The regulation of Phosphoenolpyruvate (PEP) metabolism via Phosphoenolpyruvate Carboxylase (PEPC) in P-deficient roots and nodules of *Virgilia divaricata*

by

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Declaration

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Summary

Plants exhibit a flexible array of morphological, physiological and biochemical adaptations during phosphorous limitation. Legumes are vulnerable to P deficiency, because it affects their ability to fix atmospheric nitrogen (N_2). In particular, legumes from nutrient-poor ecosystems, such as the Fynbos in the Cape Floristic Region (CFR) evolved on P deficient soils and may therefore display unique adaptations to soil P stress. In general, very few studies on legumes have focussed on the belowground structures of nodules as a plant organ. Moreover, even less is known about the P stressed responses in nodules from legumes in nutrient-poor ecosystems. The aim of this research was to investigate the metabolic flexibility of organic acid and amino acid metabolism in the nodulated root system of the Fynbos legume *Virgilia divaricata*, during low P stress.

Virgilia divaricata, which grows in the Cape Floristic Region, was used in this study to enhance our knowledge regarding the vital role that the cytosolic enzyme, phosphoenol pyruvate carboxylase (PEPC) plays in phosphoenol pyruvate (PEP) metabolism, in roots and nodules of this legume during phosphate stress. *V. divaricata* was grown under glasshouse conditions (20 - 25˚C) in sterilized quartz sand for 2-3 months whilst being inoculated with the nitrogen fixation bacteria, *Burkholderia phytofirmans,* which was isolated from *V. divaricata* nodules grown in fynbos soil. Two phosphate treatments, 5 µM and 500 µM, were applied simulating low-phosphate and high phosphate conditions respectively using a modified Long Ashton Nutrient Solution to simulate a low nutrient ecosystem such as the Cape Floristic Region. Roots and nodules were then analysed for growth kinetics, nutrient acquisition and distribution, enzyme activity and genetic responses. It was shown that during phosphate deficiency, *V. divaricata* nodules experienced less P*i* stress than roots, due to increased metabolic phosphate conservation reactions during organic acid synthesis via an increased PEPC activity. The increased PEPC activity resulted in an increase in downstream metabolic products such as organic acids, (malic acid and citric acid), and amino acids (glutamate, aspartate and asparagine). Although the biological nitrogen fixation (BNF) declined, the high efficiency of BNF may be underpinned by these altered phosphate conservation pathways and enhanced resource allocation during growth particularly under low phosphate (LP) conditions. Therefore, it can be concluded that the efficiency of the nodules via an increased allocation of resources and P acquiring mechanisms in *V. divaricata* may be the key to the plant's ability to adapt to poor P environments and thus sustaining its reliance on BNF. From the data obtained as well as previous findings, it has been established that the phosphate conservation mechanisms in

roots and nodules, involve the non-adenylate requiring PEPC-bypass route. ^{13}C Nuclear magnetic resonance (NMR) gave us a better understanding regarding the incorporation rates of the PEPC-derived C into malate, α-ketoglutarate and asparagine. It therefore is suggested that *V. divaricata* nodules may use their large PEPC-derived malate pool to prevent large declines in BNF under low phosphate conditions. The nodules of *V. divaricata* were able to offset an excessive drop in BNF, despite a decline in inorganic phophosphate (P*i*) levels. It therefore appears that nodules have evolved to acquire different mechanisms than roots to adapt to phosphate deficiency in order to maintain their function. This was achieved via increased regulation of nodule PEPC and its downstream products. This implies that compared to roots under low P, nodules alter the metabolism of PEPC derived C, in order to maintain nodule respiration and amino acid synthesis. This trait could be observed in the synthesis of larger ^{13}C malate pools of nodules compared to roots, from PEPC, which was underpinned by their different regulation mechanisms of enzyme activity, of the same protein isoform. Since malate is a potent inhibitor of PEPC activity, roots appear to have invested in more PEPC protein compared to nodules. In contrast, nodules with lower PEPC protein, achieved greater enzyme activity than roots, possibly due to higher phosphorylation in order to reduce the malate effect. The subsequent metabolism of this PEPC-derived malate, caused roots and nodules to synthesise asparagine via different pathways. These findings imply that roots and nodules under P stress, synthesise their major export amino acid, asparagine, via different routes. This research has generated new knowledge regarding the physiological impact of the organic and amino acid metabolism, derived from PEPC-C in the roots and nodules of legumes growing in nutrient poor ecosystems. It has demonstrated for the first time that the nodules of legume from a nutrient-poor ecosystem rely on improved resource allocation, P*i* distribution, and PEPC-derived organic acids to maintain the efficient functioning of N assimilation under P stress. This may be a consequence of having evolved in a nutrient-poor ecosystem, so that nodule-bacteroid respiration and N metabolism can be maintained in P-poor soils such as the Fynbos.

Opsomming

Tydens fosfaat stremming maak plante gebruik van buigsame kombinasies van morfologiese, fisiologiese en biochemiese aanpassings. Peulplante is sensitief vir fosfaat tekorte omdat dit die vermoë om atmosferiese stikstof te kan fikseer, grootliks beïnvloed. Peulplante vanuit ekosisteme met mineraal-arme gronde, soos Fynbos binne die Kaapse Blommeryk, het ontwikkel in grond met lae fosfaatvlakke en mag dus unieke aanpassings tot fosfaat tekorte toon. Oor die algemeen is daar baie min peulplant studies wat fokus op die ondergrondse strukture van wortelknoppies as ‘n plant orgaan. Nog minder inligting is beskikbaar oor wortelknoppies, van peulplante, vanuit mineraal-arme ekosisteme, se reaksie teenoor ‘n fosfaat tekort. Die doel van hierdie navorsing was om die metaboliese buigsaamheid van organiese- en aminosuur metabolisme in die (nodulated) wortelknoppie-wortelstelsel van die Fynbos peulplant *Virgilia divaricata*, tydens fosfaat tekort te ondersoek.

Virgilia divaricata wat voorkom in die Kaapse Blommeryk, was gebruik in hierdie studie om die huidige kennis te verbeter van die essensiële rol wat die sitisoliese ensiem, fosfo-enol piruvaat karboksilase (PEPC) in fosfo-enol piruvaat metabolisme tydens ‘n fosfaat tekort speel binne die wortels en wortelknoppies van hierdie peulplant. *V. divaricata* was gegroei onder glashuis toestande (20 - 25˚C) in gesteriliseerde kwartssand vir 2-3 maande. Die plante was geïnokuleer met die stikstoffikserende bakterie, *Burkholderia phytofirmans,* wat geïsoleer is vanaf *V. divaricata* wortelknoppies wat in Fynbos grond gegroei is. Twee fosfaatbehandelings, 5µM and 500µM, was toegedien om lae en hoë fosfaat toestande, onderskeidelik, na te boots deur gebruik te maak van ‘n aangepasde Long Ashton voedingstofmengsel om ‘n ekosisteem, soos die Kaapse Blommeryk, met lae voedingstofvlakke na te boots. Die wortels en knoppies was geanaliseer ten opsigte van die groeikinetika, opname en verspreiding van voedingstowwe, ensiemaktiwiteit en genetiese aanpassings.

Dis is bewys dat tydens fosfaat tekort *V. divaricata* wortelknoppies minder fosfaat stres ervaar as die wortels, as gevolg van die verhoogde metaboliese fosfaat bewaringsreaksies tydens organise suur sintese via die styging in PEPC aktiwiteit. Die styging in PEPC aktiwiteit lei tot ‘n verhoging in stroomaf metaboliese produkte soos organiese- (appel- en sitroënsuur) en aminosure (glutamaat, aspartaat en asparagien). Alhoewel biologiese stikstoffiksering verlaag het, kan die hoë doeltreffendheid daarvan ondersteun word deur díe aangepasde fosfaat bewarings weë asook verhoogde hulpbron toekenning tydens groei onder lae fosfaat omstandighede. Dit kan dus afgelei

word dat die doeltreffendheid van die wortelknoppies via die verhoging in belegging van hulpbronne en fosfaat opname meganismes in *V. divaricata* moontlik die sleutel is tot die plant se vermoë om aan te pas tot omgewings met lae fosfaatvlakke en sodoende die afhanklikheid van biologiese stikstofbinding te kan onderhou.

Data in hierdie as ook vorige studies, wys dat die fosfaat bewaringsmeganismes in wortels en wortelknoppies die PEPC-ompad roete, wat nie adenilaat benodig nie, gebruik. ^{13}C NMR het meer lig gewerp aangaande die vaslegging van koolstof vanaf PEPC na malaat, α-ketoglutaraat en asparagien. Dit word voorgestel dat *V. divaricata* knoppies ‘n groot hoeveelheid malaat, afkomstig van PEPC-werking, gebruik om groot dalings in biologiese stikstofbinding tydens fosfaat tekort, te verhoed. Die wortelknoppies van *V. divaricata* kon ‘n oormatige verlaging in biologiese stikstofbinding voorkom ten spyte van die verlaging in fosfaatvlakke. Dis wil voorkom dat wortelknoppies ander meganismes as die wortels ontwikkel het om aan te pas tot fosfaat tekort en sodoende dus hul funksie behou. Dit word bereik deur ‘n verhoging in die regulering van PEPC en die stroomaf produkte in die wortelknoppies. Dit blyk dat wortelknoppies tydens fosfaat te kort, in vergelyking met wortels, die metabolisme van die koolstof vanaf PEPC verander om sodoende respirasie en aminosuursintese te onderhou. Dit wil voorkom dat hierdie meganismes verskil van die van wortel meganismes.

Hierdie eienskap kan toegeskryf word aan die produksie van ‘n groter hoeveelheid van ^{13}C malaat vanaf PEPC in die wortelknoppies teenoor die wortels, wat ondersteun word die verskillende reguleringsmeganismes van ensiemaktiwiteit van dieselfde proteïen isoform. Malaat is ‘n kragtige inhibeerder van PEPC-aktiwiteit, dus blyk dit dat die wortels belê in meer PEPC proteïene as die wortelknoppies. In teenstelling, toon die wortelknoppies met laer PEPC proteïene, ‘n hoër ensiem aktiwiteit as die wortels. Dit kan wees as gevolg van hoër fosforilasie om die effek van malaat te verlaag. Die metabolisme van die malaat vanaf PEPC het die sintese van asparagien in die wortels en wortelknoppies via verskillende roetes tot gevolg gehad. Dit impliseer dat tydens ‘n tekort aan fosfaat, wortels en wortelknoppies hul hoof uitvoer aminosuur, asparagien, deur verskillende roetes sintetiseer.

Hierdie studie het nuwe kennis aangaande die fisiologiese impak van organiese- en aminosuur metabolisme met koolstof vanaf PEPC in die wortels en wortelknoppies van peulplante wat voorkom in ekosisteme met lae voedingstofvlakke, voortgebring. Vir die eerste keer is dit bewys dat die wortelknoppies vanaf peulplante wat voorkom in mineraal-arme ekosisteme, staatmaak op verbeterde hulpbron beleggings, fosfaat verspreiding en organiese sure vanaf PEPC om die

doeltreffendheid van funksionele stikstofassimilasie tydens fosfaat tekort, te onderhou. Dit mag die gevolg wees van, om in ‘n voedingstof arme ekosisteem te ontwikkel sodat die wortelknoppie-bakteroïed respirasie en stikstofmetabolisme onderhou kan word in fosfaat arme grond soos die Fynbos.

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List of abbreviations

Å	Angstrom
ACN	Acetonitrile
ADP	Adenosine 5‘-diphosphate
APase	Acid phosphatase
AS	Asparagine synthetase
ATP	Adenosine 5‘-triphosphate
BNF	Biological nitrogen fixation
CAM	Crassulacean acid metabolism
CAN	Ceric ammonium nitrate
DCDP	3,3-Dichloro-2-dihydroxy-phosphinoylmethyl-2-propenoate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FA	Formic acid
FeMo	Iron molybdenum
FeMoco	Iron molybdenum cofactor
Fig	Figure
FW	Fresh weight
g	Gram
g	Relative centrifugal force
GC-MS	Gas chromatography mass spectrometry
GDH	Glutamate dehydrogenase
GS/GOGAT	Glutamate synthase/Glutamine oxoglutarate aminotransferase

Glc-6-P	Glucose-6-phosphate
h	Hour
HP	High phosphate
K_m	Michaelis constant
kDa	Kilo Dalton
LC-MS	Liquid chromatography mass spectrometry
LP	Low phosphate
MDH	Malate dehydrogenase
ME	Malic enzyme
mg	Milligram
mM	Millimolar
min	minutes
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NDFA	Nitrogen derived from atmosphere
OAA	Oxaloacetic acid
P	Phosphorous
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
ppm	Parts per million
P*i*	Inorganic phosphate
PFK	Phosphofructokinase
PK	Pyruvate kinase
PP*i*	Pyrophosphate

PSI	Orthophosphate-starvation inducible
PSR	P*i*-starvation response
PVPP	Polyvinylpolypyrrolidone
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-test	Statistical student‘s *t* distribution
TCA	Tricarboxylic acid
TCEP	Triscarboxyethyl phosphine
TFA	Trifluoroacetic acid
TMS	Trimethylsilyl
UDP	Uridine diphosphate
µl	Microliter
µM	Micromolar
δ15N	Nitrogen isotopic ratio

CHAPTER 1: Literature review

1. Legumes

The Family *Leguminosae* (or *Fabaceae*) is the third largest family of flowering plants after *Orchidaceae* (the orchid family) and *Asteraceae* (Compositae; the sunflower family), with over 600 genera and 20 000 species. They are classified under the Kingdom, Plantae, the Division, Angiosperms and the Order Fabales. It is an extraordinary diverse family of worldwide distribution occurring from alpine and arctic regions to the equatorial tropics. Legumes include herbaceous plants, such as the temperate to subtropical crop species *Pisum sativum* (pea), *Cicer arietinum* (chickpea), *Vicia ervilia* (vetch), *Glycine max* (soybean), and *Phaseolus vulgaris* (common bean), *Trifolium* (clovers), *Medicago sativa* (alfalfa), *Lupinus* (lupins), *Lens culinaris* (lentils), *Arachis hypogaea* (peanuts), *Ceratonia siliqua* (carob) and *Aspalathus linearis* (rooibos) as well as large, woody lianas, such as Wisteria and 100-meter tall trees of tropical forests. One characteristic of these plants is the fact they develop nodules on their roots in which symbiotic bacteria called nitrogen fixation bacteria live. These nitrogen fixation bacteria produce nitrogen compounds that help the plant to grow. The fruit of this family is technically called a legume or pod which is composed of a single seed-bearing carpel that splits open along two seams. The importance of legumes cannot be underestimated as they are very important both ecologically and agriculturally. They are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms such as ammonia, nitrate, and organic nitrogen (Brockwell *et al*. 1995). Biological nitrogen fixation (BNF) and especially legumes are of great practical importance because the use of nitrogenous fertilizers (which apparently is applied to soil in an irresponsible manner) has resulted in unacceptable levels of water pollution i.e. the increasing concentrations of toxic nitrates in drinking water supplies and the eutrophication of lakes and rivers (Dixon and Wheeler 1986, Sprent and Sprent 1990, Al-Sherif 1998).

1.1 Nitrogen fixation

Despite the fact that our atmosphere is almost 80% nitrogen gas, the element, nitrogen is unavailable to plants and most living organisms in the inert gaseous state due to the triple bond which exists between the two nitrogen atoms. Nitrogen is the fourth most common

element in plant tissue and is essential to plants as it is the building blocks for nucleic acids, enzymes, proteins, structural components and pigments. Nitrogen is transported by the plants transport system i.e. xylem and phloem. Some higher plants have developed the ability to live in symbiosis with microorganisms that can fix N_2. The nitrogenase enzyme complex is at the core of biological nitrogen fixation (BNF). Only some microorganisms, i.e. diazotrophic prokaryotes, possess this enzyme complex and are able to fix N_2. The processes of nitrogen fixation, nitrification and ammonification make nitrogen available to autotrophic plants and ultimately to all members of the ecosystem. Nitrogen fixation can be regarded as a remarkable prokaryotic skill in which inert atmospheric nitrogen gas is combined with hydrogen to form ammonia (NH_3) employing the enzyme, nitrogenase. The reaction for nitrogen fixation is:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow \quad 2NH_3 + H_2 + 16ADP + 16Pi$$

This process occurs in the absence of oxygen due to the fact that oxygen inhibits the nitrogenase enzyme complex irreversibly by reacting with the iron component of the complex (Dixon, Wheeler 1986). BNF is essential for life because fixed nitrogen is required to biosynthesize the basic building blocks of life, i.e. nucleotides for DNA and RNA and amino acids for proteins. BNF takes place while N_2 is bound to the nitrogenase enzyme complex. The proteins that form this complex are structurally and functionally quite conserved among the different N_2-fixing organisms (Igarashi and Seefeldt 2003, Zehr *et al.* 2003).

1.1.1 The nitrogenase enzyme

Nitrogenase is composed of two ferrosulphoproteins (Fig. 1). Component I, dinitrogenase also referred to as the FeMo protein, is an α2β2 heterotetramer consisting of 4 (Fe_4S_4) clusters plus a cofactor containing Fe and Mo, where the catalytic site is located and has a molecular weight of about 220 kDa. Component II is a homodimer of about 60 kDa containing a single Fe_4S_4 cluster (Kaminski *et al.* 1998). The α and β apo-subunits of component I are encoded by the *nif*D and *nif*K genes, respectively, whereas component II polypeptides are encoded by *nif*H. Three other genes,

*nif*B, *nif*E and *nif*N are required for the biosynthesis of the FeMo cofactor (Fischer 1994). *nif*E and *nif*N encode proteins that are used as scaffold for the assembly of the cofactor, which also include the product of *nif*B (Fig. 1) (Paustian *et al.* 1989, Lanzilotta and Seefeldt 1996, Igarashi and Seefeldt 2003).

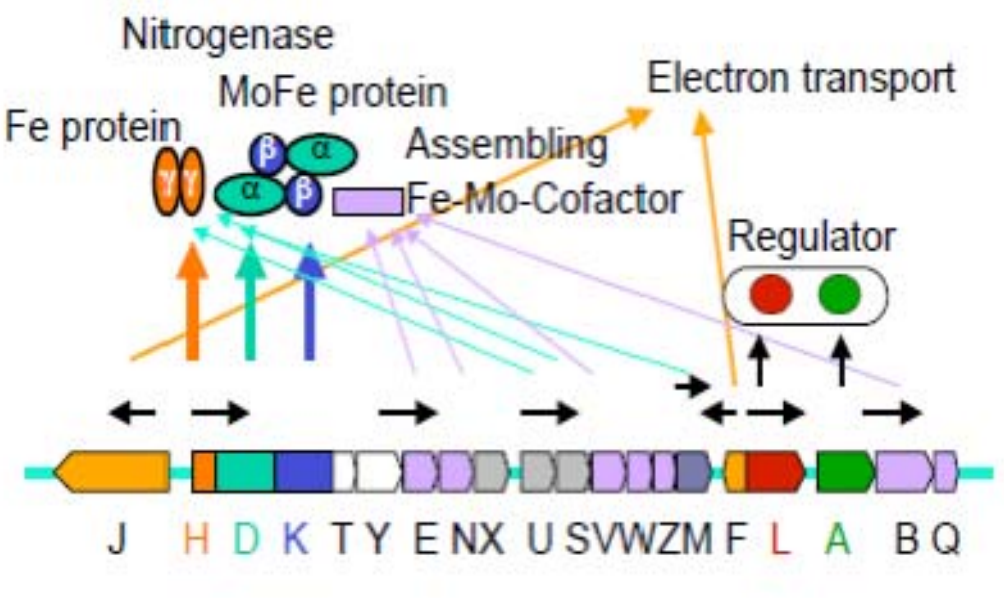

Figure 1. The physical association of *nif* genes encoding the nitrogenase enzyme complex of *Klebsiella pneumonia* (Sylvia *et al.* 2005).

The FeMo protein contains two atoms of molybdenum and has oxidation-reduction centers of two distinct types i.e. two iron-molybdenum cofactors called FeMoco and four Iron-sulphur centers. The FeMoco is regarded as the active site for nitrogenase activity. The Fe protein is reduced by electrons donated by ferredoxin. The reduced iron protein then binds ATP and reduces the FeMo protein. In return this protein then donates electrons to N_2, producing HN=NH. In two further processes, the HN=NH is reduced to $H_2N\text{-}NH_2$ and in return is reduced to $2NH_3$ (Postgate 1998).

1.2 Legume nodulation

1.2.1 Bacteria involved in nodulation

Early studies on bacterial nodulating legumes revealed that there are many differences among these legumes. Subsequently, two categories were identified, i.e. fast-growing and slow-growing bacteria nodulating legumes. These two categories were then called *Rhizobium* and

Bradyrhizobium, respectively (Jordan 1982). In addition to the growth difference, other differences were also observed. These include the production of acid or alkali when grown on suitable media as well as the location of the nodulation (*nod*) and nitrogen-fixing (*nif*) genes. In *Rhizobium* and *Sinorhizobium* these are located on extrachromosomal DNA (plasmids). In *Bradyrhizobium* they are chromosomal and in *Mesorhizobium*, they are located on symbiotic islands within the chromosome. Other differences, such as preferred carbon substrates, salt tolerance and antibiotic tolerance, were also observed. All the bacteria that are known to nodulate legumes fall into the category Gram-negative Proteobacteria which are grouped and divided into 5 sections coded by Greek letters (Sprent 2009).

1.2.2 Nodulation

Nitrogen fixing bacteria cannot fix nitrogen unless it invades the plant root. In contrast, the soil bacterium *Azotobacter* can fix nitrogen without the assistance of a host plant. In order for nitrogen fixation to take place in the plant, the plant needs to develop nodules on its roots in which the common Gram-negative, rod shaped soil bacterium, *Rhizobium* and other nitrogen fixing bacteria live. Two types of nodules are found i.e. determinate nodules and indeterminate nodules. Determinate nodules are found on tropical legumes, such as those of the genera *Glycine* (soybean), *Phaseolus* (common bean), *Lotus,* and *Vigna.* These nodules lose meristematic activity shortly after initiation. Growth is due to cell expansion resulting in mature nodules which are spherical in shape. Indeterminate nodules are found on temperate legumes such as *Pisum* (pea), *Medicago* (alfalfa), *Trifolium* (clover), and *Vicia* (vetch). They maintain an active apical meristem that produces new cells for growth over the life of the nodule. This results in the nodule having a generally cylindrical shape. Nodule formation occurs due to the fact that legumes release compounds called flavonoids, such as luteolin and naringenin (Charrier *et al*. 1995, Shirley 1996, Bladergroen and Spaink 1998) that serve as chemo attractant to *Rhizobia* (Caetano-Anolles *et al.* 1988). These compounds also activate a set of rhizobial genes that are involved in the synthesis of a compound molecule that sets off the process of root nodule formation (Zuanazzi *et al.* 1998). A number of biochemical and morphological changes happen once this nod factor is sensed by the root. The formation of a root nodule requires that two major processes are set in motion. The first step is that the cortical cells have to be dedifferentiated to form a nodule primordium and secondly the bacteria have to enter the plant, a process under strict control of the plant.

Root hair growth is redirected and winds around the bacteria multiple times until it fully encapsulates one or more bacteria. The encapsulated bacteria start multiplying and form a micro colony. These bacteria then enter the developing nodule through a structure called an infection thread, which grows through the root hair into the basal part of the epidermis cell and onwards into the root cortex where they further multiply. They are then surrounded by a plant-derived membrane (peribacteriod membrane) and differentiate into bacteroids. It was found that the bacteriods are enclosed in a compartment known as a symbiosome which prevent a plant defense response and also facilitates the movement between the bacteriods and the plant (Verma 1992, Verma and Hong 1996). The plant provides all the necessary nutrients and energy for the bacteria (via carbon-sources obtained through photosynthesis) and in return the bacteria supply the plant with the essential products of nitrogen fixation (Figs. 2, 3). The exchange of nutrients between the plant and bacterium is accomplished as the nodules are connected by the xylem and phloem which is again connected to the vascular system of the plant. The symbiotic relationship between plants and bacteriods also prevents toxic ammonium from entering plant cells. Ammonium is a primary product of nitrogen fixation which is excreted by the bateriods. The bacteriods prevent assimilation of it as ammonium is immediately incorporated into amino acids (Day *et al.* 2001).

Initially these nodules are white to grey on the inside indicating that they are not yet capable of fixing nitrogen. As they grow and increase in size, they become pink or red which is caused by leghemoglobin, indicating that nitrogen fixation has started. Nodules turn green when they cease to fix nitrogen and might be discarded by the plant (Crespi and Galvez 2000, Lindemann and Grover 2003). The bacteroids have an increased demand for oxygen as they produce ATP by means of oxidative phosphorylation. However, as mentioned, oxygen is fatal for the nitrogenase enzyme. In order to regulate the oxygen levels, it was found that oxygen needs to pass a variable oxygen diffusion barrier (Witty *et al*. 1986).

In addition it was also found that infected cells of the nodules contain high levels of leghemoglobin, which supplies just the right concentration of oxygen to the bacterium to satisfy their needs. This leghemoglobin is similar to the myoglobin found in muscles and facilitates the transfer of oxygen to the bacteriods in a controlled manner (Dixon and Wheeler 1986). The amount of nitrogen fixation varies tremendously within legumes. The common bean is an example of a poor nitrogen fixer which fixes less nitrogen than is required by the plant. Beans need to grow in fertilized soil in order to produce a good harvest. In contrast to beans, plants like peanuts and soybeans are good nitrogen fixers which fix all their nitrogen needs.

The nitrogen fixation process can be affected negatively by various factors like, temperature and water shortage. When a plant experiences a lack of certain nutrients such as phosphorus, potassium, zinc, iron, molybdenum and cobalt, it undergoes stress, resulting in a reduction of nitrogen fixation. However, these stress conditions can be corrected by the addition of fertilizers to the soil to which the legume will respond directly to the nutrient supplied and indirectly to the increased nitrogen nutrition fixation. (Lindemann and Grover 2003, Crespi and Galvez 2000).

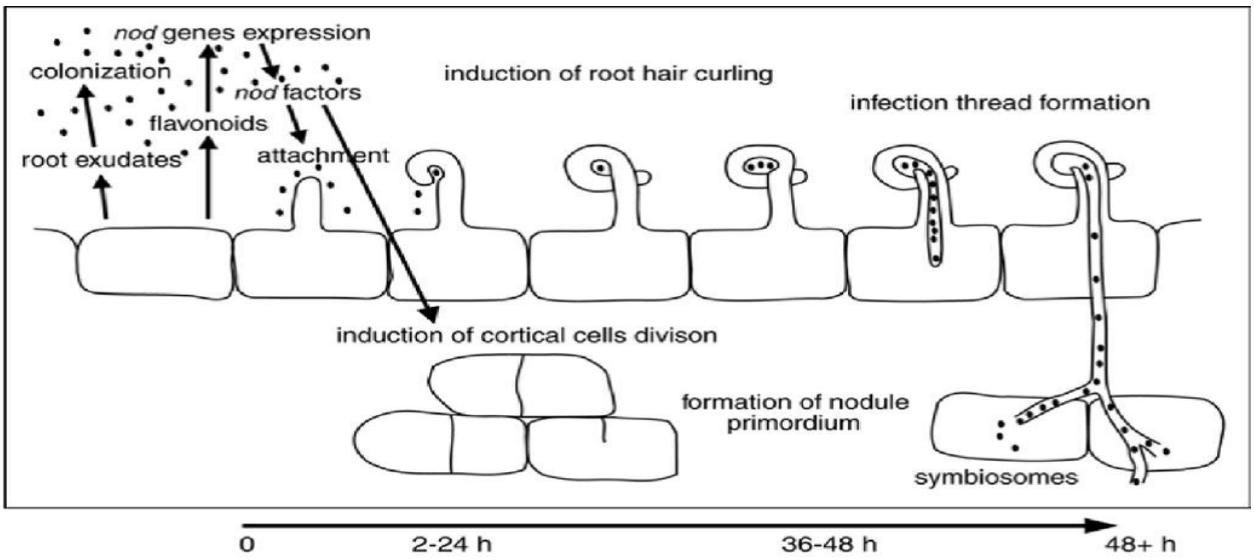

Figure 2. The infection process through the root hairs and the simultaneous formation of the nodule primordium (Amâncio and Stulen 2004).

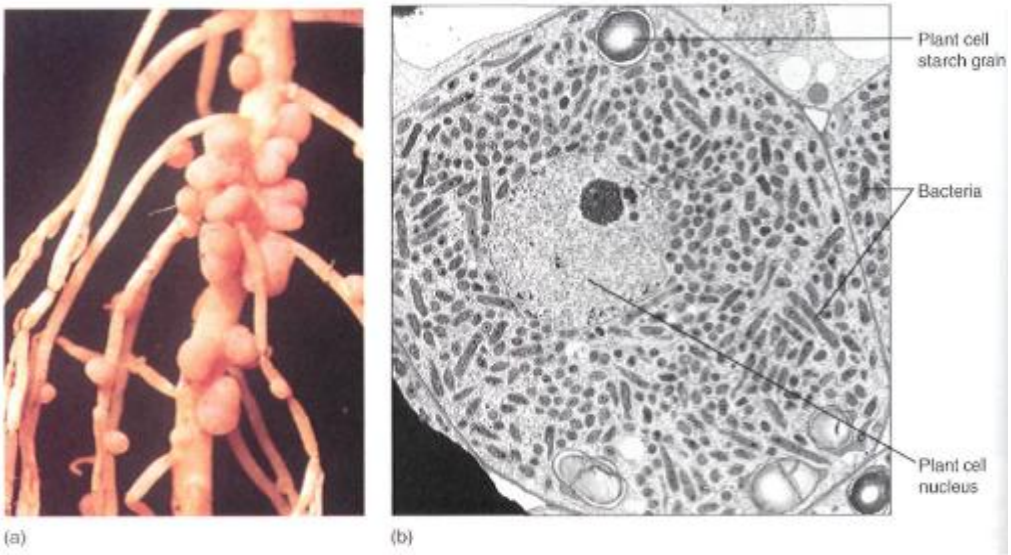

Figure 3. (a) Nodules on alfalfa containing bacteria, (b) Bacteria in an infected root of cowpea (Mauseth 1998).

1.3 Legumes response to various stress factors

It was found that N_2 fixation is strongly related to the physiological state of the host plant. Therefore, any particular rhizobial strain cannot fix nitrogen to its full capacity if stress conditions, such as unfavorable soil pH (too saline or too alkaline) nutrient deficiency, mineral toxicity, extreme temperatures, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, grazing and pesticides, prevail. These major stress factors suppress the growth and symbiotic characteristics of most rhizobia, however, several strains, distributed among various species of rhizobia, are tolerant to stress effects. Some strains of rhizobia form effective (N_2-fixing) symbioses with their host legumes under salt, heat, and acid stresses, and can sometimes do so under the effect of heavy metals (Brockwell *et al.* 1995, Peoples *et al.* 1995, Thies *et al.* 1995).

1.3.1 Soil salinity

As the salt concentration of soil increases, the possibility is very high that it might have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate 1995). As is the case with most cultivated crops, the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties, and the stage of growth (Cordovilla *et al.* 1995). The symbiotic relationship between legume and *Rhizobium*, appears to be more sensitive to salt or osmotic stress affecting nodule formation, compared to Rhizobia which do not live in symbiosis which are less sensitive (Zahran and Sprent 1986, El-Shinnawi *et al.* 1989, Velagaleti *et al.* 1990, Zahran 1991) Salt stress inhibits the initial steps of *Rhizobium*-legume symbioses. The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules (Ikeda *et al.* 1992, Delgado *et al.* 1994, Walsh 1995).

1.3.2 Soil Acidity

Soil acidity is a significant problem facing agricultural production in many areas of the world resulting in loss of legume productivity and thus nitrogen fixation (Graham 1992, Clarke *et al.*

1993, Bordeleau and Prevost 1994, Correa and Barneix 1997) Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N_2 fixation (Brockwell *et al.*1991, Bordeleau and Prevost 1994). Legumes and their rhizobia exhibit varied responses to acidity. Studies on acidity stress, indicated that the pH-sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils (Vargas and Graham 1988, Caetano-Anolles *et al.* 1989).

1.3.3 Nutrient Deficiency Stress

Mineral toxicity and especially ion toxicity usually occurs in conjunction with acidity and salinity in soil leading to nutrient deficiency and nutrient disorders in legumes and other plants. It was shown that nodulation and nodule development in cowpea were strongly depressed at low pH (4.5 - 5.5), low calcium concentration (0.05 - 2.5 mM) and stress conditions may inhibit nodulation of legumes through the inhibition of genetic activity (Alva *et al.* 1990).

1.3.4 Fertilizer application

Legumes obtain the majority of combined nitrogen in the form of NO_3^-, which is formed by the oxidation of ammonium from residual fertilizers and the mineralization of organic nitrogen. It was shown that both NO_3^- assimilation and N_2 fixation of legumes are strongly dependent on the plant cultivar, bacterial strain, ontogeny, and environmental factors as described above. NO_3^- assimilation requires less energy and allows the plant to conserve its energy, since in overall terms more energy is required to fix N_2 than to utilize NO_3^-. It is therefore necessary that the plant be able to detect the presence and level of NO_3^- in the rooting medium and to adjust its N_2 fixation accordingly. However, application of fertilizers should be done in a very responsible controlled manner to avoid downstream consequences. One of the most important and potentially limiting factors to BNF is the use of herbicides, fungicides, and other pesticides. It was reported that when the active ingredients in herbicides, bentazone and 4-chloro-2-methylphenoxyacetic acid are applied to leguminous crops, it constitutes a potential hazard to the establishment and performance of the N_2-fixing root nodules and ultimately nitrogenase activity and thus nitrogen

fixation (Ljunggren and Martensson 1980).

1.3.5 Drought:

Studies have shown that soil water limitations inhibit nodule initiation growth and development as well as nodule functioning. Thus the entire N_2 fixation process is retarded during drought (Serray *et al.* 1999a, Vadez *et al.* 2001, Streeter 2003, Pimratch *et al.* 2008) Furthermore, different legumes respond differently to drought stress. Legumes such as soybean, cowpea and pigeon pea, which export ureides (allantoin and allantoic acid) in the nodule xylem, are more sensitive to drought compared to legumes such as alfalfa, broad bean, chick pea and pea which export amides (glutamine and asparagine) (Sinclair and Serraj 1995). It was also found that nodulation and N_2 fixation will be affected depending on the stage of plant growth. Water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproduction stage (Pena-Cabriales and Castellanos 1993).

1.3.6 Phosphate stress

One major stress condition which plants encounter is that of P deficiency. Plants have unique ways of overcoming P limitations and these will be discussed in detail.

2. The role of phosphate (P)

Phosphorus is one of the most important elements for the development of the plant and its sustainability as it is the core element for many compounds such as structural components for phospholipids, nucleic acids, nucleotides, coenzymes and phosphoproteins. In addition to this, it also plays a key role in many biochemical processes which include; energy generation, photosynthesis, glycolysis, respiration, redox reactions, signaling, nucleic acid metabolism, carbohydrate metabolism, nitrogen fixation, membrane synthesis and stability.

