# Phospho*enol*pyruvate (PEP) metabolism in roots and nodules of *Lupinus angustifolius* under P stress

**by**

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(Botany Department)



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**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.

Signature :

Date :

## SUMMARY

This study investigated the activities of several of the enzymes involved in the alternative route of PEP metabolism via PEPc (EC 4.1.1.31). This reaction circumvents the adenylate-controlled PK (EC 2.7.1.40) reaction of the conventional glycolytic network under conditions of P stress. It was hypothesized that the synthesis of pyruvate under P*i* stress would induce the PEPc alternative route and that C for pyruvate synthesis would primarily be imported via this route. This was assessed by looking at how total enzyme activities are perturbed under P stress and also by following the route of radioactive labelled $^{14}CO_2$ under sufficient (2 mM) and deficient P (2 μM) conditions in either roots or nodules. The significance of the pathway under P stress, was further assessed by determining pool sizes of pyruvate that was synthesized from PEPc-derived C. The experiments were conducted under glasshouse conditions, as two separate studies: one to investigate the phenomenon of P*i* stress and its consequences for PEPc-derived C metabolism, and the other one to study the enzymes involved. 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 $PO_4$ (control) and air containing 360 ppm $CO_2$.

Roots experienced pronounced P stress with a greater decline in P*i*, compared to nodules. LP roots synthesized more pyruvate from malate than LP nodules, indicating the engagement of the PEPc route under P*i* stress. In this regard, pyruvate is considered as a key metabolite under P*i* stress. The role of pyruvate accumulation under P*i* stress, was

further highlighted by the metabolism of PEP via both the PK and PEPc routes. The enhanced PK activities supported these high pyruvate levels. 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.

The novelty of our results for symbiotic roots demonstrates that using metabolically available P*i* is indeed a more sensitive indicator of P stress. These results show that under P*i* stress, nodules are able to maintain their P*i* and adenylate levels, possibly at the expense of the root. It is suggested that nodules do not experience P stress to the same extent as roots or alternatively function optimally under conditions of low P availability. The increase in concentration of pyruvate synthesized from malate, indeed suggest that under LP conditions there is an increase requirement for pyruvate. It is clear from this data that the operation of bypass route in nodules should be investigated further. Nevertheless, this study provided incentives for understanding the role of C pathways in $N_2$-fixation, in particular under conditions of P limitation.

# OPSOMMING

Die doel van hierdie studie was om die aktiwiteite van verskeie ensieme van die alternatiewe metaboliese roete *via* phospho*enol*pirovaat karboksilase (PEPc, EC 4.1.1.31) te ondersoek. Dié reaksie omseil die adenilaat-beheerde pirovaatkinase (PK, EC 2.7.1.40) reaksie van die konvensionele glikolitiese weg onder toestande van fosfaat (P) stremming. Dit is gepostuleer dat die sintese van pirovaat onder toestande van P-stremming die alternatiewe roete via PEPc sou induseer en dat die koolstof (C) vir pirovaatsintese gevolglik hoofsaaklik vanaf hierdie roete sou kom. Dit is bepaal deur die veranderinge in die totale ensiemaktiwiteite wat sou plaasvind onder P-stremming te ondersoek. Daar is ook gekyk na die roete wat radioaktiewe C ($^{14}CO_2$) sou volg in wortles en wortelknoppies wat behandel is deur blootsteling aan eerder lae fosfaat (2 µM) of genoegsame fosfaat (2 mM; kontrole). Die betekenis van die alternatiewe roete is ook ondersoek deur die poel-groottes van pirovaat, soos gesintetiseer *via* die PEPc reaksie, te bepaal.

Twee eksperimente is in 'n glashuis uitgevoer. Eerstens is die verskynsel van P-stremming, asook die invloed daarvan op PEPc-afgeleide C-metabolisme, bepaal. Tweedens is die betrokke ensieme bestudeer.

Sade van *Lupinus angustifolius* (cv. Wonga) is geïnokuleer met *Rhizobium* sp. (*Lupinus*) bakterieë en in 'n waterkultuur gekweek. Die houers 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, anders as wortelknoppies, het 'n betekenisvolle afname in anorganiese P (P*i*) ervaar. Onder P-stremming, het lae fosfaat wortels meer pirovaat vanaf malaat gesintetiseer as wortelknoppies, wat 'n definitiewe bydrae vanaf die PEPc roete impliseer. Hiervolgens is pirovaat 'n sleutel metaboliet onder P-stremming. Die belangrikheid van die akkumulering van pirovaat onder P-stremmende toestande is verder beklemtoon deur die toename in metabolisme van PEP via beide die PK- en die PEPc-reaksies. Die toename in PK-aktiwiteite is goed gekorreleer met die verhoogde produksie van pirovaat. Onder toestande van P-stremming het die aktiwiteit van PEPc in wortels verhoog, maar nie in wortelknoppies nie. Dit was nie die gevolg van 'n verhoogde uitdrukking van die ensiem nie. Wortel- en wortelknoppie- uitdrukking van PEPc is derhalwe nie gereguleer deur die uitdrukking daarvan nie, maar eerder deur post-tranlasie kontrole.

Hierdie resultate vir wortels met wortelknoppies demonstreer dat metaboliese P*i* 'n beter maatstaf is om P-stres aan te dui. Hierdie resultate toon dat wortelknoppies beter daartoe instaat is om hul P*i*-vlakke en adenilaatvlakke te reguleer, en dit mag ten koste van die gasheerwortel wees. Ons stel voor dat wortelknoppies nie P-stremming tot dieselfde mate ervaar as die gasheerwortel nie en dat dié knoppies optimaal funksioneer by lae P*i* vlakke. Die verhoogde konsentrasie van pirovaat, wat vanaf malaat gesintetiseer is, impliseer dat daar 'n groter vereiste is vir dié metaboliet onder toestande van P-stremming. Hierdie studie het die rol van koolstofmetabolismein stikstofbindende organismes, spesifiek onder toestande van fosfaat-tekort , beklemtoon.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | degrees celsius |
| $^{14}C$ | radiolabelled carbon |
| cMDH | cytosolic malate dehydrogenase |
| $^{14}CO_2$ | radiolabelled carbon dioxide |
| AMP | adenosine 5'- monophosphate |
| ANOVA | analysis of variance |
| ATP | adenosine 5'- triphosphate |
| $dH_2O$ | distilled water |
| DIC | dissolved inorganic carbon |
| DTT | 1,4 – dithiothreitol |
| DW | dry weight |
| EDTA | ethylenediaminetetraacetic acid |
| FW | fresh weight |
| HPLC | high performance liquid chromatography |
| LSD | least significant difference |
| (NAD)-MDH | malate dehydrogenase, oxidized form (EC 1.1.1.37) |
| (NADH)-MDH | malate dehydrogenase, reduced form (EC 1.1.1.37) |
| (NAD)-ME | malic enzyme (EC 1.1.1.40), cofactor in oxidized form |
| (NADH)-ME | malic enzyme (EC 1.1.1.40), cofactor in reduced form |
| NAD | 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 |
| neMDH | nodule-enhanced malate dehydrogenase |
| 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 |
| SE | standard error |
| TCA cycle | tricarboxylic acid cycle |
| Tris | 2–amino–2–(hydroxymethyl)–1,3- propanediol |

***Dedicated to my parents***

*"As befits an expanding research area, there are no clear conclusions, no broad generalizations; instead there are problems galore, loose ends to be taken up, and central problems to be attacked. What more could a research scientist ask for?"*

***D.D. Davies***

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# Chapter 1

## 1.1 General Introduction

Nitrogen management is a key component to agricultural production. This management would involve the use of some biologically fixed $N_2$, which would be less prone to volatilisation, denitrification and leaching (Graham & Vance, 2000). However, recently Brennan and Evans (2001) have warned that legume N can be cheaper or more costly than fertilizer N, depending on the legume used. In addition different outcomes can be obtained when different time horizons are used in assessing the cost of N (Brennan and Evans 2001). Therefore legume $N_2$ fixation should be viewed as a complementary source of N rather than a substitute for mineral N fertilizers. Nevertheless, the inherent capacity for $N_2$ fixation is a mainstay in cost-effective, ecologically sound approaches to sustainable agricultural practices (Vance and Lamb, 2001).

It has been estimated that annually about $90 \times 10^9$ kg of $N_2$ is fixed symbiotically (Vance, 1998). Nitrogen fixed by legumes is a valuable resource in agriculture, with crop legumes estimated to contribute as much as 20%, which amounts up to $17 \times 10^6$ tons of the N requirements, of the world's grain and oilseed crops (Herridge *et al.*, 2000). Legumes are grown on approximately 275 million hectares, or nearly 11% of arable land worldwide and provide at least 33% of human protein requirements, but in the tropics and subtropics legumes can provide up to 80% of protein needs (Vance, 1998). Grain legumes are important as food and feed proteins and in many regions of the world they are the only supply of protein in the diet because of the high price of animal protein (Duranti and

Gius, 1997). The symbiosis between legumes and their specific root-nodule symbionts has been employed to improve agricultural productivity for most of the 20$^{th}$ century. Many research studies conducted to assess the impact of various constraints to the efficiency of the system provided invaluable insight into the biochemistry of the symbiosis and the measurement of $N_2$ fixation. In addition the advent of molecular techniques has increased our understanding of plant and bacterial genomes as well as plant and bacterial signalling. Molecular signalling between legume roots and specific rhizobia, for instance, is fundamental to the symbiosis.

Superimposed on these aforementioned limitations is that the survival of the intracellular symbionts, *i.e.* bacteroids, and their performance in improving legume productivity will, in part, be determined by their ability to access mineral nutrients from the soil rhizosphere and host plant root (O'Hara, 2001). Some essential nutrients have been shown to have specific roles in the development and functioning of $N_2$ –fixing symbioses (*see overview by* O'Hara, 2001). Essential nutrients required by root nodule bacteria are those with a direct involvement in the metabolic functioning of the system as a whole. In this regard, P falls into a category of essential nutrients that seem to have more significant effects on symbiotic $N_2$ fixation. Deficiencies of essential macronutrients (*e.g.* P) are known to limit plant vigour, but particularly in leguminous plants, such deficiencies have more pronounced effects on rhizobia and their interaction with legumes (Israel, 1987; O'Hara, 2001). At present there is only a limited level of understanding of the effects of many nutrient deficiencies on the *legume-rhizobium* symbiosis. The availability of

adequate levels of essential nutrients, such as P, is fundamental for the efficient functioning of *legume-rhizobia* symbioses.

It has been demonstrated that leguminous plants that are dependent on $N_2$ as the sole source of N, require more P than plants that is fed inorganic nitrogen either as $NH_4^+$ or $NO_3^-$ (Israel, 1987). This comes as no surprise, since $N_2$ fixation is an energy intensive process and because P has a key role in energy metabolism in cells, that P deficiency is certain to adversely affect the energy status of legume nodules and ultimately nodule function (Sa and Israel, 1991). Yet, although nodules are strong sinks for P, it is not certain to what extent nodules experience P stress compared to roots. Evidence suggests that whole nodules compared to whole roots, experience a similar decline in P*i* and AEC levels (Sa and Israel, 1991). However, when the energy status and P*i* concentration of isolated bacteroids were assessed, it was found that the bacteroids maintained their P levels (Sa and Israel, 1991; Al Niemi *et al.*, 1998) and it was suggested that nodules have some sort of strategy which allowed them to regulate the influx of P even when the host is P stressed (Tang *et al.*, 2001). Al Niemi and co-workers (1998) were of the opinion that nodules, or rather bacteroids, operate a more effective mechanism for P uptake and proposed that it allowed them to be aggressive scavengers of P from the surrounding medium. In addition, it was also demonstrated that free-living bacterial cultures require less than 5 µM for optimal growth and survival (*see review by* O'Hara *et al.*, 1989). Although this was only sufficient to maintain a few life cycles of the bacteria, it certainly opens up the question of whether bacteria in symbiosis do not also adhere to such strict P requirements. Similarly certain legumes have been reported to show signs of P toxicity,

when subjected to P concentrations in excess of 15 μM (Bell *et al.*, 1989). There is no consensus on the specific requirements for P in symbiotic legumes. It is likely that different symbioses differ in their requirements for P.

Although other workers have studied P levels in symbiotic roots (Sa and Israel, 1991; Al-Niemi *et al.*, 1997, 1998), results reported here are distinctly different to those reports. Sa and Israel (1991) compared total P of P sufficient and P starved nodules instead of metabolically active P. Similarly Al-Niemi *et al.* (1997) determined P levels using $^{32}P$ orthophosphate by digestion of the entire nodule to determine distribution of labelled P. The cellular- and cytosolic P*i* data presented here, were a more accurate measure of assessing P status since it explored the metabolically available P for tissue.

