Respiratory and photosynthetic C and N metabolism of nodulated Lupin roots during phosphorus deficiency

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis, entitled: "***Respiratory and photosynthetic C and N metabolism of nodulated Lupin roots during phosphorus deficiency***" is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree. All the sources I have used or quoted have been indicated and acknowledged by means of complete references.

M.R. Le Roux

Signature : ..... Date : .....

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ABSTRACT

Growth of symbiotic legume hosts is P limited, because of the high energetic requirements associated with N_2 fixation. Attempts to overcome P deficiency in soils where legumes are grown involve addition of P-based fertilisers. However, these are produced from finite, non-renewable resources that could be exhausted in the next 50-80 years. For this and other prudent reasons, viable alternatives are sought that include producing genetically enhanced plants with better P use efficiency (PUE). There exist some inter- and intraspecific genetic variation for associated traits of PUE in various legumes and these will have to be exploited to realize the development of P efficient cultivars. With the advent of sophisticated molecular tools, good progress has been made to understand the molecular response of some common physiological and morphological functions observed under LP. The research aims here were to investigate the energy costs and the alternative metabolic routes associated with C and N metabolism under LP in legumes, which is very scant in literature. We also investigated the recovery responses of nodulated roots upon P alleviation. Consequently, improvement strategies to produce legume varieties for better adaptation in poor P soils are envisaged.

We have demonstrated varying degrees of sensitivity between the amide and ureide legume systems being investigated under short-term LP. The species-specific responses were ascribed to differences related to the agro-climatic origins, nodule morphologies and the type of N containing export product of the different legume

types. These different responses also underscore possible different regulatory mechanisms under LP. Lupins were probed further, because of its apparent tolerance to P deficiency. Lupin nodules had between 3 to 5-fold higher P_i concentrations compared with soybeans under LP and HP, respectively. The maintenance of P_i levels, as oppose to a decline in the total P pool, is discussed in relation to its role in maintaining N_2 fixation in lupins. Under LP, an effective P_i recycling mechanism in nodules is proposed to occur via the induction of the PEPc-MDH-ME route. This route also enhanced the capacity of root nodules to procure high malate concentrations that are used to fuel bacteroid respiration and N_2 fixation. Two distinctly different cMDH proteins, one corresponding to HP and another corresponding to LP, were identified. The high malate concentrations reported here are speculated to have arisen through LP-induced cMDH.

Metabolically available P_i decline developed gradually as P deficiency progressed. This coincided with a 15% decline in the %Ndfa. Moreover, under prolonged P deficiency the disproportionate synthesis of organic acids, most notably malate, that occurred at the expense of amino acids was proposed to account for this decline. The recovery in response to alleviation from LP involved alterations in the allocation of respiratory costs to growth and nutrient acquisition. Under LP, smaller nodules were formed and nodule metabolism revolved around accentuating PUE. Thus, there is considerable potential for improvement of P efficiency in legumes through manipulation of root:shoot partitioning.

KEYWORDS

Bacteroids

C-metabolism

Dicarboxylic acids

Lupinus

Malate

Nodules

Photosynthesis

P_i deficiency

Nitrogen

N_2-fixation

Respiration

LIST OF ABBREVIATIONS

aa	amino acids
AAT	aspartate amino transferase (E.C. 1.1.23)
ABC	ATP-binding cassette
AEC	adenylate energy charge
AI	alkaline invertase
Ala	alanine
AMP	adenosine 5'- monophosphate
ANOVA	analysis of variance
APs	acid phosphatases
ATP	adenosine 5'- triphosphate
ARA	acetylene reduction assay
Asn	asparagines
Asp	aspartate
BNF	biological nitrogen fixation
°C	degrees celsius
^{14}C	radiolabelled carbon
CA	carbonic anhydrase (EC; 4.2.1.1)
cMDH	cytosolic malate dehydrogenase
$^{14}CO_2$	radiolabelled carbon dioxide
Dct	dicarboxylate transport system
dH_2O	distilled water
DIC	dissolved inorganic carbon

DTT	1,4 – dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
ESTs	expressed sequence tags
EURS	utilisation efficiency for rhizobial symbiosis
FW	fresh weight
GOGAT	glutamate synthase
GS	glutamine synthetase
HB	Haber-Bosch
HPLC	high performance liquid chromatography
IRGA	infrared gas analyser
LDC	less developed countries
LP	low phosphate
LSD	least significant difference
LWR	leaf weight ratio
Mal	malate
MDH	malate dehydrogenase
NA	^{15}N natural abundance
(NADH)-GOGAT	glutamate synthase, reduced form
(NAD)-MDH	malate dehydrogenase, oxidized form (EC 1.1.1.37)
(NADH)-MDH	malate dehydrogenase, reduced form (EC 1.1.1.37)
(NAD)-ME	malic enzyme (EC 1.1.1.40), cofactor in oxidized form
(NADH)-ME	malic enzyme (EC 1.1.1.40), cofactor in reduced form

NAD	β – nicotinamide adenine dinucleotide
NADH	β – nicotinamide adenine dinucleotide, reduced form
NADP	β – nicotinamide adenine dinucleotide phosphate
NADPH	β – nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National Center for Biotechnology Information
N.D.	not determined
Ndfa	nitrogen derived from atmosphere
neMDH	nodule-enhanced malate dehydrogenase
NID	^{15}N isotope dilution
nt	nucleotide
OAs	organic acids
OAA	oxaloacetate
PBM	peribacteroid membrane
PBS	peribacteroid space
PCR	polymerase chain reaction
PEP	phospho*enol*pyruvate
PEPc	phospho*enol*pyruvate carboxylase (EC 4.1.1.31)
PHB	polyhydroxy-butyrate
P_i	inorganic phosphate
PK	pyruvate kinase (EC 2.7.1.40)
(PN_{max})	maximum net photosynthetic rate
PUE	phosphate use efficiency
PVPP	polyvinylpolypyrrolydine

QTLs	quantitative trait loci
RGR	relative growth rate
RG_w	growth respiration per unit dry weight
RG_t	growth respiration per unit time
RM_t	maintenance respiration per unit time
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
SE	standard error
SNPs	single nucleotide polymorphisms
SNF	symbiotic nitrogen fixation
SPAR	specific P absorption rate
SPUR	specific P utilisation rate
sRNA	small ribonucleic acid
SSA	Sub-Saharan Africa
Suc	sucrose
Susy	sucrose synthase
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TE	Tris-EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
W_c	construction costs
XDH	xanthine dehydrogenase

BIOGRAPHICAL SKETCH

Marcellous Remarque Le Roux was born of Daniel and Elizabeth Le Roux in Oudtshoorn, Western Cape, South Africa, on the 24th February 1978. He attended Sacred Heart Primary School and matriculated at Bridgton Senior Secondary School in 1995. He enrolled at the University of Stellenbosch in 1996 and obtained a BSc degree with Botany and Genetics as majors in 2000. He furthered his studies at the same institution, first completing a BSc (Hons.) in 2001 and immediately after, enrolling for an MSc (Plant biotechnology) in 2002. He obtained his MSc degree in 2005.

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CHAPTER 1

General Introduction

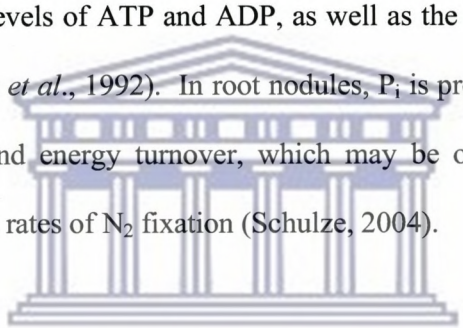
1.1 Background

It is acknowledged that leguminous plants encompass key characteristics that make them suitable to be considered to help advance efforts towards sustainable agricultural practices (Graham & Vance, 2003). The symbiosis of legumes with rhizobia enables leguminous host plants, which comprise some economically important crops, including bean plants, peas, lentils and others to grow in soils with little available nitrogen (N). The rhizobial endosymbionts capable of N_2 fixation, known as bacteroids, are housed in newly formed root nodules. In here, bacteroids are buffered against any fluctuations in the environmental conditions their legume hosts might be experiencing.

Even though evidence for the positive contribution of legumes in crop rotations and in enhancing soil fertility continues to increase, breeding for better N_2 fixation has so far had limited success, hence the poor acceptance from even some of the most supportive farmers. Furthermore, most legumes studied to date have shown highly variable traits related to N_2 fixation. Thus, breeding for enhanced N_2 fixation may require a more holistic approach in conjunction with other factors associated with nutrient deficiencies, enhancing tolerance, etc. (Graham & Vance, 2000).

Moreover, nutrient deficiency is of particular relevance, since it is considered that efforts to improve N_2 fixation through appropriate genetic means, will all be in vain if viable solutions to addressing soil fertility are not sought first (Sanchez, 2002). Mineral stress is regarded as critical in understanding the

causal effects of global climate changes on plant growth in affected soils (Lynch & St Clair, 2004). For this reason, several research efforts have tried to evaluate the relationship between various mineral nutrients, most notably inorganic phosphorus (P_i) and N_2 fixation (Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998; Olivera *et al.*, 2004). Nitrogen fixation in the various legumes studied to date is severely compromised by inadequate levels of P_i in soils (Vadéz *et al.*, 1996; Drevon & Hartwig, 1997). Inorganic phosphorus is known to regulate bio-energetic processes in plants by being one of the substrates for photo- and oxidative phosphorylation. Lack of P_i has been found to decrease the levels of ATP and ADP, as well as the adenylate energy charge in roots (Rychter *et al.*, 1992). In root nodules, P_i is proposed to be involved in C metabolism and energy turnover, which may be of pivotal importance to maintaining high rates of N_2 fixation (Schulze, 2004).



Furthermore, it emerged from several research studies looking at the effects of P stress on the primary metabolism of plants that under limiting P concentrations as observed for such studies, alternative non-phosphorylating pathways may be induced to conserve P_i and partially maintain cellular metabolism/function (Duff *et al.*, 1989; Theodorou & Plaxton, 1993). One such point of divergence, under P deficiency, is at the branched nexus of phospho*enol*pyruvate (PEP). At this point, phospho*enol*pyruvate carboxylase (PEPc), malate dehydrogenase (MDH) and malic enzyme (ME) sequentially process phospho*enol*pyruvate (PEP), presumably when P_i starvation is induced. Whilst the PEPc-MDH-ME alternative route may become increasingly important over the conventional pyruvate kinase (PK) route,

under P_i-deficient conditions, it also represents a key branch-point where N and C metabolism diverts in root nodules of legumes (Vance & Gantt 1992; Vance *et al.*, 1994). PEPc contributes a significant portion of C for assimilation of the export products of legumes, particularly those that export amides in their xylem stream. It is thought for amide-exporting legume types, that between 25 to 30% of C may be re-assimilated via the PEPc reaction (Vance *et al.*, 1985). In addition, the PEPc-MDH-ME alternative route may siphon some C to dicarboxylic acid synthesis in an attempt to supplement the TCA cycle, under the imposed restricted conditions of P deficiency.

Nodules are known to be strong sinks for P and this may be related to the role of P in C metabolism and energy turnover (Schulze, 2004). Colebatch *et al.*, (2004) have proposed a model, which demonstrates the capacity of bacteroids as active P_i scavengers, presumably in an effort to maintain N_2 fixation efficiency. This P scavenging 'tactic' asserted by the bacteroids may fulfil an important function, which is to activate/induce the PEPc-MDH-ME pathway to produce more malate, which is the preferred carbon source for symbiotic rhizobia (Rosendahl *et al.*, 1990; Schulze *et al.*, 2002). This may also corroborate observations of higher P concentrations in nodules than in either shoots or roots (Jakobsen, 1985; Israel, 1987; Israel, 1993; Tang *et al.*, 2001). Furthermore, at least the bacteroid fraction of nodules of the symbiosis may be able to maintain sufficient adenylate energy charge (AEC), under P stress, demonstrating its inherent capacity to proceed with N_2 fixation relatively unperturbed (Sa & Israel, 1991). Regardless of P concentration in the

subtending host root, bacteroids secrete phosphatases capable of solubilizing bound P, into their surrounding environment (Al-Niemi *et al.*, 1997; 1998).

1.2 Justification

Notwithstanding the major inroads made into unmasking the role of dicarboxylic acids in symbiosis, there still seem to be major deficiencies in our understanding of nutrient flux in and out of legume nodules. The most obvious reason for this is that measurements assessing transport of nutrients across the symbiosome have been achieved with isolated bacteroids, which have been disconnected from their 'in-house' regulatory features and tend to give a misrepresentation of what actually happens *in vivo* (Lodwig & Poole, 2003). In addition, the tendency to extrapolate from free-living bacteria of the same genera known to go into symbiosis effectively may be null and void as these have been shown to respond indifferently to the same conditions (McKay *et al.*, 1988). Therefore, such results should be viewed with caution, despite having illuminated our understanding of some intricacies of rhizobia-legume symbiosis (Lodwig & Poole, 2003).

Leading from this, the legume-rhizobia symbiosis, which has been widely viewed as a simplistic exchange of nitrogen (N) for carbon (C), imparts a heavy C tax on the plant partner. However, this notion of a simplistic exchange has also recently been dispelled and it was proposed that a more complex interplay, involving some amino acids or derivatives thereof from the plant host, exists (Prell & Poole, 2006). Moreover, this exchange of metabolites has to be negotiated under severely compromised conditions, most

notably within narrow limits of oxygen (O_2) (Hunt & Layzell, 1993), whilst rhizobia need to maintain high rates of fixation in order to benefit their legume host. This is because nitrogenase (N_{ase}) may be irreversibly damaged at high O_2 concentrations, inevitably resulting in low N-transfer to legume host, thus effectively nullifying the benefits of the symbiosis. The plant fraction of symbiosis is particularly affected by any such perturbations, i.e. O_2 restriction and P limitation, in primary metabolism and it is thought that under these prevailing conditions C is delivered to bacteroids in the form of dicarboxylic acids, most notably malate, via the aforementioned PEPc-MDH-ME alternative route (Rosendahl *et al.*, 1990; Vance & Heichel, 1991; Schulze *et al.*, 2002). Malate uptake has been correlated with the rate of respiration of bacteroids and O_2 uptake (Li *et al.*, 2001). The high malate flux is also the result of an MDH iso-enzyme shown to be more prevalent in nodules as opposed to other tissue (Miller *et al.*, 1998). This nodule-enhanced expression of MDH may account for as much as 50% of the total malate pool in root nodules, compared with the 20% contribution by a cytosolic MDH, in *Medicago sativa* (Miller *et al.*, 1998).

Highlighted in the discussion above are several crucial features impinging on the efficiency of N_2 fixation. As stated above root nodules appear to be at the mercy of the plant partner, even though the actual centre for N_2 fixation may be relatively safely tucked away on the inner core of the nodule. However, nodules may still be negatively affected by various stresses that affect the legume host. The most noteworthy of these is P_i, because of its integral place in C- and energy metabolism. Although the effects of P on N_2 fixation have

been extensively studied there remain some crucial gaps, which need resolving before any headway is to be made in manipulating the system for future benefits. In addition, most of these aforementioned studies evaluating the role of P in symbiosis have assessed its impact at steady state conditions where the legume host would have adapted to its conditions. One would only tentatively be able to venture an answer to the question of where primary metabolic regulation takes place, whilst the response to P stress would be species specific and will also depend on the duration of the stress (Høgh-Jensen *et al.*, 2002).

1.3 Aims

The one question pertinent to this study was: how will nodulated legumes alter their metabolism of organic acids (OAs) and nitrogen (N) in response to P deficiency? An overarching aim is to ascertain the responses to P deficiency during short-term (14 d) as opposed to prolonged (24 d) exposure.

These questions were addressed by assessing the following:

i) What role do the various phosphorus fractions (e.g. P_i, PP_i, total P) have in relation to sustaining symbiotic nitrogen fixation (SNF)? This will be addressed in the two dominant legume types, i.e. amide-exporting lupins versus ureide-exporting soybeans.

ii) Secondly, it is known that nodules accumulate high levels of OAs, in particular malate. Therefore, the role and regulation of malate metabolism in nodules, under P deficiency, was assessed.

iii) Finally, the plasticity of physiological responses to P deficiency during alleviation of P deficiency in nodulated root systems was examined.

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CHAPTER 2

Literature Review

2.1 The role of legumes in sustainable agriculture

2.1.1 Problems associated with current agricultural practices

Globally, agriculture has entered a phase where increased amounts of energy expenditure have little influence over any further increases in crop yields (Tomczak, 2006). Since 1960, the global population more than doubled, whereas world agricultural area per person decreased two-fold (Tilman *et al.*, 2001). This situation may have its roots in the Green Revolution, which dates back to the early 1900's. Through this, crop yields were increased dramatically due to the extensive use of fertilisers, rich in nitrogen (N). In general, crop yields were increased up to 50%, whereas 80% yield increases were implemented for cereals (Smil, 1991). The impact of N-rich fertiliser production on agricultural development cannot be denied and the exponential growth of the human populace bears testimony to this successful period in agriculture (Smil, 1997, 1999). It is no exaggeration to say that N is arguably one of the most limiting factors for global food crop production and inevitably human growth (Tilman, 1999).

Fertiliser is produced through the energy-intensive Haber-Bosch (HB) process (Cheng, 2008), which produces about 80 metric ton (Mt) ammoniac (NH_3) annually and accounts for 30% of the energy cost in modern-day agriculture (Graham & Vance, 2000). Non-renewable raw materials, most notably mineral oil and gas, are utilised in fertiliser production, with further expenditures incurred when fertiliser has to be transported to farms that often lack reliable infrastructure, i.e. good roads and so forth, particularly in less developed countries (LDC). Thus, fertilizer production, transportation and

other agrochemical costs are inextricably linked to similar increases in the oil price, because these are produced using the same oil-based energy sources. To offset high production costs, suppliers often increase the price of fertilisers.

Therefore, farmers, especially in LDC, do not purchase fertiliser even though there may be an acute need to boost crop growth (Sanchez, 2010). For this reason, the adoption of revolutionary industrial-scale mechanisation and access to sufficient fertiliser, to boost crop productivity is still eluding most farmers in the developing world. To date, disparities among farmers of the developed and developing worlds persist and in many instances are made worse by current trends in trade policies. Often smallholder farmers are not active participants in such technological developments, but rather passive recipients of its final products (Puente-Rodriguez, 2009). Human dependency on finite resources, e.g. oil and gas, has created a futile cycle, which is not sustainable. Unless there is a drastic shift away from the current patterns of production (and consumption) and a purposeful move towards resource-conserving practices and technologies, mankind will not be able to sustain their agricultural needs (Mafongoya *et al.*, 2006; Østergård *et al.*, 2009)

Pressures of global food markets, but also poor farm management practices, mean that fertiliser is often applied in excess of that required by crops for maximal growth. Effectively crops only absorb about half of the applied N in the first year of application, whilst most is lost through nitrification and leaching (Sanchez, 2010). Consequently, the run-off from agricultural land

pollutes groundwater, subsequently causing the eutrophication of rivers and lakes (Carpenter *et al.*, 1998; Beman *et al.*, 2005).

The aforementioned Haber-Bosch process may also contribute significantly to the present day global warming phenomenon, through liberating major greenhouse gases, such as CO_2, methane and nitrous oxide, as by-products. (Erisman *et al.*, 2008) There is no refuting the fact that the demand on modern agriculture has increased (Tilman *et al.*, 2001). However, mankind's attempts to increase food production, have also to consider conservation of land used for such purposes so as to prevent it from becoming barren due to insufficient amounts of nutrients to sustain crop growth and consequently rendering the land unfit for use (Vitousek *et al.*, 2009).

2.1.2 Food security concerns

One of the eight developmental goals envisaged by all of the member states of the United Nations (UN) at the start of this millennium, is to half the proportion of people who suffer from hunger by the year 2015 (Delmer, 2005). Efforts to rectify this have been met with stern challenges in recent years when towards the end of 2008, high food and fuel prices nurtured fears of food insecurity for many, especially in less developed countries (LDC). Uncertainties surrounding food security were propelled further to the forefront on the political agendas of most governments of the world when a subsequent economic crisis in 2009 hit financial markets.

Food security, often equated to insufficient food intake, is multifactorial, with several factors (e.g. buying power; diet; etc.) interplaying at one stage or another. The question of food security is not just one of not having enough to eat, but also relates to an imbalanced diet. For instance, many people from LDC, most notably Africa and Asia, are reliant on high calorie food sources, e.g. maize and rice, which over the long haul may contribute significantly to health issues. Focus needs to be on nutrition security, rather than food security and increased food production *per se* (Shetty, 2010). In turn, good nutrition is also strongly correlated to good health and may contribute to effective treatments for various diseases, including HIV (Shetty, 2010). The basis for the alleviation of HIV through good nutrition remains largely unfounded, though. Nevertheless, in recent times there have been incessant pleas to redress the disconnect between agriculture and health (Sanchez, 2010).

Although there is a need to increase productivity of farmlands, it is increasingly becoming an issue of growing food crops that will supply the nutritional requirements to the end user in a well-balanced manner. In addition to providing food and/or feed, crops considered for cultivation should have multiple uses not just that pertaining to their core function in order to minimise wastage. To this end, legume crops that are capable of forming root symbioses with rhizobia, which release a virtually free nitrogen (N) source to the legume host, are ideal and warrant consideration. The N 'fixed' is ultimately converted into plant biomass rich in protein, which forms the basis of legumes as feed and food sources for animals and humans alike (Graham &

Vance, 2003). Thus, legumes hold the potential to address problems related to nutrient deficiencies, amongst other things and critically contribute to enriching soils through the N rich plant matter left after harvesting. Legumes used as intercrops or in rotations are known to relay benefits to subsequent growing crops (Stern, 1993). These capabilities infer to legumes the potential to rehabilitate depleted soils through N enrichment. Thus, legumes are self-sufficient at acquiring N through symbiosis under optimal conditions and can grow in soils that have little or no available N, consequently reducing the need for excessive application of N fertilizers. In fact, any residual N in soils may be detrimental to the process of symbiotic nitrogen fixation (SNF) (Carroll & Mathews, 1990).

With regard to agricultural importance, the legume family at present plays second fiddle to genera in the Poaceae plant family, based on area harvested and total production (Gepts *et al.*, 2005). However, this is anticipated to change as it is from the diverse pool of legume plants that it is hoped agriculture will draw as current agricultural practices are compelled to reform drastically in the face of challenges related to global warming, decline in arable land and currently high external inputs to increase productivity (Kennedy & Cocking, 1997). Therefore, farmers are encouraged to grow legumes as intercrops, cover crops, or in rotation with 'traditional crops' such as cereals and in this way lessen the burden of systems relying heavily on mineral fertilisers to increase productivity.

2.1.3 Factors exacerbating food insecurities

A number of factors negate attempts to increase food production including population growth, global warming (and all its associated effects), declining renewable resources, soil degradation due to overuse, extensive fertiliser usage and the practice of tilling (Doös, 1994). The world population is steadily increasing and is set to peak at 9 billion in the not too distant future (Tilman *et al.*, 2001). The carrying capacity of the environment, that is the number of people it can potentially support, be it via land acreage for food production and/or living, is increasingly diminishing.

With regard to food production, agriculture will have to accelerate the production of food if any headway is to be made in mitigating the effects of potential global food shortages. If not, restricted food production would invariably lead to higher food prices and place the health and inevitably the lives of the majority of people especially in LDC at risk as they are bound to succumb to malnutrition. High food prices in conjunction with declining food supplies have been at the centre of recent upheavals in large parts of several LDC, but also a handful of other more developed countries (Vidal, 2007). Food trading has suffered tremendously in the wake of this, with the majority of exporters inclined to reduce the export of some important staples so as to safeguard themselves against a shortfall in terms of food provision to their own citizens (Vidal, 2007).

Another important and radical recent development, potentially impacting on the food insecurity of millions, especially in the LDC, is growing important

food staples for bio-fuel. Bio-fuels are touted as a solution to rising fuel prices, growing energy demands and the need to curb emissions of greenhouse gases. However, conservative estimation suggests that some of the more common bio-fuel crops (e.g. maize, wheat) are not always less efficient in terms of their demands on resources (De Vries *et al.*, 2010). For critiques of the practice of converting already scarce arable agricultural lands for the purpose of growing bio-fuel crops on it, it is considered that this is essentially diverting the expenditure away from food production and distribution efforts, which could serve the larger populace better. It is proposed that non-food crops with inherently resource-efficient characteristics may be more suited for the purpose of bio-fuel production (Johnston *et al.*, 2009; De Vries *et al.*, 2010).

The dynamics of food insecurity are complex and another key driving force of global food supply is diet preference. It has recently been proposed that one's diet may be responsible for up to twice as many greenhouse gas emissions as driving a vehicle about 19 000 km with 9 *l*/100 km petrol usage (Trivedi, 2008). Red meat and dairy products, which constitute approximately one third of humanity's protein intake, emerged as the most emissions-intensive foods due to the number of stages involved from production to processing. The projected increase in the global population and demands for diets richer in meat by a wealthier populace are projected to double global food demand by 2050 (Tilman *et al.*, 2001). This is despite associated problems relating to cardiovascular disease with over indulging in animal foods high in fat (Sinha *et al.*, 2009).

A recent report by the Food Climate Research Network was critical of meat diets and went as far to suggest that governments need to enforce rations of meat to be taken by consumers so as to prevent dire climate change events (Jowit, 2008). The report noted that awareness-raising campaigns would no longer suffice to effect difficult changes regarding people's diet preferences. It is therefore vital that humans balance their dietary preferences so that they consume less meat (Popkin, 2009). In this regard, nutritionists and environmentalists advocate plant-based proteins, simply because they believe these proteins to be healthier and normally not detrimental to the environment (Guillon & Champ, 2002).

2.1.4 Constraints to utilisation of legumes

The adoption of legumes has been tentatively pursued even though literature is strewn with evidence of its benefits for cropping systems and legume crop yields, for the most part, are still lagging behind cereal crop production initiatives. Several environmental and biological factors impact negatively on the performance of legume-*Rhizobium* symbioses in cropping systems. These range from soil acidity, nutrient deficiency and toxicity to the genetic potential of the organisms involved and how they interact with components of the environment (Bohlool *et al.*, 1992). Predictably, attempts to improve nitrogen fixation by legume hosts, still proof to be difficult, in part because the mutualism exists between two diverse groups of organisms, which have evolved a finely tuned relationship to make the benefits tangible to both partners involved. Indeed, responses to the array of stresses experienced, may

affect the partners involved differently. Thus, there needs to be an understanding of how changes to any one of the symbiotic partners in the legume-*Rhizobium* association are impeding on the efficiency of the mutualism as a means to satisfy the needs for both partners.

Most cereal crops do not form any integral collaborative associations of any kind, certainly not to the extent that legumes do, and therefore any improvement to such crop characteristics as increased vigour and yield, normally only has to consider the plant. Although massive strides have been achieved in understanding numerous leguminous host plant systems, the successes of biotechnological innovations have been hindered in part by the indifferent response to manipulation of the two partners involved. It has long been realized that in order to sustain legume production and stimulate the expanded use of legumes in cropping systems, there is a need to increase legume yield further (Sinclair, 2004). Recent advances made by molecular tools to improve N_2 fixation have not amounted to much in the field. Irrespective, the improvement of N_2 fixation through genetic manipulation may not come to its full potential unless issues of soil fertility are addressed first (Sanchez, 2002).

Thus, notwithstanding the challenges mentioned, legumes should form an integral part of progressive thinking strategies to assure a sufficient, secure and equitable global food supply (Tilman *et al.*, 2001). Current improvement strategies are progressively shifting towards reducing input, while simultaneously optimizing output (Østergård *et al.*, 2009). Alternative food

crops considered should be nutritious and definitely sustainable over a long period, a dual role, which legumes clearly fulfil. Thus, the renewed enthusiasm with which legume-based research has been adopted has everything to do with the sustainability practices it encompasses and the holistic approach needed in agriculture to address issues relating to diminishing renewable resources (Cannon *et al.*, 2009). It seems a logical extension then, to entertain the idea of legume usage to supplement diets of many malnourished individuals in the developing world.

2.2 Food security: the situation in Sub-Saharan Africa

The situation on the African continent with regard to food production capabilities is particularly cumbersome, because although Africa is regarded as being rich in resources it lags in progressive development initiatives unlike other underdeveloped countries. The population in Africa has reached one billion, roughly one seventh of the global population and the majority live in rural areas, not conducive to commercial agriculture (2009 World Population Data Sheet). This can be ascribed to the high birth rate, which is double (5.3) that of the global average of 2.6 children per woman (2009 World Population Data Sheet). This rate of population expansion is bound to strain food security issues in the region. In general, about one quarter of children in developing countries are chronically underweight because of under nourishment as it is recognised that some of the first casualties of an impoverished lifestyle are protein and vegetables (Sheeran, 2008). Thus, an imminent nutritional crisis is at hand, which would have dire consequences for children and their development (Sheeran, 2008). Evidence is fast emerging that early

malnutrition damages intellectual development and productivity irreversibly (Ruel & Hoddinott, 2008).

Legume crops are of paramount importance to protein deficient food diets of much of the rural and urban populations of the developing world and the cultivation thereof as food staples extends into marginal areas, even more so than it does in mainstream agriculture. Subsistence farmers in Africa generally cultivate grain-, forage- and tree legumes on marginal lands but have to contend with low yields due to soil depletion of important nutrients for growth (Dakora & Keya, 1997). Leguminous trees of the genera *Sesbania*, *Tephrosia*, *Crotalaria* and others are interplanted as fallows during dry seasons alongside young maize seedlings. The quantities of N accumulated (typically 100 to 200 kg N) through N_2 fixation, during short- to long term, are as much as that applied as fertiliser in developed countries (Keyser & Li, 1992; Ledgard & Steele, 1992). These high N concentrations contribute to increased maize yields and merit the use of legumes as an economically sound approach to subsistence farming in Africa (Graham & Vance, 2000). By virtue of incorporating legumes into their farmlands, smallholder farmers are at the forefront of innovative ways of addressing soil fertility replenishment. Moreover, rural communities have already inadvertently become key role players at managing their own food security by starting to grow vital alternative food crops, such as legumes.

The problem for SSA, but also Africa as a whole, is that it represents the last region of the world where per capita food production has remained stagnant

over the past 40 years (Sanchez, 2002). This is in spite of the fact that agriculture engages over 70% of all Africans, most of who are women. Insufficient food production in SSA has direct bearing on food security and hence, farming practices in Africa have come into sharp focus. Several research and development projects are underway to restore some confidence back in agricultural development in Africa, which has been neglected for a number of decades. The higher priority envisaged for agricultural development in African countries saw leaders commit themselves to increase their allocation of their national budgets to agricultural development by up to 10% (Ngongi, 2008).

The aforementioned rural households on the African continent depend heavily on marginal farmlands and other natural resources for their livelihoods. Poor food production is inextricably linked to impoverished soils that are characteristic of SSA. This prompted an initiative early in 2009 to start mapping African soils digitally for their constituent nutrient content, type of soil and pH (Rouwé, 2009). It is hoped that this will guide decision-making with regard to how to manage agricultural soils better for the purpose of preserving them for long-term use.

Impoverished soils arise through the practise of removing in excess of what is put in by conventional means, which in developing countries is normally either through manure or fertiliser. Despite the innate ability of nodulated legumes to accumulate high concentrations of N during pod filling (most of which may be removed during harvesting) a net depletion may occur when

more N is removed than what is provided by the symbiosis (Al-Falih, 2002). Thus, under subsistence farming conditions this will over time exacerbate the problem of soil fertility depletion. Depletion of soil fertility is the single most important factor cited to hinder the adoption of genetically improved varieties (Sanchez, 2002). Although Africa has an unsurpassed diversity in micro-ecologies concomitant with food crop varieties, adoption of new, improved varieties lags well behind that in Asia, Latin America and the Middle East (Sanchez, 2002; Ngongi, 2008).

As mentioned the use of N fertiliser in recommended quantities still eludes farmers of developing countries. The loss of important nutrients, critical to plant production has been estimated to equate to US$4 billion dollars in fertiliser (Sanchez *et al.*, 1997). However, the use of fertilisers is considered taboo as its costs far outweigh its benefits to the predominantly subsistence farming community of SSA. In fact, fertilisers are twice to six times pricier to the African farmer than to their counterparts elsewhere in Europe, North America and Asia (Sanchez, 2002). Nevertheless, for many smallholder farmers from developing countries the envisaged shift to low external input agriculture may be to their benefit as it may eventually be the 'primer' for them to break into world agriculture.

2.3 The importance of nitrogen provision by legumes

Nitrogen (N) is a vital component of chlorophyll, amino acids, nucleic acids, proteins and enzymes and the lack thereof during critical periods of plant growth may cause less nutritious plant crops (Miller & Cramer, 2004). The

importance of N to plants is highlighted by the various novel adaptations of different plant species to obtain this scarce nutrient. An example of this in the tropics, where soils are prone to N deficiency is, some plants have evolved eloquent ways to attain N through trapping insects, which they digest to release invaluable N (Adamec, 1997). Similarly, plants that grow in areas typified by almost year-round snow cover like in the arctic make use of organic-N in the form of amino acids instead of inorganic-N such as ammonia (NH_4^+) or nitrate (NO_3^-) (Kielland, 1994, Miller & Cramer, 2004). Well in excess of 50% of the N obtained by alpine plants and native plants growing in the arctic tundra may be in the form of organic-N (Kielland, 1994). Other forms of N, which can temporarily occur in the soil at high concentrations, are urea via urine excretion from animals in pastures or, as previously noted, fertiliser N applications (Andrews *et al.*, 2004). Nevertheless, almost all forms are usually rapidly converted to NH_4^+ and NO_3^- by soil microorganisms.

Another exquisite adaptation to acquire N, which is pivotal to future agricultural practices, is the association of legume plants with endosymbiotic bacteria collectively referred to as rhizobia. Rhizobia convert N_2 to a usable form, normally NH_4^+, readily available to their legume hosts. This process is confined to a selective few in the plant kingdom and allows the plant hosts to tap into an apparently inexhaustible reservoir of N. Symbiotic nitrogen fixation (SNF) is estimated to contribute approximately 65% of the total annual fixation (Herridge & Rose, 2000), with more N added to agricultural ecosystems globally than the total amount of fertiliser N (Rengel, 2002). Depending on the plant host, the amount of N fixed by agriculturally

important legumes may vary from very low to amounts as high as 60 Mt annually (Graham & Vance, 2003). Furthermore, unlike other forms of N fertilizers (NH_4^+/NO_3^-), symbiotically fixed N is less prone to volatilization, denitrification and leaching (Graham & Vance, 2000).

This capability of engaging in symbiosis may stand legume hosts in good stead, even under global warming conditions, which are leading in particular to an increase in CO_2. Elevated CO_2 has been shown to have no apparent deleterious effects on N constituents of symbiotic soybean. This legume host was able to acclimate to increased N demand at elevated [CO_2] to match increase in C gain (Rogers *et al.*, 2006). In contrast, Högy *et al.* (2009) showed that growth under elevated CO_2 altered a number of grain traits that determine nutritional value and market quality of wheat, despite yield improvements under these conditions. This deterioration in grain quality might also have some negative effects on industrial processing. Both aforementioned studies (Rogers *et al.*, 2006; Högy *et al.*, 2009) were conducted over at least two growing seasons. It has also been demonstrated that cassava (*Manihot esculenta*), after exposure to simulated CO_2 conditions envisioned under global warming conditions, accrued more toxins in their edible leaves with a concomitant decrease in yield (Gleadow *et al.*, 2009). This has dire implications to people from poorer regions that consume the tubers, but particularly the leaves of this crop as a protein supplement.

