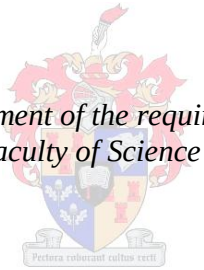
Do cluster roots contribute to the costs of carbon and nitrogen metabolism during variations in phosphate supply in the legume *Lupinus albus*?

by
Rochelle Thuynsma



Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

Supervisor: Prof. Alexander Joseph Valentine
Co-supervisor: Dr. Aleysia Kleinert

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DECLARATION

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ABSTRACT

The generally low concentrations of P and N in the soil, causes most plants to experience nutrient deficiency during their life cycle. Lupins can rely on both cluster roots and nodules for P acquisition and biological nitrogen fixation (BNF) respectively. The legume Lupinus albus is able to survive under low nutrient conditions, because it has two specialized belowground organs for the acquisition of N and P. In this regard, cluster roots increase P uptake and root nodules acquire atmospheric N2 via biological nitrogen fixation (BNF). Although these organs normally tolerates low P conditions, very little is known about their physiological and metabolic flexibility during variations in P supply. Furthermore, the resource allocation (C, N and P) between cluster roots and nodules has also been largely understudied. The aim of this investigation was therefore to determine the resource allocation, physiological and metabolic flexibility of these organs during variations in P supply.

Although variation on P supply had no effect on the total biomass, there were significant differences in specialised below-ground organ allocation to cluster roots and nodule formation. Cluster root formation and the associated C-costs increased during low P supply. In contrast to the cluster root decline at high P supply, there was an increase in nodule growth allocation and corresponding C-costs. Since cluster roots were able to increase P acquisition under low P conditions, this below-ground investment may also have benefited the P nutrition of nodules. These findings provide evidence that when lupins acquire N via BNF in their nodules, there may be a trade-off in resource allocation between cluster roots and nodules.

The short-term elevated P supply, caused an increased allocation of C and respiratory costs to nodules, at the expense of cluster roots. This alteration was also reflected in the increase in nodule enzyme activities related to organic acid synthesis, such as Phosphoenol-pyruvate Carboxylase (PEPC), Pyruvate Kinase (PK), Malate Dehydrogenase (NADH-MDH) and Malic Enzyme (ME). In cluster roots, the elevated P conditions, caused a decline in these organic acid synthesizing enzymes. This suggests that during short-term elevated P supply, there is a great degree of physiological and metabolic flexibility in the lupin nutrient acquiring structures.

OPSOMMING

Die algemeen lae konsentrasies van fosfaat en stikstof in die grond , veroorsaak dat die meeste plante voedingstekorte ervaar tydens hul lewensiklus. Lupiene kan staatmaak op beide groep-wortels en wortel-knoppies vir P verkryging en biologiese stikstofbinding onderskeidelik. Die peulplant *Lupinus albus* is in staat om te oorleef onder lae voedings toestande , as gevolg van die twee gespesialiseerde ondergrondse organe vir die verkryging van stikstof en fosfaat. In hierdie verband verhoog groep-wortels fosfaat opname en wortel-knoppies verkry atmosferiese stikstof via biologiese stikstofbinding. Alhoewel hierdie organe normaalweg lae fosfaat toestande verdra , is baie min bekend oor hul fisiologiese en metaboliese buigsaamheid tydens variasies in fosfaat aanwending. Daar is verder ‘n tekort aan die studie van hulpbron toekenning tussen groep-wortels en wortel-knoppies. Die doel van hierdie ondersoek was dus om die toekenning van hulpbronne , fisiologiese en metaboliese buigsaamheid van hierdie organe tydens variasies in fosfaat aanwending te bepaal.

Variesie in fosfaat verskaffing het geen invloed op die totale plant biomassa gehad nie, maar daar was wel ‘n beduidende verskil in gespesialiseerde ondergrondse toekenning tussen groep- wortels en wortel-knoppies. Groep-wortel vorming en die gepaardgaande koolstof koste het toegeneem met lae fosfaat verskaffing. In teenstelling met die groep-wortel daling met hoë fosfaat verskaffing, was daar 'n toename in groei van wortel-knoppies en die ooreenstemmende koolstof koste daarvan. Aangesien groep-wortels in staat was om fosfaat verkryging te verhoog onder lae fosfaat toestande, mag hierdie ondergrondse belegging bygedra het tot die voeding van wortel-knoppies . Hierdie bevindings bewys dat lupiene afhanklik van wortel-knoppies ‘n wisselwerking in toekenning van hulpbronne, tussen groep-wrotels en wortel-knoppies handaaf.

Kort termyn verhoogde fosfaat aanwending veroorsaak 'n verhoogde toekenning van koolstof en respiratoriese energie na wortel-knoppiess, ten koste van groep-wortels . Hierdie verandering is ook weerspieël in die toename in wortel-knoppie ensiem aktiwiteit in verband met organiese suur sintese (PEPC PK,MDH,ME) . In groep-wortels, het die verhoogde P toestande verder 'n afname in die organiese suur produserende ensieme veroorsaak. Dit dui aan dat tydens kort termyn verhoogde P aanwending, daar 'n groot mate van fisiologiese en metaboliese buigsaamheid in die lupiene voedingstowwe verkryging strukture plaasvind.

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%	percentage
% NDFA	percentage nitrogen derived from atmosphere
˚C	degrees Celsius
ADP	adenosine 5’-diphosphate
Al	aluminium
ANOVA	analysis of variance
APase	(intracellular) acid phosphatase
Asn	asparagine
Asp-AT	aspartate-aminotransferase
AS	asparagine synthase
ATP	adenosine 5’-triphsophate
ATPase	adenosine triphosphate hydrolase
BNF	biological nitrogen fixation
Ca^{2+}	calcium
cm^2	square centimetre
Co^{2+}	cobalt
CO_2	carbon dioxide
CS	citrate synthase
d	day
DW	dry weight
Fe	iron
Fd	ferredoxin

g	grams
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthase
H^+	hydrogen ion, proton
H_2	dihydrogen
H_2O	water
dH_2O	distilled water
ddH_2O	double distilled water
HCO_3	bicarbonate
$H_2PO_4^-$	dihydrogen phosphate ion
HPO_4^{2-}	phosphoric acid ion
K	potassium
kDa	kilodalton
M	molar
MDH	malate dehydrogenase
ME	malic enzyme
Mg^{2+}	magnesium
m^2	square meter
ml	millilitre
mg	milligram
mM	millimolar
Mn^{2+}	manganese

Mo-Fe	molybdum-iron complex
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidised form
NADP(H)	nicotinamide adenine dinucleotide phosphate, reduced form
N	nitrogen
N_2	dinitrogen
NH_3	ammonia
NH_4^+	ammonium ion
NO_3^-	nitrate
O_2	oxygen
PEPC	phosphoenolpyruvate carboxylase
PEP	phosphoenolpyruvate
Pi	inorganic phosphate
pH	acidity
PK	pyruvate kinase
Pmax	maximum rate of photosynthesis
PNUE	photosynthetic nitrogen use efficiency
ppm	part per million
PPP	phosphate pentose pathway
PPUE	photosynthetic phosphate use efficiency
RGR	relative growth rate

s	seconds
SNAR	specific nitrogen acquisition rate
SNK	Student Newman Kuehl's multiple-range test
SPAR	specific phosphate acquisition rate
SNUR	specific nitrogen utilisation rate
SPUR	specific phosphate utilisation rate
TCA-cycle	tricarboxylic acid cycle
T-test	statistical student's t distribution
µmol	micromole
µl	microliter
µM	micromolar
δ15N	nitrogen isotopic ratio

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Legumes appeared around 60 million years ago, in the late Cretaceous period after the great K/T extinction when many angiosperm families also appeared. They are the third largest of the flowering families and also contain some of the most variable species (Sprent, 2006). They can be divided into three sub families, *Ceasalpinioideae*, *Mimosoideae* and *Fabiodeae*/*Papilionoideae* (Sprent, 2006). Within these sub families are approximately 18 000 species of legumes. The focus here will be on the genus *Lupinus*. *Lupinus* or Lupins, from the sub family *Fabiodeae*/*Papilionoideae*, are the only legumes not known to form mycorrhizal associations (Sprent, 2006). Lupins are often characterized by their elongated flowers that come in an assortment of colours (Doyle and Luckow, 2003).

Legumes are second only to the Graminiae (grasses/ cereals) in their importance to humans as a food, forage, agronomic and ecological contributor (Polhill *et al*., 1981). Within the 670 genera and 18 000 – 19 000 species of legumes, many important agricultural grain, forage and forestry legumes are used today (Polhill *et al*., 1981). Legumes, including grain and forage legumes, are grown on some 180 million Ha worldwide and constitutes 27% of the primary crop production worldwide (Vance *et al*., 2000). Legumes can be divided into old world (Mediterranean and East African) and new world (American) legumes, which can again be divided into temperate and tropical legumes (Sprent, 2006). The major difference in the afore mentioned groups is the choice of fixed N translocation (Doyle and Luckow, 2003). Temperate legumes (lupins, pea) translocate fixed N as amides, predominantly asparagine and glutamine while tropical (soybean, cowpea, common bean) legumes export ureides such as allantoin and allantoic acid (Doyle and Luckow, 2003).

They are thus highly variable and their symbiotic relationship with rhizobia characterizes most of their evolutionary importance. For nodulation to have developed communication between plant and bacteria is crucial (Geurts *et al*., 2012). The first step in this evolutionary process would have been the establishment of communication between a plant and bacteria followed a modified infection process. The infection process removed more complex root hair formation signals allowing bacterial infection, without a host immune response (Geurts

et al., 2012). It is thus essential to look at this process in detail to better understand the integral part nodulation plays in legume development.

1.2 Nodulation of plant root systems

Legumes are well known for their relationship with unicellular bacteria responsible for nodule formation and biological nitrogen fixation (Indge, 2000). There are several groups of N-fixing bacteria that can broadly be divided into free living and symbiosis forming groups. Free living N-fixing bacteria are characterised by aerobic (*Azotobacter*) and anaerobic bacteria (*Clostridium*) (Chenn, 1999), while symbiosis forming bacteria can be divided into legume (*Rhizobia*) and non-legume nodulating (*Frankia*) bacteria (Chenn, 1999). *Rhizobia* symbiosis is the most common occurring symbiosis and almost entirely restricted to legumes (Geurts *et al*., 2012). These proteo-bacteria can be divided into alpha and beta groups depending on their evolutionary origin (Sprent, 2006). Nodulating proteo-bacteria can then again be divided into classical or other/unknown. Classical proteo-bacteria include *Mesorhizobium* and *Rhizobium*, whereas other/unknown refers to uncharacteristic proteo-bacteria isolated from root nodules that have yet to be identified (Sprent, 2006).

Biological nitrogen fixation is the entry point for N into the natural N cycle (Valentine et al., 2011). Through the process of nodulation, *Rhizobacteria* colonize the plant root system and allow for BNF with the N_2-fixing enzymes being present only in the symbiotic bacteria and not in the plant itself (Valentine *et al*., 2011). This symbiosis ultimately involves the exchange of fixed N from the bacteria, in the form of NH_4^+, for C resources and nutrients from the plant (Vance, 2002). Nodulation is however not obligatory, with both plant and bacteria being free living. Once the bacteria become an endosymbiont however, they cannot return to a free-living form (Geurts *et al*., 2012). Symbiotic bacteria are housed in specialised root derived structures known as nodules. Nodules are formed from primordial cells in the root cortex (Moran, 2000). At the core of these structures are large amounts of infected bacterial cells, surrounded by an exclusion membrane known as the symbiosome. The symbiosome is crucial in the plant-bacteria nutrient exchange and facilitates the exchange of N for C (Geurts *et al*., 2012).

1.2.1 Initiation of nodulation

Nodulation comprises of two processes, infection of the root cells and nodule formation. These processes occur simultaneously, but distinctly during nodule formation (Geurts *et al*., 2012). Both plant and bacteria produce signals and express genes to initiate and control the nodulation process. Genes involved in nodule formation in *Rhizobia* are referred to as nodulation (nod) genes. These are often carried on symbiotic plasmids known as sym-plasmids within the bacteria (Geurts *et al*., 2012). The bacteria also carry the genes required for BNF, including the *nif* and *fix* genes. Nodulation genes are responsible for the production of lipochitooligosaccharides (LCO's)/Nod factors which signal the plant to allow nodulation to occur (Geurts *et al*., 2012). Plant genes involved in nodulation are referred to as nodulin (Nod) genes. NSP1 and NSP2 are essential GRAS-type transcription factors found in the host plant. These transcription factors are responsible for almost all Nod-factor plant responses (Geurts *et al*., 2012). There are common (nodA, nodB, nodC) and host specific (nodQ, nodH, nodF, nodE and nodL) nod genes (Spaink, 2000). nodD is constitutively expressed due to its regulatory function. It functions as the activator of the signalling cascade and produces the

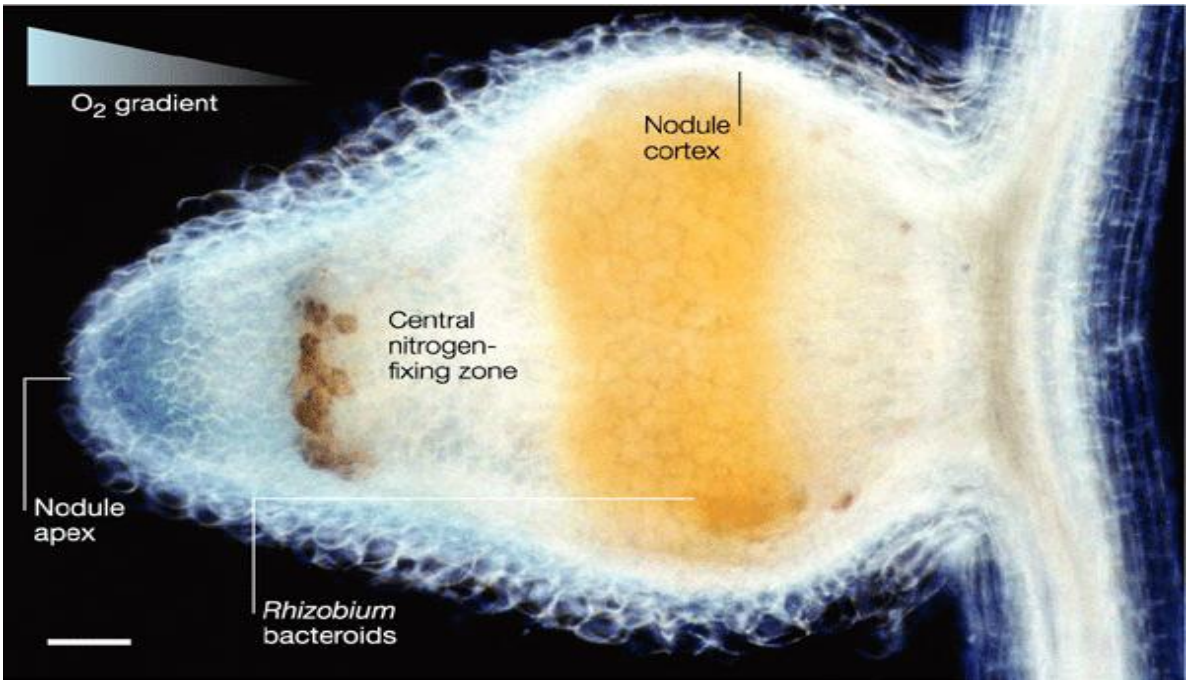

Figure 1.1 Morphology of a plant root nodule, hosting rhizobium bacteria. The nodule cortex, nitrogen fixing zone, apex and bacteroids are indicated along with the decreasing O_2 concentration where BNF occurs (Dixon and Kahn, 2004).

other nodulation factors by binding to the highly conserved nod box, present in the promotor region of the other nod genes. It functions by binding to the highly conserved nod box, present in the promotor region of the other nod genes. nodA, B and C are all structural

enzymes required for the production of the basic nodule structure. NodE, F and L are host specific nod factors and determine different structure formation depending on the host organism (Spaink, 2000).

Nodulation starts with the migration of the bacteria to the roots of the host plant due to chemotaxis. The roots exude a mix of isoflavonoids and betaines which activate nodD. The bacteria attach to the root hairs due to nod activation and instigate root hair curling around a single bacterium. The infection thread is now formed. The infection thread is a long tube-like structure derived from the plasma membrane, due to fusion of Golgi-derived membrane vesicles at the site of infection (Geurts *et al*., 2012). The tube grows by addition of vesicles to the tip and serves to move bacteria to the nodule primordia (Sprent, 2006). After primary infection, bacteria cells start to divide until an undetermined plant signal stops this division. The bacteria now enlarge and differentiate into N_2 fixing bacteroids. Bacteroids are micro aerobic environments required for N_2 fixation which also develop a vascular system (for N transport) and an O_2 exclusion zone (Geurts *et al*., 2012). The bacteria are now effectively compartmentalised.

Mature nodules can be classified as either determinate or indeterminate. Indeterminate nodules are formed in the majority of legumes including pea, lotus and alfalfa (Sprent, 2009). These nodules maintain an active apical meristem allowing cell biogenesis leading to active growth during the nodule lifecycle. They are often cylindrical in shape and form highly branched structures (Sprent, 2009). Determinate nodules are formed in temperate legumes, such as soybean, common bean and lotus. They lose meristimatic activity after initial nodule formation and grow via cell expansion. Determinate nodules are often spherical in shape (Sprent, 2009).

1.2.2 The process, associated cost and enzymes involved in BNF

The atmosphere comprises of 78% N (Vance, 2001; Valentine *et al*., 2011), yet it is unavailable to most organisms due to the strong diazo-bond that binds the molecule together. Due to the strength of this bond, only a few organisms are able to break this bond. These diazotrophs, carry the nitrogenase enzyme, which can fix N_2 in a highly exothermic and oxygen sensitive process known as biological nitrogen fixation (Garg and Geetanjali, 2007). The overall reaction being written as:

$$N_2 + 8H^+ + 8 \text{ electrons} + 16 \text{ ATP} = 2NH_3 + H_2 + 16 \text{ ADP} + 16 \text{ Pi}$$

Nitrogen is an essential building block in amino acids, nucleic acids and proteins (Vance 2001, Valentine et al., 2011). It is however also the most limiting nutrient for plant growth (Vance, 2001). Plants can obtain nitrogen either via BNF or alternatively through direct uptake mechanisms in the root system via various high and low affinity NO_3^- and NH_4^+ transporters (Vance, 2001). These transporters are either constitutively expressed or induced during low N supply.

Nitrogen must however be added to soil via fertilizer for effective plant uptake and use (Vance, 2001). Biologically fixed N is therefore regarded as a safer option when compared to fertilizer N (Valentine *et al*., 2011). N fertilizer run off creates large ecological problems due to eutrophication and hypoxia of water supplies. It can also easily enter ground-water supplies, causing illness in humans (Vance, 2001). Biological N however is gradually released through decomposition, with nitrification producing NH_3 and denitrification finally releasing N_2 back into the atmosphere, forming part of the natural N-cycle (Graham and Vance, 2003).

1.2.2 (a) The nitrogenase enzyme

Bacterial nitrogen fixation is carried out by the nitrogenase enzyme complex. The nitrogenase enzyme is comprised of two components 1) the Fe protein and 2) the MoFe protein (Rees and Howard, 2000). These two components are separately functionally inert and only gain nitrogen fixing capabilities when conjoined. nitrogen fixation is a very energetically expensive process, needing in total 12 ATP per N_2 molecule. Fd (Ferredoxin) serves as the electron donor to the Fe protein (Kim and Rees, 1994).

The Fe protein is the smaller of the two proteins and comprises of two identical subunits. These subunits range in size from 30 – 72 kDa depending on the bacterial species. Each subunit is in turn comprised of 4Fe and 4S2- molecules and catalyses the redox reaction that converts N_2 to NH_3, by activation (reduction) of the Mo-Fe protein. In the presence of O_2, the Fe protein is irreversibly inactivated by O_2 (Kim and Rees, 1994).

