THE FUNCTIONAL RESPONSES OF PHOSPHATE-DEFICIENT LUPIN NODULES AS MEDIATED BY PHOSPHOENOLPYRUVATE CARBOXYLASE AND ALTERED CARBON AND NITROGEN METABOLISM

by

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DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

In soils, the concentration of available phosphate (P) for plants is normally very low (*ca*. 1 µM in the soil solution), because most of the P combines with iron, aluminium and calcium to form relatively insoluble compounds. Inorganic P (P_i)-deficiency is thought to be one of the limiting factors of nitrogen fixation due to the high energy requirement for nitrogenase function of plants taking part in nitrogen fixation. P_i-deficiency has important implications for the metabolic P_i and adenylate pools of plants, which influence respiration and nitrogen fixation. An alternative route of pyruvate supply during P_i stress has been proposed involving the combined activities of phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase (MDH) and NAD-malic enzyme (ME) supplying pyruvate to the mitochondrion during P_i stress. Previously, three isoforms of PEPc were isolated from lupin nodules and roots, with two forms being nodule specific. The aim of this project was to determine the effect of P_i stress on these PEPc isoforms in *Lupinus luteus* at transcript and protein expression level with a view to produce genetically modified crops for nutrient-poor soils.

Cytosolic P levels were measured over a time course to give an indication of temporal development of P stress in nodules. The changes in enzyme activities of PEPc, MDH and PK (pyruvate kinase) under P stress were measured and the downstream effect on amino and organic acid pools were analysed. Two novel PEPc isoforms, LUP1 (AM235211) and LUP2 (AM237200) were isolated from nodules, followed by transcriptional and protein expression analyses.

Nodules under P stress had lower amounts of metabolically available P_i and as P stressed developed, the amount of P_i decreased. This decline in P_i levels was associated with lower growth, but higher biological nitrogen fixation (BNF). A greater proportion of root-nodule respiration was devoted to nutrient acquisition than to new growth. A typical P-stress response is higher anaplerotic carbon fixation via PEPc. However, in this study, no significant differences were found for PEPc, MDH or PK in P-stressed plants compared to P-sufficient plants which would lead to an increase in organic acids. An increase in key amino acids was reported along with unchanged levels of organic acids. These levels of organic and amino acid are in congruence with the increases in BNF under P-starvation.

No significant differences were found in expression of *PEPC1* or *PEPC2* at 12 and 20 days for both P-sufficient and P-stressed plants which further supported the lack of engagement of the PEPc-MDH-ME bypass. PEPc activity appeared not to be regulated by gene expression or phosphorylation indicating that other post-translational modifications such as a decrease in protein degradation may be of importance.

OPSOMMING

Die konsentrasie van fosfaat (P) beskikbaar vir opname deur plante vanuit die grond is gewoonlik baie laag (in die omgewing van 1 µM) aangesien die P onoplosbare komplekse vorm met katione soos yster, aluminium en kalsium. ‘n Tekort aan anorganiese P (P_i) word gereken as een van die beperkende faktore van stikstofbinding as gevolg van die hoë energie behoefte wat nitrogenase plaas op plante wat van gefikseerde stikstof gebruik maak. Hierdie P-tekort het ook belangrike betrekking op die metaboliese fosfaat- en adenilaatpoele wat weer op hul beurt respirasie en stikstofbinding beÏnvloed. ‘n Alternatiewe roete van pirovaatvoorsiening aan mitochondria tydens fosfaatstres is voorgestel wat bestaan uit die aktiwiteite van fosfoenolpirovaat karboksilase (PEPc), malaat dehidrogenase en NAD-malaat ensiem. Vantevore is drie isovorme van PEPc uit *Lupinus luteus* wortelknoppies en wortels geïsoleer, met twee van die isovorme wat wortelknoppie-spesifiek was. The doel van hierdie projek was om die invloed van P-tekort op die transkripsie en proteien uitdrukkingsvlak van hierdie PEPc isovorme te bepaal met die doel van gemodifiseerde gewasse vir arm gronde ingedagte.

Sitoplasmiese P konsentrasies is gemeet oor tyd om ‘n aanduiding te gee van die ontwikkeling van P-tekort oor tyd. Veranderinge in ensiemaktiwiteite van PEPc, MDH en pirovaatkinase (PK) is gemeet gedurende P-tekort as ook die moontlike effek van hierdie ensiemaktiwiteite op aminosuur en organiese suur poele. Twee nuwe PEPc isovorme, LUP1 (AM235211) en LUP2 (AM237200) is uit wortelknoppies geïsoleer en gekarakteriseer. Transkripsie en proteïenuitdrukking is geanaliseer.

Wortelknoppies wat P-tekort behandeling ontvang het, het laer vlakke van metabolise beskikbare P_i gehad en soos die P-tekort ontwikkel het oor tyd, het die P_i vlakke gedaal. Hierdie afname in vlakke van P_i was geassosieer met laer groei, maar met ‘n toename in biologiese stikstofbinding. ‘n Groter proporsie van respirasie is toegestaan aan minerale opname as aan nuwe groei. ‘n Tipiese reaksie op P-tekort is hoër anaplerotiese koolstofbinding via PEPc. Alhoewel, in hierdie studie is geen gevind betekenisvolle verandering gevind in die aktiwiteite van PEPc, MDH en PK nie in plante wat P-tekort ervaar het nie. Verhoogde aktiwiteit van hierdie ensieme sou verhoogde organise suur konsentrasies tot gevolg hê. ‘n Toename in aminosuur konsentrasies is gevind tesame met onveranderde vlakke van organiese sure. Hierdie toename in aminosure word onderskryf deur die verhoogde biologiese stikstofbinding tydens P-tekort.

Geen betekenisvolle verskille is gevind in die geenuitdrukking van *pepc1* en *pepc2* by beide 12 en 20 dae van P-tekort nie, wat verder die afwesigheid van die PEPc-MDH-ME alternatiewe roete beaam het. Dit blyk dat PEPc aktiwiteit nie deur geenuitdrukking of proteïenfosforilering beheer word nie, maar eerder dat ander post-translasie modifikasies soos ‘n verlaagde afbraak van proteïen ‘n rol speel.

Ke Nako - It's time

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LIST OF ABBREVIATIONS

%	percentage
(v/v)	volume per volume
(w/v)	weight per volume
˚C	degrees Celsius
AAT	aspartate amino transferase (EC 2.6.1.1.)
ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BNF	biological nitrogen fixation
bp	nucleic acid base pair
BTPC	bacterial-type phosphosphoenolpyruvate carboxylase
C	carbon
C:N	carbon to nitrogen ratio
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CTAB	Cetyl trimethylammonium bromide
dH_2O	distilled water
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
g	gram
HPLC	high performance liquid chromatography
IAEA	International Atomic Energy Agency
kb	kilo base pair
kDa	kilodalton

l	liter
LR-PCR	long range polymerase chain reaction
LSD	least significant difference
M	molar
MDH	malate dehydrogenase (EC 1.1.1.37)
miETC	mitochondrial electron transport chain
ml	milliliter
mM	millimolar
mm	millimetre
N	nitrogen
N_2	dinitrogen
NAD	nicotinamide adenine dinucleotide, oxidised form
NADP	nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
ng	nanogram
nM	nanomolar
Nm	nanometer
O_2	oxygen
P	phosphate
PBM	peribacteroid membrane
PBS	peribacteroid space
PEPc	phosphoenolpyruvate carboxylase (EC 4.1.1.31)
PHB	poly-β-hydroxybutyrate
P_i	inorganic phosphate
Pi	iso-electric point
PITC	phenylisothiocyanate
PK	pyruvate kinase (EC 2.7.1.40)

pmole	picomole
PP2A	protein phosphatase type 2A
PPCK	PEPc protein kinase
PP_i	inorganic pyrophosphate
Ppm	parts per million
PTPC	plant-type phosphoenolpyruvate carboxylase
PVPP	polyvinylpolypyrrolidine
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
TCA	tricarboxylic acid cycle
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-phosphate
x g	gravitational force
δ15N	nitrogen isotopic ratio
µl	microliter
µM	micromolar
µmole	micromole
pH	acidity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Legumes globally and in southern Africa

Legumes are grown on approximately 275 million hectares or nearly 11% of arable land worldwide (Van Kessel and Hartley 2002) and provide at least 33% of human protein requirements. In the tropics and subtropics legumes can satisfy up to 80% of protein needs. Grain legumes are important as food and feed proteins and in many regions of the world they are the only supply of protein in the diet (Duranti and Gius 1997), because of the high price of animal protein. All legume seed proteins are relatively low in sulphur-containing amino acids and tryptophane, but the amount of lysine, another essential amino acid, is much greater than in cereal grains (Ampe *et al.* 1986). Most food in the developing countries of the tropics is grown on small, family-managed farms where, especially in Africa and South America, the use of fertilizer and other agro-chemicals is minimal. Growing N_2-fixing grain legume crops in such circumstances provides excellent opportunities for producing high protein food sources without inputs of N fertilizer (predominantly in the form of NH_4^+) (Boddey *et al.* 1997). It takes 1.3 tons of fossil fuel to manufacture one ton of nitrogen fertilizer. Since legumes do not require nitrogen as fertilizer, the production of these plants as a source of protein would not consume non-renewable fuels. The reliance on legumes is therefore basic to sustainable and economic production of food and feed proteins (Howieson *et al.* 2000).

In southern Africa, indigenous leguminous crops are used as food and as a feed source for livestock. They may also be economically important, as they allow smallholder farmers to trade with the surplus.

These crops include the following (Van Wyk and Gericke 2000):

- Wild coffee bean (*Bauhinia petersiana*) and jack bean (*Canavalia ensifolia*) — frequently used as a coffee substitute, but the seeds and pods may also be used as a source of food
- Pigeon pea (*Cajanus cajan*) — used for seeds and also as a green vegetable; it is grown almost exclusively for subsistence, with only small quantities reaching local and international markets

- Copalwood (*Guibortia coleosperma*) — the seeds are a primary food source to the !Khu Bushmen of north-eastern Namibia, who use its oily arils for food during periods of famine, and various parts of the plant for traditional medicine
- Karoo boer-bean (*Schotia afra* var. *afra*)
- Marama bean (*Tylosema esculenta*) — has a large woody below-ground tuber with a moisture content of 81 %, which makes it a valuable source of water during emergencies. The young pods are eaten as a vegetable and the large seeds are eaten roasted. It forms an important part of the diet of rural people in the Kalahari, the Kaokoveld and Mozambique
- Bambara groundnut (*Vigna subterranea*) — grown exclusively as a protein source, and included in many traditional recipes. The immature beans can be eaten raw or cooked, while ripe beans can be pounded into a flour, or soaked and then cooked (Van Wyk and Gericke 2000). Bambara is considered a substitute for meat and the ripe beans are very nutritious (Venter and Coertze 1996). One of the most under-rated and under-developed crop plants in the world, it produces reasonably well under extreme conditions such as drought and poor soil (Van Wyk and Gericke 2000).
- Cowpea (*Vigna unguiculata*) and mung bean (*Vigna radiata*) — these important food sources in southern Africa are popular for their beans and also as green vegetables
- Wild sweetpea (*Vigna vexillata*) (Van Wyk and Gericke 2000).

1.2 Environmental limitations

Worldwide, more than 50 % of crops are lost due to abiotic stress such as nutrient deficiency, drought, salinity and aluminium toxicity with an approximate 40 % reduction in soybean yield ascribed to drought (Valentine *et al.* 2010). The impoverishment of the soil is a growing constraint on sustainable development in the Third World, particularly in sub-Saharan Africa. The use of fertilizer in this part of the continent averages only 5–10 kg h^{-1}, with many soils progressively being denuded of their nutrients (Graham and Vance 2000). The concentration of available phosphorus for plants is normally very low in soils, because most of the element combines with iron, aluminium and calcium to form relatively insoluble compounds (Aono *et al.* 2001). Phosphorus is present in the soil water in the ionic forms $H_2PO_4^-$ and HPO_4^{2-} (Wild 2003). Smallholders in southern Africa apply less inorganic phosphorus fertilizer (P_i, as inorganic mineral salts) than is removed during harvesting, thereby

depleting soil P reserves (Holford 1998). P_i -deficiency is thought to be one of the limiting factors of nitrogen fixation (Aono *et al.* 2001) owing to the high energy requirement of plants engaged in nitrogen fixation for nitrogenase function (Al Niemi *et al.* 1997). P_i -deficiency has important implications for the metabolic P_i and adenylate pools of plants, which influence respiration and nitrogen fixation (Theodorou and Plaxton 1995, Plaxton and Podesta 2006). An alternative route of pyruvate supply during P_i stress has been proposed by Theodorou and Plaxton (1995). This involves the combined activities of phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase and NAD-malic enzyme supplying pyruvate to the mitochondrion during P_i stress (Plaxton and Podesta 2006).

Nodules impose an energy cost on host resources. The *Rhizobium* bacteria inside the nodules reduce N_2 to NH_4^+ in exchange for reduced carbon compounds from the plant. Sucrose from the shoot is the principal source of reduced carbon for the nodule (Kouchi and Yoneyama 1984). This sucrose is metabolised via the action of sucrose synthase and glycolytic enzymes and it is generally accepted that organic acids are the main products of sucrose degradation supplied to bacteroids to support nitrogen fixation in most legumes (Udvardi and Day 1997). The carbon costs of N_2 fixation may vary with host species, bacterial strain and plant development. Nodules may consume up to 50% of photosynthates produced by N_2 fixing plants. For example, average values of carbon costs for different N_2 fixing legumes range as follows: 36–39% of carbon is required for nodulation, nodule growth and maintenance respiration, 42–45% for nitrogenase activity, and 16–22% for amino acid synthesis from NH_4^+ assimilation and subsequent export (Marschner 1995). About 50% of photosynthates consumed by nodules are respired as CO_2 and nodules are able to reassimilate between 25 and 30% of this respired CO_2 via PEPc. This anaplerotic activity of PEPc can provide up to 25% of the carbon required for amino acid synthesis (Marschner 1995). Oxaloacetate, the product of PEPc refixation, can be used in bacterial metabolism or exported to the host in the form of amino acids (Pathirana *et al.* 1997). Legumes are categorized as amide or ureide exporters depending on the amino acids exported from the nodules. This is important for carbon costs because amide exporters rely more heavily on the activity of PEPc than ureide exporters (Streeter, 1991).

1.3 Progress in biotechnological modifications of legumes

Herbicide-resistant soybeans, which are ureide exporters, were the first genetically modified legumes made commercially available in 1996. More than 1000 of these glyphosate-resistant varieties are available (Owen 2000). Recent years have seen sequenced the genomes of two model legumes, *Medicago truncatula* and *Lotus japonicus*, as well as the important crop plant, *Glycine max*. This knowledge places scientists in an advantageous position to engineer the seeds of legume crop plants to include desired traits eg. resistance to soybean cyst nematodes for increasing crop yields or improving the oxidative stability of soybean which would improve its nutritional value as well its application in the biofuel industry (O'Brian, Vance and VandenBosch 2009). The focus to date has mostly been on the papilionoid subfamily where most of the model and crop legumes group together in one large clade, but this clade represents less than half of the species diversity for legumes. An emerging model plant for the mimosoid subfamily is *Chamaecrista fasciculate* and having a complete genome sequence would enable comparison with other model legumes which would shed more light on development, genome evolution and symbiosis (O'Brian, Vance and VandenBosch 2009).

Direct molecular modification of host plants or bacteria has not yet resulted in improvement of N_2 fixation (Graham and Vance 2000), but we propose that modification of the host component of the nodule could lead to improved seed protein content, which would be a great advantage to those who depend on these seeds as a primary source of protein. The interaction between carbon and nitrogen metabolism in the symbiotic system is regulated at three levels (Layzell and Atkins 1998) by key enzymes, which offer possibilities for genetic modification. The first level of regulation involves glutamine synthetase and glutamate synthase, which control the assimilation of NH_4^+ into glutamate and glutamine. The second level of regulation is made up of the combined activities of aspartate aminotransferase and asparagine synthetase, which regulate the flow between organic and amino acids. The third level of regulation is composed of the PEPc and malate dehydrogenase enzymes, which control the replenishment of the organic acid pool with anaplerotic carbon (Layzell and Atkins 1998). In our studies, *Lupinus luteus* serves as a model system for studying the physiology and metabolism of these levels of regulation, with a view to applying such knowledge to African legume crops.

Marczewski (1989) purified three isoforms of PEPc from lupin nodules and roots, with two forms being nodule specific. The same study also reported that these two forms, one of which appeared to be closely associated with N_2 fixation, differed substantially in their kinetic properties and were also different from a third form of PEPc. The two nodular isoforms of PEPc could be the products of separate genes, or could be from the same gene, but may undergo different post-translational modification (Pathirana *et al.* 1992). The mutiple PEPc isoforms could possibly contribute to different metabolic mechanisms in the nodule (Pathirana *et al.* 1997).

For southern African food security it is imperative that the protein quality of local legumes is improved under financially constrained farming practices. In this regard the biotechnology focus on inorganic carbon metabolism via PEPc could facilitate a platform of control for the carbon used during amino acid synthesis. The aim of this research is the investigation of the phenomenon of P stress in legume root systems and how it influences the nodular PEPc isoforms and pyruvate synthesis. These objectives would address the level of control associated with the replenishment of the organic acid pool.

The aim of this work was to investigate

1) how P stress affects the biological nitrogen fixation in terms organic and amino acid synthesis and the organic acid supply for nodule metabolism (Chapter 3)
2) how P stress affects the biological nitrogen fixation in terms of photosynthetic and respiratory carbon metabolism (Chapter 4)
3) the response of nodules to P-stress via the isoforms of PEPC (Chapter 5).

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nitrogen (N) is a limiting element for crop production, but makes up 78 % of Earth's atmosphere. It occurs in a stable form, dinitrogen (N_2), and only diazotrophs are capable of breaking the strong triple bond between the N atoms. Breaking this strong triple bond enables the atoms to react with other atoms, such as hydrogen, to form ammonia. The reaction is an exothermic one and has to take place under anoxic conditions, because nitrogenase is irreversibly inactivated by oxygen (O_2) (Valentine *et al*. 2010).

The industrial fixation of N_2 into NH_3 is known as the Haber-Bosch method. This industrial process was pivotal in the success of the Green revolution, which ensured N fertilizer for crops used as food, as well as industrial uses (Valentine *et al*. 2010). The Haber-Bosch method contributes 65 % of the total N fixed on Earth (Conley *et al*. 2009) and the fertilizer derived from this process supports *ca*. 40 % of the global population (Fryzuk 2004, Valentine *et al.* 2010). Nitrogen and phosphorus are major pollutants, which have detrimental effects on freshwater bodies and aquatic life, as well as disturbing the ecological equilibrium of terrestrial food webs (Valentine *et al*. 2010). To ensure agricultural sustainability, the amount of N available has to be managed efficiently. This management would involve the use of some biologically fixed N_2, which would be less prone to volatilisation, denitrification and leaching (Graham and Vance 2000). According to Vance (1998), up to 80 % of this biologically fixed N_2 utilised in agriculture comes from symbioses involving leguminous plants and species of *Rhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Allorhizobium*. In 1994, the estimate of arable land covered by legumes was 250 Mha, which would fix about 90 Tg of N_2 per year (Kinzig and Socolow 1994).

2.2 Nitrogen fixation

2.2.1 Nitrogenase characteristics

The biological reduction of N_2 to NH_3 is a highly endergonic reaction requiring an energy minimum of *ca*. 960 $kJ.mol^{-1}$ N^{-1} fixed (Sprent and Raven 1985). Nitrogenase (EC 1.18.6.1) is the enzyme responsible for the conversion of N_2 to NH_3 and is unique to N_2-fixing microorganisms. Nitrogenase not only catalyses the reduction of N_2, but also substrates such as H^+ and C_2H_2. The reaction for dinitrogen reduction is as follows:

$N_2 + 8H^+ + 8e^- + 16Mg.ATP \rightarrow 2NH_3 + H_2 + 16Mg.ADP + 16\ P_i$

The actual ATP requirement for N_2 fixation is considerably more than 16 mol per mol N_2 fixed and as a rule between 25 and 30 mol ATP are required (Bergersen 1991).

This enzyme has been found in aerobic and anaerobic bacteria, cyanobacteria, as well as root nodules of legumes and nonlegumes. The enzyme consists of two oxygen-sensitive nonheme iron proteins. The Fe-protein is the smaller of the two proteins and has a molecular mass between 52 and 73 kDa. This Fe-protein also consists of two subunits and a single Fe_4S_4 cluster. The MoFe-protein is the larger of the two proteins and has an approximate molecular mass of 240 kDa. This MoFe-protein consists of four subunits and contains 30 Fe and 2 Mo atoms (Thorneley 1992). Some diazotrophic bacteria have a nitrogenase, which contains vanadium instead of molybdenum (Rosendahl *et al.* 1991), whereas other forms of nitrogenase appear to contain neither of the two elements (Eady *et al*. 1987).

2.2.2 Anatomy of symbiosis

Nitrogen fixation occurs in a number of different free-living bacteria, but the symbiotic association between rhizobia and *Leguminosae* makes the largest contribution. This association comprises the infection of a legume by rhizobia, such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium* (Lodwig and Poole 2003). The development of nodules can be loosely divided into 3 stages: 1) pre-infection, 2) nodule initiation and 3) nodule differentiation. The phase of pre-infection occurs before the host plant and suitable *Rhizobium* strain recognise each other. The roots and seed coats of legumes, similar to other plants, contain a large and diverse amount of flavonoids. These flavonoids are released by the legumes, which serve as chemoattractants and in turn induce expression of *Rhizobium nod*

genes. These *nod* genes are expressed at very low levels or not at all in free-living rhizobia (Hirsch 1992).

Rhizobia usually infect the host legumes via root hairs, but in some species they infect via surface cracks (Lodwig and Poole 2003). Recently formed root hairs are the most susceptible to rhizobia infection, but rhizobia attach to root hairs all over the root. Attachment to susceptible root hairs consists of two steps. Rhizobia attach to a plant receptor and this attachment is facilitated by rhicadesin, a protein on the bacterial surface (Smit *et al.* 1987). Cellulose fibrils (Smit *et al.* 1987) or fimbriae (Vesper and Bauer 1986) then enable tighter adherence. The rhizobia bacteria seem to enter the root hair at the tip and this could be because of the thinner cell wall and fewer cross linkages. The deformation of susceptible root hairs depends on functional *Rhizobia nod* genes. The root hair deformation can occur in various shapes such as corkscrews, branches, twists and spirals. Some of the infected/inoculated root hairs may form characteristic curls known as shepherd's crooks by coiling 360˚ (Hirsch 1992).

Nod factor induces root hair formation and cortical cell division by 1) the binding of N-glucosamine residues of the Nod factor with the sugar-binding site of a receptor, presumably a lectin; 2) the strength of the interaction between this Nod factor and receptor regulates early nodulation events (Hirsch 1992). Rhizobia then attach to the thin cell wall at the tip of the young root hair. Receptor molecules bind to the Nod factor on the membranes of Rhizobia and these receptor molecules are able to move laterally in the plasma membrane of the root hair. The receptor molecules bound to the rhizobia patch together to form a 'cap' analogous to the binding of antibodies to specific proteins on the plasmamembrane. The transmembrane proteins attached to the receptor molecules also 'cap' in the membrane. Receptor-mediated endocytosis then takes place, which is the basis of infection thread formation. After the rhizobia have entered the root hair, numerous subcellular changes take place within the root hair. These changes include membrane permeability and arrangement of the cytoskeleton (Hirsch 1992). Each of these may be part of, or may generate, the next signal for inducing cortical cell divisions as proposed by Long and Cooper (1988). Cortical cell divisions may also be stimulated by other molecules, such as plant hormones (Hirsch 1992, Oldroyd and Downie 2008), which include cytokinins.