Some data indicate a direct effect of P deficiency on nodule development and function (Israel, 1987, Tang *et al.*, 2001), whereas other data show the effects are indirect via changes in plant growth and metabolism (Vance *et al.*, 2000). Hence, it will be interesting to see whether symbiotic bacteria maintain their P levels, even when P stressed and if this is the case, how this will affect their partitioning of C via the divergent pathways of interest. The metabolic changes that accompany P deficiency in roots associated with nodules are important for the understanding of C and N metabolism in the host and symbiont. In this regard PEP is situated at a major branch-point of C and N metabolism of higher plants, in particular leguminous plants (Vance *et al.*, 1985; Snapp and Vance, 1986; *see review by* Chollet *et al.*, 1997).

A fundamental role of glycolysis is the production of pyruvate for the TCA cycle. However, because of the decline of adenylates and P*i* under P stress, pyruvate synthesis from PEP via pyruvate kinase (PK) should be restricted. Furthermore, the control of carbon flow during glycolysis is very flexible (Duff *et al.*, 1989, Theodorou and Plaxton, 1993). This flexibility is achieved in part by engaging routes, which circumvents the adenylate-controlled PK reaction (Duff *et al.*, 1989, Theodorou and Plaxton, 1993). In plant cells, it has been proposed that two alternative routes can metabolise PEP in order to bypass the reaction of PK to produce pyruvate (Plaxton, 1996). It is possible for PEP to be metabolized in the vacuole via PEP phosphatase (PEPp) (*see diagram below*). This route is considered unlikely since it would be energetically expensive under P deficiency.

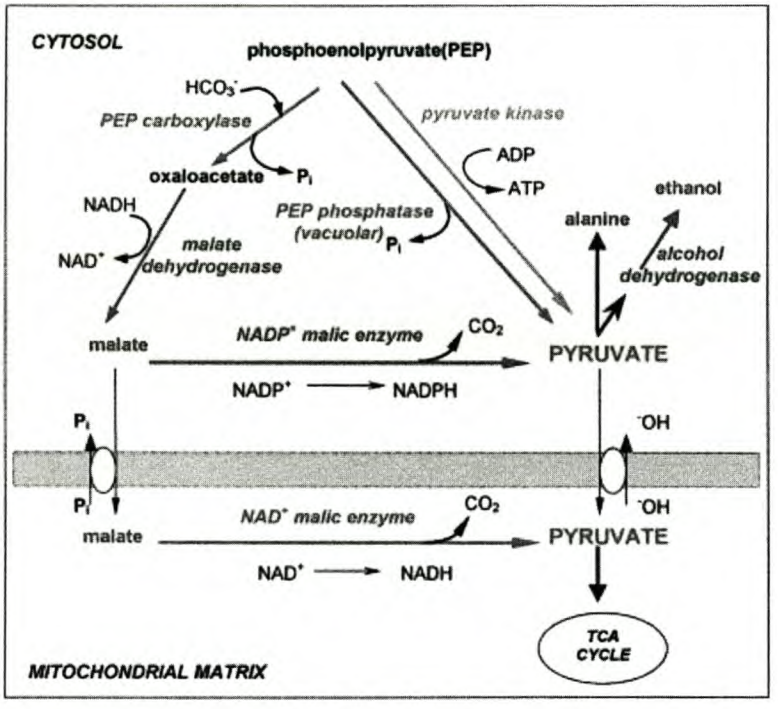


***Diagram***. A model depicting alternative pathways of glycolytic carbon flow during nutritional P*i* deprivation of higher plants. This metabolic flexibility permits plants to circumvent the adenylate- and P*i*-dependent reactions of respiration, thus facilitating respiration by P*i*-deficient plants. The reactions relevant to this study are: *PEP phosphatase*, *cytosolic PK*, *PEPc*, *malate dehydrogenase*, *$NADP^+$-malic enzyme* (cytosolic form) and *$NAD^+$-malic enzyme* (mitochondrial form). Reproduced from *Jusczuk and Rychter* (2002).

PEP could alternatively be converted to malate through the concerted action of PEPc and malate dehydrogenase (MDH) (*see diagram*). Malate is considered to form a large proportion of the product of $O_2$ restricted metabolism, such as prevails in nodules (McCloud *et al.*, 2001). It is then also considered that malate is the predominant dicarboxylic acid of the reaction via PEPc that 'fuels' bacterial respiratory metabolism, in the symbiosome (Rosendahl *et al.*, 1990). Since under P stress, PEPc activity is induced (Johnson *et al.*, 1996 a,b; Toyota *et al.*, 2003) and this increased activity is correlated with dark $CO_2$ fixation (Christeller *et al.*, 1977) and $N_2$ fixation (Coker and Schubert, 1981), it is felt that the dark fixation route of PEPc will be favoured as a means by which malate and subsequently pyruvate is formed. While this adaptation may occur as a normal metabolic pathway, in many instances it is associated with adaptation to stress such as nutrient limitation (Theodorou and Plaxton, 1993; Plaxton, 1996). Thus, a major alternative route is the concerted activity of PEPc, cytosolic MDH and mitochondrial NAD-ME, which appears to function as an adenylate-independent bypass to the ATP-generating PK reaction under P*i* stress (Nagano *et al.*, 1994; Theodorou *et al.*, 1991; Theodorou and Plaxton, 1995). In particular, PEPc activity of *Brassica nigra* and *Catharanthus roseus* suspension cells under P*i* limitation was found to be more than double the P sufficient controls (Duff *et al.*, 1989; Nagano *et al.*, 1994). Furthermore, in proteoid roots of *Lupinus albus*, the dark fixation rates increased under P*i* limitation, while respiration declined (Knowles *et al.*, 1990). Plaxton (1996) suggested that the demands for pyruvate and the generation of P*i* were the likely causes of these enhanced PEPc activities. More recently, the role of the alternative route via PEPc for pyruvate synthesis was studied (Juszczuk and Rychter 2002) in P starved un-nodulated bean roots.

Although Juszczuk and Rychter (2002) showed that the activities of pyruvate producing enzymes, via the PEPc route had increased with P*i* stress, they also found an increase in PK activity. It was suggested (Juszczuk and Rychter 2002) that the accumulation of pyruvate was related to its role in reducing oxidative stress under P*i* deficiency. The PEPc reaction is also beneficial for plants that are P stressed, because P*i* is released as a by-product of the reaction and in this way P*i* is recycled within a P limited cell environment (Duff *et al.*, 1989).

Prior to the work by Juszczuk and Rychter (2002), the research on the alternative route of PEP metabolism via the combined PEPc-MDH-ME suite (Nagano *et al.*, 1994; Theodorou *et al.*, 1991; Theodorou and Plaxton, 1995; Duff *et al.*, 1989; Knowles *et al.*, 1990), focused on the activities of PEPc and formation of organic acids. No published data are available on the contribution of this alternative route in nodulated root systems under P deficiciency. This is particulary pertinent since PEPc has a vital role in the C provision to legumes for nitrogen assimilation and bacterial respiration. Moreover, the role of PEPc-derived C in pyruvate synthesis under these P limiting conditions is unknown, and will therefore be the focus of this project. The value of this information will be of great significance to the understanding of respiratory C metabolism in P limited legume root systems, where the fate of PEPc-derived C and the route of pyruvate synthesis are currently unknown. It is seen, then, that the route followed for pyruvate synthesis is governed by the conditions, which prevails at a certain stage in the plant. Therefore the selection for increased dark $CO_2$ fixation may be a feasible means of increasing legume productivity (Groat *et al.*, 1984).

The aim of this study was the investigation of PEP metabolism of P deficient roots and nodules. It was hypothesized that under P limitation, the synthesis of pyruvate would induce the PEPc alternative route, so that pyruvate would be synthesize mainly from PEPc-derived C.

The objectives in this study were to:

(1) assess the changes in adenylate and P*i* levels as well as enzymatic changes that occur in roots and nodules under P stress.

(2) establish the route of pyruvate synthesis from PEP in roots and nodules under P stress.

(3) assess the importance of the PEPc alternative route in roots and nodules, under P deprivation.

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# Chapter 2

## Literature Review

### 2.1 **Infection**

Legumes include some of the most agronomically important crops such as beans and peas. Beside these crops this plant group also comprises of other, but by no means less important plants, such as clover and lupin. Much of this importance is owed to the fact that legumes provide up to 33% of the worldwide protein intake (Vance, 2001). This increases substantially in the tropical regions where it is believed that they can provide up to 80% of the needed protein. Yet, it is rather their ability to fix atmospheric nitrogen ($N_2$) that has resulted in them being pursuit so relentlessly by researchers. This system provides a means to determine the restrictions on N-assimilation processes, which adversely affect plant dynamism out in the field. Biological nitrogen fixation is restricted to prokaryotic organisms, either free-living or in a symbiotic association. With regard to the latter, the capability of crop plants to form mutualistic associations with soil microbes is crucial for attaining higher yields in a sustainable manner (Vance, 2001).

Nitrogen-fixing bacteria in concert with legumes fix atmospheric nitrogen ($N_2$), which is then made available to the infected plant. The resulting association 'dictates' that the plant provides carbon (C) compounds to support the growth and maintenance of the bacteria (in the nodule), and the bacteria in turn provide nitrogen (N) compounds for the plant. This in effect minimizes the need for nitrogen fertilizer application drastically, so much so that leguminous plants can achieve vigorous growth without the nitrogen

fertilizer that are required by most other plants. This particular reaction is catalyzed by the microbial enzyme, nitrogenase (EC 1.7.99.2).

As discussed by Dakora (2003), effective $N_2$ fixation (and N contribution) by root nodules is often viewed as the major role of symbiotic legumes in cropping systems. As a result, successful nodulation of these leguminous species is regarded as the first step to achieving the potential N benefits from these bacterial symbioses (Dakora, 2003). The interaction between rhizobia and the legume root begins with an exchange of chemical signals in the soil. Some findings suggest that in the symbiotic interaction with legumes, rhizobia (the term rhizobia as used here refers to the nitrogen-fixing genera of the family Rhizobiaceae; this include *Rhizobium*, *Bradyrhizhobium*, *Mesorhizobium* and *Azorhizobium*) are initially recognized as 'intruders' (Vasse *et al.*, 1993, Santos *et al.*, 2001). However, the interaction between rhizobia and legumes is finely regulated by signal molecules, which are perceived by receptors that activate a signal transduction cascade (Dakora, 2003). This inevitably leads to the activation of target genes and the products these genes encode stimulate the development of tumor-like nodules on the roots. The process of nodule formation in legumes involves the production of flavonoids, betaines by the plant and aldonic acids in its seed and root exudates as signals to the microbial symbiont (Dakora, 2003). In compatible associations these compounds interact with Nod protein of the rhizobial cells and induce the expression of nodulation (*nod*) genes. The rhizobia, in turn, respond by releasing lipo-chito-oligosaccharide Nod factors that cause morphological changes in legume root hairs, leading to infection thread formation, nodule development and ultimately $N_2$ fixation. Infection of rhizobia is usually

very species specific and a rhizobial strain which infects one plant, will not always effectively nodulate another.

## 2.2 **Classification**

$N_2$-fixing legumes can be classified as either amide exporters (*e.g. Pisum, Vicia* and *Lupinus*) or ureide exporters (*e.g. Phaseolus, Vigna* and *Glycine*) (*see review by* Streeter, 1991). This depends mainly on the composition of the xylem exudate collected from nodules or nodulated root systems. The production of ureides, the main compounds translocated by tropical legumes, are considered to be energetically less expensive with regard both to their biosynthesis and translocation as oppose to amide-exporting legumes and are therefore regarded as a more efficient form of N transport (Schubert, 1986). Thus, it is to be expected that these two groups will show differences in their major pathways of C metabolism since asparagine (ASN) can be formed from OAA, whereas allantoin and allantoic acid are products of purine metabolism (Rawsthorne *et al.*, 1980). Furthermore, nodule shapes vary and can be elongated lobes or round. The former, is termed indeterminate nodules, and are usually associated with amide-exporting legumes. These type of nodules have undifferentiated meristems and can continue to divide throughout the life cycle of their particular host. In contrast, determinate nodules have well defined meristems, which means that such nodules will die and reform on roots each year.