The second component of the nitrogenase enzyme is the Mo-Fe protein. This protein comprises of four subunits, with a combined molecular mass of between 180-235kDa. Each individual subunit comprises of two Mo-Fe-S clusters (Rees and Howard, 2000). The Mo-Fe protein reduces N_2 to NH_3, with the evolution of H_2. The evolution of H_2 is a natural by-product of the reaction but is energetically wasteful, with up to 60% of the carbon supplied to

the bacteria being used for this process. A total of 4 ATP is needed per H_2 molecule evolved. As with the Fe protein, the Mo-Fe protein is irreversibly inactivated by O_2 (Rees and Howard, 2000).

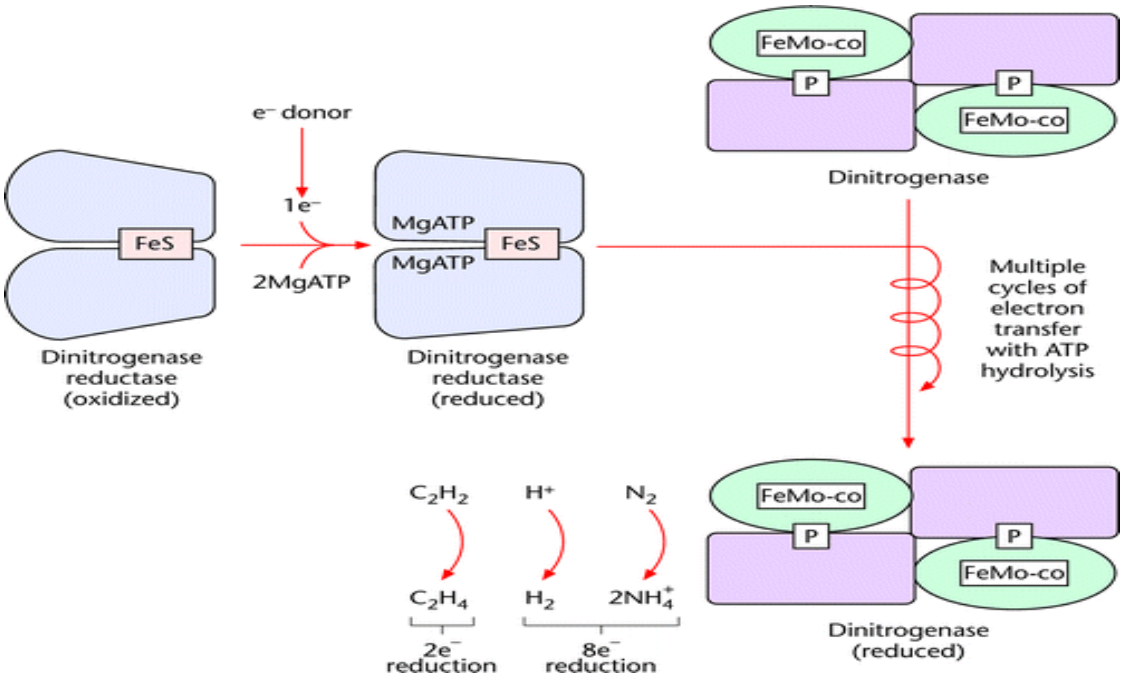

Figure 1.2 The functioning of the various components of the nitrogenase enzyme complex during BNF. After dimerising the proteins are active and use Fd as an electron donor (Rubio and Ludden, 2005).

BNF is an energetically expensive process, for the production of 2 NH_3 molecules, 16 ATP is needed combined with obligatory H_2 evolution, thus 20 ATP in total. This translates to vast amounts of energy and P required for the production of one N containing amino acid. Comparatively, direct uptake mechanisms of N may indirectly consume 15 ATP per reaction.

1.2.2(b) Assimilation of ammonia

High levels of cellular NH_3 is toxic to plant cells and thus must quickly be transported and converted to amino acids. The primary path of ammonia assimilation is the sequential action of the GS/GOGAT system to produce glutamine and glutamate respectively (Robinson *et al*., 1991). Other enzymes involved in ammonium assimilation include GDH, asparagine synthase (AS) , aspariginase and Asp-AT. Both GS and GOGAT catalyse irreversible reactions (Robinson *et al*., 1991).

Glutamine synthase (GS) is responsible for the ATP-dependent reaction, whereby glutamate and ammonia is converted to glutamine (Lam *et al*., 1996). The reaction requires the

consumption of 1 ATP and the presence of a divalent cation such as Mg^{2+}, Mn^{2+} or Co^{2+}. GS is present in two forms GS1 and GS2. GS1 is localized in the cytoplasm with a molecular mass of 38-40 kDa and primarily produces glutamine for nitrogen transport. GS2 is localized in plastids with a molecular mass of 44-45 kDa and produces amides for local tissue consumption. GS is not only involved in BNF but can also utilize different forms of absorbed N (Lam *et al*., 1996).

The second enzyme in this pathway is glutamate synthase (GOGAT). This reaction is the reduction driven transport of an amide group from glutamine to 2-oxoglutarate, to produce two glutamate molecules (Lea and Miflin, 2010). The increase in glutamine concentration due to the action of GS, serves as the stimulant for GOGAT activity. Two forms of glutamate synthase occur, one Fd and the other NAD(H) dependent and both in plastids. The Fd dependent GOGAT is active in the chloroplast under high light conditions, where it can use the light as direct reductant (Lea and Miflin, 2010). The NAD(H) dependent form is not located in photosynthetic active cells and receives reducing agents through the Pentose Phosphate Pathway (PPP) (Lea and Miflin, 2010).

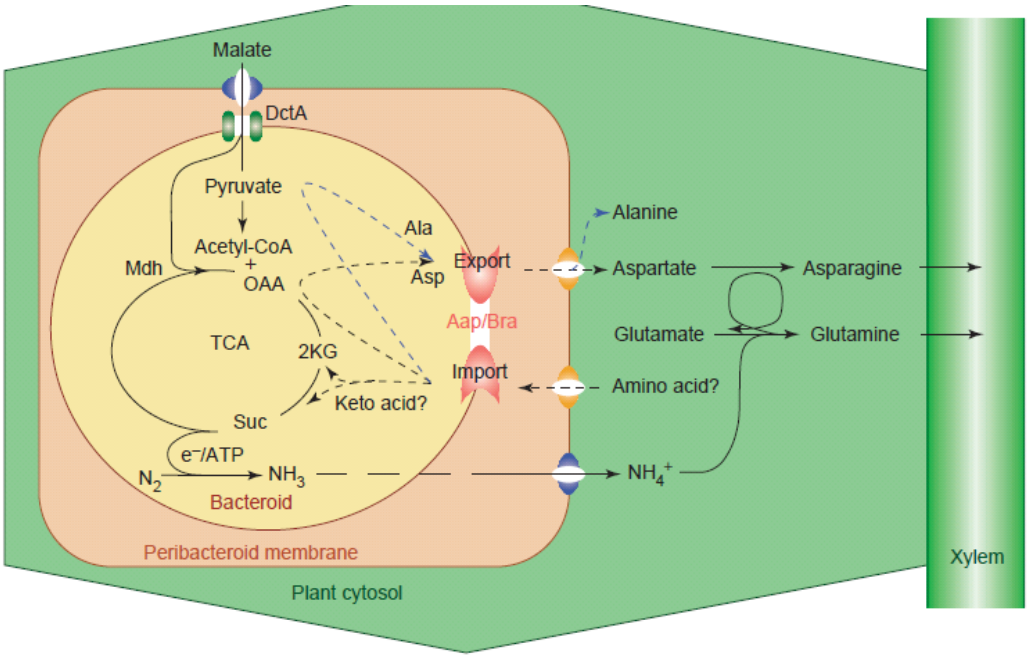

Figure 1.3 The role of amino-acid cycling in nodules. Only reactions directly involved in amino-acid cycling in the bacteroid and plant are shown. Transport systems from the peribacteroid membrane that have been kinetically but not genetically characterized are shown in blue, while those that are hypothetical are in yellow (Lodwig *et al*., 2003).

Glutamate dehydrogenase (GDH) does not play a direct role in ammonium assimilation, but does play a role in deaminating glutamate during nitrogen reallocation. It requires NAD(H) as a coenzyme and is found in the mitochondria with a NADP(H) dependent form being localized in the chloroplasts of photosynthetic organs. GDH catalyses the reaction of ammonium with 2-oxoglutarate to produce one glutamate molecule (Scott *et al*., 1976).

The final step in ammonium assimilation is the incorporation of nitrogen into other amino acids via transamination reactions. Aspartate aminotransferase (Asp-AT) is an example of an aminotransferase in the production of aspartate. Aspartate is produced when Asp-AT catalyses the reaction of glutamate and oxaloacetate. In this reaction the amino group from glutamate is transferred to the carboxyl group of aspartate (Lea and Miflin, 2010).

Asparagine synthase and Aspariginase are another two enzymes involved in nitrogen metabolism (Lea and Miflin, 2010). Although neither of these enzymes is involved in primary ammonium assimilation, their activity is important under physiological stress. Under many physiological stresses plants divert glutamine to asparagine instead of glutamate (Lea and Miflin, 2010). Asparagine synthase catalyses the ATP-dependent transfer of an amide group from glutamate to aspartate to produce one molecule of glutamate and asparagine. Aspariginase is activated when the stress conditions have subsided and hydrolyses aspartate, releasing the ammonium group, which is then fed back into the GS/GOGAT system (Lea and Miflin, 2010).

Due to the complexity of the nitrogen fixing machinery it is not surprising that various biotic and abiotic stress factors have a pronounced effect on BNF and nodule formation. Soil temperature, soil-water status, soil N concentration, C demand, seasonal growth changes and nutrient deficiency (P deficiency) all effect BNF or nodulation on some level.

1.3 Abiotic factors affecting biological nitrogen fixation

Biological nitrogen fixation is a sensitive process with many biotic and abiotic factors affecting the functioning of the BNF machinery and subsequent BNF rates (Valentine *et al*., 2011; Liu *et al*., 2011). These can include environmental conditions such as temperature, drought, N source and supply and rhizosphere pH. Biotic factors can include plant nutritional status pertaining to C metabolism, N metabolism, mineral nutrient status such as K, Al and P, but also intrinsic genetic variation in N-fixing capacity (Liu *et al*., 2011). These factors may all subsequently directly or indirectly affect nodule growth and nitrogenase function.

1.3.1 Temperature

Soil temperature can affect nodule formation, growth and function if temperatures are either too high or too low (Whitehead, 1995). Between minimum and maximum temperatures, a range of temperatures exists where nodulation is favoured and enhanced (Liu *et al*., 2011). The optimal temperature for nodulation is however very species specific. Schomberg and Weaver (1992) showed that *Trifolium vesiculosum* (arrowleaf clover) nodulation was enhanced at 25˚C, in *Glycine max* (soybean) however the most nodules were produced between 20-25 ˚C (Lindemann and Ham, 1979) and in *Trifolium repens* (white clover) between 10-35 ˚C (Whitehead, 1995).

Nitrogenase activity is also affected by temperature, with low temperatures significantly inhibiting function (Liu *et al*., 2011). The minimum range for nitrogenase function is between 2-10 ˚C, with maximum functioning at 20-25 ˚C and an upper limit of 35-40 ˚C (Liu *et al*., 2011). Tropical and subtropical legumes tend to exhibit higher minimum temperatures for optimal nitrogenase activity, when compared to temperate legumes. The effect of temperature on the nitrogenase enzyme is however plant and microbial species specific (Liu *et al*., 2011).

1.3.2 Soil N source and supply

Mineral soil N has been shown to inhibit nodulation and nitrogenase activity, regulating BNF (Serraj *et al*., 1995; Abdel Wahab *et al*., 1996). This is due to the reduced energy cost of direct N uptake mechanisms when compared to BNF (Cannell and Thornley, 2000). Increasing soil N concentration is directly linked to the severity of nodulation and N-fixing inhibition (Macduff *et al*., 1996)

Certain concentrations of N can also enhance nodulation, known as soil "starter N" (Gulden and Vessey, 1997; Gan *et al*., 2004). Starter N stimulates nodule formation at varying concentrations, with concentrations generally being less than 4 mM for NH_4^+ and 2 mM for NO_3^- for plants grown in soil (Gan *et al*., 2004). The time point of starter N application also plays a crucial role. In *Pisum sativum* (pea) external N application caused less inhibition of nodule growth and BNF rates, when applied after nodule establishment (Waterer and Vessey, 1993). The inhibitory effect of N is also dependent on the source of N supplied. White clover, pea and soybean showed greater inhibition of N-fixation when supplied with NO_3^- compared to NH_4^+, although high concentrations of either N-source had an inhibitory effect (Bollman and Vesey, 2006; Gan *et al*., 2004).

1.3.3 Drought

Drought can affect nodule growth and N_2-fixation (Vadez *et al*., 2000). Several factors can affect nitrogenase activity during drought stress, including decreased ATP supply (due to decreased C supplied from the host plant), feedback inhibition by ammonia, carbon supply limitation and O_2 permeability (Serraj, 2003).

Nodules and nodule function is extremely sensitive to drought stress (Galves *et al*., 2005; Ladrera *et al*., 2007). Photosynthetic rates are known to decrease during drought stress, with a subsequent decline in C-compound production (Valentine *et al*., 2011). N_2-fixation rate decline often precedes declines in photosynthetic rates and thus indicates decreased C demand from nodules before declines in host plant C supply to the nodules (Verdoy *et al*., 2004). The main form of C supplied to the bacteroids is sucrose, transported from the source, to the sink organ via the phloem. This sucrose is then converted in the infected cells to malate, the energy currency of the bacteroids (Valentine *et al*., 2011). This is achieved through the action of sucrose synthase (SS) (Horst *et al*., 2007). It was shown that SS activity is greatly decreased during drought stress in soybean nodules (Gonzalez *et al*., 1995), leading to increased sucrose accumulation (Streeter *et al*., 2003). This accumulation of sucrose could inhibit the functioning of the tricarboxylic acid (TCA) cycle , thus affecting malate production and supply to bacteroids (Valentine *et al*., 2011).

The permeability of O_2 is also decreased during drought stress. This may lead to decreased rate of respiration and thus decreased rates of BNF and amino acid production (Valentine *et al*., 2011). The effect of drought stress has been described for broad bean (Guerin et al., 1990) and common bean (Ramos *et al*., 2003; Verdoy *et al*., 2004). Drought stress seem to cause structural changes in nodules including folding and dehydration of the cell wall, damage to the plasma-, bacteroid- and peribacteroid membranes and organelles, decreased air spaces in the bacteroids often leading to no senescence of bacteroids (Guerin *et al*., 1990; Ramos *et al*., 2003; Verdoy *et al*., 2004).

Ureide-exporting legumes are more sensitive to drought stress than amide-exporting legumes (Serraj *et al*., 1995). During drought stress BNF rates decline, however large amounts of ureides accumulate in nodules and shoots (Charlson *et al*., 2009). This could be due to decreased N demand from the host plant and subsequent decreases in xylem loading of N compounds (Charlson *et al*., 2009), leading to accumulation. Due to the complexity of ureide export and the multiple cells involved, including transport, it is proposed that the amount of

amides and amino acids are the actual N-feedback mechanisms (Valentine *et al*., 2011. This was shown to be true for soybean, where asparagine showed a faster reduction in BNF rates when compared to allantoic acid (Serraj *et al*., 1999). For amide-exporting plants, N-feedback inhibition would be expected to occur more strongly, due to the location of both the N_2-fixing and amide synthesis pathways in the same cell (Gonzalez *et al*., 1998). Pea nodules under drought stress showed decreased rates of GS and AAT activity (Galvez *et al*., 2005), and alfalfa decreased GOGAT activity (Naya *et al*., 2007). This could point to feedback inhibition by N in amide transporting legumes as well (Valentine *et al*., 2011).

1.3.4 Aluminium toxicity

Acidic soils, prevalent on 40% of the world's arable land surface, feature both toxicity and deficiency related problems (Ligaba *et al*., 2004; Liao *et al*., 2005). The acidity causes Al^{3+} to become soluble in the soil, causing Al toxicity in plants. This acidity also causes N and P to become deficient. Both Al toxicity and P deficiency have been linked to major effects on plant growth in acidic soil conditions (Zheng *et al*., 1998; Ligaba *et al*., 2004). It was shown by Silva and Sodek (1997) that soybean nodules exposed to Al caused a 90% reduction in BNF and loss of nodule biomass, leading to decreased ureides in the xylem sap. The composition of amino acids in the xylem sap also changes, with increased asparagine and decreased glutamine levels. This may also indicate decreased dependence on BNF (Silva and Sodek, 1997).

Both soybean and common bean exude large amounts of citrate for Al^{3+} toxicity tolerance (Miyasaka *et al*., 1991; Silva *et al*., 2001; Yang *et al*., 2001). The exudation of citrate was shown to enhance P-acquisition in Al^{3+} toxic soil during short-term P-deficiency in soybean (Nian *et al*., 2003). Thus it is expected than P-efficient genotypes are more tolerant to Al^{3+} toxicity due to the large amount of organic acids exuded. The combination of Al and P-deficiency however causes decreases in organic acid exudation (Valentine *et al*., 2011). The overexpression of nodule MDH, PEPC (Tesfaye *et al*., 2001) and CS (Barone *et al*., 2008) in alfalfa, during Al^{3+} toxicity, does however restore organic acid exudation thus conferring Al tolerance.

1.3.5 Phosphate deficiency

Phosphate is the second most limiting nutrient resource for plants, after N (Ragothama, 1999; Vance, 2001; Vance *et al*., 2002). For legumes however, P can be considered as the most

limiting nutrient for plant growth. Plants require P for a variety of functional, structural and regulatory processes (Ragothama, 1999; Vance *et al*., 2002). These include photosynthesis, energy metabolism, ATP production, synthesis of cell wall proteins, amino acid biosynthesis and gene regulation via activation/repression (Ragothama, 1999; Vance *et al*., 2002). It is not surprising then that P also plays a large role in nodule formation and function and ultimately influences BNF.

Nodules are known as strong sinks for P (Al-Niemi *et al*., 1997; Tang *et al*., 2001) because nodule formation and function require large amounts of P for maintenance and the process of BNF. Phosphate deficiency can ultimately both directly influence nodule growth and functioning, or indirectly. Direct influences include limited P supply which leads to diminished nodule growth. Smaller nodules have increased surface area which has been shown by Schulze *et al.* (2006) to allow increased O_2 permeability. Increased O_2 concentrations in nodules can lead to irreversible inactivation of the nitrogenase enzyme and thus a decrease in BNF. Indirect influence can include decreased photosynthate supply from the host plant to the nodules, due to decreased rates of photosynthesis under P deficient conditions (Valentine *et al*., 2010).

Nodules also do not readily release P back to the host plant (Israel., 1993). During P deficiency root and shoot P levels can decrease dramatically, while nodule P levels stay constant (Le Roux *et al*., 2006). With short term P-deficiency, plants preferentially allocate nutrients to nodules, to maintain BNF, even at the expense of plant growth. This scenario however changes during long-term P-deficiency (more than 30 days), when the nodule mass is adjusted for decreased P supply (Drevon and Hartwig., 1997; Olivera *et al*., 2004). Decreased plant growth ultimately leads to decreased BNF due to a reduced need for N (Le Roux *et al*., 2008).

Metabolic adaptations to avoid Pi use must also be considered here. During P-deficiency, Pi and adenylate pools decline and alternative, non-Pi requiring metabolic paths are initiated as alternatives to glycolysis and respiratory Pi-requiring steps (Theodoru and Plaxton, 1993). The PEPC-pathway is induced under long term P stress (more than 25 days) to circumvent the pyruvate kinase P-requiring reaction of the glycolytic pathway (Valentine *et al*., 2011). For nodules under limited P supply this pathway can however be problematic. The PEPC pathway competes for carbon skeletons and causes a shift from organic acid to amino acid

metabolism (Le Roux *et al*., 2008). The problem is further compounded by malate being the carbon substrate of the growing bacteroids.

