (280 CO_2 μL L^{-1}) depended on the level of NPK supply (Grünweig and Körner, 2003). The magnitude of the photosynthetic response to increasing N supply is modulated by the level of P supply. This modulation of the photosynthetic response to N supply by P may take place either directly or indirectly. Direct modulation of photosynthetic activity by P appears to be affected through the influence of P on the ribulose-1, 5- bisphosphate carboxylase oxygenase (RuBPCO) activation (Marcus and Gurevitz 2000). Alternately, indirect control of photosynthetic rates by P supply could be exerted by through the chloroplast phosphate shuttle. Phosphate recycling between the chloroplast and cytoplasm has been observed to modulate photosynthetic rate by influencing the rate of export of photosynthate from the chloroplast (Mächler *et al.* 1984, Rao and Terry 1989, Rao *et al.* 1989a, b, Usuda and Shimogawara 1991, Rao and Terry 1995). P supply may also indirectly modulate photosynthetic rate by influencing sink demand for photosynthate (Pieters *et al.* 2001).

In general, the proposal that photosynthesis is usually co-limited by both N and P

supply is consistent with the observations of recent studies that yield maximization in various crops was influenced by N:P supply stoichiometries (Ågren 2004, Sadras 2006). It has also been reported that enhanced biomass production in response to prolonged exposure to elevated CO_2 was dependent on an increase in N and P supply (Grünweig and Körner, 2003). The objective of this study was to investigate the effects of the C:N:P supply ratios on light saturated photosynthesis (P_{max}) and quantum yield efficiency (α). The experiments focused primarily on how N and P supply rates influence photosynthetic responses to short term increases in the CO_2 supply concentration in plants that had been grown under normal ambient CO_2 concentrations.

1.3 Overview of the legume-*Rhizobium*-AM fungi relationship

The value of microbiological symbiont inoculum as *bio-fertilizer aid* for augmenting or enhancing a legume crop positive growth response to chemical crop fertilization needs to be quantitatively evaluated. The ecology of symbiotic micro-organisms and their impact on crop productivity have been reviewed over the past decade (Friese and Allen 1991). However, information on the effects of the tripartite symbiotic association in broad bean (*Vicia faba L.*) growth is scarce. Therefore, objective of this study was to quantify the effects of different combinations of microbial endosymbiotic associations on *Vicia faba L.* growth responses to high and low nitrogen supply rates under conditions of low phosphorus availability.

The biological basis for the agronomic advantages derived from legume crops arises as a consequence of the tripartite (legume: *Rhizobium*: arbuscular mycorrhizal fungi) symbiotic association (Gray 1996). The agro-economic advantages arising as

consequence of this tripartite symbiotic association for legume crops under low nitrogen and phosphorus fertilizer inputs needs to be fully quantified. It has been well established that the legume tripartite symbiotic association improves both the uptake of phosphorus and fixation of dinitrogen and this in turn results in increased plant growth and yield under low fertilizer input agricultural conditions (Azcón-Aguilar *et al*. 1979, Azcón, *et al*. 1991, Barea *et al*. 1992, Koide *et al*.1989). It is also well established that phosphorus availability is an important limiting factor in nitrogen fixation and legume crop production. In the tripartite symbiotic association the phosphorous mobilizing capacity of the arbuscular mycorrhizal fungi (AMF) in the rhizosphere of leguminous plants increases the dinitrogen (N_2) fixing capacity of the legume *Rhizobium* endosymbiont and its this positive synergy between the two microbial endosymbionts that promotes legume crop growth under nitrogen and phosphate limiting conditions (Smith and Read 1997, Sa and Israel 1991). In general, AMF infection results in enhanced levels of legume root nodulation under phosphorus limiting conditions (Barea and Azcón-Aquilar 1983, Skot *et al*.1986). In the absence of mycorrhizal infection supplementary phosphorus fertilization of legume crops is generally necessary maintenance of *Rhizobium* N_2 fixation rates at levels for economically viable crop production (Andrade *et al*. 1998).

Resource acquisition and allocation in legumes is dependent on a complex set of exchanges between three structural functional components: the legume; the *Rhizobium* nodules; the AMF association. The biological basis for the superiority of legume crops derives from the three-way resource exchanges among members of the tripartite (nodules, AM fungi, legume) symbiotic association (Bethlenfalvay and Newton 1991; Barea *et al.* 1992). Growth of legumes under limiting nitrogen (N) and phosphorus (P)

regimes is facilitated by the resource acquisitions efficiencies and capacities of the two microsymbionts, *Rhizobium* and AM fungi. Each of the symbionts in the legume system functions as both sinks and sources for C, N, and P. Therefore, responses of the symbionts to nutrient stress must be considered in the context of a complex, three-way source-sink relationship, where the allocation of resources may also be subject to intersymbiont competition (Bayne *et al.* 1984, Bethlenfalvay 1992). The two microsymbionts may be regarded as the primary sources of P and N to legumes growing in soils deficient in plant available forms of these two nutrients (Azcón *et al.* 1979; Barea and Azcón-Aguilar 1983, Azcón *et al.* 1988, Piccini *et al.* 1988, Cihacek 1993). From the legume side of the symbiotic association, the exchange of resources involves the allocation of carbon to nodules in exchange for reduced N and to arbuscular mycorrhizal fungi (AMF) in exchange for P. Phosphorus, the major plant growth limiting factor apart from N, is required for both photosynthesis in the leaves and nitrogen fixation in the root nodules (Israel 1987, Haaker 1988,). The contribution of AM fungi to the tripartite symbiotic association is particularly significant for nodulated legumes growing under a soil regime where available inorganic nitrogen in the form of ammonium or nitrate is limiting: the reason being the high P requirement for nodulation (Daft 1978, Bethenfalvey and Yoder 1981) and N_2 fixation (Bergersen 1971). AM fungi symbiotic associations with plant roots generally improves plant growth by enhancing the uptake of inorganic phosphorus (Jayachandran *et al.* 1992).

With respect to above ground and below ground source-sink interactions in legume systems, the roots, nitrogen fixing nodules and AM fungi all compete for a share of the below ground carbon allocation. An appreciation of the potential three-way below ground competition for carbon in legumes brings a new perspective to the

conceptualization of source-sink dynamics in legumes. It has been reported that 42% or more of daily net photosynthate can be allocated to the belowground legume-*Rhizobium*-AMF association (Paul and Clark 1989). Paul and Kucey (1981) reported that 60% of the photosynthetic carbon flux was partitioned into the below ground Root-Nodule-AMF association. This below ground fraction of daily carbon allocation is nearly evenly distributed (12, 13 and 17%) to nodules, the root, and the AM fungi (Paul and Clark 1989). Cralle *et al.* (1987) reported that the photosynthate allocation schedule in growing alfalfa plants, the partitioning of the carbon in the following proportions to the major organ systems was observed: 26.2 % to the main stem; 12.7% to shoot apex unexpanded leaves on the main stem; 0.8% to the fully expanded leaves on the main stem; 27.1% to the auxiliary bud shoots on the main stem; 6.5% to the crown shoots; 3.8% to the crown; 19.1% the roots; 3.5% to the nodules. If the crown is included as part of the taproot, then 73.3 % of photosynthate is allocated to the shoot, and the remaining 26.7% of fixed carbon is allocated to the roots and nodules. The carbon allocation to AM fungi, which is an obligant symbiont, can constitute between 4-20% of host photosynthate as indicated in several studies on single host-fungus combinations (Azcón and Ocampo 1984, Doubs *et al.* 1988, Pearson and Jakobsen 1993). Observations on the supply of photosynthate in legume systems confirm that photosynthate production is in excess of carbon demand by the nodules (Gordon *et al.* 1985, Kouchi *et al.* 1985, Hostak *et al.* 1987, Vance and Heichel 1991). In a study investigating the carbon economy of soybean – *Rhizobium* - *Glomus* symbiotic associations, Harris *et al.* (1985) found that carbon was allocated in the following proportions: 30.49% to leaves; 20.52% to stems and petioles; 6.3% to shoot respiration; 7.8% to roots; 2.0% to nodules; 2.7% to AMF; 5.2% to root and soil respiration; 13.7% to AMF respiration; 9.4% to nodule respiration. In this study

approximately 42.6% of photosynthate was allocated to below ground sinks. Of this 42.6% below ground carbon allocation, 38.6% went to the AMF component, 26.6% to the nodule component and 30.6% to the roots.

While there is excess photosynthate supply capacity in legumes such as alfalfa as evidenced in the accumulation of starch in the taproots in these legumes (Vance and Heichel 1991) the above examples do demonstrate that legume root symbionts represent substantial carbon sinks. Taken together a series of observations (Hostak e*t al.* 1987, Walsh *et al.* 1987) may indicate that the growth of legumes such as alfalfa or soybean is not limited by source capacity but rather by symbiont sink strength. This becomes especially significant if the flux of carbon to the microsymbionts is regulated by the plant in exchange for P or N. This idea of microsymbiont carbon sink strength defined in terms of carbon demand being coupled to the symbiont capacity to supply N or P to the legume needs to be more fully developed. In this context an interesting consideration is the question of what resource exchanges limit or constrain legume growth: Is it the carbon demand of the microsymbiont sinks that limits legume growth or is it the magnitude of the capacity of the microsymbionts to exchange P or N for C that limits legume growth? Given that the supply of P or N or both are factors that in general limit plant growth then all influences that the effect C, N, and P exchange dynamics among symbionts with have an impact on legume growth rates. There is evidence that relative amounts of carbon allocated into storage or growth depends on the supply of N and P to the legume (Greenwood *et al.* 1991).

1.4 *Rhizobium* nodule phosphorus requirements

Phosphorus deficiency is a major limiting factor for N_2-fixing plants. Specific nitrogenase activity decreases with the onset of P-deficiency. Several physiological and metabolic properties were associated with lower specific nitrogenase activity in nodules of P-deficient plants: Bacteroid mass per unit nodule mass, bacteroid N concentrations, plant cell ATP concentrations, and energy charge were significantly lower in nodules of P-deficient plants. Indeed, because nitrogenase is localized in the bacteroids, lower bacteroid mass per unit nodule mass and N concentration could account for decreased specific nitrogenase activity under P-deficiency (Sa and Israel 1991). Legumes dependent on symbiotic N_2 fixation have a higher internal P requirement for optimum nitrogen assimilation compared to plants dependent on combined inorganic nitrogen in the form of nitrate and ammonium (Israel 1987). Soybean grown with limiting P supply showed a reduction in: total nodule numbers; total nodule mass; individual nodule mass; total plant mass (Israel 1993). The growing nodule is a major sink for P in legumes and in soybean the total nodule P concentration is 3-fold higher than in the rest of the plant. In fact, phosphorus deficiency resulted in decreases of *Rhizobium* bacteroid dry mass per unit nodule dry mass by an average of 20% relative to P-sufficient controls in soybean; P and N concentrations in bacteroids from P-deficient plants averaged 9 and 95 mg g^{-1} dry mass bacteroid respectively (Sa and Israel 1991). The latter P and N concentrations were 25 and 17% lower, respectively, than the P and N concentrations in bacteroids from P-sufficient plants. Nodule nitrogenase activity is decreased by plant P deficiency independently of the effectiveness of the rhizobium strain with non-limiting P supply (Singleton et al 1985).

1.5 Legume host carbon economy in the tripartite symbiotic relationship.

Exchanges of photosynthate, N and P between the symbiotic systems and the legume have important consequences for the overall plant carbon economy. The source capacity of leaves measured as daily gross photosynthetic output will be dependent on the nitrogen and phosphorus concentrations in the leaf tissues. Leaf nitrogen and phosphorus concentrations in legumes are influenced by the degree of nodulation and intensity of AM fungal infection and the extent to which the external AMF mycelium explores the soil volume surrounding the root. The ratio of AMF P-supply and nodule N-supply to the respective carbon demand can gives a quantitative index of host carbon expenditure necessary for the acquisition of nitrogen and phosphorus via these two symbiotic associations (Twary and Heichel 1991; Bethlenfalvay 1992).

Photosynthetic rates in the host plant of the *Glycine-Glomus-Rhizobium* symbiont system increased linearly with increasing leaf P or N concentration. Brown and Bethlenfalvay (1988) showed that the rate of photosynthetic CO_2 assimilation per unit leaf N or P was significantly greater in symbiotic than in nonsymbiotic plants. The experimental results of Brown and Bethlenfalvay (1988) provide sufficient evidence to counter the argument that the enhancement of photosynthetic nutrient-use efficiencies (N and P) in plants with microsymbionts can be explained as being exclusively due to increased stomatal conductance resulting from AMF colonization of the roots as suggested by Koide (1991). They found that soybean plants which had an association with one (*Glomus* or *Rhizobium*) or both microsymbionts always had greater photosynthetic rates per unit leaf N or P than nonsymbiotic plants with similar leaf N or P concentrations. Also efficient nutrient utilization by the N- and P-deficient symbiotic plants relative to the N- and P-sufficient non-symbiotic plants is shown by higher CO_2 assimilation rates in the former. The threshold of N- deficiency and P-deficiency for

youngest mature soybean leaves has been defined as < 40 mg N and < 1.5 mg P per g of leaf dry mass (De Mooy *et al.* 1973). Brown and Bethlenfalvay (1988) reported that most of the leaves of their symbiotic plants were below these values. The mechanisms responsible for the greater photosynthetic N- and P-utilization efficiencies that have been observed in N- deficiency and P-deficient symbiotic plants remain obscure.

A formal-analytical approach may help to elucidate the fundamental conceptual issues underlying plant nutrient-utilization efficiencies. One approach for achieving this involves the *decoupling* of photosynthesis and growth, photosynthesis involves nonstructural carbon or photosynthate (starch and sucrose) production from growth as structural biomass production.

1.6 Nitrogen-utilization efficiency

Under any given ambient CO_2 partial pressure, the upper limit of light-saturated photosynthetic rates is fixed by a relatively small set of physical and biochemical factors, for example: the stomatal conductance; concentration of activated rubisco catalytical sites; steady-state concentrations of sugar-phosphate intermediates of the Calvin cycle; chlorophastic orthophosphate concentration. Ågren (1985) provides a useful summary of the potential rates of biomass production theoretically achievable when the rate of CO_2 assimilation is calculated as a function of one of the following factors: irradiance; water supply; ambient CO_2; nitrogen. He concluded that nitrogen tissue concentration set the upper limit to plant productivity. In order to develop an AMF analysis of how nitrogen contributes to plant productivity, it is useful to begin with a quantitative analysis of the physical characteristics of the leaf photosynthetic

system.

The general response of light saturated rates of photosynthesis to increasing leaf nitrogen (g N m^{-2}) is generally curvilinear with two critical response regions: 1) a lower limit for photosynthetic rates corresponding to a certain threshold of leaf nitrogen concentration below which photosynthetic rates approach zero; and 2), an upper limit for photosynthetic rates which corresponds to threshold leaf nitrogen concentrations above which no increase in photosynthetic rate occurs (Sinclair and Horie 1989). In soybeans the rate of photosynthesis is zero at 1.0 g N m^{-2} and increases linearly for leaf nitrogen concentration between 1.0 and 2.4 g N m^{-2}; above 2.4 g N m^{-2} the response is curvilinear reaching a maximum of 1.6 mg CO_2 m^{-2} s^{-1} (Sinclair and Horie 1989).

1.7 Phosphate-utilization efficiency

Plant nutrient sufficiency levels for phosphorus in alfalfa on a percent dry mass basis ranges from 0.26 to 0.7 % (Miller and Donahue 1990 pp 370-371). Higher P tissue concentrations result in toxic effects. An interesting consideration is the relationship or correlation between the legume's relative growth rate and the capacity of the external hyphal system of the AMF to acquire and transfer phosphorus from the surrounding soil volume into the plant root system. It has been proposed that only 20% of the total root mass is involved in nutrient acquisition (Robinson 1986) and the most probable that the AMF-root association is restricted to this fraction of the total root mass. The fraction of root dry mass attributable to mycorrhizal fungi ranges from 12% to 14% for AMF legumes (Sieverding 1991). Functional relationships exist between specific growth rates, and the uptake rate per unit root mass of any mineral nutrient (Garnier et al., 1989).

When plant are grown at steady-state nutritional supply rate it has been shown for P (Ericsson and Ingestad 1988, Eissenstat *et al.* 1993) and for N (Cromer and Jarvis 1990, Ingestad and Agren 1991) that the slope of the relative growth rate (*RGR*) *vs* plant nutrient concentration is linear until a maximum *RGR* is reached. With steady-conditions state conditions of phosphate supply during exponential growth, tissue phosphorus concentration remains constant.

For sustained steady-state exponential growth a dynamic functional equilibrium must exist between the size and the specific activities of the microbial symbiotic associations and the plant. Maintenance of this dynamic functional equilibrium involves the regulatory control by the plant of the proportion of carbon lost in exchange for the quantity of phosphate necessary for sustaining exponential growth. Therefore with regard to phosphate limited growth, the maintenance of a constant tissue phosphorus concentration above a P-deficiency threshold is a necessary condition for sustained steady-state exponential growth. The constraint that plant tissue P-concentration imposes on plant growth is mediated via the effects that P has on key growth driving metabolic and physiological processes such as the Calvin cycle and phloem loading. Low-P treatment (plants grown on 0.05 mM phosphate) has a greater impact on plant biomass production (60% reduction compared to P-sufficient plants) than on the rate of photosynthesis; low-P treatment effected photosynthesis much less at low irradiances than at high radiances relative P-sufficient plants (plants grown on 1.0 mM phosphate), light saturated rates in leaves of P-deficient plants were decreased by 35% (Rao and Terry 1989). P-deficiency may decrease light saturated rates of photosynthesis by decreasing RuBP regeneration capacity and/or decreasing the concentration of activated rubisco (Brooks 1986; Brooks *et al.*1988). Under low-P conditions RuBP concentrations

declined to half the rubisco binding site concentration (Rao *et al*. 1989).

Maintenance of optimal rates of photosynthetic carbon assimilation, photosynthate translocation and nitrogen fixation during exponential growth of legumes requires the maintenance of an optimal constant concentration of phosphate in the leaf tissue and root nodules. Put differently, in the legume symbiotic system, phosphorus uptake rates must satisfy the metabolic phosphate demands of the Calvin cycle, phloem phosphate-loading system and the N_2-fixing nodule system, which are the necessary P-demands for sustaining optimal relative growth rates. Tight coupling between the relative growth rate and nitrogen supply rates have been shown to exist (Hirose *et al.*, 1988; MacDuff *et al.*, 1993), the same tight coupling applies between growth rates and phosphate supply rates (Ericsson and Ingestad 1988).

1.8 Functional relationships among the tripartite symbionts

The role of AMF association as a determining factor in legume growth becomes the basis for a reformulation of Davidson (1969) original expression defining the functional equilibrium between sizes and specific activities of the above ground and below ground components. In terms of the tripartite legume association the functional equilibrium or functional homeostasis between the components of the association needs to be defined with respect to the size and specific activities of the plant system (shoot and root) and the symbiont systems (AMF association and the nitrogen fixing nodules). The size and specific activity of the AMF association can be defined in terms of the sizes and specific activities of the shoot and the nitrogen fixing root nodule system.

AMF symbiotic associations form only with the actively growing younger tissues of the root system and the fraction of the root that is actively engaged in the foraging for nutrients within the soil. Older root tissue will be surrounded by a nutrient depletion zone. In order to define a functional relationship between the legume and its AMF component its useful to assume that the growth of the two symbionts is dependent on the resource exchanges (C and P) that take place between the two during exponential growth. It has been proposed that plant phosphate demand is a function of the inherent growth capacity of the plant as well as the minimum tissue phosphorus concentrations necessary to sustain maximum growth (Koide 1991).

If during balanced exponential growth a functional equilibrium is maintained between the steady-state rates of resource acquisitions (C, N, P) then it logically follows that the concentrations of various substrates (C, N, P) within the plant tissues will remain constant and the relative growth rates of the various components and associations can be expressed as a function of one or more of these substrate concentrations. The values of the different substrate concentrations will at any given time be determined by the rate of acquisition the external environmental. Rate of acquisition will be depended on the available supplies of the resources.

1.9 The motivation, aims and objectives of the present investigation

To conclude, legumes grown as crops provide a variety of benefits, primarily because of their input of fixed N_2, improving the physical and chemical properties of the soil. Symbiotic N_2 fixation of a legume genotype is influenced by the partner strain of *Rhizobium*. This N_2 fixation involves complex metabolic interactions between the

cytoplasm of the infected host plant cell and the *Rhizobium* bacteroids (Skot *et al.* 1986).

Arbuscular mycorrhizal fungi (AMF) play an important role in regulating carbon fluxes between biosphere and the atmosphere. AMF have wide host ranges, yet certain host and fungus combinations are more effective from either the perspective of the fungus, *i.e.* greater spore/hyphae production or from that of the host, *i.e.* enhanced growth, nutrient acquisition, or pathogen resistance (Schwab *et al*. 1991).

The roots of most high plants form arbuscular mycorrhizas with AM fungi. The legumes, such as *Vicia Faba L.* are also associated with *Rhizobium*. AMF infection can alleviate plant response to N and P content, even N–fixing bacteria, i.e. *Rhizobium* in the soil. Some authors have suggested that AMF infection may be even more important to plant growth under poor soil i.e. low N or low P conditions than plentiful N or P in the soil. The performance above each symbiosis (*Rhizobium* or AMF) in the legumes can be affected by various environmental and physiological factors, of which soil P availability is most studied (Bethlenfalay 1982). However, the performance of the tripartite legume-*Rhizobium*-Mycorrhizal symbiotic association that includes the effects of temperature, irradiance, nitrogen phosphorus supply is less studied.

1.9.1 The aims

The aims of this research were to study the influence of the tripartite legume – *Rhizobium* - AMF symbiotic association on photosynthetic parameters, and growth yield in *Vicia Faba L.* It was also to study the effect of N and P accumulation, elevated CO_2 and temperature on *Vicia faba* plants inoculated with an AMF, *Rhizobium* or both

(AMF and *Rhizobium*) compared with non-inoculated ones.

This study involves a number of core allocation hypotheses which are not really new with regard to our understanding of allocation in plant growth. However these hypotheses will be tested quite rigorously in a number of experiments and the results will be applied in the development of a new allocation model. The core allocation hypotheses involve the following propositions:

1). The existence and maintenance of a functional balance or functional equilibrium between the shoot activities and the root activities of the plant is the central assumption of the allocation model.

2). The balanced exponential growth or steady-state exponential growth of all plant components (leaves, stems and roots) depends on the existence and maintenance of this functional equilibrium between shoot and root.

3). The shoot provides substrate carbon (Wc) and the root provides substrate nitrogen (Wn) for plant growth. Given the existence and maintenance of: 1) a functional equilibrium between shoot and root activities; and 2) steady-state exponential growth of all plant components; it follows that concentrations of substrate carbon (C) and substrate nitrogen (N) within the plant tissues will remain constant at a given value which will be determined by external environmental factors.

4). To develop an optimization principle for an allocation strategy that will maximize growth for a given supply level of soil nitrogen, an appropriate optimization

problem must be formulated and solved. The plants specific growth rate (μ_w) needs to be maximized within the constraints of a given level of soil nitrogen supply. The optimization problem is: "what is the optimal root and shoot mass that ensures maximum specific growth rates (μ_w) for a given supply of soil nitrogen?" The solution of this optimization problem is the ultimate aim in this study. It involves defining the optimal allocation strategy for a given soil nitrogen regime.

1.9.2 Objectives

1). The overall guiding objective is to develop a model for the carbon economy of the tripartite legume-*Rhizobium*-AMF symbiotic association that includes the effects of temperature, irradiance and nitrogen supply. The inclusion of the micro-symbiont is the major objective of the research project.

2). This objective also involves conducting additional experiments that will contribute to the analysis of the carbon budget or carbon costs associated with N and P acquisition by the micro-symbiont associated and non-symbiotic association with the legume system.

3).The objectives of the project involve the data required for the development of this carbon optimal economy model for the legume system.

The experiments will provide the carbon cost associated with P and N acquisition with and without the micro-symbiont. The experiments will also provide data for estimating nitrogen use efficiency (NUE) and phosphate use efficiency (PUE). These efficiencies

are defined as the increment of biomass produced per unit of N or P supplied. The data will also show how P and N interact in photosynthesis and plant growth. However this last point requires additional multi-factorial experiments.

1.9.3 Experimental approach

A. Destructive experiments

i. Different nitrogen supply (0, 10, 25, 50, 100, 250 and 500ppm soil N): The plants will be harvested 3 times (T_1, T_2 and T_3) at 7 days harvesting interval. The first harvest (T_1) will commences 6 weeks or any appropriate period after germination. Harvest intervals (T_1-T_2-T_3) represent intervals for destructive harvesting for the determination of: LA; SLM; SLA; N and P content of all the components; relative growth rate (RGR); leaf, stem, root, old-seed and total mass, and W_l, W_s, W_r and W_g allometric coefficients and so on.

ii. Different nitrogen supply (0, 10, 25, 50, 100, 250 and 500ppm soil N) with two phosphorus (0.05 and 1.6 mmol P) concentration applied. Apart from the determination of variables will be done as same in (i.), the effects of the C:N:P supply ratios on light-saturated photosynthesis (P_{max}) and quantum efficiency (α) and how N and P supply rates influence photosynthetic responses to short-term increases in the CO_2 supply in plants that had been grown under normal ambient CO_2 concentrations.

iii. Tripartite symbiosis experiment:

The plants will be inoculated with *Rhizobium*, arbuscular mycorrhizal fungi (AMF), and

Rhizobium-AMF under two concentrations of N supply (10ppm and 250ppm soil N) and a P supply of 0.05mg P L^{-1} (1.61 mM NaH_2PO_4). The plants will be harvested twice at 7 days harvesting interval. The first harvest will be 6 or 7 weeks when the plants are growing exponentially. The leaf, stem, root, old seed and nodule, and N and P concentrations of different components for every harvest will be determined. Photosynthetic rate and respiratory rate for the whole plants under the tripartite symbiosis will be measured using IRGA or CIRAS. The measuring cuvette (0.63 m^2) for leaf photosynthetic parameters and the chamber for whole plant photosynthetic measurement of IRGA were designed by Dr. V.M. Gray (Picture 1.1). *Rhizobium* and AMF infections will beinvestigated.

B. Non-destructive experiments

The design of experiments and harvesting intervals (T_1-T_2-T_3) are the same as the destructive experiments (parallel experiments). Between the harvesting intervals, the IRGA (infra-red gas analyzer) or CIRAS will be used to determine the effects of nitrogen supply, temperature, irradiance and symbioses of the following photosynthetic parameters/variables: quantum yield efficiency (α), photosynthetic parameters (P, P_n, P_{Nmax}), respiratory rate (N), maintenance respiration (N_m) and growth efficiency (Y_G).

1.9.4. Experimental design and methods

The exercise to be undertaken in this project will be significantly different from previous models, and the distinguishing features of the proposed model are as follows:

Table 1.

External factors:	Plant parameters/variables:
Nitrogen supply	- leaf area during vegetative period
	- leaf, stem, root, seed and total mass
	- leaf N concentration (g/m^2)
	- relative growth rate (RGR)
	- photosynthetic parameters (P_{max})
	- respiration
	- specific leaf mass
	- allocation coefficients
Irradiance	- quantum yield efficiency (α)
	- photosynthetic parameters (P_{max})
Temperature	- quantum yield efficiency (α)
	- photosynthetic parameters (P_{max})
Tripartite symbiosis	-quantum yield efficiency (α)
	- photosynthetic parameters (P_{max})
	- relative growth rate (RGR)
	- respiration
	- N concentration inoculated with *Rhizobium*
	- P concentration inoculated with AMF
	- Nodule mass in response to low N levels

In this project the effects of the: 1) external abiotic factors namely Nitrogen supply concentrations, temperature, irradiance; and 2) Symbiotic associations on the variables listed in Table 1.will be investigated.

1.9.5 Variables and parameters data for modelling

The experiments of nitrogen supply will be repeated twice or three times, so as to facilitate the continuous monitoring of the above variables and parameters of temperature and irradiance in a range of nitrogen regimes. The list given below summarizes the data required to meet the empirical and modelling objectives of the project. It must be noted that variables and parameters can be subdivided into those which are measured directly and those where are derived directly from measurements. Linear and non-linear regression involving best fit curves to data points are the standard procedures used to generate values for variables and parameters from directly measured data.

1.9.6 Variable and parameter data listing

Daily leaf area increment (LA/d)

Daily stem mass increment (Sw/d)

Daily root mass increment (Rw/d)

Daily leaf mass increment (Lw/d)

Specific leaf area (SLA, m^2/g)

Specific leaf mass (SLM, g/m^2)

Leaf area - plant mass allometries

Leaf N content (mgN/gLDM) in N treatment

Stem N content (mgN/gSDM) in N treatment

Root N content (mgN/gW_r) in N treatment

Total N content (mgN/gW_g) in N treatment

R/S (shoot = stem + root mass) versus total N content (mgN/gW_g)

R/S versus N production (1/N/W)

R/S versus RGR (g/g/week)

LA (cm^2/plant) in N treatment

W_l/W_g in N treatment

$F_{sh} = F_l + F_r$, (Wsh/Wg) in N treatment

F_r, (W_r/W_g) in N treatment

Biomass (W_g) in N treatment

W_l leaf mass (g/plant) in N treatment

W_{st} stem mass (g/plant) in N treatment

W_r root mass (g/plant) in N treatment

W_{sd} seed mass (g/plant) in N treatment

Biomass (Wg) production (g/plant)

Specific leaf nitrogen content (g N/m^2)

GRG versus N treatment (ppm)

GRG versus specific leaf N content (g/m^2)

GRG versus tissue N content (mgN/g DM)

GRG versus total N content (mgN/W_g)

α versus total N content (g/W_g)

α versus leaf N content (g/g DM)

P_{max} versus specific leaf N content (g/m^2)

P_{max} versus plant tissue N content (g/gDM)

P_{max} versus total N content (g/W_g)

In the investigation of tripartite symbiosis, variables and parameters data will be added:

W_{nod} the nodule system dry mass under the tripartite symbiosis in N treatment

W_{AMF} the AMF dry mass under the tripartite symbiosis in N treatment

P concentration under the tripartite symbiosis in N treatment

The above list provides data with respect to physiological and morphological variables and parameters. These data will be derived from destructive or nondestructive experiments. The different allocation of biomass to leaves, stems and roots depends on the nitrogen supply. If the nitrogen supply rate is kept a constant over time, then a functional equilibrium will be established between the shoot and root. A consequence of the existence of a functional equilibrium will be derived during steady-state exponential growth, so that the ratios for W_l/W_g, W_s/W_g and W_r/W_g and the concentration in the leaf (mgN/gLDM), stem (mgN/SDM) and root (mgN/gRDM) remain constant. As long as the analysis of variance for both ratios and concentrations will be conducted, and if they are not significantly different for each treatment between harvest intervals, this will imply that the ratios and concentrations remain constants in exponential growth period is verified.

If the weekly leaf area, mass and the stem root mass increment is obtained, the balance exponential growth or steady-state exponential growth will be defined. If all above data or parameters and the relations derived by empirical and mathematical computation, a series of saturating functions or hyperbolic curves is observed and the fact that allocation of N and C to the leaf, stem and root will be confirmed. It may be follow the pattern best described by the "**Law diminished marginal returns**". In other words, the hypothesis will be supported with the statistical approach and mathematical approach. Finally, an optimization principal for an allocation strategy maximizing growth for a given supply level of soil nitrogen and appropriate optimization problem must be formulated and solved. The plant growth model under the different environmental conditions will be determined.

1.10 Structure of the thesis

The format in which this thesis is written by published papers. The body of the thesis consists of published 5 papers. It is stated in the Faculty of Science University of the Witwatersrand that a thesis by means of publication of papers should be contain: (i) an abstract of a specified length, (ii) a general introduction and literature review, (iii) the main body of the thesis consisting of publication, (iv) an overall discussion and conclusion, and (v) appendices. Although this thesis follows these guidelines, and the publications were presented in as similar format as possible to the original published form, it was necessary to make some editorial changes. This included figure numbering, unit and references format. It must also be understood that having individual published papers lends itself to some repetition, particularly in introduction and referencing of the individual papers.

