THE ROLE AND REGULATION OF PEPcarboxylase IN DISSOLVED INORGANIC CARBON METABOLISM UNDER P*i* STARVATION IN LEGUME ROOT SYSTEMS

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

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April 2004

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

This study aimed to assess the contribution of anaplerotic C provision via phospho*enol*pyruvate carboxylase (PEPc, EC 4.1.1.31), during P_i stress in the root and nodule components of *Lupinus angustifolius*. The role of PEPc in DIC metabolism in roots and nodules of phosphate-starved plants was studied. The symbioses involving leguminous plants and species of *Rhizobium* and *Bradyrhizobium* bacteria form an integral part of effective management of N in the environment. In agricultural settings, roughly 80% of this biologically fixed N_2 comes from this type of symbiotic relationship. Nitrogen-fixing bacteria in concert with legumes fix atmospheric nitrogen, which is then available to the infected plant. Worldwide, legumes are grown on approximately 250 Mha and they fix about 90 Tg (90 billion tons) of N_2 per year. The overall stoichiometry for nitrogen assimilation in the nodule requires one molecule of oxaloacetate to be converted to one molecule of asparagine per dinitrogen molecule fixed. One possible source for the required oxaloacetate is the reaction catalysed by PEPc. The reaction catalysed by PEPc is a major source of anaplerotic carbon for the plant and it is expected that this reaction will be even more important to plants under P_i stress, as the reaction is not ATP-dependent.

Seeds of *Lupinus angustifolius* (cv. Wonga) were inoculated with *Rhizobium* sp. (*Lupinus*) bacteria and grown in hydroponic culture. Tanks were supplied with either 2 μM PO_4 (LP) or 2 mM PO_4 (HP) and air containing 360 ppm CO_2. Roots experienced pronounced P stress with a greater decline in P_i, compared to nodules. Under P stress, PEPc activities increased in roots but not in nodules and these changes were not related to the expression of the enzyme. Root and nodular PEPc were not regulated by

expression, but possibly by posttranslational control. LP roots also synthesised more pyruvate from malate than LP nodules. The role of pyruvate accumulation under P_i stress, was further highlighted by the metabolism of PEP via both the pyruvate kinase (PK, EC 2.7.1.40) and PEPc routes. The enhanced PK activities supported these high pyruvate levels.

The results show unequivocally that nodules do not experience P stress to the same extent as roots. Implications of the findings are that nodules require low P to function normally. Maintenance of phosphate levels in nodules may be at the expense of host. It can be suggested that when nodules are P-starved they can become aggressive scavengers for available P and even out-compete roots.

OPSOMMING

Die doel van hierdie studie was om die bydrae van anaplerotiese koolstof-voorsiening via fosfo-*enol*pirovaatkarboksilase (PEPc, EC 4.1.1.31), tydens fosfaatstremming in die wortels en wortelknoppies van *Lupinus angustifolius* te bepaal. Die rol van PEPc in die metabolisme van opgeloste anorganiese koolstofdioksied in fosfaat-beperkte wortels en wortelknoppies is ondersoek. Die simbiose tussen peulplante en spesies van *Rhizobium* en *Bradyrhizobium* bakterieë vorm 'n integrale deel van die doeltreffende bestuur van stikstof in die omgewing. In die landbou word ongever 80 % van biologies-gefikseerde stikstof deur hierde simbiotiese verhouding geproduseer. Stikstofbindende bakterieë, in simbiose met peulplante, fikseer atmosferiese stikstof, wat dan beskikbaar is vir die geïnfekteerde plant. Wêreldwyd fikseer peulplante ongeveer 90 biljoen ton stikstof per jaar. Die algehele stoïgiometrie vir stikstof-fiksering in wortelknoppies vereis dat een molekule oksaalsuur na een molekule asparagien omgesit word per stikstofmolekule wat gefikseer word. Een moontlike bron vir die benodigde oksaalsuur is die reaksie wat deur PEPc gekataliseer word. Die reaksie wat deur PEPc gekataliseer word is 'n belangrike bron van anaplerotiese koolstof vir die plant en dit word vermoed dat hierdie reaksie van nog groter belang sal wees vir plante onder fosfaatstremming, omdat die reaksie nie ATP-afhanklik is nie.

Sade van *Lupinus angustifolius* (cv. Wonga) is geïnokuleer met *Rhizobium* sp. (*Lupinus*) bakterieë en gekweek in waterkultuur. Tenke is voorsien met óf 2 µM PO_4 (LP), óf 2 mM PO_4 (HP) en lug wat 360 ppm CO_2 bevat het. Wortels het skerp fosfaatstremming ervaar, met 'n groter afname in P_i, vergelykbaar met wortelknoppies.

Tydens fosfaatstremming het die aktiwiteit van PEPc toegeneem in wortels, maar nie in wortelknoppies nie en hierdie veranderinge was nie verwant aan die uitdrukking van die ensiem nie. PEPc van wortels en wortelknoppies is nie gereguleer deur uitdrukking nie, maar moontlik deur post-translasie kontrole. Wortels onder 'n lae-fosfaat voorsiening het ook meer pirodruiwesuur vanaf malaat gesintetiseer as wortelknoppies. Die rol van pirodruiwesuur-akkumulering tydens fosfaatstremming is verder beklemtoon deur die metabolisme van PEP *via* beide die pirovaatkinase- (PK, EC 2.7.1.40) en PEPc- roetes. Die verhoogde PK-aktiwiteite verklaar hierdie hoër vlakke van pirodruiwesuur.

Die resultate toon ondubbelsinnig dat wortelknoppies nie tot dieselfde mate fosfaatstremming ervaar as wortels nie. Dit impliseer dat wortelknoppies min fosfaat benodig om normal te funksioneer. Handhawing van fosfaatvlakke in wortelknoppies mag ten koste van die wortel wees. Dit is moontlik dat, wanneer wortelknoppies fosfaatbeperk is, hulle aggressiewe opruimers word vir beskikbare fosfaat en selfs beter funksioneer as die wortels.

ACKNOWLEDGEMENTS

I dedicate this thesis to my family. To my parents for their unwavering support and commitment to my education, both financially and in the form of encouragement and emotional support. To my brother, Robert, for being one of my best friends during our years at university and for encouraging me to achieve because I know you are only one step behind.

I am deeply indebted to my supervisor, Alex Valentine, for his commitment to my project, constant encouragement and enthusiasm.

I also wish to thank my co-supervisor, Frikkie Botha, for lively discussions and always having a suggestion for what to do next.

Thanks also to M.R. Le Roux for invaluable assistance with experimental procedures.

Furthermore, I am grateful to Prof. Carroll Vance for supplying me with PEPc antibody.

To NF Hiten and Dr. F Lynen for technical assistance with the molecular work and HPLC analysis respectively.

Thank you to Mr. L. Wille from AGRICOL, Pty. Ltd. for generous donation of seeds and sound advice on growth and maintainance.

Lastly, I thank the NRF for funding.

To see a world in a grain of sand

And a heaven in a wild flower,

Hold infinity in the palm of your hand

And eternity in an hour.

(William Blake 1757-1827)

LIST OF ABBREVIATIONS

°C	degrees centigrade
^{14}C	radio-labelled carbon
$^{14}CO_2$	radio-labelled carbon dioxide
ADP	adenosine 5'- diphosphate
AEC	adenylate energy charge
AMP	adenosine 5'- monophosphate
ANOVA	analysis of variance
ATP	adenosine 5'- triphosphate
BSA	bovine serum albumin
dH_2O	distilled water
DIC	dissolved inorganic carbon
$DI^{14}C$	radio-labelled dissolved inorganic carbon
DTT	1,4 – dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
HEPES	4 – (2 – hydroxyethyl) – 1 – piperazineethanesulfonic acid
HP	high phosphate
LP	low phosphate
LSD	least significant difference
MES	(2 – [N – Morpholino] ethanesulfonic acid)
mRNA	messenger ribonucleic acid
(NAD)-MDH	malate dehydrogenase (EC 1.1.1.37), cofactor in oxidized form
(NADH)-MDH	malate dehydrogenase (EC 1.1.1.37), cofactor in reduced form

(NAD)-ME	malic enzyme (EC 1.1.1.40), cofactor in oxidized form
(NADH)-ME	malic enzyme (EC 1.1.1.40), cofactor in reduced form
NAD	ß – nicotinamide adenine dinucleotide
NADH	ß – nicotinamide adenine dinucleotide, reduced form
NADP	ß – nicotinamide adenine dinucleotide phosphate
NADPH	ß – nicotinamide adenine dinucleotide phosphate, reduced form
OAA	oxaloacetate
PEP	phospho*enol*pyruvate
PEPc	phospho*enol*pyruvate carboxylase (EC 4.1.1.31)
PEPck	phospho*enol*pyruvate carboxylase kinase (EC 4.1.1.49)
PEPp	phospho*enol*pyruvate phosphatase (EC 3.1.3.60)
P_i	inorganic phosphate
PK	pyruvate kinase (EC 2.7.1.40)
PVPP	polyvinylpolypyrrolydine
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecylsulphate
SE	standard error
TCA	tricarboxylic acid
TCA cycle	tricarboxylic acid cycle
Tris	2–amino–2–(hydroxymethyl)–1,3- propanediol

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Chapter 1

1.1 General introduction

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

The symbiotic association, which develops when legume roots are infected with an appropriate strain of *Rhizobium* is able to convert atmospheric N_2 into ammonia. Legume nodules can be classified into two groups, determinate nodules generally export ureides, whereas indeterminate nodules export amino acids - mostly in the form of asparagine (Streeter 1991).

In *Lupinus angustifolius*, an amide exporter, ammonia is assimilated into amino acids, which are used for plant growth. However, any consumption of acids of the TCA cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate (OAA), and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a build-up of acetyl-CoA and the input of the TCA cycle would stop. An alternative source of oxaloacetate is

therefore needed. If it is accepted that oxaloacetate is the carbon skeleton and asparagine the aminated product, then the overall stoichiometry for nitrogen assimilation in the nodule requires one molecule of oxaloacetate to be converted to one molecule of asparagine per dinitrogen molecule fixed. One possible source for the required oxaloacetate is the reaction catalysed by the enzyme phospho*enol*pyruvate carboxylase or PEPc (EC 4.1.1.31). The PEPc in roots is located in the cytoplasm, as are the root and nodule enzymes for ammonia assimilation. In addition, it has been shown that PEPc is present in the root nodule of broad bean, and that bean nodules take up CO_2 (Christeller, Laing & Sutton, 1977).

The high turnover of carbon in the nodule is reflected by a substantial requirement for newly fixed carbon continuously provided by the shoot, and by about a two-fold higher respiration rate per unit dry weight of nodulated compared with non-nodulated legume roots. The carbon costs of N_2 fixation vary with host species, bacterial strain and plant development. At certain stages of the growth period nodules may consume as much as 50% of the photosynthates produced by legume plants (Pate and Herridge, 1978). About half of this 50% is respired as CO_2. However, between 25 and 30% of the respired CO_2 can be reassimilated by the nodules via PEPc providing up to 25% of the carbon needed for malate and aspartate synthesis, required for the assimilation of NH_3 and export to the host plant. On a fresh weight basis, PEPc activity is between 20 times (pea) and 1000 times (soybean) higher than in the roots (Marschner, 1988; Schulze *et al.*, 1998; Vance, Stade & Maxwell, 1983).

Thus, as can be expected, the metabolism of the plant and the metabolism of the bacteria are interconnected and even show signs of interdependence. The *Rhizobium* / legume symbiosis is based on the exchange of nutrients between the symbionts. Besides the exchange of carbon and nitrogen, other nutrients are important for this plant-microbe association to function optimally. Studies with soybean have consistently shown a positive response to inorganic phosphorous (P_i) fertilisation. Nutrient flux in and out of the legume nodule is of great importance to nitrogen fixation, but little information is available regarding solute uptake or flow to the bacteroid (Streeter, 1991).

Mineral nutrients may influence symbiotic nitrogen fixation of leguminous plants at any of four phases of the overall process: (a) host plant growth, (b) growth and survival of rhizobia, (c) infection and nodule development, and (d) nodule function. The influence of P_i on symbiotic dinitrogen (N_2) fixation in leguminous plants has received considerable study but its role in the process remains unclear (Israel, 1987). Leidi & Rodríguez-Navarro (2000) found that nutrient limitation might be a major constraint on legume N_2 - fixation and yield. There is a pH-dependency of plant P_i uptake, with maximum rates between pH 5.0 and 6.0. Root-induced changes of rhizosphere pH, caused by processes such as differential uptake of anions and cations, root respiration or organic acid exudation, might strongly affect P_i uptake.

Inorganic phosphate (P_i) is known to regulate bio-energetic processes in plants by being one of the substrates for photo - and oxidative phosphorylation. Lack of P_i has been found to decrease the levels of ATP and ADP, as well as the adenylate energy charge in leaves and roots. In some plants, P_i limitation lowers photosynthetic activity, but in others it does not influence the rate of photosynthesis. During prolonged P_i limitation, a

decreased activity of the cytochrome pathway and an increased participation of the cyanide-resistant pathway were observed. The activity of the alternative, non-phosphorylating pathway allows the functioning of the Krebs cycle and operation of the mitochondrial electron transfer chain with limited ATP production and thereby may contribute to the survival of P_i -deficient plants (Juszczuk & Rychter, 1997. Mikulska, Bomsel & Rychter, 1998).

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

Increased PEPc activity in C_3 plants has been associated with increases in internal cellular pH or a high demand for C skeletons, such as during amino acid biosynthesis, N assimilation, and exudation of organic acids. It has been suggested that changes in cytosolic pH may modulate PEPc activity by directly or indirectly regulating its phosphorylation status. The phosphorylation status of PEPc in soybean root nodules appears to be modulated by photosynthate transported from shoots. In legumes, root nodule C_3 non-photosynthetic PEPc is both transcriptionally and post-transcriptionally

regulated, as well as being regulated by phosphorylation. Phosphorylation of root nodule PEPc reduces the sensitivity of the enzyme to malate inhibition. This is a fundamental characteristic of an enzyme that functions in an environment in which malate synthesis is high, such as root nodules or proteoid roots. Enhanced synthesis of organic acids is dependent on continued high PEPc activity (Johnson *et al.*, 1996).

To summarise, the legume / *Rhizobium* symbiosis is expensive in terms of carbon cost to the plant. The plant provides the bacteroids in the nodule with respiratory carbon in the form of organic acids, mainly malate. The plant also uses oxaloacetate to assimilate the NH_3 produced by the bacteroids. Depletion of these TCA cycle organic acids has detrimental effects on the plant. Thus, the plant needs to find a way to supplement its production of organic acids for the symbiosis to be beneficial. The reaction catalysed by PEPc is a major source of anaplerotic carbon for the plant and it is expected that this reaction will be even more important to plants under P_i stress, as the reaction is not ATP-dependent. This study aims to assess the contribution of anaplerotic C provision via PEPc, during P_i stress in the root and nodule components.

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Chapter 2

2.1 General concepts of the legume / *Rhizobium* symbiosis

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

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

Within the infected cells, the symbiotic bacteria (called *bacteroids*) occupy enclosures (*symbiosomes*) surrounded by a plant-derived membrane called a *peribacteroid* or *symbiosome membrane*. The bacteroids differ from the free-living bacteria in that they are larger and express a complement of genes that are not expressed in the free-living

form. Plant organelles, including mitochondria, plastids and peroxisomes, tend to be localised near gas-filled *intercellular spaces* that form a network throughout the entire central zone and are thought to play a role in providing a low-resistance diffusion pathway for O_2 supply to, and H_2 and CO_2 removal from, the metabolically active cells within the central zone (Layzell *et al.* 1990).