2.1 Accessibility of P

From results obtained it appears that the concentration gradient from the soil solution of P to plants cells exceeds 2000-fold with an average P*i* of 1 µM in the soil solution. This concentration reflects a well below K_m for plant uptake (Bieleski 1973, Schachtman *et al.* 1998). It is quite ironic that P is available in such abundance but is unavailable to plant uptake posing a potential threat to plants. Phosphorous becomes inaccessible to plants as it forms insoluble complexes with cations, like aluminum and iron which prevails during acidic conditions. It appears that P availability to plants is controlled by the types and amount of clay and metal oxides. In addition, its availability also depends on soil pH solution, ionic strength, concentrations of P and the metals Fe, Al and Ca as well as the presence of competing ions which include organic salts (Sanyal and DeDatta 1991) Fertilizers which are rich in P*i* is one possible solution to boost the P concentration of soil to sustain plant growth and development and ultimately stimulating crop yield (Vance *et al.* 2002).

Worldwide it is a common situation where enormous amounts of P*i*-containing fertilizers are used to boost the soluble P*i* concentration in soil, indicating that the P*i* levels of soil is sub-optimal for plant growth. Currently the major source of P*i* is rock P, which is currently being depleted at an alarming rate. Projections show that there might not be enough rock P to sustain this utilization past the end of the century (Vance *et al.* 2003). In addition, it appears that the application of fertilizers is inefficient, as the plants absorb less than 20% of the applied fertilizers during their first growing season due to retention by the soil making P immobile or due to leaching into nearby water and rivers (Russel 1973). This excessive use of P*i* poses a major problem as it can be regarded as a primary factor in the eutrophication of lakes and marine estuaries. It also results in the rapid blooming of toxic cyanobacteria (Runge-Metzger 1995, Bumb and Baanante 1996).

2.2 Importance of P

Phosphate plays a crucial role in the nitrogen fixation process. Plants acquire P as $H_2PO_4^-$ or $H_2PO_4^{2-}$ (depending on pH of the soil) and utilize it in its fully oxidized and hydrated form as P*i*. A small portion of inorganic phosphorous is converted to an organic state upon entry to the plant and then transported to the shoots. It was also shown that plants can absorb soluble organic P in the form of nucleic acids (Clarkson 1980).

The element, P is a trivalent resonating tetraoxyanion and serves as a binding site for many molecules. In its P*i*-state, it can be further condensed to form oxygen-linked polyphosphates. These unique properties of P make it possible to form water stable anhydrides and esters. These molecules are of utmost importance to the plant for energy transfer and storage in biochemical processes. The most important ones for plants are ADP and ATP. The transfer of P molecules from energy transforming processes to ATP and the transfer from energy requiring molecules from ATP in the plant is known as phosphorylation. A portion of the energy which the plants obtain via photosynthesis is utilized by phosphorylation to convert ADP to ATP in a process called photophosphorylation. During respiration, energy is also released and plants utilize this energy in a process called oxidative phosphorylation. Furthermore, P is released from organic compound by means of enzymes, collectively known as phosphatases. P is also a structural component for phospholipids, nucleic acids, nucleotides, coenzymes and phosphoproteins. Phospholipids are crucial for membrane structure, nucleic acids which carry genetic material and in its mono-ester state it serves as a ligand in enzyme catalysis (Clarkson 1980.)

2.3 Effect of P deficiency to roots

The plant's root system is responsible for the primary uptake of the plants mineral element requirement, from where it is distributed to the rest of the plant. During P limitation, the plant responds by allocating more carbon compounds to the roots. This leads to increased root growth, enhanced lateral root formation, greater exploration of the root surface and an increased length and density in root hairs as well as the amount of root hairs. This allows the plant to explore larger areas such as topsoil with the possibility to acquire P (Lynch 1995, Gilroy and Jones 2000, Liao *et al.* 2001, Lynch and Brown 2001, Williamson *et al.* 2001). P deficiency also leads to enhanced P transporters (Muchhal *et al.* 1996, Leggewie *et al.* 1997, Smith *et al.* 1997, Liu *et al.* 1998, Liu *et al.* 2001) and the exudation of organic P and organic acids which thus increases the P availability to plant areas where the demand for P is crucial (Duff *et al.* 1991, Tadano and Sakai 1991, Johnson *et al.* 1994, 1996a, Dinkelaker *et al.* 1997, Gilbert *et al.* 1999, Neumann and Romheld 1999). During periods of P deficiency, certain plants also form cluster roots to obtain P. However, these type of plants usually do not form mychorrhizal associations (Skene 1998, Pate and Watt 2001).

From studies performed on roots it appears that roots have a reservoir of organic compounds

which include simple sugars, organic acids, phenolics, quinines (iso)-flavonoids, growth hormones, proteins and polysaccharides. These compounds are exuded by the roots and accomplish many functions where needed in the plant (Curl and Truelove 1986, Marschner 1995). These exuded compounds differ in their functionality and are involved in a variety of processes such as signaling in plant microbe interaction, allelophathy and nutrient acquisition (Curl and Truelove 1986, Marschner *et al.* 1986, Marschner 1995, Harrison 1997). It was found that these exuded compounds can be adjusted both in quality and quantity, depending on the stress condition which the plant experiences (Marschner 1995, Ryan *et al.* 2001, Neurmann and Martinola 2002). During nutritional stress such as P deficiency, Al-toxicity, Fe-deficiency, and exposure to heavy metals, roots show enhanced synthesis and exudation of several organic acids in the form of anions to compensate for the current stress situation (Dinkelaker *et al.* 1989, Delhaize *et al.* 1993, Delhaize and Ryan 1995, Ryan *et al.* 1995a, b, 1997, Larsen *et al.* 1998, Neumann *et al.* 2000). Studies on plant responses to low P and high aluminum conditions are well documented and there is currently convincing evidence that the exudation of malate and citrate are the principle mechanisms that the plant uses to alleviate the P deficiency and aluminum toxicity. In practice it means that the release of these organic acids allow for the chelation of Al^{3+}, Fe^{3+} and Ca^{2+} resulting in the release of P from a bound form (Gerke 1994, Jones 1998, Hinsinger 2001, Ryan *et al.* 2001). In addition this mechanism also causes organic P to be more susceptible to be hydrolysed through acid phosphatases (Gerke 1994, Braum and Helmke 1995). The release of organic acids also protects the plant from Al-toxicity. Al^{3+} ions are chelated as the plant releases these organic acids and thereby prevents Al^{3+} entering the roots.

2.4 Effect of P deficiency on carbon metabolism

P*i* plays a crucial role in metabolic pathways (Fig. 4) and will have a negative impact on biochemical pathways when P*i*-limitations arise. Enzymes responsible for the conversion of certain substrates to end products cannot fulfill their duty as crucial P*i* is needed. Despite the crucial role that P*i* plays in these pathways, the plant continues to generate energy and produce carbon skeletons. Plants must have adapted in a remarkable way to still execute the necessary production of certain life sustaining products despite P limitations. Unlike other eukaryotes, plants are very flexible in adjusting their metabolic rates and utilizing alternative metabolic pathways. It was shown that plants can skip P*i*- or ATP-requiring steps of

glycolysis (Fig. 4) under environmental stress conditions such as P deficiency (Duff *et al*. 1989b, Mertens 1991, Theodorou *et al*. 1992, Theodorou and Plaxton 1996). There are reports that ATP- and ADP-levels decline in P stress conditions (Ashihara *et al.* 1988, Duff *et al.* 1989b), however, the levels of pyrophosphate (PP*i*) appear to be buffered during such conditions (Duff *et al*. 1989b, Dancer *et al.* 1990). Initial reports indicated that PP*i* cannot be used as energy source for alternative pathways, however, it became known that PP*i* can indeed serve as energy donor in the plant cytosol (Duff *et al*. 1989b, Plaxton 1996, Plaxton and Carswell 1999). Studies have shown that PP*i* can serve as energy donor in an alternative glycolytic pathway which utilizes the enzyme phosphofructokinase (PFK). This alternative pathway bypasses the conventional ATP-dependent PFK to yield fructose 1,6 biphosphate (Fig. 4) (Theodorou *et al*.1992, Theodorou and Plaxton 1996, Plaxton and Carswell 1999). It is assumed that other processes such as the cleavage of sucrose in the sucrose synthase pathway might also use PP*i* as alternative energy source. In addition the H^+ pump in the tonoplast can also make use of PP*i* to actively pump protons into the vacuole (Plaxton and Carswell 1999).

From literature it appears that PP*i* can be regarded as a secondary product of plant-metabolism and anabolism and accumulates in bulk during biosynthesis. It can thus be used in the plant cytosol to increase the energy demand of several cellular processes as energy donor in order to conserve ATP pools under limiting conditions (Duff *et al.* 1989b, Theodorou *et al.* 1992, Plaxton 1996, Theodorou and Plaxton 1996, Plaxton and Carswell 1999, Plaxton and Tran 2011).

Plants can also utilize another alternative glycolytic pathway which uses a non-phosphorylating NADP-dependent glyceraldehyde 3-P dehydrogenase (NADP-G3PDH). This pathway bypasses the conventional P*i*-dependent NAD-G3PDH phosphoglycerate kinase (Fig. 4) (Duff *et al.* 1989b, Theodorou *et al.* 1992, Plaxton and Carswell 1999).

A third alternative glycolytic pathway in plants was also identified. In this pathway, plants make use of a combined action of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and a NAD malic enzyme to catalyze the alternative pathway reactions of glycolysis, feeding its products into the TCA cycle. In the process, the pyruvate kinase enzyme which requires P*i* and ADP is bypassed and carbon flow is maintained. Not only is carbon flow through glycolysis (Fig. 4) maintained, but P*i* is also generated through this bypass pathway (Theodorou *et al*. 1992, Plaxton and Carswell 1999). These alternative

pathways are plant species specific as all plants do not make use of these pathways during P stress conditions (Plaxton and Carswell 1999). Experiments have shown that during P stress conditions, phosphofructokinase (PFK) activity could not be found in plants that form symbiotic association with mycorrhizal fungi (Murley *et al*. 1998).

P stress also has a negative impact on mitochondrial respiration. It is currently assumed that electron transport in the mitochondrion also makes use of alternative pathways but unlike the alternative glycolytic pathways which are well documented, not much is known regarding alternative mitochondrial pathways (Rychter and Mikulska 1990, Theodorou and Plaxton1993). It is believed that during ADP and P*i* limitations, the plants' cytochrome pathway is inhibited, resulting in a high ATP:ADP ratio which inhibits respiration. This phenomenon is known as the adenylate control of respiration (Bryce *et al*. 1990). There are also alternative pathways, which can bypass energy requiring sites. These include: rotenoneinsensitive NADH dehydrogenase (Fig. 4) (Rasmusson and Moller 1991) and the alternative oxidase (Fig. 4) (Lambers 1985, Lance *et al.* 1985, Day and Wiskich 1995). These alternative pathways are both nonphosphorylative pathways. Alternative oxidase catalyses the oxidation of ubiquinol and the reduction of oxygen to water, however, it bypasses the two energy requiring sites that is usually used in the cytochrome pathway (Fig. 4). It was shown that alternative oxidase is regulated by a sulfhydryl disulfide redox system (Siedow and Umbach 2000) and by allosteric activation via α-keto acids, especially pyruvate (Miller *et al.* 1993, Hoefnagel *et al*. 1995). From these results it is proposed that alternative oxidase is activated due to elevated levels of PEPC which occurs during P stress.

Despite this apparent lifeline that plants make use of as alternative for P consumption, it was shown in white lupin that mitochondrial respiration can decrease up to 60% during P stress. This reduction is also accompanied by an increased NADH:NAD ratio (Johnson *et al.* 1994, Neumann *et al.* 1999). It can be assumed from these results that alternative oxidase does not fully compensate as an alternative for the cytochrome respiration pathway. However, further investigations are required to determine the regulation and expression of genes during P stress.

2.5 Additional adjustments of plants to P limitation

It is well known that plants switch from primary metabolism to secondary metabolism during nutrient stress. It was shown that during P stress, plants have the tendency of accumulating

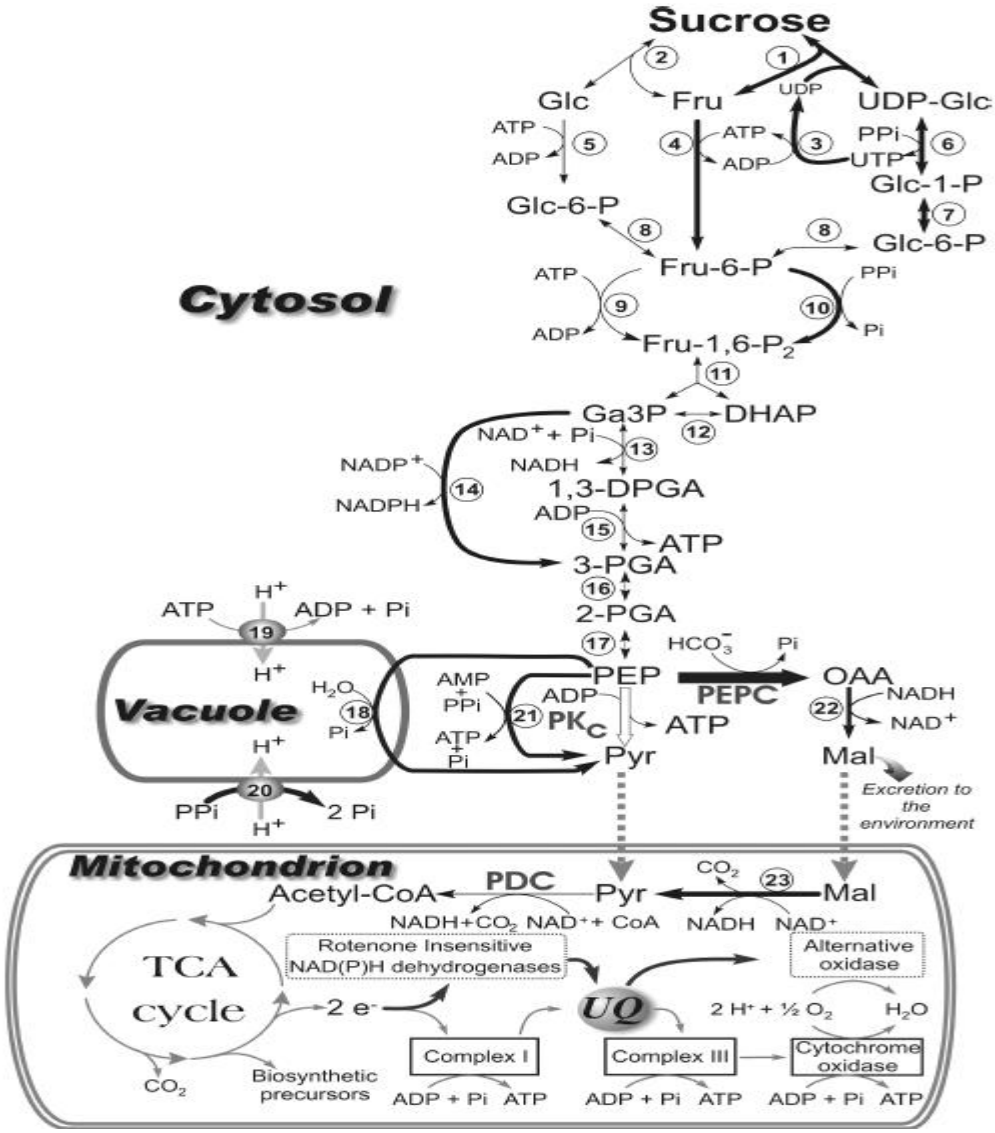

Figure 4. Alternative pathways of cytosolic glycolysis, miETC, and tonoplast H^+- pumping processes that may facilitate respiration and vacuolar pH maintenance by P*i*-deprived plant cells. Enzymes that catalyze the numbered reactions are as follow: 1, sucrose synthase; 2, invertase; 3, NDP kinase; 4, fructokinase; 5, hexokinase; 6, Uridine diP-glucosepyrophosphorylase; 7, P-glucomutase; 8, P-glucose isomerase; 9, ATP-PFK; 10, PP*i*-PFK; 11, aldolase; 12, triose-P isomerase; 13 and 14, phosphorylating and non-phosphorylating glyceraldehyde 3-P dehydrogenase, respectively; 15, phosphoglycerate kinase; 16, phosphoglyceromutase; 17, enolase; 18, PEP phosphatase; 19, H^+-ATPase; 20, H^+-PP*i*ase; 21, pyruvate P dikinase; 22. Malate dehydrogenase. 23, NAD-ME. (DHAP = dihydroxyacetone-P; Ga3P = glyceraldehyde-3-P; 1,3-DPGA =1,3-diphosphoglycerate; 2-PGA = 2-phosphoglycerate; UQ = ubiquinone) (O'Leary *et al.* 2011).

secondary metabolites such as flavonoids and indole alkaloids (Plaxton and Carswell 1999) as well as anthocyanins. The increase in anthocyanins is presumed to reduce the photoinhibitory damage to the chloroplast (Takahashi *et al.*1991). It appears that P limitation also results in the alteration of the lipid composition of the photosynthetic membranes (Essigmann *et al.* 1998, Yu *et al.* 2002).

These thylakoid membranes generally consist of monogalactosyldiacyl-glycerol, diacylgalactocyldiacyl-glycerol, phosphaidyl-glycerol and sulfoquinovocyldiacyl-glycerol. However, under P limiting conditions, the thylakoid membranes show a decrease in phospholipids and an increase in sulfolipids and sulfiquinovosyl diacylglycerol (Benning 1998, Yu *et al.* 2002). It is possible that the change in the thylakoid membranes' sulfolipid: phospholipid ratio conserves P while also maintaining the membranes functionality and therefore photosynthesis can continue (Essigmann *et al.* 1998).

2.6 Biochemical and molecular changes during P stress

Due to the fact that many of the biochemical and molecular changes that take place during P deficiency occur in synchrony, it is believed that all these processes are controlled by a common regulatory system. The genes responsible for the expression of the various products during P stress are believed to be expressed by this common regulatory system. It was suggested that regulation resides in a P regulatory system which is similar to those found in bacteria and yeast (Goldstein 1992, Schachtmann *et al.* 1998, Raghothama 1999). A plant type, two-component signaling system has been identified in plants which are involved in the perception and response to ethylene, cytokinin and osmoticum (Sakakibara *et al.* 1999, Urao *et al.* 2000, Hwang and Sheen 2001, Lohrmann and Hartner 2002). It appears that this two-component plant system is very complex involving a multistep process and at least 3 proteins. The process functions in the following manner. The input domain of membrane bound histidine kinase is signaled and autophosphorylates an aspartate residue in the transmitter domain. The transmitter domain then phosphorylates a histidine residue on a phosphotransfer (HPt) protein which is transported into the nucleus and transfers the P to an aspartate residue of the response regulator. Once the response regulator is phosphorylated, it undergoes conformational change which enables it to bind to *cis* elements within the promoter region of genes that needs to be activated depending on the type of signal received (Sakakibara *et al.* 2000, Urao *et al.* 2000, Chang and Stadler 2001, Gilroy and

Trewawas 2001, Lohrmann and Harter 2002). It appears from literature that no conclusive data have been found to link this system with signaling low or high P concentration, but was there is evidence that it can be implicated in nitrogen signaling (Sakakibara *et al.* 1999) and osmotic stress (Urao *et al.* 1999).

2.7 Genes and expression during P stress

Over the years, plants have adapted to limited P conditions and show a complex array of morphological, physiological and biochemical changes collectively known as the P*i*-starvation response (PSR). This response is partially induced by the coordinated induction of hundreds of orthoP-starvation inducible genes (PSI). These PSI genes code for enzymes to be activated which reprioritize internal P*i* use and maximize external P acquisition (Vance *et al.* 2003, Ticconi and Abel 2004, Fang *et al.* 2009, Lin *et al.* 2009, Nilsson *et al.* 2010). It is well known that many elements of the PSR are controlled at transcriptional and translational level and that under P*i* starvation plants remodel their transcriptome and proteome in ways that enable them to meet the demand for the metabolic and morphological adaptations (Plaxton and Hue 2011). Another interesting fact surfaced regarding posttranscriptional mechanisms in the control of PSI enzyme expression and activity. Studies performed on rice, corn and *Arabidopsis*, demonstrated that transcript abundance of various genes is not always an indication of the amount of protein that will be expressed during P deprivation (Fukuda *et al.* 2007, Li *et al.* 2008a, Trans and Plaxton 2008).

The *Arabidopsis* PSR regulatory module has been studied in great detail and serves as a good model for understanding these post transcriptional mechanisms. This regulatory module consists of the transcriptional factor PHR1, PHO2 (that encodes the E2 ubiquitin conjugase UBC24), the microRNA399 (miR399) and the non-coding RNA At4 (Bari *et al.* 2006, Fang *et al.* 2009, Lin *et al.* 2009, Nilsson *et al.* 2010). The miR399 is responsible for regulating P*i* homeostasis by controlling UBC24 expression. UBC24 is important during P*i* limitation as it promotes the proteolytic turnover of PSI proteins which includes the high affinity Pi transporters of the plasmalemma. PHR1 is induced by P*i* starvation and in return activates expression of miR399 which is transported in the phloem. Upon binding of miR399 to the complementary bases of UBC24, the resulting transcript leads to the destruction of UBC24 mRNA. This action results in low levels of UBC24's E2 ubiquitin conjugase activity and the accumulation of its downstream

protein targets (Bari 2006). These results show that shoot-derived miR399 serves as long distance signal to suppress the expression of UBC24 in the roots.

It was shown that when prolonged periods of P*i* starvation exists, then the ribo-regulator At4 is strongly induced (Fang *et al.* 2009, Lin *et al.* 2009, Nilsson *et al.* 2010). At4 binds to complementary bases of miR399, and silences the UBC24 mRNA. This allows UBC24 levels to adjust to the dynamic balance required for P*i* supply and demand. In addition, PSR also has to maintain posttranslational mechanisms of metabolic control, as P*i* serves as an allosteric- activator or inhibitor of many important enzymes controlling intermediary plant metabolism (Plaxton and Podesta 2006).

A visible common feature of PSR is the development of dark-green or purple shoots due to anthocyanin accumulation. This anthocyanin accumulation occurs due to PSI anthocyanin biosynthetic enzymes (Vance *et al.* 2003, Fang *et al.* 2009). Anthocyanin protects the nucleic acids from ultraviolet damage and the chloroplast from photoinhibitory damage (Zeng *et al.* 2010). PSR is also responsible for the up-regulation of high affinity tissue specific P*i* transporters of the plasma membrane. These transporters obtain their energy via an ATP- dependent proton efflux and thus ensuring active P*i* assimilation against a concentration gradient (Fang *et al.* 2009, Lin *et al.* 2009)

2.8 Enzymes involved during P stress

Plants also show an increase in phosphatase enzymes (APases) during P limitation. These enzymes are present in the plant throughout its growth as they are involved in many processes for the plant's development (Duff *et al.* 1994).These processes include the following;

- Provides P during seed germination from stored phytate (Biswas and Cundiff 1991, Brinch-Pedersen *et al.* 2002)
- They remobilize internal P (Duff *et al.* 1991, del Pozo *et al.* 1999, Baldwin *et al.* 2001)
- They release P from soil organic P esters by exuding enzymes into the rhizosphere (Goldstein *et al.* 1988, Lefebvre *et al.* 1990, Miller *et al.* 2001)
- The synthesis of glycolate from phospho-glycolate (Christeller and Tolbert 1978)

- The synthesis of glycerate from 3-phosphoglyceric acid during photorespiration (Randall *et al.* 1971)

APases play an important role during P stress in the alleviation thereof. It was shown that the primary function of intracellular APases, which are induced during P deficiency, is to release P from senescent tissue as well as to bypass P-requiring steps in the processes of carbon metabolism (Fig. 4) (Duff *et al.* 1989b, Plaxton and Carswell 1999). In contrast, root extracellular APases are involved in the acquisition of P from the surrounding soil (Marschner *et al.* 1986, Tarafdar and Claasen 1988).

Plants also have the ability to affectively increase the use of P*i* during P*i* starvation via the up-regulation of a wide array of PSI hydrolases that seizes and recycle P*i* from intra- and extracellular organic compounds (Vance *et al.* 2003, Fang *et al.* 2009, Nilsson *et al.* 2010, Tran *et al.* 2010a). It was also found that P-starved plants also scavenge and conserve P*i* by replacing their membrane phospholipids with amphipatic sulfolipids and galactolipids (Fang *et al.* 2009, Lin *et al.* 2009). Plants universally respond to P stress by the up-regulation of intracellular and secreted APases. These APases are enzymes that hydrolyse P*i* from a wide range of P*i* monoesters (Plaxton and Tran 2011).

One of the amazing features about plants is the fact that plants can adjust their metabolic pathway in such a way that the same step of a metabolic pathway can also be accomplished in a different way leading to the same final product (Fig. 4). This feature of plant metabolism allows them to respond dynamically to their continuous changing and stressful environment (Plaxton and Podesa 2006).

To date, at least six P*i* and adenylate-independent glycolytic bypass enzymes as well as the inorganic pyrophosphate (PP*i*)-dependent H^+-pump (H^+-PP*i*) of the tonoplast were identified. These enzymes are up-regulated by P*i* starving plant cells (Duff *et al.* 1989, Palma *et al.* 2000, Vance *et al.* 2003, Plaxton and Podesa 2006).

During severe P stress conditions, when the adenylates and P*i* are very low, these PSI bypasses facilitate glycolytic flux and vascular pH maintenance. During these processes PP*i* is consumed to perform cellular work while ATP and P*i* are recycled at the same time. The P*i*-stress enzymes such as the PP*i*-dependent phosphofructokinase (PP*i*-PFK), PEPC, pyruvate P dikinase and the tonoplast H^+-PP*i*ase also promote intracellular P*i* recycling, as P*i* is a by-product of their reaction (Plaxton and Tran 2011).

The PEPC-malate dehydrogenase-malic enzyme glycolytic bypass allows the P*i*-stress plants to

maintain the conversion of PEP to pyruvate flux when the cytosolic pyruvate kinase (PKc) becomes ADP limited (Fig 4).

In recent studies it was shown that PEPC and PEPC protein kinase are up-regulated in P-stressed plants which are under control of multiple transcription factors. This up-regulation was shown in *Arabidopsis* cell cultures and seedlings, rice leaves, and white lupin proteiod roots (Vance 2003, Plaxton 2006). These studies provide evidence that regulatory enzyme phosphorylation contributes to the plant PSR, which agreed with other findings that the most prominent PSI genes of *Arabidopsis* include the PEPC protein kinase-encoding genes *AtPPCK1* and *AtPPCK2* (Fang *et al.* 2009, Gregory *et al.* 2009, Lin *et al.* 2009, Nilsson *et al.* 2010). Studies have also shown that BHLH32 is a transcription factor that operates as a negative regulator of several PSI genes in *Arabidopsis,* which include *AtPPCK1* and *AtPPCK2* (Chen *et al.* 2007).

2.9 Two major enzymes involved during P stress

Nitrogen fixation comes at a huge energy cost, which is sustained by the plant via products released during photosynthesis. It was found that nitrogen fixation can consume up to 20% of photosynthate in certain leguminous plants (Haysteadt *et al.* 1980). This energy consumed by nitrogen fixation is derived from saccharose which is transported from the leaves to the nodules via the phloem. In the nodules it is hydrolysed to monosaccharides via glycolysis to eventually form phosphoenolpyruvate (PEP). Phosphoenolpyruvate carboxylase (PEPC) is an enzyme which occurs in abundance in the nodules and can reach up to 2% of total nodule protein (Pathirana *et al.* 1992). PEPC is responsible for the metabolic breakdown of PEP to oxaloacetate (Fig. 4) and will be discussed to further detail.

Another enzyme, i.e. malate dehydrogenase (MDH) is an enzyme in the citric acid cycle that hydrolyses oxaloacetate to the dicarboxylic acid, malate using NAD^+ (Fig. 4). Malate is a major energy source for bacteriod- and plant respiration and is used for ammonium assimilation as carbon skeleton in the glutamine synthetase/glutamate synthase pathway (Poole and Alloway 2000, Stitt *et al.* 2002).

3. Phosphoenolpyruvate carboxylase (PEPC)

3.1 Structure of PEPC

PEPC is a ubiquitous cytosolic enzyme found in higher plants, algae, photosynthetic bacteria and most non-photosynthetic bacteria and has been studied in great depth. Two types of PEPCs were identified, i.e. Class 1 and Class 2. The Class 1 plant-type PEPC, with a molecular weight of ca100 kDa consists of four identical subunits (homotetramer), compared to plant type Class 2 PEPC which does not have identical subunits and exists as a hetero-octamer. The four identical subunits of PEPCs are arranged in a "dimer of dimer" form resulting in an overall square arrangement (Fig. 5) (Izui *et al.* 2004, Kai *et al.* 2003). The primary structure of PEPC consists of 40 α helices and eight β-strands and make up a β–barrel formation. The α-helices comprise 65% of the polypeptide and most of them are located on the C-terminal of the β barrel while a few are located on the N terminal. The unit comprises a catalytic site which is located near the C-terminal side of the β–barrel. This is the binding site for L-aspartate, an allosteric inhibitor, whose function is to regulate the enzyme activity about 20 Angstrom away from the catalytic site. The β strands occupies 5% of the peptide and include 40 residues. Several mobile loops can be observed due to the binding of either an allosteric inhibitor (Kai *et al.* 1999), a metal cofactor (Matsumura *et al.* 1999), or a PEP analog (Matsumura 2002). All of these show significant catalytic or regulatory functions. A model for these reaction mechanisms as well as the allosteric activation could be established which is based on these structure studies in conjunction with other related biochemical studies (Naide *et al.* 1979, Morikawa *et al.* 1980, Ishijima *et al.* 1986, Terada *et al.* 1991)

3.2 Binding to PEPC

Proteolytic cleavage by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) could established that the residual 702-708 at the C-terminal region of the β barrel showed little or no electron density, indicating that the loops, are freely mobile when unligated or exist in multiple conformations. This loop from K702 to G708 is likely to be the active site of the enzyme (Yoshinaga, 1974). R703 is conserved in all PEPCs, and K702 and R704 are conserved well in the form of either lysine or arginine (Yoshinaga, *et al.* 1974). L-Aspartate is one of the allosteric inhibitors, which is tightly bound and 20 Angstrom away from the C-terminus of the β barrel. Four amino acid residuals, i.e. Arg587 (647), Lys773 (835), Arg832 (894), and Asn881 (968), directly

participate in the binding of Asp (Fig. 6). R587 is conserved in all PEPCs as one of the catalytically essential residues and locates in the unique sequence GRGGXXGR587GG (XX=TV, SI, or SV). R587 is ion-paired to the carboxyl group of the aspartate. This indicates that arginine is "trapped" by the negatively charged allosteric effectors. It appears that binding to PEPC is highly specific as it was found that when R587 is replaced by serine, it resulted in a significant loss of catalytic activity of PEPC. K773 is strictly conserved in all PEPC sequences and its replacement by alanine causes the complete loss of enzymatic activity. K773 is ion paired to the carboxyl group of the aspartate through extending its side chain. R832, which is also highly conserved in all PEPCs, also serves as a salt-bridge to the same carboxyl group as K773 is. Aspartate binding to the complex is supported by the action of N881 (Fig. 6). N881 is also highly conserved in all PEPCs (Toh *et al.* 1994).

Reports indicate that Mn^{2+} or Mg^{2+} binds directly to PEP or its enolate intermediate in the catalytic site (Fig. 7) (Miller, 1968, Miziorko, 1974, Miziorko *et al.* 1974).

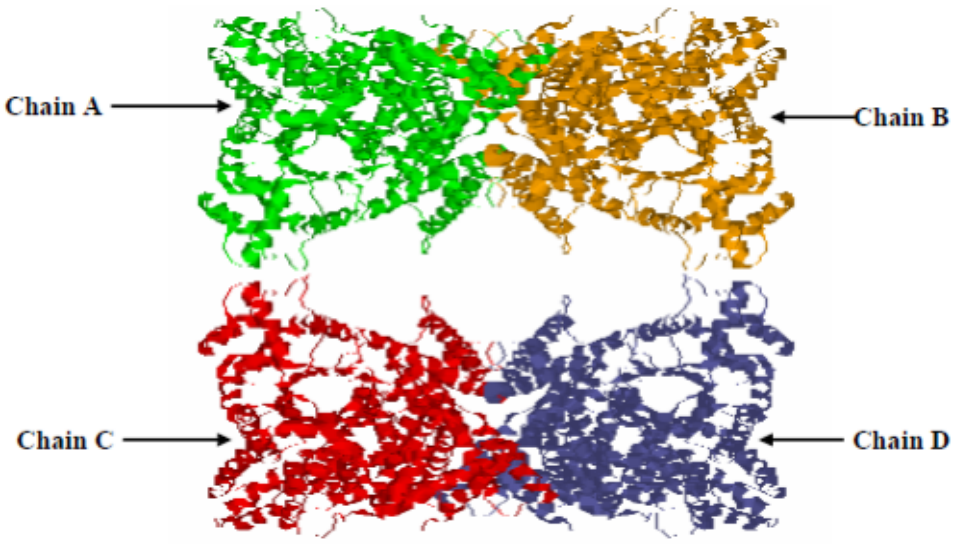

Figure 5. Dimer of dimer formation of PEPC (Kai *et al.* 1999).

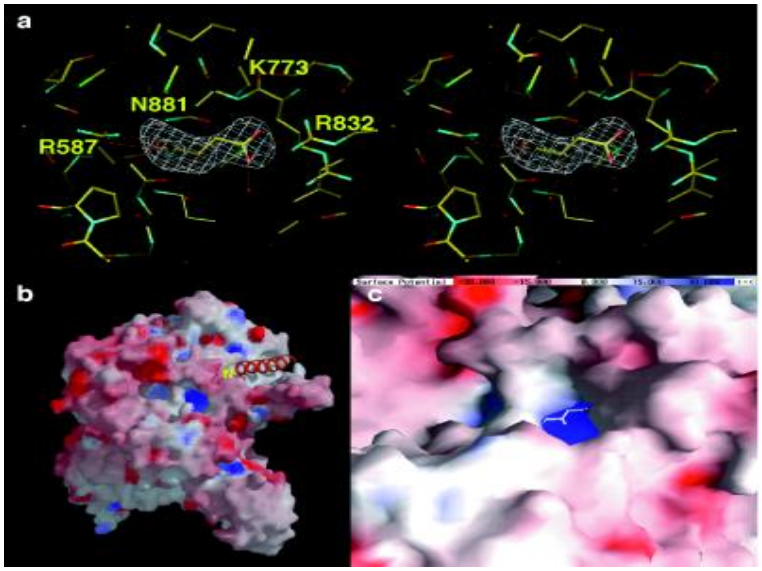

Figure 6. Aspartate binding site of *E. coli* PEPC (Kai *et al.* 1999).

Mn^{2+} binds to the oxygen atoms of the carboxyl groups of Glu506 (566) and Asp543 (603) at the C-terminal of β-5 and β-6 strands (Fig. 7). Studies indicate that PEPC is similar to pyruvate kinase (PK) and pyruvate phospho-dikinase. Both these enzymes form a PEP-binding domain at the α/β barrel. Furthermore it was shown that the structure of the β barrel in PEPC is also similar to those of PK and pyruvate phospho-dikinase. All these three PEP-utilizing enzymes require divalent cations such as Mn^{2+} or Mg^{2+} for enzymatic activity. Despite their common cofactor preference, studies have shown that there are no significant sequence similarities between them.