The thesis consists of eight chapters which can be divided into two sections. The first section is made up of (i) different nitrogen supply (0, 10, 25, 50, 100, 250 and 500ppm soil N) and the plants will be harvested 3 times (T_1, T_2 and T_3) at 7 days harvesting interval and (ii) different nitrogen supply (0, 10, 25, 50, 100, 250 and 500ppm soil N) with two phosphorus (0.05 and 1.6 mmol P) concentration applied and the *Vicia faba* seeds were planted in 1600 cm^3 pots (150 mm diameter) filled with autoclaved river sand (medium grained with sieve mesh 2.5 mm) in order to non-producing the nodules like normal cereal crops. The plants will be harvested as same as (i). The main determination of: LA; SLM; SLA; N and P content of all the components; relative growth rate (RGR); leaf, stem, root, old-seed and total mass, and W_l, W_s, W_r and W_g

allometric coefficients and so on. Apart from the determination of variables will be done as same in (i.), the effects of the C:N:P supply ratios on light-saturated photosynthesis (P_{max}) and quantum efficiency (α) and how N and P supply rates influence photosynthetic responses to short-term increases in the CO_2 supply in plants that had been grown under normal ambient CO_2 concentrations, as shown in chapter 1, 2 and 3.

The second section is tripartite symbiosis experiment including chapter 5 and 6. The experimental design was a randomized complete block with four replications for each harvest interval, with combinations of treatment factors randomly assigned to pots in the block. The concentration of nitrate-N for the low N (LN) treatments was 0.71 mM KNO_3 (10 ppm N) and 17.86 mM KNO_3 (250 ppm N) for the high N treatments (HN). All treatments received a phosphorus supply of 0.05mg P L^{-1} (1.61 mM NaH_2PO_4). The various treatments were: (a) two different N supply rates without any microbial symbiotic associations, LN and HN; (b) two different N supply rates plus AMF association, LNM and HNM; (c) two different N supply rates plus *Rhizobium* association, LNR and HNR; and (d) two different N supply rates plus both AMF and *Rhizobium* symbiotic associations: LNMR and HNMR. Plants were harvested at 54 d (4 October, T_1) and 63 d (13 October, T_2) after planting. The effects of nitrogen supply, temperature, irradiance and symbioses of the following photosynthetic parameters/variables: quantum yield efficiency (α), photosynthetic parameters (P, P_n, P_{Nmax}), respiratory rate (N), maintenance respiration (N_m) and growth efficiency (Y_G) were measured with Destructive and non-destructive experiments. Chapter 7 and chapter 8 are discussion and appendices respectively for whole research.

Picture 1. Photosynthetic Instruments used in the experiments.

A-1. Chamber for measuring photosynthesis and respiration of the whole plant.

A-2. ADC infra-red gas analyzer (IRGA) machine.

A-3. water bath device for regulating temperature of the chamber.

B. Portable CIRAS-1, PP IR gas analysis system.

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CHAPTER TWO:

INTERRELATIONSHIPS BETWEEN NITROGEN SUPPLY AND PHOTOSYNTHETIC PARAMETERS IN *VICIA FABA* L.

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Chapter 2:

Interrelationships between Nitrogen Supply and Photosynthetic Parameters in *Vicia faba* L.

2.1 Abstract

The influence of nitrogen uptake and accumulation on the values of photon saturated net photosynthetic rats (P_{Nmax}), quantum yield efficiency (α), intercellular CO_2 concentrations (C_i) and carboxylation efficiency (C_e) was determined in *Vicia faba* L. As leaf nitrogen concentration (N_L) increased, the α converged onto a maximum asymptotic value of 0.0664±0.0049 µmol (CO_2) µmol (Quantum)$^{-1}$. Also, as N_L increased the C_i value fell to an asymptotic minimum of 115.80±1.59 µmol mol^{-1}, and C_e converged onto a maximum asymptotic value of 1.645±0.054 µmol (CO_2) m^{-2} s^{-1} Pa^{-1} and declined to zero at a N_L-intercept equal to 0.596±0.096 g (N) m^{-2}. The α fell to zero for an N_L-intercept of 0.660±0.052 g (N) m^{-2}. As N_L increased, the value of P_{Nmax} converged onto a maximum asymptotic value of 33.400±2.563 µmol (CO_2) m^{-2} s^{-1}. P_N fell to zero for an N_L-intercept for 0.710±0.035 g (N) m^{-2}. Under variable daily meteorological conditions the values for $N_{L,}$ specific leaf area (δ_L), root mass fraction (R_f), P_{Nmax} and α remained constant for a given N supply. A monotonic decline in the steady-state value of R_f occurred with increasing N supply. δ_L increased with increasing N supply or with increasing N_L.

Key words: carboxylation efficiency; faba bean; intercellular CO_2 concentration; net photosynthetic rate; quantum yield efficiency; root mass fraction.

2.2 Introduction

In general, the overall relationship between the photosynthetic rate and leaf nitrogen content (N_L) follows a curvilinear pattern, with the linear portion of the curve originating from a positive N_L-intercept (Schmitt and Edwards 1981, Sage and Pearcy 1987, Sinclair and Horie 1989, Meinzer and Zhu 1998). As N_L increases, the photosynthetic rate converges onto an asymptotic maximum value. This curvilinear relationship arises as a direct consequence of various rate limiting processes which play a role in setting the upper limit for the maximum possible values obtainable for photosynthetic parameters such as the photon-saturated net rate of photosynthesis (P_{Nmax}) and the quantum yield efficiency (α) as N_L increases. For example, the maximum value for P_{Nmax} is directly proportional to the flux of CO_2 from the atmosphere. The rate of CO_2 diffusion into the leaf is directly proportional to the atmosphere-leaf CO_2 concentration gradient, the stomatal conductance, the mesophyll conductance, and the carboxylation efficiency of the leaf (Charles-Edwards 1978, Fisher *et al*. 1981, Evans and von Caemmerer 1996, Katul *et al.* 2000). The carboxylation efficiency (C_e) is dependent on the concentration of the various enzyme catalytic sites available for the different reactions involved in photosynthetic metabolism. Theoretically the upper limit to the maximum value for P_{Nmax} which also determines the plant potential photosynthetic capacity is proportional to the number of active catalytic sites in chloroplasts that are involved in the reductive assimilation of CO_2. The total number of these catalytic sites involved in CO_2 assimilation is dependent on the proportion of the total N_L allocated to the chloroplasts.

The asymptotic relationship between the P_{Nmax} or α and N_L indicates that as the number

of catalytic units increases, other processes become in turn rate limiting. As N_L increases, these processes will in turn fix the upper limit of the plant N efficiency for biomass production. In this respect, the rate of carbon dioxide diffusion into the leaf will be a factor limiting the plant N dependent capacity for biomass production (Evans and von Caemmerer 1996, Katul *et al*. 2000). Other transport processes such as the rate of phosphate recycling between the chloroplast and cytosol also play a prominent role in fixing the upper limit of the plant capacity for biomass production (Cockburn *et al*.1967a, b, Usuda and Edwards 1982, Pradet and Raymond 1983, Mächler *et al*. 1984, Rao and Terry 1989, 1995, Rao *et al*. 1989a, b).

If the concentration of nitrogen supply (N_s) to the roots remains constant while the plant exponential growth, then steady-state content of N within the plant can only be maintained if the capacity for N accumulation increases exponentially during plant growth (Hirose 1986, Garnier *et al*. 1989). In this context, the term balance exponential growth has been used to describe the situation where under constant conditions of nutrient supply, moisture availability, and saturating irradiances, all extensive variables (*e.g.* plant biomass and leaf area) increase exponentially at a constant growth specific growth rate (μ_w). Under these conditions all variables that are either ratios or rates (*e.g.* plant N concentration) remain constant (Thornley 1998). Under balanced exponential growth, it is expected that μ_w or specific photosynthetic rate would be determined by the plant steady-state N_c. This was an important consideration in this study. Under balanced exponential growth, it would also be expected that ratios such as the root mass fraction (R_f) and the specific leaf area (δ_L) would remain constant. Information on these predictions does not exist for *Vicia faba*. For *V. faba* there are no reports on how N_L influences P_{Nmax} and α. Therefore, the objectives of this study were: (1) determination

of the asymptotic values and associated intercept values for P_{Nmax} and α with respect to $N_{L;}$ (2) provision of information on the minimum value of N_L below which P_{Nmax} and α equal zero; (3) establishing whether intensive variables or ratios such as P_{Nmax}, α, N_c, N_L, δ_L and R_f remain constant under steady-state N_s. The latter information will help decide whether these intensive variables or ratios can be treated as unchanging plant growth parameters whose values are governed by the steady-state N_s.

2.3 Material and Methods

2.3.1 Plant

We used the cold-hardy *Vicia faba* L. cv. Aquadulce Claudia. Early spring or autumn planted cold-hardy varieties require approximately 130-150 d for crop development, while over-wintering cultivars require approximately 240 d to reach dry seed maturity. Faba beans germinate and grow well in cool soil. For out-door experimental pot trials seeds were sown on the 23rd February and all measurements were done in April. During April, the daily average extraterrestrial global irradiance was 28.7 MJ m^{-2}, average photoperiod was 11.99 h, average daily maximum temperature was 21.6℃, average daily minimum temperature 9.1℃, and average A-pan evaporation was 6.3 mm d^{-1}.

Faba bean seeds were planted in 1600 cm^3 pots (150 mm diameter) filled with autoclaved river sand (medium grained with sieve mesh 2.5 mm). Two seeds were planted in each pot and after germination one seedling per pot was selected so as to give an initial uniform population of seedlings. All pots were watered every second day with tap water until emergence of cotyledons. Once the seedlings had emerged, they were

watered every second day with a modified Long Ashton nutrient solution. The modifications involved the application of the nitrogen in the form KNO_3 at the following concentrations of N: 0, 0.714, 1.786, 3.571, 7.143, 17.857, 35.714 mM N (equivalent to 0, 10, 25, 50, 100, 250, 500 g (N) m^{-3}). All zero nitrogen treatments were watered using N-free Long Ashton nutrient solution. Dry biomass, nitrogen, and photosynthesis were measured for three harvest intervals: 44 (T_1, 8th April), 51 (T_2, 15th April) and 58 (T_3, 22nd April) d after planting (DAP).

2.3.2 Biomass determination

At each harvesting interval, the roots were rinsed carefully with tap water to remove sand. The plants were divided into leaf, stem and root components, respectively. Before drying, the leaf area was measured using a *Li-Cor 3100* area meter (*Li-Cor,* Lincolin, NE USA). The leaves, stems and roots were dried in the oven at 105℃ for 15 min and then at 65℃ for 3 d for dry mass determinations.

2.3.3 Tissue N content analysis

After the determination of dry mass, the dried leaves, stems and roots were milled for N analysis. 0.100±0.001g milled plant material were digested by the Kjeldahl procedure. The total N content of the digests was determined using colorimetric assays (Dorich and Nelson 1983, Anderson and Ingram 1993). For the Kjeldahl digestion mixture, 0.42 g of selenium powder and 14 g lithium sulphate was added to 350 cm^3 of 30% hydrogen peroxide, and 450 cm^3 of concentrated sulphuric acid was added slowly to the mixture that was kept cool in an ice bath.

For the colorimetric determination of ammonium nitrogen, two reagent solutions were used. The first reagent solution was made up of 34 g sodium calculate, 25 g of sodium citrate and 25 g sodium tartarate dissolved together in 750 cm^3 H_20. To this mixture 0.12 g sodium nitroprusside was added and when dissolved the solution was made up to 1000 cm^3 with H_2O. The second reagent solution was made of 30g sodium hydroxide dissolved in 750 ml H_2O. After cooling, 10 cm^3 of sodium hypochlorite was added and the solution was brought up to 1000 cm^3. For the ammonium assay, a 0.1 cm^3 ammonium sample was added to a 25 cm^3 Erlenmeyer flask and 5.0 cm^3 of both the first and second reagent solutions were added. After 1 h the absorbance was measured at 665 nm.

2.3.4 Gas exchange measurements

Photosynthetic gas exchange rates were determined at the three harvest intervals on equal-aged cohorts. All plants were fully acclimatised to full sunlight. For the measurement of C_i and C_e, the portable *CIRAS-1, PP IR* gas analysis system (*PP system*, Hitchin, Hertfordshire, U.K.) was used. For the estimation of P_{Nmax} and α, an *ADC* Infra-red gas analyser (IRGA) *225-2B-SS* (*Analytical Development*, Hoddesdon, Hertfordshire, England) on differential mode was used. These values were measure at 44, 51 and 58 DAP. Leaf surfaces were exposed to photosynthetically active photon flux densities (PPFD) ranging from 75 to 1800 µmol (Quantum) m^{-2} s^{-1} by adjusting the height of a 400 W halide (*Power Star HQ1*) lamp above the leaf surface. The PPFD at the leaf surface was measured with a *LI-188B* quantum meter. P_N was measured on the youngest fully expanded leaves. The trifoliate leaflet was clamped into and sealed in a

Perspex leaf chamber that allowed for the irradiance of 0.63 cm^2 of leaf area. The chamber was surrounded by a water jacket connected to a temperature regulated water bath water (*SS-CD-5, Specht Scientific*, Johannesburg, SA). Leaf temperature was continuously monitored by means of a thermocouple (Model *BAT-12, Bailey Instruments*, NJ, USA) touching the underside of the lamina within the leaf chamber. The leaf temperature was maintained at 25^oC for all PPFDs. Average barometric pressure during photosynthetic measurements was 83.5 kPa.

2.3.5 Experimental design and statistical analysis

The experiment involved a completely randomized 3x7 factorial design. The experimental factors were three different harvest intervals (T_1, T_2 and T_3) and seven N treatments. A replicate consisted of one faba bean plant per container. The appropriate data were analysed using ANOVA. A Tukey's multiple comparison test was performed to determine which treatments differed. When appropriate, means ± SE (standard error) were calculated, and when the F ratio was significant, the least significant differences were evaluated by the Tukey HSD-test to examine differences among the harvesting intervals (T_1, T_2 and T_3) in each treatment. *SYSTAT* version *8.0* was used for all data analyses.

2.4 Results

2.4.1 Effects of N supply on biomass production and N accumulation

For each nitrogen supply concentration (N_s) the nitrogen contents (N_c) in leaves, stems, and roots remained constant over the three harvest intervals (Fig 1). While the total

quantity of elemental N accumulated per plant (N_t) increased with increasing N_s (Fig.3), N_c for the different tissue components always remained constant over time for each N_s N_s (Figure 2.1). Hence that the plant capacity to accumulate N increased exponentially or kept pace with the rate of biomass production. Also under conditions of N_s, the specific leaf nitrogen (N_L), specific leaf area (δ_L), root mass fraction (R_f), and P_{Nmax} all remained constant over the three harvest intervals (Figure 2.2). All these results are consistent with the balanced exponential growth hypothesis. Increasing N_s promoted increases in the production of total biomass, total plant leaf area, and total accumulation of nitrogen (Figure 2.3).

2.4.2 Effects of N_s on R_f, δ_L and N_L

Both R_f and δ_L declined slightly with increasing N_s (Figure 2.2). However, these slightly declining differences in R_f and δ_L in response to increasing N_s were significantly different at the 5% level. There were no significant differences at the 5% level among three harvest intervals within the specific N treatments. Even though both total biomass and leaf area production increased significantly with increasing N_s, the values for R_f and δ_L remained constant over the three harvest intervals. The δ_L also declined slightly with increasing N_L. N_L expressed on a leaf area basis also remained constant over time for a given steady state N_s (no significant differences at the 5% level among three harvest intervals within the N_L treatments). These results are consistent with the balanced exponential growth hypothesis that predicts that if N_s is constant then intensive variables such as R_f, δ_L, and N_L will also remain constant during plant growth and the capacity to accumulate N also increases exponentially. Therefore in modelling N uptake during plant growth R_f, δ_L, and N_L should be used as parameters.

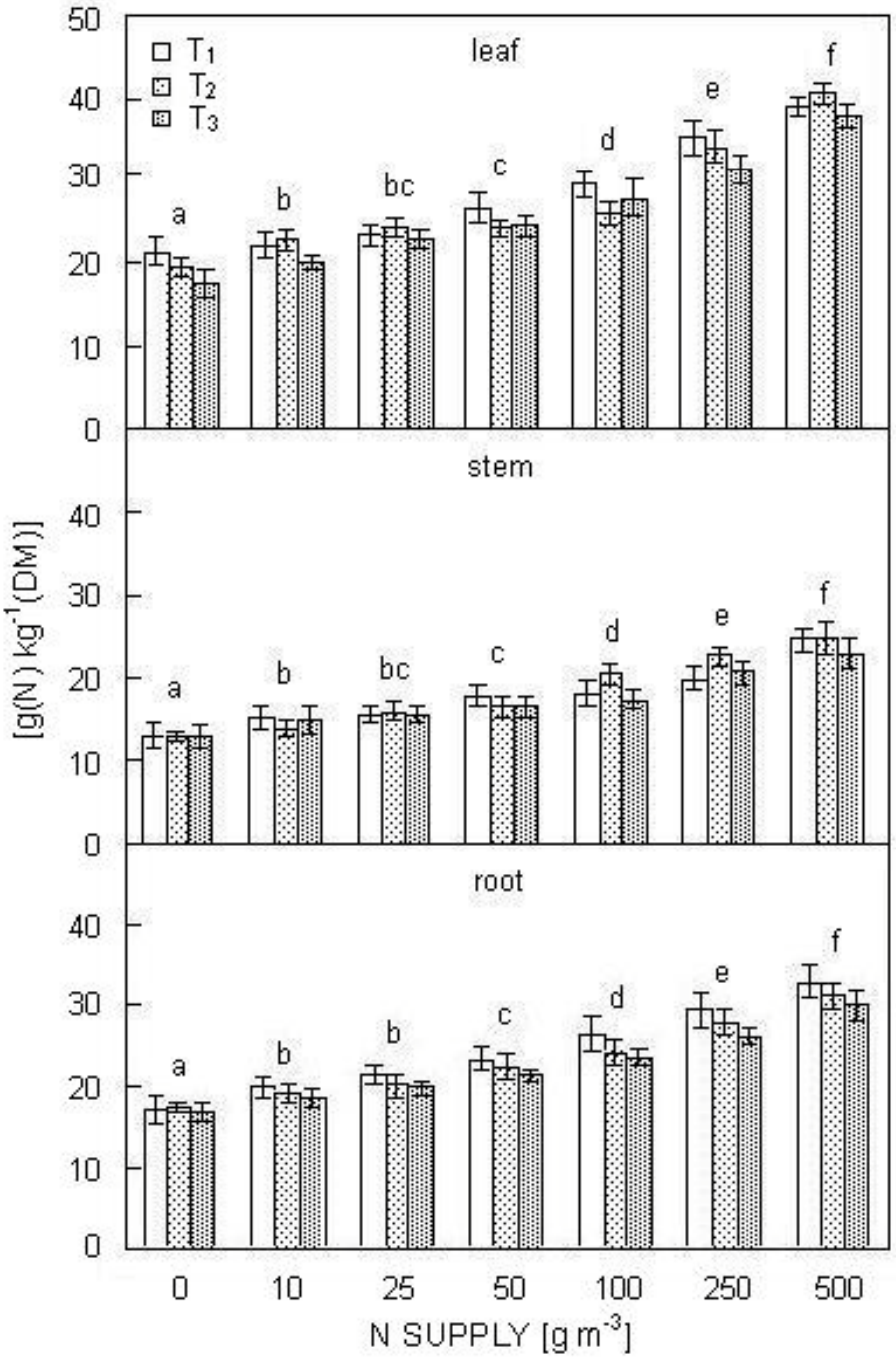

Figure 2.1. Steady-state leaf, stem, and root N contents (N_c) at the three harvests (T_1, T_2, and T_3) in response to different N supply (0, 10, 25, 50, 100, 250, and 500 g m^{-3}). *ANOVA* results for tissue N_c did not indicate significant differences (at the 5 % level) among the harvest intervals within the individual N treatments, but the differences among the N treatments were significant. *Different letters* indicate significant different among treatments assessed by Tukey HSD-test ($p < 0.05$).

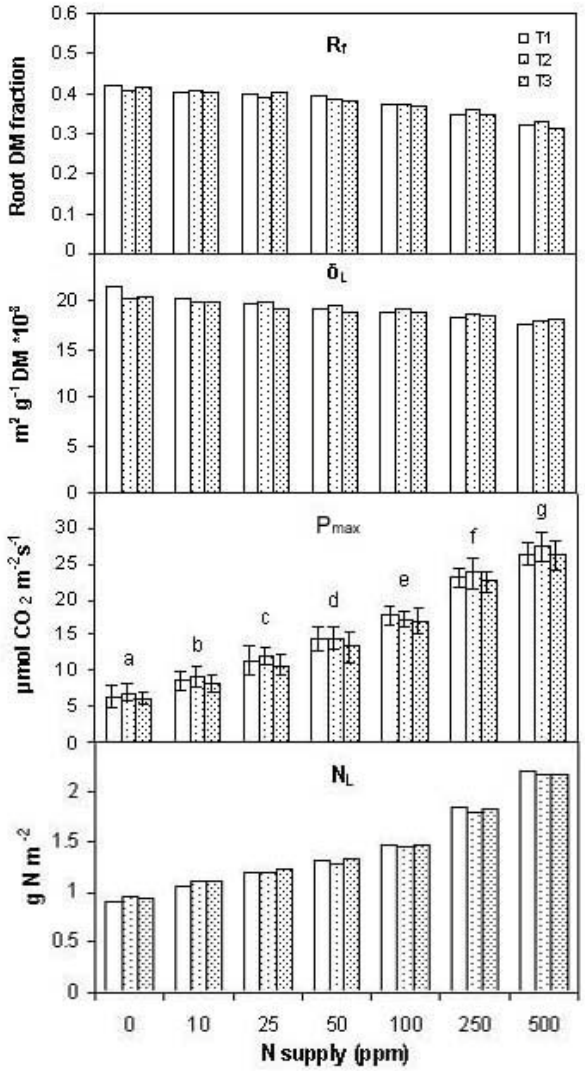

Figure 2.2. The influence of increasing N supply on root dry mass fraction (R_f), specific leaf area (δ_L), photon-saturated net photosynthetic rate (P_{Nmax}), and specific leaf N content (N_L) at the three harvest intervals (T_1, T_2, and T_3). *ANOVA* results did not indicate significant differences (at the 5 % level) among the harvest intervals for individual N treatments, but the differences among the N treatments were significant. For P_{Nmax} results, *different letters* indicate significant different among treatments assessed by Tukey HSD-test ($p < 0.05$).

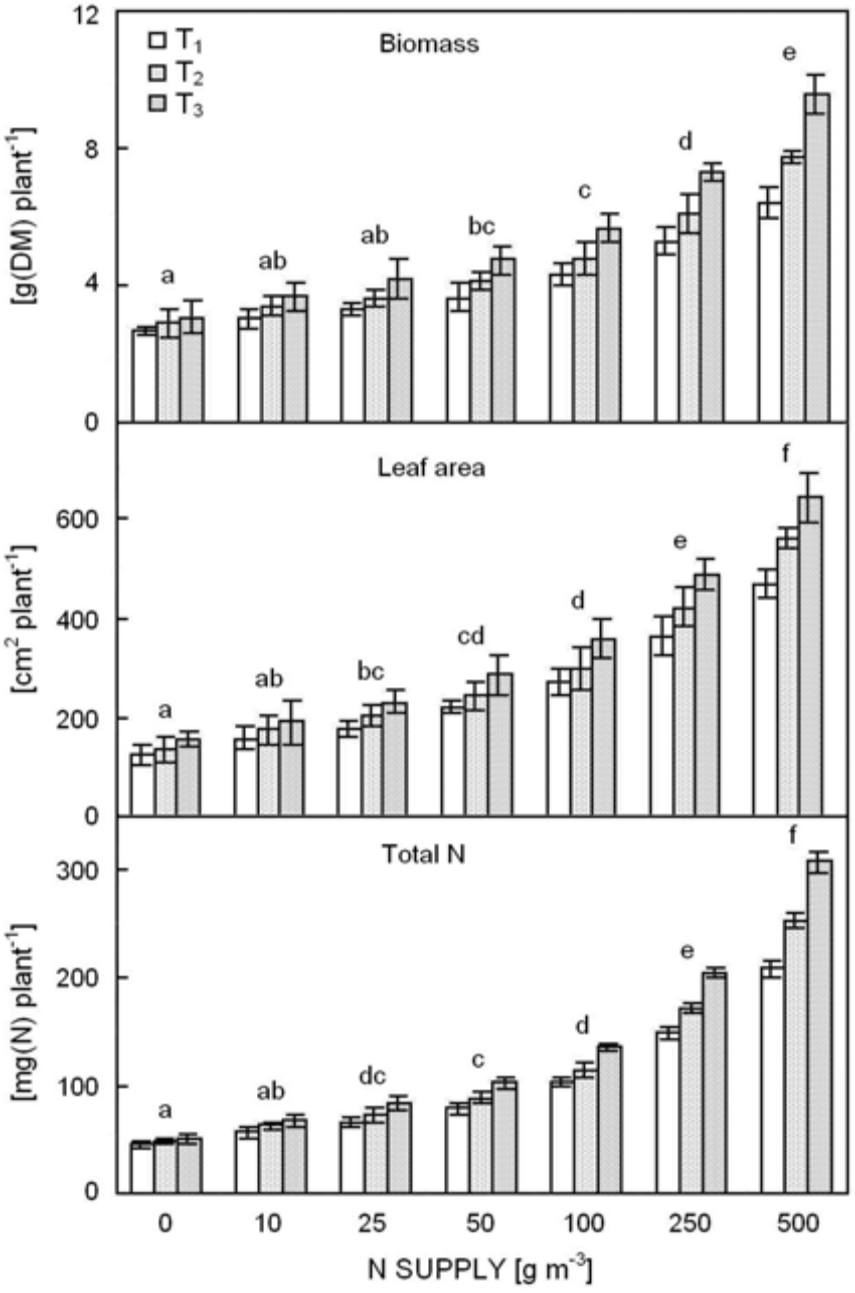

Figure 2.3. The influence of increasing N supply on total biomass production, leaf area production, and total plant N accumulation (N_t) measured at the three harvests (T_1, T_2, and T_3). *ANOVA* for all the results indicated (*1*) significant differences (at the 5 % level) among the N treatments, and (*2*) significant differences among the harvest intervals within the N treatments. *Different letters* indicate significant different among treatments assessed by Tukey HSD-test ($p < 0.05$).

2.4.3 α versus N_s

How does leaf N_c affect α, given the fact that the total leaf area per plant and the total number of catalytic units per unit leaf area increases markedly with increasing N_s (Fig. 3)? For *V faba,* α showed a curvilinear response to increasing N_L (Figure 2.4). The empirical equation of Sinclair and Horie (1989) was used for generating the asymptotic value for α and the N_L-intercept value for α = 0. With increasing N_L, α converged onto a maximum asymptotic value of 0.0664±0.0049 μmol (CO_2) μmol（quantum）$^{-1}$. α fall to zero for N_L-intercept of below 0.6600±0.0515 g(N) m^{-2}. For a given N_s α remained constant over all three harvest intervals (values not shown); this confirms the validity of the premise that a photosynthetic parameter such as α will also remain constant under steady-state or constant N_s. The curvilinear relationship between α and N_L for faba bean was quantitatively very similar to those reported for soybean, wheat and maize by Sinclair and Horie (1989).

2.4.4 P_{Nmax} versus N_s

Again it may be asked: How does N content affect the light saturated rate of photosynthesis, given the fact that the total leaf area per plant and the total number of catalytic units per unit leaf area increases markedly with increasing N supply? Light saturated CO_2 assimilation increased in a curvilinear fashion with respect N_L (Fig. 4) and the empirical equation of Sinclair and Horie (1989) was used for deriving the asymptotic value for P_{Nmax} and the N_L -intercept value for P_{Nmax} = 0. With increasing leaf N content the value of P_{Nmax} converged onto a maximum asymptotic value of 33.4

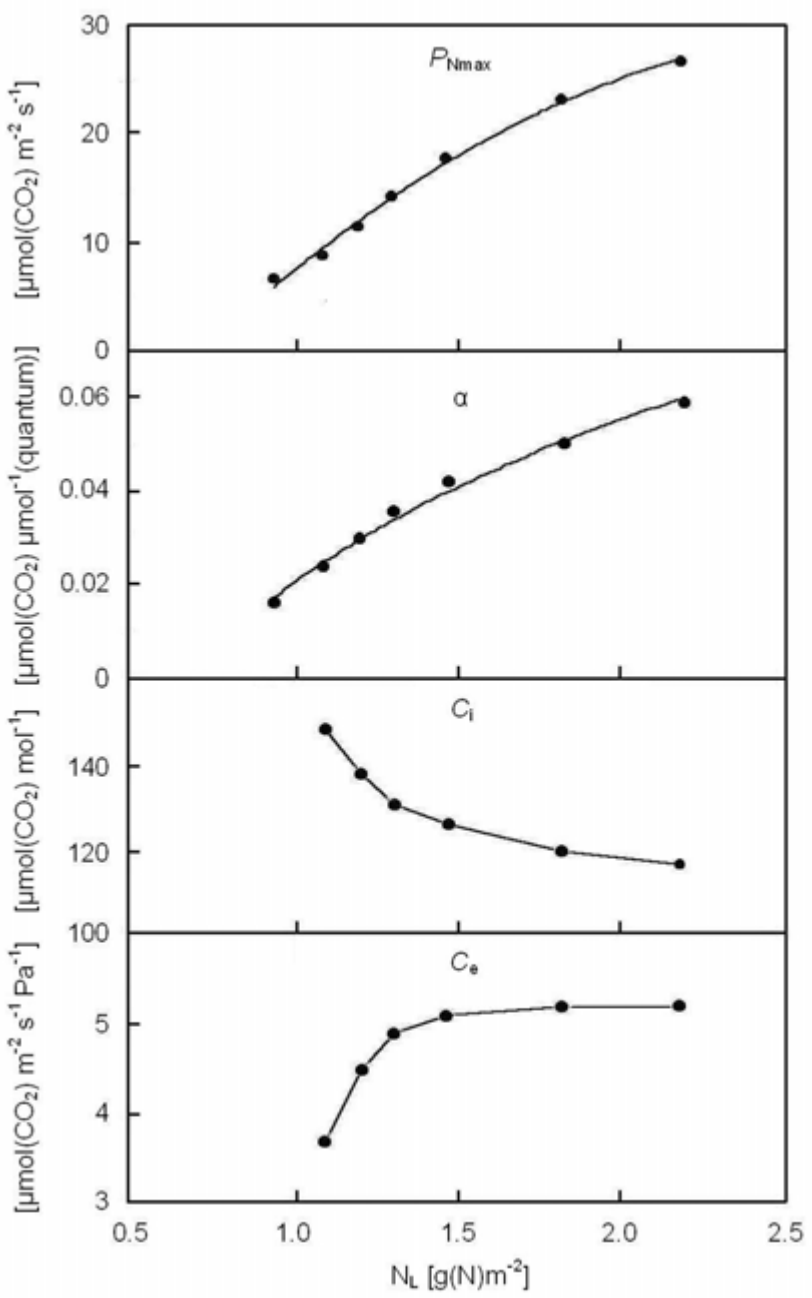

Figure 2.4. The influence of increasing specific leaf nitrogen (N_L) on photon saturated CO_2 assimilation rate (P_{Nmax}), quantum yield efficiency (α), leaf intercellular CO_2 concentration (C_i), and the carboxylation efficiency (C_e).

± 2.563 µmol CO_2 m^{-2} s^{-1}. Maximum asymptotic values with increasing N_L measured for maize, soybean and rice were 57, 36 and 34 µmol CO_2 m^{-2} s^{-1} respectively (Sinclair and Horie 1989).