Limited availability of P can also directly influence BNF via an N-feedback mechanism (Valentine *et al*., 2011). Low levels of P induce increased asparagine (Asn) synthesis, which in turn inhibited BNF in white clover plants (Hogh-Jensen *et al*., 2002). Increased Asn concentrations, up to 35-fold have been shown to accumulate in soybean during applied N treatment (Bacanamwo and Harper, 1997). Lupins showed a two-fold increase in Asn concentration upon defoliation (Hartwig and Trommler, 2001). It was also found that asparagine synthetase was down-regulated during low P supply, with Asn possibly acting as a feedback mechanism (Keller *et al*., 2002)

Phosphate thus plays an important role in nodule initiation, formation and function. During P-deficient conditions, nodule formation and function is drastically impaired. This decline is not only attributed to direct P deficiency, but also to the secondary regulatory, metabolic and physiological changes initiated to conserve P. Nodule function is clearly favoured during short-term P-deficiency. The plant can however not maintain sufficient nutrient supply to the bacteroids, while ensuring growth, for long periods of time. Ultimately reduced plant growth and nodule size, leads to reduced BNF.

1.4 Phosphate (P) and P deficiency

Phosphate (P) is an essential macro-nutrient for plant growth (Lambers *et al*., 2006; Peret *et al*., 2011). It is involved in a myriad of processes ranging from energy metabolism to structural function (Lambers *et al*., 2006). It is however one of the most limiting nutrients for plant growth (Ragothama, 1999; Vance *et al*., 2003). It is a key component in plant growth and dependent on many factors of both the ability of the plant to acquire the nutrient as well as the availability in the environment (Hogh-Jensen *et al*., 2002). The many adaptive responses by plants to P deficiency, shows the necessity of this element for plant growth and development (Ragothama, 1999).

Plants require P for a host of functional and structural processes including photosynthesis, respiration, glycolysis, nucleic acid synthesis, enzyme activity modulation, redox reactions, internal signalling, synthesis and stability of membranes and BNF (Ragothama, 1999; Vance *et al*, 2003; Lambers *et al*., 2006). Although P is abundant in many soils, it's unavailability for uptake places constraints on plant acquisition (Ragothama, 1999). Most of the P in soil is

bound in inactive metal or organic complexes and must be remobilised or mineralised to release useable P (Vance *et al*., 2003). The problem is further exacerbated in the acid-weathered soils of the tropics and subtropics, where legumes are cultivated (Vance, 2001).

Nearly 40% of the planets arable land surface is deficient in P (Vance, 2001). The most common solution is the application of large amounts of P fertilizer (Vance *et al*., 2003). This is however ineffective as only 20% of applied P is available for immediate plant use, the rest being retained in the soil (Schachtman *et al*., 1998). This can lead to the application of four times the amount of fertilizer usually required (Ragothama, 1999). Phosphate not mobilised and removed by plants can easily be eroded from soil particles by water, releasing large amounts of P into water supplies. Enriching of water ecosystems by P can cause considerable damage to the environment and humans' a like (Ragothama 1999, Vance, 2001, Vance *et al*., 2003).

Plants have thus developed various ways to locate, mobilise and ultimately acquire P from the soil (Ragothama, 1999; Vance *et al*., 2003). Phosphate acquisition is however energetically and nutritionally expensive. The production of ATP, transporter proteins and C expended for uptake, all contribute to the cost of P acquisition. Furthermore, excretion of organic acids and various extracellular enzymes from roots and cluster roots, to mobilise soil P, increases the P acquisition cost.

1.4.1 Phosphate uptake and the associated costs

Plants acquire P from the soil in the orthophosphate form ($H_2PO_4^-$ and HPO_4^{2-}). It readily reacts with metal cations, clay particles and organic matter to form inert complexes (Schachtman *et al*., 1998; Vance *et al*., 2003). The P molecules must be chelated from these cations, or mineralised from organic matter, to become available for plant uptake (Ragothama, 1999). Phosphate is further only directed to plant roots via diffusion, with concentrations of 0.1-10 µmol in most soils (Hinsinger, 2001). This leads to slow rates of P uptake from the soil, due to the large difference in P concentration between plant tissue and soil (Schachtman *et al*., 1998). Internal cellular environments can have concentrations of Pi, 1000 times that of the soil, leading to specialised Pi transporters and efflux systems (Ragothama, 1999).

The transport of P from soil to cell is thus mediated via P transporters specifically expressed in plant roots during P deficiency (Smith *et al*., 2001). There are two broad classes of P

transporters, high and low affinity (Smith *et al*., 2000). High affinity transporters are expressed mainly during P-deficiency as a means for increasing P uptake from the soil. Low affinity transporters are constitutively expressed, to allow for continuous P uptake (Smith et al., 2000). Phosphate transporters have been characterised in tomato (LePT1), potato (StPT1), *Arabidopsis thaliana* (PHT family) and white lupin (LaPT1 and LaPT2). Due to the unfavourable nature of the P concentration gradient, P must be moved into the cell at the expense of ATP, coupled to the movement of protons (H^+) (Vance *et al*., 2003).

The nature of P uptake involves the expenditure of energy in the form of carbon (C). Carbon is the common currency for plants; it's investment in various organs indicating the effectiveness of uptake and use by these organs (Lynch and Ho, 2005). Carbon is also dynamic and involved in primary metabolism such as photosynthesis and respiration (growth and maintenance). Growth respiration is mainly concerned with the synthesis of new tissue, whereas maintenance respiration refers to maintenance of tissues (enzymes, membranes and transporters) (Lambers *et al*., 2002). During P deficiency plants expend C on various strategies to enhance P uptake. These include root exudates, membrane transport and root morphology changes. Acidification of the rhizosphere via citrate and malate exudation requires large amounts of TCA-skeletons (Hinsinger, 2001; Ryan *et al*., 2001; Vance *et al*., 2003). In cluster root producing species, these exudates can consume 5-25 % of daily photosynthate produced (Johnson *et al*., 1996). It was also shown by Johnson *et al*. (1996) that root exudates of lupins were 70% derived from phloem-translocated sugars, the rest being supplied by root carbon fixation via PEPC. Direct proton extrusion is another form of rhizosphere acidification used by plants to increase P uptake. Hydrolysis of ATP at the plasma membrane requires the functioning of H-ATPase's (Smith, 2001) and subsequent transport via transporters could amount to substantial energy expenditure, although this has not been quantified (Lynch and Ho, 2005). Plants can also preferentially allocate C to their roots for increased respiration and growth. This is a common strategy due to the immobility of P in the soil and leads to an increased root: shoot ratio (Ragothama, 1999; Lambers *et al*., 2002; Vance *et al*., 2003; Lynch and Ho, 2005). The increase in root growth however can place a large respiratory burden on plant growth and leads to growth decreases in other plant organs. Common bean grown in P deficient conditions can expend up to 40% of net daily carbon assimilation on root respiration, compared to 20% for plants supplied with sufficient P (Lynch and Ho, 2005).

1.4.2 Adaptations of plants to P deficiency

Plants have developed various strategies, involving physiological, morphological and biochemical changes to cope with the unavailability and slow transport of P in the soil (Ragothama, 1999). These strategies are broadly divided into (1) increasing the acquisition of P via soil exploration, acidification of the rhizosphere and exudation of hydrolytic enzymes and (2) increasing internal phosphate recycling via decreased growth, breakdown of P containing compounds and alternative (non P-requiring) pathways of glycolysis (Vance, 2001).

1.4.2.1 Metabolic adaptations

Phosphate deficiency affects whole plant physiology and metabolism. Primary plant metabolism requires large amounts of P for sugar-P, ATP and TCA cycle intermediate production (Theodoru and Plaxton, 1993). Photosynthesis directly uses P in the production of ATP and with the production of sugar-P (Theodoru and Plaxton, 1993). During P deficiency, export of triose-P from the chloroplast to the cytosol declines. The synthesis of sugar-phosphates is down-regulated, while the synthesis of non-sugar phosphates is up-regulated (Rao and Terry, 1995). This leads to changes in the sugar-P: non-sugar-P ratio. The synthesis of larger P-free carbon compounds, such as starch, is now favoured (Rao and Terry, 1995). This ultimately leads to a build-up of starch in the leaves of plants under short- term P stress. The response of photosynthesis to P deficiency is however highly species specific. Some plants show a clear expected decline in photosynthesis rates (Ragothama, 1999), while other maintain near control levels of photosynthesis. During long term P stress there is however a switch to conserve and recycle phosphate so as to maintain functional levels of photosynthesis (Rao and Terry, 1995). Photosynthetic rates decline and plant growth may be impaired to compensate for P deficiency. Carbon is now partitioned to the roots to facilitate respiration and so allow for lateral root formation. Increased lateral root formation will lead to increased soil discovery and absorptive area for P uptake. Carbon thus accumulates mainly in the leaves and roots of P-starved plants, although it has been shown to also accumulate at the whole plant level (Rao and Terry, 1995; Ragothama, 1999).

Due to the metabolic shifts caused by P deficiency, changes in C partitioning and up-regulation of enzymes such as PEPC, MDH and CS, organic acid production is increased in roots and cluster roots (Theodoru and Plaxton, 1993; Neumann *et al*., 1999). These organic

acids are exuded into the rhizosphere where they solubilize P in the soil, through acidification (Vance, 2001). They are exuded in exudation zones, found a few centimetres behind the root tip (Grierson, 2002). These exudation zones are predominantly in contact with rock P, which can be solubilized. By excreting organic acids only in the presence of rock P, valuable C resources are used efficiently to allow for increased P acquisition (Hoffland *et al*., 1992). Citrate and malate are the predominant organic acids exuded by roots and cluster roots during P deficiency (Ryan *et al*., 2001). Increased expression and activity of PEPC in roots allows not only for increased production of OAA, but also increased root CO_2 fixation to sustain organic acid synthesis (Neumann *et al*., 1999). The reaction of HCO_3^- with PEP via PEPC, furthermore also releases Pi. Oxaloacetate can now be converted to citrate via CS or alternatively to malate via MDH in the TCA cycle (Theodoru and Plaxton, 1993). These steps mainly seek to circumvent P requiring steps, such as PK, in the glycolytic pathway and maintain the function of the TCA cycle, during P deficiency.

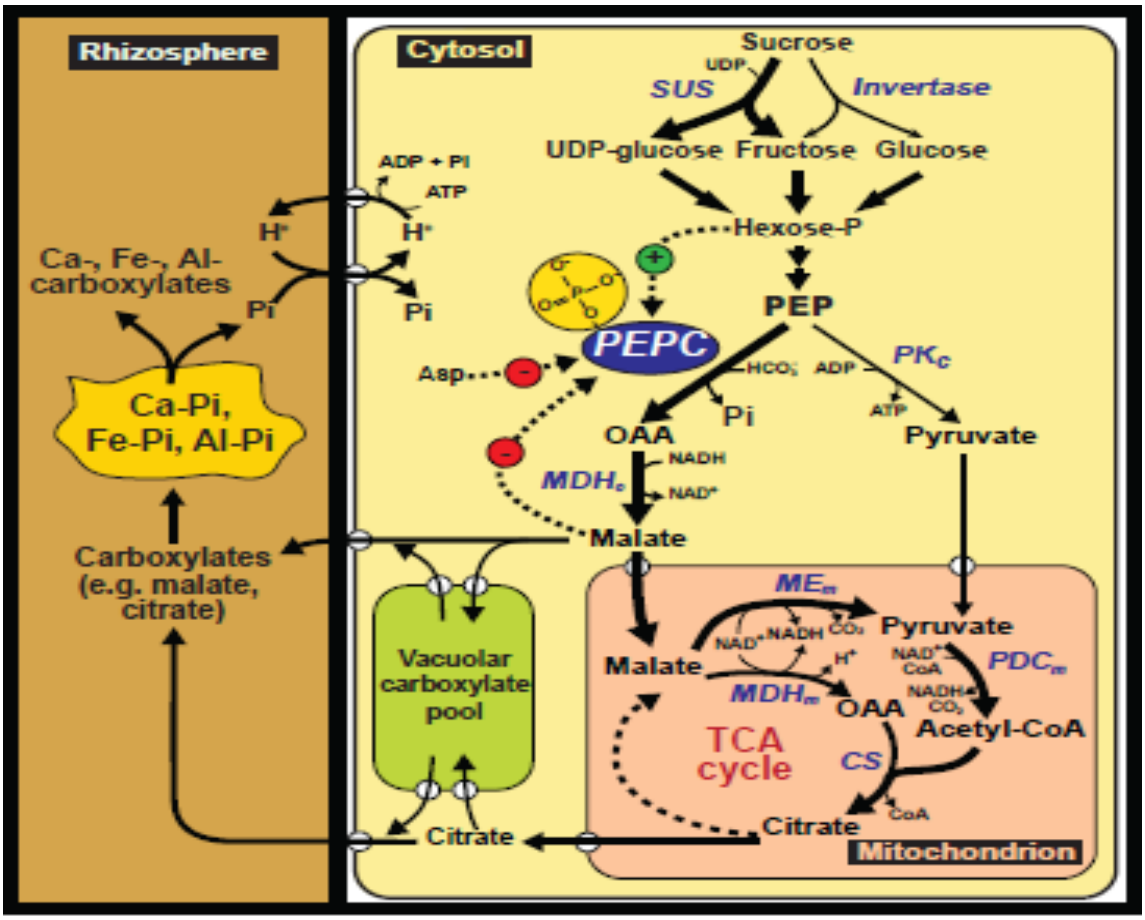

Figure 1.4 Model highlighting PEPC's metabolic functions during plant acclimation to nutritional Pi deprivation. This includes bypassing ADP-limited cytosolic pyruvate kinase (PKc), metabolic Pi recycling, and controlling the anaplerotic production of organic acid anions (*e.g*., malate, citrate) (Shane *et al*., 2013).

1.4.2.2 Below-ground physiological adaptations

Root growth and development is primarily affected by P-deficiency (Williamson *et al*., 2001). One of the major physiological responses to P deficiency is modifications of root architecture including morphology, topology and distribution patterns of the root system (Vance, 2001). Plants usually allocate more resources (C and nutrients) to their root systems during P-deficiency (Lynch and Brown, 2001). This allows for increased root growth, lateral root formation, soil exploration, root hair number and lengths (Gilroy and Jones, 2000; Lynch and Brown, 2001), expression of P transporters (Liu *et al*., 1998; Liu *et al*., 2001), exudation of organic acids (Johnson *et al*., 1996; Gilbert *et al*., 1999) and extracellular enzymes (Vance, 2001), which all function to increase P availability and acquisition from the soil.

1.4.2.2 (a) Root hairs

Below-ground changes in root architecture for P acquisition mainly include increased root hair and cluster root production. Root hairs can be responsible for up to 90% of total Pi acquired from the soil (Ragothama *et al*., 1999). They are formed from epidermis cells and are tubular in shape, growing through tip extension (Gilroy and Jones, 2000). They have an increased absorptive area due to a decreased diameter and grow perpendicular to the root axis (Ragothama, 1999). Root hairs are the primary site for nutrient uptake in plants that lack mycorrhizal colonisation (Jungk, 2001).Phosphate deficiency is also known to cause elongation of root hairs (Vance, 2001). Liu *et al*. (1998) showed that Pi transporter genes are preferentially expressed in the epidermis and root hairs of tomato plants grown during P-deficiency.

The formation and growth of root hairs is largely influenced by N (NO_3^-) and P (Gilroy and Jones, 2000). Root hair production is inversely related to P concentration, with increases in internal P, causing decreased root hair growth. This was shown to be true for rapeseed, tomato, spinach (Jungk, 2001), *Medicago truncatula* (Vance, 2001) and barley (Gahoonia and Nielson, 1998). *Arabidopsis thaliana* produces five times more, and three time longer root hairs during P deficiency (Bates and Lynch, 2000; Ma *et al*., 2001). Low P supply furthermore stimulated epidermis differentiation into root hair cells, increasing the likelihood of root hair formation (Ma *et al*., 2001).

1.4.2.2 (b) Cluster roots

Cluster root formation is the third major root architecture change induced during P-deficiency, after AM symbiosis and root hair growth (Neumann, 2001). Cluster or proteoid roots are defined as densely clustered rootlets of defined length that form on the lateral root axis at defined points (Dinkelaker *et al*., 1995; Johnson *et al*., 1996). There are 28 known plant species that form cluster roots and these include from the genus *Leguminose* (Dinkelaker *et al*., 1995). One of the best documented species of plant cluster root formation under P deficiency is the legume *Lupinus albus* (*L.albus*) or white lupin (Marschner *et al*., 1987; Johson *et al*., 1996). Cluster roots have a myriad of functions in these plants including mobilizing mineral P, extracting P from organic material, obtaining Fe^{2+} and Mn^{2+} from alkaline soil and the uptake of organic N (Dinkelaker *et al*., 1995). Cluster root morphology can be classified as either a single cluster, as is the case in *L. albus* or as a complex cluster. A complex cluster forms when a root within a cluster becomes the site for another lateral cluster to form; this is not the case in a single cluster system (Watt and Evans, 1999).

Cluster root formation is controlled by both internal and external signals and not by external P concentration alone (Marschner *et al*., 1987). Although cluster root formation can be repressed with foliar P application, internal P levels control initiation of cluster root formation. *L. albus* produces cluster roots irrespective of nutritional P status, although significantly fewer cluster roots are formed under sufficient P nutrition (Watt and Evans, 1999). Plant dry matter in response to cluster root formation during P limitation is seemingly unchanged, which leads to the conclusion that these roots can form under non-P limiting conditions as well (Keerthisinghe *et al*., 1998). For *L. albus* the greatest number of cluster roots is formed at 1-10 mM P and the least amount at concentrations of 25 mM P and higher (Keerthisinghe *et al*., 1998).

Cluster roots have various adaptations for improving P uptake from the soil. These include increased surface area (soil exploration) and P transporter expression (Liu *et al*., 2001), exudation of organic acids (Shane *et al*., 2004) including altered C metabolism and exudation of acid phosphatases for breakdown of organic P (Watt and Evans, 1999). The formation of cluster roots increases the absorptive area of the root system. This increase leads to increased uptake of water and mineral nutrients from the soil (Shane *et al*., 2003). Cluster root proliferation is not random however, proliferation only occurs where nutrients might be available, as was shown in *Banksia prionoles* (Lamont *et al*., 1984). Cluster roots only

proliferated in the top organic layers of the soil where organic P was readily available as compared to the rest of the soil which was P deficient.

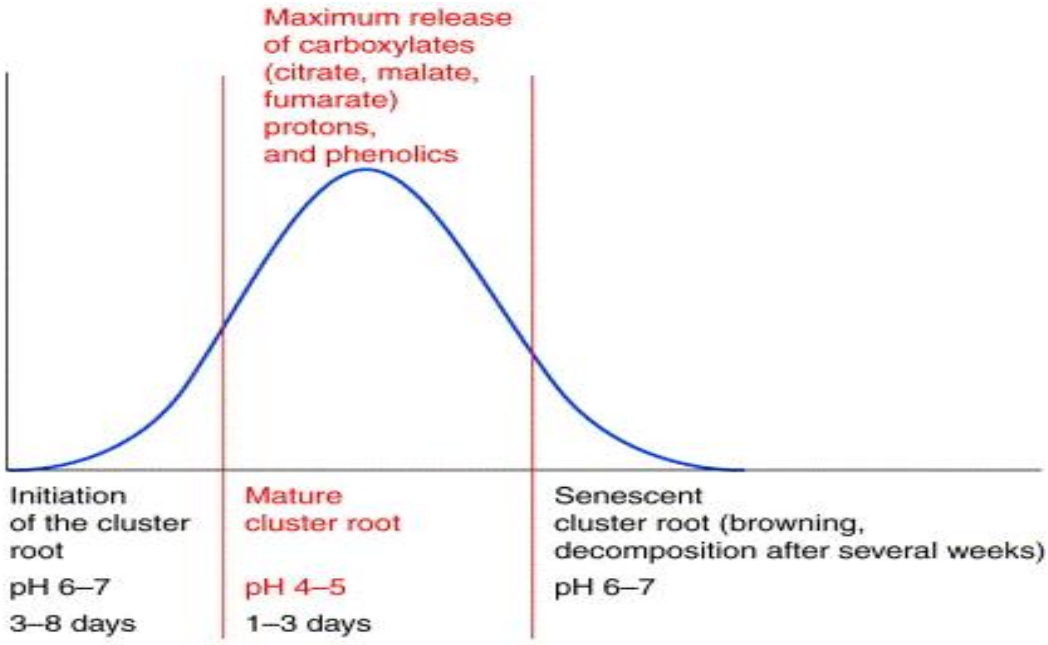

Figure 1.5 Temporal changes during cluster root development and root exudation in *Lupinus albus* (Neumann and Martinoia, 2002).