It was found that substrate analogs of PEP, such as 3,3-dichloro-2-dihydroxy-phosphinoylmethyl-2-propenoate (DCDP) (Fig. 8), also ligate tightly to the active site of the T state PEPC due to its high affinity for PEPC. This is actually quite strange as allosteric enzymes in the T state are usually believed to have a low affinity for their substrates (Jenkins et al.1987). The active site is located at the C-terminal side of the β barrel, and the electron density associated with Mn2+ and DCDP appears in this region. It was observed that the negatively and positively charged residues which interact with the phosphoryl and carboxyl groups of DCDP form clusters. DCDP interacts with R396, R699, R713 as well as Mn2+. Despite this interaction, a small conformational change of the side chains of Asp543 and Arg581 is needed to avoid a van der Waal clash. These side chains facilitate the interactions between the side chains of R396, R581 and R713 and the phosphoryl group of PEP. R396 and R713 have been found to be essential for PEPC catalytic function by site-directed mutagenesis (Gao and Woo 1996). R581 is the first arginine in a glycine-rich GR581GGXXGRGG sequence which is highly conserved and unique to PEPC. It was also

found that a hydrophobic pocket consisting of Trp248 (288), Leu504 (564), and Met538 (598) exists around the methylene group of PEP. This pocket is assumed to be the space where free CO_2 is fixed onto the enolate form of pyruvate (Matsumura *et al.* 1999).

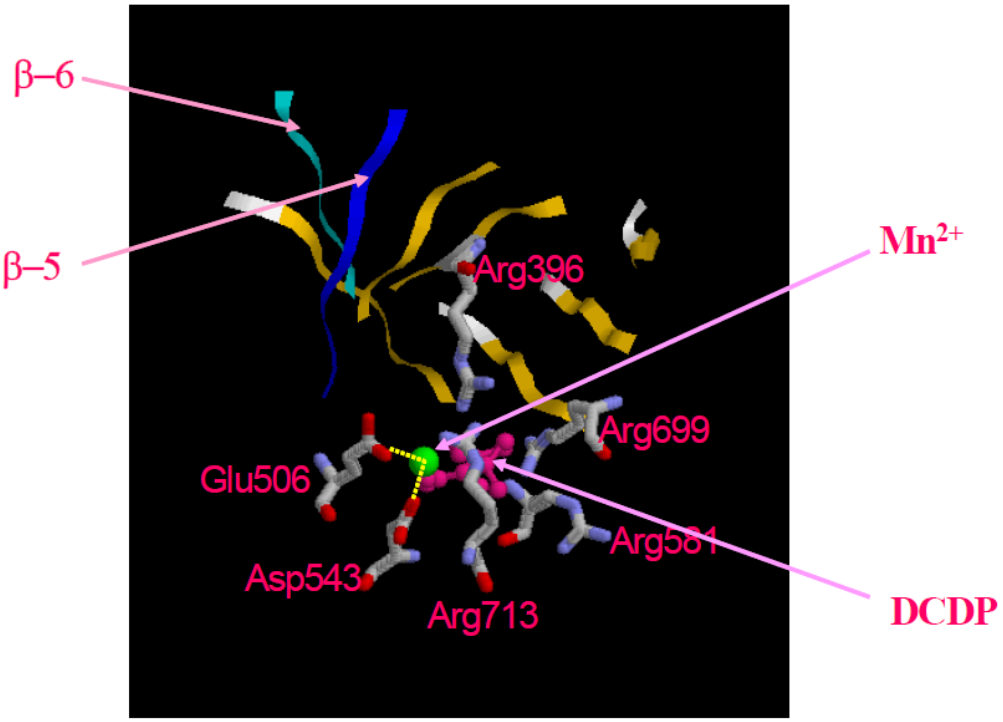

Figure 7. Mn^{2+} and 3,3-dichloro-2-dihydroxy-phosphinoylmethyl-2-propenoate (DCDP) binding (Kai *et al.* 1999).

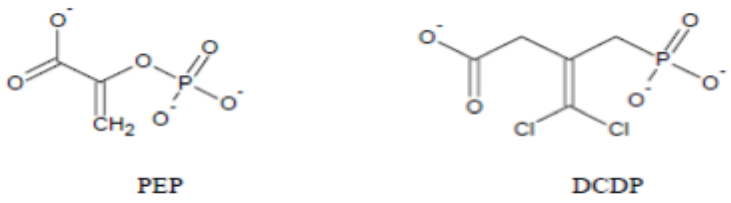

Figure 8. PEP and its analog 3,3-dichloro-2-dihydroxy-phosphinoylmethyl-2-propenoate (DCDP) (Jenkins *et al*. 1987).

3.3. Role of PEPC

PEPC plays diverse roles in plants and in addition fulfills complex regulation. It has been shown that PEPC is actively involved in the following:

- Primary carbon metabolism
- Controlling of plant glycolysis and respiration
- Produces malate as an alternative respiratory substrate for symbiotic N_2-fixing bacteriods of legume root nodules
- Carbon – nitrogen interactions (will be discussed in detail)
- Photosynthate partitioning in developing seeds and organic acid metabolism of germinating seeds
- Mediation of organic acid accumulation by developing fruit and expanding cells
- Help plants acclimatize to abiotic and biotic stress (Huppe and Turpin 1994, Stitt *et al.* 2002, Foyer *et al.* 2006, Plaxton and Podesta 2006)

In order to achieve these functions, it was found that PEPC belongs to a small multigene family encoding several closely related plant type PEPCs, along with a bacterial type PEPC (O'Leary *et al.* 2011). To date it is not certain what the functions of all these genes are.

3.4 Functions of PEPC

PEPC catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO^-_3 to yield oxaloacetate (OAA) and P*i* using Mg^{2+} as co-factor. The conventional way of plant cells to fix atmospheric carbon dioxide into carbohydrates, via photosynthesis is through the Calvin cycle. This pathway is also referred to as the C-3 cycle because it produces 3-phosphoglycerate which is a 3-carbon intermediate. Unfortunately the Calvin cycle cannot function when the CO_2 levels are below $5x10^{-5}$, or being repressed by photorespiration. To curtail this, certain plants have evolved to develop an additional photosynthetic pathway that conserves CO_2. Studies have shown that this pathway can function even if the CO_2 concentration reaches a low level of $5x10^{-6}$. This pathway is referred to as the C-4 cycle because it incorporates CO_2 into a four carbon intermediate, OAA (Fig. 9) (Garrett and Grisham 1999).

The key reaction in this pathway is the carboxylation of PEP, by PEPC, to form OAA using Mn^{2+} or Mg^{2+} as a co-factor. OAA is converted into malic acid, which is then broken down to CO_2 and pyruvate, and then converted back to PEP. The reaction is executed in the following organized way in which reactant binding to the enzyme takes place. First a bivalent cation Mn^{2+} or Mg^{2+} is bound, then PEP and finally bicarbonate (Chollet et al. 1996). Initially PEP binds to PEPC which is already bound to the divalent cation Mn^{2+}. The bicarbonate ion nucleophilically attacks the phosphoryl group of PEP and forms a carboxy phosphate intermediate as well as enolate from pyruvate. In this step the positively charged pocket dissipates the negatively charged P group of PEP (Fig. 10).

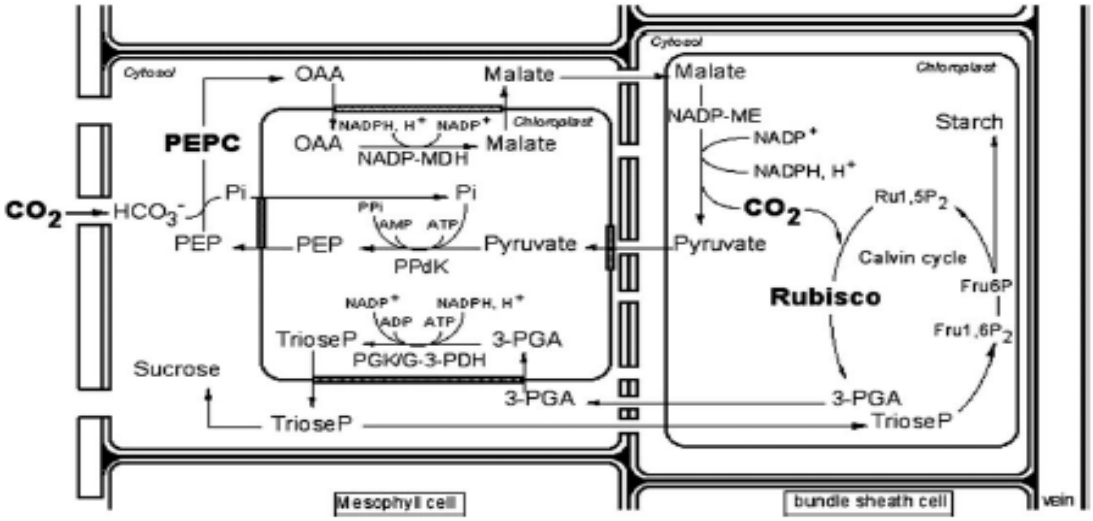

Figure 9. Carbon assimilation in C4 plants (Lepiniec *et al.* 2003).

In the process the phosphorus atom becomes more electrophilic and easier to be attacked by the nucleophilic carbon of bicarbonate (Westheimer 1987). However, the carbonyl carbon of the carboxy phosphate intermediate poses a problem of binding as it is quite a distance from the C-3 of enole. A conformational change of the enzyme takes place in order for the two carbon atoms to get closer to each other. In the process the carboxyl group of the carboxy-phosphate is deprotonated by an enzyme base. Once this is accomplished, the carboxy-phosphate intermediate decomposes to enzyme bound CO_2 and P*i*. Finally the CO_2 combines to the metal stabilized enolate and interacts with it to form OAA (Matsumura et al. 2002). PEPC also plays a fundamental role in the fixation of atmospheric CO_2 during C4 and CAM photosynthesis. However, it fulfills an important role in non-photosynthetic pathways too, as it carboxylases PEP to replenish the TCA cycle carbon skeletons that are withdrawn to support anabolism. In the

presence of malate dehydrogenase and a NAD^+-dependent malic enzyme it converts OAA to malate and ultimately pyruvate. Employing this step, PEPC thus replaces PK. This pathway is a very distinguishing feature of the anaplerotic PEPC reaction as it represents a highly flexible aspect of primary plant metabolism.

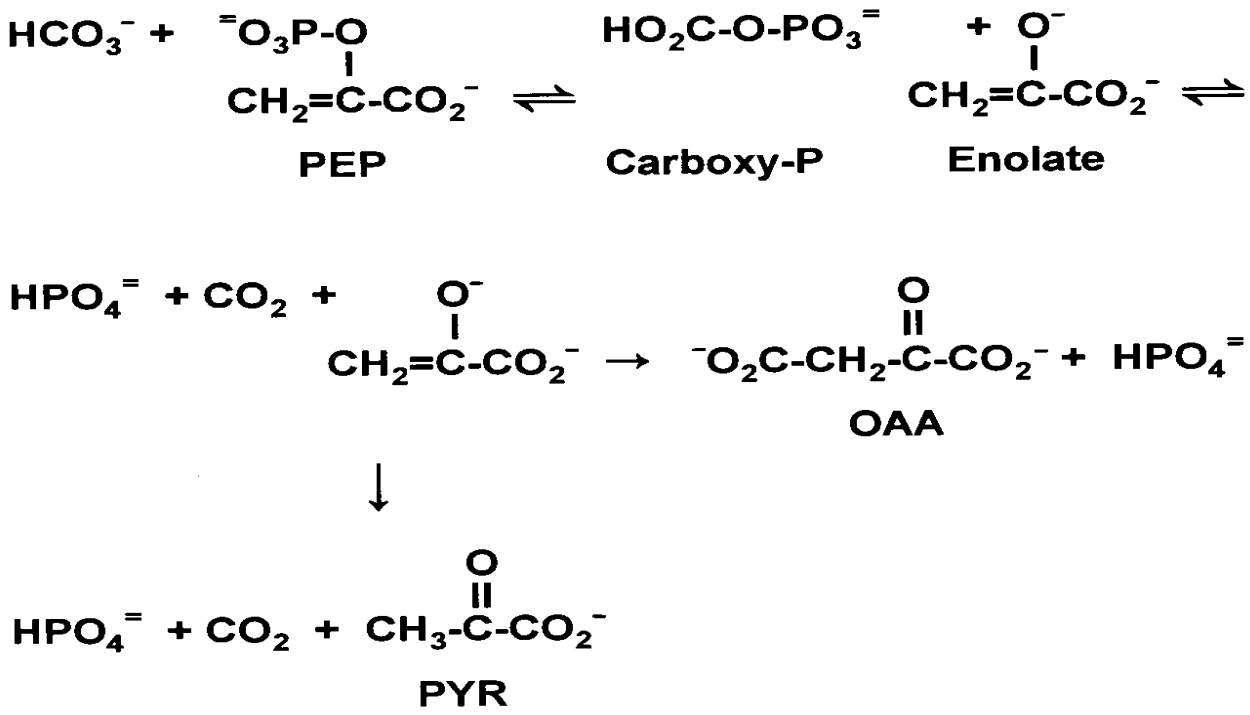

Figure 10. Reaction mechanism of PEP carboxylation to form OAA (Chollet *et al.*1996)

This highly flexible mechanism is activated when the plants need to adapt to certain environmental conditions, such as a change in oxygen concentrations (Williams *et al.* 2008), temperature (Williams *et al.* 2010), glucose availability (Rontein *et al.* 2002) and nitrogen source (Junker *et al.* 2007). When P stress is experienced, some enzymes are upregulated to release P*i* which is used for recycling and to maintain glycolytic activity in the absence of ATP. From this, it can be concluded that C3 PEPC acts an alternative to the pyruvate kinase pathway which operates in conjunction with malate dehydrogenase in the cytosol (Toyota *et al.* 2003). It is therefore of utmost importance that plants synchronise their metabolism during changing environmental conditions, especially at branch points like PEP in the glycolytic pathway. It was shown that control of PEPC activity occurs at both the transcriptional and translational level as well as at post-translational levels (O'Learly *et al.* 2011).

A variety of plant systems show that plant glycolysis is controlled from the bottom up with

primary and secondary regulation exerted at the levels of PEP and fructose 6-P utilization respectively (Plaxton and Podesa 2006). This bottom up regulation agrees with findings that plant ATP-PFKs show a potent allosteric feedback inhibition by PEP. This allosteric feedback inhibition causes the upliftment of PEP inhibition on ATP-PFK, which results in the increase of the glycolytic flux from hexose P. It is known that reduced cytosolic PEP levels cause elevated levels of the signal metabolite fructose 2,6-bisphosphate. It also results in the activation of the PP*i*-dependent phosphofructokinase, since PEP is a potent inhibitor of plant 6-phospho-fructo-2-kinase (Plaxton and Podesta 2006). It is suggested that a possible advantage of this bottom-up regulation of glycolysis is that it allows plants to control the net glycolytic flux independently from the other metabolic processes such as the Calvin-Benson cycle and sucrose-starch interconversion (Plaxton and Podesta 2006). It was found that the amount of flux control exerted by ATP-PFK on glycolysis and respiration is much lower compared to the amount exerted on the metabolism of PEP. From these findings it can be derived that PEP inhibit, to a certain degree, the action of ATP-PFK, PEPC and PK and may play a central role in the overall regulation of plant respiration. The control of their activities in return will ultimately dictate the rate of mobilization of sucrose or starch for respiration. The controlling of PEPC and PK also results in the simultaneous controlling of the provision of respiratory substrates such as pyruvate and/or malate for ATP production via oxidative phosphorylation as well as feeding the TCA cycle with carbon skeletons needed for nitrogen assimilation as biosynthetic precursor (O'Leary *et al.* 2011).

3.5 Role of PEPC in nodulated plants

It was found that PEPC also plays a crucial role in that regulation of carbon flux in vascular plant tissue which actively assimilates nitrogen (Huppe and Turpin 1994, Stitt *et al.* 2002, Foyer *et al.* 2006, Plaxton and Podesta 2006). Enhanced levels of PEPC were found in all nodulated legumes as well as the non-legume *Alnus glutinosa* which fixes nitrogen in symbiosis with *Frankia* (Deroche and Carrayol 1988). PEPC yields several organic acids and the acids are vital for nitrogen metabolism. These organic acids include α-Oxo acids, especially OAA and 2-oxoglutarate, which are important carbon skeletons on to which ammonium is assimilated and exported to other tissue. The OAA formed as a result from the action of PEPC has several metabolic pathways into which it is incorporated (Fig. 4). These include its use in the TCA cycle, where malate and succinate are formed which are imported into bacteriods and used as a carbon

energy source. In addition, aspartate for the bacteriods is also produced through the transamination reaction of aspartate amino-transferase. It was found that in amine-transporting plants like alfalfa, that much of the aspartate is converted to asparagine through the action of asparagine synthetase. Asparagine is transported into the xylem from the nodules to the shoots as a translocated form of nitrogen (Ta *et al.* 1986).

In nodule forming plants, PEPC also provides malate, which is the major respiratory substrate which is supplied to symbiotic rhizobia bacteria that reduces N_2 to ammonium (Schultze *et al.* 1998, Nomura *et al.* 2006).

In addition, malate and citrate may also act as counterions to replace nitrate and in the process they maintain cytosolic pH (Sakano 1998). A specific plant type PEPC isoenzyme is found in legumes, which is enhanced by nodule formation and up-regulated and activated by phosphorylation during active N_2 assimilation by the root nodules (Pathirana *et al.* 1997, Suganuma *et al.* 1997, Nakagawa *et al.* 2003, Nomura *et al.* 2006). In addition to the role that PEPC plays in respiratory regulation, it is also involved in active nitrogen assimilation, especially when post translationally modified by phosphorylation (Foyer *et al.* 2006, Plaxton and Podesta 2006). From literature it appears that lupin and soybean nodule PEPC's are activated by hexose and triose phosphates and inhibited by dicarboxylates (Christeller *et al.* 1977, Marczewski 1989, Schuller *et al.* 1990). However, activation by triose- and hexose-phosphates could possibly result in a forward fed regulation for the conversion of photosynthate to organic acids required by the bacteroids for nitrogenase activity (Schuller *et al.* 1990). Despite this it was also found that high cellular malate concentration in nodules is a potent inhibitor of PEPC under physiological conditions (Streeter 1987, Schuller *et al.* 1990).

Studies indicated that when the malate concentration is high in maize, that PEPC activity is also high, but only when its phosphorylated. This can serve as an indication that both protein phosphorylation and metabolite activation or protection interact synergistically (Gao and Woo 1996). Literature indicates that all plant PEPCs investigated thus far show varying degrees of inhibition by malate, which is usually relieved by the activator glucose-6-phosphate (Glc-6-P) or through protein kinase-mediated phosphorylation. It is suggested that a feed forward activation of PEPC by Glc-6-P may help to balance sucrose availability with the flux of PEP carboxylation to dicarboxylic acids via PEPC (Law and Plaxton 1995, Chollet *et al.* 1996, Law and Plaxton 1997, Moraes and Plaxton, 2000, Blonde and Plaxton, 2003, Nimmo 2005, Tripodi *et al.* 2005).

Similar to studies found in C4 plants, the PEPC in nodules are also subjected to post translational

regulation by phosphoenolpyruvate carboxykinase (PEPCk) and is allosterically regulated by metabolites. It is activated by Glc-6-P and triose phosphate, but inhibited by malate and aspartate (Schuller *et al.* 1990, Schuller 1993, Vance *et al.* 1994, Zhang *et al.* 1995, Zhang 1997). However, the mechanism of the metabolic regulation of the interdependence of PEPC and nitrogenase is still obscure. Furthermore, it was shown that PEPC phosphorylation in roots and leaves responds to the carbon/nitrogen due to the action of PEPCk (Champigny and Foyer 1992, Duff and Chollet 1995, Nimmo 2003, Fukayama *et al.* 2006, Chen *et al.* 2008). Phosphorylation by PEPC also occurs at different times of day depending on plant specie. It was shown that C4-PEPC is phosphorylated in the daytime, and CAM-PEPC is phosphorylated at night when the ambient CO_2 is actively captured for photosynthesis, thus PEPC is regulated by a circadian clock in CAM plants (Nimmo 2003).

Therefore, the role of P especially in the regulation of PEPC and the generation of subsequent downstream PEPC-derived metabolic products seems to be vital, especially during P stress. Despite numerous studies done on PEPC, there appears to be limited information regarding the vital role that this enzyme plays during P in a poor nutrient Mediterranean type ecosystem such as the CFR. *V. divaricata* may have evolved to develop various strategies for the maintenance of nodules and thus sustaining various plant functions such as BNF in nutrient poor ecosystems and therefore may serve as a model for local Fynbos species.

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CHAPTER 2: General introduction

2.1 Phosphate (P)

2.1.1 The role of P in plants

Plants acquire P as $H_2PO^-_4$ or $H_2PO^{2-}_4$ (depending on pH of the soil) and utilize it in its fully oxidized and hydrated form as P*i*. P*i* occurs in very low concentrations in soil i.e. 0.1–10 µM (Hinsinger 2001). These molecules are of utmost importance to the plant for energy transfer and storage in biochemical processes. The most important ones for plants are ADP and ATP.

Phosphorus plays a crucial role in the nitrogen fixation process in legumes. In addition, some legumes develop proteoid roots during P stress. These proteoid roots accumulate citrate which is then released in the rhizosphere of P starved plants. The release of citrate and other organic acids, which are synthesized via C3 PEPC, allow for the chelation of Al^{3+}, Fe^{3+} and Ca^{2+}, resulting in the release of P from a bound form. As a result, the immediate surrounding then becomes acidified resulting in the chelation of ions, which normally would be inaccessible to plants (Raghothama 1999). The accumulation of citrate in proteoid roots also results in metabolic changes that take place in the plants. These changes involve the altering of enzymes which are involved in organic acid synthesis and catabolism (Gerke 1994, Jones 1998, Hinsinger 2001, Ryan *et al.* 2001). From studies done on the roots of white lupins under P deficiency it was shown that C3 PEPC is activated which supports the above-mentioned theories (Johnson *et al.* 1994, Neuman and Römheld 1999).

2.1.2 Acquisition of P

Plants have multiple plasmalemma P*i* transporters which are differently expressed under varying P*i* nutritional requirements. Currently it is widely accepted that plants make use of the dual P*i* uptake system. This dual uptake system refers to the constitutive low affinity and P*i*-starvation inducible (PSI) and high-affinity Pi transporters. These P*i* transporters function at low and high concentrations of P respectively. It is most likely that the high affinity P*i* transporters play a crucial role in the acquisition of limiting P availability in P*i* starved plants from external P. In addition, to acquire P, P-starved plants use acid phosphatases (APase) to function as intracellular or extracellular P*i* salvage systems which catalyses the hydrolysis from P*i* from P monoesters.

There are also several PSI glycolytic bypass enzymes i.e. phosphofructokinase, phosphoenolpyruvate phosphatase and phosphoenolpyruvate carboxylase which might aid in the recycling of internal P*i* during P stress. These enzymes accomplish this recycling, as P*i* is a by-product of the reactions catalysed by them. In addition to recycling P*i*, these enzymes may also be involved in facilitating respiration and/or organic acid excretion, while generating P*i* for reassimilation into the metabolism of the P*i* cells. Plants also adapt to limiting P conditions by utilizing the non-phosphorylating alternative pathways of the electron transport chain. In addition it was also found that increased levels of alternative oxidase protein could be found in P starved plants. This adjustment allows the continuation of the citric acid cycle as well as the respiratory electron transport chain with limited ATP (Plaxton 2004). In studies performed on *L. albus*, it appears that plants use specialized metabolic systems where carbon flow is directed to certain targets. The observations made on *L. albus* showed that genes which involve citrate synthesis are up-regulated when citrate exudation occurs from cluster roots. They also observed that cytosolic PEPC is a key enzyme which facilitates the exudation of carboxylates such as malate and citrate. In addition PEPC mediated non-photosynthetic CO_2 fixation provided a quarter of the carbon exuded as citrate from cluster roots. Increased levels of PEPC activity, expression of mRNA encoding PEPC and PEPC protein abundance are then also found when these high levels of carboxylates occurs in plants (Shane *et al.* 2004).

2.2 PEPC

2.2.1 PEPC and its role in plants/nodules

PEPC catalyzes the irreversible β-carboxylation of phosphoenolpyrovate (PEP) in the presence of HCO^-_3 to yield oxaloacetate (OAA) and P*i* using Mg^{2+} as co-factor. From studies performed on PEPC's from photosynthetic and non-photosynthetic plant tissue on C3, C4 and CAM plants, it was found that PEPC exists in multiple isoforms, or at least 4 at this point of time. These isoforms are the C4 photosynthetic PEPC, the C3 PEPC in leaves of C3, C4 and CAM plants, the CAM PEPC in the leaves of CAM plants and a non-autotrophic PEPC found in the roots of all plants (Ting and Osmond 1973a,b). PEPC is actively involved in primary carbon metabolism and plays a crucial role at the PEP branch point in glycolysis. Not only does it provide the plant with alternative pathways but also participates in the control of glycolysis and respiration. In addition it also produces malate as an alternative respiratory substrate for symbiotic N_2-fixing bacteriods of

legume root nodules. However, little is known of the mechanisms that regulate root nodule PEPC enzyme activity or synthesis.

The energy needed for the bacteriod's nitrogenase activity in the nodules is provided from carbon derived from photosynthesis which is transported to the nodules via the phloem. To date it was only shown in *Phaseolus vulgaris* that sugars are being transported across the symbiosome membrane. In other plant nodules, transport occurs apparently only by diffusion. This scenario does not support sufficient support for maintaining nitrogen fixation. Since this might be the case, it is currently widely accepted that dicarboxylic acids and not sugars are supplied to bacteriods. (Herrada *et al.* 1989, Udvardi and Day, 1997, Day *et al.* 2001).

In studies done on the nodules of various legumes, it was found that PEPC activity is significantly higher compared to the surrounding roots. This might be an indication that PEPC plays a crucial role in providing carbon skeletons to the bacteriods for nitrogen assimilation as well as for bacteriod metabolism to fuel nitrogenase activity (Pathirana *et al.*1992). It appears that nodule survival depends on the activity of PEPC, which necessitates an understanding of how PEPC is regulated.

2.2.2 PEPC activation and inhibition

PEPC is activated by a Ca^{2+}-independent PEP carboxylase kinase that phosphorylates it on a serine residue. It was found that when the N-terminal domain from the PEPC polypeptide is removed, it caused a remarkable decrease in the *in vitro* phosphorylatability and sensitivity of PEPC to its negative allosteric effector L-malate (Cholet and Vidal 1996). When a decrease in the phosphorylation of PEPC occurs, it makes the enzyme more sensitive to malate inhibition. It is thus important that PEPC remains highly phosphorylated in legume nodules, to ensure continued synthesis of malate (Nimmo 1993, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). It was shown in the nodules of lupin and soybean that PEPC is activated by hexose and triose phoshates and inhibited by dicarboxylates (Christeller *et al.* 1977, Marczewski 1989, Schuller *et al.* 1990). The activation of PEPC by the triose- and hexose-P may represent a feedforward regulation for the conversion of photosynthate to organic acids which are required by the bacteroids for nitrogenase activity (Schuller *et al.* 1990). In contrast, high cellular malate concentration in nodules may be a potent inhibitor of PEPC under physiological conditions. It was suggested that protection of PEPC against such feedback inhibition might be important if dark CO_2 assimilation was to be executed

during nitrogen fixation in nodules (Streeter 1987, Schuller *et al.* 1990). It was found in maize that PEPC's activity can only remain high during high malate concentrations if it is phosphorylated. This gives an indication that both protein phosphorylation and metabolite activation/protection interact synergistically in the regulation of maize PEPC (Gao and Woo 1996).

Studies performed on C4 and CAM plants using pharmacological and molecular based approaches have led to the dissection of a highly complex signaling cascade which controls the levels of phosphoenolpyruvate carboxylase kinase in these plants. After this discovery it appears now that there is a similar mechanism in the regulation of phosphoenolpyruvate carboxylase kinase in C3 plants. It can be assumed that phosphorylation needs to modulate the metabolic regulation of photosynthetic PEPC in such a way to ensure that the enzyme is being protected against feedback inhibition by malate (Vidal and Cholet 1997). However the manner in which it is performed remains unclear. In addition *in vitro* results have shown that these processes in C4 plants (activity of PEPC, the phosphorylation process and metabolic control) are highly sensitive to pH (Echevarria *et al.* 1994, Gao and Woo 1996).

2.2.3 PEPC derived metabolites

Sucrose serves as major energy source in the symbiotic nodules of legumes, for the anaplerotic CO_2 fixation by PEPC for downstream nitrogen fixation (Reibach and Streeter 1983, Day and Copeland 1991, Vance and Heichel 1991). Sucrose can be converted to fructose and glucose via sucrose invertase, and/or be converted to UDP-glucose and UDP-fructose via sucrose synthase (SS) (Akazawa and Okamoto 1980, Morell and Copeland 1985). These generated hexoses can be metabolized via the glycolytic pathway to eventually yield PEP or can be used for diverse downstream pathways, which are critical for cell functioning (Chourey and Nelson 1976, Delmer and Amor 1995, Ruan *et al.* 1997). PEP can follow either the conventional adenylated pathway via PK or it can follow the non-adenylated route (especially during P-stress) via PEPC for the generation of downstream products. PEP-derived malate via PEPC is used as major energy source in nodules for respiration and also plays a crucial role in N fixation as it provides the carbon skeletons that are needed for the subsequent assimilation of NH_4 into amino acids (Vance *et al.* 1994). Experiments done on the oxidation of dark carbon dioxide fixed products in soybean root nodules showed that the dicarboxylic acids produced by PEPC is capable of providing up to 48% of the required energy needed for nitrogenase activity. PEPC produces OAA from PEP which is

then reduced to malate via malate dehydrogenase. OAA can also be transaminated to aspartate via aspartate transferase. The malate produced from PEPC can be utilized in different ways in nodule metabolism. It can be used as carbon source for the energy requirements for the nitrogen fixing bacteriods. In addition, PEPC was shown to play a pivotal role in the regeneration of metabolic compounds in the TCA cycle, which are replenished when these compounds become exhausted for other downstream metabolic functions such as nitrogen assimilation and amino acid biosynthesis, especially when plants experiences stress of various forms, such as P-stress. In addition malate could also play a role in adjusting the charge balance in the vacuoles and the xylem fluids. Furthermore elevated levels of organic acids such as malate and citrate in the rhizosphere was found when plants experience P-stress (Huppe and Turpin 1994, Stitt *et al*. 2002, Foyer *et al*. 2006, Plaxton and Podesta 2006, O'Leary *et al.* 2011a). There appears to be a heavy reliance of nodules on PEPC functioning, as previous work indicated that PEPC derived dicarboxylic acids are the main respiratory substrates of bacteroids in nodules (Tajima *et al*. 1990, Streeter 1991). In addition, these dicarboxylic acids are also the main carbon skeletons for transamination by aspartate aminotransferase (AAT) to produce aspartate (Asp). It was found in *Lotus japonicus* that most of the produced aspartate is converted to asparagine by asparagine synthase (AS), which is the major amide which is loaded into the xylem stream from nodules to shoots as the translocation form of fixed nitrogen in amide- transporting legumes (Ta and Joy 1986, Tajima *et al.* 2004).

2.3 Problem Statement

During PEP metabolism via PEPC, it is not known how PEPC derived C becomes metabolized to downstream metabolic products in P-stressed nodules of non-model legume species, such as *V. divaricata*. Most previous work on PEP metabolism via PEPC has been carried out on non-legume models species such as *Arabidopsis.* The PEP metabolism via PEPC in legumes has largely focused on the roots of herbaceous model legumes such as soybean (*Glycine max*), barrel-medic (*Medicago truncatula*) and common bean (*Phaseolus vulgaris*). The nodule has therefore remained understudied with regards to PEPC-derived carbon P deficiency.

2.4. Proposed investigation

2.4.1 Working Hypothesis

During P deficiency, the metabolism of PEPC-derived C is crucial for the organic acid and amino acid supply, to sustain nodule N metabolism.

2.4.2 Aim

Understanding the levels of PEPC regulation will enhance our knowledge of the integration of key organic acid and nitrogen metabolism in P-deficient nodules of an indigenous legume of the Cape Flora.

2.4.3 Motivation for using *Virgilia divaricata*

Virgilia has evolved to fix N_2 in low P- soils of Fynbos. Understanding the mechanisms used by this legume may enhance our knowledge of N nutrition in P-poor ecosystems. An indigenous nodule-forming bacterium, which is normally associated with this legume was used for BNF.

2.4.4 Workplan

Three major experiments were conducted with the legume species, *V. divaricata*, which will be inoculated with the nitrogen fixing bacterium *Burkholderia phytofirmans.* Plants were grown under glasshouse conditions in sterilized quartz sand for 2-3 months. P treatments were applied as a range between 500 uM to 5 uM P supply, to represent the control and deficiencies.

2.4.4.1 Experiments 1:

Objective: How flexible is the role of PEPC in BNF during P deficiency?

Motivation: Since PEPC is pivotal to BNF for organic acid supply for bacteroid respiration and amino acid metabolism, the range of PEPC's flexibility was tested under various P supplies.

Methodology: Two levels of P supply (500 µM and 5µM) were used.

a) BNF via stable isotope discrimination
b) Enzymatic assays of organic acid synthesis (PEPC, MDH, PK, ME)
c) Nodule PEP-derived organic acid concentration

2.4.4.2 Experiments 2:

Objective: How is PEPC-derived C metabolised into amino acids and downstream organic acids of P-deficient nodules?

Motivation: Previous studies have not used NMR to determine the flux of PEPC-derived C in nodules. Le Roux *et al.* (2006, 2007) used ^{14}C isotopes to trace the PEPC-derived C in organic acid and amino acid pools, but could not calculate the metabolism of specific compounds in these pathways. It was reported that NMR spectroscopy could be exploited to calculate the flux of metabolites in plant cells (Chang and Roberts 1989).

Methodology: ^{13}C feeding to nodules as labelled-HCO_3^-, the substrate of PEPC. The metabolic flux of PEPC-derived C^{13} carbon was calculated from different time-sequenced harvests:

a) NMR of ^{13}C in the organic and amino acid profiles

b) Enzyme assays of organic acid pathways (PEPC, PK, MDH, ME)

c) Calculating the metabolic fluxes

2.4.4.3 Experiments 3:

Objective: How are PEPC enzymes (plant and plant-bacterial type) or their isoforms regulated in P-deficient nodules?

Motivation: The simultaneous characterization of PEPC types and their isoforms have not been conducted in nodules. This is essential to determine PEP metabolism via PEPC, during P-deficiency in nodules.