2.4 Alternative uses for legumes

Nodulated legumes can serve a multitude of purposes in sustainable agriculture. Food legumes, most commonly beans and peas, are important components of farming systems and these have received considerably more attention than any of the other lesser known leguminous plants. This is because such food legumes are directly correlated to human well-being. However, legumes accruing high N concentrations through SNF also have an importance in other industries, including agroforestry, the medical profession and the meat and dairy industries (Morris, 1997; Graham & Vance, 2003). Legumes can provide speciality products, which can be used as natural pharmaceutical remedies for ailments (Graham & Vance, 2003). Furthermore, their use as a source of fuelwood, fiber, gums, dyes and oils as well as windbreaks and for stabilizing of sand dunes and eroding hillsides is well documented. Thus, some if not all legumes may have multiple purposes, which may extend beyond their use as a source of N in agricultural soils. All these encompassing traits of legumes make their incorporation into even well established farming systems, desirable (Vance, 2001).

2.5 Legumes and rhizobia

2.5.1 Rhizobial infection

There are some, indications that in the symbiotic interaction with legumes, rhizobia are initially recognized as intruders (Dakora *et al.*, 1993; Stougaard, 2000). Not to be deterred, rhizobia eventually overcome plant defence responses and colonize the roots. Symbiosis development in root nodules of legumes is one of the most intensely studied symbiotic relationships between

microorganisms and higher plants. The idea is to 'unmask' much of the mystery surrounding potential benefits of N_2 fixation so as to transfer these to other economically valued cereal crops (Webster *et al.*, 1997; Cocking *et al.*, 1997). This has, so far, eluded the best proponents of bio-engineering, but recent discoveries surrounding the intricacies of the legume-rhizobia partnership have given fresh insight to allow researchers to pursue this goal.

Several reviews have, in recent times highlighted, the methodical precision of the infection process of the legume host by rhizobia (Gage & Margolin 2000, Rengel, 2002). The interaction between rhizobia and the legume root system begins with an exchange of chemical signals in the soil, which leads to colonization of the root hairs and initiation of root cortex cell division. The plant initiates its association with rhizobia through secreting phenolic compounds, most notably flavonoids, from its roots into the rhizosphere (Cage *et al.*, 2000, Rengel, 2002). These phenolic signals also incur some degree of host specificity to the rhizobia. In turn, competent rhizobia 'cross talk' by secreting nodulation (*nod*) factors. What follows is a complex signalling pathway, which is characterised by calcium spiking in susceptible root hairs (Ehrhardt *et al.*, 1996). Rhizobia are entrapped in the root hairs, which upon receiving the right signals, deform and curl around the rhizobia. Once inside the cell, rhizobia proliferate in infective threads.

This association is both selective and very specific. Individual species of rhizobia have a distinct host range allowing nodulation of only certain legumes (Stougaard, 2000). Formation of a successful symbiotic association

therefore requires a coordinated expression of both bacterial and plant genes in the appropriate ecological niche (Hungria & Stacey, 1997). At the end of these processes, the bacteroids are harboured in a symbiosome, surrounded by a plant-derived membrane, called the peribacteroid membrane (PBM). This serves as a physical and functional barrier over which nutrient exchange will take place over the duration of the symbiosis (Szafran & Haaker, 1995; Udvardi & Day, 1997).

2.5.2 Nodule structure and function

Leguminous plants are conveniently divided into two distinct groups according to the N source translocated from the root system to the shoots of a particular legume host (Schubert, 1986). This is closely related to the shape of the nodules induced on the root surfaces of the legume host. In tropical regions, the predominant forms of N found in the xylem sap of legume hosts are ureides (allantoin & allantoic acid), which are produced in spherically-shaped determinate nodules with a closed meristem that at nodule maturity is unable to divide any further. Nodules from ureide-exporting legumes are spherically shaped and its infected cells lack vacuoles (Schubert, 1986). Furthermore, determinate nodules are known to harbour several bacteroids, which come about by fusion of separate symbiosomes and/or bacteroids dividing continually within an existing symbiosome (Prell & Poole, 2006).

In contrast, legumes from more temperate environments transport amide amino acids (glutamine & asparagines) and their nodules are typically indeterminate. Nodules are characterised by an open meristem, which allows

for continual divisions right through the plants life cycle. This gives rise to a cylindrically shaped nodule in which the infected cells are vacuolated (Schubert, 1986). Nodules of this type only contain one bacteroid per symbiosome (Prell & Poole, 2006).

Although, various nodules differ in some structural details, the general organization of tissue regions is similar, with the infection zone normally confined to the central region of the nodule. The two types of nodules present an intriguing difference in metabolism. This forms the basis of intense research to discern differences in nodular metabolism, which cannot be easily extrapolated between the two systems and even less so between different species (Prell & Poole, 2006). There is no consensus over which of these is actually the more efficient way of transporting N, although some consider that exporting N in the form of ureides is more efficient, since their C:N ratio is 1:1 as opposed to 2:1 for amides (Smith & Atkins, 2002).

1.6 P metabolism and root nodule symbiosis

1.6.1 Importance of P_i in plant metabolism

Although it appears that legumes are self-sufficient at acquiring N, for the symbiosis to be effective, other minerals, in particular phosphate (P_i), are also required. Thus, the inclusion of legumes in cropping systems does not always ensure the attainment of optimal levels of SNF (Serraj & Adu-Gyamfi, 2004). It has been consistently demonstrated that low P, in particular, impedes upon SNF, even more so than other environmental stresses. The reasons for this remain obscure, as it is often difficult to isolate the effects of low P on plant

growth and/or rhizobial function. Nodulated legumes are known to require more P, than those plants that depend on mineral N fertilizer. This may be related to the energy requirements of the N_2 fixing process under severely compromised aerobic glycolysis.

Generally in plant metabolism (as in other organisms), P has an important intermediary role acting as the energy carrier. Adenosine triphosphate (ATP) is the most important P_i compound in this regard. As such, P_i is intimately involved with metabolic processes. The importance of P_i in plant metabolism can be attributed to a variety of factors including: (i) the unique physical characteristics of the P-P bond and its role in energy transduction, (ii) the pivotal role P_i plays in fine control of metabolism as an allosteric effector of enzymatic activity (Iglesias *et al.*, 1993; Theodorou & Plaxton, 1993) or by the reversal phosphorylation of key regulatory enzymes (Plaxton, 1996), (iii) its function as an important constituent of macromolecules, e.g. phospholipids and nucleic acids and (iv) its vital role in regulating the exchange of triose phosphate between the plastid and cytosol via the P_i translocator (Preiss, 1984; Walker & Sivak, 1986).

1.6.2 Adaptive strategies under P_i deprivation

In soil, the concentration of available P_i for plants is usually very low as most of the P_i is bound to iron (Fe^{3+}), aluminium (Al^{3+}) and calcium (Ca^{2+}), to form sparingly soluble compounds. Consequently, plants have developed various ways to overcome the shortage of P_i in their immediate environment.

A typical response to low P content of soil is increased relative biomass allocation to roots, which ultimately results in an increase in root:shoot ratio (Paul & Stitt, 1993). In turn, this enhances the P acquisition from the growth medium. However, this may also result in retarded growth rates because of the diversion of C to the production of heterotrophic rather than photosynthetic tissues. Nielsen *et al.*, (2001) remarked that the allocation of carbohydrates to various plant parts and functions is a governing parameter of plant survival and success. Thus, the success of plants under stress conditions may be determined by their ability to control carbohydrate utilization for metabolic energy.

Several plants, including legumes, are known to exude organic acids, of which citrate, malate and succinate constitute the dominant forms, in response to P_i deprivation (Hoffland *et al.*, 1989; Imas *et al.*, 1991; Johnson *et al.*, 1996a,b; Tang *et al.*, 2001). The organic acids exuded by the roots displace the elements to which P_i is bound and solubilize the P_i. The P_i released in such a way may be taken up by roots either on its own or as a chelated complex to the organic acids exuded. The increased rate of organic acid exudation was ascribed to an increased synthesis of the organic acids mentioned. The non-photosynthetic carbon fixation route, via phospho*enol*pyruvate carboxylase (PEPc), has been implicated as the pathway through which the increased synthesis of these particular organic acids occurs. The activity of non-photosynthetic PEPc in the roots was enhanced by P_i starvation (Pilbeam *et al.*, 1993; Johnson *et al.*, 1994). Johnson *et al.*, (1996a) in a subsequent study revealed that this increase in PEPc activity coincided with the increase in

PEPc transcripts and the amount PEPc proteins. Hence, it was concluded that the expression of PEPc in response to P_i deprivation was, at least in part, under transcriptional control (Johnson *et al.*, 1996b).

Another strategy to acquire P_i and an increase in the P_i uptake rate in response to P_i starvation has been observed in roots and cultured cells (Drew *et al.*, 1984; Aono *et al.*, 2001). Furihata *et al.*, (1992) proposed a dual mechanism model, composed of two kinetically different uptake systems; one a high-affinity transport system and the other a low-affinity transport system. As most soils are deficient in P_i, the high-affinity transport system is thought to be primarily functional in roots.

Studies undertaken to investigate plant adaptation to P starvation have further revealed that plants induce alternative pathways of glycolysis and mitochondrial electron transport (Duff *et al.*, 1989; Theodorou & Plaxton, 1993). This is seen as an adaptive strategy to facilitate respiration by P_i deficient plant cells. Phosphate deficiency causes a decline in cytosolic P_i and adenylates and under these conditions the increased engagement of these bypasses negate the necessity for adenylates and P_i which are utilized under normal, non-stressed conditions (Duff *et al.*, 1989; Theodorou & Plaxton, 1993).

2.6.3 Nodule metabolism and P_i

It is becoming evident that in addition to functioning at low oxygen (O_2) tension (typically 5-20 nM, Hunt & Layzell, 1993; Kuzma *et al.*, 1993)

rhizobia often live and function where their host is P limited (Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998; Tang *et al.*, 2001; Colebatch *et al.*, 2004). Evidence from expressed sequence tags (ESTs) probing has recently strengthened claims from physiological studies (Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998) that bacteroids possess the genes related to P remobilization and recycling (Colebatch *et al.*, 2004). Nodules also possess plant originated galactolipids cell membranes (substitutes for phospholipids), a feature until recently only thought to occur in higher plant cells. This appears to be a strategy to secure P_i for other essential cellular processes (Gaude *et al.*, 2004). Phosphate has specific roles in nodule initiation, growth and functioning. Several key parameters related to the capabilities of legumes to fix N_2 effectively, which may be deemed to give them a competitive advantage over other crops, are adversely affected by P deficiency (Jakobsen, 1985; Israel, 1993, Olivera *et al.*, 2004). These parameters include nitrogenase activity, nodule number, nodule mass and plant N accumulation of N_2 fixing species.

Although P deficiency and its role in the N_2 fixation process have received considerable attention (Israel 1987; Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998; Tang *et al.*, 2001; Ribet & Drevon, 1995; Drevon & Hartwig, 1997; Jebara *et al.*, 2005; Schulze & Drevon, 2005), the relationship between low phosphate (LP) supply and N_2 fixation is not clear. Low and limiting P supply eventually reduces plant growth and thus N demand and subsequent N_2 fixation. Nitrogen fixation rate in P deficient plants may exert effects on the growth of the host plant, on the growth and functioning of the nodule or on the

growth of both the plant and nodule (Jakobsen, 1985; Israel, 1993; Sa and Israel, 1991).

Phosphate-deficient plants form smaller nodules compared with control plants supplied with sufficient P (Ribet & Drevon, 1995). This translates into an increased nodule surface area to nodule volume for nodules under P deprivation (Ribet & Drevon, 1995). Unlike other abiotic stresses studied thus far, P deficiency increases the permeability to O_2, with a concomitant increase in respiration. The permeability of nodules is due to ultrastructural changes manifested in the cortex of infected cells (Ribet & Drevon, 1995). Dinitrogen fixation remains unchanged even with this apparent disturbance in O_2 flux into nodules (Ribet & Drevon, 1995).

The fact that legumes are particularly sensitive to P_i deficiency may be related to its role in energy turnover within root nodules. The nitrogenase enzyme complex catalyzes the six-electron reduction of N_2 to NH_4^+, including the reduction of $2H^+$ to H_2 that uses 16-18 molecules of ATP (Dixon & Kahn, 2004). Thus, because N_2 fixation requires a huge amount of energy and because P has a key role in energy metabolism in cells, P deficiency may adversely affect the energy status of legume nodules and ultimately nodule function (Sa & Israel, 1991). However, bacteroids are able to maintain their adenylate energy charge (AEC) even when subjected to prolonged P deficiency, whereas the AEC in the plant fraction of the nodule was reduced (Sa & Israel, 1991).

Alternatively, sensitivity of N_2 to P_i deficiency may be because of altered N metabolism in nodules. On a quantitative basis, N and P are the two mineral elements required most by plants and a deficiency in either one of these elements impacts negatively on various processes of plant metabolism. Furthermore, since the metabolism of these elements is integrally linked, a deficiency in one adversely affects the processing of the other. This might be even more apparent in leguminous plants where it has been reported that an improvement in P status of legumes solely dependent on symbiotic N_2 fixation, increased tissue N concentration as well as overall host plant growth (Israel, 1987). Greater stimulation of symbiotic N_2 fixation with added P is associated with an enhancement in the specific nitrogenase activity of the nodules (Jakobsen, 1985; Israel, 1987). Soils devoid of extractable P, which are encountered globally, may negate the benefits associated with high N_2 fixation rates of leguminous hosts. This is of particular relevance to subsistence farmers who remove P during harvesting which strains soil P reserves (Vance, 2001).

The initial assimilation step of N_2 to NH_4^+ appears to be more sensitive to P deficiency than the subsequent incorporation of soluble reduced N into protein and nucleic acids (Israel & Rufty, 1988; Sa & Israel, 1995). This conclusion was derived from results that showed that initial P deficiency conditions resulted in low levels of soluble reduced N (Israel & Rufty, 1988). This effect gradually disappeared when increasing the P nutrition of the nodule (Israel & Rufty, 1988). Similarly, the adverse effect that P deficiency had on N_2 fixation might have been as a result of poor nodule initiation, growth and

specific nitrogenase activity (Israel, 1987; 1993). In addition, it must be realized that P deficiency may also directly or indirectly influence other steps in the N assimilatory pathway of N_2 fixing plants. Furthermore the activities of nitrogenase and nitrate reductase, both enzymes of nitrogen assimilation, have been shown to increase with P nutrition (Carling *et al.*, 1978).

Nodules are composed of heterogeneous, but distinct, regions and cell types; an outer uninfected cortex (with vascular tissue) and a central region with infected and uninfected cells. Thus, an altered distribution pattern among this diverse population of cells for metabolites such as P is envisaged. Indeed, ^{31}P-nuclear magnetic resonance (NMR) studies have demonstrated the variable ditribution of P in nodules (Rolin *et al.*, 1989). In addition, this distribution pattern might differ between the different N-exporting legumes, since it is known that bacteroids from ureide-exporters such as soybean are non vacuolated as oppose to vacuolated cells of amide-exporting legumes (e.g. lupins) (Schubert, 1986). Thus, cells with less pronounced vacuolar spaces, as is the case for ureide exporters, would considerably limit the storage capacity for nutrients, including P_i.

Furthermore, slow growing strains of rhizobia appear more tolerant to low P levels than do fast growing strains, despite having similar capacities for P uptake. Both may increase their uptake efficiency several times faster than cells grown with adequate P (Beck & Munns, 1984). In general, both free-living and symbiotic rhizobial cultures respond to P stress by increasing their P transport capacity and inducing alkaline- and acid phosphatases (Al-Niemi

et al., 1997; 1998). Alternatively, cultured rhizobia demonstrated the capacity to store P and utilize it accordingly under P starved conditions (Cassman *et al.*, 1981).

2.7 Trade-offs in a mutualistic symbiosis

2.7.1 C and N exchange during symbiosis

The major exchange of nutrients between the symbiotic partners in the legume-*Rhizobium* association is reduced carbon (C) from the plant, for fixed N from the bacteroid (Rosendahl *et al.*, 1990; Udvardi & Day, 1997). A much more complex nature of this exchange has been hypothesised, though, which involves amino acid cycling (*see later discussion; Section 1.7.2*) (Lodwig *et al.*, 2003; Prell & Poole, 2006; White *et al.*, 2007).

Nitrogen fixation requires a large input of energy and reducing equivalents. Carbon supplied to the bacteroids is ultimately derived from photosynthate, transported to nodules as sucrose (Suc). However, once in the nodule, Suc can be cleaved by either sucrose synthase (SuSy, EC 2.4.1.13) or alkaline invertase (AI, EC 3.2.1.26). Mutants in which SuSy activity has been removed, although forming viable bacteroids seemingly capable of N_2 fixation, were unable to fix N_2 (Craig *et al.*, 1999; Gordon *et al.*, 1999). This appears to be the result of impaired C and energy metabolism and demonstrates the need for SuSy in assuring N_2 fixing capacity for the host.

There is considerable evidence that Suc breakdown occurs in the uninfected cell region of the nodule where O_2 levels may still be sufficiently high to drive

the metabolism of Suc breakdown. Once in the infected cells, the microaerobic conditions that prevail might prevent mitochondrial respiration from supplying Suc as a C source to the bacteroids at a sufficient rate to support any sunbstantial N_2 fixation activity (Day & Copeland, 1991). In addition, SuSy and AI activities are markedly up-regulated in uninfected cells compared with the surrounding roots (White *et al.*, 2007).

One possible fate of the hexoses (e.g. UDP-glucose and fructose) derived from Suc cleavage is that they could be metabolised through the glycolytic pathway to form phospho*enol*pyruvate (PEP), which in turn is converted to malate (Mal) via the concerted action of phospho*enol*pyruvate carboxylase (PEPc, EC 4.1.1.31) and malate dehydrogenase (MDH, EC 1.1.1.37). The oxidation of sugars liberates energy in the form of ATP and also reducing equivalents (NAD[P]H, NAD, etc.).

Phospho*enol*pyruvate (PEP) is situated at a major branch-point for N and C metabolism of higher plants, particularly in leguminous plants (Vance *et al.*, 1985). At this PEP branch-point, there is constant competition for C skeletons between the organic acid pool (for TCA cycle intermediates and energy) and the amino acid pool (for N assimilation). Both these pathways form essential parts of a leguminous plant's high nutritional status, which has made it such an important source of dietary protein globally. Simply stated, an impeded supply of C skeletons to assimilated N could well be expected to be detrimental to the incorporation of N rich compounds in the seeds, which forms the basis of the protein richness in legumes. Thus, there are grounds to

consider C and N metabolism simultaneously, since it appears the metabolism of the two metabolites is so integrally linked, particularly in legume root nodules.

While previous research has proposed that bacteroids in symbiosis trade NH_4^+ in exchange for the C offered by the plant partner, one study, in particular, casts some doubt on the type of N metabolite released to the host (Waters *et al.*, 1998). These authors reported alanine (Ala) instead of NH_4^+ as the sole secretion product of bacteroids in soybean (Waters *et al.*, 1998). However, in a subsequent study the amount of Ala secreted depended on the relative amount of NH_4^+ build up in the medium (Allaway *et al.*, 2000). Nevertheless, results from the first study (Waters *et al.*, 1998) in which this was demonstrated have not been repeated since (Li *et al.*, 2001), whereas subsequent mutational studies refuted the importance of Ala dehydrogenase in other symbioses where N_2 fixation proceeded unperturbed (Allaway *et al.*, 2000; Kumar *et al.*, 2005). Alanine dehydrogenase is the enzyme responsible for converting pyruvate to Ala in bacteroids and the principal route for Ala synthesis. Thus, due to the inconsistency in results demonstrated, NH_4^+ is still considered the main excretory product of the root-nodule symbiosis.

In the root nodule, the generated NH_4^+ is processed via the coupled activity of glutamine synthetase and glutamate synthase (GS/GOGAT) after being excreted into the plant cytosol. The first step in this assimilatory pathway is the catalysis by glutamine synthetase (GS), which requires ATP and forms glutamine (Gln). In excess of 90% of the total GS activity is in the plant

tissue, where it has been estimated to account for approximately 2% of the total soluble protein (Cullimore & Bennett, 1998). Moreover, the activity of this enzyme is increased compared with that in uninoculated roots (Trepp *et al.*, 1999). The Gln that was formed by the former reaction is subsequently converted to glutuamate (Glu) by glutamate synthase (NADH-GOGAT) (Cullimore & Bennett, 1998).

It would appear, that the legume host might be able to alter the assimilatory route of N under P stress conditions (Olivera *et al.*, 2004). Common bean plants, which are deemed ureide-exporting legumes, subjected to P stress, showed a negative correlation between xanthine dehydrognase (XDH) and uricase, the enzymes involved in glutamate (Glu) assimilation into ureides. On the other hand a strong positive correlation was demonstrated for aspartate amino transferase (AAT), an enzyme normally associated with Glu incorporation into aspartate (Asp) in amide-exporting legumes (Olivera *et al.*, 2004).

2.7.2 The role of amino acid cycling in attaining symbiotic efficiency

A new and groundbreaking theory has probed the complex interchange of C and N between the legume host and the endosymbiotic rhizobia it harbours. This new hypothesis proposes that in addition to releasing C to the rhizobia, the host plant also trades an N source in the form of amino acid(s) (Lodwig *et al.*, 2003).

The exchange of amino acids in symbiosis is proposed to function by shutting down bacteroid ammonium (NH_4^+) assimilation and allowing the flow of fixed N to the host (Lodwig *et al.*, 2003). As far as this is concerned, there is substantial evidence for amino acid metabolism in nodules. Isolated *Bradyrhizobium japonicum* bacteroids incubated anaerobically with ^{14}C malate or succinate accumulated 20-40 % of the labeled C in Glu (Salminen & Streeter, 1987). Furthermore, labeling studies with $^{14}CO_2$ in which isolated bacteroids of detached nodules for both pea and soybean were used also showed similar distribution patterns of the amino acid fraction in Glu (Salminen & Streeter, 1992). The Glu may have been derived from Ala, which was rapidly turned over. In addition, long-term exposure to ^{14}C malate of isolated peribacteroid units of pea accumulated 60% of the amino acid fraction in Ala and 40% in aspartate (Asp) (Rosendahl *et al.*, 1992).

In contrast to ^{14}C labelling studies, whole plant labelling studies with ^{15}N revealed no such high levels of Glu (or Ala) in bacteroids of pea plants, but rather showed asparagine (Asn) to be the dominant amino acid compound (*see* Lodwig & Poole, 2003). Nevertheless, although Glu appears to support N_2 fixation in isolated soybean bacteroids (Bergersen & Turner, 1988; Kouchi *et al.*, 1991) it seems that the isolated PBM of soybean, bean and pea is impermeable to Glu (Price *et al.*, 1987; Udvardi *et al.*, 1988; Herrada *et al.*, 1989; Hernández *et al.*, 1996). In addition, Asp is not a dominant amino acid in bacteroids and has not consistently been demonstrated as an excretory product at high levels by peribacteroid units of pea (Rosendahl *et al.*, 1992). Instead Ala is the principal amino acid detected in soybean bacteroids (Waters

et al., 1998; Allaway *et al.*, 2000). Lodwig and Poole (2003) proposed, on the grounds of increased activity of aspartate aminotransferase (AAT, E.C. 1.1.23) in the peribacteroid space (PBS) (Rosendahl *et al.*, 2001), that the transamination of Asp might well occur in the PBS. Aspartate aminotransferase catalyses the reversible conversion of Asp and 2-oxoglutarate to glutamate and OAA (Lodwig & Poole, 2003).

Although there is substantial labelling evidence showing intracellular amino acid synthesis and metabolism by bacteroids, the precise role of amino acids in the N_2 fixation process remains elusive (Walshaw & Poole, 1996; Hosie *et al.*, 2001). The question arises, then, if bacteroids were able to accept amino acid(s) from the plant host, how would such an exchange contribute to the efficiency of the symbiotic process? Indeed, the exchange of amino acids, such as Asp, Glu, Ala, and potentially others, between rhizobia and their host plants appears to be critical for successful symbiosis (Lodwig *et al.*, 2003).

Propositions of a malate-aspartate shuttle between the plant- and bacteroid fractions through which reducing equivalents are shunted to the nitrogenase system for N_2 fixation by bacteroids have surfaced in earlier studies (Kahn *et al.*, 1985; Appels & Haaker, 1991). It was suggested that malate and glutamate would be imported into the bacteroid in exchange for 2-oxoglutarate and Asp, respectively (Kahn *et al.*, 1985). Recently, this has been probed by assessing bacterial mutants which had two broad range amino acid ABC-type transporters (*aap* and *bra*) have been knocked out (Lodwig *et al.*, 2003). These amino-acid transporters have also shown low-affinity exporting activity

(Walshaw & Poole, 1996; Hosie *et al.*, 2001). These double mutants displayed a marked reduction in shoot dry weight (DW) and shoot N and typically responded to the N deficit by increasing nodule numbers and mass (Lodwig *et al.*, 2003). Interestingly, the double mutants were able to grow on minimal medium, suggesting that they exuded amino acids into the medium for subsequent uptake. Also, the nodules produced by such mutants retained their capacity to fix N_2 at similar rates or even higher rates than the wild-type parents (Lodwig *et al.*, 2003).

Based on the evidence available and the stoichiometric relationship (or lack thereof) of a conventional malate-aspartate shuttle, it appears more likely that a modified malate-alanine shuttle is active in root nodules (Lodwig & Poole, 2003). However, some of this may be related to the isolation technique used, which is important since improper energisation of the bacteroid membrane once it has been isolated affects the transport of various nutrients (Lodwig & Poole, 2003). Energy is a prerequisite for transport of dicarboxylic acids in *Rhizobium leguminosarum*, *Bradyrhizobium. japonicum*, *R.meliloti* (Glenn *et al.*, 1980; Reibach & Streeter, 1984; McRae *et al.*, 1989). Although this proposed model still has a few challenges in terms of identifying which amino acids are traded between the partners, it provides evidence for the co-dependence of the two symbiotic partners and evolutionary pressure for symbiosis to prevail (Lodwig *et al.*, 2003). Nevertheless, this is an exiting proposal and has added another dimension to how complex the liaison between the legumes and their rhizobial partners actually is and the possible consequences this may have for exploiting the symbiosis are far-reaching.

2.7.3 Respiratory substrates for bacteroids in symbiosis

The supply of photosynthate-derived C and energy-bearing substrates to bacteroids residing in the root nodules of legumes is the subject of extensive investigation because the supply of 'essential' nutrients to bacteroids (N_2 fixation hub) in relation to nutrient partitioning in nodule tissue and nutrient transport to the symbiosome may affect the effectiveness of N_2 fixation (McRae *et al.*, 1989). Contrary to common belief, symbiotic rhizobia may have a nutritional complexity unsurpassed by other free-living bacteria, judged solely on the higher incidence of ATP-binding cassette (ABC) transporters identified by genomic studies (Gaude *et al.*, 2004). This dispels the notion that rhizobia in symbiosis may have limited access to a variety of nutrients, either from the soil or in the plant rhizosphere (Prell & Poole, 2006).

As strictly respiratory organisms, differentiated bacteria in the symbiosome (bacteroids) must get their energy from the respiratory chain by oxidizing energy rich substrates and reducing O_2. The first casualty, however, of the symbiosis is the luxury O_2, which in the nodular environment has now become almost limiting, at least for baseline glycolysis. Once in symbiosis, bacteroids are dependent on the plant host for all compounds crucial for an efficient metabolism. The low O_2 tension in the root nodule environment has implications for the catabolism of C, not likely to be encountered under aerobic conditions. Legume nodules maintain a low O_2 concentration of between 3 and 22 nM (Hunt & Layzell, 1993, Minchin, 1997). This is achieved by expressing high affinity terminal oxidases and also by

maintaining high levels of O_2 binding leghemoglobin protein. This sets the tone for further symbiosis, because if the O_2 concentration is not kept at a sufficiently low level, N_2 fixation, which is realised through an oxygen-labile nitrogenase, would fail to occur.

Compromised C metabolism under microaerobic conditions has given further credence to the proposal that instead of C being metabolised to pyruvate, which would happen under fully aerobic conditions, C is converted to malate via the PEPc reaction. Symbiotic N_2 fixing rhizobia favour malate (and other related dicarboxylic acids) as C source. Neither sugars nor amino acids elicit significant acetylene reducing activity over a wide range of O_2 concentrations (Miller *et al.*, 1988). Moreover, dicarboxylic acids are transported via the C_4 dicarboxylate transport system (Dct), at significant rates, consistent with their role as principal C source for bacteroids, whereas there are seemingly no carriers on the peribacteroid membrane (PBM) for sugars and amino acids (McRae *et al.*, 1989; Udvardi *et al.*, 1990). The Dct system, consists of three genes: *dctA* encoding a transport protein and two sensory regulators, *dctB* and *dctD*, which activate the transcription of *dctA* in reponse to C_4 dicarboxylic acids (Reid & Poole, 1998; Yurgel & Kahn, 2004). This transport system has been shown to be indispensable for N_2 fixation, with mutants defective in it registering a Fix^- phenotype (Yurgel & Kahn, 2005).

Dicarboxylic acids accumulate to millimolar (mM) concentrations in legume root nodules (McRae *et al.*, 1989; Rosendahl *et al.*, 1990). Labeling data on whole nodule tissue further support dicarboxylates (e.g. malate, succinate and

fumarate) as the C source for bacteroids due to them being rapidly labeled in plant cytosol when nodules are exposed to $^{14}CO_2$ and rapidly incorporated into bacteroids as malate (Kouchi & Yoneyama, 1986; Rosendahl *et al.*, 1990). Malate may make up as much as 50% of the total organic acid pool (Rosendahl *et al.*, 1990).

In vitro studies in a flow chamber have shown a complex relationship between dicarboxylic acid concentration, O_2 concentration and N_2 fixation in soybean bacteroids (Li *et al.*, 2001). The exchange of malate between the legume host and inhabitant rhizobia is central to the efficiency of symbiotic relationship. Among other things, malate provides a significant portion of C skeletons for fixed N assimilation. It has also been suggested that malate helps in the regulation of the oxygen diffusion barrier via an osmoelectrical mechanism (Gálvez *et al.*, 2000). Further evidence of the pivotal role that malate plays in root nodules is the fact that ineffective nodules, whether induced by changes in either bacterial or plant genotype, have strikingly reduced levels of malate as compared with effective nodules (Schulze, 2004). Moreover, mutations in rhizobia that block organic acid use, result in ineffective nodules, while those that block amino acid- and carbohydrate use generally have no effect on N_2 fixation (Ronson *et al.*, 1981; Driscoll & Finan, 1993).

De Vries *et al.*, (1980) proposed at least three roles for the malate pathway in nodule metabolism. According to De Vries *et al.*, (1980), a part of the malate and possibly of other OAs could be used as a carbon and energy source for the N_2 fixing bacteroids. Another part of the malate could enter the Krebs cycle of

mitochondria and produce energy, in the form of ATP, reducing power and C skeletons used during the functioning of nitrogenase and biosynthesis of amino acids from ammonium. Lastly, and no less important, malate could also be involved in the adjustment of charge balance in vacuoles and in xylem fluid. Malate has been shown to balance the majority of the excess inorganic cation charge of xylem sap (approximately 75%), with most of the remaining charge being balanced by allantoate (in tropical legumes) and aspartate (Israel & Jackson, 1982).

2.8 Enzymes of malate metaboilism

2.8.1 Malate dehydrogenase (MDH)

There is widespread consensus that the predominant C_4 dicarboxylic acid in root nodules of legumes is malate, which is synthesized by the concerted action of PEPc and malate dehydrogenase from oxaloacetate (OAA) and NADH. Under physiological conditions, such as in the plant cytosol, the equilibrium of the reaction catalyzed by MDH is completely in favour of malate and NAD^+. Malate dehydrogenase activity in root nodules has consistently been shown to be higher than that in roots (Lawrie & Wheeler, 1975; Appels & Haaker, 1988). As shown by tracer experiments, metabolite concentrations and by enzyme activity determinations, OAA is rapidly reduced into malate by MDH or transaminated into aspartate by aspartate aminotransferase (AAT). The latter pathway leads to asparagine synthesis and is the source of the main amide translocated from nodules to shoots in temperate legumes. However, an increase in plant cell cytosolic malate concentration due to reduced malate uptake by the bacteroids could favour

PEP metabolism via PK over PEP metabolism via PEPc (McCloud *et al.*, 2001). Reduced malate uptake by the bacteroids could occur when nitrogenase is inhibited by treatments that perturb plant C and N metabolism, e.g. detopping, Ar/O_2 (80/20, v/v) treatment (Curioni *et al.*, 1999).

The enzyme MDH occurs ubiquitously as multiple forms that differ in sub-cellular localization and co-factor specificity (Miller *et al.*, 1998; Schulze *et al.*, 2002). The presence of these various forms of MDH is indicative of its diverse role in plant as well as microbial metabolism (Schulze *et al.*, 2002; Lodwig *et al.*, 2003). Five distinct cDNAs for MDH were cloned (Miller *et al.*, 1998) and this was the first report of a cDNA that encoded nodule-enhanced MDH (neMDH). This particular transcript, although distributed in other tissues, was mainly localized in the nodules. This substantiated earlier biochemical reports of a nodule specific form of MDH in pea and lupin nodules (Appels & Haaker, 1988; Ratajczak *et al.*, 1989). Biochemical characterisation of the novel neMDH in alfalfa revealed that it had approximately 7-fold greater affinity for OAA than its comparative form in the cytosol (cMDH). Similarly, neMDH enzyme turnover rates for malate oxidation and oxaloacetic acid (OAA) reduction were 4-fold and 30-fold greater, respectively, than those for cMDH (Schulze *et al.*, 2002). Specificity constants for OAA and NADH were also increased by up to 100-fold compared with specificity constants for malate and NAD. What this shows, is that the neMDH catalyses high *in vitro* rates of malate production. Thus, neMDH provides an attractive target to develop plants with modified malate

metabolism. Immunoprecipitation experiments showed that neMDH makes up about 50% of the total nodule MDH (Miller *et al.*, 1998).

Furthermore, it has previously been shown that malate can form up to 32% of the exuded C in proteoid roots under P deficiency (Johnson *et al.*, 1996a). This reflects the role MDH plays in providing organic acids for exudation from P stressed roots and is seen as an adaptive strategy by these P deficient roots to mobilize bound P from their immediate environment. Thus, malate has a pivotal role in acquisition of N through symbiotic N_2 and P through root exudation. In addition, increased synthesis of malate (and citrate) confers plant tolerance to aluminium toxicity (Delhaize & Ryan, 1995). In the light of all this evidence, it has been proposed that malate forms a critical component of improved plant nutrition through its effects of increased nutrient acquisition and adaptation to environmental stress (Schulze *et al.*, 2002). Two transgenic lines with increase capacity for malate synthesis via the neMDH have been developed (Tesfaye *et al.*, 2001). This increased malate synthesis was correlated with greater MDH activity and expression of the enzyme. The transgenic plants with enhanced neMDH in root tips also showed a significant increase in nodule efficiency, a parameter related to potential for improved N_2 fixation.