## 2.3 **Mineral constraints of leguminous plants**

In managing legumes, one needs to identify the constraints that draws back on the efficacy of the symbiosis and which seriously depresses legume yields below their

maximum potential. It should be clear, then, that nodulated plants although N-sufficient, will almost certainly suffer from similar nutrient deficiencies as other plants. In this regard P has specific roles in initiation, growth and functioning of nodules, in addition to its role in host plant growth (Israel, 1987). It is a well established fact that because nitrogen fixation is a real energy intensive process, leguminous plants have a high demand for P. It has also been demonstrated that legumes solely dependent on $N_2$ require more P than plants fed with either $NO_3^-$-N or $NH_4^+$ -N (Israel, 1987). Sa and Israel (1991) demonstrated that bacteroids maintained their P levels even when subjected to P deficiency. Al Niemi and co-workers (1998) similarly showed bacteroids to maintain their P levels when P stressed, suggesting that nodules (or rather bacteroids), have a more efficient system to take up P. Free-living cultures have been shown to require approximately 5 μM P for optimal growth and survival (O'Hara, *et al.*, 1988). At the other end of the extreme, P levels in excess of 15 μM were detrimental to certain leguminous species (Bell *et al.*, 1990). It seems, then, that a fine balance needs to be struck between P demand and P supply in legumes with $N_2$ fixing capabilities. Hence, it was suggested that somehow nodules regulate the influx of P in such a way that when P is limited there is not that high a demand for it (Tang *et al.*, 2001). Insofar as control availability is concerned this P demand does not seem to change much so that P levels are maintained, since control would probably have some toxicity effects on bacterial growth, host plant growth or both (Tang *et al.*, 2001).

It must be noted that limitations to legume production do not result only from deficiencies in the more common macronutrients such as P (also K and S), but also of micronutrients such as Fe, Mo and B (see O'Hara *et al.* 1988). However, this discussion

will deal only with the constraining effects of P deprivation and the reader is referred to O'Hara *et al.* (1988) for a more extensive overview of the effects of other minerals.

### 2.3.1 **Importance of P*i* in plant metabolism**

Although plants take up P*i* in much smaller amounts than N, the importance of P*i* must not be underestimated. Despite this discrepancy between the two aforementioned mineral elements, P*i* is found in a large variety of compounds in plants. Phosphate has a very important intermediary role in acting as the energy carrier in plant metabolism. ATP is the most important P*i* compound in this regard. As such P*i* is therefore intimately involved with metabolic processes. The importance of P*i* in plant metabolism can be attributed to a variety of factors including: (i) the unique physical characteristics of the P-P bond and its role in energy transduction, (ii) the pivotal role P*i* plays in fine control of metabolism as an allosteric effector of enzymatic activity (Iglesias *et al.*, 1993, cited in Theodorou and Plaxton, 1993) or by the reversal phosphorylation of key regulatory enzymes (Plaxton, 1996), (iii) its function as an important constituent of macromolecules, e.g.. phospholipids and nucleic acids, and (iv) its vital role in regulating the exchange of triose phosphate between the plastid and cytosol via the P*i* translocator (Preiss, 1984; Walker and Sivak, 1986).

### 2.3.2 **Metabolic adaptations to P*i* deprivation**

In soils, the concentration of available P*i* for plants is usually very low as most of the P*i* is bound to iron, aluminium and calcium, to form insoluble compounds. Subsequently

plants have developed various ways to overcome the shortage of P*i* in their immediate environment.

A very common response to low P content of soil is increased relative biomass allocation to roots, which ultimately results in an increase in root:shoot ratio (Rychter and Mikulska, 1990; Paul and Stitt, 1993;). In turn this enhances the P acquisition from the growth medium. However, this may also result in retarded growth rates because of the diversion of C to the production of heterotrophic rather than photosynthetic tissues (Freeden *et al.*, 1989). Nielsen *et al.* (2001) remarked that the allocation of carbohydrates to various plant parts and functions is a governing parameter of plant survival and success. The success of plants under stress conditions may be determined by their ability to control carbohydrate utilization for metabolic energy (Nielsen *et al.*, 2001).

Several plants, including legumes, are known to exude organic acids (OAs), of which citrate, malate and succinate constitute the predominant forms, in response to P*i* deprivation (Hoffland *et al.*, 1989; Imas *et al.*, 1991; Johnson *et al.*, 1996 a,b). These exuded organic acids chelate the elements to which P*i* is bound and at the same time solubalize these P*i* compounds. The increased rate of OA exudation was ascribed to an increased synthesis of the OAs mentioned. The non-photosynthetic carbon fixation route, via phospho*enol*pyruvate carboxylase (PEPc), has been implicated as the pathway through which the increased synthesis of these particular OAs occurs. The activity of non-photosynthetic PEPC in the roots was enhanced by P starvation. (Pilbeam *et al.*, 1993; Johnson *et al.*, 1994). Johnson *et al.* (1996) in a subsequent study revealed that this increase in PEPc activity coincided with the increase in PEPc transcripts and the amount

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

As another strategy to acquire P*i*, an increase in the P*i* uptake rate in response to P*i* starvation has been observed in roots and cultured cells (Furihata *et al.*,1992; Aono *et al.*, 2001). Aono *et al.* (2001) demonstrated a dual mechanism model, composed of two kinetically different uptake systems; one a high-affinity transport system and the other a low-affinity transport system. In light of the P-status of most soils, these authors (Aono *et al.*, 2001) were of the opinion that the high-affinity transport system would be primarily functional in roots.

Studies undertaken to investigate plant adaptation to P starvation have further revealed that plants induce alternative pathways of glycolysis and mitochondrial electron transport (Duff *et al.*, 1989; Rychter *et al.*, 1992). This is seen as an adaptive strategy to facilitate mitochondrial respiration by P-deficient plant cells. P deficiency causes a decline in cytosolic P*i* and adenylates (Duff *et al.*, 1989; Rychter *et al.*, 1992) and under these conditions the increased engagement of these bypasses neutralize the necessity for adenylates and P*i*, utilized under normal, non-stressed conditions.

## 2.4 **The C – N – P linkage**

The reduction of $N_2$ and the subsequent assimilation of $NH_4^+$ require large quantities of photosynthate. The photosynthates, which are translocated from the leaves, generate C skeletons, reducing power and energy required for symbiotic nitrogen fixation. The

dependence of C from photosynthesis may increase substantially under unfavourable (e.g. temperature extremes, low $NO_3$-supply, dark grown plants) growing conditions (Ching *et al.*, 1975; Bouma *et al.*, 1997a; Van der Werf *et al.*, 1992). In nodulated roots this C flow from the shoots would be to support the increased respiratory burden of nodulated roots and to provide C skeletons necessary for the synthesis of the organic forms of nitrogen, which are exported from the nodule to the aboveground biomass (Schubert, 1986).

Free-living (*Brady*)*Rhizobium* bacteria have a wide range of C sources available to them. However, several reports indicate that utilization of carbohydrates in *Rhizobium* bacteroids in symbiosis is limited (Day and Copeland, 1991; Vance and Heichel, 1991). There are only a few reports showing sucrose to be the dominant sugar incorporated into nodules (Kouchi and Yoneyama, 1986). This might be a question of it being the exception rather than the rule. It is a widely held view (backed by good evidence) that TCA cycle intermediates, particularly malate and succinate, act as substrates for bacteroid respiration (De Vries *et al.*, 1980; Reibach and Streeter, 1983; Rosendahl *et al.*, 1990). Initial products of $^{14}CO_2$ fixation in roots and nodules, efficient in $N_2$ fixation, of alfalfa and birdsfoot trefoil showed more of the incorporated radioactivity in the organic acid fraction, whereas the export of fixed $^{14}C$ from the same tissue was amino acids (Anderson *et al.*, 1987; Maxwell *et al.*, 1984). However, if nodules were inefficient in $N_2$-fixation, $^{14}C$ was transported as organic acids. In short then, plant C metabolism in legume root nodules primarily provides (a) respiratory substrates to actively growing bacteroids, and (b) dicarboxylic acids, which act as C skeletons for the incorporation of fixed N into amino acids (Curioni *et al.* 1999). Yet it must be noted that most of the capacity for

carbohydrate utilization was observed in the plant fraction of nodules (Copeland *et al.*, 1989b).

Low soil fertility due to nitrogen (N) and phosphorus (P) deficiencies is an overriding constraint in many ecosystems and are probably the major factors limiting plant growth. The metabolism of these elements is highly integrated in plants, even more so in leguminous plants in which it has been frequently reported that $N_2$-fixation to be dependent on a good supply of P for efficient functioning (Israel, 1987, Almeida *et al.*, 2000). In the former study (Israel, 1987), the supply of P has been shown to contribute to the higher $N_2$-fixation ability and nodule mass of soybeans, rather than to plant growth. Almeida *et al.* (2000) later demonstrated that severe P deficiency prevented nodulation or stopped nodule growth when the P deficiency occurred after plants had formed nodules. Furthermore, it was shown that root respiration of bean plants grown under low P conditions, represented as a fraction of the whole plant C budget, was approximately twice that of plants grown under moderate P stress (Nielsen *et al.*, 1998). Root respiration can be partitioned into costs for growth, maintenance and ion uptake.

Israel and Rufty (1988) proposed the initial assimilation step of $N_2$ into $NH_4^+$ to be more sensitive to P-deficiency compared to the subsequent incorporation of soluble reduced N into protein and nucleic acids. This was confirmed in a later study undertaken by Sa and Israel (1991). This conclusion was derived from results that showed that initial P-deficiency conditions resulted in low levels of soluble reduced-N. This effect was gradually succumbed when increasing the P nutrition of the nodule (Israel and Rufty, 1988). In addition, the adverse effect that P deficiency had on $N_2$ fixation might have

been as a result of poor nodule initiation, growth and specific nitrogenase activity (Israel, 1987, 1993). Phosphorus deficiency may also directly or indirectly influence other steps in the N assimilatory pathway of $N_2$-fixing plants (Israel and Rufty, 1988). Furthermore, nitrogenase and nitrate reductase activities, both enzymes of nitrogen assimilation, have been shown to increase with P nutrition (Carling *et al.*, 1978).

Nitrogen fixation rate in P deficient plants is reduced because of the effect of low P supply on the growth of the host plant, on the growth and functioning of the nodule or on the growth of both the plant and nodule (Israel, 1993; Sa and Israel, 1991). A minimum requirement of 12 ATP`s for each mole of $N_2$ reduced has been deduced from kinetic studies with purified nitrogenase and physiological studies with several $N_2$-fixing bacteria (Shanmugam and Valentine, 1975). Thus, because $N_2$ fixation requires a huge amount of energy, and because P has a key role in energy metabolism in cells, P deficiency is certain to adversely affect the energy status of legume nodules and ultimately nodule function (Sa and Israel, 1991).

## 2.5 **Flexibility of the glycolytic network**

Cytosolic glycolysis is a complex network containing alternative enzymatic reactions. The cytosolic glycolytic network may provide an essential metabolic flexibility that facilitates plant development and acclimation to environmental stress (*reviewed by* Plaxton, 1996). Glycolysis developed primarily as an anaerobic pathway, which oxidizes hexoses to generate ATP, reductant. Apart from its function as the major producer of building blocks for anabolism, it can also function as an amphibolic pathway, which

when operational in the reverse could generate hexoses from various low-molecular compounds in energy-dependent gluconeogenesis. Moreover, glycolysis is the predominant pathway that 'fuels' plant respiration. The plant mitochondrial respiratory chain is branched, with bypasses of all the three energy transducing (proto-translocating) sites associated with the conventional chain. These bypasses act as non-phosphorylating alternative routes of electron flow from NADH to $O_2$ (Moore and Rich, 1985), and allow plant respiration to proceed under conditions of high cellular energy charge levels (Lambers, 1985). The non-phosphorylating branches can be distinguished by their insensitivity to the inhibitors rotenone and KCN. Nodule mitochondria have been shown to be more sensitive to these inhibitors and it was concluded that the non-phosphorylating pathways therefore is absent from nodules, at least in soybean (Day *et al.*, 1986).

The integration of cellular metabolism necessitates controlled interactions between pathways sequestered in various cellular compartments (Plaxton, 1996). Forming symbiotic associations may further contribute to this metabolic complexity.

Thus 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). The cytosolic glycolytic network is proposed to furnish plants with the requisite metabolic options needed to facilitate their development and acclimation to unavoidable environmental stresses such as anoxia and P*i* deprivation (Plaxton, 1996).

## 2.6 **Enzymes involved in pyruvate synthesis**

There are several enzymes that comprise the alternative route (via PEPc) of C oxidation. These enzymes allow channelling of C into mitochondria to allow respiration to proceed under conditions at which conventional glycolysis is restricted, *e.g.* P deficiency. When biosynthetic activity of growing plant cells is high, which may almost certainly be the case in root nodules, the major flux through the glycolytic pathway may serve a dual purpose. In addition to supplying substrates for mitochondrial energy metabolism it may also be to supply biosynthetic intermediates to match the high demand (Dennis and Greyson, 1987). In general the rate of glycolysis is controlled carefully by PEP metabolism. PEP is situated centrally of what could be seen as the major point of C partitioning in higher plant cells. One of the effects of a decline in PEP is that it increases the activity of PFK that is believed to be a major rate-limiting step of glycolysis for animals. This is considerably different for plants, where there is a concerted effort to funnel hexose phosphates away from starch and sucrose synthesis and ultimately towards and increased rate of glycolysis (Davies, 1979). Carbon from both these processes can enter into the glycolytic pathway of plants at midpoint, rather than at the top as for animals. This is owed to the presence of two glycolytic pathways, one in the cytosol and the other in the plastid. Hence, our understanding of the regulation of glycolysis is limited by knowledge (or lack thereof) with regard to the manner in which the concentration of PEP is regulated.