2.2 Symbiotic biological N_2 fixation

The symbiotic association, which develops when legume roots are infected with an appropriate strain of *Rhizobium*, is able to convert atmospheric N_2 into ammonia. The ammonia is then assimilated into amino acids, which are used for plant growth.

Atmospheric N_2 is reduced by the microbial enzyme nitrogenase (EC 1.7.99.2) expressed by *Rhizobium* and *Bradyrhizobium* bacteria during symbiotic N_2 fixation. This reaction is energetically expensive, as it requires 16-20 mol of ATP per mol N_2 fixed (Vance 1990; Layzell *et al.* 1990). The complex series of events leading from bacterial colonisation of the legume rhizosphere to fixation of N_2 and export of that fixed N requires controlled co-ordinated expression of both bacterial and host plant genes. The contribution of these genes to symbiosis can be grouped into several functions including: recognition, root hair division, infection thread growth, nodule differentiation, carbon assimilation, organic acid metabolism, ammonia assimilation and possibly, suppression of host plant defence responses (Vance 1990).

Primary assimilation of NH_4^+ involves complex intermingling with C metabolites, especially C-4 acids produced by the TCA cycle. However, any consumption of acids of the TCA cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate, and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a

build-up of acetyl-CoA and the input of the TCA cycle would stop. An alternative source of oxaloacetate is therefore needed. If it is accepted that oxaloacetate is the carbon skeleton and asparagine the aminated product, then the overall stoichiometry for nitrogen assimilation in the nodule requires one molecule of oxaloacetate to be converted to one molecule of asparagine per dinitrogen molecule fixed. One possible source for the required oxaloacetate is the reaction catalysed by phospho*enol*pyruvate carboxylase or PEPc (EC 4.1.1.31). The PEPc in roots is located in the cytoplasm, as are the root and nodule enzymes for ammonia assimilation. In addition, it has been shown that PEPc is present in the root nodule of broad bean, and that bean nodules take up CO_2 (Christeller *et al.* 1977).

The high turnover of carbon in the nodule is reflected by a substantial requirement for newly fixed carbon continuously provided by the shoot, and by about a two-fold higher respiration rate per unit dry weight of nodulated compared with non-nodulated legume roots. The carbon costs of N_2 fixation vary with host species, bacterial strain and plant development. At certain stages of the growth period nodules may consume as much as 50% of the photosynthates produced by legume plants. About half of this 50% is respired as CO_2. However, between 25 and 30% of the respired CO_2 can be reassimilated by the nodules via PEPc providing up to 25% of the carbon needed for malate and aspartate synthesis, required for the assimilation of NH_3 and export to the host plant. On a fresh weight basis, PEPc activity is between 20 times (pea) and 1000 times (soybean) higher than in the roots (Marschner 1988; Schulze *et al.* 1998; Vance *et al.* 1983).

As the metabolism of the plant and the metabolism of the bacteria are interconnected, legume nodules include two different sets of genes and two different sets of metabolic

capabilities. Superimposed on this metabolic complexity is a system of specialised tissues and compartments within cells, and superimposed on most of the system is a very low free oxygen concentration (Streeter 1991). The large potential of nodulated legumes for N_2 fixation is mainly based on three factors: Direct supply of photosynthates to the N_2 - fixing bacteroids in the nodules; effective maintenance of very low O_2 concentrations in the interior of the nodules for protection of nitrogenase; and rapid export of the fixed nitrogen (Marschner 1988).

2.3 The effects of Pi deficiency on symbiotic N_2 fixation

The *Rhizobium* / legume symbiosis is based on the exchange of nutrients between the symbionts. Besides the exchange of carbon and nitrogen, other nutrients are important for this plant-microbe association to function optimally. Studies with soybean have consistently shown a positive response to inorganic phosphorous (P_i) fertilisation; whole plant nitrogen (N) concentration, plant dry matter, nodule number, nodule mass, nitrogenase activity and nodule specific nitrogenase activity were found to increase as a result of P_i fertilisation. Increased nodule specific nitrogenase activity resulting from the addition of P_i implies more efficient nitrogen fixation, but the reasons for this P_i response are not understood. It has also been observed that soybeans grown with fertiliser nitrogen have a lower P_i requirement than when nitrogen is obtained from symbiosis, suggesting optimum symbiotic interaction between the host plant and rhizobia depends on efficient allocation and use of available P_i. Nutrient flux in and out of the legume nodule is of great importance to nitrogen fixation, but little information is available regarding solute uptake or flow to the bacteroid (Al-Niemi, Kahn & McDermott 1998; Streeter 1991).

Mineral nutrients may influence symbiotic dinitrogen fixation of leguminous plants at any of four phases of the overall process: (a) host plant growth, (b) growth and survival of rhizobia, (c) infection and nodule development, and (d) nodule function. The influence of P_i on symbiotic dinitrogen (N_2) fixation in leguminous plants has received considerable study but its role in the process remains unclear. It has been found that combined nitrogen increased the growth response of nodulated subterranean clover plants to P_i. Thus, P_i increased symbiotic dinitrogen fixation in subterranean clover by stimulating host plant growth rather than by exerting specific effects on rhizobial growth and survival or on nodule formation and function. Studies on soybean plants have suggested more specific effects of P_i on nodule initiation, growth, development and function (Israel 1987).

2.3.1 Respiration in symbiotic root systems

Legume nodules have a much higher rate of respiratory O_2 consumption than most other plant tissues; about four fold higher than that observed in a similar biomass of root tissue. This O_2 is required to meet the large demands for ATP within the bacteroids and the ATP requirements for the synthesis of the exported organic solutes of N, in addition to the other needs for cell growth and maintenance. However, O_2 is a very potent, irreversible inhibitor of the nitrogenase enzyme. Therefore, legume nodules must provide a very high flux of O_2 to the bacteria-infected cells, but at an extremely low O_2 concentration (Layzell *et al.* 1990).

The respiration of nodulated roots is intense and has often hidden their fixation of CO_2. In leguminous plants, the photosynthates are transported from leaves to roots and nodules. In this way, the energy, the reducing power and the carbon skeletons required for the symbiotic nitrogen fixation are provided to nodules (Deroche *et al.* 1981). The overall

function of legume nodules requires very large amounts of reducing equivalents, more specifically, reduced carbon. Estimates vary widely, but there seems to be a consensus for a requirement of about 12.2 g of carbohydrate for each gram of nitrogen fixed by soybean nodules (Streeter 1991; Gordon & James 1997). ^{14}C-labelled photosynthates, translocated to the nodules from the shoot, are rapidly utilised in the formation of amino acids from ammonia produced by nitrogen fixation. The widespread occurrence in higher plant tissues of enzymic mechanisms for the dark fixation of CO_2 into organic acids suggests that a proportion of the carbon skeletons in the nodules used to form amino acids may be produced by this means also. Carboxylation of phospho*enol* pyruvate (PEP) catalysed by phospho*enol*pyruvate carboxylase (PEPc) is probably the most important reaction for dark CO_2 fixation in plant tissues with subsequent reduction and transamination of oxaloacetate to produce malate and aspartate respectively. Many workers have suggested that the organic acids produced by CO_2 fixation may replace or complement Krebs cycle acids used in biosynthetic reactions in the cell. They attributed increased nodulation and nitrogen fixation to dark fixation of CO_2 by the root system, resulting in increased levels of keto-acids in the nodules which sequestered the ammonia produced by N_2 - fixation (Lawrie & Wheeler 1975).

P_i limitation has profound effects on plant metabolism. In respiration, during prolonged P_i limitation, a decreased activity of the cytochrome pathway and an increased participation of the cyanide-resistant pathway were observed. The activity of the alternative, nonphosphorylating pathway allows the functioning of the Krebs cycle and operation of mitochondrial electron transfer chain with limited ATP production and thereby may contribute to the survival of P_i-deficient plants (Juszczuk *et al.* 1997; Mikulska *et al.* 1998).

2.3.2 C & N budgets in symbiotic root systems

In legumes, the phloem provides the nodule with reduced carbon in the form of sucrose, whereas the xylem removes the products of nitrogen fixation as either ureides (allantoin and allantoic acid) or as the amide asparagine. Sucrose provided to the nodules is not only a source of C for growth, but provides oxidisable substrates needed for plant and bacteroid respiration. In addition, sucrose provides the C skeletons required in the synthesis of asparagine and ureides. Evidence indicates that C4 acids, principally malate, are synthesised in the cytosol of the infected cell and transported across the plant symbiosome membrane and the bacterial plasma membrane where they are metabolised by a bacterial TCA cycle. PEPc is a key enzyme in the infected cell. Together with malate dehydrogenase (EC 1.1.1.37), it generates the malate for the bacteria, and in nodules that export asparagine, it provides the 4-C skeleton for asparagine synthesis (Layzell *et al.* 1990).

In lupin, asparagine is the main compound exported from the N_2-fixing nodule tissue, together with smaller amounts of aspartate, glutamine, glutamate and threonine. Both asparagine and aspartate can derive their carbon skeletons from oxaloacetate, while glutamate and glutamine can derive their carbon from α-ketoglutarate. Oxaloacetate and α-ketoglutarate are tricarboxylic acid cycle intermediates. However, any consumption of acids of the TCA cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate, and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a build-up of acetyl-CoA and the input of the TCA cycle would stop. An alternative source of oxaloacetate is therefore needed. One possible source is the reaction catalysed by PEP carboxylase (PEPc). The PEPc in roots is located in the cytoplasm, as are the root and nodule enzymes for ammonia assimilation. In addition, it has been shown that PEPc is

present in the root nodule of broad bean, and that bean nodules take up CO_2 (Christeller *et al.* 1977).

Although sinks and sources for C and N respond in concert to increased supply of photosynthetic products, there is evidence that N accumulation lags C accumulation as photosynthesis increases. It was found that the quantity of nitrogen fixed by clover could be greatly increased, if the photosynthetic activities of the plant were accelerated by increases in the available CO_2 concentration (Wilson, Fred & Salmon 1933).

Although quantitative estimates of the spatial and temporal relationships between carbon and nitrogen economy are undoubtedly more informative, indirect evidence - based on manipulations of the plant or its environment - provides some insight into the competitive restraints under which the nodules must function, and of the potential of various symbioses when relieved of these restraints. Thus, manipulations - which impair photosynthesis (e.g. defoliation or decreased light intensity) - invariably decrease the rate or duration of N_2-fixation, whilst those which promote photosynthesis (e.g. supplementary light) typically lead to increased fixation activity. The short-term effect of altering carbon supply is on fixation efficiency (the rate of N_2 - fixation per unit nodule dry weight), whilst in longer term changes in nodule mass per plant, in the rate of growth of the population of nodules, in the onset of senescence and in the rate of degeneration of the nodule population are likely to become more important. However, such 'manipulation' studies are complicated by endogenous control mechanisms, which allow the magnitude of N_2 - fixation to reflect, if not always meet, the demands of the host plant. Whether or not roots and nodules can benefit directly from CO_2 enrichment of the soil is debatable. The relative magnitude of the effect of CO_2 enrichment depends on when and

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

A continuous supply of photosynthates to the nodules is a prerequisite of N_2 - fixation. However, the rate of fixation may be controlled by endogenous mechanisms other than the rate of photosynthesis. An analysis of the data from atmospheric CO_2 enrichment studies suggests that the resultant improvement in fixation rate is often due to increases in overall plant growth rather than an immediate increase in nodule efficiency. Carbon and nitrogen budgets for nodulated roots suggest that 40-50% of total photosynthate is consumed by below-ground organs during vegetative growth and 63-64% of this is used in respiration, 16-22% is used in growth and 14-20% in the production and export of organic nitrogenous products of fixation (Minchin, *et al.* 1981; Gordon *et al.* 1997).

The nodule and its subtending root system represent one of the strongest sinks, receiving an estimated 15-30% or more of the net photosynthate of the plant. This supply of photosynthate transported via the phloem is used for energy-yielding substrates and carbon skeletons to support (a) the growth and maintenance of the nodule tissue; (b) the energy-consuming reactions associated with the reduction of N_2 in the endophyte and the assimilation of the NH_4^+ produced in the host cytosol; and (c) the synthesis of N-containing organic compounds for export from the nodule (Schubert 1986).

Ciereszko, Milosek & Rychter (1999) found that bean plants grown for 2-3 weeks on nutrient medium without phosphate show no effect in their net photosynthetic rate. However, increases in ^{14}C-assimilate translocation from the shoot to the root were observed. Furthermore, analysis of the phloem exudate from phosphate deficient leaves also indicated the stimulation of sucrose export. The greater assimilate transport and sugar accumulation in the roots appears to be an early plant response to phosphate deficiency and may be involved in plant acclimation to low nutrient conditions (Ciereszko *et al.* 1999; Ciereszko, Zambrzycka & Rychter 1998). It was suggested that phosphate-deficient plants might allocate more photosynthates from the leaves to the roots to improve their capacity for absorbing phosphate ions (Jeschke *et al.* 1997). Nodules contain very high concentrations of carbohydrates relative to other plant tissues and the major compound is generally sucrose. Perhaps the most important feature of carbohydrate composition is the rapid increase of all compounds just at the onset of N_2 - fixation; concentrations become much higher than in the adjacent roots, but what initiates this rapid change in sink activity is not known (Schubert 1986).

Although sucrose is the major carbohydrate found in nodules, malate is the primary plant carbon source used by bacteroids, and is also a factor in plant adaptation to P and Al stress (Graham *et al.* 2000; Gordon *et al.* 1997). Citrate exudation into the rhizosphere increases the P_i available to the plant by mobilizing the sparingly soluble mineral P_i and, possibly, organic P sources (Johnson *et al.* 1996). Amino acid composition of nodules has also been studied, and one of the most striking features is the high glutamate concentration in bacteroids (Schubert 1986).

Many investigators have suggested that one of the major effects of increased CO_2 concentrations is to increase the rates of CO_2 fixation in nodules which is catalysed primarily by PEPc forming the C_4 acid, oxaloacetate (McClure, Coker & Schubert 1983). P_i deficiency has been shown to stimulate the activity of C_3 PEPc in leaves and nonphotosynthetic PEPc in roots. In addition to supplying anaplerotic C to replenish TCA-cycle intermediates, elevated PEPc caused by P_i limitation may be a response to increased demands for pyruvate and/or a greater need for P_i recycling. PEPc in the roots of P_i -deficient plants provides as much as 25% of the C for citrate and 34% of the C for malate for exudation (Johnson *et al.* 1996; Vance & Stade 1984).

PEPc clearly plays a key role in amino acid biosynthesis. This is especially true in nodules of amide-exporting plants (McClure *et al.* 1983).

Leidi *et al.* (2000) found that nutrient limitation might be a major constraint on legume N_2 - fixation and yield. There is a pH-dependency of plant P_i uptake, with maximum rates between pH 5.0 and 6.0. Root-induced changes of rhizosphere pH, caused by processes such as differential uptake of anions and cations, root respiration or organic acid exudation, might strongly affect P_i uptake.

Inorganic phosphate (P_i) is known to regulate bio-energetic processes in plants by being one of the substrates for photo - and oxidative phosphorylation. Lack of P_i has been found to decrease the levels of ATP and ADP, as well as the adenylate energy charge in leaves and roots. In some plants, P_i limitation lowers photosynthetic activity, but in others it does not influence the rate of photosynthesis. During prolonged P_i limitation, a decreased activity of the cytochrome pathway and an increased participation of the cyanide-resistant pathway were observed. The activity of the alternative, non-

phosphorylating pathway allows the functioning of the Krebs cycle and operation of mitochondrial electron transfer chain with limited ATP production and thereby may contribute to the survival of P_i -deficient plants (Juszczuk *et al.* 1997; Mikulska *et al.* 1998).