1.8.2 Malic enzyme (ME)

The malate pool size is the result of both malate synthesis and utilisation. In this regard, activities of two malic enzymes (ME), NAD-ME and NADP-ME have been demonstrated in *Bradyrhizobium japonicum* (Copeland *et al.*,

1989). Malic enzymes decarboxylate malate to pyruvate (Lodwig & Poole, 2003). The two enzymes have similar activities in *B. japonicum*. Yet in *Sinorhizobium meliloti* the activity of NAD-ME is much higher than NADP-ME. This lower activity is correlated with a lower expression (approx. 20% relative to free-living cells), whilst NAD-ME activity was unaffected (Copeland *et al.*, 1989). Mutation of NAD-ME in *S. meliloti* produced bacteroids with a Fix^- phenotype, whereas a mutation in NADP-ME had no effect on symbiosis. Purified NAD-ME has a K_m of 9.4 mM for Mal and K_ms of 89 µM and 1.56 mM for NAD^+ and $NADP^+$, respectively. In contrast, the K_m of $NADP^+$- ME for malate was 2.6 mM and 33 µM for $NADP^+$ (Voegele *et al.*, 1999).

Furthermore, it was shown that the *in vitro* NAD^+-ME affinity for Mal was stimulated by sucrose and fumarate and inhibited by OAA, pyruvate and acetyl-CoA. $NADP^+$-ME showed no such regulation. Allosteric regulation of NAD^+-ME may be important in maintaining a balanced input of Mal and subsequent acetyl-CoA into the TCA cycle of mitochondria or bacteroids. When acetyl-CoA concentration is low, sucrose and fumarate might accumulate, stimulating NAD^+-ME to produce more pyruvate and subsequent acetyl-CoA for consumption in the TCA cycle. An increase in acetyl-CoA down regulates NAD^+-ME activity to ensure Mal is available for MDH.

The activities of both bacteroid MEs play a pivotal role in regulation of metabolism through balancing the input of reducing equivalents. Redox balance of TCA is essential. For example, if the NADH/NAD ratio is either

too high or too low, the cycle is inhibited. A ratio of 0.83 for *B. japonicum* bacteroids was shown to be inhibitory for citrate synthase activity *in vitro*. (Tabrett & Copeland, 2000). This indicates that during symbiotic N_2 fixation enzyme activities would be markedly reduced *in planta*.

2.9 Carbon diversion and β-polyhydroxy-butyrate (PHB) metabolism

As discussed in earlier sections of this review, it is clear that bacteroids have a high demand for C to meet their respiratory needs. This insatiable C need may be second only to its other strong sink capacity for P. Indeed, the demand for either C or P may be interrelated (Olivera *et al.*, 2004). Earlier, we alluded to the need for maintaining the sensitive balance between C (and energy) inputs and outputs in the light of the non-growing nature of bacteroids. Bacteroids have adapted an eloquent way of circumventing dire complications when a surplus of C is sensed. In symbiosis, rhizobia respond to an oversupply of C by accumulating much of the excess C in reserve polymers.

In nodules, the main C storage polymer is polyhydroxy-butyrate (PHB). Interestingly, the C storage polymer PHB has only been shown to occur in bacteroids of determinate nodules such as *B. japonicum, R. etli* and *R. leguminosarum*. This C polymer accumulates and may form as much as 70% of the bacteroid dry weight under microaerobic conditions in symbiotic cells (Lodwig & Poole, 2003). Similarly, when cultured cells were supplied with an excess of malate, N_2 fixation was inhibited until such time as the substrate level was lowered either through bacteroid metabolism or through washing away from the medium (Bergersen & Turner, 1990; Bergersen & Turner,

1992). In free-living cells any alteration(s) to growing conditions that reduce(s) the flow of C into biosynthetic pathways or respiration results in concomitant increases in the PHB content of such cells (Lodwig & Poole, 2003).

In vitro studies using isolated *B. japonicum* bacteroids showed that bacteroids are able to fix N_2 when no substrate is supplied, suggesting the usage of endogenous reserves. As much as 90% of the ^{14}C malate supplied to bacteroids accumulated in PHB within 5 h. When substrate is omitted PHB reserves are depleted by 9% over a similar 5 h-period and it was shown that most of the respired CO_2 was derived from PHB (Bergersen & Turner, 1992). Furthermore, *B. japonicum* bacteroids degrade PHB when the supply of C is impeded by stem girdling (Bergersen *et al.*, 1991). An interesting observation is that significant changes in PHB content can be detected in hours, suggesting a rapid flux of C through PHB. Moreover, it is clear from this observation that exogenous substrate is used primarily to supplement endogenous reserves such as PHB, which in turn may provide support for N_2 fixation.

A link between PHB synthesis and N_2 fixation has been demonstrated for various strains. For instance, a *R. etli* mutant devoid of *nifA* produced little or no PHB (Cermola *et al.*, 2000). Additionally, there is substantial evidence to show that under certain circumstances bacteroid PHB metabolism fuels N_2 fixation. Strains of *R. leguminosarum* and *R. lupini* showed an inverse correlation between PHB content and the rate of nitrogenase activity with both showing daily antagonistic fluctuation (Kretovich *et al.*, 1977). Furthermore,

PHB content of lupin bacteroids is high (about 13-14% of dry weight) at the start when nitrogenase activity has not yet commenced. However, as development progresses and nitrogenase capacity is gained, there is a substantial decline (3-4% DW at the period of maximum N_2 fixation) in PHB content. Polyhydroxy butyrate content reaches its peak again once seed ripening is realised (Romanov *et al.*, 1980).

It has been suggested that bacteroid PHB metabolism may serve to divert reductant and C away from the TCA cycle under microaerobic conditions. This in turn would alleviate TCA cycle enzyme inhibition (*see section on inhibition of TCA cycle by redox poise*), whereas removal of C would secure a balanced supply to the TCA. This evidence is suggestive of the hypothesis, which claims that PHB metabolism operates separately or alternatively competes with the nitrogenase enzyme complex for reductant and energy (Lodwig & Poole, 2003). This evidence is provided by studies with a rhizobial mutant strain unable to produce PHB where it was found that this did not impact negatively on the symbiosis. In fact, there was an increase in N_2 fixation and the mutation prolonged the capacity for N_2 fixation of bean nodules (Cevallos *et al.*, 1996). In addition, plants inoculated with this mutant strain increased their dry weights and they produced more seeds with a higher N content (Cevallos *et al.*, 1996). Polyhydroxy-butyrate content and activity of PHB metabolic enzymes increase during nodule development during which there is a gradual decline of O_2 uptake as the bacteroids differentiate to maturity.

2.10 Other regulatory features of symbiotic N_2 reduction

Some studies indicate that regulation takes place in the photosynthetic apparatus, thereby affecting production and supply of non-structural carbohydrates (CHO) to nodules (Jakobsen, 1985; Sa & Israel, 1991). Estimates suggest that the N_2 reduction process requires approximately 2.9 mg C. mg^{-1} fixed N, which amounts up to 30% of the current photosynthetic rate (Warembourg & Roumet, 1989). In contrast, other studies seem to indicate that regulation of N_2 fixation has direct effects on nitrogenase activity in nodules (Ribet & Drevon, 1995; Almeida *et al.*, 2000). These have recently been reviewed and emphasises the reality that that no one means of regulation for the process of N_2 fixation exists (Schulze, 2004). From this account, there are currently three theories, which are central to explaining differences in N_2 fixation regulation and which will be explained in brief below.

2.10.1 C-induced limitation of N_2 fixation

In a number of earlier studies, various manipulations, which impair photosynthesis (e.g. defoliation, decreased light intensity, etc.), invariably decreased the rate of N_2 fixation. Similarly, treatments that promote photosynthesis typically increase N_2 fixation. This has implicated C supply to nodules in regulating N_2 fixation activity.

Superimposed on this is the notion that nodules are also prone to low levels of other metabolites, in particular P, which constitutes a major element for energy metabolism in the N_2-fixing process. There is strong evidence that

nodules retain adequate levels of P, whether the host plant is sufficiently supplied with P or not (Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998, Le Roux *et al.*, 2006). Furthermore, the effects of P nutrition, more specifically the deficiency thereof, on C diversion have received considerable attention (Freeden *et al.*, 1989; Raghothama, 1999). It is unknown at this stage how P deficiency affects C partitioning in nodules, i.e. how is C partitioned between host plant cell fraction, where effects of P deficiency are clear, and the bacteroids in the symbiosome, which seem resilient to even the harshest P deficiency in the plant host. And so, even with this wealth of information available to us, we remain in the dark on the precise nutritional conditions under which bacteroids are maintained by the plant (Lodwig & Poole, 2003). Thus, it is vitally important to define the support requirements of and practical limitations on symbiotic N_2 fixation.

1.10.2 The O_2 paradox

Oxygen has a prominent role in root nodule symbiosis as a signalling molecule in the early establishment of the symbiosis and as a substrate for bacteroid respiration. However, the nitrogenase complex responsible for the conversion of N_2 to NH_4^+ has a very low O_2 tolerance and may get damaged irreversibly if exposed to excess O_2 (Hunt & Layzell, 1995). On the other hand, O_2 is necessary to fuel the highly active respiratory processes, including that of bacteroids, which provides energy to the entire nitrogen fixation process, from incorporation to translocation. These occur aerobically in the plant fraction and bacteroid compartments. Thus, nodules are confronted with an O_2

dilemma and nodule metabolism is negotiated within safe of O_2 limits to sustain efficient N_2 fixation (Layzell *et al.*, 1993).

Legume nodules will vary their permeability to O_2 diffusion in response to various treatments that alter the metabolic activity of the nodules (Minchin, 1997). To date, P deficiency is only of these treatments investigated that shows an increase in O_2 permeability (Schulze & Drevon, 2005). The reason for this remains obscure. The variable component of nodule O_2 permeability involves changes in the distribution of air spaces within the nodule inner cortex, resulting from an occlusion of intercellular spaces and/or volume of some of the cells (Serraj & Adu-Gyamfi, 2004). Although there is considerable evidence in support of the variable nodule O_2 permeability as a factor controlling nitrogenase activity, the site, nature and mode of action of such a mechanism is still elusive (Hunt & Layzell, 1993). Features of such a regulatory mechanism include 1) the rapid response to treatments that perturb apparent nitrogenase activity, 2) the reversal of the inhibitory effect by increase in partial O_2 pressure around the nodule.

2.10.3 N feedback regulation of N_2 fixation

Product feedback regulation is a common feature of biochemical pathways. Surprisingly, this was only developed as a concept for N_2 fixation regulation in legumes in the last 15 years (Parsons *et al.*, 1993; Serraj *et al.*, 1999). Despite its recent inception, it is fast emerging as perhaps the most credible explanation for the decrease in N_2 fixation activity of a number of legume species (Hartwig, 1998; Almeida *et al.*, 2000; Høgh-Jensen *et al.*, 2002;

Schulze, 2004; Sulieman *et al.*, 2008). It is suggested that if N_2 fixation proceeds unabated to the extend that N accumulates in excess of what is required for growth by the legume host, then the feedback inhibition that may arise from this would firstly assure that C expenditure for N assimilation ceases. This would relate to the cost-effectiveness, both in terms of C costs and energy efficiency, of the symbiotic process. Secondly, this N feedback regulation may avoid the build up of toxic NH_4^+, the first stable product of the symbiosis, because plants have very limited capacity to store any excess N (Hartwig, 1998). Various independent studies have identified accumulated asparagine (ASN) in the phloem as the main signal for this feedback inhibition to commence (Almeida *et al.*, 2000; Høgh-Jensen *et al.*, 2002; Schulze, 2004; Sulieman *et al.*, 2008).

2.11 Concluding Remarks

Legumes may soon become part of the transformed agricultural landscape envisaged in response to dire changes in global climate and ill-advised agricultural management practices. Although we have benefited hugely from the Green Revolution of the 20th century, this exacerbated conditions for farming. Thus, the focus will now have to shift to the development of sustainable, environmentally friendly crop production systems. The role of leguminous plants as one of the pillars of sustainable agricultural practices is well documented.

However, legumes have shown particular sensitivity to environmental stresses of which P deficiency is probably the most extensively studied. Nevertheless, a clear picture of how P deficiency affects the process of N_2 fixation is slowly emerging. Furthermore, the newly proposed model by Lodwig *et al.*, (2003) for how exchange of C and N occurs within nodules reiterates the need for understanding the basic biochemistry and physiology of nodule function. These will subsequently need to be reconciled with genetic regulation incurred by the symbiosis. The knowledge gained from this study is important for the development of novel crops and provides impetus for both fundamental and applied research in the area of root nodule symbiosis.

1.12 References

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CHAPTER 3

Nitrogen and carbon costs of soybean and lupin root systems during phosphate starvation

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3.1 Abstract

Phosphate (P) starvation is one of the most limiting nutrients to N_2 fixation in legumes. Soybeans and lupins present different climatic origins, nodule morphologies and metabolic complexities, which may have various adaptive responses to short-term P starvation. Lupins and soybeans were cultivated hydroponically for 3 weeks. Short-term P starvation was induced for 14 days by switching the P supply to 2 μM P. During P starvation, the lupins showed a lower decline in nodular P concentrations and maintained their biological N_2 fixation (BNF), in contrast to the soybeans. The lupins also maintained their photosynthetic rates and the nodular construction and growth respiration costs under P starvation, whilst soybeans showed a decrease in photosynthetic rates and an increase in nodular construction and growth respiration costs under P starvation. There was also a shift towards more organic acid synthesis, relative to amino acid synthesis, in lupin nodules than in soybean nodules under P starvation. The lupins had higher amino acid concentrations in their nodules, whilst the soybean nodules maintained their ureide levels at the expense of a decline in amino acids. These results indicate that lupins may be better adapted to maintaining BNF during short-term P starvation than the soybeans.

3.2 Introduction

P limitation is one of the most notable environmental constraints for legumes (Jakobsen, 1985, Israel, 1987, Høgh-Jensen *et al.*, 2002). The high sensitivity of the N_2 fixation process to environmental conditions may be attributed to the C costs (Mengel, 1994). Legumes relying on N_2 fixation require more P than when N is acquired from soil mineral N (Sa & Israel, 1991; Ribet & Drevon, 1995; Al-Niemi *et al.*, 1997; 1998; Tang *et al.*, 2001). The high requirement of P may be linked to its role in nodule carbon and energy metabolism, with at least the plant cell fraction being energy limited under low P supply (Sa & Israel, 1991).

The effect of P starvation on N_2 fixation and nodule O_2 permeability has been demonstated for both amide and ureide exporting nodule types (Ribet & Drevon, 1995; Drevon & Hartwig, 1997; Schulze & Drevon, 2001). Most temperate legumes (e.g. lupin, pea, clover) translocate fixed N as amides, notably asparagine and glutamine (Streeter, 1991), whilst tropical legumes (e.g. soybean, cowpea, common bean) export ureides, most commonly allantoin and allantoic acid. Lupins and soybeans also differ in other means, such as being of temperate and tropical origin and having morphologically distinct nodules. However, as noted by Streeter (1991), the two different sets of metabolic capabilities of amino acid and ureide exporting legumes, present a metabolic complexity that is perhaps unsurpassed by other more typical plant systems. Although the ureide exporters require several more enzymes for ureide biosynthesis than the few for asparagine synthesis the ATP and

reductant expenditure per N assimilated is similar to that of amide exporting legumes (Smith & Atkins, 2002).

Due to the energy costs of N_2 fixation, overall C expenditure is high under non-stress conditions and varies considerably among legume species and varieties (Vance, 1998; Schulze, 2004). Since lupins and soybeans are from temperate and tropical origins and present a difference in N-metabolism and nodule morphology, it is likely that they may show different physiological responses to P starvation. The aim of this study was to draw comparative analyses between the nitrogen and carbon costs imposed on the two legume systems during short-term P starvation.

3.3 Materials and methods

3.3.1 *Plant material and growth conditions*

Seeds of *Lupinus angustifolius* (cv. Tanjil) and *Glycine max* (var. PAN 626) were germinated in vermiculite. At planting, seeds were inoculated with a rhizobial inoculum specific for each species obtained from a commercial seed company. Seeds were coated in a saturated sucrose solution and 2 g of inoculum / 150 seeds were added and mixed. The seeds were spread out, away from direct sunlight to allow the inoculum to dry, before the seeds were planted.

Seedlings were transferred seven to 10 d after germination to 20 *l* hydroponic tanks that were purged with ambient air. The nutrient solution consisted of 4 mM $CaCl_2$, 1.5 mM $MgSO_4$, 2 mM K_2SO_4, 2 mM NaH_2PO_4/Na_2HPO_4,

139 µM H_3BO_3, 21 µM $MnSO_4$, 2 µM $ZnSO_4$, 3 µM $CuSO_4$, 0.2 µM Na_2MoO_4, 89 µM FeEDTA and no N (pH 5.8) (Hewitt, 1966). Solutions were changed once a week. Seedlings were inserted through holes in the lids of the tanks and fixed in the lid of the hydroponic tank with foam rubber at their bases. Since the aim of the experiments was to investigate the effects of abrupt P deprivation, plants were initially grown on a non-limiting P supply (2 mM P) for approximately three weeks, after which the P supply was withdrawn from half of the plants. Phosphorus starvation was induced for 14 days, after which plants were harvested. Although this represents a short period of P starvation after 3 weeks of normal growth, the control group of plants, was maintained at sufficient P supply, so that the short-term departure from these sufficient conditions could be physiologically recorded. The plants were grown in an east-facing glasshouse in Cape Town, South Africa. The range of midday irradiances were between 500 and 670 µmol $m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 21/16 °C and 34/73%, respectively.

3.3.2 *Photosynthesis*

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

3.3.3 *P, N and C determination*

Inorganic orthophosphate (P_i) and pyrophosphate (PP_i) measurements were made on fresh nodules. Approximately 0.5 g of fresh nodule mass was ground to a fine powder in liquid nitrogen and was extracted in 500 µl 10% trichloroacetic acid (TCA) at 4 °C as described by Rychter & Mikulska (1990). This was diluted 3 times with cold 5% TCA and centrifuged for 10 min at 2 500 g at 4 °C. The supernatant was removed and centrifuged a further 10 min at 13 000 g at 4 °C. The resultant supernatant was kept on ice (or stored -20°C) until ready for use. PP_i and P_i concentrations were determined indirectly via pyrophosphatase activity, which was assayed by incubating 10 µl of extract with 190 µl of 50 mM Tris-HCl (pH 8.0) containing 2.4 U/assay inorganic pyrophosphatase, 20 mM $MgCl_2$ and 1.3 mM Na-PP_i for 15 min (Mustroph *et al.*, 2005).

For total C, N and P concentrations, dried and milled samples were analysed by a commercial laboratory (BemLab, De Beers Rd, Somerset West, South Africa), using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with Spectrascan standards (Norway).

3.3.4 *Construction cost and growth respiration*

Tissue construction costs (mmol C. g^{-1} DW) and growth respiration, (mol CO_2. d^{-1}) were calculated according to Mortimer *et al.,* (2005, 2008), as modified from the equations used by Peng *et al.*, (1993). Construction costs represent the C required for tissue growth, whilst growth respiration is the daily respiration associated with new growth (Peng *et al.*, 1993).

3.3.5 *Nitrogen fixation*

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town, using a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. The seed corrected $\delta^{15}N$ values (Boddey *et al.* 1995) were used to determine the percentage N derived from the atmosphere (%Ndfa). Ndfa was calculated according to Shearer & Kohl (1986):

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

Where *B* is the $\delta^{15}N$ natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N free culture, according to Shearer & Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76‰.

3.3.6 *Amino acid and Ureide concentrations*

Amino acid concentrations were determined according to the ninhydrin method by Rosen *et al.*, (1957) using leucine as a standard. Ureides were measured as allantoin concentration according to Trijbels & Vogel (1966).

3.3.7 Enzyme activities

The extraction of the organic acid synthesizing enzymes, *phospoenol pyruvate carboxylase* (PEPc) and NADH-*malate dehydrogenase* (MDH) was performed

according to Ocaña *et al.* (1996). The procedure was modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (m/v) insoluble polyvinylpoly pyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 ml of buffer. The extract of N assimilating enzymes, *aspartate amino transferase* (AAT), *glutamine synthetase* (GS) and *glutamate synthase* (GOGAT) was prepared according to the method of Olivera *et al.* (2004). All extractions were carried out at 4 °C and the crude extract was used in colourometric assays for PEPc, MDH, GS, GOGAT and AAT according to Le Roux *et al* (2006).

3.3.8 Statistical analysis

The percentage data were arcsine transformed (Zar, 1999). Significant differences between treatment means were separated using a *post hoc* Student Newman Kuehls (SuperAnova, Abacus Concepts, USA), multiple range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

3.4 Results

3.4.1 *Nodular P concentration and utilization efficiency*

Nodular P_i (Fig. 1b) and PP_i (Fig. 1c) concentrations remained unchanged during P deficiency, but the total P (Fig. 1a) concentrations declined and this decline was more pronounced for soybeans (60%) than for lupins (35%).

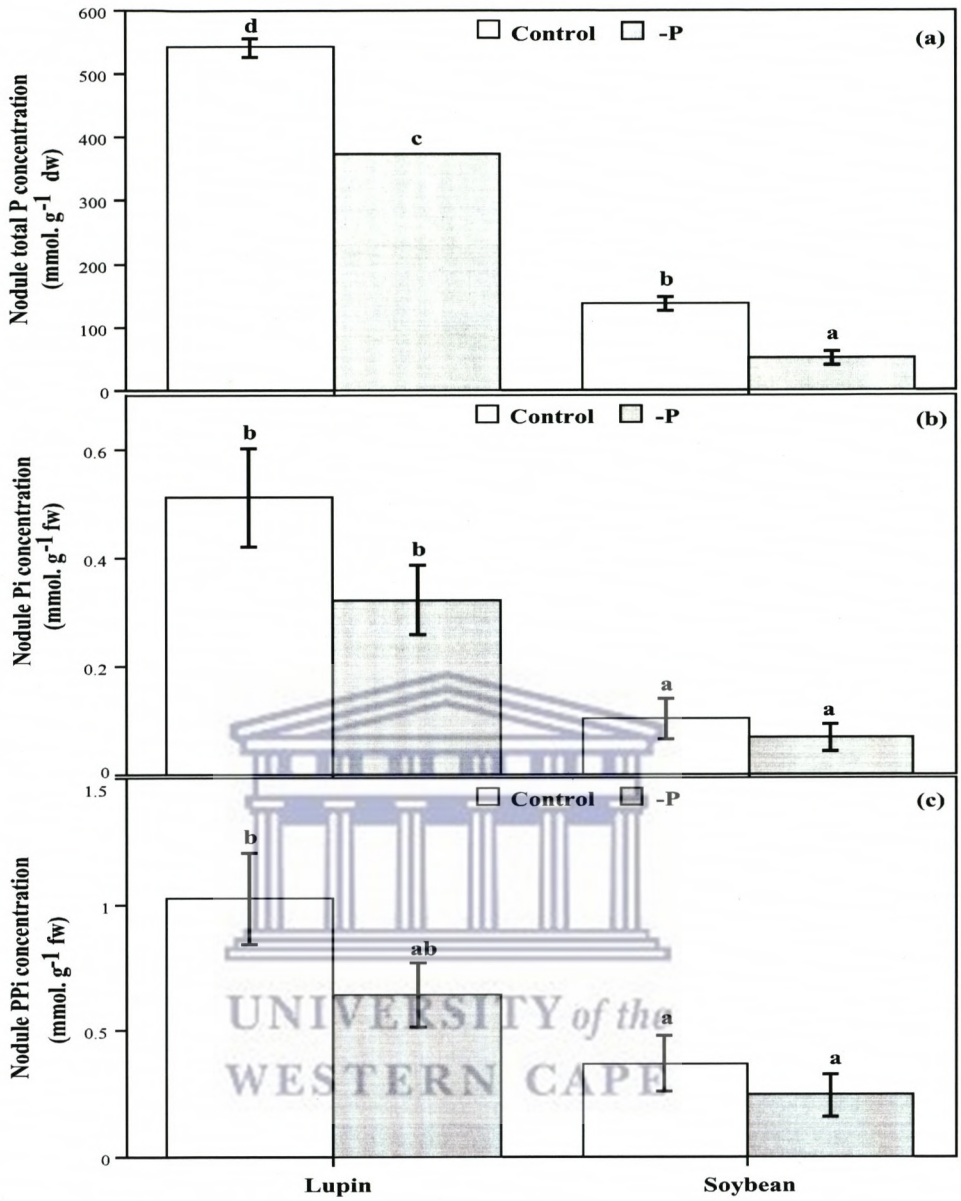

Figure 1. (a) The nodular total phosphate (P), **(b)** orthophosphate (P_i), and **(c)** and pyrophosphate (PP_i) concentrations of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

The specific total P (Fig. 2a) and P_i (Fig. 2b) utilisation rates for soybean nodules, increased significantly under P starvation, compared with the soybean controls and the P starved lupins.

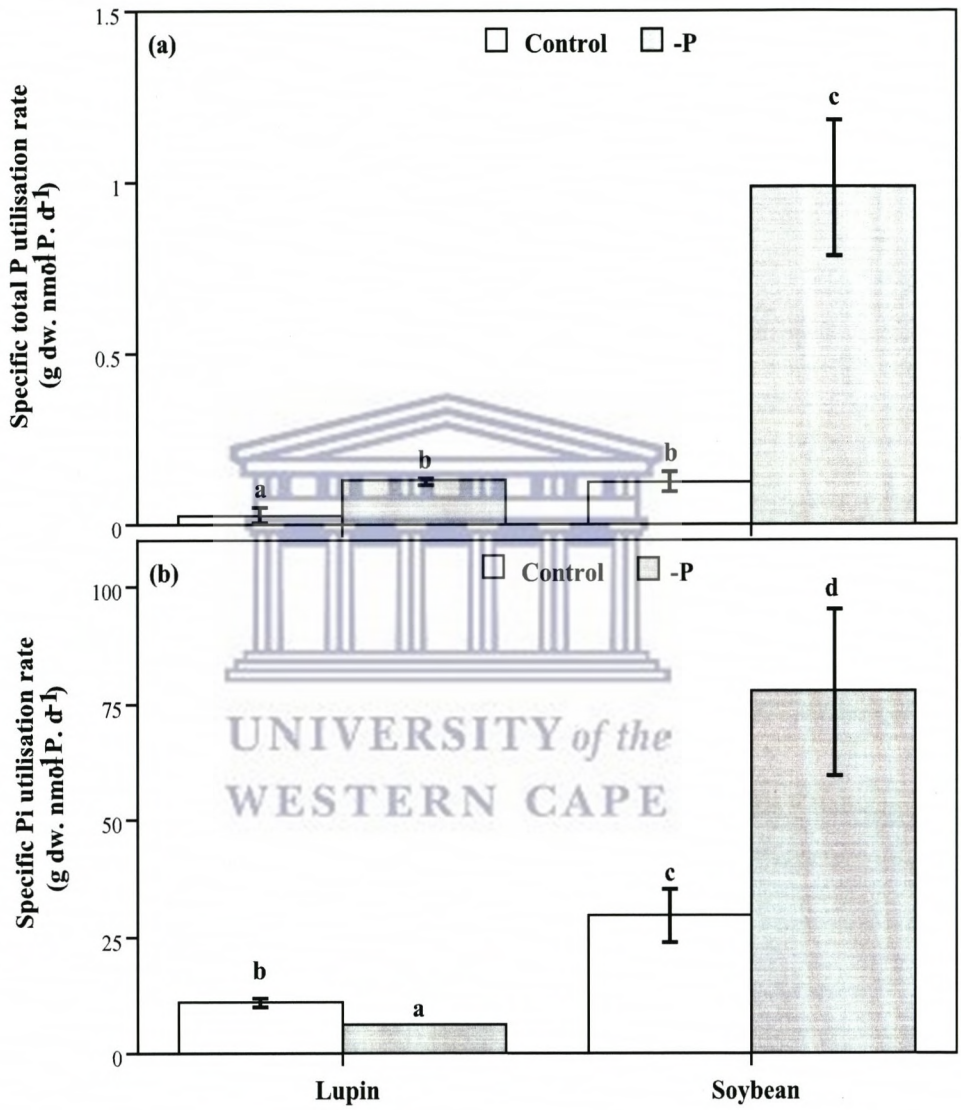

Figure 2. (a) The specific total phosphate (P) utilisation rate and **(b)** specific orthophosphate (P_i) utilisation rate of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Indeed, in spite of the decline in root growth under P starvation, the growths of nodule and shoot remained unaffected in both legumes (Table 1, *see page 96*).

3.4.2 *Nodule respiration and photosynthesis*

During P starvation, the nodule construction costs and growth respiration of lupins remained unchanged, in contrast with those of soybeans, which increased with P deficiency (Table 1). Lupins generally had higher photosynthetic rates than soybeans (Fig. 3). Although the photosynthetic rates in lupins remained unchanged during P deficiency, soybeans showed a decrease during P starvation (Fig. 3).

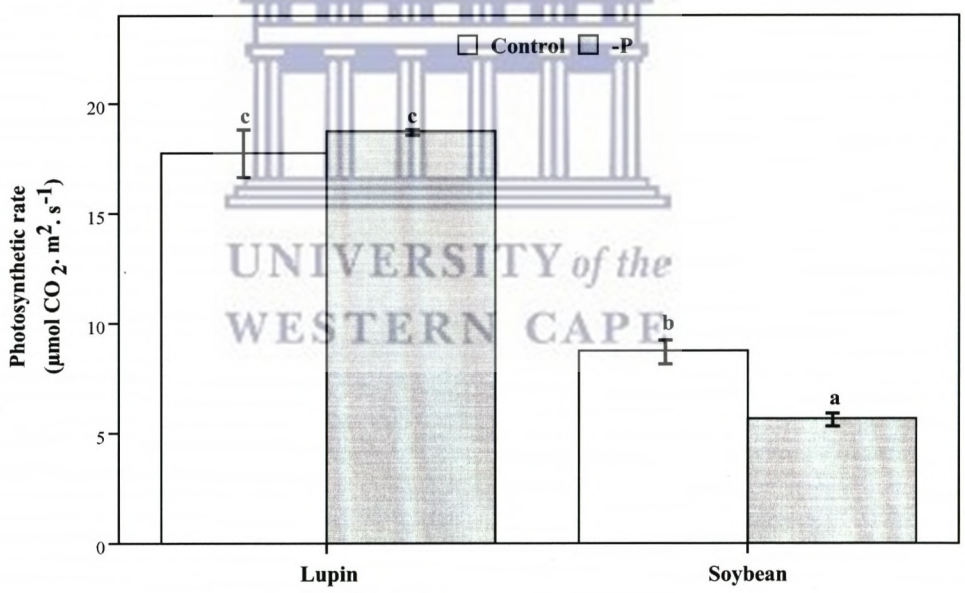

Figure 3. The photosynthetic rates of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Table 1. Biomass and nutrients of lupins (*Lupinus angustifolius*) and soybeans (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 in the root zone. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Biomass	Lupin				Soybean			
	Control		-P		Control		-P	
Plant growth								
Plant DW (g. plant^{-1})	0.90	**c**	0.77	**b**	0.75	**b**	0.62	**a**
Shoot DW (g. plant^{-1})	0.65	**b**	0.58	**b**	0.30	**a**	0.25	**a**
Root DW (g. plant^{-1})	0.18	**b**	0.12	**a**	0.40	**d**	0.33	**c**
Root:Shoot	0.28	**a**	0.26	**a**	1.60	**b**	1.21	**b**
Nodule growth								
Nodule DW (g. plant^{-1})	0.07	**b**	0.06	**ab**	0.04	**a**	0.04	**a**
Nodule number	171	**b**	169	**b**	12	**a**	15	**a**
Normalised nodule weight (g. g^{-1} shoot)	0.10	**a**	0.10	**a**	0.16	**b**	0.13	**ab**
Nodule C cost								
Nodule construction cost (mmol C. g^{-1} DW)	281.28	**c**	295.87	**c**	218.51	**a**	264.83	**b**
Nodule growth respiration (mol CO_2. g^{-1})	0.28	**a**	0.27	**a**	0.22	**a**	0.37	**b**
N concentrations (mmol N.g^{-1} DW)								
Root N: Shoot N	0.35	**a**	0.40	**a**	0.72	**c**	0.60	**b**

3.4.3 *Nitrogen metabolism*

During P starvation, lupins maintained the percentage nitrogen derived from atmosphere (%Ndfa), compared with soybeans, which showed a decline in the %Ndfa (Fig. 4).

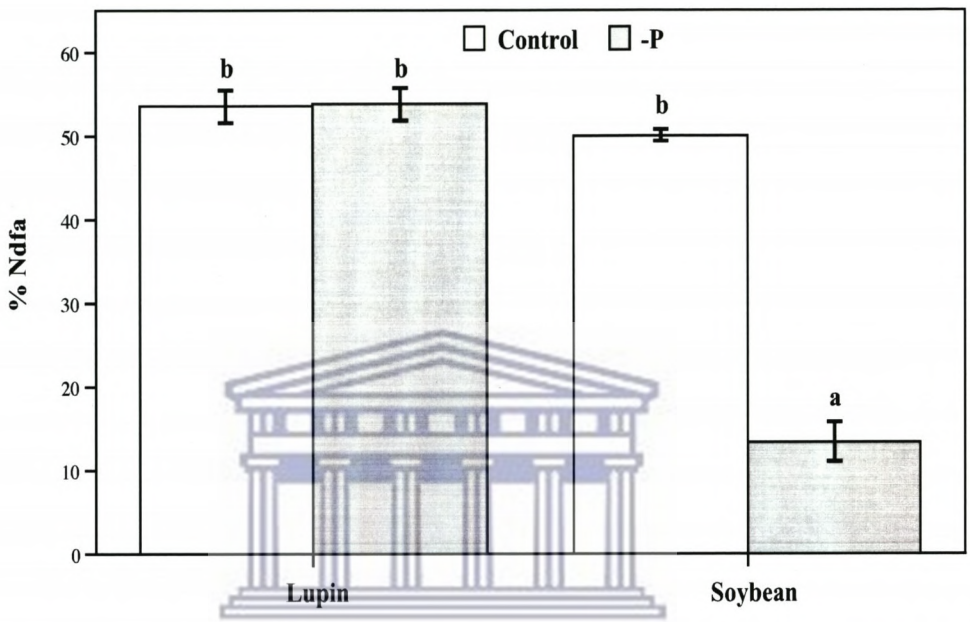

Figure 4. The percentage of nitrogen derived from atmosphere (%Ndfa) of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*.). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

The tissue N levels remained largely unchanged for lupins, except for the nodule N, which increased under LP. The N concentrations of the aerial parts of soybeans were unaffected, whereas the belowground portions, that is the root and nodule N concentrations had increased significantly (Fig. 5a,b,c).