However, for one to truly appreciate to which extent the alternative route is engaged it is necessary to know how the conventional glycolytic pathway is regulated in conjunction with the alternative route. Here, we will only consider the enzymes at the PEP branch-

point and also for sake of simplicity discuss them separately. It must be noted, though, that metabolism does not exist in boxes as depicted here and that these pathways interact with each other in order to bring about concerted action for regulating metabolism.

### 2.6.1 ***Pyruvate kinase*** (PK)

PK is an enzyme, which in vascular plant tissue (and algae) exists as both cytosolic (PKc) and plastid (PKp) isozymes that differ markedly in their respective physical, immunological and kinetic properties. These isozymes are regulated quite distinctly from each other and provides plants with a 'safety net' unlike for any other organisms (e.g. yeast and animals). Here we will mainly consider PKc although it is interesting to note that the plastid form PK is suggested to provide ATP for biosynthetic purposes within the organelle (Sangwan *et al.*, 1992). PKp increased relative to PKc during development of the seed in *Brassica* (Sangwan *et al.*, 1992). This is an oilseed which requires large amounts of ATP for fatty acid synthesis.

PK catalyzes the formation of pyruvate from PEP, with the concomitant synthesis of ATP from ADP. The abnormal growth, C partitioning and dark respiration of transgenic tobacco lacking cytosolic PK in their leaves underscores the importance of this enzyme in the control of plant C flow and subsequent energy metabolism (Knowles *et al.*, 1998).

Furthermore, plant PK is of interest because considerable evidence indicates that it is a primary site for control of glycolytic flux to pyruvate (Plaxton, 1996; Smith *et al.*, 2000). Insofar as symbiotic root systems are concerned, PK involvement in metabolism is poorly documented and subsequently very little is known about the regulation of legume nodule PK. PK has been demonstrated to be more closely related to eukaryotes than prokaryotes,

which suggest that the nodular PK form may be distinctly different to that of root PK (Blakeley *et al.*, 1992).

Recently McCloud and co-workers (2001) devised a method, by which pure extractions of PK, *i.e.* free of any contamination by PEPc, were made. These authors (McCloud *et al.*, 2001) demonstrated that at normal physiological pH 7.0, and in the presence of elevated malate concentrations (5 mM) the activity of PK was unaffected. It was proposed that reduced uptake of malate by bacteroids, as a result of reduced $N_2$ fixation, may favour PEP metabolism by PK over PEP metabolism by PEPc. This decreased $N_2$ fixation may be initiated by treatments (*e.g.* detopping, exposure to $Ar:O_2$ gas) that perturb C and N metabolism (Curioni *et al.*, 1999). It is proposed here that the conventional glycolytic route via PK would become restricted under P deficient conditions and that the PEPc alternative pathway would rather predominate under prevailing conditions.

On the other hand, it was shown that isolated mitochondria of cowpea and soybean nodules were able to oxidize both malate and pyruvate, although lacking malic enzyme activity (Rawsthorne and La Rue, 1986 a,b; Bryce and Day, 1990). This allowed for speculation as to whether nodules of these plants operate a truncated version of the TCA cycle (McCloud *et al.*, 2001). Since it is further generally accepted for the TCA cycle to become restricted under low oxygen tension conditions such as prevails in nodules, and also that elevated malate levels would inhibit PEPC activity, it was suggested that pyruvate would be synthesized predominantly via PK in root nodules (McCloud *et al.*, 2001).

### 2.6.2 ***Phosphoenolpyruvate carboxylase*** (PEPc)

We now know that plants in the wild are subjected to various forms of stresses, including mineral constraints such as N and P deficiencies. It is well documented that under these conditions that the plant engage alternative pathways, which are not directly subjected to adenylate control (Theodorou and Plaxton, 1993; Jusczuk and Rychter, 2002). One such branchpoint is at PEP and PEPc is regarded as the main anaplerotic enzyme under stress conditions, which replenishes intermediates to the TCA (Chollet *et al.*, 1997). PEPc catalyses the irreversible carboxylation of PEP to oxaloacetate (OAA). The enzyme can comprise up to 2 % of the soluble protein in alfalfa root nodules (Vance and Stade, 1984) and plays an important role in partitioning C towards either N assimilation or mitochondrial energy metabolism. In the case of a symbiotic system such as root nodules it may even take on a further role in that it funnels C for bacteroid respiration (Rosendahl *et al.*, 1990).

PEPc, together with PK, are key enzymes that are situated at a major branch point of C and N metabolism in higher plants and green algae (Schuller *et al.*, 1990). The intermediary product, OAA of the PEPc reaction condenses with acetyl-CoA to yield citrate. The acetyl-CoA for this reaction is synthesized from pyruvate. The relevancy of this relates back to a key question of this study and that is what route pyruvate synthesis follows under P deficiency conditions. OAA may alternatively act as a precursor for assimilating the N compounds of bacterial metabolism.

It is not known at this stage if, within a symbiotic set-up, roots or nodules respond differently with regard to engaging alternative routes to adapt to P deficiency.

PEPc activity has consistently been demonstrated to be higher in nodules when compared to uninoculated roots (Lawrie and Wheeler, 1975; Christeller *et al.*, 1977; Duke *et al.*, 1979; Deroche *et al.*, 1983). These estimates are all based on the fresh weight of sample material. These differences were not so apparent when enzyme activities of nodules and young roots (root apices) were compared (Smith, 1985). Both of the aforementioned tissue types are meristematic and have controlrotein contents per gram fresh weight and therefore much smaller differences are reported when these two tissue types are compared versus a comparison made between nodules and cortices (Smith, 1985). The higher PEPc activity in nodules compared with roots may in fact be related to the observation that the legumes studied so far have always had one more isoenzyme in their nodules than in their related young roots (Deroche *et al.*, 1983; Vance and Stade, 1984; Marczewski, 1989).

In plants most studies have focused on the role of PEPc in $C_4$ and CAM species where the enzyme provides an effective mechanism for concentrating $CO_2$ within leaves (Wedding *et al.*, 1989). Rapid advances in molecular techniques have been useful in lifting the veil of mystery around PEPc in $C_3$ and non-autotrophic tissue. Through these procedures it has come to light that PEPc in $C_3$ plants fulfills a similar role as for its counterpart(s) in $C_4$ and CAM plants in that it refixes $CO_2$ released by the mitochondria to ensure a more effective metabolism. Carbon dioxide fixation in root nodules is integrally associated with $N_2$ fixation (Christeller *et al.*, 1977; Laing *et al.*, 1979; King *et al.* 1986). Results of labeling studies are consistent with the hypothesis of a primary role for PEPc in $CO_2$ assimilation (Laing *et al.*, 1979; Schubert, 1981). Shoot harvest, which implies the

removal of the main photosynthetic and carbon source for nodules, resulted in a sharp decline of PEPc activity of alfalfa nodules (Vance *et al.*, 1983). Christeller *et al.* (1977) have shown that there is a significant correlation between rates of $CO_2$ fixation and $N_2$ fixation in developing lupin nodules. It was subsequently proposed that the primary role of dark $CO_2$ fixation in lupin nodules was to provide the precursors for the synthesis of aspartate and asparagine, the predominant forms of organic N in the xylem exudate of lupins (Christeller *et al.*, 1977). However, the regulation of PEPc is suggested to change quite drastically in legumes, which mainly export their N in the form of ureides, as was shown for soybeans (Schubert, 1986). These plants would not require as much $C_4$-acids for the synthesis of its major organic N exporting component. This of course does not imply that PEPc becomes less important in these plants. King *et al.* (1986) demonstrated no marked effect on dark $CO_2$ fixation with a mixture of $Ar:O_2$ (80:20, v/v) of soybean nodules, although $N_2$ fixation was hampered quite considerably. On the contrary, Anderson *et al.* (1987) showed that alfalfa plants were much more sensitive to an $Ar:O_2$ atmosphere, with a reduction of $N_2$ fixation to 51%, nodule $CO_2$ fixation to 45% and the respiration rate to 55%. Similarly, the glycolytic flux was decreased. This is probably the direct result of the different pathways through which the major exporting compounds (ureides for soybean and amides for alfalfa) of these two plants get synthesized. PEPc has been shown to concomitantly increase with glutamine synthetase, glutamate synthase, aspartate aminotransferase and asparagine synthethase. All of the aforementioned enzymes are involved in the synthesis of amino acids aspartate and asparagine, the major transport compounds in lupins and other amide transporting species (King *et al.*, 1986;

Maxwell *et al.*, 1984; Rosendahl *et al.*, 1990). All this implies a central role for PEPc in $N_2$ fixation and ammonia assimilation in legume root nodules.

In summary, PEPc have been assigned various roles in the physiology and metabolism of a number of legumes and include aspects pertaining to: (a) its role in recovering some of the respired $CO_2$ (Vance *et al.*, 1983); (b) a means by which dicarboxylic acids is synthesized which inevitably get used as respiratory substrates by the bacteroids (Rosendahl *et al.*, 1990); (c) its availability as a source of C skeleton for $NH_4^+$ assimilation (Christeller *et al.*, 1977; Rosendahl *et al.*, 1990), and (d) it constituting a mechanism to maintain the charge balance and pH in the cell and xylem fluid (Deroche and Carrayol, 1989).

### 2.6.3 ***Malate dehydrogenase*** **(MDH)**

The enzyme is known to exist as both cytosolic and organellar forms. Malate dehydrogenase activity in root nodules has consistently been shown to be higher than that of roots (Lawrie and Wheeler, 1975; Appels and Haaker, 1988). This increased activity is associated with a nodule-specific MDH (neMDH) that has been demonstrated for *Pisum sativum* (Appels and Haaker, 1988). A similar unique nodule-enhanced form of MDH has also been reported for alfalfa (*Medicago sativa* L.). Interestingly, interspecies similarity (> 90 % with alfalfa and soybean) of the neMDH form exceeds intraspecies (< 30 % homology with chloroplast) similarity (Fedorova *et al.*, 1999), although it does share some commonality with another chloroplast-targeted MDH of *Arabidopsis thaliana* (Berkemeyer *et al.*, 1998). Evidence suggests that the neMDH is in fact another plastid-

targeted form of MDH (Miller *et al.*, 1998). Western blots of nodule protein probed with antibodies to neMDH recognized a single polypeptide of approximately 35 kDa (Miller *et al.*, 1998). Similarly antibodies produced to cytosolic MDH (cMDH) recognized two polypeptides in nodule extracts of approximately 35 and 33 kDa, suggesting that there are two isoforms of this polypeptide (Miller *et al.*, 1998). In addition, the increased expression of neMDH occurring well after the onset of nitrogen fixation suggests that other additional signals (*e.g.* $O_2$ restriction, high $NH_4^+$ concentration) inside the nodule may be important in gene expression of neMDH of a mature, effective nodule (Fedorova *et al.*, 1999). This neMDH favors production of malate required for nodule metabolism (Appels and Haaker, 1988; Miller *et al.*, 1998). The subcellular localizations of each of these aforementioned proteins have yet to be determined.

Malate is regarded as the major $C_4$ dicarboxylic acid in root nodules of legumes, which is synthesized by the concerted action of cytosolic PEPc and MDH from oxaloacetate (OAA) and NADH, substrates for which it has been demonstrated to have high affinity for (Appels and Haaker, 1988). Under physiological conditions in the plant cytosol (*i..e.* pH 7.0), the equilibrium of the reaction catalyzed by MDH is completely in favour of malate and $NAD^+$. In this case it would be expected that MDH would catalyze a very substantial reduction of OAA to malate under average cytoplasmic physiological conditions. As shown by tracer experiments, or by enzyme activity determination, OAA is rapidly reduced into malate by MDH or transaminated into aspartate by aspartate aminotransferase. The latter pathway leads to asparagine synthesis and is the source of the main amide translocated from nodules to shoots in lupin. The aspartate

aminotransferase is very active in legume nodules and mainly localized in the plant cytosol (Grimes and Turner, 1971, Reynolds and Farnden, 1979). However, the asparagine synthethases, which catalyze the ATP-dependent and glutamine dependent transfer of an amide-group from aspartate to asparagine, should in light of the hypothesis of our study be considerably hampered under conditions of P stress. An increased engagement of the PEPc → MDH → ME- route is therefore envisaged.