Furthermore, because P_i is necessary for nucleotide synthesis, severe P_i deficiency can result in decreased total RNA biosynthesis. In addition, P_i deficiency induces a specific RNase in tomato and Arabidopsis. This RNase functions to liberate P_i from RNA, facilitating its remobilisation. A decrease in total RNA concentration due to P_i deficiency may be the result of decreased biosynthesis, increased degradation or a combination of both (Johnson *et al.* 1996). Johnson *et al.* (1996) also found that although the concentration of total RNA was reduced by P_i deficiency, roots continued to have enhanced expression of PEPc mRNA, which suggests preferential synthesis and/or stability of PEPc mRNA. Furthermore, they found that increased PEPc specific activity was related directly to an increase in PEPc mRNA and PEPc enzyme, providing strong evidence that this non-photosynthetic PEPc is in part under transcriptional regulation.

Increased PEPc activity in C_3 plants has been associated with increases in internal cellular pH or a high demand for C skeletons, such as during amino acid biosynthesis, N assimilation, and exudation of organic acids. It has been suggested that changes in cytosolic pH may modulate PEPc activity by directly or indirectly regulating its phosphorylation status. The phosphorylation status of PEPc in soybean root nodules appears to be modulated by photosynthate transported from shoots. In legumes, root nodule C_3 non-photosynthetic PEPc is also regulated by transcriptional and translational events, as well as by phosphorylation. Phosphorylation of root nodule PEPc reduces the

sensitivity of the enzyme to malate inhibition. This is a fundamental characteristic of an enzyme that functions in an environment in which malate synthesis is high, such as root nodules or proteoid roots. Enhanced synthesis of organic acids is dependent on continued high PEPc activity (Johnson *et al.* 1996).

2.4 The central role of PEPc and its regulation

Phospho*enol*pyruvate carboxylase (PEPc, E.C. 4.1.1.31) is a homotetrameric enzyme that catalyses the β-carboxylation of PEP by HCO_3^- in the presence of a divalent cation to yield P_i and oxaloacetate. The tetramer is comprised of subunits of 100 to 110 kDa and several isoforms of PEPc have been detected in both CAM and C_4 plants (Pathirana *et al.* 1992; Vidal & Chollet 1997; Pathirana *et al.* 1997; Chollet, Vidal & O'Leary 1996). The PEPc enzyme is thought to be encoded by a single gene or a small gene family (Schulze *et al.* 1998; Chollet *et al.* 1996).

The activity of the various isoforms of plant PEPc is subject to allosteric control by a variety of positive [e.g. glucose-6-phosphate (G6P), Triose-P] and negative (e.g. malate, aspartic acid) metabolite effectors. Although changes in the cytosolic levels of these opposing allosteric effectors and H^+ are likely to contribute to the overall regulation of PEPc activity *in vivo*, research has primarily focused on the reversible phosphorylation of the enzyme (Chollet *et al.* 1996; Paterson & Nimmo 2000). Post-translational regulation of PEPc is achieved through phosphorylation of the protein, oligomerisation as affected by PEP, malate and G-6-P and protein turnover (Pathirana *et al.* 1992).

This reversible phosphorylation plays a central role in controlling flux through the enzyme *in vivo*. Upregulation/phosphorylation of the target enzyme is catalysed by a

highly regulated, specific Ca^{2+}-independent PEPc protein kinase, which significantly reduces the sensitivity of PEPc to inhibition by malate and thereby increases the catalytic activity. Downregulation/dephosphorylation proceeds via a typical mammalian-type protein phosphatase 2A (Paterson *et al.* 2000; Pasqualini *et al.* 2001; Plaxton 1996).

There is now convincing evidence that the reversible phosphorylation of PEPc is widespread, if not ubiquitous. Complementary measurements of *in vivo* changes in PEPc activity and/or malate sensitivity under near-physiological assay conditions have underscored the regulatory nature of this covalent modification in nodules.

Furthermore, related *in vitro* studies have established that PEPc kinase activity is present in soybean and alfalfa root nodules and have demonstrated this kinase's similarity to the C_4 and CAM enzymes with respect to its Ca^{2+} independency, chromatographic properties and catalytic subunit(s). The activity state of this PEPc kinase is modulated reversibly *in vivo* by a complex interaction between photosynthesis and N (C_3 leaves) or photosynthate supply to N_2-fixing root nodules (Chollet *et al.* 1996).

Phospho*enol*pyruvate carboxylase kinase or PEPck (EC 4.1.1.49) is a cytosolic enzyme that catalyses the reversible reaction:

$$OAA + ATP \leftrightarrow PEP + ADP + CO_2$$

However, because of its low affinity for CO_2 *in vivo* it probably acts as a decarboxylase. Thus, the direction of the reaction *in vivo* is generally the reverse of the reaction catalysed by PEPc, i.e. PEPck usually acts as a decarboxylase, whereas PEPc acts as a carboxylase. The importance of these two reactions in plants is that they play a key role in mediating

the interconversion of glycolytic intermediates and organic acids and both may play anaplerotic roles in metabolism (Leegood *et al.* 1999).

The presence of both these enzymes in the cytosol means that they require strict regulation in order to avoid a potentially futile cycle, which leads to the hydrolysis of ATP. Recent evidence suggests that, in many plant tissues, both these enzymes are regulated by phosphorylation (Leegood & Walker 1999; Leegood *et al.* 1999).

PEPck forms a central point in metabolism, where metabolism of lipids, organic acids and amino acids can give rise to oxaloacetate and sugars. Other organic and amino acids and secondary metabolites can then be formed from PEP (Leegood *et al.* 1999).

PEPck is a small (30-32 kDa) protein kinase with no recognisable regulatory domain or phosphorylation site and it appears to have a short half-life. It therefore seems likely that control of transcription of the PEPck gene may be the principal method of regulating PEPck activity and hence PEPc phosphorylation in most plant tissues (Walker *et al.* 2002; Nimmo, Wilkins & Nimmo 2001).

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Chapter 3

Carbon fixation by PEPc during P_i starvation in legume root systems

To be submitted to *Plant, Cell and Environment*.

Chapter 3

Carbon fixation by PEPc during P_i starvation in legume root systems

Running title: **PEPc in legume roots**

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3.1 Abstract

The role of phospho*enol*pyruvate carboxylase (PEPc, EC 4.1.1.31) in DIC metabolism in roots and nodules of phosphate-starved plants was studied. Seeds of *Lupinus angustifolius* (cv. Wonga) were inoculated with *Rhizobium* sp. (*Lupinus*) bacteria and grown in hydroponic culture. Tanks were supplied with either low phosphate (2 μM PO_4) or adequate phosphate (2 mM PO_4) and air containing 360 ppm CO_2. Roots experienced pronounced P stress with a 60 % decline in P*i*, whilst nodules only had a 22 % decline. Under P stress, PEPc activities and DIC metabolism increased in roots but not in nodules. Compared to roots, nodules did not experience P stress and therefore, PEPc activities and DIC metabolism were unaffected.

Key words: lupin plants, PEPc, P_i -deficiency, DIC incorporation.

3.2 Introduction

The symbiotic association, which develops when legume roots are infected with an appropriate strain of *Rhizobium*, is able to convert atmospheric N_2 into ammonia. The respiration of nodulated roots is intense and has often masked their fixation of CO_2. In leguminous plants, the photosynthates are transported from leaves to roots and nodules. In this way, the energy, the reducing power and the carbon skeletons required for the symbiotic nitrogen fixation are provided to the nodules (Deroche & Carrayol 1988; Deroche *et al.* 1981; Streeter 1991). The overall function of legume nodules requires very large amounts of reducing equivalents, more specifically, reduced carbon. Estimates vary widely, but there seems to be a consensus for a requirement of about 12.2 g of carbohydrate for each gram of nitrogen fixed by soybean nodules (Streeter 1991. Gordon & James 1997). ^{14}C-labelled photosynthates, translocated to the nodules from the shoot, are rapidly utilised in the formation of amino acids from ammonia produced by nitrogen fixation. The widespread occurrence in higher plant tissues of enzymic mechanisms for the dark fixation of CO_2 into organic acids suggests that a proportion of the carbon skeletons in the nodules used to form amino acids may be produced by this means also. Carboxylation of phospho*enol*pyruvate (PEP) catalysed by phospho*enol*pyruvate carboxylase (PEPc) is probably the most important reaction for dark CO_2 fixation in plant tissues with subsequent reduction and transamination of oxaloacetate to produce malate and aspartate respectively. Many workers have suggested that the organic acids produced by CO_2 fixation may replace or complement Krebs cycle acids used in biosynthetic reactions in the cell. They attributed increased nodulation and nitrogen fixation to dark fixation of CO_2 by the root system, resulting in increased levels of keto-acids in the nodules, which sequestered the ammonia produced by N_2-fixation. Furthermore, dark CO_2 fixation is widespread in the root nodules of legumes and has been estimated to recycle 9

to 30% of nodule respiratory carbon in soybean. It has been suggested that reassimilation of respired CO_2 may increase the apparent energy use efficiency of legume symbioses and that selection for increased dark CO_2 fixation may be a feasible means of increasing legume productivity (Anderson, Heichel & Vance 1987; King, Layzell & Canvin 1986; Lawrie & Wheeler 1975; McClure, Coker, III & Schubert 1983).

The carbon costs of N_2 fixation vary with host species, bacterial strain and plant development. At certain stages of the growth period nodules may consume as much as 50% of the photosynthates produced by legume plants. About half of this 50% is respired as CO_2. However, between 25 and 30% of the respired CO_2 can be reassimilated by the nodules via PEPc providing up to 25% of the carbon needed for malate and aspartate synthesis, required for the assimilation of NH_3 and export to the host plant. PEPc activity is higher in nodules than in roots. On a fresh weight basis, PEPc activity is between 20 times (pea) and 1000 times (soybean) higher in nodules than in the roots (Anderson *et al.* 1987; Deroche *et al.* 1988; Marschner 1988; Schubert 1986).

Besides the exchange of carbon and nitrogen, other nutrients are important for this plant-microbe association to function optimally. Studies with soybean have consistently shown a positive response to inorganic phosphorous (P_i) fertilisation. Nutrient flux in and out of the legume nodule is of great importance to nitrogen fixation, but little information is available regarding solute uptake or flow to the bacteroid (Al-Niemi, Kahn & McDermott 1998; Streeter 1991).

The influence of P_i on symbiotic dinitrogen (N_2) fixation in leguminous plants has received considerable study but its role in the process remains unclear (Israel 1987). It has been found that nutrient limitation might be a major constraint on legume N_2-fixation and yield. It has also been observed that soybeans grown with fertiliser nitrogen have a lower P requirement than when nitrogen is obtained from symbiosis, suggesting optimum symbiotic interaction between the host plant and rhizobia depends on efficient allocation and use of available P (Al-Niemi *et al.* 1998; Al-Niemi, Kahn & McDermott 1997; Leidi & Rodríguez-Navarro 2000). There is a pH-dependency of plant P_i uptake, with maximum rates between pH 5.0 and 6.0. Root-induced changes of rhizosphere pH, caused by processes such as differential uptake of anions and cations, root respiration or organic acid exudation, might strongly affect P_i uptake.

Inorganic phosphate (P_i) is known to regulate bioenergetic processes in plants by being one of the substrates for photo - and oxidative phosphorylation. Lack of P_i leads to a decrease in the levels of ATP and ADP, as well as the adenylate energy charge in leaves and roots. During prolonged P_i limitation, the activity of the cytochrome pathway decreases and participation of the cyanide-resistant pathway increases. The activity of the alternative, nonphosphorylating pathway allows the functioning of the Krebs cycle and operation of mitochondrial electron transfer chain with limited ATP production and thereby may contribute to the survival of P_i-deficient plants (Ciereszko, Milosek & Rychter 1999; Juszczuk & Rychter 1997; Mikulska, Bomsel & Rychter 1998). P_i starvation has been shown to stimulate the activity of C_3 PEPc in leaves and non-photosynthetic PEPc in roots. In addition to supplying anaplerotic C to replenish TCA-cycle intermediates, elevated PEPc caused by P_i limitation may be a response to increased demands for pyruvate and/or P_i recycling. PEPc in the roots of P_i-starved

plants provides as much as 25% of the C for citrate and 34% of the C for malate exudation (Johnson, Vance & Allan 1996). PEPc clearly plays a key role in amino acid biosynthesis; this is especially true in nodules of amide-exporting plants (McClure *et al.* 1983).

Since the PEPc reaction can provide C for bacteroid respiration and the plant's assimilation of the NH_3 produced by the bacteroids, the role of PEPc as an alternative route during P_i stress may comprise these anaplerotic provisions of C to root and nodule components. The aim of this study was to assess the contribution of anaplerotic C provision via PEPc, during P_i stress in the root and nodule components.

3.3 Materials and Methods

3.3.1 Plant growth conditions

All seeds were grown in vermiculite, which was commercially irradiated by a cobalt C-60 source of gamma radiation at a dose of 18 kGray.

Pots measuring 10 cm in diameter were washed in Ekon-D and rinsed in distilled water, then dried. The pots were then filled with vermiculite.

For all experiments, seeds of *Lupinus angustifolius* (cv. Wonga) were inoculated with a rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria. Seeds of lupins were coated in a saturated sucrose solution and 2 g of inoculum / 150 seeds was added and mixed. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in the pots containing vermiculite.

Seeds were germinated during May and June 2002 in an east-facing glasshouse at the University of Stellenbosch, Stellenbosch, South Africa. The range of midday irradiances was between 540-600 μmol m^{-2} s^{-1} and the average day/night temperatures and humidities were 23/15 °C and 35/75 % respectively. Pots were watered daily with distilled water until seeds germinated. Upon germination seedlings were watered once every two days for three to four weeks, until nodule formation had occurred. Once nodule formation was established, the seedlings were transferred to 22 litre hydroponic tanks under the same glasshouse conditions. The tanks contained a modified Long Ashton nutrient solution modified to contain 1 mM NH_4^+, 2 mM PO_4 and 0.05 mM MES (pH 6). Solutions were changed every 3-4 d. The hypocotyls of seedlings were wrapped

with foam rubber at their bases and inserted through holes in the lids of the tanks. Each tank was supplied with an air supply line, which bubbled air containing 360 ppm CO_2. Once nodules were established and of adequate size, usually in the third or fourth week of hydroponic growth, plants were divided into two treatments. Half of the hydroponic tanks were supplied with nutrient solution containing 2 mM PO_4 (HP treatment), the other half were supplied with nutrient solution containing 2 µM PO_4 (LP treatment). P_i starvation occurred for 10-12 days, after which plants were harvested.

3.3.2 Inorganic phosphate and adenylate level determinations

P_i levels were determined using a modified Fiske-Subbarow method (Rychter & Mikulska 1990). For adenylate level determinations, samples were extracted using a method modified from Stitt *et al.* 1983. The resulting supernatant was placed at room temperature in a rotary evaporator (Speed Vac® Plus SC 110 A, Savant) for approximately 24 hours to yield a dry pellet. The pellet was re-dissolved in the 200 µL of 10 mM HEPES (pH 8.0). Samples were analysed by HPLC using a Phenomenex Aqua 5µ C18 125A, 150 x 4.6 mm column. A sample volume of 20 µL was injected onto the column. Buffer A (0.1 M K_2HPO_4/KH_2PO_4 and 5 mM tetrabutylammonium hydrogen sulphate, pH 6.0) was passed through the column at a rate of 0.8 ml/min and buffer B (70% A, 30% methanol) was passed through the column at a rate of 0.7 ml/min. The detection took place in the UV range at 254 nm and at a column temperature of 30°C.