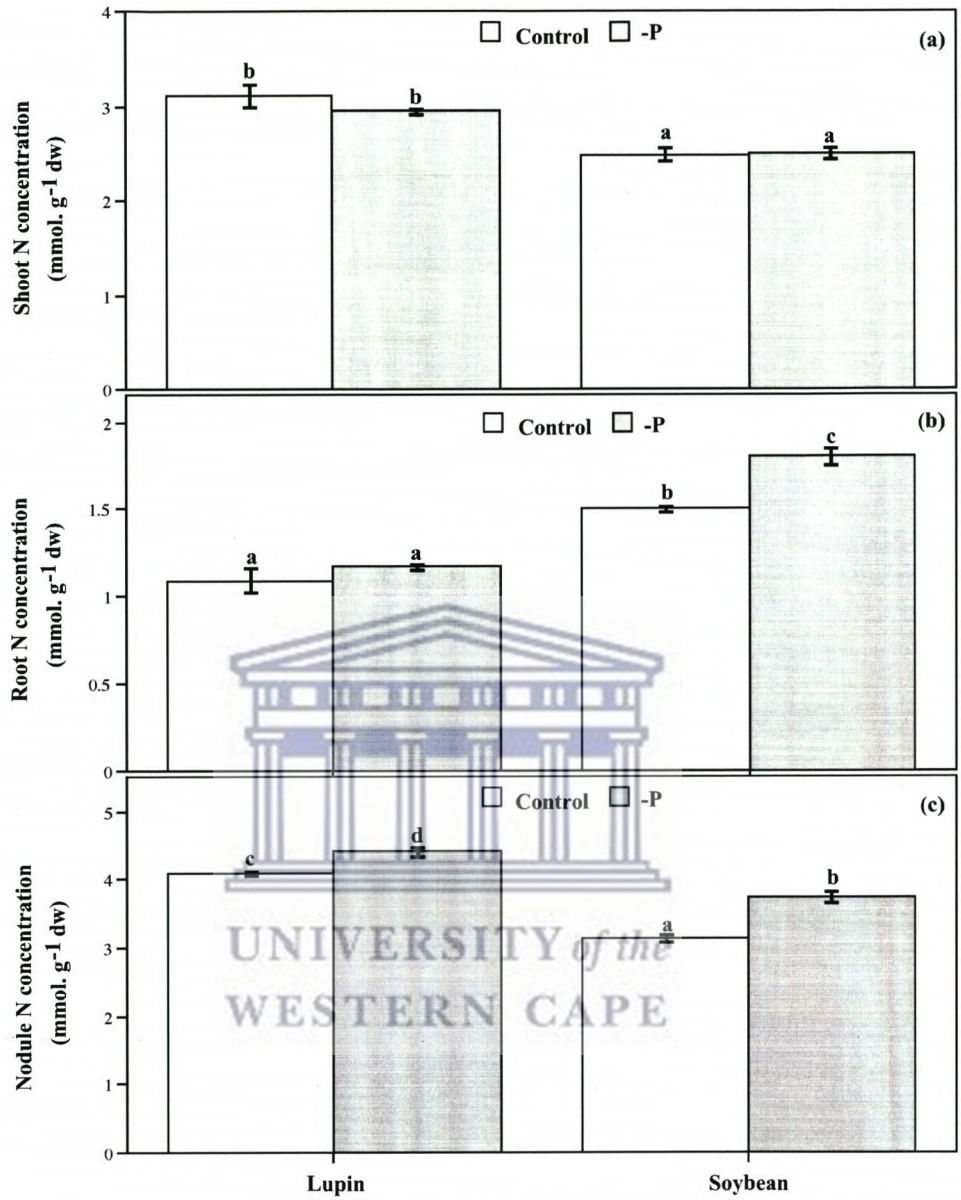

Figure 5. (a) Shoot N, **(b)** Root N, and **(c)** Nodule N concentrations of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Furthermore, the ratio of N between roots and shoots was unaffected by P starvation in lupins, but in soybeans the root N: shoot N ratio declined (Table 1).

The activities of the enzymes associated with organic acid acid synthesis, PEPc (Fig. 6a) and MDH (Fig. 6b) increased for both lupin and soybean nodules under P starvation. In addition, there were generally also increases in the amino acid synthesizing enzyme, GS (Fig. 6c), GOGAT (Fig. 6d) and AAT (Fig. 6e), with P starvation in both lupin (except for unchanged GOGAT activity) and soybean nodules. In spite of these increases in both organic acid and amino acid synthesizing enzymes, the ratio of the major organic acid synthesizing enzyme, MDH to the amino acid synthesizing enzymes, was enhanced in lupins, but not in soybeans (Fig. 7a,b,c).

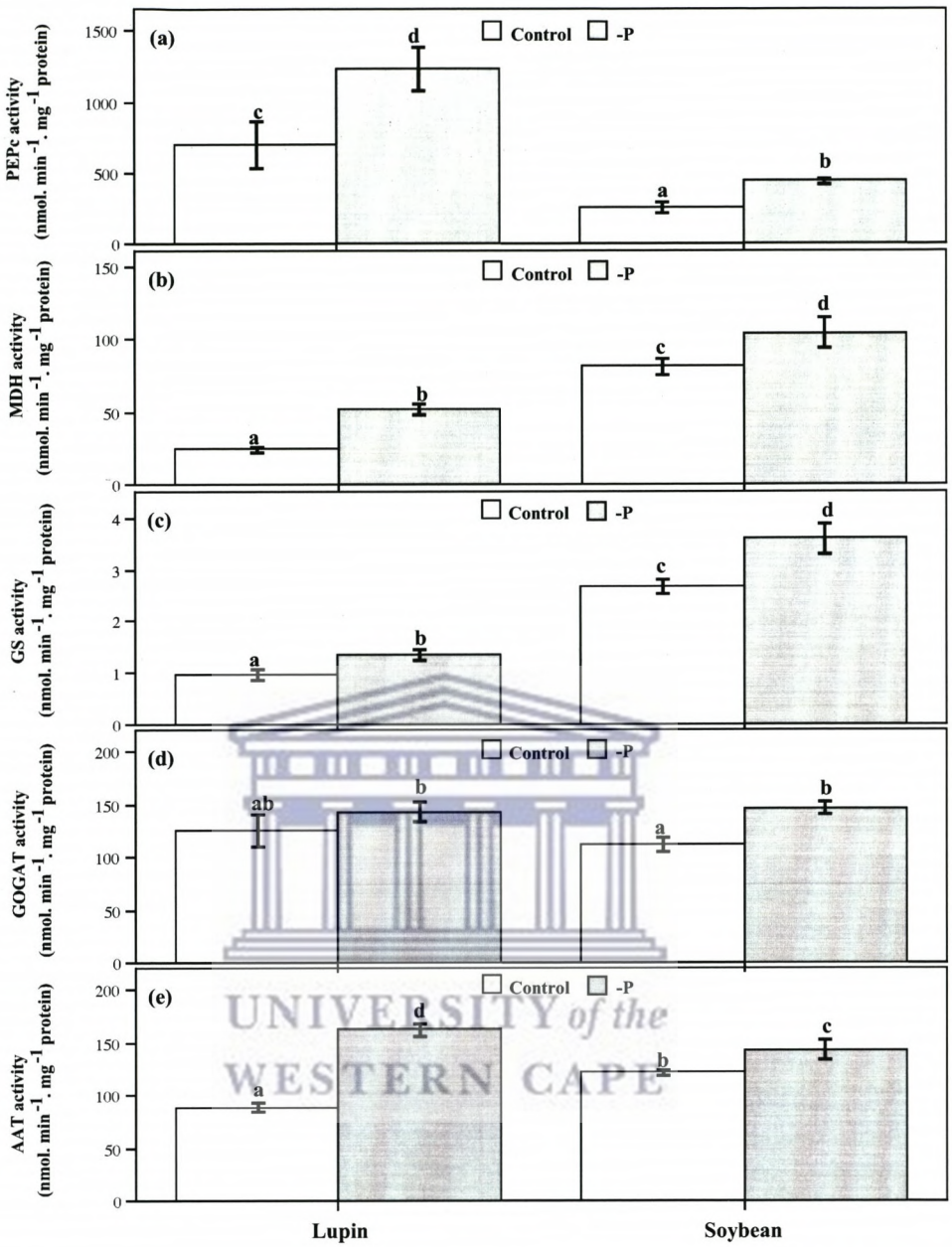

Figure 6. *In vitro* specific activities of **(a)** PEPc, **(b)** MDH, **(c)** GS, **(d)** GOGAT, and **(e)** AAT of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

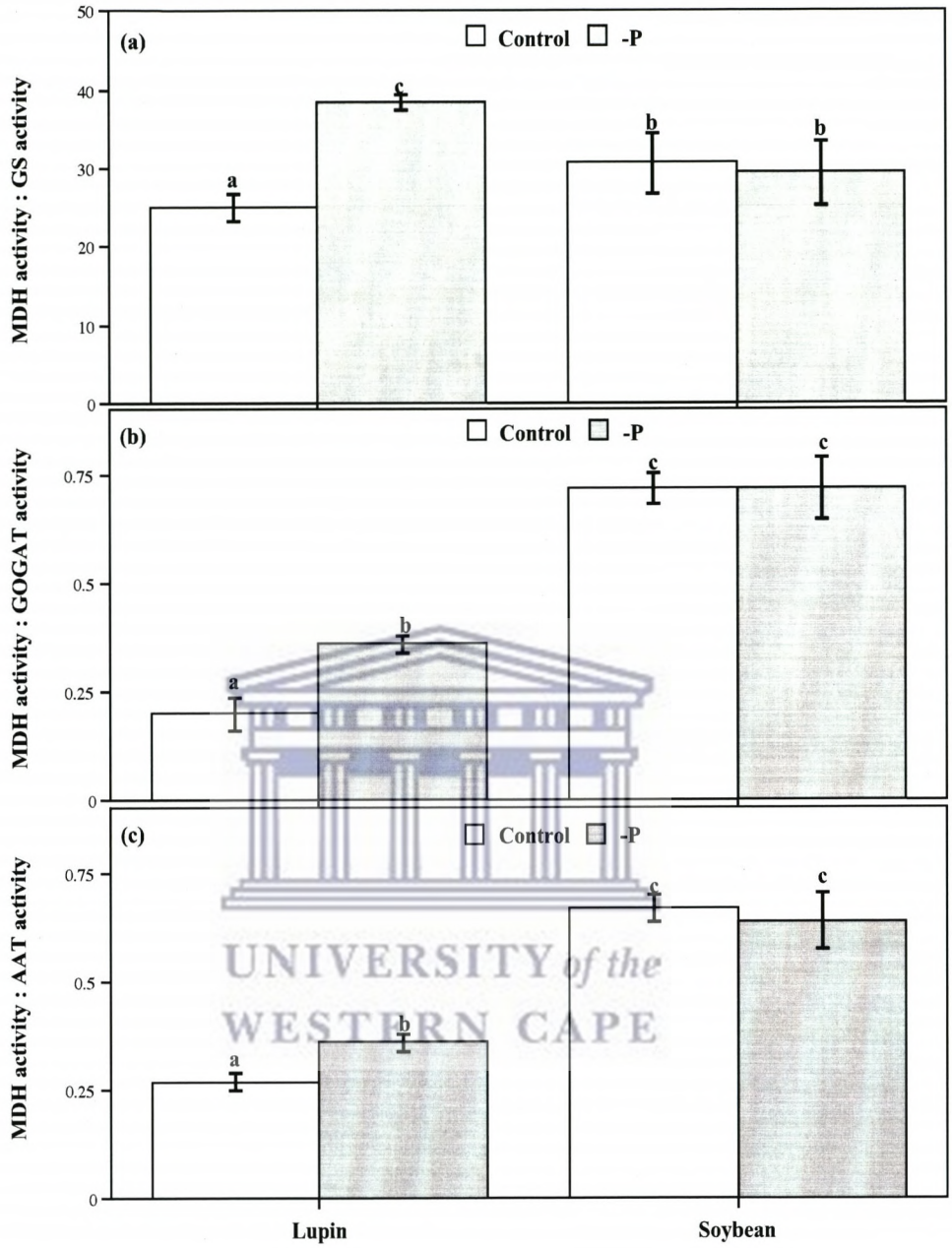

Figure 7. The relative ratios of *in vitro* specific activities of **(a)** MDH to GS, **(b)** MDH to GOGAT, and **(c)** MDH to AAT of lupin (*Lupinus angustifolius*) and soybean (*Glycine max.*). All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

These increased activities of amino acid synthesizing enzymes during nodular P deficiency, were associated with the enhanced concentrations of nodular amino acid levels in lupins (Fig. 8a), but not in soybeans (Fig. 8b). The maintenance of the nodule ureide concentrations in soybeans under P starvation appeared to have been at the expense of amino acid levels in soybean nodules (Fig. 8c). This accounted for the increase in the ratio of ureides: amino acids concentrations in soybean nodules during P deficiency (Fig. 8d).

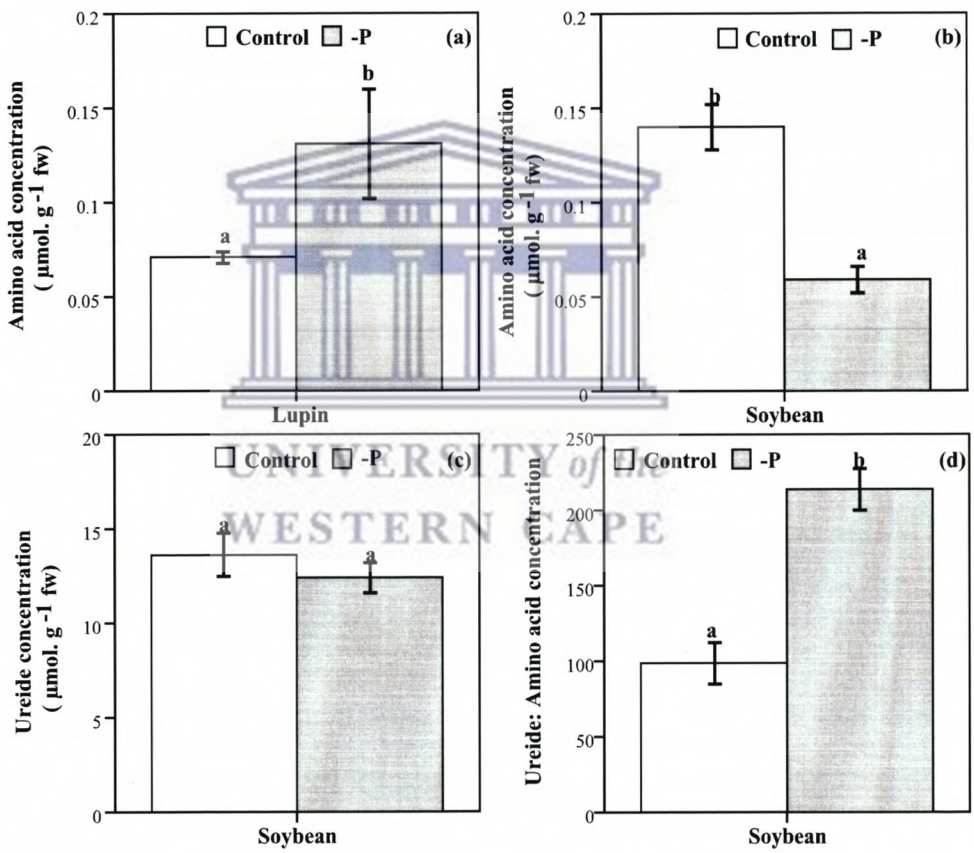

Figure 8. The nodular amino acid concentrations of **(a)** *Lupinus angustifolius* **(b)** *Glycine max*, **(c)** nodular ureide concentrations of *Glycine max*, and **(d)** nodular ureide:amino acid ratio of *Glycine max*. All plants were grown 3 weeks in hydroponic culture with N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

3.5 Discussion

During short-term P starvation, the lupins were physiologically better adapted for maintaining biological N_2 fixation (BNF) than the soybeans. The maintenance of metabolic P_i (Fig. 1b) and PP_i (Fig. 1c) concentrations in nodules of both legumes under P starvation, suggest that nodules function optimally at these P levels (Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002; Colebatch *et al.*, 2004; Le Roux *et al.*, 2006). Lupins showed a lower decline (35%) in nodular total P concentration (Fig. 1a) than the soybeans (64%) and this may be related to lupin nodules being more efficient at P recycling and having a greater sink strength during P starvation. Nodules are known to have a strong sink capacity for P incorporation during P starvation (Israel, 1987; Høgh-Jensen *et al.*, 2002).

Although soybeans had fewer nodules per plant than lupins (Table 1), the soybean nodules had a greater efficiency to utilise P for growth under limiting P conditions (Fig. 2a,b). This increase in P utilisation efficiency of soybean nodules may be compensating for the relatively, more pronounced decline in the total P concentration of soybean nodules, compared with lupin nodules (Fig. 1a). The enhanced nodule P utilization efficiency is considered to be a pivotal coping strategy during P starvation (Vadéz *et al.*, 1999). The alteration in biomass allocation is another strategy for coping with P starvation (Høgh-Jensen *et al.*, 2002; Le Roux *et al.* 2006).

The maintenance of nodule and shoot growth for both lupins and soybeans appeared to have been at the expense of root growth (Table 1). Such

adaptations in legumes have been reported previously, e.g. where growth of nodules was less affected than the growth of other plant organs such as roots after abrupt P withdrawal in white clover plants (Høgh-Jensen *et al.*, 2002).

The %N derived from atmosphere (%Ndfa) in lupins and soybeans may have been directly affected by a limitation in P supply during P starvation, or indirectly by means of other physiological responses. During P starvation, the unchanged %Ndfa in lupins compared with the decline in %Ndfa in soybean (Fig. 4), may be related to lupins having a lower decline in total nodular P, compared with soybean. However, the P starved lupins were also able to maintain nodule growth C costs, leaf photosynthetic rates and to increase the activities of enzymes related to the synthesis of organic acids, relative to amino acids.

In this regard, the C-costs of nodule growth (Table 1) in lupins did not compete with C costs of BNF (Fig. 4), as it seems to have been the case with soybeans. This has been found in common bean where C costs associated with growth were able to compete for C with BNF (Mortimer *et al.* 2008). Furthermore, the unchanged shoot and root N (Fig. 5a,b) concentrations and the constant root N: shoot N concentrations of lupins under P starvation (Table 1), may have underpinned the sustained photosynthetic rates (Fig. 3). By maintaining shoot N allocation relative to the roots N under P starvation, it is likely that the sustained photosynthetic rates in lupins were able to provide C to the nodules for BNF.

Although both lupins and soybean had an increase in nodular organic acid and amino acid synthesizing enzymes under P starvation (Fig. 6), only lupin nodules showed a shift of increasing the activities of the major organic acid synthesizing enzyme MDH, relative to amino acid synthesizing enzymes (Fig. 7) during P starvation. This shift in lupin nodules during P starvation may have provided more organic acids in the form of malate for the maintenance of bacterial metabolism and the synthesis of certain amino acids. This concurs with previous work (Le Roux *et al.* 2006) where a shift towards organic acid metabolism specifically malate, occurred in lupin nodules under P starvation. This may explain the increase in amino acid synthesis of lupin nodules, compared with soybean nodules (Fig. 8a,b).

In spite of the decline in %Ndfa in soybeans, their nodules accumulated more ureides relative to amino acids under P starvation. This was primarily achieved due to a decline in amino acid synthesis, rather than an increase in ureide production (Fig. 8b,c,d). The amino acid (lupins) and ureide (soybeans) exporting legumes represent an unusual metabolic complexity among plant systems (Streeter, 1991). In addition, it has also been suggested that different regulatory principles might have to be adopted for amino acid exporting and ureide exporting legumes (Schulze, 2004) and some of these may be of importance to P starvation adaptation. At this stage it is not possible to conclude whether one strategy for N assimilation is more cost effective than the other because this needs to be substantiated by more comprehensive comparative analyses (Smith & Atkins, 2002). Furthermore,

lupins and soybeans also differ in other means, such as being of temperate and tropical origin and having determinate and indeterminate types of nodules.

In conclusion, the ability of lupins to maintain BNF under short-term P starvation compared with soybean, may be related to a variety of differences between the species. The data from the current study show that lupins have more physiological alteration than soybean during P starvation, which include the changes in nitrogen and carbon metabolism and showing a relatively lower decline in total P concentration of nodules. Although the findings are based on two weeks of P starvation, they represent the adaptations to short-term P stress.

3.6 References

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CHAPTER 4

Detection of novel cytosolic malate dehydrogenases (cMDH) in *Lupinus angustifolius*

4.1 Abstract

Malate, synthesised via malate dehydrogenase (MDH, EC 1.1.1.37), accumulates in root nodules under P deficiency. Cytosolic- and nodule-enhanced MDH (cMDH and neMDH, respectively) form the bulk of MDH activity observed in root nodules. The objective of this study was to investigate the role of the cMDH and neMDH isoforms in malate supply under P deficiency. Nodulated lupins (*Lupinus angustifolius* var. Tanjil) were hydroponically grown at adequate P (+P) or low P (-P). Malate metabolism and its impact on physiological processes in nodules and how these are regulated on a genetic level were assessed. Total P concentration in nodules decreased under P deficiency, which coincided with an increase in total MDH activity and a subsequent increase in malate concentration. Here, no measurable neMDH presence could be detected by PCR. In contrast, gene-specific primers detected two 1 kb amplicons of cMDH, designated MDH1 (corresponding to +P) and MDH2 (corresponding to -P), respectively. Sequencing analyses of these cMDH amplicons showed them to be 96% identical on an amino acid level. There was a high degree of diversification between proteins detected in this study and other known MDH proteins, particularly those from other leguminous plants.

Keywords: MDH, isoforms, P deficiency, cytosolic, nodules

4.2 Introduction

There is widespread consensus that malate is the preferred carbon (C) source for bacteroids in almost all legume-*Rhizobium* symbioses studied to date (Rosendahl *et al.*, 1990; Driscoll & Finan, 1993; Schulze *et al.*, 2002). The high apparent flux of malate in root nodules is the result of active synthesis *via* malate dehydrogenase (MDH). This enzyme catalyses the reversible reduction of oxaloacetate (OAA) to malate. In root nodules, though, the kinetics of the reaction suggest that this reaction is favored in the forward direction towards malate synthesis (Miller *et al.*, 1998). This may further depend on the physiological state of the cell and the subcellular location of the enzyme within the cell (Schulze *et al.*, 2002). Nodule MDH has to maintain a fine balance between malate as respiratory source and optimal functioning of bacteroids and malate for conversion to oxaloacetic acid (OAA) and subsequent use in N assimilation.

Numerous earlier immunological and biochemical studies have detected novel forms of the MDH enzyme (Appels & Haaker, 1988; Ratajczak *et al.*, 1989), but it was only much later that several of these forms were isolated and characterized (Miller *et al.*, 1998). This is highly indicative of the importance of the enzyme in several metabolic pathways (Schulze *et al.*, 2002). Of special interest in root nodules is the isolation of an unique nodular enhanced MDH (neMDH) isoform, which was shown to account for up to 50% of the total MDH activity in root nodules of alfalfa but the sub-cellular location of which has yet to be determined (Miller *et al.*, 1998). Conversely, approximately 20% of the MDH activity is found in the cytosolic fraction of

root nodules (Miller *et al.*, 1998). The lower cMDH activity in root nodules may be as a result of the microaerobic conditions, which impede complete oxidative phosphorylation of more complex C substrates as one progresses to the inner core of the nodule (Kaur & Singh, 1999; Cabrerizo *et al.*, 2001).

Recently, it was proposed that C provision to bacteroids in root nodules is acquired through eloquent manipulation of the surrounding host cell cytoplasmic P_i concetration (Colebatch *et al.*, 2004). This model (Colebatch *et al.*, 2004) notes the tendency of bacteroids in root nodules actively to scavenge P_i, probably through the excretion of acid phosphatases (APs), from the surrounding plant cell fraction (Al-Niemi *et al.*, 1997; 1998). High levels of APs have been shown even under conditions of apparent P sufficiency, which suggests that bacteroids, in addition to functioning optimally at low O_2 levels, also operate within narrow limits of P_i concentration (Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998). Nevertheless, in so doing bacteroids induce the alternative pathway via the concerted action of the non-phosphorylating enzyme reactions phospho*enol*pyruvate carboxylase (PEPc) and MDH to metabolize PEP in the plant cell fraction (Colebatch *et al.*, 2004). Implicit to this model would be the extent to which nodules acquire such high malate levels. Secondly, the sink strength of nodules for P can also be inferred from this, which would explain why nodules are almost always unaffected by even very acute P deficiencies of host root. Nodules consistently maintain higher P levels than either root or shoots under P deficiency (Israel, 1993; Sa & Israel, 1991; Tang *et al.*, 2001). MDH activity of the crude extract has been

induced under P deficient conditions in lupins and soybean (Le Roux *et al.*, 2006, 2009; Schulze *et al.*, 2006).

The objective of this study was to assess if expression of novel MDH isoforms is induced under P stress conditions in *Lupinus angustifolius*. Subsequently, it was to ascertain how, if at all, these forms were to exist, how they would differ at the genetic level and then to explore any differences in expression patterns under P stressed conditions.

4.3 Materials and methods

4.3.1 *Plant material, growth conditions and harvesting*

Seeds of *Lupinus angustifolius* (L. cv. Tanjil) were germinated in vermiculite. At planting seeds were coated with a genus-specific rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria (Agricol, Western Cape, South Africa). Seedlings were transferred by 7 to 10 days after planting to an aerated nutrient solution (4 mM $CaCl_2$, 1.5 mM $MgSO_4$, 2 mM K_2SO_4, 2 mM NaH_2PO_4/Na_2HPO_4, 139 µM H_3BO_3, 21 µM $MnSO_4$, 2 µM $ZnSO_4$, 3 µM $CuSO_4$, 0.2 µM Na_2MoO_4, 89 µM FeEDTA and no N). The nutrient solution was changed once a week and the pH adjusted to 5.8 daily by addition of either 1 M HCl or 1 M NaOH. Plants were initially grown in a non-limiting P supply (2 mM P, designated +P) for approximately three weeks, after which half of the plants were switched to a limiting P supply (2 µM P, designated -P). Phosphorus starvation was induced for fourteen days at which time nodule tissues from the +P and -P plants were harvested. Upon excising nodules from adjacent roots, they were frozen in liquid nitrogen and stored at

-80°C until further analysis. Roots were oven-dried to a constant weight at 72°C for 48 h and the dry weights recorded. The dried root material was sent to a commercial laboratory (Bemlab, De Beers Road, Somerset West, South Africa), where it was milled and the P concentrations determined, using inductively coupled mass spectrometry (ICP-MS) with suitable standards. Total P concentration was expressed per root dry weight.

4.3.2 *Determination of $\delta^{15}N$*

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample using standards as defined by Farquhar *et al.*, (1989). Oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, California, USA). Samples of approximately 200 mg dried shoot sample were weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

The $\delta^{15}N$ natural abundance of the legumes was corrected for the seed N, according to Boddey *et al.*, (1995). The seed corrected $\delta^{15}N$ values were used to determine the percentage N derived from the atmosphere (%Ndfa) according to Shearer & Kohl (1986):

$$\%Ndfa = 100 \times [(\delta^{15}N_{\text{reference plant}} - \delta^{15}N_{\text{legume}})/(\delta^{15}N_{\text{reference plant}} - B)]$$

Where *B* is the $\delta^{15}N$ natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N free culture, according to Shearer & Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76‰.

4.3.3 *Malate concentration and MDH activity*

Malate concentrations and NADH-MDH activities, which equate to cytosolic MDH, were determined in accordance with methods from Le Roux *et al.*, (2006). Roots of plants with nodules still attached were used in feeding experiments. These attached root nodule pieces were submerged in the same nutrient solutions (15 ml) in which plants were grown, but modified to contain 1% sucrose, to compensate for the loss of photosynthate supply via the shoot. The system was aerated with ambient air (360 ppm CO_2) for the duration of the experiment. Root nodule pieces were equilibrated for 5 min, prior to feeding with 10 µl of ^{14}C label in the form of $NaHCO_3$. After 5 min had elapsed the experiment was stopped by discarding the incubation solution. The various segments were bagged, quenched in liquid N_2 and stored at -80°C. Components were homogenised with 80% (v/v) ethanol and separated into

soluble and insoluble components. The soluble component was subsequently separated into chloroform-soluble and water-soluble components, of which the latter was fractionated into amino acid, organic acid, and carbohydrate fractions, as described by Atkins & Canvin (1971).

High performance liquid chromatography (HPLC) separations were made isocratically on a 30 × 0.78 cm Bio-Rad Aminex Ion Exclusion HPX-87H organic acid column. HPLC analysis was carried out on an Alliance 2690 Separations Module equipped with a 996 Photodiode array detector (Waters). The mobile phase consisted of 30 mM H_2SO_4 at a flow rate of 0.6 $ml.min^{-1}$. Eluting peaks were detected by ultraviolet absorption at 247 nm and at a column temperature of 50°C. The system was calibrated with known standards (0-100 mM of Malate) for quantification and determination of retention time, and was co-chromatographed with the sample for identification. Data analysis was done using Millenium^{32} Chromatography software (Waters). In addition the organic acid fractions of interest were manually collected for the determination of radioactivity in the specific organic acid, i.e. malate. Radioactivity measurements were made on a liquid scintillation counter.

Malate dehydrogenase was assayed as described by Appels and Haaker (1988). Blank reactions were set up beside actual MDH determination assays and comprised of a reaction medium without OAA. The reaction was initiated by adding 30 µl crude extract to the reaction mixture in a total volume of

250 µl. Initial reaction rates have been shown to be proportional to the concentration of enzyme under the conditions used. The activity of MDH activity was expressed per fresh mass of nodule.

4.3.4 *RNA isolation*

Nodules were homogenized in liquid nitrogen using pre-chilled mortars and pestles and total RNA extracted with 1 ml of Trizol reagent per 100 mg nodule tissue. The homogenate was incubated at room temperature for three to five minutes. Thereafter, 200 µl of chloroform was added to the homogenate, which was inverted carefully to emulsify and incubated for a further two to three minutes at room temperature. This was centrifuged at ≥16 000 g for 20 min and the resulting supernatant was carefully transferred to a new tube to which equal volumes of 75% cold ethanol were then added. The homogenate was mixed with a pipette and subsequently transferred to an RNeasy spin column (Qiagen, Hilden, Germany), attached to a collection tube. The RNA was purified in accordance with the manufacturer's instructions and afterwards resuspended in 30 µl water. The eluate was aliquoted in 4 µl volumes and stored at -80°C.

4.3.5 *Semi-quantitative reverse transcription (RT)-PCR*

Total RNA extracted was reverse transcribed using an ImprompII Reverse Transcription (RT) system provided by Promega (Madison, WI, USA). Degenerate primers were designed to hybridize with conserved regions of cMDH using multiple alignments (CLUSTAL X, version 1.81) of various nucleotide sequences of other legumes identified from the genetic database,

National Center for Biotechnology Information (NCBI; Altschul *et al.*, 1997). A 36-mer gene-specific forward primer (*cMDHFw*) (ATAC**GCGATCGCC**ATGGCCAAAGACCCAGTTCGTGT) and a 33-mer gene-specific reverse primer (*cMDHR*ev) for cMDH (GTGC**GTTTAAAC**CTAAGAGAGGCAAGAGTGAGC) were compiled. Primers were designed to include sites for two restriction enzymes (Sgf I at the 5′-end and Pme I at the 3′-end; *in bold and underlined*). Attempts to detect neMDH with various gene-specific primer sets in the same extract were unsuccessful and subsequently abandoned.

Reverse transcription-PCR for the synthesis of cDNA included 1 µl of the reverse primer (10 µM) to which 4 µl of RNA was added. Magnesium concentration was optimized for +P treatment at 4 mM (3.2 µl $MgCl_2$ in a final volume of 20 µl) and for -P treatment at 8 mM (6.4 µl $MgCl_2$ in a final volume of 20 µl). The water was adjusted accordingly, that is 5.3 µl and 2.1 µl of nuclease-free H_2O for +P and –P, respectively. Each reaction contained 5 µl RNA, 4.0 µl ImprompII buffer, 1.0 µl dNTPs, 0.5 µl RNasin and 1.0 µl RT. Reactions were done as follow: reverse transcription at 25ºC for 5 min, 42ºC for 60 min and 70ºC for 15 min.

The cDNA was subsequently amplified in a polymerase chain reaction (PCR) performed with Promega's Flexi GoTaq DNA Polymerase in the buffer supplied and a final volume of 50 µl. The degenerate primers (5 µl each) were used at a final concentration of 10 µM in PCR reactions. Nucleotides (dNTPs, 1 µl) were added to final concentrations of 0.2 mM. The PCR reaction was

started by adding GoTaq DNA polymerase enzyme in the assay at a final concentration of 1.25 U. The PCR program was 94°C for 2 min, 1 cycle, 95°C for 20 sec, 65°C for 30 sec, 72°C for 90 sec and 35 cycles and a final extension of 72°C for 5 min, 1 cycle. Products from the RT-PCR were separated by 0.8% (w/v) agarose gel electrophoresis.

4.3.6 *DNA sequencing and cloning*

Sequencing of the purified PCR product (Wizard, Promega, Madison, WI, USA) was conducted using the custom designed primers. The cMDH cDNA of both +P and –P inserts from positive clones were cloned into *pGEM-T* Easy vector (Promega Corporation, Madison, WI, USA) and transformed into JM109 *Escherichia coli* cells. Prior to plating transformed cells, LB agar plates were supplemented with 50 µl of a 100 mg/ml Ampicillin (AMP^+) solution, 40 µl of a 2% X-Gal solution and 7 µl of a 20% IPTG solution. All the reagents were freshly made on the day. Transformed cells were plated and left overnight (16-24 h) at 37°C. A single white colony was picked from LB agar plates and inoculated into 5 ml LB broth, containing 1 µl/1 ml AMP^+, overnight at 37°C. Plasmid DNA was purified using the PureYield plasmid miniprep system from Promega (Promega Corporation, Madison, WI, USA). About 3 ml of bacterial cells were pelleted for 30 sec at maximum speed. The supernatant was discarded and the pellet resuspended in 600 µl of TE (Tris-EDTA) buffer. One hundred microlitres of cell lysis buffer was added to the resuspended pellet and the tube was inverted six times to mix its contents. At this stage a pre-chilled neutralization solution was added to the mixture and again inverted to mix thoroughly. Cell debris was subsequently pelleted at

maximum speed and approximately 900 µl of the supernatant resulting from this was carefully transferred to a PureYield minicolumn to which a collection tube was attached and then centrifuged. This was followed by a wash step whereby 200 µl of endotoxin removal wash was added to the minicolumn, followed by a second wash with 400 µl column wash solution. Finally, the DNA was eluted from the column with 30 µl of elution buffer and stored at -20°C for further analysis.

After digestion of 10 µl of DNA with 1 µl of *Eco*R1, the resulting fragments were separated by agarose gel electrophoresis. At least two independent clones, for each treatment were identified. DNA sequencing of all isolated clones was done at Inqababiotech (Pretoria, RSA) and the sequences of the full-length clones were corroborated with those sequences previously obtained from partial clones [Central Analytical Facility (CAF), Stellenbosch, RSA]. The nucleotide sequences of the complete open reading frames (ORFs) were deposited in GENBANK, for *Lang*MDH1 (corresponding to +P) and *Lang*MDH2 (corresponding to -P) (*accession numbers were not available at the time this manuscript went to print*).