The 'capping' at the infection site causes a change in the direction of growth, so that the plasmamembrane of the root hair invaginates to form an infection thread. The

rest of the root hair continuous to grow and eventually grows around the infection site (Hirsch 1992, Oldroyd and Downie 2008). Rhizobia seem to grow along plant-derived infection threads, initially remaining exterior to the plant cell, before being released into newly divided plant cells. A symbiotic organelle-like structure is then formed through division and/or differentiation of both the plant membrane and the bacteria. This structure is known as the peribacteroid unit (PBU), or the symbiosome (Roth *et al*. 1988), and consists of the plant-derived peribacteroid membrane (PBM), the bacteriod (differentiated bacteria capable of nitrogen fixation), and the peribacteroid space (PBS) (Lodwig and Poole 2003).

2.2.3 Determinate vs indeterminate nodules

The type of nodule formed depends on where the initial cell division takes place. This initiation of nodule formation usually takes place through anticlinal cell divisions. These cell divisions can occur in the inner or the outer cortex of the root. The host plant and not the rhizobial strain determine the type of nodule that will be formed (Newcomb 1981). The two major types of nodule structure are determinate and indeterminate. These two nodule types have large developmental differences in vascularization, persistence of nodule meristem and bacteroid development (Lodwig and Poole 2003).

Determinate nodules are found on plants such as bean, soybean and *Lotus japonicus*. These nodules have several bacteroids contained within one peribacteroid membrane. Indeterminate nodules found on pea, alfalfa and *Medicago trancutula*, have a single bacteroid surrounded by a peribacteroid membrane (Lodwig and Poole 2003). Determinate nodules grow until they reach maturity after which development stops and N_2 fixation commences. The nodules have homogenous inner tissues consisting of a parenchymatic core of infected cells with uniform ontogeny. Indeterminate nodules which are found in most of the symbiotic legume species exhibit a more complex structure comprising the following: zone I, the meristematic region situated in the apex of the nodule; zone II, the infection zone where the symbiosome is formed by bacteria-containing infection threads that are engulfed by plant cells; interzone II-III where bacteria are physiologically transformed to bacteroids; zone III where most N_2-fixation takes place and zone IV, the senescence zone (Valentine *et al*. 2010). Intermediate nodules continue growing with zones changing from their current state to the next as the zone matures, whereas in determinate nodules, nodular core cells develop simultaneously with the whole

nodule senescing afterwards (Van de Velde *et al*. 2006). Legumes deviated into two distinct clades or groups, tropical and temperate nodules, approximately 50 million years ago. The main difference between the two clades is the type of nitrogenous compounds (amides or ureides) translocated to the shoots. Both clades have legumes with indeterminate and determinate nodules (Valentine *et al.* 2010).

There are also differences in the carbon metabolism of bacteroids in determinate and indeterminate nodules and one of the most striking differences is the large accumulation of the reserve polymer poly-β-hydroxybutarate (PHB) in determinate nodules and its absence in indeterminate nodules. It is also likely that there will be differences in the regulation of the TCA cycle between the bacteroids of the determinate and indeterminate nodules (Lodwig and Poole 2003).

2.2.4 Regulating the O_2 environment

Mature bacteroids reduce atmospheric nitrogen in an extremely energy-intensive process that is fuelled by provision of carbon from the plant. Nodules require energy for the fixation of atmospheric N, synthesis of exported organic solutes of N as well as for growth and maintenance (Layzell and Atkins 1998). Large amounts of O_2 are required to generate this energy and nodules therefore have a considerably higher rate of O_2 consumption than other plant tissues (Layzell and Atkins 1998). Oxygen inhibits the functioning of nitrogenase and the regulation of bacteroid metabolism in the oxygen-limited environment of the nodule presents the bacteroid with problems. Therefore, energy, reductant and carbon pools must be carefully balanced to ensure optimum rates of nitrogen fixation (Lodwig and Poole 2003).

The oxygen concentration in infected cells of nodules is in the range of 4 to 70 nM O_2, whereas the outer cells in the nodule cortex, which are in equilibrium with atmospheric air, may have an intercellular O_2 concentration 10 000 times higher despite having only a millimeter of space between the different types of cells (Layzell and Atkins 1998). The mechanism of O_2 control in legumes has not yet been fully elucidated, but regulation appears to consist of two components:

1) nodule metabolism in O_2 limited which provides some flexibility for additional O_2 consumption
2) nodules are capable of adjusting their resistance to O_2 diffusion (Layzell and Atkins 1998).

The innermost region of the nodule cortex consists of densely packed cells with few intercellular spaces and it was found that long term changes in O_2 concentrations brought about concomitant changes in the number of cell layers in this region. Changes in nodule cortex structure would not be possible for short-term fluctuations in O_2 (Layzell and Atkins 1998). Displacement of gas-filled spaces with water or a glycoprotein in the nodule inner cortex would achieve short-term regulation of oxygen diffusion (Iannetta *et al*. 1995). Infected cells can regulate oxygen diffusion by terminating the oxygenated leghemoglobin gradient which will impede leghemoglobin facilitated diffusion (Thumfort *et al*. 1995). Additionally, mitochondria clustered around gas spaces may consume most of the oxygen before diffusion to the symbiosomes can take place (Millar *et al*. 1995; Layzell and Atkins 1998).

2.2.5 Nitrogen provision to the host

Ammonium is the primary stable product of nitrogen fixation (Bergersen 1965) and was initially thought to be the sole nitrogen source secreted by bacteroids. The levels of the ammonium assimilating enzymes, GS and GOGAT, in the bacteroid are insufficient to support the rates of glutamate and glutamine synthesis required by the plant (Cullimore and Bennet 1991, Temple *et al.* 1998, Lodwig and Poole 2003). Waters *et al.* (1998) found that soybean bacteroids isolated on sucrose gradients synthesised and secreted alanine as the sole nitrogen product. These authors suggested that ammonium might have been detected as the sole nitrogen product excreted in previous studies performed *in vitro,* because bacteroids were contaminated with plant extract, which contains alanine deactivase (Lodwig and Poole 2003). Allaway *et al.* (2000) found that the secretion products of bacteroids isolated from pea nodules on Percoll gradients were dependent on cell density. Only ammonium was produced at low bacteroid densities, while alanine and ammonium were secreted at high bacteroid densities (Lodwig and Poole 2003). Assays where the bacteroid densities are not that high or the ammonium is removed such as in flow chambers, may result in alanine synthesis that is not significant. Consequently, differences in assay conditions between Bergersen and Turner (1990a) and Waters *et al.* (1998) may have been responsible for the discrepancy in results (Lodwig and Poole 2003). Pea bacteroids produce alanine from NH_4^+ and pyruvate via the enzyme L-alanine dehydrydrogenase (AldA). Allaway *et al.* (2000) found that an AldA mutant of *Rhizobium leguminosarum* still fixed nitrogen and this indicates that the synthesis of alanine from NH_4^+ is not crucial for nitrogen export in pea nodules (Lodwig and Poole 2003). Bacteria possess high affinity aspartate and alanine

transporters, but the transport across symbiotic membranes may not be fast enough to be considered a source of reducing equivalents or fixed nitrogen (Whitehead *et al*. 1998).

Ammonia is probably transferred from the bacteroid cytoplasm to the plant cytoplasm by diffusion across the membranes due to a large concentration gradient that exists between the bacteroid and host cytoplasm (Lodwig and Poole 2003). This large concentration gradient is due to the high activities of ammonia-assimilating enzymes in the host, which suggests that rapid movement of ammonia could be accomplished by diffusion (Streeter 1989, Lodwig and Poole 2003). Several authors have reported that free-living rhizobia have an ammonium uptake system under conditions of ammonium starvation, but in all cases studies the uptake system was shown to be inoperative in the symbiotic state (Gober and Kashket 1983, Glen and Dilworth 1984, Howitt *et al.* 1986, Lodwig and Poole 2003). Tate *et al.* (1998) identified an ammonium transporter (AmtB) in *Rhizobium etli*, which is inactivated during bacteriod differentiation presumably by the high concentration of ammonium in the nodule (Lodwig and Poole 2003). When the Amt system was ectopically expressed in *R. etli* bacteroids, host cell invasion and peribacteroid unit differentiation were disrupted (Lodwig and Poole 2003). From this it can be concluded that the movement of ammonium is passive, at least across the bacteroid membrane and into the peribacteroid space. It is presumed that the lack of an uptake system in the symbiotic state prevents the bacteroids from competing with host cells for ammonium in the peribacteroid space (Udvardi and Day 1997, Howitt and Udvardi 2000, Lodwig and Poole 2003). The transport of ammonium across the peribacteroid membrane may be facilitated by a H^+-ATPase pump, which would create an electric potential across the membrane and acidify the peribacteroid space (Udvardi and Day 1989, RojasOjeda *et al.* 1998). The acidification of the peribacteroid space would promote the formation of NH_4^+ from NH_3, which would maintain a concentration gradient for the diffusion of NH_3 from the bacteroid into the peribacteroid space. Subsequent movement of NH_4^+ to the plant cytosol may be through a channel on the peribacteroid membrane, but this still has to be proven (Lodwig and Poole 2003).

Lodwig *et al.* (2003) proposed a more complex amino acid cycle between bacteroids and host plants than was previously anticipated where dicarboxylates from the host are oxidised to provide energy for N_2 assimilation. The plant provides amino acids (most probably glutamate or a precursor to it), which is transported into the bacteroids in addition to the dicarboxylates. The glutamate or precursor would enter

the bacteroids via amino-acid transporters and then act as a transamination donor to produce aspartate or possibly amino acids such as alanine, which would then be secreted to the plant for asparagine synthesis (Lodwig *et al.* 2003). The provision of amino acids to the bacteroids enables them to shut down their ammonium assimilation and in turn they have to secrete ammonium to the plant in order to receive the amino acids. This dependence of the bacteroids and host plant on each other would have favoured the evolution of mutualism (Lodwig *et al.* 2003).

2.3 Carbon requirements of symbiosis

2.3.1 Carbon supply to the bacteroids

The symbioses come at a cost to the host and the *Rhizobium* bacteria exchanges NH_4^+ for reduced carbon compounds from the plant. This reduced carbon is used as fuel for bacterial metabolism (Udvardi and Day 1997), generation of ATP and reducing equivalents (Whitehead *et al*. 1998). Sucrose transported via the phloem from the shoot is the principal source of reduced carbon for the nodule (Kouchi and Yoneyama 1984). Sucrose in the nodule tissue is cleaved by sucrose synthase to UDP-glucose and fructose (Lodwig and Poole 2003). Thummler and Verma (1987) found that sucrose synthase activity is nodule enhanced and that it is needed for nitrogen fixation (Craig *et al.* 1999, Gordon *et al.* 1999). These findings suggest that sucrose synthase is the principal enzyme responsible for sucrose cleaving in the nodule (Lodwig and Poole 2003). The products of this sucrose hydrolyzation are used as substrates for cellulose or starch biosynthesis and/or are metabolised by glycolytic enzymes to produce phosphoenolpyruvate (PEP) (Lodwig and Poole 2003). Various authors have found that the enzymes of the glycolytic pathway have high activity in the plant cytosol (Salminen and Streeter 1987b, Copeland *et al.* 1995, Kaur and Singh 1999, Lodwig and Poole 2003). PEP can be further metabolised to produce malate via the activities of phosphoenolpyruvate carboxylase (PEPc) and malate dehydrogenase (MDH). The concentrations of dicarboxylates, especially malate (ca. 3.4 mM), were found to be high in nodules (De Vries *et al.* 1980, Kouchi and Yoneyama 1986). Labelling experiments have shown that malate, fumarate and succinate were rapidly labelled in the plant cytosol and in turn this label was incorporated into bacteroids chiefly as malate when nodules were supplied with $^{14}CO_2$ (Vance *et al.* 1985, Kouchi and Yoneyama 1986, Lodwig and Poole 2003). Therefore, it is generally accepted that C_4-dicarboxylates are the main products of

sucrose degradation supplied to bacteroids to support nitrogen fixation in most legumes (Udvardi and Day 1997).

2.3.2 Transport of carbon compounds across the peribacteroid membrane

The transport of metabolites, in particular carbon compounds, across the symbiotic membranes of legume nodules has been reviewed extensively by Udvardi and Day (1997). The transport of substances across the peribacteroid membrane is similar to transport across the membranes of free-living cells. When host plants become infected with nitrogen fixing bacteria, the plant membrane engulfs the bacteria to give rise to nodules. Therefore, the spatial orientation of the peribacteroid membrane with regards to the bacteroids is complex, because the extracellular side of the PBM faces the peribacteroid space, while the intracellular side faces the plant cytosol. Consequently, the movement of solutes from the plant cytosol across the PBM to the peribacteroid space is similar to secretion of products by a plant membrane, while the movement of solutes from the peribacteroid space to the plant cytosol is analogous to uptake across a normal plant membrane (such as the uptake of fixed nitrogen) (Brewin 1991, Verma and Hong 1996, Lodwig and Poole 2003). Research with determinate nodules have shown that C_4-dicarboxylates are transported at high rates, which is consistent with their role as main carbon source of bacteroids, while passive movement across the PBM of some sugars and amino acids occur at much lower rates but is not sufficient to support nitrogenase activity (Udvardi *et al.* 1990, Lodwig and Poole 2003). Some studies have also been done with peribacteroid units from indeterminate nodules and they also showed rapid transport of C_4-dicarboxylic acids but not of amino acids such as glutamate (Hernandez *et al.* 1996). It is important to keep in mind when studying peribacteroid units that the isolation of what effectively a fragile intracellular structure may result in the loss of factors needed by some transport systems and that the isolated membrane might not be energised properly, which would result in selective loss of measurable uptake activity (Lodwig and Poole 2003).

2.3.3 Carbon costs of N_2 fixation

The carbon costs of N_2 fixation may vary with host species, bacterial strain and plant development and nodules may consume up to 50% of photosynthates produced by N_2 fixing plants. Average values of carbon costs for different N_2 fixing legumes are as follow: 36-39% of carbon is required for nodulation, nodule growth and maintenance

respiration, 42-45% for nitrogenase activity, and 16-22% for amino acid synthesis from NH_3 assimilation and subsequent export (Marschner 1995). About 50% of photosynthates consumed by nodules are respired as CO_2 and nodules are able to reassimilate between 25 and 30% of this respired CO_2 via phosphoenolpyruvate carboxylase (PEPc). This anaplerotic activity of PEPc can provide up to 25% of the carbon required for amino acid synthesis (Marschner 1995). The resultant oxaloacetate can be used in bacterial metabolism or exported to the host in the form of amino acids (Pathirana *et al.* 1997).

It is easier to obtain a clearer understanding of total energy requirements when quantifying carbon expenditure for nitrogen fixation (Valentine *et al.* 2010). For several amide-exporters the cost of carbon to fixed N was approximately 17:1 (Witty *et al.* 1983) while the cost for soybean (a ureide exporter) was 12:1 (Rainbird *et al.* 1984, Valentine *et al.* 2010). There are methodological problems with measuring carbon economy and more recently the carbon expenditure of nitrogen fixation was estimated to vary between 2-3 mg C per mg symbiotic N fixed. Variation can be expected between species and genotypes (Valentine *et al.* 2010).

2.4 Effect of phosphate stress on nitrogen fixation

Leguminous plants require a relatively large amount of P_i and Israel (1987) found that P_i contributed to higher nitrogen fixing ability as well as nodule mass. In soils, the concentration of available P_i for plants is usually very low because most of the P_i combines with iron, aluminium and calcium to form scarcely soluble compounds (Aono *et al*. 2001). P_i -deficiency is thought to be one of the limiting factors of nitrogen fixation (Aono *et al*. 2001). In the tropics and subtropics, very little P fertilizer is available for extensive agriculture. This lack of sufficient quantities of P fertilizer may be ascribed to a shortage of infrastructure for the production of fertilizer and transport thereof, as well as a lack of capital for purchasing fertilizer in poverty afflicted communities (Vance *et al*. 2003).

2.4.1 Effect of P deficiency on nodulation

Phosphorus plays a role in many metabolic processes such as energy generation, nucleic acid synthesis, photosynthesis, respiration, glycolysis, membrane synthesis and integrity, activation/inactivation of enzymes, redox reactions, signalling, carbohydrate metabolism as well as nitrogen fixation (Vance *et al.* 2003). Nodules

require large amounts of P, therefore P deficiency can reduce nodule growth either directly or indirectly by affecting the carbon supply to the nodules (Valentine *et al.* 2010) through a reduction in shoot growth and photosynthesis (Jakobsen 1985, Almeida *et al.* 2000). Nodule response to P stress was found to be dependant on the period of the P starvation experiment (Valentine *et al.* 2010).

The response of nodules to P stress is slower than that other plant organs with nodules of *Lupinus angustifolius* experiencing no P stress after 14 days of P starvation (Le Roux *et al.* 2006), but only experiencing a decline in P_i levels 25 days after the onset of P starvation (Le Roux *et al.* 2008). Once P stress was exhibited, a concomitant decrease in nodule weight and nodular P concentrations were observed (Valentine *et al.* 2010). These findings were supported by Drevon and Hartwig (1997) as well as Olivera *et al.* (2004). Le Roux *et al.* (2008) also reported no change in carbon construction costs for these nodules which implied that new nodule growth was restricted by P supply and not by carbon allocation. During short-term P deficiency nodule growth may be maintained at the expense of root growth (Valentine *et al.* 2010) as was reported for soybean (Le Roux *et al.* 2009), *L. angustifolius* (Le Roux *et al.* 2006, 2009) and *Trifolium repens* (Høgh-Jensen *et al.* 2002).

2.4.2 Effect of P deficiency on P_i levels

Symbiotic legumes require more P than when mineral N is taken up from the soil, but the role of P in nodule metabolism remains unclear (Valentine *et al. 2010*). Soybeans receiving nitrogen fertiliser instead of obtaining their nitrogen from symbiotic nitrogen fixation (SNF) appear to require less P (Leidi and Rodriguez-Navarro 2000). Nodules are strong sinks for P with nodules being able to obtain P from the host, but do not release P to the host root (Al-Niemi *et al.* 1998). The suggestion of nodules acting as a strong sink for P is supported by Vadez *et al.* (1996) who found three-fold higher P levels in nodules than other tissues (Valentine *et al.* 2010). Nodules may have a mechanism whereby P influx is regulated which in turn ameliorates the effects of P deficiency when P is in short supply (Valentine *et al.* 2010).

Roots experience P deficiency more severely than nodules during short-term P stress (Le Roux *et al*. 2006) with decreases in cellular P_i and ATP:ADP ratio (Valentine *et al.* 2010), whereas nodules maintain P_i and energy levels. Nodules appear to function optimally at low P_i levels (Al-Niemi *et al.* 1997, 1998, Colebatch *et al.* 2004) and bacteroids which consistently operate at low P concentrations are able

to scavenge P from the host cells to fulfil their metabolic requirements (Al-Niemi *et al.* 1997, 1998, Valentine *et al.* 2010)

2.4.3 Effect of P deficiency on carbon metabolism

There is not a lot of data available regarding carbon metabolism in P deficient nodules, however responses of non-legumes to P deficiency has been well-documented (Valentine *et al.* 2010). The type of response of nodules experiencing P stress is species dependant , which could be ascribed to differences in temperate vs tropical origin, determinate vs indeterminate, amino vs ureide exporters as well as short-term vs long-term P deficiency exposure (Valentine *et al.* 2010).

Phosphoenolpyruvate carboxylase (PEPc) is an important branchpoint between amino and organic acid metabolism and during P stress there would be competition between these two pools for carbon skeletons (Olivera *et al.* 2004, Le Roux *et al.* 2006). Malate is the major substrate for bacterial respiration and nitrogen fixation (Rosendahl *et al.* 1990) and increased levels of malate in nodules are primarily due to low oxygen and phosphorus concentrations (Colebatch *et al.* 2004, Valentine *et al.* 2010).

Le Roux *et al.* (2006) reported high malate levels in *L. angustifolius* nodules during short-term P stress associated with increased malate dehydrogenase (MDH) and PEPc activities even though the nodules did not show symptoms of experiencing P stress (Valentine *et al.* 2010). Le Roux *et al.* (2006) also found that short-term exposure to P deficiency did not affect the flux of malate-derived pyruvate via malic enzyme (ME). These findings suggest that malate production via the PEPc and MDH route is favoured to ensure high levels of nodular malate for bacteroid respiration (Rosendahl *et al.* 1990). Vance and Heichel (1991) proposed that malate rather than pyruvate would be favoured as the end product of glycolysis by the low oxygen concentrations found in nodules (Valentine *et al.* 2010).

During long-term P deficiency, Le Roux *et al.* (2008) found an even greater increase in malate levels of *L. angustifolius* nodules indicating engagement of the PEPc-MDH-ME glycolytic bypass proposed by Duff *et al.* (1989). This bypass ensured the continuation of respiration. Despite ensuring continued respiration, the high malate levels led to a decline in nitrogen fixation and shifted carbon allocation from amino acid synthesis to organic acid synthesis (Valentine *et al.* 2010).

Nodules experiencing P stress may suffer an effect on nitrogen fixation via N feedback mechanisms (Valentine *et al.* 2010). Two separate groups of researchers studying P-deficient *Trifolium repens* found an accumulation of asparagine in xylem sap, roots and nodules together with reduced SNF indicating that P inhibits SNF by reducing host growth which would have a lower requirement for nitrogen (Almeida *et al.* 2000, Høgh-Jensen *et al.* 2002). P deficiency can directly affect nitrogen metabolism with the assimilation of N_2 into NH_4^+ being sensitive to P levels and this sensitivity decreases with an increase in nodular P (Israel and Rufty 1988, Valentine *et al.* 2010). *Phaseolis vulgaris* nodules displayed a shift from ureide to asparagine (an amine) production during P stress, despite *P. vulgaris* being a ureide exporter (Olivera *et al.* 2004). Le Roux *et al.* (2009) found a similar shift in P stressed soybean nodules, but discovered that ureides were still the major export product compared to amides (Valentine *et al.* 2010).

2.4.4 Effect of P deficiency on oxygen diffusion

Both amide and ureide exporting nodules have their respective O_2 permeabilities affected by P starvation (Ribet and Drevon 1995, Drevon and Hartwig 1997, Schulze and Drevon 2005, Le Roux *et al.* 2009), but the lack of P appears not to inhibit SNF (Schulze 2004, Valentine *et al.* 2010). Soybean (Ribet and Drevon 1995), common bean (Vadez *et al.* 1996) and alfalfa (Schulze and Drevon 2005) have all shown an increased O_2 diffusion into nodules during P stress and Drevon *et al.* (1998) suggested that osmoregulatory changes in nodule cortical cells may be responsible for the increased oxygen diffusion (Valentine *et al.* 2010). The physiological purpose of this increased O_2 diffusion during P stress is unclear, but may be connected to ensuring sufficient adenylate levels for high nitrogen fixation rates (Schulze and Drevon 2005).

Other legume species such as *L. albus* control the O_2 diffusion in nodules during P stress by blocking the free spaces between cortical cells (De Lorenza *et al.* 1993, Iannetta *et al.* 1993, Schulze *et al.* 2006). Plants experiencing P deficiency may also form smaller nodules which would increase the surface:volume ratio of nodules which are supported by increased respiration rates and unchanged nitrogen fixation as reported by Schulze and Drevon (2005) (Valentine *et al.* 2010).

2.5 Plants' adaptation to phosphate stress

Plants exhibit numerous morphological, physiological and metabolic adaptations to P_i deprivation (Theodorou and Plaxton 1995). Vance *et al.* (2003) summed up plants' adaptations to low P into two main thrusts: 1) conservation of use of P and 2) enhanced acquisition or uptake of P (Lajtha and Harrison 1995, Horst *et al.* 2001, Vance 2001). A conservation of P use include processes such as a decrease in growth rate, an increased P use efficiency, the remobilization of internal P_i, alterations in carbon metabolism that would bypass P-requiring steps as well as alternative pathways in respiration (Schachtman *et al.* 1998; Plaxton and Carswell 1999, Raghothama 1999, Uhde-Stone *et al.* 2003a and 2003b, Vance *et al.* 2003). Processes leading to enhanced uptake of P involve increased production and secretion of phosphatases, greater root growth combined with modified root architecture, root surface increase due to development of root hairs and an increased P_i transporter expression (Marschner *et al.* 1986, Duff *et al.* 1994, Schachtman *et al.* 1998, Gilroy and Jones 2000, Lynch and Brown 2001, Vance *et al.* 2003). For the purpose of this study, only the modification of carbon metabolism under P_i deficiency will be reviewed further. For an extensive review on plant adaptations to phosphorus acquisition and use, please refer to Vance et al. (2003).