Organic acid (citrate and malate) exudation is a very effective method for obtaining P from the soil. Phosphate readily reacts with Al^{3+}, Ca^{2+} and Fe^{2+} in the soil forming insoluble matrixes. Organic acids chelate these metals, releasing the phosphate for uptake (Gardner et al 1982, 1983). It was shown that *L. albus* grown in calcareous (Ca-P) soil had white calcium-citrate precipitation around the rhizosphere/root system after 90 days of growing (Dinkelaker *et al*., 1989). White lupins primarily exude citrate, but small amounts of malate are also present (Shane *et al*., 2004). Citrate exudation is however a transient process. At early cluster root development little or no citrate exudation is seen. This however changes as the cluster roots mature (Watt and Evans, 1999). Between days 10-12 after formation, a diurnal pule starts (oxidative burst) and lasts 2-3 days, whereby citrate exudation is kept at a maximum. After this pulse citrate exudation returns to basal levels and the clusters start to senesce (Watt and Evans, 1999).

Citrate accumulation and exudation in cluster roots is very well documented (Keertsinghe *et al*., 1998; Neumann *et al*., 1999; Watt and Evans, 1999; Neumann and Martinoia, 2002; Lamont, 2003; Lambers *et al*., 2006). Citrate is produced via the enzyme citrate synthase (CS) through the condensation of oxaloacetate with acetyl CoA, in the TCA cycle (Theodoru and Plaxton, 1993). In the plant cell cytosol, citric acid will disassociate into citrate and H^+,

due to the high cytosolic pH (Zhu *et al*., 2005). Yan *et al*. (2002) proposed that two separate, independent plasmamembrane transport processes must exist to transport citrate and H^+ out of the cell. Protons are exuded by a membrane associated H^+-ATPase (Yan *et al*., 2002) and citrate (and malate) via two permeable channels located in the plasma membrane (Zhang *et al*., 2004). The exudation of protons with citrate has the added benefit of further acidifying the medium and thus producing favourable conditions for the chelating action of citrate. This extrusion also creates an electrochemical gradient across the plasma membrane and can possibly protect the plant against acidosis during citrate accumulation in response to P-deficiency (Zhu *et al*., 2005). White lupin plants showed increased H^+-ATPase activity in cluster roots with high rates of citrate exudation (Yan *et al*., 2002), with similar results reported for carrot cells, with induced mutations for increased CS activity (Ohno *et al*., 2003). The anti-sense inhibition of H^+-ATPase gene expression, furthermore decreased citrate exudation in carrot cells significantly (Ohno *et al*., 2004).

The continuous production and exudation of large amounts of organic acids however require changes in cluster root metabolism (Neumann *et al*., 1999, 2000; Penaloza *et al*., 2005). PEPC is a highly regulated and multi-functional enzyme that plays a role in organic acid synthesis, the supply of carbon skeletons for amino acid biosynthesis, generation of substrates for the TCA cycle and maintenance of cellular pH (Watt and Evans, 1999). It catalyses the reaction of PEP with bicarbonate ions, releasing Pi, ultimately producing organic acids and fixing C (Theodoru and Plaxton, 1993). It is well known that PEPC activity is enhanced under P deficiency in cluster roots (Johnson et al, 1996; Neumann *et al*., 2000). PEPC activity however varies along the cluster root axis. The highest activity is observed at zones of citrate exudation (Keertsinghe *et al*., 1998) and in the cortex of emerging and mature clusters (Uhde-Stone *et al*., 2003). Both PEPC mRNA and PEPC enzyme activity ensure increased citrate production and transport (Johnson et al, 1996). Under P deficiency, root CO_2 fixation by PEPC also increases, thus replenishing the C lost due to organic acid exudation (Penaloza *et al*., 2005). Johnson *et al*. (1996) showed that 25% of labelled C exuded as citrate was fixed by PEPC in cluster roots and 34% of malate. There are at least three different isoforms of PEPC present in *L. albus* cluster roots (Penazola *et al*., 2005). These PEPC transcripts are designated LaPEPC2, LaPEPC3 and LaPEPC4. LaPEPC2 is constitutively expressed in most plant tissues, whereas LaPEPC3 and LaPEPC4 are almost exclusively expressed in cluster roots and at very low levels in leaves (Penazola *et al*., 2005). The abundance of these isoforms in cluster roots is mainly controlled by the Pi concentration of

the medium (Kai *et al*., 2002). During P-deficiency LaPEPC3 and 4 are highly up regulated in cluster roots. When P sufficient conditions returns all PEPC transcripts are strongly down-regulated. The LaPEPC3 isoform is however regulated well before Pi levels change and might be regulated by a different mechanism (Penazola *et al*., 2005).

Acid phosphatase enzymes are another exudate of cluster roots. Acid phosphatase hydrolyses organic forms of P thus facilitating Pi release into the soil (Miller *et al*., 2001). It has been shown that *L. albus* has various specific acid phosphatases (mAPase) and a novel acid phosphatase (sAPase) which it exudes for P acquisition (Gilbert *et al*., 1999). This novel acid phosphatase is only exuded from cluster roots of P-deficient *L.* albus and unlike mAPase is not membrane bound (Miller *et al*., 2001). Acid phosphatases have optimal enzyme activity at an acidic pH, below 7.0 (Gilbert *et al*., 1999). The presence of citrate thus increases acid phosphatase activity, due to citrate competing for P once it is released from its organic form (Braum and Helmke, 1995). It was shown by Tadano and Sakai (1991) that P-deficient *L. albus* plants had the greatest phosphatase activity and simultaneously released 20× the acid phosphatase activity from the root system, when compared to 9 other plant species. It was further shown by Gilbert *et al.* (1999), that acid phosphatase activity and specifically sAPase activity was localised to cluster roots and exuded in large quantities.

Plants thus invest a lot of resources in the acquisition of mineral resources, primarily P. Since N is the other macro-nutrient in short-supply, it is not surprising that interplay would occur between the various strategies to acquire these resources. Cluster roots not only affect P nutrition but also N nutrition and are able to acquire various forms of organic N. Increased N supply has been shown to increase non-cluster root growth, but repress cluster-root formation (Lamont, 1973). Phosphate nutrition affects both N fixation and nodulation, and it is thus not surprising that N nutrition can effect cluster root formation, function and development.

1.5 Cluster roots and N nutrition

Nitrogen source may affect cluster root formation. In sweet gale (*Myrica gale*) it was observed that urea is more effective at stimulating cluster root formation than NO_3^-. The size of the root cluster was reduced however (Crocker and Schwintzer, 1993). In *Gymnostoma papuana* NO_3^- was more effective than NH_4^+ at promoting cluster root formation (Racette *et al*., 1990). This can however be due to faster uptake of NH_4^+ leading to increased internal N levels and cluster root suppression. Paungfoo-Lonhienne *et al.* (2009) found that *Hakea*

actites (mulloway needlebush) grown with any form of N produced no cluster roots, while plants supplied with no N produced large amounts of cluster roots. When N was however supplied in growth-limiting amounts, N-source affected cluster root production.

Nitrogen supply can also affect cluster root growth. Supplying plants with low N and low P stimulates cluster root formation, whereas supply of low P and high N supresses cluster root formation (Lamont, 2003). This was confirmed for *L. albus* by Dinkelaker *et al.* (1995). It was shown that cluster root growth was increased during low N supply and decreased during high N supply. Sas *et al.* (2002) showed that *L. albus* plants deficient in P and supplied with NH_4^+ produced the most cluster roots and N_2 fixation the least. Cluster roots grown with NH_4^+ as an N source also produced significantly more cluster root dry biomass when compared to non-cluster roots. Exudates from cluster roots are also dependent on both N and P nutrition. Increased proton extrusion during P deficiency and NH_4^+ supply has been reported for *L. albus* (Sas *et al*., 2002). The supply of NO_3^- to P-deficient *L. albus* plants caused OH^- extrusion after 21 days of treatment. Phosphate deficient and adequately supplied plants grown on NH_4^+ furthermore also exuded the same amount of protons, while NO_3^- and N_2-fixation exuded up to 10× more protons.

Although N-fixing nodules and clusters roots can co-occur in lupins, little is known about their competition for C and nutrient resources from the same host resources during nutrient acquisition by each specialized organ. Nitrogen and phosphate share a close relationship with both affecting one another on various levels. It is thus often difficult to discern the effect of one from another. Though the effect of cluster root formation is limited on N nutrition per se, P deficiency has a great impact. Phosphate deficiency causes the formation of cluster roots and thus both of these factors will interplay with N nutrition. Moreover, both organs are influenced by P nutrition and it remains unexplored how a variation in P supply at both extremes, may influence the functioning and C cost of these specialized organs in the lupin root system.

1.6 References

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CHAPTER 2

GENERAL INTRODUCTION

2.1 Legumes: Plants of global importance

Legumes are grown on some 12 to 15 % of arable land worldwide (Vance at et al., 2000). They are used as food source; forage for animals, rotation crops and in agroforestry applications (Graham and Vance, 2003). They are the second most important intensively grown agricultural crops, only second to the cereals (Graham and Vance, 2003). Due to the nutrient poor conditions of large portions of arable land, large amounts of nitrogen (N) and phosphate (P) fertiliser are applied to enhance plant growth (Vance, 2001). Most of this fertiliser is however bound in the soil and unavailable to the plant, or removed through water run-off (Ragothama, 1999). Furthermore, it is estimated that worldwide rock phosphate supply could be exhausted in the next 60-80 years (Vance, 2000). Nitrogen fertiliser production is also a concern (Vance, 2001). The production of N fertiliser involves large amounts of energy and fossil fuels for production. These vast amounts of fertiliser are further estimated to consume up to 1% of the world's energy supply (Simth, 2002).

Legumes have adapted to various environmental conditions to acquire both N and P effectively from the environment (Graham and Vance, 2003). Their ability to form symbiotic relationships with rhizobia enable N acquisition and they have various adaptations to acquire P, from even the most deficient soils. The study of legumes and their adaptations to adverse environmental conditions is thus crucial for our understanding and subsequent use of this natural resource . With increasing strain placed on arable lands, production of fertilisers and the environment, alternatives must be found to conventional agricultural methods (Vance, 2001).

2.2 Legume adaptations to varying P supply

The nutrient deficiency of both N and P has led to various adaptations by legumes to acquire these nutrients (Ragothama, 1999; Vance, 2001). These strategies are mainly aimed at either the acquisition, redistribution or conservation of the deficient nutrient (Vance, 2000). Legumes are known to form symbiotic relationships with rhizobia, leading to the formation of nitrogen fixing nodules (Valentine *et al*., 2011). The legume *Lupinus albus* (white lupin) is readily inoculated with *Bradyrhizobium* for effective nodule formation (Schulze *et al*., 2006). White lupin is also known to not form mycorrhizal associations, but rather produce cluster

roots during low P supply (Sprent, 2006). With cluster roots providing a combined strategy for P acquisition (Lambers et al., 2006).

The growth and function of both nodules and cluster roots do however come at a nutritional and C cost to the plant (Ragothama, 1999; Vance *et al*., 2003). Nodules can consume large amounts of photosynthate (up to 50%) for the reduction of N_2 to NH_4^+ in active N_2-fixing bacteroids (Marschner, 1995). The subsequent production and transport of amino acids to the plant, also requires large amounts of TCA-cycle intermediates (Valentine *et al*., 2011). Nodule function thus impacts all aspects of plant physiology, including photosynthesis, growth and nutrient status of the plant (Valentine *et al*., 2011). Nodule function can even be maintained at the expense of plant growth, with plants preferentially allocating resources to nodules during short-term P-deficiency (Hogh-Jensen *et al*., 2002).

Cluster roots are another below-ground adaptation to nutrient deficiency, specifically the acquisition of P (Neumann and Martinoia, 2002). Cluster roots acquire P via increased surface/area ratios, soil exploration, organic acid and acid phosphatase exudation (Watt and Evans., 1999; Lamont, 2003; Lambers *et al*., 2006). Organic acid exudation by cluster roots function by acidifying the rhizosphere and thus allowing P to be chelated from metal cations (Ca^{2+}, Mg^{2+}) and released for uptake (Vance et al., 2003). Acid phosphatases liberate P from organic sources of P, releasing this into the rhizosphere (Ragothama, 1999). The action of organic acids and acid phosphatases, coupled to increased absorptive area for P uptake and soil exploration lead to increased P availability for the plant (Lambers *et al*., 2006).The production of large amounts of organic acids, predominantly citrate and malate, could incur a C cost to the plant (Neumann and Martinoia, 2002; Lamont, 2003; Lambers *et al*., 2006). Citrate and malate is produced via citrate synthase (CS) and malate dehydrogenase (MDH) respectively, ultimately feeding into the TCA-cycle (Theodoru and Plaxton, 1993). The action of phosphoenolpyruvate carboxylase (PEPC), MDH and CS has been shown to be enhanced in cluster roots during P-deficiency and would supply the necessary C-skeletons for organic acid production (Neumann *et al*., 1999, 2000; Penaloza *et al*., 2005).

The proximity of clusters and nodules thus begs the question of possible interaction. During P-deficiency both nodules and cluster roots must be maintained if an adequate N and P tissue concentrations are to be maintained. Nodules and cluster roots must thus compete for both C and nutrient (N and P) resources to maintain growth and optimal function. Little is however known about how cluster roots and nodules compete for host resources during nutrient

acquisition. It is imperative that this interaction and/or cost of interaction be understood. Since most arable lands are deficient in both N and P, nodules and cluster roots will be produced by the plant. Both these organs are ultimately influenced by P nutrition, yet the influence of variations in P supply on resource requirements from the host, remains unclear. Variation in P supply at both extremes, may influence the functioning and C cost of these specialized organs in the lupin root system .

The aim of this work is:

1. To determine the carbon and nutritional costs associated with P and N acquisition via cluster roots and nodules under P-deficiency (Chapter 3).

2. To determine the carbon and nutritional costs, including the enzymes associated with P and N acquisition via cluster roots and nodules during short term high P supply (Chapter 4).

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CHAPTER 3

PHOSPHORUS DEFICIENCY AFFECTS THE ALLOCATION OF BELOW-GROUND RESOURCES TO COMBINED CLUSTER ROOTS AND NODULES IN *LUPINUS ALBUS*.

3.1 Abstract

Lupins can rely on both cluster roots and nodules for P acquisition and biological nitrogen fixation (BNF) respectively. The resource allocation (C, N and P) between cluster roots and nodules has been largely understudied during P-deficient conditions. The aim of this investigation was therefore to determine the changes in resource allocation between these organs during fluctuations in P supply. *Lupinus albus* was cultivated in sand culture for 3 weeks, with either sufficient (2 mM high) or limiting (0.1 mM low) P supply. Although variation on P supply had no effect on the total biomass, there were significant differences in specialised below-ground organ allocation to cluster roots and nodule formation. Cluster root formation and the associated C-costs increased during low P supply, but at sufficient P-supply the construction and growth respiration costs of cluster roots declined along with their growth. In contrast to the cluster root decline at high P supply, there was an increase in nodule growth allocation and corresponding C-costs. However, this was not associated with an increase in BNF. Since cluster roots were able to increase P acquisition under low P conditions, this below-ground investment may also have benefited the P nutrition of nodules. These findings provide evidence that when lupins acquire N via BNF in their nodules, there may be a trade-off in resource allocation between cluster roots and nodules.

3.2 Introduction

Phosphate (P) is one of the most limiting mineral nutrients for plant growth (Plaxton and Carswell, 1999; Ragothama, 1999, 2000). Its availability to the plant is limited by various properties of the soil itself and is largely determined by solubilisation of P containing compounds and P diffusion rates in the soil (Silberbush and Barber, 1983). Phosphate readily chelates to metal cations, clay particles and organic soil material rendering it unavailable for plant uptake (Jungk *et al*., 1993; Richardson 1994; Abel *et al*., 2002; Vance *et al*., 2003). Soil P is also influenced by pH, ionic strength, adsorption and dissolution from these particles (Vance *et al*., 2003). Slow soil diffusion rates and fast root uptake transporters, causes a rapid depletion of P in the rhizosphere, leading to irregular P distribution in the soil (Lambers *et*

al., 2006). Organic and inorganic compounds readily interact and bind to P (Raghothama, 1999). Plants display great phenotypic plasticity in acquisition strategies for macro-nutrients such as N and P, and can respond to P deficiency by means of a suite of adaptations at the morphological and biochemical level (Keertsinghe *et al*., 1998; Vance *et al*., 2003; Lambers *et al*., 2006).

It is well established that plants preferentially allocate resources to increase below ground biomass and growth under P limitation. This is often at the expense of growth and photosynthesis (Cakmak, 1994; Ragothama, 1999; Vance *et al*., 2003; Lambers, 2006) Cluster or proteoid roots are a combined physiological and morphological below-ground adaptation for phosphate (P) acquisition in P- deficient soils (Dinkelaker *et al*., 1995). The production of cluster roots will incur a C and nutrient (N and P) cost to the plant. Moreover, the cost of cluster root production must be kept to a minimum to decrease negative growth effects at the whole plant level. The root system alone can consume 11-14% of fixed carbon to maintain functionality (Kaschuk *et al*., 2009). Under P-limitation, cluster roots can constitute more than 50% of the root system (Reddell et al., 1997; Lamont, 2003). P must ultimately be transported from the cluster roots to other plant organs if plant P status is to be maintained. It was shown by Keertsinghe *et al.* (2002) that plant growth can be maintained, if cluster roots constitute more than 50% of the root system. The exact costs of cluster roots vs. roots in relation to respiratory costs, are currently unknown (Lamont, 2003), however cluster root growth and function must incur a large C burden on the plant (Lambers *et al*., 2006). Most species of plants associated with cluster root formation can symbiotically fix atmospheric nitrogen via biological nitrogen fixation (BNF) (Skene, 1998), but interestingly do not form mycorrhizal associations (Skene, 1998; Neumann and Martinoia, 2002).

Legumes are well known for their symbiotic relationship with rhizobia (Valentine *et al*., 2011). This symbiosis allows for N acquisition through BNF, bypassing the need for direct N uptake during N deficient conditions. BNF is an energetically expensive process, consuming on average 16 ATP per reaction, for the production of two NH_3 molecules (Schulze *et al*., 1999). Production of ATP is a high P requiring reaction, consuming phosphate per nitrogenase reaction. Comparatively, nitrate reduction to ammonia, after direct transporter uptake, indirectly consumes 15 ATPs (Valentine *et al*., 2011). It is furthermore, also known that nodulated plants expend more P on BNF when compared to direct N uptake mechanisms (Sa and Israel, 1991). Nodules act as strong sinks for P even under adequate P supply (Drevon and Hartwig, 1997). This is compounded during P deficiency where nodules often

exhibit higher P content when compared to roots and shoots (Drevon and Hartwig, 1997). Høgh-Jensen *et al.* (2002) also showed that P is preferentially partitioned to nodules for maintenance of BNF rates under P-deficiency, sometimes at the expense of plant growth. Apart from a strong P sink, nodules must also be supplied with photosynthate in the form of malate. Nodules thus incur a large C and P burden on the plant. The model legume, white lupin (*L. albus*) readily nodulates with *Bradyrhizobium sp*. to form effective nodules (Schulze et al., 2006) and is also one of the best documented, cluster root forming species (Watt and Evans, 1999; Neumann *et al*., 2002; Neumann and Martinoia, 2002; Lamont, 2003; Cheng *et al*., 2011). It is therefore an ideal model to use for the investigation of the costs associated with nutrient acquisition via nodules and cluster roots.