4.3.7 *Bioinformatics and statistical analysis*

Similarity searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment of *Lang*MDH1 and *Lang*MDH2 was performed with CLUSTAL X (version 1.81) (Thompson *et al.*, 1994) using default parameters and the Blosum matrix. GENEDOC software (Nicholas & Nicholas, 1997) was used for homology shading of the

aligned nucleotide and/or protein sequences. A phylogenetic tree of cMDHs was constructed using the Neighbour-joining algorithm of CLUSTAL W. The resulting phylogenetic tree was visualized in MEGA 4.1.

4.4 Results

4.4.1 *P concentrations and ^{14}C incorporation*

Phosphorus concentration in nodules of P deficient plants (LP) declined by about 20% compared with P sufficient (HP) controls over the 14 d period (Fig. 1a). In addition, nodules of plants subjected to LP incorporated significantly more $^{14}CO_2$, which constituted a two-fold increase under LP conditions compared with HP controls (Table 1). This increase was mainly associated with a markedly higher incorporation into the soluble fraction of LP nodules. Most of the incorporated ^{14}C was recovered in organic acids, which was higher at LP supply than at HP (Table 1). This coincided with increases in nodular malate levels of approximately 60% under the same P deficient conditions (Fig. 1b). Phosphorus deficient conditions also lead to a concomitant 6-fold increase in MDH activity (Fig. 1c).

4.4.2 *Nitrogen fixation*

The nitrogen fixation capacity, expressed as %Ndfa, of root nodules as assessed by the ^{15}N enrichment procedure was maintained at about 70% for both +P and -P treatments as imposed under the short-term (14 d) P deficient experimental conditions in this study (Table 1). This correlated positively with the unchanged $\delta^{15}N$ values for plants grown at either HP or LP (Table 1).

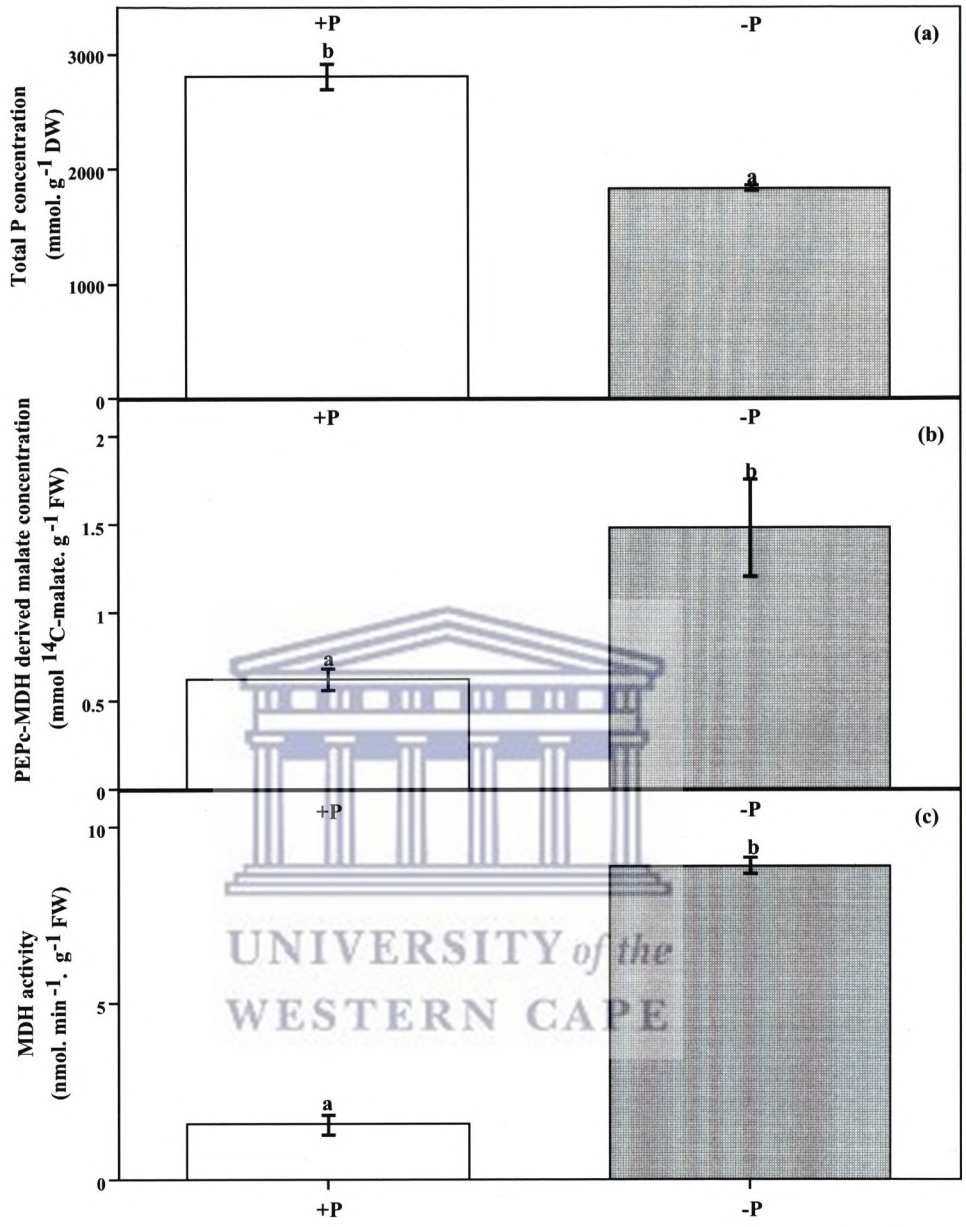

Figure 1: (a) Total phosphorus concentration (mmol. g^{-1} DW); (b) PEPc-derived malate concentration (mmol ^{14}C. g^{-1} FW) and (c) MDH activity (nmol. min^{-1}. g^{-1} FW) in nodules of *Lupinus angustifolius* either sufficiently supplied with P (+P) or deficient in P (-P). Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Table 1. Percentage nitrogen derived from atmosphere (%Ndfa), and the incorporation and assimilation of dissolved $^{14}CO_2$ in nodules attached to a piece of root from P sufficient and P deficient *Lupinus angustifolius* (cv. Tanjil) plants after 5 min of $^{14}CO_2$ labelling.

	Treatments	
Pramaters	**+P**	**-P**
Shoot parameters		
$\delta^{15}N:^{14}N$ ratio	-1.36 ± 0.12a	-1.75 ± 0.21a
% Ndfa	73.09 ± 1.91a	76.35 ± 3.38a
^{14}C concentration (nmol. g^{-1} fw)		
Soluble fraction	2.48 ± 0.14a	7.54 ± 2.19b
Insoluble fraction	3.41 ± 0.31a	2.39 ± 0.97a
Lipid fraction	0.05 ± 0.01a	0.22 ± 0.03a
Total incorporation	5.94 ± 0.49a	10.15 ± 1.43b
^{14}C fractions of total incorporation (%)		
Organic acids	50.52 ± 1.77a	60.09 ± 3.89b
Amino acids	27.64 ± 1.05a	22.96 ± 1.86a
Neutral fraction	21.85 ± 0.87a	16.95 ± 2.09a

4.4.3 *Identification of two independent MDH cDNAs*

After RT-PCR with degenerate primers, two independent amplicons of approximately 1.0 kb in size of cMDH transcripts (Fig. 2), one each from +P (*Lang*MDH1) and -P (*Lang*MDH2) root nodules, were cloned into the vector pGEM T-Easy. Sequencing of the identified amplicons showed that the MDH cDNAs of both transcripts were more similar to those reported previously for *Lupinus albus* (GENBANK accession number AF459645.1). Comparative analyses of the deduced nucleotide (nt) sequences from start (ATG) to stop (TGA) of the cMDHs isolated within this study demonstrated that both are highly homologous to *L. albus* cMDH isolated from P-deprived tissue (Uhde-Stone *et al.*, 2003).

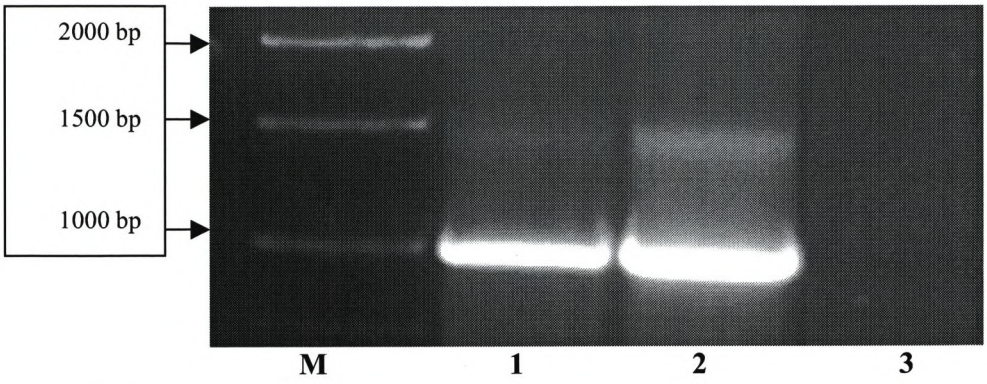

Figure 2: Agarose gel electrophoresis of PCR-amplified cDNA inserts performed on total RNA extracted from root nodules. A 1 kb ladder was used as a molecular size marker (**M**) with the sizes indicated in base pairs. The approximate sizes of the hybridization bands are indicated on the left. Lane 1 represents the signal from the isolates extracted from root nodules that served as controls, that is +P, whilst lane 2 shows a hybridization signal as depicted under conditions of P deficiency (-P). Lane 3 is a negative control in which nuclease-free water was substituted as a template.

There was a high degree of homology (92%) between the two amplicons based on nucleotide sequence. Single nucleotide polymorphism analysis showed that single nucleotide differences occurred throughout the genes of the two isolates identified within this study (Table 2). The deduced nt-sequence of the *Lang*MDH1 in this study was 88% similar to previously reported sequences, *La*MDH1 and *La*MDH2 of *L. albus*. Similarly, for *Lang*MDH2, nucleotide sequence similarity showed about 94% homology to *La*MDH1 and 91% to *La*MDH2. Both these latter isoforms were isolated from proteoid roots of plants subjected to P deficiency.

Table 2. Single nucleotide polymorphisms (SNPs) between *Lang*MDH1 and *Lang*MDH2 isolated from *Lupinus angustifolius*. [a]Nucleotide position as from the start codon.

SNP position[a]	*Lang*MDH1				*Lang*MDH2			
	A	C	G	T	A	C	G	T
45			+		+			
54	+						+	
69		+					+	
87	+						+	
96	+							+
97				+			+	
111			+			+		
192		+						+
224		+						+
243			+					+
259	+							+
330				+		+		
344		+				+		
396				+		+		
444		+						+
447				+	+			
480				+				+
498				+		+		
571				+	+			
587				+		+		
602				+		+		
609		+						+
615	+							+
666			+		+			
687	+						+	
753				+		+		
765				+		+		
766	+					+		
771				+		+		
804		+			+			
807			+		+			
816				+	+			
828	+							+
831				+	+			
835	+						+	
874	+						+	
877-878	+	+				+	+	
885			+					+
910			+			+		
936	+						+	
952			+			+		
957	+						+	
969			+		+			
972	+						+	
978			+		+			
982				+		+		

Furthermore, sequencing data showed that the *Lang*MDH1 amplicon with an open reading frame (ORF) of 999 nucleotides (nt) encoded for a predicted protein of 332 amino acids (aa) with a predicted molecular weight of approximately 35.4 kDa (Fig. 3a). The 999-nt ORF of *Lang*MDH2 encoded for a similar sized protein of 332 amino acids, with a predicted molecular weight of 35.4 kDa (Fig. 3b). Protein sequence alignment of the two isolates identified within this study, *Lang*MDH1 and *Lang*MDH2, revealed 96% similarity between them. The *Lang*MDH1 amplicon shared a higher degree of similarity (≈ 98%) based on aa-sequence to *La*MDH1 of *L. albus*, whereas the degree of similarity to *La*MDH2 was 96%. The *Lang*MDH2 amplicon shared equal similarities of 95% with both *La*MDH1 and *La*MDH2 (Table 3). The iso-electric points for *Lang*MDH1 and *Lang*MDH2 proteins were established at 5.64 and 5.99, respectively.

(a) *Lang*MDH1

```
accgctctcaggagatcttccgtaaggtcgacctgcaggcggccgcgaattcactagtgattc
gcgtcgccATG GCC AAA GAC CCA GTT CGT GTT CTT GTC ACT GGT GCT GCA
          M   A   K   D   P   V   R   V   L   V   T   G   A   A
GGG CAA ATC GGA TAT GCT CTT GTC CCC ATG ATT GCT AGG GGA GTA ATG
 G   Q   I   G   Y   A   L   V   P   M   I   A   R   G   V   M
CTG GGA TCT GAC CAG CCC GTG ATC CTC CAC TTG CTT GAC ATT CCA CCT
 L   G   S   D   Q   P   V   I   L   H   L   L   D   I   P   P
GCA GCA GAG TCA CTG AAT GGT GTT AAA ATG GAG TTG GTG GAT GCT GCA
 A   A   E   S   L   N   G   V   K   M   E   L   V   D   A   A
TTC CCC CTT CTT AAA GGT GTT GTT GCT ACA ACT GAT GCG GTT GAG GCA
 F   P   L   L   K   G   V   V   A   T   T   D   A   V   E   A
TGC ACT GGG GTC AAT ATT GCT GTA ATG GTT GGT GGA TTC CCT AGA AAA
 C   T   G   V   N   I   A   V   M   V   G   G   F   P   R   K
GAA GGT ATG GAG AGG AAA GAT GTG ATG TCT AAA AAT GTC TCT ATT TAT
 E   G   M   E   R   K   D   V   M   S   K   N   V   S   I   Y
AAG TCC CAG GCT TCT GCT CTT GAA AAG CAT GCT GCT GCT AAC TGC AAG
 K   S   Q   A   S   A   L   E   K   H   A   A   A   N   C   K
GTT CTG GTT GTT GCT AAT CCA GCA AAC ACC AAT GCA TTG ATC CTG AAG
 V   L   V   V   A   N   P   A   N   T   N   A   L   I   L   K
GAA TTT GCT CCA TCT ATC CCT GAG AAA AAC ATT TCT TGT TTG ACT AGA
 E   F   A   P   S   I   P   E   K   N   I   S   C   L   T   R
CTG GAT CAT AAC AGG GCA CTG GGT CAA ATT TCT GAA AGA CTG AAC GTT
 L   D   H   N   R   A   L   G   Q   I   S   E   R   L   N   V
CAA GTT TCT GAT GTA AAA AAT GTT ATA ATC TGG GGT AAC CAT TCA TCA
 Q   V   S   D   V   K   N   V   I   I   W   G   N   H   S   S
TCT CAG TAT CCT GAT GTC AAC CAT GCA ACT GTT ACA ACC CCA GCA GGG
 S   Q   Y   P   D   V   N   H   A   T   V   T   T   P   A   G
GAA AAG CCT GTC CGT GAA CTT GTT GCT GAT GAT GCC TGG TTG AAT TCG
 E   K   P   V   R   E   L   V   A   D   D   A   W   L   N   S
GAA TTC ATA GCT ACT GTC CAA CAA CGT GGT GCT GCA ATT ATT AAA GCT
 E   F   I   A   T   V   Q   Q   R   G   A   A   I   I   K   A
AGA AAG CTT TCA AGT GCA CTA TCT GCT GCT AGT GCT GCT TGT GAC CAC
 R   K   L   S   S   A   L   S   A   A   S   A   A   C   D   H
ATT AGA GAT TGG GTT CTT GGA ACT CCT GAG GGC ACC TGG GTC TCG ATG
 I   R   D   W   V   L   G   T   P   E   G   T   W   V   S   M
GGA GTT TAT TCT GAT GGA TCT TAC AAT GTA CCA GCT GGA CTG ATC TAT
 G   V   Y   S   D   G   S   Y   N   V   P   A   G   L   I   Y
TCA TTC CCT GTC ACC ACT CAG AAT GGG GAA TGG AAA ATA GTT CAA GGA
 S   F   P   V   T   T   Q   N   G   E   W   K   I   V   Q   G
CTT GCA ATT GAT GAG TTC TCA AGG AAA AAA TTG GAC TTG ACA GCA GAA
 L   A   I   D   E   F   S   R   K   K   L   D   L   T   A   E
GAA CTT TCT GAG GAG AAA GCT TTG GCT TAC TCA TGC CTC TCT TAG tac
 E   L   S   E   E   K   A   L   A   Y   S   C   L   S   *
gacgatggmatscscargcasccatgtcgtgctgyagctagctgaygctgsagcmaawtccgc
stattgrccggtgtctcmgtawtsaatktkcycg
```

Figure 3 (a) Nucleotide sequence of the cDNA encoding the *Lupinus angustifolius* cytosolic MDH (denoted *Lang*MDH1) of +P treatments. The catalytic active site is underlined (_). The deduced amino acid sequence is denoted below the nucleotide sequence in the standard one-letter code. The translation stop codon is designated with an asterik (*).

(b) *Lang*MDH2

```
aaccmagctccggccgccatggcggccgcgggaattcgattacgatcgcc ATG GCC AAA
                                                     M   A   K
GAC CCA GTT CGT GTT CTT GTC ACT GGT GCT GCA GGA CAA ATC GGG TAT
 D   P   V   R   V   L   V   T   G   A   A   G   Q   I   G   Y
GCT CTT GTC CCG ATG ATT GCT AGG GGA GTG ATG CTG GGT GCT GAC CAG
 A   L   V   P   M   I   A   R   G   V   M   L   G   A   D   Q
CCC GTC ATC CTC CAC TTG CTT GAC ATT CCA CCT GCA GCA GAG TCA CTG
 P   V   I   L   H   L   L   D   I   P   P   A   A   E   S   L
AAT GGT GTT AAA ATG GAG TTG GTG GAT GCT GCA TTC CCT CTT CTT AAA
 N   G   V   K   M   E   L   V   D   A   A   F   P   L   L   K
GGT GTT GTT GCT ACA ACT GAT GTG GTT GAG GCA TGC ACT GGT GTC AAT
 G   V   V   A   T   T   D   V   V   E   A   C   T   G   V   N
ATT GCT GTA TTG GTT GGT GGA TTC CCT AGA AAA GAA GGT ATG GAG AGG
 I   A   V   L   V   G   G   F   P   R   K   E   G   M   E   R
AAA GAT GTG ATG TCT AAA AAT GTC TCT ATT TAC AAG TCC CAG GCT TCT
 K   D   V   M   S   K   N   V   S   I   Y   K   S   Q   A   S
GCT CTT GAA AAG CAT GCT GCT GCT AAC TGC AAG GTT CTG GTT GTT GCT
 A   L   E   K   H   A   A   A   N   C   K   V   L   V   V   A
AAC CCA GCA AAC ACC AAT GCA TTG ATC CTG AAG GAA TTT GCT CCA TCT
 N   P   A   N   T   N   A   L   I   L   K   E   F   A   P   S
ATT CCA GAG AAA AAC ATT TCT TGT TTG ACT AGA CTG GAT CAT AAC AGG
 I   P   E   K   N   I   S   C   L   T   R   L   D   H   N   R
GCA CTG GGC CAA ATT TCT GAA AGA CTG AAC GTT CAA GTT TCT GAT GTA
 A   L   G   Q   I   S   E   R   L   N   V   Q   V   S   D   V
AAA AAT GTT ATA ATC TGG GGT AAC CAT TCA TCA ACT CAG TAT CCT GAT
 K   N   V   I   I   W   G   N   H   S   S   T   Q   Y   P   D
GCC AAC CAT GCA ACT GCT ACA ACT CCA GCT GGG GAA AAG CCT GTC CGT
 A   N   H   A   T   A   T   T   P   A   G   E   K   P   V   R
GAA CTT GTT GCT GAT GAT GCC TGG TTG AAT TCA GAA TTC ATA GCT ACT
 E   L   V   A   D   D   A   W   L   N   S   E   F   I   A   T
GTC CAG CAA CGT GGT GCT GCA ATT ATT AAA GCT AGA AAG CTT TCA AGT
 V   Q   Q   R   G   A   A   I   I   K   A   R   K   L   S   S
GCA CTA TCT GCT GCT AGT GCT GCC TGT GAC CAC ATC CGA GAC TGG GTT
 A   L   S   A   A   S   A   A   C   D   H   I   R   D   W   V
CTT GGA ACT CCT GAG GGC ACC TGG GTA TCA ATG GGA GTA TAT TCT GAT
 L   G   T   P   E   G   T   W   V   S   M   G   V   Y   S   D
GGT TCA TAC GAT GTA CCA GCT GGA CTG ATC TAT TCA TTC CCT GTC ACC
 G   S   Y   D   V   P   A   G   L   I   Y   S   F   P   V   T
GCT GCC AAT GGT GAA TGG AAA ATA GTT CAA GGA CTT CCA ATT GAT GAG
 A   A   N   G   E   W   K   I   V   Q   G   L   P   I   D   E
TTC TCA AGG AAA AAG TTG GAC TTG ACA GCA CAA GAG CTT TCT GAG GAA
 F   S   R   K   K   L   D   L   T   A   Q   E   L   S   E   E
AAG GCT TTA GCT CAC TCT TGC CTC TCT TAG tttaagcacattcactagtgaaa
 K   A   L   A   H   S   C   L   S   *
ttcgccgccgcctgcaggtcgaccatatggcagagcccccagccgcgtgcatgcatagcttga
gtattctatagtgtcacctaaatwagcttgwcgta
```

Figure 3 (b) Nucleotide sequence of the cDNA encoding the *Lupinus angustifolius* cytosolic MDH (denoted *Lang*MDH2) of -P treatments. The catalytic active site is underlined (_). The deduced amino acid sequence is denoted below the nucleotide sequence in the standard one-letter code. The translation stop codon is designated with an asterik (*).

4.4.4 *Homology to other known MDH* proteins

Both putative MDH proteins, *Lang*MDH1 and *Lang*MDH2, displayed over 90% homology to MDH proteins from other legumes, e.g. *Medicago sativa*, *M. truncatula*, *Glycine max* and *Cicer arietinum* (Table 3 & Fig. 4). The least similar of the legumes was *Sesbania rostrata*, which is known to form a mutualistic symbiosis in both its stem and root system with *Azorhizobium caulinodans*. It only shared sequence homology of 36% with both *Lang*MDH1 and *Lang*MDH2. Similarly, compared with other non-leguminous plants, *Lang*MDH1 and *Lang*MDH2 displayed the lowest similarity to *Arabidopsis thaliana* of about 70% and 71%, respectively (Table 3).

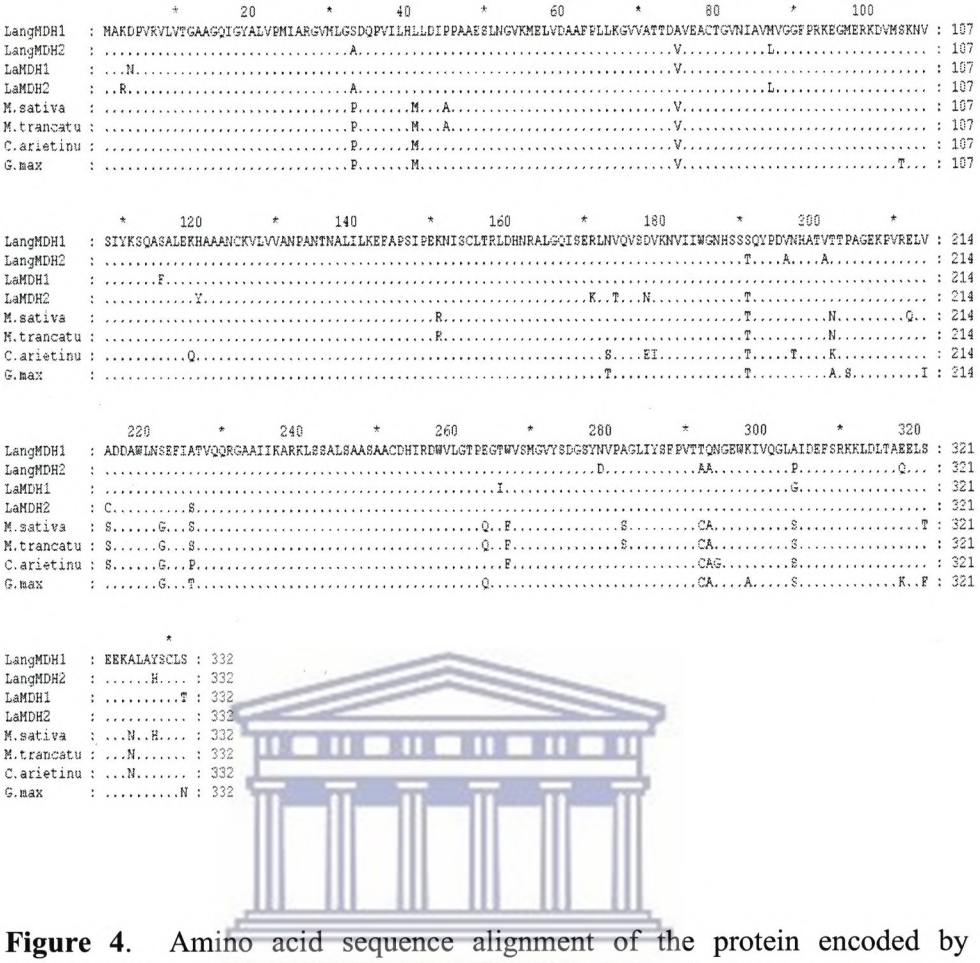

Figure 4. Amino acid sequence alignment of the protein encoded by *Lang*MDH1 and *Lang*MDH2 with deduced cMDHs of other legumes. Dots indicate identical amino acids. Numbers on the right-hand side of each sequence indicate the last amino acid number of that line. The cMDH polypeptides used in the comparison are *Lupinus albus*1 (AAO15574.1), *Lupinus albus*2 (AAO15575.1), *Medicago sativa* (AAB99756.1), *Medicago truncatula* (ACJ84983.1), *Glycine max* (AAS18241.1) and *Cicer arietinum* (CAC10208.1).

Table 3. Percentage of amino acid sequence identity between *Lang*MDH1 and *Lang*MDH2 from *Lupinus angustifolius* and MDHs from other legume- and non-leguminous plant sources. **Sesbania rostrata* is an annual woody- or short-lived perennial tropical legume, which is known to form a mutualistic symbiosis in both its stem and root system with *Azorhizobium caulinodans*.

	GENBANK accession #	% Identity to *Lang*MDH1	% Identity to *Lang*MDH2
Legumes			
*Lang*MDH1	not assigned	100	96
*Lang*MDH2	not assigned	96	100
*L. albus*1	AAO15574.1	98	95
*L. albus*2	AAO15575.1	96	95
M. sativa	AAB99756.1	93	93
M. truncatula	ACJ84983.1	94	93
G. max	AAS18241.1	94	93
C. arietinum	CAC10208.1	94	93
* *S. rostrata*	Q8W4XO	36	36
Non-legumes			
P. persica	AAL11502.1	94	92
P. trichocarpa	7456432	94	93
C. sinensis	ABK22380.1	90	90
L. chilense	ABB16910.1	92	90
P. major	CAH58641.1	93	91
G. hirsutum	ACJ11738.1	92	91
Z. mays	NP001147160.1	90	89
S. lycopersicum	AAU29199.1	83	83
A. thaliana	NP200483.1	71	71

The phylogeny of 18 cMDH genes present in GENBANK, revealed that the proteins identified in this study, formed a separate cluster with cMDHs of *L. albus* (*La*MDH1 and *La*MDH2) (Fig. 5). Some of the other known legume species in this phylogenetic tree formed clusters separate from the amide-exporting *Lupinus*.

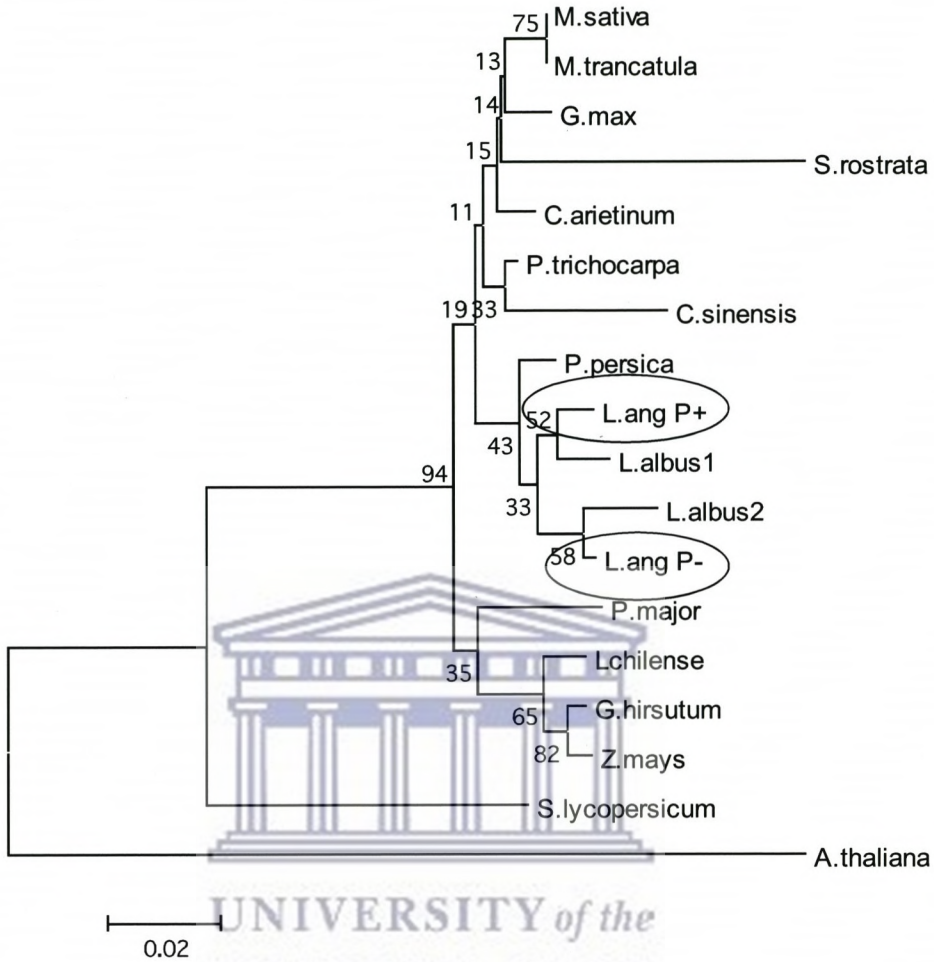

Figure 5. Evolutionary relationships of 18 taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 130 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.1 (Tamura *et al.*, 2007). The circled portions illustrate where proteins isolated in this study are located in relation to the other proteins in the tree.

4.5 Discussion

It is demonstrated here that the N_2 fixation capacity by root nodules subjected to short-term LP conditions may be attained through increasing flux via the PEPc-MDH route, which drives increased malate synthesis. This apparent increase in malate concentration in root nodules, under LP may have been the result of the distinct expression of a novel MDH under LP.

It is well established that P deprived conditions lead to conventional glycolytic metabolism being circumvented in favour of alternative routes (Theodorou & Plaxton, 1993). This is an adaptive strategy that serves the purpose of the continuation of metabolism when unfavourable conditions are encountered. In root nodules, where metabolism may already be restricted by limited O_2, P limitation may further impede the complete oxidation of sugars via conventional pyruvate kinase (PK) activity. Thus, P deficiency may lead to sugars being metabolized to dicarboxylic acids via the concerted actions of phospho*enol*pyruvate carboxylase (PEPc) and MDH. PEPc has a vital role in C provision to legumes for nitrogen assimilation and bacterial respiration (Schulze *et al.*, 1998; Le Roux *et al.*, 2006). In addition, the PEPc reaction also liberates P_i, which could buffer the metabolism against acute P deficiency (Theodorou & Plaxton, 1993). This study corroborates the notion that the alternative route for phosphoenolpyruvate (PEP) metabolism is channeled through the PEPc-MDH route under LP, as MDH activity was enhanced. Malate concentrations were increased in accordance with the associated increase in cMDH activities under LP conditions. The P deficiency-induced activation of MDH is intended for the procurement of malate for bacteroids in

root nodules as a source of respiratory C under LP (Colebatch *et al.*, 2004). In addition, the PEPc-MDH alternative route may also produce organic acids for exudation in an attempt to solubilize sparingly available P in the growth medium under LP (Johnson *et al.*, 1994, 1996; Raghothama, 1999).

After the failure to isolate a neMDH, it can only be speculated that the cMDH would be the primary reaction responsible for the synthesis of the high malate levels under LP, reported in this study. Two independent cMDHs in *L. angustifolius*, designated *Lang*MDH1 and *Lang*MDH2, for +P and -P conditions respectively, have been isolated in this study. Sequencing data showed that the MDH1 gene had an open reading frame (ORF) of 999 nucleotides (nts) encoding a predicted protein of 332 amino acids. Similarly, the *Lang*MDH2 gene contained and ORF of 999 nts, coding for exactly the same sized protein as that of the *Lang*MDH1 form. There seems to be no significant variations in the amino acid sequence between the two amplicons, with homology exceeding 90%.

These two proteins resembled the cMDH of two previously characterized isoforms from -P proteoid roots of *L. albus* (*La*MDH1 and *La*MDH2) with homology being between 98% and 95% similar for MDH1 and MDH2, respectively (Uhde-Stone *et al.*, 2003). Notably, interspecific sequence similarities exceeded intraspecies sequence similarities of the different MDHs, especially under P sufficient control conditions. Protein sequence analyses revealed a catalytic active site at residues 156 to 173 for both amplicons.

Malate plays an array of functional roles and several isoforms of MDH, i.e. the enzyme responsible for its synthesis, are expressed differentially, reflecting its varying functions in different cell types (Miller *et al.*, 1998). However, there is limited knowledge of different isozymes for cMDH that may be responsible for the variable biological functions in nodulated root systems, especially under P deficient conditions. In a previous study, two full-length cMDH cDNA clones were isolated and characterized through screening a library from P-deprived proteoid roots of *L. albus* (Uhde-Stone *et al.*, 2003). In order to identify cDNA for cMDH in *Lupinus angustifolius*, gene-specific primers were designed and used to probe total RNA from +P and -P root nodules.

Phylogenetic analysis of cMDH relatedness revealed that there is a high degree of divergence amongst leguminous species used, with almost all of the legumes compared with *Lang*MDH1 and *Lang*MDH2 forming independent clades separate from the genus *Lupinus*. This may be indicative of the highly diverse nature of the legume family as a whole. However, phylogeny based on this single cMDH gene may inaccurately reflect species relatedness and a better interpretation of this observation could only be given when more cMDH sequences are available.