2.4.5 C_i and C_e versus nitrogen supply

In Vicia *faba* as N_L increased the intercellular CO2 concentration (C_i) declined exponentially at an exponential of 3.53 ± 0.29 µmol mol^{-1} (g N m^{-2})$^{-1}$ to an asymptotic minimum C_i value of 115.80 ± 1.59 µmol mol^{-1}. A strongly rectangular hyperbolic relationship was observed to exit between the carboxylation efficiency (C_e) and N_L (Figure 2.5) with a maximum C_e asymptotic value of 1.645 ± 0.054 µmol CO_2 m^{-2} s^{-1} Pa^{-1} CO_2 and with an N_L-intercept equal to 0.596 ± 0.096 g N m^{-2}.

2.5 Discussion

2.5.1 The relationship between α and N_L

In this study the quantum yield efficiency with respect to increasing leaf N reached a maximum of 0.0664 ± 0.0049 µmol CO_2 µmol $quanta^{-1}$. Meinzer and Zhu (1998) reported that quantum yield efficiency for CO_2 uptake in sugarcane increased linearly from 0.042 to 0.075 µmol CO_2 µmol $quanta^{-1}$ with leaf N content. Approximately doubling as leaf N content, it was increased from 0.63 g m^{-2} to 1.54 g m^{-2}. This dependence of quantum yield efficiency on leaf N content was similar for different sugarcane clones. So, it is thus clear that any dynamic model of leaf photosynthesis

should not assume that the value of α is not influenced by tissue N content. Other quantum yield efficiencies measurements for CO_2 uptake (measured at leaf temperatures of 30°C, 330 cm^3 m^{-3} CO_2 and 21 kPa O_2) in various monocot and dicot plants, with different photosynthetic pathways give the following range of α values (μmol CO_2 μmol $quanta^{-1}$): C_3 dicots, 0.052 ± 0.001; C_3 grasses, 0.053 ± 0.001; C_4 (NAD-ME) dicots, 0.053 ± 0.001; C_4 (NAD-ME) grasses, 0.060 ± 0.001; C_4 (PCK) grasses, 0.060 ± 0.002; C_4 (NADP-ME) dicots, 0.061 ± 0.002; C_3 (NADP-ME) grasses, 0.065 ± 0.001; C_4 (ME-MIX) dicot, 0.057 (Pearcy and Ehleringer, 1984). Ehleringer and Björkman (1977) found α value for C_3 photosynthesis of 0.081 μmol CO_2 μmol $quanta^{-1}$ under conditions where oxygenase activity was suppressed (low O_2 or high CO_2 concentrations). At a leaf temperatures of 20°C, quantum yield efficiencies (α) of 0.066 μmol CO_2 μmol $quanta^{-1}$ and 0.044μmol CO_2 μmol $quanta^{-1}$ has been measured for field grown ryegrass and clover respectively a value of 0.044 μmol CO_2 μmol $quanta^{-1}$ (unpublished data, Papadopoulos, 1992 MSc Thesis). Thus，it is reasonable to assume that depending on leaf N content quantum yield efficiencies for CO_2 uptake in C_3 and C_4 plants can range from 0.016 to 0.075 μmol CO_2 μmol $quanta^{-1}$.

2.5.2 The relationship between P_{Nmax} and N_L

For faba bean, the N_L - intercept for saturated rates of net photosynthesis was 0.710 ± 0.0345 g N m^{-2}. In other studies the linear response of P_{Nmax} to N_L rose from: zero at 1.0 g N m^{-2} to about 2. 4 g N m^{-2} for soybean, zero at 0.3 g N m^{-2} to about 1.6 g N m^{-2} for rice, and zero at 0.2 g N m^{-2} to 0.6 g N m^{-2} for maize (Sinclair and Horie 1989). With regard to *Chenopodium album* L. (C_3) and *Amaranthus retroflexus* L (C_4), the

A_{max} intercept of the N_L axis ranged from 0.64 to 0.78 g N m^{-2} (Sage and Pearcy 1987), for a given N supply rate the values for the corresponding P_{Nmax} remained constant over all three-harvest intervals (Fig. 2). This result confirms the validity of the premise that a photosynthetic parameter such as P_{Nmax} will also remain constant under steady-state nitrogen supply conditions.

2.5.3 The relationship between C_i, C_e and N_L

The steady-state functional C_i/C_a ratio is not only determined by plant water status and stomatal conductance, the C_i/C_a ratio also influenced by N_L (where C_i and C_a represent the intercellular and atmospheric CO_2 concentration respectively). With regard to benchmarking C_i values, it is generally accepted that at 25℃, under photosynthetically saturating irradiances and ambient CO_2, the intercellular CO_2 is approximately 100 μmol mol^{-1} for C_4 species and 250 μmol mol^{-1} for C_3 species, and the compensation point (Γ) is close to 0 for C_4 versus 5.0 μmol mol^{-1} in the case of C_3 species (Evans and von Caemmerer 1996). C_i values for a C_3 plants such as *Pinus pinaster* have been reported to range from 103 to 266 μmol mol^{-1} (Warren *et al*. 2000; Warren and Adams 2001).

Carboxylation efficiency C_e has been expressed as the reciprocal of mesophyll resistance r_m (Edwards and Walker, 1983). The following range of r_m values have been reported for C_3 plants: *Glycine max* 2-3 s cm^{-1}; *Atriplex hastata* 2.6 s cm^{-1}; *Phaseolus* spp 2.6 s cm^{-1}; *Triticum aestivum* 2.8 s cm^{-1} *Solanum tuberosum* 5.4 s cm^{-1}; *Medicago sativa* 2.8 s cm^{-1}. Thus, the carboxylation efficiencies for the above C_3 plants range

from 0.0019 to 0.005 m s^{-1}. In other experiments the following carboxylation efficiency values have been reported for C_3 plants: *Helianthus* 0.0063 m s^{-1}, *Xanthium* 0.0048 m s^{-1}; *Vigna* 0.0036 - 0.0047 m s^{-1}; *Tilia* 0.0035 m s^{-1}; *Ficus* 0.0031 m s^{-1} and Citrus 0.0018 - 0.0024 m s^{-1} (Laisk and Loreto 1996).

Assuming a temperature of 20°C and air pressure of 101 315 Pa (given that under these conditions CO_2 $litre^{-1}$ = 41.6 x 10^{-6} mol CO_2 m^{-3} = 0.101 Pa CO_2) the range of reported carboxylation efficiency values reported for various C_3 plants can be recalculated to give the following range of equivalent values 0.741 to 2.595 :mol CO_2 m^{-2} s^{-1} Pa^{-1} CO_2 In general carboxylation efficiencies ranged from 1.046 to 1.478 µmol CO_2 m^{-2} s^{-1} Pa^{-1} CO_2 for C_3 *Cyperus* species and from 2.529 to 4.123 µmol CO_2 m^{-2} s^{-1} $Pa^{-1}CO_2$ for C_4 *Cyperus* species (Li 1993). Teh carboxylation efficiency values estimated for faba bean also fell within the C_3 benchmark range of values

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CHAPTER THREE:

INFLUENCE OF PHOSPHORUS AND NITROGEN ON PHOTOSYNTHETIC PARAMETERS AND GROWTH IN *VICIA FABA* L.

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Chapter 3:

Influence of Phosphorus and Nitrogen on Photosynthetic Parameters and Growth in *Vicia faba* L.

3.1 Abstract

The influence of phosphorous (P) and nitrogen (N) supply on biomass, leaf area, photon saturated photosynthetic rates (P_{max}), quantum yield efficiency (α), intercellular CO_2 concentration (C_i) and carboxylation efficiency (CE) was investigated in *Vicia faba* L. The influence of P on N accumulation, biomass and leaf area production was also investigated. An increase in P supply was consistently associated with an increase in N accumulation and N productivity in terms of biomass and leaf area production. Furthermore, P increased the photosynthetic N use efficiency in terms of P_{max} and α. An increase in P was also associated with an increase in CE and a decrease in C_i. Under variable daily meteorological conditions specific leaf nitrogen content (N_L), specific leaf phosphorus content (P_L), specific leaf area (δ_L), root mass fraction (R_f), P_{max} and α remained constant for a given N and P supply. A monotonic decline in the steady-state value of R_f occurs with increasing N supply. δ_L increased with increasing N supply or with increasing N_L. This study tests the hypothesis that P supply positively affects both N demand and photosynthetic NUE by influencing the upper limit of the asymptotic values for P_{max}, □ and □CE, and the lower limit for C_i in response to increasing N.

Additional key words: carboxylation efficiency (CE); intercellular CO_2 concentration (C_i); leaf area; nitrogen use efficiency (NUE) photon saturated photosynthetic rates

(P_{max}); quantum yield efficiency (α); root; stem.

Abbreviations: α - quantum yield efficiency; C_a - ambient CO_2 concentration; CE - carboxylation efficiency; C_i - intercellular CO_2 concentration; DM - dry mass; $[E_a]$ – content of activated Ribulose-1,5-bisphosphate carboxylase/oxygenase; h - leaf thickness; k_{cat} - Ribulose-1,5-bisphosphate carboxylase/oxygenase turnover rate; k_{cat}^{app} – P-dependent apparent catalytic constant for Ribulose-1,5-bisphosphate carboxylase/oxygenase; Km_{appCO2} - apparent Km for CO_2 at varying O_2 concentrations; K_N - Michaelis constant for N; K_N^{app}- apparent specificity constant for N; K_{NP} - kinetic coefficient; K_P - Michaelis constant for P; M_r - the maintenance respiration; N_L - leaf nitrogen per m^2; N_{plt} – total plant elemental N; NUE - nitrogen use efficiency; P_G - gross photosynthetic rate; P_L leaf phosphorus per m^2; P_{max} - photon saturated rate of photosynthesis; P_N net photosynthetic rate; PPFD – photosynthetic photon flux density, Qt – quantum; R_f - root mass fraction; R_G - growth respiration; RuBPCO - Ribulose-1,5-bisphosphate carboxylase/oxygenase; R - root dry mass; V_{max} - the maximum velocity of Ribulose-1,5-bisphosphate carboxylase/oxygenase activity; W - biomass; δ_L - specific leaf area; τ - the carboxylation constant.

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3.2 Introduction

Jia and Gray (2003) showed that for *Vicia faba* a curvilinear relationship exists between

leaf nitrogen content (N_L) and photon saturated photosynthetic rate (P_{max}). A similar relation holds for the quantum yield efficiency (α□). Carboxylation efficiency (CE) increases and intercellular CO_2 concentration (C_i) decreases in response to increasing N supply. With increasing N supply P_{max}, □Œ, and C_i all converged into asymptotic values. This study tests the hypothesis that P supply positively affects both N demand and photosynthetic N use efficiency (NUE) by influencing the upper limit of the asymptotic values for the above characters in response to increasing N supply.

3.3 Material and Methods

3.3.1 Growth conditions

The cold-hardy faba bean (*Vicia faba L.*) cultivar variety, Aquadulce Claudia (Straathof Seed Group) requiring approximately 130-150 days for crop development was used. Outdoor experimental pot trials were carried out as reported in Jia and Gray (2003). Faba bean seeds were planted in 15cm diameter (area equivalent to 0.0177 m^2) pots. The pots were filled with sterile river sand. Sand was sterilized by autoclaving at 121℃ and 103.4 kPa for 3 hours in metal buckets. Two seeds were planted in each pot and after germination one seedling per pot was selected, so as to give an initial population consisting of very similar seedlings. All pots were watered every second day with tap water until emergence of cotyledons. Once the seedlings had emerged, they were watered every second day with a modified Long-Ashton nutrient solution. The modification involved the application of nitrogen in the form KNO_3 at the following concentration of N: 10, 25, 50, 100, 250 and 500 g N m^{-3} were applied. LP and HP supply rates were 0.05 and 1.6 mM P, respectively.

3.3.2 Nutrients

A modified Long-Ashton nutrient mixture was used to supply the non-nitrogen micro- and macro-nutrients. For the micro-nutrients 100x stock solutions were made up as follows [kg m^{-3}]: $MnSO_4 \times H_2O$ 0.223, $CuSO_4 \times 5H_2O$ 0.024, $ZnSO_4 \times 7H_2O$ 0.029, H_3BO_3 0186, $(NH_4)_6Mo_7O_{24} \times 4H_2O$ 0.004, $CoSO_4 \times 7H_2O$ 0.003, NaCl 0.585. For the macro-nutrients 100x stock solutions were made up as follows [kg m^{-3}]: $CaCl_2$ 50.00, $MgSO_4 \times 7H_2O$ 36.900, K_2SO_4 21.75, FeEDTA 3.00. After adding the appropriate quantity of N and P, 10 cm^3 of the stock solutions were added. The pH of the nutrient solutions were then adjusted to pH 7.0 and made up to 1000 ml with distilled water before being applied to the pots. Because of the low water holding capacity due to the coarse texture of the river sand in the pots, it was necessary to water plants every 2 days with 150 cm^3 of a modified Long-Ashton nutrient solution. Inclusion of 0.218 kg m^{-3} K_2SO_4 to all treatments meant that K supply, while abundant to the high N treatments.

3.3.3 Biomass analysis

Dry mass (DM) and photosynthetic measurements were determined at 38 (T_1) and 45 (T_2) days after planting (DAP), respectively. After measuring the photosynthetic rate, the plants were harvested for biomass and total N content determination. The plants were divided into leaf, stem and root, respectively. The roots were rinsed carefully with tap water to remove sand. Before drying leaf area was determined with a area meter *Li-Cor 3100* (*Li-Cor*, Inc, Lincolin Nebrasha, USA). The leaves, stems and roots were

dried in the oven at 105℃ for 15 minutes and then at 65℃ for 3 days for dry mass determinations. DM was determined using electronic balance (model *JP_2-3000, chyo balance corporation* Kyoto Japan). Dried plant material was milled to sub-samples for total N and total P determinations.

3.3.4 Tissue N and P analysis

After the determination of DM of leaves, stems and roots, tissues were milled and analysed for N and P contents. Sub-samples (0.1 ± 0.001g) were digested in a hydrogen peroxide-sulphuric acid digestion mixture by the Kjeldahl procedure flowed by standard colorimetric assays (Anderson and Ingram 1993).

3.3.5 Gas exchange measurements

It was done using the portable *CIRAS-1, PP IR* gas analysis system at 38 and 45 DAP. For the estimation of P_{max} and α, the *ADC* infrared gas analyser *225-2B-SS,* (*Analytical Development Co.*, Hoddesdon, UK) on differential mode was used. Leaf surfaces were exposed to photosynthetic photon flux density (PPFD) of 75 to 2000 μmol (quanta) m^{-2} s^{-1} by adjusting the height a 400 W halide (*Power Star HQ1*) lamp above the leaf surface. The PPFD at the leaf surface was measured with a *LI-COR, LI-188B* quantum meter. Net photosynthetic rate (P_N) was measured on the youngest fully expanded leaves. The trifoliate leaflet was clamped into and sealed in a *Perspex* cuvette leaf chamber which allowed a leaf area of 0.63 cm^2 to be irradiance. The cuvette chamber was surrounded by a water jacket connected to a temperature regulated water bath *SS-CD-5* (*Specht Scientific*, Johannesburg, SA). Leaf temperature was monitored with a

thermocouple *BAT-12* (*Bailey Instruments*, Saddlebrook, NJ, USA) touching the underside of the lamina within the leaf chamber. The leaf chamber or cuvette was surrounded by a water jacket and temperature was maintained at 25℃. Average barometric pressure during photosynthetic measurements was 83.5 kPa.

3.3.6 Experimental design and statistical analysis

The experiment was a randomized complete block with four replicates for each harvest interval, with combinations of treatment factors (4 x 6 factorial) randomly assigned to pots in the block. The various treatments were six different N supply rates [10, 25, 50, 100, 250 and 500 g (N) m^{-3}] applied with two different P supply rates (0.05 mM and 1.6 mM P). A replicate consisted of one plant per container. Dry mass, leaf area and N and P contents were analysed by three-way ANOVA for main effects and interacting effects (Zar, 1984). The significance of the differences in variables as a result of the interactions between factors A (N supply concentration), B (P supply concentration) and C (harvest dates, T_1 and T_2) were tested. For the multi-factorial experiment the sources of variation were subdivided into three main effects: A (effect of N supply concentration); B (effect of P supply concentration); C (harvest intervals); three first-order interactions, A x B, A x C, and B x C; and one second-order interaction, A x B x C. Multiple comparisons of means were performed by the Tukey's HSD test ($p< 0.05$) after performing three-way ANOVA with residual estimation. The Statistica version 6.0 package was used for statistical analysis.

3.4 RESULTS

3.4.1 Summary of ANOVA

Table 3.1. The significance of differences in variables was as a result of the interactions among factors A [10, 25, 50, 100, 250 and 500 g (N) m^{-3}], B (P supplies 0.05 mM and 1.5 mM) and C (harvest internals T_1 and T_2). Biomass (DM), leaf area, total N accumulation, total P accumulation, specific leaf area (δ_L), root fraction (R_f), elemental N and P content of leaves, roots and stems were analyzed by three way ANOVA with residual estimation, for main effects and interaction effects.

Source of Variance	A	B	C	A x B	A x C	B x C	AxBxC
Degree of Freedom	5	1	1	3	3	3	3
Leaf N (mg g^{-1}DM)	***	***	ns	***	ns	ns	ns
N_L (g N m^{-2})	***	***	ns	***	ns	ns	ns
Stem N (mg g^{-1}DM)	***	***	ns	***	ns	ns	ns
Root N (mg g^{-1}DM)	***	***	ns	***	ns	ns	ns
Leaf P (mg g^{-1}DM)	***	***	ns	***	ns	ns	ns
P_L (g P m^{-2})	***	***	ns	**	ns	ns	ns
Stem P (mg g^{-1}DM)	***	***	ns	***	ns	ns	ns
Root P (mg g^{-1}DM)	***	***	ns	**	ns	ns	ns
Total N (mg N pl^{-1})	***	***	***	***	***	***	**
Total P (mg P pl^{-1})	***	***	***	***	ns	***	ns
Biomass (g pl^{-1})	***	***	***	***	**	*	ns
Leaf area (cm^2 pl^{-1})	***	***	***	***	*	ns	ns
δ_L (dm^2g^{-1})	*	***	ns	ns	ns	ns	ns
Root fraction (R_f)	***	***	ns	ns	ns	ns	ns
Photosynthesis (P_{max})	***	***	ns	ns	ns	ns	ns

where *, **, *** indicate effects that are significant at $P < 0.05$, 0.01 and 0.001 respectively; and ns indicates effects that are not significant at $P<0.05$.

Each of the treatment factors (A, B, C), when considered individually, had significant effects on different plant variables (Table 1). In relation to their combined effects, the three-way ANOVA results showed that the majority of first-order interactions had significant effects on all plant variables. The combined effects of the three factors on N and P tissue contents, specific leaf area (δ_L), root: biomass ratio (R_f), plant DM, leaf area, total N and P accumulation, are shown in Figs. 1 to 4. The effect of factor C or time on the intensive variables such as tissue N content, tissue P content, δ_L and R_f are given in Figures 3.1., 3.2. and 3.3. In relation to second-order interactions, there were

no significant differences. With regard to the effects arising from the interaction of factors, P was consistently associated with a significant increase in plant N accumulation and NUE in relation to biomass production and photosynthesis.

3.4.2 Effects of P supply on plant N accumulation

While within treatments there were significant differences in the accumulation of total elemental N and P over the interval T_2 - T_1, there were no significant differences in the contents of elemental N and P over this interval (Figures 3.1., 3.2. and 3.4.). It is not certain what precisely determines the plant demand for N (Grindlay 1997). If the amount of N accumulated reflects the plant demand for N, then the results indicate that P supply was a major factor governing the demand for N (Table3.1., Figure 3.1. and 3.4.). It is not certain how P influences N demand. An increase in the plant total transpiration surface area will tend to increase the flux of water through the soil-plant-atmosphere continuum and increase the flux of nitrate to the root system. This could be a possible explanation for an indirect effect of P supply on nitrate uptake. Increasing P supply was also consistently associated with an increase in N productivity in terms of biomass and leaf area production (Figure. 3.4.).

3.4.3 The effects of N supply on plant P accumulation

No significant difference in plant P contents within treatments over the harvest intervals (Table 3.1. and Figure 3.2.) was detected. The level of N supply was also a major factor governing the amount of P accumulation in growing plants (Figure 3.4.). While total P accumulation increased with N supply, the associated increases in DM production (Figure 3.4.) had a dilution effect on P content (Figure 3.2.2). It was not certain whether the increase in total

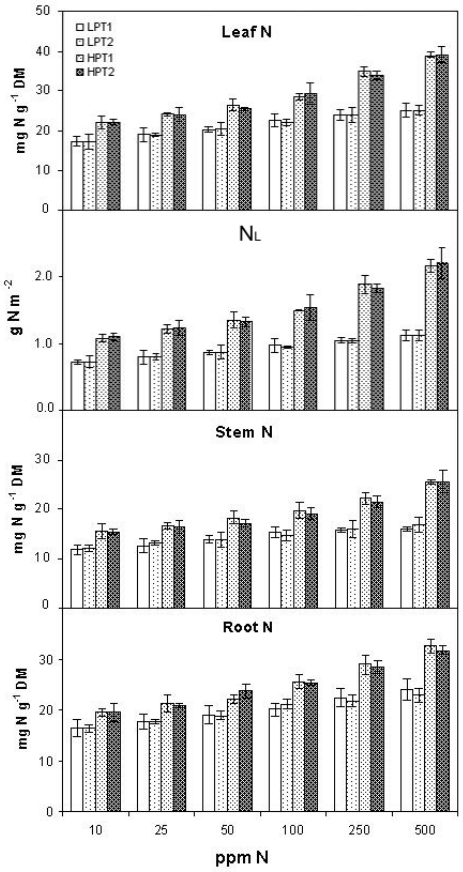

Figure 3.1. N concentration [g kg^{-1}(DM)] of leaf, stem, root and specific leaf N in *Vicia faba* as influenced by N supply [10, 25, 50, 100, 250 and 500 g (N) m^{-3}], P supply (LP and HP) at harvest intervals T_1 and T_2. Vertical bars represent SE (n = 4) of the means. Tukey's HSD test ($p<0.05$) after performing 3-ways ANOVA with residual estimation indicated significant differences among the N and P treatments, but no differences within treatments over harvest intervals .

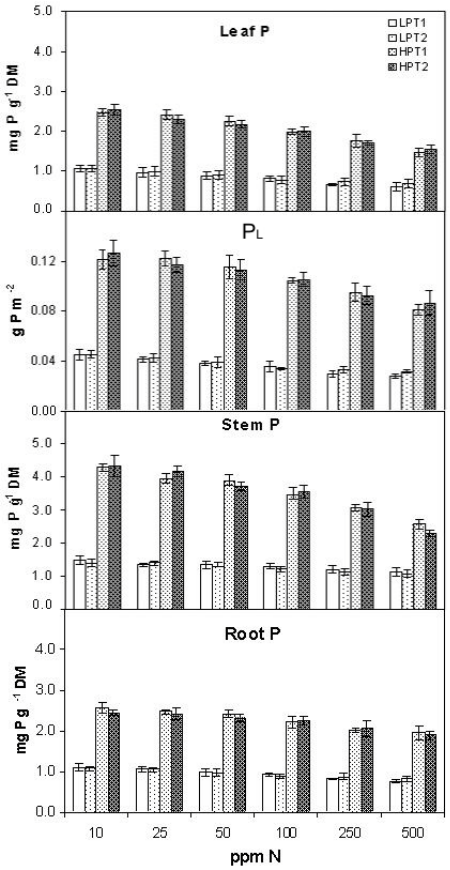

Figure 3.2. P concentration [g kg^{-1}(DM)] of leaf, stem, root and specific leaf P in *Vicia faba* as influenced by N supply [10, 25, 50, 100, 250 and 500 g(N) m^{-3}], P supply (LP and HP) at harvest intervals T_1 and T_2. Vertical bars represent SE (n = 4) of the means. Tukey's HSD test ($p<0.05$) after performing 3-ways ANOVA with residual estimation indicated significant differences among the N and P treatments, but no differences within treatments over harvest intervals.

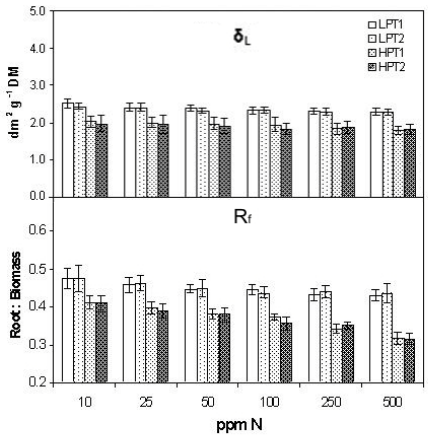

Figure 3.3. Specific leaf area and root fraction of *Vicia faba* as influenced by N supply [10, 25, 50, 100, 250 and 500 g (N) m^{-3}], P supply (LP and HP) at harvest intervals T_1 and T_2. Vertical bars represent SE (n=4) of the means. Tukey's HSD test ($p<0.05$) after performing 3-ways ANOVA with residual estimation indicated significant differences among the N and P treatments, but no differences within treatments over harvest intervals.

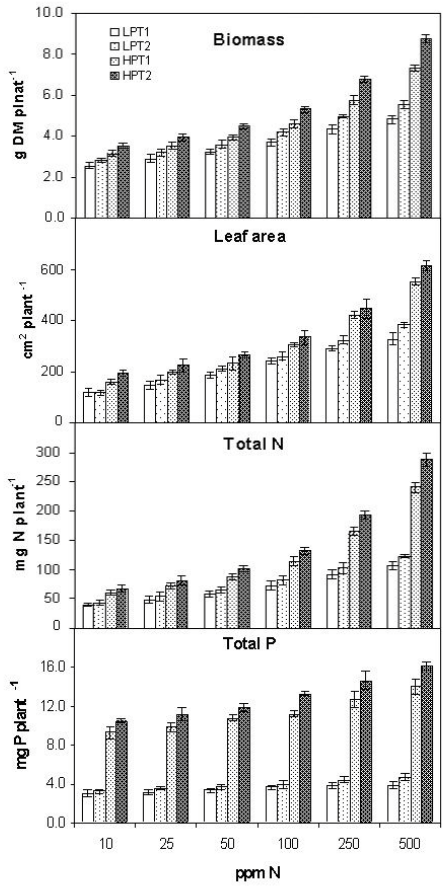

Figure 3.4. Biomass, leaf area, total elemental N and total element N and P of *Vicia faba* as influenced by N supply [10, 25, 50, 100, 250 and 500 g(N) m^{-3}], P supply (LP and HP) and at harvest intervals T_1 and T_2. Vertical bars represent SE (n = 4) of the means. Tukey's HSD test ($p<0.05$) after performing 3-ways ANOVA with residual estimation indicated significant differences among the N and P treatments, but no differences within treatments over harvest intervals .

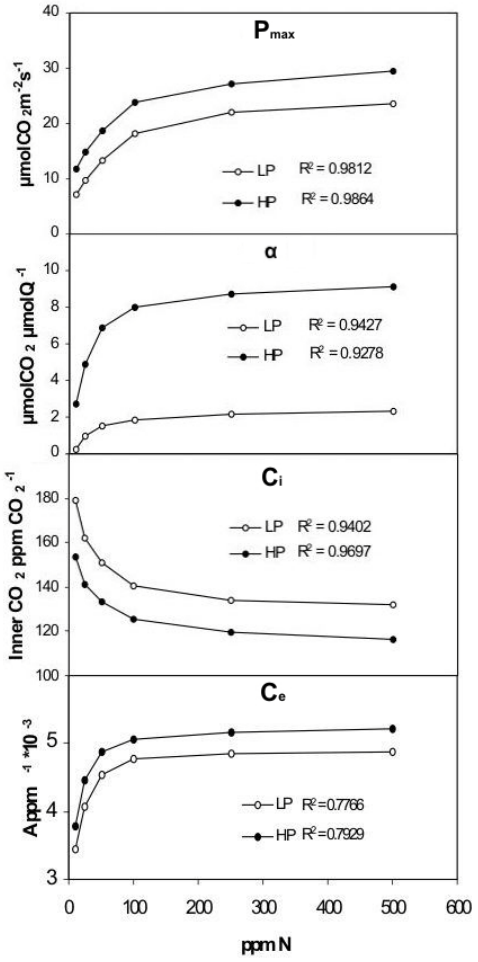

Figure 3.5. Irradiance saturated photosynthetic rate (P_{max}), quantum yield efficiency (α) internal CO_2 concentration (C_i) and carboxylation efficiency (CE) of *Vicia faba* as influenced by N supply [10, 25, 50, 100, 250 and 500 g(N) m^{-3}] and P supply (LP and HP). r^2 estimated for natural log of the plot.

P accumulation in response to N supply could also be explained as an indirect consequence of increased transpiration water fluxes delivering P to the plant root system.

3.4.4 Effects of N and P supply on plant parameters

Figs. 1, 2 and 3 show that under a regime of constant nutrient supply, intensive variables such as tissue N and P content, δ_L and R_f attained steady-state values. Both R_f and δ_L declined slightly with increasing N supply (Fig. 3). These slight declines in both R_f and δ_L as the plant N content increased were significantly different at the 5% level.

There were no significant differences at the 5% level among the harvest intervals for the N_L and P_L within treatments, indicating maintenance of steady-state values for specific leaf N and P content. N_L and P_L remained constant while DM and leaf area increased exponentially. Thus, both N and P uptake rates had also to increase exponentially during plant growth, even if the N and P supply remained constant.

Even though both DM and leaf area production increased significantly in response to P under increasing N supply rates, the values for R_f and δ_L remained constant over the harvest intervals. The δ_L declined slightly with increasing N_L. These results are consistent with the balanced exponential growth hypothesis of Thornley (1998). This hypothesis predicts that under constant nutrient supply, non-limiting moisture availability, and saturating irradiance all extensive variables (*e.g.* plant DM and leaf area) increase exponentially at a constant specific growth rate. Also, under these conditions all intensive variables that are either ratios or rates (*e.g.* N_L, P_L, δ_L and R_f) remain constant. The results corroborate the balance growth hypothesis and

consequently the intensive variables R_f, δ_L, N_L and P_L behave as plant parameters. The values of these parameters would be dependent on the amounts of the N and P supplied to the plant. For the balanced growth hypothesis should also hold for intensive variables such as the content of activated Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO). Thus, for a given constant supply of N and P the content of activated RuBPCO should remain constant. This conclusion was important for analyzing the relationship between N supply and photosynthetic activity, and the role that P may play in this relationship [see Eqs (1) to (10)].

3.4.5 The effect of N and P on C_e and C_i

A rectangular hyperbolic relationship was found between C_e and N supply for both LP and HP plants (Fig. 5). HP plant had a higher asymptotic C_e value and N_L-intercept than LP plants (Table 2). NUE in relation to C_e has been defined as the initial slope of C_e versus $N_{L,}$ HP plants had a higher value than LP plants (Table 2). As N supply increased, C_i declined exponentially to 116.1 g m^{-3} for HP plants and 131.9 g m^{-3} for low P plants (Fig.5). While plant water status and stomatal conductance are fundamental in determining the upper limits of the atmosphere-leaf CO_2 gradient (C_a-C_i), this gradient was also influenced by the proportion of P to N supply.

C_e gives an indirect indication of the content of activated RuBPCO [see Eqs. (3) and (4)]. Hence N supply influences the content of activated RuBPCO. The content of activated RuBPCO converges in a curvilinear fashion to an asymptotic value with increasing N supply; and the asymptotic content of activated RuBPCO in relation to N supply is sensitive to P. P supply can modulate the content of activated RuBPCO either

Table 3.2. The effect of low P (0.05 mM, LP) and high P (1.60 mM, HP) treatments on photosynthetic parameters in response to N supply. Q: Quantum.