2.5.1 Root exudation of organic acids

Plants have the capability of altering their rhizosphere chemistry, microbial populations in the soil, competition as well as plant growth through the exudation of organic compounds (Vance et al. 2003). These organic compounds include substances such as simple sugars, organic acids, amino acids, phenolics, quinones (iso)-flavonoids, growth hormones, proteins, and also polysaccharides which have diverse functions such as signalling in symbiotic interactions, nutrient scavenging and acquisition as well as allelopathy (Curl and Truelove 1986, Marschner *et al*. 1986, Harrison 1997, Vance *et al.* 2003). The composition of compounds making up the organic root exudates can be altered extensively in either or both quality and quantity depending on the environmental stress experienced (Marschner 1995, Ryan *et al*. 2001, Neumann and Martinoia 2002, Vance et al. 2003). Plant roots have shown increased synthesis and exudation of many organic acids (in the anionic form) when experiencing nutritional stress such as P_i limitation, Al toxicity, insufficient Fe availability as well heavy metal exposure (Dinkelaker et al. 1989, Delhaize et al.

1993, Delhaize and Ryan 1995, Ryan *et al.* 1995a and 1995b, 1997, Larsen *et al*. 1998, Neumann *et al.* 2000). To date, the responses to low P and Al toxicity are among those that have enjoyed the most attention.

The exudation of malate and citrate appears to be a major mechanism in relieving P-stress and Al-toxity and is supported by convincing evidence. The organic acids released by the plant would chelate Al^{3+}, Fe^{3+} and Ca^{2+} and in this way displace bound or precipitated P_i (Gerke 1994, Jones 1998, Hinsinger 2001, Ryan *et al*. 2001, Vance *et al.* 2003). This could cause organic P to become susceptible to hydrolysis by acid phosphatases (Gerke 1994, Braum and Helmke 1995). Malate and citrate appears to be selectively synthesised and exuded in response to P-stress, because the internal increase in organic acid concentrations does not correlate with the amounts exuded (Vance *et al.* 2003). Johnson *et al.* (1996a) found that the organic acids exuded from white lupin cluster roots under P stress came from both photosynthetic CO_2 and dark CO_2 fixation, with photosynthesis providing about 65% of the carbon for organic acid synthesis and dark CO_2 accounting for about 35% (Vance *et al.* 2003). This increase in dark CO_2 fixation in cluster roots is concomitant with an increase in specific activity of phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase (MDH) and citrate synthase (CS) (Johnson *et al.* 1996a, Vance *et al.* 2003). The dark CO_2 fixation rate in legume nodules is similar to that in cluster roots and similar increases in PEPc activity under P stress have been found by other authors in chickpea (*Cicer arietinum*), oilseed rape, and *Sesbania rostrata* (Hoffland *et al.* 1992, Moraes and Plaxton 2000, Aono *et al.* 2001, Vance *et al.* 2003).

This increase in organic acid exudation in response to P stress could also partly be due to either reduced degradation or utilisation of citrate. Neumann and Römheld (1999) found the activity of aconitase (AC) was decreased in cluster roots. In a similar fashion, other authors found that respiration rates were also decreased in cluster roots that were P deficient (Johnson *et al.* 1994, Neumann *et al.* 1999). Both the increase in organic acid synthesis and the reduced degradation or utilisation could bring about an increase in citrate available for exudation (Vance *et al.* 2003).

Several authors have reported that organic acid exudation from white lupin cluster roots coincide with rhizosphere acidification (Marschner *et al.* 1987, Dinkelaker *et al.* 1989, Neumann and Martinoia, 2002). Subsequent experiments by authors using other plants led to the conclusion that different mechanisms are involved in proton release and organic acid exudation (Vance *et al.* 2003). The release of protons into

the rhizosphere is a response that many plants use to overcome intracellular acidity (Raven and Smith, 1976) and Yan *et al.* (2002) found an increase in the activity of a plasmamembrane H^+ ATPase from white lupin cluster roots under P deficiency. This increase in plasma membrane H^+ ATPase activity could be the reason for the increase in H^+ extrusion (Vance *et al.* 2003). Sakano (2001) explained this somewhat confusing phenomenon at the hand of his revised pH-stat hypothesis of the plant cell. Plant glycolysis is regulated by a feedback process in contrast to nonplant systems, where glycolysis is regulated by a feed-forward system. By making use of this feedback system, glycolysis can only take place when the cytoplasm is sufficiently alkaline to stimulate PEPc activity, which has an alkaline pH optimum. Therefore, the active pumping out of H^+ is a possible response to prevent the inhibitory effect that cytosolic acidification could have on PEPc activity and subsequent organic acid synthesis (Vance *et al.* 2003).

The specific cell types and mechanism of organic acid exudation have not yet been characterized for the proteiod roost of white lupin (Vance *et al.* 2003), while studies on aluminium tolerance in wheat (Ryan *et al.* 1997) and maize (Kollmeier *et al.* 2001, Pineros and Kochian 2001) have pointed to the root apex as the site of organic acid exudation. Using anion channel blockers (Neumann et al. 1999), citrate exudation in P stressed cluster roots was decreased by 40 – 60 % (Vance et al. 2003). Uhde-Stone *et al.* (2003a) found that PEPc and MDH transcripts were localized in the apex and elongation zone of cluster roots. Therefore enzymes responsible for organic acid extrusion occur in the root tips (Vance et al. 2003). These findings indicate that the extrusion of organic acids in response to P deficiency may involve anion channel proteins.

The molecular steps leading to organic acid exudation have not yet been characterized, putative anion channel genes related to chloride channels have been characterized (Vance *et al.* 2003) and appear to be involved in iron-limited growth and nitrogen acquisition, but none yet for organic acid efflux. One of several ESTs isolated from cluster roots was nine-fold induced during P deficiency (Uhde-Stone *et al*. 2003b) and had high homology with an Arabidopsis putative integral membrane protein belonging to the MATE family, which are a large family of putative antiporters possibly involved in exudation of various drugs and toxins (Vance *et al.* 2003). A MATE protein in Arabidopsis is involved in plant response to iron deficiency, which makes the cluster root MATE EST a possible candidate for organic acid extrusion (Vance *et al.* 2003).

2.5.2 Alternative glycolytic pathways

There are several enzymes in the glycolytic pathway that are P_i or adenylate dependant therefore severe P stress may hinder these metabolic processes (Vance *et al.* 2003). Large reductions in cytosolic P_i levels caused significant reductions in intracellular levels of ATP, ADP and nucleoside-Ps, while pyrophosphate (P P_i) levels remain relatively unchanged (Plaxton and Podesta 2006). This could be detrimental to the carbon flux through the adenylate- and P_i – dependant enzymes of glycolysis, but despite P_i starvation, plants have to continue producing energy and carbon skeletons for pivotal metabolic pathways (Plaxton and Podesta 2006). Three glycolytic bypasses have been hypothesised to facilitate alternate routes or carbon flux during P stress (Vance *et al.* 2003).

Under certain environmental conditions, PP_i can serve as a cytosolic energy donor helping to conserve ATP pools (Theodorou and Plaxton 1996, Vance *et al.* 2003). During P_i stress a PP_i -dependant phosphofructokinase (PFK) may be able to bypass the reaction of the ATP-dependant phosphofructokinase and forming fructose-1,6-bisphosphate (Theodorou and Plaxton 1996, Vance et al. 2003). Paul *et al.* (1995) found that P_i deficient transgenic tobacco plants with reduced PP_i -PFK were not distinguishable from wild-type plants which questioned the proposal the PP_i-PFK substitutes for ATP-PFK during P_i stress (Plaxton and Podesta 2006). A subsequent study by Fernie *et al.* (2002) using tobacco plants overexpressing the mammalian 6-phosphofructo-2-kinase found that the glycolytic contribution of ATP-PFK and PP_i -PFK respectively decreased and increased during P_i stress (Plaxton and Podesta 2006). Other PP_i -utilizing processes may be sucrose cleavage by a PP_i –dependant sucrose synthase metabolic pathway as well as vacuolar proton transport by a PP_i -dependant proton pump situated in the tonoplast (Plaxton and Carswell 1999, Vance *et al.* 2003).

The second glycolytic bypass comprises the action of a nonphosphorylating NADP-dependant glyceraldehyde-3P dehydrogenase (NADP-G3PDH) as an alternative to P_i-dependant NAD-G3PDH and phosphoglycerate kinase (Duff et al. 1989b, Vance et al. 2003, Plaxton and Podesta 2006).

PEPc, together with malate dehydrogenase (MDH) and malic enzyme (ME), functions as the third possible glycolytic bypass and are the alternative for the

reaction catalysed by ADP-dependent cytosolic pyruvate kinase (PKc). The activity of this metabolic bypass during P_i stress, when the ADP supply may be limiting, would ensure continued pyruvate supply to the tricarboxylic acid cycle, while at the same time releasing P_i back into the metabolic pool (Duff *et al.* 1989, Plaxton 2004). Several authors have reported an increase in PEPc activity for P_i -stressed samples compared to P_i -sufficient controls in *Brassica nigra* (Duff *et al*. 1989), *Brassica napus* (Nagano *et al.* 1994) and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000, Plaxton and Podesta 2006).

2.5.3 Alternative mitochondrial respiration

The decline in P_i and ADP which are the results of severe P_i stress will restrict the flow of electrons through the cytochrome pathway, also known as mitochondrial electron transport (miETC), at sites of ATP coupled synthesis (Plaxton and Podesta 2006). Low ADP and P_i levels may result in an increased ATP:ADP ratio which inhibits respiration through a process termed adenylate control (Vance *et al.* 2003). Plants possess non-energy conserving pathways which allow respiratory flux to continue when levels of ADP and/or P_i are limited (Plaxton and Podesta 2006). These nonphosphorylative pathways able to bypass energy-requiring sites include rotenone-insensitive NADH dehydrogenase and the alternative oxidase (Vance *et al.* 2003).

Several authors have shown that plants adjust to P stress by increasing or upregulating the rotenone- and/or cyanide insensitive pathways of the miETC (Rychter and Mikulska 1990, Rychter *et al.* 1992, Gonzaléz-Meler *et al.* 2001, Juszczuk et al. 2001, Plaxton and Podesta 2006). This engagement of non-energy conserving pathways would ensure continued functioning of the TCA cycle and miETC when ATP production is limited. P_i stressed transgenic tobacco plants has reduced growth and failed to synthesise a functional AOX protein (Parsons *et al.* 1999, Plaxton and Podesta 2006). Alternative respiratory pathways allow plants to adapt to their growth to available P_i levels and also play a role in maintaining cellular redox and carbon balance (Plaxton and Podesta 2006).

2.5.4 Other metabolic and structural adaptations

2.5.4.1 Secondary metabolism

A typical response of plants is a shift to secondary metabolism at the expense of primary metabolism and during P deficiency secondary metabolites such as flavonoids and indole alkaloids are increased (Vance et al. 2003). Examples of other compounds that accumulate in plants experiencing P stress are putrescine in rice which would inhibit growth, anthocyanin accumulation in cell cultures of *Vitis* which most likely relieves the photoinhibitory damage to chloroplasts and the exudation of phenolic compounds into the rhizosphere to assist in releasing P_i from their insoluble complexes (Vance *et al.* 2003). Exuded phenolics include piscidic acid from *Cajanus cajan* roots (Ae *et al.* 1990), alfafuran from *Medicago sativa* roots (Masaoka *et al.* 1993) and isoflavonoids from *Lupinus albus* cluster roots (Neumann *et al.* 2000). Sclerophylly is possible also an adaptation involving increased phenolic metabolism (Vance *et al.* 2003).

Phenolic and flavonoid pathway enzymes are upregulated in P_i deficient plants (Plaxton and Carswell 1999). These secondary metabolic pathways do not consume as much P_i and can also recycle significant amounts of P_i from phosphate esters. Secondary metabolism brings about an excess of reducing equivalents and the subsequent cytosolic acidification could cause activation of the alternative oxidative and other pathways to relieve this accumulation of reducing equivalents (Sakano 2001, Vance *et al* 2003).

2.5.4.2 Modified thylakoid membranes

During P deficiency the thylakoid membranes of chloroplasts show a decrease in phospholipids and large increases in the sulfolipid, sulfiquinovosyl diacylglycerol. This may be explained by the isolation and characterisation of two *Arabidopsis* genes which encode for enzymes in sulfolipid synthesis that have significantly increased expression during P deficiency (Vance *et al.* 2003). The proposed function of the changes in the sulfolipid:phospholipid ratio in the thylakoid membrane is to preserve P while maintaining membrane stability (Essigman *et al.* 1998). Phosphatidylglycerol may be replaced by sulfolipid during P stress which would reduce the requirement for membrane-bound P while ensuring continued photosynthesis during P stress (Vance et *al.* 2003).

2.6 Phosphoenolpyruvate carboxylase characteristics

PEPc (EC 4.1.1.13) is a ubiquitous cytosolic enzyme found in vascular plants, green algae and bacteria, but is absent from animals, fungi and yeast. It is a CO_2-fixing enzyme responsible for the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- and Mg^{2+}, yielding oxaloacetate and P_i in the process (Chollet *et al.* 1996). In C4 and CAM leaves, PEPc takes part in photosynthesis by catalyzing the initial fixation of atmospheric CO2 (Latzko and Kelly 1983). The role of PEPc in C3 photosynthetic cells and non-photosynthetic tissue is primarily an anaplerotic one whereby the citric acid cycle intermediates that are utilized for energy and biosynthetic metabolism are replenished (Melzer and O'Leary 1987). This amount of carbon fixed by PEPc is significant and can provide up to 25 % of the carbon requirement of symbiotic nitrogen assimilation (Vance *et al.* 1983, Uhde-Stone *et al.* 2003). During P deficiency 25 – 34 % of this PEPc-derived C is excreted in the form of citrate and malate from proteoid roots of white lupin (Johnson *et al.* 1996).

Native plant PEPc usually occurs as a polypeptide made up of four identical subunits and ranges in size from 870 amino acid residues (100-kDa) in bacteria to 970 residues (110-kDa) in vascular plants up to 1010 (116-kDa) and 1150 (134-kDa) residues in cyanobacteria and protozoa, respectively (Izui *et al.* 2004). The bioinformatic analyses of Arabidopsis, rice and soybean genomes have led to the identification of several conserved PEPc genes in these species encoding 110-kDa polypeptides featuring plant-type PEPc (PTPC) characteristics eg. a N-terminal seryl-phosphorylation domain and a critical C-terminal tetrapeptide QNTG (Izui *et al*. 2004; Xu *et al.* 2006). Each of these genomes contain a bacterial-type PEPc (BTPC) gene which encodes a larger, approximately 116-kDa polypeptide which has low sequence identity with PTPCs and lacks the N-terminal phosphorylation site while containing a prokaryotic-like tetrapeptide at the C-terminus (Sánchez and Cejudo 2003, Gennidakis *et al.* 2007). The physiological role of these BTPCs have yet to be defined as their transcripts have been documented in various plant tissues but no functional enzyme has been characterized yet in vascular plants (Gennidakis *et al.* 2007).

PEPc plays a pivotal in primary plant metabolism and is therefore subject to fine metabolic control. This enzyme undergoes allosteric regulation with L-malate or

asparagine acting as the inhibitors. In dicot plants, PEPc is activated by glucose-6-phosphate, while in monocot plants glycine or alanine functions as activators (Izui *et al*. 2004). Furthermore, plant PEPc is also controlled by reversible phosphorylation which is catalysed by an endogenous Ca^{2+}-independent PEPc protein kinase (PPCK) while a protein phosphatase type 2A (PP2A) catalyses the dephosphorylation. This reversible phosphorylation takes place at a highly conserved N-terminal seryl residue (Chollet *et al.* 1996, Izui *et al.* 2004, Murmu and Plaxton 2007) and reduces sensitivity to malate inhibition while concurrently enhancing activation by glucose-6-phosphate.

In nodules PEPc is responsible for reassimilating a substantial amount of the respired CO_2 and the oxaloacetate derived from the PEPc-catalysed reaction has several metabolic fates (Pathirana *et al.* 1997). PEPc activity has been found to be higher in nodules than in roots (Deroche and Carrayol 1988) and is present in both infected and uninfected cells of the infected region of nodules (Streeter 1991). Three forms of PEPc have been purified from lupin nodules and roots (Marczewski 1989). Two forms, PEPc I and PEPc II, were purified from nodules and PEPc III was purified from lupin roots. PEPc I and PEPc II differed substantially in kinetic properties and these two forms were also different from PEPc III. PEPc I appeared to be closely associated with N_2 fixation (Marczewski 1989).

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CHAPTER 3

EFFECT OF P STRESS ON BIOLOGICAL NITROGEN FIXATION IN LUPINS: THE ROLE OF ORGANIC ACID AND AMINO ACID SYNTHESIS IN NODULES

3.1 Abstract

This study focussed on the effect of phosphorus (P) deficiency on key respiratory enzymes as well as the allocation between organic and amino acid pools. Phosphorus is an essential element for plant growth, but is usually largely unavailable for uptake by plants due to the formation of insoluble complexes. Nodulated *Lupinus luteus* plants were grown hydroponically on a modified Long Ashton nutrient solution containing no nitrogen for *ca*. four weeks after which one set was transferred to a P-deficient nutrient medium, while the other set continued growing on a P-sufficient nutrient medium. Phosphorus stress was measured at 12 and 20 days after onset of P-starvation. No significant differences in phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase (MDH) or pyruvate kinase (PK) activity were found between P-stressed and P-sufficient treatments. There were also no changes in organic acid synthesis during P stress, however there were increases in asparagine, aspartate, glutamine and alanine levels in P-deficient nodules compared to P-sufficient nodules. The accumulation of amino acids under P deficiency is possibly the result of an indirect feedback effect on nodule nitrogen fixation by the reduction in host growth, which would require less nitrogen (N). Although the PEPc-MDH-ME bypass was not engaged during P deficiency, the unchanged organic acid levels suggest a role for exudation, by which nodules can overcome periods of P stress.

Key words: nodules, amino acids, organic acids, phosphorus deficiency

3.2 Introduction

Phosphorus (P) is essential for plant growth and plays a role in a variety of processes eg. photosynthesis, glycolysis, respiration, energy production, nitrogen metabolism to name a few (Vance *et al*. 2003). This element is usually abundant in soils, but largely unavailable for uptake by plants (Bieleski 1973, Schachtman *et al*. 1998) due to the fact that P readily forms insoluble complexes with cations such as calcium, aluminium and iron (Aono *et al*. 2001). This is especially the case for the acid-weathered soils of both the tropics and sub-tropics (Sanchez and Uehara 1980, Vance *et al*. 2003). The prescribed course of action for P-deficient soils is the application of fertilizer P, however, in the tropics and subtropics fertilizer is often not applied due to financial constraints, transportation problems as well as a lack of available fertilizer P. The onus rests on plant biologists to find mechanisms to improve P uptake by plants, P efficiency of plants as well as suitable management of crop systems (Vance *et al*. 2003).

Following severe P-stress, cytoplasmic P_i levels in plants may decrease by as much as 50-fold. This reduction in cytoplasmic P_i in turn leads to decreases in intracellular levels of ATP, ADP and nucleoside P (Plaxton and Podesta 2006). A decrease in these molecules would impact negatively on the carbon flux through the adenylate- and P_i -dependent enzymes of the glycolytic pathway. However, regardless of P_i -stress, plants have to continue generating energy and carbon skeletons for ongoing primary metabolic processes and to this end a cluster of at least six adenylate-independent glycolytic “bypass” enzymes have been identified (Plaxton and Podesta 2006). The activities of sucrose synthase, UDP-glucose pyrophosphorylase, PP_i -PFK, nonphosphorylating NADP-glyceraldehyde-3-phosphate, phosphoenolpyruvate carboxylase (PEPc), phosphoenolpyruvate phosphatases and NAD-malic enzyme have all shown to be significantly upregulated during P_i stress and it has been proposed that these enzymes are inducible bypasses during P_i stress to ensure continuation of glycolytic flux when adenylate and P_i levels are limiting (Plaxton and Podesta 2006). The activity of PEPc, together with malate dehydrogenase (MDH) and malic enzyme (ME), functions as the glycolytic bypass for the reaction catalysed by ADP-dependent cytosolic pyruvate kinase (PKc). The activity of this metabolic bypass during P_i stress, when the ADP supply may be limiting, would ensure continued pyruvate supply to the tricarboxylic acid cycle (TCA), while at the same time releasing P_i back into the metabolic pool (Duff *et al*. 1989, Plaxton 2004). Several authors have reported an increase in PEPc activity for P_i -stressed samples

compared to P_i -sufficient controls in *Brassica nigra* (Duff *et al*. 1989), *Brassica napus* (Nagano *et al*. 1994) and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000, Plaxton and Podesta 2006).

The role of PEPc in C3 photosynthetic cells and non-photosynthetic tissue is primarily an anaplerotic one whereby the TCA intermediates that are utilized for energy and biosynthetic metabolism are replenished (Melzer and O'Leary 1987). This amount of carbon fixed by PEPc is significant and can provide up to 25 % of the carbon requirement of symbiotic nitrogen assimilation (Vance and Johnson 1983, Uhde-Stone *et al*. 2003). During P deficiency 25 – 34 % of this PEPc-derived carbon is excreted in the form of citrate and malate from proteoid roots of white lupin (Johnson *et al*. 1996).

There have been numerous studies on the effects of P on host plant growth as well as the factors controlling N_2-fixation in nodules, but there has not been the same focus on the role of P in nodule metabolism (Jakobsen 1985, Israel 1987, Sa and Israel 1991, Al-Niemi *et al*. 1997, 1998, Tang *et al*. 2001, Le Roux *et al*. 2006, 2008). Limited P supply is thought to be one of the constraints of nitrogen fixation (Aono *et al*. 2001). Phosphorus deficiency impacts negatively on the energy status of nodules: nitrogen fixation is an ATP-consuming process and the assimilation of the resulting ammonium into amino acids and ureides are also energy-dependent processes (Sa and Israel 1991). Engaging the PEPc bypass in nodules during P stress would lead to subsequent competition for carbon skeletons between the organic and amino acid pools (Valentine *et al*. 2010). The responses of nodule carbon metabolism to P_i stress appear to be species dependent. This could be attributed to differences such as tropical vs. temperate origins, determinate vs. indeterminate nodules and amino acid vs. ureide exporters (Valentine *et al*. 2010).

The main carbon source for bacteroid respiration and nitrogen fixation is C4 dicarboxylic acids, especially in the form of malate (Rosendahl *et al*. 1990, Valentine *et al*. 2010). The importance of these C4 acids is supported by the high levels of malate found in effective nodules (Streeter 1987, Rosendahl *et al.* 1990, Valentine *et al*. 2010). Metabolomics and transcriptomics have shown that the high levels of nodular malate are the result of low oxygen and phosphorus concentrations (Colebatch *et al.* 2004). Le Roux *et al.* (2006) reported high nodular malate levels in *Lupinus angustifolius* during short-term P-stress, which was the product of upregulated MDH and PEPc activities even though the nodules were not yet P-

stressed. No sign of upregulation of ME was found, which suggested that the PEPc bypass functioned for increased malate levels, which would serve as the major carbon source for bacteroid respiration (Rosendahl *et al*. 1990, Valentine *et al*. 2010). During long-term P-deficiency when the nodules experienced P-stress, Le Roux *et al*. (2009) found even higher levels of malate in the nodules.