Alternatively a build up of malate in plant cell cytosol due to reduced malate uptake by the bacteroids could favour PEP metabolism via PK over PEP metabolism via PEPc (McCloud *et al.*, 2001). Reduced malate uptake by the bacteroids could occur when nitrogenase is inhibited by treatments that perturb plant C and N metabolism (Curioni *et al.*, 1999). Curioni *et al.* (1999) reported that the harvesting of shoots and exposing nodulated alfalfa roots to $Ar/O_2$ (80/20, v/v) resulted in an increase in nodule PEP to pyruvate and PEP to malate ratios. This it seems, would suggest that PK activity and PEPc activity were being inhibited simultaneously.

The presence of $^{14}C$ in malate and aspartate integrally links MDH and PEPc in the dark $CO_2$ fixation route (Maxwell *et al.*, 1984; Vance and Stade, 1984). De Vries *et al.* (1980) proposed at least three roles for the malate pathway in nodule metabolism. According to De Vries *et al.* (1980), a part of the malate and possibly of other OAs could be used as a carbon and energy source for the $N_2$-fixing bacteroids. Another part of the malate could enter the Krebs cycle of mitochondria and produce energy, in the form of ATP, reducing power and C skeletons used in the functioning of nitrogenase and biosynthesis of amino

acids from ammonium. Lastly, and no less important, malate could also be involved in the adjustment of charge balance in vacuoles and in xylem fluid. Malate has been shown to balance the majority of the excess inorganic cation charge of xylem sap (approximately 75%), with most of the remaining charge being balanced by allantoate (in tropical legumes) and aspartate (Israel and Jackson, 1982). The capacity for MDH to engage in these aforementioned roles assigned to is not fully understood yet.

### 2.6.4 *Malic enzyme* (ME)

($NAD^+$)-ME occupies a key position in mitochondrial C metabolism, providing a means whereby import of $C_4$ acids can be partitioned between replenishment of mitochondrial pools and complete oxidation. Hence, ($NAD^+$)-ME represents a potential regulatory site for mitochondrial C metabolism. The flux through ($NAD^+$)-ME has been shown to vary considerably among various plant organs and under various conditions. For instance it has been demonstrated that the flux through maize kernels was approximately 30 % that of PK (Day and Hanson, 1977), whereas in P deficient roots the activity of ($NAD^+$)-ME increased up to 200 %. Hence, it is suggested that ($NAD^+$)-ME possess a number of regulatory properties that play a role in controlling flux from malate to pyruvate.

Early studies (Brunton and Palmer, 1973; Wedding *et al.*, 1976) provided sound evidence for the operation of dual pathways of malate metabolism in mitochondria; one through MDH to oxaloacetate, and the other through NAD malic enzyme to pyruvate. However, there are conflicting reports in the literature with regards to the existence of ME in nodules (Rawsthorne and La Rue, 1986a,b; Day and Mannix, 1988; Copeland *et al.* 1989a).

Observations of special note, is the demonstration of the absence of malic enzyme activity in both isolated cowpea and soybean nodule mitochondria, although it has been shown to oxidize both malate and pyruvate (Rawsthorne and La Rue, 1986a,b; Day and Mannix, 1988). In contrast, Copeland *et al.* (1989a) demonstrated the presence of a NAD-linked as well as a NADP-linked ME in *B. japonicum* bacteroids. These authors (Copeland *et al.*, 1989a) argued that the former probably had a more likely role in $N_2$ fixation due to its high affinity for its substrate malate. Likewise, the NADP-ME form served a maintenance role in the bacteroids providing the necessary C skeletons for the TCA cycle to remain operative.

### 2.6.5 ***PEP phoshatase*** **(PEPp)**

Acid phosphatases catalyze the hydrolysis of a wide range of orthophosphate monoesters and works best between pH 5.0 and 6.0. Environmental stresses such as P*i* or water deficiency (or salt excess) apparently cause acid phosphatase activity to increase (Pan, 1987; Goldstein *et al.*, 1988). The presence of a phosphatase specific for PEP has been inferred for many years owing to the substantial PEPp activity, which frequently interferes with the determination of plant PK activity (Sung *et al.*, 1987). Duff and co-workers (1989) were the first to successfully purify a PEPp from *Brassica nigra* (black mustard) leaf petiole suspension cells to apparent homogeneity and subsequently characterized physical and kinetic properties of the purified enzyme.

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## Chapter 3

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

To be submitted to ***New Phytologist***

# Chapter 3

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

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

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## 3.1 **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*.

P*i* limitation 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

## 3.2 **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.

## 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 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 1 mM $NH_4^+$, 2 mM $PO_4$ and 0.05 mM 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 P (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.

### 3.3.2 **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).

### 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 **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.

### 3.3.5 **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.

### 3.3.6 **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.

### 3.3.7 **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.

### 3.3.8 **Separation and collection of malate and pyruvate fractions via HPLC analysis**

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.

### 3.3.9 **Statistical analysis**

All data were analysed by single ANOVA. Percentage data were 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 **Changes in P*i* 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.

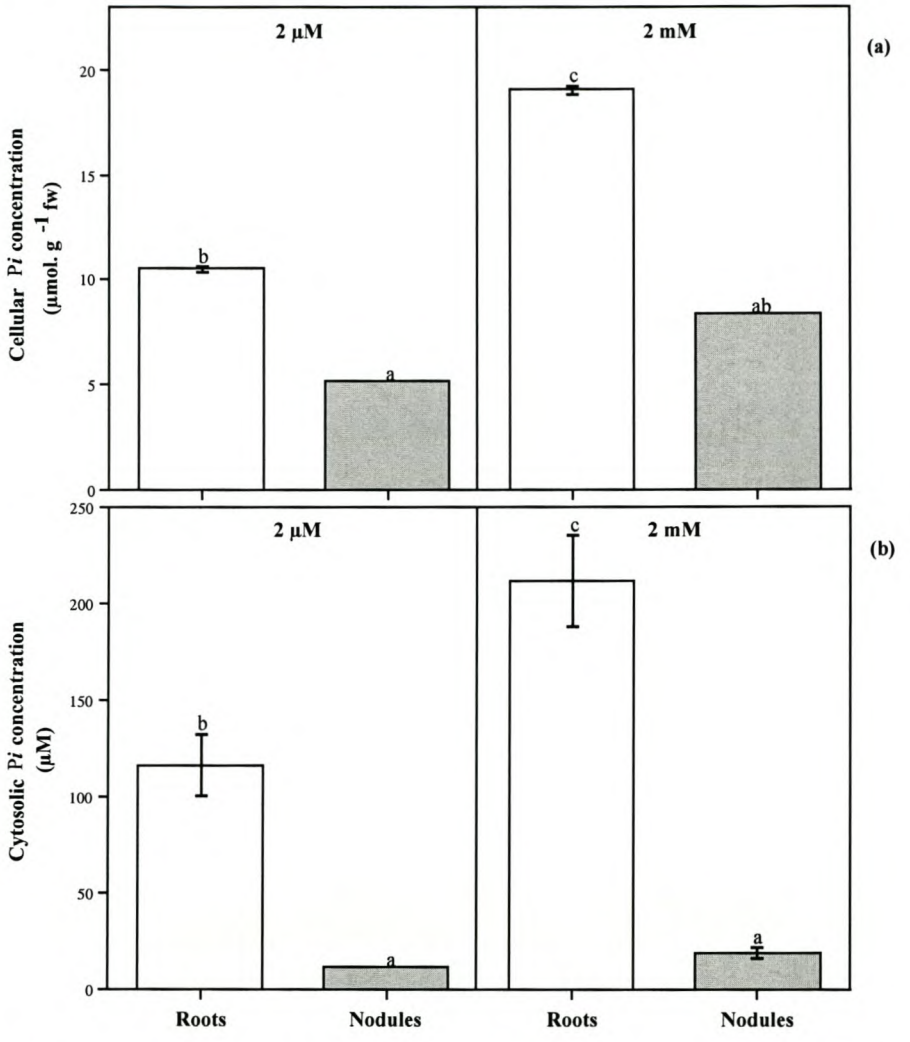


**Figure 1**. (a) Cellular P*i* concentration (µmol. $g^{-1}$ FW) and (b) cytosolic P*i* concentration (µM), of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no 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 3 replicates ($n = 3$).

### 3.4.2 **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).

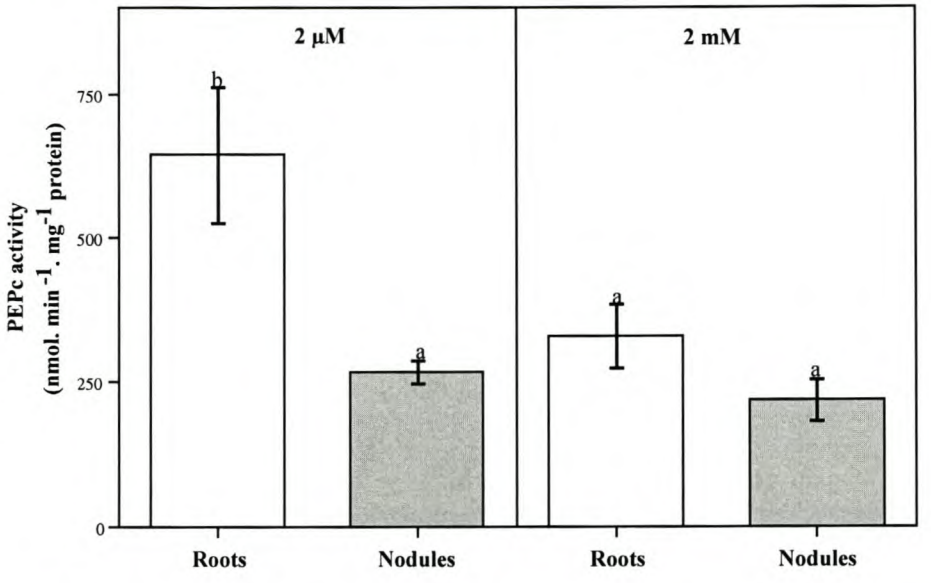


**Figure 2**. *In vitro* specific activity of PEPc (nmol. $min^{-1}$. $mg^{-1}$ protein) determined spectrophotometrically at $A_{340}$ for roots and nodules of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient 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 increased 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).

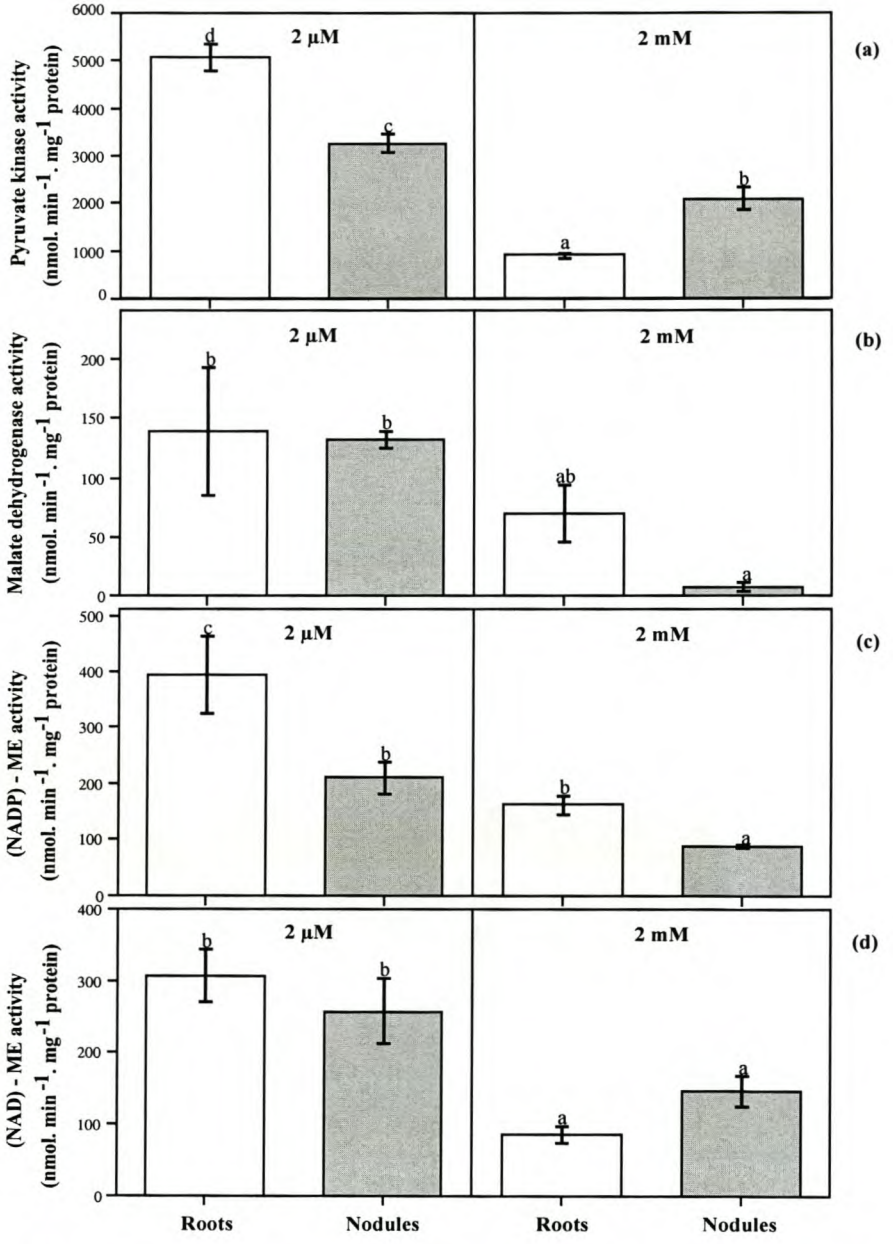


**Figure 3**. *In vitro* specific activity (nmol. $min^{-1}$. $mg^{-1}$ protein) of (a) pyruvate kinase (PK); (b) malate dehydrogenase (MDH); (c) NADP-ME and (d) $NAD^+$-ME determined spectrophotometrically at $A_{340}$ for roots and nodules of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants 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 ($n = 3$).