3.3.3 Calculations

It was assumed that in roots the cytosol occupies approximately 10 % of total cell volume and in the nodules the cytosol occupies approximately 50 % of total cell volume (Rolin *et al.* 1989). From these assumptions the cytosolic concentration of P_i was calculated for both components.

3.3.4 Phosphoenolpyruvate carboxylase extraction

The extraction was performed according to Ocaña *et al.* (1996) modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20 % (v/v) ethylene glycol, plus 2 % (m/v) insoluble polyvinylpoly pyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 ml of buffer.

3.3.5 Assay for PEPc activity

The PEPc activity was determined spectrophotometrically according to the method of Ocaña *et al.* (1996).

The soluble protein content was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

3.3.6 Whole plant ^{14}C incorporation

Roots of intact plants were supplied with 42 nmol $NaHCO_3$ containing 0.093 MBq $NaH^{14}CO_3$, and pulsed with air for 30 s thereafter every 15 minutes for an hour. In addition to the 15-minute intervals of air pulses, the solutions in the cuvettes were also swirled by hand every 5 minutes. After an hour the plants were harvested. Roots were rinsed twice in separate containers of distilled water and blotted dry. Plants were then

separated into root, nodule and shoot components, which were immediately weighed and quenched in liquid N before storage at -80°C.

3.3.7 ^{14}C labelling of detached and attached nodules

In a separate experiment plants were harvested from hydroponic tanks and the roots rinsed in distilled water. Roots of plants were subsequently cut off and the shoot sections were discarded. The roots sections were further divided into attached nodules (nodules attached to piece of root) and detached nodules (individual nodules). Approximately 0.5 g of freshly harvested material was used in feeding experiments. Erlenmeyer flasks (50 ml) were filled with nutrient solutions that were supplemented with 1 % sucrose. This was mainly in an effort to compensate for detaching nodules from their primary carbon source (i.e. shoots). Attached- and detached nodules, were pre-incubated in the Erlenmeyer flasks prior to feeding with $^{14}NaHCO_3$ label. These were subsequently transferred to vials, holding approximately 15 ml of incubation solution (see above) and through which air (360 ppm CO_2) was bubbled for the duration of the feeding experiment. Two holes, one through which label was to be fed and the other that was connected to the airline, were burnt through the lids of these vials. The various tissue segments in the vials were equilibrated for five minutes, after which the tissue was supplemented with 10 µl of $^{14}NaHCO_3$ label. The experiment was stopped by discarding the incubation solution. The various segments were bagged, quenched in liquid N_2 and stored at –80°C.

3.3.8 ^{14}C fractionation

Components were homogenised with 80% (v/v) ethanol and separated into soluble and insoluble components. The soluble component was subsequently separated into water-soluble and chloroform soluble components. The water-soluble component was further fractionated into amino acid, organic acid, and carbohydrate fractions, as described by Atkins & Canvin (1971).

3.3.9 N_2 fixation assay

Plants were harvested from hydroponic tanks, rinsed twice in two separate beakers of distilled water and blotted dry. Only the root segments were used in the experiment that followed. Nodules were carefully detached from the roots, keeping root damage down to the minimum. Similarly, nodules attached to a piece of root were separated from plants. The detached- and attached nodules were placed in vials that had been weighed prior to incubation of nodules in it. After incubation of nodules, the vials were weighed again. This was designated the fresh weight (FW) of the sample. Acetylene reduction assays for nitrogenase activity were performed at 25°C using 0.1 to 1g FW samples of either detached nodule- or attached nodule tissue in 15 ml glass vials fitted with rubber serum caps. Each vial contained 90% air and 10% acetylene (v/v) (Industrial grade; Afrox, South Africa).

After 3 h, a 1-ml gas sample was taken from each vial and ethylene concentrations were determined. Samples of 1-ml were injected with a 1-ml syringe and assayed for acetylene and ethylene, using a Varian 3400 gas chromatograph, fitted with a 6' * 1.8' column of Hayesep N 80/100 maintained at 70°C. Data were corrected for ethylene impurities in acetylene and endogenous ethylene production by plant material. The

concentrations of ethylene in samples were calculated using an ethylene standard (calibration standard mixed with 1 litre syringe LI-COR Inc., model 6000-01, USA).

3.3.10 Statistical analysis

All data was analyzed by single ANOVA. Percentage data was arcsine transformed prior to analysis and ratios were square root transformed prior to analysis. All data was then subjected to a post-hoc LSD test to determine significance.

3.4 Results

3.4.1 Inorganic phosphate and adenylate level determinations

LP roots had 60 % lower P_i levels than HP roots, whilst for nodules there was no significant decline in P_i levels from HP to LP (Fig.1a). The 60 % lower cytosolic P_i concentrations of roots and the unchanged P_i levels for nodules indicate that these two compartments were in fact P_i starved and P_i sufficient, respectively (Fig. 1b).

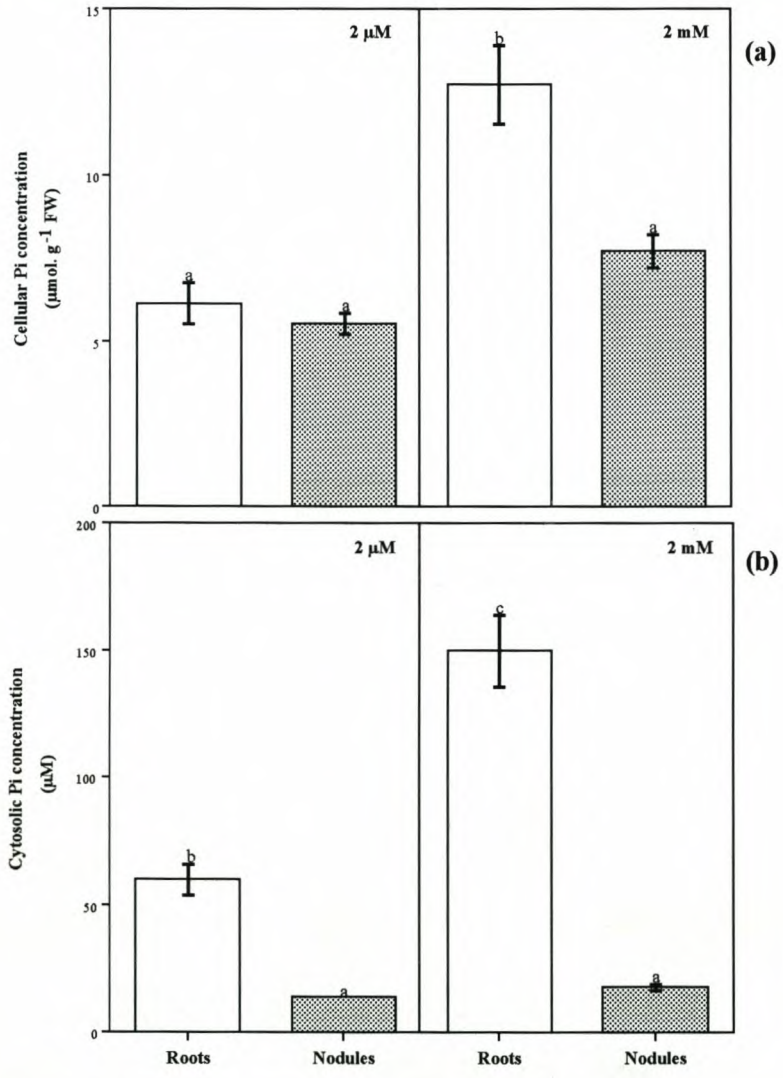

Figure 1. Cellular P_i concentration in nmol.g^{-1} FW (a) and calculated cytosolic P_i concentration in mM (b) of roots and nodules from Lupinus angustifolius (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants (control) remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates (n = 3).

Cellular ATP concentrations of plants subjected to P deprivation also showed a similar decline in levels compared to control plants (Fig. 2). This concurred well with data of the P deprivation and it similarly showed that roots experienced a more pronounced effect of P starvation, with a 5-fold decline of ATP concentration from adequate P to LP supply (Fig. 2). Roots and nodules of plants with a sufficient supply of P had correspondingly high ATP levels. When deprived of P nodule ATP levels decreased to levels of roots with sufficient P (Fig. 2).

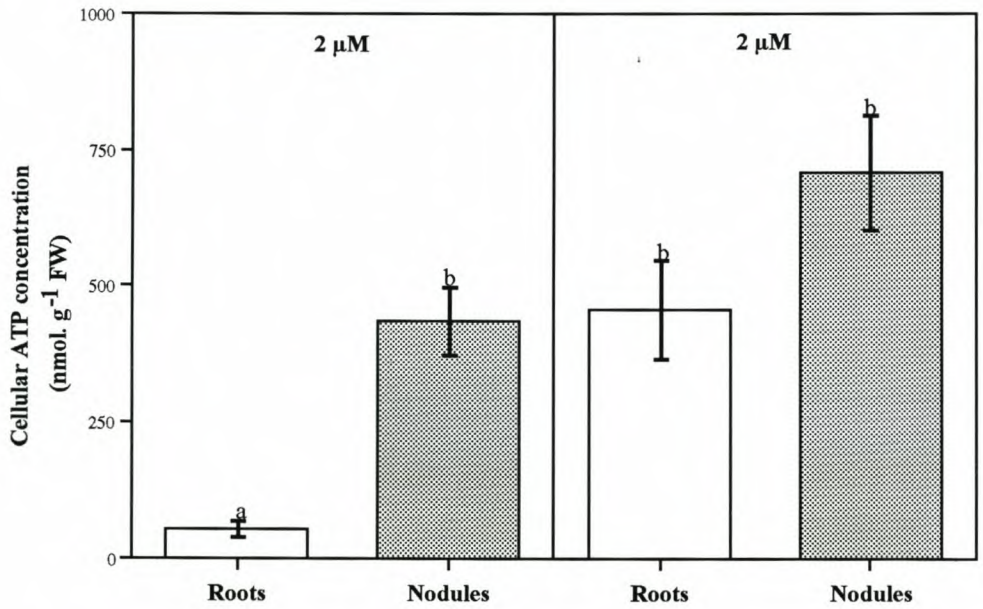

Figure 2. ATP concentration in (nmol.g^{-1} FW) of roots and nodules from Lupinus angustifolius (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants (control) remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates (n = 3).

The amount of ADP relative to ATP increased significantly in roots at LP, but not in nodules (Fig. 3). This again indicates a more pronounce P effect in roots than in nodules.

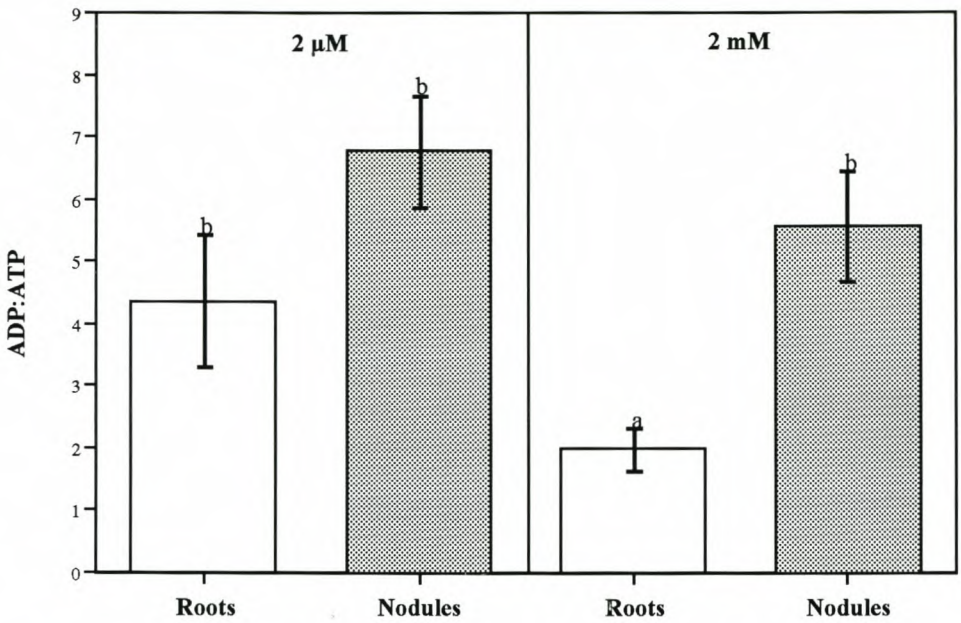

Figure 3. ADP:ATP ratio of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants (control) remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

3.4.2 PEPc activity

Nodules had a higher total protein content per unit mass than roots (Fig. 4). Hence, PEPc, activity, expressed per gram fresh weight (FW), was higher in nodules than roots (results not shown). The protein content of nodules and roots decreased significantly with LP supply (Fig. 4).

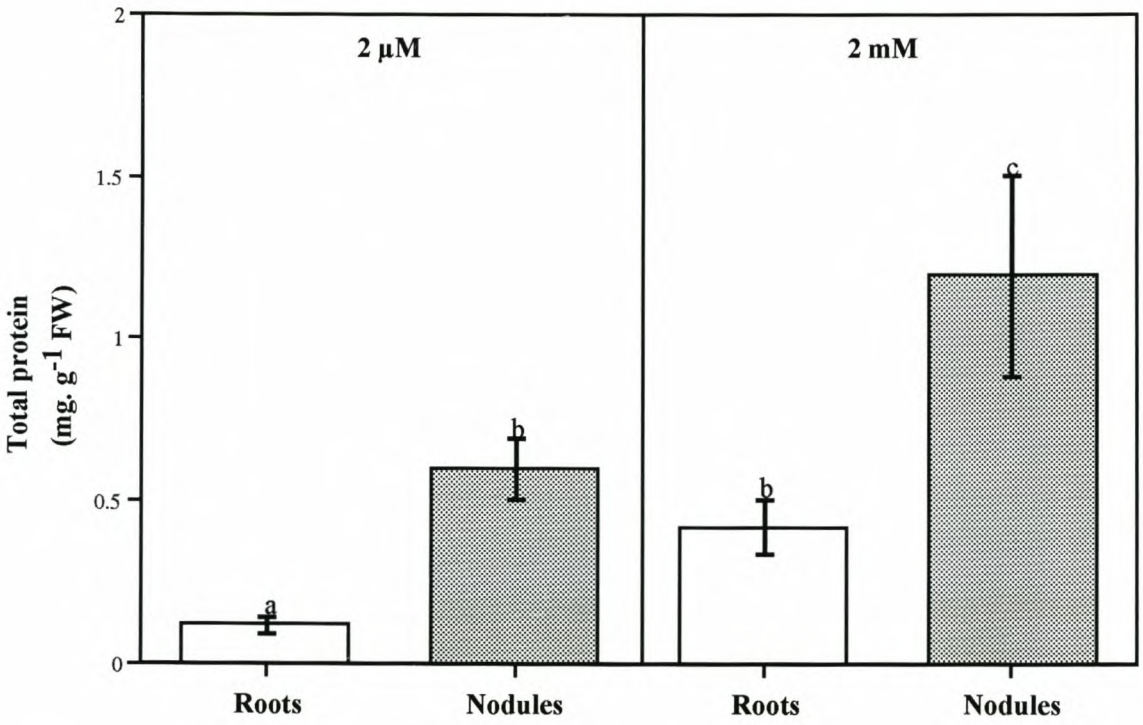

Figure 4. Total protein expressed in mg. g^{-1} FW of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$)

When expressed on a per milligram protein basis, the PEPc activity was affected more seriously in roots than in nodules (Fig. 5). Roots under P*i* stress showed PEPc activities in excess of 750 nmol. min^{-1}. mg^{-1} protein. This was a significant increase compared to roots supplied with sufficient P*i*, which had PEPc activities of 230 nmol. min^{-1}. mg^{-1} protein. Contrary to roots, nodules showed almost no change in PEPc activity, averaging 490 nmol. $min^{-1}.mg^{-1}$ protein.