Two separate groups working on P-stressed *Trifolium repens* reported an accumulation of asparagine in the xylem sap, roots and nodules (Almeida *et al*. 2000, Høgh-Jensen *et al.* 2002). This accumulation may have an indirect feedback effect on nodule nitrogen fixation under P deficiency by reducing host growth which in turn would require less N (Valentine *et al*. 2010). Olivera *et al.* (2004) and Le Roux *et al.* (2009) both reported a shift during P stress from ureide to amine production in *Phaseolus vulgaris* and *Glycine max*, respectively. However, Le Roux *et al.* (2009) reported that despite the shift from ureide to amine production, more ureides were exported to the host under P stress.

The aim of this study was to determine the effect of P deficiency on nitrogen fixation and its interaction with organic and amino acid pools.

3.3 Materials and Methods

3.3.1 Plant growth conditions

Seeds of *Lupinus luteus* var. Juno (BRF 21791, Agricol South Africa) were inoculated with 'Lupins and Serradella' inoculant containing *Bradyrhizobium* sp (*Lupinus*) bacteria (Stimuplant, Zwavelpoort, South Africa) in vermiculite. Seeds were watered with distilled H_2O (dH_2O) and *ca.* 14 days after germination (first fully expanded leaf emergence) seedlings were transferred to 22 l hydroponic tanks containing a modified Long Ashton nutrient solution (Hewitt 1966) containing no nitrogen. Prior to transfer to hydroponic culture, the roots were rinsed in dH_2O and the hypocotyls of the plants were wrapped in black closed-cell foam rubber and inserted through collars in the lids of the hydroponic tanks. The tanks were completely opaque and contained eight plants each. Nutrient solutions were strongly aerated with ambient air (360 ppm CO_2). The nutrient solutions were changed weekly and the pH of the medium was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. Plants were grown under natural light conditions in a temperature controlled (minimum 15

℃, maximum 25 ℃) greenhouse at the University of Stellenbosch during winter (June, July and August).

Plants were grown on sufficient P (2 mM) nutrient solution for ± 40 days after which the first set was harvested (this was the control treatment). The remaining plants were then split into two separate treatments, one continued receiving 2 mM P (P sufficient) nutrient solution, while the other set was grown on 5 µM P (P-stressed) nutrient solution. The transfer of plants from a P sufficient nutrient solution to a P deficient nutrient solution was deemed the onset of P starvation. Plants from both treatments were harvested 12 days after onset of P starvation and again at 20 days. At each time point, four plants of each treatment were harvested and divided into roots, shoots and nodules and weighed. The nodules were quenched in liquid N_2 and stored at -80 ℃ for later use in assays.

3.3.2 Phosphate determination

Cellular P_i concentration was determined according to Wanke *et al*. (1998). Frozen nodules were ground in 500 µl 10 % (w/v) tricholoroacetic acid (TCA) using a pre-cooled mortar and pestle and then diluted three times with 5 % (w/v) TCA. The samples were centrifuged at 2 500 x g for 10 min at 4 ˚C. The supernatant was removed and centrifuged for a further 10 min at 13 000 x g and 4 ˚C. The supernatant was kept on ice until ready for further use. Next, 700 µl of reaction mixture was added to 300 µl supernatant and vortexed. The reaction mixture consisted of a 1:6 combination of 10 % (w/v) ascorbic acid and 0.42 % NH_4^+-molybdate.$4H_2O$ in 1N H_2SO_4. The samples were then incubated for 20 min at 45 ˚C or for 1 h at 37 ˚C after which the absorbance of the samples were measured at 820 nm using a dilution range of 1 mM KH_2PO_4 as standards.

3.3.3 Enzyme assays

Frozen nodules were homogenised on ice in a pre-chilled mortar and pestle using 2 ml extraction buffer consisting of 20 mM TRIS, 100 mM sucrose, 1 mM EDTA, 10 % (w/v) PVPP (insoluble), 15 % (v/v) ethylene glycol and 5 mM DTT (Valverde and Wall 2003). The homogenate was centrifuged at 13 000 x g and 4 ˚C for 5 min and the supernatant transferred to new eppendorf tubes. The supernatants were centrifuged again at 13 000 x g and 4 ˚C for 5 min and the resulting supernatant were designated crude extracts and stored at -80 ˚C until further use.

Phosphoenolpyruvate carboxylase

The reaction mixture for PEPc consisted of 100 mM TRIS (pH 8.5), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 4 mM PEP, 0.2 mM NADH, 5 units MDH (Ocaňa *et al.* 1996). The PEPc activity was spectrophotometrically measured by coupling carboxylation with exogenous NADH-malate dehydrogenase and measuring the oxidation of NADH. The substrate, PEP, was substituted with dH_2O in the blank reactions to account for background oxidation.

Pyruvate kinase

The reaction mixture for measuring PK activity was made up of 75 mM TRIS (pH 7.0), 5 mM $MgCl_2$, 1 mM ADP, 3 mM PEP, 0.18 mM NADH, 3 units LDH (Smith 1985). To account for background oxidation blank samples were run using dH_2O instead of ADP or PEP.

NADH-Malate dehydrogenase

The MDH activity was assayed with a reaction mixture consisting of 85 mM TRIS (pH7.5), 0.2 mM OAA, 0.35 mM NADH (Appels and Haaker 1988). The blank samples were run without the addition of OAA to the reaction medium.

Aspartate amino transferase

The AAT activity was assayed using a reaction medium made up of 50 mM TRIS (pH 7.5), 4 mM $MgCl_2$, 0.2 mM NADH, 10 mM aspartic acid, 1 mM α-ketoglutarate (Olivera *et al*. 2004).

All enzymes were assayed spectrophotometrically in a final volume of 250 µl at 340 nm and 30 ˚C. Reactions were initiated by the addition of 30 µl crude extract to the reaction mixture. Protein concentrations were quantified according to the Bradford method (Bradford 1976) using a protein assay reagent and bovine serum albumin as the standard.

3.3.4 Amino acid analysis

Amino acids were extracted from nodules following an adaptation of the method used by Carpena *et al.* (2003). Nodules were dried in a freeze drier for three days after which they were ground in a chilled mortar and pestle using liquid N_2. Of the resulting finely ground powder, 0.0250 g were transferred to eppendorf tubes and 1.5 ml of 80 % (v/v) ethanol containing 1 % (w/v) sodium azide and protease inhibitor (Bio-Rad protease inhibitor cocktail tablet) were added. The tubes were vortexed and placed in a 70 ˚C water bath for 30 min after which the samples were centrifuged for 10 min at room temperature in a bench top centrifuge. The samples were transferred to an 80˚C water bath to remove excess ethanol by heating. The precipitate was resuspended with 1 ml de-ionised water and centrifuged for 5 min at room temperature in a bench top centrifuge. The supernatant was stored at -20 ˚C until used for further determinations.

Samples were thawed to room temperature and vortexed for 5-10 s after which they were centrifuged at 15 000 x g for 5 min. Next, 400 µl of the supernatant was centrifuged at 5000 g for 40 min through a 10 kDa cut-off membrane. Subsequent to this step, 50 µl of the resulting filtrate was analytically measured with a Hamilton syringe and placed into a glass hydrolysis tube and dried under vacuum for 1 h. Samples were processed further by adding 20 µl methanol: water: triethylamine (in a 2:2:1 ratio) to adjust the pH and were redried for 1 h. Samples were derivatised by addition of 20 µl derivatising solution (methanol: water: triethylamine: phenylisothiocyanate (PITC), 7:1:1:1). The samples were incubated for 10 min at room temperature and dried under vacuum for a minimum of 1 h and a maximum of 3 h or until complete dryness was reached. The derivatised dried sample was then dissolved in 200 µl of Picotag sample diluent (Waters, Millford, MA, USA) and filtered through a 0.45 µm filter. Next, 5, 10, 20 and 40 µl of the sample were subjected to HPLC using a standard method for PTC-amino acid chromatography. Quantification of amino acids was optimal using the 20 µl injection volume. Smaller or larger injection volumes were used in cases where amino acids were obscured by co-eluting compounds. Data were collected and analysed using Breeze software (Waters, Millford, MA, USA). The percentage recovery of standards was determined by analyzing standards of a known quantity. For each batch of samples, standards were dried and treated under the exact same conditions as the samples.

The amounts of amino acids present in the original extract were calculated using the following equation:

$\mu mole/\ gram = picomoles/V_i \times V_r/V_d \times V_e/weight(g) \times 10^3\ \mu l.ml^{-1} / 10^6\ pmole.\mu mole^{-1}$

where picomoles = reported amount for component

V_i = injection volume

V_r = volume in which derivatised sample is reconstituted

V_d = volume of sample derivatised

V_e = extraction volume

In total twenty samples were analysed after filtration and the following amino acids were detected: α-amino butyric acid, alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, lysine, serine, threonine, tyrosine and valine. Proline, glycine, histidine and phenylalanine were detected, but their concentrations were below the quantification level. Methionine was not detected. Tryptophan and/or ornithine were detected, but they had unresolved separation in the system used.

Chromatography revealed multiple unidentified components in the samples (not shown). These unknown compounds caused background noise that influenced the quantification of some amino acids in the samples. An unknown compound(s) with a high concentration, eluting just after leucine, was consistently found in all samples. The peak of this unknown component interfered with the detection of cysteine/cystine, isoleucine and leucine at larger injection volumes (> 5 µl). Other unknown compounds also co-eluted in certain samples with alanine, asparagine, serine, glycine, arginine, threosine and α-amino butyric acid, causing problematic quantification of these amino acids and subsequent large standard deviations. The technique used for amino acid analysis in this study was unable to distinguish the unknown compounds. All amino acids that could be detected were quantified within the range of 70 - 5000 picomole.

3.3.5 Organic acid analysis

Organic acids were extracted by adding 0.5 ml dH_2O to frozen freeze-dried samples (*ca*. 200 mg). The samples were centrifuged at 17 000 x g and 4 ˚C for 10 min. After centrifugation, 450 µl of supernatant was loaded onto a C18 column (equilibrated with MilliQ water) and then run through the column under partial vacuum using 3 ml MilliQ water to wash the sample through. The samples were collected and made up to 4 ml in volumetric flasks. Each of these samples was then filtered through a 0.45

µM filter into an HPLC vial prior to analysis. Samples were measured against standards of malate, fructose, fumarate, oxaloacetate and sucrose.

Chromatography revealed two unidentified components in the samples (not shown). One of these unknown compounds had properties like that of glucose, but did not elute where glucose would be expected. The other component could possibly have been a combination of an unknown sugar together with a small amount of glucose. When the samples were run again using a different elution buffer, the peak of the "glucose-like" sugar moved a lot more than any of the other components' peaks. It seems as if it could be more than one group or compound which are affected by changes in pH, eg. carboxylate groups. All the other components' peaks moved to the same extent as that of the standards, which further supported their proposed identities.

3.3.6 Calculations of δ15N

The δ15nitrogen (N) and carbon to nitrogen ratio (C:N) analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et al.* (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5mm mesh (Arthur H Thomas, California, USA). Between 2.1 mg and 2.2 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

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

δ15N enrichment (Seed corrected) = ((plant N X δ15N $_{plant}$) – (seed N X Ps X δ15N $_{seed}$)/ (plant N – seed N))

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

3.4 Results

The P-stressed nodules had significantly lower levels of available cellular P than the P-sufficient nodules at 12 days after onset of P starvation (Fig 3.1). These levels of available P decreased even further as P-stress developed, as seen in P-stressed nodules at 20 days after onset of P starvation.

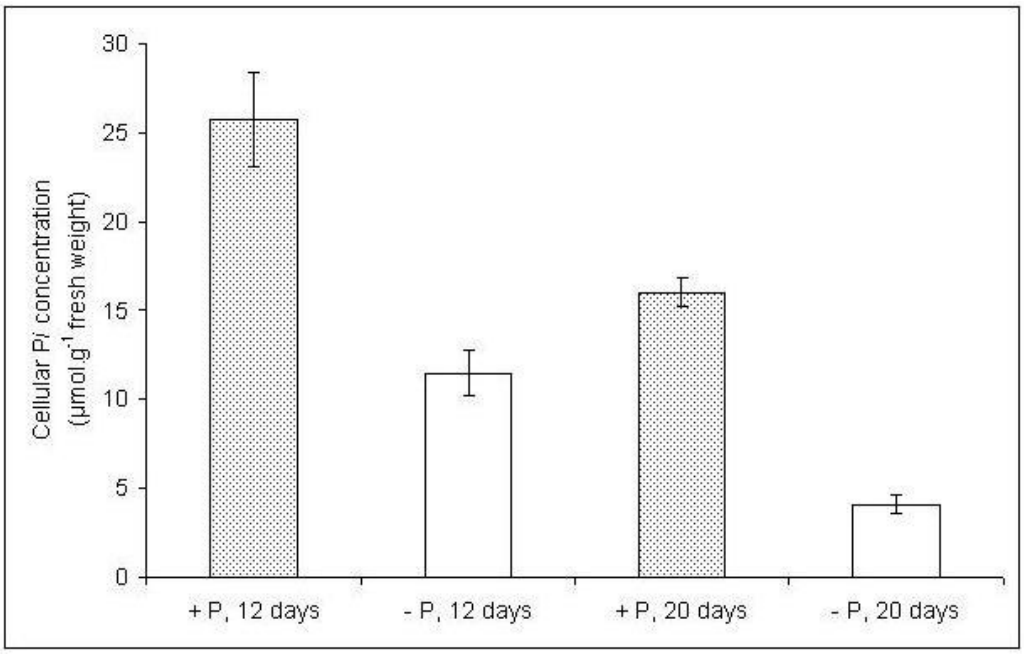

Figure 3.1 Cellular P_i concentrations (µmol.g^{-1} FW) in nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

Table 3.1 Relative growth rates (mg.g^{-1} dry weight.day^{-1}), nitrogen fixation ($\delta^{15}N$) and C:N ratio, for *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

	+ P, 12 days	- P, 12 days	+ P, 20 days	- P, 20 days
Relative growth rate (mg.g^{-1}.day^{-1})	85.87 ± 3.20 **b**	53.28 ± 6.86 **a**	84.24 ± 6.05 **b**	60.37 ± 5.08 **a**
Nitrogen Fixation δ15N (‰)	-1.09 ± 0.12 **a**	-1.72 ± 0.15 **b**	-0.84 ± 0.020 **a**	-2.05 ± 0.13 **c**
Plant C:N	9.1 ± 1.32 **b**	15.23 ± 1.21 **c**	5.34 ± 0.45 **a**	8.34 ± 1.45 **b**

The plant relative growth rates were reduced during P deficiency at 12 and 20 days, compared to P-starved plants (Table 3.1). The P deficiency at 12 and 20 days resulted in higher biological nitrogen fixation (BNF) as expressed by δ15N and also an increase in C:N ratios over the same period (Table 3.1).

Table 3.2 Nodule amino acid concentrations (mM.g^{-1} dry weight) for *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

	+ P, 12 days	- P, 12 days	+ P, 20 days	- P, 20 days
Alanine	5.87 ± 0.17 **ab**	6.28 ± 0.46 **ab**	5.24 ± 0.15 **a**	6.37 ± 0.44 **b**
Asparagine	76.01 ± 0.41 **c**	103.54 ± 7.53 **d**	42.90 ± 1.62 **a**	59.85 ± 4.9 **b**
Aspartate	8.05 ± 0.33 **b**	13.34 ± 0.73 **d**	6.47 ± 0.88 **a**	9.17 ± 1.26 **c**
Glutamate	10.24 ± 0.44 **b**	10.99 ± 0.62 **b**	7.90 ± 0.31 **a**	7.93 ± 0.65 **a**
Glutamine	1.66 ± 0.14 **cd**	2.07 ± 0.12 **d**	0.74 ± 0.04 **a**	1.13 ± 0.14 **b**

At 12 days after onset of P starvation, the asparagine and aspartate levels were significantly higher in P-stressed nodules compared to P-sufficient nodules (Table 3.2). As P starvation continued to 20 days, the asparagine and aspartate levels remained significantly higher in P-stressed nodules compared to P-sufficient nodules, albeit lower than at 12 days after onset of P stress. Alanine and glutamine levels were both significantly higher in P-stressed nodules compared to P-sufficient nodules at 20 days after onset of P starvation (Table 3.2).

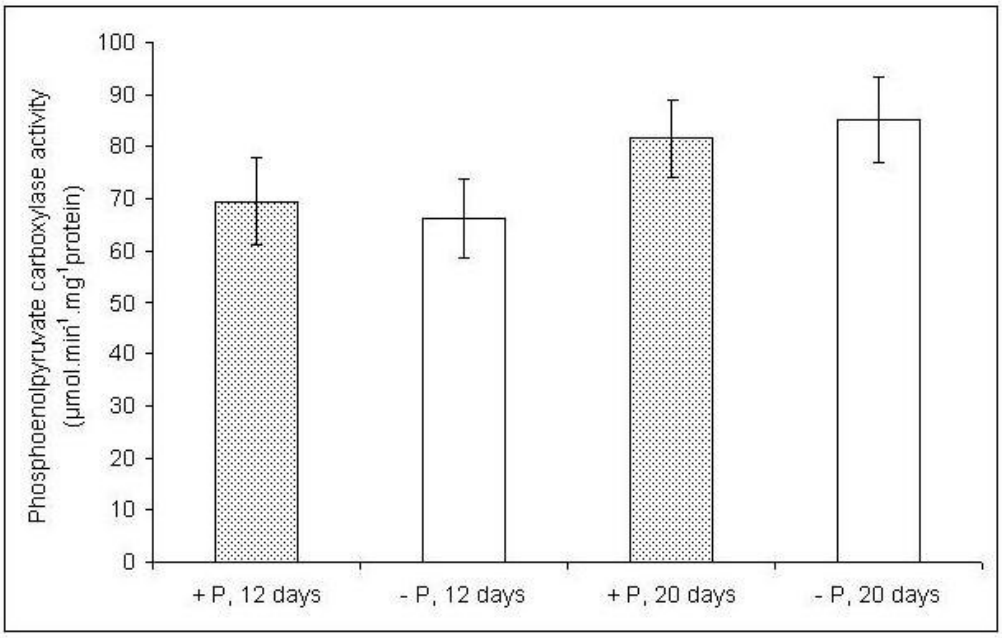

Figure 3.2 Phosphoenolpyruvate carboxylase activity (µmol.min^{-1}.mg^{-1} protein) in the nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

No significant differences in PEPc activity were found between P-stressed and P-sufficient treatments at both 12 and 20 days after onset of P starvation (Fig 3.2).

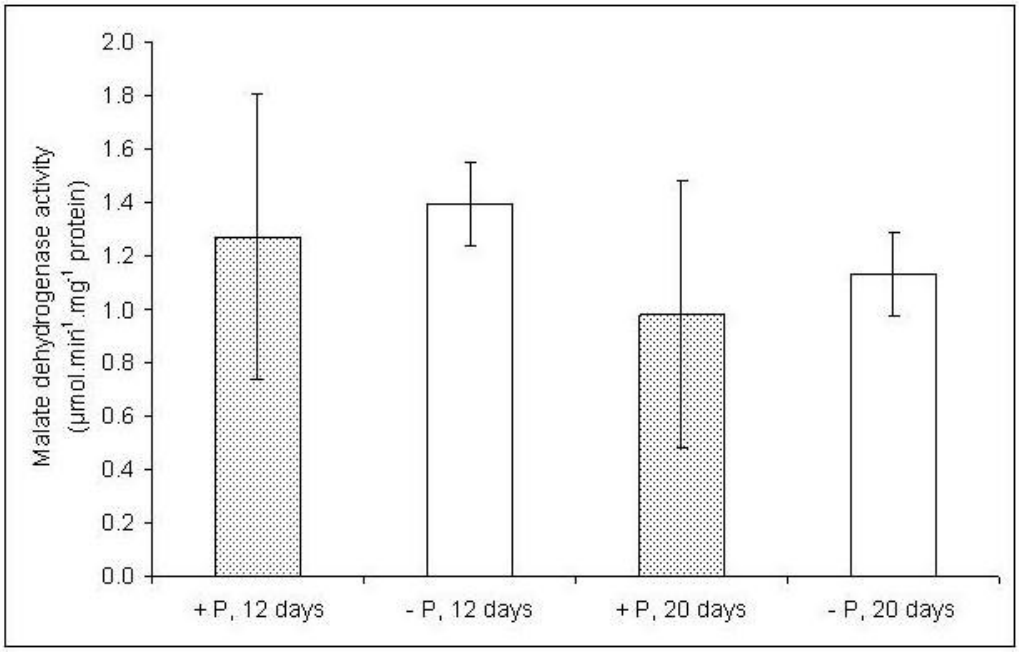

Figure 3.3 Malate dehydrogenase activity (µmol.min^{-1}.mg^{-1} protein) in the nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

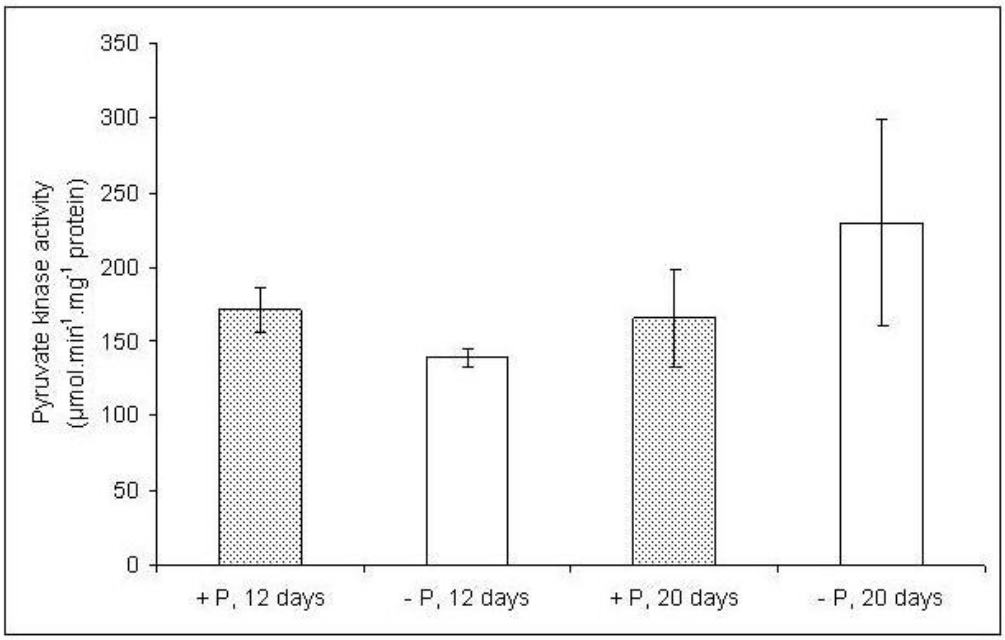

Figure 3.4 Pyruvate kinase (cytosolic) activity (µmol.min^{-1}.mg^{-1} protein) in nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

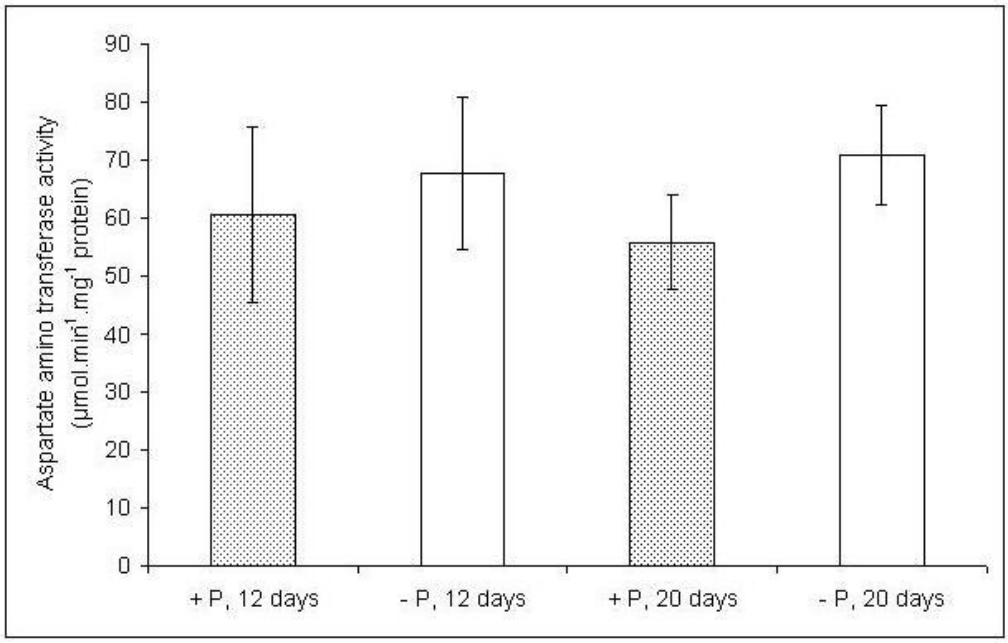

Figure 3.5 Aspartate amino transferase activity (µmol.min^{-1}.mg^{-1} protein) in nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

No significant differences in MDH (Fig 3.3), PK (Fig 3.4) or AAT activities (Fig 3.5) were found between the different treatments.