	Asymptotic		N_L-intercept		Initial slope	
	LP value	HP value	LP value	HP value	LP value	HP value
P_{max} vs N_L	20.30 ± 0.79 $\mu molCO_2\ m^{-2}s^{-1}$	30.82 ± 0.87 $\mu molCO_2\ m^{-2}$s-1	0.69±0.01 g N m^{-2}	0.80±0.02 g N m^{-2}	46.40 ± 5.20 $\mu molCO_2\ m^{-2}s^{-1}$ $(g\ N\ m^{-2})^{-1}$	19.84 ± 1.75 $\mu molCO_2\ m^{-2}s^{-1}$ $(g\ N\ m^{-2})^{-1}$
α vs N_L	0.0249 ± 0.0014 $\mu molCO_2 \mu molQ)^{-1}$	0.0909 ± 0.0018 $\mu molCO_2 \mu molQt^{-1}$	0.69±0.01 g N m^{-2}	0.98±0.02 g N m^{-2}	0.068 ± 0.012 $\mu molCO_2 \mu molQt^{-1}$ $(g\ N\ m^{-2})^{-1}$	0.168 ± 0.005 $\mu molCO_2 \mu molQt^{-1}$ $(g\ N\ m^{-2})^{-1}$
C_e vs N_L	0.0489 ± 0.0005 $\mu molCO_2\ m^{-2}s^{-1}$ $(ppmCO_2)^{-1}$	0.0510 ± 0.0005 $\mu molCO_2\ m^{-2}s^{-1}$ $(ppmCO_2)^{-1}$	0.55±0.01 g N m^{-2}	0.83±0.01 g N m^{-2}	0.032 ± 0.004 $\mu molCO_2\ m^{-2}s^{-1}$ $(ppmCO_2)^{-1}$ $(g\ N\ m^{-2})^{-1}$	0.050 ± 0.007 $\mu molCO_2\ m^{-2}s^{-1}$ $(ppmCO_2)^{-1}$ $(g\ N\ m^{-2})^{-1}$

directly (Marcus and Gurevitz 2000) or indirectly (Cockburn *et al*. 1967a, b; Usuda and Edwards 1982; Mächler *et al*. 1984; Rao and Terry 1989; Rao *et al*. 1989a,1989b; Usuda and Shimogawara 1991; Pieters *et al*. 2001).

3.4.6 Effect of P on the response of P_{max} to N

Fig. 5 shows the impact of P treatment on the asymptotic values for P_{max} in relation to N supply. Under HP and LP, P_{max} increased in a curvilinear fashion with N supply (Fig. 5). However, higher P_{max} values were achieved in the HP plants. The results in Figs. 4 and 5 are consistent with a P sensitive link between N supply, photosynthetic activity and plant growth.

Table 2 shows the relationship between P_{max} and N_L. The asymptotic value for P_{max} versus N_L and the N_L-intercept value for P_{max} = 0 were calculated using the empirical

equation of Sinclair and Horie (1989). Plants of the HP treatment had a higher asymptotic value for P_{max} compared with LP plants. However, for the HP plants the photosynthetic NUE (calculated as the initial slope of the P_{max} versus N_L plot) was less than 50% of the value for the LP plants. Also the P_{max} versus N_L-intercept value for the HP plants was considerably higher than the value for the LP plants (Table 2).

3.4.7 Effect of P on the response of α to N

Under both HP and LP, α also increased in a curvilinear fashion with respect to the N supply (Fig. 5). The curvilinear relationship between α and N_L for faba bean was very similar to the one reported for soybean, wheat and maize by Sinclair and Horie (1989). The empirical equation of Sinclair and Horie (1989) was also used for generating the asymptotic and intercept values for α versus N_L. With increasing N_L the value for α converges onto a maximum asymptotic value. Plants receiving the HP treatment had a higher asymptotic value for α value compared to the LP plants (Table 3.2.). Quantum yield efficiencies fell to zero as the N_L value approached the critical value corresponding to the N_L-intercept. This critical N_L value or N_L-intercept value for the HP plants was considerably higher than the value for LP plants. Radiation NUE defined in terms of the gradient with respect to the linear portion of α versus N_L was higher for HP plants (Table 3.2.). For a given N and P supply rate α remained constant over the two harvest intervals (data not shown), this is consistent with the hypothesis that photosynthetic parameters should remain constant when N and P supply rates are kept constant.

3.5 Discussion

Photosynthetic activity is limited by both N and P supply. During plant growth P modulates the influence of N supply on photosynthetic activity. Under balanced exponential growth the relationship among variables such as plant growth rate, rate of N accumulation and (P_N) may be formulated as follows:

$$\frac{1}{W}\frac{dW}{dt} = \frac{1}{N_{plt}}\frac{dN}{dt} = \frac{1}{W}\left[\frac{P_g L}{\delta_L} - M_r W - G_r W\right] = P_n\left(1 - R_f\right) \qquad (1)$$

where, W is plant biomass, N_{plt} is total plant elemental N, P_g is gross photosynthetic rate, L is leaf mass, δ_L is the specific leaf area, M_r is the maintenance respiration, G_r is the growth respiration. Under saturating irradiance, P_n tends to P_{max} and for a given fixed stomatal and mesophyll constant, P_{max} can be defined as follows (Charles-Edwards 1978; Fisher *et al*. 1981; Evans and von Caemmerer 1996; Katul *et al*. 2000):

$$P_{max} = h\tau\left[C_a - C_i\right] = C_e\left[C_a - C_i\right] \qquad (2)$$

where h is leaf thickness, τ is the carboxylation constant (s^{-1}), CE = h τ is the carboxylation efficiency, C_a is the ambient CO_2 concentration, and C_i is the intercellular CO_2 concentration. When irradiance is not limiting the relationship between the carboxylation efficiency, active RuBPCO content and P_{max} can be defined. For example CE can be expressed as function of the maximum velocity of RuBPCO activity (V_{max}) or P_{max} as follows:

$$C_e = \frac{V_{max}}{C_i - Km_{appCO_2}} = \frac{P_{max}}{\left[C_a - C_i\right]} \quad (3)$$

where Km_{appCO2} is the apparent Km for CO_2 at varying O_2 concentrations. Carboxylation efficiency is proportional to the content of activated RuBPCO catalytic sites, E_a, and the latter can be expressed as follows:

$$E_a = \frac{V_{max}}{k_{cat}} = \frac{P_{max} k_{CO_2}}{k_{cat}} \quad (4)$$

where k_{cat} represents the RuBPCO turnover rate (3.3 s^1) and k_{CO2} has been defined as follows:

$$k_{CO_2} = \frac{\left[C_i + Km_{appCO_2}\right]}{\left[C_a - C_i\right]} \quad (5)$$

From the above equations the relationship between E_a and the rate of N accumulation may be formulated as follows:

$$\frac{dN}{dt} = N_{plt} P_{max}\left(1 - R_f\right) = N_{plt} \frac{E_a k_{cat}\left(1 - R_f\right)}{k_{CO_2}} \quad (6)$$

As the reaction rates of the Calvin cycle are controlled by P recycling, P supply to the plant modulates the content of activated RuBPCO, P_{max} may then in relation to N and P supply be expressed as follows;

$$P_{max} = \frac{1}{N_{plt}\left(1 - R_f\right)} \frac{k_{cat}^{app} K_N^{app} E_a N}{\left(k_{cat}^{app} + K_N^{app} N\right)} \tag{7}$$

where

$$k_{cat}^{app} = \frac{k_{cat} K_P P}{k_{cat} + K_p P} \tag{8}$$

has been defined as the P dependent apparent catalytic constant for RuBPCO and K_P represents the Michaelis constant for P; and

$$K_N^{app} = \frac{K_N K_{NP} P}{K_N + K_{NP} P} \tag{9}$$

has been defined as the apparent specificity constant for N. In Eq. 9) K_N represents the Michaelis constant for N and K_{NP} represents a kinetic coefficient. Substituting Eq. (7) for P_{max} into Eq. (6) gives the following relationship for N, P and E_a:

$$\frac{dN}{dt} = \frac{k_{cat}^{app} K_N^{app} E_a N}{\left(k_{cat}^{app} + K_N^{app} N\right)} \tag{10}$$

Finally, with regard to photosynthetic activity, the results of this investigation show that P supply determines the upper limit of the asymptotic values for P_{max}, and CE in relation to N supply; and Eqs. (1) to (10) give a possible kinetic description of these results.

In conclusion, we found a tight coupling between the effects of P and N on photosynthesis and plant growth. A decline in the supply of N (Paul and Driscoll 1997)

or P (Pieters *et al.* 2001) results in an immediate decline in photosynthetic activity. This study has focused primarily on the influence of N and P on carbon source dynamics, but increasing N and P supply also stimulates photosynthetic activity by increasing down-stream utilization of Calvin cycle end-products (Paul and Pellny 2003). Sugars alone probably do not mediate sink regulation of photosynthesis (Paul and Foyer 2001). Rather it is the whole plant nutrient balance in the form of the C: N: P supply ratios that mediate both source and sink regulation of photosynthesis.

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CHAPTER FOUR:

THE INFLUENCE N AND P SUPPLY ON THE SHORT-TERM RESPONSES TO ELEVATED CO_2 IN FABA BEAN (*VICIA FABA L*)

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Chapter 4:

The Influence N and P Supply on the Short-term Responses to Elevated CO_2 in Faba bean (*Vicia faba L*)

4.1 Abstract

In this study the effects of the inorganic nutrients carbon dioxide, nitrogen and phosphorus on light-saturated photosynthetic rates (P_{max}) and quantum yield (α) were investigated in faba bean. Both P_{max} and α increased asymptotically in response to increasing N supply. However the maximums that were achieved for the asymptotic P_{max} and α values in relation to N depended on both P and CO_2 supply. Also, the short-term photosynthetic responses to the increasing concentrations of CO_2 were observed to be co-limited by both N and P supply. These findings support the proposal that the N:P supply ratio controls the plant's photosynthetic capacity in response to elevated CO_2 concentrations.

Keywords: Carbon dioxide; Light-saturated photosynthetic rates (P_{max}); Nitrogen; Phosphorus; Quantum efficiency (α)

4.2 Introduction

It has been observed that the responses of plant communities to global warming and elevated CO_2 were influenced by leaf N: P ratios (Hedin, 2004), which are in turn

dependent on soil N and P supply. This proposal has received support from experiments that show that increases in biomass production in Plants acclimatized to elevated CO_2 (440 and 600 CO_2 μL L^{-1}) relative to control plants (280 CO_2 μL L^{-1}) depended on the level of NPK supply (Grünweig and Korner, 2003). These results are consistent with the observations that photosynthesis is co-limited by both N and P supply (Jia and Gray, 2003; Jia and Gray, 2004; Jia et al., 2004). In *Pinus pinaster* the magnitude of growth, photosynthetic rates and N partitioning into ribulose-1, 5-bisphosphate carboxylase oxygenase (RubisCO) in response to increasing N supply was also positively modulated by P supply (Warren and Adams, 2002). This modulation of the photosynthetic response to N supply by P may take place either directly or indirectly. Direct modulation of photosynthetic activity by P may be facilitated through the influence of P on RubisCO activation (Marcus and Gurevitz, 2000). Alternatively, indirect control of photosynthetic rates by P supply could be exerted through the chloroplast phosphate shuttle. Phosphate recycling between the chloroplast and cytoplasm has been observed to modulate the photosynthetic rate by influencing the rate of export of photosynthate from the chloroplast (Cockburn *et al.*, 1967a, b; Usuda and Edwards, 1982; Machler *et al*., 1984; Rao and Terry, 1989; Rao *et al.*, 1989a, b; Usuda and Shimogawara, 1991; Rao and Terry, 1995). P supply may also indirectly modulate photosynthetic rate by influencing sink demand for photosynthate (Pieters *et al.* 2001). In general, the proposal that photosynthesis is usually co-limited by both N and P supply is consistent with the observations of recent studies that yield maximization in various crops was influenced by N:P supply stoichiometries (Agren, 2004; Sadrass, 2006). The objective of this study was to investigate the effects of the C:N:P supply ratios on light-saturated photosynthesis (P_{max}) and quantum efficiency (α). The experiments undertaken in this study focused primarily on how N and P supply rates influence photosynthetic

responses to short-term increases in the CO_2 supply in plants that had been grown under normal ambient CO_2 concentrations.

4.3 Material and methods

4.3.1 Growth conditions

The cold-hardy faba bean cultivar, Aquadulce Claudia (Straathof Seed Group) which requires approximately 130–150 days for crop development was used in these experiments. Outdoor pot trials were carried out as reported in Jia and Gray (2004). Faba bean seeds were planted in 15 cm diameter (area equivalent to 0.0177 m^2) pots. The pots were filled with sterile river sand. Sand was sterilized by autoclaving at 121 °C and 103.4 KPa (15 Psi) for 3 h in metal buckets. Two seeds were planted in each pot and after germination one seedling per pot was selected, so as to give an initial plant population consisting of seedlings showing similar levels of growth. All pots were watered every second day with tap water until the emergence of cotyledons. Once the seedlings had emerged, they were watered every second day with a modified Long–Ashton nutrient solution (Hewitt, 1966). Six different N (10, 25, 50, 100, 250 and 500 g m^{-3} N-KNO_3) and two phosphorus (0.05 and 1.6 mmol P) concentrations were applied. The experimental design was a randomized complete block with four replications for each treatment, with combinations of treatment factors randomly assigned to pots in the block.

4.3.2 Nutrients

A modified Long–Ashton nutrient mixture was used to supply the non-nitrogen micro and macro-nutrients. For the micro-nutrients 100×stock solutions were prepared in g L^{-1} as follows: $MnSO_4 \cdot H_2O$ 0.223, $CuSO_4 \cdot 5H_2O$0.024, $ZnSO_4 \cdot 7H_2O$0.029, H_3BO_3 0.0186, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.004, $CoSO_4 \cdot 7H_2O$ 0.003, NaCl 0.585, FeEDTA 3.00. For the macro-nutrients 100× stock solutions were prepared in g L^{-1} in separate containers as follows: $CaCl_2$ 50.00, $MgSO_4 \cdot 7H_2O$ 36.9, K_2SO_4 21.75. Nutrient mixtures were prepared by adding 10 ml of the stock solutions to distilled water which was adjusted to 900 ml and to which the appropriate quantities of N and P for each treatment were added. The pH of the nutrient solutions was then adjusted to pH 7.0 and made up to 1000 ml with distilled water before being applied to the pots. Due to the low water holding capacity of coarse textured river sand used in the pots, it was necessary to water the plants every 2 days with 150 ml of the nutrient solution. To ensure that all treatments had a sufficient supply of potassium, 0.218 kg m^{-3} K2SO4 was applied to all treatments.

4.3.3 Tissue N and P analysis

The leaves, stems and roots were dried in the oven at 105°C for 15 min and then at 65 °C for 3 days. For total N and P analysis, dried plant material was first milled to a fine power and 1±0.001 g samples were then digested in a hydrogen peroxide–sulphuric acid digestion mixture as per the Kjeldahl procedure. Standard colorimetric assays were used to determine N and P (Anderson and Ingram, 1993). All N and P measurements gave the total elemental N and P (organic plus inorganic) present in the plant tissue.

4.3.4 Gas exchange measurements

Photosynthetic measurements were performed between 38 and 45 days after planting. Photosynthetic activities were determined on equal-aged cohorts. All plants were fully acclimatised to full sunlight (2000 μmol m^{-2} s^{-1}). Net photosynthesis under light saturating conditions was measured with a portable CIRAS-1, PP systems, infrared gas analyzer (Jia and Gray, 2003). Photosynthetic measurements were carried out on eight different youngest fully expanded leaves for each treatment. Leaf area was determined with a Li-Cor area meter (Model Li-Cor 3100, Inc, Lincoln, Nebraska, USA).

4.4 Results and discussion

Leaf area production in response to increasing N supply was strongly influenced by P supply, indicating that leaf area production was co-limited by both N and P (Figure 4.1). Uptake and accumulation of P increased more markedly in response to increasing N when P supply was non-limiting (Figure 4.2). At limiting P supply concentrations the increase in P uptake and accumulation in response to increasing N supply was only marginal. Uptake and accumulation of N in leaves in response to increasing N supply were strongly influenced by P supply (Figure 4.3). Specific leaf N content did not decrease with increasing leaf area per plant (Figures 4.1 and 4.3). However, as leaf area per plant increased in response to increasing N supply, specific leaf P content declined irrespective of P supply concentration (Figures 4.1 and 4.4). Implications of these results can be summarized as follows: under non-limiting N conditions an increase in P supply enhances both leaf N content and plant growth measured as leaf area generation. Similarly, under non-limiting P conditions any increase in N supply enhances both leaf N and plant growth. The hypothesis that N and P are co-limiting with regard to plant

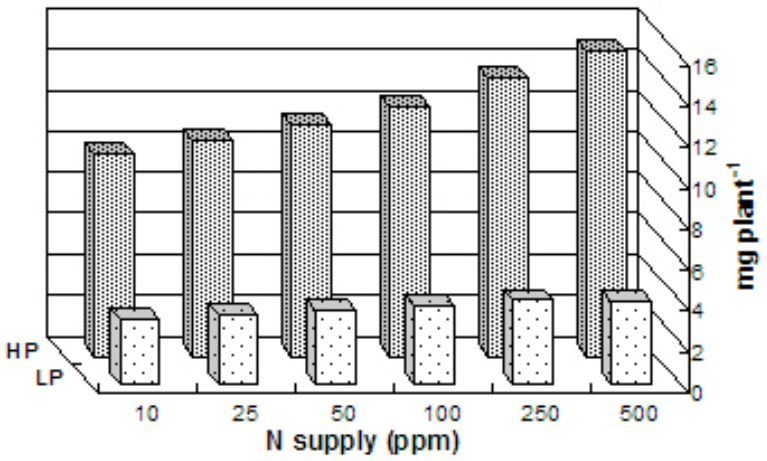

Figure 4.1. The influence of P supply (LP: 0.05 mmol P; HP: 1.6 mmol P) on the response of leaf area production (cm^2 per plant) to the concentration of N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}).

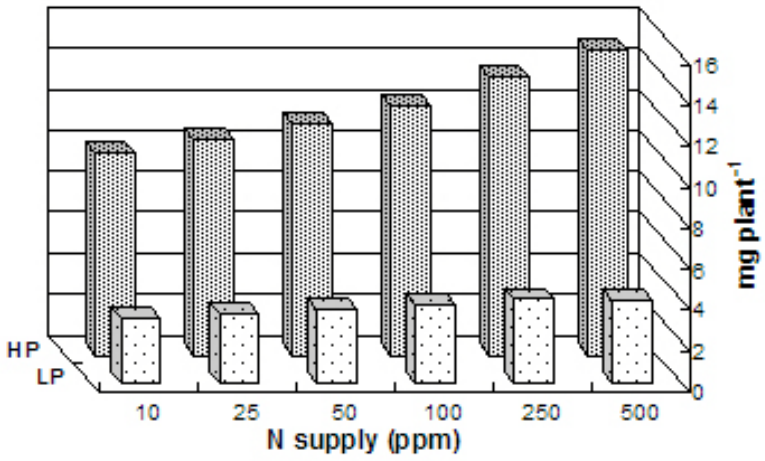

Figure 4.2. The influence of P supply (LP: 0.05mmol P; HP: 1.6mmol P) on total P accumulation per plant (mg per plant) in response to the concentration of N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}).

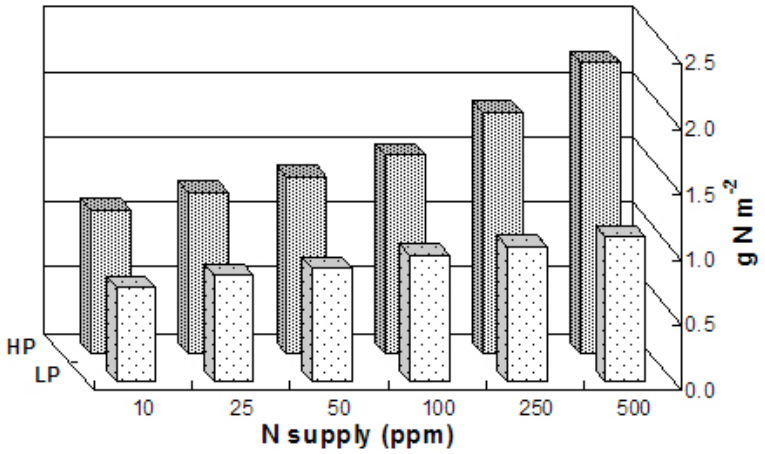

Figure 4.3. The influence of P supply (LP: 0.05 mmol P; HP: 1.6 mmol P) on the accumulation on N in leaves expressed as specific leaf N(g N m^{-2}) in response to the concentration of N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}).

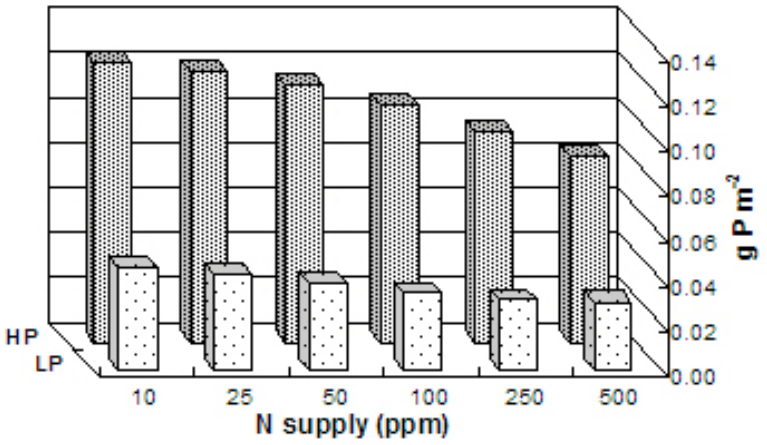

Figure 4.4. The influence of P supply (LP: 0.05 mmol P; HP: 1.6 mmol P) on the accumulation on P in leaves expressed as specific leaf P(g P m^{-2}) in response to the concentration of N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}).

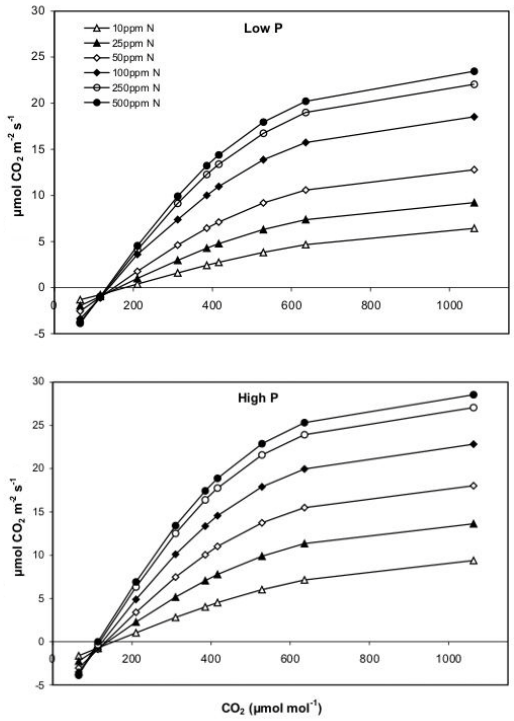

Figure 4.5. The influence of N and P supply levels on net photosynthesis for individual leaves that were exposed to increasing concentrations of CO_2 under an irradiance of 2000 µmol quanta m^{-2} s^{-1} . The points on the graph indicate means (n=4).

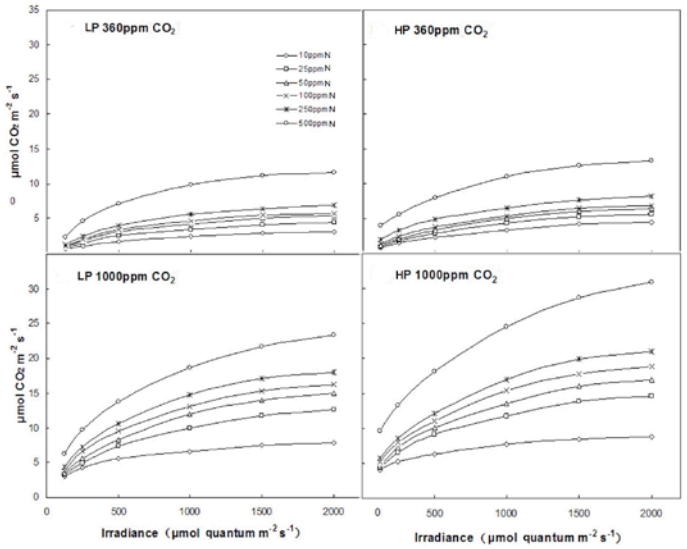

Figure 4.6. The response of net photosynthetic rate to increasing irradiance (100 to 2000 µmol quantum s) as influenced by: N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}); P supply (LP: 0.05mmol P; HP: 1.6 mmol P); and carbon dioxide concentration (360µmol mol^{-1}; and 1000 µmol mol^{-1}). The points on the graph indicate means (n=4).

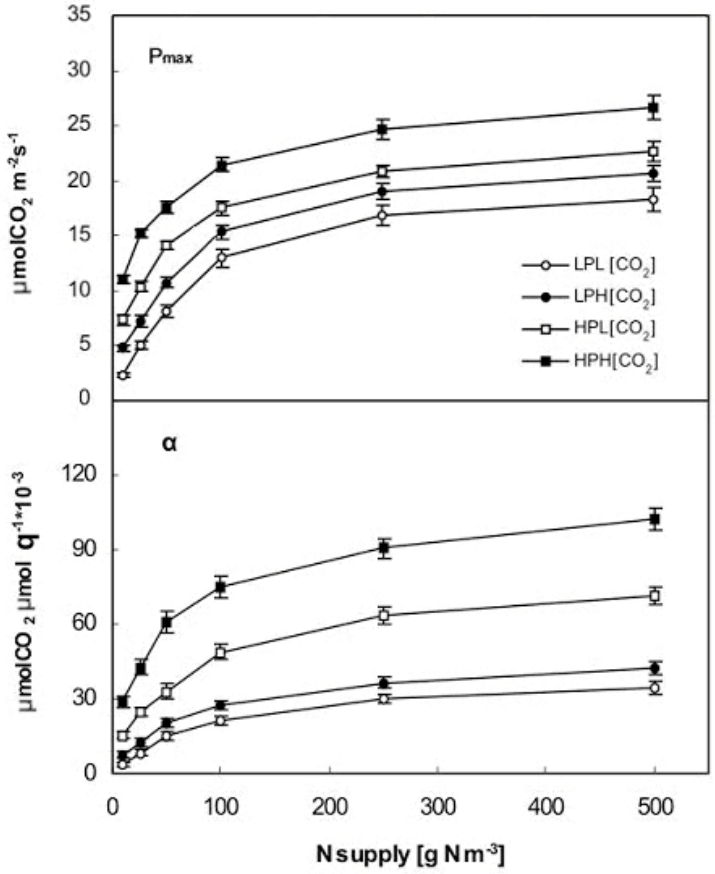

Figure 4.7. Photon-saturated net photosynthesis rate (P_{max}) and quantum efficiency (α) as influenced by: N supply (10, 25, 50, 100, 250 and 500 g N m^{-3}); P supply (LP: 0.05 mmol P; HP 1.6 mmol P) and carbon dioxide concentration (L[CO_2]: 360 µmol mol^{-1}; H[CO2]: 1000 µmol mol^{-1}). The points of the graph indicate the means (n=4) and vertical bars represent SE (n=4) of the means.

growth. Figure 4.5 shows the effects of the N and P supply levels on net photosynthesis for individual leaves that were exposed to increasing concentrations of CO_2. With increasing CO_2 concentration it appears that N and P supply levels determined the upper limits that were attained for individual leaf photosynthetic rates. This conclusion is consistent with the hypothesis that N and P supply are rate limiting factors for photosynthesis under elevated CO_2 conditions. Or alternatively expressed, these results show that, with increasing CO_2 supply, photosynthetic rates are co-limited by N and P supply. The photosynthetic–irradiance response curves show that the light-saturated photosynthetic capacity was co-limited by N, P and CO_2. From the data presented in Figure 4.6 the values for P_{max} and quantum yield (α) can be derived. Figure 4.7 shows the impact of P supply and CO_2 concentration on the net photosynthetic rates achieved for P_{max} in relation to N supply. For both CO_2 concentrations, plants receiving the high P treatment had the higher net photosynthetic rates for P_{max} compared with plants receiving low P supply. Under both high and low P supplies, quantum yield (α) also increased in a curvilinear fashion as a function of N supply (Figure 4.7).

The asymptotic maximum with increasing N supply and the magnitude of the asymptotic maximum were in turn shown to be dependent on P supply. This indicates that the impact of N supply on photosynthetic rate was modulated by P supply.

4.5 Conclusions

The optimum N:P supply ratio has been defined as the N:P ratio where net photosynthesis or plant growth is equally limited by N and P (Sterner and Elser, 2002; Agren, 2004). Even though an extensive literature now exists on the optimal N:P stoichiometries for crop growth (Sadrass, 2006), not much is known about the optimum

C:N:P ratios for photosynthesis or plant growth. However, the study of Grünweig and Korner (2003) suggested that with long-term exposure to elevated CO_2, plant growth becomes co-limited by both N and P supply. The results in Fig. 5 indicate that under short-term exposure to elevated CO_2 plants adapted to ambient CO_2 levels show responses consistent with photosynthesis being co-limited by C, N and P supply. In addition, if the photosynthetic catalytic machinery in terms of leaf N concentration determines the source capacity of the plant canopy and P concentration influences the energetic efficiency of CO_2 assimilation into plant biomass, then the stoichiometric ratio of N:P (as a % of dry biomass) will determine plant productivity levels in response to CO_2 supply. Therefore, in general, it could also be argued that with increasing CO_2 supply, the sink demand of actively growing tissues for additional reduced carbon and source capacity for assimilating additionalCO_2 wouldalsobecontrolledbytheN:P supply ratio.

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CHAPTER FIVE:

THE INFLUENCE OF RHIZOBIUM AND ARBUSCULAR MYCORRHIZAL FUNGI ON NITROGEN AND PHOSPHORUS ACCUMULATION BY *VICIA FABA* L.

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Chapter 5:

The Influence of *Rhizobium* and Arbuscular Mycorrhizal Fungi on Nitrogen and Phosphorus Accumulation by *Vicia faba* L.

5.1 Abstract

- *Background and Aims* The aim of this study was to investigate the effects of the interactions between the microbial symbionts, *Rhizobium* and arbuscular mycorrhizal fungi (AMF) on N and P accumulation by broad bean (*Vicia faba* L.) and how increased N and P content influence biomass production, leaf area and net photosynthetic rate.
- *Methods* A multi-factorial experiment consisting of four different legume–microbial symbiotic associations and two nitrogen treatments was used to investigate the influence of the different microbial symbiotic associations on P accumulation, total N accumulation, biomass, leaf area and net photosynthesis in broad bean grown under low P conditions.
- *Key Results* AMF promoted biomass production and photosynthetic rates by increasing the ratio of P to N accumulation. An increase in P was consistently associated with an increase in N accumulation and N productivity, expressed in terms of biomass and leaf area. Photosynthetic N use efficiency, irrespective of the inorganic source of N (e.g. NO_3^- or N_2), was enhanced by increased P supply due to AMF. The presence of *Rhizobium* resulted in a significant decline in AMF colonization levels irrespective of N supply. Without *Rhizobium*, AMF colonization levels were higher in low N treatments. Presence or absence of AMF did not have a significant effect on nodule mass but high N with or without

AMF led to a significant decline in nodule biomass. Plants with the *Rhizobium* and AMF symbiotic associations had higher photosynthetic rates per unit leaf area.

- *Conclusions* The results indicated that the synergistic or additive interactions among the components of the tripartite symbiotic association (Rhizobium–AMF–broad bean) increased plant productivity.

Key words: Arbuscular mycorrhizal fungi (AMF), nitrogen, phosphorus, *Rhizobium*, *Vicia faba* L.