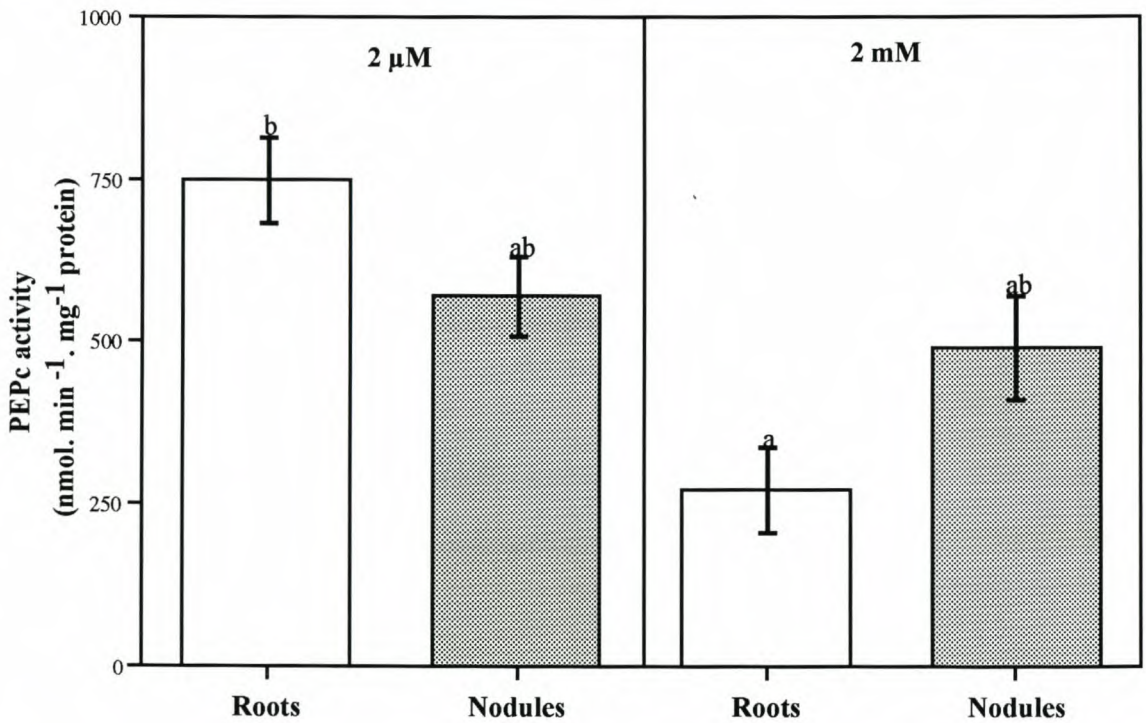

Figure 5. PEPc activity expressed in nmol. min^{-1}. mg^{-1} protein of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

3.4.3 ^{14}C incorporation and fractionation

In spite of the similar $DI^{14}C$ incorporation in the soluble fractions of both roots and nodules at either P concentration (Table 1), there were large changes in the percentage of fractionated components (Figure 6).

Table 1. Incorporation parameters of roots and nodules from *Lupinus angustifolius* (cv. Wonga) plants after 60 minutes of $DI^{14}C$ exposure. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

Incorporation parameters after 60 minutes of $DI^{14}C$ exposure	Low phosphate (2 µM)		High phosphate (2 mM)	
	Root	**Nodule**	**Root**	**Nodule**
$DI^{14}C$ concentration (µmol $C.g^{-1}$ fw)				
Soluble fractions	16.93 **ab**	11.49 **a**	23.76 **b**	19.88 **ab**
Insoluble fractions	11.11 **a**	16.99 **a**	18.13 **a**	16.84 **a**
Lipid fractions	0.17 **a**	0.11 **a**	0.18 **a**	0.33 **b**
Total incorporation	28.18 **a**	25.23 **a**	42.13 **b**	36.74 **ab**
^{14}C-C:^{14}C-N ratio				
OA:AA	4.50 **b**	2.90 **a**	4.34 **ab**	3.24 **ab**
C:N	4.63 **a**	3.27 **a**	4.48 **a**	3.36 **a**
DIC incorporation relative to root system				
Incorporation rate (µmol $^{14}C.g^{-1}$ FW.min^{-1})	0.47 **a**	0.44 **a**	0.70 **b**	0.61 **ab**
Contribution (%)	41.64 **a**	56.70 **b**	52.13 **ab**	47.87 **ab**

P deprivation did not affect $DI^{14}C$ incorporation into the organic acid fraction of either roots or nodules (Fig. 6a). However, in both P treatments, nodules had lower $DI^{14}C$ incorporated into organic acids. Although P deprivation had no effect on $DI^{14}C$ incorporation into amino acids, nodules had higher ^{14}C levels in amino acids than roots at LP (Fig. 6b).

Furthermore, the ratios of ^{14}C in organic acid : amino acids and C:N were unaffected by P deprivation in both nodules and roots. However, under low P supply, the nodules had a lower organic acid: amino acid ratio, than roots (Tab. 1). Reduced P supply had no influence on ^{14}C assimilation into the neutral fraction, but at both P concentrations, more ^{14}C resided in the neutral fraction of nodules than roots (Fig. 6c). Compared to roots, nodules had a higher percentage contribution to the total $DI^{14}C$ in the whole root system under low P supply (Tab. 1).

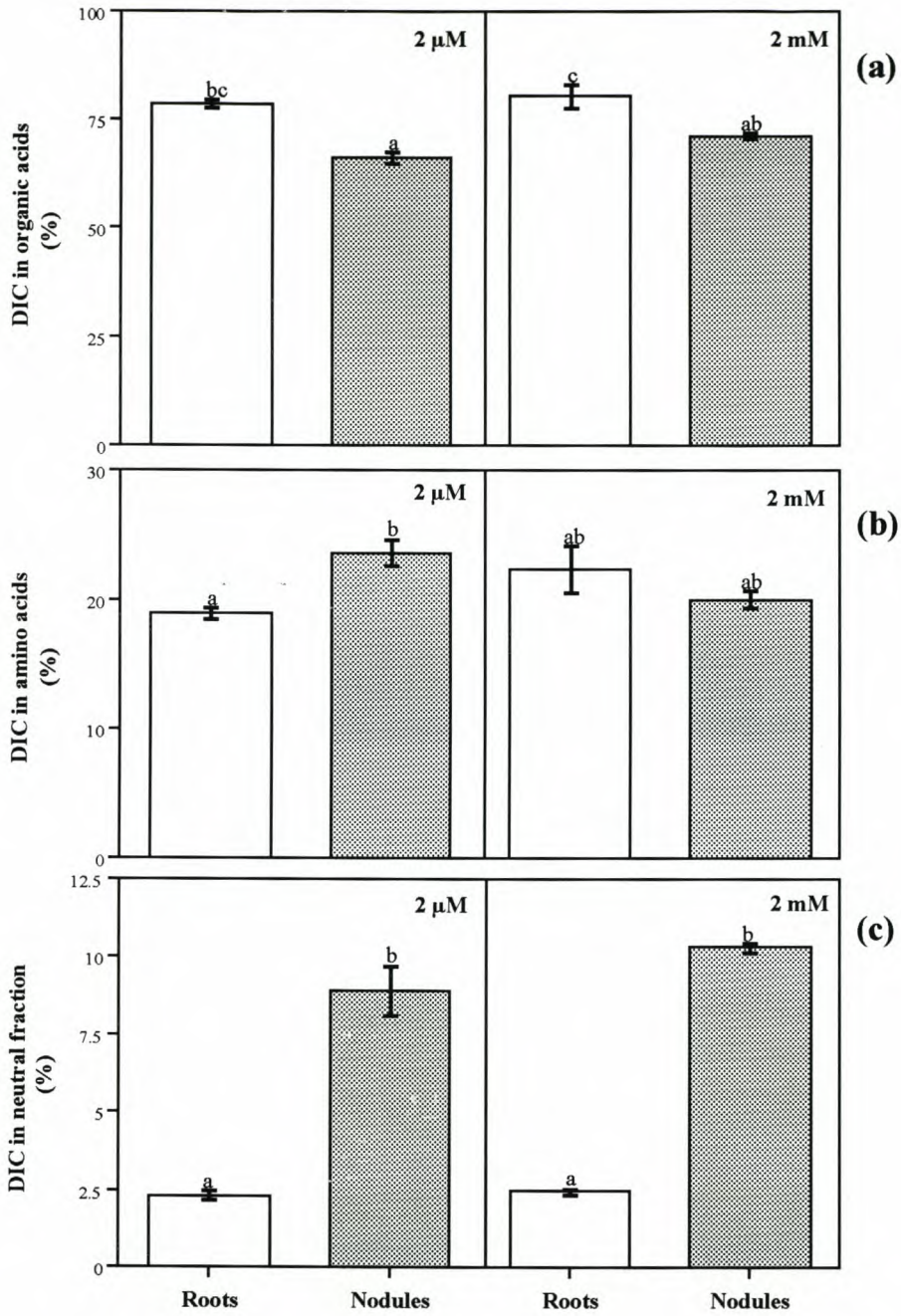

Figure 6. Percentage DIC present in the soluble fractions; Organic acids (a), amino acids (b) and carbohydrates (c) of roots and nodules from *Lupinus angustifolius* (cv. Wonga) after 60 minutes of $DI^{14}C$ exposure. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

In order to assess the extent to which nodules can incorporate $DI^{14}C$ independently of roots, excised nodules were labelled for 5 minutes. Excised nodules, in sucrose solution without a root piece, were able to incorporate ^{14}C more effectively under P deprivation than at HP (Tab. 2). Only at LP did the excised nodule, with the attached root segment, incorporate less ^{14}C. Although the ^{14}C in the amino acid fraction was similar between HP and LP, excised nodules had a higher percentage of ^{14}C in organic acids. Consequently, ^{14}C in organic acid : amino acid and C:N was higher in excised nodules at LP than HP.

Table 2. Incorporation parameters, of roots and nodules from *Lupinus angustifolius* (cv. Wonga) plants after 60 minutes of $DI^{14}C$ exposure. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

Incorporation parameters after 5 minutes of $DI^{14}C$ exposure	**Low Phosphate (2 µM)**		**High phosphate (2 mM)**	
	Detached Nodule *	**Attached Nodule ***	**Detached Nodule ***	**Attached Nodule ***
$DI^{14}C$ concentration (µmol $C.g^{-1}$ fw)				
Soluble fractions	5.86 **b**	1.26 **a**	2.82 **a**	2.48 **a**
Insoluble fractions	10.57 **b**	1.46 **a**	3.40 **a**	3.27 **a**
Lipid fractions	0.29 **c**	0.19 **bc**	0.12 **ab**	0.05 **a**
Total incorporation	19.11 **b**	2.14 **a**	6.32 **a**	6.17 **a**
DIC in soluble fractions (%)				
Amino acids	21.59 **a**	26.91 **a**	26.57 **a**	28.10 **a**
Organic acids	65.70 **b**	54.17 **a**	54.23 **a**	50.40 **a**
^{14}C-C:^{14}C-N ratio				
OA:AA	2.90 **b**	2.48 **ab**	1.85 **a**	1.84 **a**
C:N	3.51 **b**	2.70 **ab**	2.59 **a**	2.74 **ab**
DIC incorporation relative to root system				
Incorporation rate (µmol $^{14}C.g^{-1}$ $FW.min^{-1}$)	2.34 **b**	0.43 **a**	2.00 **b**	1.31 **ab**
Contribution (%)	64.87 **b**	35.13 **a**	58.04 **b**	41.96 **a**

3.4.4 N_2 – *fixation of detached- and attached nodules*

There was a decline in acetylene reduction activity of LP attached nodules versus the same tissues of plants at optimal P (Fig. 7). Detached- and attached nodules from both treatments, however, had similar N_2 –fixation capabilities based on a FW basis.

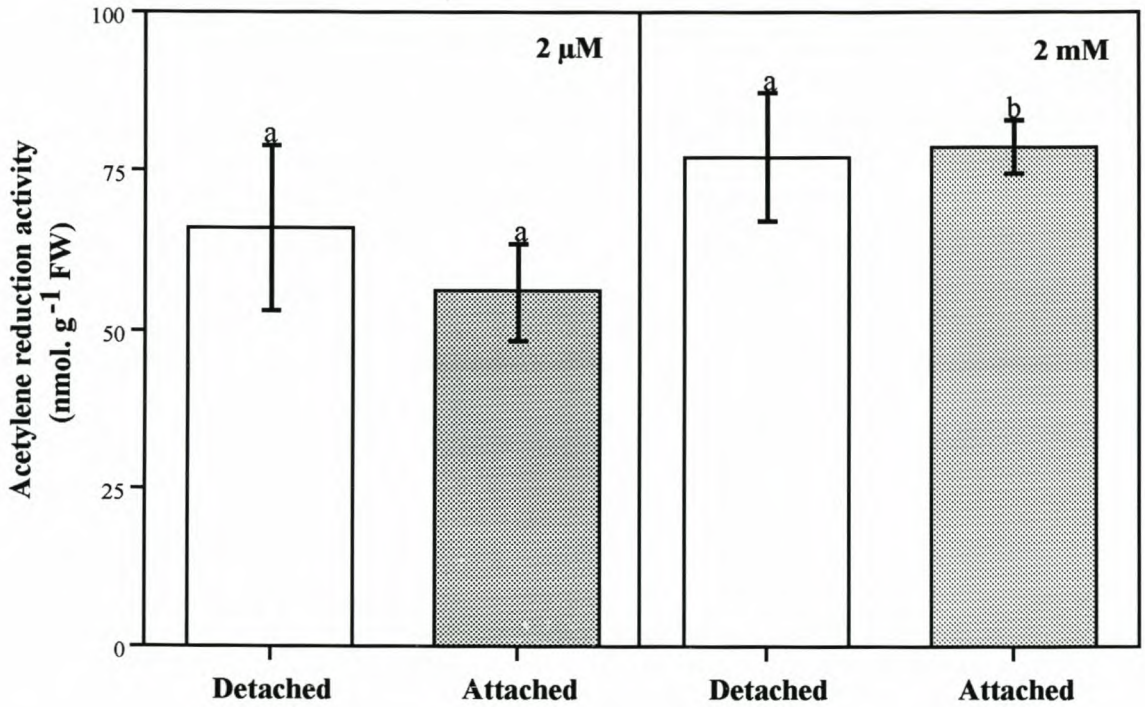

Figure 7. Acetylene reduction activity in nmol. g^{-1} FW of detached (excised from root) and attached nodules (nodules attached to root piece) from *Lupinus angustifolius* (cv. Wonga. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of low (2 µM P), whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

3.5 Discussion

The 60% decline in cellular and cytosolic P*i* concentrations in the host roots indicate that the root fraction of the symbiosis was under P*i* stress. This corresponds with the decrease in root ATP concentration and the increase in ADP:ATP ratio under low P supply. These findings concur with previous P stress studies (Fredeen *et al.*, 1991; Theodorou *et al.*, 1991), of significant declines in ATP relative to ADP under P limitation. In contrast to roots, the nodules maintained optimal P*i* levels and subsequently constant ATP concentrations, despite the changes in P supply of the medium. These constant P*i* levels indicate that metabolic P*i* concentrations are maintained for optimal nodular function. This is consistent with findings by O'Hara *et al.*, (1988), that free-living nodular bacteria require approximately 5 μM P for growth and survival.