Up-regulation of several proteins related to C (including MDH) and N metabolism in root nodules has been induced when compared with uninfected roots (Colebatch *et al.*, 2002). Le Roux *et al.* (2006; 2009) have previously demonstrated that MDH activity is markedly increased under LP conditions. Selection for increased dark CO_2 fixation is recognized as a feasible means of

increasing legume productivity (Groat *et al.*, 1984). It is still unclear how the MDH protein expressed under P deficiency may differ from that of the one expressed under non-stressed conditions. Thus, further analyses will be completed to gain insight into how the expression patterns of these two forms identified will differ if subjected to conditions of +P and -P.

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CHAPTER 5

Organic acid accumulation may inhibit N_2 fixation in P-stressed lupin nodules

Publication citation:

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5.1 Summary

- Nodulated lupins (*Lupinus angustifolius* var. *Tanjil*) were hydroponically grown at low phosphate (LP) or adequate phosphate (HP) to assess the effect of phospho*enol*pyruvate carboxylase (PEPc)-derived organic acids on N assimilation in LP nodules.
- LP conditions are linked to altered organic acid metabolism by the engagement of PEP metabolism via PEPc. In LP nodules, the enhanced organic acid synthesis may reduce the available organic C for N assimilation. The diversion of C between the organic acid and amino acid pools were assessed through key nodular enzymes and $^{14}CO_2$ metabolism.
- Under LP conditions, increased rates of organic acid synthesis via PEPc and MDH, coincided with reduced N assimilation via AAT, AS, GS/GOGAT activities. There was a preferential metabolism of nodular $^{14}CO_2$ into organic acids and into particularly malate. High malate levels were associated with reduced N_2 fixation and amino acids synthesis.
- These results indicate that P deficiency can enhance malate synthesis in nodules, but that excessive malate accumulation may inhibit N_2 fixation and N assimilation.

Key words: Legume; P deficiency; N_2 fixation; organic acids, amino acids, nodules

5.2 Introduction

Nodulated legumes are self-sufficient at acquiring nitrogen but are particularly sensitive to a wide range of other environmental limitations. In studies on mineral requirements of the legume-rhizobia symbiosis, P has received considerable attention due the greater responsiveness of traits associated with biological N_2 fixation than host plant growth (Israel, 1987; Olivera *et al.*, 2004). In spite of this, the relationship between low P supply and N_2 fixation remains obscure, primarily because of an indifferent response to P deficiency by some legume species (Vadéz *et al.*, 1996). Furthermore, the responses to sub-optimal P supply differ between the individual partners involved in the symbiosis, with nodules being less affected by fluxes in P supply than either roots or shoots (Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002; Olivera *et al.*, 2004; Le Roux *et al.*, 2006).

Although numerous studies report on the effects of P nutrition on host plant growth and parameters that quantify N_2 fixing capacity of legumes, very few consider P in nodule metabolism (Jakobsen, 1985; Israel, 1987; Sa & Israel, 1991; Al-Niemi *et al.*, 1997; 1998; Tang *et al.*, 2001; Le Roux *et al.*, 2006). Low P supply eventually reduces plant growth, with subsequent reduction in N demand and N_2 fixation. Other responses to limiting P in the growth medium in non-legumes range from an increase in root carbohydrate content (Rychter & Randall, 1994) to decline in total respiration rate and root ATP concentrations (Wanke *et al.*, 1998; Rychter *et al.*, 1992). Furthermore, for non-legumes a typical response to P deprivation at the physiological level is C channeling via alternative routes (Duff *et al.*, 1989; Theodorou & Plaxton,

1993). These are engaged as an adaptive measure to facilitate mitochondrial respiration by P_i deficient plant cells (Plaxton, 1996; Sieger *et al.*, 2005). For C metabolism, a major point of divergence in glycolysis is at PEP. It involves the engagement of non-adenylate requiring steps via the sequential action of PEPc and MDH, which serve to circumvent the conventional adenylate requiring pyruvate kinase (PK; EC 2.7.1.40) route. Similar data for symbiotic legumes are scant. Evidence suggests that P deficiency impacts negatively on the energy metabolism of root nodules for optimal nodule functioning (Sa & Israel, 1991), which may be detrimental to any one of the aforementioned assimilative routes. A potential drawback at this PEP branch-point would be the constant competition for C skeletons between the organic acid pool (for TCA cycle intermediates and energy) and the amino acid pool (for N assimilation) (Olivera *et al.*, 2004; Le Roux *et al.*, 2006).

A natural extension of microaerobic conditions is the altered metabolism of C to malate, instead of to pyruvate as under non-restricted conditions (Vance & Heichel, 1991). The major C substrates for bacteroid respiration and N_2 fixation are dicarboxylic acids, primarily in the form of malate with effective nodules accumulating high levels of this metabolite (Kouchi & Yoneyama, 1986; McRae *et al.*, 1989; Rosendahl *et al.*, 1990). Furthermore, malate is rapidly labeled when nodules are exposed to $^{14}CO_2$, which is indicative of the importance of this particular metabolite in nodules (Streeter, 1987; Rosendahl *et al.*, 1990). Moreover, metabolomic and transcriptomic analyses indicate that elevated levels of malate in root nodules are mainly the result of very low oxygen and phosphorus concentrations (Colebatch *et al.*, 2004).

Given the role of organic acids in nodule metabolism, it is unclear how P deficiency affects C partitioning between the organic acid- and amino acid pools in nodules. The aim of the present study was to assess the effect of PEPc-derived organic acids on N assimilation nodules under prolonged P_i deprivation. This was assessed through the diversion of C between the organic acid- and amino acid pools through key nodular enzymes and $^{14}CO_2$ metabolism.

5.3 Materials and methods

5.3.1 *Plant growth conditions*

All seeds were germinated in vermiculite, which had been commercially irradiated by a cobalt C-60 source of gamma radiation at a dose of 18 kGray. Pots measuring 10 cm in diameter were washed in Ekon-D and rinsed in distilled water, then dried. The pots were then filled with vermiculite. For all experiments, seeds of *Lupinus angustifolius* (cv. Wonga) were inoculated with a rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria. Seeds of lupins were coated in a saturated sucrose solution and 2 g of inoculum / 150 seeds was added and mixed. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry. Once dry, the seeds were planted in pots containing vermiculite.

Seeds were germinated in an east-facing glasshouse at the University of Stellenbosch, Stellenbosch, South Africa. The range of midday irradiances was between 540-600 µmol m^{-2} s^{-1} and the average day/night temperatures and humidities were 23/15°C and 35/75% respectively. Pots were watered daily

with distilled water until seeds germinated. Seedlings were transferred 10 d after germination to 22 *l* hydroponic tanks under the same glasshouse conditions. The tanks contained a modified Long Ashton nutrient solution containing no N, 2 mM PO_4^{3-} and 0.05 mM MES (pH 5.8). Solutions were changed every 3-4 d. The hypocotyls of seedlings were wrapped with foam rubber at their bases and inserted through holes in the lids of the tanks. Each tank was supplied with an air supply line, which bubbled air containing 360 ppm CO_2. After two weeks, once nodules were established, plants were divided into two treatments. Half of the tanks were maintained at an adequate P concentration (2 mM PO_4^{3-}; HP treatment), while the other half were switched to a low P nutrient regime (2 µM PO_4^{3-}; LP treatment) for a further 24 d, after which plants were harvested.

5.3.2 *Inorganic phosphate determination*

P_i levels were determined using a modified Fiske-Subbarow method (Rychter & Mikulska 1990). Approximately 0.5 g of root or nodule was homogenized in 500 µl 10% trichloroacetic acid (TCA) at 4°C. This was diluted 3 times with cold 5% TCA and centrifuged for 10 min at 2 500 g at 4°C. The supernatant was removed and centrifuged a further 10 min at 13 000 g at 4°C. The resultant supernatant was kept on ice (or stored -20°C) until ready for use.

5.3.3 *Protein extraction and determination*

The extraction of PEPc, PK, MDH and ME was performed according to Ocaña *et al.*, (1996) modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (m/v) insoluble polyvinylpoly pyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 ml of buffer. Crude extracts of N assimilating enzymes, GS/GOGAT, AAT were prepared according to the method of Olivera *et al.* (2004). Aspartate synthetase (AS) was extracted according to Egli *et al.*, (1989). The protein concentration was determined by the procedure of Bradford (1976) using a protein assay reagent (Bio Rad) and bovine serum albumin (BSA) as standard. Amino acid concentrations were determined according to the ninhydrin method of Rosen *et al.*, (1957) using leucine as a standard. In the same extract NH_4^+ was measured using a phenol-hypochloride method (Solorzano, 1969).

5.3.4 *Enzyme Assays*

Phosphoenolpyruvate carboxylase (PEPc): PEPc activity was determined spectrophotometrically by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 30 °C. The standard assay mixture contained 100 mM Tris (pH 8.5), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 4 mM PEP, 0.20 mM NADH and 5 U MDH (Ocaña *et al.*, 1996). The blanks consisted of a reaction medium without PEP.

Pyruvate kinase (PK): PK activity was assayed in a buffer containing 75 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and lactate dehydrogenase (3 U) (Smith 1985). The blanks consisted of one

reaction in which ADP was omitted and then another from which PEP was absent.

NADH-Malate dehydrogenase (MDH): MDH was assayed as described by Appels and Haaker (1988). The reaction mixture contained 25 mM KH_2PO_4, 0.2 mM NADH and 0.4 mM OAA. The pH was adjusted to 7.5 with 1 mM HCl (Appels & Haaker 1988). The blanks consisted of a reaction medium without OAA.

Malic enzyme (ME): This assay monitored the increase in absorption at 340 nm due to the formation of NADPH or NADH. The assay mixture contained 80 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 1 mM malate and 0.4 mM NADP or NAD^+ (Appels & Haaker 1988). The blanks consisted of a reaction medium without malate.

Aspartate amino transferase (AAT): AAT activity was determined in a reaction medium containing 4 mM $MgCl_2$, 10 mM aspartic acid, 0.2 mM NADH and 1 mM 2-oxoglutarate in 50 mM Tris-HCl buffer (pH 8.0) (Olivera *et al.*, 2004).

Glutamine synthetase (GS): GS activity was measured in a final volume of 250 µl of imidazole (pH 7.2), 20 mM $MgCl_2$, 25 mM hydroxylamine, 100 mM glutamate and 10 mM ATP in accordance with Olivera *et al.*, (2004). Absorbance was determined at 540 nm.

Glutamate synthase (GOGAT): GOGAT activities were measured spectrophotometrically, monitoring absorbance due to NADH at 340 nm. NADH-GOGAT were assayed in 100 mM KH_2PO_4 (pH 7.6) containing 0.1% (v/v) 2-mercaptoethanol. NADH-GOGAT activity was measured using 100 mM NADH, 2.5 mM α-ketoglutarate, 10 mM L-glutamine and 1 mM

aminooxyacetate (to inhibit aminotransferase activities) (Olivera *et al.*, 2004). GOGAT activities were corrected for endogenous NAD^+ (NADH) reduction (oxidation) in the absence of added substrate. All measurements of NADH-GOGAT activity were made within 3 to 4 h of extraction.

Nodule host plant enzyme activities were determined as initial rates of reaction at 25 to 30 °C. All the reactions were initiated by adding 30 µl crude extract to reaction mixture in a total volume of 250 µl. Initial reaction rates have been shown to be proportional to the concentration of enzyme used under the conditions used.

5.3.5 *Whole plant ^{14}C incorporation*

Roots of intact plants were supplied with 42 nmol $NaHCO_3$ containing 0.093 MBq $NaH^{14}CO_3$, and pulsed with air for 30 s thereafter every 15 minutes for an hour. In addition to the 15-minute intervals of air pulses, the solutions in the cuvettes were also swirled by hand every 5 minutes. After an hour the plants were harvested. Roots were rinsed twice in separate containers of distilled water and blotted dry. Plants were then separated into root, nodule and shoot components, which were immediately weighed and quenched in liquid N before storage at -80 °C.

5.3.6 *^{14}C fractionation*

Components were homogenised with 80 % (v/v) ethanol and separated into soluble and insoluble components. The soluble component was subsequently separated into water soluble and chloroform soluble components. The water-

soluble component was further fractionated into amino acid, organic acid and carbohydrate fractions, as described by Atkins & Canvin (1971).

5.3.7 *C costs determinations*

Nodule construction cost (mmol C. g^{-1} DW) were calculated according to Mortimer *et al.*, (2005). Cw = [C + (*k*N/14) x (180/24)](1/0.89)(6 000/180), where Cw is the construction costs of the tissue (mmol C. g^{-1} DW), C is the carbon concentration (mmol C. g^{-1} DW), *k* is the reduction state of the N substrate (NH_4^+, derived from N_2 reduction, was taken as the source of N for amino acid synthesis, therefore *k* is -3) and N is the organic nitrogen content of the tissue (g. g^{-1} DW). The constant (1/0.89) represents the fraction of the construction cost that provides reductant that is not incorporated into biomass (Williams *et al.*, 1987; Peng *et al.*, 1993) and (6 000/180) converts units of g glucose per g^{-1} DW to mmol C. g^{-1} DW. Growth respiration (mmol CO_2. g^{-1} DW) representing the C respired for the biosynthesis of new tissue was calculated according to Peng *et al.*, (1993).

5.3.8 *Measurement of nodule respiration*

Oxygen uptake by nodulated root segments was measured at 25 ºC for 2-5 min using a Clark oxygen electrode. Although excision can affect the nodule metabolism, we attempted to reduce the impact of the detachment. This was achieved by using nodulated root segments and limiting the time of the assay to under 5 minutes after detachment. According to Vessey (1994) these precautions can reduce the potential artifacts associated with nodule excision. Detached nodulated root segments were rinsed in distilled water and placed in

a cuvette containing 3ml aerated nutrient medium (sufficient or deficient). Upon completion of the measurements, nodules were oven dried at 80ºC for 48 h. the nodules were weighed and respiration expressed on a dry weight basis.

5.3.9 *Nitrogenase activity*

Nitrogenase activity of nodules (attached to a piece of root) was measured according to Parsons and Baker (1996). Ethylene and acetylene concentrations were measured using a Varian gas chromatograph with a 1200 x 1.6 mm Porapak T (80`100) mesh column. Gas samples were injected directly into the gas chromatograph and analysis was completed within 5 min. Although excision can affect the nodule metabolism, we attempted to reduce the impact of the detachment. This was achieved by using nodulated root segments and limiting the time of the assay to under 5 minutes after detachment. According to Vessey (1994) these precautions can reduce the potential artifacts associated with nodule excision.

5.3.10 *Statistical analysis*

Averages for the data are means of separate replicates (n = 4). All data were analysed by single ANOVA. Percentage data was arcsine transformed prior to analysis and ratios were square root transformed prior to analysis. All data was then subjected to a post-hoc LSD test to determine significance.

5.4 Results

5.4.1 *Growth and respiration following P starvation*

The concentration of P_i declined markedly after 25 d of P starvation in both roots and nodules (Fig. 1a,b). The P_i decline for both compartments was approximately 40%.

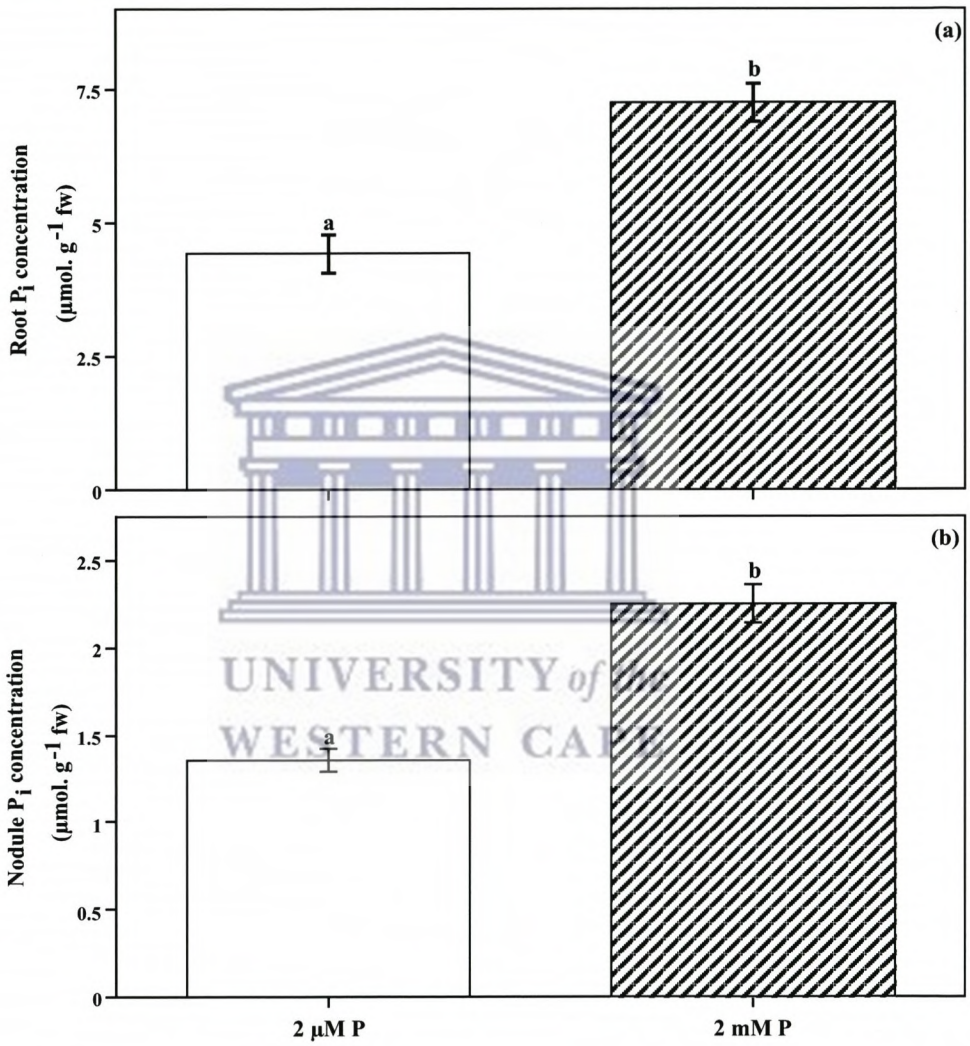

Figure 1. (a) Root P_i- and (b) nodule P_i concentrations of P starved and P sufficient lupin plants. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P (LP). The P sufficient plants remained at 2 mM P (HP) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Nodule biomass accumulation growth respiration costs declined with LP, although the construction costs of tissues remained unchanged (Table 1).

5.4.2 *Nitrogen metabolism*

Nodules at LP supply showed a decline in nitrogenase activity via the acetylene reduction assay (ARA), both on a fresh mass basis and expressed per milligram protein (Table 1).

Table 1. Nodular growth, biomass and respiratory costs and nitrogen nutrition of P starved and P sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P. The P sufficient plants remained at 2 mM P supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Nodule biomass parameters	**2 µM P**	**2 mM P**
Nodule dry weight (g. plant^{-1})	0.047 **a**	0.071 **b**
Construction costs (mmol C. g^{-1} DW)	3413 **a**	3614 **a**
Growth respiration costs (mmol CO_2. g^{-1}. day^{-1})	6059 **a**	7535 **b**
Nodule nitrogen nutrition		
N_2 fixation via Acetylene Reduction Activity (nmol. g^{-1} FW)	60.70 **a**	78.23 **b**
N_2 fixation via Acetylene Reduction Activity (nmol. g^{-1} protein)	15.45 **a**	67.15 **b**
Nitrogen concentration (mmol N. g^{-1} DW)	41.42 **a**	56.11 **b**

There was a marked decline in the concentration of amino acids and NH_4^+ in the nodules of plants that were P deprived (Fig. 2a, b).

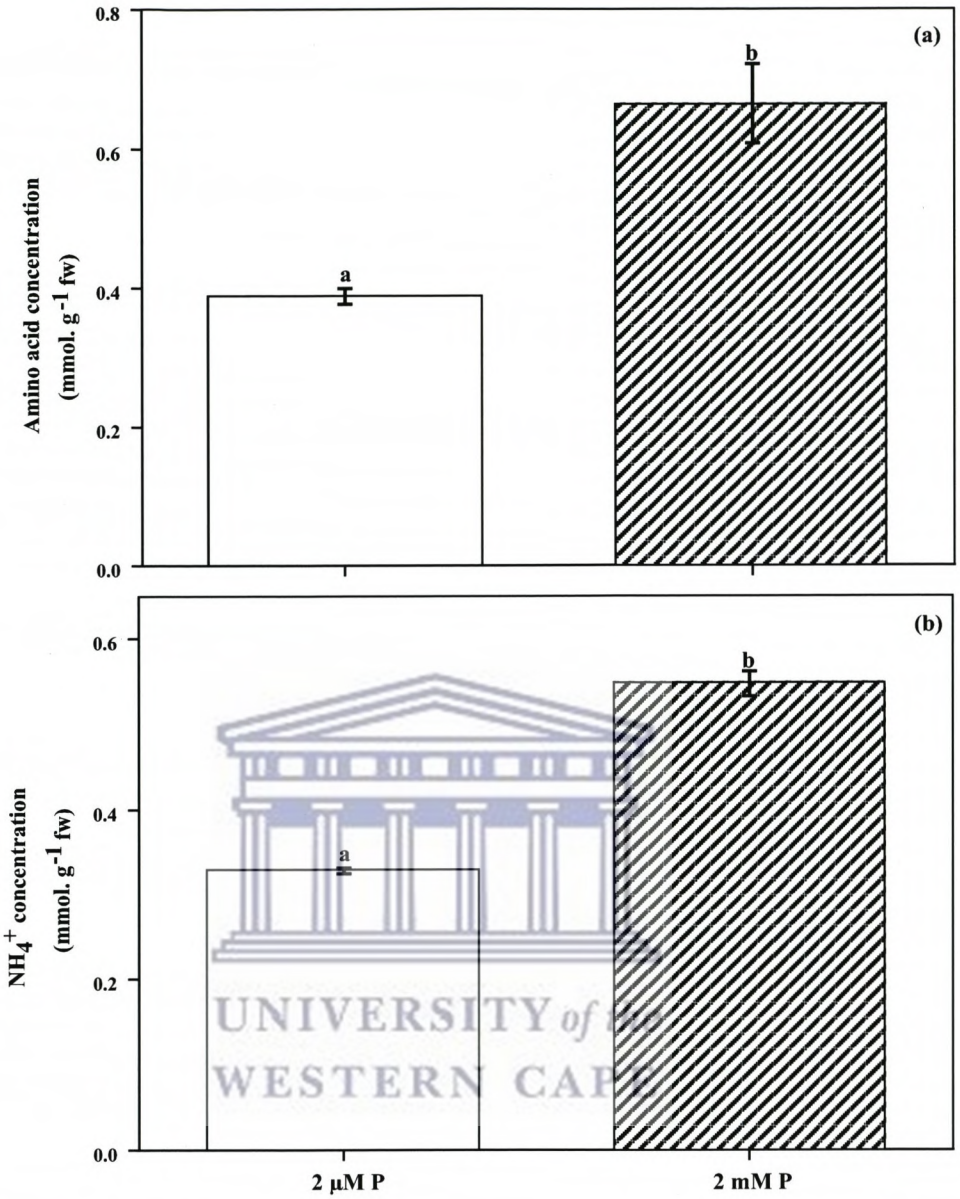

Figure 2. (a) Amino acid- and (b) NH_4^+ concentrations of P starved and P sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P (LP). The P sufficient plants remained at 2 mM P (HP) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

In all P treatments, GS activity was approximately 2 to 3-fold higher than GOGAT enzyme activity. However, both enzyme activities were unaffected by P treatment (Table 2). Similarly, AAT activity remained unchanged under P deficient conditions (Table 2).

Table 2. Specific activities of selected enzymes involved in amino acid and organic acid synthesis of P starved and P sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P. The P sufficient plants remained at 2 mM P supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Nodular enzyme activities	**2 µM P**	**2 mM P**
Amino acid synthesizing enzymes (µmol. min^{-1}. mg^{-1} protein)		
Aspartate amino transferase **(AAT)**	32.51**a**	28.39**a**
Glutemine synthetase **(GS)**	1.26**a**	1.19**a**
Glutemate oxogluterate amino transferase **(GOGAT)**	0.64**a**	0.40**a**
Organic acid synthesizing enzymes (µmol. min^{-1}. mg^{-1} protein)		
Phospho*Enol* Pyruvate Carboxylase **(PEPc)**	0.254**b**	0.169**a**
Malate Dehydrogenase **(MDH)**	0.15**b**	0.06**a**
Pyruvate Kinase **(PK)**	0.12**a**	0.28**b**
Malate dehydrogenase: amino acid synthesizing enzymes		
MDH:AAT	0.004**b**	0.002**a**
MDH:GS	0.115**b**	0.053**a**
MDH GOGAT	0.240**b**	0.156**a**

5.4.3 *C metabolism*

Enzymes of the alternative route of PEP metabolism (i.e. PEPc; MDH) were both increased markedly under P_i deprivation. PEPc registered an increase of approximately 2-fold in activity as compared with PEPc of nodules at sufficient P (Table 2). The activity of MDH was also increased about 3-fold under P deficiency (Table 2). By comparison the activity of PK, which constitutes the conventional route of C incorporation, declined 2-fold under P deficient conditions. Under LP conditions, this PK activity constituted approximately 50% that of the PEPc activity, whilst at HP supply, PK activity was almost 2-fold higher than PEPc activity. There was an increase in the ratio of MDH activities compared with all the enzyme activities (GS, GOGAT, AAT) of the amino acid-synthesizing pathway (Table 2).

5.4.4 *Nodular ^{14}C fractionation and respiration*

There was a higher incorporation of $^{14}CO_2$ in P_i-deficient nodules and their constituent fractions (Table 3). During P_i deficiency the ratio of incorporation of ^{14}C shifted towards non-nitrogenous organic compounds (Table 3).

Table 3. Incorporation and assimilation of dissolved $^{14}CO_2$ in detached nodules from P starved and P sufficient *Lupinus angustifolius* (cv. Wonga) plants after 5 minutes of $^{14}CO_2$ labelling. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 μM P. The P sufficient plants remained at 2 mM P supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

DI^{14}C concentration (nmol ^{14}C. g^{-1} FW)	**2 μM P**	**2 mM P**
Soluble fractions	5.86**b**	2.82**a**
Insoluble fractions	10.57**b**	3.40**a**
Lipid fractions	0.29**b**	0.12**a**
Total incorporation	14.11**b**	9.32**a**
^{14}C incorporation C:N ratio	4.70**b**	2.23**a**

Most of the incorporated ^{14}C was recovered in organic acids, which were higher at LP supply than at HP (Fig. 3a). The percentage of organic acids incorporated into nodules at LP supply was approximately 2 times higher than that for amino acids under the same condition (Fig. 3a,b,c). This increase in organic acid concentration was inversely related to the amino acid concentration in nodules (Fig. 3a,b,c). The higher incorporation into organic acids coincided with a higher fixation rate of $^{14}CO_2$ in LP nodules than HP nodules (Fig. 3d).

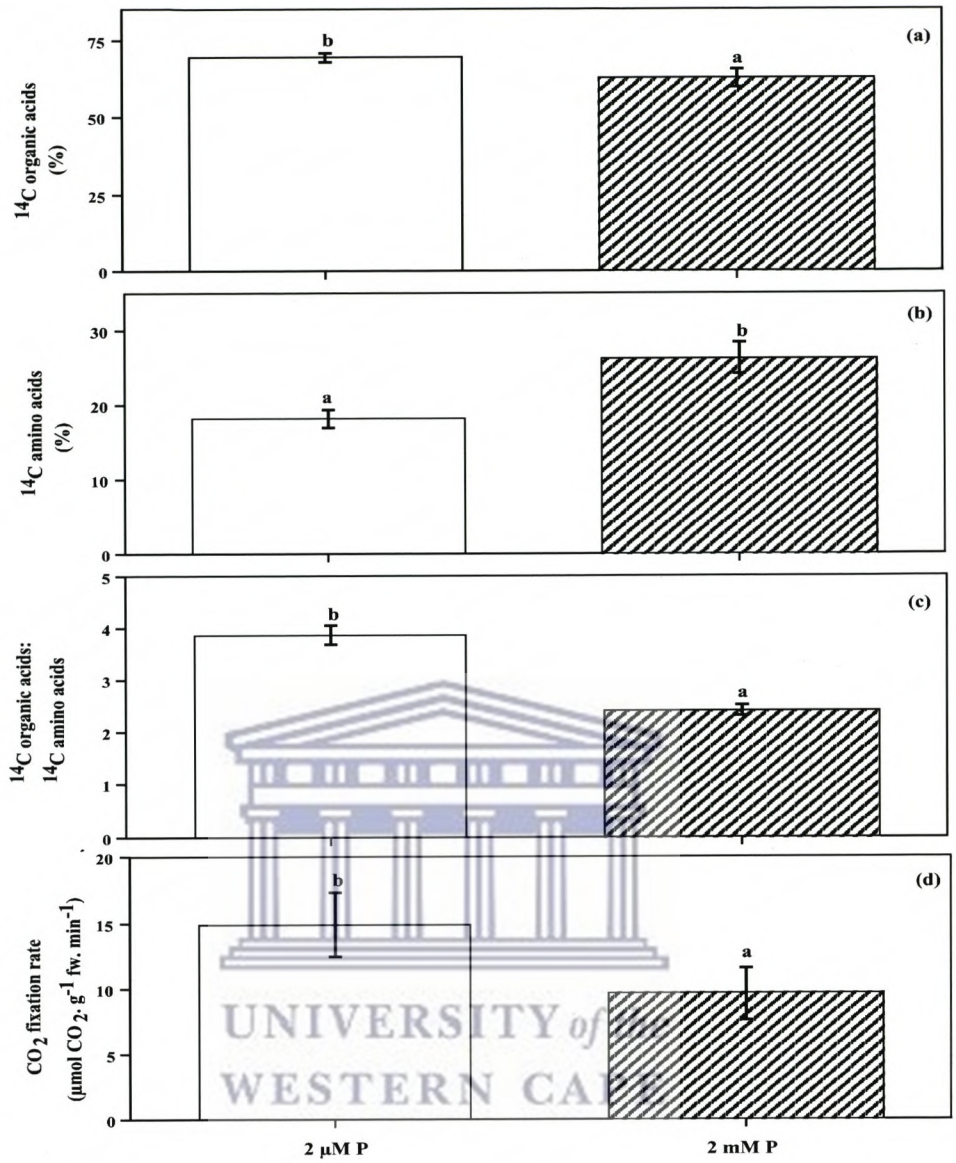

Figure 3. Dark $^{14}CO_2$ fixation into (a) organic acid fraction and (b) amino acid fractions, (c) the ratio of organic acids to amino acids and (d) the $^{14}CO_2$ fixation rate of detached nodules from P starved and P sufficient lupins after 5 minutes of $^{14}CO_2$ labelling. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P (LP). The P sufficient plants remained at 2 mM P (HP) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

The long-term P starvation period also caused an increase in O_2 consumption rate of nodules (Fig. 4).

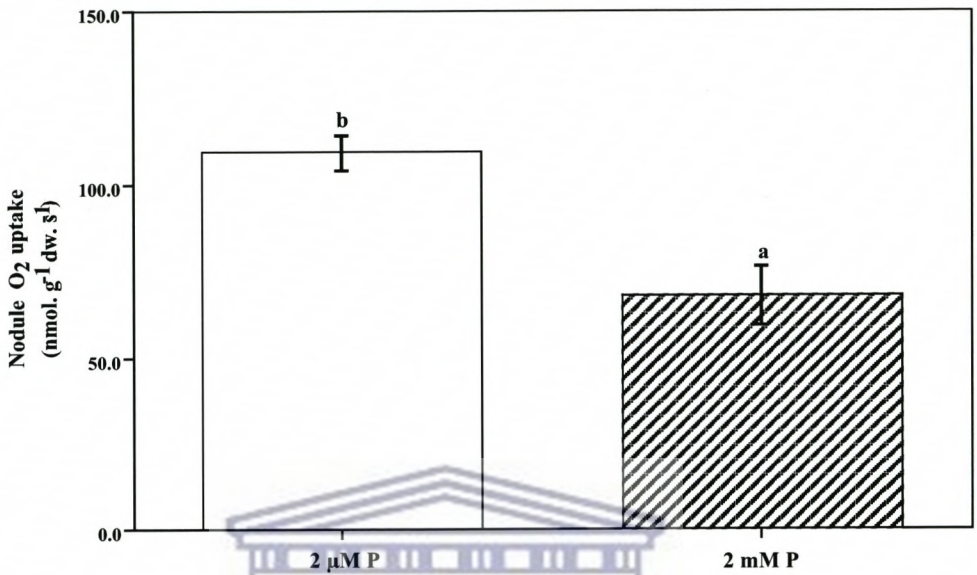

Figure 4. O_2 uptake rate of P starved and P sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P (LP). The P sufficient plants remained at 2 mM P (HP) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

There was a pronounced increase in total malate levels (derived from PEP and TCA cycle) in nodules subjected to LP conditions as opposed to nodules of P sufficient controls (Fig. 5a). However under LP conditions, most of the malate appears to be derived from PEP, as determined by $^{14}HCO_3^-$ incorporation via PEPc (Fig 5b).

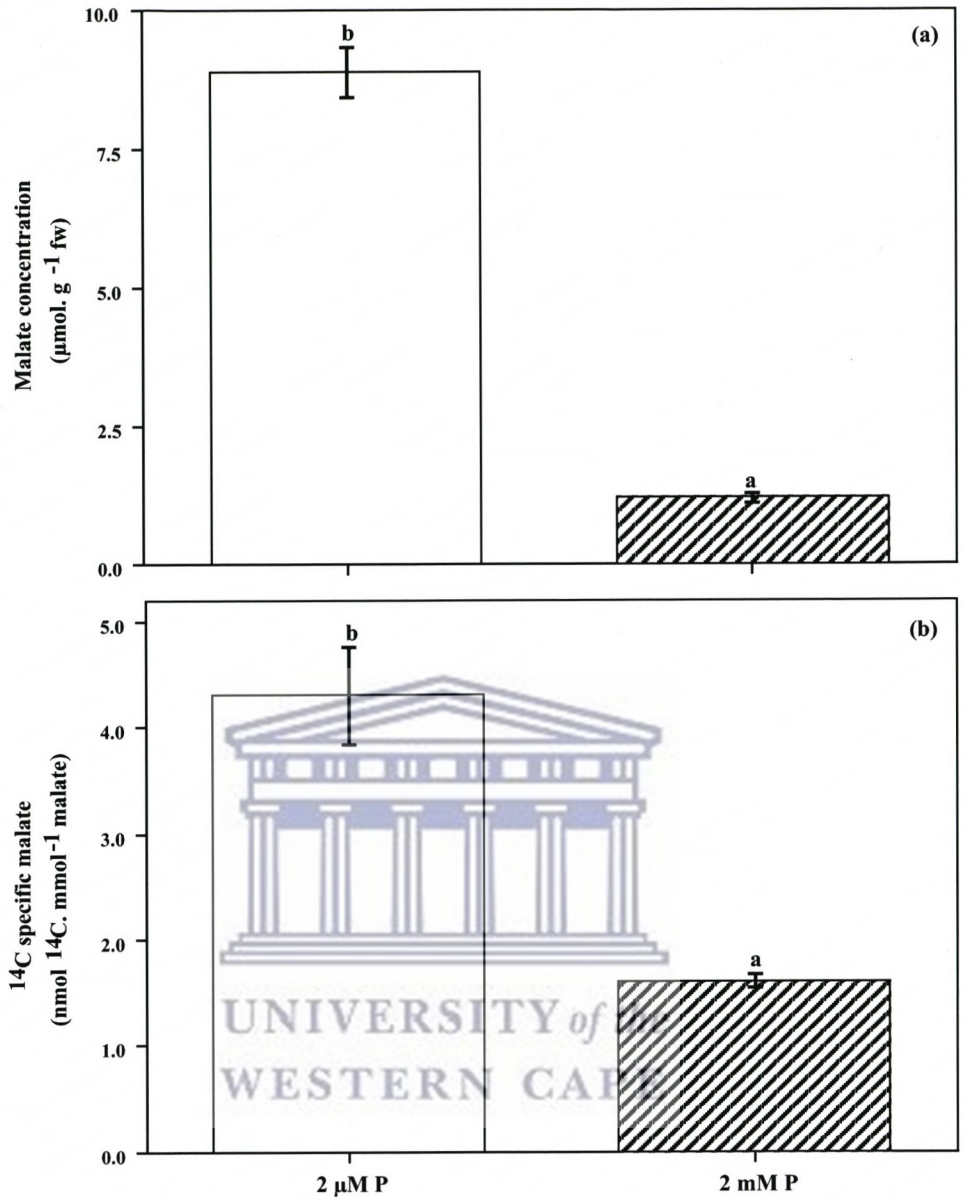

Figure 5. (a) Total malate concentration, and (b) ^{14}C enriched malate of P starved and P sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 weeks in hydroponic culture in a N free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 ppm CO_2 at the roots zone. A low P condition was induced for 24 days in the P starved plants by switching the P supply to 2 µM P (LP). The P sufficient plants remained at 2 mM P (HP) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

5.5 Discussion

During long-term P deficiency of nodules, the reduced N_2 fixation and N assimilation were associated with an increase in the allocation of PEP-derived C into organic acids, relative to amino acids.