5.2 Introduction

Under low soil P concentrations, most plant species are dependent on a symbiotic association with arbuscular mycorrhizal fungi (AMF) for the acquisition of P (Smith and Read, 1997). Under low N fertilizer inputs, soil P availability is usually the major factor limiting the rate of N_2-fixation in legume crops (Toro et al., 1998) and, in the absence of AMF infection, supplementary P fertilization is generally necessary for the maintenance of N_2-fixation rates by *Rhizobium* at the levels required for economically viable crop production (Andrade et al., 1998). In legumes the positive synergistic interactions among the members of the tripartite symbiotic association (*Rhizobium*–AMF–legume) result in improved rates of P uptake N_2-fixation and crop biomass production under conditions of reduced N and P fertilizer inputs (Azcón et al., 1991; Xavier and Germida, 2002, 2003). However, there is little information on the influence of P on N productivity or photosynthetic N use efficiency. Nitrogen productivity has been defined as the rate of biomass production per unit biomass N content (Ågren, 1985), whereas the photosynthetic N use efficiency is the amount of

CO_2 fixed (mol CO_2 m^{-2} s^{-1}) per unit biomass N content. In general, photosynthetic and Specific growth rates increase with increasing plant tissue N concentration or N supply in a curvilinear fashion (Hirose and Werger, 1987; Sinclair and Horie, 1989; Jia and Gray, 2004).

This study tests the hypothesis that the synergistic interactions among the members of the tripartite symbiotic association improve legume productivity through positive effects on the rates of photosynthetic CO_2 assimilation, N_2-fixation and P uptake. Furthermore, it is proposed that these three processes are interdependent or even tightly coupled. For example, the rate of photosynthetic CO_2 assimilation is influenced by the rates of N and P supply, and the rate of N_2-fixation is influenced by the rates of photosynthate and P supply to the nodules. Thus, the main objectives of this study were to investigate: (a) how the interactions between the microbial symbionts, *Rhizobium* and AMF affected the rate of N and P accumulation in broad bean; (b) how N productivity, irrespective of the inorganic source of N (e.g. NO_3^-or N_2) was affected by increased P supply due to AMF; and (c) how photosynthetic N use efficiency, irrespective of the in organic source of N (e.g. NO_3^- or N_2), was affected by increased P supply due to AMF colonization.

5.3 Materials and Methods

5.3.1 Plant material and growth conditions

The cold-hardy Vicia faba L. 'Aquadulce Claudia' (Straathof Seed Group), requiring 130–150 d for crop development, was used. Outdoor pot trials were carried out in spring from August to October (mean daily extraterrestrial global insolation 24.4–39.1 MJ m^2;

mean day length 10.78–12.97 h; mean daily maximum temperature 19.3–23.8℃; mean daily minimum temperature 6.4–11.3℃and mean A-pan evaporation 8.0–6.4 mm d^{-1}). Initially three seeds were planted per pot in sand in 1.6-L pots. The sand (25 mm sieve mesh) used in all the treatments was first thoroughly washed with running tap water and then autoclaved for 3 h at 121℃ and 1034 kPa in metal buckets. The initial pH of the sand was 70. After germination and shoot emergence, one seedling of uniform size per pot was selected and the others removed.

5.3.2 Experimental design

The experimental design was a randomized complete block with four replications for each harvest interval, with combinations of treatment factors randomly assigned to pots in the block. The concentration of nitrate-N for the low N (LN) treatments was 0.71 mM KNO_3 (10 ppm N) and 17.86 mM KNO_3 (250 ppm N) for the high N treatments (HN). All treatments received a phosphorus supply of 0.05mg P L^{-1} (1.61 mM NaH_2PO_4). The various treatments were: (a) two different N supply rates without any microbial symbiotic associations, LN and HN; (b) two different N supply rates plus AMF association, LNM and HNM; (c) two different N supply rates plus *Rhizobium* association, LNR and HNR; and (d) two different N supply rates plus both AMF and *Rhizobium* symbiotic associations: LNMR and HNMR. Plants were harvested at 54 d (4 October, T_1) and 63 d (13 October, T_2) after planting.

5.3.3 Nutrients

A modified Long-Ashton nutrient mixture was used to supply the non-nitrogen micro- and macro-nutrients. For the micronutrients 100 × stock solutions were made up as follows: NaH_2PO_4 20.8 g L^{-1}, $MnSO_4 \cdot H_2O$ 0.223 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.024 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.029 g L^{-1}, H_3BO_3 0.019 g L^{-1}, $(NH_4)Mo_7O_{24} \cdot 4H_2O$ 0.004 g L^{-1}, $CoSO_4 \cdot 7H_2O$ 0.003 g L^{-1}, NaCl 0.585 g L^{-1}. For the macro-nutrients, 100× stock solutions were made up as follows: $CaCl_2$ 500 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 36.9 g L^{-1}, K_2SO_4 21.75 g L^{-1}, FeEDTA 3 g L^{-1}. After adding the appropriate quantity of KNO_3 (HN or LN), 10 mL of the stock solutions were added. The pH of the nutrient solutions was then adjusted to pH 7.0 and the solution was made up to 1000 mL with distilled water before being applied to the pots. Because of the low water-holding capacity of the sand-filled pots, it was necessary to water plants every 2 d with 150 mL of the modified Long-Ashton nutrient solution. Inclusion of 0.218 g L^{-1} of K_2SO_4 in all treatments meant that potassium supply, while abundant for the high nitrogen treatments was not limiting in the low nitrogen treatments.

5.3.4 AMF inocula

For AMF infection, the mycorrhizal inoculum consisted of the roots of an *Eragrostis* sp. from the grassland close to the university campus. The pH of the grass land soil was 7.0. The inoculum was prepared by homogenizing and mixing surrounding soil with the *Eragrostis* sp. root systems, forming a mixed inoculum in terms of species and propagules. The inoculum was applied as a 1 cm layer to the surface of the soil, and covered with 1 cm layer of sand. Non-mycorrhizal treatments received inoculum that had been autoclaved at 121℃ for 20 min. No colonization of non-myorrhizal treatments by external sources of AMF spores was detected during the course of the

experiment.

5.3.5 *Rhizobium* inocula

The original *Rhizobium leguminosarum* bv. *Viciae* inoculum was obtained from the active nodules of plants of *Vicia faba* L. that had been infected previously with this strain (STIMUPLANT CC: Reg. No. CK89/04756/23; PO Box 11446, Brooklyn, South Africa 0011). *Rhizobium* colonies were maintained on solid culture medium consisting of 0.5g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H2O$, 0.1 g NaCl, 10 g mannitol, 0.4 g yeast extract, 8 g agar and 0.25 % Congo Red, dissolved in 1000 mL distilled water and autoclaved at 121℃ for 20 min. *Rhizobium* inoculum was prepared by culturing selected colonies in liquid culture medium. The cultures were grown in 250 mL Erlenmeyer flasks and incubated at 25℃ until the bacterial cell optical density measured 0.6 at 600 nm (which corresponded to approx. 15×10^9 colony forming units per mL). Seven days after planting, each pot for the *Rhizobium* treatments was inoculated three times with 100 mL of a ten-fold dilution of the *Rhizobium* inoculum. To maintain uniformity of nutrient supply, all other non-*Rhizobium* treatments were inoculated with autoclaved Rhizobium inoculum (121℃ for 20min). No nodules formed on plants inoculated with autoclaved inoculum.

5.3.6 Biomass measurements

At each harvest, four replicates of each treatment were selected randomly. The plants

Picture 2. The process of incubating and development of *Rhizobium*.

a. Screening and incubating the Rhizobium clolonies in liquid culture medium.

b. The *Vicia facia L.* plants built under no *Rhizobium* and *Rhizobium* conditions.

C. Magnified root of plants in b.

d. The root with nodules of 10 ppm in NRM treatment.

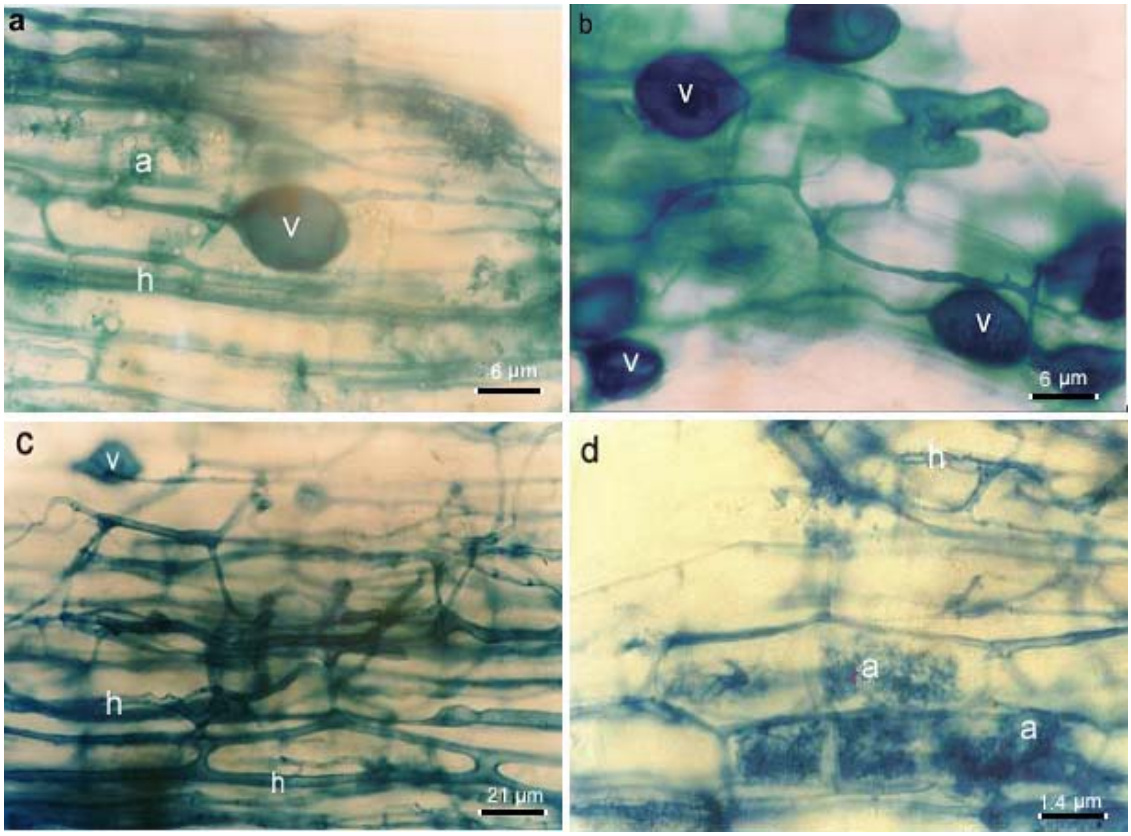

Plate 1. AM fungal infection: Intraradical AM Fungal structures in the roots of the *Vicia faba L.* at harvest between 54 days and 63 days after planting.

A. Vesicles (v), arbuscules (a) with intercellular hyphae (h), the scale bars = 6 µm; B. Vesicles (v) of high level of infection with intercellular hyphae (h), the scale bars = 6 µm; C. Hyphae (h) of high level of infection, the scale bars = 21µm; D. High power view of arbuscules (a), the scale bars = 1.4µm.

were separated into leaves, stems, roots and nodules, and oven-dried at 65℃ for 3 d. At each harvest, a root sub-sample from each treatment was taken before oven drying for estimation of AMF infection of the root tissue. The fresh mass of the sub-sample was recorded so that the dry mass of the sub-sample could be added to the total root dry mass.

5.3.7 N and P analysis

After the determination of dry mass, tissues were milled and analysed for total N and P concentrations. Sub-samples (0.1 – 0.001 g) were digested in a hydrogen peroxide – sulfuric acid digestion mixture by the Kjeldahl procedure followed by standard colorimetric assays (Anderson and Ingram, 1993). All N and P measurements represent total elemental N and P (organic plus inorganic) present in plant tissues.

5.3.8 AMF detection and quantification

AMF colonization was evaluated from a random sub-sample of approx. 150 root segments per plant. Roots were cleared in 25 % KOH (90℃) for 45 min, acidified in 1% HCl for 15 min, stained with 0.05 % Trypan Blue in acid glycerol (90 C) for 45 min, and then stored in acid glycerol according to the procedure of Koske and Gemma (1989). Randomly selected root fragments were mounted in a permanent mounting medium on slides (Omar *et al*., 1979) without squashing the root pieces. Percentage colonization by arbuscules, vesicles and total hyphae were recorded, at 250 × magnification using the magnified intersections method of McGonigle *et al*. (1990).

5.3.9 Photosynthetic studies

Photosynthetic gas exchange rates were determined at each harvest on equal-aged cohorts. All plants were fully acclimatized to full sunlight (2000 mmol m^{-2} s^{-1}). Net photosynthesis under light saturating conditions was measured with the portable CIRAS-1, PP systems, infrared gas analyser (Jia and Gray, 2003), using eight different youngest fully expanded leaves for each treatment.

5.3.10 Statistical analysis

AMF% infection data were transformed using arcsine square root to satisfy normal distribution and homogeneity of variance assumptions for three-way ANOVA. Dry mass and elemental (N and P) concentrations of leaves, stems, roots and nodules were analysed by three-way ANOVA for main effects and interactional effects (Zar, 1984). The significance of the differences in variables because of the interactions between factors A (N, NM, NR and NRM), B (N supply LN and HN) and C (harvest date T_1 and T_2) were tested. For the multifactorial experiment, the sources of variation were subdivided into three main effects: A (presence or absence of microbial symbiont(s); B (low and high nitrogen supplies); and C (harvest dates); three first-order interactions, A×B, A×C, and B×C and one second-order interaction, A×B×C. Multiple comparisons of means were performed by the Tukey HSD test ($P < 005$) after performing three-way ANOVA with residual estimation. The STATISTICA (version 6.0) package was used for statistical analysis.

5.4 Results and discussion

5.4.1 Effects of treatment factors on plant variables

Each of the treatment factors (A, B, C), when considered individually, had significant effects on all of the different plant variables listed Table 1. In relation to their combined effects, the three-way ANOVA results showed that the majority of first-order interactions had significant effects on all plant variables. The combined effects of the three factors on microbial colonization, plant biomass, rates of N and P accumulation, leaf area and photosynthetic rates are shown in Figures 5.1-5.5, and the combined effects of the three factors on N and P concentrations are shown in Table 5.2. In all instances, microbial colonization of plants was associated with significant increases in the magnitudes of plant variables such as biomass, leaf area, photosynthetic rate, N and P content. The effect of harvest date on the variables such as tissue N concentration and tissue P concentration is presented in Table 5.2. Except for leaf area, stem P content and root P content, there were no significant second-order interactions (Table 5.1).

5.4.2 Effects of N supply and *Rhizobium* on mycorrhizal colonization

Table 5.1 shows that first-order interactions (A×B) had significant effects on AMF colonization. Colonization levels of AMF were higher in low N treatments (Figure 5.1). With regard to arbuscular colonization, two kinds of mycorrhizal root colonization response were evident (Figure 5.1). The presence of *Rhizobium* infection resulted in a significant decline in %AC, %VC and %HC at low and high concentrations of N supply. Low concentrations of N in the presence of *Rhizobium* infection (LNMR) resulted in %AC values similar to those associated with high rates of N supply in the absence of

Rhizobium infection (HNM). At low N supply, the presence of *Rhizobium* infection (LNMR) resulted in about a 50 % reduction in %VC (Figure 5.1). However, at high concentrations of N there was no effect of *Rhizobium* infection on %VC. The presence of *Rhizobium* infection and/or high concentrations of N supply were each associated with 50 % or lower %VC compared with the roots of LNM plants. In the case of hyphal colonization (%HC), the presence of *Rhizobium* infection was associated with a significant decline in %HC (Figure 5.1). Whenever N supply was not limiting a significant reduction in %HC occurred. For example, under high N *Rhizobium* infection resulted in further significant decline in %HC (Figure 5.1). A similar pattern was observed for %AC. In all treatments, the presence of a mycorrhizal association had a positive impact on nutrient uptake and biomass production. For example, when the treatments for HN, HNM and HNMR are compared, the plants had similar total root biomass (Figure 5.2), but, irrespective of the level of percentage AMF colonization (Figure 5.1), the presence of an AMF association always resulted in enhanced nutrient uptake and total biomass production (Figures 5.2 and 5.3).

5.4.3 Effects of N supply and AMF on *Rhizobium* infection

Table 5.1 shows that first order interactions (A×B) had significant effects on nodule dry mass. Figure 5.2 shows the impact of AMF and N supply on nodule mass. Unlike the Rhizobium effects on AMF colonization, AMF infection did not have a significant effect on nodule mass. However, high N supply was associated with a large reduction in nodule dry mass relative to low N plants (Figure 5.2). At low N there was a doubling in nodule dry mass between T_1 and $_{T2}$ but only a slight increase over the same period at high N, indicating that high concentrations of nitrate inhibited nodule growth. A

significantly larger quantity of P accumulated in LNR and LNMR nodules between harvests compared with the HNR and HNMR nodules (Table 5.2 and Figure 5.3). The concentration of P in the LNR and LNMR nodules was also significantly higher than in the HNRand HNMR nodules (Table 5.2). In the case of low N treatments, P concentration was significantly higher in LNMR nodules than in LNR nodules (Table 5.2).

5.4.4 The effect of the N:P ratio on biomass

The level of P supply had a significant effect on plant biomass (Figure 5.2), root nodulation (Figure 5.2), N accumulation rate (Figure 5.3), leaf area (Figure 5.4), net photosynthetic rate (Figure 5.5) and tissue N concentration (Table 5.2). Increase in P supply as a consequence of AMF colonization was consistently associated with a significant increase in N accumulation and biomass production (Figures 5.2 and 5.3). For the LNMR plants the N:P ratio was 11 mg N d^{-1}: 1 mg P d^{-1}, and 14 mg N d^{-1}: 1 mg P d^{-1} for the HNMR plants. For HNM and HN the N:P ratios were 10.8m g N d^{-1}: 1 mg P $d^{-1}$and 21.7 mg N d^{-1} : 1 mg Pd^{-1} , respectively. These results indicate that the rate of biomass production increased as the proportion of total P accumulated increased relative to the total N accumulated. AMF contributed to an increase in N productivity in terms of biomass production by increasing the supply of P.

5.4.5 Effect of P on total N accumulation

The amounts of external hyphae in the pots were not determined, nor the metabolically active proportion of the internal colonization. In LNM the %HC was almost 100 %, whereas for HNMR it was approx. 60 %, although the HNMR plants accumulated

significantly more P than did the LNM plants. Plants with both *Rhizobium* and AMF associations (LNMR and HNMR) accumulated the most P. In low N treatments AMF colonization had a significant positive impact on the rate of nitrate-N accumulation (Figure 5.3), and in high N treatments, non-AMF plants such as the HNR plants accumulated significantly less N than did HNMR plants. These results are consistent with the findingn of Tobar *et al*. (1994a, b) that AMF colonization increased plant nitrate-N uptake.

P-limitation of N_2-fixation rates appears to be the reason for the lower rates of N accumulation in LNR relative to LNMR plants (Figure 5.3). Nitrate was the major N source for HNMR plants mainly because of their lower nodule mass compared with the LNR and LNMR plants (Figures 5.2 and 5.3). The N_2-fixing LNMR plants differed only slightly from the nitrate assimilating HNMR plants in rate of total elemental N accumulation. For example, at T_1, HNMR had accumulated 7.0 % more N than LNMR and, at T_2, HNMR had accumulated 5.3 % more N.

It is not clear whether the differences in nitrate accumulation rates among the LN, HN, LNM, HNM and HNMR plants were a direct consequence of increased uptake and mobilization of nitrate-N by the external hyphae, or a indirect consequence of an increase in the plant P content. If the latter held, then plant N productivity in terms of leaf area production would have been enhanced by an increase in P accumulation. Increase in the plant's total transpirational surface area would tend to increase the flux of water through the soil-plant-atmosphere continuum and increase the flux of nitrate to the plant root system. This could be a possible explanation for indirect effects of AMF colonization on plant nitrate uptake.

Root biomass did not appear to be a major factor in either P or N uptake. Since, apart from the LN plants, root biomass did not differ significantly in the other treatments (Figure 5.2) it alone could not account for the variation in the quantity of P accumulated in the different treatments (Figure 5.3). Plants with roots colonized by AMF had P accumulation rates that were 9.3–12.2 times the rates for the non-microbial low N treatments (LN versus LNM and LN versus LNMR in Figure 5.3). At high N, all plants with AMF colonized roots had P accumulation rates that were 2.5–3.2 times the rates for the non-microbial high N treatments; see HN versus HNM and HN versus HNMR in Figure 5.3. However, roots infected only with *Rhizobium* also showed an increase in P accumulation relative to the low N or high N non-mycorrhizal control plants. For example, LNR plants had a 6.6-fold increase in the level of P accumulation compared with LN plants, and there was a 1.8-fold increase in the level of P accumulation when HN plants were infected with *Rhizobium* (Figure 5.3). Here the increase in P accumulation appeared to be influenced indirectly by the association with N_2-fixing *Rhizobium* in the low N plants, or by the increased nitrate N supply in the high N treatment plants. Increased N accumulation due to N_2-fixation in LNR and HNR plants (Figure 5.3) was associated with an increase in leaf area expansion (Figure 5.4). Thus, it was possible that the AMF independent enhanced rates of P accumulation in LNR and HNR plants were due to the effects of N accumulation on leaf area production.

5.4.6 The influence of N, P and microbial symbionts on photosynthesis

Figures 5.3 and 5.5 show that photosynthetic rates increased as the ratio of P to N supply increased (note also the relationship between the N:P trend in Table 5.2 and the trend in photosynthetic rates in Figure 5.5). The impact that the microbial symbionts

had on photosynthetic rates appeared to be mediated by their effects on the plant N:P ratio. Separate experiments to test the effect of P supply on the photosynthetic N use efficiency and the quantum yield efficiency of leaf N showed that an increase in the supply of P increased the photosynthetic N use efficiency and the quantum yield efficiency of leaf N (Table 5.3). The results in Table 5.2 and Figure 5.5 are consistent with the hypothesis that increasing P supply enhances photosynthetic N use efficiency.

5.5 Conclusions

The findings of this experiment are consistent with other observations (Xavier and Germida, 2002, 2003) of the positive impact of the synergistic interactions between AMF and *Rhizobium* on legumes. The magnitude of the increases in both leaf area and biomass production with each increment in N supply was dependent on the level of P supply. Increasing P supply as a direct consequence of AMF colonization or as an indirect consequence of *Rhizobium* infection had positive effects on N accumulation, leaf area production and biomass production. Increasing P accumulation had a positive influence on photosynthetic N use efficiency. Thus, it is reasonable to conclude that, with respect to the legume tripartite symbiotic association examined here, the upper limits of nitrogen productivity or photosynthetic N use efficiency can depend on the level of P supply.

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Table 5.1. The significance of the differences in plant parameters resulting from the interactions among factors A (N, NM, NR and NRM), B (N supplies LN and HN) and C (harvest dates T_1 and T_2). Percentage AMF colonization, dry mass, leaf area, elemental N and P concentrations of leaves, stems, roots and nodules were analyzed by three-way ANOVA with residual estimation, for main effects and interaction effects.

Source of Variance	A	B	C	A x B	A x C	B x C	AxBxC
Degree of Freedom	3	1	1	3	3	3	3
Variables:							
Photosynthetic rate (μmol $CO_2 m^{-2} s^{-1}$)	*** a	***	***	***	**	**	*
AMF							
AC (%)	***	***	***	*	ns	ns	ns b
VC (%)	***	***	*	***	*	ns	ns
HC (%)	***	***	***	***	ns	ns	ns
N, P Content							
Leaf N (mg g^{-1})	***	***	***	***	***	*	ns
Stem N (mg g^{-1})	***	***	***	***	***	ns	ns
Root N (mg g^{-1})	***	***	***	***	**	*	ns
Nodule N (mg g^{-1})	***	***	***	ns	ns	*	ns
Leaf P (mg g^{-1})	***	***	***	***	**	**	ns
Stem P (mg g^{-1})	***	***	***	***	***	***	**
Root P (mg g^{-1})	***	***	***	***	***	***	**
Nodule P (mg g^{-1})	***	***	***	ns	ns	**	ns
Biomass of plant part							
Leaf area (cm^2 pl^{-1})	***	***	***	***	***	**	*
Leaf d. w. (g pl^{-1})	***	***	***	**	*	**	ns
Stem d. w. (g pl^{-1})	***	***	***	ns	*	*	ns
Root d. w. (g pl^{-1})	***	***	***	*	*	**	ns
Nodule d. w. (g pl^{-1})	*	***	***	ns	ns	***	ns
Biomass (g pl^{-1})	***	***	***	***	***	***	ns

*, **, *** Effects that are significant at P < 005, 001 and 0001, respectively; ns, effects that are not significant at P < 005.

Table 5.2. (a) N and (b) P concentration in leaf, stem, root and nodule of *Vicia faba* L. as influenced by the factors A (N, NM, NR and NRM), B (N supplies LN and HN) and C (harvest intervals T_1 and T_2), a. Variable value = N concentration (mg g^{-1}DM)

Factors			Variable		Variable		Variable		Variable	
A.	B	C	Leaf N	Tuk-t	Stem N	Tuk-t	Root N	Tuk-t	Nodule N	Tuk-t
N	LN	T_1	25.61 ± 1.07	a	11.79 ± 0.46	a	20.48 ± 0.98	a		
N	LN	T_2	25.64 ± 0.78	a	11.89 ± 0.54	a	20.15 ± 0.53	a		
N	HN	T_1	39.51 ± 0.61	cd	19.61 ± 0.59	cd	23.67 ± 1.34	bc		
N	HN	T_2	40.05 ± 0.65	de	19.69 ± 0.66	cd	23.84 ± 0.49	bcd		
NM	LN	T_1	27.14 ± 0.85	a	13.10 ± 0.65	ab	22.62 ± 0.94	ab		
NM	LN	T_2	29.47 ± 0.55	b	14.99 ± 0.53	b	25.01 ± 0.56	bcde		
NM	HN	T_1	40.61 ± 0.70	de	20.23 ± 0.68	d	24.08 ± 0.48	bcd		
NM	HN	T_2	41.80 ± 0.95	ef	21.37 ± 0.41	def	24.89 ± 0.81	bcde		
NR	LN	T_1	37.62 ± 1.10	c	17.81 ± 0.45	c	25.67 ± 0.95	cdef	51.53 ± 0.75	abc
NR	LN	T_2	41.44 ± 1.13	def	20.31 ± 0.40	de	28.60 ± 1.17	gh	54.40 ± 1.61	c
NR	HN	T_1	41.70 ± 0.64	ef	20.98 ± 0.74	def	26.68 ± 1.01	defg	49.99 ± 0.64	a
NR	HN	T_2	43.31 ± 0.92	fg	22.58 ± 0.94	f	28.40 ± 1.22	fgh	50.83 ± 1.49	ab
NMR	LN	T_1	41.97 ± 0.73	ef	22.50 ± 0.93	f	27.45 ± 1.13	efg	53.89 ± 0.93	bc
NMR	LN	T_2	45.28 ± 1.26	g	25.20 ± 0.91	g	31.14 ± 0.89	h	57.88 ± 1.40	d
NMR	HN	T_1	45.05 ± 0.95	g	22.24 ± 0.90	ef	28.22 ± 0.70	fg	52.61 ± 1.54	abc
NMR	HN	T_2	47.82 ± 0.87	h	25.06 ± 0.75	g	29.46 ± 1.36	gh	53.91 ± 1.45	bc

b.Variable value = P concentration (mg g^{-1}DM)

Factors			Variable		Variable		Variable		Variable	
A	B	C	Leaf P	Tuk-t	Stem P	Tuk-t	Root P	Tuk-t	Nodule P	Tuk-t
N	LN	T_1	1.59 ± 0.06	a	1.42 ± 0.07	a	1.69 ± 0.04	a		
N	LN	T_2	1.60 ± 0.04	a	1.41 ± 0.04	a	1.68 ± 0.07	a		
N	HN	T_1	1.71 ± 0.04	ab	1.55 ± 0.08	abc	1.73 ± 0.04	a		
N	HN	T_2	1.72 ± 0.05	ab	1.54 ± 0.12	abc	1.73 ± 0.08	a		
NM	LN	T_1	2.23 ± 0.05	de	2.11 ± 0.09	gh	2.20 ± 0.09	cd		
NM	LN	T_2	2.65 ± 0.09	gh	2.67 ± 0.10	i	2.79 ± 0.10	f		
NM	HN	T_1	2.05 ± 0.12	cd	1.84 ± 0.06	def	2.17 ± 0.09	cd		
NM	HN	T_2	2.33 ± 0.10	ef	1.97 ± 0.05	efg	2.24 ± 0.10	d		
NR	LN	T_1	1.79 ± 0.05	ab	1.54 ± 0.08	abc	1.81 ± 0.04	ab	3.84 ± 0.09	b
NR	LN	T_2	2.08 ± 0.09	d	1.66 ± 0.06	bcd	1.99 ± 0.05	bc	4.49 ± 0.23	c
NR	HN	T_1	1.77 ± 0.08	ab	1.67 ± 0.05	bcd	1.78 ± 0.07	a	3.04 ± 0.15	a
NR	HN	T_2	1.86 ± 0.06	bc	1.77 ± 0.03	cde	1.79 ± 0.05	ab	3.85 ± 0.21	b
NMR	LN	T_1	2.45 ± 0.13	efg	2.35 ± 0.12	h	2.45 ± 0.09	e	4.91 ± 0.09	cd
NMR	LN	T_2	2.87 ± 0.07	h	2.93 ± 0.14	j	2.93 ± 0.05	f	5.25 ± 0.09	d
NMR	HN	T_1	2.28 ± 0.06	de	2.06 ± 0.11	fg	2.24 ± 0.60	d	3.68 ± 0.15	b
NMR	HN	T_2	2.51 ± 0.07	fg	2.15 ± 0.08	gh	2.34 ± 0.10	de	4.73 ± 0.26	c

Values are means ± s.e, n = 8.

Different letters indicate significant differences assessed by Tukey HSD test (P < 005) after performing three-way ANOVA with residual estimation.

Table 5.3. The influence of N and P supply rats on leaf N and P concentration, total P accumulated, leaf area production, light saturated photosynthetic rate (P_{max}), and quantum yield efficiency (QYE).

Factors		SLN ($g\ N\ m^{-2}$)		SLP ($g\ N\ m^{-2}$)		Total P ($g\ plant^{-1} * 10^{-3}$)	
A	B	Mean ± SE	Tuk-t	Mean ± SE	Tuk-t	Mean ± SE	Tuk-t
10 ppm N	LP	0.721 ± 0.067	a	0.045 ± 0.004	c	3.171 ± 0.368	a
25 ppm N	LP	0.812 ± 0.070	ab	0.042 ± 0.005	bc	3.412 ± 0.377	a
50 ppm N	LP	0.871 ± 0.074	abc	0.038 ± 0.003	abc	3.571 ± 0.366	a
100ppm N	LP	0.969 ± 0.099	bcd	0.034 ± 0.004	abc	3.850 ± 0.346	a
250ppm N	LP	1.036 ± 0.098	cde	0.031 ± 0.004	ab	4.128 ± 0.628	a
500ppm N	LP	1.111 ± 0.137	de	0.029 ± 0.003	a	4.309 ± 0.522	a
10 ppm N	HP	1.098 ± 0.097	de	0.124 ± 0.010	f	9.903 ± 1.171	b
25 ppm N	HP	1.230 ± 0.087	ef	0.120 ± 0.009	f	10.525 ± 0.972	b
50 ppm N	HP	1.349 ± 0.122	fg	0.114 ± 0.008	ef	11.298 ± 1.129	bc
100ppm N	HP	1.521 ± 0.095	g	0.105 ± 0.006	e	12.178 ± 1.157	cd
250ppm N	HP	1.849 ± 0.126	h	0.093 ± 0.007	d	13.620 ± 1.012	de
500ppm N	HP	2.227 ± 0.142	i	0.083 ± 0.008	d	14.929 ± 0.996	e
n		6		6		6	

Factors		Leaf aera ($cm^2\ plant^{-2}$)		P_{max} ($\mu mol\ CO_2\ m^{-2}s^{-1}$)		QYE ($\mu molCO_2 \mu molQ^{-1} * 10^{-2}$)	
A	B	Mean ± SE	Tuk-t	Mean ± SE	Tuk-t	Mean ± SE	Tuk-t
10 ppm N	LP	117.7 ± 21.6	a	2.180 ± 0.253	a	0.230 ± 0.016	a
25 ppm N	LP	157.9 ± 24.3	ab	7.562 ± 0.620	b	1.001 ± 0.116	b
50 ppm N	LP	197.6 ± 19.5	bcd	12.110 ± 0.792	c	1.541 ± 0.144	c
100ppm N	LP	250.3 ± 20.3	e	15.039 ± 0.994	d	1.830 ± 0.180	cd
250ppm N	LP	307.1 ± 25.6	f	17.166 ± 1.248	ef	2.140 ± 0.123	d
500ppm N	LP	355.8 ± 30.8	f	18.374 ± 1.076	fg	2.320 ± 0.119	de
10 ppm N	HP	177.1 ± 21.7	bc	6.398 ± 0.516	b	2.739 ± 0.144	e
25 ppm N	HP	210.8 ± 21.8	cde	12.391 ± 0.822	c	3.861 ± 0.176	f
50 ppm N	HP	248.3 ± 27.2	de	16.145 ± 0.675	de	6.879 ± 0.379	g
100ppm N	HP	319.8 ± 30.1	f	19.108 ± 0.914	g	7.988 ± 0.610	h
250ppm N	HP	433.2 ± 31.2	g	20.875 ± 1.246	h	8.742 ± 0.496	i
500ppm N	HP	584.3 ± 38.6	h	21.692 ± 1.152	h	9.151 ± 0.498	i
n		6		8		8	

Low P (LP) plants were supplied with 0.5 mM P and high P (HP) plants were supplied with 1.6 mM P. Values are means ± s.e. and different letters indicate significant difference assessed by the Tukey HSD test ($p < 0.05$) after performing three ways ANOVA with residual estimation.