Previous work on the effects of P deficiency on legumes has found a decline in total P concentrations, during a similar period of P deprivation (Sa & Israel 1991), but cannot be easily compared to the current findings where metabolically available P*i* was used. Although Sa and Israel (1991) also found that ATP concentrations in the nodules declined with P deficiency, it cannot be directly compared to the unchanged ATP levels of the current study. This discrepancy is due to Sa and Israel (1991) using soybean, a ureide-exporting legume, whilst the current investigation used lupin, an amino-acid exporting legume. It has been proposed that for these two legume types, there would be major differences in the TCA cycle regulation (Lodwig and Poole, 2003), which should impact on ATP pools.

The changes in adenylate and P*i* concentrations consequently affected the metabolism of PEP, via PEPc. Under conditions of P*i* stress, most plants adapt their metabolism accordingly and are able to switch to alternate routes not directly subjected to adenylate control (Duff *et al.*, 1989; Theodorou and Plaxton, 1993, Jusczuk and Rychter 2002). By maintaining constant P*i* and adenylate concentrations, the unchanged nodular PEPc under P stress is a first report, whilst root PEPc increased with P deficiency as found in previous plant studies (Duff *et al.*, 1989; Theodorou and Plaxton, 1993, Jusczuk and Rychter 2002).

The similar DI^{14}C incorporation rates of nodules after 60 minutes concur with the findings of unchanged nodular PEPc activities at the two P levels. This suggests that the nodules maintain an optimal metabolism under low P*i* levels and therefore would not need an increase in PEPc activity to engage the non-adenylate requiring alternative route of PEP metabolism. The lower concentration of organic acids in nodules compared to roots may not reflect lower incorporation, but a higher utilisation of organic acids by the nodules.

This extends from the nodular requirement of organic acids such as malate, for bacterial respiration and NH_4^+ assimilation (Vance *et al.*, 1983; Rosendahl *et al.*, 1990). This possibility is mirrored by the increased amino acid percentages, indicating that organic acid may be used as carbon skeletons for NH_4^+ assimilation (Vance *et al.*, 1983). This substantiates the suggestion that nodules are functioning optimally even under low phosphate supply. The higher organic acid:amino acid ratio in LP roots than LP nodules, further indicates that organic acids are a major fuel for metabolism in nodules.

Although excised nodules were also able to fix $DI^{14}C$ (Sutton and Jepsen, 1975), the increased incorporation under LP conditions only in detached excised nodules, may be related to sucrose in the medium. Since sucrose was only present in the medium of detached excised nodules, it may have supplied more glycolytic carbon to PEPc in comparison to the attached excised nodules.

Nodular PEPc activity has been strongly associated with N_2 fixation, due to the requirement of organic acids for bacteroid metabolism (Rosendahl *et al.*, 1990) and NH_4^+ assimilation (Maxwell *et al.*, 1984). Since the lower N_2 fixing capacity of the attached nodules under LP conditions does not concur with the unchanged PEPc activities in P starved nodules, other factors such as energy status could also have influenced the ARA. Ching *et al.* (1975) found that nitrogenase activity can be limited as a consequence of the reduced energy supply as ATP, changed energy state and decreased reductants.

To conclude, under P_i stress, nodules are able to maintain their P_i and adenylate levels, possibly at the expense of the root. This results in no significant changes in the nodular incorporation of $DI^{14}C$ by PEPc. From preliminary experiments with excised nodules under P_i stress, it can be speculated that the P_i requirement of nodules for N_2 assimilation is fulfilled from host root reserves.

3.6 Acknowledgements

The authors wish to thank the University of Stellenbosch and the National Research Foundation (NRF) for financial support and the Botany Department for the use of their facilities. We are also grateful to Mr. Loubscher Wille from Agricol, Pty. Ltd.for supply of seeds, inoculum and technical advice.

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Chapter 4

Route of PEP metabolism in *Lupinus angustifolius* under P*i* stress

To be submitted to *New Phytologist*.

Chapter 4

Route of PEP metabolism in *Lupinus angustifolius* under P*i* stress

Running title: **PEP metabolism in leguminous roots**

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4.1 Abstract

Routes of phospho*enol*pyruvate (PEP) metabolism were studied in phosphate-starved lupins.

Lupinus angustifolius (cv. Wonga) seeds inoculated with *Bradyrhizobium* sp. (*Lupinus*) were cultured hydroponically and aerated with ambient air. Nutrient solutions contained either low phosphate at 2 μM (LP) or adequate phosphate at 2 mM (control). Under LP, root P*i* declined, whilst nodular P*i* remained constant. LP roots synthesized more pyruvate from malate than LP nodules. PEPc expression was unaffected by P supply, but in roots PEPc activities increased with low P*i* and malate levels.

Permanent P*i* stress in nodules resulted in lower levels of malate-derived pyruvate than in roots. Root and nodular PEPc were not regulated by expression, but possibly by posttranslational control. Enhanced PK activities supported the high pyruvate levels that are required at LP. Metabolism of PEP via both the PK and PEPc routes, indicate the importance of pyruvate synthesis during P*i* stress in roots and nodules.

Key words: *Lupinus angustifolius*, PEPc, P-deficiency, alternative route

4.2 Introduction

Although the role of dark CO_2 fixation via PEPc has been well documented in leguminous plants, this information is mainly confined to ureide-exporting legumes (Streeter, 1991). PEPc activity has consistently been demonstrated to be higher in nodules when compared to uninoculated roots in a variety of legumes (Lawrie and Wheeler, 1975; Duke *et al.*, 1979; Deroche *et al.*, 1983). This has been suggested to be the result of the presence of an additional isoform of PEPc in nodules compared to roots (Marszewski, 1989). In nodulated plants the route via PEPc can be more important, particularly in amide-exporting legumes, as one of its major roles is proposed to be the provision of C skeletons for the assimilation of NH_4^+ (Christeller *et al.*, 1977; Rosendahl *et al.*, 1990). The non-photosynthetic CO_2 fixation route via PEPc, has also been implicated as the pathway through which the increased synthesis of OAs occurs, which serve either to replenish the TCA cycle or alternatively these OAs are being exuded to solubilize bound P in a P limiting environment (Johnson *et al.*, 1996a; Neumann and Römheld, 1999). Thus the major products of the PEPc pathway are dicarboxylic acids and in legumes such products, in addition to replenishment and exudation (Johnson *et al.*, 1996a; Neumann and Römheld, 1999), also supplement the bacteroids in the symbiosome (Rosendahl *et al.*, 1990).

The activity of PEPc is usually positively induced under conditions of P deprivation (Johnson *et al.*, 1996 a,b; Toyota *et al.*, 2003). The effect of P nutrition in diverting C partitioning is well documented (Freeden *et al.* 1989; Rychter and Randall, 1994). Johnson and co-workers (1996b) showed that this increase in PEPc activity coincided with the increase in PEPc transcripts and the amount of PEPc proteins.

PEP comprises a major branchpoint for carbon (C) and nitrogen (N) metabolism in higher plants (*see review by* Chollet *et al.*, 1997). Another key enzyme known to control and integrate plant C flux through the PEP branchpoint, is PK (Smith *et al.*, 2000). This enzyme catalyzes the transfer of a phosphate group from PEP to ADP to yield ATP and pyruvate. Therefore, apart from the generation of pyruvate through the conventional route of glycolysis via the PK reaction, it can follow an alternative route, not subjected to adenylate control, via the concerted action of cytosolic PEPc and MDH and mitochondrial ME (Duff *et al.*, 1989; Jusczuk and Rychter, 2002). Another possibility is for PEP to be metabolized in the vacuole via vacuolar PEP phosphatase (PEPp) (Duff *et al.* 1989). The transport of PEP into, and pyruvate out of the cell vacuole, however, has yet to be elucidated. The concentration of pyruvate in the cell has been proposed to be the result of the dynamic processes of pyruvate synthesis (e.g. product of carbohydrate oxidation) and utilization (e.g. fermentation pathways, amino acid synthesis) in the cytosol and mitochondria (Juszczuk and Rychter, 2002). Elevated pyruvate levels in roots are thought to act as an antioxidant, acting as a scavenger of radical oxygen species produced by the electron transport chain (Juszczuk and Rychter, 2002). Similarly in symbiotic roots, bacteroids were shown to actively take up pyruvate, but this pyruvate were not used in support of nitrogenase activity (McRae *et al.*, 1984).

The success of plants subjected to unavoidable stress conditions (*e.g.* P deprivation) may be determined by their ability to control carbohydrate utilization for metabolic energy (Nielsen *et al.*, 2001). Furthermore, forming associations with soil microbes can be of critical importance in order to attain vital nutrients, which is essentially

unavailable for plant use. Evidently, forming such symbiotic associations as well as certain environmental constraints (e,g. anoxia, P starvation) dictate to a large extent the metabolic complexity of various pathways. The integration of cellular metabolism necessitates controlled interactions between pathways sequestered in various cellular compartments (Plaxton, 1996).

There is some evidence indicating that during P starvation in leguminous roots systems PEPc is subjected to phosphorylation by an endogenous protein kinase, which renders it less sensitive to inhibition by L-malate (Schuller and Werner, 1993). In spite of this evidence, the regulatory properties of non-photosynthetic PEPc are fragmented and rudimentary. An ongoing and challenging problem has been to elucidate the respective role(s), regulation and relative importance of the various alternative reactions of plant cytosolic glycolysis (Plaxton, 1996). It is postulated that the decline of adenylates and *Pi* under P stress should restrict pyruvate synthesis from PEP via pyruvate kinase (PK). To date these P stress-induced reactions have not been investigated in roots and nodules of symbiotic legume root systems.

The objective of this study is to assess the effect of P deprivation on root and nodule C metabolism at the PEP branchpoint. It will be examined whether there is a substantial difference in the way roots and nodules engage the alternative routes of PEP metabolism under P stress. This will be assessed via the routes of pyruvate synthesis, using enzyme activities and the anaplerotic ^{14}C incorporation into metabolites.

4.3 Materials and methods

4.3.1 Plant growth conditions

All seeds were grown in vermiculite, which was commercially irradiated by a cobalt C-60 source of gamma radiation at a dose of 18 kGray. Pots measuring 10 cm in diameter were washed in Ekon-D and rinsed in distilled water, then dried. The pots were then filled with vermiculite.

For all experiments, seeds of *Lupinus angustifolius* (cv. Wonga) were inoculated with a rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria. Seeds of lu*Pi*ns were coated in a saturated sucrose solution and 2 g of inoculum *per* 150 seeds was added and mixed. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in the pots containing vermiculite.

Seeds were germinated during May and June in an east-facing glasshouse at the University of Stellenbosch, Stellenbosch, South Africa. The range of midday irradiances was between 540-600 μmol m^{-2} s^{-1} and the average day/night temperature and humidity were 23/15 °C and 35/75 % respectively. Pots were watered daily with distilled water until seeds germinated. Upon germination seedlings were watered once every two days for three to four weeks, until nodule formation had occurred. Once nodule formation was established, the seedlings were transferred to 22 litre hydroponic tanks under the same glasshouse conditions. The tanks contained a modified Long Ashton nutrient solution modified to contain no inorganic N, 2 mM PO_4 and 5 μM MES (pH 6). Solutions were changed twice weekly. The hypocotyls of seedlings were wrapped with foam rubber at their bases and inserted through holes in the lids of the tanks. Each tank

was supplied with an air supply line, which bubbled air containing 360 ppm CO_2. Once nodules were established and of adequate size, usually in the third or fourth week of hydroponic growth, plants were divided into two treatments. Two of the four hydroponic tanks were supplied with nutrient solution containing adequate (2 mM PO_4) (control), the other half were supplied with nutrient solution containing low P (2 µM PO_4) (LP treatment). P starvation was induced for 14 days, after which plants were harvested.

4.3.2 Inorganic phosphate level determinations

Approximately 0.5 g of tissue (roots and nodules) were homogenized in 10 % (w/v) TCA (0.6 ml) in a pre-chilled mortar and pestle. The homogenate was diluted three times with cold 5 % (w/v) TCA. Extracts were centrifuged for 10 min at 30 000 g. The inorganic phosphate concentration in the supernatant was determined by a modified Fiske-Subarrow method as described Rychter and Mikulska (1990).

4.3.3 Calculations

It was assumed that in roots the cytosol occupies approximately 10 % of total cell volume and in the nodules the cytosol occupies approximately 50 % of total cell volume (Rolin *et al.*, 1989). From these assumptions the cytosolic concentration of P_i was calculated for both components.

4.3.4 Protein extraction

The extraction was performed according to Ocaña *et al.* (1996) modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20 % (v/v) ethylene glycol, plus 2 %

(m/v) insoluble polyvinylpoly pyrrolidone (PVPP) and one complete protease inhibitor cocktail tablet per 50 ml of buffer. Extractions were performed in a pre-chilled mortar and pestle. Resulting homogenates were centrifuged at 30 000 g for 10 min at 4 °C. The pellets were discarded and the supernatants, designated the crude extracts, were used in further assays.

4.3.5 Enzyme assays

Phosphoenolpyruvate carboxylase: PEPc activity was determined spectrophotometrically by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 30 °C. The standard assay mixture contained 100 mM TRIS (pH 8.5), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 4 mM PEP, 0.20 mM NADH, 5 U MDH (Ocaña *et al.*, 1998).

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

NADH-Malate dehydrogenase: MDH was assayed as described by Appels and Haaker (1988). The reaction mixture contained 25 mM KH_2PO_4, 0.2 mM NADH, 0.4 mM OAA. The pH was adjusted to 7.5 with 1 mM HCl (Appels and Haaker, 1988).

Malic enzyme: This assay monitored the increase in absorption at 340 nm due to the formation of NADPH or NADH. The assay mixture contained 80 mM TRIS-HCl (pH 7.5), 2 mM $MnCl_2.4H_2O$, 1 mM malate and 0.4 mM NADP or NAD^+ (Appels and Haaker, 1988).

All the reactions were initiated by adding 30 µl crude extract to reaction mixture in a total volume of 250 µl. Initial reaction rates have been shown to be proportional to the concentration of enzyme used under the conditions used.

4.3.6 Protein determination

The protein concentration was determined by the procedure of Bradford (1976) using a protein assay reagent (Bio Rad) and bovine serum albumin (BSA) as standard.

4.3.7 SDS-Polyacrylamide gel electrophoresis and western blotting

Soluble proteins in cell free extracts were separated by electrophoresis in 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose as described by Miller *et al.* (1987). Each lane was loaded with 15 µg of protein for roots and 30 µg for nodules. C.P. Vance supplied the antibody. Specificity of antibody was determined by immunotitration of PEPc activity (Miller *et al.*, 1987).

Rabbit polyclonal antibodies to alfalfa nodule PEPc were used to detect and ascertain relative PEPc enzyme protein on western blots (Miller *et al.*, 1987). In a separate experiment lanes were loaded with 5, 10, 15, 20, 25, 30 and 35 µg of protein respectively and again probed with rabbit polyclonal antibodies to alfalfa nodule PEPc.