Overall, there is very little known about the costs of combined cluster roots and nodules under P deficiency in any lupin species. Therefore, the aim of this study was to investigate the below-ground allocation of C, N and P to nutrient acquisition organs (roots, nodules and cluster roots), during P deficiency in the model legume *Lupinus albus*. In this regard, the carbon costs of both cluster roots and nodule development during P limitation was assessed, via biomass and growth kinetics, nutrient acquisition efficiencies, respiratory and photosynthetic costs.

3.3 Materials and Methods

3.3.1 Plant growth conditions

L. albus (*Lupinus albus* cv. Andromeda) seeds were germinated in vermiculite before transplantation to sand culture. Seeds were sterilized and then inoculated with a commercially available inoculum (StimuPlant cc) containing *Bradyrhizobium* sp (*Lupinus*) and grown in vermiculite for 10 days. Thereafter, plants were transplanted into 20cm pots and cultivated in quartz sand for 21 days. The plants were divided into two treatment groups, low phosphate (LP) and high phosphate (HP), each receiving a modified Long Ashton (Smith *et al*., 1983) solution containing either 2mM (HP) or 0.1mM (LP) $NaH_2PO_4.2H_2O$ as phosphate source (Keertsinghe *et al*., 1999; Le Roux *et al*., 2005, 2008). The pH of the solution was adjusted to 6.5, and 400ml was applied to the plants once a week, furthermore, the plants received distilled H_2O every other day. No N source was added to ensure nodulation and BNF. Plants were grown under glasshouse conditions in a north-facing glasshouse at the University of Stellenbosch between the months of April and June. The range of midday irradiances was between 400-600 µmol m^{-2} s^{-1} and the average night/day temperatures were 13-22 °C.

3.3.2 Photosynthesis and Gas exchange measurements

The youngest fully expanded leaf was used for photosynthetic measurements. Light-response curves were used to determine the appropriate photon flux density (800 µmol.m^{-2}.s^{-1}) at which to conduct photosynthetic measurements. Readings were taken between 11am and 4pm, using the LI-6400XT portable photosynthesis and fluorescence system (Li-Cor, Lincoln, Nebraska ,USA).

Photosynthetic CO_2 response curves were carried out in order to determine the maximum photosynthesis rate (Pmax), RuBisCO activity and electron transport. Measurements were performed on the youngest fully expanded leaves (5 replicates in each treatment per species), using a Li-6400 gas exchange system (LI-COR Inc., IRGA, Lincolin, NE, USA). Measurements were taken between 9am and 4 pm. A full response curve took 45 minutes to 1 hour to complete. The leaves were enclosed in a leaf chamber (6 cm^2), which received a steady light of 800 µmol.m^{-2}.s^{-1} at a leaf temperature of 24 ˚C. CO_2 concentrations increased according to the following increments: 50 and 100 ppm.

3.3.3 Harvesting and nutrient analysis

Seedlings were harvested at 30 days after transplantation into the sand culture. Upon harvesting, the plants were separated into nodules, roots, stems and leaves. The harvested plant material was placed in a drying oven, at 40˚C for 3 days and their dry weights (DW) were recorded. The dried material was milled with a ball mill. The milled samples were analysed for their respective C, N and P concentrations by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (BemLab, De Beers Road, Somerset West, SA.).

3.3.4 Carbon and nutrition cost calculations

Construction costs, C_W (mmol C g/DW), were calculated according to the methods of Mortimer *et al*. (2005), modified from the equation used by Peng *et al*. (1993):

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

Where C_W is the construction cost of the tissue (mmol C g/ DW), C is the carbon concentration (mmol C/g), *k* is the reduction state of the N substrate (*k*=-3 for NH_3) and N is the organic nitrogen content of the tissue (g/DW) (Williams et al., 1987). The constant (1/0.89) represents the fraction of the construction costs that provides reductant that is not

incorporated into the biomass (Williams *et al*., 1987; Peng *et al*., 1993) and (6000/180) converts units of g glucose/ DW to mmol C/ gDW.

Specific Nitrogen Absorption Rate (SNAR) (mg N/g root DW/d) is the calculation of the net N absorption rate per unit root DW (Nielson *et al*., 2001):

$$SNAR = [(M_2 - M_1 / t_2 - t_1)] \times [(\log_e R_2 - \log_e R_1)/ (R_2 - R_1)]$$

Where M is the N content per plant, t is the time and R is the root DW.

Specific Nitrogen Utilization Rate (SNUR) (g DW/ mg N/d) is a measure of the DW gained for the N taken up by the plant (Nielson *et al*., 2001):

$$SNUR = [(W_2 - W_1/ t_2 - t_1)] \times [(\log_e M_2 - \log_e M_1)/ (M_2 - M_1)]$$

Specific P Absorption Rate (SPAR) (mg N/ g root DW/d) is the calculation of the net P absorption rate per unit root DW (Nielson *et al*., 2001):

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

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

Specific Phosphate Utilization Rate (SPUR) (g DW/ mg P/ d) is a measure of the DW gained for the P taken up by the plant (Nielson *et al*., 2001):

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

Where M is the P content and W is the plant DW.

Growth respiration R_g (t) (µmol CO_2/ d) is the daily growth respiration of the plant (Peng *et al*., 1993):

$$R_g (t) = C_t - \Delta W_c,$$

C_t (µmol CO_2/ d) is the C required for daily construction of new tissue. C_t was calculated by multiplying the root growth rate (g DW/d) by tissue construction cost (C_w). ΔW_c (µmol C/ d) is the change in root C content and was calculated by multiplying the root C content and root growth rate.

3.3.5 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 $\delta=1000‰ (R_{sample}/R_{standard})$, where R is the molar ratio of the heavier to the lighter isotope of the samples and standards is as defined by Farquhar *et al*. (1989). Between 2.100 and 2.200 mg of each milled sample were weighed into 8mm x 5mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) 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 gas 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 the IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$.

%NDFA was calculated according to Shearer and Kohl (1986):

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

Where the reference plant was wheat (*Triticum aestivum*) grown under the same glasshouse conditions. The B-value is the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *Virgilia divaricata*, grown in a N-free solution. The B value of was determined as -0.71‰.

3.3.6 Statistical analysis

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the ANOVA revealed significant differences between treatments, the means (6-8) were separated using post-hoc Student Newman Kuehl's (SNK) multiple-range test ($P\leq0.05$). Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

3.4 Results

3.4.1 Plant biomass, relative growth rates and construction costs

There was no difference in above and below ground allocation of biomass between treatment groups (Fig. 3.1a) and thus no change was observed in the root: shoot ratio (Fig. 3.1b). Furthermore, there was no significant difference in the relative growth rates for roots, cluster roots, nodules or shoots between treatment groups (Fig. 3.1c). Biomass allocation to roots, shoots and on a whole plant basis, was unchanged between treatments (Fig. 3.1d).

Biomass allocation within the root system did however show a marked change between treatments. Plants subjected to P deprivation produced more cluster root biomass and less nodule biomass, when compared to plants supplied with sufficient P (Fig. 3.2a). Cluster roots furthermore constituted up to 24% of the root system in P deficient plants, but less than 5% in plants supplied with sufficient P (Fig. 3.2b). Nodules constituted up to 20% of the root system of plants supplied with sufficient P, while only 14% in the P deficient treatment group (Fig 3.2c). The construction costs for cluster roots were significantly increased in P deficient plants, while nodule construction costs were decreased. Construction costs of roots were similar for both treatment groups (Fig. 3.2d).

3.4.2 Photosynthesis and growth respiration

Maximum photosynthetic capacity was significantly lowered in P deficient plants (Fig. 3.3a). This is coupled with reduced photosynthetic phosphate (Fig. 3.3b) and nitrogen use efficiencies (Fig. 3.3c). Nodules of plants supplied with adequate P showed a significant increase in nodule growth respiration (Fig. 3.3d). There were no significant differences in the growth respiration rates for roots, cluster roots and shoots between treatment groups. It must however be noted that the majority of growth respiration investment was in shoots and the least in cluster roots (Fig. 3d).

3.4.3 Mineral nutrition

The concentration of N and P did not significantly differ for roots, cluster roots or shoots between treatment groups (Fig. 3.4a and 3.4b). There was however an increase in P concentration in the nodules of plants supplied with sufficient P (Fig 3.4a). Shoots on average contained the largest concentration of N, while roots the largest concentration of P (Fig. 3.4a and 3.4b).

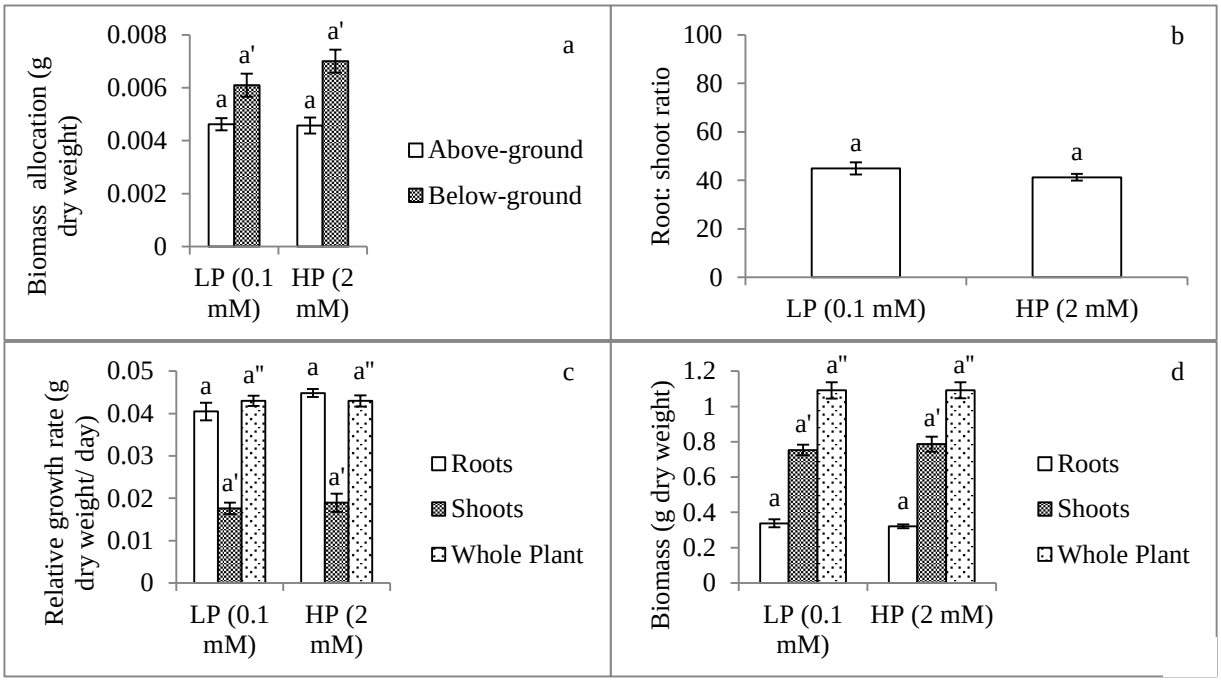

Figure 3.1 Above and below ground allocation (a), root:shoot ratio (b), relative growth rates (c) and root, shoot and whole plant biomass (d) of LP (0.1 mM) and HP (2 mM) treated *Lupinus albus* plants. Values are presented as means (n=6-8) with standard error bars, The different letters indicate significant differences among the treatments, where the prime lettering indicates the comparisons between the same organ (P≤0.05).

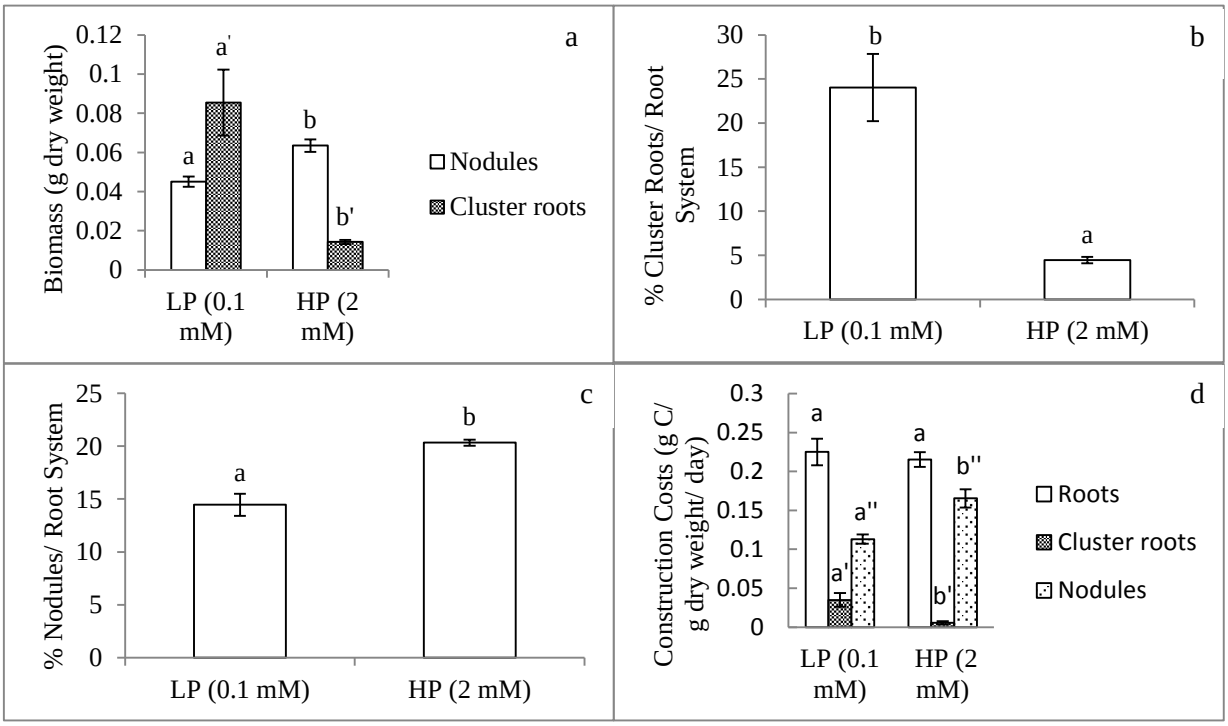

Figure 3.2 Nodule and cluster root biomass (a), percentage (%) of cluster roots (b) and nodules (c) per root system and construction costs (d) of LP (0.1 mM) and HP (2 mM) treated *Lupinus albus* plants. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments, where the prime lettering indicates the comparisons between the same organ (P≤0.05).

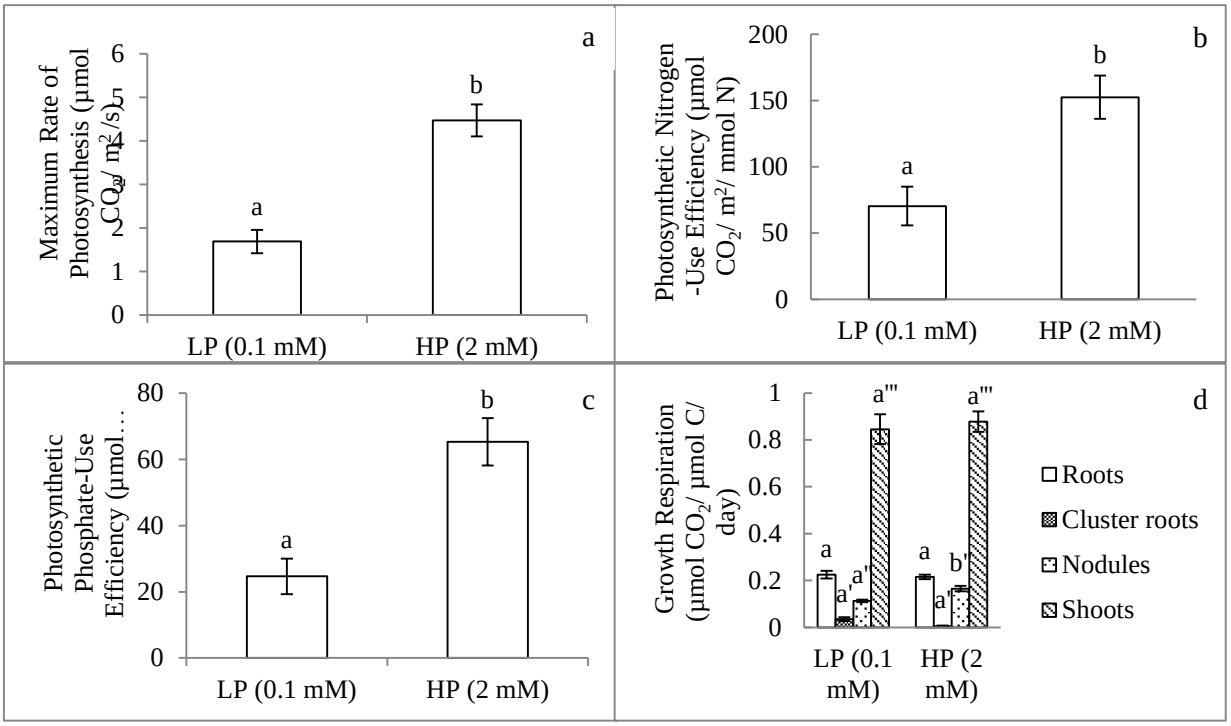

Figure 3.3 Maximum photosynthetic capacity (a), photosynthetic nitrogen (b) and phosphate (c) use efficiency and growth respiration of roots, cluster roots, nodules (d) and shoots of LP (0.1 mM) and HP (2 mM) treated *Lupinus albus* plants. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments, where the prime lettering indicates the comparisons between the same organ (P≤0.05).

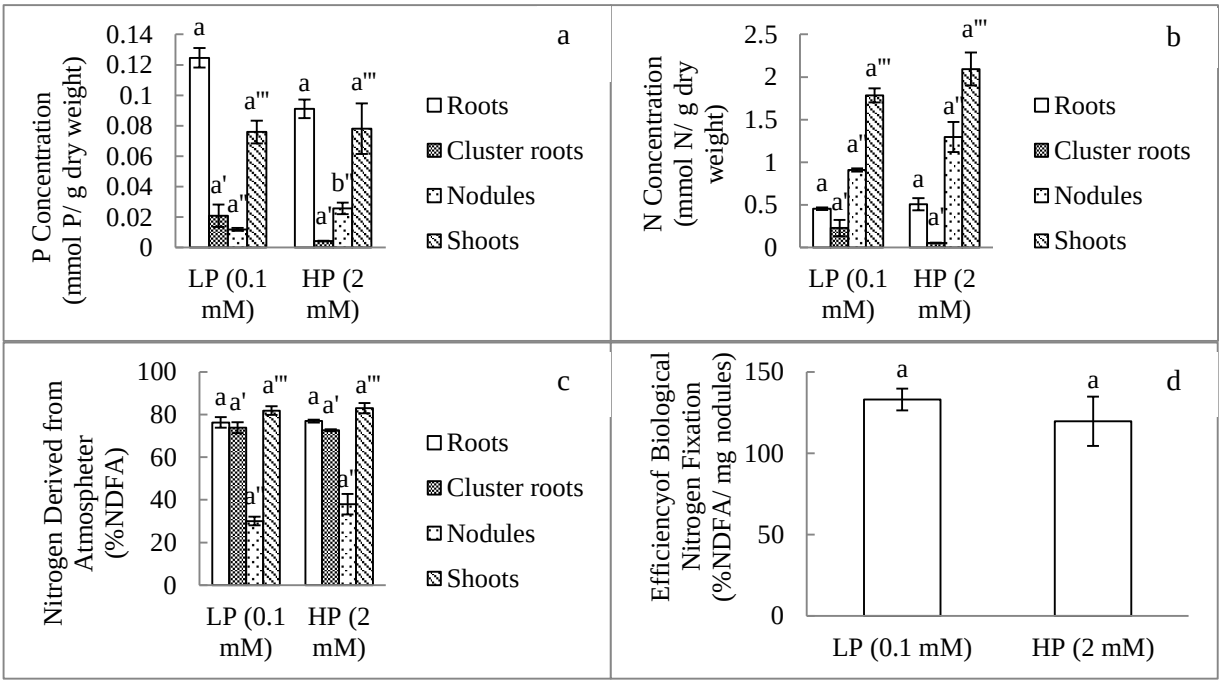

Figure 3.4 mmol P (a), mmol N (b) and nitrogen derived from atmosphere (c) of roots, cluster roots, nodules and shoots, and biological nitrogen fixation efficiency (BNF) (d) of LP (0.1 mM) and HP (2 mM) treated *Lupinus albus* plants. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments, where the prime lettering indicates the comparisons between the same organ (P≤0.05).