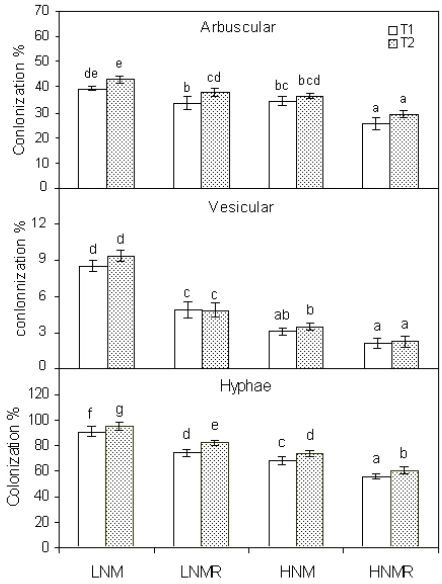

Figure 5.1. Percentage of arbuscular (AC%), vesicular (VC%) and hyphae colonization (HC%) in response to Rhizobium inoculation, low nitrogen (LN = 10 ppm) and high nitrogen (HN = 250 ppm N) supply at two harvests (T_1 and T_2). Vertical bars represent s.e. (n = 4) of the means. Different letters indicate significant differences assessed by the Tukey HSD test (P < 005) after performing three-way ANOVA with residual estimation.

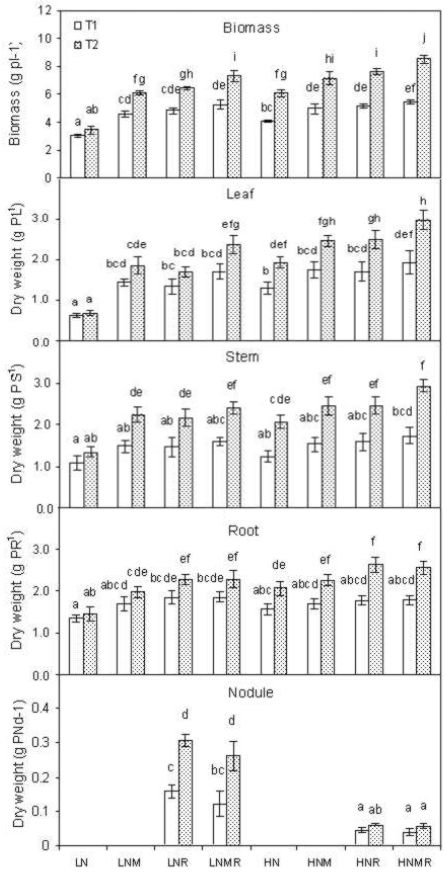

Figure 5.2. Leaf, stem, root, nodule dry weight production and total biomass as influenced by nitrogen supply (LN and HN) and microbial symbiotic association (N, NM, NR and NMR) at two harvests (T_1 and T_2). Vertical bars represent s.e. (n=4) of the means. Different letters indicate significant differences assessed by Tukey HSD test (P < 005) after performing three-way ANOVA with residual estimation.

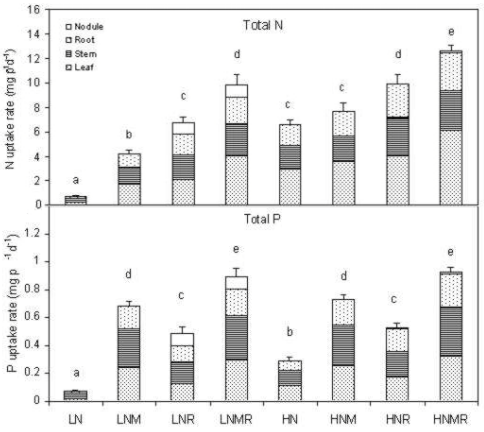

Figure 5.3. N and P accumulation rate and partitioning into leaves, stems, roots and nodules as influenced by nitrogen supply (LN and HN) and microbial symbiotic association (N, NM, NR and NMR). Vertical bars represent s.e. (n = 4) of the means. Different letters indicate significant differences assessed by the Tukey HSD test (P < 005) after performing two-way ANOVA with residual estimation. N and P accumulation rate (mg plant^{-1}d^{-1}) represents an average rate of accumulation calculated as (T_2 amount – T_1 amount)/9 d.

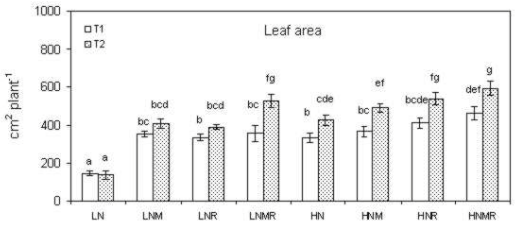

Figure 5.4. Leaf area (cm^2 $plant^{-1}$) production as influenced by nitrogen supply (LN and HN) and microbial symbiotic association (N, NM, NR and NMR) at two harvest intervals (T_1 and T_2).Vertical bars represent s.e. (n = 4) of the means. Different letters indicate significant differences assessed by Tukey HSD test (P < 005) after performing three-way ANOVA with residual estimation.

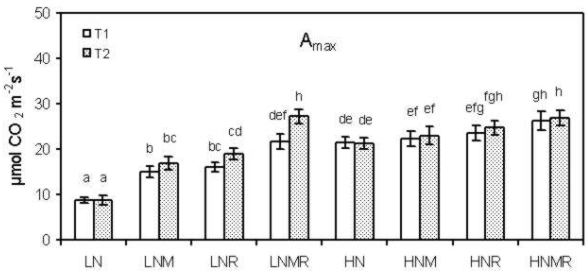

Figure 5.5. Light saturated (2000 mmol quantam^{-2} s^{-1}) photosynthetic rate as influenced by nitrogen supply (LN and HN) and microbial symbiotic association (N, NM, NR and NMR) at two harvest intervals (T_1 and T_2). Vertical bars represent s.e. (n = 8) of the means. Different letters indicate significant differences assessed by Tukey HSD test (P < 005) after performing three-way ANOVA with residual estimation.

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CHAPTER SIX:

GROWTH YIELD OF *VICIA FABA L* IN RESPONSE TO MICROBIAL SYMBIOTIC ASSOCIATIONS

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Chapter 6:

Growth yield of *Vicia faba L* in response to microbial symbiotic associations

6.1 Abstract

A multifactor experiment was undertaken to investigate the influence of *Rhizobium* and arbuscular mycorrhizal fungi (AMF) on biomass production, leaf area generation, whole plant photosynthetic and respiration rates, growth yield and maintenance respiration in *Vicia faba L.* under low phosphorous (P) supply. Treatments consisted of low (LN) or high nitrogen (HN) supply with or without microbial symbiont(s). Plants were harvested at 54 ($T_{1)}$ and 63 (T_2) days after planting. In all instances, plants colonized with both microbial symbionts had significantly higher total biomasses, leaf areas, and whole plant photosynthesis and respiration rates than plants with only one or without microbial symbionts. Similarly, plants with both microbial symbionts also had significantly higher ($\geqslant$0.7) growth yield (Y_g) values than all the other treatments. There were no significant differences in Y_g values between harvest intervals within individual treatments. Maintenance respiration rates were also highest in plants with two microbial symbionts ($>30 CO_2$ $(gDM)^{-1}$ d^{-1}). In LN plants colonized by both microbial symbionts there was evidence of compensatory increases in the photosynthetic rates in response to the carbon sink demands of the microbial symbionts. Finally, the results of this investigation are consistent with the hypothesis that the plant potential photosynthetic capacity exceeds the carbon demand of the *Rhizobium*–AMF symbiotic complex.

Key words: Arbuscular mycorrhizal fungi; Growth yield efficiency; Respiration;

Rhizobium; Tripartite symbiotic association; *Vicia faba L.*

Abbreviations: AMF, arbuscular mycorrhizal fungi; c, a maintenance coefficient; D, total whole plant daytime CO_2 influx (g CO_2 12 h^{-1} $plant^{-1}$); HN, high nitrogen treatment; HNM, high nitrogen with AMF colonization; HNMR, high nitrogen with AMF and *Rhizobium* colonization; HNR, high nitrogen with *Rhizobium* colonization; LN, low nitrogen treatment; LNM, low nitrogen with AMF colonization; LNMR, low nitrogen with AMF and *Rhizobium* colonization; LNR, low nitrogen with *Rhizobium* colonization; m, maintenance respiration coefficient (mg CO_2 (g DM) $^{-1}$ d^{-1}); N_d, total whole plantCO_2 efflux after prolonged dark exposure (gCO_2 12h^{-1} plant $^{-1}$); N_r, total night time whole plant CO_2 efflux (g CO_2 12 h^{-1} plant $^{-1}$); N_{soil}, total soil 12 h dark CO_2 efflux (g CO_2 12 h^{-1}plant $^{-1}$); W, total plant dry mass; Y_g, growth yield efficiency (g CO_2 (g CO_2) $^{-1}$).

6.2 Introduction

Arbuscular mycorrhizal fungi (AMF) and *Rhizobium* play an important role as microbial endosymbionts in the supply of P and N to legumes growing in nutrient deficient soils (Azcón et al., 1979). In exchange for P or N the two microbial symbionts receive C from the legume host. Thus the formation of the tripartite symbiotic association (legume–AMF–*Rhizobium*) is dependent on a complex three-way source-sink relation involving C exchanges for P and C exchanges for N (Brown and Bethlenfalvay, 1986). In most reported instances these exchanges have had a positive influence on legume growth (Azcón et al., 1979; Paul and Kucey, 1981; Harris et al., 1985; Brown and Bethlenfalvay, 1988).

In a study investigating the carbon economy of the tripartite soybean – *Glomus* – *Rhizobium* symbiotic association, Harris et al. (1985) found that carbon was allocated in the following proportions: 30.49% to leaves, 20.52% to stems and petioles, 6.3% to shoot respiration, 7.8% to roots, 2.0% to nodules, 2.7% to AMF, 5.2% to root and soil respiration, 13.7% to AMF respiration, 9.4% to nodule respiration. In their study approximately 42.6% of photosynthate was allocated to below ground sinks. The below ground carbon allocation was divided between the various sinks as follows: 38.6% to the AMF, 26.6% to nodules and 30.6% to roots. It appears that the complex C, N and P three-way source–sink relations between the members of the tripartite symbiotic association does not limit plant productivity when compared to non-symbiotic plants with non-limiting supplies of P and N (Brown and Bethlenfalvay, 1988). This supports the hypothesis that carbon demand by the microbial symbionts does not limit plant growth.

In the case of AMF, the sink demand created by fungal colonization could account for an extra 4–26% drain of photosynthate from the AM-infected host plant (Pang and Paul, 1980; Kucey and Paul, 1982; Snellgrove et al., 1982; Koch and Johnson, 1984; Harris et al., 1985; Douds et al., 1988; Wang et al., 1989; Jakobsen and Rosendahl, 1990; Black *et al*. 2000). The maximum hypothetical photosynthetic allocation to the AMF association may well be as high as 40%–50% of the total photosynthate production (Stribley et al., 1980). This does suggest that the carbon demand by the AMF symbiont has the potential to limit plant growth (Buwalda and Goh, 1982), and thereby bring about a decline in the plant growth yield efficiency. It has been proposed that increases in the photosynthetic rate of the AM-infected host plant fully compensates for carbon losses from the plant due to increases in AM carbon demand (Brown and Bethlenfalvay,

1988; Fitter, 1991; Wright et al., 1998a,b). In experiments where the N and P status of AM-infected plant and non-mycorrhizal plants were similar, it was found that mycorrhizal plants had higher photosynthetic rates but similar biomasses to the non-mycorrhizal, indicating that the additional photosynthetic rates had been allocated to the fungal symbiont (Wright et al., 1998a, b). Other results appear to support the conclusion that any increases in the photosynthetic rate of AM-infected plants growing under low P conditions may be mainly due to mycorrhizal-dependent increases in the plant P status (Allen et al., 1981; Fredeen and Terry, 1988; Azcón et al., 1992; Black et al., 2000). The possibility that increases in photosynthetic rates in AM-infected plants may be due to the combined effects of enhanced P status and AM-dependent carbon sink has also been acknowledged (Black et al., 2000). An increase in the photosynthetic rate in the leaves of AM-infected cucumber was found to be due to an increase in the leave P status and not due to compensatory increases in photosynthesis in response to increases in the mycorrhizal sink demand for assimilates (Black et al., 2000). In barley where there was no difference in P status between AM-infected plants and non-mycorrhizal plants, the AM-infected plants had enhanced photosynthetic rates, indicating a compensatory response to mycorrhizal colonization (Fay et al., 1996). Also, at equivalent P:N ratios AM-infected *Andropogon gerardii* had higher overall photosynthetic rates compared to the non-mycorrhizal plants (Miller et al., 2002). This enhancement of photosynthetic rates in mycorrhizal plants was found not to be indirectly related to the plant P or N status but directly due compensatory responses to fungal colonization (Miller et al., 2002).These results are similar to the ones reported by Wright et al. (1998b).

A number of attempts have been made to quantify the stoichiometric exchange of

mycorrhizal acquired phosphorus for photosynthate (Douds et al., 1988; Eissenstat et al., 1993; Pearson and Jakobsen, 1993). Schwab et al.(1991) have proposed a model involving the exchange of one triose-phosphate for one inorganic phosphate molecule. This gives a carbon/phosphorus exchange ratio of 3. A positive correlation has been observed to exist between the capacity of AMF to stimulate the growth of the host plant and the radiant flux density incident on the plant (Same et al., 1983; Son and Smith, 1988). Thus it could be argued that any enhancement of additional photosynthetic capacity above the levels of P non-limited non-mycorrhizal plants would be strongly dependent on the P supply from AMF.

In the case of rhizobial endosymbiont, dinitrogen(N_2)fixation plus nodule growth also creates like AMF a sink demand for photosynthate (Bethlenfalvay and Brown, 1985; Brown and Bethlenfalvay, 1986). A number of estimates evaluating the carbon energetic cost of N_2-fixation have been attempted (Salsac et al., 1984; Twary and Heichel, 1991; Vance and Heichel, 1991). Total energy costs including energy utilized for the growth of nodulated roots, maintenance respiration of nodules, N_2-fixation and ammonia assimilation range from 0.4 to 19.4 g C g^{-1}N (Salsac et al., 1984). If the energetic costs associated with the growth and maintenance of nodules are omitted, then the estimates of the energy costs for N_2-fixation range from 2.5 to 8.0 g C g^{-1} N (Atkins, 1984; Salsac et al., 1984). Gross and net carbon requirements for N_2-fixation in alfalfa range from 3.5 to 11.9 g C g^{-1} N and 2.5 to 8.8 g C g^{-1} N, respectively (Twary and Heichel, 1991). The average value from over 35 estimates for the carbon cost of N_2-fixation was approximately 6.0 g C g^{-1} N (Vance and Heichel, 1991).

In general the evidence indicates that the C:P and C:N exchanges between the host and

the two microbial symbionts under P and N limiting conditions does not diminish legume productivity relative to plants that are not nutrient limited (Azcón et al., 1979; Paul and Kucey, 1981; Harris et al., 1985; Brown and Bethlenfalvay, 1988; Gray, 1996). In general, plant growth is usually co-limited by both N and P supply (Jia and Gray, 2004). This observation is consistent with recent studies that have investigated the relationship between N:P stoichiometries and yield maximization in various crops (Agren, 2004; Sadras, 2006). In a previous study we have shown that the ratio of P to N was a major factor in determining the level of productivity in *Vicia faba L* (Jia et al., 2004). Plants that were part of tripartite symbiotic association (V. faba – AMF – *Rhizobium*) had significantly higher elemental P to N compared to plants with no symbiotic association. These results also confirmed the original observations of Brown and Bethlenfalvay (1988) that plants colonized by both AMF and *Rhizobium* had significantly higher photosynthetic nitrogen-use efficiencies and photosynthetic phosphorus-use efficiencies. Both P and N use efficiency has been shown to be strongly dependent on the P to N supply ratio (Jia and Gray, 2004).

The objective of this study was to investigate what affects the tripartite symbiotic association had on the host growth yield and maintenance respiration under P and N limiting conditions. The authors note that for legumes there have been several studies on maintenance respiration and growth yield (McCree and Silsbury, 1978; Irving and Silsbury, 1987), however these studies did not include the effects of microbial symbiotic partners. Also, while many other studies have focused on the apparent carbon costs induced by the tripartite symbiotic association, they have not directly investigated the impact of these costs on the growth yield (Azcón et al., 1979; Paul and Kucey, 1981; Harris et al., 1985; Douds et al., 1988). Therefore it was the goal of this study to fill in

some of these gaps in our knowledge by investigating how the two microbial symbionts influence maintenance respiration and growth yield.

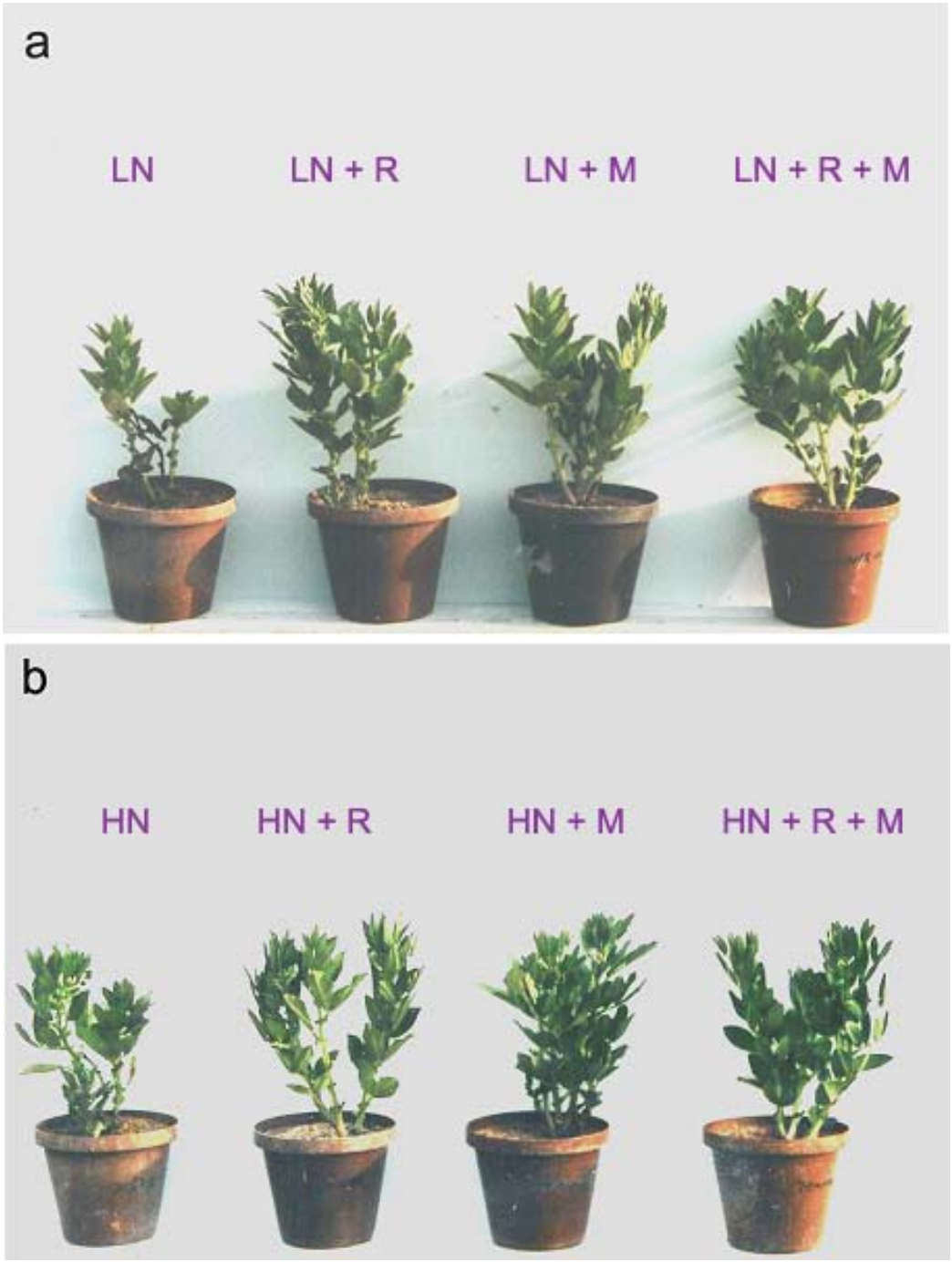
6.3. Materials and methods

6.3.1 Plant material and growth conditions

In this study AMF infection, *Rhizobium* inoculum preparation and application were the same as in Jia et al. (2004). *Rhizobium leguminosarum* biovar *viciae* inoculum was obtained from the active nodules of *V. faba* L. plants that had been previously infected with this strain. Plants were grown under outdoor environmental conditions. Day length and mean daily extraterrestrial radiation ranged from 10.78 to 12.97h and 24.4 to 39.1 MJm^{-2} respectively. Thus, plants with microbial symbiotic associations were not grown under light-limiting conditions. Consequently, for the photosynthesis, respiration and growth yield measurements the plants were pre-adapted to photosynthetically active irradiance intensities corresponding to full sunlight. Plants were also adapted to atmospheric vapour demands corresponding to daily A-pan evaporation rates ranging from 6.4 to 8.0 mm d^{-1}. For the duration of the experiment mean daily maximum and minimum temperatures ranged from 19.3 to 23.8℃ and 6.4 to 11.3℃, respectively.

6.3.2 Experimental design

At each harvest, four replicates of each treatment were randomly selected. The plants were separated into leaves, stems, roots and nodules, and oven-dried at 65℃ for three days. Also at each harvest, a root sub-sample from each treatment was taken to verify



Picture 6.1 Photographs of *Vicia faba L.* under different nitrogen supplies. a. Low nitrogen (LN) treatments: 0.71 mM KNO_3 (10 ppm N); b. High nitrogen (HN) treatments: 17.86 mM KNO_3 (250 ppm N). R = *Rhizobium*, M = arbuscular mycorrhizal fungi.

presence or absence of AMF infection (data not shown). The experimental design was a randomized complete block with four replications for each harvest interval, with combinations of treatment factors randomly assigned to pots in the block. The concentration of nitrate-N for the low N (LN) treatments was 0.71mM KNO_3 (10 ppm N) and 17.86mM KNO_3 (250 ppm N) for the high N treatments (HN). All treatments received a phosphorus supply of 0.05 mg P l^{-1} (1.61μM NaH_2PO_4). A modified Long-Ashton nutrient mixture was used to supply the non-nitrogen micro-and macro-nutrients (Jia et al. 2004).

The treatments consisted of low or high nitrogen supply with or without microbial symbiont(s). Application of different combinations of factors were used to generate the following 8 treatments: 1) low nitrogen supply without any microbial symbionts-LN; 2) high nitrogen supply without any microbial symbionts-HN; 3) low nitrogen supply with AMF infection-LNM; 4) high nitrogen supply with AMF infection-HNM; 5) low nitrogen supply with *Rhizobium* inoculation-LNR; 6) high nitrogen supply with *Rhizobium* inoculation-HNR; 7) low nitrogen supply with both AMF and *Rhizobium* present-LNRM; 8) high nitrogen supply with both AMF and *Rhizobium* present-HNRM. Plants were harvested at 54 (T_1) and 63 (T_2) days after planting.

6.3.3 Measurement of CO_2 exchange

The procedure for monitoring whole plant CO_2 exchange was adapted after Jia and Gray (2004). The modification involved replacing the leaf cuvette with a whole plant CO_2 assimilation chamber. Intact potted plants were placed in a specially constructed glass chamber (internal diameter 150 mm and height 300 mm) surrounded by a water

jacket. A small fan within the chamber ensured that the atmosphere within the chamber was well mixed. The chamber contained a thermocouple that was fixed with sticky tape to the underside of a leaf within the plant canopy. The temperature of the water in the water jacket was adjusted until the leaf temperature remained stable at 25℃. Whole plant net CO_2 uptake (D) was measured continuously over a 12-h day at high photon flux density (2000 μmol m^{-2} s^{-1}). Thereafter plants were kept in darkness for 12 h and CO_2 efflux (N_r) was measured continuously. In addition, the CO_2 efflux was measured continuously under prolonged darkness (36 h) until the efflux had decayed to a constant rate (N_d). For the soil respiration experiments, plants including roots were removed from the soil by passing soil through a 5-mesh sieve. For quantification of soil respiration rates, soil was carefully repacked into the pots after which CO2 production was measured for 12 h and after a prolonged dark period until the efflux rate had decayed to a constant rate. Soil CO2 effluxes (N_{soil})were then subtracted from both N_r and N_d to give plant only CO_2 effluxes.

6.3.4 Growth yield

The procedure of McCree and Silsbury (1978) was used to calculate values for the growth yield (Y_g) and maintenance respiration coefficient (m). Y_g depends ultimately on the plant net diurnal CO_2 fluxes and can thus be computed from the ratio of respiratory carbon efflux to photosynthetic carbon influx. This ratio which has been designated as parameter k has been defined as follows (Irving and Silsbury, 1987):

$$k = \frac{N_r - N_d}{D + N_d} \qquad (1)$$

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where *k* has the dimensions g g^{-1} . The different CO_2 fluxes used to compute k have been defined as follows: N_r represents the total night time carbon efflux (g CO_2 12 h^{-1} $plant^{-1}$); *D* represents the total daytime photosynthetic carbon influx (g CO_2 12 h^{-1} $plant^{-1}$); N_d represents12h rate of CO_2 efflux after a prolonged dark period (g CO_2 12 $h^{-1}plant^{-1}$). It has been experimentally established that with regard to N_d the following relationship holds (McCree, 1970, 1974):

$$N_d = cW \qquad (2)$$

where *W* represents plant dry mass (DM) and *c* is a maintenance coefficient. The relationship between parameter *k* and the growth yield Y_g has been defined as follows (McCree and Silsbury, 1978):

$$Y_g = \frac{1-k}{1+k} \qquad (3)$$

where the dimensions of Y_g are g CO_2 (g CO_2) $^{-1}$.

6.3.5 Maintenance respiration

The dark decay procedure of Irving and Silsbury (1987) was used to measure maintenance respiration. The maintenance respiratory coefficient (m) with dimensions mgCO2 (gDM) $^{-1}$ d^{-1} was estimated as follows:

$$m = \frac{2N_d}{W} \quad (4)$$

6.3.6. Statistical analysis

Dry mass of leaves, stems, roots and nodules were analysed by three-way ANOVA for main effects and international effects. The significance of the differences in the various variables resulting from interactions between factors A (N, NM, NR and NRM), B (N supply LN and HN) and C (harvestdateT1 andT2) were tested. Multiple comparisons of means were performed by the Tukey HSD test ($p < 0.05$) after performing three-way ANOVA with residual estimation. The STATISTICA (version 6.0) package was used for statistical analysis.

6.4 Results

6.4.1 Effects of treatment factors on plant variables

Table 1 summarizes the effects arising from interactions between the three sets of factors. In relation to their combined effects, the three-way ANOVA results showed that the majority of first-order interactions had significant effects on all plant variables. Details of the combined effects of the three factors on plant leaf area, plant leaf mass, total plant biomass (including roots) are shown in Table 2. In all instances, microbial colonization of plants was associated with significant increases in the magnitudes of total biomass and leaf area.

6.4.2 Microbial effects on whole plant CO_2 influxes

After a prolonged period of darkness the CO_2 efflux decayed to a constant rate (Figure 6.1) and these values were then used to estimate N_d. The values that were measured for D, N_r and N_d and which were used for computing parameter k are shown in Table 6.2. The magnitudes of the total day time whole plant photosynthetic carbon influxes D were influenced by the level of N supply and microbial associations (Table 6.2). At each harvest interval the D values for LNM and LNR plants were not significantly different (Table 6.2). Similarly, at each harvest interval the D values for HNM and HNR plants were also not significantly different. However, the D values for HNM and HNR plants were significantly higher than the values for the LNM and LNR plants. At the second harvest interval the D values for HN, LNM and LNR plants were not significantly different. In contrast to this, at both harvest intervals the D values for HNM and HNR plants were significantly higher than the values for HN plants. At the first harvest interval, LNM, LNR, HN, HNM and HNR plants all had similar biomasses and leaf areas (Table 6.2). But at the second harvest interval the biomasses and leaf areas of the LNM and LNR plants were significantly lower than the HNM and HNR plants. The lower D and plant biomass values for the LNM and LNR plants appears to be consistent with N and P being co-limiting. This putative N and P co-limitation of D and biomass production appears to have been overcome in the LNMR plants.

The highest D values were achieved for the LNMR and HNMR plants (Table 6.2). At the first harvest interval the D values were similar, but at the second harvest interval the values for the LNMR plants were significantly higher than the HNMR plant values. However, at both harvest intervals the LNMR plant biomasses and leaf areas were not

significantly different from the HNMR values (Table 6.2). This result provides some evidence of compensatory increases in the photosynthesis of LNMR plants in response to the carbon sink demands of the microbial symbionts, especially at low N supplies.

6.4.3 Microbial effects on night time CO_2 effluxes

The magnitude of the total night time carbon effluxes, N_r were dependent on plant size. Night time carbon effluxes were significantly higher in plants that had one or more microbial symbiotic association. As was the case with *D*, LNM and LNR plants gave similar values for N_r (Table 6.2). The pattern was repeated for the HNM and HNR plants. Plants that were part of a tripartite symbiotic complex, LNMR and HNMR, had the highest N_r. At the second harvest interval while LNMR had a significantly higher *D* value, both LNMR and HNMR plants had similar Nr values.

The trend for CO_2 effluxesafter aprolonged darkperiod, N_d mirrored the patterns observed for *D* and N_r. Soil respiration rates, N_{soil}, were subtracted from total measured respiration rates before being used in the calculations for N_r and N_d. Soil respiration rates where highest for treatments having a microbial symbiotic association (Table 6.2). The highest rates of soil respiration occurred with LNRM plants.