4.3.8 Separation and collection of malate and pyruvate fractions through HPLC analysis

High performance liquid chromatography (HPLC) separations were made isocratically on a 30 × 0.78 cm Bio-Rad Aminex Ion Exclusion HPX-87H organic acid column. HPLC analysis was carried out on an Alliance 2690 Separations Module equipped with a 996 Photodiode array detector (Waters). The mobile phase consisted of 30 mM H_2SO_4 at a flow rate of 0.6 $ml.min^{-1}$. Eluting peaks were detected by ultraviolet absorption at

247 nm and at a column temperature of 50°C. The system was calibrated with known standards (0-100 mM of Malate and Pyruvate) for quantification and determination of retention time, and was co-chromatographed with the sample for identification. Data analysis was done using Millenium[32] Chromatography software (Waters). In addition the organic acid fractions of interest were manually collected for the determination of radioactivity in the specific organic acids (*i.e.* malate and pyruvate). Radioactivity measurements were made on a LSC.

4.3.9 Statistical analysis

All data was analysed by single ANOVA. Percentage data was arcsine transformed prior to analysis and ratios were square root transformed prior to analysis. All data was then subjected to a post-hoc LSD test to determine significance.

4.4 Results

4.4.1 Changes in Pi levels of roots and nodules

There were 48 % lower cellular P*i* levels from the adequate phosphate (2 mM) treatment to the low phosphate (2 µM) treatment for roots, whilst for nodules the P*i* levels for the corresponding treatments remained unchanged (Fig.1a). A similar pattern was observed in the cytosol of roots and nodules with LP. P*i* levels within nodules ranged from 10-20 µM in the cytosol.

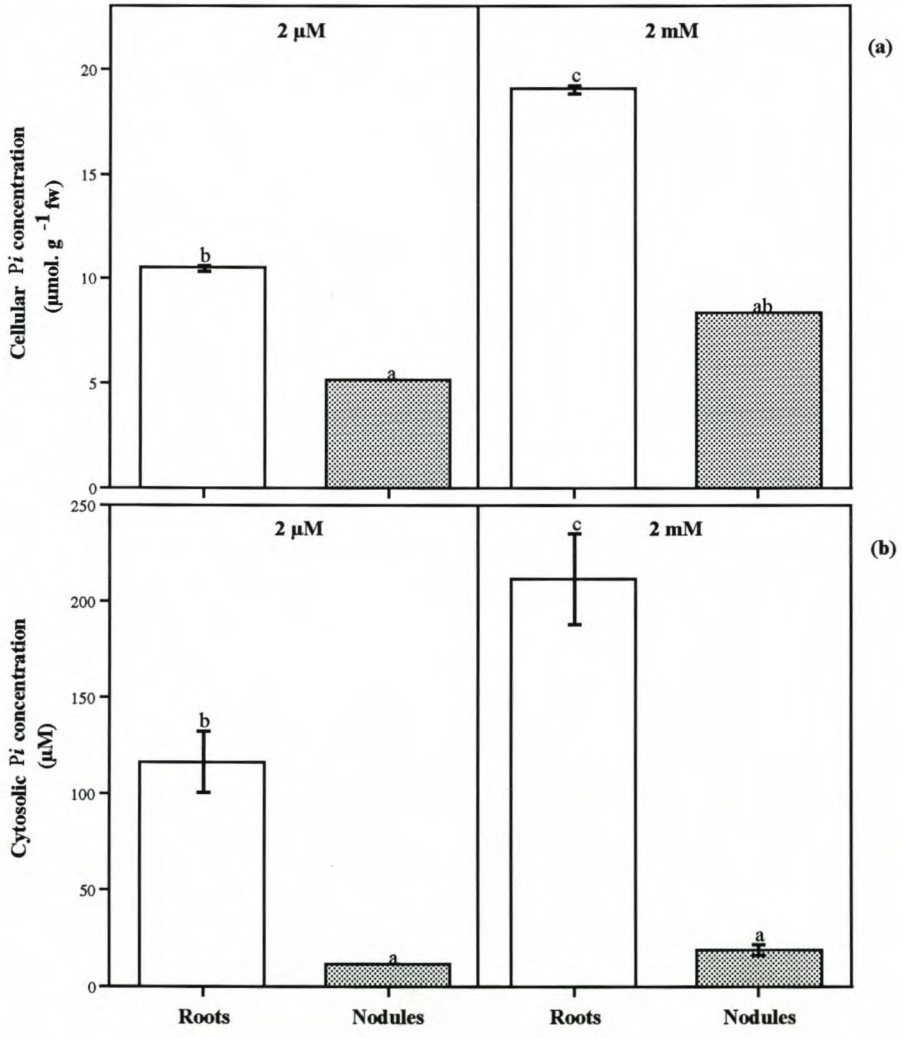

Figure 1. (a) Cellular P*i* concentration (µmol. g^{-1} FW) and (b) cytosolic P*i* concentration (µM), of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 5 replicates ($n = 5$).

4.4.2 Enzymes involved in pyruvate production

The decline in P*i* levels induced a 2-fold increase in the PEPc activity of roots, whilst for nodules PEPc activity remained constant between LP treatment and adequate P (Fig. 2).

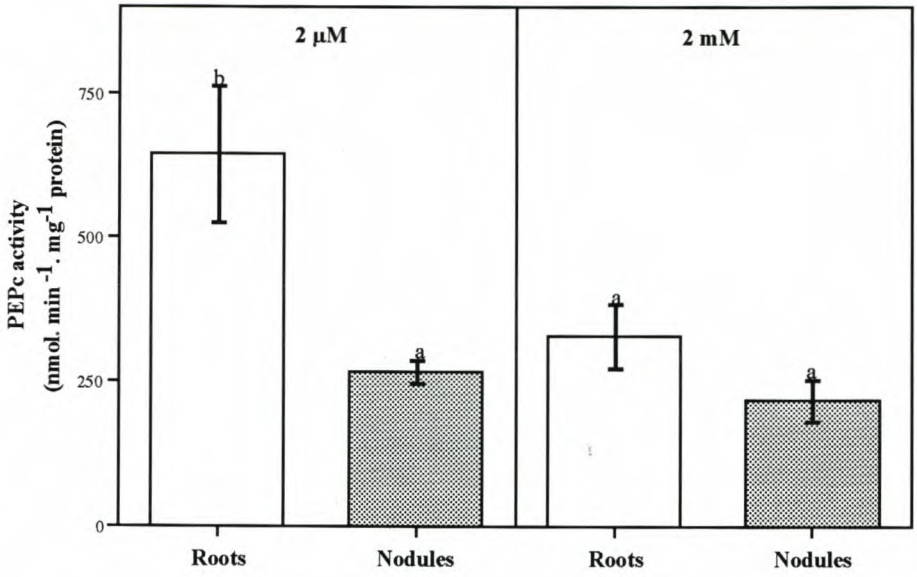

Figure 2. *In vitro* specific activity of PEPc (nmol. min^{-1}. mg^{-1} protein) determined spectrophotometrically at A_{340} for roots and nodules of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

A concomitant increase in the activities of PK was observed for roots (Fig. 3a). This represented an increase in activity of approximately 80% in roots. This increase in activity was less pronounced in nodules, with an observed increase of approximately 35% under LP supply (Fig. 3a).

MDH activity showed no change during P starvation of roots (Fig. 3b), whilst for LP nodules the activity of MDH increased by 90%. During LP, (NADP)-ME (cytosolic form) activities were higher in both roots and nodules deprived of P (Fig. 3c). This represented a 55% increase in activity for both these compartments, *i.e.* roots and

nodules, under P limitation. The mitochondrial form, (NAD^+)-ME activity of roots was stimulated by 70 % under LP conditions (Fig. 3d). The percentage increase in the activity of (NAD^+)-ME under LP conditions was less pronounced in nodules, which only showed an increase by 40 % (Fig. 3d).

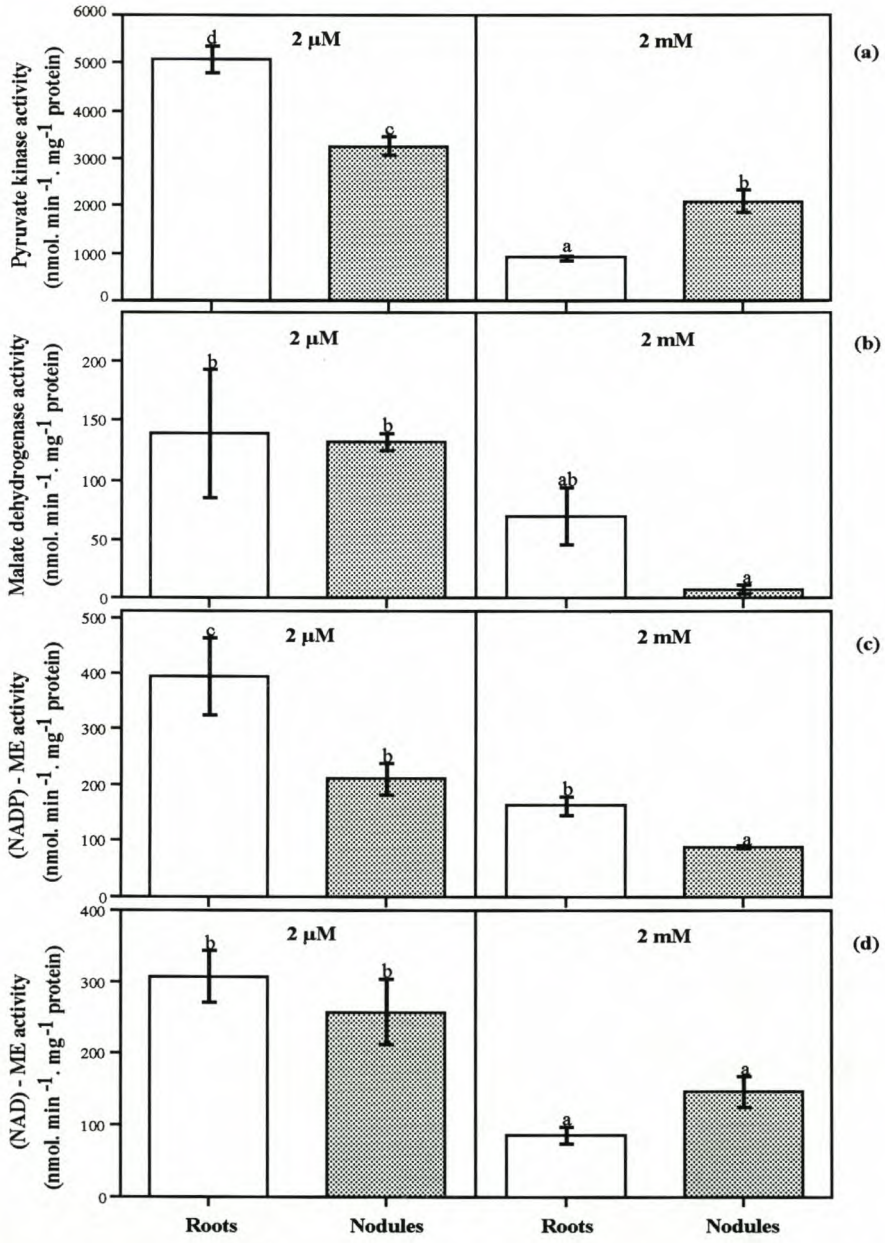

Figure 3. *In vitro* specific activity (nmol. min^{-1}. mg^{-1} protein) of (a) pyruvate kinase (PK); (b) malate dehydrogenase (MDH); (c) NADP-ME and (d) NAD^+-ME determined spectrophotometrically at A_{340} for roots and nodules of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

4.4.3 Western blot analysis of PEPC protein

Western blot analysis of PEPc protein indicates that there are two polypeptide bands present in nodules ranging from 100-110 kD in weight (subunit molecular weight of PEPc is between 100 and 110 kD, Miller *et al.*, 1997) whilst for roots there is only one (Fig. 4 a & b). Furthermore, the PEPc polypeptides in roots and nodules were unaffected by a decline in available P (Fig. 4 a & b).

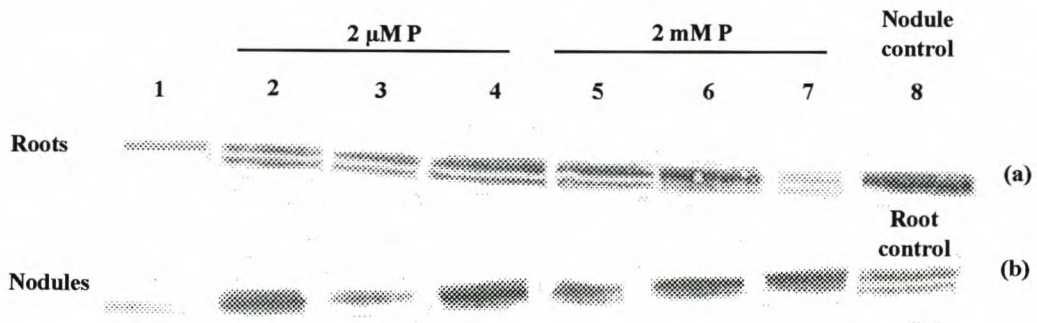

Figure 4. Western blot analysis of (a) root PEPc and (b) nodular PEPc with a polyclonal PEPc antibody from alfalfa nodule PEPc. Lane 1 contains the standard marker. Each lane was loaded with 15 µg of protein for roots and 30 µg for nodules. Lanes 2-4 indicate PEPC from plants that was subjected to LP (2 µM). Lanes 4-6 show plants that was subjected to adequate P (2 mM). Lane 8 contains the nodular control and root control, for (a) and (b), respectively.

4.4.4 Metabolite concentrations and flux through the PEPc bypass

Phosphate supply had varying effects on malate and pyruvate concentrations, in roots and nodules. Under LP supply malate concentrations in roots increased by at least 10-fold, yet for nodules malate concentrations remained constantly low, regardless of P supply (Fig. 5a). A similar pattern was observed with pyruvate conentrations for both compartments, with roots showing a 60% increase in concentrations under LP supply as oppose to the unchanged levels in nodules (Fig. 5b).