No significant difference in %NDFA was observed between treatment groups in any plant organ (Fig. 3.4c). BNF efficiency was also similar for both treatment groups (Fig. 3.4d).

Cluster roots of P deficient plants showed a significant increase in P acquisition rates when compared to cluster roots of plants supplied with sufficient P (Fig. 3.5c). The utilisation rates of both N and P were however not significantly different for roots and shoots between treatments (Fig. 3.5b and 3.5d). Cluster roots of plants supplied with adequate P had a significantly increased P utilisation rate (Fig. 3.5d).

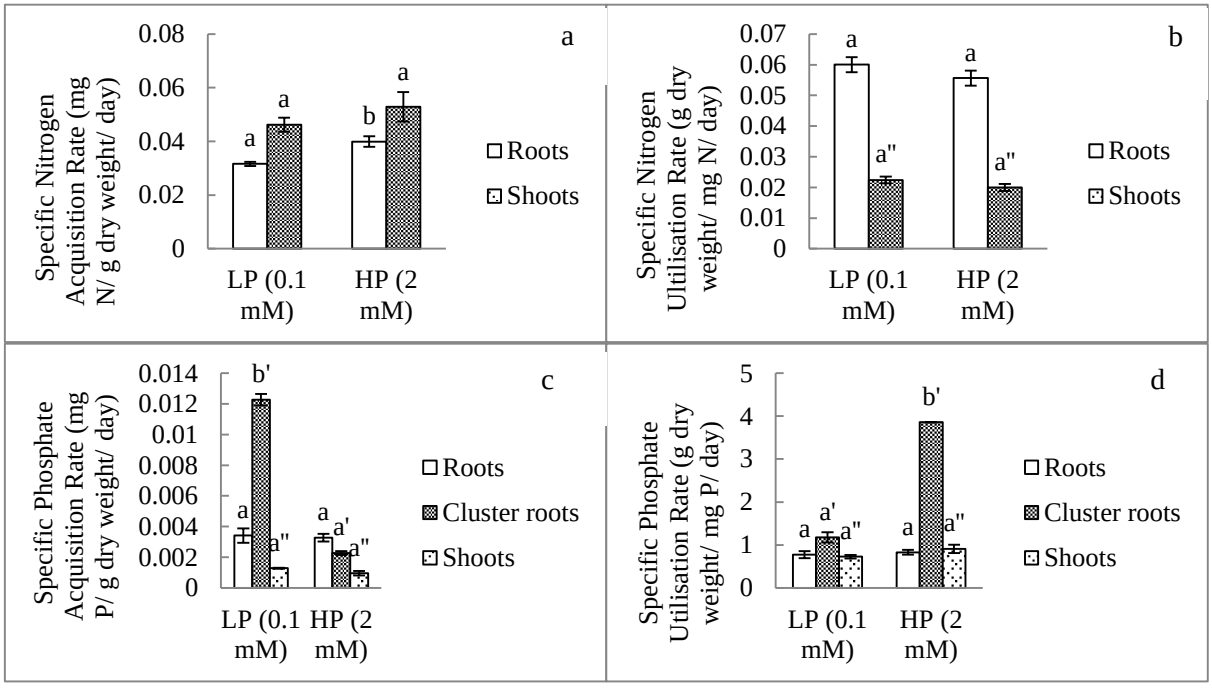

Figure 3.5 Specific nitrogen acquisition rate (a), specific nitrogen utilisation rate (b), specific phosphate acquisition rate (c) and specific phosphate utilisation rate (d) of roots, cluster roots and shoots of LP (0.1 mM) and HP (2 mM) treated *Lupinus albus* plants. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments, where the prime lettering indicates the comparisons between the same organ (P≤0.05).

3.5 Discussion

When plants are dependent on BNF via symbiotic nodules, there may be a trade-off in resource allocation between cluster roots and nodules. Both cluster roots and nodules require resources, in the form of P, N and C for maintenance, growth and functionality. When any of these resources are deficient, competition can arise between sinks organs for valuable resources (Lynch and Ho., 2005). This is evident from the increase in cluster root formation during P deficiency, but decline during P sufficient conditions. The opposite is true of nodules, where more nodules were formed during adequate P supply.

During P-deficiency, the absence of any effects on gross biomass accumulation or allocation to below and above-ground organs in *L. albus*, may indicate the extreme adaptation of lupins to P-deficient conditions. This concurs with previous work, where Schulze *et al.* (2006) found that *L. albus* grown with no added P, showed no effect on biomass accumulation when compared with plants supplied with sufficient P for up to 27 days of growth. Similar results have also been reported by Abdolzadeh *et al.* (2010), where there was no significant difference in gross biomass for *L. albus* before 32 days of LP treatment. This could be attributed to cluster root mobilisation and uptake of P, compensating for decreased P supply. These temperate legumes are also mainly found in nutrient-poor, acidic soils, conditions that would limit growth in most plants (Gilbert *et al*., 1998).

Despite the absence of changes in total biomass, significant difference in specialised below-ground organ allocation to cluster roots and nodules, suggest that these adaptations may have underpinned the plant's tolerance of P-deficient conditions. The greater biomass allocation to cluster roots during P-deficiency specifically concurs with previous work. Keertsinghe *et al.* (1998), Watt and Evans (1999) and Neumann *et al.* (2000), all report increased biomass allocation to cluster roots during P-deprivation. The reduction of cluster root growth during sufficient P supply, thus points to the plasticity of these specialised roots for P-acquisition. This functional plasticity is however associated with C-costs, since with sufficient P-supply, construction and growth respiration costs of cluster roots declined. It has been shown by Shane *et al.* (2003) and Li and Liang (2005) that cluster root formation is reduced in plants supplied with sufficient P. This decline in cluster root formation can be attributed to a threshold shoot P level that supresses cluster root formation during sufficient P supply (Shen *et al*., 2005) leading to decreased demand for C. The main functions of cluster roots are the location and acquisition of P during low P supply (Lamont, 2003). Once favourable P conditions return, cluster root production declines, since roots themselves are capable of P acquisition via P-transporters (Marschner *et al*., 1996). Cluster roots consumed more P during favourable conditions instead of acquiring P. This can be seen from the large increase in P utilisation rate, yet decrease in P acquisition rate and biomass of cluster roots under sufficient P supply. Cluster roots thus appear to function optimally at low P supply. During high P supply nutrient resources are diverted from cluster root production and function to other plant organs, mainly nodules.

Increased C-costs under deficient P-supply can be associated with more than just growth and construction costs for cluster roots; it also includes the functional advantage of these organs

under nutrient deficient conditions. The improved P acquisition in cluster roots is underpinned by the specific P acquisition rates (SPAR) of these organs. In this regard, the high uptake rate of P might be a factor of increased surface area or high P-transporter activity in cluster roots as shown in previous studies (Liu *et al*., 2001; Lamont., 2003). Keertsinghe *et al.* (1998) showed that cluster roots increased the uptake of P from soils, by increased mining of the rhizospehere depletion zone. Cluster roots also increase the absorptive area of nutrient uptake by an increased (more than a 100 fold) surface/area ratio due to their fine structure and bottlebrush-like architecture (Schulze *et al*., 2002; Lamont, 2003). Liu *et al*. (2001) reported increased expression of phosphate transporters, LaPT1 and LaPT2, in cluster roots of P-deficient *L. albus*. LaPT1expresssion was dramatically increased in cluster roots (compared to normal roots) during P deficiency and lead to enhanced P acquisition by cluster roots. LaPT1 is further only expressed under P-deficient conditions in both normal roots and cluster roots, whilst LaPT2 is uniformly expressed in both roots and cluster roots, irrespective of P supply (Liu *et al*., 2001). The functional and growth costs of these cluster roots, were however not reflected as a sink stimulation of leaf photosynthesis. Instead, increased nodule growth, once cluster root growth subsided at sufficient P levels, appears to have been a larger sink for photosynthesis stimulation. Nodules can cause a sink stimulation of photosynthesis due to increased phloem loading of photosynthates, associated with the C-costs of this symbiosis (Kaschuk *et al*., 2009). This is verified by the absence of any increases in leaf P, during sufficient P conditions.

The increase in nodule biomass investment coincides with a decrease in cluster root production, during adequate P supply. This increase however, did not translate into an increased plant dependence on BNF, or improvement of the plant's nitrogen nutrition. The unchanged BNF efficiencies under P supply may imply optimal functioning of nodules under low P supply, as found in *L. angustifolius* where no change in BNF efficiencies or %NDFA was observed during short term P deprivation (Le Roux *et al*., 2006; 2009). Legumes are known to produce more, but smaller nodules during P-deficiency (Schulze *et al*., 2006). These nodules are less effective due to increased O_2 diffusion caused by an increase in the surface/area ratio. Due to the number of nodules produced however, similar BNF rates, comparable to non-stressed plants, can be maintained (Schulze et al., 2006). It may also be possible that the cluster roots may have improved the P nutrition to the nodules as evidenced by previous work on white lupins (Schulze *et al*., 2006; Mortimer *et al*., 2008). In spite of their unchanged function, the increase in nodule growth allocation may have imposed a sink

stimulation, due to increased construction costs and growth respiration at high P supply. Nodules are known to act as strong sinks for both C and P resources, even under sufficient P supply. Nodulated legumes have been shown to require more P compared to unnodulated legumes (Høgh-Jensen *et al*., 2002), with nodules consuming up to 4-16% of recently fixed photosynthate (Kaschuk *et al*., 2009). This can lead to sink stimulation via photosynthesis for increased C supply, as is evident in this study.

These findings suggest that when plants are dependent on BNF via nodules, a physiological trade-off may exist between cluster roots and nodules during low P supply. Valuable resources in the form of C and P are redirected from cluster roots to nodules during adequate P supply. This was evident in the increased photosynthetic rates, P concentration and growth of nodules during high P supply. Nodules thus appear to have adapted to maintain function and efficiency of BNF, despite changes in P nutrition and costs associated with cluster roots, during fluctuations in P supply. This increase in the costs of cluster roots during P deficiency, appears to have improved the P nutrition of nodules in order to maintain their function under P stress.

3.6 References

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CHAPTER 4

SHORT-TERM SUPPLY OF ELEVATED PHOSPHATE ALTERS THE BELOWGROUND CARBON ALLOCATION COSTS AND FUNCTIONS OF LUPIN CLUSTER ROOTS AND NODULES

4.1 Abstract

The legume *Lupinus albus* is able to survive under low nutrient conditions, because it has two specialized belowground organs for the acquisition of N and P. In this regard, cluster roots increase P uptake and root nodules acquire atmospheric N_2 via biological nitrogen fixation (BNF). Although these organs normally tolerates low P conditions, very little is known about their physiological and metabolic flexibility during short-term changes in P supply. The aim of this investigation was therefore to determine the physiological and metabolic flexibility of these organs during short-term supply of elevated P nutrition. *Lupinus albus* was cultivated in sand culture for 4 weeks at 0.1 mM P supply, and then supplied with 2mM P for 2 weeks. The short-term elevated P supply, caused an increased allocation of C and respiratory costs to nodules, at the expense of cluster roots. This alteration was also reflected in the increase in nodule enzyme activities related to organic acid synthesis, such as Phosphoenol-pyruvate Carboxylase (PEPC), Pyruvate Kinase (PK), Malate Dehydrogenase (NADH-MDH) and Malic Enzyme (ME). In cluster roots, the elevated P conditions, caused a decline in these organic acid synthesizing enzymes. P recycling via Acid Phosphatase (APase), declined in nodules with elevated P supply, but increased in cluster roots. Our findings suggest that during short-term elevated P supply, there is a great degree of physiological and metabolic flexibility in the lupin nutrient acquiring structures, and that these changes are related to the altered physiology of these organs.

4.2 Introduction

Phosphate (P) is a key macro-element involved in a wide array of plant processes, from photosynthesis to gene regulation (Abel *et al*., 2002; Vance *et al*., 2003; Lambers *et al*., 2006). It is however the second most limiting nutrient for plant growth, due to strong soil particle interaction (Jones, 1998; Ragothama 1999). Phosphate readily binds to cation metals (K^+, Ca^{2+}, Fe^{2+}) in the soil, rendering it unavailable for plant uptake and use (Schachtman *et al*., 1998) . Plants have thus developed a wide array of adaptive strategies to mobilize, enhance uptake and recycle P, both in the rhizosphere and in planta. These strategies may

involve production and exudation of organic acids (Johnson *et al*., 1996; Keertsinghe *et al*., 1998; Pearse *et al*., 2006), modifications and preferential allocation of resources to the root system (Ragothama, 1999; Lambers *et al*., 2006), coupled to internal P recycling (Rao and Terry, 1995) and induction of alternative metabolic pathways (Theodoru and Plaxton., 1993).

Lupins employ a dual strategy for both N and P acquisition. *Lupinus albus* is known to form both nodules and cluster roots during N and P deficiency, respectively (Lamont, 2003; Schulze *et al*., 2006). Nodules are small outgrowths on the root that house bacteria capable of BNF, via the action of the nitrogenase enzyme (Hogh-Jensen *et al*., 2002). Nodules supply the host plant with N, in the form of amino acids, in exchange for C and nutrient resources (Drevon and Hartwig, 1997). They are however strong sinks for P, due to the energetically expensive (ATP requiring) breakdown of the N_2 molecule (Le Roux *et al*., 2008). Plants even favour BNF during P deficiency, sometimes at the expense of photosynthesis and/or growth (Drevon and Hartwig, 1997; Hogh-Jensen *et al*., 2002).

It is well documented that plants preferentially allocate resources to the roots during early P deficiency. This allows for increased soil exploration and nutrient (N and P) uptake (Ragothama *et al*., 1999, Vance *et al*., 2003). Cluster roots are a specialised root adaptation for P uptake and is well documented in *Lupinus albus* (white lupin) (Watt and Evans, 1999; Neumann and Martinoia 2002; Lamont, 2003). Cluster roots are small rootlets of defined length formed on the root axis, producing small bristle-like structures along the length of the root (Watt and Evans., 1999). These rootlets are formed in response to P and Fe deficiency (Dinkelaker *et al*., 1995; Hagstrom *et al*., 2001). White lupin produces cluster roots irrespective of internal P concentration, however significantly less cluster roots are formed during P sufficient conditions (Watt and Evans, 1999).

The acquisition of P via cluster roots, is a combined strategy for effective P location, liberation and absorption from recalcitrant soils (Watt and Evans, 1999; Lambers *et al*., 2002; Lamont, 2003). Organic acid exudation by cluster roots enhances solubilisation and mobilisation of soil bound P (Watt and Evans 1999; Lambers *et al*., 2002; Uhde-Stone *et al*., 2003), while the increased root surface area, coupled with increased P transporter production (Liu *et al*., 2005), allows for effective P uptake. During P deficiency the root system of *L. albus* can comprise of up to 50% cluster roots (Lamont, 2003). This allows for adequate P uptake to maintain photosynthesis and growth rates similar to plants supplied with adequate P, for up to 28 days after emergence. The uptake of P via cluster roots, also

supply nodules with sufficient P for the maintenance of biological nitrogen fixation (BNF) (Schulze *et al*., 2006). Schulze *et al*. (2006) reported nodule formation in close proximity to cluster roots during P deficiency in *Lupinus albus*.

Plants exhibit great morphological and metabolic changes during P deficiency (Goldstein *et al*., 1989; Duff *et al*., 1991). Changes in enzyme activity and alternative pathways of glycolysis point to the flexibility of plant metabolism during high P supply (Theodoru and Plaxton, 1993). It was found that the degree of activation of phosphofructokinase (PFP) by fructose 2,6 bis-phosphate (Fru-2,6-P_2), in *Brassica nigra* (black mustard) cells supplied with high P, correlated with production of the α-subunit of PFP (Theodoru *et al*., 1992). Thus reduced production of the α-subunit of PFP during high P-supply represses enzyme activity (Theodoru *et al*., 1992). This points to the fine regulation and metabolic flexibility required by plants during P variations in P supply.

Phosphate starvation responses are aimed at the acquisition of P from the soil (Rao and Terry, 1995). These adaptations can include increases in root growth, soil exploration and mobilising soil P (organic and inorganic) (Plaxton and Carswell, 1999; Uhde-Stone *et al*., 2003) and adaptations for the conservation and recycling of internal P (Rao and Terry., 1989; Duff *et al*., 1994). This is achieved by increasing production of P-free carbohydrates (starch, sucrose, glucose) and phosphatases, while decreasing the production of P-containing molecules (sugar-phosphates, adenylates etc.) (Rao *et al*., 1995).

There are countless articles relating to P deficiency as compared to P sufficient conditions (Johnson *et al*., 1996; Keertsinghe *et al*., 1998; Neumann *et al*., 1999; Hogh-Jensen *et al*., 2002; Hernandez *et al*., 2009). Little regard is however given to the short term supply of high P (Israel, 1993; Rao and Terry., 1995; Tang *et al*., 2001). This is surprising, as most legumes are agriculturally important crops, grown in P deficient soils (as in the case of *L. albus*), which in turn is treated with fertilizer to increase available soil P. Moreover, the addition of high P would also indicate the degree of flexibility of the low P physiological syndrome.

The aim of this study was to investigate the flexibility of lupin physiology and metabolism to high P addition to the normal low P growth conditions. This was investigated following the supply of high P to low P *L. albus* plants, and subsequently assessing biomass and growth kinetics, nutrient acquisition efficiencies, respiratory growth costs and enzyme activities.

4.3 Materials and Methods

4.3.1 Plant growth conditions

L. albus (*Lupinus albus* cv. Andromeda) seeds were germinated in vermiculite before transplantation to sand culture. Seeds were sterilized and then inoculated with a commercially available inoculum (StimuPlant cc) containing *Bradyrhizobium* sp (*Lupinus*) and grown in vermiculite for 10 days. Thereafter, plants were transplanted into 20cm pots and cultivated in quartz sand for 30 days and received a modified Long Ashton solution (Smith et al., 1983) containing 0.1mM $NaH_2PO_4.2H_2O$ (Control) as P source. After the 30 day growth period half of the treatment group was supplied with a high P Long Ashton solution (Smith et al., 1983) containing 2 mM $NaH_2PO_4.2H_2O$ (Control + HP) as P source (Keertsinghe at al., 1998; Le Roux et al., 2006, 2008) for 14 days, while the rest of the plants continued receiving the modified Long Ashton solution (Smith et al., 1983) containing 0.1mM $NaH_2PO_4.2H_2O$ (Control) as P source. The pH of the solution was adjusted to 6.5, and 400ml was applied to the plants once a week, furthermore, the plants received distilled H_2O every other day. No N source was added to ensure nodulation and BNF. Plants were grown under glasshouse conditions in a north-facing glasshouse at the University of Stellenbosch between the months of April and June. The range of midday irradiances was between 400-600 µmol m^{-2} s^{-1} and the average night/day temperatures were 13-22 °C.

4.3.2 Harvesting and nutrient analysis

Seedlings were harvested 6 weeks after transplantation into the sand culture. Upon harvesting, the plants were separated into nodules, roots, stems and leaves. The harvested plant material was placed in a drying oven, at 40˚C for 3 days and their dry weights (DW) recorded. The dried material was milled with a ball mill. The milled samples were analysed for their respective C, N and P concentrations by a commercial laboratory, using inductive coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (BemLab, De Beers Road, Somerset West, SA).

4.3.3 Cost and efficiency calculations

Construction costs, Cw (mmolC/g DW), were calculated according to Mortimer *et al*., (2005), modified from the equation used by Peng *et al*. (1993):

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

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

Growth respiration, Rg(t) (µmol CO_2/d), is the daily growth respiration for the plant (Peng *et al*., 1993):

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

Where Ct (µmol CO_2/d) is the C required for daily construction of new tissue. Ct was calculated by multiplying the root growth rate (gDW/d) by tissue construction cost (Cw). ΔWc (µmol C/d) is the change in root C content and was calculated by multiplying the root C content and the root growth rate.