### 3.4.3 **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 number of PEPc polypeptides in roots and nodules was unaffected by a decline in available P (Fig. 4 a & b).

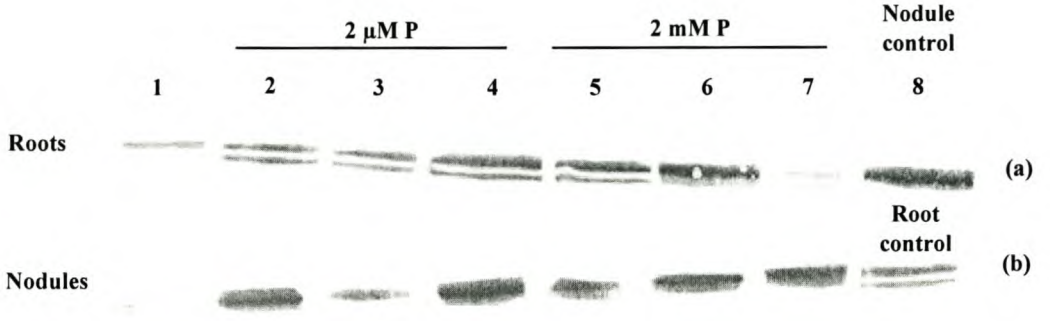


**Figure 4**. Western blot analysis of (a) root PEPc and (b) nodular PEPc with a polyclonal PEPc antibody from alfalfa nodule PEPc. Lane 1 contains the standard marker. Each lane was loaded with 15 μg of protein for roots and 30 μg for nodules. Lanes 2-4 indicate PEPC from plants that was subjected to 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.

### 3.4.4 **Metabolite concentrations and flow 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 concentrations for both compartments,

with roots showing a 60% increase in concentrations under LP supply as oppose to the unchanged levels in nodules (Fig. 5b).

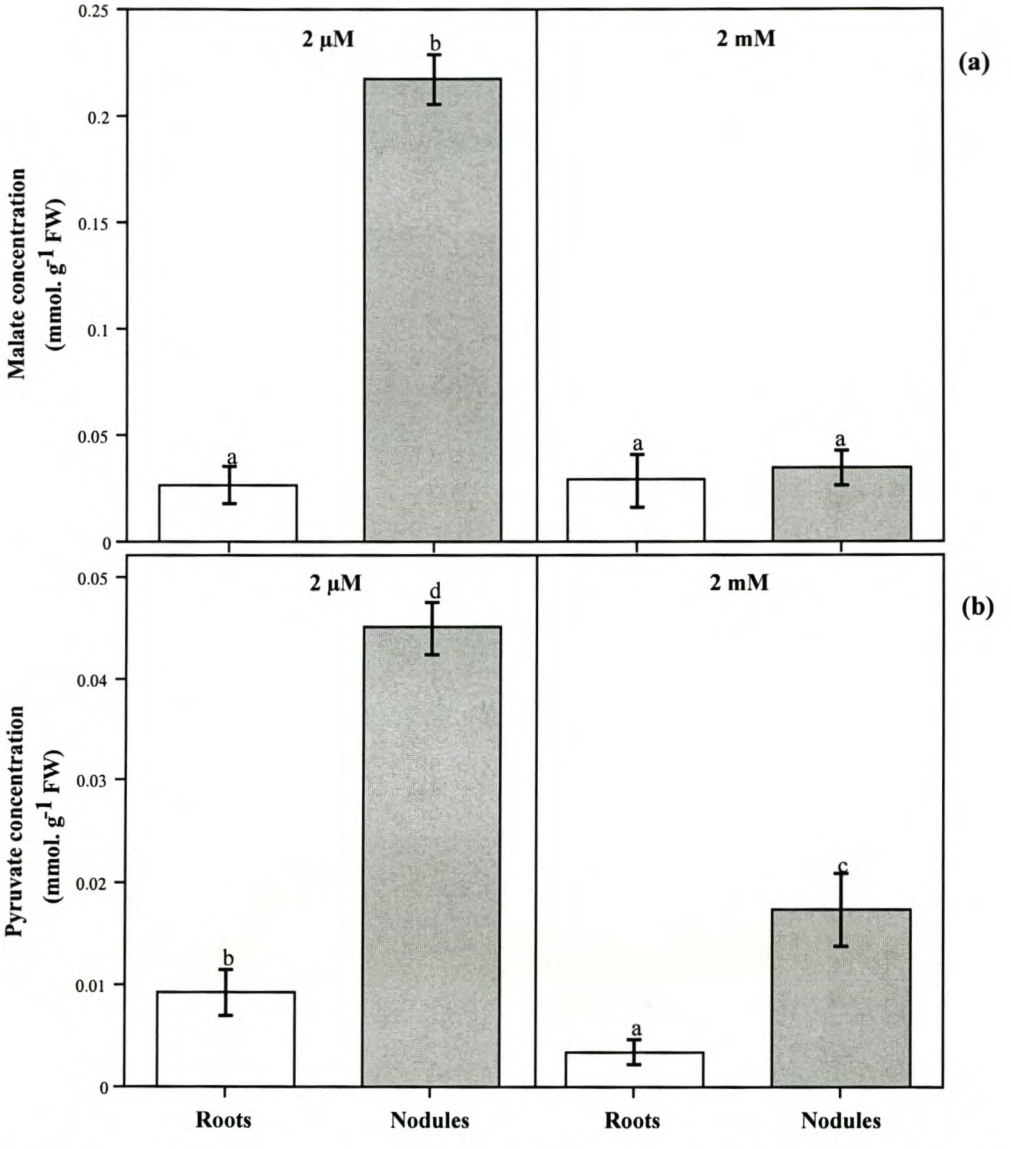


**Figure 6**. (a) Malate- and (b) pyruvate concentrations (mmol. $g^{-1}$ FW) of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. 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.

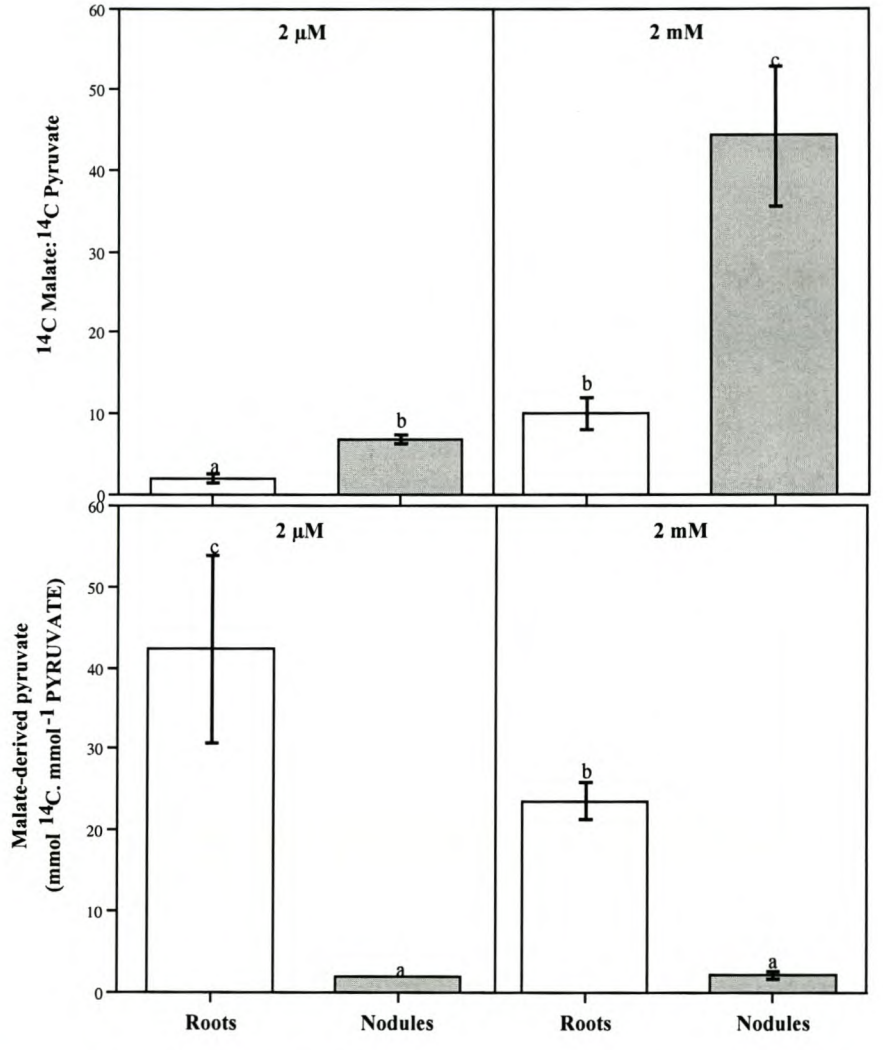


**Figure 7**. Specific malate-derived pyruvate concentration (mmol $^{14}C$. mmol$^{-1}$ PYRUVATE) of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic 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 ($n = 3$).

## 3.5 **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 was 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 bacteroids at the expense of the host roots. Bacteroids in nodules have been shown to take up P from its 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. This 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 malates. 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

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# Chapter 4

## Carbon fixation by PEPc during P*i* starvation in legume root systems

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# Chapter 4

## 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 are able to withstand 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 *et al.* 1981; Deroche & Carrayol 1988; 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 *et al.*, 1987; King *et al.*, 1986; Lawrie & Wheeler 1975; McClure *et al.*, 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 *et al.*, 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.*, 1997; Al-Niemi *et al.* 1998; 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, 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 and Rychter, 1997; Mikulska *et al.*, 1998; Ciereszko *et al.*, 1999). 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 *et al.*, 1996a). 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.

## 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 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. Two of the four hydroponic tanks were supplied with nutrient solution containing adequate P (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 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.

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 *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.

4.3.5 *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.

4.3.6 *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.

4.3.7 *$^{14}C$ 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.

### 4.3.8 *$^{14}C$ 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).

### 4.3.9 *$N_2$ 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).

*4.3.10 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.

## 4.4 **Results**

### 4.4.1 *Inorganic phosphate and adenylate level determinations*

LP roots had approximately 50 % lower P*i* levels than roots supplied with adequate P whilst for nodules there was no significant decline in P*i* levels from control plants with adequate P to LP (Fig.1). A similar pattern was observed in the cytosol of roots and nodules under the same conditions (data not shown).

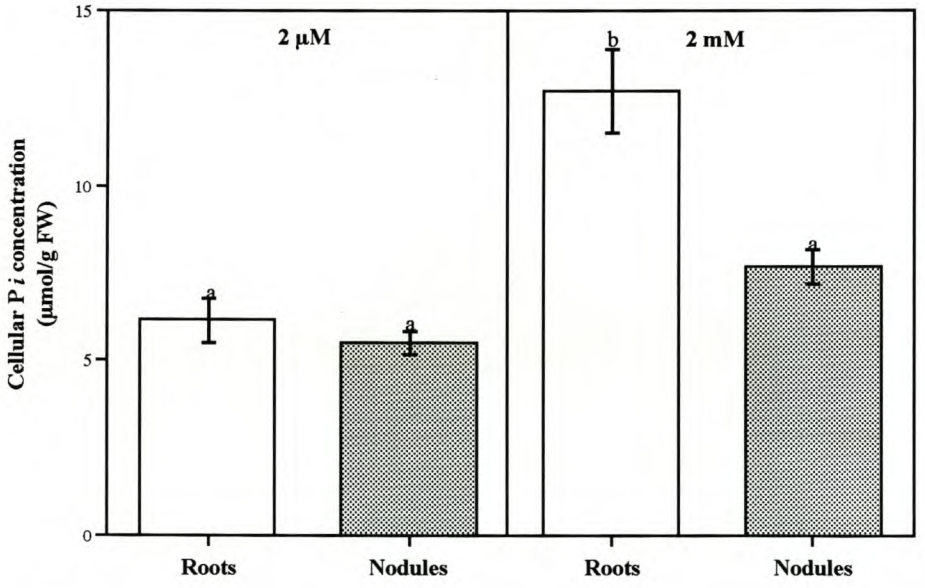


**Figure 1**. P*i* concentration in (µmol.g$^{-1}$ FW) of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated 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 ($n = 3$).