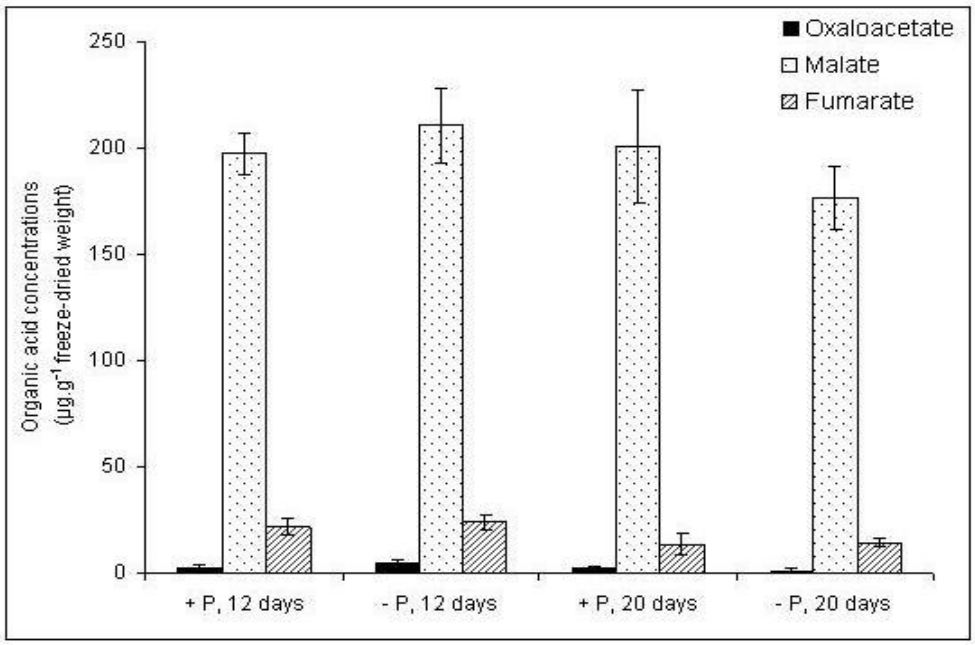

Figure 3.6 Organic acid concentrations (µg.g^{-1} freeze-dried weight) in nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

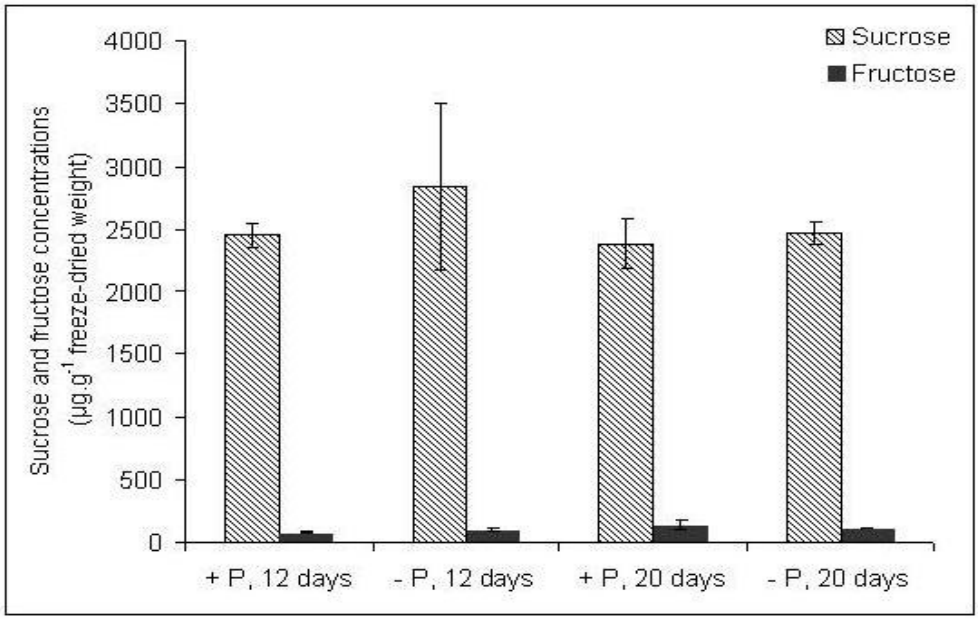

Figure 3.7 Sucrose and fructose concentrations (µg.g^{-1} freeze-dried weight) in nodules of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

No significant differences were found in the measured organic acids (Fig 3.6) or sucrose and fructose concentrations (Fig 3.7) between the P-stressed and P-

sufficient plants at either day 12 or 20 after initiation of P stress.

3.5 Discussion

In this study, 12 and 20 days of P starvation at a concentration of 5 µM was sufficient to induce a decrease in P stress in *Lupinus luteus* nodules (Fig 3.1). This concurs with the results of Sa and Israel (1991) who used 50 µM in their starvation studies on soybean nodules. Previous work done on *Lupinus angustifolius* used 2 µM P as the P-deficient treatment and Le Roux *et al.* (2006) reported no change in nodular P_i after 14 days of P stress, whereas Le Roux *et al.* (2008) reported a decrease in nodular P_i levels after 25 days of P stress. The decrease in nodular P_i levels found here would influence the down-stream allocation of carbon to either the organic acid pool (for bacteroid respiration) or the amino acid pool (for nitrogen assimilation) (Valentine *et al*. 2010).

The decrease in nodular P_i levels did not elicit an increase in *in vitro* PEPc (Fig 3.2) and MDH activities (Fig 3.3), two enzymes which together with ME, act as a P_i - dependent glycolytic bypass for cytosolic PK (Duff *et al.* 1989, Plaxton 2004, Plaxton and Podesta 2006). This is in contrast with the findings of several other authors who reported increased PEPc activity during P stress in *Brassica nigra* (Duff *et al.* 1989), *Brassica napus* (Nagano *et al.* 1994) and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000). Furthermore, Le Roux *et al.* (2006, 2008) reported increased PEPc and MDH activities in P-stressed *Lupinus angustifolius* nodules. The possible explanations for this apparent lack of engagement of the PEPc bypass in *Lupinus luteus* nodules may be due to several reasons, as discussed below.

In this study, the 20 days of P starvation using a 5 µM concentration of P may not have been sufficient to engage the glycolytic bypass enzymes. Despite a significant decrease in nodular P_i at 12 and 20 days of P stress, it might not have been low enough to warrant a P-starvation response via the glycolytic enzymes in these nodules. In fact, previous studies have shown that nodules may function optimally at low P_i concentrations (Al-Niemi *et al.* 1997, 1998, Le Roux *et al*. 2006). Nodules appear to have a mechanism whereby P influx can be regulated, allowing nodules to diminish the effect of P deficiency during periods of low P supply (Jakobsen 1985, Tang *et al.* 2001, Valentine *et al*. 2010). Moreover, the bacteroid fraction of the nodule may also be able to regulate its P-stress response. Several physiological studies have shown that bacteroids constantly operate at low P levels (Sa and Israel

1991, Al-Niemi *et al.* 1997, 1998) and are able to scavenge P from host cells (the cytosolic fraction) to fulfil their own P requirements (Al-Niemi *et al.* 1997, 1998).

The PEPc-MDH-ME bypass is not the only possible bypass or response during P stress. Another possible route of PEP catabolism and pyruvate production for the TCA cycle is the vacuolar phosphoenolpyruvate phosphatase (Plaxton 2004). The PEP branchpoint is important because here it is determined whether carbon skeletons are allocated to the organic acid or the amino acid pools. In contrast with other studies which reported increased organic acid synthesis (Le Roux *et al.* 2006, 2008), here no change in organic acid synthesis (Fig 3.6) was found, but rather increases in the levels of key amino acids significant to amide exporters such as *L. luteus*. The stable organic acid levels during P starvation also concur with the unchanged sucrose and fructose concentrations, from which these organic acids are largely derived. Organic acid levels such as malate, may serve as the energy source for bacteroid metabolism and nitrogen fixation (Le Roux *et al.* 2006, 2008). The unchanged organic acid levels during P starvation, may be maintained to support the increased nitrogen fixation. In addition, this accumulation of organic acids may suggest the potential for organic acid exudation during P deficiency (Le Roux *et al.* 2006, 2008, Valentine *et al*. 2010)

During P-deficiency, the increase in aspartate, asparagine, glutamine and alanine levels (Table 3.2), concurs with the increase in biological nitrogen fixation (BNF) under the same P conditions and indicates an accumulation of key amino acids that are derived from BNF. Although previous reports have found a decrease in lupin BNF with P-deficiency, these reported effects have been due to longer term P deprivation (25 days) than the current study (Le Roux *et al*. 2008). These authors found that the effects of P starvation on nodule metabolism were only visible at 25 d after P starvation (Le Roux *et al*. 2008), compared to a previous study where 14 d of P withdrawal had no apparent effect on Pi concentrations of nodules (Le Roux *et al*. 2006).

The mechanism for nodules to increase their BNF during low Pi concentrations, are not clear, but it appears that they are able to function optimally under low Pi levels, as supported by previous findings (Al Niemi *et al*. 1997, 1998, Colebatch *et al*. 2004, Le Roux *et al*. 2006). It therefore appears that nodules may have a strategy to regulate P influx, allowing nodules to minimize effects of P deficiency when supply is low (Jakobsen 1985, Tang *et al*. 2001). Furthermore, the bacteroid fraction of the

nodule may also be able control its response to P stress inside the nodule. From physiological studies (Sa and Israel 1991, Al Niemi *et al*. 1997, 1998) it is clear that bacteroids in the nodule consistently operate at low P concentrations and can fulfill their P requirements by scavenging from the host cells (Al Niemi *et al*. 1997, 1998).

3.6 Conclusion

The decline of nodular P_i levels in *L. luteus* was not associated with the engagement of the P-starvation metabolic bypass at the PEPc branchpoint. This may indicate that lupin nodules are able to function optimally under moderate P-starvation and thereby to resist changes in C and N metabolism.

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CHAPTER 4

EFFECT OF P STRESS ON BIOLOGICAL NITROGEN FIXATION IN LUPINS: THE ROLE OF PHOTOSYNTHETIC AND RESPIRATORY CARBON METABOLISM

4.1 Abstract

The role of P stress on biological nitrogen fixation (BNF) was investigated via the respiratory and photosynthetic C and nutrient costs. BNF can impose a considerable sink stimulation on host respiratory and photosynthetic C, but the energy costs of BNF during P deficiency has not yet been studied. Nodulated *Lupinus luteus* plants were grown in sand culture, using a modified Long Ashton nutrient solution containing no nitrogen for *ca*. four weeks after which one set was exposed to a P-deficient nutrient medium, while the other set continued growing on a P-sufficient nutrient medium. Phosphorus stress was measured at 20 days after onset of P-starvation. During P stress the decline in nodular P levels was associated with lower BNF and nodule growth. There was also a shift in the balance of photosynthetic and respiratory C. In this regard, the shift was towards a loss of C during P stress. During P stress the below-ground respiration declined, but the plants under P stress shifted the proportion of respiratory energy from maintenance to growth respiration. This was to support the increase in root growth under P stress. These results show that in spite of the decline in respiratory and photosynthetic C metabolism during P stress, the affected plants can alter the allocation of respiratory energy from nutrient uptake to growth.

Key words: P deficiency, nitrogen fixation, respiration, photosynthesis, C and N costs

4.2 Introduction

Phosphate (P) is usually abundant in soils, but largely unavailable for uptake by plants (Bieleski 1973, Schachtman *et al*. 1998) due to the fact that P readily forms insoluble complexes with cations such as calcium, aluminium and iron (Aono *et al*. 2001). This is especially the case for the acid-weathered soils of both the tropics and sub-tropics (Sanchez and Uehara 1980, Vance *et al*. 2003). Limiting supply of P is thought to be one of the major constraints of biological nitrogen fixation (BNF) (Aono *et al*. 2001, Vance *et al*. 2003).

Legumes that rely on BNF have a high requirement for phosphate (Jakobsen 1985, Israel 1987, Sa and Israel 1991, Al-Niemi *et al*. 1997, 1998, Tang *et al*. 2001, Le Roux *et al*. 2006, 2008) and it is known that P_i contributes to a higher nitrogen fixing ability as well as nodule mass (Jakobsen 1985, Israel 1987, Sa and Israel 1991). Since the role of P deficiency in other plants has revealed the interaction of P stress and intracellular levels of ATP and ADP (Plaxton and Podesta 2006, Duff *et al*. 1989, Plaxton 2004), it has become more clear as to how P deficiency may negatively impact on the energy status of nodules to affect BNF (Sa and Israel 1991, Le Roux *et al*. 2006).

It is clear from the aforementioned studies that in legumes there have been many investigations on the role of P in nodule metabolism (Jakobsen 1985, Israel 1987, Sa and Israel 1991, Al-Niemi *et al*. 1997, 1998, Tang *et al*. 2001, Le Roux *et al*. 2006, 2008). However nodule C and N metabolism during P stress are likely to be intricately linked to whole plant physiological events, which can be measured in terms of growth, photosynthesis and respiration. During P-stress the role of plant growth affecting the requirement of BNF has been proposed as a means of BNF regulation. The accumulation of BNF-derived amino acids in the xylem sap, roots and nodules (Almeida *et al*. 2000, Høgh-Jensen *et al.* 2002), may have an indirect feedback effect on nodule nitrogen fixation under P deficiency, because the reduction in host growth would require less N (Valentine *et al*. 2010). Furthermore, the sink effect of nodules at various levels of developmental and functional stages, have been observed via alterations in photosynthesis and respiration (Mortimer *et al*. 2008, 2009). In these studies, the photosynthetic and respiratory C costs of BNF and nodule growth were considerable contributions to the overall C budget of the symbiosis. However, these reports did not investigate the role of P stress in such an overall C budget of legumes (Mortimer *et al*. 2008, 2009).

These effects of P stress on the C and N interactions of BNF, should be greatly influenced by the C balance in leaf photosynthesis and root-nodule respiration. During P stress, this has not been investigated in great detail for respiration (Le Roux *et al*. 2006, 2008) and certainly not in combination with photosynthesis. Therefore, the aim of this study was to determine the effect of P deficiency on nitrogen fixation as mediated by N and C interactions during respiration and photosynthesis.

4.3 Materials and Methods

4.3.1 Plant growth conditions

Seeds of *Lupinus luteus* var. Juno (BRF 21791, Agricol South Africa) were inoculated with 'Lupins and Serradella' inoculant containing *Bradyrhizobium* sp (*Lupinus*) bacteria (Stimuplant, Zwavelpoort, South Africa) in vermiculite. Seeds were watered with distilled H_2O (dH_2O) and *ca.* 14 days after germination (first fully expanded leaf emergence) seedlings were transferred to sand culture, using quartz sand (500 µm grain size) as a medium and a modified Long Ashton nutrient solution (Hewitt 1966) containing no nitrogen. Plants were grown under natural light conditions in a temperature controlled (minimum 15 ℃, maximum 25 ℃) greenhouse at the University of Stellenbosch during winter (June, July and August).

Plants were grown on sufficient P (2 mM) nutrient solution for ± 40 days after which the first set was harvested (this was the control treatment). The remaining plants were then split into two separate treatments, one continued receiving 2 mM P (P sufficient) nutrient solution, while the other set was grown on 5 µM P (P-stressed) nutrient solution. The transfer of plants from a P sufficient nutrient solution to a P deficient nutrient solution was deemed the onset of P starvation. Plants from both treatments were harvested at 20 days after onset of P starvation At that time point, four plants of each treatment were harvested and divided into roots, shoots and nodules and weighed. The gas exchange measurements were taken prior to harvesting on the same plant material.

4.3.2 Phosphate determination

Cellular P_i concentration was determined according to Wanke *et al*. (1998). Frozen nodules were ground in 500 µl 10 % (w/v) tricholoroacetic acid (TCA) using a pre-

cooled mortar and pestle and then diluted three times with 5 % (w/v) TCA. The samples were centrifuged at 2 500 x g for 10 min at 4 ˚C. The supernatant was removed and centrifuged for a further 10 min at 13 000 x g and 4 ˚C. The supernatant was kept on ice until ready for further use. Next, 700 µl of reaction mixture was added to 300 µl supernatant and vortexed. The reaction mixture consisted of a 1:6 combination of 10 % (w/v) ascorbic acid and 0.42 % NH_4^+-molybdate.$4H_2O$ in 1N H_2SO_4. The samples were then incubated for 20 min at 45 ˚C or for 1 h at 37 ˚C after which the absorbance of the samples were measured at 820 nm using a dilution range of 1 mM KH_2PO_4 as standards.

4.3.3 Photosynthesis

The youngest fully expanded leaf for each plant was used for the photosynthetic determinations. Light-response curves were used to determine the irradiance (1200 $\mu mol.m^{-2}.s^{-1}$) at which to conduct the photosynthetic rates. The photosynthetic readings were taken at midday, using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA, England).

4.3.4 Root respiration

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

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

4.3.5 Cost and efficiency calculations

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

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

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

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

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

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

Specific P utilization rate (SPUR) (g dw.mg^{-1}P.d^{-1}) is a measure of the dw gained for the P taken up by the plant (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.

4.3.6 Calculations of δ15N

The δ15nitrogen (N) and carbon to nitrogen ratio (C:N) analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar *et*

al. (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5mm mesh (Arthur H Thomas, California, USA). Between 2.1mg and 2.2mg of each sample was weighed into 8mm by 5mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$.

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

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

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

4.3.7 Statistical analysis

The percentage data and ratio data were arcsine transformed prior to any statistical tests. The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (SuperAnova Software). Where the ANOVA revealed significant differences between treatments the means (n=4) were separated using a *post hoc* least significant difference (LSD) multiple range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

4.4 Results

The starvation of P for 20 days caused a decline in cellular P_i levels of nodules (Table 4.1) and this was interpreted as a P stress condition. The effect of this decline in metabolic P_i was evident in various physiological parameters in this study.

Table 4.1 Relative growth rates (mg.g^{-1} dry weight.day^{-1}), nitrogen fixation ($\delta^{15}N$) and C:N ratio, for *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 20 days after onset of P stress. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

Parameters	+ P 20 days		- P 20 days	
Nodule P_i (mmol.g^{-1} FW)	19.23	±2.11 **b**	11.67	±1.52 **a**
Dry mass (g)				
root	0.253	±0.017 **a**	0.417	±0.015 **b**
nodule	0.035	±0.001 **b**	0.021	±0.001 **a**
shoot	1.395	±0.020 **b**	0.934	±0.017 **a**
root:shoot	0.181	±0.010 **a**	0.447	±0.019 **b**

The biomass showed an alteration of growth, which is typical of P stress. In this regard, there was a increase in the root:shoot ratio and a decline in the total biomass of P stressed plants (Table 4.1). Nodule growth under P stress was lower than during P sufficient conditions (Table 4.1). Despite the reduced growth of nodules during P stress, BNF was increased (Table 4.3) under these conditions.

Table 4.2 Root-nodule respiration, maintenance and growth components of CO_2 respiration, nodule respiration and root-nodule construction costs of *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 20 days after onset of P stress. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

Parameters	+ P 20 days		- P 20 days	
Root-nodule CO2 release (mmol CO_2.g^{-1} dw.s^{-1})	175.93	±8.84 **b**	89.02	±9.56 **a**
Maintenance respiration (% of CO_2 release)	64.67	±8.35 **a**	36.42	±3.27 **b**
Growth respiration (% of CO_2 release)	35.33	±6.38 **a**	63.58	±4.82 **b**
Nodule O_2 uptake (mmol O_2 g^{-1} dw.s^{-1})	85.34	±7.34 **b**	62.23	±5.22 **a**
Root-nodule construction costs (mmol C .g^{-1} dw)	2645.23	±210 **a**	2875.56	±124 **a**

This increase in BNF under P deficiency occurred in spite of a lower energy status of these nodules as reflected in the decline in respiratory CO_2 flux from the root-nodule system (Table 4.2) and the nodule O_2 uptake rates (Table 4.2). Since the tissue

construction costs of nodules remained unchanged by P stress, the higher % of CO_2 flux devoted to growth respiration during P stress (Table 4.2) concurs with the increase in root growth. Consequently, with a greater proportion of respiratory energy (expressed as CO_2 flux) allocated to growth, there was a decline in the % maintenance respiration during P stress (Table 4.2). The lower % of CO_2 flux devoted to maintenance respiration during P stress (Table 4.2) is an indication of less energy being expended on nutrient acquisition.

Table 4.3 Nitrogen fixation, N and P utilization rates for *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 20 days after onset of P stress. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

Parameters	+ P 20 days			- P 20 days		
Nitrogen Fixation δ15N (‰)	-1.07	±0.12	**b**	-2.78	±0.73	**a**
Specific N utilisation (g dw.mg^{-1} N.d^{-1})	0.078	±0.003	**b**	0.056	±0.002	**a**
Specific P utilisation (g dw.mg^{-1}P.d^{-1})	0.041	±0.002	**b**	0.064	±0.004	**a**

This is in accordance with the lower rate of BNF and the specific N utilisation for growth during P stress (Table 4.3). Although the specific P utilisation rate for tissue growth during P stress had increased, this was not reflected in the maintenance respiration.

Table 4.4 Leaf photosynthesis, respiration and the ratio of photosynthesis to respiration for *Lupinus luteus* plants grown with 2 mM PO_4^{2-} (+ P) or 5 µM PO_4^{2-} (- P). Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 20 days after onset of P stress. Mean ± SE (n=4) are followed by letters indicating whether significant differences existed between treatments (ANOVA with post-hoc LSD tests).

Parameters	+ P 20 days			- P 20 days		
Photosynthesis (µmol CO2.m-1.s-1)	10.78	±2.45	**b**	6.42	±1.33	**a**
Leaf respiration (µmol CO2.m-1.s-1)	-1.34	±0.22	**a**	-2.67	±0.33	**b**
photosynthesis: respiration	8.04	±1.45	**b**	2.40	±0.98	**a**

For leaf gas exchange, the decline in the ratio of photosynthesis to leaf respiration during P stress, is an indication of less C gain for growth and nutrition (Table 4.4).

This was achieved by the proportionally greater increase in leaf respiration relative to the decline in photosynthesis (Table 4.4).