Specific P utilization rate (SPUR) (g DW/mg P/d) is a measure of the DW gained for the P taken up by the plant (Mortimer *et al*., 2008):

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

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

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

Belowground allocation represents the fraction of new biomass partitioned into new roots and nodules over the given growth period. This was calculated according to Bazzaz and Grace (1997):

$$df/dt = RGR\ (\partial - Br/Bt)$$

Where RGR is the relative growth rate (mg/g/d) and ∂ is the fraction of new biomass gained during the growth period. Br/Bt is the root weight ratio, based on total plant biomass (Bt) and root biomass (Br).

4.3.4 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 $\delta=1000‰ (R_{sample}/R_{standard})$, where R is the molar ratio of the heavier to the lighter isotope of the samples and standards is as defined by Farquhar *et al*. (1989). Between 2.100 and 2.200 mg of each milled sample were weighed into 8mm x 5mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) 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 gas 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 the IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$.

%NDFA was calculated according to Shearer and Kohl (1986):

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

Where the reference plant was wheat (*Triticum aestivum*) grown under the same glasshouse conditions. The B-value is the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *Virgilia divaricata*, grown in a N-free solution. The B value was determined as -0.71‰.

4.3.5 Protein extraction and determination for PEPC, PK, NADH-MDH and ME

The extraction was performed according to Ocaña et al. (1996) modified so that 0.6 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Randburg, South Africa) per 50 ml of buffer. The protein concentration was determined by the procedure of Bradford (1976) using a protein assay reagent (Bio-Rad Laboratories, Johannesburg, South Africa) and bovine serum albumin (BSA) as standard (1:1).

4.3.6 Protein extraction and determination for intracellular APase

The protein extraction was performed according to Hurley *et al*. (2010). Tissues were homogenized (1:2 w/v) in ice-cold extraction buffer composed of 20 mM sodium acetate (pH

5.6), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethlysulfonyl fluoride, 5 mM thiourea, and 1% (w/v) insoluble polyvinyl (polypyrrolidone). Homogenates were centrifuged at 4 ˚C and 15,000g for 10 min, and the supernatants reserved as clarified extract.

4.3.7 Enzyme assays

Enzyme assays for PEPC, PK, NADH-MDH and ME were performed according to Le Roux *et al.* (2006, 2008). Enzyme assays for APase were performed according to Hurley *et al.* (2010). All reactions were initiated by adding 30 µl crude extract to reaction mixture in a total volume of 250 µl and read continuously, spectrophotometrically at 340 nm and 25°C for a total of 5 minutes.

Phosphoenol-pyruvate carboxylase (PEPC) The PEPC activity was determined by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation. 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, 5 U MDH (Ocaña *et al*., 1996). The blanks consisted of a reaction medium without PEP.

Pyruvate kinase (PK) The PK activity was assayed in a buffer modified to contain 75 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 20 mM KCl, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and lactate dehydrogenase (LDH) (3 U) (McCloud *et al.*, 1985). The blanks consisted of one reaction without ADP and another without PEP.

NADH-Malate dehydrogenase (MDH) The MDH activity was assayed as described by Appels & Haaker (1988). The reaction mixture contained 25 mM KH_2PO_4, 0.2 mM NADH, 0.4 mM oxalo-acetic acid (OAA) (Appels and Haaker, 1988). The blanks consisted of a reaction medium without OAA.

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

Acid Phosphatase (APase) APase activity was measured by coupling the hydrolysis of PEP to pyruvate to the LDH reaction and continuously monitoring NADH oxidation (Hurley *et al*., 2010). The assay mixture contained 50 mM Na acetate (pH 5.6), 5 mM PEP, 10 mM $MgCl_2$,

0.2 mM NADH, and 3 units of rabbit muscle LDH. The blanks consisted of reaction medium without PEP (Hurley *et al*., 2010).

4.3.8 Statistical analysis

The effects of the factors and their interactions were tested with a Tukey's Student T-test (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the T-test revealed significant differences between treatments, the means (6-8) were separated using post-hoc Student Newman Kuehl's (SNK) multiple-range test ($P \leq 0.05$). Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

4.4 Results

4.4.1 Biomass and growth

Control plants supplied with low P, produced less nodule biomass, but had increased cluster root formation when compared to control plants supplied with high P (Fig. 1a). There was no significant difference in shoot or whole plant biomass between treatment groups. Relative below-ground allocation of resources to roots, nodules and cluster roots, was however significantly different (Fig. 4.1b). The composition of the root system of control plants supplied with high P, constituted 25% less cluster roots, but 50% more nodules when compared to control plants supplied with low P (Fig. 4.1b). Roots constituted up to 49% of the total root system in control plants supplied with high P, but only 30% in low P supply control plants (Fig. 4.1b).

There was no significant increase in daily growth respiration rates for roots and shoots between treatment groups (Fig. 4.2a). Control plants supplied with low P however showed increased daily growth respiration (Fig. 2a) and relative growth rates (Fig. 4.2b) for cluster roots, but decreased growth respiration (Fig. 4.2a) and relative growth rates (Fig. 4.2b) for nodules, when compared to control plants supplied with high P (Fig. 4.2a and b).

4.4.2 N and P concentrations in plant organs

The concentration of P in cluster roots was greatly increased for control plants supplied with low phosphate, however control plants supplied with high P showed a significant increase in nodule P concentration (Fig. 4.3a). Furthermore, shoots, nodules and roots of control plants supplied with high P had an increased N concentration, when compared to control plants

only supplied with low P (Fig. 4.3b). There was no significant increase in N concentration for cluster roots between treatment groups (Fig. 4.3b). Specific N (Fig. 4.3c) and P (Fig. 4.3d) acquisition rates were also significantly increased for control plants supplied with high P.

4.4.3 Nodules and biological nitrogen fixation

Nodules of control plants supplied with high P showed a 33% increase in N derived from atmosphere (Fig. 4.4b) and a 20% increase in BNF efficiency, when compared to control plants (Fig. 4.4a). Daily C construction costs only significantly differed for shoots of control plants supplied with high P (Fig 4.5).

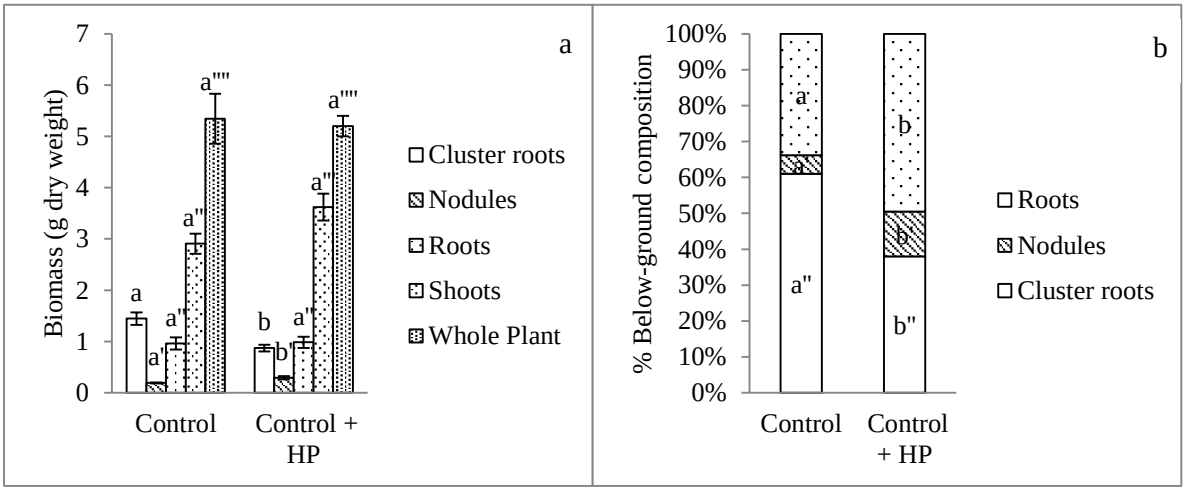

Figure 4.1 Biomass of cluster roots, nodules, roots, shoots and whole plant (a) and percentage (%) below-ground allocation of roots, nodules and cluster roots of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

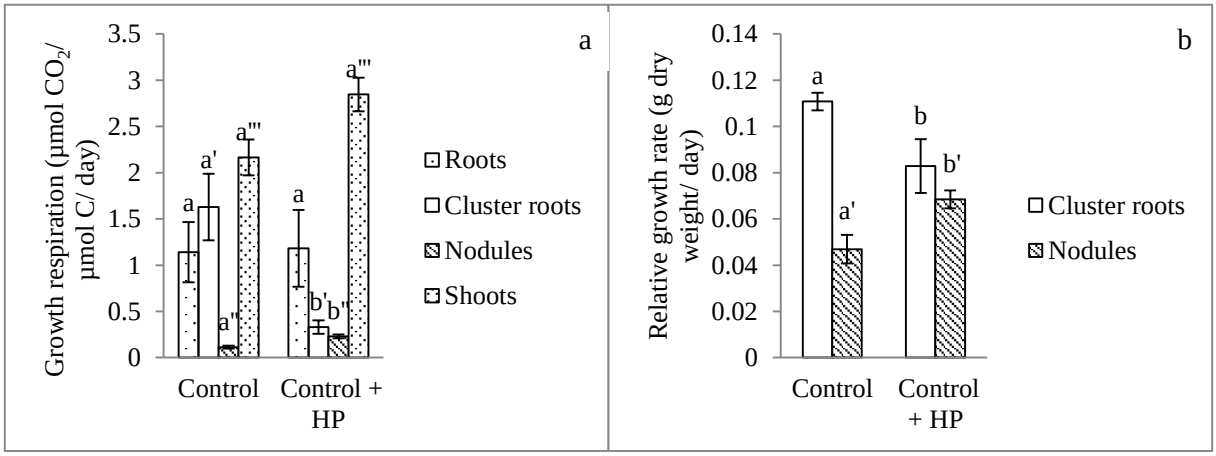

Figure 4.2 Daily growth respiration for roots, cluster roots, nodules and shoots (a) and relative growth rates of cluster roots and nodules (b) of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

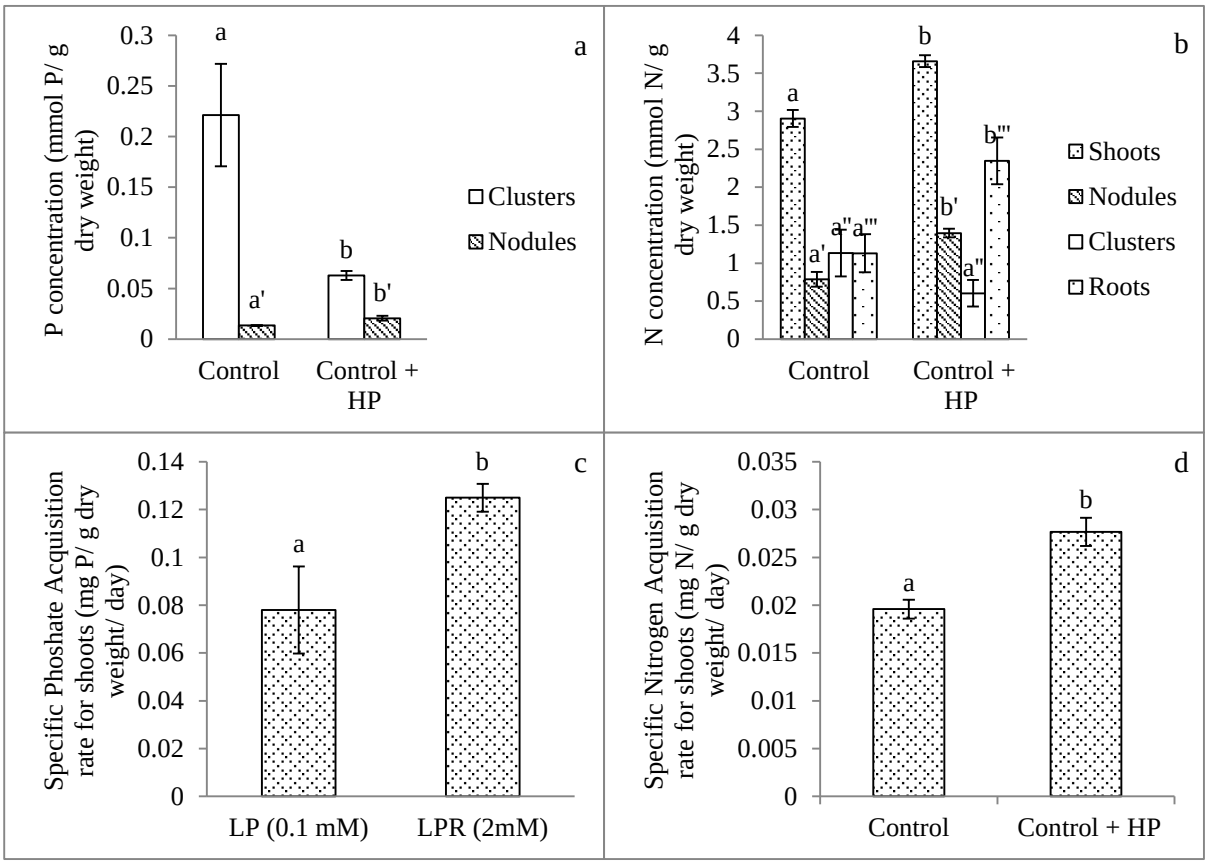

Figure 4.3 P concentration in clusters and nodules (a), N concentration in shoots, roots, cluster roots and nodules (b), Specific P (c) and N (d) acquisition rate of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

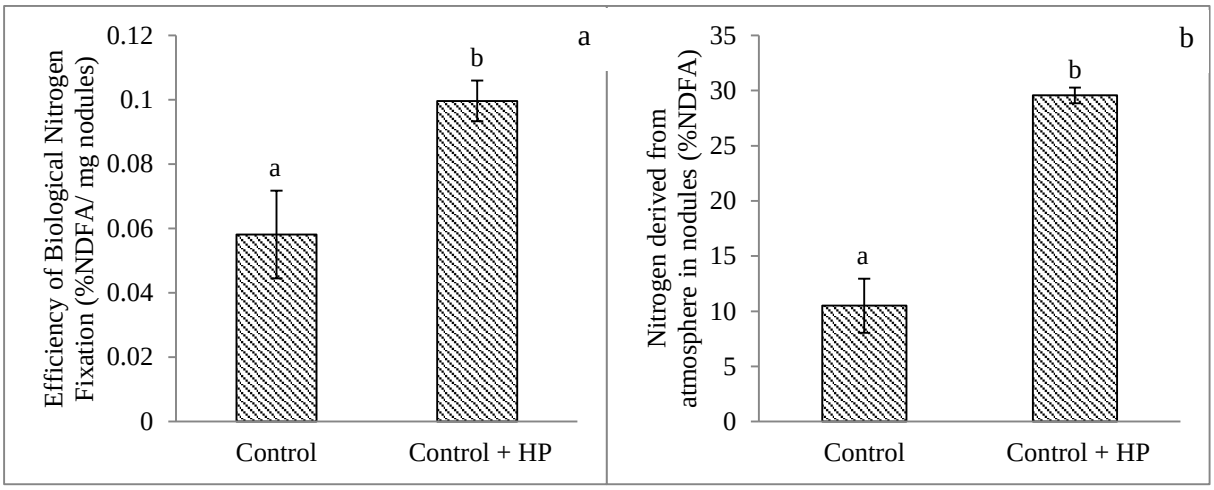

Figure 4.4 The efficiency of biological nitrogen fixation (a) and the nitrogen derived from atmosphere (b) in nodules of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

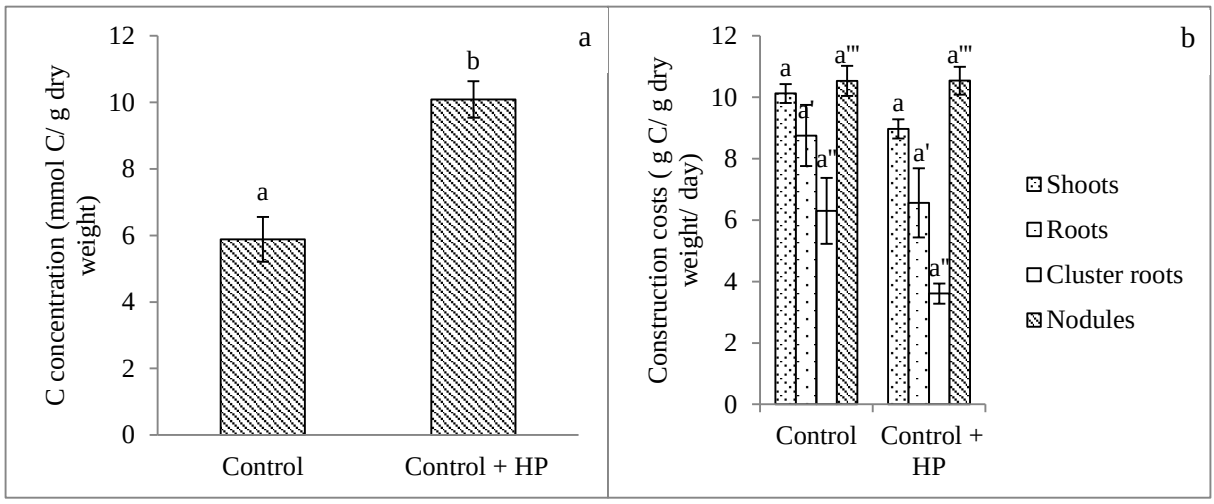

Figure 4.5 Carbon concentration in nodules (a) and carbon construction costs of shoots, roots, cluster roots and nodules of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

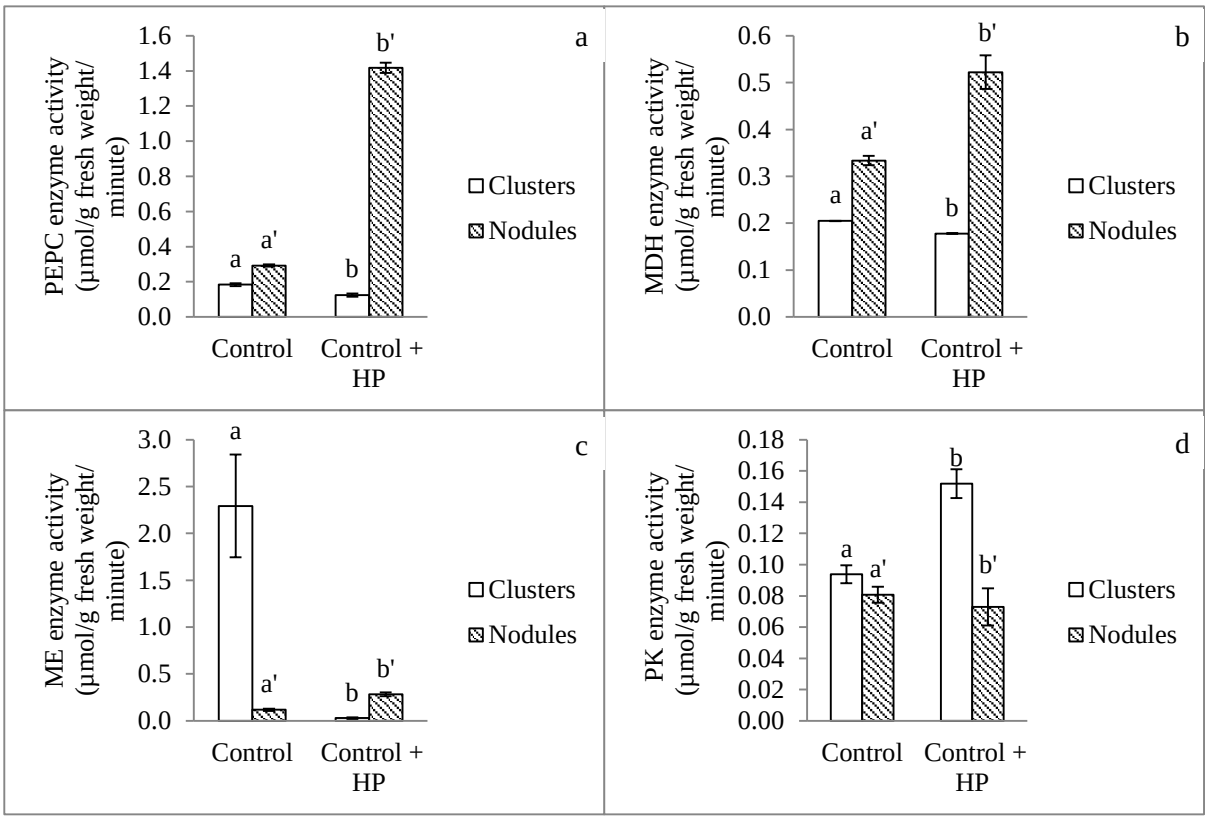

Figure 4.6 Enzymatic activity of Phosphoenol-pyruvate Carboxylase (a), Malate Dehydrogenase (b), Malic enzyme (c) and Pyruvate kinase (d) from clusters and nodules of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

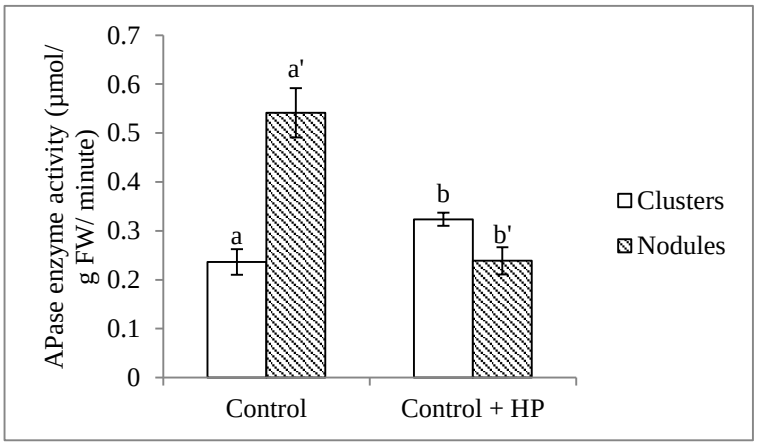

Figure 4.7 Enzymatic activity of intracellular acid phosphatases (APases) from clusters and nodules of *L. albus* control plants and plants supplied with high phosphate. Values are presented as means (n=3) with standard error bars. Different letters indicate significant differences between treatments, where the prime lettering indicates the comparisons between the same organs.