Methodology: Proteome & phosphorylated proteome sequencing

a) Phosphorylation by immuno-blots studies of PEPC

b) PEPC identification, phosphorylation & quantification via orbitrap mass spectrometry,

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Chapter 3:

Roots and nodules responded differently to P starvation in the Mediterranean-type legume *Virgilia divaricata.*

3.1 Abstract

The legume, *Virgilia divaricata*, which is indigenous to the Cape Floristic Region (CFR) was investigated regarding its ability to grow in poor nutrient soils. Plants were grown under conditions of high and low phosphate supply. A comparison of roots and nodules was conducted to evaluate the plants ability to cope under P stress. Plants use various strategies in order to cope with low phosphate stress, which include, an increased allocation of resources, increased reliance on BNF and enhanced enzyme activity, especially PEPC. Nodules appeared to maintain their P status, as nodules had a lower percentage decline in P compared to roots, probably to uphold its metabolic functions. These strategies may hold the key to the manner in which *V. divaricata* can sustain growth despite low P (LP) conditions. Although the number of nodules declined with LP, their biomass remained unchanged in spite of a plant decline in dry weight. This was due to the increase in plant allocation towards nodules under LP. This may be achieved via the high efficiency of BNF under P stress. Under LP conditions, the nodules and roots declined in total P. The decline in nodules was less, at 34% than that observed in the roots (88%) during P stress. This may have been due to P conservation strategies in LP nodules. This P conservation strategy may have been due to the increase in a metabolic bypass that operates at the PEP branch point in glycolysis. The enhanced activities of nodule PEPC, MDH and ME, combined with a decrease in PK, suggest that under LP conditions an adenylate bypass was in operation either to synthesize more organic acids or to mediate pyruvate via a non-adenylate requiring metabolic route. Both possibilities represent a P-stress adaptation route. Although this has been demonstrated in the nodules of an annual model legume, this is the first report of its kind for legume trees that are indigenous to low P, acid soils. This bypass may have been able to buffer the deleterious effects of P deficiency on nodule function. Although BNF declined by a small percentage during LP, this P conservation was evident in the unchanged BNF efficiency per weight, and the increase in BNF efficiency per mol of P. It appears that legumes that are indigenous to acid soils, may be able to continue their reliance on BNF via increased allocation to nodules and also due to increase their efficiency for BNF on a P basis, owing to P-saving mechanisms such as the organic acid routes.

3.2 Introduction

The CFR, found in the south western area of South Africa can be regarded as one of the highest P-impoverished regions of the world and simultaneously also a Global Biodiverse Hotspot (Lambers and Shane 2007). The CFR regions resembles a typical Mediterranean-type ecosystem usually characterised by sandstone-derived soils (Goldblatt and Manning 2000), which are acidic with insufficient nutrients (especially N and P) to sustain normal plant growth (Bordeleau and Prevost 1994, Von Uexkull HR and Mutert E 1998, Grigg *et al.* 2008). In particular, legume species reliant on BNF are highly dependent on P supply, more so than legumes growing on mineral N (Drevon and Hartwig 1997). For legumes, P not only affects the the formation of nodules (Israel 1993), but limiting P also impacts negatively on the nitrogen fixation process (Schultze *et al.* 2006, Tsvetkova and Georgiev 2007). The tree species, *V. divaricata* (Adamson) is a native legume to the CFR and is distributed over a wide range of P-poor soils. This range includes the relatively richer forest margins to poorer Fynbos soils (Coetsee and Wigley 2013). This implies that the indigenous species may have a range of mechanisms to adapt to variable P supply.

These mechanisms, have evolved adaptations to function optimally under these limiting P conditions (Vance *et al.* 2003). Some strategies are aimed at conserving the use of P, whereas others are directed toward enhanced acquisition and uptake of P (Lajtha and Harrison 1995, Raghothama 1999, Horst *et al.* 2001, Vance *et al*. 2003). Adaptations that conserve the use of P involve a decrease in growth rate, increased growth per unit of P uptake, remobilisation of internal P*i*, modifications in C-metabolism that bypass P-requiring steps and alternative respiratory pathways (Schachtman *et al.* 1998, Plaxton and Carswell 1999, Raghothama 1999, Uhde-Stone *et al.* 2003*a*, 2003*b*). In legumes, adaptations leading to enhanced P acquisition entail the expression of genes that result in the production of cluster roots. Cluster roots increase the root surface area. This enhances nodule efficiency for P utilisation (Le Roux *et al.* 2008), root exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters (Gilbert *et al.* 2000, Gilroy and Jones 2000, Lynch and Brown 2001, Neumann and Martinoia 2002, Lamont 2003, Uhde-Stone *et al.* 2003*a*, Vance *et al.* 2003). The exudation of organic acids such as malate and citrate stimulated by P stress has mostly been reported in non-mycorrhizal species such as lupin (Dinkelaker *et al.* 1995, Jones 1998, Hinsinger 2001, Ryan *et al.* 2001, Le Roux *et al.* 2008).

The high sensitivity of legume plants, and indeed the N_2- fixation process to environmental conditions such as acidic soils associated with P deficiency, may result in higher C costs (Mengel

1994). This concurs with Le Roux *et al.* (2008), who showed that lupin nodules under P stress acted as stronger C sinks. Nodules are known to have a strong sink capacity for P assimilation during P starvation (Hogh-Jensen *et al*. 2002). The enhanced nodule cost for P utilisation is considered to be an essential coping strategy during P stress (Le Roux *et al.* 2008). The C sink was found to be more pronounced in plants during double symbiosis under low-P conditions (Mortimer *et al.* 2008). This was shown by a greater growth respiration of low-P plants than high-P plants (Mortimer *et al.* 2008). The sink effect was also evidenced by the higher photosynthetic rates of host plant (Mortimer *et al.* 2008). In the case of P stress, the most direct currency is P itself or growth parameters related to P accumulation (Koide and Kabir 2000).

Virgilia is a small tree genus that includes two species *V. divaricata* (Adamson) and *V. oroboides* (P.J. Bergius Salter) and two subspecies. It is confined to the south-western and southern coastal regions of the CFR (Greinwald *et al.* 1989). Studies have been conducted on growth and adaptations of legume species native to Mediterranean-type fynbos ecosystems that occur on naturally acidic soils (Muofhe and Dakora 1999, Spriggs and Dakora 2008, Power *et al*. 2010, Kanu and Dakora 2012). However, information on the physiology of N and P uptake, efficiency and utilisation in legume trees in fynbos soils is largely unknown.

Physical changes to roots (adjustment of root architecture, root growth, root system composition and mycorrhiza infection) that take place, are complimented by the exudation of a variety of organic compounds (carboxylate anions phenolic, carboxylates, amino acids, enzymes and other proteins), as well as inorganic compounds (protons, phosphate and nutrients) into the rhizosphere to aid in the adaption for a particular nutrient stressed environment (Crowley and Rengel 1999). The family *Fabaceae* develop cluster roots which are stimulated during phosphate stress. Not only do these species develop cluster roots, but also exude carboxylates which releases P from its bound form, making P more accessible for root uptake (Lambers and Shane 2007). It was found that during P deficiency, plants exude carboxylates such as citrate, malate, malonate, acetate, fumerate, succinate, lactate and oxalate in various concentrations (Rengel 2002). White lupin exudes large amounts of carboxylates in the form of malic- and citric acid to the immediate soil surrounding to release P from its bound form in the soil. These excreted organic acids have the ability to chelate metal cations such as Al^{3+} and Ca^{2+} and immobilize P*i* in the soil, which results in higher P*i* concentrations in the soil of up to 1000 fold (Gardner *et al.* 1983, Dinkelaker *et al.* 1989, Neumann *et al.* 2000). The production of these exudates is accomplished by the concerted action

of a variety of enzymes, such as the PP*i* dependent phosphofructokinase (PP*i*-PFK), PEP phosphatase and PEPC. Pyruvate, which is the precursor to many of these substances, can be generated in the cytosol and in the mitochondria. Cytosolic pyruvate is produced from PEP during the glycolytic conversion of ADP to ATP which is catalysed by PK (Plaxton 1996). It is suggested, that when plants experiences P stress, that pyruvate synthesis from PEP via PK is restricted (Theodorou and Plaxton, 1993, Plaxton 1996). However, pyruvate can also be generated from malate when plants make use of a "bypass" route especially during P-limitations. In this "bypass" route, PEP is hydrolysed to OAA by PEPC and OAA is subsequently converted to malate by MDH. Mitochondrial ME converts malate into PEP (Plaxton 1996).

Although the CFR has a high legume diversity found on the P-poor soils (Goldblatt and Manning 2000), not much is known about the functional mechanisms which underpin N nutrition within the nodules of these indigenous legumes. The adaptation to P stress may involve a variety of morphological and biochemical mechanisms that are related to enhancing acquisition of soil P, recycling of internal P*i* and conserving available internal P. Recent work from our group has shown that *Virgilia* uses a variety of strategies to adapt to low P conditions. Magadlela *et al.* (2014) compared two species within the genus *Virgilia*, and demonstrated that *V. divaricata* maintained a high efficiency of BNF, owing to a greater allocation of biomass towards nodules during P deficiency. Vardien *et al.* (2014) showed that nodules have a high functional plasticity during variable P supply, by recycling organic P via acid phosphatase enzymes and redistributing Fe within the nodule. Adaptive mechanisms to conserve P, such as the internal P-use efficiency and metabolic bypasses of P-requiring reactions of the nodules under P deficiency, have not been explored to date.

Our hypothesis is that in P-poor soils, adaptive mechanims which conserve internal P may be important for maintaining nodule function during P deficiency. We therefore investigated the root system engagement of a non-P requiring metabolic bypass and its implications to nodule efficiency of the indigenous legume *V. divaricata,* during variable P supply.

3.3 Materials and Methods

3.3.1 *Plant growth*

Seeds of *V. divaricata* (Silverhill Seeds, Kenilworth, Cape Town, South Africa) were incubated for 1 h in water containing a smoke disc, after which they were placed for 5 h in a water bath, (50 ºC), in order to enhance germination. Seeds were then washed with distilled water (10x) and allowed to germinate in sterile filter sand in seed trays. Germination and growth of these seedlings took place under natural light conditions in a north facing glass house where plants were exposed to sunlight for a 10 h period per day. The temperature of the glass house varied between 15 ºC at night and 25 ºC during the day. After 2 weeks, when the sprouting of leaves was observed, the seedlings were transferred to pots which contained sterile filtered sand. The nodule forming bacteria, *Burkholderia phytofirmans*, (which was grown on yeast mannitol agar) was used for nodulation and 500 µl of culture was used for inoculation, which was repeated twice after the first inoculation. Plants were divided into 2 groups i.e. low (5 µM) –and high (500 µM) phosphate according to the Long Ashton nutrient treatment. The low phosphate treatment (LP), consisted of; Macronutrients ($MgSO_4/K_2SO_4/CaCl_2$)(50 ml), Phosphate (50 ml pH 5.5), Micronutrients ($H_3BO_3/MnSO_4/ZnSO_4/CuSO_4/NaMoO_4$)(2.5 ml), Iron (6 ml), NH_4NO_3 (10 ml). The high phosphate treatment (HP) consisted of Macronutrients ($MgSO_4/K_2SO_4/CaCl_2$),(50 ml), Phosphate (5 ml pH 5.5), Micronutrients ($H_3BO_3/MnSO_4/ZnSO_4/CuSO_4/NaMoO_4$) (2.5 ml), Iron (6 ml), NH_4NO_3 (10 ml). Plants received the respective treatments twice per week and allowed to grow for 8 weeks before they were harvested.

3.3.2 *Harvest*

After 8 weeks plants were harvested and divided into leaves, stems roots and nodules which were respectively weighed for their fresh weights. Liquid nitrogen was added to nodules in microfuge tubesand caution was taken that the nodules should not pop by piercing a hole in the lid of the tube. Nodules were then frozen at -80 °C for further analyses. The leaves, stems and roots were dried in an oven at 50 °C for a week and their dry weights were then determined.

3.3.3 *Protein extraction*

Proteins from roots and nodules were extracted according to the method of Kottapalli *et al.* (2008) for running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant containing the extracted proteins was then frozen at -20 °C. Standard SDS-PAGE (10%) gels were prepared. Gels were run on a Biorad Mighty tan gel electrophoresis apparatus and separation of various proteins were made visible by Coomassie stain. Proteins from roots and nodules were extracted according to the methods used by Ocaña *et al.* (1996) which was modified to an extent 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 Ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Randburg, South Africa) per 50 ml of buffer. The protein concentration was determined by the NanoDrop Lite Spectrophotometer (Thermo Scientific) - the extraction buffer was used as standard.

3.3.4 Enzyme assays

All enzymatic reactions were performed in a multi-well plate reader at a wavelength of 340 nm. The reactions started by adding 30 µl of the crude extraction mixture in a total volume of 250 µl. The various initial reaction rates have been shown to be proportional to the concentration of the extracted enzymes used under the conditions used (Ocaña *et al.* 1996).

3.3.4.1 *Phosphoenolpyruvate carboxylase*

PEPC activity was determined by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 25 °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.* 1996). All reactions were performed in triplicate. The 9 blanks that were used in the assay consisted of a reaction medium without PEP.

3.3.4.2 *Pyruvate kinase*

The PK activity that was performed in triplicate was assayed in a buffer modified to contain 75 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 20 mM KCl, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and lactate dehydrogenase (3 U) (McCloud *et al.* 2001). Blanks consisted of the buffer without ADP.

3.3.4.3 *Malic enzyme*

The Malic enzyme (ME) activity was assayed at 340 nm in triplicate and the reaction mixture consisted of 80 mm Tris-HCl (pH 7.5), 2 mm MnCl2, 1 mm malate and 0.4 mm NADP or NAD^+ (Appels & Haaker, 1988). The blanks consisted of a reaction medium without malate.

3.3.4.4 *NADH-Malate dehydrogenase*

MDH activity that was also performed in triplicate 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). The 3 blanks consisted of a reaction medium without OAA.

3.3.5 *Amino acid analyses*

The standard procedure for the hydrolysis of amino acids was performed according to the methods as set out in Official method of analysis of AOAC international 17th Edition, Revision 2, 2003. Triplicate samples of roots and nodules (HP and LP) were used for digestion. After hydrolysis, the samples were stored at -20 °C for the analysis of amino acids by liquid chromatography mass spectrometry. Amino acid content in the roots and nodules of LP and HP plants were determined by liquid chromatography mass spectrometry using a Waters API Quattro Micro and performed in triplicate. The samples were first subjected to the Waters AccQ Tag Ultra Derivitization Kit, prior to injection. In brief, 10 ul of the undiluted sample was added to the Waters AccQ Tag Kit constituents and placed in a heating block at a temperature of 55 ˚C, for ten minutes. For the mobile phase, the following was used, Solvents A1 and A2: Eluent A2 (100 ml Eluent A concentrate and 900 ml Water) Solvents B1 and B2: Eluent B (Supplied by AccQ Tag Kit). Samples (1 µl) were separated on an AccQ Tag C18, 1.7 um, 2.1x100 mm column. Separation was achieved by an ESI+ source with a Capillary voltage of 3.5 kV and a Cone voltage of 15 V. The source temperature was set at 120 ºC and the desolvation temperature at 350 ºC. The desolvation gas was set at a flow rate of 350 L/h and the cone gas at 50 L/h.

3.3.6 *Citric- and malic acid determination*

Citric- and malic acid content for HP and LP nodules and roots were determined using a photometric analyser (Arena 20XT, Thermo Electron Oy, Finland), which measures the amount of

product formed after an enzymatic reaction. The reactions were performed in triplicate. The pH of the samples was adjusted to between 8 and 10 at room temperature with NaOH. Reactions inside the instrument were performed at 37 °C. Citrate and malic acid concentrations were determined by the enzymatic conversion of citrate and malate. In the process, NADH is oxidised which is stoichiometric to the amount of citrate and malate, respectively. NADH is then photometrically determined at 340 nm.

3.3.7 *Phosphate (P) determination*

Phosphate analysis was performed on HP and LP samples of roots and nodules For the determination of total P, approximately 0.25 g of the sample material was digested in 7 ml HNO_3 in a Mars CEM microwave digester, then diluted into 50 ml deionised water. P was measured on a Thermo ICAP 6300 ICP-AES after calibration of the instrument with NIST-traceable standards.

3.3.8 *Isotope analysis*

Analyses of δ15N was done at the Archeometry Department at the University of Cape Town, where the isotopic ratio of δ15N was calculated as δ=1000‰ (Rsample/Rstandard). R refers to the molar ratio of the heavier to the lighter isotope of the samples. Standards were similar to those as described by Farquhar *et al.* (1989). In brief, between 2.100 and 2.200 mg of each milled sample was placed in 8 mm x 5 mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) and weighed on a Sartorius microbalance (Goettingen, Germany). Combustion of the samples were performed in a CHN analyser (Fisons NA 1500, Series 2, Fisons instruments SpA, Milan, Italy) and the δ15N values for the nitrogen gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. The sample values were corrected by the use of three standards due to drifting of the machine that can occur. Two in-house standards (Merck Gel and Nasturtium) were used and the third was the IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$. The methodology used for the calculation of % NDFA was according to Shearer and Kohl (1986), where % NDFA = 100((δ15Nreference plant - δ15Nlegume)/ (δ15N reference plant –B). The wheat (*Triticum aestivum*), was used as reference plant which was grown under the same glasshouse conditions. The B-value (which was determined as -0.71‰.) refers to

the δ15N natural abundance of the N derived from biological N-fixation of the above-ground tissue of *V. divaricata*, grown in a N-free solution.

3.3.9 *Calculations*

3.3.9.1 *Specific Nitrogen absorption rate:*

Specific N absorption rate (SNAR) (mg N g^{-1} root DW d^{-1}) is the net N absorption rate per unit root DW as outline in Nielson *et al.* (2001), where:

SNAR = [(M2 - M1 /t2 - t1)] x [(loge R2 – loge R1)/ (R2 – R1)]

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

3.3.9.2 *Below ground allocation:*

Belowground allocation refers to the fraction of new biomass partitioned into new roots and nodules over the given growth period. The calculations were done according to Bazzaz and Grace (1997):

df/dt = RGR (∂-Br/Bt)

Where RGR is the relative growth rate (mg.g^{-1}.day^{-1}) and ∂ is the fraction of new biomass gained during the growth period. Br/Bt is the root weight ratio, based on total plant biomass (Bt) and root biomass (Br).

3.3.10 *Statistical analysis*

The effects of the factors and their interactions (between HP and LP) were tested using the *t*-test. Different letters indicate significant differences between treatments (P≤0.05), as revealed by the *t*-test.

3.4 Results

3.4.1 *Biomass*

The DW of the roots and nodules were much lower in the LP treatment compared to the HP treatment. The relative growth rate for roots was much higher for the LP treatment and slightly higher in nodules of the LP treatment compared to the HP treatment. However, nodulation in the LP treatment was much lower. The plants allocated more of its resources to roots in LP than in

HP. The allocation of resources in nodules was also higher in the LP treatment. In addition, more than twice the amount of nodules were formed in the HP treatment. Nodule DW was also higher in the HP treatment (Fig. 3.1). A decline in % NDFA was observed in the LP nodules, however the nodules were more efficient in BNF per P concentration. There was also an increase in SNAR in LP nodules (Fig. 3.3).

3.4.2 *Phosphate (P) and Inorganic phosphate (Pi)*

Higher P values were obtained in the HP treatment of roots and nodules. The P values were higher in LP nodules compared to roots. Higher P*i* values were obtained in nodules compared to roots, irrespective the treatment (Fig. 3.2).

3.4.3 *Amino- and organic acids*

Similar amounts of amino acids were found for both the HP- and LP treatments in roots and nodules. However the concentration of aspartate was much higher in root and nodules which received the LP treatment. Aspartate and glutamate levels were the highest of all the amino acids as expressed in the LP-nodules and roots with a 4- and 2-fold increase in concentration of these two amino acids respectively in both the HP and LP treatments. In addition histidine was twice the amount in the LP treatment of roots and nodules. In contrast, serine and glutamate were much higher in the HP treatment. Organic acids were higher in roots and nodules receiving the HP treatment. The citric acid concentration in HP roots was more than double the amount compared to LP roots. Almost double the amount of citric acid was found in nodules for the HP treatment compared to the LP treatment. The malic acid content was also more than double the amount for the HP treatment in both the roots and nodules (Fig. 3.4).

3.4.4 *Protein and enzymes*

There was an increase in protein concentration in the LP treatment of roots and nodules compared to roots of the HP treatment. The protein concentration of the LP nodules was more than double that of the HP and LP treatment in roots. The nodules of the LP treatment yielded the highest PEPC activity per FW in both roots and nodules (Fig. 3.5a). Higher PK activity per FW was obtained in both the HP treated roots and nodules compared to the LP treated roots and nodules, with the highest activity obtained in the HP treated roots. The PK activity was lower in the LP

treated nodules compared to the LP treated roots and slightly lower in the HP treated nodules compared to the HP treated roots (Fig 3.5b).

ME activity per FW was found to be the highest in the LP treatments of nodules and roots. The LP treated nodule ME activity was more than double the amount as found in the HP treated nodules. (Fig. 3.5c). Higher MDH activity per FW was found in the LP treatment of roots compared to the the HP treatment for roots. Higher LP treated nodule activity was also obtained compared to HP treated nodules (Fig. 3.5d).

3.5 Discussion

During P deficiency, *V. divaricata* nodules experienced less P*i* stress than roots, due to increased metabolic P conservation reactions during organic acid synthesis. Although the BNF declined, the high efficiency of BNF may be underpinned by these altered P conservation pathways and enhanced resource allocation during growth.

Higher resource allocation for the belowground organs such as roots and nodules, under LP conditions was possibly due to their potential for greater contribution to mineral nutrition. This concurs with other species during P stress **(**Almeida *et al.* 2000**)** and also with legumes from nutrient poor ecosystems (Vardien *et al*. 2014, Magadlela *et al*. 2014). Although there was a decline in the number of nodules in the LP treatment, the unchanged total nodule mass may indicate that plants allocate more resources to existing nodules, possibly to increase or maintain their efficiencies during LP conditions. This is supported by the efficiency of nodule functioning (compared to roots), under LP conditions, as reflected in the maintenance or proportionally lower decline of P levels during P stress. This lower decline in P concentration in nodules may also be attributed to the fact that nodules are P scavengers (mostly from roots) as reflected in the higher amounts of P and P*i* in nodules compares to roots in the LP-treatment, probably to maintain its functioning (Jacobsen 1985, Israel 1993, le Roux *et al.* 2006). Similar findings also indicated that nodule growth and functioning may not limited by P-deficiency in white clover (Almeida *et al.* 2000) and also concurs with findings for *Medicago truncatula* where the P concentration in nodules seem to be unaffected as most of the P was allocated to the nodules (Sulieman *et al.* 2010). This may serve as an adaptation of this species to the naturally low P environment.

Despite the slight decline in BNF, there was an increase in BNF efficiency per weight, as well as

an increase in BNF efficiency per mole P. This apparent unaffected BNF efficiency is a further indication that nodules can remain unaffected by conserving their P status despite low P conditions. It was suggested that the decrease in nitrogen fixation in P stressed plants, should be viewed in correlation with whole plant growth, while specific nitrogenase activity is still maintained (Schultze 2003). This idea is supported by experimental evidence that it is the plant N status which regulates nitrogen fixation rates (Schultze 2003). In addition, it appears that *V. divaricata* might also shift its acquisition of N from BNF to soil N acquisition. This is reflected in higher mineral N uptake of nodulated roots as evidenced by the increase in specific root system N acquisition rate during P deficiency when BNF declines. This is in contrast to findings by Vardien *et al.* (2014), where roots showed a decline in mineral N uptake during P deficiency, compared to the current increase of mineral N in the nodulated root system. These differences may reside in the fact that in the current system, both roots and nodules may have contributed to mineral N uptake from soil. It is known that in a nodulated root system, both roots (Magadlela *et al*. 2014) and nodules can seperately acquire and assimilate mineral/soil N (Becana and Sprent 1987).

Soil derived N is usually taken up in the form of NO_3^- (Lambers and Shane 2007) and it might be that the roots increase their contribution to acquire N under P limitation. Although root nitrate uptake by roots could be beneficial to plant metabolism, it could also impact negatively on BNF as it might inhibit nitrogenase activity in legume plant nodules. It was shown that nitrate impacts negatively on *Rhizobium*-infection as well as on the ratio of the nodule dry mass to the whole plant mass (Luciñski *et al.* 2002). As BNF is a costly process, it may be more beneficial for legumes from low nutrient ecosystems to take up N via its roots and to reduce energetically costly BNF (Magadlela *et al*. 2015). Similar trends were also observed in white clover where N concentration was unaffected by P deficiency. It was found in white clover that N_2 fixation increased strongly under P deficiency and that approximately 30% of N was assimilated due to N_2 fixation (Almeida *et al.* 2000). Similar to the BNF in white clover, it was calculated that the nodules in the LP treatment experiment derived approximately 32% of the N from the atmosphere. The remaining approximately 68% of N might be from soil uptake, (whether directly by the nodules or via roots) as the plants were fed with nutrient solution containing NH_4NO_3. In support of above we obtained higher specific nitrogen acquisition rate values for naked roots compared to nodulated roots, irrespective of the treatment. This could be an indication that the plant would rather utilise soil N instead of utilising the costly BNF route, which could explain the abovementioned findings.

In spite of variable P supply, the unchanged N levels are also reflected in the elevated levels of all major amino acids found in the nodules of the LP treatment compared to the nodules of the HP treatment, and both the treatments for roots. A similar trend was seen under P deficient white clover (Almeida *et al.* 2000) and *Medicago truncatula* (Sulieman *et al.* 2010), where elevated levels of all major amino acids were founds, especially asparagine. It appears that aspartate, which serves as a precursor to asparagine, also plays a key role in the maintenance of these processes, as it was the predominant amino acid found in this study (Maxwell *et al.* 1984, King *et al.* 1986, Rosendahl *et al.* 1990). Asparagine, which is usually found in elevated levels during low P conditions, can act as a possible N-feedback regulator to the nodules during P-stress, as it flows from the shoots to the nodules and conveys the message of the shoot nitrogen status to the nodules and modulates their activity according to nutrient status of the plant (Sulieman *et al.* 2010). In this way the nitrogenase activity can be regulated by asparagine and this trend is also similar in other legumes and non-legumes plants under stress (Steward and Larher 1980, Lea *et al.* 2007).

The key to these generated amino acids and other metabolic products during P stress might lie in the operation of the non adenylated PEPC bypass route. Various studies have implicated this non adenylated PEPC-bypass route to increase the PEP metabolism during P deficiency. (Duff *et al.* 1989, Theodorou and Plaxton 1993). Those studies have also found that the PEPC-activity may lead to an increase malate production. Malate could serve as C fuel in bacteriods, which is generated by the combined action of CA, PEPC and MDH (Vance and Heichel, 1991). In addition, malate can be transformed into OAA through MDH and serves as C skeleton to generate asparagine, which serves as the principle N export compound in temperate legumes (Schultze *et al.* 2006). The higher accumulation of malate in the nodules compared to the roots, (irrespective the treatment) might implicate its role as C fuel for nodules to sustain nodule activity. Similar findings were also observed in white lupin, where higher malate concentrations were also found in nodules compared to roots (Schuller and Werner 1993). In addition to its role as fuel for nodules, malate as well as citrate can be excreted by roots to chelate metal cations such as Fe^{3+}, Fe^{2+}, Al^{3+}, and Ca^{2+} and in the process it releases P from these cations, especially during low P conditions (Neumann and Romheld 1999). The larger amount of citrate accumulation in roots compared to nodules may be an indication that *V. divaricata* also follows this trend to acquire P.

Although a combined action of all three enzymes (CA, PEPC, MDH) are needed to generate organic acids for bacterial fuel and for exudation, literature highlights Class1 PEPC as playing a crucial role in the anaplerotic replenishment of tricarboxylic acid cycle intermediates where carbon

skeletons are removed for other metabolic functions like nitrogen assimilation and amino acid biosynthesis especially during P-deficiency. (Uhde-Stone *et al.* 2003, Vance *et al.* 2003, Shane *et al.* 2004a, O'Leary *et al.* 2011a). When extremely low levels of P in the plant are reached, PEPC (in conjunction with MDH and ME) can theoretically function as a glycolytic enzyme by indirectly bypassing the conventional ADP dependent PK reaction to facilitate continued pyruvate supply to the TCA cycle. In the process, P*i* is also generated and recycled in the P-starved cells (Nagano *et al.* 1994, Plaxton and Carswell 1999). *In vitro* root-MDH activity (LP treatment) appears to be the only enzyme to show higher activity over that of nodule-MDH activity. A direct result of this elevated LP root MDH activity might have been the export of malate to nodules which gave rise to the higher malate concentration in nodules, compared to roots.
These findings give an indication that P deficiency may impact negatively on the root's metabolic processes, resulting in the lower biomass obtained for roots compared to the apparently unaffected nodule metabolism, resulting in an increase in biomass for nodules under P-stress.

3.6 Conclusion

The efficiency of the nodules via an increased allocation of resources and P acquiring mechanisms in *V. divaricata* may be the key to the plant's ability to adapt to poor P environments and thus sustaining its reliance on BNF.

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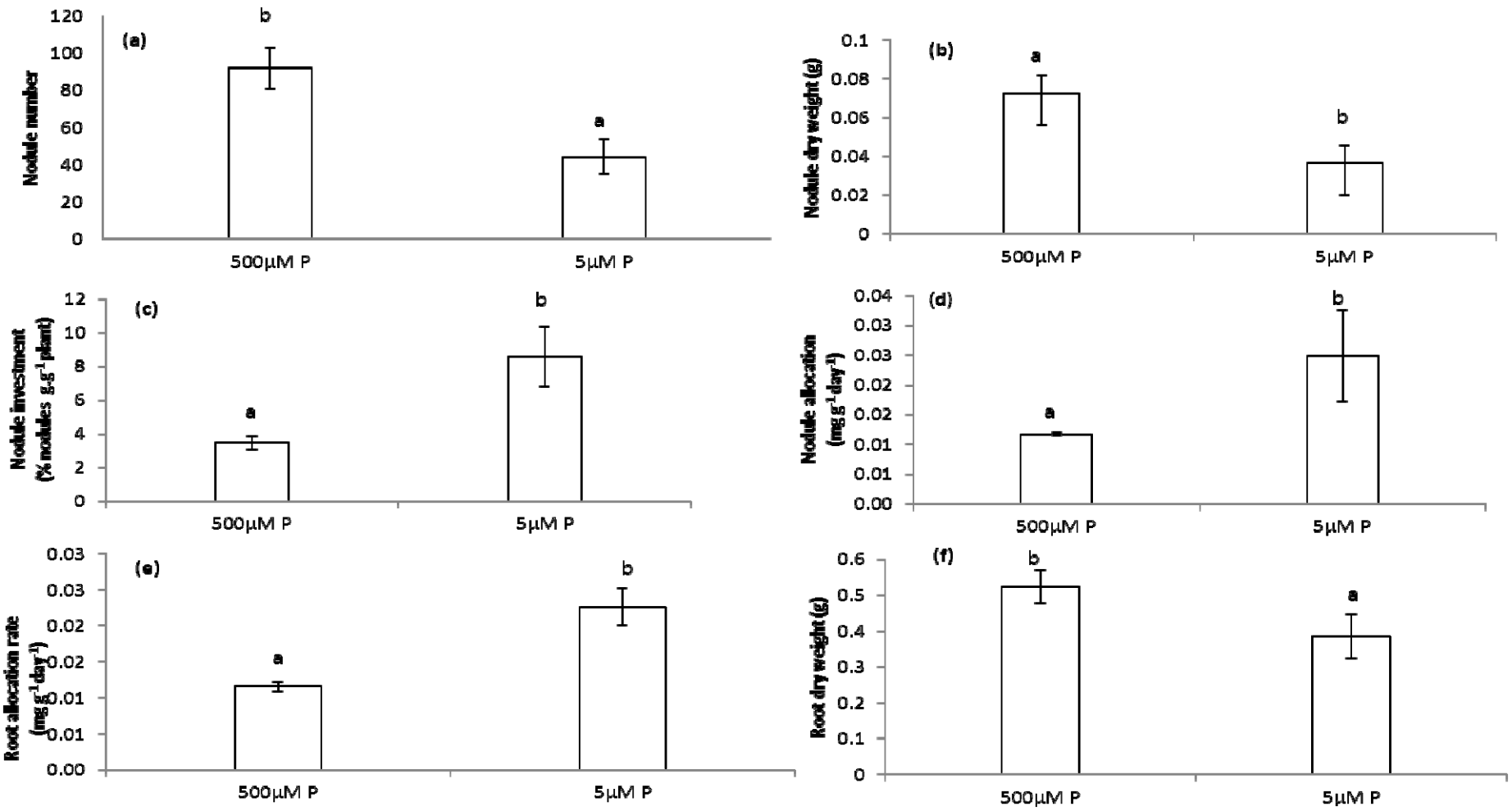

Figure 3.1. (a) Number of nodules on roots, (b) Dry weight of nodules, (c) Nodule % of plant dry weight, (d) Nodule allocation rate, (e) Root allocation rate, (f) Root dry weight, from roots and nodules of *V.divaricata* grown under high phosphate (500 µM P) and low phosphate (5 µM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

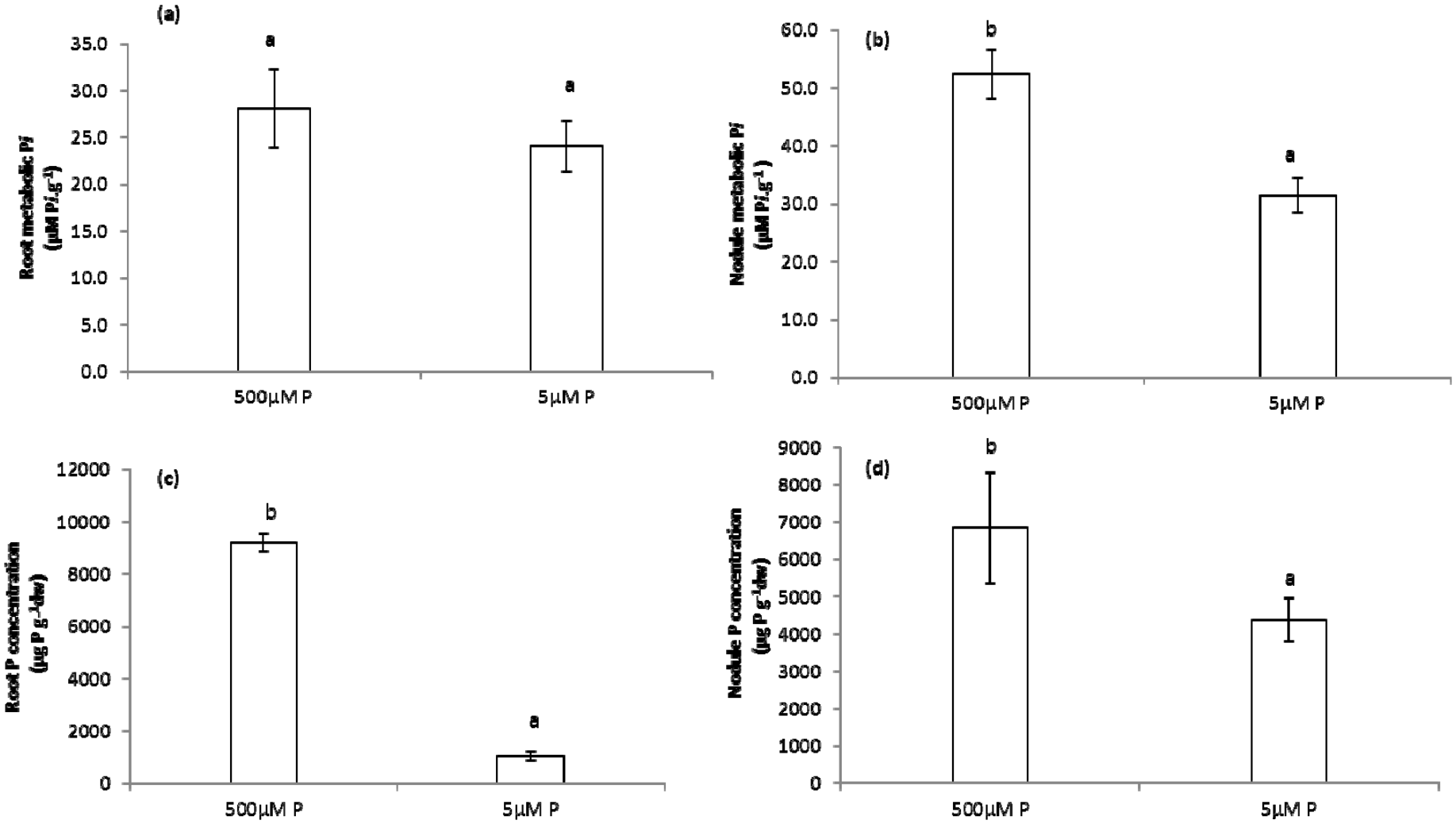

Figure 3.2. Inorganic P*i* of (a) roots, (b) nodules. Phosphate concentration in (c) roots, (d) nodules of *V. divaricata* grown under high phosphate (500 µM P) and low phosphate (5 µM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