Nodules are known to be strong sinks for P, particularly under conditions of P deficiency (Al-Niemi *et al.*, 1997; 1998; Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002). It has been reported that nodules often respond slower to P deficiency than roots (Israel, 1993; Le Roux *et al.*, 2006). This is evidenced by the delayed decline of P_i in root nodules only 25 d after P starvation in this study, compared with a previous study where 14 d of P withdrawal had no apparent effect on P_i concentration of nodules (Le Roux *et al.*, 2006). Here, prolonged P deficiency decreased nodule DW, which concurs with previous reports of the effects of low P supply on legumes (Drevon & Hartwig, 1997; Olivera *et al.*, 2004). Although the growth respiration, an indication of the respired C associated with the biosynthesis of new tissue (Peng *et al.*, 1993), declined with the decreased nodular growth, the construction costs of these nodules were unaffected. Since construction costs represent the amount of simple carbohydrate required to build new tissue (Peng *et al.*, 1993), this implies that new nodular growth was limited not by C allocation, but by P supply.

The low P induced decreases in nodular growth have been attributed to the decline of N_2 fixation (Ribet & Drevon, 1995) but the present study implicates changes in C allocation between amino acid and organic acid pools. The decline in P_i and adenylates during P stress can increase the engagement of alternative routes to circumvent the adenylate and P_i requiring pathways of

conventional glycolysis and mitochondrial respiration (Rychter *et al.*, 1992; Theodorou & Plaxton, 1993). In nodules, the subsequent increased engagement of an alternative glycolytic route at a low P_i concentration, most notably PEP metabolism via PEPc, is thought to contribute to the elevated malate levels typical for nodules (Colebatch *et al.*, 2004; Le Roux *et al.*, 2006). Previous work on shorter-term P deficiency in nodules found no engagement of this PEPc-bypass, when cellular P_i levels were maintained for 14 days of P starvation (Le Roux *et al.*, 2006). However, the P_i decline in nodules under 25d of P starvation in the present study was correlated with an increase in PEP metabolism via PEPc.

The increase in oxygen consumption with decreasing nutritional P supply concurs with the respiratory responses of previous work on P deficient plants (Usuda & Shimogawara, 1993; Bingham & Farrar, 1989). This was mainly ascribed to O_2 consumption being mediated via the non-phosphorylating alternative pathway (Rychter *et al.*, 1992). In addition, P deficiency may upset the favorable energy status of the plant cell fraction where N assimilation into amino acids and ureides occur (Sa & Israel, 1991; Israel, 1993).

In this regard, the present study found that nodular enzymes associated with N metabolism (GS/GOGAT, AAT, AS) had decreased activities under LP conditions, which resulted in lower N assimilation. Despite the current study's focus on amino acid exporting legumes, a similar down-regulation of similar N assimilation enzymes, has previously been reported for the ureide exporting *Phaseolus vulgaris* under LP conditions (Olivera *et al.*, 2004). Although

Almeida *et al.*, (2000), found that nitrogenase activity undergoes regulatory inhibition by an N feedback mechanism under LP conditions, the present study's decline in total amino acid and NH_4^+ concentrations were not associated with this predicted relief of the inhibition of nitrogen fixation at LP. Furthermore, it was found that accumulation of specific amino acids (e.g. asparagine), rather than changes in total amino acids could elicit a decline in specific nitrogenase activity (Almeida *et al.*, 2000).

The nodule metabolite levels and enzyme activities strongly suggest that LP nodules have an increased C flux towards organic acids. Since nodules have a high requirement for dark CO_2 fixation (Rosendahl *et al.,* 1990), the enhanced uptake and assimilation of dark CO_2 under P deficiency and the subsequent preferential incorporation into organic acids, concurs with the engagement of the P-deficient bypass in plant cells (Theodorou & Plaxton, 1993). The PEP-derived organic acids, particularly malate, are widely accepted to be the primary source of C for bacteroids (Rosendahl *et al.*, 1990; Kaur & Singh, 1999). In the present study, this shift in C metabolism towards organic acid synthesis was at the expense of C assimilation into amino acids. Therefore, it is proposed here that altered organic acid metabolism may account for the reported decline in N_2 fixation in this study. In particular, the increase in malate synthesis and dark CO_2 incorporation into malate can further substantiate this assertion. This increased malate concentration in nodules of plants grown at LP is matched by the increase in MDH activities of these nodules. Elevated malate concentrations at LP may have been inhibitory to N_2 fixation.

In vitro studies have demonstrated that even modest increases (0.5 to 2 mM) in malate supply to isolated bacteroids could heavily impede N_2 fixation (Bergersen & Turner, 1990; 1993). This effect on N_2 fixation was only reversed once substrate levels were lowered in isolated bacteroids (Bergersen & Turner, 1990; 1993). Although there has been a study on whole nodules to resolve the malate concentrations between the cytolsolic and bacteroid components, these findings were not implicated in N_2 fixation (Rosendahl *et al.* 1990).

In conclusion, the engagement of the PEPc bypass route in P_i-limited nodules may ensure the continuation of respiration but the preferential shift of PEPc-derived C to organic acid synthesis may have resulted in malate accumulation to inhibit symbiotic N_2 fixation. Furthermore, it is clear that more detailed information is required on the malate distribution in various nodular tissues in order to assess the relationship between malate levels and N_2 fixation.

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CHAPTER 6

Physiological responses to and the recovery from P deficiency in *Lupinus angustifolius*

6.1 Summary

- The effects of P deficiency on growth, N_2 fixation and photosynthesis in nodulated lupins (*Lupinus angustifolius* var. *Tanjil*) developing and recovering from P deficiency were investigated.
- Lupins were grown in pots on a non-limiting P (2 mM P; HP) supply for three weeks. A subset of these HP plants was supplemented with a low phosphorus (2 μM P; LP) nutrient solution, whereas the rest served as HP control plants for a further 24 days. Two weeks after P treatments were induced in the LP plants, a subset of these were re-supplied with a HP solution (with the rest of the plants serving as P deficient controls) to their medium and the recovery responses evaluated after ten days.
- LP arrested nodule growth and caused a substantial decline in %Ndfa. Similarly, the leaf weight ratio (LWR) decreased, but the rate of photosynthesis per unit specific leaf mass or area was unaffected by LP. Respiratory costs of nodulated roots were diverted to growth respiration at the expense of maintenance respiration in nodulated roots under LP. All the parameters that showed a decrease under LP, responded positively to P re-supply and recovered to HP control levels.
- Thus, LP impairs the functions associated with nodule growth and metabolism. Physiological adaptations could involve a switch to a highly efficient nodule respiratory metabolism related to the optimisation of N_2 fixation under LP.

***Key words**: Lupins*, legumes, P deficiency, N_2 fixation; PUE, root nodules, respiration, maintenance, growth

6.2 Introduction

Lupinus is a temperate legume with great agronomic potential due to the high protein content of its seeds and its positive contribution to restoring depleted soils through N enrichment (Fernández-Pascual *et al.*, 2007). Lupins appear to have certain key morphological and physiological adaptations that make them suitable for nutrient poor soils (Schulze *et al.*, 2006). This may confer tolerance to several abiotic stresses, amongst which is a high tolerance to prolonged periods of P stress (Fernández-Pascual *et al.*, 2007; Schulze *et al.*, 2006).

The model lupin species that has been used to address issues related to modifications of root physiology and biochemistry under P deficiency, is white lupin (*Lupinus albus*). White lupin develops proteoid (cluster) roots, which are positively correlated with the excretion of high amounts of organic acids, in response to P deficiency (Johnson *et al.*, 1996; Keerthisinghe *et al.*, 1998). However, this adaptation does not make it easy to separate the physiological responses of cluster roots to P deficiency from those of nodulated root phenomena, which might be very different. In this regard, it might be more desirable to use a lupin species that does not form cluster roots and ascertain P effects on root nodule physiology independently of any other contributing factors.

Phosphate deficiency is known to induce numerous changes in plant metabolism and its importance may encompass parameters related to C economy and photosynthetic activity (Freeden *et al.*, 1989; Liao *et al.*, 1999;

Nielsen *et al.*, 2001). Translocation of photosynthate to the nodules was severely compromised under P deficiency (Jakobsen, 1985). However, how C limitation impacts on N_2 fixation remains largely unknown and numerous authors have proposed this to be a secondary response related to O_2 permeability effects (Vance & Heichel, 1991; Hartwig, 1998; Schulze, 2004). Indeed, some of these studies describe the down regulation of N_2 fixation to occur in advance of any changes in host plant growth and glycolytic flux (Israel, 1993; Curioni *et al.*, 1999). Carbon resources acquired by plants ultimately have various fates, including construction of new plant tissue, utilization in respiration and export to the rhizosphere.

There exists a complex interplay between carbohydrate and P metabolism, with P stress induced lupin genes down regulated in response to sugar addition (Liu *et al.*, 2005). This may be related to the role of P in the energy metabolism and in the C turnover in root nodules of leguminous plants. The C burden that N_2 fixation incurs on its leguminous host may well be high under non-stressed conditions given the energy-intensive nature of the symbiosis. However, it could be further exacerbated by mineral stress, such as P deficiency. The partners in the symbiosis are required to optimise their energy needs in response to P deficiency. Thus, respiration that drives the energy metabolism of nodulated roots will be modulated to sustain a highly efficient symbiosis. In this regard, root nodule CO_2 fixation is of pivotal importance for N_2 fixation in legume-rhizobia symbioses (Fischinger & Schulze, 2010). Nodules can re-fix some of the respired C through the expression of enzymes such as phospho*enol*pyruvate carboxylase (PEPc; EC 4.1.1.31) and carbonic

anhydrase (CA; 4.2.1.1). These and other enzymes related to N and C metabolism, including other recycling mechanisms (e.g. H^+ uptake) in root nodules, may severely impact on C efficiency of root nodule symbiosis and make experimental analyses of respiratory costs difficult at best. All these factors may account for the highly variable values of C expenditure for N_2 fixation for a range of legume hosts in literature.

Furthermore, differences in belowground C allocation, between root respiration and growth may account for P efficient metabolism of varying P efficient genotypes of non-symbiotic common bean plants (Nielsen *et al.*, 2001). Therefore, better performances by certain plants may be related to their ability to amend growth with low yields or alternatively their capacity to utilize absorbed P more effectively in the production of biomass, so-called phosphate use efficiency (PUE) (Vadéz *et al.*, 1999). The plant may expend in excess of 20% of the C required via photosynthesis in root respiration under optimal conditions (Lambers *et al.*, 1996). Various studies highlight how this respiratory burden may be altered for legumes dependent on root symbiosis (Warembourg *et al.*, 1982; Warembourg & Roumet, 1989; Walsh *et al.*, 1992; Schulze *et al.*, 1994). However, none of these consider what role P limitation in the growth medium plays in relation to maximizing symbiotic efficiency in this regard. Under such conditions, the way in which plants are able to govern how carbohydrates are being allocated to the various pathways related to optimising metabolic energy synthesis is paramount to their survival (Nielsen *et al.*, 2001). Root respiration can be partitioned into costs for growth and maintenance. Growth respiration is defined as the amount of carbohydrates

respired in processes related to a net gain in plant biomass and includes ATP production and reductant for biosynthetic processes, transport processes and nutrient uptake and reduction (Van Iersel & Seymour, 2000). In turn, maintenance respiration is the respiration required to provide energy for all plant processes, which do not result in a net accumulation of plant dry matter (Peng *et al.*, 1993; Van Iersel & Seymour, 2000).

This study aimed to ascertain responses of symbiotic nitrogen fixation (SNF) to P deficiency in the growth medium and the associated partitioning of photosynthetic and respiratory carbon metabolism under prolonged P deficiency conditions (24 d) imposed in this study. The recovery responses to P re-supply (14 d) was also assessed. This may have implications for agriculture in marginal soils.

6.3 Materials and methods

6.3.1 *Plant growth conditions*

Seeds of *Lupinus angustifolius* (cv. Tanjil) were germinated in vermiculite and were inoculated with a rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria at planting. Seeds of lupins were coated in a saturated sucrose solution and 1 g of inoculum / 80 seeds was added and mixed. Seed germination was carried out in a controlled environment with the range of midday irradiances between 500 and 670 μmol. m^{-2}. s^{-1} and the average day/night temperatures and humidities were 21/16 °C and 34/73%, respectively.

Subsequent to the first visible signs of nodulation (≈ 7 d) seedlings were transferred to pots (30 × 30 mm) (one plant per pot) containing silica sand. Plants were watered daily with 300 ml of Long Ashton solution modified to contain a high phosphorus (HP; 2 mM P) solution. The nutrient solution consisted of no N, 2 mM phosphorus and 0.05 mM MES [2-(*N*-morpholino) ethane-sulfonic acid] buffer. The pH of the solution was appropriately adjusted with either 1 mM HCl or 1 mM NaOH to 5.8 prior to watering. A switch to a low phosphorus (LP; 2μM) treatment started once nodules were established and of adequate size, usually in the third or fourth week of growth. After 14 d of growth of the plants on the LP treatment, a subset of these plants were re-supplied with a HP solution, constituting the re-feed treatment, whereas the rest of the plants were either kept at LP or HP and served as plants under prolonged (i.e. 24 d) of P-deficiency and P-sufficient control treatments, respectively. A group of plants were harvested prior to inducing any of the treatments and served as a reference to assess variation in P treatments from the point of measurement. For the groups of plants from HP and LP treatments, measurements started at the point of P withdrawal and measurements were carried out once a week for a 3-week period. In the case of the re-feed treatment, measurements were only made once and that was at the final harvest after 24 d. The LP plants at 14 d served as the point of departure for plants that were re-supplied with HP and on which calculations were based.

6.3.2 *Harvesting and nutrient analysis*

Harvest intervals occurred at 14 and 24 days after P deficiency was induced. Upon harvesting the plants (n = 4) were separated into roots, nodules, stems and leaves. The harvested material was dried at 72 °C for 48 h to a constant weight and dry weights (DW) were recorded. The dried plant material was milled using a 0.5 mm mesh (Arthur H Thomas, California, USA). The milled samples were analyzed for their respective C, N and P concentrations, by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyzer with suitable standards (BemLab, De Beers Rd, Somerset West, South Africa).

6.3.3 *Measurements of CO_2 assimilation*

All CO_2 assimilation measurements were conducted at an ambient leaf temperature, using a portable infrared gas analyzer (IRGA; LCA-Pro, ADC, Herts SG12 9TA, England). The IRGA comprised of a photosynthetic leaf chamber featuring light, temperature and humidity control. Humidity, in the leaf chamber was maintained at ambient conditions. Photosynthetic rates were measured at an irradiance of 1200 µmol photons. m^{-2}. s^{-1}, derived beforehand from light response curves. The youngest fully expanded leaves for each plant were used for photosynthetic determinations.

The portable infrared gas analyzer was also used to measure the amount of CO_2 released from the belowground whole root systems. The analyzer was fitted with an adaptable soil hood and the total root respiration was measured

over a period of 5 min at 15 s intervals. A constant flow rate in the cuvette space was maintained at 300 ml. min^{-1}.

6.3.4 *Calculations of $\delta^{15}N$*

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of $\delta^{15}N$ was calculated as δ = 1000‰ $[R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample with standards as defined by Farquhar *et al.,* (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, California, USA). Between 2.1 and 2.2 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

The $\delta^{15}N$ natural abundance of the legumes was corrected for the seed N, according to Boddey *et al.*, (1995):

$$\delta^{15}N \text{ enrichment (Seed corrected)} =$$

$$((\text{plant N} \times \delta^{15}N_{\text{plant}}) - (\text{seed N} \times Ps \times \delta^{15}N_{\text{seed}})/ (\text{plant N} - \text{seed N}))$$

Where plant N and seed N represent the respective N concentrations of the plant and seed, $\delta^{15}N$ plant and $\delta^{15}N$ seed represent the respective $\delta^{15}N$ values of the plant and seed and Ps is the proportion of the seed N that was assimilated by the legume.

The seed corrected $\delta^{15}N$ values (Boddey *et al.* 1995) were used to determine the percentage N derived from the atmosphere (%Ndfa). Ndfa was calculated according to Shearer & Kohl (1986):

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

Where *B* is the $\delta^{15}N$ natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N free culture, according to Shearer & Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76‰.

6.3.5 *C costs determinations*

Tissue construction costs (mmol C. g^{-1} DW) were calculated according to Mortimer *et al.*, (2005). Cw = [C + (*k*N/14) × (180/24)](1/0.89)(6 000/180), where Cw is the construction costs of the tissue (mmol C. g^{-1} DW), C is the carbon concentration (mmol C. g^{-1} DW), *k* is the reduction state of the N substrate (NH_4^+, derived from N_2 reduction, was taken as the source of N for amino acid synthesis, therefore *k* is -3) and N is the organic nitrogen content of the tissue (g. g^{-1} DW). The constant (1/0.89) represents the fraction of the

construction cost that provides reductant that is not incorporated into biomass (Williams *et al.*, 1987; Peng *et al.*, 1993) and (6 000/180) converts units of g glucose per g^{-1} DW to mmol C. g^{-1} DW. Growth respiration (mmol CO_2. g^{-1} DW) representing the C respired for the biosynthesis of new tissue was calculated according to Peng *et al.*, (1993).

The specific P utilization rate (SPUR) (g DW. mg^{-1} P. d^{-1}) is a measure of the DW gained for the P taken up by the roots (Nielsen *et al.*, 2001) over the time interval (t_1 to t_2) and is described by the equation:

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

Where M is the P content of the roots and W is the nodulated root DW.

6.3.6 *Statistical analysis*

Averages for the data are means of separate replicates (n = 4). All data were analysed by single analysis of variance (ANOVA) (SuperAnova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). All data were then subjected to a post-hoc Student Newman Kuehls (SNK) multiple range test ($P \leq 0.05$) to determine significant differences, which are indicated by different letters.

6.4 Results

6.4.1 *Symbiotic nitrogen fixation and dry matter yield*

Judging from the %Ndfa, legume hosts under prolonged LP showed a decline of about 16% in their fixation capacity compared with HP controls (Fig 1). During the 10 d of P re-supply to the medium of plants formerly P starved, such plants regained all of this capacity, reaching a %Ndfa similar to the HP controls (Fig. 1).

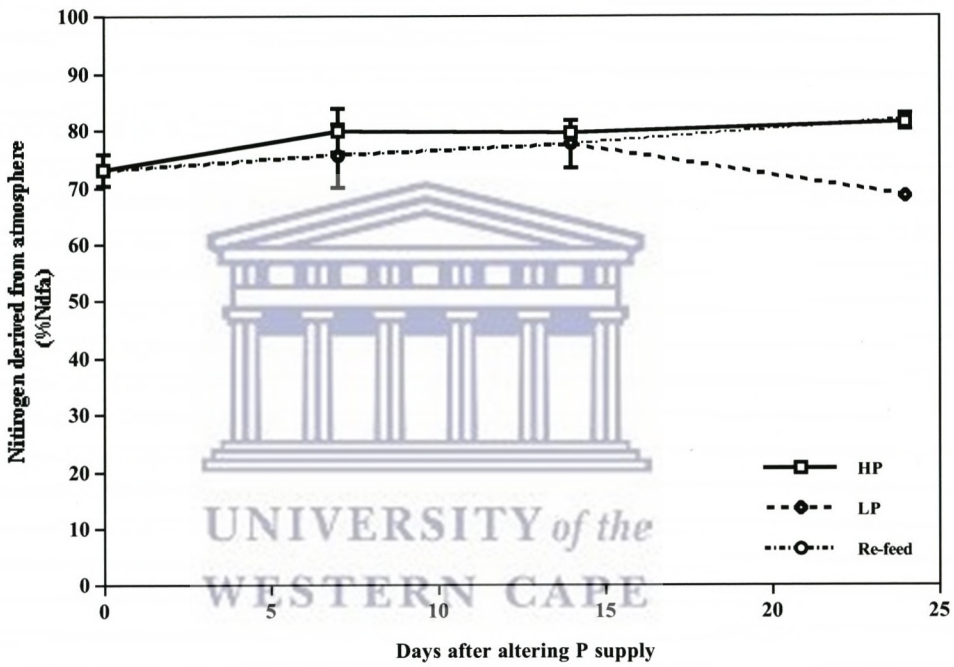

Figure 1: The percentage nitrogen derived from the atmosphere (%Ndfa) of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 μM P (LP). For the P re-feed treatment (Re-feed), half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Error bars denote the standard error ($n = 4$).

In general, plant growth was unaffected by prolonged P deficient conditions (Table 1). Shoot growth was largely unaffected by P treatment but did manifest an increase in construction costs (W_c) and growth respiration per unit shoot dry weight (RG_w), under LP conditions (Table 1). Nodulated roots increased their relative growth rate (RGR) by 56% under prolonged P deficient conditions imposed in this study (Table 1). Thus, plants subjected to LP, responded typically by increasing root biomass at the expense of shoot biomass, with a concomitant higher root:shoot ratio (Table 1). Nodulated root relative growth rate was restored to levels resembling that of P sufficient controls after P was re-introduced to the growth medium (Table 1). Dry matter yield of nodulated roots increased by approximately 32% and plants gained only about 12% of the dry weight after P re-supply at the end of the experimental period (Table 1). Thus, dry matter accumulation was still not back to levels of P sufficient controls by the end of the 10 d re-supply period (Table 1).

Nodule biomass appeared to have been more responsive to P withdrawal from the medium, than either shoots or roots (Table 2). Various nodule parameters, i.e. nodule weight, mean nodule mass, nodule number, normalised nodule weight and the percentage that nodule weight contributed to the root system, decreased after 24 d of LP conditions (Table 2). However, these same parameters, with the exception of nodule number, responded rapidly (within 10 d) and recovered to levels similar to HP controls after P was re-introduced into the medium at 14 d of growth on LP (Table 2).

Table 1: Plant biomass parameters of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 μM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Paramaters	Treatments HP		LP		Re-feed	
***Biomass* (g. plant^{-1})**						
Plant DW (g)	0.84 ± 0.07	a	0.97 ± 0.02	a	0.88 ± 0.11	a
Shoot DW (g)	0.70 ± 0.02	a	0.71 ± 0.06	a	0.69 ± 0.08	a
Root DW (g)	0.15 ± 0.01	a	0.27 ± 0.01	c	0.22 ± 0.02	b
Root (including nodules) : Shoot	0.29 ± 0.01	a	0.43 ± 0.04	c	0.38 ± 0.01	b
Shoot growth						
Relative growth rate (RGR) (mg. g^{-1}. d^{-1})	173.96 ± 0.01	a	176.62 ± 0.02	a	179.26 ± 0.03	a
Construction costs (W_c) (mmol C. g^{-1} DW)	229.99 ± 7.22	a	261.71 ± 5.14	b	234.91 ± 5.16	a
Growth respiration (RG_w) (mmol C. g^{-1} DW)	214.86 ± 4.57	a	237.03 ± 3.93	b	217.68 ± 6.82	a
Nodulated root growth						
Relative growth rate (RGR) (mg. g^{-1}. d^{-1})	24.91 ± 0.01	a	57.27 ± 0.01	b	30.94 ± 0.02	a
Construction costs (W_c) (mmol C. g^{-1} DW)	105.06 ± 8.28	b	72.01 ± 10.88	a	65.07 ± 2.52	a
Growth respiration (RG_w) (mmol C. g^{-1} DW)	44.05 ± 2.84	b	23.18 ± 5.29	a	23.43 ± 0.55	a

Table 2: Nodule biomass parameters and N-fixed per P concentration (mg N. g^{-1} DW. mg^{-1} P) or per nodule DW (mg N. g^{-1} DW) of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 µM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

	Treatments		
Nodule paramaters	**HP**	**LP**	**Re-feed**
Nodule DW (mg. $plant^{-1}$)	50.23 ± 8.15 b	27.29 ± 0.31 a	39.06 ± 4.86 b
Mean nodule mass (mg)	4.44 ± 1.16 b	1.10 ± 0.09 a	2.65 ± 0.29 b
Normalised nodule weight (mg. g^{-1} shoot DW)	72.37 ± 36.03 b	39.15 ± 3.03 a	56.33 ± 3.76 b
Nodule weight per nodulated root system (%)	24.71 ± 4.40 c	9.07 ± 0.24 a	14.92 ± 1.28 b
Nodule number	12 ± 1.53 a	25 ± 1.73 b	29 ± 2.08 b
Root nodule use efficiencies			
N fixed / P concentration	13.87 ± 1.74 a	43.27 ± 1.22 b	14.12 ± 1.97 a
N fixed / nodule DW	14.30 ± 0.91 a	20.90 ± 1.35 b	14.90 ± 0.64 a

In assessing whether N_2 fixation changed in accordance to P deficiency the shoot biomass was plotted as a function of its nodule biomass. The slope of the regression analysis, only if it was significantly correlated ($R \geq 0.65$), is an indication of the utilisation efficiency for rhizobial symbiosis (EURS) (Kouas *et al.*, 2008).

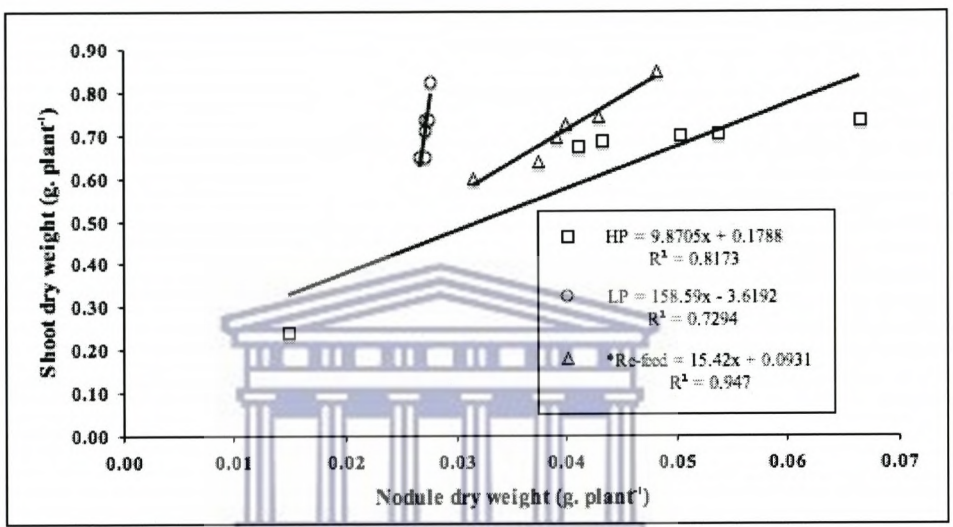

Figure 2: The effect of P nutrition on utilisation efficiency for rhizobial symbiosis (EURS; the regression parameter of shoot as a function of nodule) of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil). Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either (□) 2 mM P (HP) or (○) 2 µM P (LP). For the P re-feed (Δ) treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Data are individual values of plants harvested 24 d after P was altered to LP or maintained at HP, whereas it constituted 10 d after the P was introduced into the medium of formerly starved plants.

Here, a positive correlation was observed for all the treatments under review in this study (Fig. 2). The slope for plants subjected to LP was approximately 16 times higher at 159 g shoot DW (g nodule DW)$^{-1}$ than about 10 g shoot DW (g nodule DW)$^{-1}$ for plants with sufficient P. At P-re-supply of formerly

P starved plants, the EURS of plants returned to a level similar to that of HP plants of about 19 g shoot DW (g nodule DW)$^{-1}$. Thus under P deprived conditions, plants were able to increase their utilisation efficiency of the rhizobial symbiosis considerably for shoot growth (Fig. 2). This is confirmed by the increases in both W_c and RG_w, expressed per unit weight for shoots (Table 1). Both the W_c and the RG_w settled back to HP control levels after P re-supply (Table 1).

6.4.2 *Nutritional analyses and use efficiencies*

Shoot P concentrations increased above those of HP controls after the first 7 d of P deficiency, but declined steadily from this point onwards. After 14 d of P deficiency this trend was reversed and shoot P of LP plants had declined to levels significantly less than that of HP controls (Fig. 3a). By the end of the 24 d experimental period shoot P concentrations under LP had reached levels (≈ 2 mg P. g^{-1} shoot DW) similar to what was recorded before any of the treatments were induced (Fig. 3a). At the same time, P accumulated to concentrations of 6.4 mg P (g shoot DW)$^{-1}$ at HP, which constituted an increase of 64% from the time (day 24) that treatments were started to the time of final harvest (Fig. 3a). This was approximately 3 times higher than the concentrations recorded under LP conditions at 24 d (Fig. 3a). After P was re-introduced into the medium of plants under LP at 14 d, shoot P concentrations were increased to levels close to that for HP controls (4.9 mg P. g^{-1} shoot DW) but were still markedly less (Fig. 3a).

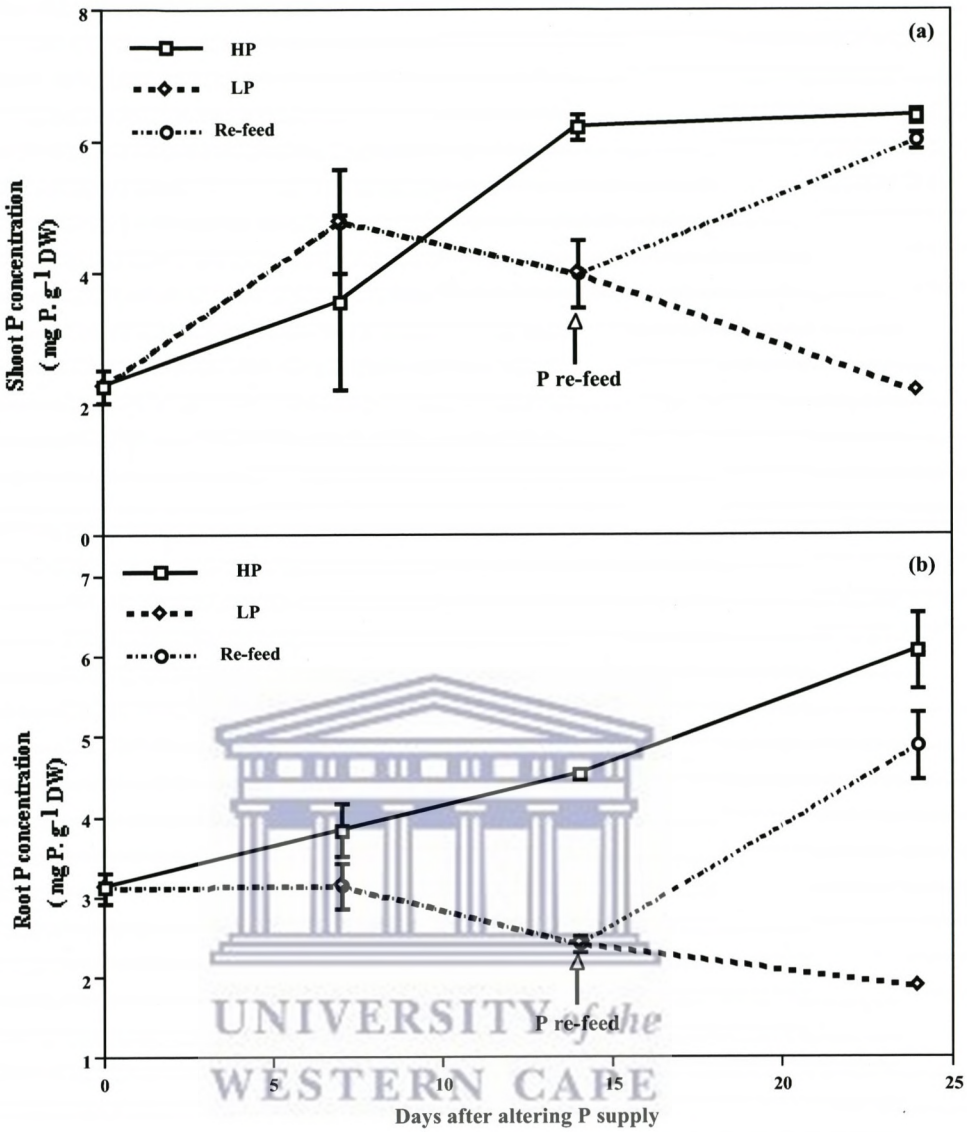

Figure 3 (a) The shoot total P concentration and (b) root total P concentration of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 μM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Error bars denote the standard error ($n = 4$).

In contrast, nodulated roots responded quicker to P starvation, with marked declines in P concentrations already notable after 7 d of P withdrawal (Fig. 3b). Nodulated roots accumulated P steadily under HP, whereas the reverse was true for nodulated roots under LP, with a steady decline in P accumulation reaching P concentrations 74% less than those of HP control plants (Fig. 3b). After P re-supply nodulated root P concentrations recovered to levels 29% less than those of HP control plants (Fig. 3b). However, this was still markedly less than the P-sufficient controls by the end of the 24 d trial period.

The lower P concentration of roots under LP by day 24 was accompanied by a 90% decrease in specific phosphorus absorption rate (SPAR) (data not shown). The diminished capacity for P uptake resulted in higher P utilisation efficiencies though, as reflected in both the higher specific phosphate utilisation rate (SPUR) and the root nodule phosphorus use efficiency (PUE) for growth (Figs. 4a,b). The SPUR corresponded to a 3.5 fold increase over HP controls (Fig. 4a), whereas PUE represented a 3 fold increase (Fig. 4b). These increases in SPUR and PUE under LP were readily reversed and reached levels similar to that of P sufficient controls once P was re-supplied (Figs. 4a,b).