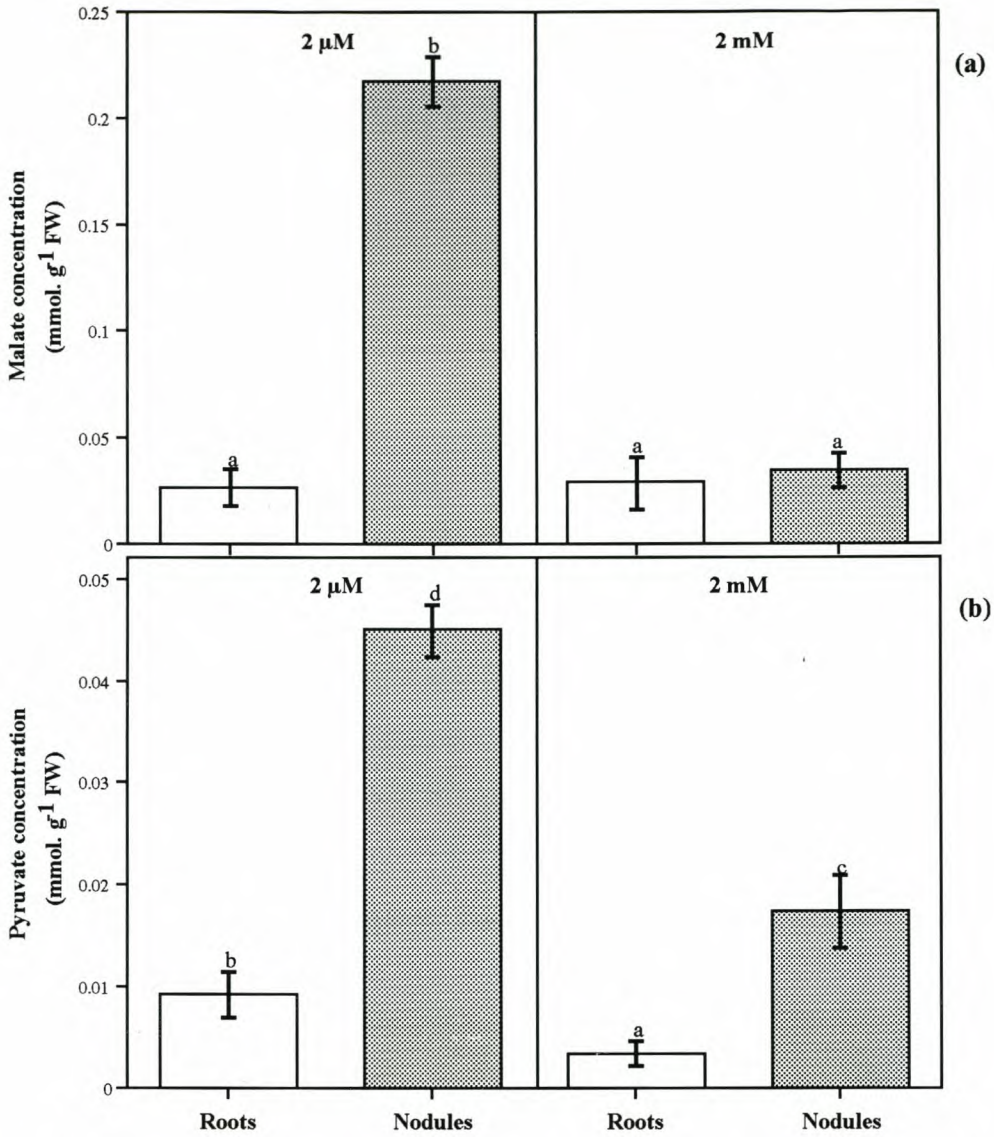

Figure 6. (a) Malate- and (b) pyruvate concentrations (mmol. g^{-1} FW) of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the adequate P plants (HP) remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

The exposure to LP influenced the ^{14}C partitioning into pyruvate. This increase was more pronounced in roots at LP, whilst the malate derived pyruvate pool remained low for nodules, regardless of the P levels. Roots had higher ^{14}C enrichment in pyruvate at LP concentrations compared to adequate P.

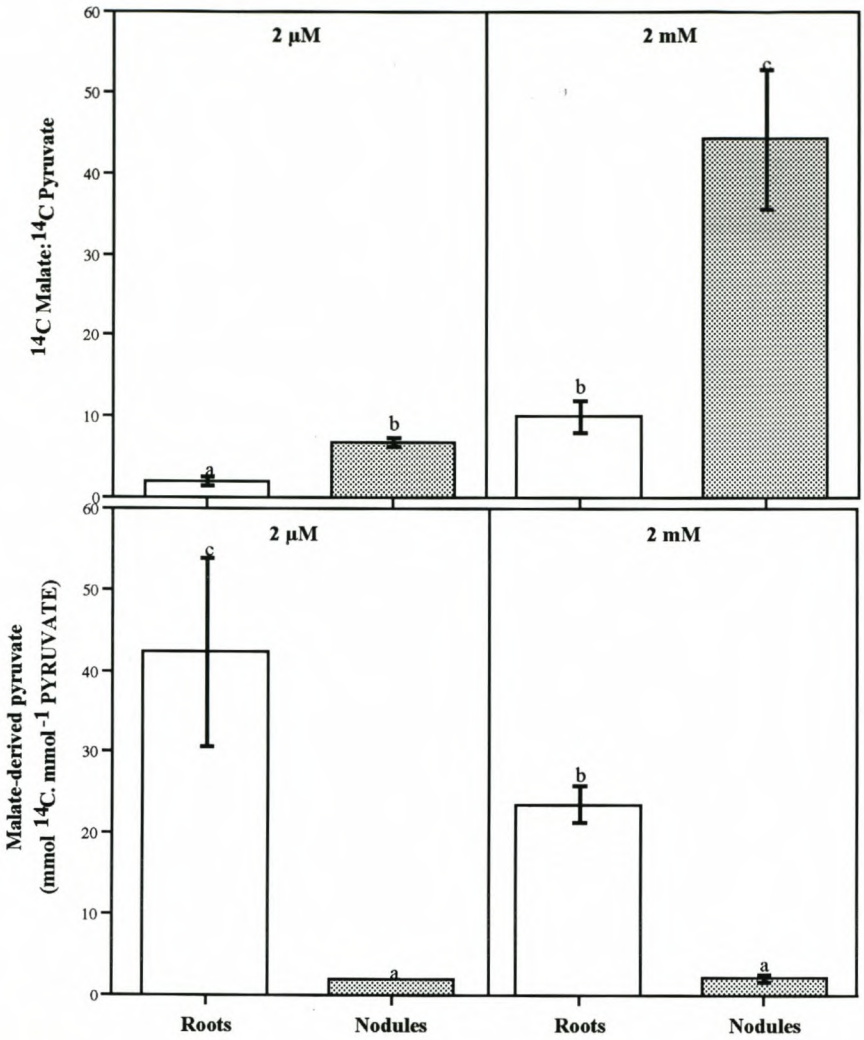

Figure 7. Specific malate-derived pyruvate concentration (mmol ^{14}C. mmol^{-1} PYRUVATE) of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm CO_2. P starvation in was induced for 14 days in the low P plants (LP) with the supply of 2 µM P, whilst the control plants (HP) remained at 2 mM P supply. Different letters indicate significant differences between each treatment ($P \leq 0.05$), based on a SNK multiple range test. The values represent the means of 3 replicates ($n = 3$).

4.5 Discussion

The constant P levels (10-20 μM) observed for nodules at normal (2 mM) and low (2 μM) P supply indicate that metabolic P*i* concentrations are maintained for optimal nodular function. This concurs with other findings that free-living nodular bacteria require approximately 5 μM P for growth and survival (O'Hara *et al.*, 1988). Furthermore, in legume tissues, total P concentrations of 15 μM were found to be toxic to the host plant only when inorganic N was limiting (Bell *et al.*, 1990). It therefore appears that nodules have a strategy in place to regulate P influx. This allows nodules to minimize effects of P deficiency when supply is low and avoid excess when P is high (Tang *et al.*, 2001). Due to the low P*i* concentrations required by the bacteria (O'Hara *et al.*, 1988), the optimal P*i* levels are maintained either by efficient P*i* utilisation under low P supply (Beck and Munns, 1984), or storage as polyphosphates under normal P supply (Cassman *et al.*, 1981).

Furthermore, it is also possible that these constant P*i* levels in the nodules are maintained by the bacteriods at the expense of the host roots. Bacteroids in nodules have been shown to take up P from their immediate environment, by utilising high-affinity uptake systems (Smart *et al.*, 1984; Bardin *et al.*, 1996; Al-Niemi *et al.*, 1997). Moreover, under conditions of limiting P supply, the nodules have been shown not to make the acquired P available to the host plant (Al-Niemi *et al.*, 1998).

Based on the evidence presented here, the changes in PEPc activities may be related to P*i* levels. The constant nodular PEPc activity concurs with the unchanged P*i* status of nodules during P limitation and adequate P supply. It follows naturally that PEPc activity remains constant since numerous workers (Johnson *et al.*, 1996; Juszcuk and

Rychter, 2002, Toyota *et al.*, 2003) reported that PEPc is sensitive to P*i* limitation. The root P*i* stress induced the increased PEPc activities as a possible alternative route to circumvent the adenylate-requiring PK reaction (Johnson *et al.*, 1996; Juszcuk and Rychter, 2002, Toyota *et al.*, 2003).

A consequence of the PEPc reaction is the synthesis of malate via MDH (Duff *et al.*, 1989; Plaxton 1996), which can subsequently be metabolised to pyruvate via ME (Juszcuk and Rychter, 2002). Malate is an allosteric inhibitor of PEPc activity (Schuller and Werner 1993). Therefore the constant concentrations of malate in roots at both P limiting and adequate supply levels, in spite of high PEPc activity at low P, suggests that malate accumulation is rapidly prevented. The high root pyruvate concentrations, along with the increases in both forms of ME, indicate that malate may be converted to pyruvate. This concurs with the lower ratio of PEPc-derived ^{14}C in malate than pyruvate and the increase in malate-derived pyruvate (PEPc-incorporated ^{14}C) increased under P starvation. Furthermore, the unchanged P*i* levels in nodules, favoured the consistent synthesis of pyruvate from PEPc-derived malate.

Pyruvate accumulation in roots may not only arise to prevent allosteric inhibition of PEPC via malate. Pyruvate is the substrate for oxidative and/or fermentative processes and its utilization may influence the pyruvate pool (Vanlerberghe *et al.*, 1997). In the absence of data regarding utilization of pyruvate it is proposed that pyruvate levels increased as a result of its synthesis via the PEPc bypass route. These increased pyruvate levels may have acted as a mechanism to oxidize accumulating reducing equivalents (Jusczuk and Rychter, 2002). Under certain stress conditions different fermentation pathways are engaged to regenerate NAD^+, which is utilized in limited

glycolytic ATP production. The increase in the flow via PK, even under LP conditions, is unexpected since the low P*i* concentrations may not favour the ATP dependant route via PK (Theodorou and Plaxton 1993; Duff *et al.*, 1989). However, the high requirement of pyruvate under LP conditions (Tadege *et al.*, 1999), suggests the PEPc reaction may have liberated P*i* for this ATP dependant PK route.

At low P supply some of the root malate may be sequestered to the nodule, as is evident in the high nodular malate concentration. It is known that organic acids from the host can be exported to the bacteroid fraction of the nodules to synthesize malate (Rosendhal *et al.*, 1990; Vance *et al.*, 1985) for bacterial respiratory fuel. It has also been proposed that the low oxygen concentration in nodules would favour malate, rather than pyruvate as the main end-product of glycolysis (Vance and Heichel, 1991). For nodules exposed to low P, the increases in enzyme activities leading to pyruvate synthesis and the lower ratio of PEPc-derived ^{14}C in malate than pyruvate, suggest that pyruvate accumulates at the expense of malate. This may be to prevent malate allosterically inhibiting nodular PEPc, as found by Schuller and Werner (1993).

The current Western blots concur with Johnson *et al.* (1996), who also found little to no differences in root PEPc levels of P starved *Lupinus albus* plants. PEPc regulation may not only reside from the amount of protein but more likely at the post-translational level (Schuller *et al.* 1990, 1993). The findings show that PEPc may be regulated by phosphorylation from an endogenous PEPck (Schuller *et al.* 1990, 1993). Phosphorylation of nodular PEPc has been shown to reduce its sensitivity to malate inhibition (Schuller *et al.* 1990, 1993, Zhang *et al.* 1995). Furthermore, it should be considered that the absence of P stress effects on Western blots might not have revealed

substantial information regarding PEPc activity, since the blot does not distinguish between active and inactive forms of the enzyme.

In conclusion, nodules are able to maintain constant P*i* levels amid the changes in P supply and thereby had constant PEPc activities and malate-derived pyruvate synthesis. Roots however, experienced a decrease in P*i* levels with P starvation. A consequence of this P stress, was in the increased engagement of the PEPC bypass which synthesized a greater proportion of pyruvate from PEPc-derived malate. Furthermore, during P limiting conditions, the metabolism of PEP via the PK and PEPc route participation, indicate that pyruvate synthesis has a key role in metabolic homeostasis during cellular P*i* stress

4.6 Acknowledgements

The authors would like to thank the NRF for funding. We also wish to thank Fletcher Hiten for technical assistance with the western blot analysis and Dr. F Lynen for help with HPLC analysis. We would like to convey our utmost gratitude to Mr. Loubscher Wille for generous donation of seeds.

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Chapter 5

5.1 General Discussion

Plants dependent on symbiotic N_2 fixation have variable P and ATP requirements for nodule development and functioning. This P requirement is essential to maintain the established symbiosis where it serves to provide energy for N_2 fixation in the form of ATP. Furthermore the phenomenon of P stress has been investigated and it has been shown that low P supply induces P stress in nodules (Al-Niemi *et al.*, 1998). These studies reported total P and not metabolically available *Pi* as an indicator of P deficiency vs. P sufficiency. Duff *et al.* (1989) stressed that metabolically available P*i* and not total P as previously reported by other workers (Sa and Israel, 1991; Al Niemi *et al.*, 1998) gives a more accurate account of P status of tissue under evaluation.

Our results show unequivocally that nodules do not experience P stress to the same extent as roots. Implications of our findings are that nodules require low P to function normally. Maintenance of phosphate levels in nodules may be at the expense of the host root. It was demonstrated by Al-Niemi *et al.* 1998 that when nodules are P-starved they can become aggressive scavengers for available P and even out-compete roots. Low P supplied nodules take up P from the medium at a much higher rate than roots and unlike roots do not make this P available for export to shoots. These findings place the metabolic reaction of nodules in response to low P in the medium in a new light.

The main carbon branch point in glycolysis, the metabolism of PEP, has often been shown to be significantly influenced by phosphate stress (Johnson *et al.* 1996; Toyota *et al.* 2003; Freeden *et al.* 1989; Rychter & Randall 1994). In this regard, it has been demonstrated that PEPc activity levels increase under P stress in roots (Johnson *et al.*

1996), but data for nodules are absent. Our results for roots concur with previous findings, but our nodule data presents new ground that should be further explored. As there was no change in P_i levels between P treatments in nodules, it was to be expected that no increase in PEPc activity was observed. The absence of increases in PEPc activity for nodules concurs with similar DIC incorporation levels, as found in P stressed nodules. However, we expected to observe an increase in DIC incorporation in P-stressed roots, due to their increased PEPc activity. However, no increase was observed. This might be due to the fact that PEPc is substrate limited when supplied with low CO_2 (360 ppm) (Ward & Valentine, unpublished results).

Our findings of the regulation of PEPc protein levels concur with previous studies in that PEPc is thought to be at least partially under post-translational regulation as no changes were observed between P treatments (Johnson *et al.* 1996; Schuller & Werner 1993). It has long been known that PEPc is an allosteric enzyme inhibited by malate and activated by glucose-6-P (Pathirana *et al.* 1992). However, more recently a posttranslational regulatory mechanism was proposed involving a reversible phosphorylation cycle. This finding reinforced the status of PEPc as a highly regulated key enzyme in plants.

Subsequent studies were devoted to the identification of the phospho*enol*pyruvate carboxylase protein kinase (PEPck) and the signalling mechanisms controlling its activity. PEPck catalyses a reaction, which opposes the irreversible reaction catalysed by PEPc. The presence of both these enzymes in the cytosol means that they require strict regulation in order to avoid a futile cycle, which leads to the hydrolysis of ATP. Recent evidence suggests that, in many plant tissues, both these enzymes are regulated

by phosphorylation. Thus, PEPck is implicated in the co-ordination of carbon and nitrogen metabolism in plants in much the same manner as is already recognised for PEPc (Leegood & Walker 1999; Leegood *et al.* 1999) (Walker *et al.* 2002; Nimmo, Wilkins & Nimmo 2001).

The phosphorylated form of PEPc is less susceptible to feedback inhibition by malate and more susceptible to activation by the allosteric modulator glucose-6-P. Comparatively few metabolites have large effects on the activity of PEPck (Chollet *et al.* 1996).

To summarise, PEPc shows no change in levels of expression under phosphate starvation. However, this study did not investigate the different isoforms (one in the root and two in the nodules). It is possible that, although there was no observed change in nodule PEPc protein levels, this was due to the fact that the blot distinguished neither between the two isoforms nor between active and inactive enzyme. Further work should aim to investigate whether there is a differential expression/regulation of PEPck between isoforms and compartments, similar to that proposed for PEPc.

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