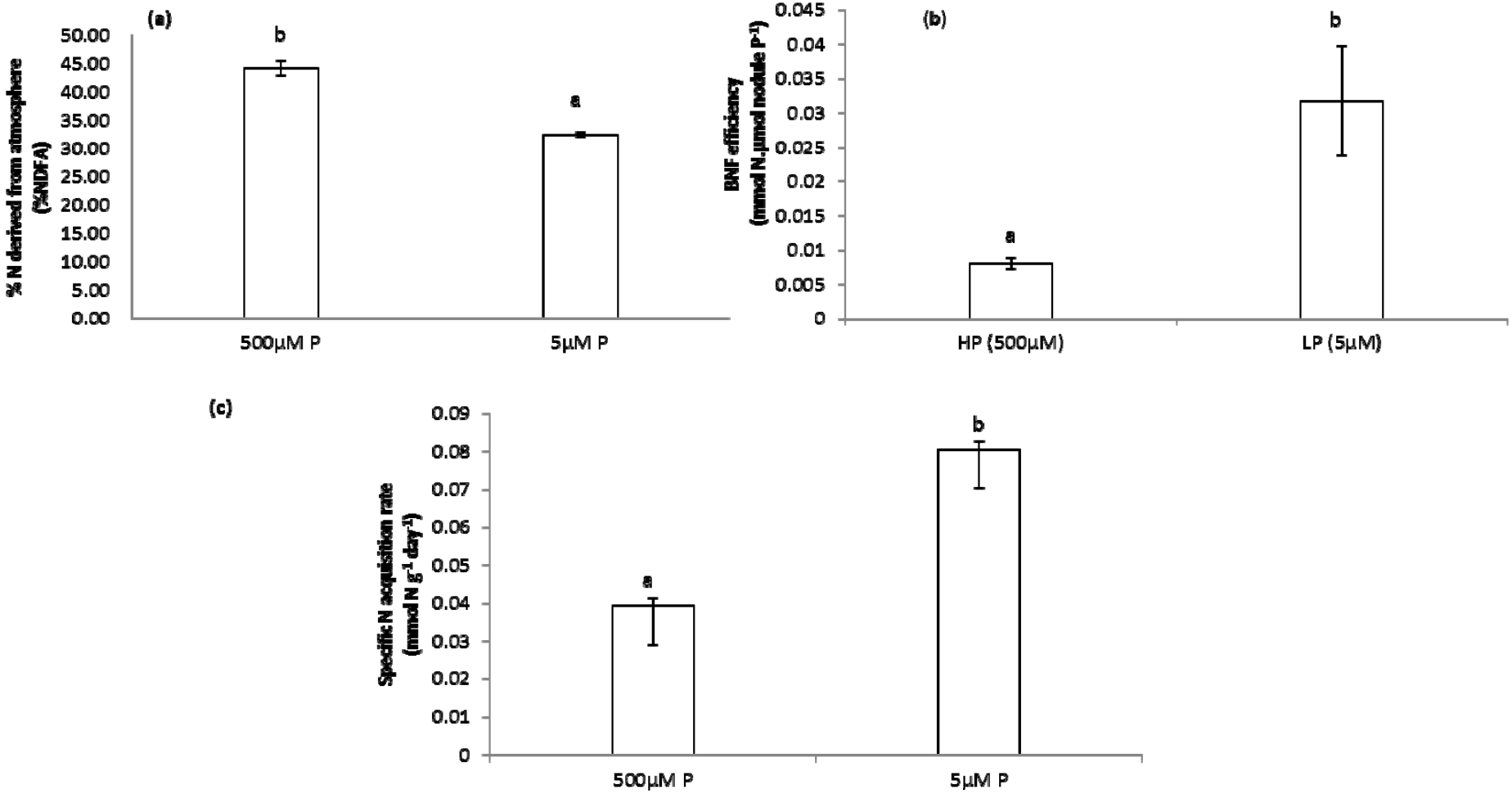

Figure 3.3. (a) Percentage nitrogen derived from atmosphere, (b) Nitrogen fixation efficiency per P concentration, (c) Specific N acquisition rate (SNAR) in nodulated roots of *V. divaricata* , grown under high phosphate (500 µM P) and low phosphate (5 µM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

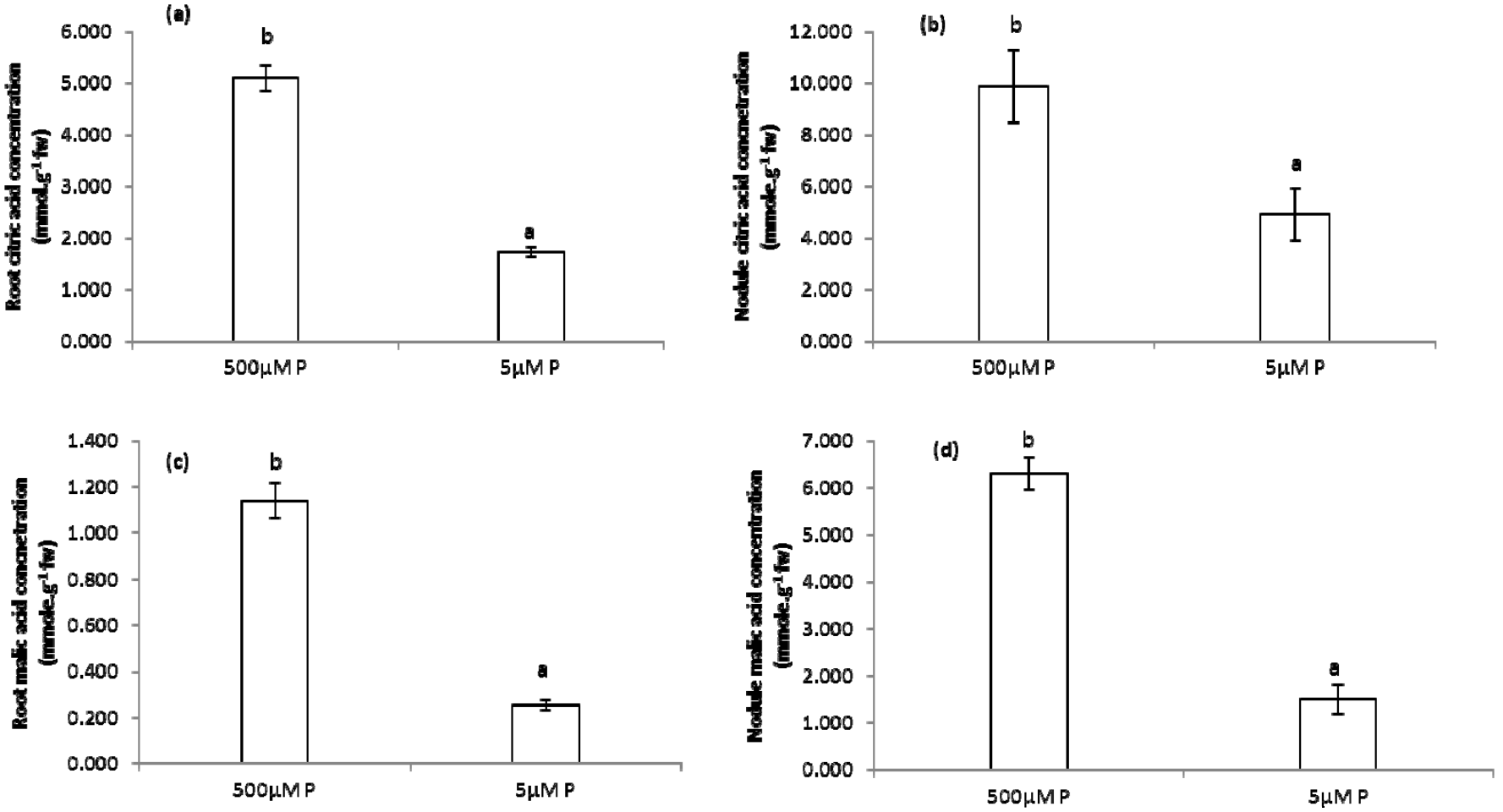

Figure 3.4. Various organic acids concentrations by GC-MS analysis in roots and nodules of *V. divaricata*, grown under high phosphate (500 µM P) and low phosphate (5 uM P) conditions. Citric acid concentration in (a) roots, (b**)** nodules. Malic acid concentration in (c) roots (d),nodules. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

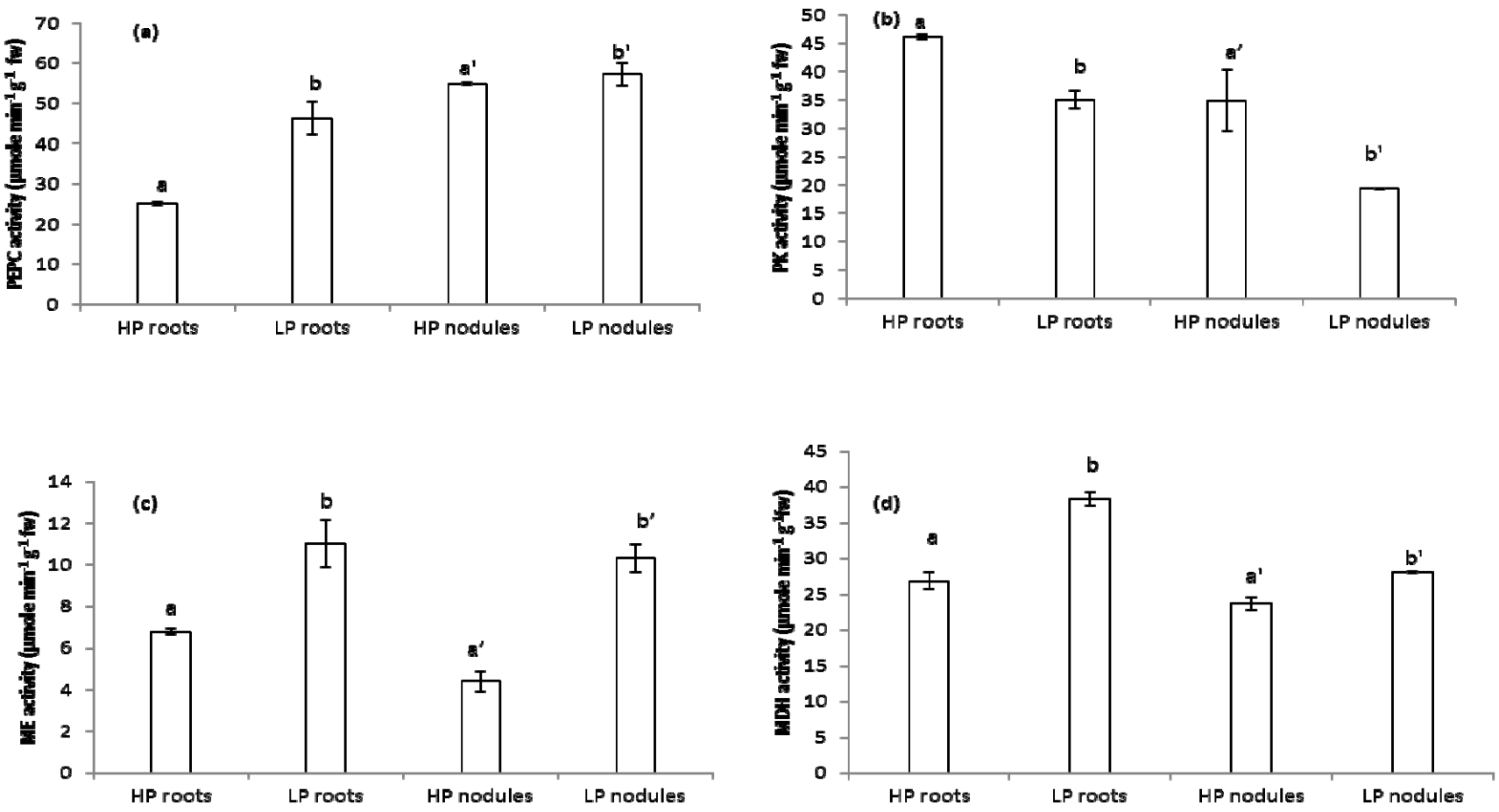

Figure 3.5. Root and nodule enzyme activities of *V. divaricata*, grown under high phosphate (HP 500 µM P) and low phosphate (LP 5 µM P) conditions. (a) Phosphoenolpyruvate carboxylase (PEPC) activity, (b) Pyruvate kinase (PK) activity, (c) Malic enzyme (ME) activity, (d) Malate dehydrogenase (MDH) activity. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Chapter 4:

The fate of PEPC derived carbon in phosphate stressed roots and nodules of *V. divaricata* by means of ^{13}C NMR.

4.1 Abstract

The legume, *Virgilia divaricata*, is indigenous to the Cape Floristic Region (CFR) and acquires soil and atmospheric N via its nodulated root system in P-poor soils. The reliance of N assimilation on organic acids during P limitation has not been investigated in a legume from a nutrient-poor ecosystem. The enzyme PEPC plays a pivotal role in linking organic and amino acid metabolism, but may have different implications for roots and nodules of the same root system. Therefore the goal of this study was to determine the fates of the PEPC-derived C into organic and amino acids in roots and nodules under P deficiency. PEPC incorporation of its inorganic substrate HCO_3^- was investigated in roots and nodules using NMR. ^{13}C NMR revealed differences regarding the incorporation of PEPC-derived carbon into organic acids, amino acids and other metabolic compounds between these two organs. Two profound peaks were identified as the organic acids, malate and citrate in both roots and nodules. These two organics acids appeared to be the first major metabolic products from PEPC derived carbon. The concentrations of these organic acids remained unchanged after 1h exposure to ^{13}C after which a sharp decline was observed after 2 h exposure in both roots and nodules. These organic acids were further metabolised to α-ketoglutarate, as the presence of this keto group could be established at ~216 ppm, especially in LP roots and nodules. In addition, the LP treatment also revealed the presence of asparagine in both roots and nodules. It appears that this amino acid became rapidly converted in nodules to other metabolic compounds, as no asparagine could be detected after 2 h. PEPC appears to be at the core of these generated downstream products, and the functioning of the PEPC-bypass route was established via PEPC, MDH, and ME activity, which was much higher in the LP treatment of roots and nodules compared to the HP treatment. In addition, higher internal P*i* was found in LP nodules compared to roots and it appears that plants allocate more of their resources to nodules during LP conditions. Furthermore, *V. divaricata* nodules may use their large PEPC-derived malate pool to prevent large declines in BNF under low P. This may enable nodules to maintain their BNF function under low P conditions.

4.2 Introduction

Phosphoenolpyruvate carboxylase (PEPC) catalyses the conversion of phosphoenolpyruvate and bicarbonate to OAA and P*i* (Chollet *et al*. 1996) and is believed to play a pivotal role in carbon metabolism in symbiotic nodules of legume roots (Rawstone *et al*. 1980, Day and Copeland 1991). The PEPC derived OAA can be converted to malate, via malate dehydrogenase (MDH). The generated malate can be fed into the mitochondrial TCA cycle for further metabolism, or metabolised to pyruvate via malic enzyme (ME). PEPC plays a crucial role in the assimilation of atmospheric CO_2 during C4 and CAM photosynthesis. In addition PEPC has been implicated to replenish the TCA cycle intermediates when carbon skeletons are removed for other metabolic functions like nitrogen assimilation and amino acid biosynthesis when plants experiences stress of various forms, such as P-stress. The induction of PEPC during P-stress also results in elevated levels of organic acids such as malate and citrate in the rhizosphere (O'Leary *et al*. 2011a). It was found that PEPC derived dicarboxylic acids are the main respiratory substrates of bacteroids in nodules (Tajima *et al*. 1990, Streeter 1991). In addition, these dicarboxylic acids are also the main carbon skeletons for transamination by aspartate aminotransferase to produce aspartate. Most of the produced aspartate is converted to asparagine by asparagine synthase (AS) (Ta and Joy 1986). Furthermore, N_2 fixation comes with a high CO_2 loss (Pate *et al*. 1979a), which could account for more than 60% of the carbon allocated to the nodules (Voisin *et al*. 2003a). Plants manage to reincorporate this CO_2 as intermediates to the TCA cycle, and to fuel nodule metabolism, by the combined actions of carbonic anhydrase and PEPC (Vuorinen and Kaiser 1997, Flemetakis *et al*. 2003).

PEPC, which uses the substrate HCO_3^-, naturally discriminates between $H^{12}CO_3^-$ and $H^{13}CO_3^-$ (Raven and Farquhar 1990). Furthermore, an increase in cytoplasmic pH stimulates the synthesis of malate. It was thus derived from this scenario that when the plant is fed with $^{13}CO_2$, that the C4 of malate can be regarded as a direct product of PEPC (Gout *et al*. 1993). Similar results were also observed in maize root tips (Chang and Roberts 1989) and intact leaves of *Kalanchoe tubiflora* (Stidham *et al*. 1983).The discriminatory effect of PEPC makes it ideal to study the fate of its derived ^{13}C-molecules via ^{13}C-NMR.

NMR spectroscopy has been established as a powerful technique to obtain physical, chemical, electronic and structural information about molecules in solution as well as in their solid state. This allows for the characterization of the metabolic activities in plant cells by coupling NMR with stable isotope labelling. It was shown that this technique could be exploited to determine the

metabolite flux in plant cells making it suitable to establish the conditions and compartmentation of these metabolites in plant cells (Chang and Roberts 1989).

Gilbert *et al*. 2011, reported on the successful application of ^{13}C NMR for the determination of intramolecular $\delta^{13}C$ values in fructose from plant sucrose samples. One remarkable feature of plants is the fact that photosynthetic CO_2 fixation discriminates against ^{13}C. The $^{13}C/^{12}C$ ratio of organic matter in plants can be measured as it is relative to the $^{13}C/^{12}C$ ratio of inorganic carbon from which the cell organic matter has been formed. This implies that when ^{13}C is fed to plants, the resultant organic matter formed, becomes naturally depleted of ^{13}C as compared to atmospheric CO_2. Apparently there is a more rapid fixation of ^{13}C-inorganic C by carboxylates which use CO_2, compared to HCO_3 carboxylates and transport processes where less discrimination is found (Troughton *et al.* 1974, O'Leary *et al.* 1992). Furthermore it was found that ^{13}C fractionation associated with the net CO_2 assimilation also differs between the various CO_2 assimilation pathways.

The aim of the study was to get a better understanding of how PEPC-derived C is metabolised into amino acids and downstream organic acids of P-deficient nodules, using ^{13}C NMR spectroscopy, providing information on how nodules and roots manage to sustain their functioning during P-stress.

4.3 Materials and methods

4.3.1 *Plant growth*

Seeds of *Virgilia divaricata* (Silverhill Seeds, Kenilworth, Cape Town) were incubated for 1 hour in water containing a smoke disc, after which they were placed for 5 h in a water bath (50 ºC), in order to enhance germination. Seeds were then washed with distilled water (10x) and allowed to germinate in sterile filter sand in seed trays. Germination and growth of these seedlings took place under natural light conditions in a north facing glass house where plants were exposed to sunlight for a 10 h period per day. The temperature of the glass house varied between 15 ºC at night and 25 ºC during the day. After 2 weeks, when sprouting of leaves was observed, the seedlings were transferred to pots which contained sterile filtered sand as well as the nodule forming bacteria, *Burkholderia phytofirmans* (which was grown on yeast mannitol agar).

Plants were divided into 2 groups i.e, low (5 µM P) – and high (500 µM P) phosphate according to the Long Ashton nutrient treatment. The low phosphate treatment (LP), consisted of;

Macronutrients ($MgSO_4/K_2SO_4/CaCl_2$, 50 ml), Phosphate (50 ml pH 5.5), Micronutrients ($H_3BO_3/MnSO_4/ZnSO_4/CuSO_4/NaMoO_4$, 2.5 ml), Iron (6 ml), NH_4NO_3 (10 ml). The high phosphate treatment (HP) consisted of Macronutrients ($MgSO_4/K_2SO_4/CaCl_2$, 50 ml), Phosphate (5 ml pH 5.5), Micronutrients ($H_3BO_3/MnSO_4/ZnSO_4/CuSO_4/NaMoO_4$, 2.5 ml), Iron (6 ml), NH_4NO_3 (10 ml). Plants received the respective treatments twice per week and allowed to grow for 8 weeks before they were harvested.

4.3.2 *Feeding of ^{13}C*

Two month old plants of *V. divaricata* were fed with ^{13}C in the following manner. A solution of sodium bicarbonate-^{13}C (Sigma Aldrich Cat # 372382-1G, 99 atom % ^{13}C) [0.215 g/L] was prepared at pH 6.8. A solution of KOH (250 mM) was also prepared and placed in the trays of the pots to absorb CO_2 which could escape through the soil. Soda lime was placed in 5 ml tips to serve as trap for CO_2 in the head space between the lid and soil in pot. Lids and holes were sealed off and made air tight with Bostik Prestik (BOSTIK SA). Lids were placed on pots immediately after 300 ml of $NaH^{13}CO_3$ solution was fed, and were made completely airtight with Bostik Prestik. The run-off volumes of the $NaH^{13}CO_3$ were collected and measured. Pots were then placed on clean lids and KOH was added to the lid. Plants were harvested at 1 h and 2 h intervals after feeding of $NaH^{13}CO_3$. All metabolic processes were stopped by placing nodulated roots in liquid N.

4.3.3 *^{13}C NMR*

Sample preparation was done as described in Gout *et al.* (2000). Briefly, 9 g of roots and 1g nodules were frozen in liquid N and ground to a powder in 1 ml of 70% ($^{v}/_{v}$) perchloric acid. The frozen powder was allowed to thaw at -10 ºC. The thick slurry was then centrifuged at 15000*g* for 10 min and the supernatant neutralized with 2 M $KHCO_3$ to pH 5. The supernatant was then centrifuged at 10000*g* for 10 min to remove $KClO_4$ and then lyophylised and stored in liquid N. The lyophylised sample was redissolved in 2.5 ml water which contained 10% ($^{v}/_{v}$) D_2O. The solution was neutralized to pH 7.5, buffered with HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) and CDTA (1,2-cyclohexanediaminetetraacetic) (50 – 100 µM) was added to chelate divalent cations.

NMR was performed at 25 ºC in D_2O on a Varian 600 mHz instrument with an Inova

spectrometer. The conditions were as follow: Pulse sequence: s2pul, Number of scans: 1303, Receiver gain: 60, Relaxation delay: 1.0000, Pulse width: 0.0000, Acquisition time: 0.8688, Spectrometer frequency: 150.88.

Stronger signals and larger peak areas did not necessary reflect a higher concentration of sample. Samples concentrations were corrected by dividing peak areas into the ^{13}C bicarbonate peak area at 161 ppm. These relative concentrations were used to calculate the ^{13}C flow via PEPC into various compounds. ^{13}C compounds were expressed per gram FW and then divided by 60 or 120 min to calculate the metabolic flux.

4.3.4 *Protein extraction and determination*

Proteins from roots and nodules were extracted according to the methods used by Ocaña *et al.* (1996), modified to the extent 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 Ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 20 % (v/v)ethylene glycol, 2 % (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Randburg, South Africa) per 50 ml of buffer. The protein concentration was determined by the NanoDrop Lite Spectrophotometer (Thermo Scientific), where the extraction buffer was used as standard.

4.3.5 *Enzyme assays*

All enzymatic reactions were performed in a multi-well plate reader at a wavelength of 340 nm. The reactions started by adding 30 µl of the crude extraction mixture in a total volume of 250 µl. The various initial reaction rates have been shown to be proportional to the concentration of the extracted enzymes used under the conditions used (Ocaña *et al*. 1996).

4.3.5.1 *Phosphoenolpyruvate carboxylase*

The PEPC activity (in triplicate) was determined by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 25 °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*. 1996). The 9 blanks that were used in the assay consisted of a reaction medium without PEP.

4.3.5.2 *Pyruvate kinase*

PK activity (in triplicate) was assayed in a buffer containing 75 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 20 mM KCl, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and lactate dehydrogenase (3 U) (McCloud *et al*. 2001). The 3 blanks consisted of the buffer without ADP.

4.3.5.3 *Malic enzyme*

The Malic enzyme (ME) activity was assayed at 340 nm in triplicate and the reaction mixture consisted of, 80 mm Tris-HCl (pH 7.5), 2 mm $MnCl_2$, 1 mm malate and 0.4 mm NADP or NAD^+ (Appels & Haaker, 1988). The blanks consisted of a reaction medium without malate.

4.3.5.4 *NADH-Malate dehydrogenase*

MDH activity, (in triplicate) 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). The 3 blanks consisted of a reaction medium without OAA.

4.3.6 *Isotope analysis*

Analyses of δ15N was done at the Archeometry Department at the University of Cape Town, where the isotopic ratio of δ15N was calculated as δ=1000‰ (Rsample/Rstandard). R refers to the molar ratio of the heavier to the lighter isotope of the samples. Standards were similar to those as described by Farquhar *et al*. (1989). Briefly, between 2.100 and 2.200 mg of each milled sample was placed in 8 mm x 5 mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) and weighed on a Sartorius microbalance (Goettingen, Germany). Combustion of the samples were performed in a CHN analyser (Fisons NA 1500, Series 2, Fisons instruments SpA, Milan, Italy) and the δ15N values for the nitrogen gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct for drifting that might occur in instrument. Two in-house standards (Merck Gel and Nasturtium) were used and the third was the IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$. The methodology used for the calculation of %NDFA was according to Shearer and Kohl (1986), where %NDFA = 100(δ15Nreference plant - δ15N legume)/ (δ15N reference plant –B). *Triticum*

aestivum was used as reference plant grown under the same glasshouse conditions. The B-value (which was determined as -0.71‰.) refers to the δ15N natural abundance of the N derived from BNF of the above-ground tissue of *V. divaricata*, grown in a N-free solution.

4.3.7 *Calculations*

4.3.7.1 *Specific nitrogen absorption rate:*

Specific N absorption rate (SNAR) (mg N g^{-1} root DW d^{-1}) is the net N absorption rate per unit root DW as outline in Nielson *et al*. (2001), where:

SNAR = [(M2 - M1 /t2 - t1)] x [(loge R2 – loge R1)/ (R2 – R1)]

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

4.3.7.2 *Below ground allocation:*

This allocation refers to the fraction of new biomass partitioned into new roots and nodules over the given growth period. The calculations were done according to Bazzaz and Grace (1997): df/dt = RGR (∂-Br/Bt).

4.3.8 *Inorganic Pi determination*

A modified method of Fiske and Subbarow (1925) was used to determine P*i* concentration (Rychter and Mikulska 1990). Briefly, 0.1 g of roots/nodules were ground in 100 µl 10 % trichloroacetic acid ($^{v}/_{v}$). Samples were then diluted 3x with 5 % ($^{v}/_{v}$) trichloroacetic acid and then centrifuged for 10 min at 4 ºC at 2500 *g*. The supernatant was then removed and centrifuged for a further 10min at 4 ºC at 13000 *g*. The supernatant was then removed and 700 µl reaction mix (10% ($^{v}/_{v}$) $C_6H_8O_6$ + 0.42% $[NH_4]6Mo_7O_{24}.4H2O$ in a 1:6 [H_2SO_4 1N] ratio) was added to 300 µl supernatant. Absorbance of the samples was determined on a multiwell plate reader at 820 nm.

4.3.9 *Statistical analysis*

The effects of the factors and their interactions (HP and LP) were tested using the *t*-test. Different letters indicate significant differences between treatments ($P \leq 0.05$), revealed by the *t*-test.

4.4 Results

4.4.1 *Biomass and Inorganic Pi data*

There was a decline in %NDFA and in the LP treatment compared to the HP. However, nodules appeared to be more BNF efficient per P concentration during LP conditions compared to HP conditions (Fig 1a). Higher values were obtained for the allocation of resources in the LP treatment compared to the HP treatment for roots and nodules (Fig 4.1c). Higher P*i* values were obtained in the HP treatment for both roots and nodules. (Fig. 4.1d).

4.4.2 *^{13}C NMR*

An array of various resonances of various structures were obtained from the ^{13}C spectra, with the most significant peaks between 175 – 181 ppm throughout all the treatments (Figs 4.2, 4.3, 4.4, 4.5). Chemical shifts were identified by running reference solutions of these organic acids, (solubilised in D_2O at pH 7.5) under the same running conditions as the samples. These two peaks were assigned to the organic acids malate and citrate respectively. Much higher relative malate concentrations were found compared to citrate (Fig. 4.6). Incorporation of ^{13}C was very noticeable during the first hour with higher relative concentrations and a sharp decline in relative concentration after 2 h of exposure to ^{13}C, especially citrate. Malate levels remained almost unchanged in HP conditions, however a sharp decline was observed in LP conditions in both roots and nodules. The presence of a keto-group (at 200 – 220 ppm) could also be observed in the LP root and nodule spectra (but not in HP spectra), which can be assigned to that of α-ketoglutarate (Figs 4.3, 4.5). Asparagine could also be identified at ~125 ppm on the same principle as the above peaks were assigned. Asparagine could only be detected in the roots and nodules under LP conditions (Figs 4.3, 4.5). It appears that asparagine became converted to other metabolic compounds as it could not be detected after 2 h in LP nodules (Fig 4.5). The peak at 161 ppm, which was present in all the samples (except in the control samples) can be assigned to ^{13}C bicarbonate which was present in the perfusion medium (Gout *et al.* 2000). The chemical shifts and consequent identification of these organic acids was shown to be pH- and solvent sensitive, as different shifts were observed with different pH and solvents when the reference samples were run. Therefore it appears that chemical shifts might differ from previous work, and that similar running conditions should always be maintained, by keeping the samples solutions at the same pH.

4.4.3 *Enzyme assays*

Higher PEPC, ME and MDH (Figs. 4.7a,c,d) values in LP conditions were obtained for these assays compared to HP conditions. The highest PEPC activity was obtained in LP nodules. PEPC activity in LP nodules was almost double compared to HP nodules (Fig. 4.7a).

The highest ME (Fig. 4.7c) activity was obtained in LP nodules which was more than double of that in HP nodules. Higher activity was also found in LP roots compared to HP roots.

PK activity (Fig 4.7b) was higher in the HP conditions compared to the LP conditions.

MDH activity was higher in the LP treatment for roots and nodules. Nodule MDH activity was higher compared to roots in the LP treatment (Fig. 4.7 d).

4.5 Discussion

The P-conservation mechanisms in roots and nodules, are known to involve the non-adenylate requiring PEPC-bypass route (Chapter 3). In the current study, the incorporation rates of the PEPC-derived C into malate, α-ketoglutarate and asparagine, revealed that *V. divaricata* nodules may use its large PEPC-derived malate pool to prevent large declines in BNF under low P.

One advantage of ^{13}C NMR, is that it enables us to trace the PEPC-derived C in downstream organic acid and other compounds. Due to the discriminatory action of PEPC between ^{13}C and ^{12}C (Raven and Farquhar 1990) it can be assumed that all labelled ^{13}C peaks on the various spectra are generated via PEPC and metabolised to various downstream compounds. The rapid incorporation of ^{13}C into malate and citrate during the first hour of exposure, gives an indication of the reliance of the plant of these respiratory C-compounds (especially malate) presumably generated via PEPC. We observed a sharp decline of the relative concentrations of both organic acids. The decline of citrate in roots, might implicate its role for exudation by roots, as reported in previous work (Neumann and Romheld 1999). The steep decline of the relative malate concentrations in LP conditions of roots and nodules (after 2 h) can serve as an indication of the intricate role that malate plays in plant metabolism, as it is incorporated into various downstream compounds. Malate was found to fulfil various functional diverse roles in plants which include, respiration and energy generation, nitrogen fixation and amino acid biosynthesis and uptake of P (Schultze *et al.* 2002). Former work also showed how malate became rapidly labelled when nodules are exposed to $^{14}CO_2$, (Streeter 1987, Rosendahl *et al*. 1990). It was shown that these PEPC derived

dicarboxylic acids are the main respiratory substrates of bacteroids, as PEPC has the ability to generate dicarboxylic acids by recycling CO_2 (Tajima *et al*. 1990, Streeter 1991). The generated ^{13}C labelled malate might also serve as an indication of the plant's metabolic preference to favour the PEPC-bypass route instead of the conventional pyruvate route via PK, especially during LP conditions. It was evident that this scenario is more applicable to LP conditions, as higher PEPC activity was recorded.

Furthermore it was also proposed that this PEPC bypass route is favoured especially in nodules where LP conditions prevail (Le Roux *et al*. 2006, Streeter 1987, Rosendahl *et al*. 1990). The reliance of nodules and roots on these PEPC derived organic compounds during P-stress conditions, became evident in the higher PEPC, MDH, and ME activity obtained compared to the HP treatment. The engagement of the non-adenylated PEPC bypass route was shown to be vital as indicated by $^{14}CO_2$ and $^{15}N_2$ studies done on nodules and shoots, highlighting the C feeding of the bacteriod and C skeleton provision for N assimilation. (Rosendahl *et al.* 1990, Vance and Heichel 1991, Day *et al.* 2001). In addition, it was also shown that PEPC activity is closely related to nitrogen fixation rates (Schultze *et al.* 1998b). The generation of this PEPC derived C to downstream metabolic products was also reflected in the detection of the labelled ^{13}C α-ketoglutarate peak at ~216 ppm. Previous work implicated α-ketoglutarate as a key compound in the TCA cycle and obligatory substrate for α-ketoglutarate-dependant dioxygenases (Lancien *et al.* 2000). The α-ketoglutarate concentration also showed a decreasing trend after 2 h, which was also observed in the citrate- and malate concentration, thus implicating its role especially for N metabolism. It was found that this TCA intermediate could serve as a co-factor for a diverse range of enzymes involved in amino acid, glucosinolate, flavonoid, alkaloid, and gibberelline metabolism (Araújo *et al.* 2014).

Despite the engagement of the PEPC bypass route, a decline in BNF during low P conditions was observed. This could serve as an indication that nodule functioning is hardly impaired during P-stress. Furthermore, the sustainability and efficiency of nodules during LP conditions are also reflected in the higher internal P*i* values obtained in nodules compared to roots as well as the higher resource allocation to LP nodules compared to high P conditions. The higher internal P*i* concentrations in nodules during LP conditions are in agreement with previous work where higher internal P*i* values were obtained under similar conditions (Al-Niemi *et al*. 1997, 1998, le Roux *et al.* 2006). It was therefore suggested that nodules function optimally under LP conditions (Al-Niemi *et al*. 1997, 1998). Nodules are also known to be P scavengers as it has been shown that

nodules do scavenge P from their host cells and have a natural tendency of holding on to the extracted P and therefore it was suggested that they may act as sinks for P under LP conditions (Al-Niemi *et al.* 1998).