Nodulated roots increased the amount of N_2 fixed per concentration of P accumulated by 3-fold under LP at 24 d (Table 2). Therefore, for a lesser concentration of P accumulated in their roots (Fig. 3b), LP plants were more efficient at utilising N derived from symbiosis, compared with HP controls (Table 2). In addition, LP plants increased the amount of N_2 fixed per nodule

dry weight significantly compared with HP plants (Table 2). The amount of N_2 fixed for both these aforementioned parameters declined to levels of HP controls after P re-supply (Table 2).

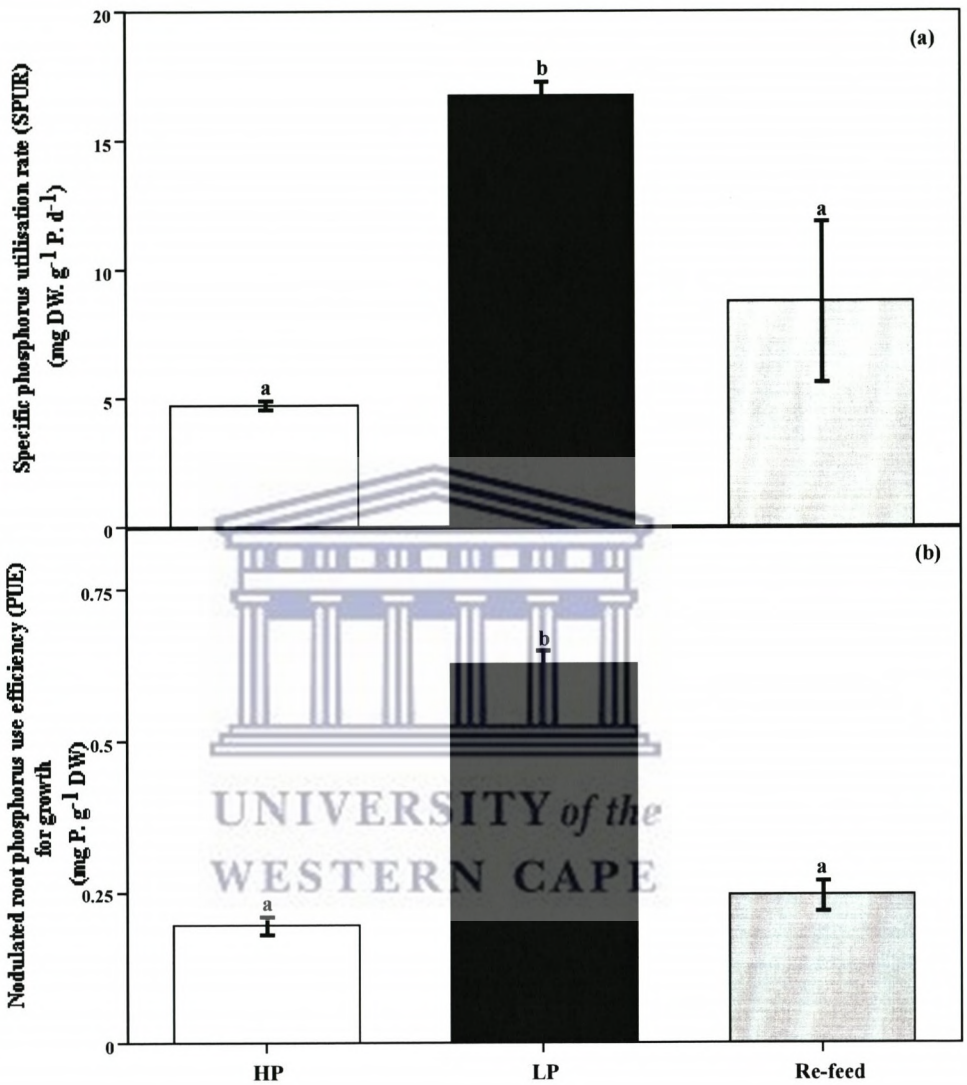

Figure 4: (a) The specific total phosphorus utilisation rate (SPUR) and (b) nodulated root total P use efficiency (PUE) for growth of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 μM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means with the error bars denoting the standard error (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

6.4.3 *Respiratory costs*

There was also a 55% decline in total CO_2 evolution from nodulated roots as measured in LP soils (Fig. 5).

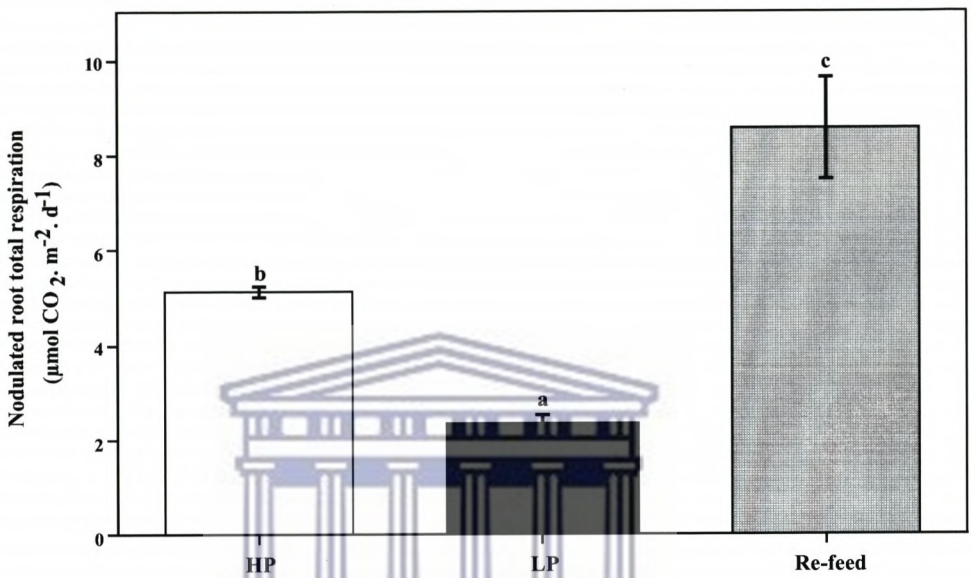

Figure 5: The respiration, measured as CO_2 evolution, in the root space of soil grown nodulated *Lupinus angustifolius* (cv. Tanjil) plants during recovery from and onset of phosphorus deficiency. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 µM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means with the error bars denoting the standard error (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

This was subsequently used to calculate the allocation between growth respiration (RG_t) and maintenance respiration (RM_t) over time. Root growth respiration (RG_t), as assessed over the period of the experiment, was increased to 12% under LP compared with HP controls of 3% and after P re-supply respiratory costs settled back to levels corresponding to P sufficient controls

(Fig. 6a,b,c). Concurrently, the RM_t, i.e. respiration related to upholding ion gradients across membranes and the resynthesis of degraded organic compounds, was decreased under P limitation (Fig. 6b). Maintenance respiration always represented proportionately more of the total respiration than growth respiration at any of the given P regimes (Fig. 6).

6.4.4 *Photosynthetic parameters*

Leaf dark respiration was unaffected by P supply over the assessment period in this study, remaining constant at approximately 1.25 μmol. mol. s^{-1} (Table 3). Similarly there were no changes observed for any of the other leaf parameters related to photosynthetic capacity (e.g. specific leaf area, specific leaf mass, etc.) under the P limiting conditions imposed in this study. All these translated into no changes in the net photosynthetic rate (PN_{max}) either expressed per specific leaf mass or per area of leaf under LP (Table 3). Likewise, the biomass-based net assimilation rate showed a similar trend (Table 3).

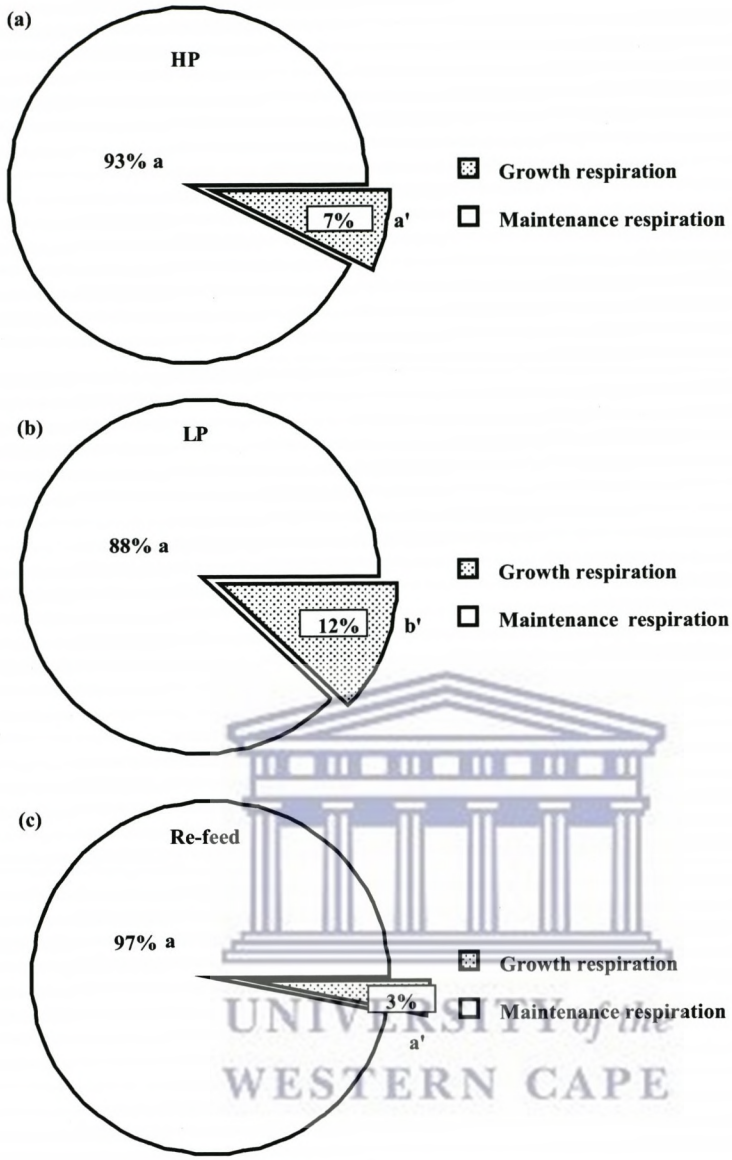

Figure 6: The growth and maintenance respiration as a percentage (%) of the total respiration of nodulated roots for (a) P sufficient (HP) control plants, (b) P deficient (LP) plants, and (c) P re-feed of formerly starved plants. Nodulated *Lupinus angustifolius* (cv. Tanjil) plants were soil grown and the recovery from and onset of phosphorus deficiency assessed. Plants were supplemented twice weekly over the course of 24 d with a N free Long Ashton nutrient solution containing either 2 mM P (HP) or 2 μM P (LP). For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means (n = 4). Primed letters indicate significant differences between each treatment ($P \leq 0.05$).

Table 3: Leaf dark respiration (µmol. mol^{-1}. s^{-1}), LA (m^2. $plant^{-1}$), LAR (m^2. g^{-1}), SLA (m^2. g^{-1}), SLM (g. g^{-1}), LWR (g. g^{-1}), LER (m^2. day^{-1}), NAR (g. m^{-2}. day^{-1}), Net photosynthesis rate (PN_{max})/per leaf dry weight (µmol CO_2. m^{-2}. s^{-1}. DW), Net photosynthesis rate (PN_{max})/per LA (µmol CO_2. m^{-2}) of plants grown either at HP or LP. For the P re-feed treatment, half of the plants that were formerly given a LP solution were re-supplied with a HP solution at 14 d. The other half was kept at LP and served as P-deficient plants. These were compared with P sufficient plants, which remained at HP for the duration of the experiment. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

	Treatments		
Leaf Paramaters	**HP**	**LP**	**Re-feed**
Leaf dark respiration	1.22 ± 0.32 a	1.17 ± 0.20 a	1.37 ± 0.57 a
Leaf area (LA)	6.87 ± 0.50 a	6.20 ± 0.47 a	6.33 ± 0.35 a
Leaf area ratio (LAR)	7.06 ± 0.40 a	7.48 ± 0.72 a	7.34 ± 0.76 a
Specific leaf area (SLA)	0.04 ± 0.01 a	0.03 ± 0.01 a	0.04 ± 0.01 a
Specific leaf mass (SLM)	0.019 ± 0.01 a	0.021 ± 0.01 a	0.017 ± 0.01 a
Leaf weight ratio (LWR)	0.33 ± 0.01 a	0.40 ± 0.01 b	0.37 ± 0.01 ab
Leaf expansion rate (LER)	0.14 ± 0.01 a	0.13 ± 0.01 a	0.13 ± 0.01 a
Net assimilation rate (NAR)	41.33 ± 1.56 a	40.29 ± 5.89 a	41.26 ± 9.30 a
Net photosynthetic rate (PNmax) per SLM	0.65 ± 0.07 a	0.52 ± 0.08 a	0.66 ± 0.05 a
Net photosynthetic rate (PNmax) per LA	18.24 ± 1.23 a	16.87 ± 1.29 a	18.02 ± 1.02 a

6.5 Discussion

Several morphological (e.g. smaller nodules) and physiological adaptations, including rapid adjustment of nodule biomass features and concurrent amendment of maintenance and growth respiration geared towards sustaining efficient N_2 metabolism as a response to and during recovery from P deficiency were observed for *Lupinus angustifolius*. In this study, the N_2 fixation response to and the subsequent recovery from P deficiency may be correlated to morphological and physiological changes apparent in nodules after prolonged P deprivation. Some of these responses are consistent with earlier reports, where it was found that P stress might increase nodule number and also that nodule mean mass is particularly sensitive to P deficiency (Israel, 1993; Ribet & Drevon, 1995).

Lupin plants in this experiment were well established and responses to P deficiency were expected to occur after some delay. This was indeed the case for dry matter accumulation, as total dry matter yield was not reduced significantly within the duration of the experiment. In contrast, physiological responses were more rapid despite having started off with a reasonably high internal P concentration.

Several studies have proposed a functional optimum for N_2 fixation at a given shoot P concentration (Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002; Schulze *et al.*, 2006). For instance, under LP, white lupin requires a shoot P of about 3.05 mg P. g^{-1} DW for N_2 fixation to function optimally for a similar period of assessment (21 d; Schulze *et al.*, 2006) to that reported here. The optimum P

level in shoots for the continuation of sufficient N_2 fixation at HP in this study is 3 times higher than the levels reported under LP, which corresponded to a marked decline in N_2 fixation under P deficiency conditions imposed here. Indeed, the value reported for this study of 2.0 mg P. g^{-1} DW of shoot for LP corresponded to a decline in N_2 fixation of 15% (Fig. 2). During recovery of SNF from P-deficiency at 24 d, the shoot P concentration was altered to levels similar to those for HP plants and this translated into a concomitant increase in N_2 fixation (Fig. 2). The discrepancy in levels of optimal P in shoots for *L. albus* (≈ 8.0 mg P. g^{-1} shoot DW; Schulze *et al.*, 2006) and *L. angustifolius* (≈ 6.2 mg P. g^{-1} shoot DW, this study) under HP control conditions might be species related. Alternatively, it might be a reflection of metabolism as it pertains to cluster root formation in the case of *L. albus* in that study (Schulze *et al.*, 2006). Cluster roots would optimise P acquisition via organic acid exudation into the surrounding medium (Johnson *et al.*, 1996). Nevertheless, these data support the notion that legume host plants may require an internal P concentration for optimal N_2 fixation and physiological function (Israel, 1987; Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002; Schulze *et al.*, 2006).

Furthermore, the increased PUE and SPUR of nodulated roots found in this study may also reflect the low P demand of *Bradyrhizobium* spp. in general. *Bradyrhizobium* sp. Vigna has previously been shown to be a P-efficient strain; that is, it was able to maintain a reasonable amount of N_2 fixation even at low levels of P (Beck & Munns, 1984). This concurs with the higher amount of N fixed expressed either per concentration P of nodulated root or per nodule dry weight, for P-deficient plants (Table 2) than for P sufficient

plants. The tendency for increased efficiency under LP has been noted previously for tolerant varieties of common bean, which consistently demonstrated a 3 times higher N-fixed/P concentration over time than plants with adequate P (Vadéz *et al.*, 1999).

Furthermore, results from this study suggest that lupins are able to withstand abrupt P withdrawal for up to at least 14 d, with a decline of symbiotic nitrogen fixation (SNF) capacity, judging from the altered %Ndfa associated herewith, at 24 days, (Fig. 1). This is in agreement with a previous study, which observed N_2 fixation to be unaffected by LP and only manifesting late (≈ 27 d) in the growth cycle of *L. albus* under P deficient conditions (Schulze *et al.*, 2006). The %Ndfa recovered within 10 d to levels similar to those of P sufficient controls after P re-supply midway (14 d) through P deficiency (Fig. 1). A similar response of N_2 fixation recovery as observed for lupin was noted for soybean, in which seven days was the turn around time for recovery of N_2 fixation from P deficient conditions (Israel, 1993). Responses to abiotic stress, apart from being species specific, will also be a reflection of other features, including nodule anatomy (Walsh, 1995). Although these two systems represent contrasting nodule types, the evidence here and elsewhere (Israel, 1993), suggest that these responses to P re-supply may be similar and may be related to direct consequences of P effects on nodule functioning (Israel, 1993; Le Roux *et al.*, 2008).

One of the more pertinent questions of this study was to ascertain how respiratory costs would be altered to sustain N_2 fixation efficiency. Lower

root respiration, measured as CO_2 evolution, was demonstrated under LP conditions. However, this may not reflect true metabolic activity as it prevails in nodulated roots, since a great amount of the released CO_2 may very well be refixed via PEPc, especially under LP. Alternatively, some P efficient bean genotypes are known to invest in adventitious roots under LP, which would have higher specific root length and are associated with lower metabolic construction costs (Lynch, 2007). In the absence of detailed root anatomical and morphological assessments, this conclusion is at best speculative in this study but seems plausible.

However, the decline in total respiration with the onset of P deficiency concurs with previous work on LP versus HP in mychorrhizal roots (Peng *et al.*, 1993). Similarly, net nodulated root respiration as reported here, has previously been shown to decline under LP for white lupin (Schulze *et al.*, 2006). In that instance, a much higher CO_2 fixation rate of nodulated roots under LP was eventually able to offset the lower net CO_2 evolution of LP plants. We have previously reported (Le Roux *et al.*, 2008) that the CO_2 fixation rates increase dramatically under LP conditions due to the action of PEPc-MDH-ME alternative route engagement and therefore it seems plausible that although there was a decline in root respiration in general, this may have been transient and that a related mechanism, as observed for *L. albus*, may be at work in *L. angustifolius*. It was proposed that plants that have a higher efficiency of P acquisition per unit C spent for root construction and maintenance would perform better under LP (Nielsen *et al.*, 2001). This may

hold true here with lower root construction costs associated for nodulated lupins in this study.

During the vegetative growth phase of plants, it is thought that a larger fraction of total respiration may be expended on maintenance at the expense of growth (Amthor, 1989). Due to the protein-richness of legumes in general the maintenance respiration component is expected to increase since the C for building of proteins require relatively more maintenance as opposed to that for lignin and cellulose (Penning de Vries, 1975; Johnson, 1990). The components of total root respiration revealed a greater percentage of respiratory energy devoted to maintenance respiration than to growth respiration. However, with P deficiency, the allocation toward maintenance respiration declined in favour of growth respiration. This concurs with the increase in root growth under LP, suggesting that proportionally more respiratory energy was allocated to growth of new tissue. Moreover, the decline in maintenance respiration under LP is underpinned by the lower P uptake and SNF rates. Upon re-feeding, the total root respiration increased with a simultaneous increase in the percentage of maintenance respiration, reflecting that energy was spent on BNF and P uptake.

The changes that occur during response to P deficiency in nodulated roots versus the maintenance of all photosynthetic parameters (except for the leaf weight ratio [LWR]) in our study apparently support the view that P deficiency elicits a direct response from root nodule functioning rather than from host plant growth (Tables 1 & 3). Leaf weight ratio increased when

subjected to LP (Table 3). These observations with *L. angustifolius* starkly contrast those for *L. albus* where it was proposed that plant growth was more significantly affected (Schulze *et al.*, 2006). The tendency for an increased LWR under LP was instantly reversed once the roots were supplemented with P again. The higher LWR under P deficiency reflects the investment into leave biomass and may be interpreted as optimising light harvesting for whole plant photosynthetic capacity to proceed unperturbed. This was previously observed for P-efficient potato genotypes (Balemi, 2009). Indeed, results here corroborate this notion in that there was no effect of P supply on net photosynthetic rate expressed both per unit leaf area (PN_{max}/LA) and per unit specific leaf mass (PN_{max}/SLM) under LP (Table 3). In addition to unchanged photosynthetic rates, biomassed-based net assimilation rate (NAR) and leaf dark respiration also remained unchanged at LP (Table 3).

In conclusion, lupins show clear adaptive features, encompassing both physiological and morphological parameters associated with nodule function. Physiological adaptations could involve a switch to a highly efficient nodule metabolism related to the optimisation of N_2 fixation under LP and an increase in growth respiration relative to investment in maintenance respiration.

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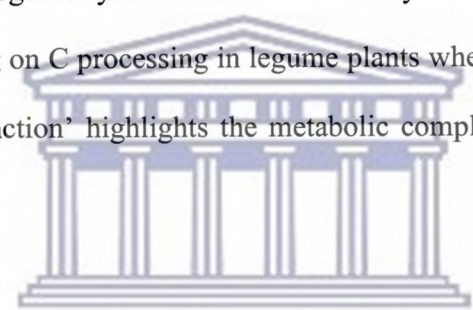
CHAPTER 7

General Conclusion

7.1 Overview

The effects of P deficiency have received considerable attention in legumes, due to the greater responsiveness of the N_2 fixing process to P as depicted by the numerous studies in Table 1. Most such studies addressed the perturbations in biological nitrogen fixation (BNF) in response to changes in various host growth effects, the oxygen diffusion barrier, amino acid feedback regulation, photosynthetic supply, and proton efflux from nodules. Some notable shortcomings of the aforementioned studies are a lack of investigation of both the energy costs and the alternative metabolic routes associated with C and N metabolism in legume systems under P deficiency. Phosphorus deficiency has specific bearing on C processing in legume plants where its interaction with N at the PEP 'junction' highlights the metabolic complexity in such symbiotic systems.



This thesis examined the alterations in photosynthetic and respiratory C metabolism in response to P deficiency and its associated affects on N_2 fixation. This study ascertained the 'late' responses (*see* Hammond *et al.*, 2004), which affect morphological, physiological or metabolic changes in legumes, as these are seen to be more specific in response to the stress imposed on the system (Nilsson *et al.*, 2010). Moreover, these were contextualised in terms of short-term P deficiency (14 d) (Chapters 3 & 4) versus prolonged (24 d) P deficiency responses (Chapters 5 & 6). The recovery after subsequent P alleviation was also assessed (Chapter 6). Furthermore, the accumulation of organic acids, most notably malate, was closely scrutinised in relation to its role in the N_2 fixation process.

Table1: The effects of P deficiency on various aspects of nodule development and nodule metabolism. The information was sourced from various publications. ***Note***: * P deficiency is the only known abiotic stress that increases nodule permeability. [a] *Not determined*

P deficiency effect	Legume species	References
	Nitrogen fixation	
Increased	*Phaseolus vulgaris spp.; Vigna unuguilata*	Jebara *et al.*, 2005
Unchanged	*Lupinus angustifolius*	Le Roux *et al.*, 2006; 2009
Decreased	*Medicago sativa; Glycine max; Lupinus albus*	Drevon & Hartwig, 1997; Schulze *et al.*, 2006
	O_2 metabolism/Respiration	
Increased *	*Phaseolus vulgaris spp.; Vigna unuguilata; Medicago truncatula*	Schulze & Drevon, 2005; Alkama *et al.*, 2009; Sulieman *et al.*, 2008
Unchanged		-
Decreased		-
	N metabolism	
Increased	*Phaseolus vulgaris; Vigna unguiculata*	Sulieman *et al.*, 2008
Unchanged	*Phaseolus vulgaris; Lupinus angustifolius*	Olivera *et al.*, 2004; Le Roux *et al.*, 2009
Decreased	*Glycine max*	Le Roux *et al.*, 2009
	C metabolism	
Increased	*Lupinus albus, Lupinus angustifolius*	Schulze *et al.*, 2006; Le Roux *et al.*, 2008
Unchanged	*N.D.*[a]	-
Decreased	*Trifolium repens*	Høgh-Jensen *et al.*, 2002
	H^+ release	
Increased	*Phaseolus vulgaris spp.; Vigna unuguilata*	Tang *et al.*, 2001; Kouas *et al.*, 2008; Alkama *et al.*, 2009
Unchanged	*N.D.*	-
Decreased	*N.D.*	-
	Nodule biomass	
Increased	*N.D.*	-
Unchanged	*Lupinus angustifolius, Glycine max*	Le Roux *et al.*, 2009
Decreased	*Glycine max; Medicago sativa*	Drevon & Hartwig, 1997, Schulze *et al.*, 2006

7.2.1 Key findings

This study demonstrated some common responses of symbiotic legumes to LP, albeit that we have considered the dynamics of such responses after abrupt P withdrawal as oppose to steady-state conditions. Under the latter, P stress is imposed on the system since the seedlings emerge up until they are harvested. Thus, discrepancies abound with results from various microrray studies reflecting the different approaches attempted to address effects of P deficiency on growth and metabolism (Misson *et al.*, 2005; Morcuende *et al.*, 2007). To this end, we highlight here some key new features, which we propose should be exploited in future research studies that follow below.

- This study demonstrated species-specific responses of N_2 fixation to P deficiency, which may also underpin different regulatory mechanisms. For instance, feedback inhibition of N_2 fixation by accumulated N metabolites, probably ureides or specific amino acid(s), is emerging as the regulatory trigger under LP in the ureide-exporting legume types, including soybean from our study (Vadéz *et al.*, 1999; Almeida *et al.*, 2000; Tang *et al.*, 2001; Olivera *et al.*, 2004).
- Under short-term LP, an efficient metabolic P_i recycling mechanism, through the engagement of the alternative PEPc-MDH-ME route was implicated in the maintenance of N_2 fixation in the apparent more tolerant lupin. Radio isotope ^{14}C tracer studies and subsequent organic acid fractionation, confirmed the induction of the aforementioned alternative route in P-starved nodules. These are in accordance with previous studies (Theodorou & Plaxton, 1993, Le Roux *et al.*, 2006).

- The observed induction of the PEPc-MDH-ME route in nodulated root lupins increased the capacity of nodules to procure malate for bacteroid respiration, which serves to sustain SNF. Two novel cytosolic MDHs (cMDHs), one for HP controls and another for plants grown under LP were identified in our study. The high malate concentrations reported here are speculated to have arisen through the apparently distinct form of cMDH under LP. Future expression studies with the isolated cMDHs will elucidate this.

These aforementioned responses to P deficiency mainly reflect the dependence on plant growth history and effects on nodulation and nodule anatomy as noted by Walsh (1995). In addition, the responses to P deficiency are directly related to the severity and duration of the stress (Walsh, 1995; Høgh-Jensen *et al.*, 2002). What follow relates the adaptive responses of lupin plants, because of its perceived robustness to LP in the short-term, to the extent and intensity of P deficiency. Lupin plants were well developed before the P stress treatments were induced in our studies and responses were expected to occur after some delay.

- The most notable lagged response was observed for the decline in %Ndfa, which is directly proportional to the rate of N_2 fixation. Similarly, other related parameters (e.g. photosynthetic activity) remained largely unchanged, likely due to increases in associated shoot construction costs and growth respiratory costs under LP, which upheld growth.

- An observed increase for the leaf weight ratio (LWR) may have been an adaptation to optimize light interception for the purpose of driving photosynthate (C) supply from the shoot to the nodules. In addition, the EURS, an indication of the utilisation efficiency of the rhizobial symbiosis for shoot growth, may be a useful parameter to consider in programmes, which attempt to breed legumes with tolerance for LP.
- Root respiration and N_2 fixation per unit root mass were lower under LP, with most of the assimilates partitioned to the root system used to increase the size of the root system. This is evidenced by a typical response under LP of a preferential distribution of biomass to roots as oppose to shoots; thus an increase in root:shoot ratio under LP. This is further confirmed by the considerable decoupling of the legume host nutrient uptake and plant growth, with an observed increase in nodulated growth respiration relative to maintenance respiration under LP. Thus, changes in relative growth rate, particularly that of nodulated roots, rather than changes in resource uptake are of consequence here.
- The perceived increased ratio of non-photosynthetic CO_2 fixation to N_2 fixation under prolonged LP conditions, reflects the interrelationship between organic acid synthesis and N_2 fixation and gives strong credence to the proposal that accumulation of organic acids (or specific organic acids; e.g. malate) above a certain threshold will be inhibitory to N_2 fixation.

- Respiratory costs involved in sustaining N_2 fixation efficiency of nodulated roots, expressed both as O_2 consumption and CO_2 evolution were impaired under prolonged LP conditions. A possible attempt to offset impaired nodulated root respiration was through producing smaller nodules. Legumes that form smaller nodules under LP increase their surface: volume ratio, which are supported by increased respiration rates and unchanged SNF as reported by Schulze & Drevon (2005). Alternatively, initial declines of respiration could eventually be offset by increased CO_2 fixation (Schulze *et al.*, 2006). Our observed results are supportive of this conclusion.

Finally, this work has shown that as P deficiency develops under prolonged LP, whole plant morphological responses are initiated in lupins. These morphological adjustments included a proportional increase in leaf mass and reduction in nodule mass. The observed phenotypic plasticity of leaves and nodules was also noticeable after alleviation of P limitation. Therefore, regulatory responses to P deprivation in lupin plants involved increasing partitioning to nodulated roots (for P acquisition) and leaves (for C acquisition) and by reducing nodule mass (N acquisition), similarly to that for white clover plants (Høgh-Jensen *et al.*, 2002). Moreover, attempts to optimise nodule metabolism was through accentuated use efficiencies for P (e.g. PUE; SPUR), under LP.

7.2.2 *Measuring plant-associated nitrogen fixation*

Several studies involving legumes are concerned with evaluating N_2 fixation in host plants, because of its importance in cropping systems and its consequent effects on soil N dynamics (Danso *et al.*, 1992). The methodologies for quantifying N_2 fixation may be instantaneous (*e.g.* acetylene reduction assay [ARA], hydrogen evolution), which gives a measure of activity only at the time of sampling. In contrast, the ^{15}N natural abundance (NA) and ^{15}N isotope dilution (NID) methods feature among the more common ^{15}N isotope methods, because it estimates time-integrated rates of symbiotic N_2 fixation during the growing period of crops. In recent times, there has been increased interest in the NA method, because of its perceived accuracy and ease of use on various large-scale fields (Gathumbi *et al.*, 2002; Pons *et al.*, 2007). Methodologically it is important to consider the choice of technique and the limitations of each for measuring N_2 fixation capacity.

The NA method utilises the small differences, expressed as $\delta^{15}N$, in ^{15}N natural abundance between N_2 fixing and non-fixing plants. Variations of $\delta^{15}N$, signified as the relative deviation (in ‰), arise from small discrepancies of ^{15}N abundance of atmospheric N_2. For plants growing non-symbiotically and at high mineral N, the $\delta^{15}N$ is set to nil, because they derive all their N from the soil. One possible limitation of the NA technique is the wide temporal and spatial variations of soil $\delta^{15}N$, due to heterogenous distribution of soil N and also processes such as nitrification, denitrification, and mineralization occurring in soils. The other problem relates to finding a

suitable reference plant, having soil N acquisition characteristics similar to those of the N_2 fixing plants being assessed (Boddey *et al.*, 2000).

We have assessed N_2 fixation through both these methods in studies undertaken here, and although not directly comparable, we have found a small degree of variability, based only on the percentage decline of N_2 fixation capacity under the same LP conditions. The decline of biomass-based N_2 fixation as assessed through ARA amounted to 22%, whereas for the ^{15}N isotope natural abundance this decline was only 15% under LP in relation to HP control plants. Both the ARA and ^{15}N natural abundance isotope techniques, then, appears to have been sensitive diagnostic tools for detecting N_2 fixation capacity where plants were either grown in pot culture or hydroponics. In any case, the main objective of our studies was merely to compare relative differences between different legume species and induced treatments (*i.e.* HP vs. LP). Nevertheless, we extended the understanding of the sensitivity of N_2 fixing systems to the nutritional variable of LP. Such pot and hydroponics experiments under glasshouse conditions will still need to be validated under field conditions, because a poor correlation between legumes grown under these varying conditions (Hernandez *et al.*, 1993).

7.3 Future work

Although N_2 fixation may be highly responsive to any alterations in P concentrations, a lagged response to LP could be due to the high accumulation of P_i as proposed here. This may be an innate ability of more tolerant legume varieties. In this regard, the use efficiencies, both for P (and N), are key

determinants in adaptability of legumes to changes in the environment. This study may serve to inform management strategies to improve nutrient use efficiencies (P and/or N) of various agronomically important legumes and is regarded as an important strategy to address the problem of declining arable land.

Furthermore, there is a need to link transcriptional and biochemical responses with regulatory mechanisms involving quantitative trait loci (QTLs) associated with tolerant cultivars. This remains crucial to determining the relevance of those genes to tolerance traits. The advent of tailored genomic techniques has placed researchers ever closer to identifying patterns of gene expression and gene function. In this regard, a study on the regulatory role of small RNA (sRNA), most notably miR399, in response to P deficiency is currently underway. The plasticity of regulatory and signalling mechanisms involved in P acquisition will be useful for the ultimate manipulation of legume systems.

Integrated analyses involving transcriptomics, metabolomics, proteomics and enzyme biochemistry is key to identifying important roles of certain genes/pathways of symbiotic host plants to P deficiency. The induction of the alternative PEPc-MDH-ME route is one such case in point. We confirmed the key role of malate in regulating N_2 fixation activity. This should pave the way for exploitation because of its central role in metabolism and its apparent utilization in almost every major organelle in which it features, and in symbiotic plants in particular. Already, the over-expression of a neMDH in alfalfa plants have been shown to contribute to microbial diversity and

increased availability of several macronutrients, including P, in the rhizosphere of plots on which such transgenic plants were grown (Tesfaye *et al.*, 2003).

7.3 Concluding remarks

From the onset, this manuscript argues for the need to integrate legumes more prominently into modern-day farming systems, because they have a role to moderate the environmental impact of agriculture. However, in many low-input agricultural systems, P_i is one of the most limiting mineral nutrients for plant production in general, but for legumes in particular. The renewed upsurge in research on plant mineral nutrition applying molecular biology and plant physiology to dissect the mechanisms underlying the nutrient acquisition and utilisation is great. This is especially important in a complex mutualistic relationship of this nature, which poses a challenge with regard to how mineral resources are shared for the benefit of both partners, but ultimately for the betterment of the ecology of an entire system. Molecular tools will be imperative to advance this agenda. In this way, as the molecular physiology of the mechanisms underlying the regulation of mineral acquisition and utilisation emerges it should pave the way for the improvement of legume crops related to improved P acquisition efficiencies.

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