6.4.4 Microbial effects on parameter *k*

The impact of the treatment factors on parameter *k* have been given in Table 2. Lower *k* values tended to be associated with plants having the highest biomass and leaf areas. In most instances *k* remained constant over the two harvest intervals. Plants without any

microbial symbiotic association had significantly higher *k* parameter values. In the absence of any microbial associations, HN plants had lower *k* values than LN plants. Thus an increase in N supply reduces the value of *k*. Colonization by one or two microbial symbionts always resulted in a significant decline in the *k* value relative to LN and HN plants. Plants with a single microbial association, either with AMF or *Rhizobium* always had similar *k* values irrespective of the N supply level. Plants colonized with both AMF and *Rhizobium* also had similar *k* values irrespective of the N supply level.

6.4.5 Microbial effects on the growth yield

Increases in plant productivity were associated with a significant increase in Y_g. For broad bean the Y_g values ranged from 0.44 to 0.78 (Table 6.2). Growth yield were significantly higher in plants that had one or more microbial symbiotic association. All the plants with one microbial symbiotic had similar Y_g values irrespective of N supply level. Plants with two microbial associations had significantly higher Y_g values ($\geqslant$0.7) than all the other treatments. There were no significant differences in Y_g values between harvest intervals within individual treatments. This finding has theoretical significance for plant growth modelling because it is often assumed that under steady-state conditions physiological parameters such as Y_g remain constant over time. In this study the experimental conditions in terms of nutrient supply were close to a steady-state situation. The results for Y_g show that, even where there were complex interactions between organisms such as the tripartite symbiotic association (legume–AMF–*Rhizobium*), the parameters such as the growth yield remain constant under steady-state conditions.

6.4.6 Microbial effects on maintenance respiration

There were no significantly differences in the maintenance respiration rates for plants (m) with one microbial association (Table 6.2). Their values fell between 12 and 36 $CO_2(gDM)^{-1}d^{-1}$. Maintenance respiration rates for the HN and HNMR plants were also not significantly different. Maintenance respiration rates were highest (>30 Y_g) in LNMR plants. As with Y_g, the m rates also remained constant over the two harvest intervals for individual treatments. However, if instead of using Eq. (4) and the instantaneous total plant dry mass, the m rates were calculated from the slope of the N_d versus total plant dry mass plot, then the values for m were found to be slightly higher, ranging from 16 to 43 $CO_2(gDM)^{-1}d^{-1}$ (see footnote to Table 2).

6.5 Discussion

Eq. (3) shows that high k values result in low Y_g values. The importance of *k* arises from the fact that it is a directly measurable parameter, where as Y_g is a derived parameter. In this study the values measured for *k* and Y_g were higher and lower respectively, relative to the values reported for *Trifolium subterraneum* L by McCree and Silsbury (1978). They reported temperature dependent values for *k* which ranged from 0.120 at 10℃ to 0.175 at 35℃ for plants exposed to irradiances of 2000 μmol m-2 s-1 while growing under non-limiting nutrient conditions. In their experiments Yg values ranged from 0.70 at 35℃ to 0.78 at 15℃. In this study low P supply in many of the treatments has possibly contributed to the k values being higher than those reported by McCree and

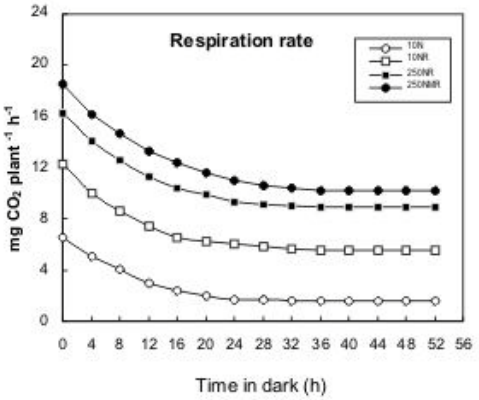

Figure 6.1. Examples of whole plant respiratory CO_2 efflux patterns after prolonged dark exposure (10N: un-inoculated plants receiving 10 mg N L^{-1}; 10NR: plants inoculated with *Rhizobium* and receiving 10 mg N L^{-1}; 250N: un-inoculated plants receiving 250 mg N L^{-1}; 250NR: plants inoculated with *Rhizobium* and receiving 250 mg N L^{-1}).

Table 6.1. The significance of the differences in plant variables and parameters resulting from the interactions among factors A (N, NM, NR and NRM), B (N supplies LN and HN) and C (harvest dates T_1 and T_2).

Source of Variance	A	B	C	A x B	A x C	B x C	AxBxC
Degree of Freedom	3	1	1	3	3	3	3
Variables:							
Biomass and leaf area							
Leaf area (cm^2 $plant^{-1}$)	***	***	***	***	***	**	*
Leaf dry mass (g $plant^{-1}$)	***	***	***	**	*	**	ns
Biomass (g $plant^{-1}$)	***	***	***	***	***	***	ns
D, N_r, N_d and N_{soil}							
D (g CO_2 $plant^{-1}$ $12h^{-1}$)	***	***	***	***	***	***	ns
N_r (g CO_2 $plant^{-1}$ $12h^{-1}$)	***	***	***	***	***	*	ns
N_d (g CO_2 $plant^{-1}$ $12h^{-1}$)	***	***	***	***	***	*	ns
N_{soil} (g CO_2 $plant^{-1}$ $12h^{-1}$)	***	*	ns	***	ns	ns	ns
k, Y_q and *m*							
k (g g^{-1})	***	***	***	***	ns	***	ns
Y_q (g CO_2 (g CO_2)$^{-1}$)	***	**	***	***	ns	*	ns
m (mg CO_2 (g DM)$^{-1}$ d^{-1})	***	***	**	***	ns	ns	ns

All variables and parameters were analysed by three-way ANOVA with residual estimation for main effects and interaction effects.

*, **, *** Effects that are significant at $p < 0.05$, 0.01 and 0.001 respectively;

ns: effects that are not significant at $p < 0.05$.

Table 6.2. The influence of N supply, *Rhizobium* and AMF on leaf area, biomass, whole plant photosynthesis, growth yield efficiency, respiration rates and respiratory parameters at two harvests (T_1 and T_2) in *Vacia faba* L.

Treatment	Leaf area (cm2plant-1)				Leaf dry mass (g plant-1)			
	T1		T2		T1		T2	
N	149.14 ± 10.35	a	140.73 ± 17.58	a	0.617 ± 0.043	a	0.677 ± 0.053	a
LNM	356.41 ± 14.21	bc	408.99 ± 21.07	bcd	1.424 ± 0.085	bcd	1.841 ± 0.168	cde
LNR	337.33 ± 12.88	b	390.10 ± 19.68	bcd	1.309 ± 0.090	bc	1.716 ± 0.065	bcd
LNMR	358.39 ± 31.74	bc	526.16 ± 33.40	fg	1.580 ± 0.107	bcd	2.487 ± 0.175	efg
HN	333.03 ± 22.72	b	427.69 ± 26.31	cde	1.493 ± 0.125	b	1.727 ± 0.122	def
HNM	367.10 ± 24.97	bc	491.11 ± 17.02	ef	1.804 ± 0.109	bcd	2.450 ± 0.124	gh
HNR	411.98 ± 220.6	bcde	538.99 ± 30.44	fg	1.723 ± 0.158	bcd	2.402 ± 0.194	gh
HNMR	461.31 ± 28.80	def	592.92 ± 33.29	g	2.029 ± 0.231	def	2.875 ± 0.181	h
n	4		4		4		4	
	Biomass (g plant^{-1})				D (g CO_2 plant^{-1} 12h^{-1})			
	T_1		T_2		T_1		T_2	
LN	3.044 ± 0.110	a	3.459 ± 0.212	ab	47.49 ± 5.27	a	49.22 ± 3.92	a
LNM	4.593 ± 0.171	cd	6.059 ± 0.214	fg	155.72 ± 8.65	b	202.52 ± 6.79	cd
LNR	4.779 ± 0.233	cde	6.465 ± 0.166	gh	156.17 ± 8.27	b	204.84 ± 7.54	cd
LNMR	5.139 ± 0.247	de	7.427 ± 0.338	i	258.81 ± 10.43	fgh	319.19 ± 13.93	i
HN	4.278 ± 0.146	bc	5.848 ± 0.268	fg	189.85 ± 11.91	c	189.43 ± 12.91	c
HNM	5.024 ± 0.303	de	7.149 ± 0.415	hi	222.23 ± 9.71	de	230.42 ± 14.46	def
HNR	5.142 ± 0.136	de	7.537 ± 0.155	fg	224.15 ± 13.40	de	239.05 ± 16.66	efg
HNMR	5.577 ± 0.119	ef	8.408 ± 0.256	j	264.44 ± 14.47	gh	282.37 ± 16.80	h
n	4		4		4		4	
	N_r (g CO_2 plant^{-1} 12h^{-1})				N_d (g CO_2 plant^{-1} 12h^{-1})			
	T_1		T_2		T_1		T_2	
LN	45.67 ± 6.08	a	47.26 ± 4.99	a	19.22 ± 2.54	a	22.52 ± 3.52	a
LNM	109.84 ± 6.06	b	142.23 ±7.80	d	58.86 ± 8.86	b	84.60 ± 7.64	cde
LNR	114.74 ± 6.24	bc	147.14 ± 7.03	de	66.35 ± 4.90	bc	94.49 ± 8.627	ef
LNMR	143.32 ± 11.58	d	190.17 ± 11.16	fg	84.26 ± 5.53	cde	133.91± 10.70	h
HN	125.18 ± 6.68	bcd	147.97 ± 14.50	de	58.42 ± 4.50	b	80.07 ± 7.45	cde
HNM	135.64 ± 9.32	cd	170.07 ± 8.37	ef	73.36 ± 9.11	bcd	105.46 ± 9.34	fg
HNR	135.41 ± 10.78	cd	183.41 ± 13.18	fg	75.91 ± 6.58	bcde	115.85 ± 10.63	gh
HNMR	148.83 ± 10.92	de	206.88 ± 12.33	g	87.67 ± 6.23	def	134.45 ± 5.77	h
n	4		4		4		4	

	k (g g^{-1})				Y_G (gCO_2 ($g CO_2$) $^{-1}$			
	T_1		T_2		T_1		T_2	
LN	0.399 ± 0.023	g	0.346 ± 0.018	f	0.435 ± 0.035	a	0.493 ± 0.042	ab
LNM	0.240 ± 0.017	cde	0.201 ± 0.017	bc	0.615 ± 0.032	cde	0.666 ± 0.029	cde
LNR	0.218 ± 0.022	bcd	0.176 ± 0.016	b	0.644 ± 0.037	cde	0.700 ± 0.023	ef
LNMR	0.174 ± 0.021	b	0.126 ± 0.014	a	0.707 ± 0.025	ef	0.779 ± 0.048	f
HN	0.271 ± 0.013	e	0.252 ± 0.013	de	0.576 ± 0.041	bc	0.600 ± 0.038	cd
HNM	0.214 ± 0.016	bcd	0.194 ± 0.013	b	0.652 ± 0.038	cde	0.676 ± 0.041	de
HNR	0.198 ± 0.023	bc	0.193 ± 0.016	b	0.669 ± 0.032	de	0.680 ± 0.027	de
HNMR	0.176 ± 0.021	b	0.174 ± 0.019	b	0.703 ± 0.030	ef	0.704 ± 0.047	ef
n	4		4		4		4	
	m (mg CO_2 ($g CO_2$)$^{-1}$ d^{-1})				N_{siol} ($g CO_2$ $plant^{-1}$ $12h^{-1}$)			
	T_1		T_2		T_1		T_2	
LN	12.625 ± 1.563	a	12.992 ± 1.607	a	0.150 ± 0.011	a	0.151 ± 0.016	a
LNM	25.762 ± 1.738	b	27.982 ± 1.656	bcd	0.178 ± 0.012	a	0.181 ± 0.012	a
LNR	27.774 ± 1.639	bcd	29.242 ± 1.919	bcde	0.335 ± 0.016	b	0.344 ± 0.019	bc
LNMR	32.830 ± 1.147	ef	36.026 ± 1.443	f	0.499 ± 0.031	e	0.509 ± 0.020	e
HN	27.321 ± 1.427	bc	27.443 ± 1.382	bc	0.153 ± 0.013	a	0.153 ± 0.013	a
HNM	29.316 ± 2.062	bcde	29.532 ± 1.710	bcde	0.178 ± 0.017	a	0.183 ± 0.015	a
HNR	29.545 ± 1.366	bcde	30.754 ± 1.328	cde	0.376 ± 0.017	bcd	0.387 ± 0.017	cd
HNMR	31.485 ± 1.504	de	31.987 ± 1.240	e	0.412 ± 0.018	d	0.417 ± 0.016	d
n	4		4		4		4	

Values are means ± s.e. (n = 4). Different letters indicate significant differences assessed by Tukey HSD test ($P<0.05$) after performing three-way ANOVA with residual estimation.

Silsbury (1978). However, the *k* values measured for LNMR and HNMR plants, 0.120 and 0.174 respectively, were similar to those obtained for *T. subterraneum* L. From various legume studies the average values reported for Y_g have been: 0.75 for *T. subterraneum* L (McCree and Silsbury, 1978), 0.7 for field bean, 0.72 for Lucerne and 0.68 for chick pea (Irving and Silsbury, 1987). In the current study LNMR and HNMR plants achieved values for $Y_g > 0.7$. This shows that the tripartite symbiotic complex can overcome the constraints on growth efficiency that arise as a consequence of low N and P supply.

Maintenance respiration estimates obtained for field bean, lucerne, chick pea, pea and kidney bean have been as follows: 21.35, 24.11, 28.65, 26.53, 16.51 mg CO_2 $(gDM)^{-1}d^{-1}$ respectively (Irving and Silsbury, 1987). For *T. subterraneum* L maintenance respiration rates ranged from 14 mg CO_2 $(gDM)^{-1}d^{-1}$ for plants grown at 10℃ to 64 mg CO_2 $(gDM)^{-1}d^{-1}$ for plants grown at 35℃ (McCree and Silsbury, 1978). In this study the maintenance respiration rates fell within the ranges reported for other legumes.

While both total plant dry mass and leaf area increased significantly over the two sampling intervals, the values for Y_g and m, and dimensionless constants *k* within treatments showed a tendency to remain constant between harvest intervals. These results are consistent with the balanced exponential growth hypothesis of Thornley (1998). This hypothesis predicts that under constant nutrient supply, non-limiting moisture availability and saturating irradiances all extensive variables (plant dry mass and leaf area) increase exponentially at a constant specific growth rate. Also under these conditions all intensive variables that are either ratios or rates remain constant. Thus in plant respiration equations m and Y_g have been treated as constants.

In this study experimental evidence indicated that the values of intensive variables related to plant respiration such as m and Y_g may be treated as time invariant constants. However their specific values as constants are determined by factors such as symbiotic associations and nutrient availability. Finally, the results of this study are consistent with the hypothesis that the plant's potential photosynthetic capacity exceeds the carbon demand of the *Rhizobium*–AMF symbiotic complex.

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CHAPTER SEVEN:

DISCUSSION AND CONCLUSION

7.1 Influence of N supply on photosynthetic parameters and growth without symbiotic association for 3 harvest intervals

The results obtained in this study have shown the clear-cut effects of the various factors which the data with respect to physiological and morphological variables and parameters by non-symbiotic association in faba bean (*Vicia faba* L.). It is prime importance and essential to ensure that the influence and interrelationship of N and P supply and photosynthetic parameters, different allocation of biomass and growth by non-symbiotic association clearly before research on the tripartite (legume - Rhizobium - AMF) symbiosis, because of the symbiotic N_2 fixation of faba bean genotype by the partner strain of *Rhizobium*. Another, it is usually assumed that the most important effects on the host plant due to mycorrhizal colonization are the improvement of macronutrient (Maschner and Bell 1994) and micronutrient acquisition directly or indirectly related to enhanced P uptake by the Arbuscular mycorrhizal fungi (AMF) for any plants (kothari *et al.* 1991, Li *et al.* 1991). The data were derived from destructive or non-destructive experiments without tripartite (legume-Rhizobium-AMF) symbiotic association under the 7 different N and normal P supplies by 3 harvest intervals in the experiment. We definitude that some relationships and effects on N supply and its results were almost same as another authors under the non-symbiotic association after autoclaving the soil in chapter 1.

7.1.1 The relationship between α and N_L

In Chapter 1, the quantum yield efficiency (α) with respect to increasing leaf N (N_L) reached a maximum of 0.0664 ± 0.0049 μmol CO_2 μmol Qt^{-1}. Meinzer and Zhu (1998) reported that α for CO_2 uptake in sugarcane increased linearly from 0.042 to 0.075 μmol CO_2 μmol Qt^{-1} with leaf N_c. Other α measurements for CO_2 uptake [measured at leaf temperatures of 30℃] in various monocot and dicot plants, with different photosynthetic pathways, give the following range of α values (μmol CO_2 μmol Qt^{-1}): C_3 dicots, 0.052 ± 0.001; C_3 grasses, 0.053 ± 0.001; C_4 (NAD-ME) dicots, 0.053 ± 0.001; C_4 (NAD-ME) grasses, 0.060 ± 0.001; C_4 (PCK) grasses, 0.060 ± 0.002; C_4 (NADP-ME) dicots, 0.061 ± 0.002; C_3 (NADP-ME) grasses, 0.065 ± 0.001; C_4 (ME-MIX) dicot, 0.057 (Pearcy and Ehleringer, 1984). Ehleringer and Björkman (1977) found α value for C_3 photosynthesis of 0.081 μmol CO_2 μmol Qt^{-1} under conditions where oxygenase activity was suppressed (low O_2 or high CO_2 concentrations). At a leaf temperatures of 20°C, α of 0.066 μmol CO_2 μmol Qt^{-1} and 0.044μmol CO_2 μmol Qt^{-1} has been measured for field grown ryegrass and clover respectively. It is thus reasonable to assume that depending on leaf N_c, α for CO_2 uptake in C_3 and C_4 plants can range from 0.016 to 0.075 μmol CO_2 μmol Qta^{-1}.

7.1.2 The relationship between P_{Nmax} and N_L

In Chapter 1, the N_L-intercept for the saturated net photosynthetic rate (P_{Nmax}) was 0.7100 ± 0.0345 g N m^{-2}. In other studies the linear response of P_{Nmax} to N_L rose from: zero at 1.0 g N m^{-2} to about 2. 4 g N m^{-2} for soybean, zero at 0.3 g N m^{-2} to about 1.6 g N m^{-2} for rice, and zero at 0.2 g N m^{-2} to 0.6 g N m^{-2} for maize (Sinclair and Horie 1989). In *Chenopodium album* (C_3) and *Amaranthus retroflexus* (C_4), the P_{Nmax} intercept of the N_L axis ranged between 0.64 to 0.78 g N m^{-2} (Sage and Pearcy 1987),

for a given N supply rate the values for the corresponding P_{Nmax} remained constant over all three harvest intervals (Figure 1.2). This confirms the validity of the premise that a photosynthetic parameter such as P_{Nmax} will also remain constant under steady-state N_s conditions.

7.1.3 The relationship between C_i, C_e and N_L

In Chapter 1, the steady-state functional C_i/C_a ratio is not only determined by plant water status and stomatal conductance, the C_i/C_a ratio also influenced by N_L (where C_i and C_a represent the intercellular and atmospheric CO_2 concentration respectively). With regard to benchmarking C_i values, it is generally accepted that at 25°C, under saturating PPED and ambient CO_2, C_i are approximately 100 µmol mol^{-1} for C_4 species and 250 µmol mol^{-1} for C_3 species. The CO_2 compensation point (Γ) is close to 0 for C_4 *versus* 5.0 µmol mol^{-1}, for C_3 species (Evans and von Caemmerer 1996). C_i values for a C_3 plants such as *Pinus pinaster* range from 103 to 266 µmol mol^{-1} (Warren *et al*. 2000; Warren and Adams 2001).

Carboxylation efficiency (C_e) has been expressed as the reciprocal of mesophyll resistance r_m (Edwards and Walker, 1983). The following range of r_m values [s cm^{-1}] have been reported for C_3 plants: *Glycine max* 2.0-3.0, *Atriplex hastata* 2.6, *Phaseolus* spp 2.6, *Triticum aestivum* 2.8, *Solanum tuberosum* 5.4, and *Medicago sativa* 2.8. Thus the carboxylation efficiencies for the above C_3 plants range between 0.002 to 0.005 m s^{-1}. (Laisk and Loreto 1996).

Assuming a temperature of 20°C and air pressure of 101.315 Pa [under these conditions CO_2 $litre^{-1}$ = 41.6 x 10^{-6} mol CO_2 m^{-3} = 0.101 Pa (CO_2) the range of C_e values reported

for various C_3 plants can be recalculated to give the following range of equivalent values 0.741 to 2.595 μmol CO_2 m^{-2} s^{-1} $Pa(CO_2)^{-1}$. In general, C_e ranged between 1.046 to 1.478 μmol CO_2 m^{-2} s^{-1} $Pa(CO_2)^{-1}$ for C_3 *Cyperus* species and between 2.529 to 4.123 μmol CO_2 m^{-2} s^{-1} $Pa(CO_2)^{-1}$ for C_4 *Cyperus* species (Li 1993). Thus the C_e values estimated for faba bean fell within the C_3 benchmark range of values.

7.2 Influence of N and P supply on Photosynthetic Parameters and Growth without symbiotic association for 2 harvest intervals

In chapter 2 and 3, the experiment was designed by 6 different N supplies and 2 P supplies for 2 harvest intervals. the photon saturated photosynthetic rates (P_{max}), quantum yield efficiency (α), intercellular CO_2 concentration (C_i) and carboxylation efficiency (CE) were limited by both N and P supply under non-symbiotic association in *Vicia faba* L. The influence of P on N accumulation, biomass (W) and leaf area (cm^2 $plant^{-1}$) production were also investigated. An increase in P supply was consistently associated with an increase in N accumulation and N productivity in terms of biomass and leaf area production. Furthermore, P increased the photosynthetic N use efficiency (NUE) in terms of P_{max} and α. An increase in P was also associated with an increase in CE and a decrease in C_i. Under variable daily meteorological conditions specific leaf nitrogen content (N_L), specific leaf phosphorus content (P_L), specific leaf area (δ_L), root mass fraction (R_f), P_{max} and α remain constant for a given N and P supply. A monotonic decline in the steady-state value of R_f occurs with increasing N supply. δ_L increased with increasing N supply or with increasing N_L. This study tests the hypothesis that P supply positively affects both N demand and photosynthetic NUE by influencing the upper limit of the asymptotic values for P_{max}, □ and □CE, and the lower limit for C_i in

response to increasing N (Grinday 1997). These results are consistent with the balanced exponential growth hypothesis of Thornley (1998). This hypothesis predicts that under constant nutrient supply, non-limiting moisture availability, and saturating irradiance all extensive variables (*e.g.* plant DM and leaf area) increase exponentially at a constant specific growth rate. Also, under these conditions all intensive variables that are either ratios or rates (*e.g.* N_L, P_L, δ_L and R_f) remain constant.

7.3 Influence of N and P supply on short-term response to elevated CO_2 and irradiance (PPFD) with non-symbiotic association for 2 harvest intervals

The effects of the N and P supply levels on net photosynthesis (P_N) for individual leaves that were exposed to increasing concentrations of CO_2. With increasing CO_2 concentration it appears that N and P supply levels determined the upper limits that were attained for individual leaf photosynthetic rates. This conclusion is consistent with the hypothesis that N and P supply are rate limiting factors for photosynthesis under elevated CO_2 conditions. Or alternatively expressed, these results show that, with increasing CO_2 supply, photosynthetic rates are co-limited by N and P supply.

The photosynthetic–irradiance (PPFD) response curves show that the light-saturated photosynthetic capacity was co-limited by N, P and CO_2. From the data presented in Figure 3.6 the values for P_{max} and quantum yield (α) can be derived. Figure 3.7 shows the impact of P supply and CO_2 concentration on the net photosynthetic rates achieved for P_{max} in relation to N supply. For both CO_2 concentrations, plants receiving the high P treatment had the higher net photosynthetic rates for P_{max} compared with plants receiving low P supply. Under both high and low P supplies, quantum yield (α) also increased in a curvilinear fashion as the functions of N supply (Figure 3.7). The

asymptotic maximum with increasing N supply and the magnitude of the asymptotic maximum were in turn shown to be dependent on P supply. This indicates that the impact of N supply on photosynthetic rate was modulated by P supply.

7.4 **The influence of *Rhizobium* and AM Fungi on N, P, photosynthetic parameters and growth in *Vicia faba* L.**

7.4.1 The developments of *Rhizobium* and AMF dependent upon active inocula and accurate experiments

The study conducted here, we have the problem that arose in the study are inoculums for *Rhizobium* and AMF. At beginning, we employ a large number of pots with poor soil from the grassland close to Wits University campus due to the symbiotic N_2 fixation of faba bean genotype characteristic by the natural strain of *Rhizobium*, and AMF inoculums consisted of the normal grass root from same grassland. When we tested, there were no nodules or a few in many pots. After testing AMF, we could not found any arbuscular, vesicular and hyphae colonization, but found the contamination in the roots. Later we cultured the active nodules of plant from Vicia faba L. and selected active AM fungal clover (*Trifolium repens L.*) root after examining 13 crops for different treatments. The infect methods for both inocula as described in chapter 4.

Our observations suggest that regulation of mycorrhizal development is not only dependent upon the host plant, as indicated by Douds *et al*. (1988) studies, but also upon the colonizing fungus. The fact that specific stages in the plant-fungus interaction are affected suggests that definite AMF characteristics are involved in the restriction of colonization. The induction of compatibility or incompatibility reactions is determined

by the particular host-fungus combination in further studies.

7.4.2 The promotions of *Rhizobium* and AMF and an inhibition between them on the tripartite (legume-Rhizobium-AMF) symbiosis

It is generally accepted that AM fungi have the ability to transport P and other nutrients into host plant (Koide 1991, Schwab *et al.* 1991). Under low soil P concentrations, most plant species are dependent on a symbiotic association with arbuscular mycorrhizal fungi (AMF) for the acquisition of P (Smith and Read, 1997). Under low N fertilizer inputs, soil P availability is usually the major factor limiting the rate of N_2-fixation in legume crops (Toro et al., 1998) and, in the absence of AMF infection, supplementary P fertilization is generally necessary for the maintenance of N_2-fixation rates by *Rhizobium* at the levels required for economically viable crop production (Andrade et al., 1998). In legumes the positive synergistic interactions among the members of the tripartite symbiotic association (Rhizobium–AMF–legume) result in improved rates of P uptake N_2-fixation and crop biomass production under conditions of reduced N and P fertilizer inputs (Azcón et al., 1991; Xavier and Germida, 2002, 2003).

The results in this study indicated that AMF promoted biomass production and photosynthetic rates by increasing the ratio of P to N accumulation. An increase in P was consistently associated with an increase in N accumulation and N productivity, expressed in terms of biomass and leaf area. Photosynthetic N use efficiency, irrespective of the inorganic source of N (e.g. NO_3^- or N_2), was enhanced by increased P supply due to AMF. The presence of *Rhizobium* resulted in a significant decline in AMF colonization levels irrespective of N supply. Without *Rhizobium*, AMF colonization levels were higher in low N treatments. Presence or absence of AMF did

not have a significant effect on nodule mass but high N with or without AMF led to a significant decline in nodule biomass. Plants with the *Rhizobium* and AMF symbiotic associations had higher photosynthetic rates per unit leaf area.

7.4.3 **Growth yield of *Vicia faba L* in response to the tripartite (legume – Rhizobium - AMF) symbiosis**

This study was to investigate what affects the tripartite symbiotic association had on the host growth yield and maintenance respiration under P and N limiting conditions. Some authors note that for legumes there have been several studies on maintenance respiration and growth yield (McCree and Silsbury, 1978; Irving and Silsbury, 1987), however these studies did not include the effects of microbial symbiotic partners. Also, while many other studies have focused on the apparent carbon costs induced by the tripartite symbiotic association, they have not directly investigated the impact of these costs on the growth yield (Azcón et al., 1979; Paul and Kucey, 1981; Harris *et al.*, 1985; Douds *et al.*, 1988). We undertook to investigate the influence of *Rhizobium* and AMF on biomass production, leaf area generation, whole plant photosynthetic and respiration rates, growth yield (Y_g) and maintenance respiration in *Vicia faba L.* under low phosphorous (P) supply. It was showed that in all instances, plants colonized with both microbial symbionts had significantly higher total biomasses, leaf areas, whole plant photosynthesis and respiration rates than plants with only one or no microbial symbionts. Similarly, plants with both microbial symbionts also had significantly higher ($\geqslant 0.7$) growth yield (Y_g) values than all the other treatments. There were no significant differences in Y_g values between harvest intervals within individual treatments. Maintenance respiration rates were also highest in plants with two microbial symbionts ($>30 CO_2$ $(gDM)^{-1}$ d^{-1}). In LN plants colonized by both microbial symbionts there was

evidence of compensatory increases in the photosynthetic rates in response to the carbon sink demands of the microbial symbionts.

7.5 C**onclusion**

7.5.1 **Influence of N and P supply without symbiotic association**

We determined for *Vicia faba* L. the influence of N uptake and accumulation on the values of photon saturated net photosynthetic rats (P_{Nmax}), quantum yield efficiency (α), intercellular CO_2 concentrations (C_i) and carboxylation efficiency (C_e). As leaf nitrogen concentration (N_L) increased, the α converged onto a maximum asymptotic value of 0.0664±0.0049 μmol (CO_2) μmol (Quantum)$^{-1}$. Also, as N_L increased the C_i value fell to an asymptotic minimum of 115.80±1.59 μmol mol^{-1}, and C_e converged onto a maximum asymptotic value of 1.645±0.054 μmol (CO_2) m^{-2} s^{-1} Pa^{-1} and declined to zero at a N_L-intercept equal to 0.596±0.096 g(N) m^{-2}. α fell to zero for an N_L-intercept of 0.660±0.052 g(N) m^{-2}. As N_L increase, the value of P_{Nmax} converged onto a maximum asymptotic value of 33.400±2.563 μmol (CO_2) m^{-2} s^{-1}. P_N fell to zero for an N_L-intercept for 0.710±0.035 g(N) m^{-2}. Under variable daily meteorological conditions the values for N_L, specific leaf area (δ_L), root mass fraction (R_f), P_{Nmax} and α remained constant for a given N supply. A monotonic decline in the steady-state value of R_f occurred with increasing N supply. δ_L increased with increasing N supply or with increasing N_L.

We found a tight coupling between the effects of P and N on photosynthesis and plant growth. A decline in the supply of N (Paul and Driscoll 1997) or P (Pieters *et al.* 2001) results in an immediate decline in photosynthetic activity. This study has focused

primarily on the influence of N and P on carbon source dynamics, but increasing N and P supply also stimulates photosynthetic activity by increasing down-stream utilization of Calvin cycle end-products (Paul and Pellny 2003). Sugars alone probably do not mediate sink regulation of photosynthesis (Paul and Foyer 2001). Rather it is the whole plant nutrient balance in the form of the C: N: P supply ratios that mediate both source and sink regulation of photosynthesis.

The optimum N:P supply ratio has been defined as the N:P ratio where net photosynthesis or plant growth is equally limited by N and P (Sterner and Elser, 2002; Agren, 2004). Even though an extensive literature now exists on the optimal N:P stoichiometries for crop growth (Sadrass, 2006), not much is known about the optimum C:N:P ratios for photosynthesis or plant growth. However, the study of Grünweig and Korner (2003) does suggest that with long-term exposure to elevated CO_2, plant growth becomes co-limited by both N and P supply. The results in Fig. 5 indicate that under short-term exposure to elevated CO_2 plants adapted to ambient CO_2 levels show responses consistent with photosynthesis being co-limited by C, N and P supply. In addition, if the photosynthetic catalytic machinery in terms of leaf N concentration determines the source capacity of the plant canopy and P concentration influences the energetic efficiency of CO_2 assimilation into plant biomass, then the stoichiometric ratio of N:P (as a % of dry biomass) will determine plant productivity levels in response to CO_2 supply. Therefore, in general, it could also be argued that with increasing CO_2 supply, the sink demand of actively growing tissues for additional reduced carbon and source capacity for assimilatingadditionalCO_2 would also be controlled by the N:P supply ratio.