Cellular ATP concentrations of plants subjected to P deprivation also showed a similar decline in levels compared to control plants (Fig. 2). Roots and nodules of plants with a sufficient supply of P had correspondingly high ATP levels compared to roots and nodules supplied with LP (Fig. 2). Furthermore, the ATP concentrations observed for roots concur 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). The decline in ATP concentration in nodules was not as severe as in roots under the same conditions (Fig. 2).

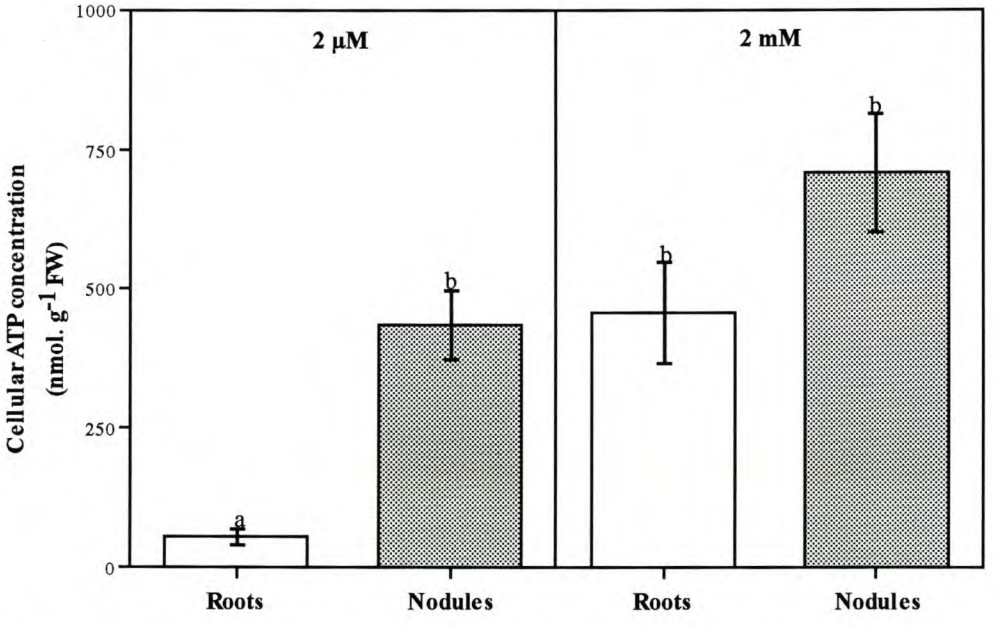


**Figure 2**. ATP concentration in (nmol.$g^{-1}$ FW) of roots and nodules from Lupinus angustifolius (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated 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.

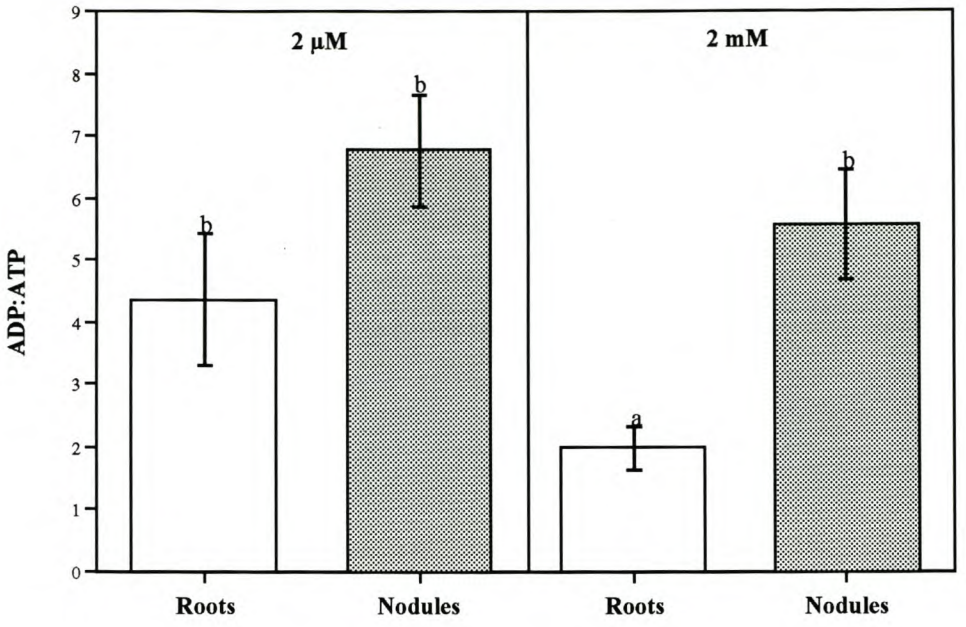


**Figure 3**. ADP:ATP ratio of *Lupinus angustifolius* (cv. Wonga) plants. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated with ambient air consisting of 360 ppm $CO_2$. 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 ($n = 3$).

### 4.4.2 *PEPc activity*

The protein content of nodules and roots decreased significantly with LP supply (Fig. 4). 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).

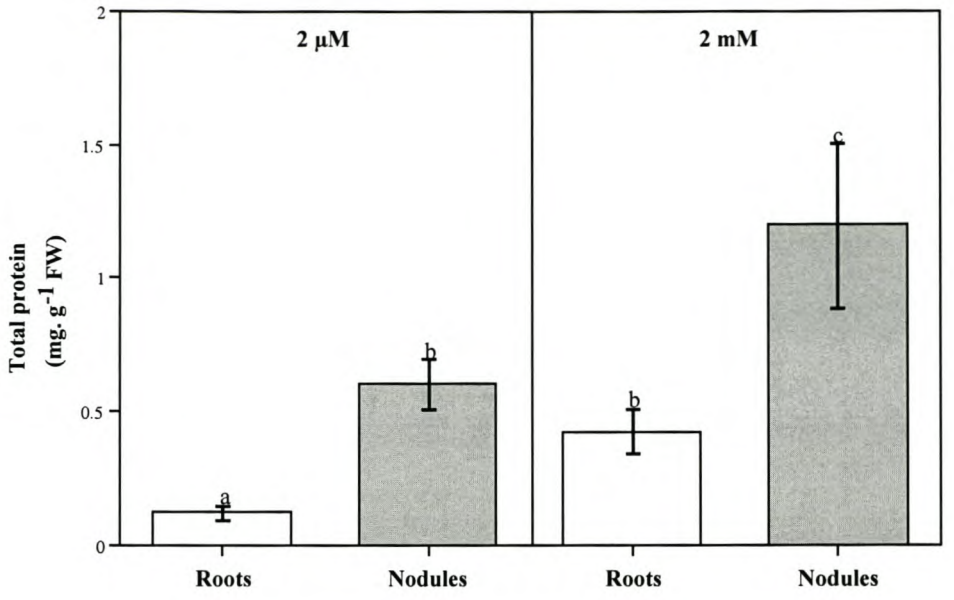


**Figure 4**. Total protein expressed in mg. $g^{-1}$ FW of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. The solution was aerated 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.

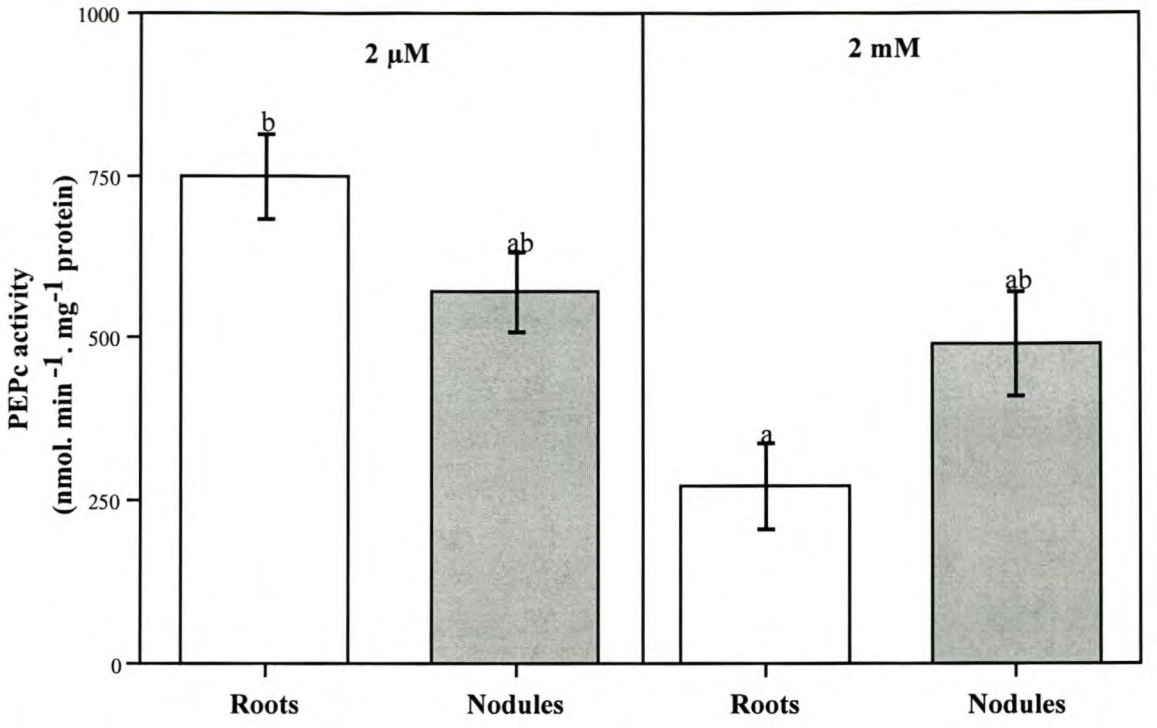


**Figure 5**. PEPc activity expressed in nmol. $min^{-1}$. $mg^{-1}$ protein of roots and nodules from *Lupinus angustifolius* (cv. Wonga). Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified to contain no inorganic N and 2 mM phosphate. 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 ($n = 3$).

### 4.4.3 *$^{14}C$ incorporation and fractionation*

After 1 hour the total incorporation of $DI^{14}C$ into organic compounds during P deprivation was lower in roots, whilst it remained unchanged in nodules (Tab. 1). Within LP and HP treatments there were also no differences in the nodule or root $DI^{14}C$ incorporation (Tab. 1).

**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) | | Adequate 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** |

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

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.