These results (and previous findings) indicate that nodules might have a strategy to regulate the P influx under LP conditions and thereby reducing the effects that LP conditions might have on them (Jakobsen 1985, Tang *et al*. 2001). Although some of these results concur with findings of model legumes that nodule growth and functioning is not limited by P deficiency (Almeida *et al.* 2000, Sulieman *et al.* 2010), this current study highlighted a potential underlying mechanism of nodule maintenance under P stress. In this regard, this is the first study of legumes from a nutrient-poor ecosystem, where the PEPC-derived organic acids were shown to underpin nodule adaptation to P stress.

4.6 Conclusion

In conclusion, the key factor that underscored the enhanced nodule adaptation to P stressed conditions to a greater extent than roots of *V. divaricata*, is that the large PEPC-derived malate pool can be used to prevent large declines in BNF under low P.

4.7 References

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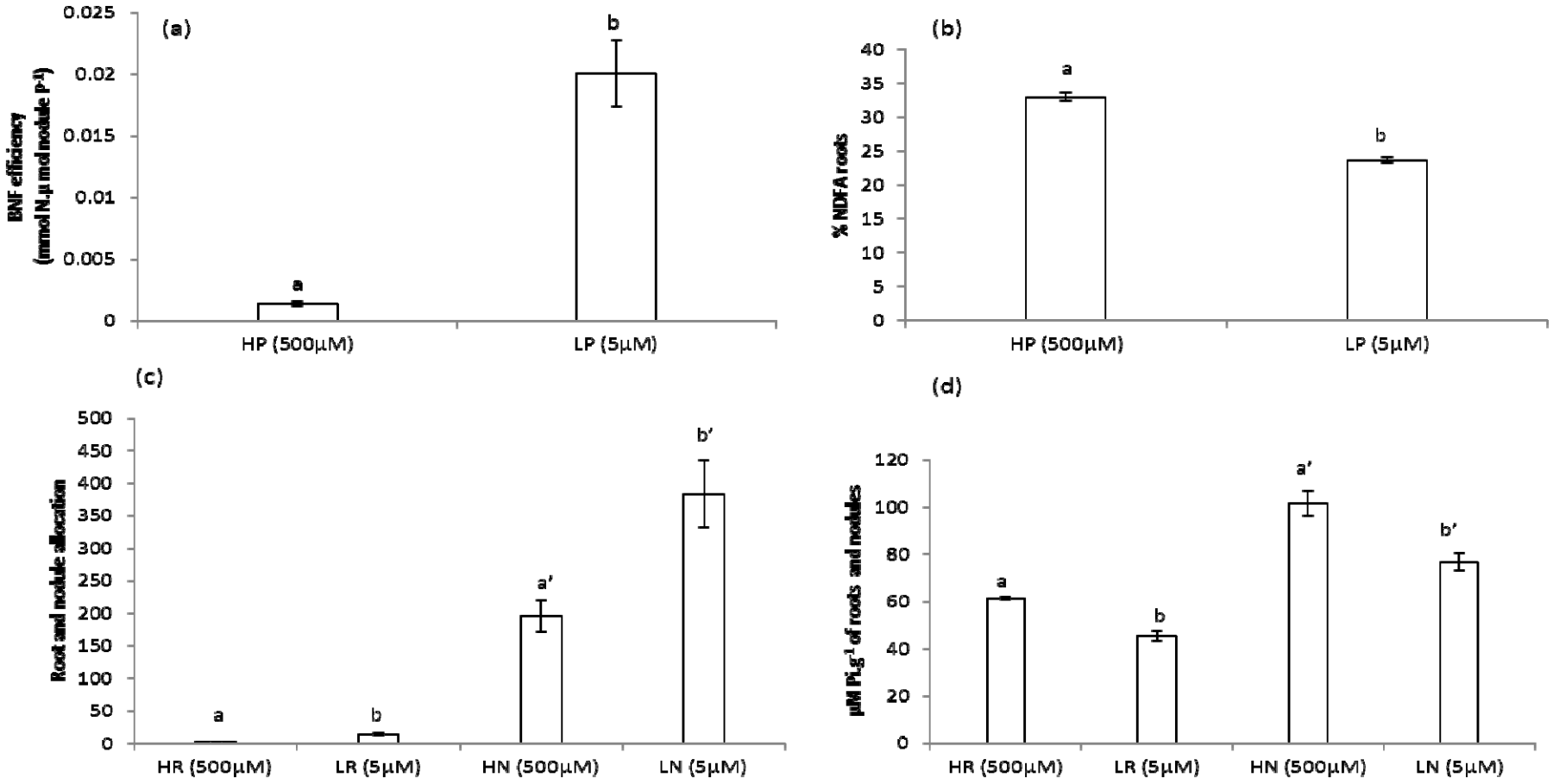

Figure 4.1. (a) Biological nitrogen fixation (BNF) efficiency per P concentration, (b) Percentage nitrogen derived from the atmosphere (% NDFA) of whole plants on a mass basis in nodules, (c) Root and nodule allocation in high (500 µM P) and low (5 µM P) P conditions, (d) Inorganic P*i* of roots and nodules of *V. divaricata*, grown under high (500 µM P) and low (5 µM P) P conditions Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

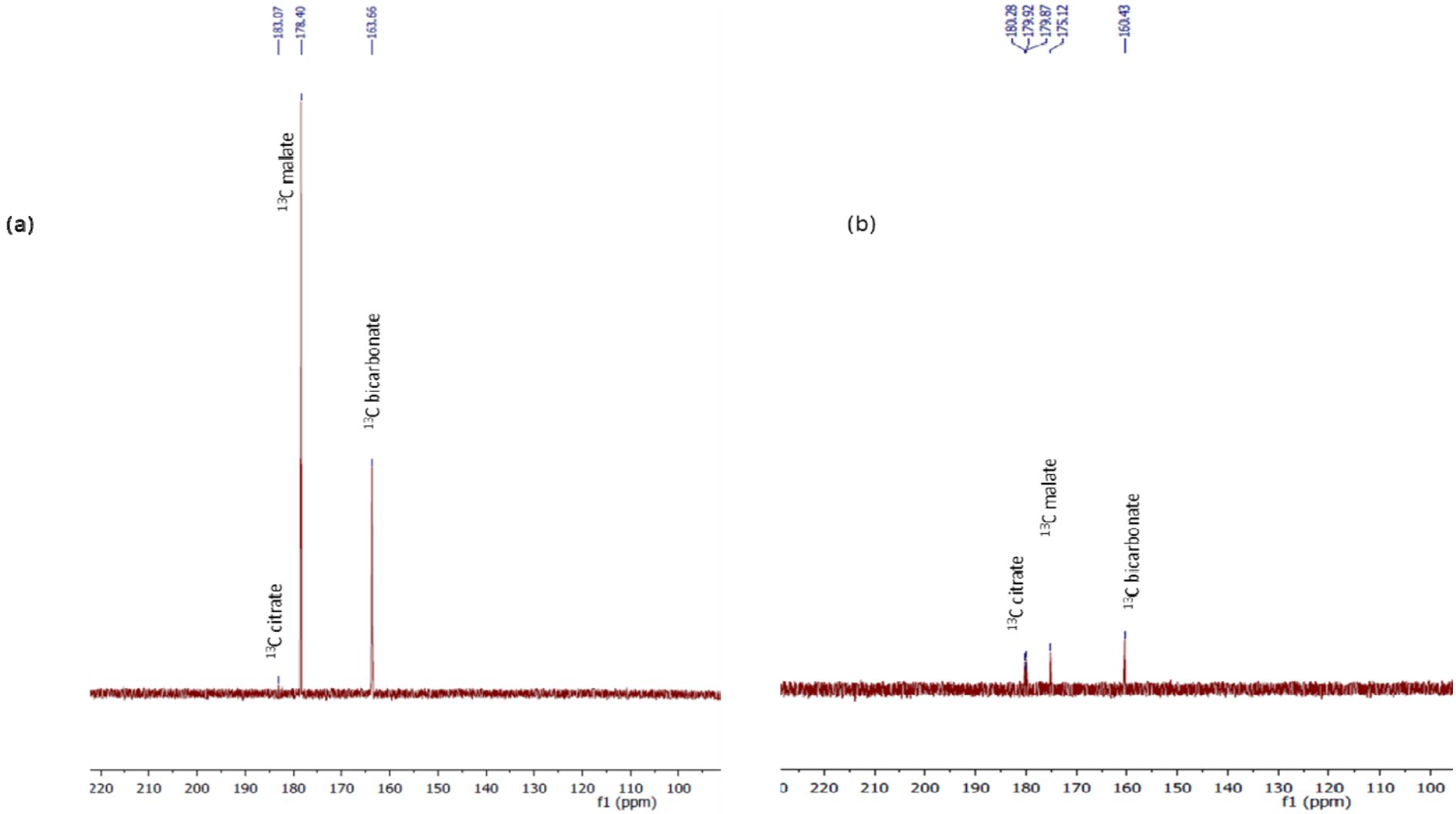

Figure 4.2. ^{13}C spectra of (a) roots of *V. divaricata* after 1h, (b) roots after 2h grown under high phosphate (500 µM P) conditions.

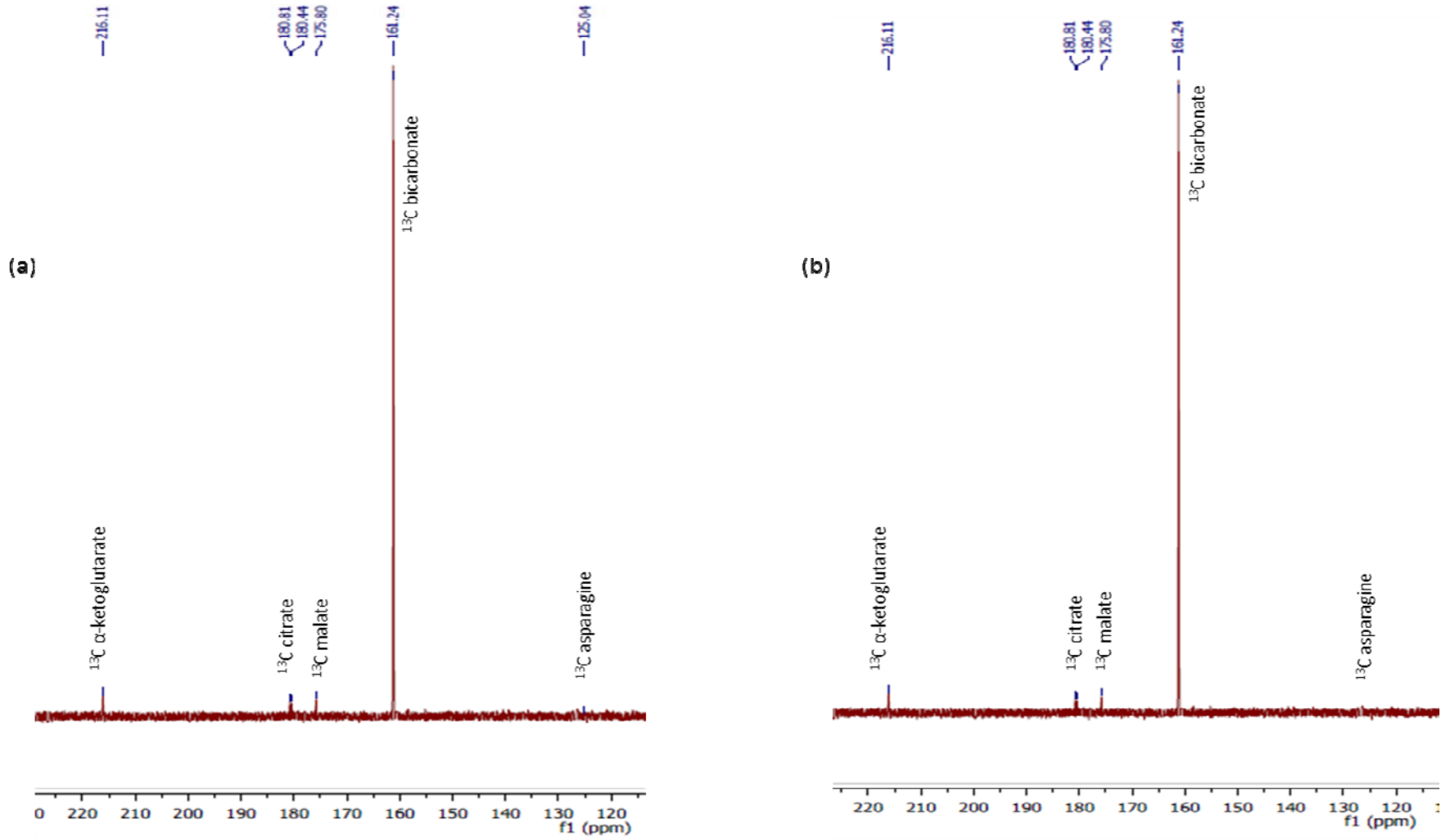

Figure 4.3. ^{13}C spectra of (a) roots of *V. divaricata* after 1h, (b) roots after 2h grown under low phosphate (5 µM P) conditions.

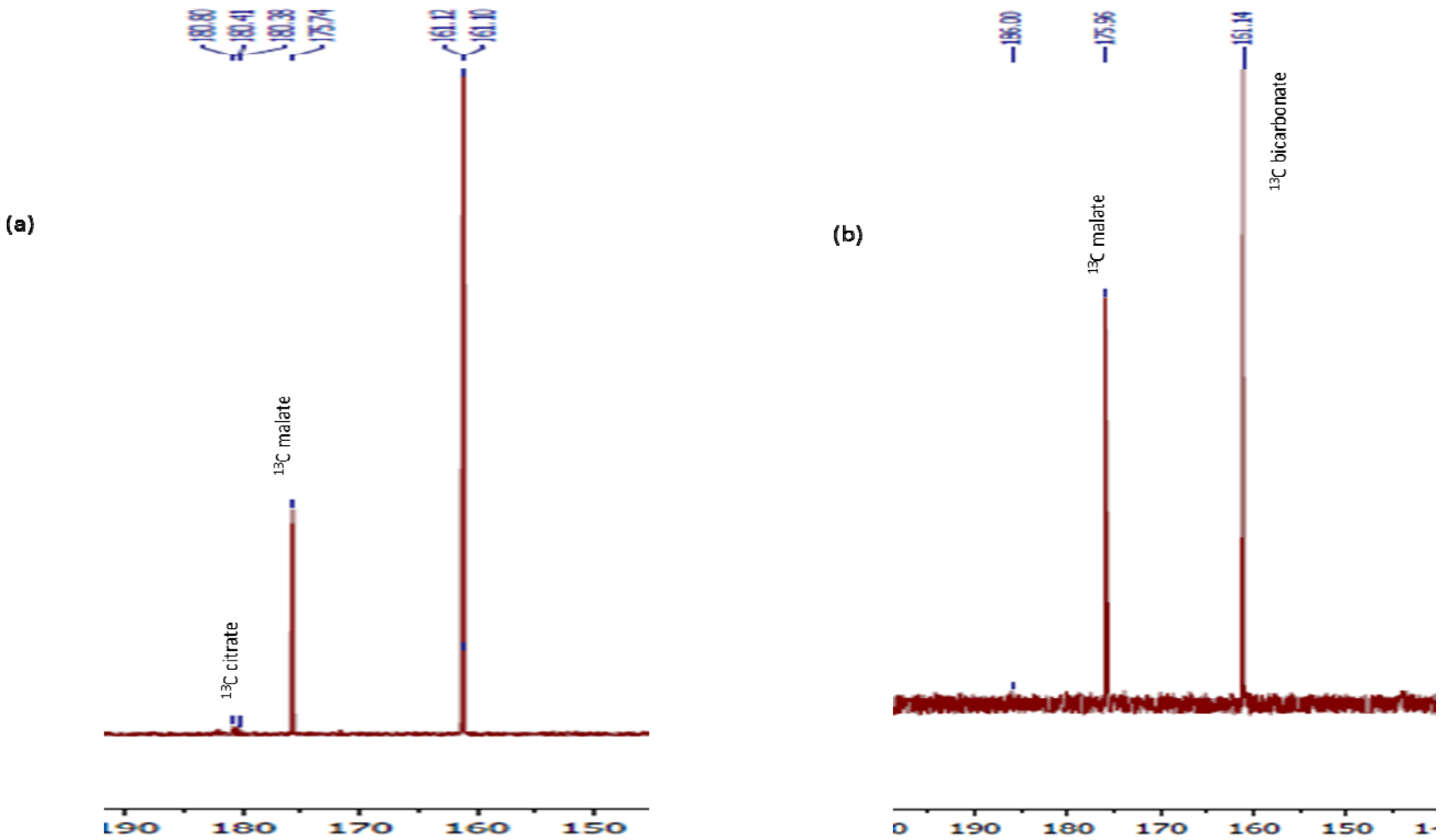

Figure 4.4. ^{13}C spectra of (a) nodules of *V. divaricata* after 1h, (b) nodules after 2h grown under high phosphate (500 µM P) conditions.

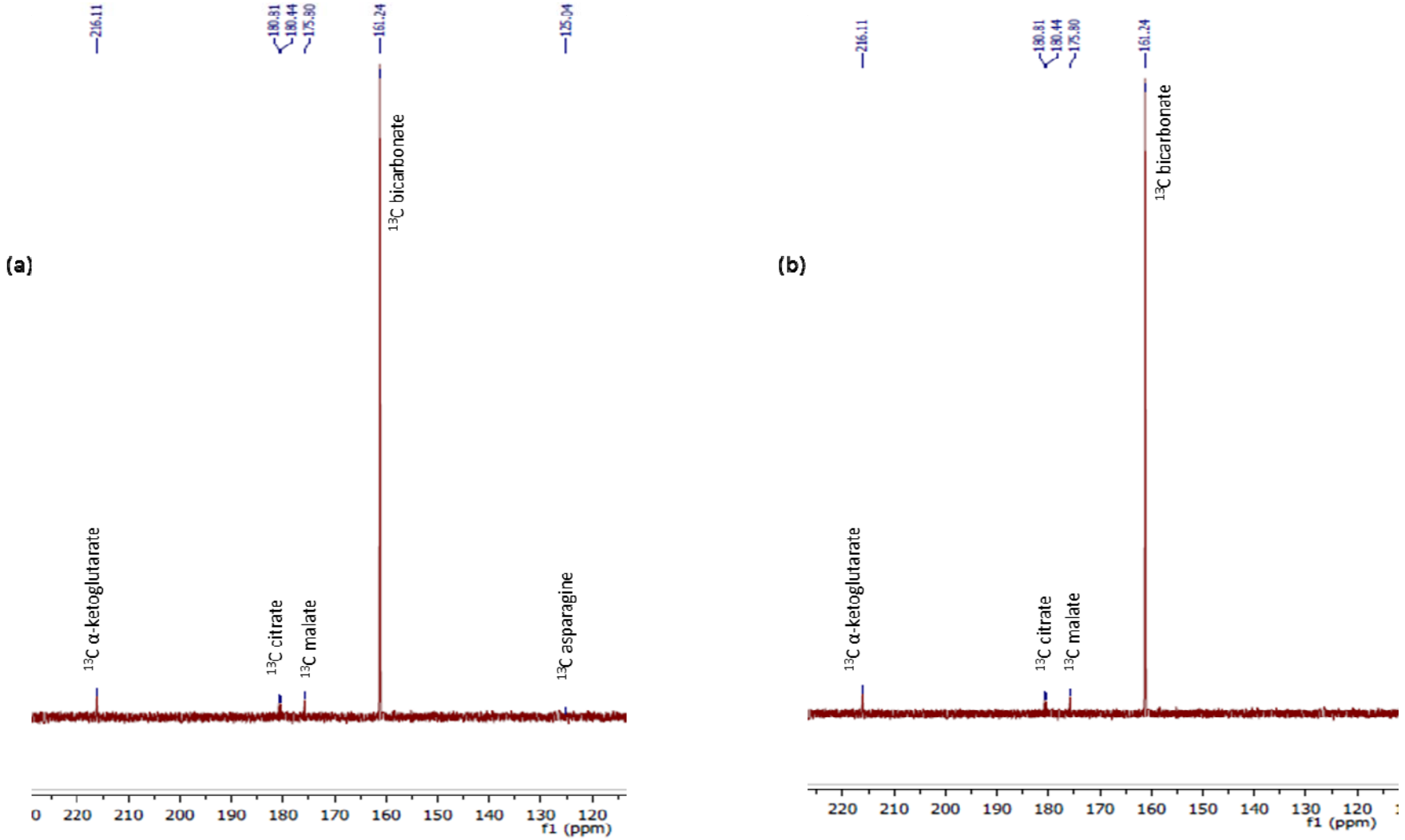

Figure 4.5. ^{13}C spectra of (a) nodules of *V. divaricata* after 1h, (b) nodules after 2h grown under low phosphate (5 µM P) conditions.

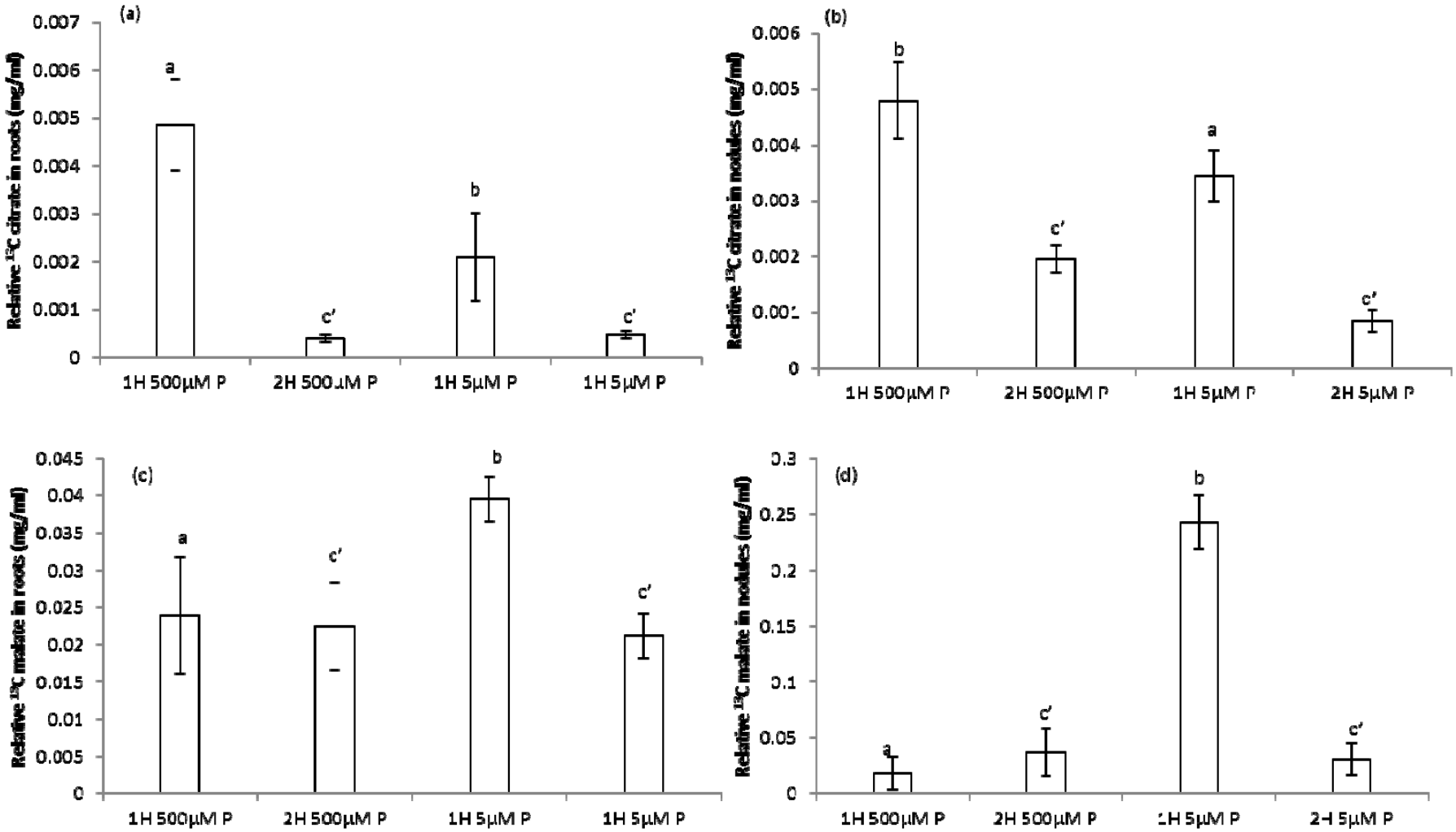

Figure 4.6. Relative ^{13}C organic acid found by ^{13}C NMR analysis in roots and nodules of *V. divaricata*, grown under high phosphate (500 µM P) and low phosphate (5 µM P) conditions (a) root malate, (b) nodule malate, (c) root citrate, (d) nodules citrate. Values are presented as means (n=3). The different letters (prime 2 hour treatment and non-prime for 1 hour) indicate significant differences among the treatments. (*P<0.05).

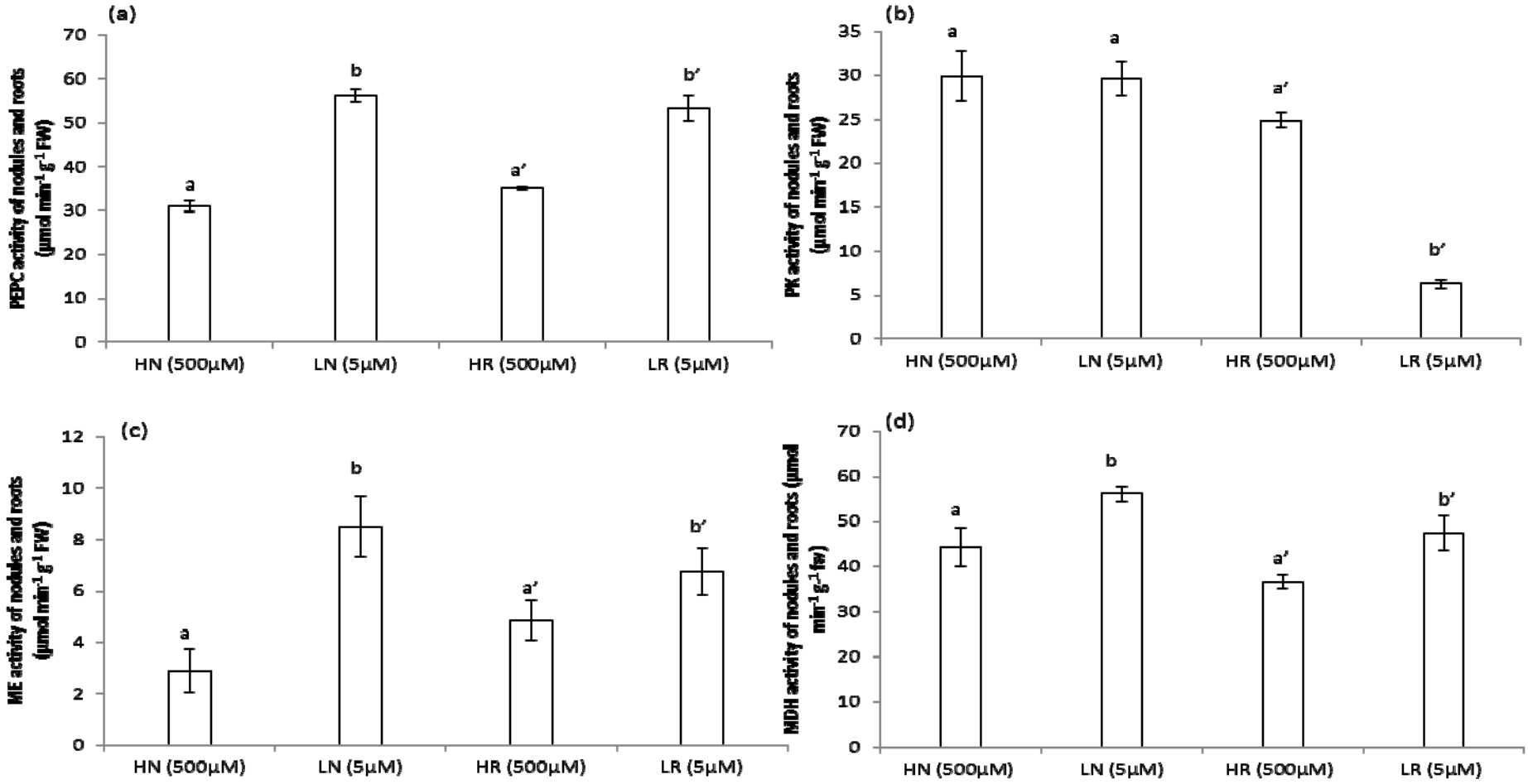

Figure 4.7. Enzyme activities in roots and nodules of roots and nodules of *V. divaricata* grown under high phosphate (500µM P) and low phosphate (5 µM P) conditions (a) Phosphoenolpyruvate carboxylase (PEPC) activity, (b) Pyruvate kinase (PK) activity, (c) Malic enzyme (ME) activity, (d) Malate dehydrogenase (MDH) activity. The different letters indicate significant differences among the treatments. (*P<0.05).

Chapter 5:

The turnover of PEPC-derived organic acids in N metabolism in P-stressed roots and nodules of *V. divaricata.*

5.1 Abstract

One of the typical signs of phosphate-stress in roots and nodules is the decline of internal P*i* in both these organs. A response to this decline in P*i* at the metabolic level is the increase in a bypass route, associated with the enzyme PEPC. This route can greatly affect the metabolism of organic acids and amino acids. In nodules in particular, this may impose on their ability to fix N_2 . However, very little is known about this. Therefore the aim of this study was to determine whether the turnover rates of PEPC-derived C from organic into amino acids can affect N assimilation under P limitation in roots and nodules and how PEPC is regulated in these organs. Nodules appear to be more capable of resisting phosphate starvation, as nodule functioning was hardly impaired. Elevated levels of sucrose were observed, which was reported to be the major carbon source in nodules for downstream metabolic pathways and enzyme activity. Higher PEPC enzyme activity was found in LP conditions, which implied that the non-adenalated PEPC bypass route was favoured above the adenylated, P*i*-requiring route, most probably to conserve P. ^{13}C NMR studies showed that the majority of PEPC-derived carbon was incorporated into malate for both nodules and roots. The ^{13}C malate was subsequently converted to α-ketoglutarate and asparagine. Nodules appear to have utilised their large PEPC-derived malate pool for bacteroid fuel respiration, but also to synthesise asparagine from keto-glutarate, since there was a higher turnover of asparagine from keto-glutarate than from malate. Roots, in contrast appeared to synthesise the asparagine from malate via oxaloacetate (OAA). Despite lower PEPC protein in nodules than in roots, nodules showed greater enzyme activity, indicating that nodules may use different mechanisms, such as phosphorylation, to regulate PEPC, enabling nodules to reduce the malate inhibitory effect of PEPC. The subsequent metabolism of this PEPC-derived malate, caused roots and nodules to synthesise asparagine via different pathways. These findings indicated that roots and nodules under P stress vary in their metabolism of PEPC-derived carbon by synthesising their major export amino acid, asparagine, via different routes.

5.2 Introduction

Phosphoenolpyruvate carboxylase (PEPC) is widely distributed in all vascular plants, algae, cyanobacteria, and photosynthetic bacteria, and also in non-photosynthetic bacteria and protozoa. Plant type PEPC, which belongs to a multigene family, exists in most vascular plants as a homotetrameric "dimer of dimer" structure composed of four identical 100 – 110 kDa subunits which is referred to as Class-1 PEPC. Vascular plants also have another isozyme, namely a bacterial-type PEPC, which is a heteromeric complex and referred to as Class-2 PEPC (Sánchez and Cejudo 2003). Many phylogenetic studies have been performed on PEPC sequences and those analyses indicate that all PEPCs stems from a single origin before it diverging into plant-and bacterial-type PEPCs (Chollet *et al.* 1996, Westhoff and Gowik, 2004, Izui *et al.* 2004).

PEPC plays an important role in the primary metabolism of these organisms and two well-known isoforms in higher plants have been identified, i.e. the photosynthetic and non-photosynthetic isoforms. The photosynthetic form catalyses the initial fixation of atmospheric CO_2 into C4-dicarboxylic acids during C4 photosynthesis and CAM metabolism (Izui *et al.* 2004, Vidal and Chollet 1997). The non-photosynthetic isoform could be found in C3 leaves and various heterotrophic sink organs such as roots, developing seeds and legume roots and nodules (Vidal and Chollet 1997). PEPCs which are ubiquitous anaplerotic enzymes in higher plants, catalyse the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- to yield oxaloacetate (OAA) and P*i*, using Mg^{2+} as co-factor (Chollet *et al.* 1996). Oxaloacetate is then converted to malate via malate dehydrogenase and could be fed into the mitochondrial TCA cycle for further metabolism, or metabolised to pyruvate via malic enzyme (Plaxton 1996). PEPC has been implicated to replenish the citric acid cycle intermediates when carbon skeletons are removed for other metabolic functions like nitrogen assimilation and amino acid biosynthesis when plants experiences stress of various forms, such as P-stress (Chollet *et al.* 1996, Izui *et al.* 2004). The importance of PEPC during P-stress has been demonstrated in a variety of plant species, such as *Arabidopsis thaliana* (thale cress) (Morcuende *et al.* 2007), *Lupinus albus* (white lupin) (Johnson *et al.* 1996), *Nicotiana sylvestris* (tobacco) (Toyota *et al.* 2003) and *Lycopersicon esculentum* (tomato) (Pilbeam *et al.* 1993). It is suggested that PEPC provides an alternative metabolic bypass route to the P-dependant pyruvate kinase route to maintain a continued supply of pyruvate to the TCA cycle whilst recycling the PEPC by-product, P*i*, for recycling into P-deficient cells (Duff *et al.* 1989). In addition, PEPC also plays a major role in the maintenance and functioning of various

plants structures, such as roots and symbiotic nodules as demonstrated by $^{14}CO_2$ labelling studies of plant cytosolic PEPC in legume nodules (Christeller *et al.* 1977, Laing *et al.* 1979, Coker and Schubert, 1981, Vance *et al*. 1983, King *et al.* 1986, Anderson *et al*. 1987, Rosendahl *et al.* 1990).

In the symbiotic nodules of legumes, PEP is generated via the glycolytic pathway with sucrose as the major C-source. PEP-derived malate via PEPC is used as the major energy source in nodules for respiration and also plays a crucial role in N fixation as it provides the carbon skeletons that are needed for the subsequent assimilation of NH_4 into amino acids (Vance *et al.* 1994)

On the gene level, it was found that plant type PEPC was found to be encoded by a small gene family and expressed in various tissues of plants (Gehrig *et al.* 2001). All these plant type PEPC's contain the conserved regions of the N-terminal seryl phosphorylation domain and the C-terminal tetrapeptide "QNTG" (Izui *et al.* 2004, Xu *et al.* 2006). These highly conserved regions are believed to be involved in the domains of the active site structure and/or the regulation of the enzyme by phosphorylation (Lepiniec *et al.* 1993, Lepiniec *et al.* 1994, Relle and Wild 1994, Toh *et al.* 1994, Nakamura *et al.* 1995). Most PEPCs (including those in legumes) are allosteric enzymes which are post-translationally regulated by a variety of positive metabolic effectors (e.g. glucose 6-P, triose-P) and negative metabolic effectors (e.g. malate, aspartate) opposing each other in order for PEPC to be activated/deactivated. The strictly conserved serine- residue near the protein's N terminus, which can be reversibly phosphorylated, is largely controlled by a transcriptional and proteolytic up-/down-regulation of a dedicated vascular plant-unique serine/threonine kinase, PEPC-kinase (Izui *et al*. 2004, Agetsuma *et al*. 2005, Nimmo 2006). In addition, it was found that when PEPC is phosphorylated, it becomes less sensitive to feedback inhibition by its inhibitor, malate and more sensitive to its activator, glucose 6-phosphate, which results in a higher activity yield of this enzyme (Vidal and Chollet 1997).