4.5 Discussion

The 20 days of P starvation was sufficient to induce a decline in cellular P_i levels in *Lupinus luteus* nodules. This is in agreement with previous studies where longer-term P starvation caused a reduction in nodular P levels for *Glycine max* (Sa and Israel 1991) and *Lupinus angustifolius* (Le Roux *et al.* 2006, 2008). The interaction between nodules and P supply may be complicated by the nodule's P requirement during BNF and ability to maintain its P levels during stress. For soybeans grown with fertiliser nitrogen, there is a lower P requirement than when nitrogen is obtained from BNF (Leidi and Rodríguez-Navarro 2000). Moreover, nodules can also take up P from the host, but do not readily release its P reserves to the host roots (Al-Niemi *et al*. 1998). This implies that nodules are strong sinks for P and this is supported by a report that nodules contain three-fold higher P concentrations compared to other tissues (Vadez *et al*. 1997). Furthermore, previous studies have shown that nodules may function optimally at low P_i concentrations (Al-Niemi *et al.* 1997, 1998, Le Roux *et al*. 2006). They also appear to have a mechanism whereby P influx can be regulated, allowing nodules to diminish the effect of P deficiency during periods of low P supply (Jakobsen 1985, Tang *et al.* 2001, Valentine *et al*. 2010). Therefore under conditions of P starvation, the decline in nodular P_i may not be an indication of P stress in nodules. However, in this study the decline in nodular P_i was associated with an increase in BNF and an alteration in respiratory and photosynthetic C metabolism.

For *Lupinus luteus* the reduction in plant growth and the increased root:shoot ratios during low P supply are typical responses for plants growing under P limitation (Rychter and Mikulska 1990, Juszczuk *et al.* 2001). Furthermore the reduced P supply may have inhibited the observed nodule growth, due to the high requirement for P in nodules (Graham and Rosas 1978). However, in some studies the unchanged number of nodules during low P supply was because the P starvation was induced only after nodule formation and indicates that no new nodules were formed during the period of P deficiency (Le Roux *et al*. 2006).

The increase in BNF during P deficiency may be the consequence of several influencing factors, during the period of decline in P levels. From physiological studies (Sa and Israel 1991, Al Niemi *et al*. 1997, 1998) it is apparent that bacteroids

in the nodule almost always operate at low P concentrations and that bacteroids can fulfil their P requirements by scavenging from the host cells (Al Niemi *et al*. 1997, 1998). Nodules may have a strategy to minimize effects of P deficiency when supply is low, by either regulating P influx or increasing the P-use efficiency for essential metabolic functions (Jakobsen 1985, Tang *et al*. 2001). Since BNF is carried out by bacteroids, this fraction of the nodule is known to consistently operate at low P concentrations by fulfilling their P requirements from scavenging the host cells (Al Niemi *et al*. 1997, 1998).

The increase in BNF does not concur with the reduction in root-nodule CO_2 release rates and nodule O_2 uptake rates. For CO_2 release, this is due to the gas-exchange measurements being a reflection of the root-nodule system and not representing nodules alone. In view of nodules increasing their BNF, it is possible that the high requirement for organic acids would have increased CO_2 refixation via PEPC (Le Roux *et al*. 2006, 2008). Furthermore, since the roots are more sensitive to perturbations in P-supply than nodules (Sa and Israel 1991, Al Niemi *et al*. 1997, 1998), it is therefore likely that the decrease in CO_2 release can be largely attributed to the roots.

A response of leaf respiration and photosynthesis to this decline in root-nodule sink activity during P stress appears to be the reduction in leaf C gain, as evidenced by the lower ratio of photosynthesis: leaf respiration. Furthermore, since the C construction costs of below-ground root-nodule tissues remained unaffected by P stress, the increase in root growth during P stress has caused a greater allocation of the below-ground CO_2 respiration flux toward growth respiration. This was at the expense of maintenance respiration, which indicates that less respiratory energy is allocated to nutrient acquisition of the root-nodule system during P stress. Since BNF has increased in nodules during P-stress, this is largely a reflection of a reduction in the root component of maintenance respiration.

4.6 Conclusion

The decline of nodular P_i levels in *L. luteus* was associated with a reduction the sink activity of the below-ground root-nodule system. In response to this, the proportionally lower allocation of below-ground respiratory energy towards maintenance respiration, suggests that less energy was spent on nutrient acquisition

during P stress. However, these changes represent the whole root-nodule system and may obscure interpretations regarding nodule sink capacity.

4.7 References

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CHAPTER 5

ISOLATION, SEQUENCING AND EXPRESSION ANALYSIS OF TWO PUTATIVE PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPc) ISOFORMS FROM *Lupinus luteus* NODULES UNDER P_i STRESS

5.1 Abstract

Phosphorus is an essential element obligatory for optimal plant growth. The concentration of available soil phosphorus is usually low, because it readily forms insoluble compounds with cations such as aluminium and iron, rendering this P unavailable for plant uptake. Therefore, phosphorus deficiency is a wide-spread agricultural dilemma. Phosphoenolpyruvate carboxylase (PEPc) plays a predominant role during phosphate deficiency, together with malate dehydrogenase and malic enzyme, as a metabolic bypass when ADP levels are limiting. The aim of this work was firstly to determine if the nodular PEPc isoforms were the products of different genes and secondly if these isoforms were differentially affected by P_i stress at the levels of transcription and translation. Two novel PEPc isoforms, *PEPC1* (AM235211) and *PEPC2* (AM237200), were isolated from *Lupinus luteus* (yellow lupine) var. Juno nodules. Transcriptional analyses using semi-quantitative PCR showed no differential expression of PEPc isoforms in P_i -stressed nodules. Nucleic acid and amino acid sequence alignments indicated higher sequence homology to nodular and C3 root isoforms than to C4 forms. Proteomic studies found more phoshorylated PEPc in P-starved nodules, which may have been due to more PEPc protein in P-starved nodules as a result of slower protein degradation.

Key words: phosphoenolpyruvate carboxylase, gene expression, phosphate deficiency, *Lupinus luteus*

5.2 Introduction

The concentration of available phosphorus (P) for plants is normally very low in soils, because most of the element combines with iron, aluminium and calcium to form relatively insoluble compounds (Aono *et al.* 2001). Inorganic phosphate (P_i)-deficiency is thought to be one of the limiting factors of nitrogen fixation (Aono *et al.* 2001) owing to the high energy requirement of plants engaged in nitrogen fixation for nitrogenase function (Al Niemi *et al.* 1997). During P_i -deficiency plants employ a variety of responses at morphological, physiological and metabolic level (Vance *et al*. 2003) as well as at the level of genetic control (Tesfaye *et al*. 2007) to obtain sufficient phosphorus. Many of these responses have been well documented in *Lupinus albus* (white lupin), a legume which develops proteoid or cluster roots in response to P_i stress (Gardner *et al.* 1983). These proteoid roots accumulate large amounts of organic acids such citrate and malate (Neumann *et al.* 1999), which upon secretion into the rhizosphere enables the release and subsequent uptake of mineral bound phosphates (Dinkelaker *et al*. 1989, Gardner *et al*. 1983) as well as release of phosphate from humic substances (Braum and Helmke 1995). *Lupinus luteus* (yellow lupin) plants, on the other hand, do not form cluster roots when growing in P_i -deficient conditions (Hocking and Jeffery 2004), but appear to utilize other strategies enabling it to take up normally unavailable P.

The reduction in intracellular levels of ATP, ADP and related nucleoside-Ps which follows a drastic decline in cytoplasmic P_i levels, as experienced during prolonged P deficiency, would inhibit carbon flux through the ATP-dependent glycolytic steps (Plaxton and Podesta 2006). However, P_i -deficient plants need to generate energy as well as carbon skeletons to maintain their core metabolic processes. To this end, a cluster of at least six adenylate-independent glycolytic “bypass” enzymes have been identified (Plaxton and Podesta 2006) with phosphoenolpyruvate carboxylase (PEPc) functioning as the bypass enzyme together with malate dehydrogenase (MDH) and malic enzyme (ME) for the reaction catalysed by ADP-limited cytosolic pyruvate kinase (PKc). The activity of this metabolic bypass during P_i stress when the ADP supply may be limiting for optimal PKc functioning would ensure continued pyruvate supply to the tricarboxylic acid cycle, while at the same time releasing P_i back into the metabolic pool (Duff *et al*. 1989, Plaxton, 2004). Several authors have reported an increase in PEPc activity for P_i -stressed samples compared to P_i -sufficient controls in *Brassica nigra* (Duff *et al*. 1989), *Brassica napus* (Nagano *et al*. 1994) and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000).

PEPc (EC 4.1.1.13) is a ubiquitous cytosolic enzyme found in vascular plants, green algae and bacteria, but is absent from animals, fungi and yeast. It is a CO_2-fixing enzyme responsible for the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- and Mg^{2+}, yielding oxaloacetate and P_i in the process (Chollet *et al.* 1996). In C_4 and CAM leaves, PEPc takes part in photosynthesis by catalyzing the initial fixation of atmospheric CO_2 (Latzko and Kelly 1983). The role of PEPc in C3 photosynthetic cells and non-photosynthetic tissue is primarily an anaplerotic one whereby the citric acid cycle intermediates that are utilized for energy and biosynthetic metabolism are replenished (Melzer and O'Leary 1987). This amount of carbon fixed by PEPc is significant and can provide up to 25 % of the carbon requirement of symbiotic nitrogen assimilation (Vance *et al*. 1983, Uhde-Stone *et al*. 2003). During P deficiency 25 – 34 % of this PEPc-derived C is excreted in the form of citrate and malate from proteoid roots of white lupin (Johnson *et al*. 1996).

Native plant PEPc usually occurs as a polypeptide made up of four identical subunits and ranges in size from 870 amino acid residues (100-kDa) in bacteria to 970 residues (110-kDa) in vascular plants up to 1010 (116-kDa) and 1150 (134-kDa) residues in cyanobacteria and protozoa, respectively (Izui *et al.* 2004). The bioinformatic analyses of *Arabidopsis*, rice and soybean genomes have led to the identification of several conserved PEPc genes in these species encoding 110-kDa polypeptides featuring plant-type PEPc (PTPC) characteristics eg. a N-terminal seryl-phosphorylation domain and a critical C-terminal tetrapeptide QNTG (Izui *et al*. 2004, Xu *et al*. 2006). Each of these genomes contain a bacterial-type PEPc (BTPC) gene which encodes a larger, approximately 116-kDa polypeptide which has low sequence identity with PTPCs and lacks the N-terminal phosphorylation site while containing a prokaryotic-like tetrapeptide at the C-terminus (Sánchez and Cejudo 2003, Gennidakis *et al*. 2007). The physiological role of these BTPCs have yet to be defined as their transcripts have been documented in various plant tissues but no functional enzyme has been characterized yet in vascular plants (Gennidakis *et al*. 2007).

PEPc plays a pivotal in primary plant metabolism and is therefore subject to fine metabolic control. This enzyme undergoes allosteric regulation with L-malate or asparagine acting as the inhibitors. In dicot plants, PEPc is activated by glucose-6-phosphate, while in monocot plants glycine or alanine functions as activators (Izui *et*

al. 2004). Furthermore, plant PEPc is also controlled by reversible phosphorylation which is catalysed by an endogenous Ca^{2+}-independent PEPc protein kinase (PPCK) while a protein phosphatase type 2A (PP2A) catalyses the dephosphorylation. This reversible phosphorylation takes place at a highly conserved N-terminal seryl residue (Chollet *et al*. 1996, Izui *et al*. 2004, Murmu and Plaxton 2007) and reduces sensitivity to malate inhibition while concurrently enhancing activation by glucose-6-phosphate.

Marczewski (1989) isolated three enzymatic forms of PEPc (PEPc I, PEPc II and PEPc III) from *L. luteus* nodules. Based on chromatography results it was concluded that PEPc I and PEPc II were nodule specific isoforms and PEPc III was root-specific. The aim of this study was to determine whether the two nodule specific isoforms were products of separate genes, or from the same gene, but undergoing different post-translational modifications as well as how changes in P supply would affect the expression of these two isoforms.

5.3 Materials and methods

5.3.1 Plant growth conditions

Seeds of *Lupinus luteus* var. Juno (BRF 21791, Agricol South Africa) were inoculated with Lupins and Serradella inoculant containing *Bradyrhizobium* sp (*Lupinus*) bacteria (Stimuplant, Zwavelpoort, South Africa) in vermiculite. Seeds were watered with distilled H_2O (dH_2O) and ca. 14 days after germination (first fully expanded leaf emergence) seedlings were transferred to 22 l hydroponic tanks containing a modified Long Ashton nutrient solution (Hewitt 1966) containing no nitrogen. Prior to transfer to hydroponic culture, the roots were rinsed in dH_2O and the hypocotyls of the plants were wrapped in black closed-cell foam rubber and inserted through collars in the lids of the hydroponic tanks. The tanks were completely opaque and contained eight plants each. Nutrient solutions were strongly aerated with ambient air (360 ppm CO_2). The nutrient solutions were changed weekly and the pH of the medium was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. Plants were grown in a temperature controlled (minimum 15°C, maximum 25°C) greenhouse at the University of Stellenbosch during winter (June, July and August).

Plants were grown on high P (2 mM) nutrient solution for ± 40 days after which the first set was harvested (this was the control treatment). The remaining plants were

then split into two separate treatments, one continued receiving 2 mM P (P-sufficient) nutrient solution, while the other set was grown on 5 µM P (P-stressed or low P) nutrient solution. The transfer of plants from a P-sufficient nutrient solution to a P-deficient nutrient solution was deemed the onset of P-starvation. Plants from both treatments were harvested 12 days after onset of P-starvation and again at 20 days. At each time point, four plants of each treatment were harvested and divided into roots, shoots and nodules and weighed. The nodules were quenched in liquid N_2 and stored at -80°C for later use in total RNA extraction.

5.3.2 Isolation of full-length cDNAs coding for phosphoenolpyruvate carboxylase

Total RNA was extracted from 200 mg frozen nodules using a 2 % CTAB buffer (White *et al*. 2008). Samples were run on a denaturing 1 % (w/v) agarose gel stained with ethidium bromide to visualize the quality of extracted RNA. These samples were treated with RNAse-free Dnase I (Invitrogen Corporation, Carlsbad, California, USA) for 1 h at 37°C to prevent amplification of genomic DNA in RT-PCR analyses. RNA samples were quantified spectrophotometrically using a ND-1000 spectrometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The SuperScript III kit (Invitrogen Corporation, Carlsbad, California, USA) was used according to the manufacturer's instructions to perform first strand cDNA synthesis.

Long-range PCR amplification (LR-PCR) of cDNAs putatively identified as LUP1 and LUP2 was carried out by designing LUP1-forward (5'- ATG GCG AAC AGG AAC TTG GAA A –3') and LUP1-reverse (5'- TTA ACC GGT GTT CTG AAG T – 3' toward the 5' UTR) as well as LUP2- forward (5'- ATG GCA AAC AAT AGG AAC ATG G-3') and LUP2-reverse (5'- TTA ACC AGT GTT CTG CAT G-3' toward the 5'- UTR). All primers used in this study were ordered from Integrated DNA Technologies, Inc., Coralville, Iowa, USA. These primers were designed based on the full-length PEPc cDNA sequences isolated from *Lupinus albus* proteoid roots (Peñaloza *et al*., 2005). These three PEPc sequences were annotated as LaPEPC2, LaPEPC3 and LaPEPC4 with ascession numbers AF459644, AY663386, AY663387 and AY663388, respectively (NCBI database).

LR-PCR reactions were performed with 5 µl cDNA template in a total volume of 50 µl containing 2 µl Buffer A, 8 µl Buffer B (Invitrogen Corporation, Carlsbad, California, USA), 1 µl dNTP (10 mM), 1 µl of each primer (LUP1-forward and LUP1-reverse, or LUP2-forward and LUP2-reverse), 1.5 µl Elongase Taq (Invitrogen) and 30.5 µl

dH_2O. PCR samples were denatured at 94°C for 30s followed by 40 cycles of amplification (94°C denaturation, 1 min; 45°C annealing, 1min; 68°C polymerization, 5min). All PCR reactions were carried out in a Perkin Elmer GeneAmp®Thermocycler 9700 (Perkin Elmer Corporation, Waltham, Massachusetts, USA). The resulting putative PEPc PCR fragments were separated on a 1 % (w/v) agarose gel stained with ethidium bromide, excised and cleaned up with a Qiagen: Gel Extraction Kit (QIAGEN Inc., Valencia, California, USA) after which the fragments were fluorometrically quantified.

5.3.3 Sequencing of the full-length putative PEPc genes

Two resulting amplicons of approximately 3 kb each were separated on a 1 % (w/v) agarose gel stained with ethidium bromide and excised. A QIAGEN: Quiaquick Gel Extraction Kit (QIAGEN Inc., Valencia, California, USA) was used to clean up the two cDNA fragments prior to fluorometric quantification. Subsequent to quantification, 50 ng and 40 ng of the respective LUP1 and LUP2 PCR fragments were ligated into the pGEM®-T Easy Vector system (Promega Corporation, Madison, Wisconsin, USA), followed by transformation in DH5α *Escherichia coli* competent cells (Hanahan 1983). Colonies were obtained for both isoforms and a master plate was made with 8 colonies of LUP1 and LUP2 each. A colony PCR was performed with the first five colonies selected for each isoform to confirm the presence of the LUP1 and LUP2 constructs in the pGEM®-T Easy Vector system. The colony PCR reactions were performed with 5 µl of cDNA in a total volume of 25 µl containing 2.5 µl 10x PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTP, 3 µl of 6 µM T7 or Sp6 primer, 1 µl of Bioline Taq (Bioline USA Inc., MA, USA) and 8.75 µl dH_2O. The samples were denatured at 94 ˚C for 3 min followed by 35 cycles of amplification (denaturation at 94 ˚C for 30 s, annealing at 50 ˚C for 30 s and polymerization at 72 ˚C for 90 s).

A Sigma GenElute Plasmid Miniprep Kit (Sigma-Aldrich Corporation, St Louis, Missouri, USA) was used to clean up the plasmids prior to fluorometric quantification. All sequencing reactions were performed on an ABI PRISM® 3100 genetic analyzer using AmpliTaq® DNA polymerase (Perkin-Elmer Life Sciences, Waltham, Massachusetts, USA) using 4 µl ABI-prism sequencing mix, 3 µl of 6 µM T7 or Sp6 primer OR 1 µl of specially designed sequencing primers, 2 µl of template plasmid and 1 µl of dH_2O in a final volume of 10 µl. Samples for sequencing were denatured at 94 ˚C for 5 min followed by 25 cycles of amplification (denaturation at 94 ˚C for 10 s, annealing at 50 ˚C for 10 s and polymerization at 60 ˚C for 4 min). Sequencing of

the LUP1 and LUP2 genes by LR- PCR was done by sequentially designing primers as represented in Table 5.1

Table 5.1 Primers designed based on ESTs of *pepc1* and *pepc2* in the pGEM-T easy vector system.

Primer name	Position on *pepc1*/*pepc2*	Sequence (5' → 3')
LUP1-FRW	1→24	ATGGCGAACAGGAACTTGGAAA
LUP1-REV	2903 → 2975	TTAACCGGTGTTCTGAAGT
LUP1-FRW-2	648 → 669	CAAGCTGCCTTCCGCACTGATG
LUP1-REV-2	2255 → 2234	AATATTCATCCGGCCATACTCC
LUP1-FRW-3	1307 → 1327	GAGGCAAGTCTCCACTTTCGG
LUP1-REV-3	1568 → 1545	GATATATGCTCCAAAGTTGTCTGG
LUP1-FRW-4	1827 → 1848	GCATGGCAGCTATATAAGGCTCAAG
LUP1-REV-4	1252 → 1226	GACCTATAACACAGTTCGAGAGGTTCC
LUP1-FRW-5	2541 → 2564	GATCTGTGGTCATTTGGGGAGCAG
LUP2-FRW	1 → 22	ATGGCAAACAATAGGAACATGG
LUP2-REV	2907 → 2889	TTAACCAGTGTTCTGCATG
LUP2-FRW-2	499 → 520	GATCTGGTTCTTACTGCTCATC
LUP2-REV-2	2282 → 2269	CTTCGCTTTGCTGGTCGACTTC
LUP2-FRW-3	1120 → 1143	GAAGTAAGGGACAGACTCTATCAG
LUP2-FRW-4	1826 → 1850	CTGCGGCATGGCAGTTATACAAG

5.3.4 Semi-quantitative reverse transcriptase – PCR analysis

RT-PCR was carried out with primers specifically designed to distinguish and amplify shorter regions of the 2 PEPc isoforms, LUP1 (424 bp) and LUP2 (300 bp). Primers for isoform LUP1 were forward 5'-GGC AGC ATT CAG ACA AGT TAT TAC-3' and reverse 5'- CGG CAG CTT TAC TTA TCT CTA TAC AC-3'. Primers for isoform LUP2 were forward 5'- AGA CCC AGG AAT AGC GGC TCT GA-3' and reverse 5'- TAG CTG ATG TAC TTT TCT CCA ATG AG-3'. Cyclophilin was used as house-keeping gene and primers were specifically designed to distinquish and amplify a region of 297 bp based on *Lupinus luteus* mRNA for cyclophilin (Y16088, NCBI database). The primers for cyclophilin were cyclo-FRW 5'- CAT CGT CAT GGA GCT GTA CGC C-3' and Cyclo-REV 5'- CTG AGA TCC ATT GGT TCC AGC TCC-3'.

Three µg total RNA of root nodules were treated with Dnase I according to manufacturer's protocol (Fermentas Inc., Glen Burnie, Maryland, USA). First strand CDNA was synthesized using the SuperScript^{TM}III kit (Invitrogen Corporation, Carlsbad, California, USA). Semi-quantitative RT-PCR reactions were performed in a final volume of 25 µl containing 1 µl cDNA template, 2.5 µl 10x PCR buffer, 0.75 µl $MgCl_2$ (50 mM), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.5 µl Bioline Taq and 18.75 µl dH_2O. RT-PCR amplification of partial isoform regions included a predenaturation step at 94 °C for 30 s then 30 cycles of amplification (94

℃ denaturation, 30 s; 60 ℃ annealing, 30 s; 72 ℃ polymerization, 30 s). PCR reactions were repeated with cyclophilin primers as “house-keeping” control and PCR products were visualized in ethidium-bromide stained 1 % (w/v) agarose gels. Spot densitometry was conducted from the Integrated Density Value (IDV)-score of each PCR fragment and the “Auto Background” option was selected using AlphaEase™ image analysis software (AlphaInnotech, San Leandro, California, USA).

5.3.5 Protein extraction and 2D gel analysis

Freeze-dried nodules were ground in a pre-chilled mortar and pestle using liquid nitrogen and incubated thereafter in chilled 10 % (w/v) TCA and 0.07 % (v/v) β-mercaptoethanol for at least 45 min at -20 ˚C (Natarajan *et al.* 2005). This protein extract was centrifuged at 14 000 x g and 4 ˚C for 10 min and the supernatant discarded. The resulting pellet was washed with 90 % acetone containing 0.07 % β-mercaptoethanol and centrifuged at 14 000 g and 4 ˚C for 10 min. This step was repeated a total of three times to remove all traces of TCA. After the wash steps the pellets were air dried to remove acetone residues before resuspension in resolubilisation buffer (8 M urea, 4 % CHAPS, 20 mM DTT, 0.5 % ampholytes plus 0.001 % bromophenol blue) and sonicated to facilitate protein extraction. Insoluble material was pelleted out by centrifugation at 14 000 x g and 10 ˚C for 5 min. The Bradford assay was used to determine protein concentration with bovine serum albumin as standard (Bradford 1976).

Following first dimension electrophoresis, the immobilised pH gradient strips were denatured with 6 M urea, 75 mM Tris (pH 8.8), 2 % (w/v) SDS, 20 % (v/v) glycerol and 100 mM DTT for 10 min and afterwards incubated in 6 M urea, 75 mM Tris (pH 8.8), 2 % (w/v) SDS, 20 % (v/v) glycerol and 135 mM iodoacetamide for another 10 min. After the second dimension electrophoresis the gels were stained overnight with 1 $g.l^{-1}$ CBB R-250 in 40 % (v/v) MeOH and 5 % (v/v) acetic acid. Subsequent to overnight staining, the gels were destained using 40 % (v/v) MeOH and 5 % (v/v) acetic acid. The protein blots were transferred to nitrocellulose membranes for immuno-blotting to determine the phosphorylated and de-phosphorylated states of PEPc according to Tripodi *et al.* (2005). Two antibodies were used for immuno-blotting, one was specific to the phosphorylated site of the PEPc enzyme and the other was specific to the non-phosphorylated site of PEPc.