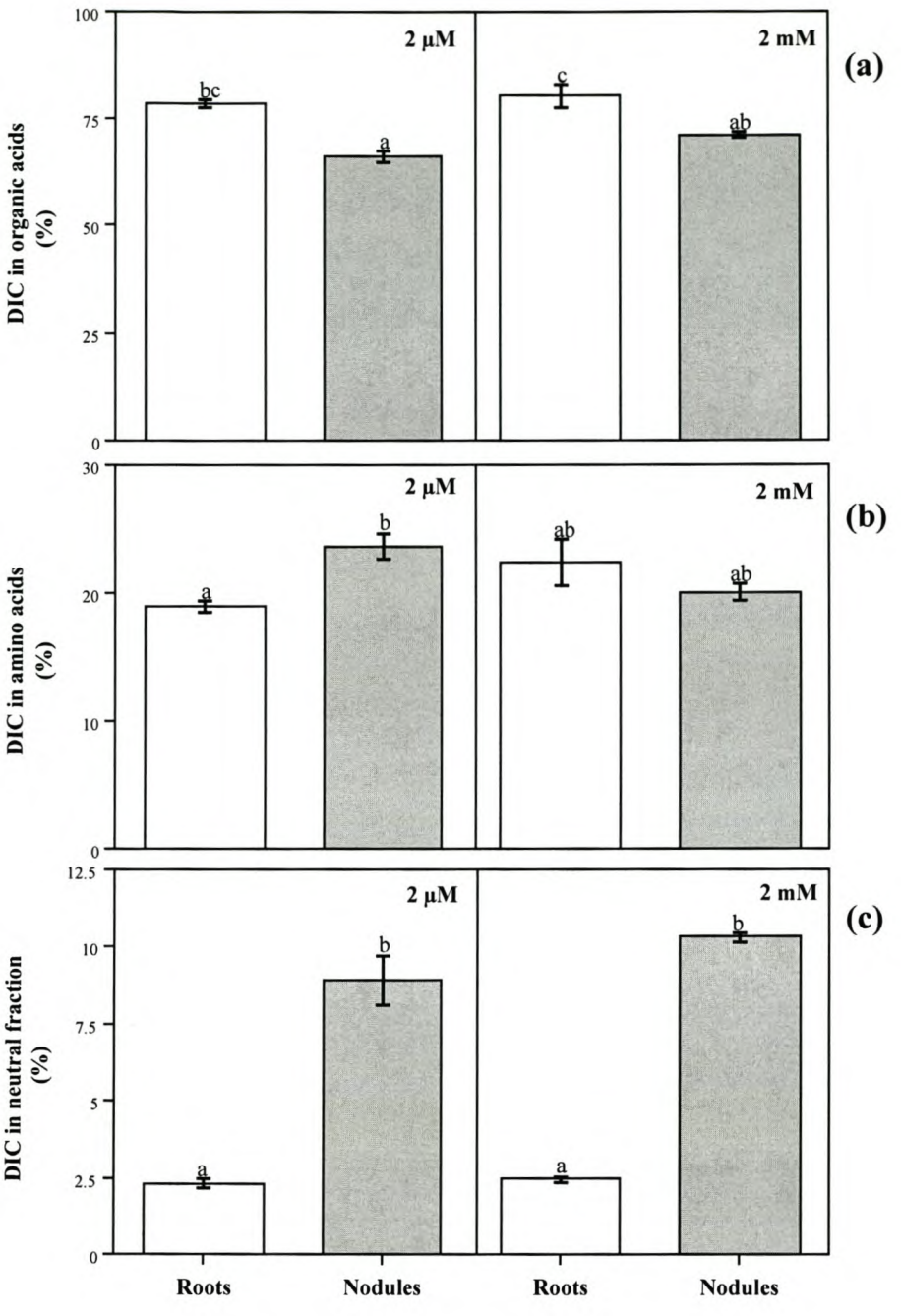


**Figure 6**. Percentage DIC present in the soluble fractions; Organic acids (a), amino acids (b) and carbohydrates (c) of roots and nodules from *Lupinus angustifolius* (cv. Wonga) after 60 minutes of $DI^{14}C$ exposure. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long 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 ($n = 3$).

**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)** | | **Adequate 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** |

### 4.4.4 *$N_2$ – 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). No changes in the ARA capacity were observed for nodules that were excised, *i.e.* detached from root piece.

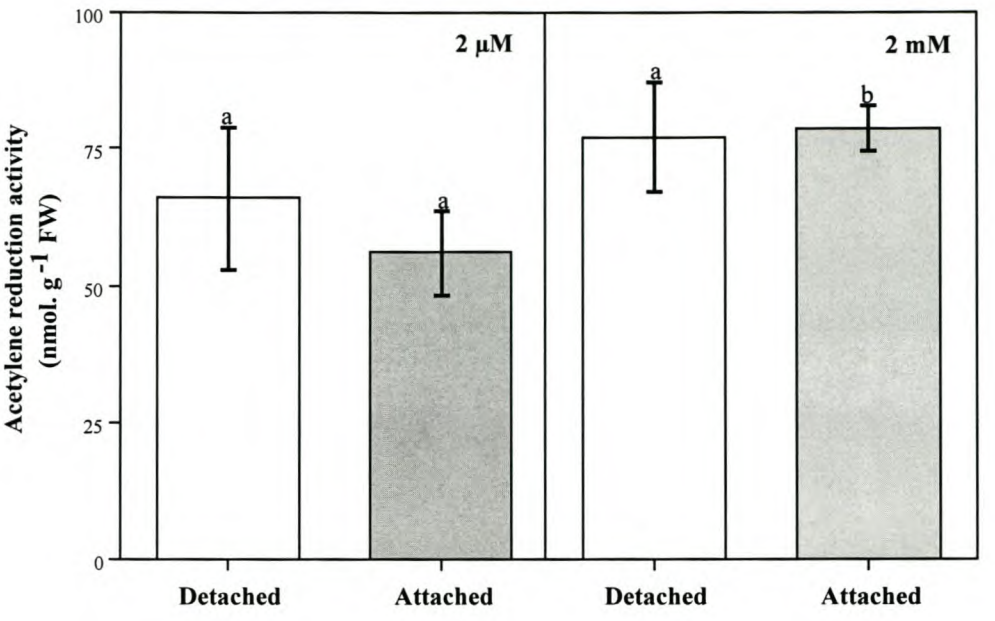


**Figure 7.** Acetylene reduction activity in nmol. $g^{-1}$ FW of detached (excised from root) and attached nodules (nodules attached to root piece) from *Lupinus angustifolius* (cv. Wonga. Nodulated plants were grown for 5 weeks in hydroponic culture in a standard Long Ashton nutrient solution modified 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 ($n = 3$).

## 4.5 **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, 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 l991), but cannot be easily compared to the current findings where metabolically available P*i* was used. Although Sa and Israel (l991) 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 (l991) using soybean, a ureide-exporting legume, whilst the current investigation used lupin, an amino-acid exporting legume. It has been proposed 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 to engage the non-adenylate requiring alternative route of PEP metabolism. In addition, 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 acids 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 (*see 'Methods and material' section*) 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 deprived 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. This is in agreement with the current findings of lower and ATP levels in nodules at LP conditions.

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 control$_i$ requirement of nodules for $N_2$ assimilation is fulfilled from host root reserves.

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# Chapter 5

# General discussion

## 5.1 Crop potential and research needs

Although research in biological N fixation has advanced at a rapid pace, the particular advances in understanding the molecular and biochemical components regulating symbiotic $N_2$ fixation have yet to be translated into applied improvements (Vance 2001). Therefore important goals for agriculture are to enhance the use of and improving the management of legume biologically fixed N (Graham and Vance, 2000). In agricultural soils, nutrient availability is a limiting factor for microbial growth and activity. In no way is this better demonstrated than with soils depleted in P. Maximum benefits from $N_2$ fixation depend heavily on P availability (Israel, 1987). Nodules (or in particular bacteroids) the engine room where $N_2$ fixing capabilities are realized, form strong sinks for P and it has been reported that symbiotically grown legumes require more P than plants grown on inorganic N (Israel, 1987). This is not related to the ability of the two separate root systems to absorb P more efficiently.

Plants dependent on symbiotic $N_2$ fixation have variable P and ATP requirements for nodule development and functioning (Ribet and Drevon, 1996). 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.

The novelty of our results for symbiotic roots demonstrates that using metabolically P*i* is indeed a more sensitive indicator of P stress. Our results show unequivocally that nodules do not experience P stress to the same extent as roots. The implications of these findings are that nodules require low P concentration to operate normally. This is in accord with the P requirement of some free-living bacterial cultures, for which it was reported that P concentrations of less than 5 μM were sufficient for growth and survival (O'Hara *et al.*, 1988).

Although other workers have studied P levels in symbiotic roots (Sa and Israel, 1991; Al-Niemi *et al.*, 1997, 1998), results reported here are distinctly different to those reports. Sa and Israel (1991) compared total P of P sufficient and P starved nodules instead of metabolically active P. Similarly Al-Niemi *et al.* (1997) determined P levels using $^{32}P$ orthophosphate by digestion of the entire nodule to determine distribution of labelled P. The cellular- and cytosolic P*i* data presented here, were a more accurate measure of assessing P status since it explored the metabolically available P for tissue.

It was also found that bacteroids within nodules could be P limited even when plants have received adequate P (Sa and Israel, 1991; Al-Niemi *et al.*, 1997). Phosphate toxicity has also been observed in legumes grown in hydroponics with P concentrations in excess of

15 μM (Bell *et al.*, 1990). These results collectively suggest that although nodules appear to be strong sinks for P, it seems as if nodules strike a fine balance to avoid excess P when available, but also to minimize the effects of P deficiency (Tang *et al.*, 2001). It is not certain whether this might be achieved through engaging a more efficient uptake mechanism than comparative roots (Al-Niemi *et al.*, 1998).

The maintenance of constant low P concentrations in nodules may have been at the expense of the host root system. Other workers have demonstrated that when nodules are P starved they can become very aggressive scavengers for available P in the medium, even out competing roots (Al Niemi *et al.*, 1998). Al Niemi and co-workers (1998) showed that when P starved, nodules incorporated P at much higher rate than roots. It was also speculated that unlike roots nodules do not readily immobilize P to host shoot (Al Niemi *et al.*, 1998). These findings of constant P levels in nodules place metabolic reaction of the nodules in response to low P in medium in a new light. Unchanged AEC of nodules, in the absence of a perceived change in P status, clearly demonstrate an energy sufficiency compared to roots subjected to P stress. The correlation of AEC with metabolically available P is a significant departure from other studies with P stress in nodules.

P*i* and adenylate levels decline in response to P stress (Duff *et al.*, 1989; Rychter and Randall, 1994) and alternative routes, which are not under adenylate control are engaged. PEP is situated at important branch-point, through which C flux to mitochondria is regulated. PEPC is regarded as the major enzyme, which supplements the TCA cycle

under P stress conditions. In leguminous root systems, particularly amide-exporting legumes, PEPC provides C skeletons for $NH_4^+$ assimilation and also fuels bacteroids in the bacterial sac (Christeller *et al.*, 1977; Rosendahl *et al.*, 1990).

Results for roots with regard to engaging the alternative route via PEPC under P stress concur with previous findings (Jusczuk and Rychter, 2002; Toyota *et al.*, 2003). Likewise, unchanged nodular PEPC activity was correlated with absence of a perceived change in P levels with P stress. The fact that the P levels of nodules were unaffected by P deprivation opens up an avenue for future research.

Furthermore, the increase in *in vitro* PEPC activity of roots was not translated into a greater capacity to incorporate DIC. This reflected either that the substrate may have been the limiting factor at low $CO_2$. In addition P supply did not affect protein expression levels. In spite of blots revealing that PEPC for roots and nodules were not regulated at translational level, the route of pyruvate synthesis was still affected by P starvation. In this regard more pyruvate was synthesized via concerted activities of PEPC, MDH and ME bypass compared to its synthesis via PK. Hence, the operation and existence of the bypass route is not disputed by our results because there was an increase in synthesis of pyruvate via malate. However, this does not imply a lack of participation via the PK route. It is proposed that under P stress this increase in flux via both pathways primarily serves to produce pyruvate. This increase in pyruvate serves to oxidize accumulating reducing equivalents (Jusczuk and Rychter, 2002).

There was a more pronounced engagement of the alternative PEPc route in roots, in agreement with lower P*i* levels, than in nodules. Interestingly, engagement of this path was not at the exclusion of the conventional route via PK. This is believed to be because of the aforementioned importance of pyruvate in oxidative stress (Jusczuk and Rychter, 2002). The operation of the alternative route may serve different purposes in roots and nodules. In roots the activation of the alternative route may be regulated by malate. The effect of malate is likely through end production inhibition of PEPc (Marzewski, 1989; Schuller *et al.*, 1990, 1993).

In nodules the increased engagement of the bypass did not increase in response to P stress because nodules did not experience P stress. For this reason increased engagement may have served to produce malate, which acts as a bacterial fuel (Rosendahl *et al.*, 1990). Alternatively pyruvate synthesis via the PEPc bypass might have served as a precursor for amino acid biosynthetic pathways, *e.g.* alanine biosynthesis. In addition, Sa & Israel (1991) suggested that nodules of P-deficient plants contain sufficient P to support the energy transducing pathways that lead to ATP synthesis, allowing them to still fix $N_2$ into $NH_4^+$. Thus, it seems that $N_2$ fixation may not have been energy limited but rather restricted by C skeletons.

In this regard, the operation of both pathways served to supply C skeletons for $NH_4^+$ assimilation, which would otherwise build up to toxic levels. Therefore it is likely that $NH_4^+$ would have accumulated under P stress since P stress may limit carbohydrate supply to nodules to greater extent than ATP supply (Almeida *et al.*, 2000). This build up of $NH_4^+$ would have required conversion to amino acids to reduce its toxic effects at high

concentrations. Since C would have been drawn from TCA, the anaplerotic provision of C via the malate route would have been imperative in this regard.

The increase in concentration of pyruvate synthesized from malate, indeed suggest that under LP conditions there is an increse requirement for pyruvate. It is clear from this data that the operation of bypass route in nodules should be investigated further. In addition the proposed assessment of this particular bypass would be incomplete without investigation of another non-adenylate requiring bypass via PEPp. Nevertheless, this study provided incentives for understanding the role of C pathways in $N_2$-fixation, in particular under conditions of P limitation. Finally, labelling studies substantiated the concept of glycolysis to PEP with further reduction to malate and subsequently pyruvate.

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