This study aimed to determine whether the turnover rates of PEPC-derived C from organic acids into amino supported N assimilation under P limitation in roots and nodules, and how the responsible enzyme, PEPC, was regulated in these organs.

5.3 Materials and methods

5.3.1 *Growth and Harvest*

Seeds of *Virgilia divaricata* (Silverhill Seeds, Kenilworth, Cape Town, South Africa) were

incubated for 1 hour in water containing a smoke disc, after which they were placed for 5 h in a water bath (50 ºC), in order to enhance germination. Seeds were then washed with distilled water (10x) and allowed to germinate in sterile filter sand in seed trays. Germination and growth of these seedlings took place under natural light conditions in a north facing glass house where plants were exposed to sunlight for a 10 h period per day. The temperature of the glass house varied between 15 ºC at night and 25 ºC during the day. After 2 weeks, when sprouting of leaves was observed, the seedlings were transferred to pots which contained sterile filtered sand The nodule forming bacteria, *Burkholderia phytofirmans*, (which was grown on yeast mannitol agar) was used for nodulation and 500 µl of culture was used for inoculation, which was repeated twice after the first inoculation. Plants were divided into 2 groups i.e. low (5 µM P) –and high (500 µM P) phosphate according to the Long Ashton nutrient treatment. After 8 weeks plants were harvested and divided into leaves, stems roots and nodules which were respectively weighed for their fresh weights. Liquid nitrogen was added to nodules in microfuge tubes and caution was taken that the tubes should not pop by piercing a hole in the lid. Nodules were then stored at -80 °C for further analyses. The leaves, stems and roots were dried in an oven at 50 °C for a week and their dry weights were then determined.

5.3.2 *Electrophoresis and immunoblotting*

Duplicate 10% SDS-PAGE molecular mass determination was performed using a Mini-PROTEAN 3 gel electrophoresis apparatus (Bio-Rad) at 180 V for 60 min (gels done in triplicate). SDS-PAGE gels were stained overnight with Biorad Coomassie staining reagent and destained with Bio-Rad destaining solution. The duplicate gel was used for immunoblotting.

Immunoblotting of the duplicated SDS-PAGE gels was performed by electroblotting proteins from gels onto a polyvinylidene fluoride (PVDF) membrane for 45 min. Addition of the primary antibody and subsequent visualization of the immnoreactive polypeptide via an alkaline-phosphatase-conjugated secondary antibody with chromogenic detection was performed as set out in Gregory *et al.* (2009). Primary antibodies (as in Gregory *et al.* 2009) were obtained from the Plaxton laboratory, Department of Biochemistry, Queen's University, Canada.

5.3.3 *LC-MS*

5.3.3.1 *In-gel-digestion*

Immunoblotting-confirmed PEPC bands were excised from the SDS-PAGE gels and further

analyses were performed at the Centre for Proteomic and Genomic Research (CPGR) in Cape Town. All reagents used in the analyses were of analytical grade or equivalent. The supplied gel slices were destained in a microfuge tube (1.5 ml) tube with 100 mM ammonium bicarbonate (Ambic, Sigma 40867F), 50 % ($^{v}/_{v}$) acetonitrile (ACN, Anatech BJ015CS) until clear. Samples were dehydrated and desiccated before reduction with 2 mM triscarboxyethyl phosphine (TCEP; Fluka 646547) in 25 mM Ambic for 15 min at room temperature with agitation. Excess TCEP was removed and the gel pieces again dehydrated. Cysteine residues were carbamidomethylated with 20 mM iodoacetamide (Sigma I6125) in 25 mM Ambic for 30 min at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25 mM Ambic followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in 20 ng/uL trypsin (Promega PRV5111) made up in 40 mM Ambic, 0.1 % octyl-β-D-glucopyranoside (Sigma O8001) and incubating at 37 °C overnight. Peptides were extracted from the gel pieces once with 50 µl 0.1 % trifluoroacetic acid (TFA, Sigma T6508). The samples were dried down and 200µl water added and concentrated to less than 20 µl to remove residual Ambic. The samples were dried and re-dissolved in 0.05 % TFA, 5 % Ceric ammonium nitrate (CAN) for LC-MS analysis.

5.3.3.2 *LC-MS-run*

LC−MS/MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. The extracted peptides were dissolved in sample loading buffer (97.5 % ($^{v}/_{v}$) water, 2.5 % ($^{v}/_{v}$) ACN, 0.1 % FA) and loaded on a C18 trap column (300 µm × 5mm × 5 µm) (Dionex/Thermo). Chromatographic separation was performed with a C18 column (75 µm × 20 mm × 3.5 µm) (Dionex/Thermo). The solvent system employed was solvent A, Water; 0.1 % FA and solvent B, Acetonitrile; 0.1 % FA. The linear gradient for peptide separation was generated at 400 nL/min as follows: Time change: 50 min, gradient change: 5-30 % Solvent B. The mass spectrometer was operated in positive ion mode with a capillary temperature of 250 °C. The applied electrospray voltage was 1.95 kV. Data was acquired running the instrument in full-scan mode with a resolution of 70,000 at m/z 200. The AGC target value was set at 3e6 with a scan range of 300-1750 m/z. The maximal injection time (ms) was set to 250.

The inclusion was switch “off” for data-dependent MS/MS and the resolution set to 17,500 (at m/z 200, with an AGC target value of 2e5 and a maximal injection time (ms) of 80. The loop count

was set at 8 with an isolation window width (Da) of 4, with a NCE (%) of 26.
The Data-dependent Settings were set to an underfill ratio (%) of 1 and the charge exclusion had charge states of 1,6-8 > 8. The instrument was set where preference was given to peptide match and the exclusion isotopes were set to "on" with a dynamic exclusion (s) of 30.

5.3.4 *Compilation of Phylogenetic tree*

PEPC peptide- sequences obtained from LC-MS data, corresponding to multiple peptide-sequence alignments were used to compile the phylogenetic tree for *V. divaricata*. LC-MS spectra for the protein identification are matched against a combination of Swissprot as well as all unreviewed PEPC sequence databases. These PEPC sequences matched those of *Sesbania rostrate, Arachis hypogaea, Medicago truncatula, Phaseolus vulgaris,* and *Medicago sativa.* A compilation of a phylogenetic tree from these peptide sequences was performed using ModelGenerator-software (Keane *et al*. 2006).

5.3.5 *^{13}C NMR*

Sample preparation was done as described in Gout *et al.* (2000). Briefly, 9 g of roots and 1 g nodules were frozen in liquid N and ground to a powder in 1 ml of 70 % ($^{v}/_{v}$) perchloric acid. The frozen powder was allowed to thaw at -10 ºC. The thick slurry was centrifuged at 15000 *g* for 10 min and the supernatant neutralized with 2 M $KHCO_3$ to pH 5. The supernatant was then centrifuged at 10000 *g* for 10min to remove $KClO_4$ and then lyophylised and stored in liquid N. The lyophylised sample was redissolved in 2.5 ml water which contained 10 % ($^{v}/_{v}$) $^{2}H_2O$. The solution was neutralized to pH 7.5, buffered with HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and CDTA (1,2 cyclohexane-diaminetetra-acetic acid) (50 – 100 µM) was added to chelate divalent cations. NMR was performed at 25 ºC in D_2O on a Varian 600mHz instrument with an Inova spectrometer. The conditions were as follow: Pulse sequence: s2pul, Number of scans: 1303, Receiver gain: 60, Relaxation delay: 1.0000, Pulse width: 0.0000, Acquisition time: 0.8688, Spectrometer frequency: 150.88.

5.3.6 *GC-MS*

5.3.6.1 *Sample preparation:*

In brief, 1.4 ml methanol was added to 100 mg of roots and nodules. In addition, Ribitol (60 µl,

0.2 mg/ml) which serves as an internal standard was added. Sugar- and amino acid standards were also prepared at 1 mg/ml. The mixtures were incubated on a shaking incubator at 70 °C for 15 min. Samples were then centrifuged for 15 min at 13000 rpm. After centrifugation, 500 µl was transferred to 1.5 ml microfuge tubes. Chloroform (250 µl) and 500 µl dH_2O was then added and then vortexed for 15 s. Samples were then centrifuged for 15 min at 4000 rpm to separate the two phases, whereafter 150 µl of the top phase was removed. The remainder of the samples was aliquotted and stored at -80 °C as a back-up.

Methoxyamine hydrochloride, 40 µl, (2 %, $^{v}/_{v}$) in pyridine was added to samples and then incubated at 37 ºC for 2 h. The tubes were then centrifuged for 1min to remove droplets from the tube cap, after which 70 µl of the derivitizing agent MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was added. The samples were then incubated for 30 min at 37 ºC. Droplets were again removed by centrifugation for 1 min and samples were then transferred to sample vials.

5.3.6.2 *GC-MS-run*

Derivitized samples (1 µl) were chromatographically separated by injecting the samples into the GC-MS (Agilent 6890 N Agilent, Palo Alto, CA, coupled to a Agilent 5975 MS mass spectrometer detector) equipped with a polar (95 % dimethylpolysiloxane) capillary column (Model Number: ZB 7HG-G027-11ZB-Semivolatiles Guardian) with the following dimensions: 30 m length, 0.255 mm ID, and 0.25 µm film thickness. The GC injector port was set at 280 ºC, operated in a split mode which was set at a ratio of 10:1. Helium, at a flow rate of 1 $ml.min^{-1}$ was used as carrier gas. The oven was set to an initial temperature of 70 ºC and was maintained there for 2 min, and there after ramped at 1 °C/min to 76 °C, then at 8 °C/min to a final temperature of 300 °C and then held for 5 min. The MSD was set to full scan mode (40 – 650 m/z) to record the mass spectral data. The ion source and the quadrupole temperatures were maintained at 240 °C and 150 °C respectively. Identification of metabolites was obtained by comparing their trimethylsilyl (TMS) derivatives mass spectra with those found in the NIST05 (National Institute of Standards and Technology, Gaithersburg) library, and/or the Wiley (275) libraries. Quantitative analysis of the metabolites was achieved by calculating the ratio (unitless) of the compounds of interest to that of the internal standard (ribitol). This was obtained by dividing the areas of the respective metabolites with that of ribitol.

5.3.7 *Protein extraction and determination*

Proteins from roots and nodules were extracted according to the methods used by Ocaña *et al.* (1996) and was modified to an extent 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 Methylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol(DTT), 20 % (v/v) ethylene glycol, 2% (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Randburg, South Africa) was used per 50 ml of buffer. The protein concentration was determined by the NanoDrop Lite Spectrophotometer (Thermo Scientific) - where the extraction buffer was used as standard.

5.3.8 *Enzyme assays*

All enzymatic reactions were performed in triplicate in a multi-well plate reader at a wavelength of 340 nm. The reactions started by adding 30 µl of the crude extraction mixture in a total volume of 250 µl. The various initial reaction rates have been shown to be proportional to the concentration of the extracted enzymes used under the conditions used (Ocaña *et al*. 1996).

5.3.8.1 *Phosphoenolpyruvate carboxylase*

PEPC activity was determined by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 25 °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*. 1996). The 9 blanks that were used in the assay consisted of a reaction medium without PEP.

5.3.8.2 *NADH-Malate dehydrogenase*

MDH activity was assayed as described by Appels & Haaker (1988). The reaction mixture contained 25 mM KH_2PO_4, 0.2 mM NADH, 0.4 mM oxalo-acetic acid (OAA). The pH was adjusted to 7.5 with 1 mM HCl (Appels & Haaker, 1988). The 3 blanks consisted of the reaction medium without OAA.

5.3.9 *Inorganic Pi determination*

A modified method of Fiske-Subbarow (1925) was used to determine P*i* concentration (Rychter and Mikulska 1990). Briefly, 0.1 g of roots/nodules was ground in 100 µl (10 %, $^{v}/_{v}$ TCA) Samples were then diluted 3x with 5 % ($^{v}/_{v}$) TCA and then centrifuged for 10 min at 4 ºC at 2500

g. The supernatant was then removed and centrifuged for a further 10 min at 4 ºC at 13000 *g*. The supernatant was then removed and 700 µl reaction mix (10 % $C_6H_8O_6$ + 0.42 % $[NH_4]_6Mo_7O_{24}.4H_2O$ in a 1:6 [H_2SO_4, 1N] ratio was added to 300 µl supernatant. Absorbance of the samples was determined on a multiwell plate reader at 820 nm.

5.3.10 *Statistical analysis*

The effects of the factors and their interactions between nodules and roots under LP conditions were tested using the *t*-test. Different letters indicate significant differences between treatments ($P \leq 0.05$), as revealed by the *t*-test.

5.4 Results

5.4.1 *Immunoblotting*

SDS-PAGE gels which were done in triplicate followed by western blotting consistently revealed a PEPC protein at ~101kDa in all 3 western blots. These gels consistently revealed a PEPC band which cross reacted with affinity-purified anti-(*B. napus* 1P*i* PEPC) (Fig. 5.1)

5.4.2 *Phylogenetics*

A phylogenetic tree compilation that was obtained from LC-MS peptide sequence data of partially purified PEPC from *V. divaricata* indicated that the PEPC of *V. divaricata* revealed a similarity which is closely related to that of *Lupinus luteus, Sesbania rostrata, Arachis hypogaea, Boerhavia coccinea, Leuenbergeria aureiflora, Salsola genistoides, Eleusine floccifolia, Eleusine multiflora,* and *Portulacaria afra* (Fig 5.2). The PEPC peptide sequences of *Arachis hypogaea* and *Sesbania rostrata* appears to have the closest relation to the PEPC peptide sequence of *V. divaricata* after the compilation of a phylogenetic tree (Fig. 5.2; Fig 5.3).

5.4.3 *LC-MS*

Various PEPC peptide sequences were obtained from the LC-MS sequence analyser, which could indicate that more than one PEPC isoform might be present. The highest score of the most unique peptide sequence matched the PEPC peptide sequence of *A. hypogaea* and *S. rostrata.*

In addition, the data also revealed that plants grown under LP conditions (5 µM P) had a PEPC up-regulation with regard to roots. In nodules the opposite was found, i.e. a down-regulation of PEPC peptides for plants grown under low phosphate conditions (5 µM P) (Fig 5.4).

5.4.4 *GC-MS*

5.4.4.1 *Amino- and organic acids*

The concentrations of aspartate and glutamate were much higher in the LP treatment of nodules compared to the LP treatment of roots. Asparagine was found to be only slightly higher in nodules compared to roots (Table 5.1).

Higher concentrations of malate- and citrate were found in the LP treatment of nodules compared to roots and higher oxaloacetate concentrations were found in LP roots compared to LP nodules (Table 5.2). The higher malate concentration in LP roots and nodules in conjunction with ^{13}C concentrations shifted the focus of the experiment to compare roots and nodules.

5.4.4.2 *Sugars*

The sucrose concentration in LP nodules was much higher compared to LP roots, probably implicating its role as major C-fuel source for nodules. In addition, the hexoses, glucose and fructose were also much higher in nodules than in roots which served as an indication that nodules have a higher preference for the hexoses (Table 5.3).

5.4.5 *Protein content*

There was an increase in protein concentration in the LP treatment of roots and nodules compared to roots of the HP treatment. The protein concentration of the LP nodules was almost double compared to the LP treatment in roots (Table 5.4). The higher protein content of nodules which appeared as much thicker and more concentrated bands on SDS gels, may also have contributed in identifying the PEPC protein on the immunoblot (Fig 5.1)

5.4.6 *Inorganic Pi*

The P*i* concentration in P-stressed plant organs followed an expected decline compared to HP organs. Higher intercellular P*i* values were recorded for low phosphate nodules compared to low phosphate roots. There was also a greater decline in P*i* in LP roots compared to LP nodules (Table 5.4).

5.4.7 *Enzyme assays*

The highest PEPC activity, was recorded in the LP of nodules. MDH activity followed a similar trend as PEPC activity (Table 5.5).

5.4.8 *^{13}C NMR*

Chemical shifts were identified by running reference solutions of these organic acids, (solubilised in D_2O at pH 7.5) under the same running conditions as the samples. The most significant peaks between 175 – 181 ppm were identified as those of the organic acids, malate and citrate (Fig. 5.5). The presence of a keto-group (at 200 – 220ppm) could also be observed in the LP root and nodule spectra, which can be assigned to that of α-ketoglutarate (Fig. 5.5). Asparagine could also be identified at ~125ppm and could only be detected in the roots and nodules under LP conditions (Fig. 5.5). The asparagine signal could not be observed in the 2 h ^{13}C spectra of nodules, which could possibly due to the high demand for it in nodules and therefore not detected. Higher incorporation of ^{13}C to malate was found in nodules than roots, however, in roots higher ^{13}C incorporation to α-ketoglutarate and asparagine was found (Table 5.6). Calculating the various conversions from this data revealed that nodules had a higher conversion rate from malate to α-ketoglutarate and asparagine, and a lower conversion rate from α-ketoglutarate to asparagine, compared to roots (Table 5.7). Nodules showed a higher turnover rate between α-ketoglutarate and asparagine, but were lower between malate to α-ketoglutarate and malate to asparagine compared to roots (Table 5.8). Stronger signals in the ^{13}C spectra and larger peak areas did not necessary reflect a higher concentration of sample. Samples concentrations were corrected by dividing peak areas into the ^{13}C bicarbonate peak area at 161ppm. These relative concentrations were used to calculate the ^{13}C flow via PEPC into various compounds. ^{13}C compounds were expressed per gram fresh weight and then divided by 60 or 120 min to calculate the metabolic flux.

5.5 Discussion

In spite of the decline in P*i* levels, nodules of *V. divaricata* were able to offset an excessive drop in BNF decline. This ability of nodules to maintain function under low P conditions involved different mechanisms compared to roots. This was achieved via increased regulation of nodule PEPC and its downstream products. This implies that compared to roots under low P, nodules alter

the metabolism of PEPC-derived C, in order to maintain nodule respiration and amino acid synthesis.

One of the typical signs of a P-stressed plant could be observed in the decline of internal P*i* in both LP roots and nodules. The decline in internal P*i* was more severe in LP roots than nodules, possibly indicating that nodules were more capable in resisting P-starvation (Jebara *et al*. 2005, Kouas *et al*. 2005, Sulieman and Tran 2015). This may be attributed by the fact that nodules scavenge P*i* especially from roots and functioning is hardly impaired as found by various studies (Jacobsen 1985, Israel 1993, Almeida *et al.* 2000, le Roux *et al.* 2006, Sulieman *et al.* 2010). Although P*i* levels were reduced in both organs during P stress, this decline is known to occur with an accumulation of sugars.

Furthermore, GC-MS results of LP roots and nodules revealed that sucrose was the dominant sugar which might indicate that it is the preferred respiratory sugar in nodules to be used as C-source. This is supported by the idea that photosynthates, mainly sucrose, are translocated to the nodules via the shoots and provide the nodules with nutrients for the bacteroids, and C- skeletons for the assimilation of fixed $NH4^+$ (Morell and Copeland 1984). It was reported that nodules have a preference for sucrose (above other root reserves) to be used in the anaplerotic CO_2 fixation by PEPC for downstream nitrogen fixation (Reibach and Streeter 1983, Day and Copeland 1991, Vance and Heichel 1991). Therefore under low P conditions, this sucrose requirement may be enhanced. Sucrose can be converted to fructose and glucose via sucrose invertase, and/or be converted to UDP-glucose and UDP-fructose via sucrose synthase (Akazawa and Okamoto 1980, Morell and Copeland, 1985). These generated hexoses can be fed into the glycolytic pathway to yield phosphoenol pyruvate or it can be used for diverse downstream pathways, which are critical for cell functioning Chourey and Nelson 1976, Delmer and Amor 1995, Ruan *et al*. 1997) These two hexoses (glucose and fructose) which were found in reduced levels compared to sucrose, might have been a result of the metabolised sucrose via the nodule enhanced enzyme, sucrose synthase. A much higher glucose concentration, compared to fructose concentration was found and might suggest a higher demand for this hexose in PEP generation, or it could also indicate that fructose becomes metabolised more readily. Glucose- and fructose concentrations were also found to be higher in the LP treatment of nodules compared to roots indicating, that there might be a higher demand for these hexoses during P-stress in nodules to maintain downstream metabolic pathways. In addition, the higher glucose and fructose concentration in nodules might serve as continued activation of PEPC via fructose-6-phosphate and glucose-6-phosphate. It was found that

fructose-6-phosphate and glucose-6-phospate are able to modulate PEPC activity in nodules (soybean and lupin) by a feed-forward regulation for the generation of organic acids by this enzyme. However, the main organic acid product of PEPC, malate, might also inhibit its action when the malate concentrations become too high (Christeller *et al*. 1977, Marczewski 1989, Schuller *et al*. 1990).

In spite of the high malate concentrations under low P, the PEPC enzyme activity results concurred with previous findings where it was shown that during LP conditions, the non-adenalated PEPC bypass route is favoured above the adenylated, P*i* requiring route, most probably to conserve P (Rychter *et al.* 1992, Theodorou & Plaxton 1993). OAA, which is the first downstream product of PEPC, can subsequently be converted to malate by malate dehydrogenase. The generated malate can be exported or fed into the TCA cycle via malic enzyme and has been widely accepted as the primary C-source for bacteroids, especially in the assimilation of fixed N (Rosendahl *et al.* 1990) and bacteroid respiration (Lodwig and Poole 2003). Higher PEPC activity was found in LP nodules compared to roots, which might indicate that nodules need to produce higher downstream PEPC-derived metabolic products. However, there appears to be strict control in this bypass pathway regarding the activation and inhibition of PEPC.

^{13}C NMR studies showed that the majority of PEPC-derived carbon was incorporated into malate for both nodules and roots, which is in agreement with previous studies where elevated levels of malate, especially in nodules may be a direct result of the action of the phosphorylated PEPC which is less sensitive to malate inhibition. In this way nodules ensure a continued metabolic flow via PEPC under low P conditions (Streeter 1987, Rosendahl *et al*. 1990, Le Roux *et al*. 2006). ^{13}C NMR studies also showed that there was a higher incorporation rate of PEPC derived carbon into α-ketoglutarate under LP conditions, which plays an important role providing carbon skeletons for ammonia assimilation through the Glutamate synthase/Glutamine oxoglutarate aminotransferase (GS/GOGAT) pathway (Scheible *et al.* 2000) or via Glutamate dehydrogenase (GDH) (Lehmann *et al*. 2011). PEPC apparently only plays a smaller role in ureide transporting legumes such as soybean, compared to amino acid exporting legumes (Coker and Schubert 1981, King *et al*. 1986). Since it was found that much higher productions of C-skeletons are needed to be incorporated into fixed nitrogen production in amide transporting legumes such as alfalfa and lupin, these C-skeletons can subsequently be converted into asparagine and glutamine (Christeller *et al*. 1977, Laing *et al*. 1979, Vance *et al.* 1983, Anderson *et al.* 1987). This study also reflects similar results, as the nodules had a tendency to accumulate higher amounts of aspartate and glutamate under LP

conditions indicating a higher demand for these amino acids during LP conditions compared. These two amino acids could not be detected during HP conditions. In addition, ^{13}C studies revealed that there was a higher incorporation rate of the PEPC-generated α-ketoglutarate into asparagine. Therefore it appears that there is a greater reliance of nodules and roots for PEPC-derived C to be generated under LP conditions in order to sustain downstream amino acid synthesis. Furthermore, the asparagine declined significantly after 2 hours, perhaps indicating that the PEPC-derived asparagine in nodules was translocated to other organs at a faster rate after 1h, compared to roots. This could be explained by former findings that dicarboxylic acids are the main carbon skeletons for transamination by aspartate aminotransferase to produce aspartate (Maxwell *et al.* 1984, King *et al.* 1986, Rosendahl *et al.* 1990). Nodules appear to follow a different strategy than roots regarding the utilisation of PEPC-derived organic acids. In nodules, the large PEPC-derived malate pool may have been used to fuel bacteroid respiration, but also to synthesise asparagine from keto-glutarate. This was evidenced by the higher turnover of asparagine from keto-glutarate than from malate. In this case keto-glutarate can be used for asparagine synthesis by being converted to glutamate via GDH (Lehmann *et al*. 2011) or GOGAT (Scheible *et al*. 2000). Roots, in contrast, appeared to synthesise the asparagine from malate via oxaloacetate (OAA) (Yoshioka *et al*. 1999). In the case of roots, the OAA is used to first synthesise aspartate, and then the aspartate pathway leads to asparagine synthesis. For both roots and nodules, the asparagine is the major amide which is loaded into the xylem stream from nodules to shoots as the translocation form of fixed nitrogen in amide-transporting legumes such as *Lotus japonicus* (Tajima *et al*. 2004), where the majority of the aspartate is converted to asparagine by asparagine synthetase (AS) (Ta and Joy 1986). Given the importance of PEPC–derived C in amino acid synthesis, it may be prudent to explore the PEPC isoforms and their regulation in roots and nodules.

We confirmed the presence of PEPC in our extracted protein samples by immuno-blotting as well as PEPC activity studies. The identified PEPC band on the western blot was that of the phosphorylated form of the enzyme, as the primary antibody was a polyclonal antibody raised against a synthetic phosphopeptide matching the conserved N-terminal Ser-11 phosphorylation domain of PEPC (Gregory *et al.* 2009). It was shown that when PEPC is phosphorylated, it becomes less sensitive to feedback inhibition by malate and more sensitive to activation by its activator, glucose 6-phosphate (Vidal and Chollet 1997). A constant supply of sucrose would therefore be required in order to generate glucose-6-phosphate via sucrose invertase and sucrose synthase. This concurs with the higher PEPC activities in nodules, where sucrose levels were also

elevated. The variety of PEPC sequences revealed by the LC-MS sequence data might serve as an indication of more than one isoforms of this enzyme co-existing in roots and nodules. Other studies observed 2 isoforms of PEPC in *Phaseolus vulgaris* and *Lupinus luteus* nodules (Deroche *et al.* 1983, Marczewsky 1989). It appears that PEPC activity and subsequent downstream metabolic production in the various organs are not restricted to one specific isoform, but that multipleisoforms might act in synergy, contributing to the maintenance of the organ and the plant. Previous work also indicated that the activity of the various isoforms of plant PEPC are subject to allosteric control by a variety of activators (e.g. glucose 6-P, fructose 6-P), and inhibitors (e.g. L-malate, aspartate). However, these studies also indicated that if malate, which is a potent inhibitor of PEPC, exceeds 0.1 mM malate then it would inhibit the phosphorylated enzyme in vivo (Streeter 1987, Schuller *et al.* 1990, Vance and Heichel 1991). In addition, studies on the nodules of *Vicia faba* revealed that when glucose-6-phosphate were lower or equal to that of the malate concentration reversed the inhibitory effect on PEPC, caused by malate. This effect was found to be more effective at pH 7 and where the glucoe-6-phosphate and malate concentrations were less than 1mM (Ocaña *et al.* 1996). Surprisingly, despite elevated protein levels in low phosphate nodules, LCMS data revealed that PEPC protein levels are downregulated under LP conditions. The key to these observations might lie in the phosphorylation status of PEPC, since the antibody used, was raised against a phosphorylated PEPC peptide PEPC. The post translational modification of PEPC by phosphorylation was shown to play a pivotal role in the regulation of this enzyme (Chollet *et al*. 1996, Nimmo 2000, Vidal and Chollet 1997). This activation/inhibition of PEPC, which is probably the major posttranslational mechanism, occurs via changes in the phosphorylation/dephosphorylation state of a serine residue near the N-terminus which is accomplished by a highly regulated protein-Ser/Thr kinase (Jiao and Chollet 1990, Jiao *et al.* 1991, Baur *et al.* 1992, Terada *et al.* 1990, Wang *et al* 1992, Duff *et al.* 1995, Zhang and Chollet 1995) It therefore appears that the PEPC expression levels do not necessarily accompany the protein activity level, since higher expression levels were observed in high phosphate nodules, despite lower activity when compared to low phosphate nodules. It appears that the phosphorylation status of PEPC is only one of the mechanisms used by the plant to prevent inhibition. Previous work also indicated that the activity of the various isoforms of plant PEPC are subject to allosteric control by a variety of activators (e.g. glucose 6-P, fructose 6-P), and inhibitors (e.g. L-malate, Aspartate). There appears to be one or more protection/activation of PEPC-mechanisms that nodules employ to accomplish this. It therefore appears that protection of

PEPC by protein phosphorylation and metabolite activation/inhibition would act in synergy to modulate PEPC activity.

5.6 Conclusion

The synthesis of large ^{13}C –malate pools of roots and nodules to PEPC-derived C, under low P conditions, was underpinned by their different regulation mechanisms of enzyme activity, of the same protein isoform. Since malate is a potent inhibitor of PEPC activity, roots appear to have invested in more PEPC protein compared to nodules. In contrast, nodules with lower PEPC protein, achieved greater enzyme activity than roots, possibly due to higher phosphorylation in order to reduce the malate effect. The subsequent metabolism of this PEPC-derived malate, caused roots and nodules to synthesise asparagine via different pathways. These findings imply that roots and nodules under P stress synthesise their major export amino acid, asparagine, via different routes. This may be a consequence of having evolved in a nutrient-poor ecosystem, so that nodule-bacteroid respiration and N metabolism can be maintained in P-poor soils such as the Fynbos..

5.7 References

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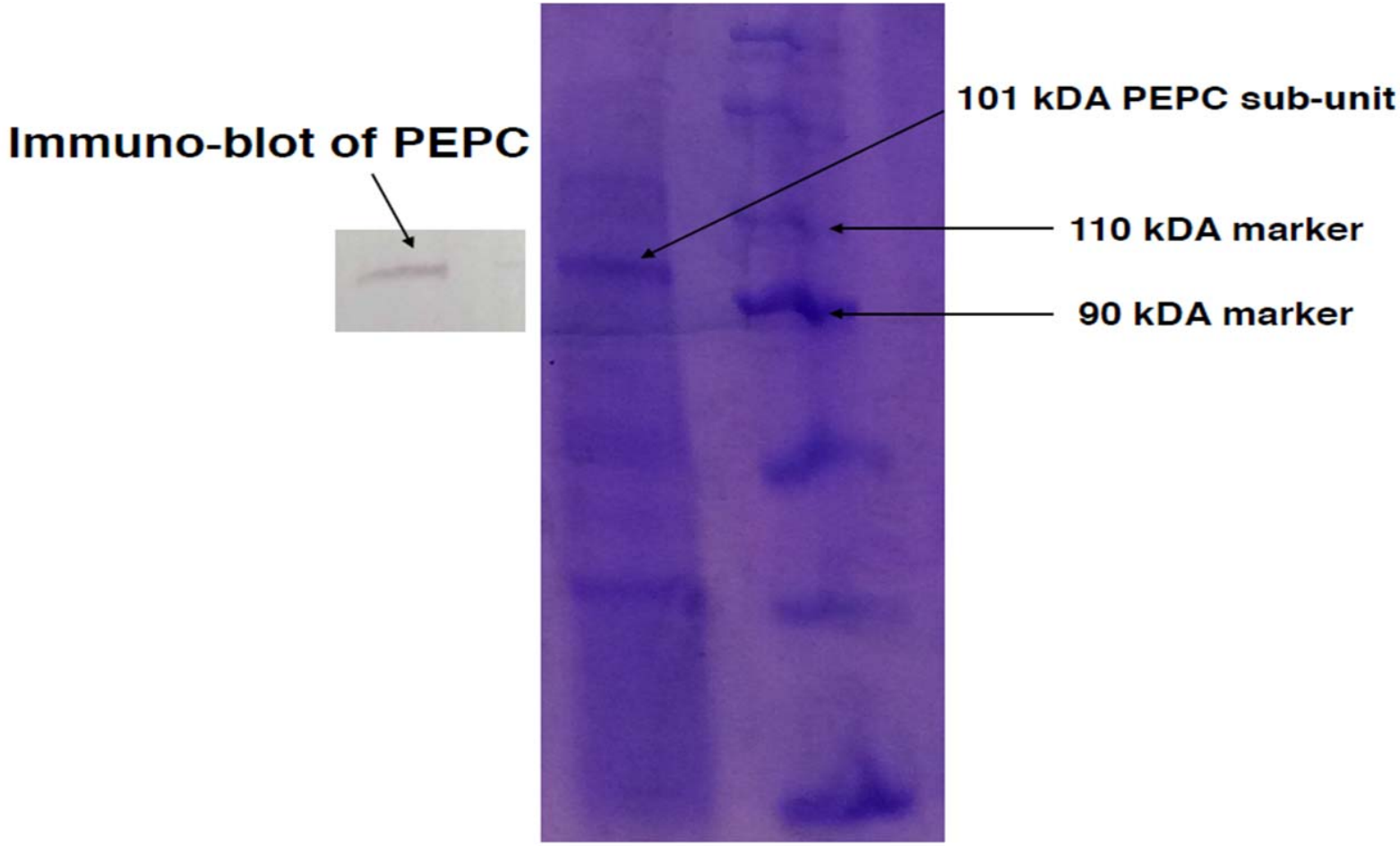

Figure 5.1. Representative Immuno-blot of PEPC (nodules of *V. divarivata*) from the roots and nodules of *V. divarivata* grown under high phosphate (500µM P) and low phosphate (5µM P) conditions.