4.4.4 Enzyme activity

There was a significant increase in nodule PEPC activity of control plant supplied with short term high P, however a decrease in cluster roots PEPC activity was observed for the same treatment (Fig. 4.6a). The activity of MDH showed a similar trend in activity for both cluster and nodules between treatments (Fig. 4.6b). MDH activity was significantly increased for nodules of control plants supplied with short term high P; however cluster root MDH activity declined (Fig. 4.6b).

Enzymatic activity of ME was drastically increased in the cluster roots of control plants (Fig. 4.6c) with low activity observed in nodules of control plants, nodules and cluster roots of control plants supplied with high P. There was however a small, but significant increase in ME activity in nodules of control plants supplied with high P (Fig. 4.6c).

Cluster roots of control plants supplied with high P showed a significant increase in PK enzyme activity when compared to control plants (Fig. 4.6d). There was however no significant change in PK nodule activity between treatments (Fig. 4.6d).

APase activity was significantly increased in nodules, but decreased in cluster roots of plants supplied with low P (Fig. 4.7). Cluster roots of plants supplied with high phosphate showed increased APase activity when compared to plants supplied with low P.

4.5 Discussion

White lupin plants grown with low P supply, preferentially allocated C and P resources to nodules after high P supply, at the expense of cluster roots. This alteration in physiological allocation during high P supply, was also reflected as metabolic changes in the activities of enzymes related to organic acid synthesis.

The variation in P supply can affect the growth and physiology of nodules and cluster roots. Phosphate deficiency can affect BNF through effects on host plant growth, nodule functioning and nodule formation (Tang *et al*., 2001). It was shown by Hogh-Jensen *et al.* (2002) that white clover plants preferentially allocated resources for increased nodule mass during the first 9 days of low P supply. After this period however, nodule biomass was adjusted for the decreased P supply (Hogh-Jensen *et al*., 2002). Phosphate deficiency has also been shown to decrease nodule biomass in common bean (Vadez *et al*., 1996), pea (Jakobsen, 1985) and alfalfa (Drevon and Hartwig, 1997). Schulze *et al.* (2006) showed that *L. albus*

plants grown under P deficiency (no P supply) for 21 days produced comparable amounts of nodule biomass. This however changed after 37 days of P deficiency, when significantly less nodule biomass was produced when compared to plants grown with adequate P supply (0.5 mM).

Cluster root production during low P supply is well documented for *L. albus* (Dinkelaker *et al*., 1989; Johnson *et al*., 1996; Watt and Evans, 1999; Lamont, 2003; Schulze *et al*., 2006). Cluster roots function as a combined P acquisition strategy by combining increased root surface area (Neumann *et al*., 1999), transporter production (Liu *et al*., 2001), organic acid and acid phosphatase exudation (Vance *et al*., 2003). Cluster roots supply the whole plant, including nodules with P. This is evident in observations by Schulze *et al.* (2006), that nodules form above/near cluster roots in P deficient *L. albus*. This allows for adequate P supply to the nodules for the maintenance of BNF and nodule growth. Cluster roots are however only favourable during low P conditions. Exudation of large amounts of organic acids, in the form of citrate and malate, is futile once P sufficient conditions return as is further cluster root formation. Thus during short term high P supply normal root growth is favoured over cluster root formation. This can be seen in the below-ground composition of the root system, where roots constituted 23% more of the root system, during short term high P supply, with a subsequent decrease in cluster root formation. Roots however did not show any significant increase in P uptake. This could be due to the action of cluster roots in the early stages of high P supply. Due to increased P supply and subsequent uptake of P from the rhizosphere via cluster roots, internal plant P levels increase and thus supress the low P response. To avoid P toxicity no further P must be obtained from other sources until the cluster roots have senesced.

The C costs associated with changes in allocation of resources between cluster roots and nodules are reflected in the growth respiration and relative growth rates for each organ. Nodules of plants supplied with high P produced 33% more dry weight per day, with a subsequent 50% reduction in cluster root RGR. Furthermore, the nodules of these plants showed a 42% increase in daily growth respiration, while cluster root growth respiration decreased by 44%. There is thus a clear shift in C resource allocation from cluster roots to nodules during short term high P supply. This possibly points to a change in allocation of available C resources, as there was no difference in construction costs for nodules between treatment groups. Bacteroids that contain active *rhizobia* are large consumers of C skeletons in the form of malate (Driscoll and Finan, 1993). Malate is used as the main energy source

and is used to maintain growth, BNF and the production of amino acids for export/use (Rosendahl *et al*., 1990; Schulze *et al*., 2002; 2006). *Lupinus albus* on average requires 3.64 g C/g N fixed (Layzell *et al*., 1979), with *rhizobia* consuming 4-16% of the daily photosynthate to maintain BNF function (Kaschuk *et al*., 2009). This translates into vast amounts of C being supplied to the nodules by the host plant. This is substantiated by nodule biomass showing a significant increase after short term high P supply, when compared to roots, shoots and cluster roots.

Plants supplied with short term high P also had increased N and P nutrition in nodules, roots and shoots. Although there was no increase in shoot biomass, shoots of high P supplied plants acquired both N and P at an increased rate. Interestingly there was no significant increase in shoot P, however N concentration was increased. In spite of the unchanged shoot P levels, the increased N concentrations in high P supply shoots, could point to P being utilised for photosynthesis and/or sugar-P production, while increased N could point to increased protein production required for these processes. Tang *et al*. (2001) showed that *Medicago truncatula* (barrel clover) nodules, grown with increasing P supply (1- 8 µM), showed a 50% increase in nitrogenase activity. Nitrogenase activity is generally repressed during P deficiency, due to the effects of limited P supply on nodule O_2 permeability (Layzell *et al*., 1990; Ribet and Drevon, 1995). Nodules of smaller size are formed during P deficiency, albeit more are formed, allowing for increased surface: area ratio and thus facilitating oxygen diffusion into the nodule (Ribet and Drevon, 1995). Increased O_2 permeability during P deficiency has been shown for common bean (Valdez *et al*., 1996), alfalfa (Schulze and Drevon., 2005) and soybean (Ribet and Drevon., 1995). Increased BNF efficiency rates and %NDFA for plants supplied with high P, could indicate increased nitrogenase activity and can explain the increase in shoot N concentration and acquisition.

Nodules act as strong sinks for P and C, readily acquiring both from the host (Al-Niemi *et al*., 1998). Legumes that solely rely on BNF have a greater demand for P, due to this energetically expensive process (Sa and Israel, 1991; Tang *et al*., 2001). During P deficiency, successful host-rhizobia interaction can depend largely on the efficiency of P allocation and use (Valentine *et al*., 2010). Le Roux *et al.* (2008) found that nodules experiencing P deficiency induced alternative non-adenylate requiring steps for basic pyruvate metabolism via PEPC and MDH. This allowed for the accumulation of large amounts of malate. This in turn inhibited BNF and caused a shift in C allocation, from amino acid to organic acid synthesis. Another possible reason for the lowered BNF rate of P deficient plants is inhibition

of BNF via a N-feedback mechanism. Hogh-Jensen *et al.* (2002) showed that *Trifolium repens* grown under P deficiency accumulated asparagine in roots and nodules, with a subsequent reduction in BNF. This was attributed to a reduced N demand from the host plant, which would ultimately supress BNF rates in nodules. Nodules from P deficient *Phaseolus vulgaris* (common bean) also showed increased activity of AAT (amino acid transferase) and increased asparagine (amine) production (Oliveira *et al*., 2004).

Plants supplied with short term high P also showed increased organic acid synthesis, specifically malate, via increased PEPC-MDH and ME enzyme activities. The engagement of both routes for enhanced malate synthesis in nodules, may be related to the increase in BNF rates with higher P nutrition. The higher malate in this regard, may serve as a bacteroid C source or a source of organic acid skeletons for amino acid synthesis as discussed earlier (Rosendahl *et al*., 1990; Schulze *et al*., 2002; 2006). This high requirement for organic acids by nodules, concurs with the general trend of the data, that organic acid synthesis, linked to malate, is much higher in nodules when compared to cluster roots during high P supply. Nodule enzyme activity of PEPC, MDH and ME was all reduced during low P, while cluster root enzyme activity was increased. This indicates the necessity of cluster root function during low P supply, even at the expense of BNF rates.

Cluster roots are clearly adapted for organic acid synthesis during low P supply. This however changes with supply of short term high P. This is evident from the high activities of MDH, PEPC and ME in cluster roots, at low P supply, and their subsequent decline during short term high P supply. It is well documented that PEPC and MDH activity is increased during low P supply in cluster roots of *L. albus* (Johnson *et al*., 1996; Gilbert *et al*., 1998; Neumann *et al*., 1999; Massenneau *et al*., 2001). These enzymes serve to circumvent Pi requiring steps in glycolysis and so contribute to efficient metabolic use of P (Plaxton, 1998). The activity of PEPC also involves non-photosynthetic CO_2 fixation and can account for up to 35% of C released as carboxylates in cluster roots as reported by Johnson *et al.* (1994, 1996). This action could possibly compensate for the loss of C through the large scale exudation of organic acids during low P supply (Neumann and Martinoia, 2002). PK values remained unchanged for nodules, indicating no changes in conversion rates of PEP to pyruvate via PK. This however coupled to increased ME activity in nodules during high P supply, shows how the allocation of pyruvate is shifted towards malate production during times of increased energy demand.

Interestingly there was also a large increase in intracellular APase activity in cluster roots of high P supplied, even though less cluster roots were formed. Intracellular APases break down organic forms of P in the plant cell, being located mostly in the vacuole, cytosol and plasma membrane (Gilbert *et al*., 1998; Ragothama and Karthikeyan, 2005; Tang *et al*., 2013). These APases recycle internal Pi from organic molecules present in the cell, such as nucleotides, sugar-P and P-monoesters (Hurley *et al*., 2010; Tang *et al*., 2013). Tang et al., et reported increased intracellular APase activity in white lupin cluster roots after maturation and again during senescence. This is due to the need for efficient use of available P during cluster root function and the subsequent recycling of this invested P during cluster root senescence (Wasaki *et al*., 2003; Tang *et al*., 2013). Due to the large decrease in cluster roots upon short term high P supply, senescence of cluster root would occur, thus explaining the increased APase activity during short-term high P supply in cluster roots. Nodules however showed the reverse trend with higher APase activities in nodules of plant supplied with low P. Penheiter *et al*. (1997) showed that APase activity in soybean nodules increased with nodule development, but decreased with nodule age. Due to the ubiquitous function of APases their role in nodules during Pi deficiency is largely uncharacterised (Penheiter *et al*., 1997). Soybean nodules grown with low P has significantly increased APase activity, when compared to plants grown with sufficient P (Penheiter *et al*., 1997). It is thought that nodule APases function to recycle P in Pi starved nodules, to maintain BNF rates, through the supply of P for amino acid biosynthesis and nodule metabolism (Penheiter *et al*., 1997).

Our findings suggest that there is a great degree of functional flexibility in the lupin nutrient acquiring structures, during short-term P supply. Although no changes in whole plant biomass was observed with short-term supply of high P, our findings indicate that overall plant P and N nutrition was improved at the physiological and metabolic level. Nitrogen nutrition was especially favoured due to increased nodule biomass and function, albeit at the expense of cluster root production and function

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CHAPTER 5

GENERAL DISCUSSION

5.1 Introduction

The availability of P in the soil is a major constraint for plant growth (Vance *et al*., 2003; Ragothama and Karthikeyan, 2005; Lambers *et al*., 2006). Phosphate is a key element and is involved in various physiological, biochemical and metabolic processes in the plant (Marschner, 1995; Uhde-Stone *et al*., 2003; Vance *et al*., 2003). The generally low concentrations of P in the soil, however causes most plants to experience P deficiency during their life cycle (Lamont, 2003; Lambers *et al*., 2006). Soil P concentrations rarely exceed 10 µM due to the reactivity of P with other metals, predominantly Ca^{2+} and Mg^{2+}, forming insoluble complexes or interaction with clay particles, rendering uptake impossible (Ragothama 1999; Vance *et al*., 2003; Ragothama and Karthikeyan, 2005). This problem is further exacerbated in the acid weathered soils of the temperate Mediterranean, where calcareous, nutrient poor soils predominate (Zhu *et al*., 2005).

White lupin (*Lupinus albus*) is a temperate, intensively studied, cluster root forming, agricultural legume (Watt and Evans, 1999). It's extreme tolerance to P deficient conditions has led to white lupin becoming a model system for studying plant responses to nutrient deficient conditions (Vance, 2001; Neumann and Martinoia, 2002; Shane and Lambers, 2005; Rath *et al*., 2010). Schulze *et al.* (2006) reported normal growth patterns for both Pi deficient (no P supplied) and Pi sufficient plants, for up to 30 days after treatment initiation. The formation of specialised cluster roots intimately linked to this extreme tolerance of Pi deficiency. Instead of forming a mycorrhizal association white lupin plants initiate a co-ordinated expression of genes leading to the production of cluster roots, organic acid and acid phosphatase exudation and increased P transporter production (Neumann and Martinoia, 2002; Vance *et al*., 2003; Uhde-Stone *et al*., 2003). This leads to increased availability and uptake of P from the soil, allowing white lupin to maintain growth under P deficient conditions. This study focused on the effects of variations in P supply and the allocation of below-ground resources (Chapter 3) and how this alters the belowground C allocation costs and functions (Chapter 4) of cluster roots and nodules.

5.2 Consequences of short term and long term variation in P supply on nodule and cluster root function

This study found that white lupin can maintain growth even when grown in P deficient conditions as was shown by the unchanged above and below ground allocation of biomass and biomass on a whole plant basis, even with reduced photosynthetic capacity. This concurs with work done by Schulze *et al.* (2006) that shows the high tolerance of *L. albus* to Pi deficient conditions and the maintenance of BNF rates. Although no changes were observed in biomass on a whole plant basis, root system architecture did change with variations in P supply. There was a clear increase in cluster root biomass, with a subsequent suppression of nodule biomass in P deficient plants. This was largely due to decreased internal P concentrations in nodules, although BNF rates were maintained. This could be due to preference of maintaining nodule function even during P deficiency. This is supported by previous studies showing that nodules almost always operate at low P concentrations (Sa and Israel, 1991; Al Niemi *et al*., 1997, 1998). Their P requirements were being fulfilled by scavenging P from host cells (Al Niemi *et al*., 1997, 1998). Nodules also have strategies to minimize the effects of P deficiency by either (a) regulating P influx or (b) increasing the phosphate use efficiency, for essential metabolic functions (Jakobsen, 1985; Tang *et al*., 2001, Colebatch *et al*., 2004). Furthermore after short term high-P supply, nodule biomass, P concentration and BNF efficiency increased, with subsequent declines in cluster root biomass and P concentration. Nodules thus appear to have adapted to maintain function and efficiency of BNF during fluctuations in P supply. This suggests a physiological trade-off may exist between cluster roots and nodules during variations in P supply, with maintenance of nodule function being favoured.

Changes at the metabolic level were more flexible than overall plant physiology. Plants supplied with short term high P showed increased malate synthesis, via increased PEPC, MDH and ME enzyme activities. The engagement of both metabolic routes for increased malate synthesis may be related to the increase in BNF rates with higher P nutrition. The increased malate production could serve as a C source for nodule function and growth or as a source of organic acid skeletons for amino acid biosynthesis (Rosendahl *et al*., 1990; Schulze *et al*., 2002, 2006). Cluster roots are clearly adapted for organic acid synthesis during low P supply. This is evident from the increased activities of MDH, PEPC and ME in cluster roots, at low P supply, and their subsequent decline during short term high P supply. Increased activities for PEPC and MDH during low P supply in cluster roots of *L. albus* is well

documented (Johnson *et al*., 1996; Gilbert *et al*., 1998; Neumann *et al*., 1999; Massenneau *et al*., 2001). These enzymes serve to circumvent Pi requiring steps in glycolysis and so contribute to efficient metabolic use of P (Plaxton, 1998). The activity of PEPC also involves non-photosynthetic CO_2 fixation. Non-photosynthetic CO_2 fixation can account for up to 35% of C released as organic acids by cluster roots (Johnson *et al*., 1994,1996). This activity could compensate for the loss of C through the large scale exudation of organic acids during low P supply (Neumann and Martinoia, 2002). This indicates the necessity of cluster root metabolism and function during low P supply for the acquisition, conservation and recycling of P.

5.3 Future studies

This study highlights the importance of investigating the interplay of nutrient acquisition strategies employed by plants in response to variations in P supply, at a physiological and metabolic level. Although this work has been the first to investigate the below-ground root system responses to the variation in P supply, the parameters were mostly physiological and biochemical. A natural progression of this work in our laboratory would be to investigate this regulation at the transcriptomic and proteomic levels.

In addition, the following research areas warrant further investigation by researchers in this field.. There are several other bypass reactions induced during P deficiency , which should also be studied. These include the (a) PPi-phosphofructokinase (PFP) bypassing the reaction of the ATP-dependant PFP and (b) non-phosphorylating NADP-glyceraldehyde-3P dehydrogenase (NADP-G3PDH) as an alternative to Pi dependant NAD-G3PDH and phosphoglycerate kinase route (Plaxton and Podesta, 2006). Furthermore alternative pathways of respiration can be induced in nodules and roots during low P supply. These pathways (cyanide insensitive pathways) consume less P due to decreased synthesis of adenylates in the electron transport chain (mitochondria) (Gonzaléz-Meler *et al.*, 2001; Juszczuk *et al*., 2001, Plaxton and Podesta, 2006). This could be an important pathway in cluster roots for conservation of P during formation and growth and requires further investigation.

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