7.5.2 **The influence of *Rhizobium* and AM Fungi in *Vicia faba* L.**

The association between nitrogen-fixing *Rhizobium* and leguminous plants is a very well-known symbiotic relationship. However, in the 'real world' of the soil, other symbiotic relationships also occur, such as those between plant roots and AM fungi. This raises a whole series of questions. Can two rather different micro-organisms form mutualistic relationships with the same leguminous plant? Do they interact with each other as well as with the plant? What is the overall effect on plant nutrition and growth? These questions have been addressed in a very thorough study by our research (Chapter. 5). We have looked at a range of interactions (of which we focus here on a selection) between *Vicia faba*, an AMF and *Rhizobium leguminosarum*. Plants infected with the AMF showed a greater uptake of P (as has been shown for other AMF–plant symbioses) which in turn led to enhanced N uptake, increased photosynthesis and greater dry matter accumulation. One of the most obvious effects of the establishment of the *Rhizobium*–plant symbiosis was an increase in plant N; this was correlated with increased P-uptake, which seemed in this instance to be a result of, rather than a cause of, the increase in plant N. As with the AMF infection, photosynthetic efficiency and plant biomass were also increased. In relation to the interaction between the *Rhizobium* and the AMF, prior infection with *Rhizobium* reduced the infectivity of the AMF but, interestingly, the reverse was not true. The lower AMF infection rate in the presence of *Rhizobium* may have been related to the higher N-status of the plant since, in general, AMF colonization was greater in low-N conditions. Finally, productivity was greatest in plants infected with both AMF and *Rhizobium* and it is possible that the two symbionts act synergistically rather than just additively.

In this study (Chapter 5) experimental evidence does indicate that the values of

intensive variables related to plant respiration such as m and Y_g may be treated as time invariant constants. However their specific values as constants are determined by factors such as symbiotic associations and nutrient availability. Therefore it was the goal of this study to fill in some of these gaps in our knowledge by investigating how the two microbial symbionts influence maintenance respiration and growth yield.

7.5.3 Future directions for study

Most theories that relate N mass fraction of leaves assume that photosynthesis can be maximal at N mass fraction of 15-20 $mg.g^{-1}$. However, legumes often have much higher N mass fractions (up to 40-50 $mg.g^{-1}$, see p.12). A possible explanation is their N-demanding life style (an argument introduced by McKey). Also legumes have higher P mass fractions, related to N fixation, so possibly the general relationships for plants are somewhat different for N-fixing legumes. Ågren's statement that N is generally limiting is true for temperate ecosystems (young soils with enough P that is not yet occluded or fixed after long weathering, N-mineralisation being relatively low due to low temperatures), but not for the tropices where P is usually limiting.

The findings of this experiment are consistent with other observations (Xavier and Germida, 2002, 2003) of the positive impact of the synergistic interactions between AMF and *Rhizobium* on legumes. The magnitude of the increases in both leaf area and biomass production with each increment in N supply was dependent on the level of P supply. Increasing P supply as a direct consequence of AMF colonization or as an indirect consequence of *Rhizobium* infection had positive effects on N accumulation, leaf area production and biomass production. Increasing P accumulation had a positive influence on photosynthetic N use efficiency. Thus, it is reasonable to be a bio-fertilizer

for *Rhizobium*, AMF or both of them as inoculums to promote plant productivity. How to pure and culture them? It is a new project at front of us in future.

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CHAPTER EIGHT:

APPENDICES

Appendix 1.

Long-Ashton Nutrient Medium (stock 100x in 2.5 L)

Solution 1 Micronutrients

Chemical	**g / 2.5 L**
*NaH_2PO_4 (Sodium dihydrogen orthophosphate anhydrous)	52.0
$MgSO_4 \cdot 7H_2O$ (Magnesium sulphate heptahydrate)	92.25
$MnSO_4 \cdot H_2O$ (Manganese sulphate tetrahydrate)	05575
$CuSO_4 \cdot 5H_2O$ (Cupric sulphate)	0.0600
$ZnSO_4 \cdot 7H_2O$ (Zinc sulphate)	0.0740
H_3BO_3 (Boric acid)	0.4650
$(NH_4)_6MO_7$ $7O_{24} \cdot H_2O$ (Ammonium molybdate)	0.00875
$CoSO_4 \cdot 7H_2O$ (Cobaltous sulphate)	0.007
NaCl (Sodium chloride)	1.4625

Solution 2

*KNO_3 (Potassium nitrate)	126.25

Solution 3

*$Ca(NO_3)_2$ (Calcium nitrate) have no using it in all experiments	205.0

Solution 4

FeEDTA (Ethelenediamine tetra-acetic acid ferric monosodium salt) 7.5

Solution 5

$CaCl_2$ (Calcium chloride dihydrate) 125

Solution 6

K_2SO_4 (Potassium sulphate) 54.375

* Modified Long-Ashton nutrient solution with low P < 0.05ppm (0.05ppm P, 0.00161 mMol NaH_2PO_4 in the experiment), added 0.0484 mg NaH_2PO_4 into the stock 100x in 2.5 L for lower P treatment.
* $Ca(NO_3)_2$ (Calcium nitrate) have no using it in all experiments.
* KNO_3 (Potassium nitrate) solutions of 10ppm N and 250ppm N treatments were made separately for the stock 100x in 2.5 L. Put 3.611 g KNO_3 (10ppm) and 90.176 g (250ppm) into the stock 100x in 2.5 L respectively.

The nutrient solution was diluted taking out 50 ml stock (100x in 2.5 L, e.g. solution1, 4, 5 and 6, without KNO_3 and $CaNO_3$) into 5 liter tap water container. Solution of 10ppm N and 250ppm N was same methods that taken 50 ml 10ppm and 250ppm stocks and put them into 5 liter tap water containers separately. Mixed them well and applied to different treatments.

8.2 Appendix 2.

***Rhizobium* Selection and Growth Media** (pH 6.8 -7.0)**:**

1. Make up in 1000 ml

K_2HPO_4 .....0.5 g

$MgSO_4 \cdot 7H_2O$ .....0.2 g

NaCl ..... 0.1 g

Mannitol .....10.0 g

Yeast extract .....0.4 g

*Agar .....5.0 g

Distilled H_2O .....1000 ml

*Congo red (0.25% mv) ..... 10.0 ml

*These components were not included in the liquid growth medium.

2. Pour into 250 ml *Erlenmeyer* flask and place cotton bung in top.

3. Autoclave at 121℃ and 15 psi for 20 minutes.

8.3 Appendix 3.

Permanent mounting medium for AM fungi

Polyvinyl Lactic Acid

Polyvinyl alcohol (PVA) ..... 1.66 g

Water ..... 10 ml

Lactic acid ..... 10 ml

Glycerol ..... 1.0 ml

PVA is dissolved in water and lactic acid first, and then glycerol is added.

8.4 Appendix 4.

Nitrogen and Phosphorus Assay

These are carried out using the Dorich and Nelson (1983) method which is a colorimetric method, using a hydrogen peroxide-sulphuric acid digestion step and determine the nitrogen and phosphorus in the digests as follows:

Digestion

The first step in the determination was to digest the plant material. For this, approximately 0.1g of plant material was added (weight and record) and 4.4 ml digestion mixture in a digestion tube. These samples were then digested at 360℃ in a fume cupboard until the solutions became clear. They were then allowed to cool and 95.6 ml de-ionized water was poured into the digestion tube about 3 or 4 times while mixing by shaking, and then poured into 100 ml *Erlenmeyer* flasks and mixed well. Blanks were also prepared for use as standards. Allow to settle so that a clear solution can be taken from the top of the flask for N and P analysis. Compensate the working standards by addition of 4.4 ml digestion blank.

8.4.1 Colorimetric determination of ammonium

Reagents

N_1: Dissolved 34 g sodium salicylate, 25 g sodium citrate and 25 g sodium

tartrate together in about 750 ml de-ionized water. Add 0.12 g sodium nitroprusside and when dissolved make up to 1000 ml de-ionized water and mix well.

N_2: Dissolved 30 g sodium hydroxide about 750 ml de-ionized water in a volumetric flask. Allow to cool, add 10 ml sodium hypochlorite solution and make up to 1000 ml with de-ionized water and mix well.

(**Note**: Reagents **N_1** and **N_2** should be made at least 24 hours before use and stored in the dark).

Standards

1. Dry about 7 g ammonium sulphate at 105℃ for 2 hrs. Cool in a desiccator.
2. Dissolved 4.714 g dry ammonium sulphate in water and make up to 1000 ml in a volumetric flask. This is a 1000 µg/ml NH_4^+ - N stock solution.
3. Pipette 50 ml of the 1000 µg/ml NH_4^+ - N stock solution into 500 ml volumetric flask and make up to the mark with water. This is a 100µg/ml NH_4^+ - N solution.
4. Pipette 0, 5, 10, 20, and 25 ml of the 100 µg/ml NH_4^+ - N solution into labelled 100 ml volumetric flasks. Compensate the standards as indicated in the solution preparation stage. Make up to the mark with water and mix well. These are the working standards and contain 0, 5, 10, 20, and 25 µg/ml NH_4^+ - N respectively.

Procedure

a. Transfer 0.1 of each standard and sample into suitably marked test tubes.
b. Add 5.0 ml of reagent **N_1** to each test tube, to mix well and leave for 15 minutes.
c. Add 5.0 ml of reagent **N_2** to each test tube, to mix well and leave for 1 hour for full colour development.

d. The colour is stable for the day only. Read each standard and sample absorbance at 655 nm.

Calculation

Chose the standard data to perform regression analysis, a set of standard concentrations represented independent variables (x) and affecting absorbance were dependent variables (y). The relationship between concentration and absorbance is a linear estimate. With on independent variables, repression analyses plots a line of best fit (regression line) through a scatter plot of each independent-dependent value pair. It was conformed to:

$$y = a + bx$$

linear equation. Where y = absorbance (nm), x = standard concentration (µg/ml), a = constant, b = regression coefficients of the concentration. Subtract the mean blank value of absorbance (nm) from each unknown. According to the linear equation, the corrected concentration of samples could be gotten from: x = (y + a)/b equation. For digestion and measurement procedures, each standard and sample were diluted for 100 times (see standard 3). So, nitrogen concentration of plant materials was:

$$\text{Nitrogen (mg/g)} = C * 100/W/1000$$

Where C = calculated concentration (µg/ml, *i.e.* the values from x = (y + a)/b equation), w = weight (g) of sample. Because 1000 µg equals 1 g, so it was divided 1000 in the calculation.

8.4.2 Colorimetric determination of phosphorus

Reagents

a. **Ascorbic acid**: 1%, make a fresh solution every day.
b. **Molybdate reagent**: dissolve 4.3 g ammonium molybdate in 400 ml water in a 1000 ml measuring cylinder. Dissolve 0.4 antimony sodium tartrate in 400 ml water, then add to the 54 ml H_2SO_4 carefully. Allow to cool and make up to 1000 ml with water. Mix well stable for 4 weeks at 2℃.

Standards

1. Dried about 7 g KH_2PO_4 at 105℃ for 2 hours. Cool in a desiccator.
2. Dissolved 4.394 g dry KH_2PO_4 in water and make up to 1000 ml in a volumetric flask. This is a 1000 µg/ml P stock solution.
3. Pipette 10 ml of the 1000 µg/ml P stock solution into a 500 ml volumetric flask and make up to the mark with water. This is a 20µg/ml P solution.
4. Pipette 0, 5, 10, 20, and 25 ml of the 20 µg/ml P solution into labelled 100 ml volumetric flasks. Compensate the standards if required, as indicated in the solution preparation stage. Make up to the mark with water and mix well. These are the working standards and contain 0, 5, 10, 20, and 25 ml of 5µg/ml P respectively.

Procedure

a. Pipette 1 ml standard or sample into a test tube.

b. Add 4.0 ml ascorbic acid solution.

c. Add 3.0 ml molybdate reagent and mix well.

d. Leave for 1 hour for the colour to develop fully.

e. Read the standard and sample absorbance at 880 nm.

Calculation

The calculation of P concentration was as same as for N. because each standard and sample was diluted for 100 times during digestion and measurement procedures (see standard 3). So, the phosphorus concentration of plant materials was:

Phosphorus (mg/g) = C * 20/W/1000

Where C = calculated concentration (µg/ml, i.e. the values from x = (y + a)/b equation), w = weight (g) of sample. Because 1000 µg equals 1 g, so it was divided 1000 in the calculation.

References:

Dorich JA, Nelson DW. (1983) Direct colorimetric measurement of ammonium in potassium chloride extracts in soil. *Soil Science Society of America Journal* **55:** 171–178.

8.5 Appendix 5.

Staining of Root for AM Fungi Infection

Roots were stained according to the method described in Koske and Gemma (1989). This method is an improvement on several of the earlier methods employed from the point of view that potentially harmful chemicals (particularly phenol and lactic acid in staining and de-staining procedures) are eliminated where possible. In addition, the number of ingredients is kept to a minimum. The fresh root sub-sample taken from each plant in each pot was fixed and stored (overnight usually) in 20 ml 50% ethanol (This step was not necessary if the roots were to be used immediately for staining). The actual clearing and staining were carried out as follows:

8.5.1 Tissue clearing

In order to disrupt the cell membranes, so that cell contents are lost, the root material was placed in 20 ml of 2.5% KOH (potassium hydroxide w/v) and heated at 90℃ for 45 minutes. This treatment was found to be the most effective in clearing the tissue, without causing too much damage to the cortex. The more rapid approach of autoclaving the samples for three minutes were found to be very damaging to the cortex.

8.5.2 Rinsing and bleaching

Following the tissue clearing step, the roots were rinsed several times with distilled water and bleached with alkaline hydrogen peroxide (3ml 20% NH_4OH in 30 ml 3%H_2O_2) for 45 minutes. This was followed by a thorough rinsing in distilled water.

Although this step to lighten dark roots is optional (Koske and Gemma, 1989), it was carried out routinely on all the samples in this study as it was found to give enhanced clarity when viewing under a microscope.

8.5.3 Acidification

Inadequate acidification is reported to result in poor binding of the trypan blue to the AMF structure (Koske and Gemma, 1989). It was therefore necessary to acidify the samples which were left highly alkaline by the preceding two stages. In order to achieve this acidification, the root sampled were transferred to 20 ml of 1% hydrochloric solution and left overnight

8.5.4 Staining

The material was stained in trypan blue (0.05% trypan blue in acid glycerol) at 90℃ for 45 minutes. Because of the absence of phenol, this stain has the advantage of having relatively low toxicity. It also stains the fungus blue which has greater contrast than certain of the alternatives such as acid fuscin. The roots were then de-stained in acid glycerol (500 ml glycerol, 450 ml water, 50 ml 1% HCl).

8.5.5 Estimation of Intra-radical AM Fungi Colonization

There are essentially three methods for assessing levels of infection based on microscopy (as opposed to chemical estimation of chitin). The most elementary (and least accurate) approach is a sample subjective estimation of infection levels. Another approach is to base the estimation on the ratio of an Olympas microscope fields of view with infection to the total number of fields considered. Although less subjective than the previous approach it can easily be demonstrated (McGonigle *et al*., 1990) that the

approach often leads to overestimation of infection. The last group of methods is based on permutations of a grid or line-intersect method, where the infection level is determined from the ratio of infected intersection to uninfected intersections. The approach followed in this work was described available.

Stained roots were cut into 1 cm long pieces and 5 of these root sections were mounted parallel to the long axis of microscope slides (75 x 22 mm) with No. 1 cover-slips (22 x 22) in the permanent mounting medium (polyvinyl lactic acid, Omar for *et al*., 1979, Appendix 3). AM infection fungi were observed at a magnification of x 200 using Olympus microscope. The field of view was moved across the slide to make 5 complete intersection of the crosshair with a root segment perpendicular to its long axis for 10 root segment. There were thus a total of 50 intersections counted per slide, and 4 slides per replicate. Therefore, a total of 200 intersections were recorded per species per replicate. There were 4 replicates per treatment in this observation.

Except where the cortex was missing, all intersections between roots and the eyepiece crosshair were recorded. Rotation of the crosshair ensured that each intersection was at right angles to the long axis of the root. At each intersection, the roots were scored into one of six categories:

a. **Negative**: crosshair did not cut through any hyphae, nor arbuscules and nor vesicles.
b. **Arbuscules (plate 1):** crosshair cut through at least one arbuscule.
c. **Vesicles (Plate 1):** crosshair cut through at least one vesicle.
d. **Hyphae only:** crosshair cut through at least one hypha, but no arbuscules or vesicles.
e. **Mycorrhizal hyphae:** crosshair cut through at least on hypha that is visibly attached to a vesicle or arbuscule.

f. **Total:** total number of intersections examined. Arbuscular colonisation (AC) and vesicular colonisation (VC) were calculated as sum of the categories 2, 5 and 3, 5 above respectively and divided by 6. Hyphal colonisation (HC) was calculated as the sum the categories 2, 3, 4 and 5, and divided by 6.

8.5.6 References

Koske RE, Gemma NJ. (1989) A modified procedure for staining roots to detect VA myckorrhiza. *Mycological Research* **92:** 486–505.

McGonigle TP, Miller ML, Evans DG. 1990. A new method which gives an objective measure of colonization of roots by vesicular-Mycorrhizal fungi. *New Phytologist* **115**: 495–501.

McGonigle TP, Miller ML, Evans DG. 1990. A new method which gives an objective measure of colonization of roots by vesicular-Mycorrhizal fungi. *New Phytologist* **115**: 495–501.

Omar MB, Bolland L, Heather WA. (1979) A permanent mounting medium for fungi. *Bulletin of the British Mycological Society* **13**: 31–32.

8.6 Appendix 6.

Calculation of Photosynthetic Rate

8.6.1 The calculation of photosynthetic rate (P_{max}) for single leaf by the measurement in Cuvette of IRGA (mmol CO_2 m^{-2} s^{-1})

According to the equation as following:

$$P_{max}\ (\mathbf{mmol\ CO_2\ m^{-2}\ s^{-1}}) = \frac{\Delta ppm * 10^{-6}}{LA} * \frac{Flow\ Rate}{22.416} * \frac{273.15}{Ta} * \frac{Pa}{1013}$$

∵ Room Temperature: 24°C, ∴ Ta = 273.15°C + 24°C = 297.15°C

LA (Leaf Area in Cuvette): 63mm^2 = 0.63cm^2 = 0.000063m^2

Flow rate: One of two exhaust tubes CO_2 on the flow meter was used, so, the flow rate is 800/2 = 400 (ml min^{-1}). Here: the *Flow Rate* = 400/60 = 6.66667 (mlCO_2 s^{-1}), Pa = 840, the calculation as following:

$$P_{max}\ (\mathbf{mmol\ CO_2\ m^{-2}\ s^{-1}}) = \frac{\Delta ppm * 10^{-6}}{LA} * \frac{Flow\ Rate}{22.416} * \frac{273.15}{Ta} * \frac{Pa}{1013}$$

$$= \frac{\Delta ppm * 10^{-6}}{0.000063} * \frac{400/60}{22.416} * \frac{273.15}{297.15} * \frac{840}{1013}$$

$$= \frac{\Delta ppm}{60 * 63} * 17.8443 * 0.9192 * 0.8292$$

$$= \Delta ppm * 13.6009 / 3780$$

$$= \Delta ppm * 0.00359812\ (\mathbf{mmol\ CO_2\ m^{-2}\ s^{-1}})$$

∵ 1 mmol = 1000 μmol.

∴ P_{max} (**μmol CO_2 m^{-2} s^{-1}**) = Δppm *3.598 (**μmol CO_2 m^{-2} s^{-1}**)

For instance:

The Leaf No. 6 of measurement for 500ppm N treatment by IRGA

T°C = 24, the ratio of indicator / amaranths (120/17.5 = 6.86)

Measured: 55, 55, 56, 58, 59, 58, 58, 57, 56; n = 9, $\bar{x}$ = 56.89

∴ Δppm = 56.89 / 6.86 = 8.293 (ppm CO_2)

P_{max} (μmolCO_2 m^{-2} s^{-1}) = Δppm *3.598 = 8.293 * 3.598 = 29.84 (μmol CO_2 m^{-2} s^{-1})

8.6.2 The calculation of photosynthetic rate (P_{max}) for whole plant leaves by the measurement in Cuvette of IRGA (μmol CO_2 m^{-2} s^{-1})

According to the equation as following:

$$\mathbf{P_{max}}\ (\mu mol\ CO_2\ m^{-2}\ s^{-1}) = \frac{\Delta ppm * 10^{-6}}{LA} * \frac{Flow\ Rate}{22.416} * \frac{273.15}{Ta} * \frac{Pa}{1013}$$

1) The flow rate is 710 (ml min^{-1}), 710/60/22.416 = 0.52789674 (mlCO_2 ≅ s^{-1})
   (42600ml hr^{-1} = 42.6l hr^{-1}).
2) ∵Room temperature: 25°C, ∴ Ta = 273.15°C + 25°C = 298.15°C
   273.15/Ta = 273015/298.15 = 0.916149589
3) Leaf Area of whole plant: 353.68cm^2 = 3.5368dm^2 = 0.035368m^2 (e.g.: 250NMR^{-1} treatment)

4) Δppm (CO_2) = 349.44-124.8 = 224.64ppm (CO_2)

5) Pa/1013 = 840/1013 = 0.829220138

The calculation $\mathbf{P_{max}}$ as following:

$$\mathbf{P_{max}}\ (\mu mol\ CO_2\ m^{-2}\ s^{-1}) = \frac{\Delta ppm * 10^{-6}}{LA} * \frac{Flow\ Rate}{22.416} * \frac{273.15}{Ta} * \frac{Pa}{1013}$$

$$= \frac{\Delta ppm * 10^{-6}}{353.68(cm^2)} * \frac{710/60}{22.416} * \frac{273.15}{298.15} * \frac{840}{1013}\ \textbf{(μmol CO₂ m}^{\textbf{-2}}\ \textbf{s}^{\textbf{-1}}\textbf{)}$$

$$= \frac{224.64 * 10^{-6}}{353.68(cm^2)} * 0.52789674 * 0.916149589 * 0.829220138\ \textbf{(μmol CO₂ m}^{\textbf{-2}}\ \textbf{s}^{\textbf{-1}}\textbf{)}$$

∵ $1m^2 = 100dm^2 = 10{,}000cm^2$

$$\therefore = \frac{0.22464 * 10^{-3}}{0.035368(m^2)} * 0.40103771\ \textbf{(mmol CO₂ m}^{\textbf{-2}}\ \textbf{s}^{\textbf{-1}}\textbf{)}$$

$$= \frac{0.22464}{35.368} * 0.40103771\ \textbf{(mmol CO₂ m}^{\textbf{-2}}\ \textbf{s}^{\textbf{-1}}\textbf{)}$$

= 0.0063515*0.40103771 = **0.002547191**(mmol CO_2 m^{-2} s^{-1})

∵ 1 mmol = 1000 μmol.

∴ P_{max} (μmol CO_2 m^{-2} s^{-1}) =0.002547191(mmol CO_2 m^{-2} s^{-1}) *1000

= 2.547191 (μmol CO_2 m^{-2} s^{-1}) ≈ **2.55** (μmol CO_2 m^{-2} s^{-1})

∵ $1m^2 = 100dm^2 = 10{,}000cm^2$, 1 hour = 3600 seconds, 1 mmol CO_2 = 44 mg (12 +16*2 = 44) and 1 mmol = 1000 μmol.

∴ 0.00255(mmol CO_2 m^{-2} s^{-1}) * 44mg * 3600 / 100

= 0.00255(mmol CO_2 m^{-2} s^{-1}) * 44 * 36

= 0.00255(mmol CO_2 m^{-2} s^{-1}) *1584

= **4.0392** (mg CO_2 dm^{-2} hr^{-1})

® **4.0392** (mg CO_2 dm^{-2} hr^{-1}) / 44 / 3600 * 100

= 4.0392 / 44/ 3600 * 100

= **0.00255** (mmol CO_2 m^{-2} s^{-1})

∵ **0.00255 (mmol CO_2 m^{-2} s^{-1}) *1000**

∴ = **2.55** (μmol CO_2 m^{-2} s^{-1})

8.6.3 The calculation of photosynthetic rate (P_n), Respiration Rate of whole plant by the measuring Chamber of IRGA ($mgCO_2 \cdot dm^{-2} \cdot hr^{-1}$)

An Absolute Type Measurement of Photosynthesis

Formula 8.6.3-1). P_n Photosynthetic Rate for whole plant leaves ($mgCO_2 \cdot dm^{-2} \cdot hr^{-1}$**)**

$$P_n\ (mgCO_2 \cdot dm^{-2} \cdot hr^{-1}) = \frac{[CO_2]_{-in} - [CO_2]_{-out}}{10_{-6}} * \mathbf{F} * \frac{273.15}{Ta} * \frac{Pa}{1013} * \frac{100}{LA} * \frac{44}{22.416}$$

Where: Room temperature: 25°C, Ta = 273.15°C + 24°C = 297.15°C; LA: Leaf area for whole plant (*e.g.* 250NMR-1 treatment 353.68cm^2/plant); ∵ 1m^2 = 100 dm^2 =

$10000cm^2$, ∴ $353.68cm^2$ (*e.g.*: $250NMR^{-1}$ treatment) = $3.5368dm^2$; **F**: flow rate, There is one exhaust tubes (CO_2) on the flow meter, so, the flow rate is 710 (ml min^{-1}), 710 * 60 = 42600 (ml hr^{-1}), Pa = 840, 1 mol CO_2 = 12 + 16*2 = 44g, 1 μmol CO_2 = 44μg; $[CO_2]$-in: Air CO_2 concentration, normally there is 350ppm $[CO_2]_{-in}$, It is 349.44ppm measured at that time; $[Co_2]$-out: measured CO_2 concentration; $[CO_2]_{-out}$, there is 124.8ppm CO_2 after measuring the plant of 250NMR treatment. So,

$$\Delta ppm = [CO_2]_{-in} - [CO_2]_{-out} = 349.44 - 124.8 = 224.64 \text{ ppm } [CO_2]$$

$$= \frac{\Delta ppm * 100}{10^6 * LA} * \frac{710 * 60}{22.416} * \frac{273.15}{298.15} * \frac{840 * 44}{1013}$$

$$= \frac{\Delta ppm}{10^4 * LA} * \frac{42600}{22.413} * 0.916149589 * 36.48568608$$

$$= \frac{\Delta ppm}{LA} * \frac{42600}{224130} * 0.916149589 * 36.48568608$$

$$= \frac{\Delta ppm}{LA} * 0.190068264 * 0.916149589 * 36.48568608$$

$$= \frac{\Delta ppm}{LA} * 6.352437329 (\approx 6.35)$$

$$\mathbf{P_n} \ (mgCO_2 \bullet dm^{-2} \bullet hr^{-1}) = \frac{\Delta ppm}{LA} * 6.352437329 = \frac{224.64}{353.68} * 6.352437329 \ (\approx 6.35)$$

$$= 0.635150418 * 6.35 = 4.034753226 \approx 4.035 \ (mgCO_2 \bullet dm^{-2} \bullet hr^{-1})$$

Formula 8.6.3-2). D: Photosynthetic Rate for whole plant ($mgCO_2\ plant^{-1} \bullet hr^{-1}$**)**

$$P_n\ (mgCO_2 \bullet plant^{-1} \bullet hr^{-1}) = \frac{\Delta ppm}{10^6} * \mathbf{F} * \frac{273.15}{Ta} * \frac{Pa}{1013} * \frac{44}{22.416}$$

$$= \frac{[CO_2]_{-in} - [CO_2]_{-out}}{10^6} * \mathbf{F} * \frac{273.15}{298.15} * \frac{840}{1013} * \frac{44}{22.416}$$

Where: Room temperature: 25°C, Ta = 273.15°C + 25°C = 298.15°C; **F**: flow rate, There is one exhaust tubes (CO_2) on the flow meter, so, the flow rate is 710 ($ml\ min^{-1}$), 710 * 60 = 42600 ($ml\ hr^{-1}$), Pa = 840, 1 mol CO_2 = 12 + 16*2 = 44g, 1 μmol CO_2 = 44μg; $[CO_2]$-in: Air CO_2 concentration, normally there is 350ppm $[CO_2]_{-in}$; $[Co_2]$-out: measured CO_2 concentration; $[CO_2]_{-out}$. For example (250NR treatment), “A” the ratio for needle of IRGA is 364/175 = 2.08, the measurement is 65, 64.5, 64.5, 64, 65, 65.5, 66, 66, 66; n = 9, $\bar{x}$ = 65.16667, 65.16667 * 2.08 = 135.54667, there is 135.55ppm CO_2 after measuring the plant of 250NR treatment. So,

$\Delta\ ppm$ = $[CO_2]_{-in} - [CO_2]_{-out}$ = 350 – 135.55 = 214.45 ppm $[CO_2]$

$$= \Delta ppm * \frac{42600}{10^6} * \frac{273.15}{298.15} * \frac{840}{1013} * \frac{44}{22.416}$$

$$= \Delta ppm * 0.0426 * 0.916149589 * 0.829220138\ 8 * 1.962883655$$

$$= \Delta ppm * 0.063524468$$

D: Photosynthetic Rate of 250NR treatment ($mgCO_2 \bullet plant^{-1} \bullet hr^{-1}$) = 214.45 * 0.063524468 = 13.62282216

Formula 3-3). Respiration Rate for whole plant ($mgCO_2\ plant^{-1} \bullet hr^{-1}$**)**

N: Respiration Rate ($mgCO_2 \bullet plant^{-1} \bullet hr^{-1}$) = $\frac{\Delta ppm}{10^6} * \mathbf{F} * \frac{273.15}{Ta} * \frac{Pa}{1013} * \frac{44}{22.416}$

$$= \frac{[CO_2]_{-out} - [CO_2]_{-in}}{10^6} * \mathbf{F} * \frac{273.15}{298.15} * \frac{840}{1013} * \frac{44}{22.416}$$

Where: Room temperature: 25°C, Ta = 273.15°C + 25°C = 298.15°C; **F**: flow rate, There is one exhaust tubes (CO_2) on the flow meter, so, the flow rate is 710 ($ml\ min^{-1}$), 710 * 60 = 42600 ($ml\ hr^{-1}$), Pa = 840, 1 mol CO_2 = 12 + 16*2 = 44g, 1 μmol CO_2 = 44μg; $[CO_2]$-in: Air CO_2 concentration, normally there is 350ppm $[CO_2]_{-in}$; $[Co_2]$-out: measured CO_2 concentration; $[CO_2]_{-out}$. For example (250NR-2 treatment), "B" the ratio for needle of IRGA is 668/132 = 5.0606060606, ≈ 5.0601, the measurement is 100, 98.5, 98.5, 99, 99, 99, 99, 99.5, 99.5, 99.5, 99.5, 99.5; n = 12, $\bar{x}$ = 99.208333, 99.20833 * 5.061 ≈ 502.432, there is 502.432ppm CO_2 after measuring the plant of 250NR treatment. So,

$\Delta\ ppm$ = $[CO_2]_{-out} - [CO_2]_{-in}$ = 502.432 – 350 = 152.432ppm $[CO_2]$

$$= \Delta ppm * \frac{42600}{10^6} * \frac{273.15}{298.15} * \frac{840}{1013} * \frac{44}{22.416}$$

= Δppm * 0.0426 * 0.916149589 * 0.829220138 8 * 1.962883655

= Δppm * 0.063524468

= 9.6831602

N: Respiration Rate of 250NR treatment ($mgCO_2 \cdot plant^{-1} \cdot hr^{-1}$) = 502.432* 0.063524468 = 9.683 (mg CO_2 plant $^{-1}$ hr^{-1})

Specific Respiration Rate (mg CO2 g^{-1}LDM hr^{-1})

∵ Leaf dry mass: 1.851 g

∴ 9.683 (mg CO2 plant $^{-1}$ hr^{-1}) / leaf dry mass

= 9.683 / 1.851

= 5.231 (mg CO2 g^{-1}LDM hr^{-1})

References:

Coombs J and Hall D. O. (1982) Techniques in Bioproductivity & Photosynthesis. Printed in Great Britain by A Wheaton & Co., Ltd.