5.3.6 Sequence analyses

The programs BLAST (Altschul *et al*. 1990 and 1997) and CLUSTAL W (Higgins *et al.* 1994) were used for all similarity searches and alignment analyses between sequences.

5.4 Results and Discussion

5.4.1 Identification of full-length cDNAs by sequence homology

Primers were designed against consensus sequences constructed from three phosphoenolpyruvate carboxylases isolated from *Lupinus luteus*, namely, *LaPEPC2*, *LaPEPC3* and *LaPEPC4* (accession numbers AY663386, AY663387 and AY663388, respectively). These primers enabled the amplification of two full-length cDNA's of approximately 3 kb each from RNA isolated from lupin nodules (Fig 5.1).

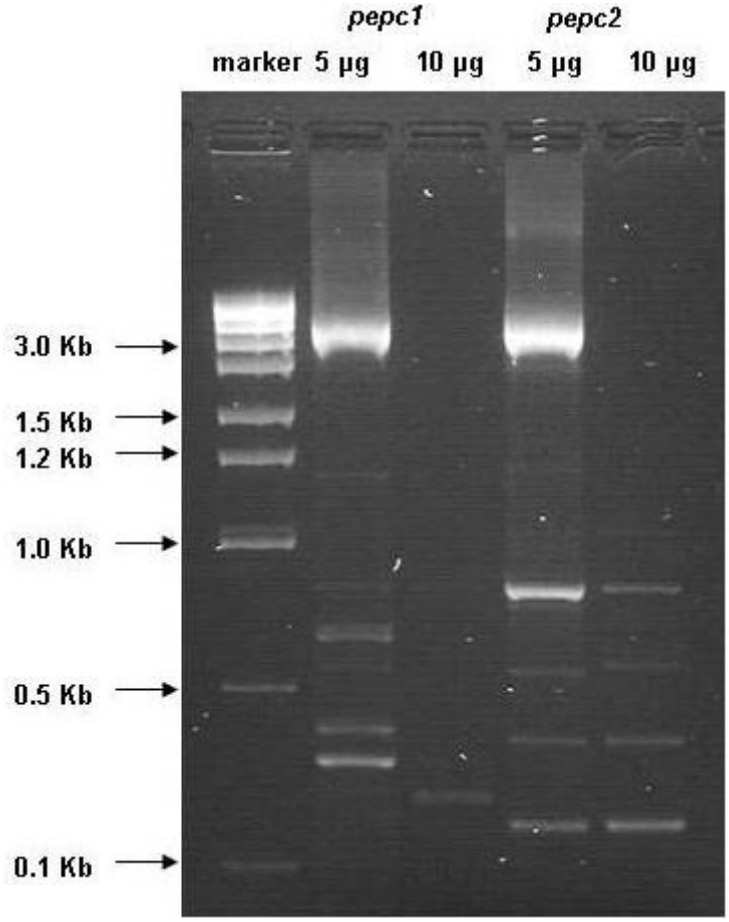

Figure 5.1 Amplification of full-length cDNA's of putative *PEPC1* (approximately 3 Kb) and *PEPC2* isoforms (approximately 3 Kb each) from single stranded cDNA. The marker is the 2-log DNA marker (New England BioLabs #N3200S).

These two putative PEPc isoforms were denoted *PEPC1* and *PEPC2.* Subsequent sequencing of *pepc1* and *pepc2* indicated an open reading frame of 2904 bp and 2907 bp, respectively and translated to proteins of 967 and 968 amino acids each. At both nucleotide and amino acid level there were high levels of sequence identity (>86 %) between the putative *L. luteus* PEPc sequences and the confirmed *L. albus* sequences (Table 5.2). Therefore, it was assumed that these two fragments represented PEPc in *Lupinus luteus*. *Pepc1* showed the highest percentage nucleotide and amino acid identity with *LaPEPc3* and *LaPEPc4* from *L. albus* (>90 %), whereas *pepc2* showed the highest percentage sequence identity with *LaPEPc1* and *LaPEPc2* (>90 %) (Table 5.2). Both *pepc1* and *pepc2* showed the highest percentage identity (> 83 %) at nucleotide and amino acid level with PEPcs from *L. albus*, *Medicago sativa*, *Glycine max* (plant-type PEPc) and *Pisum sativum*, which are all legumes. Comparing *pepc1* and *pepc2* with the bacterial-type PEPc from *Arabidopsis thaliana* and *G. max* showed very low percentage identity (<40 %). The percentage identity of *pepc1* and *pepc2* with C_3 (*A. thaliana* and *Oryza sativa*) and C_4 grasses (*Saccharum officinarum* and *Zea mays*) were lower than that of legume PEPcs, but not as low as the bacterial-type PEPc, which further supports the assumption that these two genes and translated proteins are plant-type PEPcs. These results are supported by phylogenetic analysis of the PEPc gene family which have shown that bacterial and plant PEPc form two different subgroups in the gene family with C_3 and C_4 PEPc forming two branches in the plant PEPc subgroup (Gehrig *et al*. 1998, Sánchez and Cejudo 2003).

Table 5.2 Percentage sequence homology of two novel *Lupinus luteus* phosphoenolpyruvate carboxylase sequences with that of phosphoenolpyruvate carboxylases from several other species. Accession numbers are given in brackets for each species. Sequence alignments for both nucleotide and amino acid similarity were carried out using ClustalW multiple alignment.

	% Nucleotide base identity		% Amino acid residue identity	
	pepc1	***pepc2***	***pepc1***	***pepc2***
Arabidopsis thaliana ppc1 **(AJ532901)**	76	77	86	82
A. thaliana ppc2 **(AJ532902)**	73	74	83	81
A. thaliana ppc3 **(AF071788)**	76	77	83	86
A. thaliana ppc4 **(AJ532903) (BTPC)**	32	29	40	39
Glycine max **PEPc17 (AY563043) (BTPC)**	30	30	38	39
G. max **PEPc4 (AY563044)**	78	78	84	86
Lupinus albus **PEPc1 (AF459644)**	86	95	94	89
L. albus **PEPc2 (AY663386)**	87	96	87	97
L. albus **PEPc3 (AY663387)**	92	87	94	89
L. albus **PEPc4 (AY663388)**	96	87	95	88
Medicago sativa **(M83086)**	86	84	89	89
Oryza sativa **(AY187619)**	74	75	81	85
pepc1 **(AM235211)**		87		87
pepc2 **(AM237200)**	87		87	
Pisum sativum **(D64037)**	85	83	88	87
Saccharum officinarum **(AJ93346)**	66	66	73	74
Zea mays Z561 **(FJ415327)**	67	67	75	76

An important distinction between bacterial and plant PEPc proteins is the presence of a serine residue at the N terminal which is the catalytic site for reversible phosphorylation by PEPc kinases (Vidal and Chollet 1997), a key regulatory mechanism in carbon metabolism. Plant PEPc also has a strictly conserved C-terminal tetrapeptide (QNTG) which plays an important role in catalysis and negative allosteric regulation (Xu *et al*. 2006). In bacteria and archaeal type PEPc this C-terminal is not as tightly conserved. Both *pepc1* and *pepc2* contain the serine residue as well as the highly conserved QNTG plant motif at the C terminal of the translated protein (Appendices 5.1 and 5.2). These findings, together with the higher level of identity of other plant PEPc enzymes provide strong evidence that *pepc1* and *pepc2* encode for plant-type PEPcs.

5.4.2 Transcript expression of PEPc in nodules

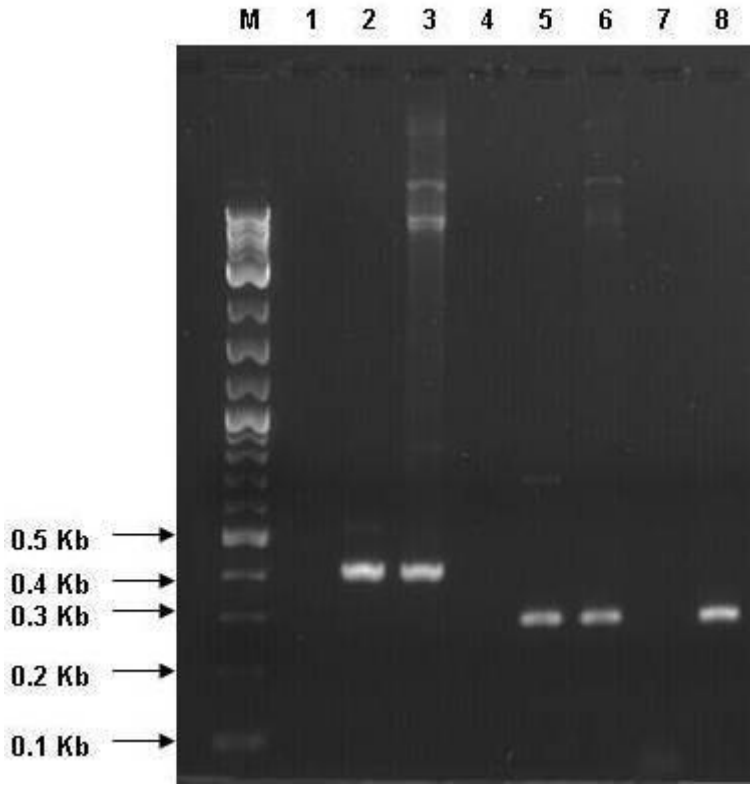

Figure 5.2 Analysis of the specificity of the primers used in the semi-quantitative RT-PCR experiments. M is the 2-log DNA marker (New England BioLabs #N3200S). Lanes 1, 4 and 7 contained dH_2O as a negative control with LUP1, LUP2 and cyclophilin primer pairs, respectively. Lane 2 indicated cDNA fragments amplified by the LUP1 primer pair and lane 3 showed cDNA amplified from the pGEM-Teasy plasmid containing *pepc1* with the LUP1 primer pair as positive control. Lane 5 indicated cDNA fragments amplified by the LUP2 primer pair and lane 6 showed cDNA amplified from the pGEM-Teasy plasmid containing *pepc2* with the LUP2 primer pair as positive control. Lane 8 contained cDNA fragments amplified with the *cyclophilin* primer pair.

Prior to transcript analysis, the specificity of the different primer pairs were tested to ensure that no cross amplification would take place. The specificity of these different primer pairs were shown by amplification of the pGEM-T easy plasmid containing *pepc1* and *pepc2*, respectively, as well as amplifying fragments of the expected size for identification of *pepc1* (424 bp), *pepc2* (300 bp) and *cyclophilin* (297 bp) (Fig 5.2). Primers were designed to amplify less conserved exon regions and therefore it was assumed that these PCR products were three unique cDNAs, because no cross amplification was seen between the gene-specific primer pairs.

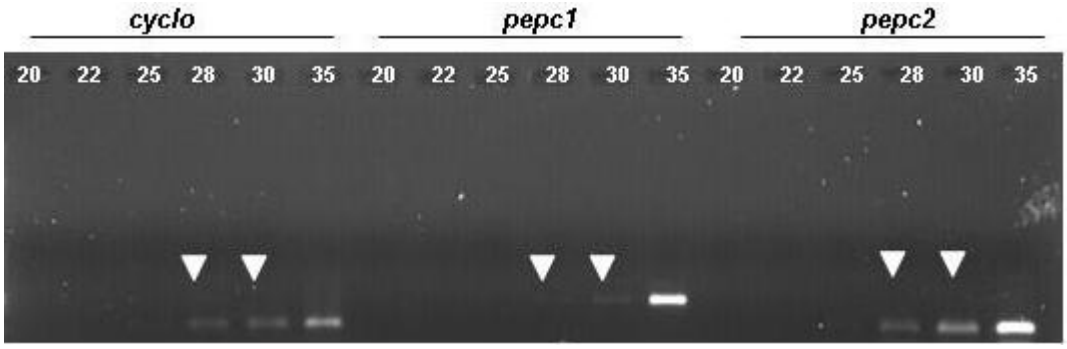

Figure 5.3 An ethidium-bromide stained gel of *cyclo*, *pepc1* and *pepc2* transcripts amplified by semi-quantitative RT-PCR using gene-specific primers. Arrows indicate the cycle number resulting in a similarly intense amplification product. Cyclophilin transcript serves as the internal control.

Transcript analysis showed amplification in the linear range from cycles 28 and 30 (Fig 5.3) and further semi-quantitative RT-PCR analysis were carried out using 30 cycles of amplification for *pepc1*, *pepc2* and *cyclo*.

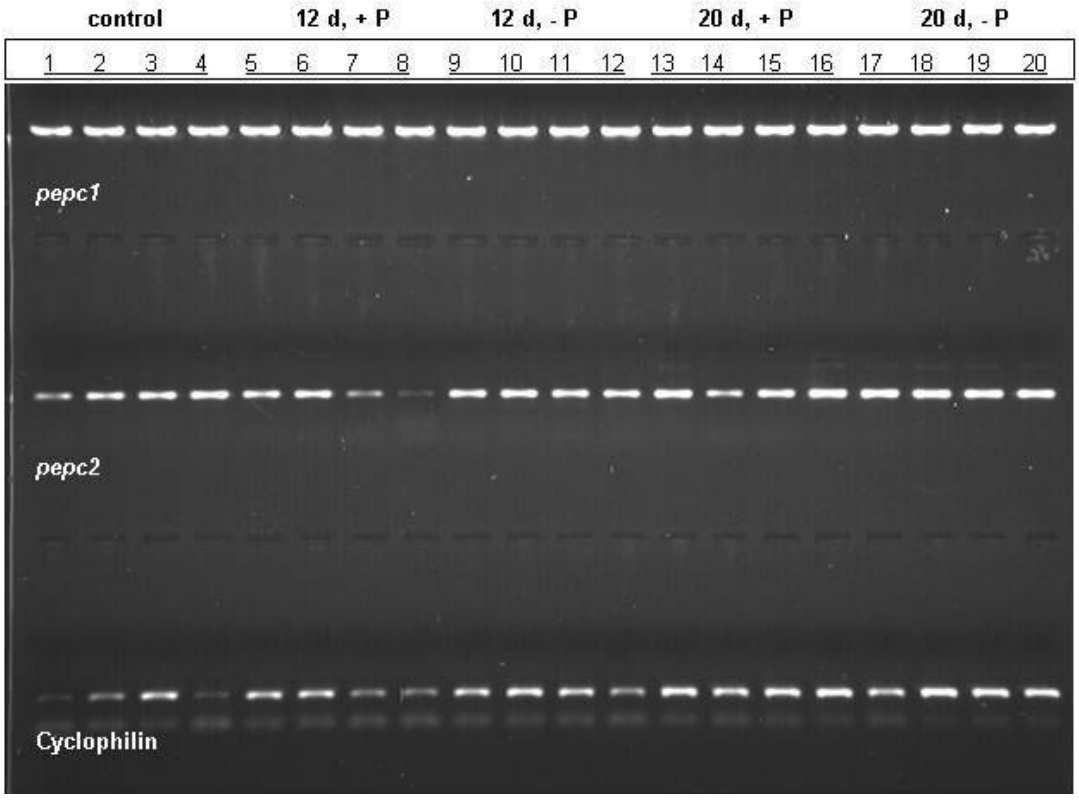

Figure 5.4 Ethidium-bromide stained gel of semi-quantitative RT-PCR analysis of *pepc1* and *pepc2* transcripts in *Lupinus luteus* nodules. Gene specific primers were used for each cDNA. *Cyclophilin* transcript serves as the internal control. Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days.

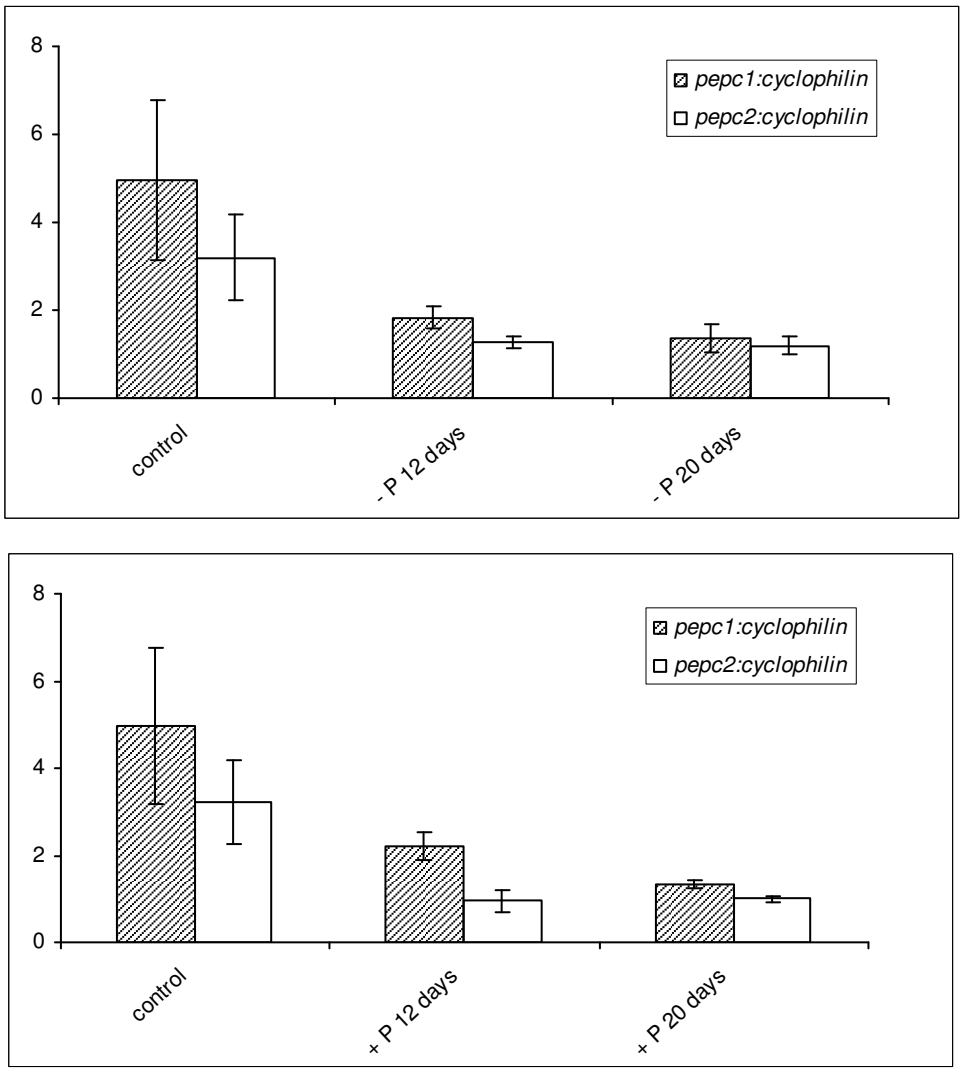

Figure 5.5 Comparison of expression ratios of *pepc1* and *pepc2* to *cyclophilin*. Plants were grown on 2 mM PO_4^{2-} for ± 40 days (control) after which the treatments were initiated. Plants were harvested 12 days after onset of treatments and again at 20 days. Error bars denote the standard error (n=4).

Semi-quantitative RT-PCR was used to distinguish between the different PEPc genes and to detect transcripts with low abundance. Based on spot densitometry values (Fig 5.5) these results showed a decrease in expression of both *pepc1* and *pepc2* at 12 and 20 days for both P-stressed and P-sufficient plants compared to the control set. This may have been due to nodule senescence, because at this stage the lupin plants had started flowering. No significant differences were found in expression of *pepc1* or *pepc2* after 12 and 20 days for both P-deficient and P-sufficient plants. These findings are in contrast with that of several other authors who have found an increase in PEPc gene transcripts in response to P_i deficiency in various species such as *Arabidopsis*, white lupin and tobacco (Gregory *et al*. 2009, Peňaloza *et al.* 2005, Toyota *et al*. 2003). A possible explanation could be that 20

days of phosphate starvation may not have been long enough to have an effect on the vacuolar stores of phosphate reported by Schachtman *et al*. (1998). Toyota *et al*. (2003) found that tobacco plants first responded to P_i deficiency by inducing P_i transporters and acid phosphatases to enhance P_i uptake by roots. The induction of PEPc was a second step in adaptation to P deficiency.

5.4.3 Protein expression of PEPc in nodules

Plant PEPc is controlled by reversible phosphorylation catalysed by a PEPc protein kinase (PPCK) while a protein phosphatase catalyses the dephosphorylation of this protein.

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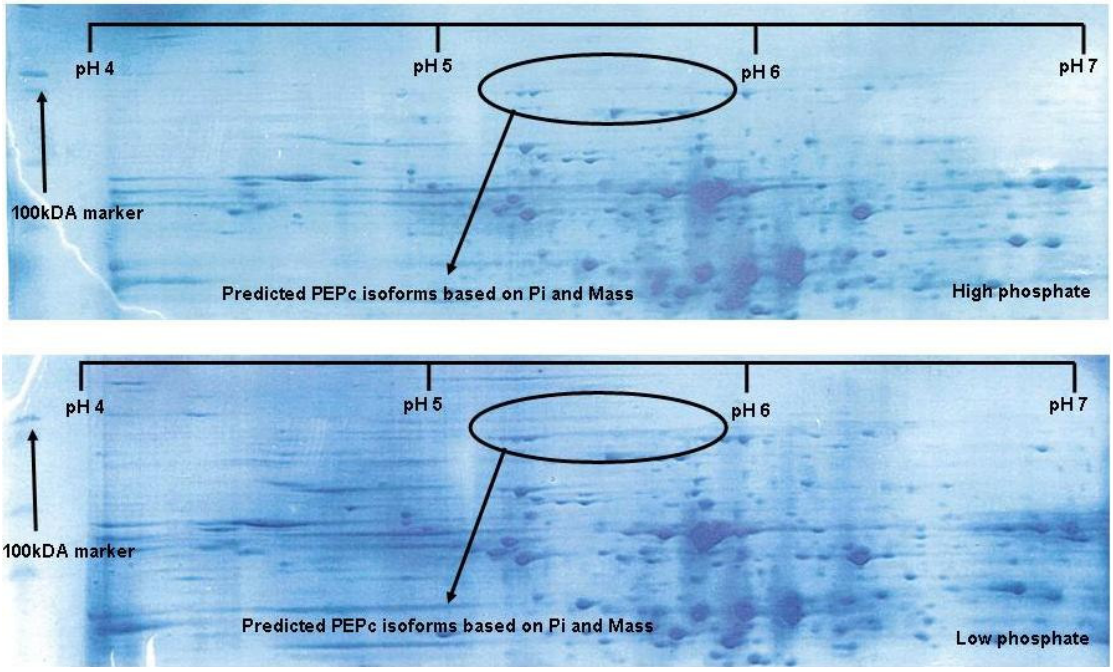

Figure 5.6 Predicted positions of phosphoenolpyruvate carboxylase (PEPc) isoforms based on the iso-electric points (Pi) and mass (kDa) of the two isoforms (LUP1 and LUP2) of nodules grown at high (HP) and low phosphate (LP).For PEPc-1 the Pi = 5.77 and mass =110.820 kDA. For PEPc-2 the Pi = 5.85 and mass =110.684 kDA.