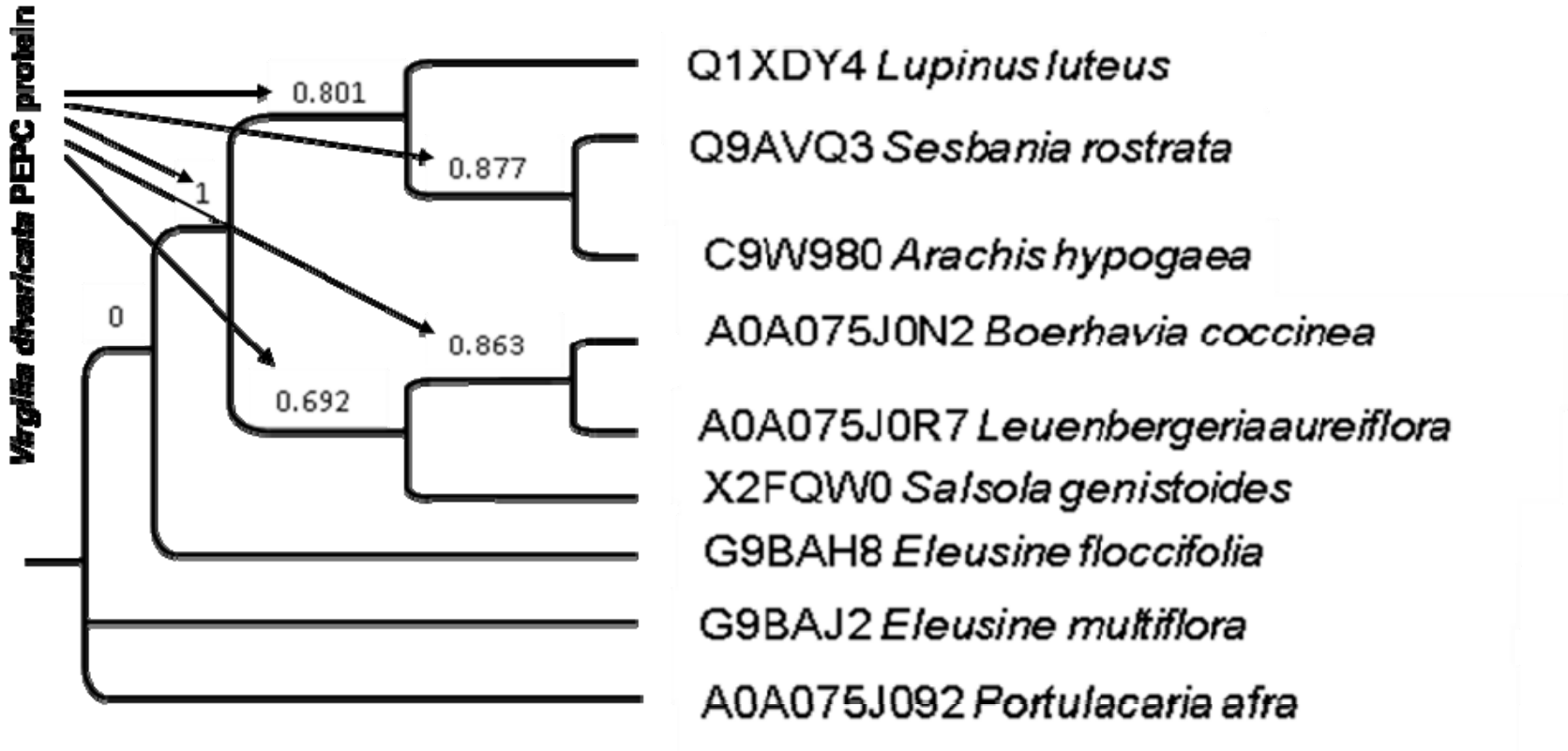

Figure 5.2. Proposed phylogenetic tree of *V. divaricata* PEPC grown under high phosphate (500µM P) and low phosphate (5µM P) conditions of roots and nodules from LC-MS peptide sequences data. The generated PEPC peptide sequence of *V. divaricata* appears to be closely related to that of *Sesbania rostrata* and *Arachis hypogaea*.

(a) *Arachis hypogea*

(b) *Virgilia divaricata*

(c) *Sesbania rostrata*

Figure 5.3. Comparison of flower and leaf structure of (a) *Arachis hypogea*, (b) *Virgilia divaricata*, (c) *Sesbania rostrata*

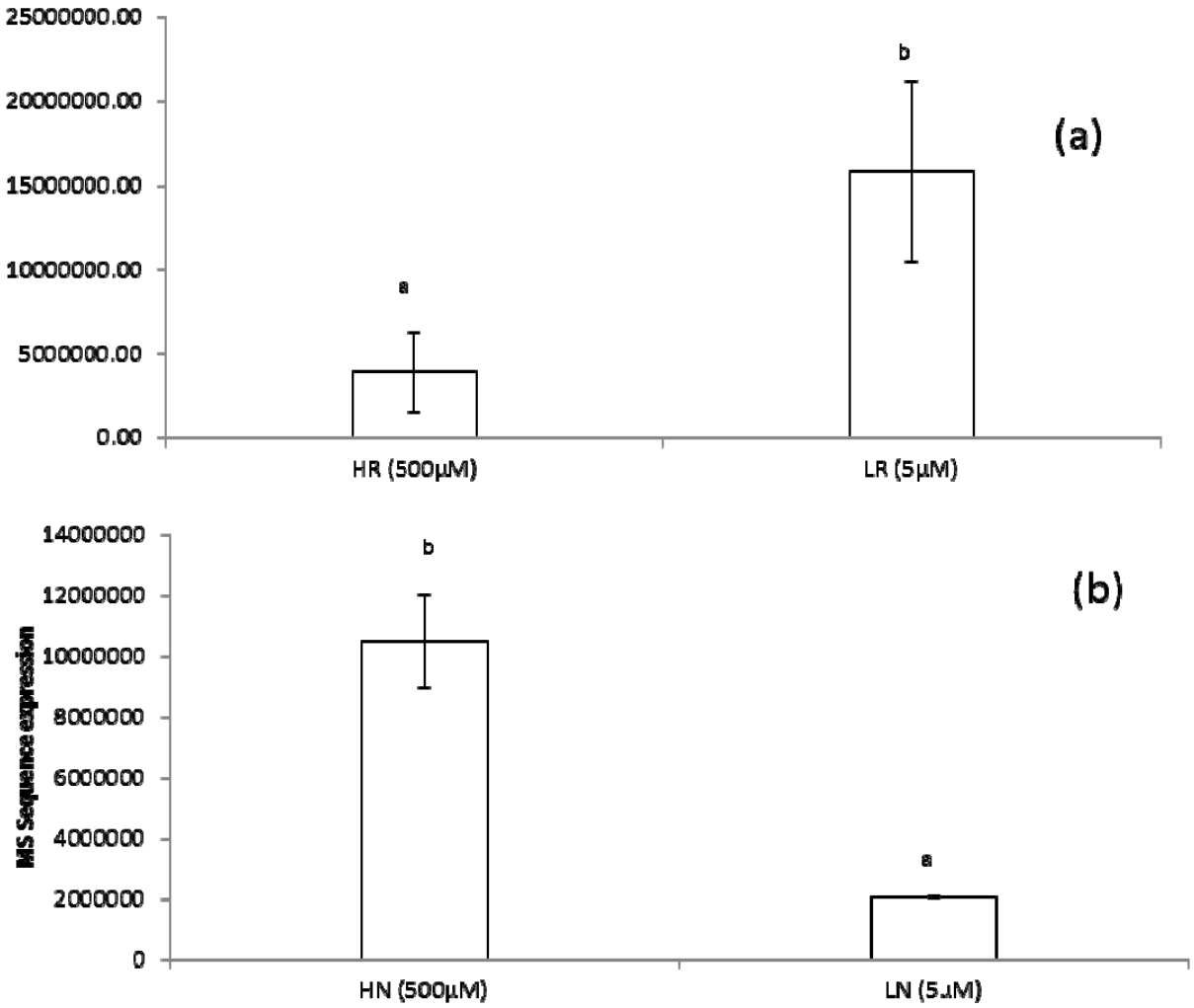

Figure 5.4. PEPC peptide expression as detected by LC-MS (a) in roots (b) in nodules (b) of *V. divaricata* grown under high phosphate (500 uM P) and low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Table 5.1. Amino acids detected by GC-MS analysis in roots and nodules of *V. divaricata*, grown under low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Various amino acid concentrations(mg/ml) detected in elevated amounts			
Treatment 5 µM P	Glutamate	Aspartate	Asparagine
Roots	0.081 ± 0.017 a	0.106 ± 0.016 a'	0.171 ± 0.045 a''
Nodules	0.367 ± 0.275 b	0.236 ± 0.157 b'	0.211 ± 0.208 b''

Table 5.2. Various organic acid concentrations (mg/ml) found by GC-MS analysis in roots and nodules of *V. divaricata*, grown under low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Various organic acid concentrations(mg/ml) detected in elevated amounts						
Treatment						
5 µM P	Malate		Citrate		Oxaloacetate	
Roots	3.982	± 0.475 a	3.246	± 0.077 a'	0.098	± 0.090 b''
Nodules	5.343	± 0.472 b	8.097	± 0.023 b'	0.018	± 0.010 a''

Table 5.3. Free sugars found by GC-MS analysis in roots and nodules of *V. divaricata*, grown under low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$), where the letters with primes are within the same treatment comparison.

Various sugar concentrations(mg/ml) detected in elevated amounts			
Treatments 5 µM P	Fructose	Glucose	Sucrose
Roots	1.936 ± 0.069 a	2.467 ± 0.102 a'	52.291 ± 2.357 a''
Nodules	8.452 ± 0.844 b	25.495 ± 1.917 b'	89.638 ± 4.952 b''

Table 5.4. Root and Nodule inorganic phosphate (P*i*) and protein concentration of *V. divaricata*, grown under high phosphate (500 uM P) and low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$), where the letters with primes are within the same treatment comparison.

Protein and Inorganic P*i* concentrations in roots and nodules		
Treatments	Protein concentration (mg/ml)	Inorganic P*i* concentration (µM P*i*/g)
500 µM P		
Roots	6.415 ± 0.086 a	62 ± 0.439 a''
Nodules	15.065 ± 0.277 b	73.8 ± 2.250 b''
5 µM P		
Roots	9.808 ± 0.028 a'	43.3 ± 0.514 a'''
Nodules	16.985 ± 0.234 b'	58.7 ± 2.10 b'''

Table 5.5. Root and nodule enzyme activities of *V. divaricata*, grown under low phosphate (5 uM P) conditions. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$), where the letters with primes are within the same treatment comparison.

PEPC and MDH activity(µmol.min^{-1} .g $^{-1}$ fw)		
5µM P Treatment	PEPC	MDH
Roots	53.234 ± 0.944 a	50.866 ± 3.486 a’
Nodules	56.089 ± 0.497 b	56.075 ± 1.519 b’

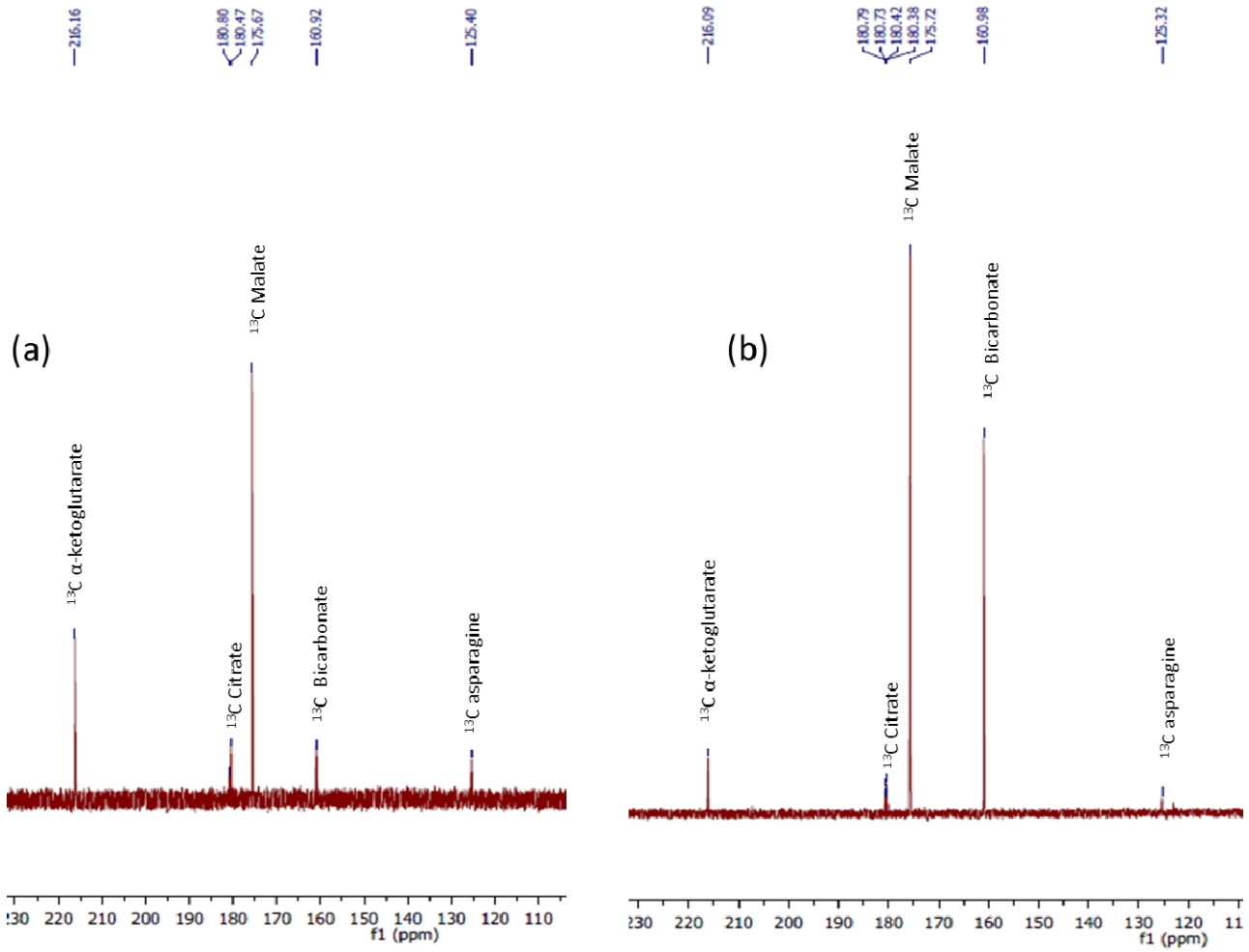

Figure 5.5. ^{13}C NMR spectra of (a) nodules and (b) roots of *V. divaricata* grown under low phosphate conditions (5 µM P).

Table 5.6. ^{13}C incorporation into various metabolites in roots and nodules of *V. divaricata* grown under low phosphate (5 µM P) conditions. Values of 3 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$), where the letters with primes are within the same treatment comparison.

	^{13}C Incorporation into various metabolites (mg/ml)											
Treatment	Malate (1 h)		Malate (2 h)		α-ketoglutarate (1h)		α-ketoglutarate (2 h)		Asparagine (1 h)		Asparagine (2 h)	
5 µM P												
Roots	0.3341	± 0.1165 a	0.1581	± 0.0224 a'	0.042	± 0.0011 b''	0.1126	± 0.0426 b'''	0.0929	± 0.0119 b''''	0.0196	± 0.0060
Nodules	1.1594	± 0.6191 b	0.1754	± 0.0923 b'	0.016	± 0.0012 a''	0.0041	± 0.0042 a'''	0.0191	± 0.0006 a''''		

Table 5.7. ^{13}C metabolic ratios to represent the proposed conversion of metabolites from PEPC-derived incorporated $H^{13}CO^{-}_{3}$ under low P (5 µM P) conditions in roots and nodules of *V. divaricata*. Values of 4 replicates are presented as means ± SE. Different letters indicate significant differences between treatments ($P \leq 0.05$), where the letters with primes are within the same treatment comparison

Ratios of ^{13}C -metabolites in roots and nodules (mg/ml)						
Treatment	Malate :Asparagine		Malate : α- ketoglutarate		α-ketoglutarate : Asparagine	
5 µM P						
Roots	0.334	± 0.1165 b	0.158	± 0.022 a'	0.042	± 0.0011 b''
Nodules	1.159	± 0.6191 a	0.175	± 0.092 b'	0.016	± 0.0012 a''

Table 5.8. ^{13}C metabolic turnover rate to represent the proposed conversion of metabolites from PEPC-derived incorporated $H^{13}CO^{-}_{3}$ into organic- and amino acids under low P (5 µM P) conditions in roots and nodules of *V. divaricata*. Regressions of 4 replicates are being represented for each organ at the low P treatment.

Turnover rate of ^{13}C-labelled metabolites (nmol ^{13}C/g fresh weight/min)			
Treatment	**Asparagine from α-Ketoglutarate**	**Asparagine from malate**	**α-Ketoglutarate from malate**
5µM P			
Roots	0.0196	0.0488	0.0198
Nodules	0.8751	0.0134	0.0076

The turnover rates were calculated from the slopes of regression lines with the following equations.

a) Asparagine from α-Ketoglutarate: roots ($y= 0.0003e^{0.1879x}$ $R^2= 0.611$); nodules ($y= 0.0141e^{0.0574x}$ $R^2= 0.53$)

b) Asparagine from malate: roots ($y= 0.0006e^{0.773x}$ $R^2= 0.509$); nodules ($y= 0.0002e^{0.276x}$ $R^2= 0.902$)

c) α-Ketoglutarate from malate: roots ($y= 0.0003e^{0.209x}$ $R^2= 0.883$); nodules ($y= 0.0001e^{0.606x}$ $R^2= 0.86$)

Chapter 6: General discussion and conclusion

6.1 Background of the study

PEPC-derived C has been investigated for its vital role in nodule organic and amino acid metabolism during phosphate stress in *Virgilia divaricata,* a legume that is native to P-poor soils in the Fynbos ecosystem. This study was done to obtain a better understanding of how PEPC-derived carbon and subsequent downstream metabolic products, such as organic- and amino acids, are integrated into nitrogen metabolism in P-deficient nodules of an indigenous legume of the Cape Flora, which was achieved by three experimental chapters (Chapters 3-5).

This study is the first of its kind to investigate the physiological role that PEPC plays in organic acid and amino acid metabolism in P-deficient nodules of nutrient-poor ecosystems. Previous work by our group has investigated the role of PEPC-derived C in P stressed lupin nodules where labeled ^{14}C was traced from PEPC incorporation into organic and amino acid pools (Le Roux *et al*. 2006, 2008). However, these former studies had the limitation of only determining the allocation of PEPC-derived C into the metabolite pools of non-specific organic and amino acids. Since no information was provided about the specific organic acids or amino acids that were metabolized in P-stressed nodules, it was not possible to determine the fate of the PEPC-derived C in N metabolism during P stress. Therefore, a more in-depth understanding about the specific organic and amino acids derived from PEPC along with turnover rates, was required. This is particularly important for legumes that are indigenous to ecosystems with P-poor soils, because they may display unique metabolic adaptations, owing to their evolution on these P-poor soils. Our main findings in this study are that nodules of *V. divaricata* (Adamson), are better adapted to function under low P than roots, and that they use the PEPC-derived C more efficiently to prevent large declines in BNF. This is because the P-stress bypass route involving the synthesis of PEPC-derived C, enabled these nodules to utilize this C mainly for synthesis of the organic acids malate and α-ketoglutarate, and the export amino acid asparagine.

The legume, *V. divaricata*, which grows in the south western parts of South Africa, referred to as the Cape Floristic Region (CFR) is native to this area and is distributed over a wide range of phosphate deficient soils. The CFR resembles a typical Mediterranean type ecosystem, characterized by acidic sandstone soils where especially phosphate- and nitrogen availability is rather poor to sustain normal plants growth. (Bordeleau and Prevost 1994, Von Uexkull and Mutert 1998, Grigg *et al.* 2008). Legumes which rely on biological nitrogen fixation (BNF) to sustain growth, require much higher amounts of phosphate compared to their counterparts growing on mineral nitrogen in order to sustain BNF. (Drevon and Hartwig 1997). In additions, insufficient phosphate impacts negatively on nodule growth and on the nitrogen fixation process (Schultze *et al.* 2006, Tsvetkova and Georgiev 2007). Currently, not much research has been done regarding the physiology of nitrogen and phosphate uptake, efficiency and utilization of legume trees in Fynbos soils, despite numerous studies which have been conducted on legumes regarding their growth and adaptations in these soils (Muofhe and Dakora 1999, Spriggs and Dakora 2008, Power *et al*. 2010, Kanu and Dakora 2012). *Virgilia* has evolved to fix N_2 despite a low phosphate environment and appears to be well adapted in a typical Mediterranean soil type, which is characterized by various growth-limiting factors. Although previous studies by our group have highlighted the adaptive mechanisms of P tissue allocation and P recycling (Magadlela *et al.* 2014, Vardien *et al*. 2014, Magadlela *et al.* 2015) within the nodules of these plants, these findings do not represent other P stress adaptations by nodules, such as the operation of PEPC as a potential metabolic bypass..

6.2 Summary of completed work

The current study, therefore, focused on the metabolism of PEPC-derived C, which represents a P conservation mechanism. Elucidation of the P conservation mechanisms used by this legume will enhance our understanding of N nutrition in P-poor ecosystems. The findings of the thesis, spanning three integrated studies (Chapters 3-5), have attempted to address this for the first time:

a) The first set of experiments (Chapter 3) dealt with the role of PEPC activity in BNF during P deficiency, since it is known that PEPC plays a vital role in BNF. This is due to PEPC providing the carbon skeletons for the downstream assimilation of ammonium into amino acids (Vance *et*

al. 1994). Under P deficiency, it was found that *V. divaricata* nodules experienced less P*i* stress than roots. Although the BNF declined, the observed high efficiency of BNF was underpinned by altered P conservation pathways and enhanced resource allocation during growth. This found that there is a tendency of a higher contribution of resources to the roots and nodules of *V. divaricata*, under LP conditions, as reflected in the allocation and efficiency studies. This concurs with other findings of *V. divaricata* which followed a similar trend (Magadlela *et al.* 2014). Although there was a decline in nodule number under LP conditions, it appears that the plant would rather invest in efficiency above quantity of the nodules, which requires much more energy. The efficiency of nodules is also reflected in the higher P- and P*i* values as well as the lower percentage decline under LP conditions, when compared to roots. It therefore may serve as an indication that nodules resisted the changes in P concentration during P stress. This is in agreement with findings which indicate that nodules have a strong sink capacity for P assimilation during P starvation (Hogh-Jensen *et al.* 2002), scavenging the P from roots as found in various studies (Jacobsen 1985, Israel 1993, le Roux *et al.* 2006). Despite the apparent ability of nodules to resist the change of P concentration, it was also found that there was a slight decline in BNF for all the organs. However, an increase of BNF efficiency per fresh weight as well as an increase in BNF efficiency per mole P was observed, which further demonstrates the nodule's ability to remain unaffected irrespective the P status. In addition, the decrease of BNF during P stress should rather be viewed in correlation with whole plant growth and the N status (which regulates BNF), while nitrogenase activity is still maintained as suggested and also found by Schultze *et al.* (2003). An alternative to plant BNF would be to increase the nitrate uptake by roots, as this is less cost effective. Although this alternative may be beneficial to plant metabolism, it may also impact negatively on BNF as it inhibits nitrogenase activity (Luciñski *et al.* 2002). Therefore there appears to be a close correlation between BNF and N uptake by roots, which enable the nodules to maintain their functioning. The tendency to switch to a higher N uptake by roots appears to be evident in the higher SNAR values obtained for naked roots compared to nodulated roots, irrespective the treatment.

The apparent consistent N levels are also reflected in the elevated levels of all major amino acids (especially glutamate, aspartate and asparagine) found in the LP nodules. Significant lower values of these amino acid concentrations were observed in HP nodules as well as LP-and HP-

roots and nodules. Asparagine, which was found in elevated levels in nodules, may fulfil many roles, of which feedback regulation of the nitrogenase enzyme appears to be very important in leguminous and non-leguminous plants during various stresses (Steward and Larher 1980, Lea *et al.* 2007). Asparagine, which serves as the principle N export compound in temperate legumes (Schultze *et al.* 2006), can be generated via the PEPC-bypass route, where malate provides the C-skeleton for its production. Malate can be generated by the action of three enzymes, i.e. PEPC, MDH and ME, which appears to be vital, especially during LP conditions, in order to maintain metabolic pathways. Higher malate concentrations were also found in nodules compared to roots during LP conditions. We observed a higher MDH, PEPC and ME activity per fresh weight in LP nodules and roots compared to HP nodules and roots. This might be an indication that the PEPC-bypass route is active during P stress. This scenario is supported by findings that PEPC (in conjunction with MDH and ME) can theoretically function as a glycolytic enzyme by indirectly bypassing the conventional ADP-dependent PKc reaction to facilitate continued pyruvate supply to the TCA cycle during extremely low P conditions. In the process, P*i* is also generated and recycled in the P-starved cells). In addition, PEPC in nodules had higher activity during LP conditions compared to roots, which indicates that the bypass route is more active in nodules during LP conditions than in roots and that PEPC activity is not compromised during LP conditions. These findings may link nodule maintenance directly to PEPC and that nodules have developed strategies to maintain the bypasss route without PEPC being inhibited by malate. These results (Chapter 3) highlight the important role that PEPC plays during P stress for the functioning and maintenance of roots and especially nodules, which maintain their metabolic functions despite P limitations. However, the fate of the PEPC-derived C into specific organic acids and amino acids, remained unknown.

b) The second set of experiments (Chapter 4) addressed the fate of the PEPC-derived carbon via ^{13}C NMR into various metabolic compounds such as organic acids and amino acids. It was reported that ^{13}C NMR spectroscopy could be exploited to calculate the flux of metabolites in plant cells (Chang and Roberts 1989). The ability of PEPC to naturally discriminate between PEPC $H^{12}CO_3^-$ and $H^{13}CO_3^-$ (Raven and Farquhar 1990) enabled us to determine PEPC-derived downstream metabolic products. Two profound ^{13}C NMR peaks of malate (~175ppm) and citrate (~181ppm), indicated the importance of these PEPC organic acids during P stress. PEPC-derived

^{13}C malate, which is the second metabolic product in the PEPC bypass pathway, was found in higher concentrations in LP nodules compared to roots, supporting previous findings, as it serves as main respiratory fuel for bacteriods in nodules (Tajima *et al.* 1990, Streeter 1991). ^{13}C NMR also revealed a sharp decline in malate concentrations after 2 h, indicating its downstream metabolic role, of providing C-skeletons for incorporation into other metabolic products. Our results indicated a rapid incorporation of this PEPC-derived ^{13}C malate into α-ketoglutarate. Furthermore, the generated ^{13}C derived α-ketoglutarate was shown to be incorporated into asparagine at a higher rate in nodules compared to roots during low P conditions. This is in agreement with findings where ^{14}C aspartate was exogenously applied to effective alfalfa nodules and became rapidly converted to asparagine, showing the importance of this amino acid in nodules as an exported substrate for N fixation (Maxwell *et al.*1984, Snapp and Vance 1986). ^{13}C NMR was therefore able to provide valuable information regarding the incorporation of PEPC-derived C into downstream metabolic products and therefore the dependence of these organs on this enzyme to function under P stress. However, the fate of the PEPC-derived C into specific organic acids and amino acids, remained unknown.

c) The third set of experiments (Chapter 5) gives us an indication of the preferred C-fuel in roots and nodules subjected to P stress, as well as an insight of how the PEPC enzyme may be regulated. Throughout literature it appears that the regulation of PEPC isoform expression in other non- legume species appears to be via transcription factors and their interactions associated with the gene expression networks. To our knowledge, studies have not yet been undertaken in P- deficient nodules, and this study will address it. GC-MS data revealed that elevated levels of sucrose occurred in nodules, which was much higher than in roots of P stressed plants. This might implicate sucrose as the preferred photosynthetic sugar in nodules to be used as C-source, for downstream nitrogen fixation via PEPC, as shown in previous work (Reibach and Streeter 1983, Day and Copeland 1991, Vance and Heichel 1991). The generated metabolic products of sucrose i.e. glucose-6-phosphate and fructose-6-phosphate via sucrose invertase and sucrose synthase, can serve as downstream C-sources and it could also be used as a feed-forward activator of PEPC. Aspartate and glutamate (PEPC inhibitors) which were also found in higher levels than in roots, may serve as precursors for asparagine, most probably to be translocated to shoots. Similar results were also found in *Lotus japonicus* (Tajima *et al*. 2004), where the

majority of the aspartate is converted to asparagine by AS, which is regarded as the major exporting amide of fixed nitrogen in amide transporting legumes. In addition, the elevated level of malate (compared to roots) appears not to inhibit the action of PEPC as activity was shown for this enzyme, which was higher than the PEPC activity of roots. It therefore appears that PEPC in nodules circumvents the inhibitory effect of malate to a large extent, under LP conditions, since the enzyme remains active. This is in agreement with similar finding of soybean and alfalfa, where the phosphorylated PEPC becomes less sensitive to feedback inhibition by its inhibitor, malate and more sensitive to its activator, glucose 6-phosphate which results in a higher activity yield of this enzyme (Vidal and Chollet 1997). The phosphorylation and dephosphorylation of PEPC is accomplished on the biochemical level by the strictly conserved serine-residue near the protein's N terminus, which can be reversibly phosphorylated is largely controlled by a transcriptional and proteolytic, up-/down-regulation of a dedicated vascular plant-unique Ser/Thr kinase, PEPC-kinase (Izui *et al.* 2004, Agetsuma *et al.* 2005, Nimmo 2006). The post-translational regulation of PEPC in nodules (during LP conditions) might hold one of the keys, as to how nodules manage to be unaffected by the elevated malate concentration, which would normally inhibit PEPC action.

6.3 Contribution of completed work in context of the current field

This investigation, spanning three interlinked papers (Chapters 3-5), has shown that there are various ways in which plants adapt to conserve the use of P. These adaptations include, a decrease in growth rate, increased growth per unit of P uptake, remobilisation of internal P*i*, modification in C-metabolism that bypass P-requiring steps and alternative respiratory pathways, higher resource allocation and increase in BNF efficiency (Schachtman *et al.* 1998, Plaxton and Carswell 1999, Raghothama 1999, Uhde-Stone *et al.* 2003*a*, 2003*b*, Magadlela *et al.* 2014, Vardien *et al.* 2014). Furthermore, legumes induce genes during P stress which results in the production of cluster roots, thereby increasing the roots surface area leading to enhanced P acquisition. Nodule efficiency for P utilization is enhanced in the process (Le Roux *et al.* 2008), leading to root exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters (Gilbert *et al.* 2000, Gilroy and Jones 2000, Lynch and Brown 2001, Neumann and Martinoia 2002, Lamont 2003, Uhde-Stone *et al.* 2003*a*, Vance *et al.* 2003). Since

legumes are highly sensitive to P stress conditions, it follows naturally that there will be a high carbon cost involved in order to sustain growth, especially the nitrogen fixation process (Mengel 1994). This scenario was also observed in white lupin, where nodules acted as strong carbon sinks during P stress (Le Roux *et al.* 2008), In addition, it is known that nodules do have a strong sink capacity for P assimilation during P starvation (Hogh-Jensen *et al.* 2002). LC-MS/MS revealed that various PEPC isoforms might be present in roots and nodules and that PEPC-levels and its regulation appears to differ between roots and nodules under P-stress. There was an apparent up-regulation of PEPC during P-stress in roots and a down regulation of this enzyme in nodules. This apparent down-regulation in nodules despite high activity of this enzyme suggests that PEPC levels are not necessarily related to its activity. It was found in in *Lupinus luteus* that PEPC might not be regulated by gene expression or phosphorylation under P-stress and that the higher levels of phosphorylated PEPC protein found in P-stressed nodules might have been due to slower protein degradation. However, those findings also yielded unchanged PEPC activity under P-stress (Kleinert *et al.* 2010). The higher activity values in our study and the apparent various isoforms present in nodules, might suggest that not all of the PEPCs might be active but rather act in synergy depending on their individual active or inhibition state.

The new findings of this thesis have addressed the limited information regarding the role of PEPC-derived C metabolism during P stress in legumes and non-legumes. In particular, this thesis focused on these limitations in roots and nodules of *V. divaricata*, a legume which is indigenous to the nutrient-poor Fynbos ecosystem. The context of these findings in the areas of legumes and P nutrition in Mediterranean-type nutrient-poor ecosystems, has been summarized in Table 6.1. Although our group has made progress in the study P-deficient adaptations of legumes that are indigenous to nutrient-poor ecosystems such as Fynbos (Magadlela *et al.* 2014, Magadlela *et al.* 2015, Maistry *et al.* 2015a, b, c, Vardien *et al.* 2014), none of these former studies have investigated the comparative responses of nodules and roots to PEPC-derived C metabolism under P stress. From Table 6.1, it is apparent that an increase in PEPC activity for legumes and non-legumes usually leads to an increase organic acid synthesis. From this table of previous studies, there appears to be an exception in *Lupinus luteus* where PEPC activity remained unchanged, but with an increase of metabolic products under low P conditions. Although *Lupinus angustifolius* shows an increase in PEPC activity, there was a decrease in the

amount of amino acids. A similar trend was observed in non-legumes where an increase in PEPC activity always leads to an increase of various metabolic products under P stress, with the exception of *Catharanthus roseus*, which showed a decrease in malate. A major limitation to all these studies is that not all amino acids and organic acids have been identified, or correlated with PEPC activity. This study of *V. divaricata*, employing analytical techniques such as GC-MS, ^{13}C-NMR and LC-MS, yielded a greater understanding of the relationship between PEPC-derived organic- and amino acids and the incorporation thereof.

6.4 Conclusions and future perspectives

The metabolism of PEPC-derived C in *V. divaricata* is different in the roots and nodules of this legume. The results presented in this study shows that PEPC plays a major role in the N assimilation and maintenance respiration of the nodules, and to a greater extent than in roots of this P-stressed legume.

The pivotal role of PEPC during P-stress in the legume *V. divaricata* appears to be a vital adaptation, in order to maintain various N assimilating pathways in P poor soils. The enhanced activities of nodule PEPC, MDH and ME, whilst PK declines, leading to downstream PEPC-derived metabolites, suggests that under LP conditions an adenylate bypass was in operation either to synthesize more organic acids or to mediate pyruvate via a non-adenylate requiring metabolic route. ^{13}C-NMR supported the idea for the operation of the PEPC bypass route, especially in roots and nodules under P stress conditions. The PEPC-bypass metabolic route which aids in the formation of the subsequent downstream metabolic compounds, gives an indication of the ability of a plant to overcome the detrimental effect which low phosphate conditions might impose on roots and nodules. Studying this legume gives us a better insight to use alternative methods for enhancing BNF in crop legumes which, since it is estimated that the global P supply might not last till the end of this century (Vance *et al.* 2003). Therefore, following a metabolic approach has shed light on how this legume manages to sustain growth under limiting P conditions in such nutrient-poor ecosystems. However, potential future work should address more protein-level work in order to elucidate the cellular control on this important enzyme. There appears to be a close correlation between the activation and inhibition

of this enzyme on biochemical level, which seems to be determined by the various levels of its activators and inhibitors. This regulation of PEPC on the protein level by various physiological conditions, would therefore shed more light of the apparent intricate interaction that exists between PEPC gene activation- and inhibition during P stress. These experiments should include:

- the determination of the gene sequence of highly-expressed isoforms of PEPC,
- the relationship between the bacterial –and plant type PEPC,
- the regulation of PEPC expression,
- the regulation of PEPC protein,
- the phosphorylation/dephosphorylation status of the enzyme and to which extent it is being protected from inhibition by various factors,
- purifying the enzyme and performing its enzyme kinetics.

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Table 6.1 A comparison of organic acid and amino acid metabolism in legumes and non-legumes during P-stress.

Effect of P-stress on legumes and non-legumes										
Legumes	**Organ**	**PEPC**	**Sucrose**	**OA**	**Citrate**	**Malate**	**AA**	**Asp**	**Glu**	**Reference**
L. luteus	Nodules	Unchanged	Increase	Unchanged	Unchanged	Unchanged	Increase	Increase	Increase	Kleinert *et al* 2010
S. rostrata	Roots	Increase	ND	Increase	Increase	Increase	ND	ND	ND	Aono *et al* 2001
L. albus	Roots	Increase	ND	Increase	Increase	Increase	ND	ND	ND	Johnson *et al* 1994
L. angustifolius	roots/nodules	Increase	ND	Increase	ND	Increase	Decrease	ND	ND	le Roux *et al* 2006
Non-legumes										
H. prostrata	Roots	Increase	ND	Increase	Increase	Increase	ND	ND	ND	Shane *et al* 2004
N. tabacum	Seedlings	Increase	Increase	Increase	Increase	Increase	Increase	ND	ND	Toyota *et al* 2003
B. napus	Roots	Increase	Increase	Increase	Increase	Increase	Increase	ND	ND	Hoffland *et al* 1990
A. thaliana	Cell suspension	Increase	ND	ND	ND	ND	ND	ND	ND	Gregory *et al* 2009
C. roseus	Cell suspension	Increase	ND	ND	ND	Decrease	ND	ND	ND	Nagano *et al* 1994

OA = Organic acids; AA = Amino Acid; Asp = Aspartat; Glu = Glutamate; ND = Not determined