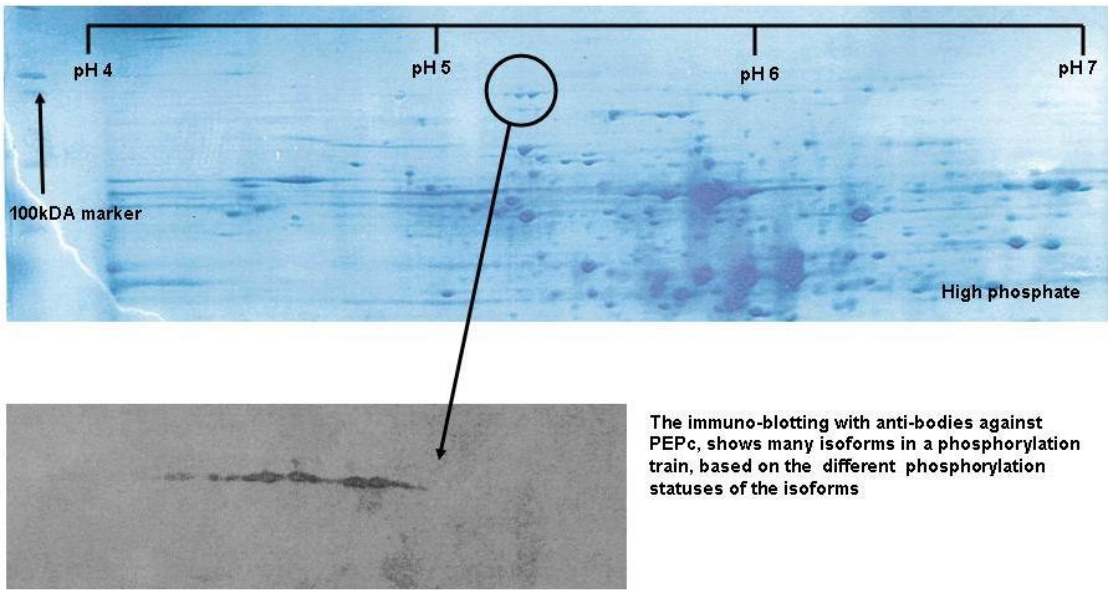

Figure 5.7 The predicted region of phosphoenolpyruvate carboxylase (PEPc) localisation in the 2D gel (based on the iso-electric points and mass of the two PEPc isoforms), was excised and subjected to immuno-probing with anti-bodies (courtesy of Prof Bill Plaxton, Queens University, Canada) against PEPc, extracted from nodules grown under high phosphate (2 mM).

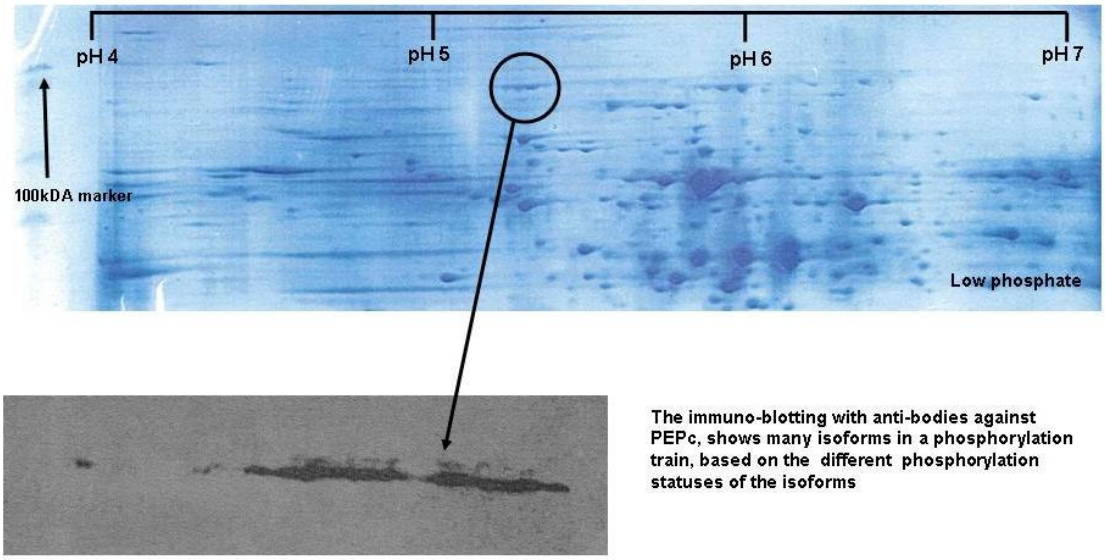

Figure 5.8 The predicted region of phosphoenolpyruvate carboxylase (PEPc) localisation in the 2D gel (based on the iso-electric points and mass of the two PEPc isoforms), was excised and subjected to immuno-probing with anti-bodies (courtesy of Prof Bill Plaxton, Queens University, Canada) against PEPc, extracted from nodules grown under low phosphate (2 µM).

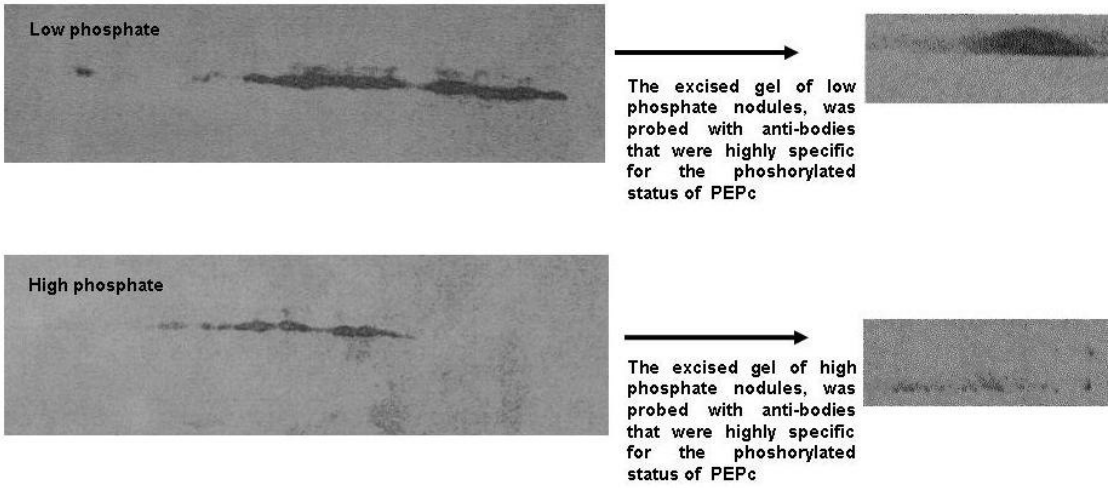

Figure 5.9 Immuno-probing with anti-bodies against phosphoenolpyruvate carboxylase (PEPc) nodules grown at low and high phosphate nutrition. The predicted region of PEPc localisation in the 2D gel (based on the iso-electric points and mass of the two PEPc isoforms), was excised and probed with monoclonal anti-bodies (courtesy of Prof Bill Plaxton, Queens University, Canada). The monoclonal anti-bodies were specific for PEPc, as tested against an extract of purified PEPc protein (courtesy of Prof BillPlaxton, Queens University, Canada) (data not shown). The monoclonal antibodies were stripped from the excised gel and probed with highly specific anti-bodies for the phosphorylated status of the highly-conserved phoshorylation-site of PEPc site at the N-terminus (courtesy of Prof Bill Plaxton, Queens University, Canada).

Since the two PEPc isoforms could not be distinguished by the two dimensional seperation and the subsequent immuno-probing, they will be referred to as the PEPc isoforms in the following discussions. The 2D-gels of nodules grown at low P and high P indicate a region between pH 5-6 and at approximately 100 kDa, where the predicted PEPc isoforms are located (Fig 5.6). This region was excised from a duplicate 2D-gel (which was not stained with Coomasie-blue) and probed with monoclonal PEPc antibodies, to detect the presence of PEPc protein in nodules grown at high P (Fig 5.7) and grown at low P (Fig 5.8).

A comparison between the immuno-blots of low P nodules and high P nodules, indicate more pronounced immuno-reaction against PEPc in nodules grown at low phosphate than at high phosphate. This may be indicative of more PEPc protein at low phosphate, due to either an increase in expression or decreased breakdown (Fig 5.9). The immuno-probing with phosphorylation-specific anti-bodies show that PEPc is more phosphorylated in nodules grown at low phosphate compared to high phosphate. This difference in phosphorylation status can be affected by the concentration of PEPc protein, and therefore the higher levels of PEPc protein at low phosphate may have influenced the phosphorylation status (Fig 5.9).

These data indicate that during P starvation, the nodular PEPc enzyme isoforms may not be regulated by gene expression, but post-translationally either by phosphorylation or protein degradation. During P-starvation, the accumulation of more PEPc protein may be a result of reduced degradation, because there was no difference in the expression of the PEPc isoforms. Furthermore, it appears that there is more phoshorylation of PEPc in P-starved nodules, but this interpretation may be confounded by the fact that there was more PEPc protein in P-starved nodules.

5.5 Conclusion

The two isolated PEPc isoforms appear to be the product of different genes and the expression of these two genes appear to be unaffected by P stress. The two nodular PEPc isoforms showed the highest sequence homology with other legume plant PEPc's which indicated evolutionary conservation between plant species. Higher levels of phoshorylated PEPc was found in P-starved nodules, this may have been due to more PEPc protein in P-starved nodules due to slower protein degradation.

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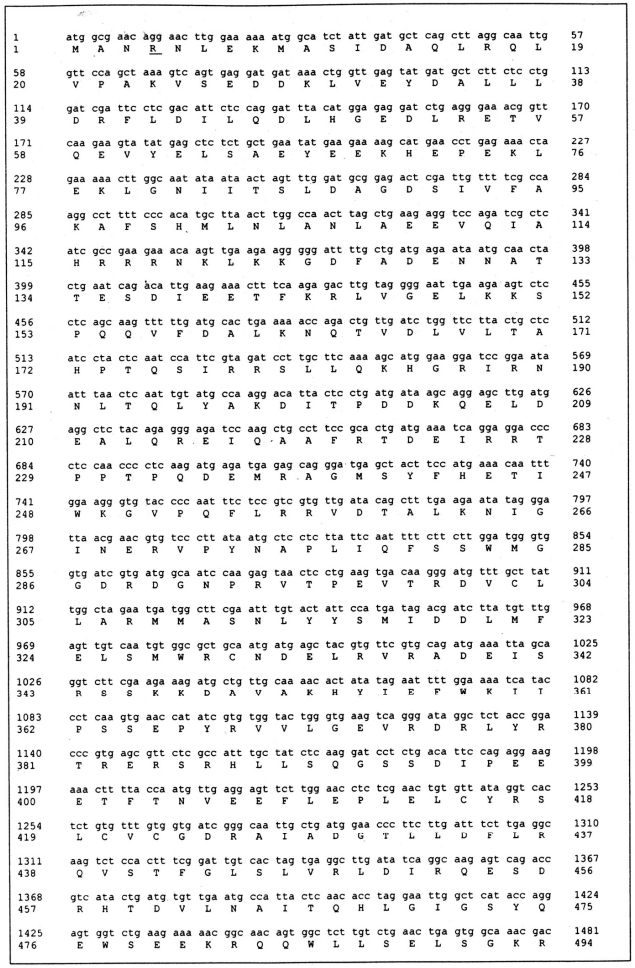
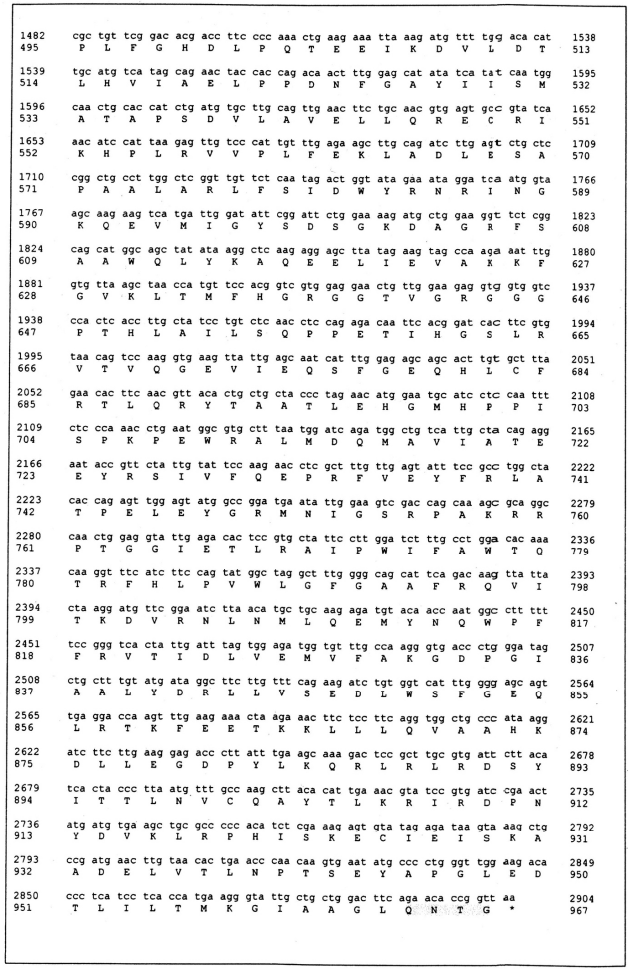
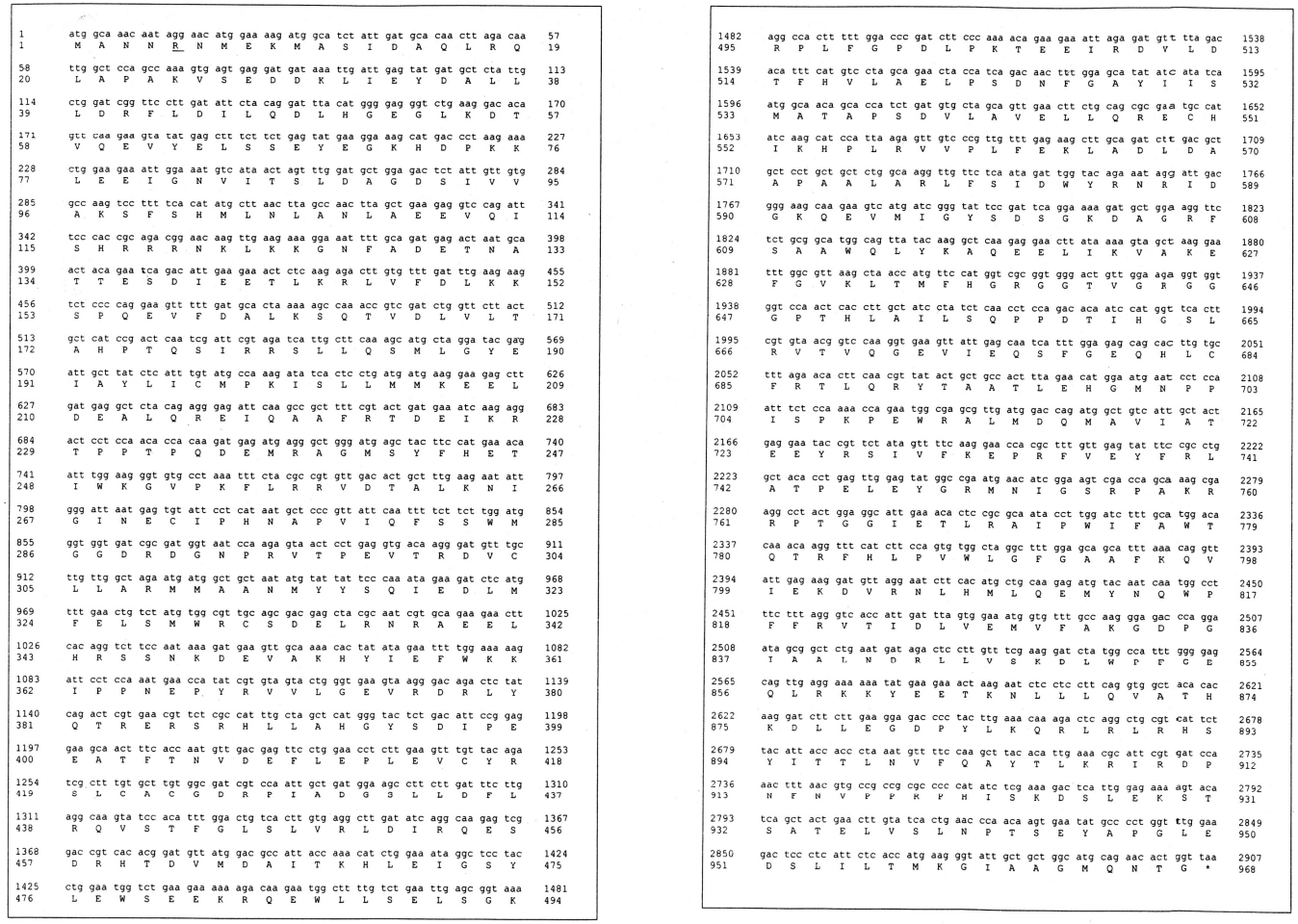




Appendix 5.1 Nucleotide and amino acid sequence of a novel full-length Lupinus luteus cDNA with a putative identity as phosphoenolpyruvate carboxylase (*pepc1*/LUP1)



Appendix 5.2 Nucleotide and amino acid sequence of a novel full-length *Lupinus luteus* cDNA with a putative identity as phosphoenolpyruvate carboxylase (*pepc2*/LUP2)

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

In many regions of the world grain legumes are the only supply of protein in the diet (Duranti and Gius 1997), because of the high price of animal protein. Smallholder farmers in southern Africa usually apply less inorganic phosphorus fertilizer (P_i, as inorganic mineral salts) due to capital and infrastructure constraints (Vance *et al*. 2003), than what is removed from the soil during harvesting thereby depleting soil P reserves (Holford 1998). P_i -deficiency is thought to be one of the limiting factors of nitrogen fixation (Aono *et al.* 2001) owing to the high energy requirement of plants engaged in nitrogen fixation for nitrogenase function (Al Niemi *et al.* 1997). Modification of the host component of the nodule could lead to improved seed protein content, which would be a great advantage to those who depend on these seeds as their primary source of protein. This study focused on the effect of P stress on organic and amino acid synthesis (chapter 3), the effect of P stress on the biological nitrogen fixation in terms of photosynthetic and respiratory carbon metabolism (chapter 4), and the organic acid supply for nodule metabolism as well as the response of nodules to P stress via the isoforms of PEPC (chapter 5).

6.2 The response of phosphate-starved *Lupinus luteus* nodules

This study found that during prolonged P stress, the decline in P_i levels was associated with a lower growth rate and an increase in the root: shoot ratio, which is a typical stress response in plants (Rychter and Mikulska 1990). The lower requirement for total growth was not reflected in the biological nitrogen fixation (BNF), since growth may have been limited by C supply from photosynthesis and not N from BNF. It is therefore conceivable that the decline in photosynthetic C supply would have limited the C required to utilize the products of BNF for growth. Although carbon metabolism via photosynthesis and root respiration was also reduced, the adaptation to P stress was in the components of root respiration, because there was a greater proportion of respiration devoted to nutrient acquisition than to new growth. This shift away from new growth was an indicator of all the non-growth resources required for the acquisition of nutrients such as phosphate.

The increase in BNF is supported by previous studies (Sa and Israel 1991, Al Niemi *et al*. 1997, 1998) showing that bacteroids in the nodule almost always operate at low P concentrations and that bacteroids can fulfil their P requirements by scavenging from the host cells (Al Niemi *et al*. 1997, 1998). Furthermore, it has been proposed that nodules have a strategy to minimize effects of P deficiency when supply is low, by either regulating P influx or increasing the P-use efficiency for essential metabolic functions (Jakobsen 1985, Tang *et al*. 2001, Colebatch *et al*. 2004).

Our finding of an increase in BNF with *Lupinus luteus* contradicts the work of Le Roux *et al.* (2008) who reported a reduction in nitrogen fixation using *Lupinus angustifolius*. However, these authors limited the P-stress exposure of plants to 14 days, whereas in the current study P starvation was measured up to 20 days. Le Roux *et al.* (2009) used the acetylene reduction activity of nitrogenase as an indication of nitrogen fixation, whilst this study used stable isotope discrimination. Another study by Le Roux *et al.* (2009) on *Lupinus angustifolius* and *Glycine max,* reported no change in the root: shoot ratio of P-stressed lupin plants despite a decrease in nodule total P concentrations as well as no change in the percentage of nitrogen derived from the atmosphere. The reason for these contrasting findings may be the length of exposure to P stress.

The increase in BNF and root growth during P stress reported in this study was associated with an accumulation of key export amino acids, but also with unchanged levels of organic acids which serves as the primary metabolic fuel for nodules. These organic acids, may also serve in exudation for P-acquisition from the rhizosphere. Le Roux *et al.* (2006) reported no change in total amino acids or organic acids during P stress, but this may have been due to the short time of P-stress exposure.

The two isolated PEPc isoforms appeared to be the product of different genes and the two nodular PEPc isoforms showed the highest sequence homology with other legume plant PEPc's, which indicated evolutionary conservation between plant species. The molecular characterization of the two PEPc isoforms confirmed the lack of the PEPc-malate dehydrogenase (MDH)-malic enzyme (ME) glycolytic bypass operation, by showing that its primary enzyme, PEPc and its isoforms, are not regulated by gene expression or phosphorylation under P-stress. Higher levels of phosphorylated PEPc were found in P-starved nodules, but this may have been due to more PEPc protein in P-starved nodules due to slower protein degradation. This indicated that other post-translational modifications may be more important.

The lack of engagement of the PEPc bypass in *Lupinus luteus* nodules after 20 days of 5 µM P stress may not have been long enough and/or severe enough to induce the bypass or may have been because these plants employ other methods of surviving P stress. The decline in BNF and growth concurs with the finding of other authors, but the constant organic acid levels suggests that there might be other factors to consider in *Lupinus luteus* when studying P stress. Hocking and Jeffery (2004) mentioned that *L. luteus* appears to have some mechanism enabling it to access soil P usually unavailable to other crops.

6.3 Future studies

The current research has highlighted the importance of investigating the P stress responses of legumes at the levels of plant physiology, biochemistry and molecular biology. Although these functional levels were investigated separately in this current study, a more integrated approach may be of much greater insight into the responses to P stress. However, the value of having investigated the functional levels separately is that many questions still abound regarding the adaptation to P stress. The more pertinent of these questions, which were raised during this study, are discussed in below.

At the biochemical level, several other plant adaptations to P stress exist (Vance *et al.* 2003) and need to be investigated. For example, the two other glycolytic bypasses, a PP_i -dependant phosphofructokinase (PFP) bypassing the reaction of the ATP-dependant phosphofructokinase and a nonphosphorylating NADP-dependant glyceraldehyde-3P dehydrogenase (NADP-G3PDH) as an alternative to P_i -dependant NAD-G3PDH and phosphoglycerate kinase (Plaxton and Podesta 2006), will have to be studied. The investigation of these alternate glycolytic bypasses would utilize a range of metabolic biochemical techniques to study enzyme regulation of these pathways.

At the physiological level, whole organ respiratory gas exhange has been carried out in many studies using either oxygen or carbon dioxide fluxes. However, the possibility of engaging alternate pathways of respiration during P stress, may also represent an elegant adaptation of nodules. Such pathways that have a low P_i requirement because of the existence of limited adenylate synthesis (rotenone- and/or cyanide insensitive pathways) in the mitochondrial electron transport system

have been studied to a limited degree (Rychter and Mikulska 1990, Rychter *et al.* 1992, Gonzaléz-Meler *et al.* 2001, Juszczuk et al. 2001, Plaxton and Podesta 2006), but should receive fresh attention, using the suite of modern genomics tools available.

At the molecular biological level, this study has only addressed the regulation of one enzyme, PEPc, which is considered to be pivotal to BNF and P stress responses. However, further work should investigate the post-translational regulation of PEPc via phosphorylation control. In addition to PEPC, other genomic regulation may also be revealed via expression of genes coding for transcription factors, small RNA's, and enzymes that function to recycle and remobilize sparsely available P within and outside the nodules during P stress. In the absence of a micro-array platform for lupins, the use of qRT-PCR or RNA-seq may be appropriate. However, the use of model legumes would facilitate these studies to a much greater degree. In this regard, the use of model legumes such as *Medicago truncatula* and *Glycine max* may serve as the best platforms for integrating various functional levels in a systems biology approach.

Since the suite of adaptations may involve a network of interactions among the levels of functional organization, such as whole plant physiological, biochemical and molecular biological responses, the future research must attempt to integrate several levels of function using a single approach. A systems biology approach for P stress in legumes should be aimed at integrating the various "omics" technologies, such as transcriptomics, metabolomics, proteomics and the physiological phenotyping known as phenomics. This will enable the elucidation of networks of regulation among these systems.

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